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To the loving memory of Jari

ABSTRACT

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Coordinated and stoichiometric expression of contractile apparatus genes during the differentiation of L6 and C2C12 cells was studied. Expression of the genes initiated prior to fusion, and coordination in-between amply expressed actin and tropomyosin (TM), and among fast members of a troponin complex, was apparent. Pronounced synthesis of members of this complex initiated slightly later than that of actin and TM. Expression seemed to start with troponins I (TnI) and C (TnC), whereas troponin T (TnT) showed variation in the cell lines. The order and abundance of expression is supported by the regulatory sequences that the genes have. The results suggest that assembly of thin filament starts with the interaction of α-actin and TM. Initial basic TnI may interact with slightly later accumulating acidic TnC, and the complex may be then joined through a basic TnT to TM-actin structure. α-actin and TM were shown to be more stable proteins than the members of the troponin complex, especially TnC. The shorter half-life member of a TM dimer may determine the renewal of the TnT-TM-actin structure, whereas the most labile TnC may have the same impact on the troponin complex.

The other goal of the study was to disturb the normal expression of TnT in L6 cells and TnC in C2C12 cells by using antisense oligonucleotides (ODNs) and/or antisense RNA. Treatment of L6 cells with 25 μM antisense AUG-TnT 3'end phosphorothioated ODNs (3'S-ODN), and C2C12 cells with 50 μM antisense TnC unmodified ODNs (umODNs) for 12 h or antisense TnC RNA, reduced the synthesis of the respective proteins by 20-50%. Antisense cap-TnC umODNs were more effective than antisense AUG-TnC umODNs or any other site along the mRNA-binding ODNs. Treatment with antisense TnT 3'S-ODN also altered cellular mRNA levels of actin, TM, and TnC, and its effects on contraction apparatus protein synthesis were greater than by treatment with antisense TnC. However, phosphorothioated antisense S-ODNs against the internal region of c/sTnC mRNA (int-c/sTnC) unexpectedly increased the synthesis of both TnC proteins in C2C12 cells, when treated for 48-72 h with 20-100 μM concentration of S-ODNs. The reason for the increase may be due to the induction of Sp1 by S-ODNs. An Increase was apparent also in around 120 h differentiated myotubes treated with antisense-c/sTnC umODNs together with Actinomycin D for 3 h. C/sTnC synthesis also increased, when in vitro transcribed c/sTnC mRNA was translated in the presence of either antisense cap-c/sTnC or AUG-c/sTnC umODNs, or sense int-c/sTnC umODN in rabbit reticulocyte lysate. Translation with antisense AUG-c/sTnC umODN in wheat germ extract, however, reduced the synthesis. Antisense cap-c/sTnC umODN had no effect. The results suggest that ODNs may open the folding of c/sTnC mRNA to a more translatable form. The unusual folding of the mRNA is also supported by its long half-life. The differentiation study also suggested, that TnC is post-transcriptionally regulated. RNAse H activity in C2C12 myocytes withdrawn from the division cycle may also decline during differentiation, which may be one reason for the differing results of the antisense cap-c/sTnC umODN treatments.

Keywords: Antisense oligodeoxynucleotide; contraction apparatus; muscle proteins; myogenesis; troponin C; troponin T.

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Contents

Abbreviations

a	atrial form
AcD	actinomycin D
ADP	adenosine diphosphate
a/e	atrial/embryonic form
APS	ammonium persulphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
c	cardiac form
cAMP	cyclic adenosine monophosphate
CIP	calf intestine phosphatase
CMV	cytomegalovirus
cpm	counts per minute
c/s	cardiac/slow form
c/e	cardiac/embryonic form
ds	double stranded
DMSO	dimethylsulfoxide
DTT	dithiotreithol
2D-PAGE	two-dimensional polyacrylamide gel electrophoresis
e	embryonic form
F	filamentous
f	fast form
G	globular
hnRNA	heteronuclear RNA
IEF	isoelectric focusing
int	internal region
iRNA	inhibitory RNA
kD	kilo Dalton
MHC	myosin heavy chain
MLC	myosin light chain
M-MLV	Moloney murine leukemia virus
NBT	nitroblue tetrazolium salt
NEPHGE	nonequilibrium pH gradient gel electrophoresis
nm	non-muscle form
ODN	oligodeoxyribonucleotide
p	perinatal isoform
PAA	polyacrylamide
PKA	polynucleotide kinase A
PKC	polynucleotide kinase C
PNK	polynucleotide kinase
RB	retinoblastoma protein
PPO	diPhenyloxazole
s	slow isoform
S-ODN	phosphorothioated oligodeoxyribonucleotide
3'S-ODN	3' end phosphorothioated oligodeoxyribonucleotide

SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sk	skeletal muscle isoform
sm	smooth muscle isoform
snRNA	small nuclear ribonucleic acid
TCA	trichloroacetic acid
TnC	troponin C
TnI	troponin I
TnT	troponin T
TM	tropomyosin
umODN	unmodified oligodeoxyribonucleotide
UTR	untranslated region
v	ventricular form
v/s	ventricular/slow form

1 INTRODUCTION

Mammalian skeletal muscle development begins from the induction of mesodermic stem cells from the primary ectoderm (Buckingham 1985, Mohun 1992, Sassoon 1993). The determination and the gene hierarchies that are involved in it, are poorly understood. The mesodermic stem cells can generate proliferating determined myoblasts, which can differentiate and fuse to multinucleated myotubes in response to different trans- and/or cis-acting signals during the development. Different positively and/or negatively affecting factors and signals can also affect a set of genes, which are in turn able to activate the coordinated and stoichiometric synthesis of contractile apparatus and other muscle specific proteins (Bandman 1992, Mohun 1992, Olson 1992, Stockdale 1992). Thus muscle tissue can offer a good model system for studies of cell differentiation and its regulation, especially since myofibre formation can also be induced in cell culture systems.

The mechanisms of assembly of the contraction apparatus, or on the other hand, its renewal are not fully understood. Moreover, all of the muscle contraction apparatus proteins exist as multiple forms (Epstein & Fischman 1991, Bandman 1992). Most of them are coded by chromosomally scattered multigene families, which genes have gained throughout the evolution relatively little alterations (Buckingham 1985, Rubenstein 1990, Lees-Miller & Helfman 1991). Thus different forms are produced from separate genes, but other options to create particular isoforms are usage of different promoters for a single gene or alternative splicing of a primary transcript. The reason why there exists different protein forms is not known. Possible explanation may be that it gives for the organism the capability to adjust to different conditions or different regulatory signals more rapidly - ancient gene duplications and dispersions have placed the muscle genes in different regulatory modules and each gene family probably has its own regulatory program. Alternative splicing is, in turn, determined at least partially by the cell type and its splicing factors (Breitbart & Nadal-Ginard 1987,

Lees-Miller & Helfman 1991, Bandman 1992). Expression of two or more of muscle genes or isoforms may also facilitate the accumulation of the needed protein rapidly, although it is not known, what effect of a particular protein form has for instance on contraction, especially with the combination of the other muscle proteins. Development as such may also require functionally different protein forms. Nevertheless, different forms of the proteins are expressed in muscles in diverse combinations, according to a developmental stage and specific signals (Buckingham & Minty 1983, Breitbart & Nadal-Ginard 1986, Rubenstein 1990, Bandman 1992, Epstein & Bernstein 1992, Stockdale 1992).

The purpose of this work has been to clarify further the described complicated muscle development in skeletal muscle originated rat L6 and mouse C2C12 continuous cell lines. The main aspect is the coordinated regulation of contraction apparatus genes during differentiation, and another purpose is to create a model of an assembly of a thin filament. To reach these goals, the normal pattern of contractile apparatus gene expression during differentiation and the half-lives of the respective muscle specific cellular mRNAs and proteins were estimated. The antisense ODN or antisense RNA techniques in turn were used to the targeted regulation of expression of three muscle specific genes, fast and cardiac/slow forms of troponin C and fast isoform of troponin T, in an effort to investigate the relationships between different muscle specific proteins and, also, between protein forms.

2 REVIEW OF THE LITERATURE

2.1 Contraction apparatus

2.1.1 Structure and formation of contraction apparatus

Muscle contraction apparatus is formed of thin and thick filaments (Fig. 1), which are both composed of several proteins existing as multiple isoforms. Individual muscles have been shown to express different combinations of these proteins according to their developmental stage. Adult muscles generally contain several different fibre types, which have characteristic myosin heavy chain gene products (Emerson, Jr. & Bernstein 1987, Miller 1990). These fibre types determine whether the muscle belongs to the slow twitching muscle type I, or to the fast twitching muscle type II, which has also A, B and C subclasses (Buckingham & Minty 1983, Hoh et al. 1989, Wade & Kedes 1989). The different fibre types, however, show some flexibility in expressing other contraction apparatus proteins/isoforms depending of specific signals (Jolesz & Sréter 1981, Buckingham & Minty 1983, Härtner & Pette 1990). Nevertheless, strict protein stoichiometry is essential for contraction apparatus function at any developmental stage, although the signals that regulate this stoichiometry and coordinated expression are not fully understood (Epstein & Bernstein 1992). The main regulation probably occurs at the transcriptional level, but some regulation exists in some cases also at the translational level as well as at the transcript and/or protein stability level (Emerson, Jr. & Bernstein 1987, Olson 1992).

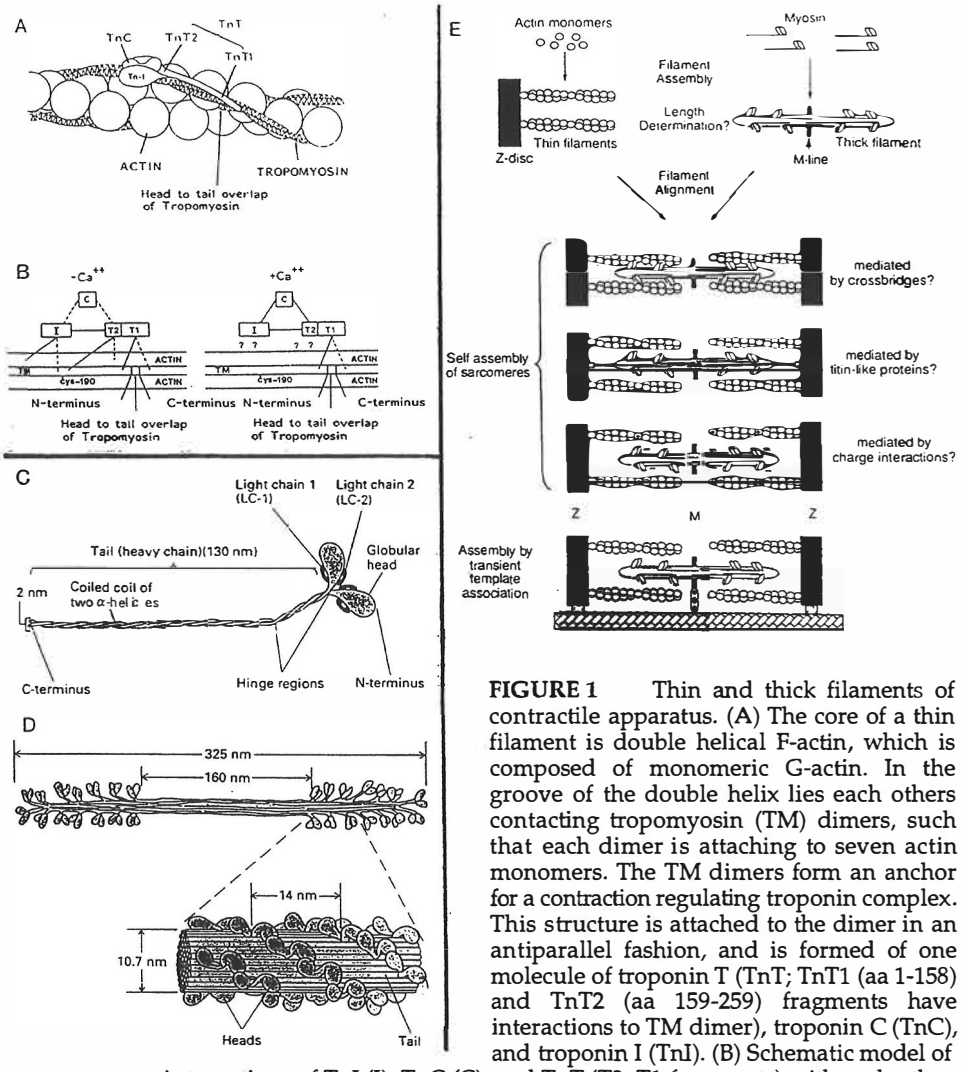

FIGURE 1 Thin and thick filaments of contractile apparatus. (A) The core of a thin filament is double helical F-actin, which is composed of monomeric G-actin. In the groove of the double helix lies each others contacting tropomyosin (TM) dimers, such that each dimer is attaching to seven actin monomers. The TM dimers form an anchor for a contraction regulating troponin complex. This structure is attached to the dimer in an antiparallel fashion, and is formed of one molecule of troponin T (TnT; TnT1 (aa 1-158) and TnT2 (aa 159-259) fragments have interactions to TM dimer), troponin C (TnC), and troponin I (TnI). (B) Schematic model of interactions of TnI (I), TnC (C), and TnT (T2, T1 fragments) with each other, TM, and actin in the presence and absence of Ca^{2+}. Solid lines represent strong interactions, dotted lines weaker ones, and the question mark possible weak interactions depending on Ca^{2+} concentration (Heeley et al. 1987, Lees-Miller & Helfman 1991). (D) The thick filament is arranged of several large, hexameric myosin complexes, where (C) one complex includes two intertwined myosin heavy chain subunits, of which both globular head portions interact with one alkali myosin light chain and one regulatory light chain subunits (Darnell et al. 1990). (E) Models of a contraction apparatus formation (Fyrberg & Beall 1990).

It is known that physiological stimuli such as hormones and innervation have influence on synthesis of particular muscle proteins/isoforms in living organism, at least partially, through the change of the balance of functional transcriptional factors (Jolesz & Sréter 1981, Izumo et al. 1986, Emerson, Jr. & Bernstein 1987). Thus the coexpression of genes and gene sets may be regulated through distinct

cardiac, fast skeletal- and slow skeletal-specific control elements as well as control elements, that coordinate isoform activation in embryonic muscles. Many of these variety of positive and/or negative cis-acting sequences binding transcription effecting factors have already been identified, but many of them as well as the additional elements, are in all likelohood still unknown.

Several alternative splicing regulating cis-acting elements have been identified for example in troponin T and tropomyosin pre-mRNAs, but the cellular factors that control their tissue-specific splicing are not known, although it seems that some of the splicing factors are induced during myogenesis (Andreadis et al. 1987, Breitbart et al. 1987). The relative affinities of different splicing sites for each others is determined by the distinct splicing environment of the cell: in one cell type or at particular developmental stage a specific donor and acceptor site has a strong functional affinity for each other and are efficiently spliced together, while in other cell type or developmental stage the same pair is ignored by the splicing machinery (Breitbart & Nadal-Ginard 1986, Breitbart & Nadal-Ginard 1987, Wieczorek et al. 1988).

The influence of particular protein/isoform on contraction apparatus and its function is not completely understood (Buckingham & Minty 1983, Epstein & Fischman 1991). Previously mentioned fibre types have distinct speed and power, and myosin heavy chain seems to determine those characters by modulating the two regulatory steps in a cross-bridge cycle: the rate of development of force and the maximal velocity of shortening. Fast fibers have a higher threshold for Ca^{2+}-activated force and a steeper force-pCa relation than do slow fibres. This difference is connected to the cooperativity in the attachment of cross-bridges, and thus it seems that different contractile proteins/isoforms, especially of troponin complex, have influence on this cooperativity. It is known, for instance, that both troponin C proteins have distinct Ca^{2+}-binding capacities and both have differences in their abilities to regulate the excitation-contraction coupling. The exact molecular bases of these functional differences of troponin C are not known (Adelstein & Eisenberg 1980, Moss et al. 1986, Warrick & Spudich 1987, Morimoto 1991, Metzger & Moss 1992).

2.1.1.1 Actin

There exists at least six different extraordinarily conserved functional actin genes (Vanderkerckhove & Weber 1984, Rubenstein 1990). The reason for the conservation may be that practically every amino acid in actin molecule has specific, important functions - it has been shown that most mutations in actin cause gross disruption of the muscle (Pollard & Cooper 1986). However, these genes are independently regulated and expressed in a specific pattern, often overlappingly, in tissues and during development (Minty et al. 1986). The general expression pattern of different actin genes has been used as a basis for dividing them to two different classes: class I contains cytoplasmic β- and γ-forms, and class II has muscle α-actin forms (Rubenstein 1990). Striated muscle express mainly α-skeletal (αsk-actin) and/or α-cardiac (αc-actin) proteins, whereas smooth muscle has two

types, α-smooth muscle (αsm-actin) and γ-smooth muscle (γsm-actin) actin. Cytoplasmic β- and γ-actins (βnm-, γnm-actin) are also expressed in striated muscle and they constitute up to 40-50% of the actins (Buckingham 1985, Rubenstein 1990). The high conservation between these proteins is apparent, since cardiac and skeletal muscle actins differ from each other only by four amino acids out of 375, smooth and skeletal muscle actin by 6 or 8, striated muscle and non-muscle actin by 24 or 25, and smooth muscle and non-muscle actin by 22 or 23 (Vanderkerckhove et al. 1986, Rubenstein 1990).

The thin filament of striated muscle is formed of αsk- and/or αc-actin globular actin molecules (G-actin), which are arranged to double-stranded, α-helical fibrous actin (F-actin) (Fig. 1). Monomeric G-actin (42 kD) has two protein domains, small and large. Both of them are divided to two subdomains. The cleft between small and large domains has an adenosine nucleotide (ATP or ADP) site, where ATP can enhance F-actin formation. The cleft also has a high affinity Ca^{2+}-binding site, which functions as a stabilizer of globular conformation of the molecule. There also exist several low affinity-binding sites for mono-, di- or trivalent cations. The myosin interacting region of actin is located to its NH_2-terminal acidic segment, and this area is the most diverse among actin forms (Pollard & Cooper 1986, Korn et al. 1987).

The coding regions of different actin genes are highly homologous. The main differences exist in positions and sequences of introns, and also in the sequences of 5' and 3' flanking and untranslated regions (UTR), which all seem to contain regulatory sequences. However, different actin genes have their own sets of regulatory elements, which facilitate the independent expression of these genes (Buckingham & Minty 1983, Minty et al. 1986, Muscat et al. 1988, Chow & Schwartz 1990, Sartorelli et al. 1990, Lee et al. 1991). Important sequences for αsk-actin expression and regulation include positively acting factor-binding sites, such as an E-box (consensus sequence CANNTG), four CArG-boxes (consensus sequence $CC(A/T)_6GGC$) of which two are muscle regulatory elements, MREs (sequence [U/C]YGUCCAAATA[A/T]GG[A/C]G) surrounded with G+C rich sequences, a T+A-rich region, M-CAT (sequence CATTCCT) and a CAAT-box (consensus sequence GGC/TCAATCT) (Minty & Kedes 1986, Chien-Tsung Hu et al. 1986, Phan-Dinh-Tuy et al. 1988, Alonso et al. 1990, Chow & Schwartz 1990). Skeletal muscle expression of αc-actin, on the other hand, requires at least its Sp1-site, MRE/CArG-box and E-box (Sartorelli et al. 1990, French et al. 1991, Pari et al. 1991). The mouse αc-actin gene also has an NFe-binding site, which may function as an additive regulatory site, as well as normal CAAT- and TATA-boxes (Buckingham & Minty 1983, Minty & Kedes 1986, Yu & Nadal-Ginard 1989).

Sarcomeric αsk- and αc-actin genes are coexpressed in striated muscle, but their relative proportions in skeletal and cardiac muscles depend on the species, the muscle type and the stage of development (Bains et al. 1984, Vanderkerckhove et al. 1986). In adult mouse, for instance, the respective protein is the dominating one in skeletal and cardiac muscle (Alonso et al. 1990). Promoter regions of αsk- and αc-actin genes have at least one common regulatory element, the CArG-box, which seems to be involved for coregulation of these genes by binding the same transcriptional factor (Muscat et al. 1988). As mentioned earlier, αsk-actin has four

of these sequences, and it has been proposed that high level expression of it in skeletal muscle may require that all of these sites are occupied with the factor, whereas the high level expression of αc-actin is modulated with additional factors, which are not abundant in skeletal muscle (Muscat et al. 1988, Alonso et al. 1990, Chow & Schwartz 1990, French et al. 1991).

2.1.1.2 Tropomyosin

Tropomyosins (TM) are a family of structural proteins that exist in all eukaryotic cells. They associate with actin in thin filaments of muscle cells (Fig. 1) and in microfilaments of non-muscle cells. The function of TM in skeletal and cardiac muscle is, in association with the troponin complex, to regulate the Ca^{2+}-sensitive interaction of actin and myosin, and also rigidify the thin filament. Biological functions of smooth muscle and non-muscle TMs are poorly understood. TMs are elongated proteins that have highly conserved α-helical coiled-coil structure (Wieczorek et al. 1988, Liu & Bretscher 1989, Lees-Miller & Helfman 1991). This structure is based on a repeated pattern of seven amino acids with hydrophobic residues at the first and fourth positions and it facilitates two TM monomers to bind to themselves in a head-to-tail manner (monomer 33 kD, homo- or heterodimer 66 kD) (Heeley et al. 1987, Lees-Miller & Helfman 1991, Parmacek & Leiden 1991). In thin filament, these TM dimers contact seven actin monomers, possibly through the NH_2-terminal region of TM (Fig. 1). In COOH-terminal region, TM has a troponin T-binding area. TM is also acetylatable, which enhances TM-troponin complex binding to actin (Heeley et al. 1987, Wieczorek et al. 1988, Hill et al. 1992).

Tropomyosins are generated from α-TM, β-TM, TM-30 and TM4 genes, but the main source of variety and differences between TM forms are a result of the alternative splicing of primary transcripts (Muthuchamy et al. 1993). Moreover, the α-TM gene has two alternative promoters to produce two different primary transcripts. Rat has at least 12 TM forms: distinct proteins are found in muscle, brain and various non-muscle cells. The striated muscle α-TM protein is prominent in cardiac and in fast twitch skeletal muscles, while skeletal β-TM is abundant in slow twitch and smooth muscles. The α-TM and β-TM subunits, however, are also developmentally regulated (Wieczorek et al. 1988, Briggs et al. 1990, Lees-Miller & Helfman 1991, Wang & Rubenstein 1992, Muthuchamy et al. 1993). Two additional forms termed γ and s have been identified in slow twitch muscles of rat, which are named as slow twitch α-TM forms (Lees-Miller & Helfman 1991). The main structural differences amongst skeletal, cardiac and smooth muscle TM proteins seem to match to functional domains, including troponin and actin binding regions and sequences involved in head-to-tail polymerization (Wieczorek et al. 1988).

The α-TM gene (28 kb) encodes at least ten isoforms and it is considered the most complex of the vertebrate TM genes (Ruiz-Opazo & Nadal-Ginard 1987, Wieczorek et al. 1988, Lees-Miller et al. 1990, Muthuchamy et al. 1993). Rat α-TM gene contains 15 exons of which exons 3, 4, 5, 7 and 8 are common to all mRNAs

expressed from the gene. Exons 1a and 1b are associated with separate promoters, which results in mRNAs with two different NH_2-termini. Furthermore, exon 1a can be spliced either to exon 2a or 2b. Exons 9a-d are needed for creating a unique 25 to 29 amino acid length COOH-terminus (Wieczorek et al. 1988, Lees-Miller & Helfman 1991, Graham et al. 1992). Striated muscle α-TM isoform (36.5 kD) seems to be encoded from exons 1a, 2b, 3, 4, 5, 6b, 7, 8 and 9a; smooth muscle uses exons 2a, 6a and 9d. Exon 9b can also be selected, but it is spliced to exon 9a, providing only the 3' UTR. Exons 6b and 9a are essential, since both of them encodes binding domain for troponin T. Sequences in exons 9a and 1a are also involved to head-to-tail polymerization between TM molecules. The polymerization process is also affected by phosphorylation of serine by a TM kinase (Lees-Miller & Helfman 1991).

The murine β-TM gene (10 kb) has 11 exons, that encode skeletal muscle β-TM (39 kD), fibroblast/smooth muscle β-TM1, nonmuscle/smooth muscle β-TM2 and β-TM3, and also striated muscle β-TM4 isoforms. The exons have close homology to α-TM exons, and the major differences between rat α- and β-TM genes seems to be that the β-TM gene does not contain exons comparable to 1a, 2a and 9c of α-TM. The internal promoter of β-TM is associated to use exon 1b. All β-isoforms also have exons 2b, 3-5, 7 and 8. Exons 6b and 9a are used exclusively in skeletal muscle β-TM, but interestingly, β-TM4 has both 6a and 6b (Wang & Rubenstein 1992, Guo & Helfman 1993, Muthuchamy et al. 1993).

The cellular factors that control the tissue-specific use of exons are not known. Some of the factors may be the same that are involved for troponin T splicing. Apparently some of these specific splicing factors are induced during myogenesis (Breitbart & Nadal-Ginard 1987, Wieczorek et al. 1988, Guo & Helfman 1993, Muthuchamy et al. 1993). Furthermore, it has been suspected that actin type and/or interaction with troponin may guide the different exon usage of TM in different cells (Lees-Miller & Helfman 1991).

The promoter of the α-TM gene resembles more housekeeping promoters than muscle specific ones by its structure and sequence organization. It lacks canonical sequence elements and it is active in most cell types. Therefore, the TM isoforms, which are produced from the primary transcript, are determined by the splicing environment of the cell type. One difference is also, compared to the muscle specific genes, that the α-TM gene has multiple, closely spaced transcription start sites within a single 5' exon. Furthermore, the TATA- and CAAT-boxes are not in their characteristic locations in both α-TM and β-TM genes as well as the 5' UTR is not interrupted by one or two introns as it is generally the case with sarcomeric genes. However, α-TM has potential M-CAT (CATACCTT) and E-box elements, which may participate in regulation of α-TM expression (Wieczorek et al. 1988). β-TM, on the other hand, has two E-box sequences and four CCACCC-sequences in the intron between exons 9a and 9b, although their function is unknown (Wang & Rubenstein 1992).

2.1.1.3 Troponin T

Troponin T (TnT) exists as a 30 kD monomer in a troponin complex, connecting TnC and TnI to TM. Its exact functions during contraction are not known, but it may have some regulatory qualities (Breitbart & Nadal-Ginard 1986, Schachat et al. 1987, Reiser et al. 1992). TnT is expressed in different muscles in various forms, which are encoded by three genes. These genes produce fast and slow skeletal, and cardiac and/or embryonic skeletal muscle TnT proteins/isoforms (fTnT, sTnT, and c/eTnT, respectively). The main source for TnT diversity is, however, the alternative splicing of primary transcripts: the fTnT gene can produce 64 different isoforms through alternative splicing, whereas c/eTnT can be spliced into at least two isoforms, as can human sTnT. The amino acid sequences of these different forms when compared to different species are quite dissimilar, which is contrary to the otherwise highly conserved contractile proteins (Cooper & Ordahl 1985, Breitbart & Nadal-Ginard 1986, Gahlmann et al. 1987, Mar et al. 1988, Jin et al. 1992).

The rat fTnT gene (16.6 kb) is composed of 18 exons, and it produces only one type of primary transcript (Breitbart & Nadal-Ginard 1986). The primary transcript exhibits three types of splicing. Exons 1-3, 9-15 and 18 exist in all processed transcripts, but each of the exons 4-8 can be individually included or excluded from the mature mRNA in a combinatorial fashion. This type of splicing can generate as many as 32 different NH_2-terminal regions for the proteins. More variety to these transcripts gives COOH-terminal regions coding exons 16α and 17β, which are alternatively spliced in a mutually exclusive manner. These exons rise the number of different TnT isoforms to 64, and also divides them to TnTα and TnTβ classes (Breitbart & Nadal-Ginard 1986, Pan & Potter 1992).

Rat c/e TnT is also a single copy gene (19.2 kb) containing 16 exons. Its DNA sequence, especially 5' upstream structure, and exon organization differs from the fast skeletal muscle one. It is expressed as two forms during cardiac muscle development, a larger, more acidic embryonic isoform and a smaller, more basic adult cardiac isoform. The primary transcript, however, seems also to be spliced to a third poorly characterized isoform, but there may exist others, too. Nevertheless, the cardiac isoforms are generated from one transcript by alternative mRNA splicing and the difference between them is due to inclusion or exclusion of exon 4 (Jin & Lin 1988, Saggin et al. 1988). Exon 12 (9 bp) is also alternatively spliced, but the functional significance of variation of this exon usage is unknown (Jin et al. 1992). Splicing pattern of exon 12 do not appear to be linked to developmental changes in muscle, but it is suspected that expression of the exon is specific to mammalian hearts (Cooper & Ordahl 1985, Breitbart & Nadal-Ginard 1986, Gahlmann et al. 1987, Jin et al. 1992). Exon 12 may be a connecting segment between two functional domains of TnT, which interacts with TnC, TnI and TM. The fetal muscle isoform of c/eTnT seems to have a different transcription initiation site (Jin et al. 1992).

The rat fTnT gene has a single canonical promoter region where TATAA- and CCAAT-sequences are present in a G-rich region. It also has four potential E-boxes and a M-CAT motif, which is important for its skeletal muscle expressi-

on, but for some reason is not recognized in cardiac muscle. Moreover, the first intron has several E-box sequences as well as three CCACCC-motifs and two T+A-rich areas (Breitbart & Nadal-Ginard 1986). The c/eTnT gene, on the other hand, has two M-CAT motifs, and in their close proximity a Sp1-site, and the normal TATA- and CAAT-boxes. The M-CAT motifs are required for its skeletal muscle specific expression. The gene has also a nominal CArG-box/MRE and an E-box, although both are dispensable for the muscle specific transcriptional activity. Furthermore, the c/eTnT gene has two putative MEF-2 motifs, which are required at least for its expression in cardiac muscle, but they may also have some importance for its expression in embryonic muscle (Mar & Ordahl 1988, Mar et al. 1988, Gosset et al. 1989, Mar & Ordahl 1990, Iannello et al. 1991, Jin et al. 1992). An NFe-site has been detected, too, and it may function as an additive transcription regulatory region (Yu & Nadal-Ginard 1989).

The differential splicing of TnT in cell lineages is a specifically regulated process, but the cellular factors, that control the tissue-specific use of exons are not known. The regulation seems to involve diffusible, tissue and developmental specific trans-acting factors as well as small nuclear RNAs (snRNA), such as U3B and U1 (Cooper & Ordahl 1985, Breitbart & Nadal-Ginard 1986, Breitbart & Nadal-Ginard 1987, Cooper et al. 1988, Cooper & Ordahl 1989, Kuo et al. 1991, Xu et al. 1993). Several cis-acting elements of the primary sequence have already been identified, and these sequences, to which regulatory proteins or snRNAs can bind, exist at least in introns and in all likelihood also in exons. These sequences can be short and lack distinctive symmetry, and in the case of alternative exons, they may also be redundant (Breitbart & Nadal-Ginard 1986, Andreadis et al. 1987, Cooper & Ordahl 1989, Weiner 1993). However, the known cis-elements cannot unambiguously differ alternative from constitutive exons: all exons are flanked by conventional donor and acceptor splice sites, which can hybridize to U1 snRNA. Moreover, the split codon structure of exons 4 to exon 8 of fTnT shares unique symmetry, which allows these exons to be interchangeably spliced in different combinations and still maintain the proper reading frame (Andreadis et al. 1987, Breitbart & Nadal-Ginard 1987, Jin et al. 1992, Weiner 1993). Nevertheless, considering that especially the fTnT primary transcript is remarkably long, there must also exist local stem-and-loop base-pairings and more distant intramolecular interactions, which are generated during and after transcription. Some of the splicing modulating factors may recognize these special secondary structures (Breitbart & Nadal-Ginard 1986, Andreadis et al. 1987, Jin et al. 1992).

Different cell lineages seem to have their specific patterns of TnT protein form expression, which is partially modulated by nerves. The effects of the different TnT proteins on contraction regulation are not fully understood. It has been suspected that different protein forms may transfer distinctively the conformational change of TnC after Ca^{2+}-binding to TM. This in turn would regulate TM interactions with actin and, furthermore, influence the actin-myosin interactions (Moore et al. 1987, Bucher et al. 1988, Härtner et al. 1989, Briggs et al. 1990, Pan & Potter 1992, Reiser et al. 1992). The most variable NH_2-terminal tail portion of TnT forms (approximately 30 aa of NH_2-terminus) interacts with the head-to-tail overlap region of adjacent TM dimers, whereas reasonably conserved

COOH-terminal ends of TnT forms (approximately 14 aa near the COOH-terminus) interact with the central region of TM and with TnI and TnC (Fig. 1). The COOH-terminus is also involved in anchoring the troponin-TM complex to F-actin and, in addition, it seems to have some influence on TM-TM interactions (Breitbart & Nadal-Ginard 1986, Heeley et al. 1987, Leszyk et al. 1988, Hill et al. 1992).

As mentioned previously, the TM binding areas of TnT are located at or near domains that are encoded by differentially spliced exons. Therefore, there may exist some coordinate regulation of mRNA splicing in the case of TnT and TM, which can modulate the interfaces between these two proteins. It is not known, however, whether the same factors are involved in splicing both TnT and TM. Moreover, the different COOH-terminal end owning fTnTα and fTnTβ isoforms, which are expressed in a tissue-specific and developmentally regulated manner, seem to have differing affinity to TM. The affinity of fTnTα is higher for α-TM and it binds more strongly to TnC than fTnTβ isoform. It has also been shown that the Ca^{2+}-affinities of TnC in the fTnTα-TnC complex are threefold higher than in fTnTβ-TnC complex. Thus the sequence difference between these two fTnT classes may have functional significance, possibly by determining the Ca^{2+}-sensitivity of muscle fibres (Breitbart & Nadal-Ginard 1986, Hill et al. 1992, Pan & Potter 1992).

2.1.1.4 Troponin C

Troponin C (TnC) member of a troponin complex functions in a muscle thin filament as a regulator of excitation-contraction coupling by its ability to bind Ca^{2+}. It belongs to a multigene family of ^{2}Ca -binding proteins, which also includes for instance calmodulin, parvalbumin and myosin light chains 1 and 2 (Kretsinger 1980, Buckingham & Minty 1983, Collins 1991). TnC exists as two proteins (both 18 kD), which are encoded by two different genes. Fast skeletal TnC form (fTnC) is expressed only in skeletal muscle and slow skeletal or cardiac gene product (c/sTnC) is typical for slow twitching and heart muscle (Parmacek & Leiden 1989, Gahlman & Kedes 1990, Parmacek et al. 1990). The c/sTnC gene is also expressed transiently during development in neonatal fast skeletal muscle and, surprisingly, in several human fibroblast cell lines (Toyota & Shimada 1981, Gahlmann et al. 1988).

Mouse c/sTnC (161 aa) and fTnC (160 aa) protein forms share 70% homology (Parmacek & Leiden 1989, Parmacek et al. 1990, Parmacek & Leiden 1991). The NH_2-terminus of c/sTnC (aa 1-18) has an α-helical region, which shares only 33% identity with fTnC. The function of this region is not known. Low-affinity Ca^{2+}-binding site I, which is nonfunctional in c/sTnC, is located to aa 28-39 and has 42% identity to fTnC. Low-affinity-binding site II is also located to NH_2-terminus and it is functional in both TnC proteins. Both of the domains contains hydrophobic residues, which are important for forming the domain itself as well as stabilizing Ca^{2+}-binding affinity of the domains. The least homologous region (26% identity) between c/sTnC and fTnC is located to COOH-terminus (aa

117-131 of c/sTnC). It forms an α-helical area in-between high-affinity Ca^{2+}-binding sites III and IV, and is partially involved interacting with TnI (Parmacek et al. 1990). Aa 90-100 of TnC, which are also involved in TnI-binding, are interacting with aa 175-178 of TnT in a noncompetitive manner, too (Leszyk et al. 1990). However, the binding sites III and IV are functional in both TnC proteins and they are always occupied by Mg^{2+} or Ca^{2+} (Parmacek & Leiden 1991).

It seems that myofibrillar ATPase activation is mainly mediated through low-affinity Ca^{2+}-binding site II (Grabarek et al. 1992). Ca^{2+}-binding site I, on the other hand, seems to function in regulation of the interactions of weakly binding cross-bridges with the thin filament (Sweeney et al. 1990). Both low-affinity sites are also affected by ATP: absence of ATP enhances their Ca^{2+}-binding ability (Morimoto 1991). In addition, the sites I and II seem to have cooperative interactions, which are mediated through intramolecular or intermolecular disulfide bonding. These interactions can change the protein conformation to such a direction, that it resembles Ca^{2+}-bound form of TnC, making possible c/sTnC functioning in fast skeletal muscle fibres (Grabarek et al. 1992). The sites III and IV, on the other hand, are suspected of having a role in stabilizing the troponin complex, probably through the affinity for TnI (Parmacek & Leiden 1991, Negele et al. 1992).

The mouse fTnC gene is split into six exons (Parmacek et al. 1990). The number of exons is the same in a c/sTnC gene, but the gene is larger (Parmacek & Leiden 1989). Introns interrupt the exons randomly, splitting also the Ca^{2+}-binding domain sequences to a nonfunctional form. Comparison of the intron-exon borders of fast and slow forms or even rat or chicken calmodulin genes or rat parvalbumin genes shows a high degree of conservation. The main difference is in the border of exon 1 and intron 1 (Parmacek & Leiden 1989, Parmacek et al. 1990). The promoters of fTnC and c/sTnC genes are located in their 5' flanking sequences, but a high level gene expression of either of them is controlled by potent transcriptional enhancer elements, which are activated during differentiation. The fTnC gene has a 855 bp enhancer fragment, whereas the size of the muscle-specific c/sTnC enhancer is only 145 bp. Both of them are located within the first intron of the respective gene. As enhancer elements of fTnC may be the three potential AP-2-binding sites and a TACAA-sequence, which may function as a TATA-box. Skeletal muscle specific expression of c/sTnC, instead, requires at least a consensus MEF-2 motif, a MEF-3 site and a consensus CCACCC-box, which can bind at least Sp1, but possibly also myocyte nuclear factor MNF, among other one or two additional transcriptional factors. The additional factors are not related to Sp1 but are identified in muscle cells. However, c/sTnC has also a consensus AP-1 site as well as a potential NF-κB binding site in its 5' flanking region (Parmacek & Leiden 1989, Parmacek et al. 1990, Parmacek et al. 1994). Cardiac specific expression of c/sTnC, on the other hand, requires other regulatory elements, which include cardiac enhancer factors 1 and 2 (CEF-1, CEF-2) -binding sites and cardiac promoter factors 1, 2 and 3 (CPF1, CPF2, CPF3) -binding sites, of which the CPF-3 site resembles of CCAAT-binding transcription factor/nuclear binding factor 1 (CTF/NF-1) -binding site. The 5' end of the CEF-2-site also has a CCACCC-box (Parmacek et al. 1992, Parmacek et al. 1994).

Common to fTnC and c/sTnC genes is that both lack a CAAT-box, which is typical to other myofibrillar genes, and an E-box. MyoD-family members, however, may activate other transcription factors that then regulate TnC expression. Nevertheless, both of these genes may have additional, not yet identified transcriptional regulatory elements (Parmacek & Leiden 1989, Parmacek et al. 1990, Parmacek & Leiden 1991, Parmacek et al. 1994).

2.1.1.5 Troponin I

The contractile regulatory protein troponin I (TnI) functions in a troponin complex by preventing actin and myosin interactions with each other and thus keeping the muscle in a relaxed state. Ca^{2+}-binding to TnC releases the inhibitory action of TnI, freeing TM to move and allowing cross-bridge formation (Van Eyk & Hodges 1988, Koppe et al. 1989). TnI exists as three different genes: the fast skeletal TnI (fTnI) gene produces a 21 kD protein, the slow skeletal gene product (sTnI) is a 27 kD, and also expressed in neonatal heart, and the cardiac TnI gene (cTnI) forms a 31.5 kD protein, which is present only in cardiac muscle (Nikovits et al. 1986, Koppe et al. 1989, Sheng et al. 1992, Gorza et al. 1993, Ausoni et al. 1994). Studies in vitro have shown that fTnI is the most efficient of the TnI forms in inhibiting actomyosin ATPase (Sheng et al. 1992).

Individual muscle fibres have predominantly either fTnI or sTnI: fast muscle fibres express fTnI and slow muscle sTnI (Toyota & Shimada 1981, Koppe et al. 1989, Härtner & Pette 1990). Regulation of transcription of these genes is poorly understood. Neural input can influence and direct expression of a particular TnI gene, although it is not essential for expression of either of them (Koppe et al. 1989, Härtner & Pette 1990, Lin et al. 1991, Leeuw & Pette 1993). In any case, appropriate TnI protein seems to be translated in excess, forming a pool of unassembled protein subunits. The reason for the accumulation is not known, but it differs from the way that other muscle specific proteins like TnC, TnT and myosin are synthesized (Martin 1981, Koppe et al. 1989).

Mouse cTnI and rat sTnI share 64% identity. This high homology is not surprising, since both of them interact with c/sTnC. The overall homology between the protein sequences of the TnI forms is about 60% (Ausoni et al. 1994). The highest diversity between the proteins is located to their NH_2-terminal halves, which interact with TnC and possibly also with TnT. Thus this TnI region may be involved in maintaining the stability of the troponin complex. However, the interaction between TnI and TnC is dependent of Ca^{2+}/Mg^{2+}-binding to TnC high-affinity domains (Baldwin et al. 1985, Van Eyk & Hodges 1988, Koppe et al. 1989, Lan et al. 1989, Leszyk et al. 1990, Wang et al. 1990, Sheng et al. 1992, Ausoni et al. 1994). The cTnI protein has also a phosphorylatable extension (27-33 residues) in its NH_2-terminal end, which seems to have influence in contraction regulation: phosphorylation of the extension can be induced by β-adrenergic agents, which in turn decreases the affinity of TnC for Ca^{2+} (Saggin et al. 1989, Gorza et al. 1993, Ausoni et al. 1994).

The highly homologous COOH-terminal halves of different TnI proteins contact with TnC at least through their inhibitory domains (rabbit aa 104-115). The interaction is dependent of Ca^{2+}-binding to the low-affinity-binding sites of TnC. In addition, there seems to exist another Ca^{2+}-dependent interaction region to TnC in COOH-terminus, which also has the actin-binding sites. These latter two regions of TnI are thought to interact with each other and thus are involved in regulation of actomyosin-ATPase activity (Baldwin et al. 1985, Van Eyk & Hodges 1988, Koppe et al. 1989, Lan et al. 1989, Leszyk et al. 1990, Wang et al. 1990, Sheng et al. 1992, Ausoni et al. 1994).

The complete gene structures of murine fTnI and sTnI are not known - only mouse cTnI gene is fully characterized (Koppe et al. 1989, Ausoni et al. 1994). Thus this review concentrates mainly on structures of the quail and chicken fTnI genes (Baldwin et al. 1985, Nikovits et al. 1986). The proper developmental regulation of the quail/chicken fTnI eight exon gene (4.5 kb) requires at least two cis-acting regulatory element complexes that seem to function in a concerted fashion: the 5' flanking region has regulatory sequences that are required for proper quantitative expression of fTnI, whereas the other main enhancer region (1.5 kb), called an internal regulatory element (IRE), is located in the first intron. To elicit its full enhancer activity, IRE requires three elements, namely an E-box, and the sites I and II. The central portion of site I (CCCCAGCC) resembles an AP-2 element, whereas site II has a CCACCC-sequence. However, studies with chicken fTnI have also reveal that there exists regulatory sequences in the 5' flanking region, which are located upstream from the promoter region. The regulatory sequences are located in the first exon, although the exon 1 complex is not considered as an enhancer. The upstream region has an M-CAT like sequence (CATTGGT), CCAT- and CCACCC-sequences, whereas exon 1 has two CTF/NF-1-binding sites resembling sequences as well as an untranslated binding protein 1 (UBP-1) -binding sequence. Furthermore, all these three regions have a long terminal repeat binding protein 1 (LBP-1) -binding site homology. Nevertheless, both upstream region and exon 1 sequences are required to elicit the full promoter activity in chicken in the absence of IRE (Konieczny & Emerson 1985, Konieczny & Emerson 1987, Yutzey et al. 1989, Nikovits et al. 1990, Lin et al. 1991).

