SARCOPLASMIC RETICULUM ATPase AND SARCOLEMMAL Ca^{2+}-ATPase MESSENGER RNA EXPRESSION DURING *IN VITRO* SKELETAL MUSCLE CELL DIFFERENTIATION

BY

JING ZHANG

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ACKNOWLEDGEMENTS

I am extremely grateful to my supervisor Dr. Angel Zarain-Herzberg for his continual support, guidance and patience throughout this thesis work. I am very grateful to the members of my advisory committee Dr. Elizabeth Kardami and Dr. Peter Zahradka for their invaluable instruction and encouragement. I also would like to thank Dr. Andrew Lassar and Dr. Theodore Y.C.Lo for providing the 10RMD and L6 cell lines.

ABSTRACT

The Ca^{2+}-pumping ATPases of skeletal and cardiac muscle cells are primarily localized at the sarcoplasmic reticulum and sarcolemmal membranes. They play a critical role in maintaining intracellular calcium homeostasis during the contraction-relaxation cycle of muscle cells. Three genes coding sarcoplasmic reticulum Ca^{2+}-ATPases (SERCA) and at least four genes coding sarcolemmal Ca^{2+}-ATPase (PMCA) have been isolated and characterized. A variety of isoforms of the calcium pumps are encoded by these genes. Although, it was previously reported that the transcription of SERCA and PMCA genes may be modulated during myogenic and neural differentiation, the precise mechanisms and functional significance for these changes remains unclear. The objective of this work was to study the mRNA expression for Ca^{2+}-ATPase isoforms during *in vitro* differentiation of skeletal muscle cell lines. To analyze the mRNA expression pattern of the SERCA and PMCA isoforms, three skeletal muscle cell lines (L6, C2C12, Sol8) were used as models. The mRNAs of these Ca^{2+}-ATPase gene were detected by a semi-quantitatively RT-PCR technique. It is generally regarded that myogenic factors such as MyoD and myogenin can directly regulate muscle-specific gene expression both *in vivo* and *in vitro*. In order to understand the relationship between the expression of MyoD and myogenin their mRNA levels was also measured during the differentiation process. The drugs 5-azacytidine (AZA) and 5-bromodeoxyuridine (BUdR) were used to further analyze the role of the above myogenic determination factors on the expression of the SR and SL Ca^{2+}-ATPases.

The results demonstrate that the mRNA levels for SERCA1a, SERCA1b and SERCA2a isoforms were increased significantly during *in vitro* differentiation from myoblast to myotube. Although low levels of mRNA for SERCA2a were detected in myoblasts, it was increased 5 to 8 -fold in myotubes. In contrast, the mRNA levels for SERCA2b were reduced in myotubes, indicating an "switch" in the mRNA distribution for both isoforms. For the PMCA isoforms, we found that the mRNAs for the PMCA1b, PMCA4b isoforms were expressed in undifferentiated myoblasts and that there was a "switch" to the PMCA1c, PMCA4a and PMCA4d during differentiation. The mRNA expression of the myogenic regulatory factors MyoD1 and myogenin were also induced during *in vitro* myogenesis of these muscle cell lines. These results led us to the hypothesis that the isoform "switch" in the expression of PMCA and SERCA isoforms results from post-transcriptional mRNA processing by mechanisms involving alternative splicing that might involve the myogenic factors MyoD and myogenin.

To further analyze the role of myogenic factors on the mRNA processing of the SR Ca^{2+}-ATPases, 5-bromodeoxyuridine (BUdR), a pyrimidine analogy which has been shown previously with the property to inhibit myogenin expression, was added to C2C12 myoblasts in culture. The results demonstrate that expression of the myogenic factors MyoD and myogenin, as well as SERCA2a, was arrested by treatment with BUdR. In contrast, PMCA1 and PMCA4 isoform expression was promoted in L6 cells supplemented with the myogenic inducer 5-azacytidine (AZA). Taken together, these results indicate that transcriptional and/or post-transcriptional processing of the PMCA and SERCA genes may be modified by MyoD and myogenin during skeletal myogenesis. We also used the 10RMD cell line derived from embryonic fibroblast C3H/10T1/2 stably

transfected with MyoD to assess the relationship between MyoD and Ca^{2+}-ATPase gene expression confirming the data obtained with AZA.

The results from this investigation, in combination with earlier observations from this and other laboratories, suggest that myogenic determination factors such as MyoD and myogenin might regulate SERCA2a gene expression at the transcriptional and post-transcriptional level. However, questions concerning the direct and /or indirect control of alternative splicing by these myogenic regulatory factors requires further study.

LIST OF TABLE AND FIGURES

I. REVIEW OF THE LITERATURE

In this chapter, particular attention will be given to three topics: 1. Ca^{2+}-transporting ATPases, 2. myogenesis and 3. processing of primary messenger RNA transcripts. These subjects are directly related to my thesis work.

1.1 Role of Ca^{2+}-transport ATPases in muscle contraction.

Skeletal, cardiac and smooth muscle cells maintain very low concentrations of free calcium [Ca^{2+}] inside the cell [10^{-7} M], whereas Ca^{2+} concentrations outside of cell are extremely high [10^{-3} M]. Therefore, even a small influx of Ca^{2+} may markedly increase intracellular concentration of free Ca^{2+}. The excitation-contraction coupling process in striated muscle cells is triggered by a transient increase in intracellular Ca^{2+} concentration. Depolarization of the sarcolemmal (SL) cell membrane results in opening of the voltage-dependent Ca^{2+} release channel that subsequently induces the opening of the sarcoplasmic reticulum (SR) membrane Ca^{2+} release channel sensitive to ryanodine (RYR). This channel releases Ca^{2+} from the lumen of the SR into the cytoplasm of the muscle cell. Increased cytoplasmic [Ca^{2+}] in turn activates muscle contraction by binding to the regulatory protein troponin C located in the thin filaments of muscle cells. To achieve muscle relaxation, calcium has to be removed from troponin C. Removal of calcium from the cytoplasm is achieved through ATP-dependent active Ca^{2+} uptake by the SR Ca^{2+}-ATPase (SERCA) and Ca^{2+} extrusion by the Na^{2+}-Ca^{2+} exchanger and the plasma membrane Ca^{2+}-ATPases (PMCA)(see **Figure 1**).

The increase in Ca^{2+} concentration inside the plasma membrane in response to external signals is one of the mechanisms for transmitting extracellular stimuli to the inside of the cell. Both SL and SR Ca^{2+}-pumps actively transport Ca^{2+} out of the cytosol. SL and SR calcium pumps belong to the P-type transport ATPases, which form a phosphorylated intermediate that results from the transfer of the "high energy" γ-phosphate of ATP to a specific amino acid residue at the catalytic site of the pump. The following section describes the properties of both Ca^{2+}-pumps in detail.

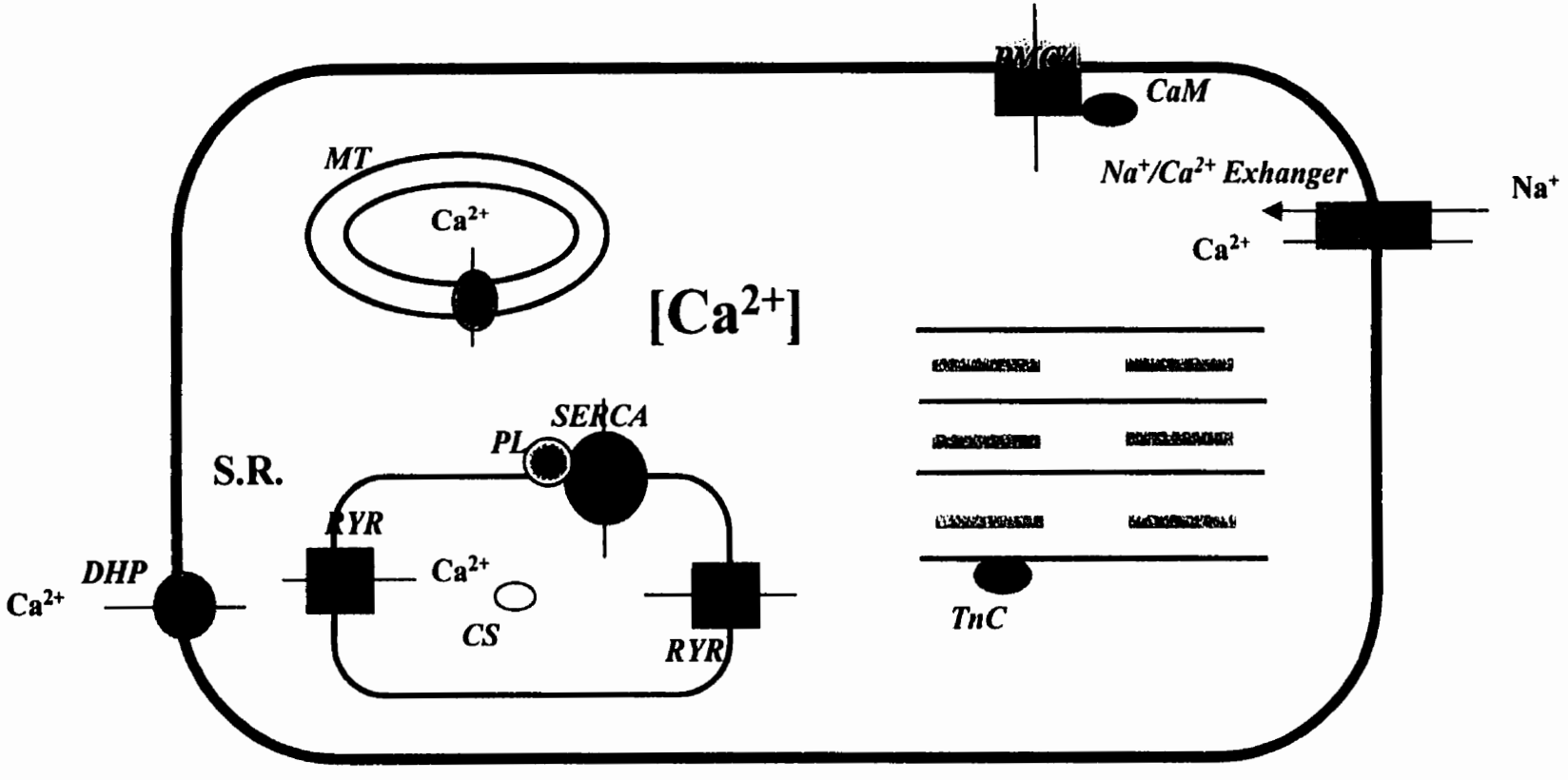

Figure 1 . Calcium Transport Mechanisms in the Muscle Cell. The proteins participating in calcium transport during the contraction/relaxation cycle of a muscle cell are indicated. SR: sarcoplasmic reticulum; MT: mitochondria; DHP:dihydropyridine sensitive voltage sensitive calcium channel; RYR2: calcium release channel sensitive to ryanodine; SERCA: sarco(endo)plasmic reticulum Ca^{2+}-ATPase; PMCA: plasma membrane Ca^{2+}-ATPase; CaM: Calmodulin; CS: calsequestrin; PL: phospholamban; TnC: Troponin C.

1.2 Sarcoplasmic reticulum Ca^{2+}-ATPases.

The movement of Ca^{2+} from the cytosol into the lumen of the SR is achieved by the SR Ca^{2+}-ATPases (SERCAs) (see **Figure 1**). These enzymes catalyze Ca^{2+} transport to the lumen of the SR by an active process. The phosphate of the ATP is transferred to aspartate-351 in the catalytic site of the Ca^{2+} SR pump (1). Enzyme phosphorylation and ATP hydrolysis cause the movement of the two enzyme-bound Ca^{2+} from a high-affinity site (E1-P) to a low-affinity site (E2-P). The two calcium ions are then released into the lumen of the SR, as shown in **Figure 2.**

The secondary structure of the SERCA2 Ca^{2+} pump was deduced for the first time from the sequence of a cDNA obtained from a rabbit slow-twitch skeletal muscle library by D.H. MacLennan and his group in 1985 (2). This enzyme is a single polypeptide of 110 kDa containing 1001 amino acid residues. In skeletal muscle fibers, about 40% of the total protein present in the SR membrane are pump protein. This group also deduced a secondary structure model for this protein, which includes three cytoplasmic domains and a set of 10 predicted transmembrane α-helixes (M1 to M10) (3) (**Figure 3 panels A and B**).