2.1.1.6 Myosin heavy chains

The core of thick filament is formed of myosin heavy chain (MHC) molecules (Fig. 1). Rat has at least seven sarcomeric MHC genes, which encode embryonic (eMHC), perinatal (pnMHC), extraocular (exMHC), fast IIA and IIB skeletal muscle MHC isoforms (MHCIIA, MHCIIB), α-MHC ventricular/atrial (v/a α-MHC) and β-MHC ventricular/slow skeletal muscle (v/s β-MHC) forms (Buckingham 1985, Mahdavi et al. 1986, Strehler et al. 1986). Different muscles are known to express MHC genes overlappingly during the development, influenced by stimuli like work, innervation and thyroid hormone (Izumo et al. 1986, Silberstein et al. 1986, Emerson, Jr. & Bernstein 1987, Nadal-Ginard & Mahdavi

1989). The genes are exceptional among otherwise scattered contractile apparatus genes by being organized to only two gene clusters (Emerson, Jr. & Bernstein 1987); one includes five skeletal muscle genes and the other two cardiac genes (Mahdavi et al. 1986). Common to all these MHC genes is that one or two upstream exons are entirely composed of nontranslated mRNA sequences and the intron positions in 5' regions are conserved. In addition, the vertebrate MHC genes have a 40 bp homologous sequence of unknown function in their 3' UTRs (Emerson, Jr. & Bernstein 1987).

One MHC molecule (200 kD) has a globular head (NH_2-terminus), which has the binding areas for two different myosin light chains (Fig. 1). The binding region continues to the hinge region, which area in turn facilitates the movement of the globular head during contraction. The COOH-terminus, on the other hand, forms the α-helical rod region that is involved in assembly of a coiled-coil structure with another MHC molecule (Fig. 1). These coiled-coil rods interact with other MHC dimers forming a bundle of MHC molecules, the core of a thick filament. MHC has binding regions for ATPase, ATP and actin, but the exact location and structure of these latter two are unknown, similarly as the precise mechanism of actin-myosin interaction on the myosin molecule is not understood (Buckingham & Minty 1983, Strehler et al. 1986, Emerson, Jr. & Bernstein 1987, Warrick & Spudich 1987, Bandman 1992). However, the globular region forming sequences of MHC genes, especially at their ATP binding areas, are highly conserved compared to their COOH-terminal sequences that have more differences (Emerson, Jr. & Bernstein 1987, Warrick & Spudich 1987). Nevertheless, products of MHC genes have physiological differences, such as enzymatic properties, but also differences in structural interactions in the sarcomere, which all has influence on the contraction property of specific muscle. Thus MHC gene expression is generally used as a base to categorize the adult muscle fibre types to slow or fast twitching as described earlier (Miller et al. 1985, Wade & Kedes 1989, Stockdale 1992).

Rat eMHC gene (24 kb) has 41 exons (Strehler et al. 1986). Its promoter is not muscle specific, but 1.4 kb of its 5'-flanking sequence is sufficient to direct the tissue-specific expression of the gene. The 5' flanking sequence has a nuclear factor-binding site (NFe-site, GTGTCAGTCA-sequence), three potential Sp1-sites, TATA- and CAAT-boxes, of which the major regulatory region seems to be the NFe-binding site. The eMHC gene has also five E-boxes, although their importance on regulation of the expression is not known (Strehler et al. 1986, Bouvagnet et al. 1987, Yu & Nadal-Ginard 1989). However, comparison of the 5' upstream sequence of the gene with the corresponding regions of other rat skeletal and cardiac MHC, MLC1, MLC2, MLC3, α-actin or TnT genes has revealed the presence of several stretches of up to 65% sequence similarity. These widely scattered stretches range in size from 20 bp to over 100 bp, although in general, they do not map in the earlier mentioned regulatory regions of eMHC (Bouvagnet et al. 1987).

The rat v/s β-MHC gene (25 kb) promoter is not able on its own to carry out sufficient transcription either (Thompson et al. 1991). The efficient transcription requires cis-acting regulatory elements, which are located in its 5' flanking region. The basal promoter has also negative control elements. Cis-acting

regulatory elements include at least β-e2 (Ap5/GT-II element, GCTGTGGAATGT-sequence) and β-e3 (NFe-binding site, TGACGACC-sequence) elements, and between these two a CCACCC-sequence (Sp1-site), which appears to function cooperatively with β-e2 in cardiocytes, and a thyroid hormone responsive element (TRE). The promoter region has also additional protein-binding areas (β-e4, β-e5, and β-e6), but their importance as regulatory regions is unknown. Nevertheless, the proper muscle-specific expression of v/s β-MHC requires at least the β-e2 and β-e3 elements in skeletal muscle, whereas its cardiac-specific expression requires in addition a CCACCC-sequence. The β-e3 element by itself is sufficient to sustain significant levels of muscle-specific expression, and, even in one copy, it is more active than for instace the triplicated β-e2 element. However, the β-e2-site is also important, since mutation of the β-e2-site can reduce 60% the activity of the β-MHC enhancer in both cardiac and skeletal muscle cells - the β-e2 element forms a M-CAT site from its noncoding strand. The respective β-e2 element of rabbit gene is also capable of binding factors and, moreover, it seems that these same factors bind to the M-CAT motif in c/eTnT gene (Thompson et al. 1991, Shimizu et al. 1992).

2.1.1.7 Myosin light chains

Myosin light chains (MLC), like TnC, belong to the superfamily of Ca^{2+}-binding proteins, that includes also for instance calmodulin and parvalbumin (Collins 1991). MLCs are classified to alkali, nonphosphorylatable MLC1/3, and regulatory phosphorylatable MLC2 gene families, which are chromosomally dispersed (Buckingham & Minty 1983). Mammalians have at least four alkali MLC genes: fast skeletal muscle MLC1/3 (fMLC1/3), ventricular/slow skeletal muscle MLC1 (v/sMLC1), atrial/embryonic skeletal muscle MLC1 (a/eMLC1) and smooth muscle/nonmuscle MLC1 (sm/nmMLC1) gene (Periasamy et al. 1984a, Barton et al. 1985, Barton et al. 1988). The MLC2 family has at least skeletal muscle (skMLC2), cardiac (cMLC2), smooth muscle (smMLC2) and nonmuscle (nmMLC2) genes (Garfinkel et al. 1982, Nudel et al. 1984, Taubman et al. 1987, Grant et al. 1990, Zhu et al. 1991a).

Adult skeletal muscles express distinct MLC proteins, which connect to globular head regions of a dimeric MHC molecule; one MHC molecule binds one alkali MLC1 (23 kD) or MLC3 (15 kD) molecule and one regulatory MLC2 (17 kD) molecule (Fig. 1) (Nudel et al. 1984, Periasamy et al. 1984b, Emerson, Jr. & Bernstein 1987). The skeletal MLC-proteins have special regions that are homologous to divalent cation-binding domains I and II. Alkali MLC-proteins have lost their Ca^{2+}-binding ability in both of the domains, but it has been suspected that they may have some other important functions, since they are conserved throughout the evolution. The MLC2 protein has one functional Ca^{2+}-binding site, domain I (Emerson, Jr. & Bernstein 1987).

The function of alkali MLC in contraction regulation is not understood (Emerson, Jr. & Bernstein 1987, Bandman 1992). Nevertheless, regulatory MLC2 associates with alkali MLC and MHC subunits through its highly conserved, basic

NH_2-terminus, which apparently changes its conformation during the contraction cycle, due to Ca^{2+}-binding to domain I and phosphorylation. The regulatory activity of MLC2 is mainly mediated by Ca^{2+}-binding, which modulates the interactions between myosin head and actin, and thus regulates the rate of force development. The phosphorylation of serine in the NH_2-terminal region may influence its modulatory activity: phosphorylation of MLC2 seems to enhance the rate of attachment of cross-bridges (Emerson, Jr. & Bernstein 1987, Lees-Miller & Helfman 1991, Metzger & Moss 1992). However, the influence of the MLCs on the enzymatic activity of the MHC is not apparent, thus the functional significance of different proteins of MLC family is not known (Bandman 1992).

The fMLC1/3 gene occupies 25 kb of DNA, about which only 4% is coding sequence (Periasamy et al. 1984a). The gene has an exceptional organization: it has two separate promoter regions and two transcription initiation sites, which facilitate the production of separate fMLC1 and fMLC3 transcripts (Strehler et al. 1985, Daubas et al. 1988). The transcription initiation sites of the isoforms are located more than 10 kb apart. The expression of rat fMLC1/3 gene is mediated through its enhancer, which is differentiation- and tissue-specific as well as promoter-independent. The enhancer is located about 2.3 kb downstream from the polyadenylation signal of the gene, thus about 24 kb and 14 kb downstream of the fMLC1 and fMLC3 promoters, respectively. It has three E-boxes, a CArG-box and a T+A-rich element in its enhancer region. The promoter region of fMLC1 (1.2 kb upstream from its 5' cap-site) has normal CAAT- and TATA-boxes, whereas fMLC3 (438 bp from its 5' cap-site) has similar CAACT- and AAATAAAA-sequences (Periasamy et al. 1984a, Cohen et al. 1988, Daubas et al. 1988, Donoghue et al. 1988, Ernst et al. 1991, Wentworth et al. 1991). The fMLC1/3 gene has also a CArG-box resembling CCTTTTATATG-sequence upstream of the fMLC1 promoter region. This sequence is common to all MLC genes and it is able to bind the same nuclear protein as the CArG-box (Cohen et al. 1988, Rosenthal et al. 1990, Uetsuki et al. 1990).

The two transcripts produced are also differentially spliced. The primary transcript of fMLC1 contains all nine exons of fMLC1/3 gene. The pre-mRNA of fMLC3, instead, lacks the first exon of fMLC1. The differential splicing of these two transcripts produces mRNAs, of which one, fMLC1, contains exons 1 and 4, and the other, fMLC3, has exons 2 and 3. Furthermore, both of these isoforms have exons 5, 6, 7, 8 and 9, which forms the common body for the isoforms. Thus the translated fMLC1 and fMLC3 proteins have different NH_2-terminal ends and identical COOH-terminal sequences (Periasamy et al. 1984a, Andreadis et al. 1987).

The alkali v/sMLC1 and a/eMLC1 forms are encoded by single copy genes, and both of them produce only one protein approximately the size of fMLC1 (Barton et al. 1985, Barton et al. 1988, Cohen et al. 1988). The major form in mouse slow type muscles is v/sMLC1, which can associate with both α-MHC and β-MHC. The v/sMLC1 gene has in its 5' flanking region an M-CAT sequence as well as a CArG-box, an AP-2 site (GCTGGGG), a TC-II motif, in addition to the CAAT- and TATA-boxes (Cohen et al. 1988, Kurabayashi et al. 1990). It has also one potential E-box in this region and a CCACCC-sequence. It is known that the CArG-box of these elements does not contribute the cell-specific expression of the

gene, but it may function as a constitutive promoter element (Kurabayashi et al. 1990). The mouse a/eMLC1 gene, on the other hand, has a TATA-like motif but lacks a CAAT-box, although a CAGCT-sequence may replace its function. Muscle specific expression of a/eMLC1, in turn, requires the MLC-sequence, but the gene has also two E-boxes and a CCACCC-sequence, although their importance for gene expression is not determined. However, translation products of v/sMLC1 and a/eMLC1, but not fMLC1, share some homology in their NH_2-terminal regions, a region thought to be involved in actomyosin interaction. All three proteins also interact with MHC through the COOH-terminus (Cohen et al. 1988, Uetsuki et al. 1990).

The rat regulatory skMLC2 gene is composed of seven exons. The promoter region of the gene has a TATA-box and a CAAT-box resembling CATT-sequence. The 3' end of the skMLC2 gene, similarly as the fMLC1/3 gene, has several potential regulatory elements, such as four E-boxes, a M-CAT sequence, a T+A-rich element, and an Ap-2 site. Other regulatory areas may be a C-rich region (43/58 residues are C), which is centered at position +1037 and a T-rich region (40/45 residues are T), which in turn is located at +2143. Moreover, the gene has a $(CA)_{22}$ sequence, starting from position +80, followed immediately by $(AG)_8$, but the function or significance of these areas is unknown. All skMLC2 introns have the same GTGAG-sequence at their 5' end and a CAG-sequence at their 3' end, but the significance of them is not clear either. The comparison of the 5' intron positions between the MLC2 and the MLC1 genes shows high conservation, which suggests that some regulatory and/or structural functions are located in these intragenic regions (Nudel et al. 1984).

2.2 Regulatory elements of muscle genes and their binding factors

2.2.1 E-box

Most of the muscle specific genes have one or more E-box motifs (consensus sequence CANNTG). It is a binding site for MyoD family (MyoD, myogenin, MRF-4, Myf-5) of nuclear myogenic determination factors, and also for other DNA-binding proteins which share comparable homology within their basic regions and adjacent helix-loop-helix motifs (bHLH-motif) (Olson & Klein 1994). These two regions mediate DNA-binding and oligomerization with other HLH-proteins (Davis et al. 1990, Voronova & Baltimore 1990, Olson 1992). However, different bHLH-proteins seem to have preferences for the binding sites, which in turn depends on the variable nucleotides within and surrounding of the CANNTG-sequence (Blackwell & Weintraub 1990, Wentworth et al. 1991, Olson 1992).

The members of the MyoD-gene family possess the ability to redirect several nonmuscle cells into the myogenic lineage, but expression of only MyoD

in a variety of cell types and tissues is not sufficient in itself to activate muscle-specific genes (Davis et al. 1987, Weintraub et al. 1991, Sassoon 1993). In myoblasts, the myogenic factors exist predominantly as non-DNA-binding monomers, which are functionally activated to mediate myogenesis when they hetero-oligomerize with widely expressed E2A HLH-proteins (E12, E47), E2A related proteins like HEB or other, still unknown positively-acting comparable proteins (Murre et al. 1989, Olson 1992, Finkel et al. 1993).

2.2.2 M-CAT element

The M-CAT element (sequence CATTCCT) is involved in MyoD-independent transcription from several cardiac gene promoters in both cardiac and early embryonic skeletal muscles. It is a binding site for ubiquitous factors, like M-CAT-binding factor (MCBF), and transcription enhancing factor 1 (TEF-1). Studies have shown, however, that MCBF and TEF-1 are closely related and possibly even identical (Mar & Ordahl 1990, Xiao et al. 1991, Farrance et al. 1992, Stewart et al. 1994). There exists also a sequence-specific single-stranded DNA-binding protein, muscle cell factor 3 (MF-3), which is possibly the same as MCBF. However, it seems to be able to bind to the M-CAT element as well as to the MRE and an E-box (Santoro et al. 1991).

According to studies with chicken, mRNA of TEF-1 is enriched in cardiac and skeletal muscles. This suggests that TEF-1 may have a role in muscle gene transcription, although TEF-1 expression is not strictly muscle-specific. TEF-1 has at least two homologues: TEF-1A and TEF-1B, which both have high affinity in a sequence-specific manner to M-CAT. TEF-1B differs from the predominant TEF-1A form by its COOH-terminal portion, which contains a 13 aa extension. The extension may influence its ability to activate transcription and therefore, TEF-1 isoforms may have different influences in the regulation of M-CAT-dependent promoters in striated muscle cells (Stewart et al. 1994). It has been suspected that the muscle-specific activity of TEF-1 may depend upon the promoter context of the genes and, also, its tissue specificity may require cell-specific coactivators (Xiao et al. 1991, Ishiji et al. 1992, Stewart et al. 1994).

2.2.3 T+A-rich element

Numerous muscle-specific enhancers and promoters have a T+A-rich element (consensus sequence [(C/T)T(A/T)(A/T)AAATA(A/G)]), although it appears to be a relatively weak enhancer element in skeletal muscle cells (Gossett et al. 1989, Cserjesi & Olson 1991, Yu et al. 1992). The element is a binding site for the myocyte-specific enhancer-binding factor 2 (MEF-2), which is not expressed in proliferating myoblasts, but is induced during differentiation by MyoD and myogenin (Gossett et al. 1989, Yu et al. 1992). Moreover, the T+A-rich element is also able to bind other MEF-2-type proteins, which are members of a group of

MADS-box transcription factors - all of these proteins share a conserved NH_2-terminal DNA-binding domain that includes the MADS homology. The ubiquitous MEF-2-type proteins are produced by alternate splicing from four genes (Pollock & Treisman 1991, Yu et al. 1992, Martin et al. 1994). At least some of the protein forms accumulate preferentially in skeletal muscle, heart, and brain. The tissue-specific activity of the forms is likely due to post-transcriptional regulation (Yu et al. 1992). Nevertheless, the myocyte-specific MEF-2 is shown to be related to ubiquitous RSRFs (serum response factors related to SRFs), which are expressed in myoblasts as well as in nonmuscle cells. RSRFs, however, are unable to induce transcription of myogenin (Gossett et al. 1989, Pollock & Treisman 1991, Edmondson et al. 1992, Yu et al. 1992).

2.2.4 CArG-box family

The CArG-box family of regulatory elements includes the muscle regulatory element (MRE), also called CAT-box associated repeat [C-BAR (consensus sequence [U/C]YGUCCAAATA[A/T]GG[A/C]G)] and a serum response element (SRE). Common to all of these CArG-box elements is a consensus sequence $CC(A/T)_6GG$. The sequence is present in the promoter region of several muscle-specific genes, such as αsk- and αc-actins and fMLC1/3, but also of growth-factor-inducible immediate early genes such as c-fos (Minty & Kedes 1986, Phan-Dinh-Tuy et al. 1988, Chow & Schwartz 1990, Ernst et al. 1991, Santoro & Walsh 1991, Tuil et al. 1993).

MREs are surrounded with negative elements and these elements are suspected to influence which factors can interact with MREs (Chow & Schwartz 1990). Nevertheless, known MRE-binding factors are striated muscle actin promoter factors I and II (MAPFI and MAPFII). These factors bind preferentially to an MRE compared to an SRE (Walsh & Schimmel 1987, Walsh 1989, Chow & Schwartz 1990). MAPFI (62 kD) is abundantly present in nonmuscle cells and myoblasts, but reduces during myogenic differentiation, whereas MAPFII (35 kD), found also from C2, L6, and Sol8 myogenic cell lines, seems to be restricted to muscle cells (Santoro & Walsh 1991). MAPFI seems to act as a repressor for αsk-actin gene transcription, whereas MAPFII is a positively-acting transcriptional factor (Walsh & Schimmel 1987, Vandromme et al. 1992).

The c-fos SRE-binding nuclear serum response factor SRF (67 kD) is also able to bind directly to the CArG-box of several muscle-specific genes and activate gene expression (Minty & Kedes 1986, Muscat et al. 1988, Phan-Dinh-Tuy et al. 1988, Boxer et al. 1989, Chow & Schwartz 1990, Ernst et al. 1991, Tuil et al. 1993). SRF, also called CBF, is an ubiquitous MADS factor expressed in L6 and C2 myoblasts and myotubes. It seems to have a role in cell proliferation, although it is also implicated in the transmission of the differentiation signal: specific inhibition of SRF in vivo can prevent the myoblast-myotube transition; inhibition of SRF also inhibits expression of myogenin, although this apparently is not mediated through direct binding to CArG-box of myogenin (Santoro & Walsh 1991, Vandromme et al. 1992).

There exists also at least three different SRF-related proteins, which have distinct DNA-binding specificities. These RSRF proteins bind to DNA as dimers, but they cannot oligomerize with SRF. RSRFs are expressed in various levels in different cells and their oligomerization may permit them to act as a link between repression and activation of genes with CArG-boxes, depending on their oligomerization partner. Furthermore, muscle genes that have a CArG-box often have functional E-box sequences. Thus CArG-box-binding proteins may act in concert with other cis-acting proteins to coordinate transcriptional regulation during myogenesis (Pollock & Treisman 1991, Santoro & Walsh 1991).

2.2.5 CCACCC-sequence and some other elements

The CCACCC-sequence is present in the transcriptional regulatory regions of many skeletal muscle genes, including murine c/sTnC, quail TnI, cardiac actin and β-MHC. Moreover, it seems that there exists cooperative interactions between the factors that binds to the CCACCC-motif and T+A-rich element, since both are required for transcriptional activation of for example c/sTnC and myoglobin genes. However, the CCACCC-motif seems to be able to bind a variety of transcription factors, such as myocyte nuclear factor MNF, Sp1 and Sp1-related factors, although the binding ability as well as the activation ability of them may depend on the context of this sequence (Chen et al. 1994, Parmacek et al. 1994).

The winged-helix family of transcription factors includes mouse MNF. Alternate splicing of the MNF transcript in myocytes produces proteins of 90, 68, and 65 kD. The MNF proteins are upregulated during the muscle differentiation in culture as well as in muscles of intact animals. MNF is also responsive to neural regulation in adult skeletal fibres: in intact muscles subjected to chronic motor nerve stimulation, the phosphorylated 90 kD form of MNF was upregulated (Bassel-Duby et al. 1994). Moreover, it seems that MNF has importance in muscle development by its ability to influence both skeletal- and cardiac-specific expression of muscle genes. It is probable that MNF requires interactions with other heterologous trancription factors, such as the previously mentioned MEF-2, to mediate efficient trans-activation in for instance muscle cells (Bassel-Duby et al. 1994).

Sp1, a ubiquitously expressed transcription factor, is capable of binding to the CCACCC-motif, but the affinity is lower than that of MNF. In developing myotubes there also are other CCACCC-motif-binding factors, which are distinct from MNF and Sp1 - the CCACCC-sequence is also homologous to the retinoblastoma control element (RCE) -motif (CCACCC or GCCACC) (Bassel-Duby et al. 1994, Chen et al. 1994). Nevertheless, retinoblastoma protein (RB) can either positively or negatively regulate expression of several genes through cis-acting elements in a cell-type-dependent manner. These kind of elements can be the RCE, or the Sp1 consensus-binding sequence CCGCCC, both of which can confer equal responsiveness to RB. Moreover, it seems that RB is involved in Sp1 DNA-binding activity, although there is no direct association between these two proteins. The mechanism, by which RB regulates transactivation seems to be

related to Sp1-I, a negative regulator(s) of Sp1: Sp1-I can specifically inhibit Sp1-binding to DNA, and the inhibition can be reversed by addition of recombinant RB protein. This indicates that RB may stimulate Sp1 mediated transactivation by liberating Sp1 from Sp1-I (Chen et al. 1994). The Ap-2-site (sequence CCCA/CNG/CG/CG/C) can also bind Sp1.

The recently identified MEF-3 element (SSTCAGGTTWC) or related sequences are present in a large number of skeletal muscle-specific transcriptional enhancers, such as in the murine c/sTnC, quail TnI, rat cMLC2 and murine myogenin genes. The MEF-3 element appears to be usually located near a MEF-2-binding site, and a muscle CCACCC-box, as in the case with c/sTnC. The MEF-3-binding site of c/sTnC includes the palindromic sequence ANCCTGNN-CAGGNT, and is flanked on either side by a CCACCC-motif (Parmacek et al. 1994).

2.3 Differentiation and regulation of some of the myogenic factors

2.3.1 Fusion and myogenic factors

Myoblasts seem to exist as diverse types of cells that can form different types of muscle as well as myotubes in culture (Stockdale 1992). They have different culture requirements and abilities to fuse (Block & Miller 1992). Differentiation of myoblasts to myotubes is considered to begin when myoblasts withdraw from the proliferative cell cycle in G1 phase, although this transition is in itself not sufficient (Nadal-Ginard 1978, Olson 1992). The proliferation-differentiation transition involves down-regulation of myoblastic components such as c-Fos, JunB, c-Myc, p21ras and cdc2 kinase, which in turn is triggered by alterations in growth factor levels, such as reduction of fibroblastic growth factor (FGF) and transforming growth factor β (TGF-β). Insulinlike growth factor II (IGF-II), on the other hand, can promote fusion of the myoblasts. Nevertheless, these changes lead to dramatic modifications in signal transduction pathways: alterations in some protein kinase activities can modify protein activities by altering their phosphorylation level and these changes in turn can modify several cellular functions, such as transcription (Mohun 1992, Olson 1992).

The differentiation process of myoblasts requires activation and expression of MyoD-family proteins and other myogenic regulatory factors. The regulation of these other factors during differentiation is poorly understood (Stockdale 1992, Edmondson & Olson 1993). However, expression of the MyoD-family of regulatory factors seems to be somewhat preferential: myogenic cell lines that express a high level of MyoD typically express a low level of Myf-5 and vice versa. Both factors are present in myoblasts, but in inactive forms. Activation of MyoD and/or Myf-5 in myogenic cell cultures leads to expression of MEF-2, and myogenin and possibly also MRF-4. Myogenic cells in somites, on the other hand,

express Myf-5 first, followed by myogenin, MRF4 and finally MyoD (Block & Miller 1992, Buckingham 1992, Hannon et al. 1992, Mohun 1992, Olson & Klein 1994).

MyoD has been suggested to have a specific role in regulating fusion-mediating genes in several cell lines (Block & Miller 1992, Edmondson & Olson 1993). High MyoD mRNA levels are correlated with fusion, for example in mouse C2C12 cells. Furthermore, BC3H-1 cells, which do not normally express MyoD, can be induced to fuse by transfecting them with exogenous MyoD. Interestingly, however, the exogenous MyoD was not able to activate endogenous MyoD in BC3H-1 cells, whereas in C3H10T1/2 cells, the endogenous gene got activated (Davis et al. 1987, Edmondson & Olson 1989, Brennan et al. 1990, Miller 1990). Nevertheless, transfection of BC3H-1 cells with MRF-4 instead of MyoD initiated endogenous MyoD expression and the cells showed fusion ability. There exists a possibility, however, that fusion and endogenous MyoD expression in BC3H-1 cells is independently initiated as the total concentration of the myogenic regulatory factors reaches high levels (Block & Miller 1992).

Regulation of fusion genes cannot be completely specific to MyoD, since rat L6 cells, which do not express MyoD, are capable of fusion. Fusion of L6 cells has been suggested to depend on high levels of Myf-5, instead. However, normal undifferentiated BC3H-1 cells express high levels of Myf-5 mRNA, but as mentioned earlier, are not able to fuse. Myf-5 expression of these cells also decreases during differentiation (Braun et al. 1989, Edmondson & Olson 1989). BC3H-1 transfected with MRF-4 express very little Myf-5 mRNA in the undifferentiated stage, but this increases during differentiation, reaching a level comparable to that in normally differentiated BC3H-1 cultures. Expression of myogenin was not affected. It is not known, however, whether MRF-4 regulates endogenous myf-5 and myoD genes directly, indirectly by activating intermediary genes or in a concert with other regulatory factors such as myogenin, E2A gene products or Id proteins (Block & Miller 1992, Mak et al. 1992). Nevertheless, BC3H-1 cells transfected with Myf-5 showed no alteration in their myogenic program by the increased level of Myf-5.

Myogenin has been detected in all examined myogenic cell lines, but its mRNA levels vary markedly among the different cell lines (Edmondson & Olson 1989, Wright et al. 1989, Miller 1990). Of cell lines examined, BC3H-1 cells express the least of myogenin. Additional expression of myogenin in myogenin-transfected BC3H-1 cells did not induce these cells to fusion either (Block & Miller 1992). It has suspected, however, that myogenin functions as an activator of the basic myogenic program. It may also provide a permissive environment for the additional actions of MyoD and MRF-4 (Block & Miller 1992, Olson & Klein 1994). Moreover, expression of myogenin appears to be important for maintaining the expression of contractile apparatus genes, whereas for instance continuous expression of MyoD is not required (Olson & Klein 1994).

2.3.2 Myogenic factors and their expression

The regulatory regions and their corresponding binding factors that control transcription of MyoD-family genes are not fully characterized. It is known that the members of the family can positively regulate expression of their own and each other directly, and probably also indirectly through induction of intermediate regulatory factors. However, since myogenin does not appear until differentiation has been triggered by withdrawl of myogenin promoter silencing growth factors or other serum factors that inhibit myogenesis, it offers a good example of complicated myogenic regulatory cascades, where members of the MyoD-family and other type of regulatory factors are involved in regulation (Edmondson et al. 1992, Edmondson & Olson 1993).

The myogenin gene (2.5 kb) expression is regulated, at least partially, by its MEF-2-binding site and also by two E-boxes, which are located to its 5' flanking sequence. Only the proximal E-box seems to have a significant affinity for MyoD, myogenin and Myf-5 - initial activation of myogenin demands the presence of active MyoD or Myf-5 and MEF-2. Thus it is suggested that either MyoD and/or Myf-5 can activate the expression of MEF-2 first, which then collaborates with MyoD or Myf-5 to induce transcription of myogenin (Edmondson et al. 1992). Furthermore, myogenin is in turn able to induce MEF-2 expression and its own expression, thus expression and regulation of myogenin and MEF-2 can function as a cascade (Cserjesi & Olson 1991). Expression of MEF-2 is probably regulated also by other factors in addition to myogenic bHLH-proteins. Nevertheless, when myogenin and MEF-2 are initially upregulated in response to a differentiation inducing signal, their coexpression could serve to stabilize expression of both, which in turn may aid amplifying and maintaining the myogenic phenotype (Edmondson et al. 1992, Edmondson & Olson 1993).

MyoD gene itself has two important regulatory areas, the proximal and distal regulatory regions. Proximal region contains a TATA-box, a CCAAT-box, a G+C-rich region that includes two consensus Sp1-sites, and an E-box in addition to a consensus Ap-2-site, and a M-CAT-resembling sequence. The proximal region is not able to produce a high level of expression in murine cells, but instead, it requires a distal regulatory region, which has three potential E-boxes and a T+A-rich region (Tapscott et al. 1992). Moreover, it is known that myogenesis can be inhibited with growth factor inducible immediate early gene products such as activated Fos and Ras. The function of Fos in inhibition of MyoD or myogenin is not completely clear, since there seems to be no association between Fos and MyoD. Activated c-Fos as well as Ras, however, seems to influence MyoD by inhibiting its transcription (Lassar et al. 1989, Gius et al. 1990, Bengal et al. 1992, Li et al. 1992a).

2.3.3 Cooperation and hetero-oligomerization of MyoD-family of transcription factors with other proteins

MyoD is known to be able to induce growth arrest. The mechanism of its cell cycle regulation is unknown, but it is independent of myogenesis. The tumor suppressor RB is also involved in producing and maintaining the terminally differentiated phenotype of muscle cells (Coppolla et al. 1990, Weinberg 1990, Riley et al. 1994). Cyclin dependent kinases, such as cdc2, are able to phosphorylate this nuclear protein: one of the early events leading to muscle cell differentiation is a change in RB phosphorylation level. RB becomes phosphorylated at the G1/S transition, whereas it is unphosphorylated in G1 phase, which is the growth-suppressing form of RB. RB is able to oligomerize with MyoD, and it has been proposed that the effects of MyoD on the cell cycle result from the direct binding of these two molecules. Furthermore, rephosphorylation of RB is inhibited by an as yet unknown mechanism. It has been suspected, however, that MyoD blocks RB in the unphosphorylated and growth factor-unresponsive state. Nevertheless, myogenesis can be inhibited through inactivation of RB (Gu et al. 1993, Thorburn et al. 1993).

The binding region of MyoD to RB is apparently located in the basic domain of its bHLH structure (Gu et al. 1993). RB also has the ability to bind to myogenin, Myf-5 and MRF-4, and possibly also to other bHLH proteins in vitro in a similar manner. It appears that MyoD has the highest affinity for RB. However, this RB-MyoD hetero-oligomer has not been shown to bind to the E-box in vitro. Instead, it has been suggested that RB may stabilize the MyoD-E-protein hetero-oligomer binding to E-box, although the stabilization mechanism is unknown (Qin et al. 1992, Gu et al. 1993). Moreover, myogenesis can be inhibited by activated Fos and Ras, which themselves do not bind RB. They can affect the other arm of the RB-MyoD interaction (Lassar et al. 1989). RB protein, on the other hand, can repress expression of the Fos-gene through the retinoblastoma control element (RCE) and thus influence also the AP-1 stimulatory activity. Contrarily, however, RB can also activate transcription of the the c-Jun gene through the Sp1-site within the c-Jun promoter (Chen et al. 1994).

Jun is known to be able to inhibit myogenesis. The repression is mediated, at least partially, by its ability to bind with its leucine zipper to bHLH-structure of MyoD and myogenin. Although the members of the Jun family seem to function differently in different biological events, the effects of JunB on myogenin are similar to that of c-Jun, even though JunB has been shown to be a negative regulator of c-Jun. But, since c-Jun-binding to MyoD results mutual inactivation of both, and also AP-1, the MyoD:c-Jun complex may serve as an allosteric regulator for either cell growth or differentiation depending on its stoichiometry (Bengal et al. 1992, Li et al. 1992a). Nevertheless, there are also other myogenesis inhibiting oncogene products such as c-Myc, or T antigen and E1A, which are able to bind RB, but these proteins can still inhibit myogenesis even after RB-binding (Miner & Wold 1991).

The inhibitor of differentiation, Id, is a negative regulator of myogenic bHLH factors and it is expressed in many cell types. Its post-transcriptional

down-regulation by serum withdrawal as well as its post-translational down-regulation are necessary for muscle differentiation, although not sufficient as such. The post-transcriptional control may serve to speed the response of the cells to serum deprivation: after cessation of Id mRNA synthesis, rapid clearing of the endogenous Id protein pools may in turn initiate the differentiation process. In mouse, Id exists in at least three variants, namely Id1, Id2 and Id3 (Benezra et al. 1990, Christy et al. 1991, Sun et al. 1991, Jen et al. 1992). Since Id lacks the basic domain that is essential for an E-box-binding, but has a HLH structure for hetero-oligomerization, it can sequester other HLH proteins from activating transcription. Proliferating myoblasts are known to have high levels of Id, which may be complexed with the E-proteins E12 and E47 - Id1 and Id2 have higher affinity to E-proteins than to MyoD (Benezra et al. 1990, Sun et al. 1991, Jen et al. 1992, Finkel et al. 1993). Id-binding to E-proteins in turn can prevent E12 and E47 from forming functionally active hetero-oligomers with MyoD, which can keep MyoD in an inactive form in myoblasts. High levels of Id may also keep MyoD in an inactive form by straight association with it. On the other hand, lower levels of Id may sequester E-proteins in myotubes, which could regulate the activity of MyoD as a transcription activator (Benezra et al. 1990, Jen et al. 1992).

2.3.4 Phosphorylation mediated regulation

Activation of muscle transcription is inhibited by a variety of peptide growth factors, such as basic FGF and TGF-β, which have influence on intracellular growth factor cascades, including signal transduction (Li et al. 1992c, Olson 1992). One important member of the signal transduction pathway is cyclic AMP (cAMP), which has been shown to regulate a variety of cellular events, including activation and repression of gene transcription - several studies have shown that the phosphorylation of transcription factors plays a direct role in their DNA-binding and oligomerization activity. It is also known that myogenic factors can be silenced by phosphorylation, either by straight inactivation, modulation of oligomerization partners, or modulation of transcription of these factors and autoregulatory loops by phosphorylation (Li et al. 1992b, Olson 1992).

The cAMP signal transduction pathway is able to inhibit differentiation of skeletal muscle cells. Substitution of cAMP with catalytic subunit C of cAMP-dependent protein kinase A (PKA) can suppress muscle-specific transcription as well, by silencing the activity of the MyoD-family of regulatory factors. MyoD has two potential serine phosphorylation sites for PKA, four for casein kinase II, and three for protein kinase C (PKC), whereas only the PKA site is within the bHLH domain and is unique for MyoD among the myogenic factors. Thus regulated phosphorylation may also modulate MyoD activity, since phosphorylation can change the MyoD homodimer/hetero-oligomer equilibrium (Li et al. 1992b, Li et al. 1992c, Mitsui et al. 1993). However, PKA has also an R subunit, in addition to a muscle-specific transcription suppressing C subunit, and these two proteins are expressed at various levels in different cell types. The excess of R subunit of PKA can potentiate the activity of myogenic regulators (Li et al. 1992b).

Myogenin also has two PKA phosphorylation sites as well as a PKC threonine phosphorylation site, all within its basic region. Phosphorylation of threonine by PKC inhibits its DNA binding ability and thus its transcriptional activation ability. The activity of myogenin can be repressed by PKA, although it seems that direct phosphorylation of its PKA sites is not required. Instead, repression seems to involve another indirect mechanism with one or more intermediate steps. The indirect repression of myogenin by PKA may be mediated through bHLH-proteins E12, E47 and HEB, although another potential mediator can be Id (Li et al. 1992b).

Growth factor stimulation and casein kinase II can also influence the phosphorylation level of SRF. The dilemma, that SRF is involved in both transcriptional activation of proliferative immediate early genes such as c-fos, and on the other hand, in the expression of muscle-specific genes, such as skeletal and cardiac α-actin during differentiation, can be at least partially explained by its ability be phosphorylated. Phosphorylation may alter the activity of SRF in a tissue-specific fashion, although, SRF can also bind various cofactors, such as TCF (62 kD). This type of SRF:TCF interaction is required for full transcriptional activation of the c-fos gene, thus other specific cofactors may regulate SRF-binding to the CArG-boxes of muscle-specific genes instead. However, there may also exist other post-translational modifications, which all may effect its binding ability and affinity to different CArG-boxes (Gius et al. 1990, Santoro & Walsh 1991, Vandromme et al. 1992, Tuil et al. 1993).

2.4 Targeted regulation of gene expression

2.4.1 Antisense RNA mediated regulation

The majority of RNA in a cell is stable RNA, such as ribosomal RNA and variety of small cytoplasmic and nuclear RNA species (Izant 1989). Some of these structural RNAs have specific secondary structures that can interact with mRNA and thus can be described as naturally occurring antisense RNAs. These RNA species have been found in both prokaryotes and eukaryotes (Khandekar et al. 1984, Green et al. 1986, Simons & Kleckner 1988, Izant 1989).
Some of them have effects on transcription and some on translation, and some RNA species may also affect DNA replication. The complementary region of antisense RNA species is usually relatively short and most commonly interacts with the target mRNA near the 5' end or the AUG-codon (Green et al. 1986, van der Krol et al. 1988, Izant 1989, Colman 1992). Naturally occurring antisense RNAs have given the idea of develop techniques to introduce synthetic antisense RNAs into cell culture systems. In many cases these antisense RNAs have been used successfully to reduce target gene expression (Green et al. 1986, Izant 1989).

2.4.1.1 Flipped gene constructs

One commonly used RNA introduction-method has been to inject in vitro produced antisense RNA into the cells. In most cases, however, a more useful and simple method is the production of antisense RNA inside the target cells. A fragment of a cloned gene can be linked to a suitable promoter, which can facilitate the transcription of antisense RNA in a cell system. Another alternative for modulating gene expression can be the antisense RNA resembling a ribozyme construct, which has an enzymatic activity to cleave RNAs at preselected sites (Haseloff & Gerlach 1988, Cotten & Birnstiel 1989, Izant 1989, Knecht 1989). The construct can be introduced transiently or stably to the cells with techniques such as transfection with the $CaPO_4$-precipitation-method, liposomes or electroporation (Knecht 1989, Chrisey 1990).

The flipped gene technique usually needs a strong promoter, which preferably can also be regulated externally, since generally high antisense to sense transcript ratio (50-100-times antisense RNA over mRNA) is required to produce an inhibitory effect, although this is not always the case. Ribozymes, on the other hand, seem to require approximately a 200-fold excess over substrate in the cytoplasm to mediate the inhibitory action (van der Krol et al. 1988, Cotten et al. 1989, Knecht 1989). Moreover, if antisense RNA can be expressed sooner than the target gene, that may also be beneficial. The best results with flipped gene transfections have been obtained when multiple copies of an antisense gene with a strong promoter have been incorporated into the cells. But, high antisense expression may have secondary effects, such as decreased expression from several unrelated mRNAs (Cotten & Birnstiel 1989, Daugherty et al. 1989).

The reason for needing a vast excess of antisense RNA or ribozyme may be the relatively inefficient hybridization of them with their targets in vivo. The duplex forming ability is in all likelihood determined by the secondary structure of the sense and antisense RNAs and also by whether regions of the RNAs are protected by proteins. The efficiency of hybridization may also partially depend on the accessibility or stability of the target mRNA as well as of the antisense RNA - some studies have shown that antisense RNA can be more labile in cells than sense mRNA. Thus antisense ribozymes may have very little activity in vivo, possibly because of the RNA-binding proteins, but also of the absence of factor(s), which may aid the cleavage (Izant 1989, Knecht 1989, Weintraub 1990).

Usually antisense transcripts are difficult to detect in cells and, even more so, antisense RNA:mRNA duplexes (Hélène & Toulmé 1990). Only very few examples of duplexes have been reported and one of these was in *Xenopus* oocyte, where antisense RNA was injected. The duplex formation can occur in cytoplasm, but in one case the duplexes of full-size antisense RNA and its target were seen only in nucleus. Duplex formation in the nucleus in turn may inhibit the normal mRNA transport to the cytoplasm. Moreover, the export of antisense RNA itself into the cytoplasm may be less efficient than that of sense RNA (70% versus 95%, respectively) (Daugherty et al. 1989, Knecht 1989). Nevertheless, if the majority of the target mRNA and antisense RNA forms a duplex, there is still a possibility that the remaining, unhybridized mRNA is translated more efficiently.

2.4.1.2 Effects of antisense RNA

Generally it is assumed that antisense RNA-techniques are most efficient in transcriptionally quiescent cells. Antisense RNA suppression, when it is successful, usually leads to 50-99% reduction of the target gene expression (Izant 1989). Both transient and stable expression of antisense RNA as well as microinjected antisense RNA has been shown to specifically inhibit expression of a wide variety of target genes, including several oncogenes, heat shock genes and axonal sheath components (Chrisey 1990, Weintraub 1990). Highly transcribed cytoskeletal genes, such as cytoplasmic actin, have also been inhibited in cultured mammalian cells (Gunning et al. 1987).

The mode of interference of antisense transcripts is in most cases obscure. The binding region of the antisense RNA to the sense mRNA in all likelihood determines the type of disturbance that the antisense transcript can cause. Antisense RNA can interfere with transcription, post-transcriptional processing or transport of the target to the cytoplasm (Daugherty et al. 1989, Knecht 1989, Hélène & Toulmé 1990, Weintraub 1990). The mode of action can also be at the level of mRNA translation, since the binding or the translocation of ribosomes on the mRNA may get disturbed. Antisense RNA:mRNA duplexes are not substrates for RNAse H, but cleavage may still occur, mediated by another RNAse, RNAse III. This enzyme can cleave both sense and the antisense strands in a RNA:mRNA duplex, at least in prokaryotes. RNAse III-like activity is also found in eukaryotic cells, but its role in blockage of gene expression by antisense RNA is uncertain. Nevertheless, cleavage of the message is not essential for inhibition (Dash et al. 1987, Walder 1988, Knecht 1989).

2.4.2 Antisense oligodeoxyribonucleotide mediated regulation

Techniques to synthesize oligodeoxyribonucleotides (ODN) have opened another possibility to regulate gene expression. Introduction of ODNs to the cells can partially replace the need for making mutants, and they can offer an attractive method for studying differentiation, coordinated regulation of gene expression and interactions between mRNAs and proteins (Hélène & Toulmé 1990, van der Krol 1990). Moreover, the benefit of antisense ODNs is that the effects of them can be evaluated in controlled environments, which allows the estimation of their possible therapeutic potential (Rothenberg et al. 1989).

2.4.2.1 Uptake of the ODNs and some other parameters

It has been shown that ODNs and their derivatives can penetrate mammalian cells in physiological conditions, although the mechanism is not fully understood (Baker et al. 1990). The main entrance mechanism seems to be receptor mediated

endocytosis: at low concentrations the uptake occurs through absorptive endocytosis mediated by a 80 kD surface protein, but, if the ODN concentration is moderate or high, the uptake mechanism seems to be fluid-phase endocytosis. The 80 kD receptor seems to be able to bind ODNs, tRNA and plasmids in physiological conditions. The binding of ODNs to the cell surface seems to be saturable, but the 5'-phosphate containing polynucleotides of any length can disturb the uptake of ODNs possibly by acting as competitive inhibitors. Different cell types have different abilities to take up ODNs and the time required for uptake may vary. A 34 kD membrane protein, identified from T-cells, has been described as another receptor candidate. This protein binds oligonucleotides (DNA or RNA), but not mononucleotides, in acidic conditions (pH 4.0-4.5). The binding of the receptor occurs rapidly, does not require Mg^{2+} or Ca^{2+} and the binding is not energy-dependent (Loke et al. 1989, Yakubov et al. 1989, Hélène & Toulmé 1990, Goodarzi et al. 1991).

The mechanism whereby ODNs are released in cytoplasm is not known (Stein & Cheng 1993). Generally, molecules that are taken up by endocytotic pathway are released from the vesicles through the lysosomic pathway. Acidic conditions in lysosomes cause the dissociation of the molecule and the cell membrane receptor, and the receptor is returned to the cell surface. Apparently, most of the ODNs that are taken up by receptor mediated mechanism, end up in lysosomes where the majority gets degraded. Only a minor amount of intact ODNs is released to the cytoplasm (Loke et al. 1989). However, the 34 kD receptor mediated uptake causes a dilemma in this release pathway, since the receptor has higher affinity for the ODNs in acidic conditions than in neutral environment. Thus the mechanism how the ODN is released from the 34 kD receptor is not known, since the acidicity of lysosomes themselves would prohibit the liberation. One explanation for this is that the acidic environment may modify the structure of the receptor molecule on the cell surface allowing the high affinity-binding of the ODN. When the formed endocytotic vacuole is internalized, the interior pH of the vacuole is changed to more neutral direction, which in turn allows ODN release from the receptor (Goodarzi et al. 1991).