This laboratory subsequently used site-directed mutagenesis to analyze the structure-function relationships of the SR Ca^{2+}-pump (4). They found that Ca^{2+} transport ability was inhibited if any six amino acids in the transmembrane regions M4 (Glu-309), M5 (Glu-771), M6 (Asn-796, Thr-799 and Asp-800) and M8 (Glu-908) were mutated. It is suggested that these regions form a channel that binds and translocates Ca^{2+}(5).

To date three sarco(endo)plasmic reticulum (SERCA) genes (named SERCA1, SERCA2 and SERCA3) have been found. A total of five isoforms of the SR Ca^{2+} pump are encoded by these three genes. The SERCA1 gene is only expressed in the fast-twitch skeletal muscle fibers. The pre-mRNA of the SERCA1 gene is alternatively spliced to yield the adult (SERCA1a) and the neonatal (SERCA1b) isoforms (6). The SERCA1 isoforms are identical proteins, except that the C-terminus of the neonatal isoform consists of 8 charged amino acids that are replaced by a single Gly residue in adult

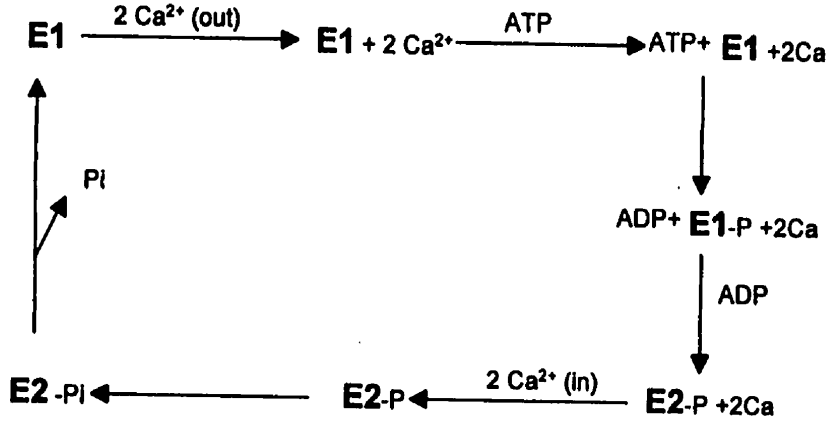

Figure 2: General schematic representation of the catalytic and transport cycle of the SR and SL Ca^{2+}-ATPases.

E1-P: phosphorylated intermediate with high energy and high affinity for Ca^{2+} .
E2-P: low energy and low affinity for Ca^{2+}.

isoform (7). The differences in the catalytic properties of the SERCA1 isoforms are not completely understood. The SERCA2 gene is mainly expressed in slow-twitch skeletal and cardiac muscle. The SERCA2 gene produces two isoforms, SERCA2a and SERCA2b by alternative splicing which removes an intron at the 3' end of the SERCA2 primary transcripts. Structurally, the only differences between the SERCA2a and SERCA2b isoforms lie in the C-terminus where the last four amino acids of SERCA2a are replaced by 49 different amino acids in SERCA2b, and in the 3'-noncoding regions of the mRNA (8) (**Figure 6**). The SERCA2a isoform is a protein of 110 kD and the SERCA2b isoform is a protein of 115 kD (see **Figures 3 panel A** and **B**). The SERCA3 gene is mainly expressed in endothelial cells and epithelial cells and encodes only one isoform (9).

The catalytic activity of SR Ca^{2+}-pumps can be regulated by a 52 amino acid polypeptide named phospholamban, which is a pentameric protein that can be phosphorylated by cAMP-dependent kinase, cGMP-dependent kinase and Ca^{2+} / calmodulin-dependent protein kinase (10). When phospholamban is not phosphorylated, it inhibits the Ca^{2+}-ATPase activity by interacting with the enzyme and decreasing the pump affinity for Ca^{2+} (11). In contrast when this protein is phosphorylated *in vitro*, phospholamban is released from its binding site and the affinity of the pump for Ca^{2+} is increased, which results in increased transport of Ca^{2+} into the SR.

In vivo, only the SERCA2 calcium pump activity is inhibited by phospholamban in its unphosphorylated state, however, the activities of the SERCA1, SERCA2a and SERCA2b can be regulated by phospholamban *in vitro*, when phospholamban is expressed in COS-1 cells (12). Therefore, the lack of sensitivity of SERCA1 to phospholamban *in vivo* is due to the absence of phospholamban gene expression in fast-twitch skeletal muscle. Phospholamban is only expressed in slow-twitch skeletal muscle, cardiac muscle and some smooth muscle cells. Phospholamban has been stably transfected in the C2C12 cell line that is derived from fast-twitch skeletal muscle, and the expression of phospholamban, which decreases the affinity of SERCA1 for Ca^{2+}, decreases the rate of Ca^{2+}-uptake in microsomes.

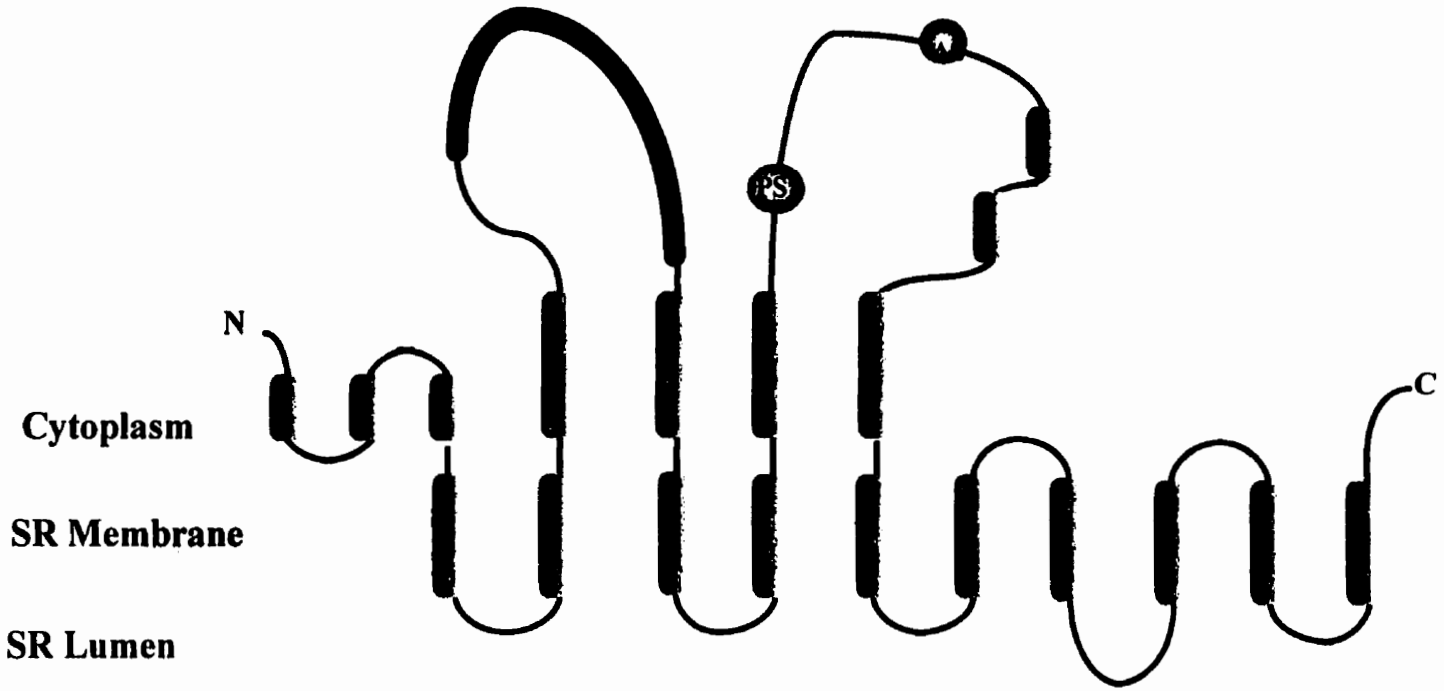

Figure 3-A. Sarcoplasmic Reticulum Ca^{2+}-ATPase secondary structure for isoforms type "a".
The predicted structure for SERCA2a isoform is illustrated. The NH2 (N) and COOH (C) ends of the enzyme are indicated. The ten transmembrane passages and the regions of alpha-helix are indicated by boxes.
The beta-sheet region is indicated by a thick line.The phospholipid (PL) and the phosphorylation sites PS and AS are indicated.

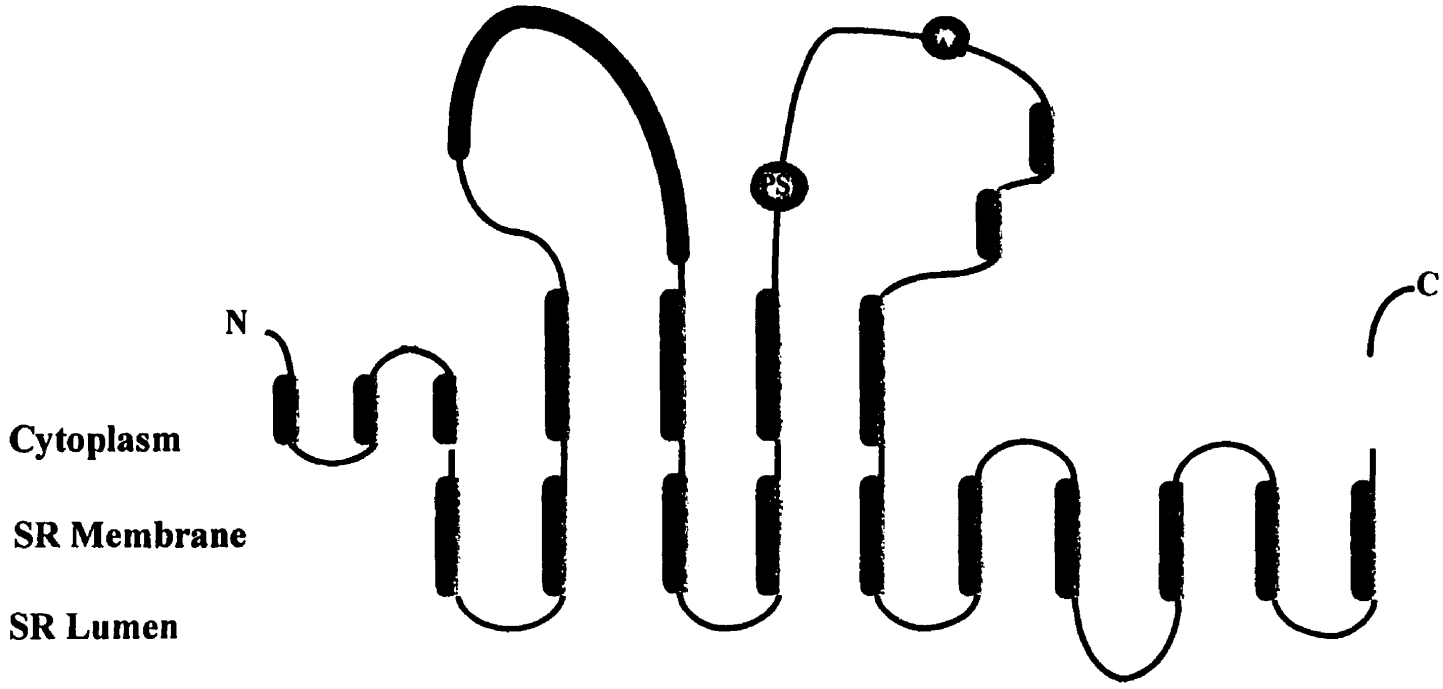

Figure 3-B. Sarcoplasmic Reticulum Ca^{2+}-ATPase secondary structure for isoforms type "b". The predicted structure for SERCA2b isoform is illustrated. The NH2 (N) and COOH (C) ends of the enzyme are indicated. The ten transmembrane passages and the regions of alpha-helix are indicated by boxes. The beta-sheet region is indicated by a thick line.The phospholipid (PL) and the phosphorylation sites PS and AS are indicated.