The internalized undegraded ODNs have been detected in cytoplasm, where the ODN-mRNA hybridization mainly occurs, and also in the nucleus (Hélène & Toulmé 1990, Goodarzi et al. 1991). Usually, to reach the maximum inhibition of the target, a 10- to 100-fold excess of antisense ODNs over the target is required (Izant 1989). However, very high ODN concentrations and long ODNs may cause nonspecific mRNA cleavage and, in some cases, ODNs may also inhibit the activity of specific enzymes. In most cases, the purity of the ODN is essential, because incorrect sequences can decrease the specific inhibition, whereas other impurities can have toxic effects. The ability of a specific ODN to hybridize depends also on its GC/AT ratio, which ratio should be relatively constant to avoid self-folding (Marcus-Sekura 1988, Wagner 1994).

Another important parameter is the length of the ODN, since large and highly charged DNA molecules are unlike to enter the cells rapidly (Fakler et al. 1994). Usually a 15mer is a good choice, since a 15 bp sequence is probable to be found only once in 500 million bp of DNA (Marcus-Sekura 1988). An 8mer has also been described as being effective. Longer ODNs would form more stable

hybrids, but increasing the length brings the problems of self-folding, nonspecific hybridization to other mRNAs and reduced solubility, which, as mentioned earlier, may lead to uptake problems (Marcus-Sekura 1988, Weintraub 1990, Vlasov & Yurchenko 1991, Stein & Cheng 1993, Fakler et al. 1994). The binding of the ODN to the correct reading frame, on the other hand, does not seem to be important: ODNs complementary to any of the three positions in the reading frame can inhibit translation. The most suitable ODNs for experiments in vivo can often be selected by using in vitro translation methods for screening (Marcus-Sekura 1988, Stein & Cohen 1988, Boiziau et al. 1991).

Control ODNs can be those with one or more bases changed from the original, sequences absent from the sequence of interest, sense molecules of the test antisense molecules or homopolymers. Nevertheless, the most recommended ones are sequences that have the same nucleotides as the antisense ODN, but in a mixed and random order. The sequence, however, should be searched for its complementarity to any known sequence in the examined organism to avoid antisense influence on another target (Marcus-Sekura 1988, Hélène & Toulmé 1990, Wagner 1994).

2.4.2.2 Inhibitory activity of the ODNs

Several indirect studies have shown that ODNs can bind to target mRNAs, although there are very few reports that show the direct mRNA and ODN duplexes or the reaction products of these duplexes. The hybridization is also temperature dependent. Most effective antisense ODNs are considered to be complementary to the 5' end or translation initiation start site, although ODNs targeted to the middle region, 3' end, splice junctions and introns or even to 3' untranslated domains of target mRNAs have all been successful in some cases (Izant 1989, Knecht 1989). ODNs may also function by forming a triple-helix with the target gene and blocking transcription, although this is considered quite unprobable to occur in vivo (Sklenar & Feigon 1990, Weintraub 1990, Duval-Valentin et al. 1992).

When ODNs bind to a target, they can mediate their inhibitory action in different ways depending on where in the cell binding occurs. In the nucleus, it may disturb mRNA splicing, other processing reactions or transport into the cytoplasm by changing the tertiary structure of the target to such a conformation that it is unrecognizable by RNA-binding proteins. In the cytoplasm, where the inhibitory action most probably usually occurs, the antisense ODN can physically disturb the translational machinery by interfering with ribosomal attachment to the message, and with the translation process (Boiziau et al. 1991, Lawson et al. 1986, Rhoads 1988, Sonenberg 1988, Izant 1989, Knecht 1989). However, the effect of the antisense ODN can depend on the accessibility and secondary structure of the target, and on the proteins that are bound to the mRNA. A limiting factor can also be, as indicated earlier, the concentration of the ODN in the cell as well as its half-life. Finally, if the ODN:mRNA duplex formation does occur, the binding of the ODN might be unstable (van der Krol et al. 1988, Izant 1989, Knecht 1989).

RNAse H is considered to be a very important mediator of inhibition by antisense ODNs, especially of those which are complementary to the coding region. RNAse H activity seems to be widely distributed among eukaryotic cell types. Its activity is located mainly in nucleus where it is involved in DNA replication, but there also exists an RNAse H like activity in the cytoplasm. RNAse H can cleave mRNA from just before and after where the DNA binds leading to mRNA degradation and possibly release of intact ODN for another round of binding. Thus RNAse H may enhance translation inhibition in antisense experiments, although there is no direct evidence for this. RNase H activity can also have some unwanted secondary effects, such as the degradation of a nontarget mRNA, which contains minor complementary regions with the ODN (Walder & Walder 1988, Furdon et al. 1989, Agrawal et al. 1990, Colman 1990, Vlasov & Yurchenko 1991, Wagner 1994).

2.4.2.3 Unmodified ODNs

Unmodified antisense ODNs (umODNs) were the first ODNs to be used for modulating gene expression. Usually they are needed in 10 to 100-fold molar excess to mediate their inhibitory effect. The benefits of the umODN are that they are relatively inexpensive and easy to produce. An important factor is also that they can mediate the degradation of the target mRNA by RNAse H, which is not the case with many modified ODNs (Izant 1989, Colman 1990).

Although pure umODNs are not considered being very toxic to cells, the major problems with them seems to be that they do not enter the cells very efficiently and they are sensitive to nucleases. The native phosphodiester linkage between bases is a substrate for DNAses and it is very often cleaved before the umODN reaches its target, for instance, the half-life of the umODNs in *Xenopus* embryos is less than a minute. The main degradative nuclease activity seems to be a 3' exonuclease which exists in serum containing medium (van der Krol et al. 1988, Izant 1989, Baker et al. 1990, Shaw et al. 1991, Wagner 1994). However, cells have been described to also contain endonucleases and 5' exonucleases in nuclei and cytoplasm (Fisher et al. 1993). Thus the therapeutic potential of unmodified antisense compounds remains questionable because of the sensitivity to nucleases, and the general requirement of a large micromolar concentration of the compound to produce effective inhibition (Marcus-Sekura 1988).

2.4.2.4 Modified ODNs

The problems with the uptake and degradation of umODNs have led to the development of more lipid-soluble and stable ODNs. A large variety of modifications has already been developed, although these analogues very often compromise one or two of the important characters for ODNs function, namely penetration, hybridization and RNAse H mediated inhibition. However, some of

these modificated ODNs can frequently be used at lower concentrations than unmodified ones to produce the same effect in a given assay; alternatively, when used at the same concentration, they can inhibit to a greater extent (Marcus-Sekura 1988, Toulmé & Hélène 1988, van der Krol et al. 1988, Colman 1990, Shaw et al. 1991).

Modified ODNs are usually generated by replacing the native phosphodiester linkage between bases with more nuclease resistant substances (Weintraub 1990). One of the nonbridging oxygen atoms in each internucleotide phosphate linkage can be replaced by a sulfur atom (phosphorothioate ODN; S-ODN), a methyl-group (methyl-phosphonate ODN), an amide-group (phosphoroamidate ODN), or an alkyl-group (alkyl-phosphotriester ODN) (van der Krol et al. 1988, Hélène & Toulmé 1990). These nonionic analogues can traverse cell membranes more efficiently, their biological life-time is usually quite long and these modifications can strengthen their binding to the targets under physiological conditions. But, the substitution of a phosphorus oxygen by a methyl group or sulfur atom generates chirality, since four different groups are attached to the phosphorus atom and the synthesis of these type of ODNs is not stereospecific. Therefore, the end result is a heterogenous mixture of molecules that have asymmetric internucleotide bonds. Thus at each base only one of the two possible stereoisomers is capable of forming a duplex and, therefore, only a subset of the analogue ODNs can bind mRNA necessitating a higher dose (Eckstein 1985, Marcus-Sekura 1988, van der Krol et al. 1988). Although the phosphodiester analogue ODNs are more stable, they are often less effective at destabilizing target mRNA, and may function primarily as blocks of processing or translation of mRNA (Izant 1989).

The most commonly used analogues are phosphorothioated ODNs (S-ODN) (Eckstein & Gish 1989, Bielinska et al. 1990). They have several benefits, such as good resistance to cleavage by nucleases, solubility and they also show some depression of Tm, and hence can be expected to hybridize well to the target (van der Krol et al. 1988). On the other hand, the uniformly substituted S-ODN can destabilize the ODN:mRNA duplex severely, because of the chirality of these ODNs. The other disadvantage is that often there exist some cytotoxicity associated with active S-ODNs (Stein & Cohen 1988, Colman 1990). They may interact with cellular proteins and enzymes, nucleic acids and other small molecules specifically or nonspecifically, and cause nonspecific effects (Wagner 1994). It has been shown that S-homopolymers can inhibit RNAse H activity (Stein & Cheng 1993), but S-ODNs can also interfere for instance with absorption, penetration or uncoating of viruses by a non-antisense mechanism. Moreover, four contiguous guanosine residues in an S-ODN sequence can have an inhibitory influence by a non-antisense mechanism (Wagner 1994, Burgess et al. 1995), whereas a 28mer S-(dC) homopolymer has been shown to inhibit HSV-2 DNA polymerase. Furthermore, the influence of S-ODNs may depend on the concentration: at low concentrations (3 µM) they inhibit HIV viral protein synthesis by inhibiting reverse transcriptase in a non-sequence-specific manner, whereas at higher concentrations (>25 µM) they appear to have antisense activity (Marcus-Sekura 1988, Rothenberg et al. 1989).

A variety of phosphotriesters and methylphosphonate antisense ODNs has also been developed (van der Krol et al. 1988). These ODNs lack ionizable groups and are uncharged and thus, fairly hydrophobic. This can increase their cell membrane permeability, but these substances can also accumulate in hydrophobic domains, such as membranes, instead of entering the cells. Nevertheless, their uptake probably occurs through passive transport across the cell membrane, independently of the 80 kD receptor mediated mechanism. Although they are nuclease resistant (Stein & Cohen 1988, Rothenberg et al. 1989), they are enzymatically depurinated with time and ethyl phosphotriesters were found to undergo degradative deethylation in cells. Another disadvantage of oligomethylphosphonates is that they are often insoluble, if they are longer than 12mers. Furthermore, they and ethyl phosphotriesters have been shown to be relatively poor at inhibiting mRNA translation in cell-free systems (Stein & Cohen 1988).

Additions of compounds like poly-L-lysine and cholesterol are often used to improve the uptake of the ODNs. However, poly-L-lysine ODNs have a tendency to aggregate, and both poly-L-lysine and cholesterol have nonspecific cellular toxic effects (Rothenberg et al. 1989, Stein & Cheng 1993). In an effort to increase ODN efficacy, they can be covalently bound to the complementary sequences by UV irradiation, although this method is seldom suitable. A more useful method is to covalently link intercalating agents such as acridine to the ODN, which can increase its affinity for the target and strengthen weak or unstable binding of ODN. They can also improve the uptake of ODNs by cells and enhance resistance to 3' exonucleases (Walder 1988, Duval-Valentin et al. 1992). Commonly used additions to increase the reactivity of ODNs are alkylating moieties and metal complexes (such as EDTA-Fe(II), o-phenantroline-Cu(I) or porphyrin-Fe(II)). These groups can generate hydroxyl radicals in the presence of a reducing agent and oxygen. These hydroxyl radicals can cleave both DNA and mRNA, although they often have other unwanted effects, such as toxicity (van der Krol et al. 1988, Rothenberg et al. 1989, Hélène & Toulmé 1990).

3 PURPOSE OF THE STUDY

The aim of the work was to clarify the coordinated expression of contraction apparatus genes and their regulation in skeletal muscle derived rat L6 and mouse C2C12 cell lines, in an effort to create a hypothetical model of events that lead to assembly of a functional thin filament. Thus synthesis of cellular mRNAs and proteins of some of the contraction apparatus protein genes during differentiation was estimated, and their half-lives measured in one timepoint. The aim of antisense ODN work against fTnT in L6 cells and fTnC and c/sTnC in C2C12 cells was to regulate their gene expression. Effects of antisense RNA against c/sTnC were also examined in stably transfected C2C12 cells. The changes induced in gene expression may elucidate further coordination and relationships between contraction apparatus members and isoforms. The questions that were asked are:

1. How coordinated and stoichiometric is expression of muscle contraction apparatus members during differentiation of L6 and C2C12 cells and does there exist special co-regulation between some members of the contraction apparatus?

2. Is it possible to at least partially explain the expression pattern of contractile apparatus genes during differentiation according to the regulatory regions that these genes have?

3. What happens to the steady state levels of muscle-specific mRNAs and proteins, when expression of one these gene products is disturbed with antisense ODN treatment or antisense RNA expression?

4. Do L6 and C2C12 cells have mechanisms to compensate the influences of antisense treatments?

4 MATERIALS AND METHODS

4.1 Differentiation experiment

4.1.1 Cell cultures

A skeletal muscle originated rapidly fusing subculture of rat L6 cells (provided by B.D. Sanwal, University of Western Ontario, London), isolated from original L6 cell line (Yaffe 1968), and mouse C2C12 (ATCC CRL 1772) cells (Yaffe & Saxel 1977, Blau et al. 1983) were cultured to 90% confluency in Dulbecco's modification of Earle's medium (DMEM), supplemented with 10% fetal bovine serum (GIBCO BRL) in all the experiments described in this study, unless otherwise indicated. The culture medium was changed every other day. Differentiation was induced with medium supplemented with 2.5% horse serum (GIBCO BRL). Cells were washed with phosphate buffered saline (PBS, pH 7.2) before lysing. The compositions of the solutions are described in Appendix 2.

4.1.2 mRNA isolation

L6 and C2C12 cells were cultured in 100 mm plates (Nunc) as described and differentiated for given periods of time (0-360 h). The washed cells were harvested by using Guanidinium thiocyanate method described by Chomczynski & Sacchi (1987). In brief, the cells were lysed with 3 ml of solution D, and removed to 15 ml polypropylene tubes (Sarstedt). The lysates were supplemented with 0.2 M sodium acetate (pH 4.0), 3 ml of water-saturated phenol and 0.6 ml of

chloroform:isoamyl alcohol (49:1) (Fisher), and vortexed at high speed for 15 s. The mixture was centrifuged with a clinical table centrifuge (7.000 g) for 30 min. after 15 min. of incubation on an ice-bath. The aqueous phase was removed and mRNA was precipitated by adding an equal volume of 100% isopropanol (-20°C) (Fisher) and incubating the mixture at -20°C overnight. The mRNA was pelleted by 15 min. of centrifugation, the pellet was then dissolved in 1 ml of lysis solution, reprecipitated with an equal volume of 100% isopropanol at -20°C for 2 h, and pelleted as described. The mRNA was washed once with 70% ethanol (-20°C), after which it was lyophilized and dissolved in RNAse free water. The yield of total mRNA was estimated by taking O.D. reading at 260 nm, 280 nm, and 320 nm with a spectrophotometer (LKB). Total mRNAs (5 or 15 µg) were analysed using RNA-hybridization analysis as described in chapter 4.5.3 (RNA-hybridization analysis).

4.1.3 Protein isolation

Cells were cultured in 24 well plates (Corning) as described. Variously differentiated L6 cultures (0-360 h) and C2C12 cultures (0-240 h) were labeled with 250 µCi/ml of [^{35}S]-Methionine (ICN) in methionine-free DMEM supplemented with 2.5% dialyzed horse serum (GIBCO BRL) during the last 3 h of incubation. The cells were washed three times with ice-cold PBS and lysed with 100 µl of IEF lysis buffer. Labeling efficiency was measured by the hot TCA-precipitation method: 5 µl of lysates were applied on Whatman 42 filter papers, dried, and boiled in 10% TCA (Fisher) for 15 min. The filters were washed once with 100% ethanol (Fisher), dried, and counted in 3 ml of Ecolume scintillant (ICN). Samples from each timepoint with an equal amount of counts were analysed in 2D-PAGE as described in chapter 4.5.4 (Protein gel electrophoresis: Two-dimensional protein gel electrophoresis (IEF, NEPHGE)).

4.2 Half-life measurements

4.2.1 Transcription inhibition with Actinomycin D

Cells were grown in 100 mm plates as described. The differentiated C2C12 myotubes (5 d) and L6 myotubes (3 d) were treated with 5 µg/ml of AcD (Boehringer Mannheim) at set intervals (0-48 h, and 0-32 h, respectively). After incubations, cultures were rinsed three times with ice-cold PBS, lysed, and the mRNA was isolated as described in chapter 4.1.2 (mRNA isolation). Equal amounts of total mRNA (2, 5 or 10 µg) from each timepoint were subjected to northern blot analysis, as described in chapter 4.5.3 (RNA-hybridization analysis).

4.2.2 Pulse labeling of proteins

C2C12 and L6 cells were cultured in 24 well plates (Corning) and differentiated for 5 d and 3 d, respectively. The cells were labeled with 250 µCi/ml of [^{35}S]-Methionine (ICN) in methionine-free DMEM supplemented with dialyzed 2.5% horse serum (GIBCO BRL) for 3 h. After labeling, the cells were washed three times with differentiation medium and chased for set periods (0-48 h). The cells were lysed with 100 µl of IEF lysis buffer, and an equal volume of cell lysate from each timepoint was used for 2D-PAGE, as described in chapter 4.5.4 (Protein gel electrophoresis: Two-dimensional protein gel electrophoresis (IEF, NEPHGE)).

4.3 Antisense oligodeoxyribonucleotide experiments

4.3.1 Labeling of ODNs

Unmodified ODNs (umODNs; 500 ng of each) were 5' end labeled in a reaction mixture supplemented with [γ-^{32}P]ATP (7000 Ci/mmol; 1 Ci=37 GBq, ICN), PNK buffer (40 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT) and PNK T4 (GIBCO BRL) at +37°C for 1 h. The sequences of the ODNs are described in Appendix 1. The labeling products were purified through a Biogel P-4 column (Bio-Rad). Samples from each water-eluted 100 µl fraction (10) were counted, and analysed in 20% PAGE (200 V, 30 min.) with 1x TBE. The gels were exposed to Kodak X-AR film. The fractions that had the purest labeling products were pooled together and used in uptake experiments or as ODN probes for northern blots or as primers for primer extension studies.

4.3.2 Experiments in vitro

Transcription in vitro. Mouse c/sTnC cDNA in pGEM-3Z plasmid (Parmacek & Leiden 1989) was linearized from its 3' end with EcoRI (GIBCO BRL). The linearized plasmid was separated in 0.8% agarose gel with 1x TAE (Appendix 2), and the correct band was excised from the gel. cDNA was purified from the gel slice by using a Gene Clean kit (BioCan) according to the manufacturer's instructions.

Purified linearized cDNA (5 µg) was transcribed with an in vitro-transcription kit (Promega) according to the large scale synthesis protocol by using SP6 polymerase. m^{7}GpppG-capping solution (20 µl; Pharmacia in vitro-transcription kit) was added according to the manufacturer's instructions. The reaction mixture was incubated at +37°C for 1 h, after which the reaction was stopped by exposing the vial to +65°C for 5 min. The cDNA template was

destroyed by adding 5 units of RNAse-free DNAse I (Pharmacia) to the mixture and incubating it at +37°C for 15 min.

Synthesized mRNA was purified by extracting the mixture with equal volume of water saturated phenol:chloroform (24:1) and twice with chloroform:-isoamyl alcohol (24:1) (Fisher). The mRNA was precipitated from the aqueous phase at -20°C overnight by adding 0.3 M sodium acetate and 2.5 volumes of 100% ethanol (-20°C) (Fisher). The mRNA was pelleted by microcentrifuge (13.000 g, 15 min.) and the pellet was rinsed once with 70% ethanol, dried, and dissolved in distilled water. The yield of mRNA was measured spectrophotometrically by taking an O.D. reading at 260 nm. The confirmation of the measurement was performed by using a DNA/RNA Dipstick kit (Invitrogen).

Translation in vitro. Samples of in vitro-produced capped c/sTnC mRNA (150 ng) were exposed to antisense or sense cap-c/sTnC umODN (15mers), antisense or sense AUG-c/sTnC umODNs (18mers), antisense or sense int-umODN, antisense int-S-ODN (18mers), jun umODN (16mer) or to a random sequence S-ODN (18mer) (Oligos Etc. Inc.) and translated in vitro in rabbit reticulocyte lysate or in wheat germ extract (Promega). The structures of the ODNs are described in Appendix 1. The ODNs (0, 5, 10, or 20 µM) were lyophilized in Eppendorf-tubes (Sarstedt) and dissolved in 1 µl of water. The reaction mixture made according to the manufacturer's instructions with 250 µCi/ml [^{35}S]-Methionine (Amersham) as well as c/sTnC mRNA was aliquoted to the ODNs containing tubes, rabbit reticulocyte lysate or wheat germ extract was added and mixed, and the mixture was incubated at +37°C for 30 min. Reaction was stopped by exposing the vials to +65°C for 5 min., after which the samples were freezed (-20°C). The translation products were analysed in 1D-PAGE, as described in chapter 4.5.4 (Protein gel electrophoresis: One dimensional protein gel electrophoresis). Gels were autofluorographed to Kodak X-AR film.

4.3.3 Antisense TnT experiments in L6 cells and antisense TnC experiments in C2C12 cells

Uptake experiment. The cells were grown in 24 well plates (Corning) as described. L6 myotubes (60 h differentiated) and C2C12 myocytes (48 h differentiated) were washed twice before the experiments with PBS, after which they were exposed to either 5' end labeled unmodified (antisense cap-c/sTnC umODN; 15mer), 3' end phosphorothioated (antisense fTnT 3'S-ODN; 15mer) (The Midland Certified Reagent Company), or completely phosphorothioated (random sequence S-ODN; 18mer) (Oligos Etc. Inc.) ODNs for different periods of time. The sequences of the ODNs are described in Appendix 1. The 5' end labeled ODNs were added to a final concentration of 5 µM to culture medium (4.5 x 10^6 cpm/ well) supplemented with heat-inactivated 2.5% horse serum (L6 cells) or normal, untreated 2.5% horse serum (L6 and C2C12 cells). The cultures were incubated with ODNs for set periods (0-24 h) in a total volume of 150 µl. Following incubation the medium was collected and the cultures were washed

six times with 0.5 ml of PBS. These solutions were collected, the cells lysed with 2x 100 µl of lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 0.005% NP-40, 0.05% SDS), and the nucleic acids from the lysates were extracted with phenol:chloroform. In brief, an equal volume of Tris-buffered phenol (Fisher) was added to the lysate, mixed, and the aqueous phase was separated with a microcentrifuge (13.000 g for 3 min.). Phenol was re-extracted with half the volume of distilled water and this aqueous phase was combined with the first one. The pooled aqueous phase was extracted once with an equal volume of phenol:chloroform (1:1) and twice with equal volume of chloroform:isoamyl alcohol (24:1) as described. The cpms of the medium, washing solutions, crude lysates and aqueous phases of phenol:chloroform extractions were counted.

Antisense oligodeoxyribonucleotide treatments and mRNA. L6 cells were cultured in α-modified Earle's medium (α-MEM; Flow) in 60 mm plates (Nunc) as described. The cells were washed twice before the experiments with PBS. The 3.5 d differentiated myotubes were exposed to 25 µM of antisense or sense fTnT 3'S-ODNs (15mer) (The Midland Certified Reagent Company) for 12 or 24 h. The sequences of the 3'S-ODNs are described in Appendix 1. The medium was changed after 6 h of incubation to a fresh ODN-containing medium. Before harvesting, the cultures were washed three times with ice-cold PBS. mRNA was isolated by using the Guanidinium thiocyanate-method (Chomczynski & Sacchi 1987) as described in chapter 4.1.2 (mRNA isolation). In brief, the cells were lysed with 0.6 ml of Solution D and the lysates were collected. The lysates were supplemented with 0.6 ml of water-saturated phenol and 120 µl of chloroform:-isoamyl alcohol (48:1). The aqueous phases were separated by microcentrifugation (13.000 g, 20 min.), precipitated with an equal volume of isopropanol (-20°C) overnight and pelleted by microcentrifugation as described. The pellets were dissolved in 150 µl of Solution D, reprecipitated with equal volume of 100% isopropanol (-20°C), washed with 70% ethanol (-20°C), and dried. Total mRNAs (10 or 15 µg) were analysed with primer extension and northern blot analyses as described in chapters 4.5.2 (Primer extension) and 4.5.3 (RNA-hybridization analysis).

Antisense oligodeoxyribonucleotides and protein labeling. L6 cells were cultured in α-MEM (Flow) in 24 well plates (Corning) as described. The cells were washed twice before the experiments with PBS. The 3.5 d differentiated myotubes were exposed to 25 µM of antisense or sense fTnT 3'S-ODNs (The Midland Certified Reagent Company) or random sequence S-ODNs (Oligos Etc. Inc.) by adding the ODNs to the cultures for 12 h. The experiments were performed in a total volume of 200 µl in α-MEM without horse serum, which was replaced by a fresh medium with fresh 3'S-ODNs after 6 h of incubation. At the 9th h of incubation, 250 µCi/ml of [^{35}S]-Methionine (ICN) was added and the cells were incubated for an additional 3 h. After labeling, cells were washed three times with 0.5 ml of ice-cold PBS and lysed with 100 µl of NEPHGE lysis buffer. The lysates were freezed at -20°C and analysed by 2D-PAGE as described in chapter 4.5.4 (Protein gel electrophoresis: Two-dimensional protein gel electrophoresis (IEF, NEPHGE)). The TnT spot was determined by western blot analysis from 2D-

PAGE (NEPHGE), as described in chapter 4.5.4 (Protein gel electrophoresis: Immunoblot analysis).

C2C12 cells were cultured in 24 well plates (Corning) as described. The cells were washed twice before the experiments with PBS. The tested umODNs against c/sTnC mRNA in the C2C12 cells were targeted to (a) the cap-region (cap-c/sTnC; 15mer), (b) just after the AUG-codon (AUG-c/sTnC; 18mer or 20mer), (c) the internal-region (int-c/sTnC; 18mer), (d) the protein coding end (20mer) or (e) the 3' UTR end (20mer or 28mer). In the antisense fTnC studies either (a) the cap-region-binding 15mers (cap-fTnC) or (b) after the AUG-codon- binding 18mers (AUG-fTnC) were used. A random sequence umODN (18mer) was used as a control. The umODNs were tested at 5, 20, or 50 µM concentrations for 6 or 12 h in 24 or 48 h differentiated cells. The cells were treated also with 5, 10, 20, 40, or 100 µM of antisense int-c/sTnC S-ODNs (Oligos Etc. Inc.). A random sequence S-ODN (Oligos Etc. Inc.) was used as a control ODN. In these experiments, variously differentiated myotubes were tested (24, 48, 72 or 96 h differentiated) as well as different incubation times (3, 6, 12, 24, 48, 72 or 96 h). The sequences of the ODNs used are described in Appendix 1. The experiments were done in a total volume of 200 µl in DMEM supplemented with heat-inactivated 2.5% horse serum (GIBCO BRL). The final experiments with umODNs were performed in 48 h differentiated myotubes, which were exposed to 50 µM of particular umODNs for 12 h. A fresh culture medium was substituted after every 3 h of incubation with the umODNs, whereas in the case of S-ODNs, the medium was not changed due their nondegradable character. During the last 3 h of ODN incubation the proteins were labeled with 500 µCi/ml of [^{35}S]-Methionine (ICN), after which the cells were washed three times with 0.5 ml of ice-cold PBS. The cultures were lysed with 100 µl of IEF lysis buffer and freezed at -20°C. The method of analysis is described in chapter 4.5.4 (Protein gel electrophoresis: Two-dimensional protein gel electrophoresis (IEF, NEPHGE)).

Antisense oligodeoxyribonucleotide and Actinomycin D treatment in C2C12 myotubes. C2C12 myotubes (5 d differentiated) in a 24 well plate (Corning) were exposed for 3 h to 5 µg/ml of AcD (Boehringer Mannheim) together with either 50 µM of antisense cap-c/sTnC, AUG-c/sTnC, cap-fTnC or c/sTnC umODNs, or random sequence S-ODNs (Oligos Etc. Inc.), and 250 µCi/ml of [^{35}S]-Methionine (ICN). The sequences of the ODNs are described in Appendix 1. After incubation, the cells were washed three times with 0.5 ml of ice-cold PBS, lysed with 100 µl of IEF lysis buffer (Appendix 2) and freezed at -20°C. The method of analysis is described in chapter 4.5.4 (Protein gel electrophoresis: Two-dimensional protein gel electrophoresis (IEF, NEPHGE)).

4.4 Transfection studies

4.4.1 Plasmid constructs

The c/sTnC cDNA in pGEM-3Z vector (Parmacek & Leiden 1989) lacked 30 bps of coding region from its 3' end, which was constructed from the umODNs. The sequences of the ODNs are described in Appendix 1. The sense coding-end 30mer of c/sTnC (EcoRI site in the 5' end and HindIII site in the 3' end) and respective antisense umODN were annealed (1 mM of each) in 100 µl of annealing solution (10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$; Fisher) by heating the mixture at +80°C and allowing it to cool for 2 h to room temperature in the same 200 ml water bath which was used for heating. The DNAs were precipitated overnight by adding 0.2 M of sodium acetate pH 5.2 and 2.5 volumes of 100% ethanol (-20°C) (Fisher) at -20°C. The precipitate was pelleted with microcentrifuge (13.000 g, 15 min.), washed once with 70% ethanol and dried modestly. The DNA was resuspended in 20 µl of water and formulated with DNA loading dye. The double-stranded ODNs were separated from the unannealed ones in a 0.7% agarose gel buffered with 1x TAE. The correct band was excised and purified with a Magic minipreps™ DNA purification column system (Promega) according to the manufacturer's instructions.

The purified 30 bp dsDNA fragments were blunt-end ligated as concatamers overnight in T4 ligase buffer (30 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP, 0.1 mg/ml BSA) with a 0.25 Weiss unit of T4 DNA ligase (Promega) at +16°C. The reaction was stopped by heating at +60°C for 5 min. The concatamers were digested with EcoRI (GIBCO BRL), the fragments were phenol:chloroform purified, and treated with calf alkaline phosphatase (Promega) in CIP buffer (1 mM $ZnCl_2$, 1 mM $MgCl_2$, 10 mM Tris-HCl pH 8.3) at +37°C for 30 min. according to Sambrook et al. (1989). The fragment was ligated with T4 DNA ligase to the 3' end with EcoRI linearized c/sTnC cDNA in the pGEM-3Z vector. The formed plasmids were multiplied in *E. Coli*, isolated and then analysed with restriction enzyme analysis with SalI (linearization of the plasmid construct), HindIII (vector, full-size c/sTnC and 30 bp fragment), and EcoRI (plasmid construct and 30 bp fragment) (GIBCO BRL). A full-size coding sequence of c/sTnC from the constructed plasmid was excised with HindIII (GIBCO BRL), purified, CIP-treated and ligated in HindIII cut pCMVp plasmid vector, downstream after a human cytomegalovirus promoter (pCMVp (mammalian expression vector) Boshart et al. 1985, Foecking & Hofstetter 1986). The vector also had the sequence (Donoghue 1982, Hanahan 1985), which is necessary for polyA-tail adding to the noncoding 3' end lacking c/sTnC construct. The formed plasmids were multiplied in *E. Coli*, isolated and analysed using restriction enzyme analysis with XbaI and EcoRI (orientation of the c/sTnC in respect to the CMV promoter), HindIII and BamHI (the size of the ligated c/sTnC fragment), and SalI (size of the linearized construct) (GIBCO BRL). The constructs that had c/sTnC in either the sense or antisense orientation were chosen for

further large scale growing. As a selection plasmid for transfectants the pSV2neo plasmid, which has SV40 early promoter upstream from neomycin resistance gene (Southern & Berg 1982) was chosen. The plain pCMVp without an insert was chosen as a control plasmid. The alkaline lysis isolation method for plasmids and subsequent pancreatic RNAse A treatment are described by Sambrook et al. (1989).

The plasmids were purified by separating the supercoiled DNA electrophoretically in 0.7% agarose gel with 1x TBE. The correct band was excised from the gel and the DNA was run out of the gel in a dialysis bag. The mixture of supercoiled DNA and 1x TBE were collected, purified with phenol:chloroform extraction and precipitated overnight with 0.2 M sodium acetate, pH 5.2, and 2.5 volumes of 100% ethanol (Fisher) at -20°C. The precipitates were pelleted with a clinical table centrifuge (7.000 g, 30 min.) and washed once with 70% ethanol. The DNAs were dried modestly and resuspended gently in sterile distilled water and the yield estimated by taking O.D. readings at 260 nm, 280 nm and 320 nm.

4.4.2 Cell cultures and transfections

C2C12 cells were plated to 60 mm plates (Nunc) and grown in DMEM supplemented with 10% fetal bovine serum (GIBCO BRL) so that next day the plates were approximately 60-70% confluent. The cells were washed 3 h before the transfections with PBS, and 3 ml of fresh growth medium was added to each plate. The cells were transfected with pCMV-antisense-c/sTnCp, pCMV-sense-c/sTnCp or pCMVp, together with pSV2neo plasmid constructs in the ratio 5:1, respectively, or only with pSV2neo. The transfections were performed by using the $CaPO_4$-precipitation method (Frost & Williams 1978, van der Eb & Graham 1980). The precipitates of the plasmid DNAs were formed as 20 µg/ml concentrations by mixing the DNA with 0.25 M $CaCl_2$, and adding an equal volume of 2x HBS (pH 7.05) in three equal volume batches. The mixing of each batch of HBS was done by passing bubbles gently with a Gilson P200 pipet, after which the precipitates were allowed to form at room temperature for 20 min. The fine precipitates were added dropwise around each plate at a concentration of 5 µg DNA/60 mm plate, after which the cultures were incubated for 5 h. After treatment, the medium was removed and the cells were washed twice with 2 ml of medium and exposed to 2 ml of glyserol shock solution for 1 min. The solution was removed, after which the cells were washed with 2 ml of 1x HBS, and the normal growth medium was added. After 1 d of recovery, the cells were trypsinazed, and each 60 mm plate was divided to two 150 mm plates. Next day, selection antibiotic G418 (GIBCO BRL), described by Davies & Jiminez (1980), was added (400 µg/ml) for 2 wk, after which the used concentration was reduced to 100 µg/ml. After 3 wk of culturing small colonies were visible.

The colonies were isolated (24 colonies/each transfection), and plated to 12 well plates (Nunc) from which frozen stocks were prepared. The selected colonies were examined by RNA-hybridization analysis. The cells were plated to 30 mm plates and let to grow to 80-90% confluency. mRNA was isolated

(Chomczynski & Sacchi 1987), and equal amounts of total myoblast mRNA were separated in MOPS-buffered 1% agarose gel electrophoresis, and northern blots were prepared (described in chapter 4.5.3 (RNA-hybridization analysis)). The northern blots were hybridized with [α-^{32}P]dCTP (3000 Ci/mM; 1 Ci=37 GBq, ICN) random primed c/sTnC cDNA (Parmacek & Leiden 1989), pCMVp or pSV2neo plasmids, or GAPDH cDNA (pRGAPDH13; Fort et al. 1985), which were labeled according to the manufacturer's instructions (Boehringer Mannheim). The DNAs were isolated from the 3 highest antisense or sense c/sTnC expressing colonies.

4.4.3 DNA isolation

DNA was isolated from the 3 colonies that had the highest expression of the antisense-c/sTnC and sense-c/sTnC. The selected colonies were cultured in 60 mm plates (Nunc) to 80-90% confluency. The cells were washed with PBS, scraped from the plates, and 0.7 ml of DNA extraction buffer, supplemented with 0.4 µg/ml proteinase K (GIBCO BRL), was added. The mixture was incubated at +55°C overnight by shaking on a Nutator.

The released DNA was purified by extracting the incubation mixtures twice with an equal volume of Tris-saturated phenol (pH 8.0) and twice with an equal volume of phenol:chloroform (24:1) mixture. The DNA was precipitated from the aqueous phase by adding 0.3 M sodium acetate (pH 5.2) and an equal volume of 100% isopropanol (Fisher) and incubating at room temperature for 2 h. The DNA was pelleted by microcentrifugation (13.000 g) for 5 min., rinsed with 70% ethanol and dried modestly. The DNA pellet was resuspended to 0.5 ml of 1x TE (Appendix 2) and reprecipitated at room temperature by adding sodium acetate as previously, and 2 volumes of 100% ethanol. Pelleting and washing were repeated as well as the resuspending to 1x TE. The pure DNA was stored at +8°C.

The samples of pure DNAs (20 µg) from pCMV-antisense c/sTnC and pCMV-sense c/sTnC transfectants were digested with HindIII (BRL), the pCMVp transfectants with EcoRI (GIBCO BRL) and the pSV2neo transfectants with BamHI (GIBCO BRL) for southern blot analysis as described in chapter 4.5.1 (DNA-hybridization analysis).

4.4.4 Protein studies of transfectants

The selected colonies of pCMV-antisense c/sTnC and pCMV-sense c/sTnC construct transfected C2C12 cells as well as the control transfectants (pCMVp and pSV2neo) were grown in 24 well plates as described, and let to differentiate for 6 d. Untransfected C2C12 cells, which were also used as a control, were cultured the same way. The differentiated cells were lysed with IEF lysis buffer (Appendix 2) and analysed with 2D-PAGE (IEF) as described in chapter 4.5.4 (Protein gel electrophoresis: Two-dimensional protein gel electrophoresis (IEF, NEPHGE)).

4.5 DNA, RNA, and protein analysis methods

4.5.1 DNA-hybridization analysis

The southern analysis was perfomed according to method described by Southern (1975) with modifications. The digested DNAs from the transfection colonies were separated in 0.8% agarose gel with 1x TBE. The separated DNAs were transferred with the aid of a TE 80 Transvac vacuum blotting unit to a Zeta-Probe nylon membrane (Bio-Rad) by using the alkaline transfer method: the agarose gel was depurinated with 0.25 M HCl under the vacuum in a blotting unit for 10 min., after which the solution was removed and replaced with a transfer solution. Transfer was stopped after 60 min., the transfer solution was removed, and the vacuum (10.16 cm of Hg) was turned off. The membrane was washed in 2x SSC at room temperature for 15 min., air-dried and baked at +80°C for 1 h.

The baked membrane was prehybridized in a hybridization solution at +42°C for 2-6 h. The solution was removed and replaced with a fresh, prewarmed one supplemented with [α-^{32}P]dCTP (3000 C1/mmol; 1 Ci=37 GBq, ICN) labeled c/sTnC cDNA (Parmacek & Leiden 1989), pCMVp (Donoghue 1982, Boshart et al. 1985, Hanahan 1985, Foecking & Hofstetter 1986) or pSV2neo (Southern & Berg 1982). The plasmid probes were random primed according to the manufacturer's instructions (Boehringer Mannheim). The probes were denaturated at +90°C for 5 min, and then cooled on ice before adding. Hybridizations were performed at +42°C with rocking for overnight. The hybridized membrane was washed twice with solution I and once with solution II. All washes were done at +60°C for 15 min. The washed membrane was slightly air-dried, covered with Saran-wrap and exposed to Kodak X-AR film.

4.5.2 Primer extension

Equal amounts of total mRNA (15 or 20 µg) from the L6 experiment with antisense and sense fTnT 3'S-ODN treatments as well as untreated mRNA were annealed with 50 ng of [γ-^{32}P]ATP (7000 Ci/mM; 1 Ci=37 GBq, ICN) 5' end labeled primer. The labeling of the ODNs is described in chapter 4.3.1 (Labeling of ODNs). The primer was the same antisense fTnT ODN as that used for the antisense treatments but unmodified (Appendix 1). The mRNA/primer mixtures in water were heated at +65°C for 10 min., after which they were cooled in an ice-bath for 15 min. and precipitated overnight with 0.3 M sodium acetate and two volumes of 100% ethanol (-20°C) at -20°C. The precipitates were pelleted by 15 min. centrifugation with microcentrifuge (13.000 g, +8°C), and the pellets were rinsed once with 70% ethanol (-20°C), dried and dissolved in equal volumes of water. Annealing of the mRNA/primer was done in a volume of 16.5 µl by adding annealing buffer and incubating the mixtures at +65°C for 10 min., after which the reactions were cooled in an ice-bath for 15 min. The mRNA/primer

mixtures were reprecipitated and washed as described. The mixture, dissolved in water, was supplemented with reverse transcription buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$), 0.5 M of each cold nucleotides (dGTP, dATP, dTTP, dCTP) and, finally, 200 U of Moloney murine leukemia virus reverse transcriptase (GIBCO BRL) was added. The reaction mixture (total volume of 25 µl) was incubated at +42°C for 1 h. Reaction was stopped by adding 0.05 M EDTA (pH 8.0) and incubating the mixture at +65°C for 10 min.

Equal volumes of reaction mixtures were formulated by adding 1x loading dye for the primer-extended samples, after which the samples were heated at +65°C for 10 min. and cooled quickly in ice-bath before loading onto the gel. The primer-extended products were separated in denaturing sequencing PAGE with 1x TBE (Maxam & Gilbert 1980). The gel was prerun at 1300-1500 V for 30 min. until the gel temperature had risen to +55°C. The denatured samples were loaded immediately after the prerun and separated in a gel with 1500-1800 V for 3 h. After the gel run, the gels were fixed with 10% acetic acid and 10% methanol for 15 min., dried and exposed to Kodak X-AR film at -80°C for 1-5 d.

4.5.3 RNA-hybridization analysis

Northern analysis was performed as described by Meinkoth & Wahl (1984) and Thinakaran & Bag (1991). Isolated cellular mRNAs (5, 10, or 15 µg) were lyophilized and to the samples were added 2 µl of water and 15 µl of formulation solution (8 µl deionized formamide, 2 µl 1x MOPS, 4 µl formaldehyde and 1 µl ethidium bromide solution (1 µg ethidium bromide/µl water)). The samples were denatured at +65°C for 15 min., cooled on ice quickly and 1.5 µl loading dye was added just before loading to 1% agarose gel, buffered with 1x MOPS. The gels were run at 60 V for 7-8 h or at 20-30 V overnight. The separated mRNAs were transferred to a Zeta-Probe nylon membrane (Bio-Rad) with 20x SSC for 30-48 h. After transfer, the membrane was rinsed briefly with 2x SSC and baked at +80°C for 2 h. The Ethidium bromide and loading dye were removed from the membrane in a stripping solution by rocking it gently at +60°C for 1 h.

The membranes were hybridized with [α-^{32}P]dCTP (3000 Ci/mM; 1 Ci=37Bq, ICN) labeled cDNA probes (α-actin; pAC269 (Schwartz et al. 1980), rat TnC; A45(TNC), α-TM; VT-TM, β-TM; A21(β-TM) (Garfinkel et al. 1982), glyceraldehyde-3-phosphate dehydrogenase (GAPDH); pRGAPDH13 (Fort et al. 1985), c-jun; pJac7 (Ryder & Nathans 1988), MyoD; pVZCIIα (Davis et al. 1987) or myogenin; pUC65-20 (Wright et al. 1988)) according to the instruction manual for Zeta-Probe blotting membranes (Bio-Rad). Labeling was performed by random priming according to the manufacturer's instructions (Boehringer Mannheim). The hybridization solution for the plasmids was added, and the membrane was prehybridized at +65°C overnight. The preheated hybridization solution was changed and supplemented with a denatured plasmid probe. The hybridizations were performed with cDNA probes, which specific activities ranged from 0.8 to 1.3 x 10^8 cpm/µg of plasmid at +65°C for 40-48 h. After hybridization, the membranes were washed once with washing solution I,

rocking vigorously at room temperature for 30 min. Additional washes were done with washing solution II, rocking vigorously at room temperature for 30 min., after which the solution was replaced, and the membranes rocked gently at +65°C for 10-20 min.

ODN hybridizations (antisense AUG-c/sTnC (murine, 18mer), cap-fTnC (murine, 15mer), fTnT (rat, 15mer), fTnI (mouse, 18mer), or sTnI (mouse, 18mer) umODNs) were performed in hybridization solution for ODNs as described by Angelini et al. (1986) in the instruction manual for Zeta-Probe blotting membranes (Bio-Rad). The 5' end labeling procedure of the ODNs is described in chapter 4.3.1 (Labeling of ODNs). The membranes were prehybridized at +65°C overnight, with hybridization solution supplemented with 0.1 mg/ml salmon sperm DNA (GIBCO BRL). The DNA was heat-denatured at +100°C for 5 min. and quickly cooled in an ice-bath before adding. The hybridization solution was also supplemented with 10% dextran sulphate (Pharmacia), and as in the previously described denaturation method treated with a mixture of salmon sperm DNA (0.1 mg/ml), purified ODN probe (50 ng/ml, specific activity 0.8 to 1.3 x 10^8 cpm/µg umODN), and 0.1 ml of O.2 M NaOH. The hybridizations were done at room temperature (fTnT), +37°C (fTnI, sTnI, and c/sTnC) or +42°C (fTnC) for 40-48 h, after which the membranes were washed. Washing was performed twice with a solution composed of 3x SSC, 5% SDS, and 25 mM $NaPO_4$ pH 7.5 at room temperature for 30 min. Final washes were done with mixture of 1x SSC and 1% SDS at room temperature for 20 min. and, finally, at +42°C for 5-10 min. (TnT) or +45°C for 5-10 min (c/sTnC, fTnC, fTnI, and sTnI).

After washing, the membranes were slightly dried, covered with Saran wrap and autoradiographed onto Kodak X-AR film at -80°C. After the exposures, the probes were stripped from the membranes with a stripping solution (Appendix 2), by rocking gently at +80°C for 1 h. The membranes were rehybridized approximately seven times.

4.5.4 Protein gel electrophoresis

One-dimensional protein gel electrophoresis. One-dimensional SDS-PAGE was performed using the modified method of Laemmli (1970). Equal volumes of rabbit reticulocyte lysates or wheat germ extracts (Promega) used in in vitro translation of c/sTnC mRNA were formulated with two volumes of 1x loading dye. The samples were heated at +65°C for 10 min. before loading to the 1 mm thick gels (Mini-protean II slab gel apparatus, Bio-Rad) which were composed of sections of 4% stacking and 15% separating PAA gels. The transcription products were run through the stacking gel (5 mm) at 100 V (10-15 min.), after which the voltage was raised to 150 V for 50-60 min. The running buffer was composed of 0.1 M Tris (ICN), 0.6 M glycine (Bio-Rad) and 0.1% SDS (Sigma), and the gels were run until the tracking dye reached the bottom of the gel.

After PAGE, the gels were fixed with fixing solution for 30 min, rinsed briefly in distilled water and enhanced as described by Bonner & Laskey (1974): the gels were dehydrated in 100% DMSO (Fisher) with gentle rocking for 2x 30

min. and treated with 22% PPO (Sigma) in DMSO with gentle rocking for 3 h, after which the PPO was precipitated by washing the gels three times with distilled water. Before drying, the gels were treated with a 10% acetic acid and 2% glyserol solution for 1 h. The dried gels were exposed to Kodak X-AR film for 3-5 d.

Two-dimensional gel electrophoresis (IEF, NEPHGE). Isoelectric focusing (IEF) and non-equilibrium pH-gradient electrophoresis (NEPHGE) were performed according to methods described by O'Farrell (1975), O'Farrell et al. (1977), Mikawa et al. (1981) and the Bio-Rad instruction manual, by using a Mini-protean II 2D minigel apparatus (Bio-Rad). Samples of [^{35}S]-Methionine labeled cell lysates (200 000 cpm or equal volume) were isoelectric-focused in first dimension gels (IEF). The IEF tube-gel mixture was degassed prior the polymerization. The loaded samples were filled to equal volumes with lysis buffer, and overlaid with three volumes of IEF overlay buffer. The upper chamber of the gel apparatus was filled with degassed 20 mM NaOH, and the lower with degassed 10 mM H_3PO_4. The proteins were isoelectric focused at 500 V for 10 min. and at 750 V for 3 h 30 min.

The non-equilibrium pH-gradient electrophoresis (NEPHGE) gels were prepared similarly to the IEF tube gels from the NEPHGE gel mixture (Appendix 2). The samples were filled to equal volume with lysis buffer and overlaid with three volumes of NEPHGE overlay solution. The upper chamber was filled with degassed 10 mM H_3PO_4 and the lower with degassed 20 mM NaOH. The proteins were separated at 200 V for 4-6 h.

After IEF or NEPHGE, the tube gels were positioned on the top of 1 mm thick second dimension gels, composed of a 5 mm layer of stacking gel and separating gel. On the top of the first dimension gels, 5 µl of tracking dye was added. The proteins were run through the stacking gel at 100 V for 10-15 min. after which the voltage was increased to 150 V for 50-60 min., until the tracking dye reached the bottom. The gels were fixed in a fixation solution for 30 min., rinsed with distilled water and finally enhanced with PPO saturated DMSO as described in previous section: One-dimensional protein gel electrophoresis.

Immunoblot analysis. Location of fTnT in 2D-PAGE (NEPHGE) autoradiograph was determined by Western blot analysis. The [^{35}S]-Methionine labeled proteins of the 4 d differentiated L6 cells were separated in 2D-PAGE (NEPHGE) as described previously. Two sets of unlabeled rabbit troponin complex proteins (Sigma) were also separated at the same time as the L6 myoblastic proteins. Western blot procedure for the L6 myotubic and myoblastic proteins and rabbit troponin complex proteins was performed according to the Mini-protean II 2D minigel apparatus instruction manual (Bio-Rad). The proteins were transferred to a Zeta-Probe nylon membrane (Bio-Rad) according to the instructions of the Bio-Rad Trans-blot cell operating manual with 25 mM Tris (ICN) and 192 mM glycine (Bio-Rad) at 60 V for 3 h with cooling. The membranes were washed according to the instruction manual for Zeta-Probe blotting membranes (Bio-Rad). The fTnT protein was detected by rabbit fTnT specific JLT12 antibody (1:200 dilution) as characterized by Lin et al. (1984) (Sigma). For detection a secondary

antibody against mouse IgG conjugated with alkaline phosphatase was used (used as 1:30 000 dilution) (Sigma). The L6 myotubic and myoblastic protein membranes as well as the rabbit troponin complex membrane were developed using BCIP/NBT color development reagents according to the manufacturer's instructions (Boehringer Mannheim), after which the two L6 protein membranes were exposed to Kodak X-AR film (-80°C). The developed autoradiographs were superimposed back onto their respective membranes, respectively, for detection of the respective radioactive protein. The L6 membranes were also compared to the rabbit troponin complex membrane, detected with the antibody, and also to the rabbit troponin complex gel, stained with Coomassie brilliant blue (Sigma).

4.6 Analysis methods

The signals from the autoradiograms and autofluorographs were analysed with a Neotech 2.1 image-analysis system (ImageGrabber® 2.1 alias Neotech version 2.1, Neotech ltd). The numerical data of the mRNAs and proteins of the differentiation experiments were used for the curves, where the data was described in y-axes and time course in x-axes. Half-lives of mRNAs and proteins were calculated by using the equation $t1/2 = 0.693:k$.

The crude uptake percentage was evaluated by dividing the cpms present in crude lysate by total cpms, which included the cpms of the incubation medium, washing solutions, and crude lysate. The result was multiplied by 100. The uptake of the cells without proteins was determined by dividing the aqueous phase of the phenol:chloform extracted cell lysate by total cpms, which result was multiplied by 100.

In the antisense ODN experiments, some of the numerical data were also analysed statistically. Paired t-test (Snedecor & Cochran 1980) and Wilcoxon signed-rank sum test (the median of the distribution of the difference between treatment and control is 0) (Conover 1980) were used to estimate the statistical significance of the differences between individual proteins of antisense ODN treated myotubes and the respective proteins of two different controls. Only matched pairs were used in these tests and the tests were performed as two-sided.

5 RESULTS AND DISCUSSION

5.1 Characterization of differentiation of L6 and C2C12 cell lines

5.1.1 Expression of contractile apparatus genes and some transcriptional factors in L6 and C2C12 cells

In the present study, skeletal muscle originated rat L6 and mouse C2C12 cell lines were used as model systems to study differentiation. These cell lines express partially different contractile apparatus proteins, which are listed in Table 1. According to the protein forms that C2C12 cells express, these cells are considered to resemble the primary culture of fetal muscle, whereas L6 cells have more resemblance to early embryonic muscle cells, although they originate from satellite cells (Nadal-Ginard 1978, Minty et al. 1986, Ernst et al. 1991, Smith & Miller 1991, Muthuchamy et al. 1992). The purpose of the study was to elucidate further the coordinated and stoichiometric expression of contractile apparatus genes, their order of expression and possible cluster type regulation during differentiation, and the similarities and differences between these cell lines. The other aim was to clarify whether the order and coordinated expression, and also expression intensity, can be explained indirectly by the specific combination of the known and potential transcriptional elements the studied contractile apparatus genes possess. The half-lives of cellular mRNAs and proteins were also determined in an effort to determine the influence of the turnover rates on the accumulation of the gene products. Thus by combining together the available information on these subjects, the main interest - assembly of a thin filament - may be further elucidated.

TABLE 1 Contractile apparatus gene isoforms expressed in differentiated C2C12 and L6 cells.

	C2C12	L6	References
αsk-actin	x	x	Bains et al. 1984, Muthuchamy et al. 1992
αc-actin	x	-	Bains et al. 1984, Muthuchamy et al. 1992
α-TM	x	x	Garrels 1979, Ruiz-Opazo & Nadal-Ginard 1987, Wieczorek et al. 1988
β-TM	x	x	Garrels 1979, Wang & Rubenstein 1992
fTnC	x	x	Parmacek et al. 1990
c/sTnC	x	x	Parmacek & Leiden 1989
fTnT	?	x	Breitbart & Nadal-Ginard 1987
c/eTnT	x	?	Jin et al. 1992
fTnI	x	x	Koppe et al. 1989
sTnI	x	x	Koppe et al. 1989
eMHC	?	x	Garfinkel et al. 1982, Periasamy et al. 1985, Weydert et al. 1987, Miller 1990, Yu & Nadal-Ginard 1989
fetal-MHC	x	-	Weydert et al. 1987
pnMHC	x	-	Silberstein et al. 1986, Weydert et al. 1987, Miller 1990
MHCIIB	x	-	Weydert et al. 1987
v/s β-MHC	x	-	Miller 1990
v/sMLC1	x	-	Barton et al. 1985, Pinset et al. 1988
a/eMLC1	-	x	Whalen et al. 1978, Garfinkel et al. 1982, Barton et al. 1988
fMLC1	x	-	Periasamy et al. 1984, Pinset et al. 1988
fMLC3	x	-	Periasamy et al. 1984, Pinset et al. 1988
sMLC1	?	-	Blau et al. 1983
skMLC2	x	x	Whalen et al. 1978

x: present; -: not present; ?: expression uncertain.

5.1.2 Results and discussion on differentiation experiments in L6 and C2C12 cells

The purpose of the study was to compare the fusion process and the initiation of transcription and translation of contractile apparatus genes in a rapidly fusing subclone of L6 cells. The influence of some of the transcriptional factors on the fusion process and gene expression was also estimated. Another purpose was to clarify the possible post-transcriptional regulation of contractile apparatus genes by comparing protein synthesis to the respective cellular mRNA accumulation during differentiation. The aim of comparing the transcriptional patterns of the contractile apparatus genes with their present known regulatory elements, in turn, was to investigate whether the transcriptional order, intensity and coordination of the genes can be explained -at least partially - by the types of regulatory sequences they have.

Results:

Cellular mRNAs of L6 cells. L6 cells were initially plated to around 40% confluency, and after around 48 h or 72 h of incubation, when the cultures had reached 80-90% confluency, the differentiation process was initiated by adding differentiation medium to the plates. The adding point was also the 0 h timepoint of the differentiation experiment. According to observations with a light microscope, the first small myotubes became apparent after about 48 h of differentiation. The fusion process was most pronounced after about 60 h, and the cells were close to 100% fused about 96 h after the change. After around 122 h of differentiation the cells had attained full maturity, but without reaching contraction ability. Shortly thereafter, some of the myotubes started to die and the very few unfused myoblast-like cells started to divide slowly, fuse, and replace the dead myotubes. Thus the experiment describes more than one life-cycle of L6 cells, where the timepoints 240 h and in particular 360 h represent fairly non-synchronous myotubes.

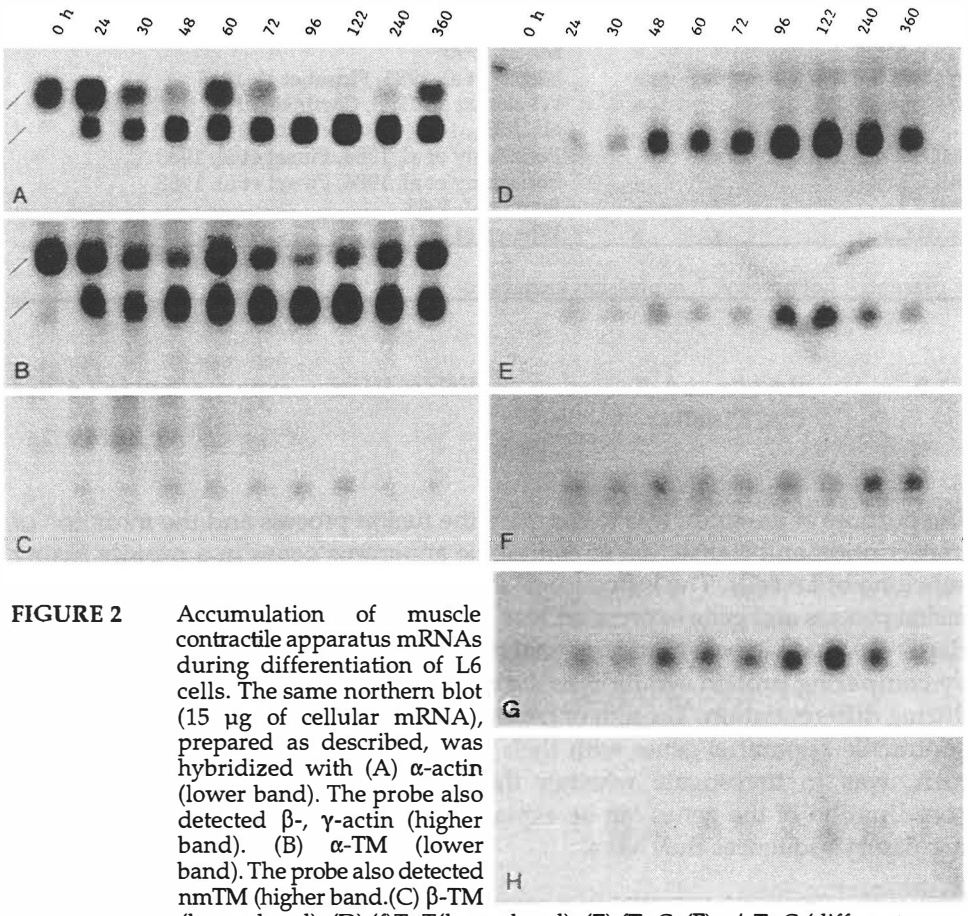

FIGURE 2 Accumulation of muscle contractile apparatus mRNAs during differentiation of L6 cells. The same northern blot (15 µg of cellular mRNA), prepared as described, was hybridized with (A) α-actin (lower band). The probe also detected β-, γ-actin (higher band). (B) α-TM (lower band). The probe also detected nmTM (higher band.(C) β-TM (lower band), (D) (f)TnT(lower band), (E) fTnC, (F) c/sTnC (different membrane, but 5 μg of mRNA from the same experiment), (G) fTnI, and (H) sTnI (same membrane as detected by c/sTnC).

Fig. 2 shows the northern blot analyses for various muscle contractile apparatus mRNAs. According to the intensity of the bands at the 24 h timepoint, the transcription of α-actin and α-TM seems to be initiated more pronouncedly, and thus probably also slightly earlier, than for instance TnT of members of the troponin complex in L6 cells; the intensities of the bands of α-actin, α-TM and TnT are otherwise quite similar at the 96 h and 122 h timepoints. There also seems to be some similarity in the accumulation of α-actin and α-TM during differentiation. The signal of the muscle isoform of β-TM is, for an unknown reason, very weak, thus definite conclusions about its expression cannot be made. Its accumulation, however, seems to resemble that of α-TM.

Initially the most markedly transcribed members of the troponin complex appear to be fTnI (15 µg mRNA) and c/sTnC (5 µg mRNA), compared to the other members of the troponin complex at the same timepoint. The initiality is also indirectly supported by the knowledge that later on, the intensities of the bands of TnI and c/sTnC are lower, compared to the respective intensities of the bands of the other members examined. This suggest that the probes used were enough sensitive for the detections. Nevertheless, a few hours later, the intensity of the (f)TnT signal is around threefold above of the signal of all the other members of the complex. Its accumulation pattern resembles, as expected, fTnC and fTnI. However, only the general accumulation of (f)TnT was estimated, since the used ODN probe (15mer) against rat fTnT was not specific to any particular fTnT isoform: the complementary region of the probe was just after the AUG-codon, covering partially the exons 2 and 3 that exist in all fTnT isoforms. The probe, however, gave only one band in the RNA-hybridization analyses of the L6 and of C2C12 cells (Fig. 2, Fig. 4), but whether it also detected other similar size TnTs is unknown. The existence and expression of the sTnT gene or its homology to fTnT or c/eTnT genes in rat or mouse is not understood, hence the possibility that the bands detected by the fTnT ODN probe in Fig. 2 and Fig. 4 represent also in next section (Proteins of L6 cells) described xTnTs remains to be determined.

The accumulations of fTnC and c/sTnC mRNAs differed from each other during differentiation. Expression of fTnC is most marked at 96 h and 122 h differentiated cells, whereas expression of c/sTnC is very moderate. Moreover, whereas accumulation of c/sTnC increases at 240 h and 360 h cultured cells, accumulation of fTnC, instead, shows a decrease, similar to that in fTnI. This also suggests that together with fTnI c/sTnC may be the first expressed member of the troponin complex. Nevertheless, the expression patterns of fTnI and sTnI have more similarity than those of fTnC and c/sTnC. Although the signal of sTnI is weak, it seems to lack the small 24 h peak that fTnI has, and its overall accumulation pattern initially resembles more that of c/sTnC.

Proteins of L6 cells. L6 cells were cultured in the same way and the cells behaved as described previously, although the cells were plated in 24 well plates instead of 100 mm plates.

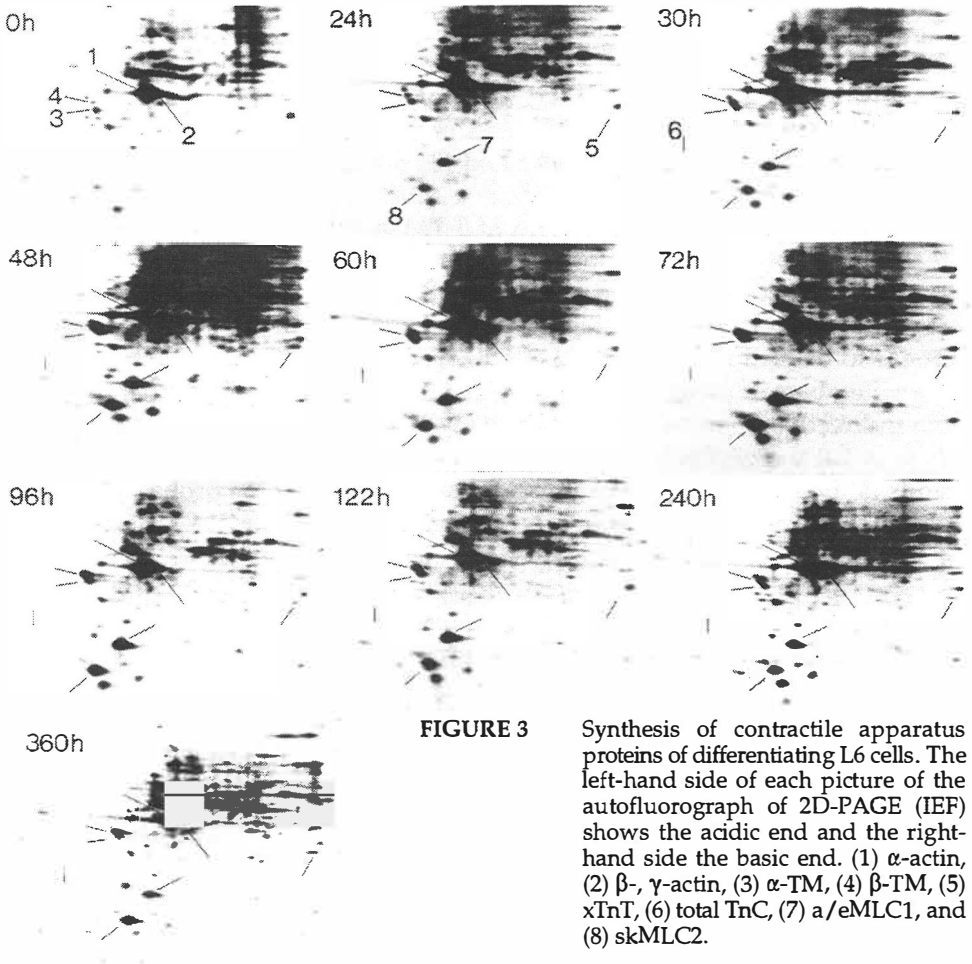

FIGURE 3 Synthesis of contractile apparatus proteins of differentiating L6 cells. The left-hand side of each picture of the autofluorograph of 2D-PAGE (IEF) shows the acidic end and the right-hand side the basic end. (1) α-actin, (2) β-, γ-actin, (3) α-TM, (4) β-TM, (5) xTnT, (6) total TnC, (7) a/eMLC1, and (8) skMLC2.

The results of the protein synthesis of α-actin and α-TM support the results of previously described mRNA study: synthesis of α-actin and α-TM is initially more pronounced and it seems to be initiated slightly earlier than the members of troponin complex. The intensity of accumulation of cellular mRNA and the respective protein of β-TM, instead, does not seem to be very comparable. The intensity of the protein synthesis of β-TM resembles of α-TM, and after 60 h of differentiation its synthesis seems to be more pronounced than that of α-TM, although initially its expression is less than of α-TM.

L6 cells have been claimed to express only one isoform of fTnT (Garfinkel et al. 1982, Breitbart & Nadal-Ginard 1987), but due to the embryonic character of L6 cells, they may also express c/eTnT, which has been detected in C2C12 cells. C2C12 cells, instead, may have also fTnT isoforms, but whether these cell lines also express sTnT isoforms is not known. In addition, the study of Vandromme et al. (1992) supports the existence of other than only one TnT isoform in L6 and in C2 cells. According to the location, L6 cells seem to express the sTnT2 isoform (Fig. 3), whereas C2C12 cells appear to express sTnT1 (Fig. 4) (Mikawa et al. 1981,

Härtner & Pette 1990, Leeuw & Pette 1993). These two proteins did not exist in myoblasts either. However, in the same area as sTnT2 are located some fTnT isoforms; thus there is a possibility that the protein is also an fTnT isoform, although it was not detectable with fTnT specific JLT12 antibody (Sigma; Lin et al. 1984). Due to the uncertainty of these proteins, they were named as xTnTs in both cell lines. The fTnT protein of L6 cells shown in Fig. 14 was localized instead by western blot analysis of 2D-PAGE (NEPHGE) of radioactively labeled proteins (data not shown) as described in chapter 4.5.4 (Protein gel electrophoresis: Immunoblot analysis). Nevertheless, the results show that the signal of fTnT is slightly weaker than that of xTnT, and also that is weaker than that of TnI (Fig. 14) or TnC (Fig. 3). This also supports the existence of an additional TnT form. Moreover, the accumulation of cellular mRNA of (f)TnT was pronounced compared, especially, to TnI (Fig. 2, Fig. 13). The intensity of the total signal of fTnT and xTnT as described above, instead, shows more similarity to TnI (Fig. 14). However, the fairly weak signal of xTnT is detectable earlier than even weaker signal of total TnC (Fig. 3).

The synthesis pattern of total TnC protein has similarities to the accumulation patterns of both fTnC and c/sTnC cellular mRNAs up to around 72 h of differentiation. After the 72 h timepoint, slight protein accumulation does not seem to be in line with to the marked mRNA accumulation of fTnC, or at the 240 h and 360 h timepoints with c/sTnC - the overall protein synthesis of TnC appears to be weak during differentiation. In another comparable L6 experiment, in which separation of fTnC and c/sTnC proteins was succesful (data not shown), fTnC was already detectable in 24 h differentiated cells, comparable to transcription. The c/sTnC protein, instead, became apparent at the 36 h timepoint, which differs from its transcriptional pattern. Protein synthesis of c/sTnC, however, was more than that of fTnC up to 72 h of differentiation, after which fTnC turned into a major protein at least up to the 96 h timepoint. Nevertheless, the synthesis of fTnC did not match to its marked cellular mRNA accumulation after 72 h, in the same way as the synthesis of c/sTnC was not related to its cellular mRNA accumulation after 60 h of differentiation.

Translation of a/eMLC1 and skMLC2 was also estimated although none of the members of the thick filament were examined at transcriptional level. Both MLCs have similar initial expression patterns with a similar intensity up to 48 h, whereas later on, the synthesis of skMLC2 is slightly less than that of a/eMLC1.

Cellular mRNAs of C2C12 cells. C2C12 cells were initially plated to approximately 40% confluency, and put into to differentiation medium after around 48 h or 72 h of incubation, when the cultures reached 80-90% confluency. This was taken as the 0 h timepoint for the differentiation experiment. The first small myotubes became apparent at about 48 h of differentiation. Fusion was most marked at about 60 h differentiated cells, and after 122 h of differentiation very little additional fusion occurred; unlike the L6 cells, the C2C12 cells never achieved 100% fusion. The L6 cells also fused faster than the C2C12 cells. However, in 122 h differentiated cultures, approximately 60-70% of the plating area was covered with multinucleated myotubes, whereas the remaining areas were covered with myoblast-like myocytes, even in 240 h differentiated cultures. The 240 h

differentiated myotubes also had contraction ability, but, shortly after, some of the myotubes started to severely vacuolize and die, and the unfused myoblast-like cells started to divide slowly, fuse, and replace the dead myotubes. Thus the experiment describes more than one life-cycle of C2C12 cells, where the timepoint 360 h shows fairly non-synchronous myotubes.

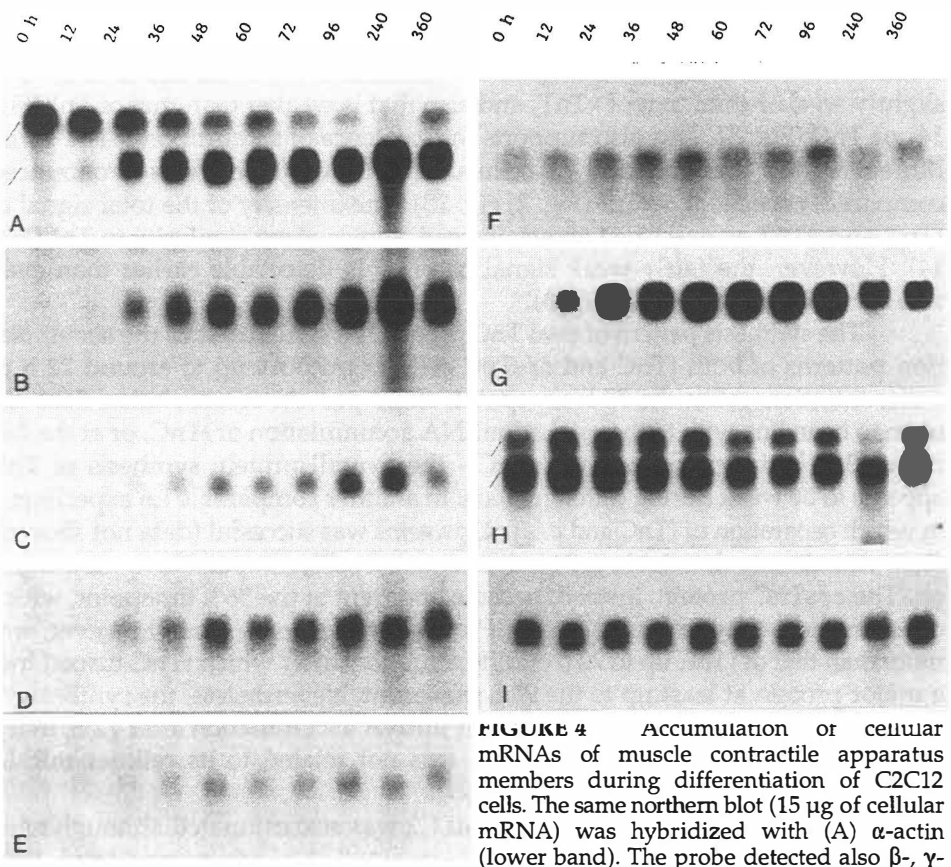

FIGURE 4 Accumulation of cellular mRNAs of muscle contractile apparatus members during differentiation of C2C12 cells. The same northern blot (15 μg of cellular mRNA) was hybridized with (A) α-actin (lower band). The probe detected also β-, γ-actin (higher band). (B) TnT, (C) fTnC, (D) c/sTnC (estimated from different membrane, where 5 μg of mRNA from the same experiment was used), (E) fTnI, (F) MyoD, (G) myogenin, (H) c-Jun, which detected two variants, and (I) GAPDH.

The cellular mRNA of α-actin becomes detectable at around 12 h and its transcription seems to be more pronounced than that of TnT (see section: Cellular mRNAs of L6 cells), which is hardly detectable at that timepoint, although their signals have otherwise similar intensity. Accumulation of α-actin seems to increase steadily from 24 h up to 48 h of differentiation, after which it remains at a rather steady level. In contraction capable cells, differentiated for 240 h, its expression is at its highest. Unfortunately estimation of α-TM in this particular experiment failed, but in another comparable study (data not shown), accumulation of α-TM had some similarities with that of α-actin: the accumulation of α-TM increased steadily up to 72 h of differentiation and also remained steady between

72 h and 96 h of differentiation, after which it showed a marked increase, at least up to 240 h timepoint.

Expression of the troponin complex appears to be initiated with fTnI also in C2C12 cells, although its signal is very faint at the 12 h timepoint. The signal of fTnI is initially comparable to that of TnT, the overall accumulation of which is more rapid and pronounced than that of fTnI during differentiation. Both TnC forms, instead, become detectable later, at the 24 h timepoint, and their expression is also weaker than of TnT. However, the observation of an also initially more pronounced expression of c/sTnC is from a different northern blot. The cellular mRNAs used were from the same differentiation experiment; however the amount used was only 5 µg compared to the 15 µg used in the main experiments.

The general patterns of accumulation of the cellular mRNAs of MyoD, myogenin and Jun during differentiation, as well as their intensities, differ from each other. The signals of myogenin and Jun are strong, whereas the expression of MyoD does not seem to be marked. Moreover, the accumulation of these factors does not completely resemble any of the studied contractile apparatus mRNAs, although, the expression of MyoD seems to have some similarity with fTnI up to around 48 h timepoint. Nevertheless, the cellular mRNA levels of MyoD increase slightly between 0 h and 12 h of differentiation, and more between 12 h and 24 h. The mRNA level stays fairly steady from 24 h to around 48 h, after which the accumulation of MyoD decreases slightly up to the 60 h timepoint, as do most of the studied contractile apparatus mRNAs. In contraction capable cells differentiated for 240 h, the expression of MyoD is at about the same level as at 0 h, whereas in 360 h cultured cells the mRNA level resembles that of 12 h differentiated cells.

Transcription of myogenin is initiated earlier than the examined contractile apparatus genes: a very faint signal is apparent already at the 0 h timepoint. The general accumulation pattern of myogenin, however, differs from that of MyoD or Jun. Its synthesis increases between 12 h and 48 h of differentiation. Thus up to about 72 h of differentiation myogenin seems to have an overall similarity with all the other contractile apparatus members. After 96 h of differentiation, the expression of myogenin decreases markedly, and in 360 h cultured cells, the expression is only slightly higher than in 240 h differentiated cells.

The expression of the activity of MyoD and myogenin regulating Jun was also estimated. The cDNA probe detects two variants of Jun, which are both expressed in these cells. Although studies have indicated that initiation of differentiation requires down-regulation of Jun, the results show that the transcription of both variants is increased slightly during the first 12 h of differentiation, thereafter decreasing slightly up to 72 h of differentiation. In contracting capable 240 h differentiated cells its expression is reduced, especially the higher band. Interestingly, however, after 36 h, the expression of MyoD and Jun shows some similarity (Fig. 6), which is not apparent with Jun and myogenin.

Proteins of C2C12 cells. In these experiments C2C12 cells were grown in the same way and the cells behaved as described previously. The cells were plated in 24 well plates instead of 10 cm plates.

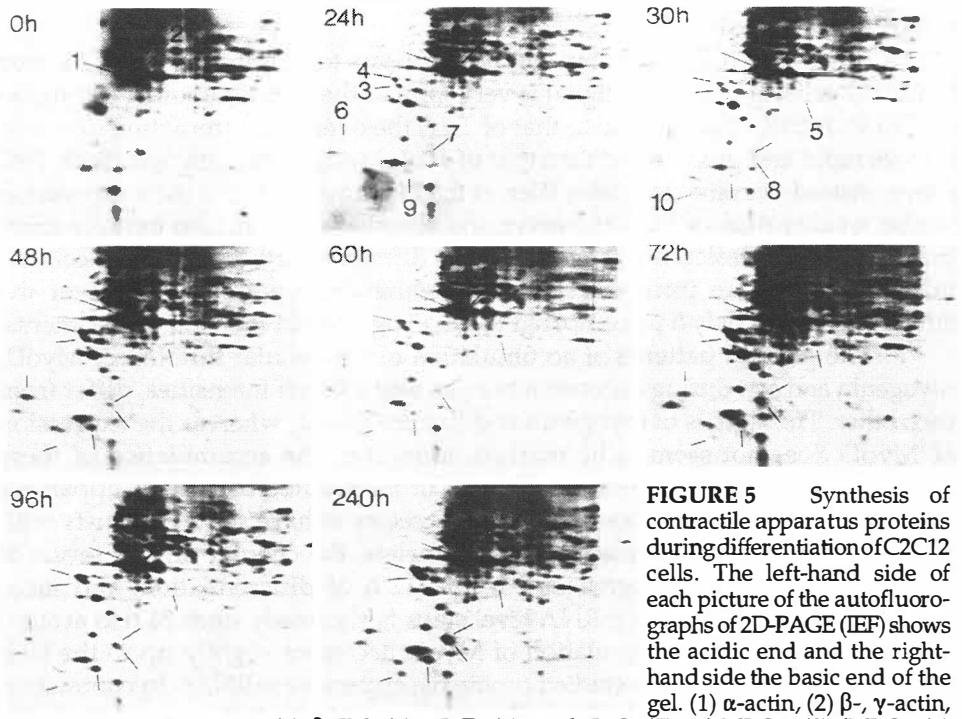

FIGURE 5 Synthesis of contractile apparatus proteins during differentiation of C2C12 cells. The left-hand side of each picture of the autofluorographs of 2D-PAGE (IEF) shows the acidic end and the right-hand side the basic end of the gel. (1) α-actin, (2) β-, γ-actin, (3) α-TM, (4) β-TM, (5) xTnT, (6) total TnC, (7) v/sMLC1, (8) fMLC1 (9) skMLC2, and (10) fMLC3.

Proteins of muscle α-TM and β-TM become apparent in 24 h differentiated cells, and their synthesis shows increase up to 48 h, when the first small myotubes are detectable (Fig. 5). Their synthesis shows a reduction at the 60 h timepoint, which is probably due to the pronounced fusion of the cells. In the case of α-actin, instead, it is not possible to estimate it by the naked eye from the autofluorograph shown in Fig. 5.

The study of the protein synthesis of the members of the troponin complex is very partial: proteins of fTnC and c/sTnC did not separate in any of the gel runs of the performed experiments, the TnI forms were not examined, and the TnT forms expressed in the C2C12 cells are imperfectly known, as already described. Nevertheless, synthesis of xTnT is very faintly detectable already after 24 h. Its expression seems to increase fairly steadily up to 72 h of differentiation. At the 240 h timepoint, the synthesis of xTnT seems to be as its highest. The results show a marked fluctuation in total TnC synthesis throughout differentiation and maturation. Total TnC becomes weakly apparent at 24 h timepoint, but more pronounced synthesis is initiated later, after 30 h of differentiation. The expression peaks exist at 48 h, 72 h, and 240 h. Thus the pattern of expression of TnC and TMs share some resemblance, but xTnT differs from them up to the 60 h timepoint.

The initial MLC forms seem to be v/sMLC1 and skMLC2, which are faintly detectable at the 24 h timepoint. Moreover, v/sMLC1 is the major MLC1 protein throughout differentiation. The transcriptional products of the fMLC1/3 gene

become apparent later: fMLC1 and fMLC3 are very weakly detectable at the 30 h timepoint. A strong fMLC1 signal becomes apparent surprisingly late, in 72 h differentiated cells. The production of fMLC1 seems to be slightly less than that of v/sMLC1, whereas the synthesis of the fMLC3 protein is around only a half of that of the fMLC1 protein throughout differentiation and maturation.

Summary of the results. The signals from the autoradiograms shown in Fig. 2 and Fig. 5 and the autofluorographs shown in Fig. 3. and Fig. 4 were image-analysed, and the results are presented in Fig. 6 and Fig. 7. However, in Fig. 6 the curves of Jun are scaled down tenfold from the intensity of the autoradiograph shown in Fig. 3. Also, the α-actin protein in the C2C12 cells, which was not possible to estimate from the autofluorograph by the naked eye (Fig. 5), was estimated by image analysis and is shown in Fig. 7. Nevertheless, the presentation facilitates the comparison of the different members of the contraction apparatus to each other during differentiation as well as cellular mRNA accumulation to the respective proteins, and it also illuminates the similarities and differences between L6 and C2C12 cells.

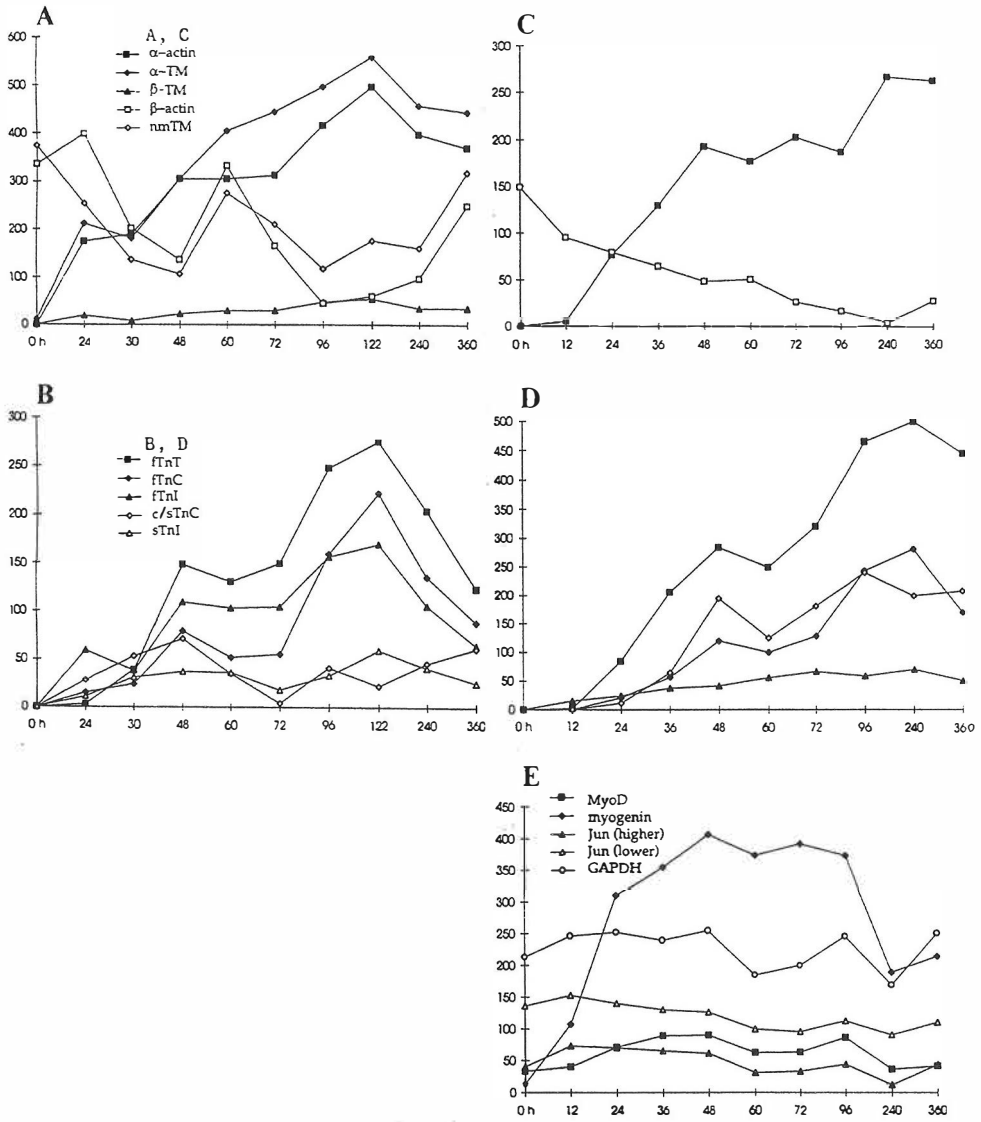

FIGURE 6 Accumulation of muscle contractile apparatus cellular mRNAs during differentiation and maturation of L6 and C2C12 cells. The curves in panels (A) and (B) describe the results of image-analyses of L6 cells, and the curves in panels (C), (D), and (E) are from C2C12 cells.

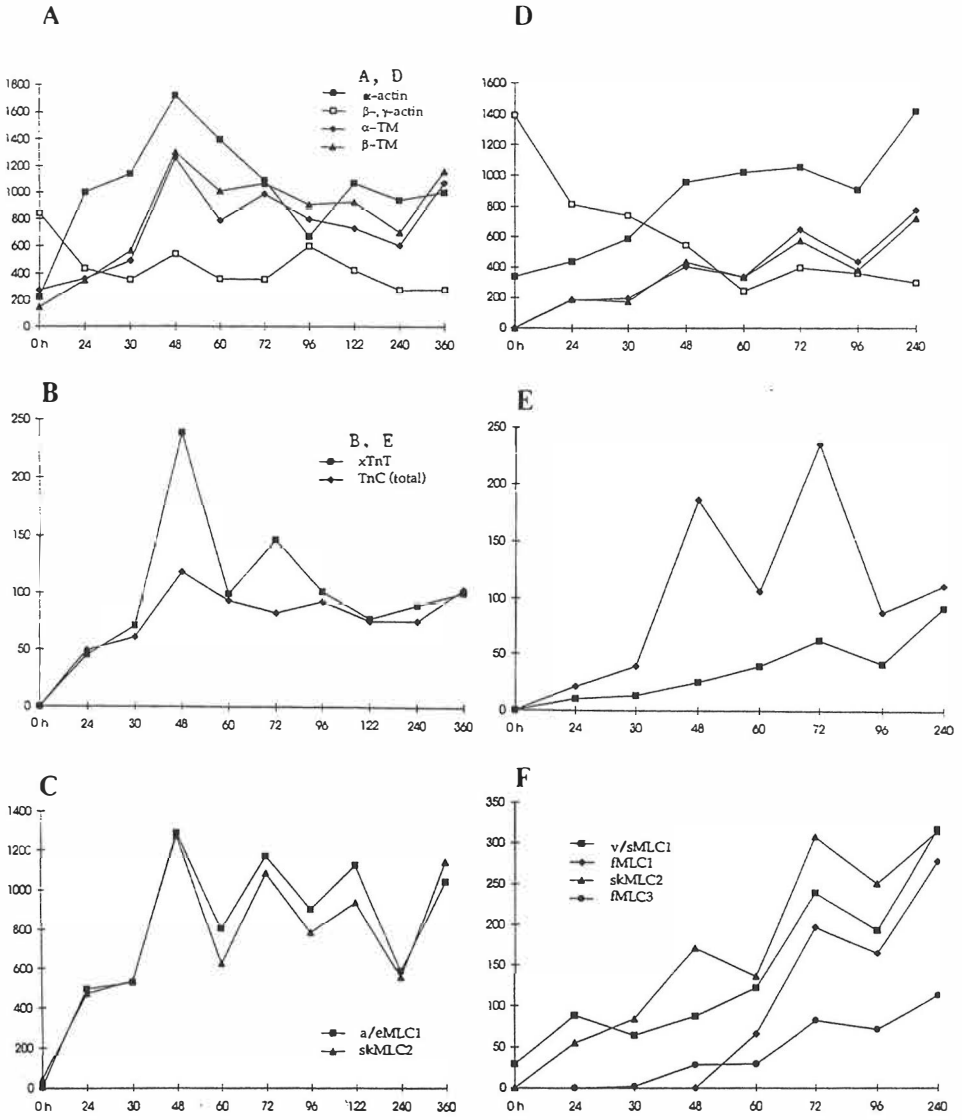

FIGURE 7 Protein synthesis during differentiation and maturation of L6 cells and C2C12 cells. Panels (A), (B), and (C) describe the results of L6 cells, and (D), (E), and (F) are from C2C12 cells.