Taken together these findings prove that phospholamban can regulate SERCA1 and SERCA2 isoforms in their membrane environment (13). Interestingly, the SERCA3 Ca^{2+}-pump is unaffected by phospholamban both *in vivo* and *in vitro* and the calcium pump conserves its sensitivity for Ca^{2+}. Analysis of the amino acid sequence in the region identified as the phospholamban binding site of the SERCA1 and SERCA2 isoforms indicates that they differ from that of the corresponding region within the SERCA3 isoform. This difference presumably explains why SERCA3 is not inhibited by phospholamban (12).

1.3 Plasma Membrane Ca^{2+}-ATPases.

The plasma membrane Ca^{2+}-ATPase was first identified in human red blood cells. In the original observation, Ca^{2+} was released from erythrocytes loaded with Ca^{2+} and ATP at a higher rate than from cells without addition of ATP. Because Ca^{2+} could be extruded even in the presence of higher Ca^{2+} concentrations outside the cells, it was clear that the extrusion was driven by ATP (14). The characteristics of the plasma membrane calcium-pump (PMCA) follows the same mechanism of other known P-type ion pumps regarding the property of transport Ca^{2+}, inhibition of pump activity, ATP and Ca^{2+} affinities of the enzyme.

The catalytic cycle of the PM Ca^{2+} pump begins with the Ca^{2+}-dependent transfer of the γ-phosphate from ATP to an aspartic residue in the catalytic site of the enzyme. A phosphorylated intermediate of the enzyme is formed by phosphate transformation (15). The typical plasma membrane calcium pump is a single polypeptide of 127-136 kD with ten transmembrane passages (see **Figure 4 panel A, B, C**). It has been suggested that in the membrane the enzyme might form homodimers and this association may be of relevance for the function of the pump *in vivo* (16). Compared to the Ca^{2+}-ATPase found in SR, the Ca^{2+}-ATPase in the plasma membrane seems more complex. A characteristic feature of PMCA is their high isoform diversity produced by at least four genes PMCA1, PMCA2, PMCA3 and PMCA4. The combination of fluorescence *in situ* hybridization (FISH) with somatic cell hybrids and genetic linkage analyses have mapped four

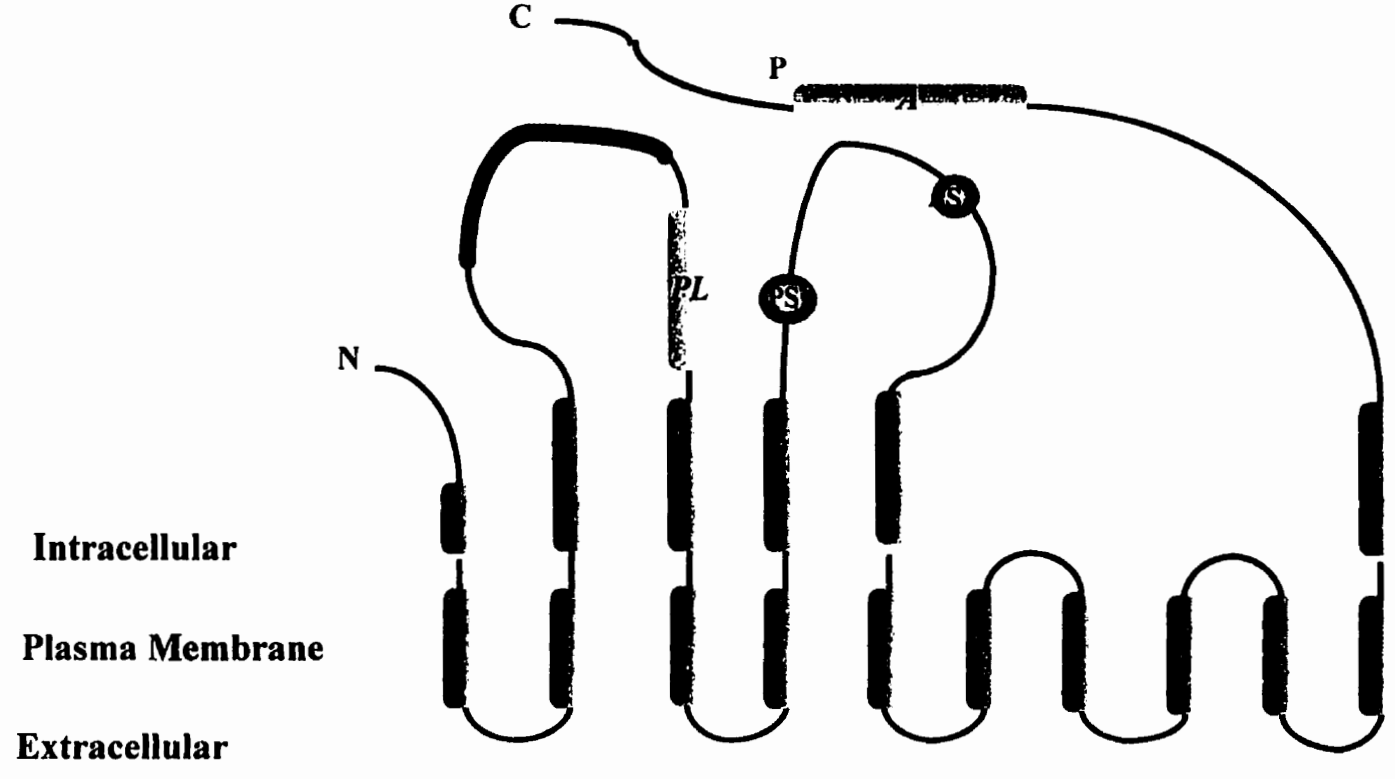

Figure 4-A Plasma Membrane Ca^{2+}-ATPase secondary structure for isoforms type "a" . The predicted structure for PMCA type "a" isoforms is illustrated. The NH2 (N) and COOH (C) ends of the enzyme are indicated. The predicted ten transmembrane passages and the regions of alpha-helix are indicated by boxes. The beta-sheet region is indicated by a thick line.The phospholipid (PL) and the phosphorylation sites PS, AS and P are indicated. The calmodulin binding domain A is also indicated.