Discussion:

Fusion and gene expression. Transcription of β-actin and nmTM forms showed an increase in 60 h differentiated L6 cells, after which expression markedly decreased. An increase in β-actin, but much weaker, was apparent also in C2C12 cells (Fig. 6). Protein synthesis of β-actin, on the other hand, was approximately at its lowest point during that time in both cell lines (Fig. 7). Nevertheless, during the fusion of muscle cells, debris generally becomes detectable on the surface of developing myotubes and in culture medium; a similar release was apparent also in C2C12 cultures, but especially in L6 cultures at around 60 h of differentiation. Some of the debris released consists of β- and γ-actin proteins (Rubenstein 1990). Thus the increase in cellular mRNA level may indicate some compensatory mechanisms in an effort to replace the released proteins, but it is possible that there also exist some translational control mechanisms. In addition, cell fusion probably changes the balance and the activity of the transcriptional factors, which may also influence the expression of the non-muscle forms of actin and TM. However, contrary to the observed increase in cellular mRNA accumulation at the 60 h timepoint, transcriptional down-regulation of β-actin has been shown to occur during differentiation. This has been suspected to be mediated through the regulatory sequence near the 3'-end of the β-actin gene, within the 40 bp upstream of the polyadenylation signal (DePonti-Zilli et al. 1988).

All the MyoD-family factors have growth-suppressing functions, as described in chapter 2.3 (Differentiation and regulation of some of the myogenic factors). Decrease in Jun, instead, has been described to be required for the initiation of differentiation, partially by its ability to regulate the activity of MyoD-family factors as described in chapter 2.3.3 (Cooperation and hetero-oligomerization of MyoD-family of transcription factors with other proteins). C2C12 cells express all the MyoD-factors, but expression of Myf-5 and MRF-4 has been described as low. L6 cells, on the other hand, lack MyoD, and expression of MRF-4 is apparently very weak (Braun et al. 1989, Miner & Wold 1991, Braun et al. 1992, Muthuchamy et al. 1992). Nevertheless, the results indicated that expression of MyoD in C2C12 cells is not pronounced either (Fig. 4), which is in line with the results described by Miller (1990). The low expression may be partially related to the culture conditions used, but since MyoD is suspected of being involved in the regulation of fusion (Brennan et al. 1990, Block & Miller 1992, Edmondson & Olson 1993), the low expression may be one reason why these C2C12 cells fused to multinucleated myotubes only moderately. Expression of myogenin (Fig. 4, Fig. 6), instead, was pronounced and increased sharply after the 12 h timepoint. Thus it did not seem to have or had only very little influence on the fusion process or expression of MyoD.

The expression pattern of MyoD and Jun showed some similarity after 36 h differentiation (Fig. 6). Interestingly, though, expression of Jun and MyoD is partially regulated by Sp1: both jun and MyoD have Sp1-binding sites - MyoD at least two, and in addition to these, one Ap-2-site. It has been shown that these Sp1-sites of MyoD are not enough for its efficient transcription as described in chapter 2.3.2 (Myogenic factors and their expresssion). Sp1 activity is regulated by Sp1-I, and RB can interact with Sp1-I and thus involve keeping Sp1 in active

form as described in chapter 2.2.5 (CCACCC-sequence and some other elements). Thus RB may regulate expression of Jun and MyoD through their Sp1-sites. The RB:Sp1-I interaction may also reduce the free RB pool. Nevertheless, as described, the accumulation of cellular mRNA of myogenin was pronounced (Fig. 4), and it did not seemed to have a marked influence on either increasing the expression of MyoD or effecting the fusion process. The lack of influence on MyoD expression suggests that it may mainly exist in an inactive form. This in turn may be influenced by highly expressed Jun, which may also have a preferential influence on MyoD-factors.

The results suggested (Fig. 6, Fig. 7) further that initiation of transcription and translation of contractile apparatus protein genes occurs slightly earlier than fusion in both cell lines. In the beginning of the fusion process at close to 48 h of differentiation, expression of the gene products increased markedly, especially in L6 cells. The overall pattern of gene expression showed similarity in both cell lines: both shared for instance a decrease in expression at the 60 h timepoint, which is probably due to the pronounced fusion process around that time. The cellular mRNA accumulation and protein synthesis, however, showed more agreement in C2C12 cells than in L6 cells throughout differentiation and maturation. The fusion and maturation process was slower in C2C12 cells, which was apparent also at the cellular mRNA and protein levels.

Muscle gene expression. *Actin.* The inital α-actin form in many skeletal muscle cell types, also in C2C12 cells but not in L6 cells, is αc-actin, but later during maturation expression of αc-actin is down-regulated and αsk-actin becomes the major actin form. The relative proportions of αsk-actin and αc-actin depend on the species, muscle type and stage of development (Caravatti et al. 1982, Bains et al. 1984, Vanderkerckhove et al. 1986). However, expression of α-actin seemed to be initiated more markedly, and thus in all likelihood slightly earlier, than for example TnT of the members of troponin in L6 and in C2C12 cells, according to initial intensity (Fig. 2, Fig. 4, Fig. 6, Fig. 7). The reason for so pronounced an expression of α-actin may lie in its vast number and selection of regulatory sequences as described in chapter 2.1.1.1 (Actin). This selection may also provide more sensitivity to varying conditions to ensure its production, which is essential to cells (Izant 1989, Weintraub 1990). Nevertheless, the level of cellular mRNA of α-actin, and the respective protein showed rather similar increase up to 48 h timepoint in L6 cells (Fig. 6, Fig. 7), whereas, later on, protein synthesis appeared to be less than the accumulation of cellular mRNA up to the 96 h timepoint. The mRNA/protein comparison suggests that α-actin is translated up to around the 48 h timepoint more efficiently than later on during differentiation. In C2C12 cells, on the other hand, the accumulation of cellular mRNA and protein of α-actin was fairly similar throughout the differentiation. The differences between these cell lines may be partially due to α-actin forms.

Studies have shown that CArG-box and Sp1-element are the key motifs for αc-actin expression: a high level of transcription of αc-actin requires CArG-box-binding factors (Muscat et al. 1988, Sartorelli et al. 1990). As described in chapter 2.1.1.1 (Actin), the promoter region of αsk-actin also has CArG-boxes. The coregulation of these genes is suggested to depend on the same transcriptional

factor (Muscat et al. 1988). Moreover, high expression of myogenin in C2C12 cells (Fig. 4) suggests indirectly that the cells have at least moderate CArG-box-recognizing SRF activity, too: it has been shown that inhibition of SRF also inhibits expression of myogenin (Santoro & Walsh 1991, Vandromme et al. 1992). The initial activation of the αc-actin gene, however, may need its E-box and MyoD expression, whereas other MyoD-family members seems to be incapable of activating it (Muthuchamy et al. 1992). Thus the lack of MyoD may be the reason for the lack of αc-actin expression in L6 cells, although, according to Hollenberg et al. (1993), even MyoD is not capable of activating αc-actin. It is probable that expression of αc-actin is also modulated by other, unknown factors, which are not abundant in skeletal muscle (Muscat et al. 1988, Alonso et al. 1990, Chow & Schwartz 1990, French et al. 1991).

Why αc-actin gene expression is reduced in skeletal muscle, and also in C2C12 cells, during maturation is unknown (Buckingham & Minty 1983, Minty et al. 1986). One reason may lie in modification to an inactive form, but the transcription environment may also change into a direction favoring more αsk-actin expression in differentiating cells. As mentioned earlier, the increase in the transcription of β-actin at the 60 h timepoint (Fig. 6) may indicate a change in the balance of the factors influencing transcription. Thus the expression or the activity of the initial factors may decrease around that time due to fusion and changes in signal transduction. These alterations in turn may activate other more downstream factors, which can induce and change, or alternatively, become more efficient, increasing contractile apparatus gene expression, such as αsk-actin, but which are not sufficient to maintain expression of the initial genes, such as αc-actin.

Tropomyosin. The results suggest that expression of α-TM is initiated more markedly as well as slightly earlier than that of members of the troponin complex in L6 cells (Fig. 2, Fig. 6, Fig. 7). The cellular mRNA level of α-TM and the respective protein increased up to the 48 h timepoint rather similarly (Fig. 6, Fig. 7). However, protein synthesis later appeared to be less than an accumulation of cellular mRNA up to the 96 h timepoint, as was the case with α-actin. In C2C12 cells, protein synthesis of α-TM (Fig. 7) seemed to be also slightly less than suggested by its mRNA accumulation, especially at the 96 h timepoint (data not shown). The results suggest that some type of translational regulation with α-TM may also occur.

The initiation and accumulation of the cellular mRNAs of α-actin, α-TM and β-TM showed similarities during differentiation, which suggests transcriptional coordination between them in both cell lines (Fig. 6). As described in chapter 2.1.1.2 (Tropomyosin), the promoter of the α-TM gene resembles housekeeping promoters. Therefore, the splicing factors that exist in cells mainly determine the α-TM isoforms that are produced from the primary transcripts. The general resemblance with α-actin, however, may be based at some level on CCACCC-type elements and the M-CAT sequence, but since the transcription influencing sequences of TMs are fairly poorly known, it is not possible to make definite estimations (Lees-Miller & Helfman 1991). One option to coordination may additionally be that the same factors that induce expression of α-actin may

induce expression of the splicing factors required for producing muscle-specific TM mRNAs.

Troponin T. It is known that muscles express preferential combinations of TnT forms during development (Breitbart & Nadal-Ginard 1987, Härtner et al. 1988, Briggs et al. 1990). As mentioned earlier in Results, the TnT studies were partial, since the TnT expressed forms are incompletely known in both cell lines. In the case of fTnT, however, the preferential expression of its isoforms depends partially on the factors influencing splicing and their expression, as with α-TM (Andreadis et al. 1987). Nevertheless, the results showed that pronounced accumulation of (f)TnT is initiated slightly later compared to α-actin and α-TM (Fig. 6). Its expression had similarities to that of α-actin in L6 cells, whereas in C2C12 cells there were some differences. The overall accumulation of cellular mRNA of TnT was fairly similar in both cell lines during differentiation (Fig. 6), although the signal of TnT was detectable already in 12 h differentiated C2C12 cells and also pronounced transcription seemed to start earlier in C2C12 cells than in L6 cells (Fig. 2, Fig. 4). The results also suggested transcriptional coordination at least with the fast members of the troponin complex, especially with fTnC (Fig. 2, Fig. 4, Fig. 6). The synthesis of the detected proteins, instead, bore more resemblance to α-TM and β-TM than to α-actin or to total TnC in both cell lines (Fig. 7).

The important sequences for the expression of the rat fTnT gene are not fully characterized, as described in chapter 2.1.1.3 (Troponin T). The transcriptional similarity and intensity with α-actin and with α-TM in L6 cells may partially depend on the M-CAT sequence - α-TM also has a potential M-CAT sequence (CATACCTT), whereas the coordination with the fast members of the troponin complex may rely more on CCACCC-like sequences. The fTnT, α-actin, and α-TM genes also have an E-box/es, but their involvement on the regulation and coordinated expression of these genes is unknown.

Troponin C. In the beginning of differentiation, fast skeletal muscle expresses both fTnC and c/sTnC genes. Synthesis of c/sTnC ceases in this muscle type later, whereas in slow skeletal muscle, expression of c/sTnC increases and becomes the major gene product (Parmacek & Leiden 1989, Parmacek et al. 1994). In C2C12 cells, c/sTnC appears to be the major protein, although they also express fTnC (Fig. 16). In L6 cells, on the other hand, both genes seem to be expressed approximately equally (data not shown). The results discussed above for the cellular mRNA accumulation of TnCs, however, are from two different membranes, but the samples used were from the same L6 and C2C12 differentiation experiments. Thus it is not possible to draw very definite conclusions and comparisons from the results. Nevertheless, both TnCs were detectable at the 24 h timepoint (Fig. 2, Fig. 4), and expression of c/sTnC (5 µg mRNA) seemed to be initially more marked than that of fTnC and TnT (15 µg mRNA) in both cell lines. Accumulation of mRNAs of c/sTnC and fTnC and protein synthesis of total TnC were also similar up to around the 60 h timepoint (Fig. 6, Fig. 7). Thereafter, transcription of both TnCs appeared to be more pronounced than total protein synthesis, although after 96 h transcription of c/sTnC and total protein synthesis started to show some respectivity again. Comparison of c/sTnC cellular mRNA with its respective protein (data not shown) suggest that translation of c/sTnC is

inefficient: cellular mRNA of c/sTnC was detectable already at the 24 h timepoint, whereas protein synthesis seemed to start slightly later. Otherwise the expression of both gene products seemed to follow each other up to around 60 h of differentiation. After that, transcription of c/sTnC decreased up to the 72 h timepoint, whereas protein synthesis increased during the same time to the same level as it had been around the 48 h timepoint. The delay in protein synthesis again suggests inefficient translation of c/sTnC. Comparison of the transcriptional pattern of fTnC to its protein synthesis (data not shown) also suggests some translational control: the protein synthesis of fTnC appeared to be very moderate after the 72 h timepoint, whereas accumulation of cellular mRNA continued the increase from 72 h to 122 h of differentiation.

In C2C12 cells, the comparison of the accumulation of cellular mRNAs of c/sTnC and fTnC to total TnC protein synthesis shows similarity up to the 72 h timepoint. At the 96 h timepoint, the protein synthesis showed a decrease, whereas the cellular mRNAs of both isoforms increased. Total protein synthesis of TnC was again increased at the 240 h timepoint (Fig. 5), similarly to the cellular mRNA of fTnC. The cellular mRNA of c/sTnC, instead, showed a slight decrease at that timepoint, although its intensity was similar to that of fTnC. However, the total protein synthesis of TnC was lower than the cellular mRNAs of both of them suggested. The difference also indicates some translational control in TnC synthesis in C2C12 cells.

The regulatory sequences of the TnC forms are described in chapter 2.1.1.4 (Troponin C). The initial similarity in expression of c/sTnC and fTnC may be due to the CCACCC-box of c/sTnC and the Ap-2-site of fTnC; the Ap-2-site can also bind some of the CCACCC-box-recognizing factors, for example Sp1. Generally the CCACCC-sequence seems to be able to bind a variety of transcription influencing factors as described in chapter 2.2.5 (CCACCC-sequence and some other elements), but probably with a different affinity, depending in all likelihood on the context of the sequence. As mentioned earlier, it has been suggested that cooperative interactions exist between CCACCC-box and some other elements, for instance CCACCC-motif and T+A-rich element are required for transcriptional activation of c/sTnC (Bassel-Duby et al. 1994, Chen et al. 1994, Parmacek et al. 1994).

The c/sTnC gene also has a MEF-3-site, flanked on either side by the CCACCC-motif, but the binding factor/s of MEF-3 are poorly characterized. MEF-3 element or related sequences are present for instance in enhancers of quail TnI and in rat cMLC2, but also in a promoter region of murine myogenin, and it appears usually to be located near an MEF-2-binding site (Parmacek et al. 1994). However, the importance of MEF-3 for the expression of myogenin is not understood. The results showed, though, that the accumulation of myogenin was marked, especially after 12 h of differentiation (Fig. 4). Thus if MEF-3 has importance for myogenin expression, MEF-3-site binding factors are likely to already exist around same time as MEF-2 factors. If this is the case, the reason for the fairly late initiated accumulation of c/sTnC cellular mRNA may not be for instance MEF-3, but instead, CCACCC-element binding factor/s. This type of more downstream factor may be for instance MNF, which binds to for example the myoglobin CCACCC-element with higher affinity than does Sp1 (Bassel-Duby

et al. 1994). Thus up-regulation of MNF may turn transcription of c/sTnC more efficient. It is not known, though, whether MNF requires other proteins to form hetero-oligomeric complexes, but it may need interactions with factors such as MEF-2 to mediate efficient trans-activation in muscle cells. Nevertheless, there also exist other CCACCC-motif-binding factors in developing myotubes, as described in chapter 2.2.5 (CCACCC-sequence and some other elements).

Troponin I. Neuronal input is one important determinant in fTnI versus sTnI specific gene expression in skeletal muscle, as is the case also with other members of the troponin complex (Koppe et al. 1989, Härtner & Pette 1990, Leeuw & Pette 1993). Both fTnI and sTnI genes are claimed to be expressed at comparable levels in differentiated L6 and C2 myotubes (Koppe et al. 1989). The results of studies of L6 cells have indicated, however, that expression of sTnI is slightly less than that of fTnI, at least in L6 cells (Fig. 2, Fig. 13). In C2C12 cells only fTnI was determined (Fig. 4), and the results suggested that the initial member of a troponin complex is fTnI. It was very faintly detectable already at the 12 h timepoint and was slightly stronger than that of TnT, the expression of which already markedly exceeded fTnI already at the 48 h timepoint (Fig. 4, Fig. 6). Although the signal of fTnI was too weak to permit definite conclusions, its expression had a similarity with that of c/sTnC. In L6 cells, on the other hand, accumulation of fTnI had an initial resemblance to α-TM with a small initial transcriptional peak at the 24 h timepoint, but later on its expression more resembled that of α-actin and TnT (Fig. 6). It also showed similarity with fTnC and TnT (Fig. 2). The accumulation patterns in L6 cells of fTnI and the very faintly detectable sTnI also showed some resemblance during differentiation (Fig. 2, Fig. 6).

Regulation of fTnI expression is discussed on the basis of the quail fTnI gene as described in chapter 2.1.1.5 (Troponin I), since only the cDNA sequences of mouse fTnI and rat sTnI are known. However, respective contraction apparatus genes are generally highly conserved in vertebrates, as seems to be the case also with their regulatory sequences, at least in some cases (Kurabayashi et al. 1990, Jin et al. 1992). The quail fTnI gene shares homologous regions also with for example the rat α-actin gene, located upstream from their promoters. The sequences align into a G+C-rich region and a T+A-rich region flanking a 4 bp central core region of a more variable sequence (Baldwin et al. 1985, Nikovits 1990). In addition, the fTnI gene has 5' and 3'-flanking transcriptional regulatory elements, of which the 5' flanking region may have influence, especially at the beginning of differentiation. However, as described earlier, IRE is required to elicit the full expression of the fTnI gene. It is composed of E-box and CCACCC-sequence owning regions I and II, and it seems that MyoD-family factors, at least initially MyoD or Myf-5, must interact with the binding proteins of regions I and II (Yutzey et al. 1989, Nikovits et al. 1990, Lin et al. 1991). It seems, though, that MRF-4 is incapable of activating the transcription of TnI (Yutzey et al. 1990, Lin et al. 1991, Mak et al. 1992). Thus the initial peak of cellular mRNA of fTnI in L6 cells (Fig. 2, Fig. 6) may be due the activation of either Myf-5 or myogenin. On the other hand, expression of myogenin showed a marked increase around 12 h and 24 h differentiated C2C12 cells, but a similar increase was not apparent with fTnI (Fig. 4). This suggested that the pronounced transcription of fTnI is activated by

a factor other than myogenin. Myogenin may also exist in a fairly inactive form in C2C12 cells. However, whether the marked transcription of fTnI requires MyoD or Myf-5 in L6 and C2C12 cells is not known, but if this is the case, the increase in L6 cells may depend on Myf-5 and its activation. The reason for the low expression of fTnI in C2C12 cells, on the other hand, may be due to the low expression of MyoD.

Myosin heavy and light chains. It is known that myotubes formed from various myoblasts express characteristic MHC forms (Miller 1990). EMHC is detectable in small, often mononucleated muscle cells, whereas late myosins are predominant in large, striated myotubes (Silberstein et al. 1986, Stockdale 1992). Regulation of MHC expression is partially intrinsic (Weydert et al. 1987), since C2C12 cells also express MHC isoforms in a certain order: 1 d differentiated cells express only the eMHC or fetal isoform of MHC, which may differ from the predominant eMHC of L6 cells. The fetal form normally disappears in vivo, but in C2C12 myotubes it remains, although its expression falls by 75% from its 1 d value by 8 d. Replacment proteins to the fetal MHC form are pMHC, MHCIIB and v/s β-MHC (Silberstein et al. 1986, Weydert et al. 1987, Miller 1990). However, the alteration in the concentration of mitogens, endocrinic stimulation, such as thyroid hormones, as well as neural stimulation can modulate the autonomous expression of MHC (Jolesz & Sréter 1981, Pette & Vrobova 1985, Izumo et al. 1986, Emerson, Jr. & Bernstein 1987). Neither Myf-5, MRF-4, nor myogenin, on the other hand, is sufficient for a complex pattern of MHC expression, but these factors may indirectly influence it (Miller 1990, Thompson et al. 1991). It seems, though, that expression of some of the skeletal MHC genes is also regulated at the translational level (Khandekar et al. 1984): the 3' end of the chicken skeletal MHC gene seems to encode a small translational control mRNA (tcRNA), which appears to influence MHC translation, apparently by acting as an antisense RNA (Dasgupta et al. 1986, van der Krol et al. 1988). Whether this is a common regulatory mechanism among MHCs or other members of the contractile apparatus is unknown.

MHC and MLC genes appear to have different responses to developmental and physiological signals, since the expression of particular MLC and MHC has shown very little correlation (Barton & Buckingham 1985). Distinct MLC forms are known to be expressed in functionally specialized muscles as well as at specific embryonic stages and adult physiological states (Barton & Buckingham 1985, Emerson, Jr. & Bernstein 1987). Nevertheless, the characterization study of L6 and C2C12 cells did not include studies of MHC forms, and MLC forms were examined only at the translational level. In the case of L6 cells, the results suggested that synthesis of a/eMLC1 and skMLC2 starts fairly simultaneously (Fig. 7). Their expression was rather similar throughout differentiation, even sharing similarities with the translation of TMs, especially of β-TM. Thus expression of these MLC genes may be partially regulated by the same factors as TMs (Andreadis et al. 1987). The regulatory sequences of the MLC genes are described more detailed in chapter 2.1.1.7 (Myosin light chains). However, the selection of a/eMLC1 regulatory sequences has similarities with of α-actin, thus the marked synthesis of a/eMLC1 may be based partially on the same factors,

such as CArG-box-binding factors (Uetsuki et al. 1990). The regulation of skMLC2 expression, on the other hand, is poorly characterized.

The initial MLC proteins in C2C12 cells appeared to be v/sMLC1 together with skMLC2 (Fig. 5). Synthesis of skMLC2 and v/sMLC1 also showed similarity to TM during differentiation (Fig. 7), despite some difference at the 48 h timepoint with v/sMLC1 (Fig. 7). Although the overall coordination of different MLCs may be mediated through their MLC-sequence or CArG-box (Uetsuki et al. 1990, Ernst et al. 1991), also in C2C12 cells, MyoD-factors may function as initial inducers of the expression of v/sMLC1 and skMLC2. CArG-box-, M-CAT- and Ap-2/CCACCC-element-binding factors, on the other hand, may have some influence on their initial rather marked expression, similarly as with α-actin. Proteins of fMLC1 and fMLC3 in turn became very faintly detectable slightly later than v/sMLC1 and skMLC2 (Fig. 5). Synthesis of fMLC3 paralleled that of fMLC1 throughout the differentiation. The signal of fMLC3, however, was around only a half of the signal of fMLC1 (Fig. 7). The result is in line with that of a study that showed rat fMLC1/3 gene to be generally expressed slightly later and, moreover, fMLC1 to be expressed earlier in development than fMLC3 (Garfinkel et al. 1982). It has been suspected that expression of fMLC1/3 may depend more on MyoD and/or MRF-4 activity, directly or indirectly, than the earlier MLCs (Muthuchamy et al. 1992): a study of MRF-4 transfected BC3H-1 cells has shown that as well as gaining fusion ability and starting to express endogenous MyoD, the cells also started to express fMLC1/3, which is normally quiescent in BC3H-1 cells (Block & Miller 1992). Thus the reason for lack of fMLC1/3 expression in L6 cells may be that the cells lack MyoD, and also that MRF-4 expression is very low. The difference in regulatory factors is probably also the reason why v/sMLC1 is not expressed in L6 cells and a/eMLC1 in C2C12 cells. Interestingly, however, at least some of the maturated C2C12 myotubes expressing v/sMLC1 and fMLC1/3 gained the contraction ability, whereas maturated L6 myotubes expressing a/eMLC1 never showed twitching abilities.

Conclusions:

Expression of contractile apparatus genes was initiated prior to fusion in both cell lines. Accumulation of gene products occurred at a slightly slower rate in C2C12 cells than in L6 cells, which was in relation to the fusion process of these cells: as expected, L6 cells fused more rapidly and completely than C2C12 cells. The overall pattern of gene expression was fairly similar in both cell lines.

The results suggested that the initial and most markedly accumulating members of the thin filament were α-actin and TMs. The first of the troponin complex members expressed at a lower rate of accumulation appeared to be fTnI and c/sTnC followed by fTnC. The initiation of expression of TnT seemed to vary in L6 and C2C12 cells, but it appeared later on to be the most pronouncely expressed member of the complex. The mRNA/protein comparison also suggested that the expression of c/sTnC and fTnC is post-transcriptionally regulated. Expression of MLCs, in turn, was more pronounced than that of members of the troponin complex as expected. Their expression showed some

coordination, but during differentiation their synthesis pattern also shared some similarity with that of TM proteins. However, to confirm the described order of expression, more sensitive detection methods, like PCR methods, are required.

The comparisons of the initial accumulation of cellular mRNAs - and partially also the protein synthesis - to the known regulatory sequences of the genes supports the transcriptional order, intensity and coordination of the genes described. The results of the comparisons suggest that although general initiation of muscle gene expression is known to require E-box binding MyoD-factors, marked expression seems to require strong enhancers such as CArG-type sequences and M-CAT, but also CCACCC/Sp1-type regions. Genes of this type are α-actin, MLCs, possibly α-TM, and fTnT, the cellular mRNA accumulation of which showed also some coordination during differentiation (Fig. 6). A common transcriptional element to most of the members of the troponin complex appears to be the CCACCC-type sequence, which may have an important role in their coordination. Other known regulatory sequences seem to be binding elements to more downstream factors: the expression of fTnI may also require an E-box, and fTnC seems to have only these CCACCC-like sequences, whereas transcription of c/sTnC also requires MEF-2- and MEF-3-sites. The binding affinity of CCACCC-type elements, E-box and T+A-sequences as well as probably others may vary depending on the exact sequence and context of that sequence. In addition, the binding factors, their modification level, and some of their hetero-oligomerization partners may vary during differentiation, which probably also influences transcription. It has been suggested, too, that at least some of these different factors interact with each other when bound to their elements.

5.1.3 Results and discussion of half-life studies of some of the contractile apparatus mRNAs and proteins

The accumulation of contractile apparatus mRNAs and proteins during differentiation and development may depend, in addition to transcription, on mRNA processing and turnover, mRNA translation, protein stability and assembly into filaments (Caravatti et al. 1982, Zhu et al. 1991). Some differences in mRNA/protein relations were also observed, especially in L6 cells during maturation (Fig. 6, Fig. 7). The variation may be partially explicable by the mRNA samples used: generally only around 1-2% of the cellular mRNA of a particular gene is translatable, but its translatable pool may vary during differentiation and maturation due, for example, to possible variation in the half-lives of mRNAs. The turnover of mRNAs may slow down during the maturation of the cultures, which in turn may have the effect of increasing the cellular mRNA pool. Nevertheless, in the present study the half-lives were estimated only from one timepoint.

The purpose of the study was to investigate the influence of the turnover rates of cellular mRNAs and proteins on their accumulation patterns during differentiation. Estimation of the turnover rates of the gene products of the contractile apparatus may also indirectly explain the assembly and renewal of the

thin filament. A further purpose of the study was to compare the gene products in two different cell lines which partially express the same contractile apparatus genes (Table 1).

Results:

The half-lives of cellular mRNAs and proteins were estimated from several experiments. Both L6 and C2C12 myotubes (3 d and 5 d differentiated cells, respectively) covered around 60-70% of the plating area, whereas the rest was covered with myoblast-like cells, estimated by light microscopy.

An example from the half-life experiment for cellular mRNAs of L6 cells is described in Fig. 8.

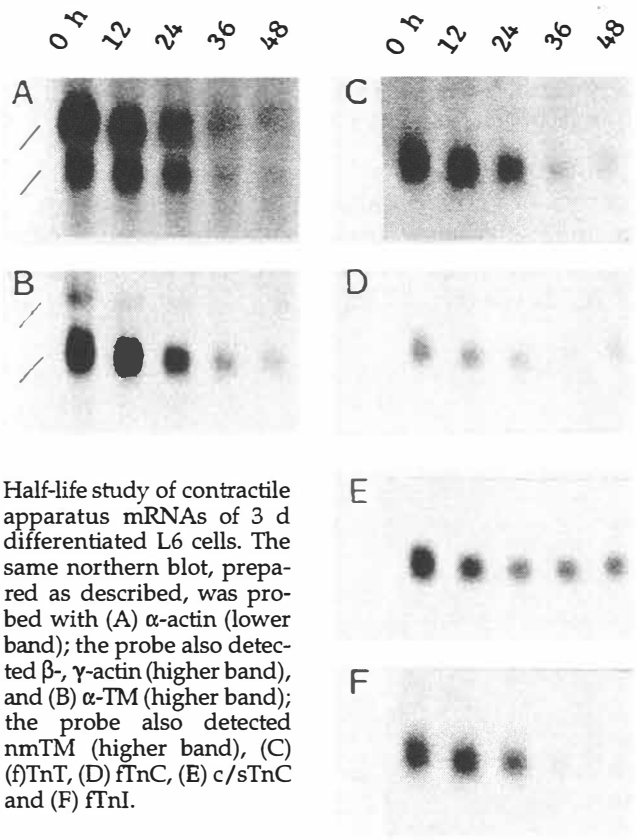

FIGURE 8 Half-life study of contractile apparatus mRNAs of 3 d differentiated L6 cells. The same northern blot, prepared as described, was probed with (A) α-actin (lower band); the probe also detected β-, γ-actin (higher band), and (B) α-TM (higher band); the probe also detected nmTM (higher band), (C) (f)TnT, (D) fTnC, (E) c/sTnC and (F) fTnI.

Light microscopy revealed that transcription inhibition with AcD damaged L6 cells during incubation. The damage was less marked, however, than in the later described similar studies in 5 d differentiated C2C12 cells. The half-life study also included estimations of proteins (Fig. 9).

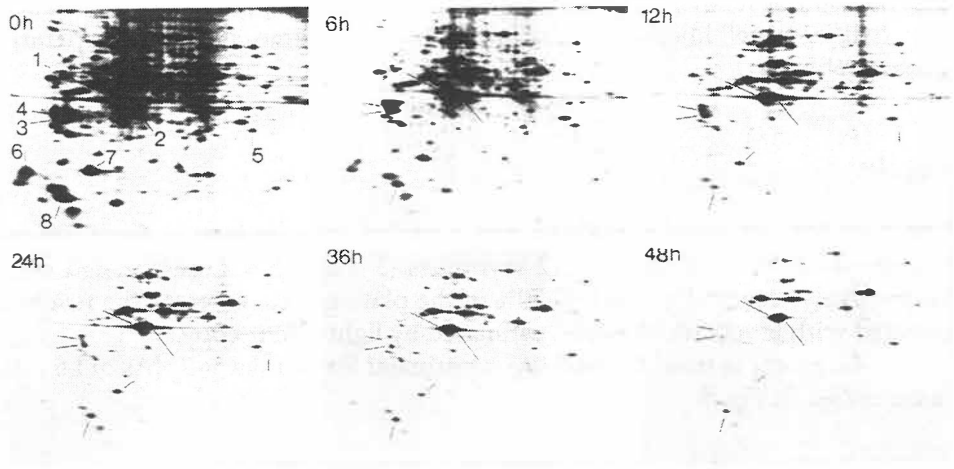

FIGURE 9 Half-life study of contractile apparatus proteins of 3 d differentiated L6 cells. Each picture of autofluorographs of 2D-PAGE (IEF) shows on the left the acidic end and on the right the basic end of the gel. (1) α-actin, (2) β-, γ-actin, (3) α-TM, (4) β-TM, (5) xTnT, (6) total TnC (7) a/eMLC1, and (8) skMLC2.

The half-lives of cellular mRNAs and proteins of L6 cells were estimated from the results of image-analyses of a few similar experiments as shown in Fig. 8 and Fig. 9. The half-lives are summarized in Table 2. The respective experiments were also performed to C2C12 cells (Fig. 10, Fig. 11).

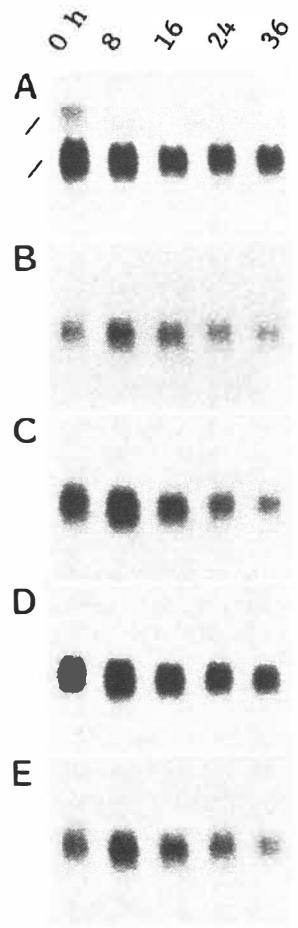

FIGURE 10 Half-life study of contractile apparatus mRNAs of 5 d differentiated C2C12 cells. The same northern blot (5 µg of mRNA) was hybridized with (A) α-actin (lower band); the probe also detected β-, γ-actin (higher band), (B) (f)TnT, (C) fTnC, (D) c/sTnC, and (E) fTnI.

The blockage of transcription with AcD caused severe damage to 5 d differentiated C2C12 cells during culture, as observed by light microscopy. The effect was more severe in myotubes, which covered 60-70% of the plating area, than in the more myoblast-like cells, which covered the rest of the area.

The half-lives of proteins were also estimated in C2C12 cells (Fig. 11).

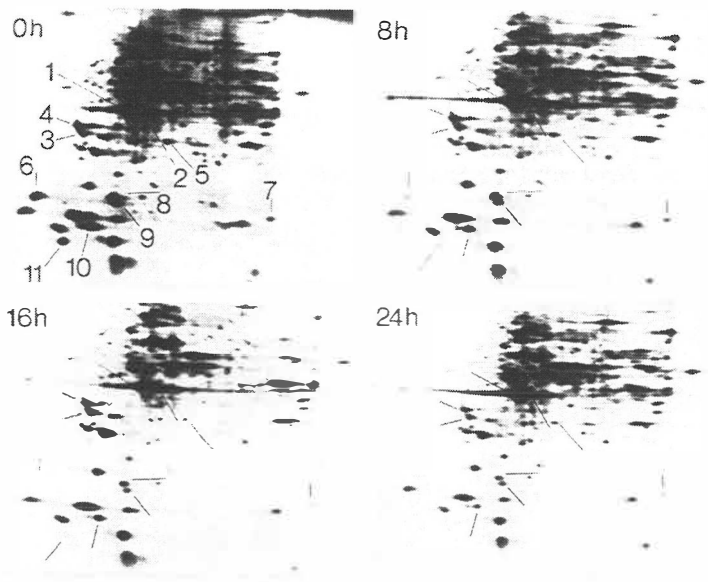

FIGURE 11 Half-life study of contractile apparatus proteins of 5 d differentiated C2C12 cells. Each picture of autofluorographs of 2D-PAGE (IEF) shows on the left the acidic end and on the right the basic end of the gel. (1) α-actin, (2) β-, γ-actin, (3) α-TM, 4. β-TM, (5) xTnT, (6) total TnC, (7) fTnI, (8) v/sMLC1, (9) fMLC1, (10) skMLC2, and (11) fMLC3.

The half-lives of cellular mRNAs and proteins of C2C12 cells were estimated from results of image-analyses. Examples from the experiments are shown in Fig. 10 and Fig. 11. The half-lives are summarized in Table 2.

TABLE 2 Half-lives of the cellular mRNAs and proteins of some of the contractile apparatus members expressed in (A) 3 d differentiated L6 and (B) 5 d differentiated C2C12 cells. }= c/sTnC and fTnC. The results are presented in hours.

(A) L6	**mRNA**			**Protein**		
	n	Mean	±SD	n	Mean	±SD
α-actin	4	9.95	0.751	3	33.96	1.464
β-actin	4	13.58	2.209	-	-	-
α-TM	3	12.92	0.715	4	11.17	3.036
β-TM	-	-	-	4	11.23	1.109
(f)TnT	4	18.77	0.828	1	8.66	-
xTnT	-	-	-	1	11.46	-
fTnC	4	15.84	0.689	3	}6.44	0.075
c/sTnC	4	29.79	4.677			
fTnI	3	12.05	0.293	2	10.52	0.184
a/eMLC1	-	-	-	4	13.29	1.131
skMLC2	-	-	-	4	10.80	2.318

(B) C2C12	mRNA			Protein		
	n	Mean	+SD	n	Mean	+SD
α-actin	4	29.61	1.099	3	28.01	1.553
β-actin	4	5.12	1.161	-	-	-
α-TM	3	23.16	4.051	3	16.89	2.880
β-TM	-	-	-	3	23.29	2.908
nm β-TM	3	9.87	0.801	-	-	-
(f)TnT	5	21.22	4.010	-	-	-
xTnT	-	-	-	2	14.81	1.230
fTnC	6	16.71	1.135	3	}6.09	0.640
c/sTnC	4	44.62	1.915			
fTnI	5	20.65	4.089	1	15.01	-
sTnI	-	-	-	1	12.89	-
v/sMLC1	-	-	-	3	10.43	1.470
fMLC1	-	-	-	3	10.03	1.193
skMLC2	-	-	-	3	14.83	0.951
fMLC3 -	-	-		3	6.42	1.395

The results show (Table 2) that the half-lives of mRNAs and proteins are rather similar to each other and that the mRNA/protein ratio is fairly similar in L6 and C2C12 cells, although the method of estimating mRNA was fairly crude. However, the results for the 5 d differentiated C2C12 cells generally shows a slightly slower turnover rate than in the case of the 3 d differentiated L6 cells.

L6 cells. The relationship between the half-lives of cellular mRNA and protein of α-actin is exceptional when compared to the other gene products examined in L6 cells: the turnover rate of cellular mRNA of α-actin is around 10 h, whereas the half-life of the protein is around 34 h; all the other studied gene products showed that the half-life of mRNA is longer or around the same as the half-life of the respective protein. The half-life of the cellular mRNA of α-actin is similar to that of α-TM, the estimated turnover rate of which is around 13 h. The α-TM protein resembles β-TM, which has a half-life of about 11 h.

The cellular mRNAs of the members of the troponin complex appear to have a slightly slower turnover rate than that of α-actin or TM, whereas the proteins of the complex seem to be more labile. The most stable member is c/sTnC, the cellular mRNA half-life of which is approximately 30 h. This differs from fTnC, which has a turnover rate of only about 16 h. On the other hand, TnC appears also to be the most unstable polypeptide of the thin filament, since its half-life is only around 6 h. The two TnCs, however, did not separate properly in any of the experiments; thus the result describes the half-life of total TnC. Other subsequently reposted studies have indicated that the ratio of fTnC and c/sTnC proteins is similar in 3 d differentiated L6 cells. Thus the estimated half-life of the total TnC protein reflects closely at least that of c/sTnC, but likely of fTnC, too.

The half-life of cellular mRNA of (f)TnT is about 19 h, whereas the turnover rate of with possible respective TnT protein detected with antibody is approximately 9 h. The half-life of the xTnT protein shown in Fig. 11 is around 11 h, resembling that of α-TM and β-TM. The third member of the troponin complex, fTnI, has, at about 12 h, the shortest cellular mRNA half-life of those

estimated the fast members of a complex. The half-life of the protein, instead, is close to that of TnT being almost 11 h. The half-lives of a/eMLC and skMLC2, in turn, are rather similar: a/eMLC1 is around 13 h and skMLC2 approximately 11 h.

C2C12 cells. The turnover rates of α-actin cellular mRNA and protein were similar in C2C12 cells, being almost 30 h and 28 h, respectively. The half-life of cellular mRNA of α-TM is around 23 h, and thus resembles that of α-actin, whereas the turnover rate of the α-TM protein is less, at only 17 h. β-TM protein, instead, shares a similarity with α-actin protein, since its half-life is approximately 23 h.

The turnover rates of members of the troponin complex were also estimated. The half-life of cellular mRNA of TnT is about 21 h, which resembles that of α-TM. The half-life of the xTnT protein was around 15 h, which is also similar to that of α-TM. Cellular mRNA of c/sTnC, on the other hand, appears to be very stable: its half-life is around 45 h, whereas that of fTnC is only 17 h. Unfortunately, determination of the turnover rates of TnC proteins failed, since they did not separate in any experiment. However, the estimation of total TnC protein shows only slightly over 6 h half-life. Other studies have indicated that in 5 d differentiated C2C12 cells the major isoform is c/sTnC. Thus it seems that the estimated half-life of the total TnC protein closely resembles at least that of c/sTnC half-life, but probably also that of fTnC. The half-lives of the gene products of fTnI, have some resemblance to those of TnT, but differ from those of TnCs: cellular mRNA of fTnI has a half-life of almost 21 h, whereas that of the protein is 15 h. The half-life of the sTnI protein, in turn, is slightly shorter than of fTnI, at around 13 h.

MLCs were estimated only at protein level. All of them seem to have rather similar turnover rates, with the exception of fMLC3. The half-lives of v/sMLC1 and fMLC1 are about 10 h, whereas that of skMLC2 is around 15 h, which is similar to that of xTnT. The half-life of the fMLC3 protein, which is only slightly over 6 h, resembles that of the TnC protein.

Discussion:

The half-lives of the mRNAs are rough estimates, since the method used was not very accurate: the ability of AcD to block transcription immediately is questionable. The results showed some increase in the accumulation of cellular mRNA at the second harvest point in C2C12 cells in almost every experiment (Fig. 10), although equal amounts of mRNA were taken from each timepoint for northern blot analysis, measured by taking optical density readings at 260 and 280 nm from the total yield of mRNA. The mechanism by which AcD increases transcription is unknown, but one option may be that AcD stabilizes the binding of the transcriptional factors, or on the other hand, it may have a stabilizing influence on mRNA. AcD may also activate some compensatory mechanisms of the cells. More accurate estimations would have demanded isolation of polyadenylated mRNA species or in situ hybridization. Moreover, only certain

time differentiated cells were used in the measurements, although variation in the turnover rates of mRNAs and proteins may exist during differentiation and maturation.

Actin and TM. The relationship of the half-lives of the cellular mRNA and protein of α-actin seems to be exceptional in L6 cells: the half-life of cellular mRNA of α-actin seems to be only about 1/3 of that of the respective protein (Table 2). The results of all the other gene products studied in 3 d differentiated L6 cells, as well as in 5 d differentiated C2C12 cells, showed that the half-life of mRNA is longer or around the same as that of the respective protein. The half-life relationship of α-actin gene products in L6 cells seems indirectly to support the marked transcription of α-actin, and thus excess in its cellular mRNA accumulation during differentiation (Fig. 6, Fig. 7). The results for C2C12 cells, however, did not indicate a similar differences in the cellular mRNA/protein relationships (Fig. 6, Fig. 7, Table 2). The reason for the marked accumulation of α-actin transcripts in L6 cells, especially later during differentiation, may be due to the favourable balance of transcriptional factors in cells, but the stability of the messages may also increase during maturation. The differences in these cells may also be due to the fact that L6 cells apparently only express αsk-actin (Muthuchamy et al. 1992), whereas αc-actin is the major form in C2C12 cells, and αsk-actin forms only the minor pool (Bains et al. 1984). Nevertheless, the stoichiometric accumulation of α-actin in L6 cells seems to be regulated at the translational level (Fig. 6, Fig. 7).

The study also indicated that L6 cells are less sensitive to AcD treatment than C2C12 cells, according to light microscopic observations. Variation may exist in the detoxification systems in these cell lines, but the difference may also be partially explicable by the α-actin mRNA/protein balance. As mentioned earlier, transcription of α-actin seemed to be in accord with translation in C2C12 cells (Fig. 6, Fig. 7), thus when the transcription of α-actin decreased due to AcD treatment, it probably also decreased protein synthesis. Moreover, since antisense mRNA studies against α-actin have shown that lack of α-actin is lethal to the cells, a decrease in the stoichiometric accumulation of α-actin protein may be the reason for the sensitivity of C2C12 cells to AcD treatment (Izant 1989, Weintraub 1990). Since, moreover, the C2C12 cells were 5 d differentiated, the renewal of the thin filament and its α-actin members may have been marked. In 3 d differentiated L6 cells, instead, the thin filament may have just formed or was still forming, and thus renewal may have not yet have become marked, due to the fairly slow turnover rate of the α-actin protein (Table 2). This in turn may protect L6 cells from thin filament breakage.