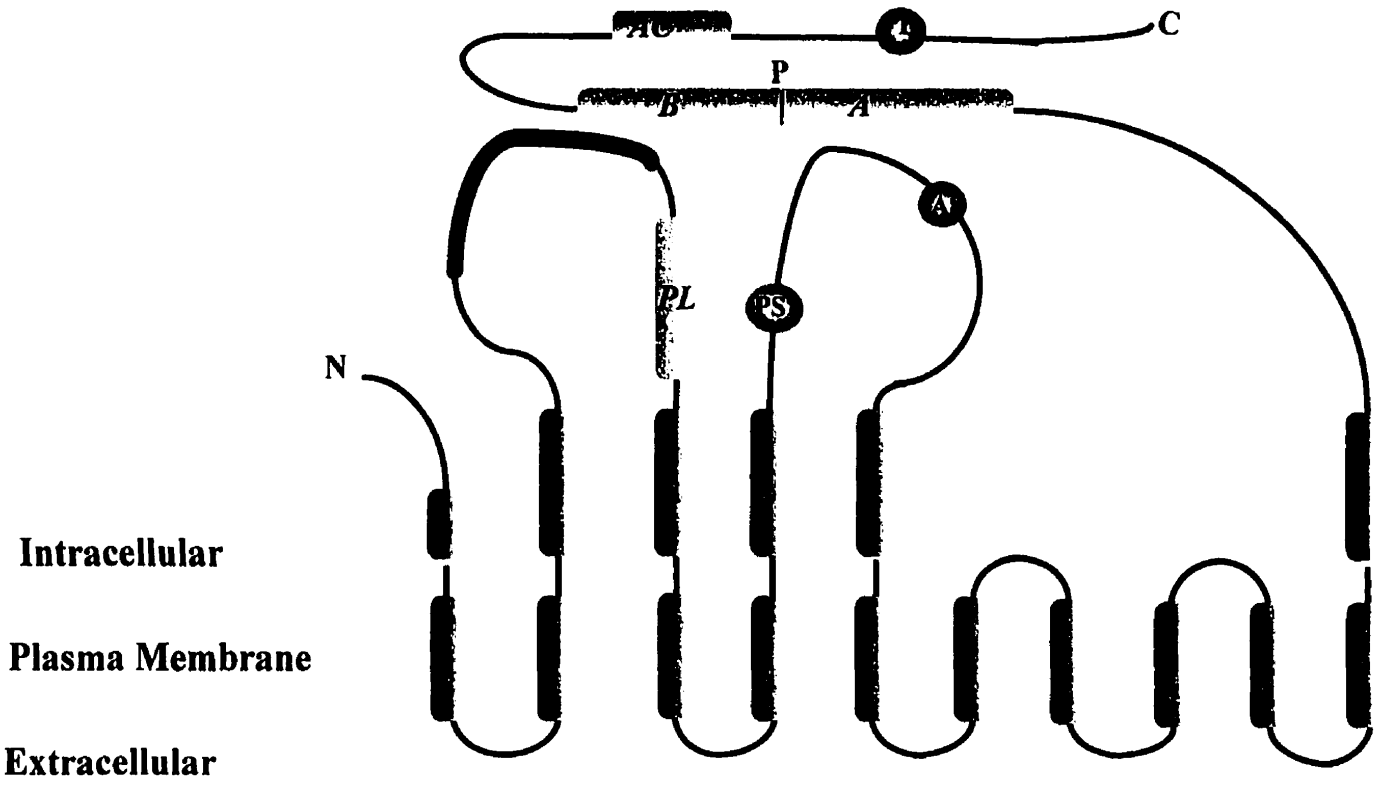

Figure 4-B. Plasma Membrane Ca^{2+}-ATPase secondary structure for isoforms type "b" . The predicted structure for PMCA type "b" isoforms is illustrated. The NH2 (N) and COOH (C) ends of the enzyme are indicated. The ten transmembrane passages and the regions of alpha-helix are indicated by boxes. The beta-sheet region is indicated by a thick line.The phospholipid (PL) and the phosphorylation sites PS, AS and P are indicated. AC: acidic amino acid stretch.The calmodulin binding domains A and B are also indicated.

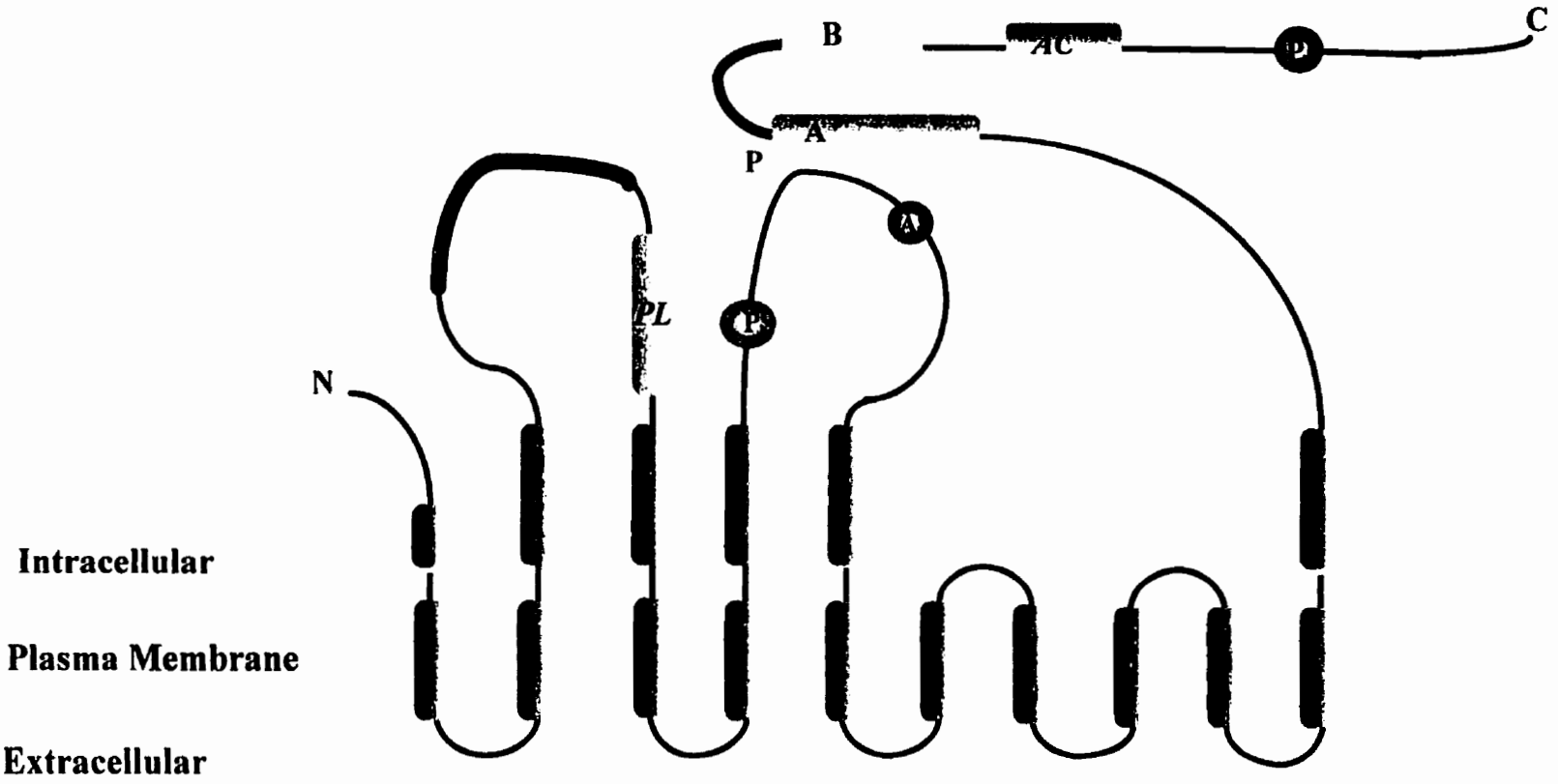

Figure 4-C.Plasma Membrane Ca^{2+}-ATPase secondary structure for isoforms type "c" and "d". The predicted structure for PMCA1 type "c" and "d" isoforms is illustrated. The NH2 (N) and COOH (C) ends of the enzyme are indicated. The ten transmembrane passages and the regions of alpha-helix are indicated by boxes. The beta-sheet region is indicated by a thick line.The phospholipid (PL) and the phosphorylation sites PS, AS and P are indicated. The only difference between isoforms 1c and 1d is the insertion of a 29 aa or 38 aa (thick red line) respectively. AC: acidic amino acid stretch. The calmodulin binding domains A and B are also indicated with letters.

human PMCA genes to chromosome 12 (PMCA1), chromosome 3 (PMCA 2), the X chromosome (PMCA3) and chromosome1 (PMCA4) (17-20).

The full-length cDNA sequence of most human and rat PMCA genes have been characterized. High homology in amino acid sequences of the corresponding isoforms exists between these two species (21). The mRNAs of all four genes encode proteins containing binding sites for ATP, Ca^{2+} and calmodulin. By Northern blot analysis it has been shown that PMCA1 is expressed in a variety of tissues, but the mRNA is most abundant in the brain (24). The expression of PMCA2 and PMCA3 mRNAs is mainly in brain, heart and skeletal muscle (22,23). The mRNA of PMCA4 was tested in the brain by using *in situ* hybridization antisense mRNA probes labeled with ^{33}P (25). cDNA cloning, Northern blot and PCR analysis have indicated that PMCA4 is also expressed in human heart, intestine and brain (24,26). Specific antibodies were generated from N-terminal sequence of the pump, and tissue distribution of the four PMCA gene products was tested by Western blot analysis (28). PMCA1 and 4 were expressed in all tissues. The PMCA2 and PMCA3 proteins were restricted to neuronal tissues. Cerebellum and cerebral cortex express PMCA2 protein in high concentrations (29).

The promoter region of the PMCA1 gene was cloned and characterized in the mouse; four sites for initiation of transcription have been identified by RNase protection assay and primer extension analysis (30). A region containing 600 bp of promoter sequences linked to a chloramphenicol acetyltransferase (CAT) gene can drive the expression of CAT in transient transfected mouse neuroblastoma cells and rat aortic endothelial cells. The promoter activity of this gene was also stimulated by phorbol esters and cyclic AMP in the order of two- to four-fold (30).

PMCA mRNA can be induced by various hormones or agonists through different second messenger pathways that include PKC, cAMP- and Ca^{2+}-dependent mechanisms. The PMCA messages of endothelial cells from rat aorta could be increased by 20-fold in response to phorbol ester and angiotensin II. Brain vessel endothelial cells only respond to stimulation by cAMP or the Ca^{2+} mobilizing agent thapsigargin. This fact suggests that hormone-induced PMCA gene expression is regulated by two distinct pathways: PKC and Ca^{2+} plus cAMP (31).

Additional isoform variability is caused by alternative splicing of primary transcripts of PMCA genes (see **Figure 7**). Two regions of the human PMCA primary transcripts, have been shown to be involved in alternative processing (27,32). For all PMCA genes, common sites within the pre-mRNA have been demonstrated to generate isoforms at two splice sites. The first site is named A and splicing at this site produces an isoform through inclusion a small exon of around 40 bp. A second site has been identified and named site C. The carboxyl-terminal exons can be alternatively spliced in the mature mRNA, generating a variety of isoforms with distinct size, kinetic properties, and sensitivity to calmodulin. The work by T. P. Keeton and coworkers examined the rat PM Ca^{2+} pump RNA by using S1 nuclease protection and PCR techniques, and the corresponding genes were analyzed to determine the tissue-specific splicing patterns at site C (32). Gene products PMCA1b and PMCA4b may represent housekeeping isoforms supplying general calcium pumping function (27). The essential conserved sequences of the PMCA transcripts maintain the basic function in transport and catalysis. These conserved segments are not involved in the alternative splicing. Alternative splicing often occurs in the sequences that can produce specific gene products, and which regulate functional specialization of the pump. For example, splice site A is close to the phospholipid-sensitive region of the enzyme and site C is within calmodulin binding domain.

Several factors have been identified as modulators of the activity of this type of calcium pump. These factors include calmodulin (33), poly-unsaturated fatty acids, acidic phospholipids (34) and protein kinases (35). Calmodulin (CaM) is the most important regulator for PMCA. The effect of CaM on the PMCA is Ca^{2+}-dependent and requires direct interaction with a specific target site on the pump molecule. Activation of PMCA by CaM is mainly due to an increase in the apparent affinity of the pump for Ca^{2+}. In the presence of proper CaM concentrations, the Km (Ca^{2+}) of the ATPase decreases markedly (36).