The accumulation of cellular mRNAs of α-TM and α-actin were similar during differentiation, at least in L6 cells (Fig. 6, Fig. 7). Some similarity existed also in their cellular mRNA half-lives, especially in C2C12 cells (Table 2), which also suggests post-transcriptional coordination between them. However, the protein synthesis of the β-TM protein seemed to be slightly more pronounced than that of α-TM in both cell lines (Fig. 3, Fig. 5). The half-lives of the α-TM and β-TM proteins, instead, were almost the same in L6 cells, whereas in C2C12 cells, the turnover rate of β-TM was longer. The half-life of α-TM had more resemblance with that of TnT in both cell lines, but β-TM was similar to α-actin in C2C12 cells (Table 2). The interesting issue is, does TM protein characterized by a longer

half-life, at least in C2C12 cells, interact more with α-actin, and on the other hand, does TnT interact more with TM protein characterized by a shorter half-life?

Troponin complex. As mentioned earlier, the studies of TnT were probably partial, since the expressed forms are incompletely known in both cell lines. Nevertheless, different mRNAs of TnT probably vary in their half-lives, but the importance of this for example on protein synthesis, variety, and proportion of the proteins/isoforms, or on the other hand, renewal and protein/isoform changes of the thin filament, is not known. But, as mentioned previously, the half-lives of the α-TM and TnT proteins were similar in both cell lines, as well as those of β-TM and xTnT proteins in L6 cells. In C2C12 cells, instead, β-TM had a similarity with α-actin. The rather similar half-lives of TnT and TM proteins suggests that TnT proteins may have preference for interaction with a particular TM in a TM-dimer, which in turn may also influence the renewal of the thin filament.

The cellular mRNA of c/sTnC appeared to be very stable in L6 and C2C12 cells: its half-life was around twice as long as that of fTnC mRNA in both cell lines (Table 2). Sequence analysis of cDNAs of c/sTnC and fTnC has revealed 68% homology, and the most different areas are located in their 5' and 3' UTRs (Parmacek & Leiden 1989, Parmacek et al. 1990). These regions may be involved in the regulation of turnover of these mRNAs, but the c/sTnC message may also be protected from degradation, for instance by exceptional folding or by bound proteins. The half-life of the total TnC protein, on the other hand, was surprisingly short in both cell lines, only slightly over 6 h (Table 2).

Although troponin complex has shown to contain each member in a ratio of 1:1:1, studies of rabbit muscle have indicated that there exists a stock of unassembled fTnI protein in cells, but also that fTnI protein is degraded relatively slowly (Martin 1981, Koppe et al. 1989). Moreover, long-term stimulation experiments in rabbit muscles have shown that the fast-to-slow transition in TnI is less marked at protein level than at mRNA level, whereas changes in the other troponin complex proteins were comparable to the respective mRNA alterations (Härtner & Pette 1990). However, the differentiation experiments indicated that fTnI is the first expressed member of a troponin complex in L6 and C2C12 cells (Fig. 6). The half-life of the fTnI protein was also slightly longer than that of total TnC, but had similarity with TnT in both cell lines (Table 2). The similarity of the turnover rate of cellular mRNA of fTnI to those of the other fast members of the complex suggests similarities in their respective translations. Thus taking together the initiality in expression, slightly longer half-life of the protein than the half-lives of the rest of the members of the troponin complex, and the similar or higher translation intensity of fTnI, indicated by the antisense study (Fig. 14), the results also supports the accumulation of a pool of unassembled fTnI.

MLC. All the MLC proteins with exception of fMLC3 had fairly similar half-life in both cell lines (Table 2), although the ratio of the half-lives of the MLC1 and skMLC2 proteins was opposite in 3 d differentiated L6 and 5 d differentiated C2C12 cells. The results also suggest that a/eMLC1 may accumulate in slight excess in L6 cells: the half-life of a/eMLC1 was approximately 2 h longer than that of skMLC2 (Table 2), and the synthesis of a/eMLC1 also appeared to be slightly more than of skMLC2 (Fig. 7). The half-life of fMLC3,

instead, resembled that of the total TnC protein, but since MLCs are related to TnCs (Collins 1991), and since fMLC3 is expressed mainly in slow-type skeletal muscle such as c/sTnC (Barton & Buckingham 1985, Parmacek & Leiden 1989), similarity at some level of expression is not surprising.

Neither mRNA nor protein half-lives of any of the MHCs were estimated. However, the half-life of cellular mRNA of eMHC has been determined in L6E9 cells and, according to results, the half-life of MHC is rather long, at about 55 h (Medford et al. 1983). Thus although comparison of the turnover rate of MHC cellular mRNA to MLC proteins is not precise, it nonetheless suggests a similar relation to that of α-actin and the troponin complex.

Conclusions:

α-actin protein appeared to be the most stable member in thin filament, whereas TMs were located in-between α-actin and the fairly labile members of the troponin complex. The pattern was similar in both cell lines, although the overall turnover rate was slower in 5 d differentiated C2C12 cells than in 3 d differentiated L6 cells; the half-lives of the mRNAs and proteins may vary during differentiation and maturation, and most probably differences also exist between cell lines. Although the half-lives of none of the MHCs were estimated, the turnover rates of MHC proteins are probably quite long compared to those of MLCs. Thus it seems that the basal thin and thick filaments are renewed at a slower rate than the other components of these structures.

5.1.4 Concluding remarks: thin filament and its formation

How thin filament is assembled (Fig. 1), or on the other hand, renewed is poorly understood (Hill et al. 1992, Willadsen et al. 1992). The aim of the present study was to illuminate this process by investigating both transcriptional and translational patterns during differentiation as well as the half-lives of the respective contractile apparatus gene products (Fig. 6, Fig. 7, Table 2).

Assembly of thin filament has been suspected to start from the interaction of actin with TM: TMs may specifically associate with themselves and/or other cellular proteins, and these associations in turn may lead to distinctive actin structures. The involvement of particular TMs with special actin structures, however, is poorly known (Ryseck et al. 1989, Lees-Miller & Helfman 1991). The present study showed that expression of α-actin and TMs increased quickly after the cells were changed to differentiation medium. Expression was also more marked throughout the differentiation than that of members of the troponin complex in L6 and C2C12 cells (Fig. 6, Fig. 7). The order, intensity, as well as coordination of expression is also supported by the regulatory sequences that these genes have. The quick and marked increase in expression of α-actin and muscle TMs compared to those of members of the troponin complex suggest that the formation of thin filament in vivo starts with α-actin and α-TM/β-TM

interactions, although the initial events of assembly in cells are poorly understood (Epstein & Fischman 1991). Assembly may start with the formation of F-actin segments (Pollard & Cooper 1986), which interact with TM-dimers, and when the concentration of these segments increases, they may interact with each other and form the initial filamentous structures (Fig. 1). The stiffening of the actin-TM structure and also anchorage of the two TM-dimers to each other has been indicated to be influenced by the acetylatable NH_2-terminus of TM. TnT has been suggested having some influence on further rigidifying the TM-actin structure by its residues 70-158, which form the elongated tail of TnT (Hill et al. 1992, Willadsen et al. 1992). Moreover, TnT overlaps two TM-dimers by its residues 1-69, which in turn may have some influence on full-size thin filament formation (White et al. 1987, Hill et al. 1992). The results of Cooper & Ordahl (1985) have also indicated that c/eTnT is important for the initial assembly of the TM-actin structure.

The relationship of a particular TnT and a particular TM or what influence they have on each other functioning is poorly understood (Briggs et al. 1987). The differentiation and half-life studies showed some variation in their relations in L6 and C2C12 cells (Fig. 7, Table 2). The protein synthesis of α-TM appeared to be slightly less than that of β-TM (Fig. 3, Fig. 5). The half-lives of both TM proteins, instead, seemed to be fairly similar and also comparable to xTnT, but differed from α-actin in L6 cells (Table 2). In C2C12 cells, in turn, the half-life of β-TM had some resemblance to that of α-actin, whereas α-TM had a similarity to xTnT (Table 2). Thus a particular TnT protein may have a higher affinity to one member in a TM-dimer, whereas the other TM protein may interact more with actin. The interactions, in turn, may cause some internal instability to the TM-dimer, as described by Hill et al. (1992), and aid the renewal of the thin filament.

Initiation of expression of TnT seemed to vary in L6 and C2C12 cells, although TnT appeared to accumulate in most of the members (Fig. 6). The first members of the complex expressed appeared to be fTnI and c/sTnC followed by fTnC (Fig. 2, Fig. 4). Protein synthesis of fTnI (Fig. 14) seemed to be similar or more marked than of TnC (Fig. 3). The difference suggests that mRNA of fTnI is translated slightly more efficiently than of TnCs, although the accumulation of cellular mRNAs of both TnCs appeared to be more marked (Fig. 2, Fig. 4). The results also suggested that expression of c/sTnC and fTnC is post-transcriptionally regulated. Nevertheless, the differences in intensities and/or initiation times of the members were only slight (Fig. 6), but shows some similarity to the results described by Bucher et al. (1988) for quail striated muscle. The half-life study, in turn, showed that the half-life of the fTnI protein is around twice as long as that of total TnC (Table 2). The described differences indirectly relate to the studies by Martin (1981) and Koppe et al. (1989) which indicated accumulation of excess TnI.

The reason for excess TnI is unknown, but one possibility is that excess fTnI is needed for trapping less synthesized TnC to form a fTnI-TnC precomplex. Morever, TnCs are acidic proteins (isoelectric point around pH 4.0), whereas TnIs and also TnTs are fairly basic (isoelectric point around pH 8.0) (Nakamura et al. 1989), which supports the formation of a TnI-TnC precomplex. It is also in line with the results of Shiraishi et al. (1992), who suggested coregulation, at least with TnI and TnC, in their troponin complex replacement studies. However, whether

fTnI and TnC interacts with free TnT, which is then joined to TM-actin structure or already TM-bound TnT, is not fully understood - nor can it be explained by the present data. The results of the C2C12 study seem to suggest, however, that the markedly transcribed and probably also markedly translated TnTs in these cells interact first with TM-dimers, after which TnC and TnI are added to the structure, although it is not possible to draw definite conclusions. Variation may also exist in these interactions and their affinities due to the variety of TMs, and particularly to the vast variety of different TnTs.

The renewal of a troponin complex may also be hierarchical: since TnC has the shortest half-life (Table 2), and it is located in-between TnI and TnT in a troponin complex (Fig. 1), TnC probably also determines the detachment of TnI. Moreover, since the half-life of fTnI is around twice as long as that of TnC, this suggests that fTnI is recycled: the released fTnI may interact with another molecule of TnC, which complex is joined to a thin filament. The half-life difference between TnC and TnI may furthermore explain the indicated pool of unassembled TnI in cytoplasm (Martin 1981, Koppe et al. 1989). The difference in the half-lives of TnC and TnT, on the other hand, suggests that TnT may be detached by itself or together with TM from a TM-actin filament. However, the described hypothetical model of assembly and renewal requires additional studies, utilizing methods such as PCR to confirm the initial order of transcription and, in addition, electronmicroscopical methods.

5.2 Targeted regulation of gene expression of contraction apparatus members

5.2.1 Antisense oligodeoxyribonucleotide and RNA-mediated regulation of gene expression of contraction apparatus members

The assembly and maintenance of myofilaments and contraction apparatus, and the mechanisms that are involved in regulating these processes are poorly understood (Fyrberg & Beall 1990, Epstein & Fischman 1991). Reducing the gene expression of one member of the contractile apparatus, for instance with antisense ODN or antisense RNA, and examining the effects of this alteration on other members of a myofilament may further elucidate these processes. Thus as the target messages selected for the antisense ODN studies were fTnT in L6 cells, and c/sTnC and fTnC in C2C12 cells - antisense RNA was targeted only against c/sTnC in C2C12 cells. The difficulty in antisense methods, however, is the possibly rather intensive transcription of the target, which may make complete blocking difficult (Izant 1989). However, both of these members of the troponin complex are transcribed to a less extent than for example α-actin, as found in the previously described differentiation studies. Moreover, the characters of these two messages are different: fTnT isoforms are spliced from a single, but long primary transcript (Breitbart & Nadal-Ginard 1987), whereas the two TnCs are

produced from two short messages of separate genes (Parmacek & Leiden 1989, Parmacek et al. 1990).

5.2.2 Results and discussion of antisense experiments in L6 and C2C12 cells

5.2.2.1 Uptake of ODNs to L6 and C2C12 cells

It is known that ODNs are internalized to cells, but the efficiency may vary depending on the cell type, the division cycle and the ODNs as such (Boiziau & Toulmé 1991). The concentration of antisense ODNs in cytoplasm or nuclei may also vary from low to moderate, which in turn may depend on the ODN type used and its length (Marcus-Sekura 1988, Knecht 1989, Weintraub 1990). Thus the uptake of different types of ODNs were estimated in almost completely fused L6 cells and fusing C2C12 cells, and the need for fresh ODN supplementation to the cultures during the antisense ODN treatments was assessed.

Results:

The uptake experiments were performed in multinucleated 3.5 d differentiated L6 myotubes and in fusing 48 h differentiated C2C12 cells, and in culture medium.

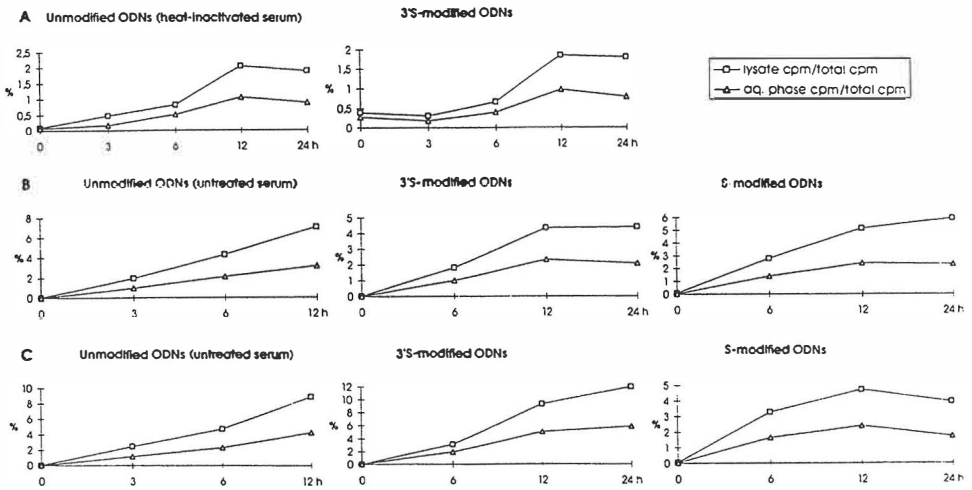

FIGURE 12 Uptake of the ODNs to L6 and C2C12 cells. 5' end labeled antisense cap-c/sTnC umODN (15mer), antisense fTnT 3'S-ODN (15mer) and random sequence S-ODN (18mer) ODNs were added to L6 cultures (5 µM; 450 000 cpms) and incubated (A) in heat-inactivated 2.5% horse serum, and (B) in

normal 2.5% horse serum for the periods shown. (C) C2C12 cells were treated similarly in normal 2.5% horse serum. The uptake is presented as a percentage of the cpms of crude lysates or prepared aqueous phases of the lysates compared to total cpms (cpms of incubation medium, washing solutions and crude lysate).

The results show that after 12 or 24 h of incubation, approximately 2-5% of the ODNs have been taken up by the cells into the aqueous phase from the culture medium, supplemented with 2.5% of normal, not heat-inactivated horse serum (Fig. 12). A slightly lower uptake percentage, around 1%, is apparent in L6 cultures exposed to 2.5% heat-inactivated horse serum during the ODN treatment. The results suggest also that the uptake of ODNs is saturatable (Fig. 12), and the modification of the ODNs has some influence on the uptake and release of ODNs to the cytoplasm (data not shown): it seemed that the signals of intact umODNs and 3'S-ODNs were weaker than those of S-ODNs in the aqueous phases in both cell lines, which may be due to degradation of some of the umODNs and 3'S-ODNs during the uptake/release process in the cells. The comparisons of the ODN signals in medium and in the aqueous phase also indicated that the increase of ODNs in the aqueous phase occurs fairly slowly, since the saturation of ODNs in the aqueous phase seemed to be reached in between 6 and 12 h of incubation in both cell lines (data not shown).

The very initial studies with heat-inactivated serum showed that around half of the 3'S-ODNs selected for the antisense fTnT ODN studies were left after 6 h of incubation in medium of L6 cultures (data not shown). The S-ODNs were also examined and the results showed that they were undegraded. Furthermore, the studies showed the presence of variation among the nuclease activities between serum batches (data not shown).

Discussion:

Although this uptake study did not show the actual uptake or location of the ODNs in the cells, the study by Leonetti et al. (1991) with mononucleated cells showed that the ODNs are mainly found in vesicular structures, such as lysosomes and mitochondria (50%), and also in nuclei (20%), whereas around 30% were located in cytosol. It has suggested that uptake may also occur as vacuoles. The results shown in Fig. 12 support the idea of vacuole mediated uptake, since the cpms in the cell lysate are higher than in aqueous phase of extracted lysate throughout the experiments. Vacuole mediated uptake would also explain the rather slow increase of ODNs in the aqueous phase, although all ODNs were detectable even after 24 h of incubation in medium of L6 cells (data not shown). In culture medium of C2C12 cells, on the other hand, umODNs and 3'S-ODNs were hardly detectable after 12 h of incubation, whereas S-ODNs were intact, as in the L6 cultures, although the culture medium used was the same. Thus C2C12 cells may have secreted nuclease and/or dephosphatase activities into medium, which may have been due to the pronounced fusion process of the C2C12 cells. The degraded ODNs have a decreasing influence on uptake, which

was also apparent in the more modest accumulation of intact umODNs and 3'S-ODNs in the aqueous phase of C2C12 cells than in L6 cells. Nevertheless, intact ODNs were detectable in the aqueous phases of L6 and C2C12 cells in every experiment (data not shown).

Conclusions:

The results suggested that for antisense fTnT ODN studies selected 3'S-ODNs need to be replenished after 6 h of incubation. According to the results of the same experiments, C2C12 cultures were supplemented with fresh umODNs after 3 h of incubation. The results also confirmed the unnecessity of the fresh supplementation of S-ODNs. It was also apparent that there was variation in nuclease activities between serum batches. The results suggested the use of heat-inactivated serum for the antisense treatments.

5.2.2.2 Effects of antisense ODNs against troponin T on L6 myotubes

The experiments to inhibit TnT expression in L6 myotubes by antisense fTnT 3'S-ODN were done to elucidate the influence of reduced TnT gene expression on the coordinated and stoichiometric accumulation of other members of the contraction apparatus. Another purpose was to elucidate possible mechanisms to compensate for a shortage of TnT.

Results:

Antisense fTnT ODNs and mRNA accumulation. The differentiation studies in chapter 5.1.1 indicated transcriptional coregulation between TnT, α-actin and TM. Accumulation of cellular mRNA of TnT had even more similarity with that of the fast members of a troponin complex. Thus the disturbance in the normal accumulation of cellular mRNA of TnT by antisense ODNs may further clarify its relation to α-actin, muscle TMs and to other troponin complex members.

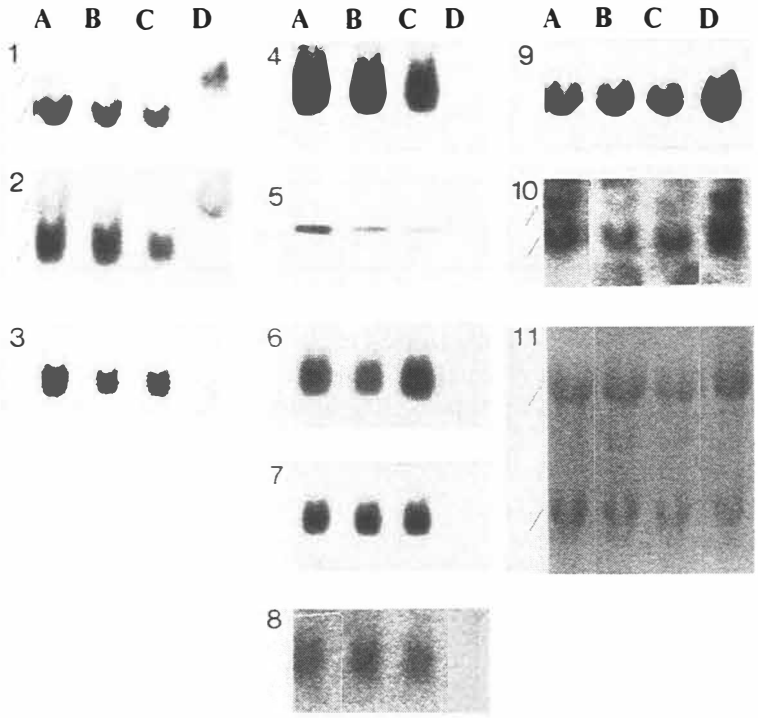

FIGURE 13 Influence of fTnT 3'S-ODN treatments on cellular mRNA accumulation of L6 cells. L6 myotubes (3.5 d differentiated) were incubated for 12 h with 25 µM of antisense fTnT or sense fTnT 3'S-ODNs. Lanes of the same northern blot (15 µg of cellular RNA): (A) untreated control myotubes, (B) sense fTnT 3'S-ODN-treated myotubes, (C) antisense fTnT 3'S-ODN-treated myotubes, and (D) myoblasts. The blot was hybridized with (1) α-actin (lower band), β-actin (higher band), (2) α-TM (lower band), nm-TM (higher band), (3) β-TM, (4) (f)TnT, (5) fTnT (primer extension), (6) total TnC, (7) fTnI, (8) sTnI, (9) EF-Tu, (10) two variants of Jun, and (11) Ethidium bromide stained mRNA.

The results of the northern blot and primer extension analyses shown in Fig. 13 were image-analysed and the results are listed in Table 3.

TABLE 3 Influence of fTnT 3'S-ODN treatment on cellular mRNA accumulation. Influence of (A) antisense fTnT 3'S-ODN treatment compared to untreated control, (B) antisense fTnT 3'S-ODN treatment compared to sense fTnT 3'S-ODN treated control, and (C) sense 3'S-ODN treatment compared to untreated control. Alterations are shown as percentages.

	(A) **as fTnT/-ODN** n=1	**(B)** **as fTnT/sense** n=1	**(C)** **sense fTnT/-ODN** n=1
mRNA	Alteration %		
α-actin	-51.33	-32.82	-27.55

α-TM	-57.87	-54.42	-7.56
β-TM	-32.86	-4.81	-29.46
(f)TnT	-39.09	-19.18	-24.56
fTnT (p.e.)	-69.47	-51.67	-36.84
TnC	11.92	50.63	-25.91
fTnI	-11.40	-9.21	-2.41
sTnI	-33.33	-40.25	11.57
Jun (higher)	-70.21	-6.67	-68.08
Jun (lower)	-7.45	8.07	-68.09
EF-Tu	-26.31	-20.74	-7.03

The results show that cellular mRNA of (f)TnT (described in chapter 5.1.2 (Results and discussion of differentiation experiments in L6 and C2C12 cells) in section Results) was reduced by approximately 40% after 12 h treatment with 25 μM of antisense fTnT 3'S-ODNs compared to untreated control and 20% when compared to a similarly sense fTnT 3'S-ODN-treated control. The results were in accord with the primer extension analyses. The results from the same northern blot show also that α-actin was reduced by around 50% or 35%, respectively, when compared to untreated or sense fTnT 3'S-ODN-treated controls and the accumulation of α-TM dropped by approximately 60% and 55%, whereas sTnI was reduced by about 30% and 40%. Expression of β-TM, in turn, was reduced by around 35%, compared to untreated control, but did not show alteration, when compared to sense fTnT 3'S-ODN-treated control. FTnI, instead, appeared to be unchanged in both comparisons. Interestingly, however, the cellular mRNA level of TnC showed a slight increase, around 10%, in comparison to untreated control, but showed around 50% increase when compared to sense fTnT 3'S-ODN-treated. Similar results were also gained in another comparable experiment.

The higher band of Jun showed approximately a 70% reduction compared to untreated control, whereas alteration was not apparent in comparison to sense fTnT 3'S-ODN-treated control. The lower band of Jun, on the other hand, did not show change in either comparison. The expression of nonmuscle-specific EF-Tu, however, showed a reduction of about 25% and 20% compared to untreated and sense fTnT 3'S-ODN-treated controls, respectively. The Ethidium bromide stained mRNA gel showed that the lanes were fairly equally loaded.

Antisense fTnT ODNs and protein synthesis. Another focus of the antisense TnT study was to illuminate whether the changes in cellular mRNA level after antisense fTnT 3'S-ODN treatment are also apparent at the protein level and whether translational coregulation of TnT with α-actin, muscle TMs and with other members of the troponin complex exists.

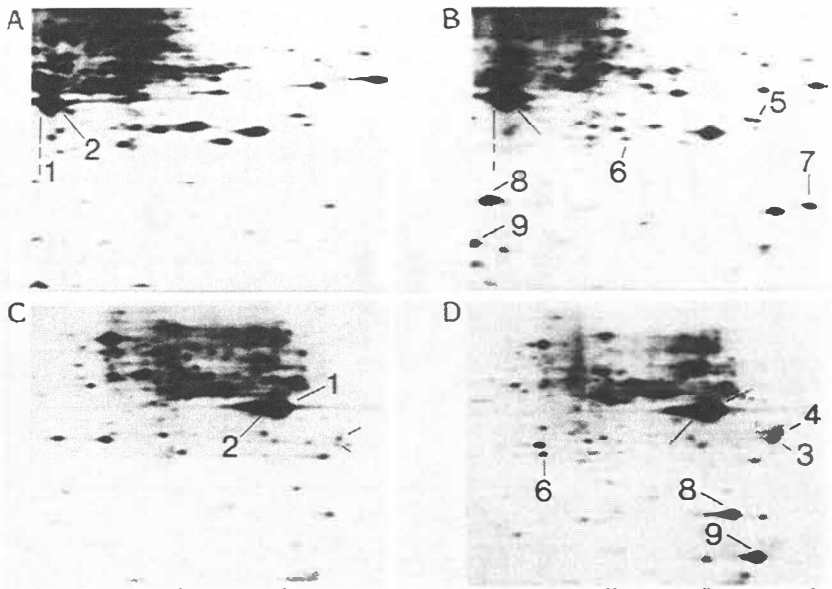

FIGURE 14 Orientation of contractile apparatus proteins in L6 cells. Autofluorograph of 2D-PAGE (NEPHGE) of (A) myoblastic proteins, and (B) myotubic proteins (3.5 d differentiated L6 cells); The left-hand side of the pictures (A) and (B) shows the acidic ends and the right-hand side the basic end of the gel. Autofluorograph of 2D-PAGE (IEF) of respective (C) myoblastic proteins, and (D) myotubic proteins; the left-hand side of the pictures (C) and (D) shows the basic ends and the right-hand side the acidic end of the gel (1) α-actin, (2) β-, γ-actin, (3) α-TM, (4) β-TM, (5) fTnT, (6) xTnT, (7) fTnI, (8) a/eMLC1, and (9) skMLC2.

The Fig. 14 shows the orientation of all the estimated proteins, except the TnC forms, in both IEF and in NEPHGE. These proteins were estimated from the autofluorograps of antisense fTnT 3'S-ODN-, sense fTnT 3'S-ODN-, random sequence S-ODN-treated, and also untreated L6 myotubes. The results of the treatments are shown as box-plots (Hoaglin et al. 1983, Tukey 1990) in Fig. 15.

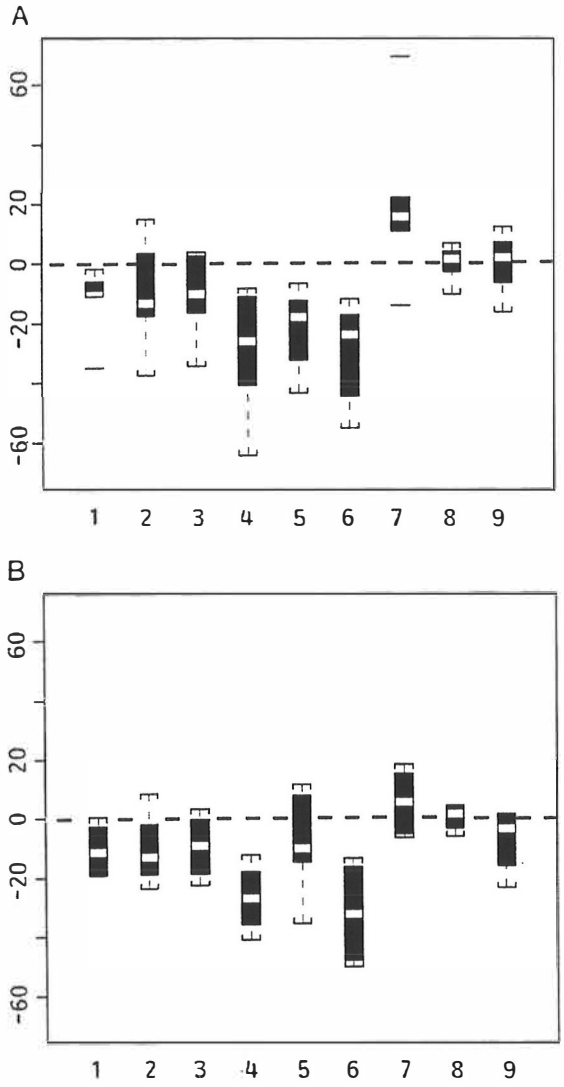

FIGURE 15 Effects of antisense fTnT 3'S-ODN treatment on the protein synthesis of L6 cells. L6 myotubes (3.5 d differentiated) were treated with 25 µM of respective ODNs as described. The prepared autofluorographs were image-analysed, and each individual protein value of antisense ODN-treated cells was compared to the respective control value and the change calculated as a percentage. The box-plots represent the change in (A) antisense fTnT 3'S-ODN-treated myotubes compared to untreated control (n=5-6), (B) antisense fTnT 3'S-ODN-treated myotubes compared to ODN-treated control (includes the results of sense fTnT 3'S-ODN and random sequence S-ODN treatments; (n=4-5). The presentation shows the means, medians, standard deviations, minimums and maximums of the results. (1) α-actin, (2) α-TM, (3) β-TM, (4) fTnT, (5) xTnT, (6) total TnC, (7) fTnI, (8) a/eMLC1, and (9) skMLC2.

Some of the respective numerical results of Fig. 15 as well as the statistical analyses are shown in Table 4.

TABLE 4 Contractile apparatus protein changes in 3.5 d differentiated L6 myotubes treated with 25 μM of antisense fTnT 3'S-ODN for 12 h. Effects of (A) antisense fTnT 3'S-ODN treatment compared to untreated control, (B) antisense fTnT 3'S-ODN treatment compared to ODN-treated control (includes the results of sense fTnT 3'S-ODN-treated and random sequence S-ODN-treated controls), (C) sense fTnT 3'S-ODN treatment compared to untreated control, and (D) random sequence S-ODN treatment compared to untreated control. The changes are presented as percentages. p(1) p-value of Paired t-test, p(2) p-value of Wilcoxon signed-rank test.

(A) Antisense fTnT 3'S-ODN treated culture/ untreated control, alteration in %

Protein	n	Mean	±SD	Median	p(1)	p(2)
actin	5	-12.90	12.85	-10.91	0.11	0.06
α-TM	5	-9.86	20.12	-13.30	0.72	0.63
β-TM	5	-10.83	15.62	-10.21	0.19	0.31
fTnT	5	-30.08	23.04	-26.16	0.05	0.06
xTnT	6	-21.66	13.74	-17.90	0.03	0.04
tot. TnC	5	-30.48	18.55	-24.00	0.03	0.06
fTnI	6	20.01	27.43	15.55	0.25	0.22
a/eMLC1	5	-0.08	6.66	1.40	0.96	1.00
skMLC2	5	-0.14	11.30	1.96	0.78	0.81

(B) Antisense fTnT 3'S-ODN treated culture/ ODN treated control, alteration in %

Protein	n	Mean	±SD	Median	p(1)	p(2)
actin	4	-10.51	9.47	-11.65	0.13	0.25
α-TM	4	-10.43	13.43	-13.19	0.47	0.38
β-TM	4	-9.50	11.45	-9.29	0.24	0.36
fTnT	4	-26.93	12.11	-27.18	0.10	0.13
xTnT	5	-8.19	18.93	-10.34	0.20	0.28
tot. TnC	4	-32.10	18.43	-32.47	0.01	0.13
fTnI	5	5.35	11.26	5.26	0.35	0.63
a/eMLC1	4	0.10	4.63	1.15	0.86	1.00
skMLC2	4	-7.41	11.68	-3.71	0.34	0.58

(C) ODN treated control culture/ untreated control, alteration in %

	Sense fTnT 3'S-ODN/ untreated control			Random seq. S-ODN/ untreated control		
Protein	n	Mean	±SD	n	Mean	±SD
actin	2	-12.61	9.60	2	6.07	11.58
α-TM	2	-1.77	3.72	2	-11.74	8.39
β-TM	2	-0.62	1.80	2	-4.07	15.42
fTnT	2	10.59	8.60	2	3.51	3.99
xTnT	2	-11.88	0.35	3	-8.52	9.95
TnC	2	-3.95	9.31	2	20.98	26.90
fTnI	2	-1.20	9.55	3	25.28	18.62
a/eMLC1	2	-0.75	10.35	2	-4.27	7.80
skMLC2	2	11.71	15.37	2	-1.21	9.97

Protein synthesis of fTnT shows decrease of about 30% after 12 h treatment with 25 μM of antisense fTnT 3'S-ODNs, compared to untreated or sense fTnT 3'S-

ODN-treated control (Table 4). Treatment for only 6 h did not alter the synthesis of fTnT (data not shown). Synthesis of xTnT also showed a reduction of approximately 20% compared to untreated control. Comparison to sense fTnT 3'S-ODN treated control, on the other hand, shows only around 10% reduction. Protein synthesis of α-actin, α-TM and β-TM also decreased by around 10-15% after the antisense fTnT 3'S-ODN treatment, which results are fairly comparable to the changes observed in the cellular mRNA level (Table 3). Synthesis of total TnC was reduced by approximately 30% compared to either untreated or ODN-treated controls, which is contrary to the results of the mRNA studies (Table 3). Synthesis of fTnI shows no change in comparison to untreated control, but shows an increase of about 20% when compared to untreated control. However, the random sequence S-ODN used appeared to have a slightly increasing influence on the synthesis of TnC but also on fTnI. Protein synthesis of a/eMLC1 or skMLC2 were unaffected (Table 4).

The results of the statistical studies show that the changes in fTnT, xTnT and total TnC, when compared to those in untreated control, were somewhat significant ($p < 0.05$ or $p < 0.10$) in both tests. Significant changes, but minor, were also observed in α-actin and fTnI ($p < 0.15$, $p < 0.25$, respectively). However, when the results are compared to ODN-treated control, the changes in fTnT, xTnT, total TnC, and α-actin have less significance ($p < 0.15$ or $p < 0.25$) in both tests than in comparison to untreated control.

Summary of the effects of antisense TnT treatments in L6 cultures. The previously described results of the antisense fTnT 3'S-ODN experiments in L6 cells (Table 3, Table 4) are summarized in Table 5.

TABLE 5 The effects of antisense fTnT 3'S-ODN treatments on gene expression of totally 4 d differentiated L6 cultures. Untr. = untreated, sense = sense fTnT 3'S-ODN, ODN = sense fTnT 3'S-ODN and random sequence S-ODN, ctrl = control.

Alterations after antisense fTnT ODN treatment in L6 cells

	Antisense fTnT 3'S-ODN (25 μM, 12 h)/			
	untr. ctrl	sense ctrl	untr. ctrl	ODN ctrl
gene	mRNA	mRNA	protein	protein
α-actin	---	--	0	0
α-TM	---	---	0	0
β-TM	--	0	0	0
(f)TnT	--	-	--	-
fTnT (p.e.)	----	---		
xTnT			-	0
TnC	0	+++	--	--
fTnI	0	0	+	0
sTnI	--	--		
a/eMLC1			0	0

skMLC2			0	0
Jun (top)	----	0		
Jun (low)	0	0		
EF-Tu	-	-		

0-15% alteration = 0; 15-30% alteration = + or -; 30-45% alteration = ++ or --; 45-60% alteration = +++ or ---; 60-100% alteration = ++++ or ----.

Discussion:

As mentioned earlier, muscles express preferential combinations of TnT forms during development (Breitbart & Nadal-Ginard 1987, Härtner et al. 1989, Briggs et al. 1990). The selection of fTnT isoforms, however, depends partially on the factors influencing splicing and their expression (Andreadis et al. 1987, Breitbart & Nadal-Ginard 1987). The variety of these factors may change and/or increase during the normal development of rat skeletal muscle, thereby also increasing the variety of fTnT isoforms. Nevertheless, the complementary region of the umODN probe used against fTnT (the same sequence as antisense fTnT 3'S-ODN) covered the end of exon 2 (14 nucleotides) and the beginning of exon 3 (1 nucleotide), which exons exist in all fTnT gene-coded 64 isoforms. The probe gave only one band in the northern blot, but in the primer extension studies, where the same ODN was used as a primer, it gave a faint additional signal, just underneath the main band (Fig. 13). As mentioned earlier, the homology of sTnT to fTnT or to the antisense fTnT ODN-binding region is unknown as are the existence and expression of sTnT in rat or in L6 cells.

The results of the antisense fTnT 3'S-ODN study showed that the reduction in cellular mRNA of (f)TnT is only a moderate 40-50% (Table 3, Table 5). This may be due to the accessibility of the message for ODN to bind: fTnT mRNA is considerably long and also probably fairly folded. The functional concentration of 3'S-ODNs may also have been inadequate inside the cells for complete blockade of the fairly marked amount of (f)TnT mRNA (Fig. 13); according to differentiation studies, the synthesis of (f)TnT is quite pronounced (Fig. 2) and also its half-life rather long, around 19 h (Table 2). It is probable that only around 1-2% of cellular mRNA of fTnT is translatable poly-A tailed mRNA; thus the non-functional pool may sequester some of the ODNs, which in turn affects the reduction in protein level. However, protein synthesis of fTnT decreased by around 30%, which suggests that some of the reduced mRNA was translatable mRNA (Table 4, Table 5). On the other hand, the half-life of both the described TnT proteins is about 9-11 h, whereas the treatment was 12 h, hence the reduction in protein level may become apparent fairly slowly. The reduction in fTnT was slightly more than that of xTnT, but since xTnT showed reduction, especially compared to untreated control, it suggests that its mRNA has some complementarity to antisense fTnT 3'S-ODN. Compensatory TnT isoforms did not become detectable in any experiment. However, the questions as to whether the unaffected translatable mRNAs are translatable for longer or the synthesized proteins are more stable in order to compensate for the reduction, cannot be answered.

The antisense fTnT 3'S-ODN treatment also had some influence on expression of the other members of the thin filament (Fig. 13, Table 3, Table 5). Accumulation of cellular mRNA of α-actin and α-TM showed a reduction of approximately 30-50%, whereas accumulation of total TnC showed a slight increase. Whether the changes are due to the reduction of TnT or the ODN used either directly or indirectly, for instance through nonspecific influence on the transcriptional factors, remains unclear. However, the results of the differentiation experiments showed expectedly (Fig. 6) that accumulation of cellular mRNA of (f)TnT had a similarity to that of fTnC and fTnI, as well as α-actin and α-TM during differentiation. Thus some coordination seems to occur already at the transcriptional level and the coregulation may rely on for instance common regulatory sequences, such as CCACCC-type motifs. Some fine-tuning to maintain proper stoichiometry among the members may also occur for example during the splicing event, at least with TnT and α-TM, but possibly also with α-actin. As mentioned earlier, the binding region of antisense fTnT 3'S-ODN covered 14 nucleotides of exon 2 and 1 nucleotide of exon 3 of fTnT; thus the ODNs may have had some influence on the splicing event of fTnT and its regulating factors. The same factors that splice the described exons of fTnT may also be involved in splicing some of the exons of α-TM - it has suspected that TnT and TM primary transcripts are spliced, at least partially, by the same factors (Andreadis et al. 1987, Breitbart & Nadal-Ginard 1987). However, α-actin is not alternatively spliced, but due to the conserved character of exons 2 and 3 of fTnT, they may be spliced by the common factors which may also be involved in splicing of α-actin. α-actin may also have been more susceptible to antisense interference, specific or non-specific, since its translation in-between cells differentiated for 48 h to 96 h showed a marked reduction (Fig. 7), whereas transcription around that time appeared to be fairly moderate (Fig. 6) - the antisense ODN treaments were initiated in cells differentiated for about 84 h and the cells had been differentiated for approximately 96 h by the end of the experiments.

The results showed that treatment with sense fTnT 3'S-ODN has a reductive influence on cellular mRNA accumulation of (f)TnT (Fig. 13). The results is partially in line with that reported by Cameron & Jennings (1991). The reduction may be caused by a partial binding of sense fTnT 3'S-ODN to other sites of fTnT mRNA as an antisense ODN. Another option may be, as mentioned earlier, at splicing level due to the sense fTnT 3'S-ODN, which was a complement to the antisense ODN used. According to Xu et al. (1993), the 5' splice site can include 3 nucleotides of the exon in addition to the sequences in intron and, on the other hand, the 3' splice site can also include the end of the intron and the first nucleotide of the exon. Thus there is the possibility that sense fTnT 3'S-ODN may have reduced the functional pool of specific factors influencing splicing, although the factors binding ability to ODNs is not known. Other, rather different explanations may also be, a triple helix formation of the ODN with the fTnT gene and disturbance of transcription by antisense ODN- binding to the non-transcribed strand (Toulmé & Hélène 1988, Weintraub 1990), or on the other hand, the sense fTnT 3'S-ODN-binding to the transcribed strand of DNA, and thus also causing a disturbance on transcription. Nevertheless, by taking together

the influences of both antisense fTnT and sense fTnT treatments, it seems that the effects of antisense fTnT 3'S-ODN have specificity, although some nonspecific influence is also probable.

Reduction of α-actin, α-TM and β-TM was only 10-15% at protein level (Table 4, Table 5). The results suggest that some of the reduced respective mRNAs were translatable mRNA. The results of the differentiation studies described earlier suggested, however, that at least α-TM may be transcribed in excess, which would partially explain the only modest reduction in protein level (Fig. 6, Fig. 7). The other reason for the differences in between mRNA and protein synthesis could be that the remaining transcripts may be translated more efficiently as a compensatory mechanism.

The slight increase of TnC after antisense fTnT treatment (Fig. 13) was not apparent at translational level, but instead, protein synthesis of total TnC showed reduction (Table 4). The sense 3'S-ODN treatment, in turn, reduced the accumulation of cellular mRNA of TnC compared to untreated control (Fig. 13), but its influence on protein synthesis was only very minor. Random sequence S-ODN, on the other hand, seemed to have slightly increasing influence on TnC protein synthesis. Nevertheless, the difference of influence of the antisense treatments on mRNA and protein synthesis suggests that translation of TnC is regulated by unknown control mechanisms. The differentiation experiments suggested the same, since marked increase in cellular mRNA of fTnC (Fig. 6) was not apparent at protein level (Fig. 7). The results suggest, too, the possible existance of some type of coregulation with (f)TnT and TnC after transcription - for instance, the importance of the cellular mRNA or the protein level of TnT as a regulator of TnC protein synthesis through for example mRNA:mRNA interactions is not known.

The results also showed that accumulation of cellular mRNA of fTnI was unaltered in either antisense or sense fTnT-treated cells, but showed a slight increase in random sequence ODN-treated control compared to untreated control (Fig. 13). Although protein synthesis of fTnI seemed to vary in the different antisense fTnT 3'S-ODN experiments, it generally showed a slight increase, especially when compared to untreated cells (Table 4). The reason for the increase may be that the ODN has some influence on the secondary structure of the fTnI mRNA, which may alter it into a more translatable form. However, one option is related to TnC: as mentioned earlier, antisense fTnT 3'S-ODN treatment seemed to increase the cellular mRNA accumulation of total TnC slightly (Fig. 13), but reduce its protein synthesis (Table 4). Thus reduction in TnC synthesis may induce more pronounced protein synthesis of fTnI. The excess of fTnI would be more efficent in trapping reduced TnC molecules to ensure the appropriate assembly of a functional thin filament, as discussed in chapter 5.1.4 (Concluding remarks: thin filament and its formation). Accumulation of cellular mRNA of sTnI, in turn, showed a slight reduction, whereas in sense ODN-treated cells sTnI showed a slight increase compared to untreated control (Fig. 13). Unfortunately sTnI protein was not detectable in any experiment.

The antisense treatment had minor influence on the protein synthesis of MLC forms. Sense treatment, instead, seemed to increase skMLC2 synthesis slightly, whereas random sequence ODN had no influence (Table 4, Table 5). The reason for the increase may be that sense fTnT 3'S-ODN may have some influence

on the folding of skMLC2 mRNA, which may improve its translatability. The increase, however, may occur already at transcriptional level, a possibility which was not examined.

The results showed a reduction in accumulation of cellular mRNA of Jun, especially of the higher band, and a slight reduction of EF-Tu (Fig. 13). The ODN treatment as such may induce the cells to differentiate faster than untreated control cells - the results of the differentiation experiment with C2C12 cells showed a reduction of Jun during differentiation (Fig. 4, Fig. 6). However, the appearance of the cells after the ODN treatments was similar to that of untreated control cells cultured in the same way. The ODN treatment, however, seemed mainly to have a reductive influence on the accumulation of muscle- specific mRNAs, whereas in normal cells, accumulation seemed to increase during differentiation (Fig. 6). The influence may indicate that ODNs have also some toxic influence on cells.

Conclusions:

The results showed that the reduction of cellular mRNA and protein of (f)TnT after antisense fTnT 3'S-ODN treatment is only moderate. Treatment with sense fTnT 3'S-ODN also had a reducing influence on the cellular mRNA accumulation of (f)TnT. Some other members of the thin filament were also affected after the treatments (Table 5), which suggests that cells may have mechanisms to balance the reduction of one member of a thin filament by reducing the synthesis of others. However, the mechanisms by which alteration of TnT can influence the stoichiometry of the gene expression of other members is unknown. One option may be that the influence is mediated for example through the mRNA:mRNA interactions of different members of the thin filament. The antisense studies also showed that blocked TnT isoform is not compensated for by an increase in the expression of the other isoform/s.

5.2.2.3 Antisense ODNs and RNA against troponin C in C2C12 cells

To further examine the coordinated and stoichiometric expression of contractile apparatus proteins, TnC was also examined. One purpose of the antisense TnC experiments was to clarify the coregulation of TnC with TnT, and on the other hand, with TnI. According to the differentiation experiment described earlier, C2C12 cultures seemed to express more c/sTnC than fTnC, and also c/sTnC seemed to be the initial one. Thus one aim was to evaluate the influence of c/sTnC shortage on the expression of fTnC. Another focus was to determine the influence of alteration of the TnC level on the normal stoichiometric and coordinated expression of other genes of the contractile apparatus proteins.

Results:

Antisense treatments in C2C12 cultures. Different types of ODNs, varying in length, binding region and modification as well as different concentrations and incubation times were tested in effort to reach maximum reduction in TnC expression. Transfection of C2C12 cells with a plasmid expressing full-length antisense TnC RNA was also carried out.

Unmodified ODNs. The first approach to modulating TnC gene expression was to use umODNs, which are somewhat unstable. Other umODNs than those used in the experiment in Fig. 16 (Appendix 2) showed very little or no effect on TnC synthesis (data not shown).

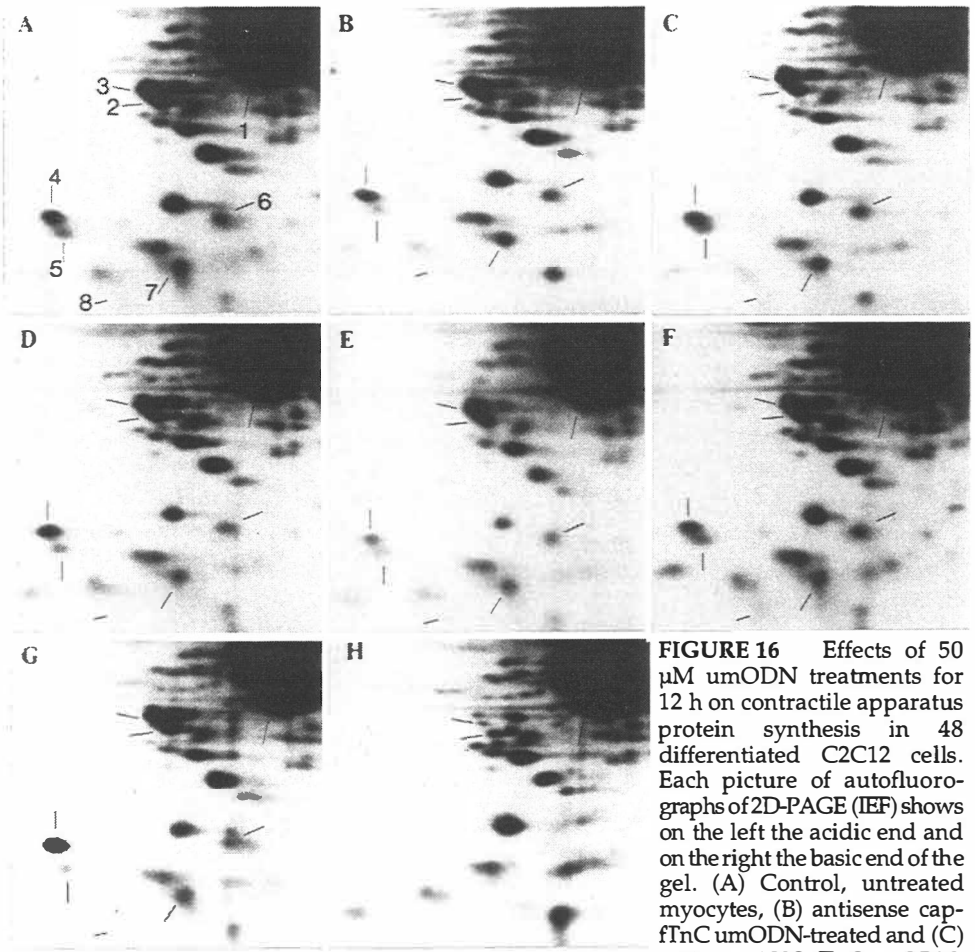

FIGURE 16 Effects of 50 μM umODN treatments for 12 h on contractile apparatus protein synthesis in 48 differentiated C2C12 cells. Each picture of autofluorographs of 2D-PAGE (IEF) shows on the left the acidic end and on the right the basic end of the gel. (A) Control, untreated myocytes, (B) antisense cap-fTnC umODN-treated and (C) antisense AUG-fTnC umODN-treated myocytes. (D) Control, untreated myocytes, (E) antisense cap-c/sTnC umODN-treated, (F) antisense AUG-c/sTnC umODN-treated myocytes, (G) control, with random sequence umODN-treated myocytes, and (H) untreated C2C12 myoblasts. (1) Actin, (2) α-TM, (3) β-TM, (4) c/sTnC, (5) fTnC, (6) total MLC1, (7) skMLC2, and (8) fMLC3.

The results of the image-analyses of the pictures presented in Fig. 16 are summarized in Table 6.

TABLE 6 Effects of antisense umODNs against c/sTnC and fTnC on accumulation of contractile apparatus proteins. Cells were treated with 50 µM of antisense umODNs for 12 h. Antisense ODN-treated cultures compared to (A) untreated control, and (B) random sequence ODN-treated control. (C) Random sequence ODN treated cells compared to untreated control. Alterations shown as percentages; n=1.

(A) Unmodified antisense ODN-treated cultures/ untreated control, alteration in %

Protein	cap-fTnC	AUG-fTnC	cap-c/sTnC	AUG-c/sTnC
α-actin	2.51	-6.31	-17.72	6.72
α-TM	11.68	-3.44	3.03	22.08
β-TM	-12.96	-15.12	3.75	12.63
xTnT	20.83	12.50	-7.14	14.29
fTnC	-53.49	4.65	-36.84	26.32
c/sTnC	-22.10	-3.16	-36.78	-3.45
tot. MLC1	-21.05	10.53	-13.04	26.09
skMLC2	-1.18	0	-16.22	2.70

(B) Unmodified antisense ODN-treated cultures/ random sequence ODN-treated control, alteration in %

Protein	cap-fTnC	AUG-fTnC	cap-c/sTnC	AUG-c/sTnC
α-actin	3.98	-4.96	-18.42	5.81
α-TM	14.04	-1.40	-16.49	-1.05
β-TM	-9.32	-11.58	-2.25	6.11
xTnT	61.11	50.00	44.44	77.78
fTnC	-20.00	80.00	-4.00	92.00
c/sTnC	-35.65	-20.00	-52.17	-26.96
tot. MLC1	-6.25	31.25	-16.67	20.83
skMLC2	13.51	14.86	-16.22	2.70

(C) Random sequence ODN-treated culture/ untreated control, alteration in %

Protein	fTnC experiment	c/sTnC experiment
α-actin	-1.42	0.86
α-TM	-12.03	23.38
β-TM	-4.01	6.14
xTnT	-25.00	-35.71
fTnC	-41.86	-34.21
c/sTnC	21.05	32.18
tot. MLC1	-15.79	4.35
skMLC2	-12.94	0

The results show (Fig. 16, Table 6) that the most marked reductions were achieved with the 5' cap-region-binding antisense umODNs by using 50 µM of ODNs in 48 h differentiated C2C12 cells for 12 h. Protein synthesis of fTnC was reduced by approximately 50% and c/sTnC by around 20% after antisense cap-

fTnC umODN treatment compared to untreated control. Synthesis of total MLC1 was also reduced by about 20%. The same treatment increased protein synthesis of xTnT by around 20% compared to untreated control (Table 6) (xTnT described in chapter 5.1.2 (Results and discussion of differentiation experiments in L6 and C2C12 cells) in section Results), whereas random sequence control umODN had a reducing effect on xTnT synthesis. Antisense AUG-fTnC umODNs had no influence on either TnCs or any other of the proteins examined.

Antisense cap-c/sTnC and AUG-c/sTnC umODN treatments reduced c/sTnC synthesis by approximately 35% and 5%, respectively, compared to untreated control. The cap-c/sTnC umODNs reduced also protein synthesis of fTnC, total MLC1, and skMLC2 (Fig. 16). However, random sequence umODN used as a control had slightly increasing effect on the synthesis of c/sTnC, whereas fTnC showed a reduction.

Phosphorothioated ODNs. Completely phosphorothioated S-ODN was targeted against the internal region of c/sTnC (antisense int-S-ODN; 18mer), but it, also, had only 2 bp mismatch to fTnC. Thus the ODN probably also interacted with fTnC mRNA. However, short treatments (3, 6, or 12 h) with 5, 10 or 20 µM concentrations of int-S-ODNs did not seem to influence the synthesis of the c/sTnC protein or others (data not shown), whereas the higher concentrations and long incubations affected gene expression as described.

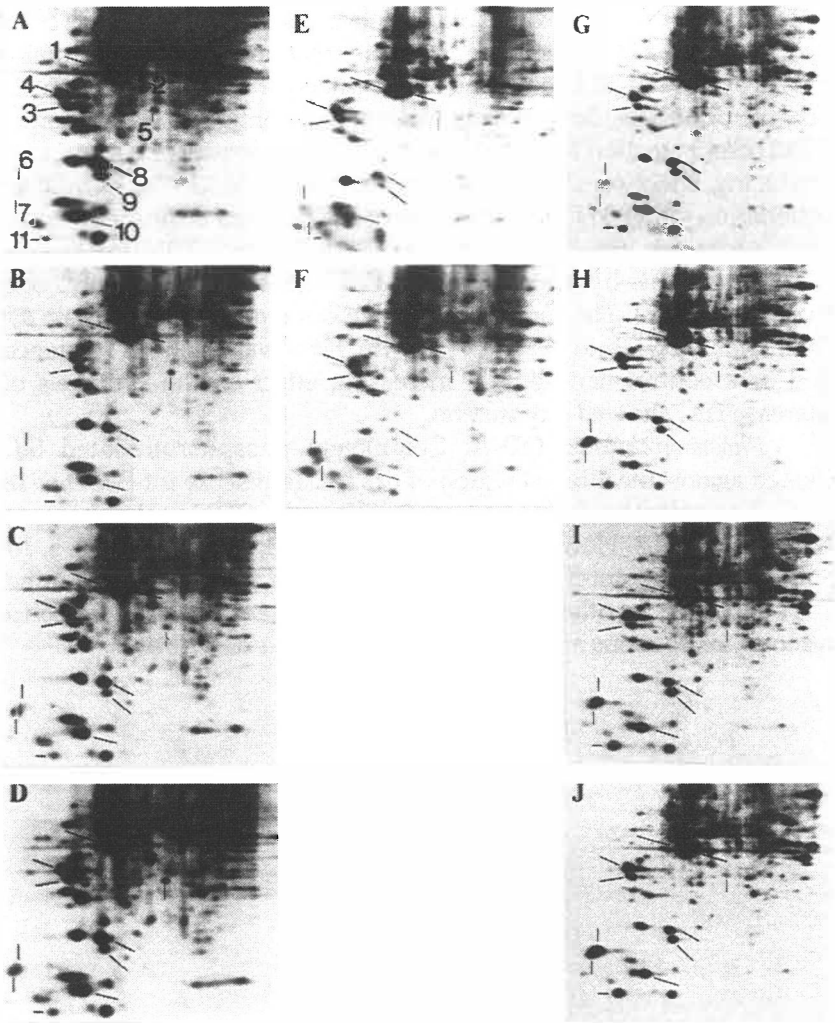

FIGURE 17 Influences of S-ODNs on C2C12 protein expression. Pictures A-D, E-F, and G-J represent separate experiments. Proteins of (A) untreated control, (B) 20 μM of antisense int-S-ODN-treated, (C) 20 μM random sequence treated control, and (D) 40 μM of antisense int-S-ODN-treated cells. Cells were 48 h differentiated and treatments lasted 48 h. Proteins of (E) untreated control, and (F) 20 μM of antisense int-S-ODN-treated cells. Cells were 24 h differentiated and treatments lasted 72 h. Proteins of (G) untreated control, (H) 40 μM of antisense int-S-ODN-treated, (E) 40 μM of random sequence S-ODN-treated control, and (J) 100 μM of antisense int-S-ODN-treated cells. Cells were 48 h differentiated and treatments lasted 48 h. Each picture of autofluorographs of 2D-PAGE (IEF) shows on the left the acidic end and on the right the basic end of the gel. (1) α-actin, (2) β-, γ-actin, (3) α-TM, (4) β-TM, (5) xTnT, (6) c/sTnC, (7) fTnC, (8) v/sMLC1, (9) fMLC1, (10) skMLC2, and (11) fMLC3.

The autofluorographs were image-analysed and the results are summarized in Table 7. However, Table 7 shows the mean of 48 h treatment with 20 μM antisense ODN in 48 h differentiated cells, although only one picture (picture B) of two experiments is presented. The results of the other experiment are very

similar. The mean of 40 μM antisense treatment is also presented, and it includes the pictures D and H. These 40 μM experiments were performed three times. The effects of a third experiment were comparable in the experiments shown in Fig. 17. Moreover, in Table 7 panel (B) shows the antisense treatments compared to random sequence-treated controls. The comparisons have been done from the pictures shown in Fig. 17, in other words, from the respective experiments.

TABLE 7 Effects of S-ODNs against c/sTnC on protein accumulation of contractile apparatus members in C2C12 cells. Int-S-ODN-treated compared to (A) untreated control, and (B) random sequence S-ODN-treated control. (C) Random sequence S-ODN-treated compared to untreated control. Alterations showed as percentages; n=1; }=total fTnC and c/sTnC.

(A) Antisense internal-c/sTnC ODN-treated culture compared to untreated control, alteration in %

Protein	20 µM as 48+48 h n=2 (mean)	20 µM as 24+72 h n=1	40 µM as 48+48 h n=2 (mean)	100 µM as 48+48 h n=1
α-actin	-18.60	-3.25	-14.46	-1.72
α-TM	-12.89	-6.40	7.46	38.82
β-TM	-16.18	2.07	3.06	43.21
xTnT	-3.90	41.38	16.89	33.33
fTnC	}-36.55	92.67	}222.98	}410.45
c/sTnC		98.10		
fTnI (n=1)	-59.09	-	36.36	-
v/sMLC1	-54.57	-66.56	-8.64	23.75
fMLC1	-37.24	-41.67	1.50	39.76
skMLC2	-23.32	-13.11	2.35	24.35
fMLC3	-32.88	-2.58	36.93	115.97

(B) Antisense internal-c/sTnC ODN-treated culture/ random sequence ODN-treated control, alteration in %

Protein	20 µM as/ 20 µM rs 48+48h n=1	40 µM as/ 20 µM rs 48+48 h n=1	40 µM as/ 40 µM rs 48+48 h n=1	100 µM as/ 40 µM rs 48+48 h n=1
α-actin	-33.40	-2.41	-15.17	10.66
α-TM	-12.62	-19.27	10.19	9.30
β-TM	-13.44	9.01	-25.96	6.04
xTnT	20.59	33.82	-33.90	-18.64
fTnC	}-46.38	}-5.88	}94.20	}43.70
c/sTnC				
fTnI	-55.00	50.00	-	-
v/sMLC1	-27.73	-4.55	14.64	12.94
fMLC1	-61.04	-27.67	22.31	-8.30
skMLC2	-12.14	-39.32	31.07	-11.46
fMLC3	-54.20	-42.57	11.47	3.21

(C) Random sequence ODN-treated control/ untreated control, alteration in %

Protein	20 µM rs 48+48 h	40 µM rs 48+48 h
α-actin	-1.89	-1.19
α-TM	1.98	27.00
β-TM	-2.65	35.05
xTnT	-10.53	8.33
TnC	}60.47	}255.22
c/sTnC		
fTnI	-9.09	-
v/sMLC1	-43.78	9.58
fMLC1	-24.17	52.41
skMLC2	-16.26	40.43
fMLC3	37.89	109.24

The result shows that treatment of 48 h differentiated C2C12 myocytes with 20 µM of int-S-ODN against c/sTnC for 48 h reduces by a moderate 35% the protein synthesis of the total TnC compared to untreated control (Fig. 17, Table 7). However, synthesis of most other proteins also shows reduction compared to untreated or random sequence ODN-treated control. The treatment of 24 h differentiated myocytes with 20 µM for 72 h, on the other hand, increased synthesis of c/sTnC and fTnC by approximately 100% and 90%, respectively, compared to untreated control (Table 7). Treatment with a higher concentration, 40 µM of ODN for 48 h, increased synthesis of total TnC even more, by around 220%. An even further increase, about 410%, is apparent compared to untreated control when concentration is increased to 100 µM for 48 h. However, the increases are more modest and varied when the results are compared to random sequence S-ODN-treated control. The increase in TnC in 40 μM antisense S-ODN-treated cells is not apparent when compared to 20 μM random sequence S-ODN-treated control (Fig. 17 C, D; Table 7), whereas in comparison to 40 µM treated control, the increase is about 95% (Fig. 17 H, I; Table 7). The comparison of 100 µM antisense treatment to 40 µM random sequence S-ODN- treated control shows an increase of around 45% in TnC synthesis (Fig. 17 I, J; Table 7). Nevertheless, Fig. 17 (G to J) shows that the results of 40 µM antisense treatment are more comparable to untreated control, and the results of 100 µM antisense treatment are more comparable to 40 µM of random sequence-treated control due to the slight loading differences.

Treatment with 20 µM of ODN for 48 h or for 72 h, or with 40 µM for 48 h does not seem to have any or only minor influence on the protein synthesis of α-actin, α-TM or β-TM. Treatment with 100 µM for 48 h does not seem to have any influence on α-actin either, but synthesis of α-TM shows an increase of about 40%, and β-TM 45%, compared to untreated control. In comparison to random sequence S-ODN-treated control, the increase is not apparent. Synthesis of xTnT shows also an increase of approximately 20-40% in all the experiments, except 20 μM antisense treatment for 48 h, compared to untreated control (Table 7). When the results are compared to the respective 40 μM random sequence S-ODN-treated control, however, both 40 and 100 µM antisense treatments show a

reduction of around 20-30% in synthesis of xTnT, whereas comparison of the results of 20 and 40 μM antisense treatments to the respective 20 μM random sequence control show an increase of about 20-30% in xTnT synthesis.

In 48 h differentiated cells treated with 20 µM antisense S-ODN for 48 h, the protein synthesis of MLC forms is affected fairly similarly as in 24 h differentiated cells treated for 72 h. In 48 h treated cells, synthesis of v/sMLC1 and fMLC1 is reduced by approximately 55% and 35%, whereas in 72 h treated cells the reduction is about 65% and 40%, respectively, compared to untreated control. Synthesis of skMLC2 and fMLC3 also shows a reduction of around 25-30% in 48 h differentiated cells, but in 72 h treated cells, they are unaffected. When the 48 h differentiated cells were treated with 40 µM of antisense S-ODN for 48 h, only synthesis of fMLC3 was affected, showing an increase of approximately 35%, compared to untreated control. Increase of the concentration to 100 µM for 48 h approximately increased the synthesis of v/sMLC1 by 25%, fMLC1 by 40%, skMLC2 almost 25%, and fMLC3 by 115%. However, when the results of the antisense treatments are compared to respective random sequence S-ODN-treated controls, the results show more variation (Table 7). In 20 μM for 48 h treated cells, v/sMLC is reduced by only around 25%, and fMLC1 and fMLC3 more markedly, by approximately 60% and 55%, compared to 20 μM random sequence S-ODN-treated control. The comparison of the results of 40 μM antisense treatment to the same control shows a reduction in synthesis of fMLC1 of about 25%, and of skMLC2 and fMLC3 by around 40%. Comparison of 40 μM antisense treatment to respective 40 μM random sequence S-ODN-treated control gives different results: v/sMLC1 and fMLC3 are unaffected, whereas fMLC1 and skMLC2 shows an increase of about 20% and 30%, respectively. Comparison of 100 μM treatment with the same control, on the other hand, does not show any changes.

Antisense RNA. C2C12 cells were also transfected with antisense c/sTnC plasmid construct. Since the transcription of a c/sTnC construct was driven by a non-muscle-specific promoter from a human cytomegalovirus (CMV) (Donoghue 1982, Boshart et al. 1985, Hanahan 1985, Foecking & Hofstetter 1986), the expression of antisense c/sTnC was constitutive, being apparent also at the myoblast stage. Transfectants were chosen according to this property: equal amounts of total myoblastic mRNA from different colonies were exposed to northern blot analysis and hybridized with random primed c/sTnC plasmid (data not shown). The colonies with the highest expression were taken for southern blot analyses, and according to the results, one colony was selected. It is likely that the selected colony had only one copy of the antisense c/sTnC construct, since the southern blot signal was very weak (data not shown) and, also because of the only very modest expression seen in the northern blot. Nevertheless, the colony was used for estimations of the influence of antisense c/sTnC RNA expression on the protein accumulation of endogenous c/sTnC and some other members of the contractile apparatus.

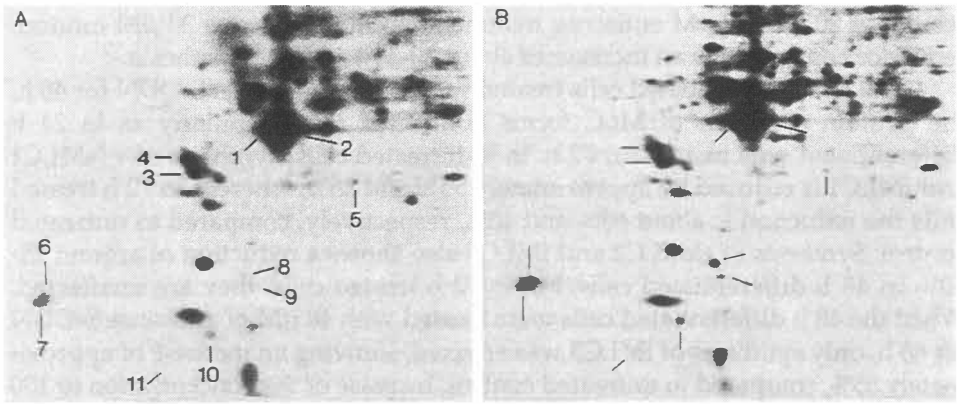

FIGURE 18 Protein expression in antisense c/sTnC transfected C2C12 myotubes. Both transfected and non-transfected C2C12 cells were differentiated 6 d. The left-hand side of both pictures of autofluorographs of 2D-PAGE (IEF) shows acidic end and the right-hand side the basic end of the gel. (A) Antisense c/sTnC transfected C2C12 myotubic proteins, (B) control, non-transfected C2C12 myotubic proteins. (1) α-actin, (2) β-, γ-actin, (3) α-TM, (4) β-TM, (5) xTnT, (6) c/sTnC, (7) fTnC, (8) v/sMLC1, (9) fMLC1, (10) skMLC2, and (11) fMLC3.

The results of the image-analyses are listed in Table 8.

TABLE 8 Effects of antisense c/sTnC RNA on contractile apparatus protein accumulation in C2C12 cells. Protein accumulation of transfected cells was compared to non-transfected controls (n=1). Results are in percentages.

	Antisense-c/sTnC transfected cells/ non-transfected control
Protein	Alteration %
α-actin	2.13
α-TM	23.22
β-TM	5.00
xTnT	17.07
fTnC	1.18
c/sTnC	-43.32
v/sMLC1	-61.54
fMLC1	-51.08
skMLC2	-18.46
fMLC3	5.77

The transfected C2C12 cells fused less efficiently than their normal non-transfected counterparts which were used as controls; thus the results may not give the correct picture of the actual changes. In both transfected and non-transfected cultures, however, approximately 60% of the plating area was covered with fused myotubes, a level of fusion which was apparent in the 5 d differentiated antisense c/sTnC transfected cells. The non-transfected cells attained that

level already on the 4th day. Visibly both transfected and non-transfected cultures were similar when the cells were harvested on the 6th day of differentiation.

The results show (Fig. 18, Table 8) that in antisense-c/sTnC transfected cells, c/sTnC protein synthesis was reduced by 45%, whereas fTnC seemed to be unaffected compared to the non-transfected control. The reduction is slightly more than that produced by the previously described antisense cap-c/sTnC umODN. Synthesis of α-actin and β-TM seemed to be unaffected (Fig. 18), whereas α-TM increased by about 25% and xTnT by around 15%. A similar increase was apparent also with earlier described antisense AUG-c/sTnC umODN-treated cells (Table 6). The protein synthesis of v/sMLC1 and fMLC1, instead, was reduced by approximately 60% and 50%, respectively, and skMLC2 by around 20% in transfected cells. Synthesis of fMLC3 seemed to be unaffected. The results for MLC forms have resemblance with the effects of the treatment of 24 h differentiated C2C12 culture with 20 μM of antisense int-S-ODNs for 72 h (Table 7).

ODN experiments in vitro. In vitro translation methods are often used for screening the most suitable ODN, and also the needed concentration of ODN for experiments in vivo (Marcus-Sekura 1988, Boiziau et al. 1991, Wagner 1994). On this occasion the method was used in an effort to find an explanation for the previously described results of the antisense TnC ODN experiments.

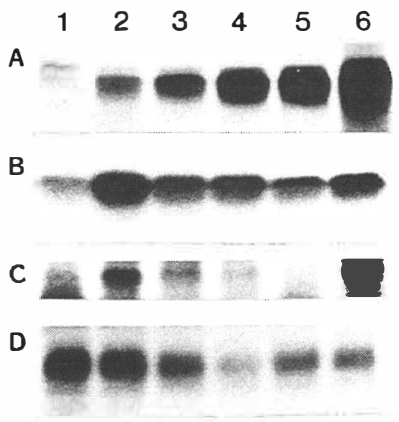

FIGURE 19 In vitro translation of in vitro transcribed c/sTnC mRNA with different ODNs. Antisense=as. (A) Rabbit reticulocyte lysate: (1) lysate without mRNA and ODN, (2) mRNA without ODN; mRNA with (3) 1 μM, (4) 5 μM, (5) 10 μM, and (6) 20 μM of as cap-c/s-TnC ODN. (B) Rabbit reticulocyte lysate: (1) mRNA without ODN; mRNA with (2) 10 μM as cap-c/sTnC, (3) 10 μM sense cap-c/s-TnC, (4) 10 μM as AUG-c/sTnC, (5) 10 μM sense AUG-c/sTnC, and (6) 10 μM jun umODNs. (C) Rabbit reticulocyte lysate: (1) mRNA without ODN, mRNA, with (2) 10 μM as cap-c/sTnC (3) 10 μM as AUG-c/sTnC, (4) 10 μM as int-c/sTnC umODNs, (5) 10 μM as int-c/sTnC S-ODN, and (6) 10 μM sense int-c/sTnC umODN. (D) Wheat germ extract: (1) mRNA without ODN; mRNA with (2) 10 μM as cap-c/sTnC, (3) 10 μM sense cap-c/sTnC, (4) 10 μM as AUG-c/sTnC, (5) 10 μM sense AUG-c/sTnC, and (6) 10 μM jun umODNs.

The results of the image-analyses of the experiments shown and some other experiments are listed in Table 9.

TABLE 9 Results of in vitro translation studies of c/sTnC in (A) rabbit reticulocyte lysate and in (B) wheat germ extract. Results are shown as percentages.

(A) Rabbit reticulocyte lysate, alteration in %

ODN	n	Mean	±SD
as cap	4	141.14	128.91
sense cap	2	58.01	24.78
as AUG	3	50.53	24.18
sense AUG	2	14.72	38.32
as internal	1	-75.06	-
sense internal	1	935.71	-
Jun	3	9.75	43.55

(B) Wheat germ extract, alteration in %

ODN	n	Mean	±SD
as cap	2	0.06	0.71
sense cap	2	-34.20	20.81
as AUG	2	-58.91	5.01
sense AUG	2	-19.52	11.55
Jun	2	-45.24	13.23

The results show unexpectedly that translation of c/sTnC increased steadily instead of decreased in rabbit reticulocyte lysates which were supplemented with an increasing concentration of antisense cap-c/sTnC umODNs compared to control, non-ODN supplemented lysate (Fig. 19). Antisense AUG-c/sTnC umODN (10 µM) also had an increasing influence on synthesis, although the increase was less compared to treatment with the same concentration of cap-c/sTnC umODN (Fig. 19, Table 9). However, comparable incubations with the respective sense umODNs or nonspecific umODNs also increased synthesis, but to a much lesser extent compared to those treated with the respective antisense umODNs. Thus some of the effects of the ODNs were nonspecific, but the increase induced by the antisense cap-c/sTnC umODN and antisense AUG-c/sTnC umODN treatments exceeded that of the controls.

Heat-denaturation, which opens the secondary structures of the native c/sTnC mRNA, was also tested; generally heat-denaturation enhances the ODN-binding ability of the mRNA (Marcus-Sekura 1988). However, the overall translation was markedly reduced, but the influences of the ODNs on translation were not affected (data not shown). Furthermore, antisense cap-c/sTnC, AUG-c/sTnC and nonspecific jun umODNs were also used for translation in vitro of the ODN-nonspecific mRNA of the poly-A-binding protein. The result showed that the protein synthesis of the poly-A-binding protein in rabbit reticulocyte lysate was not affected by the umODNs tested (data not shown).

Antisense int-umODN also slightly increased the protein synthesis of c/sTnC, whereas the respective S-ODN did not have any influence; studies have shown that S-ODNs bind quite slowly to the target compared to umODNs, possibly due their chirality (Marcus-Sekura 1988, van der Krol et al. 1988). Since

the incubation time in these experiments was relatively short (30 min.), it is possible that the antisense int-S-ODN bound only partially to the c/sTnC mRNA. The random sequence S-ODN used as a control, however, prohibited translation completely (data not shown), which may be due to the RNAse contamination of the ODN. The unmodified sense form of int-ODN, instead, markedly increased the protein synthesis of c/sTnC, by about 935%, compared to the result with untreated lysate.

The translation experiments in wheat germ extract showed that with antisense cap-c/sTnC umODN treatment induced no effect, whereas supplementation with AUG-c/sTnC umODN reduced c/sTnC synthesis by around 60%. However, comparable sense cap-, and sense AUG-c/sTnC as well as nonspecific jun umODN treatments also decreased synthesis slightly (Fig. 19).

Antisense TnC ODNs in transcription inhibited C2C12 cultures. The purpose of the study was to investigate the translatability of the TnC mRNAs after the umODN treatment, when production of new mRNAs was also inhibited. This in turn would illuminate the possible compensatory mechanisms that may exist in cells to cover the reduction in either TnC form, caused by antisense TnC umODNs in earlier described antisense TnC umODN studies.

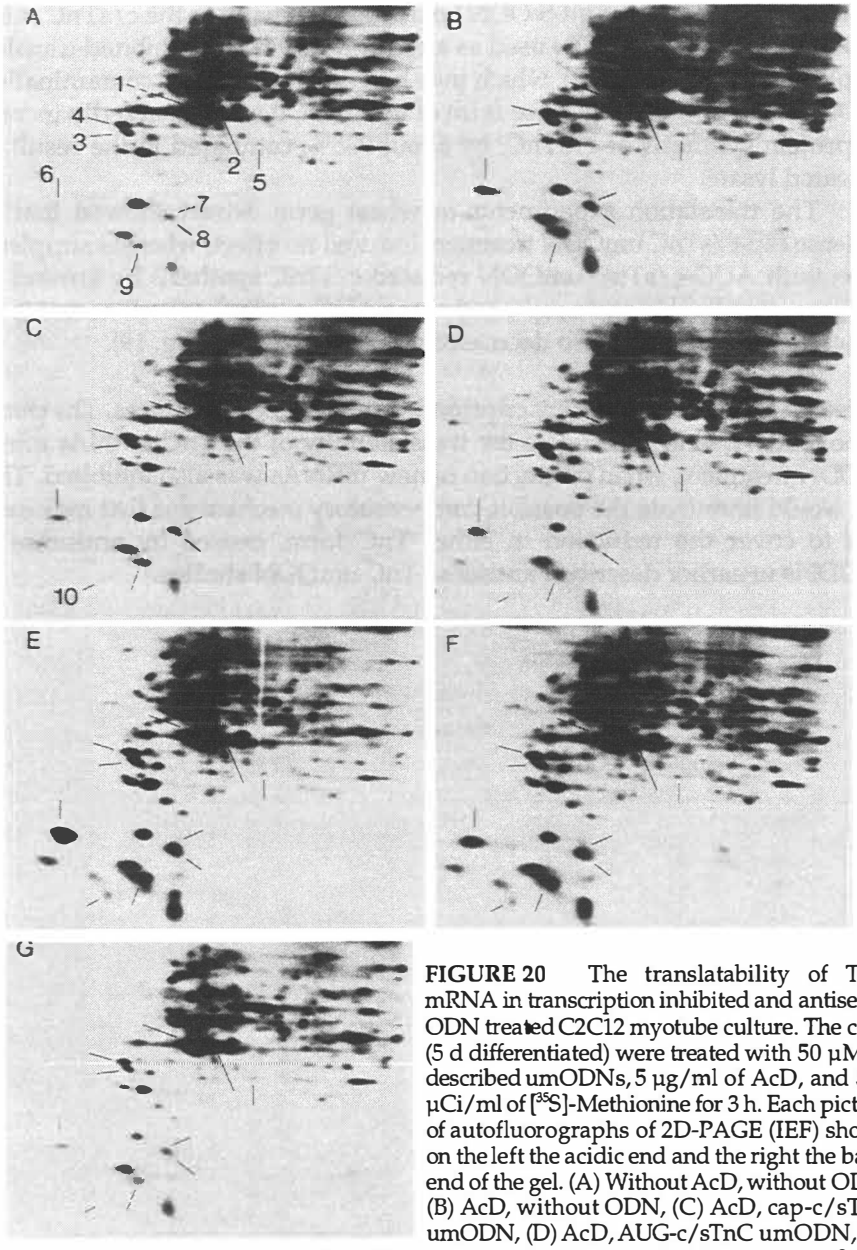

FIGURE 20 The translatability of TnC mRNA in transcription inhibited and antisense ODN treated C2C12 myotube culture. The cells (5 d differentiated) were treated with 50 μM of described umODNs, 5 μg/ml of AcD, and 500 μCi/ml of [^{35}S]-Methionine for 3 h. Each picture of autofluorographs of 2D-PAGE (IEF) shows on the left the acidic end and the right the basic end of the gel. (A) Without AcD, without ODN, (B) AcD, without ODN, (C) AcD, cap-c/sTnC umODN, (D) AcD, AUG-c/sTnC umODN, (E) AcD, cap-fTnC umODN, (F) AcD, AUG-fTnC umODN. (1) α-actin, (2) β-, γ-actin, (3) α-TM, (4) β-TM, (5) xTnT, (6) total TnC, (7) v/sMLC1, (8) fMLC1, (9) skMLC2, and (10) fMLC3.

The results of the image-analyses of the pictures in Fig. 20 are listed in Table 10.

TABLE 10 Effects of antisense umODN treatments in transcription inhibited C2C12 cells. Antisense and random sequence (rs) ODN treatments with AcD compared to (A) untreated control, and (B) AcD-treated control. (C) The difference ((A)-(B)), which describes the influence of AcD in ODN treated cultures. Results shown as percentages; n=1.

(A) ODN and AcD treated culture/ untreated control, alteration in %

Protein	fTnC cap	AUG	c/sTnC cap	AUG	rs,+AcD/ -ODN,-AcD
α-actin	-5.10	0.35	4.80	12.27	-17.97
β-,γ-act.	-16.49	2.06	-4.95	-1.03	-23.30
α-TM	10.36	4.78	15.94	9.76	-31.47
β-TM	9.49	6.46	10.30	6.46	-20.20
xTnT	4.17	16.67	4.17	22.92	-10.42
TnC	37.59	93.98	355.64	239.10	9.02
v/sMLC1	48.11	20.54	139.46	127.03	3.78
fMLC1	24.59	47.54	150.00	139.34	-4.10
skMLC2	78.89	56.67	175.56	150.00	8.89
fMLC3	33.33	48.15	174.07	144.44	9.26

(B) ODN and AcD treated culture/ AcD treated control, alteration in %

Protein	fTnC cap	AUG	c/sTnC cap	AUG	rs,+AcD/ -ODN,+AcD
α-actin	-11.70	-6.62	-2.49	4.46	-23.67
β-,γ-act.	-19.32	-1.39	-8.17	-4.38	-25.90
α-TM	-0.89	-5.90	4.11	-1.43	-38.46
β-TM	-13.14	-15.54	-12.50	-15.54	-36.90
xTnT	-10.71	0	-10.71	5.36	-23.21
TnC	-32.72	-5.15	122.79	65.81	-46.69
v/sMLC1	-11.90	-28.29	42.44	35.05	-38.26
fMLC1	-27.85	-12.20	48.78	42.44	-42.93
skMLC2	-6.94	-18.50	43.35	30.06	-43.35
fMLC3	-25.78	-17.53	52.58	36.08	-39.18

(C) Difference ((A)-(B)): influence of AcD in ODN treated and untreated cultures

Protein	fTnC cap	AUG	c/sTnC cap	AUG	-ODN,+AcD/ -ODN,-AcD
α-actin	6.60	6.97	7.29	7.81	7.47
β-,γ-act.	2.83	3.45	3.22	3.35	3.51
α-TM	11.25	10.68	11.83	11.19	11.35
β-TM	22.63	22.00	22.80	22.00	26.06
xTnT	14.88	16.67	14.88	17.56	16.67
TnC	70.31	99.13	232.85	173.29	104.51
v/sMLC1	60.01	48.83	97.02	91.98	68.11
fMLC1	52.44	59.74	101.22	96.90	68.03
skMLC2	85.83	75.17	132.21	119.94	92.22
fMLC3	59.11	65.68	121.49	108.36	79.63

The results show that AcD treatment as such had an increasing influence on protein synthesis in C2C12 myotubes (Fig. 20, Table 10). The mRNA half-life studies described earlier (Fig. 10) suggested, however, that AcD increases cellular mRNA accumulation before it gains its full functioning as a transcription inhibitor. Thus some of the increase in protein synthesis is most probably mediated either through an initial increase in transcription or the stabilizing influence of AcD on mRNA.

The results show surprisingly an almost 125% increase in total TnC protein synthesis with cap-c/sTnC umODN, and an increase of approximately 65% with AUG-c/sTnC umODN treatment, compared to AcD-treated control (Fig. 20, Table 10). Antisense cap-fTnC umODNs, instead, seem to the reduce synthesis of TnC by about 30%, whereas AUG-fTnC umODN does not seem to have any influence. The synthesis of either α-actin, α-TM, β-TM or xTnT shows no change. Treatment with antisense cap-c/sTnC or AUG-c/sTnC umODNs appears to increase synthesis of v/sMLC1, fMLC1, skMLC2 and fMLC3 by around 30-50%, compared to control treated with AcD only. Antisense cap-fTnC umODN treatment seems to have a slightly decreasing influence on fMLC1 and fMLC3 synthesis, whereas AUG-fTnC umODN treatment seems slightly to reduce the expression of v/sMLC1 and skMLC2 compared to control treated with AcD only. The results of random sequence ODN-treated control are not very accurate (Table 10) due the slight loading difference (Fig. 20).

The results seems to suggest, too, that the influence especially of antisense c/sTnC umODNs is increased by AcD (Table 10). AcD is often used in primer extension studies as a stabilizer of the ODN-primer-binding to mRNA; thus it may also function similarly in cell cultures.

Summary of the effects of antisense TnC treatments in C2C12 cultures. C2C12 cells were treated with two different types of antisense ODNs, which had varied binding regions, targeted against either c/sTnC or fTnC. Antisense c/sTnC transfection was also tested. The treatments had a rather varied influence on the protein synthesis of TnC and also on other members of the contraction apparatus. In addition, transcription/translation experiments in vitro were performed. The effects of umODNs against fTnC are summarized in Table 11.

TABLE 11 Results of the earlier described unmodified antisense ODN experiments against fTnC in C2C12 cell cultures. }= total (fTnC and c/sTnC, or v/sMLC1 and fMLC1).

Unmodified antisense ODN treatments against fTnC

	2 d diff.		5 d diff.	
	ODN/-ODN ctrl (50 μM, 12 h)		ODN,+AcD/-ODN,+AcD ctrl (5μg/ml, 50 μM, 3 h)	
Protein	cap	AUG	cap	AUG
α-actin	0	0	0	0

α-TM	0	0	-	0
β-TM	0	-	0	-
xTnT	+	+	0	0
fTnC	---	0	}--	}0
c/sTnC	-	0		
v/sMLC1	}-	}0	0	-
fMLC1			-	0
skMLC2	0	0	0	-
fMLC3			-	-

0-15% alteration = 0; 15-30% alteration = + or -; 30-45% alteration = ++ or --; 45-60% alteration = +++ or ---.

Influences of antisense RNA, umODNs and S-ODNs against the other target, c/sTnC, are summarized in Table 12.

TABLE 12 Results of the antisense c/sTnC ODN and RNA experiments in C2C12 cell cultures. (A) Antisense (as) RNA or umODN treatments against c/sTnC compared to controls (ctrl); antisense int-S-ODN treatments compared to (B) untreated control, and (C) random sequence S-ODN treated control. }=total (fTnC and c/sTnC, or v/sMLC1 and fMLC1).

(A) Antisense RNA or unmodified ODN treatments against c/sTnC compared to control

	6 d diff.	2 d diff.	5 d diff.		
	transf./ nontransf. ctrl	ODN/ -ODN ctrl (50 μM, 12h)	ODN,+AcD/ -ODN,+AcD ctrl (5μg/ml, 50 μM, 3h)		
Protein	asRNA	cap	AUG	cap	AUG
α-actin	0	-	0	0	0
α-TM	+	0	+	0	0
β-TM	0	0	0	0	-
xTnT	+	0	0	0	0
fTnC	0	-	0	}+++++	}++++
c/sTnC	--	--	-		
v/sMLC1	----	}-	}0	++	++
fMLC1	---			+++	++
skMLC2	-	-	0	++	++
fMLC3	0			+++	++

(B) Completely phosphorothioated antisense internal-c/sTnC ODN treatments compared to untreated control

	20μM	20μM	40 μM	100 μM
Protein	48+48 h	24+72 h	48+48 h	48+48 h
α-actin	-	0	0	0
α-TM	0	0	0	++
β-TM	-	0	0	++
xTnT	0	++	+	++

fTnC	}--	++++	}+++++	}+++++
c/sTnC		++++		
fTnI	---		++	
v/sMLC1	-	----	0	+
fMLC1	---	--	0	++
skMLC2	0	0	0	+
fMLC3	---	0	++	+++++

(C) Completely phosphorothioated antisense internal-c/sTnC ODN treatments compared to random sequence ODN control

Protein	20 µM/20 µM 48+48 h	40 µM/20 µM 48+48 h	40 µM/40 µM 48+48 h	100 µM/40 µM 48+48 h
α-actin	–	0	-	0
α-TM	0	-	0	0
β-TM	0	0	-	0
xTnT	0	++	--	-
total TnC	---	0	++++	++
fTnI	---	+++		
v/sMLC1	-	0	0	0
fMLC1	----	-	+	0
skMLC2	0	--	++	0
fMLC3	---	--	0	0

0-15% alteration = 0; 15-30% alteration = + or -; 30-45% alteration = ++ or --; 45-60% alteration = +++ or ---; 60-100% alteration = ++++ or ----; 100% or more = +++++ or -----

Translation studies in vitro in rabbit reticulocyte lysate generally showed increasing influence of the ODNs on the translation of c/sTnC (Fig. 18, Table 9). In particular, antisense cap-c/sTnC umODN increased protein synthesis, but sense int-umODN also had a pronouncedly increasing influence on the translation of c/sTnC. Translation studies in wheat germ extract, instead, showed only a slightly decreasing effect with antisense cap-c/sTnC umODNs, whereas antisense AUG-c/sTnC umODN fairly markedly reduced the protein synthesis of c/sTnC (Fig. 19, Table 9).

Discussion:

Decrease in TnC synthesis after the antisense treatments. A moderate reduction of about 40-50% in TnC protein synthesis was achieved in 48 h differentiated C2C12 cells treated with 50 µM of antisense cap-fTnC or antisense cap-c/sTnC umODNs for 12 h (Table 6). The results also showed, that a reduction of either TnC does not induce a more pronounced synthesis of the other. The influence of the umODNs on their targets appeared to be fairly specific (Table 11, Table 12). Both antisense fTnC umODNs seemed to have an increasing influence on xTnT synthesis (Table 12); the antisense fTnT 3'S-ODN study described earlier suggested some type of coregulation with TnT and TnC (Table 3, Table 4). Antisense cap-fTnC and antisense cap-c/sTnC treatments also slightly decreased the synthesis of total MLC1 (v/sMLC1 and fMLC1) (Table 6). However, according

to the differentiation experiments (Fig. 6), cellular mRNAs of contractile apparatus members accumulated fairly increasingly up to around the 96 h timepoint, despite the reduction around the time of most pronounced fusion, in other words, in-between 48 h and 60 h timepoints, as was also the case with TnC. Protein synthesis of xTnT and MLC1 forms, instead, showed a slight increase around that time (Fig. 7). Thus reduction in TnC expression with cultures treated with cap- and AUG-region ODNs is in all likelihood partially also due to the normal reduction in transcription and translation around those timepoints, as was the case with the increase in xTnT. The reason for the decrease in MLC1 proteins may be the nonspecific interactions of the ODNs with the respective MLC mRNAs.

The stability of the mRNAs of c/sTnC and fTnC may have some influence on the only partial inhibition of the antisense umODNs. The half-life studies showed that the half-life of cellular mRNA of c/sTnC is about 45 h, whereas that of fTnC is around 17 h (Table 2). The TnC polypeptide (fTnC and c/sTnC were not separated in the experiments), on the other hand, seemed to be one of the most unstable of the myofilament, both in the C2C12 and L6 cells (Table 2). Thus since the turnover rate of the cellular mRNA of c/sTnC, especially, is quite long compared to that of protein, it suggests that translation of the mRNA is not very efficient. The timelag and difference in intensity of transcription and translation described in the differentiation study suggested the same. The accumulating cellular mRNA of c/sTnC may also partially sequester the umODNs from binding on newly transcribed and probably translatable mRNA.

With antisense c/sTnC construct transfected, 6 d differentiated C2C12 cells showed a 45% reduction in c/sTnC synthesis (Fig. 18, Table 8). The inhibition was c/sTnC-specific. According to the results of the initial screening of the transfected constitutively antisense c/sTnC RNA expressing cells, a rather weak signal from the antisense c/sTnC transcripts was apparent in the myoblasts of the selected colony in a northern blot, compared to the control mRNA of 4 d differentiated C2C12 cells (data not shown). Thus most of the initial transcripts of endogenous c/sTnC may have become physically blocked by the antisense RNA in the beginning of differentiation. Later on, however, the transcription of c/sTnC probably exceeded the production of antisense RNA, which may partially explain the residual c/sTnC expression in the transfected cells. The bound antisense RNA block may be removed by a translation initiation complex or by other unwindase activities, which may also be developmentally regulated (Toulmé & Hélène 1988, Izant 1989, Jaramillo et al. 1991).

Synthesis of xTnT and α-TM increased slightly in the transfected cells, which accords with the results of the antisense int-S-ODN treatments (Fig. 17, Table 7, Table 12). A similar increase in synthesis was apparent also in 50 μM of antisense AUG-c/sTnC umODN-treated cells, although the approximately 15% increase in xTnT was very slight (Fig. 16, Table 6). The results seem to suggest that changes in the synthesis of c/sTnC and/or TnT can influence α-TM synthesis. The increase in α-TM may be an effort to balance for instance excess of TnT. The change in expression of TnC, in turn, may have some influence on the protein synthesis of TnT.

The protein synthesis of v/sMLC1 and fMLC1 was reduced markedly, and skMLC2 slightly in transfected cells, similarly to the changes seen in 20 µM of antisense int-S-ODNs-treated for 72 h in 24 h differentiated C2C12 cells (Table 7). The cap-c/sTnC umODN treatment had similar effects (Table 6). The cause of the changes may be that MLCs belong, like TnC, to the superfamily of Ca^{2+}-binding proteins (Collins 1991), and it is probable that MLC forms share homologous regions with TnC, which may also be detectable to antisense TnC RNA. However, the results may also indicate some delay in differentiation process, although initial v/sMLC1 showed more reduction than the slightly later expression fMLC1 in both transfected cells and in 24 h differentiated cells treated with antisense int-S-ODNs for 72 h. In the case of S-ODNs, the initial delay is supported by 20 µM of antisense int-S-ODNs-treated for 48 h in 48 h differentiated C2C12 cells, where the MLC results were opposite. Wagner (1994) has also noted that ODNs and their break-down products can disturb differentiation as well as cell proliferation.

Increase in TnC synthesis after the ODN treatments. When the transcription inhibitor AcD was used together with the same, previously mentioned antisense cap- and AUG-c/sTnC umODNs, the results unexpectedly showed a marked increase in total TnC synthesis (Table 10, Table 12). Antisense cap-fTnC umODN, instead, reduced the synthesis of total TnC slightly (Table 10, Table 11). However, according to half-life studies, treatment with AcD as such seems initially to enhance transcription (Fig. 10), which may partially explain the surprising increase. The results suggest (Table 10), however, that the antisense c/sTnC umODNs used can bind to their target sequences in cell cultures in the same way as in in vitro-experiments (Fig. 19). AcD may cause the formation of a stable ODN:mRNA duplex and prohibit the normal folding of the c/sTnC mRNA and thus improve its translatability. AcD may also reduce ODN:mRNA cleaving RNAse H activity, which may already be reduced in 5 d differentiated myotubes. Moreover, eukaryotic cells have unwindase activities other than exist in the translation initiation complex (Hélène & Toulmé 1990). Their normal biological roles are unknown. Some of these activities may not only unwind RNA:mRNA duplexes, but also be able to unwind ODN:mRNA duplexes, which would also partially explain the increase in TnC synthesis. These activities may also be developmentally regulated.

The treatment with antisense cap-c/sTnC or AUG-c/sTnC umODNs together with AcD also increased the synthesis of all MLC forms (Fig. 20, Table 10). As mentioned previously, when the umODNs were used as such, the change of expression of the MLC forms also had some parallels with TnC (Table 11, Table 12). The changes suggest some type of coregulation of MLCs and c/sTnC, but since TnC and MLCs belong to the same gene superfamily, it is likely that the genes share homologous regions and common regulatory sequences (Collins 1991). Thus antisense c/sTnC umODNs may also be able to influence the secondary structure of the mRNAs of MLC forms, especially when the stabilizing agent AcD is used. However, the question, of whether different mRNAs, like the mRNAs of TnC forms and MLC forms, interact with each other and for instance regulate each other translation remains unanswered.

The effects of antisense int-S-ODNs treatments on muscle gene expression paralleled the antisense c/sTnC umODN/AcD treatments (Fig. 17, Table 7). Whereas treatments with concentrations below 20 µM for 3 to 48 h had no or only very little influence on TnC synthesis, the treatment of 24 h or 48 h differentiated C2C12 cultures for 72 h and 48 h, respectively, with 20, 40 or 100 µM of S-ODNs induced a marked increase in TnC synthesis and also some other contractile apparatus proteins (Table 7, Table 12). Treatment with 20 µM for 48 h of 48 h differentiated cells, however, constituted an exception. However, much of the increase in TnC was nonspecific, since the random sequence S-ODN used as a control also had a general increasing influence on protein synthesis (Fig. 17, Table 12). Thus the observed increase in synthesis of TMs, xTnT, fTnI and MLCs (Table 7) probably is mainly nonspecific. However, cells treated with antisense fTnC umODNs as well as antisense c/sTnC transfected cells also showed slight increase in xTnT and α-TM synthesis (Table 11, Table 12). Thus the alterations seem to suggest that the change in the stoichiometry of one member of a contraction apparatus can also affect the expression of the others; the cells may have some mechanisms with which to balance these alterations, whether the change is specific or not.

Studies with the primary culture of chicken skeletal muscle have shown that the treatment of cells with the same sequence of antisense int-umODNs resulted in a decreased translation of TnC, while its transcription showed a slight increase (Thinakaran & Bag 1991). Thus umODNs as well as the described S-ODNs may cause the nonspecific induction of some transcriptional factors, which in turn may increase also expression of the target with a timelag. This possibility is supported by a study which has shown that S-ODNs can induce Sp1 nonspecifically (Perez et al. 1994). As described in the Review of the literature, many contractile apparatus genes have Sp1-type binding site/s or resemble regulatory sequences, such as c/sTnC (Parmacek et al. 1994); hence the increase observed in for instance c/sTnC may be partially mediated through this site. It is not known whether this Sp1 induction takes place in muscle cells, and if so, how quickly the transcriptional increase occurs. However, the difference between the treatment of 48 h differentiated cells with 20 µM for 48 h and the rest of the results, and especially that of the similar treatment initiated in 24 h differentiated cells, suggests that S-ODNs may initially have some toxic influence on fusing cells and cause delay in differentiation. After some time, the effects may be compensated for by increased transcription. When the concentration used is higher, the compensatory mechanisms may be induced more rapidly. S-ODNs may also change the balance of other transcriptional factors, which may cause further alterations in expression. The changes in the activity of transcriptional factors may also speed up the differentiation process later on.

The differentiation studies described in chapter 5.1 showed increased accumulation of cellular mRNA in both TnC forms between 60 and 96 h of differentiation (Fig. 6), which were the time limits of the ODN treatments. Protein synthesis of total TnC, on the other hand, seemed to accompany the increase only up to 72 h of differentiation, after which it showed a reduction (Fig. 7). Thus the binding of antisense int-S-ODN to c/sTnC mRNA - and probably also to fTnC mRNA - may keep it in a more translatable form by prohibiting its normal

folding. However, the binding of S-ODN to its target has been suspected to take longer than that of umODNs due to the changes in its structure and possible chirality (Marcus-Sekura 1988, Cohen 1991). But, according to the half-life studies, the half-life of c/sTnC mRNA appeared to be fairly long (Table 2), which suggests that antisense int-S-ODN has time to interact with its target before the mRNA is degraded. RNAse H activity may also gradually be reduced in fusing, non-dividing myocytes (Walder & Walder 1988). Thus the cleavage of the ODN:mRNA hybrids formed may be reduced during differentiation, although S-ODN:mRNA forms a substrate to RNAse H (Furdon et al. 1989). As mentioned previously, the ODN block in turn may be removed by either the translation initiation complex or some other unwindase activity (Toulmé & Hélène 1988, Izant 1989, Jaramillo et al. 1991).

The results of transcription/translation studies in vitro parallel both umODN/AcD studies and antisense int-S-ODN studies: when in vitro synthesized c/sTnC mRNA was translated in rabbit reticulocyte lysate with different umODNs, the results surprisingly showed a marked increase in translation, especially in antisense cap-c/sTnC umODN and sense int-c/sTnC umODN treated lysates, whereas the increase with int-c/sTnC umODN was slight (Fig. 19, Table 9). However, the last eleven nucleotides of the 3' end of the sense int-umODN were complementary to the 5' end of the c/sTnC mRNA; this complementary area is located partially in the same region where antisense cap-c/sTnC umODN binds (Appendix 1). The results suggest that antisense cap-c/sTnC umODNs and sense int-umODN - and also the antisense int-umODNs - may may keep the mRNA in a more translatable form by inhibiting its normal folding. The folding may normally cover the translation initiation codon and both cause the weak translation of the mRNA described earlier as well as protect it from degradation. Whether the possible folding of c/sTnC mRNA in the described area requires for example proteins in vivo is not known.

Whereas treatment with antisense cap-c/sTnC umODN showed no influence, antisense AUG-c/sTnC umODN, especially, seemed to decrease the protein synthesis of c/sTnC in wheat germ extracts. However, comparable sense cap- and sense AUG-c/sTnC umODNs as well as nonspecific jun umODN treatments also decreased synthesis (Fig. 19, Table 9). The results for the sense ODNs used, however, supports the idea that at least the cap-region of the c/sTnC mRNA influences on the secondary structure of the mRNA: sense cap-c/sTnC ODN may act as an antisense ODN in some other region of the mRNA. The reductions may also be due to the nonspecific cleavage of the mRNA by RNAse H - wheat germ extract has been described generally having higher RNAse H activity than reticulocyte lysate (Minshull & Hunt 1987, Colman 1990). The freezing and storing of the lysates has also been shown to decrease RNAse H activity (Vlasov & Yurchenko 1991). Another reason for the difference may be that the systems have differences in their translational factors, which in turn may have an effect on the unwinding and translation efficiency of the mRNA.

As suggested previously, there may exist interactions or secondary structure formation between antisense cap-c/sTnC ODN-binding 5' UTR and antisense int-ODN-binding areas (Appendix 1), which in turn may be involved in regulating the translation of c/sTnC mRNA. The regulatory influence of 5' UTR

of c/sTnC mRNA, in particular, compared to fTnC is supported also by the knowledge that their 5' and 3' UTRs are very different, whereas the coding sequences of mRNA of c/sTnC and fTnC are highly homologous - the homology also includes the antisense int-ODN binding sequence. The possibility also raises the question: can c/sTnC protein synthesis be regulated by different conformations of the mRNA during its life-cycle? The message may initially have an open structure but later on form a more folded and/or protein bound secondary structure, which also protects it from degradation. However, whether the secondary structure exists and whether the formation requires for instance proteins is not known. But, studies have shown that cardiac-specific expression of c/sTnC requires cardiac enhancer factors CEF-1, CEF-2, and cardiac promoter factors CPF1, CPF2, and CPF3 binding for their respective sites - the CPF3-site of these is located in the first exon, which codes the 5' UTR of the c/sTnC mRNA. This particular enhancer sequence, which also resembles the CTF/NF-1-site, covers the antisense cap-c/sTnC ODN-binding site of c/sTnC (Appendix 1). The CPF3 or related factor is also expressed in C2C12 myotubes, but its function in these cells is unknown - the rest of these cardiac factors are expressed only in heart muscle (Parmacek et al. 1992). Thus CPF3 may be involved in some level to the observed increase in TnC protein synthesis, when the cells were treated with antisense cap-c/sTnC umODN. In the normal situation CPF3 may have no influence, but on the other hand the protein may also regulate for instance mRNA transport to cytoplasm, or function as a translation regulator in skeletal muscle by influencing the secondary structure of the c/sTnC mRNA or its formation. It is not known whether the factor can bind to a single-stranded mRNA, or the mRNA:mRNA or mRNA:ODN duplex, but if it can, it may enhance mRNA translation by opening the secondary structures of the message by either acting for example as an unwindase or bind one, and aid the translation initiation complex to bind.

Conclusions:

Changes in the expression of contractile apparatus members were apparent in C2C12 cells after antisense TnC ODN treatments, and also in antisense c/sTnC transfected C2C12 cells (Table 11, Table 12). Contractile protein expression in antisense c/sTnC transfected cells had some resemblance to that in cultures treated with different antisense c/sTnC ODNs in a variety of concentrations and for a variety of incubation times. One explanation for the opposite effects of umODNs and the same umODNs together with AcD may lie in the differentiation stage of the cells: cells treated with umODNs were totally 60 h differentiated and only partially fused at the end of the experiments; thus they probably had more RNAse H activity than the 5 d differentiated fused cells that were used in the umODNs/AcD experiment. The results of translation studies in vitro of c/sTnC (Table 9) supports the involvement of RNAse H: rabbit reticulocyte lysate generally has low RNAse H activity and the results resembled both those treated with umODN/AcD and those treated with S-ODN. Translation in wheat germ

extract characterized by generally high RNAse H, instead, had a reducing influence on c/sTnC synthesis.

The studies also indicated that AcD and S-ODNs have nonspecific influences (Fig. 20, Table 12). These substances may increase transcription nonspecifically, for instance through the induction of Sp1 (Perez et al. 1994). Many contractile apparatus genes have an Sp1-type site, which may partially explain the marked increase in synthesis of some of the proteins. Sp1 induction may also change the balance of other transcription influencing factors, which in turn can for example induce the cells to differentiate more rapidly. However, the results of the antisense c/sTnC treatments suggest that the c/sTnC message is fairly folded, which would explain also partially the stability of the c/sTnC mRNA indicated by the half-life studies (Table 2). In addition to protecting the mRNA from degradation, the secondary structure may also influence its translatability. The antisense cap-c/sTnC umODN, sense int-umODN and antisense int-umODN tested may break the normal folding of the c/sTnC mRNA, altering it into a more translatable form.

5.2.3 Concluding remarks: effects of antisense TnT and TnC ODN treatments on L6 and C2C12 cultures

The attempt to alter the expression of the TnT and TnC members of thin filament by antisense ODNs and antisense RNA was done in order to investigate the influence of these alterations on the coordinated and stoichiometric accumulation of other members of the contraction apparatus. An additional purpose was to reveal the possible compensatory mechanisms following from the artificially induced alteration of TnT in L6 and TnC in C2C12 cells.

Efficacy of the ODNs. The results support those reported earlier, which have shown that antisense ODNs targeted against the cap-region or the translation initiation region are the most effective ones (Walder 1988, Izant 1989, Hélène & Toulmé 1990). The full-length antisense RNA against c/sTnC had a similar reducing effect on the protein synthesis of c/sTnC to that of the antisense cap-c/sTnC umODN. The studies have also been shown that additional dose of antisense RNA, when its production has proved possible, has not usually knocked out the residual gene activity either. Thus the results described parallel most other antisense studies also in the respect that the reduction of the target was only moderate - 100% inhibition of the target is rarely caused by either antisense ODNs or antisense RNA. The partial inhibition may resulted from some kind of compartmentalization of the target mRNA, antisense ODNs and antisense RNAs (Toulmé & Hélène 1988, van der Krol et al. 1988, Izant 1989, Knecht 1989, Stein & Cheng 1993). Thus the functional concentration of ODNs can be low inside the cells. A further reason for the only moderate inhibition may be the accessibility of the message for ODN to bind: for instance the fTnT message is rather long, and is probably fairly folded. Cellular, not translatable mRNA of fTnT, may also sequester some of the functional ODNs. Moreover, it is probable

that non-dividing cells, such as fusing and maturating C2C12 and L6 cells, have reduced RNAse H activity, which in turn can lead to inefficient cleavage of the ODN:mRNA hybrids (Walder & Walder 1988, Colman 1990). This in turn can also reduce the recycling ability of the ODNs, which remain intact after cleavage.

Both antisense ODN:mRNA and antisense RNA:mRNA hybrids can also be fairly unstable formations. It has been suggested that ODNs that are complementary to the coding region of the message can be removed by an unwinding activity. Most common unwindase activity is probably in the translation initiation complex, which may be able to remove the bound antisense RNA or ODN from the mRNA (Toulmé & Hélène 1988, Izant 1989, Jaramillo et al. 1991), although, according to some studies, the complex may not be capable to remove ODNs (Lawson et al. 1986, Rhoads 1988, Sonenberg 1988). Other unwindase activities in eukaryotic cells have also been detected such as in *Xenopus* oocytes, 3T3 fibroblasts and HeLa cells, but their normal biological roles in cells are unknown. However, some unwindase activities may not only unwind RNA:mRNA duplexes, but at least in some cases, they may also be able to covalently modify the RNAs so extensively that they are unable to rehybridize (Hélène & Toulmé 1990). It is not known, though, whether these unwindase activities can remove ODN from its target.

Contractile protein gene expression after antisense treatments. The influence of antisense fTnT 3'S-ODNs seemed to be more drastic to other members of the thin filament than treatments with antisense TnC umODNs or production of antisense c/sTnC RNA. The antisense TnC treatments seemed to have more influence on expression of the MLC forms (Table 5, Table 11, Table 12). The experiments also showed that blocked TnT or TnC does not become compensated for by increased expression of other respective protein/isoform/s. However, especially according to the results of the TnT studies, cells seem to have mechanisms by which the reduction of one member is balanced by changing the synthesis of the others, possibly in order to maintain the correct stoichiometry and also correct assembly of the contraction apparatus. The mechanisms by which the change in one member can influence the stoichiometry of the gene expression of the others is unknown. One alternative may be that this influence is mediated through mRNA:mRNA interactions of the different members of the contraction apparatus.

Studies in *Drosophila melanogaster* have shown that reduction or elimination of TM, TnT or TnI leads to disruptions in the assembly of thin filament, possibly due to the instability of the incomplete filaments (Fyrberg & Beall 1990, Beall & Fyrberg 1991). This may also be partially the case in antisense fTnT 3'S-ODN-treated L6 cells where alterations took place in most of the members of the thin filament (Table 5). The influence, however, is probably more in the renewal level of the thin filament, since most of the cells were already fused and had filamentous structures. The antisense TnC study, on the other hand, suggested that the alteration in TnC may influence on the overall assembly of the contraction apparatus due to changes in MLC expression (Table 11, Table 12). An interesting possibility is that TnC has interactions with MLC forms, and these two jointly influence the proper assembly of the full contraction apparatus (Fig. 1). The

results of studies of null mutation of MLC2 in *Drosophila melanogaster* have shown effects on the assembly and function of indirect flight muscle - the results showed that the thick and thin filaments fail to associate in a proper hexagonal formation during myogenesis (Warmke et al. 1992). A study by Morimoto & Ohtsuki (1989) in turn has suggested interactions between TnC and MLC2. Since a TnC-like protein has also been localized in the brain and in several human fibroblast cell lines (Fine et al. 1975, Gahlman et al. 1988, Parmacek & Leiden 1989), it has been suggested as having some other functions than simply those of regulating actomyosin ATPase and contraction (Parmacek & Leiden 1989).

6 SUMMARY AND CONCLUSIONS

How myofilaments and the contraction apparatus are assembled and maintained, as well as the mechanisms that are involved in regulating the coordinated and stoichiometric accumulation of the members of the contraction apparatus are poorly understood. To elucidate these processes, the normal expression patterns of some of the contraction apparatus protein genes in C2C12 and L6 cells during differentiation and maturation were characterized. The importance of each member of a myofilament on the accumulation of the others is also poorly understood. Altering the gene expression of one member, for instance with antisense ODN or antisense RNA, and examining the effects of the change on the other members may throw further light on both these relations, as well as the processes described. The targets selected for the antisense ODN studies were fTnT in L6 cells and c/sTnC and fTnC in C2C12 cells - antisense RNA was targeted only against c/sTnC in C2C12 cells.

The results of the studies show:

1. The overall pattern of gene expression was fairly similar in rapidly fusing subclone of L6 and in slower fusing C2C12 cells, both of which cells were grown in the same culture conditions. The initiation of transcription and translation of muscle-specific genes occured slightly earlier than fusion in both cell lines.

2. In both cell cultures, muscle and non-muscle actins and their respective TMs as well as the fast members of the troponin complex showed some coordination during differentiation. It seemed that the initial and the most markedly expressed genes were α-actin and muscle TMs and, slightly later and with a lower intensity fTnI and c/sTnC and other members of the troponin

complex. The expression pattern seems to be partially explicable by the regulatory sequences that the contractile apparatus genes have.

3. The half-lives of the cellular mRNAs of the members of the thin filament were fairly comparable in both cell lines with exception of α-actin - the half-life of α-actin was around threefold shorter in L6 cells than in C2C12 cells. C/sTnC, however, was exceptional in both cell lines compared to the other members of the thin filament: the half-life of cellular mRNA of c/sTnC was almost twice as long as than of fTnC or the other members of the thin filament. The most stable protein was α-actin, whereas the TnC and fMLC3 polypeptides were the least stable members.

4. Antisense TnT 3'S-ODN, antisense TnC umODNs, and antisense c/sTnC RNA were only partially effective in prohibiting the expression of their respective polypeptides. However, unmodified ODNs binding to the 5' cap-region of the respective TnC mRNA were more effective than those binding near the initiation site or at any other site along the mRNA.

5. Antisense S-ODNs against the internal region of the c/sTnC mRNA increased the synthesis of c/sTnC and fTnC, and that of some other muscle-specific proteins when C2C12 cells were treated with a high concentration of ODNs for a long period of time.

6. Protein synthesis of c/sTnC increased as well as MLC forms in C2C12 myotubes when the cells were treated with antisense cap-c/sTnC or AUG-c/sTnC umODNs together with transcription inhibitor AcD. Similar increase was not apparent in cells treated with cap-fTnC or AUG-fTnC umODNs.

7. When in vitro transcribed c/sTnC mRNA was translated with antisense cap-c/sTnC umODN or sense internal-c/sTnC umODN, the synthesis of c/sTnC showed a pronounced increase, but the increase was only slight when it was translated with antisense AUG-c/sTnC umODNs in rabbit reticulocyte lysate. Translation in vitro in wheat germ extract, on the other hand, was not altered when supplemented with antisense cap-c/sTnC umODNs, whereas AUG-c/sTnC umODNs reduced protein synthesis.

Conclusions of the study:

1. The differentiation experiments are in agreement with the model that contraction apparatus formation in living cells starts with actin and TM interactions. TnI and TnC may initially form a complex, which is then joined to free or TM-actin-bound TnT.

2. Reduction in TnT gene expression had an influence on the expression of the other members of the contraction apparatus. The influence was more pronounced than the reduction of TnC by antisense ODNs. The results suggest

that cells have mechanisms which can be used to control the stoichiometry of members of the contraction apparatus other than by transcriptional regulation.

3. The results suggest that the translation of TnC could be regulated by special folding of the mRNA, which may also protect the mRNA from degradation.

4. RNAse H activity during differentiation needs to be determined.

5. The results suggest that phosphorothioated oligonucleotides have non-specific influences.

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Yhteenveto (Résumé in Finnish)

Lihassolujen erilaistuminen in vitro ja antisense oligodeoksinukleotidien vaikutukset supistumisproteiinigeenien ilmenemiseen

Työn tarkoituksena oli karakterisoida luurankolihasperäisten L6- ja C2C12-solulinjojen supistumisrakennegeenien ilmenemistä erilaistumisen ja kehityksen aikana. Työssä tarkasteltiin lähinnä ohuen filamentin proteiinien ja vastaavien lRNA:iden koordinoitua kertymistä ja kertymisen voimakkuutta. Tuloksia tarkasteltiin myös geenien säätelyalueiden valossa. Lisäksi geenituotteiden puoliintumisajat määritettiin yhdestä aikapisteestä. Tulokset osoittivat, että supistumisrakenteen eri osien välinen suhde säilyi melko samankaltaisena koko erilaistumisen ajan, tosin proteiinisynteesin ja lRNA:iden tasojen välillä oli eroja. Erilaistumisen alun ilmentämisen voimakkuuden, geenien säätelyalueiden sekä proteiinien puoliintumisaikojen perusteella arvioitiin ohuen filamentin mahdollista kokoamistapaa ja uusiutumista soluissa. Kokoonpano saattaa alkaa aktiinin ja tropomyosiinin (TM) vuorovaikutuksilla, mikä arvio mukailee myös aikaisempia, muissa laboratorioissa tehtyjä in vitro-tutkimusten tuloksia. Ilmaantumisajankohdan ja ilmenemisvoimakkuuden perusteella, joita myös säätelyalueet tukevat, troponiinikompleksin muodostuminen saattaisi alkaa emäksisen troponiini I:n (TnI) ja happaman troponiini C:n (TnC) vuorovaikutuksilla, ja tämä esikompleksi vuorostaan liitettäisiin TM:iin tarttuneeseen tai vielä vapaaseen emäksiseen troponiini T:hen (TnT) - L6- ja C2C12-soluissa näytti olevan eroa TnT:n kertymisen alun ajankohdissa. Ohuen filamentin jäsenten puoliintumisajat puolestaan viittaisivat siihen, että TnC-proteiini määrää troponiinikompleksin uusiutumista lyhyimmällä puoliintumisajallaan.

Työn toisena tarkoituksena oli häiritä normaalisti hyvin koordinoitua ja stoikiometristä supistumisrakennegeenien ilmenemistä antisense-oligonukleotideillä (ODN) ja antisense-RNA:lla. Säätelyn kohteina oli nopean lihastyypin TnT-muoto (fTnT) L6-soluissa, sekä nopean lihastyypin ja hitaan lihastyypin/sydänlihaksen TnC-muodot (fTnC ja c/sTnC vastaavasti) C2C12-soluissa. Näiden kokeiden vain osittainen, kohteensa n. 30-50% estotulos saattaa johtua ODN:en ja RNA:n epäsuotuisasta jakautumisesta soluissa, ODN:en toiminnallisesta pitoisuudesta soluissa, sekä erilaisista unwindaasi-aktiivisuuksista, joita on havaittu eri soluissa. Lisäksi ODN:n estokykyyn vaikuttavan RNAasi H:n aktiivisuus saattaa vähetä erilaistumisen aikana solujakautumisen lakattua, mikä osaltaan saattaisi selittää tulosten vaihtelua eri ODN-kokeiden välillä, erityisesti eri antisense-TnC -kokeiden kohdalla. Käytetyillä ODN:eilla saattaa olla vaikutuksia myös transkriptiofaktoreihin - erään tutkimuksen mukaan S-ODN:t voivat indusoida Sp1:n, ja mm. c/sTnC omaa tämän faktorin sitoutumisalueen.

TnT:n estolla oli suuremmat vaikutukset ohuen filamentin muiden jäsenten geenien ilmenemiseen kuin TnC-muotojen estolla. TnT:n vähenemisen lisäksi myös aktiinin ja α-TM:in lRNA:iden kertyminen väheni, TnC nousi lievästi, kun taas fTnI oli muuttumaton. Proteiinisynteesitasolla sen sijaan α-TM oli muuttumaton, TnT, α-aktiini ja TnC vähenivät, ja TnI osoitti lievää nousua. Vaikka

koordinaation ja stoikiometrian säätelyä tapahtuu edellä kuvattujen erilaistumiskokeiden perusteella jo transkriptiotasolla, TnT-estokokeen tulokset viittaavat stoikiometrian säätelyä tapahtuvan myös translaatiotasolla, erityisesti TnC:n kohdalla. Edellä mainittujen TnC-kokeiden perusteella vaikuttaisi siltä, että ainakin c/sTnC:n translaatio saattaisi olla säädelty lRNA:n erikoisella laskostumisella, mikä selittäisi myös lRNA:n pitkän, n. 45 h puoliintumisajan ja erilaistumiskokeiden osoittaman TnC:n heikon synteesin. Estokokeissa käytetyt cap-alueen muuntamattomat ODN:t aktinomysiini D -käsittelyn yhteydessä, kuten osittain myös sisäiseen alueeseen sitoutuvat ODN:t, saattavat muuttaa tätä laskostumista translaatiolle suotuisampaan muotoon, mikä osaltaan selittäisi yllättävän nousun TnC:n proteiinisynteesissä.

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APPENDIX 1

1 Target sequence of the rat fast troponin T

```
            83                  97
5'--ACUAUGUCUGACGAGGAAACUGAACAA--3'
```

fTnT ODN:

3' ApGACTGCTCCTTTGA 5' S	antisense fTnT, 15mer (3'S-ODN)

The 3' phosphodiester bond of the ODNs was thiolated.

Control ODNs:

5' TCTGACGAGGAAACpT 3' S	sense fTnT, 15mer (3'S-ODN)
5' CGCAACATCCACAGCACC 3'	Random sequence, 18mer (umODN or S-ODN)

2 Nucleotide sequences of murine c/sTnC and fTnC isoforms, and antisense and control ODNs used

(A) Mouse cardiac/slow troponin C cDNA sequence:

```
                ***** * * **   *
  1 TGGCTGGCAA CCCCAGTAGC CTGTCCTGTG AGCTGTCTCC AGAATGGATG
 51 ACATCTACAA AGCTGCGGTA GAACAGTTGA CAGAGGAGCA GAAGAATGAG
101 TTCAAGGCTG CCTTTGATAT CTTTGTCTTG GGCGCGGAGG ATGGCTGCAT
151 CAGCACCAAG GAGCTGGGCA AGGTGATGAG GATGCTGGGC CAGAACCCCA
201 CACCTGAGGA GCTGCAGGAG ATGATCGACG AAGTAGACGA GGATGGCAGT
251 GGCACAGTGG ACTTCGATGA GTTTCTTGTC ATGATGGTTC GGTGCATGAA
301 GGACGACAGC AAAGGGAAGT CTGAGGAGGA GCTGTCGGAT CTCTTCCGCA
351 TGTTTGACAA AAACGCTGAT GGCTACATTG ACTTAGATGA GCTGAAGATG
401 ATGCTGCAGG CCACAGGTGA GACCATTACG GAAGATGACA TTGAAGAGCT
451 CATGAAGGAC GGTGACAAGA ACAACGATGG CCGAATTGAC TATGACGAGT
501 TCCTGGAATT CATGAAGGGT GTGGAGTAGA TGCTGGTCTT GCACGGTTGC
551 CTGCGCCTGT TCTCCCCCTC CACCCAGACC CCGTGGTAGG AGTGCAGCTG
601 GGCTCTCTAG ACTCTGAGCC TGCCTGTGTC CTTGAACCTT GGCCTTCCGG
651 ACTTTCTCTC CCCATTCCTG TCCTGGGGAA CGCAAATAAA TCCCTTGCTC
    CCC
```

Antisense c/sTnC ODNs:

3' CGTTGGGGTCATCGG 5'	cap-c/sTnC, 15mer (umODN)
3' CTACTGTAGATGTTTCGA 5'	AUG-c/sTnC, 18mer (umODN)
3' CCACTACTCCTACGACCC 5'	int-c/sTnC, 18mer (umODN or S-ODN)
3' AGTACTTCCCACACCTCATC 5	coding end, 20mer (umODN)
3' TTTATTTAGGGAACGAGGGG 5'	non-coding end, 20mer (umODN)
3' CCCTTGCGTTTATTTAGGGAACGAGGGG 5'	non-coding end, 28mer (S-ODN)

Top underlining shows the CPF3 site (Parmacek et al. 1992), and (*) shows the complementarity of sense int-c/sTnC (18mer).

(B) Mouse fast troponin C cDNA sequence:

```
  1 ACCTTTGGGT GGTGGAGTGC GGAGGAGACA ACCCACAGCG GAGGAGTCCC
 51 AGTCGCCAGC AACCATGACG GACCAACAGG CTGAGGCCAG GTCCTACCTC
101 AGCGAGGAGA TGATCGCTGA GTTCAAGGCT GCCTTTGACA TGTTCGATGC
151 TGATGGCGGT GGGGACATCA GCGTTAAAGA GTTGGGCACC GTGATGAGGA
201 TGCTAGGGCA GACACCCACC AAAGAGGAAT TGGATGCCAT CATCGAGGAG
251 GTGGACGAGG ATGGCAGCGG TACTATCGAC TTTGAAGAGT TCTTGGTCAT
301 GATGGTGCGC CAGATGAAAG AGGATGCGAA GGGGAAGAGC GAAGAGGAAC
351 TGGCTGAGTG CTTCCGCATC TTTGACAGGA ACGCAGACGG CTACATTGAT
401 GCTGAGGAGC TAGCTGAGAT TTTCCGGGCT TCTGGGGAGC ATGTGACAGA
451 AGAGGAGATC GAATCCCTGA TGAAGGATGG TGATAAAAAC AACGACGGCC
501 GCATTGACTT TGATGAGTTT CTGAAGATGA TGGAGGGCGT TCAGTAA
```

Antisense fTnC ODNs:

3' CACCACCTCACGCCT 5'	cap-fTnC, 15mer; umODN
3' TGCCTGGTTGTCCGACTC 5'	AUG-fTnC, 18mer; umODN

Control ODN:

5' CGCAACATCCACAGCACC 3'	Random sequence, 18mer (umODN or S-ODN)

ODNs used in the in vitro studies:

3' CGTTGGGGTCATCGG 5'	antisense cap-c/sTnC, 15mer (umODN)
5' GCAACCCCAGTAGCC 3'	sense cap-c/sTnC, 15mer (umODN)
3' CTACTGTAGATGTTTCGA 5'	antisense AUG-c/sTnC, 18mer (umODN)
5' GATGACATCTACAAAGCT 3'	sense AUG-c/sTnC, 18mer (umODN)
3' CCACTACTCCTACGACCC 5'	antisense int-c/sTnC, 18mer (umODN, S-ODN)
5' GGTGATGAGGATGCTGGG 3'	sense int-c/sTnC, 18mer (umODN)
5' GTAGCCATAAGGTCCG 3'	jun, 16mer (umODN)
5' CGCAACATCCACAGCACC 3'	Random sequence, 18mer (umODN, S-ODN)

ODN probes and fTnT primer:

3' AGACTGCTCCTTTGA 5'	antisense fTnT, 15mer; umODN (T_H +39°C)
3' CTACTGTAGATGTTTCGA 5'	antisense AUG-c/sTnC, 18mer; umODN (T_H +45°C)
3' CACCACCTCACGCCT 5'	antisense cap-fTnC, 15mer; umODN (T_H +45°C)
3' CCTCTGTCGTGGACTTCT 5'	antisense mouse fTnI, 18mer; umODN (T_H +51°C)
3' AGGGCATTTGAGTACGAC 5'	antisense rat sTnI, 18mer; umODN (T_H +49°C)

ODNs used for c/sTnC plasmid construct:

5' GAATTCATGAAGGGTGTGGAGTAGAAGCTT 3'	sense coding-end of c/sTnC, 30mer; umODN
3' CTTAAGTACTTCCCACACCTCATCTTCGAA 5'	antisense coding-end c/sTnC, 30mer; umODN

APPENDIX 2

1 Cell cultures

1x PBS (phosphate buffered saline, pH 7.2): 137 mM NaCl, 2.6 mM KCl, 8.1 mM Na_2HPO_4, 1.4 mM KH_2PO_4 (Fisher).

2 RNA studies

Solution D: 4 M guanidinium thiocyanate (Fluka) 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl (BHD), 0.1 M 2-mercaptoethanol (Sigma).
Formylation solution for RNA: 50% formamide (BHD), 25% 1x MOPS buffer, 25% formaldehyde (Fisher).
10x Loading dye for agarose gel: 0.2% bromophenol blue, 0.2% xylene cyanol (Bio-Rad), 50% glyserol (Sigma).
1x MOPS buffer: 1.675 g MOPS (Sigma), 0.27 g sodium acetate (3 H_2O), 0.14 g free acid EDTA (Fisher)/ 400 ml, pH 7.4.
1x SSC: 150 mM NaCl, 15 mM sodium citrate (Fisher).
Hybridization solution for plasmids: 1% BSA fraction V (Boehringer Mannheim), 1 mM EDTA, pH 8.0, 0.5 M $NaPO_4$, pH 7.2 (Fisher), 7% SDS (Bio-Rad).
Washing solutions for plasmids: Solution I. 0.5 % BSA fraction V (Boehringer Mannheim), 1 mM EDTA, pH 8.0, 40 mM $NaPO_4$ (Fisher), 5% SDS (Sigma). Solution II. 1 mM EDTA, pH 8.0, $NaPO_4$, pH 7.2, 1% SDS.
Hybridization solution for ODNs: 5x SSC, 20 mM $NaPO_4$, pH 7.0 (Fisher), 10x Denhardt's, 7% SDS (Bio-Rad), 10% dextran sulphate (not included in prehybridization solution; Pharmacia).
Washing solutions for ODN probings: Solution I. 3x SSC (Fisher), 5% SDS (Sigma), 25 mM $NaPO_4$, pH 7.5 (Fisher). Solution II. 1x SSC, 1% SDS.
Stripping solution: 0.1x SSC (Fisher), 0.5% SDS (Sigma).
100x Denhardt's: 1 g Ficoll, type 400 (Pharmacia), 1 g polyvinylpyrrolidone (Sigma), 1 g BSA fraction V (Boehringer Mannheim)/ 50 ml of H_2O.
Annealing buffer: 0.5 mM $NaPO_4$, 0.5 mM EDTA (Fisher).
Loading dye for acrylamide gel (primer extension): 80% formamide (BHD), 0.01% xylene cyanol, 0.01% bromophenol blue (Bio-Rad), 20 mM NaOH (Fisher).
Primer extension gel: 12% acrylamide (stock: 20% acrylamide (Sigma), 8 M urea (ICN), 1x TBE (Fisher)), 0.008% APS, 0.0008% Temed (Bio-Rad).
Urea mix: 8 M urea (ICN), 1x TBE (Fisher).
1x TBE, pH 8.3: 90 mM Tris-base, 90 mM boric acid, 2.5 mM EDTA (Fisher).

3 DNA studies

1x HBS (HEPES buffered saline, pH 7.05): 21 mM HEPES (Sigma), 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 27.5 mM dextrose (Fisher).
Glyserol shock solution: 20% glyserol (Sigma), 1x HBS.

DNA extraction buffer: 50 mM Tris-HCl, pH 8.0, 100 mM EDTA, pH 8.0, 0.5% SDS (Sigma).
6x Loading dye for DNA: 0.25% bromophenol blue, 0.25% xylene cyanol (Bio-Rad), 30% glyserol (Sigma).
1x TAE: 0.04 M Tris-acetate, 0.001 M EDTA (Fisher).
Depurination solution: 250 mM HCl (Fisher).
Southern transfer solution: 0.4 M NaOH, 0.6 M NaCl (Fisher).
Hybridization solution for southern blot: 6x SSC, 5x Denhardt's, 50% formamide (BHD), 0.5% SDS (Bio-Rad).
Washing solutions for southern blot: Solution I. 2x SSC (Fisher), 0.1% SDS (Sigma). Solution II. 0.1x SSC, 0.1% SDS.

4 Protein studies

Lysis solution: 9.5 M urea (ICN), 2% Triton-X 100 (Sigma), 5% 2-mercaptoethanol (Sigma), 1.4% Bio-Lyte pH 3-10 ampholytes, 0.6% Bio-Lyte pH 5-7 ampholytes (Bio-Rad).
Tube gel for IEF: 4% acrylamide (stock: 28.38% acrylamide (Sigma), 1.62% Bis (Bio-Rad)), 9.2 M urea (ICN), 2% Triton-X 100 (Sigma), 2% Bio-Lyte pH 3-10 ampholytes, 0.01% APS, 0.1% Temed (Bio-Rad).
Sample overlay buffer for IEF: 9.0 M urea (ICN), 0.7% Bio-Lyte pH 3-10 ampholytes, 0.3% Bio-Lyte pH 5-7 ampholytes, 0.0025% bromophenol blue (Bio-Rad).
Tube gel for NEPHGE: 4.5% acrylamide (stock: 28.38% acrylamide (Sigma), 1.62% Bis (Bio-Rad)), 9.0 M urea (ICN), 2% Triton-X 100 (Sigma), 1.6% Bio-Lyte pH 5-7 ampholytes, 0.4% Bio-Lyte pH 3-10 ampholytes, 0.01% APS, 0.1% Temed (Bio-Rad).
Sample overlay buffer for NEPHGE: 6.0 M urea (ICN), 0.8% Bio-Lyte pH 5-7 ampholytes, 0.2% Bio-Lyte pH 3-10 ampholytes (Bio-Rad).
Running buffers for first dimension: 20 mM NaOH (upper chamber for IEF, lower chamber for NEPHGE), 10 mM H_3PO_4 (lower chamber for IEF, upper chamber for NEPHGE) (Fisher).
1x loading dye for 1D: 2% SDS, 10% glyserol, 5% 2-mercaptoethanol (Sigma), 0.1 M Tris-HCl pH 6.8 (Fisher), 0.05% bromophenol blue (Bio-Rad).
Tracking dye: 0.05% bromophenol blue (Bio-Rad), 10% glycerol (Sigma).
Stacking gels: 4% acrylamide (stock: 30% acrylamide (Sigma), 0.44% Bis (Bio-Rad)), 125 mM Tris-HCl (Fisher), pH 6.8, 0.1% SDS (Sigma), 0.05% APS, 0.001% Temed (Bio-Rad).
Separating gels: 12.5% acrylamide (15% in 1D gels) (stock: 33.5% acrylamide (Sigma), 0.3% Bis (Bio-Rad)), 380 mM Tris-HCl (Fisher), pH 9.1, 0.2% SDS (Sigma), 0.05% APS, 0.0005% Temed (Bio-Rad).
Running buffer for second dimension or 1D gels: 250 mM Tris (Fisher), 250 mM glycine (Bio-Rad), 0.1% SDS (Sigma).
Gel fixation solution: 10% acetic acid, 10% trichloroacetic acid, 25% methanol (Fisher).
Gel enhancing solution: 22% biPhenyloxazole (Sigma) in 100% dimethylsulfoxide (Fisher) w/v.