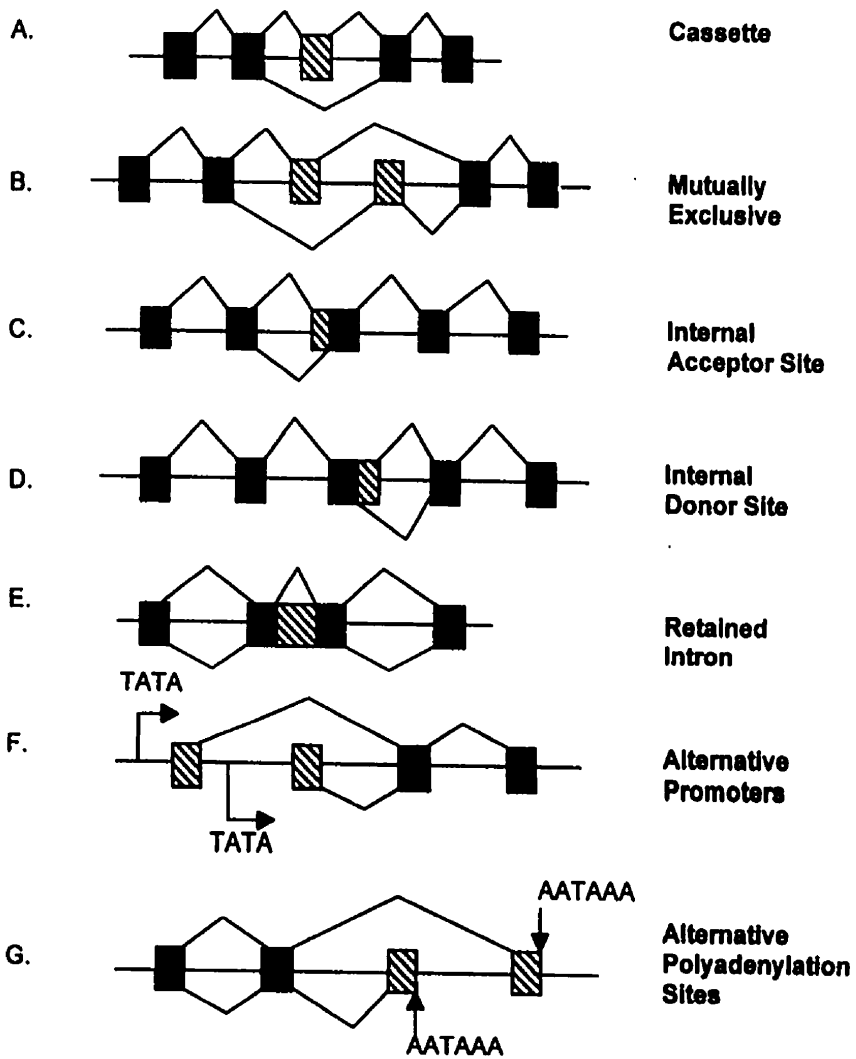

Figure 5. Mechanisms of alternative splicing. Constitutive exons (solid boxes), alternatively spliced exons (striped boxes) and introns (solid lines) are spliced following the different patterns indicated. Alternative promoters (TATA) and polyadenylation signals (AATAAA) are indicated. This schematic representation was modified from the publication by Christopher W.J. et al at Annu. Rev.Genet 1989, 23:527-577.

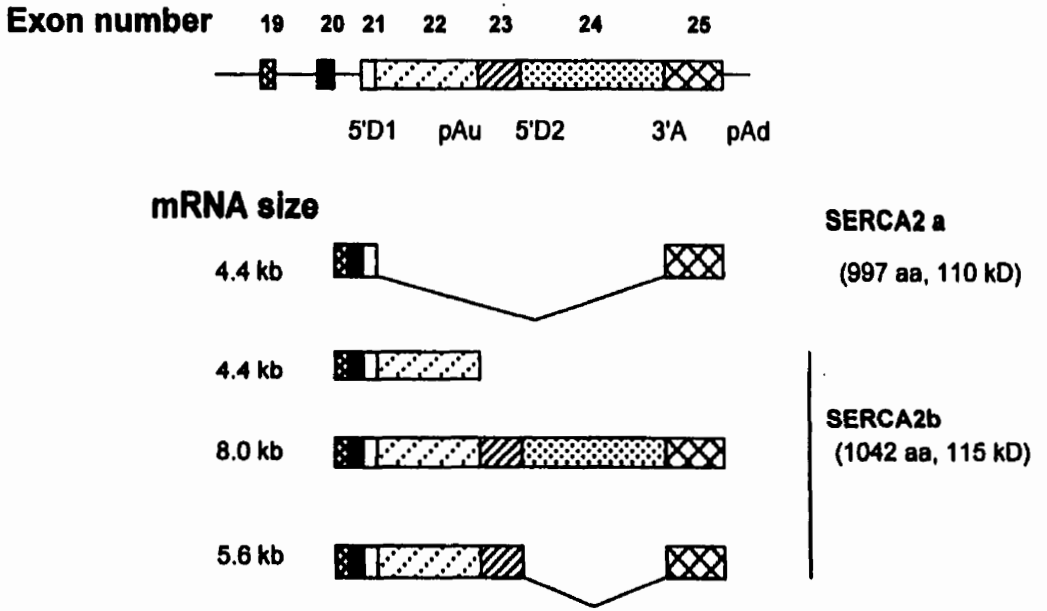

Figure 6. Schematic representation of mRNA processing for SERCA2 isoforms. Upper line represents the structural organization of the 3' end of the rabbit SERCA2 gene. The rectangles represent exons. 5'D1 and 5'D2 are the donor splice sites that compete for the single acceptor site (3'A). The upstream (pAu) and the downstream (pAd) polyadenylation sites are indicated. The four lower lines represent the 3' ends of the four species of mRNA for SERCA2 the SERCA2a and SERCA2b isoforms. Exons 19 - 21 are constitutive exons present in the four SERCA2 mRNAs. Exons 22 - 25 are optionally included or excluded. The four species of mRNA translate into only two different enzyme isoforms, SERCA2a (cardiac/slow) or/and SERCA2b (smooth/non-muscle).This schematic representation was modified from the publication by Wuytack F.L. et al in Ann N Y Acad Sci 1992, 671:82-91.

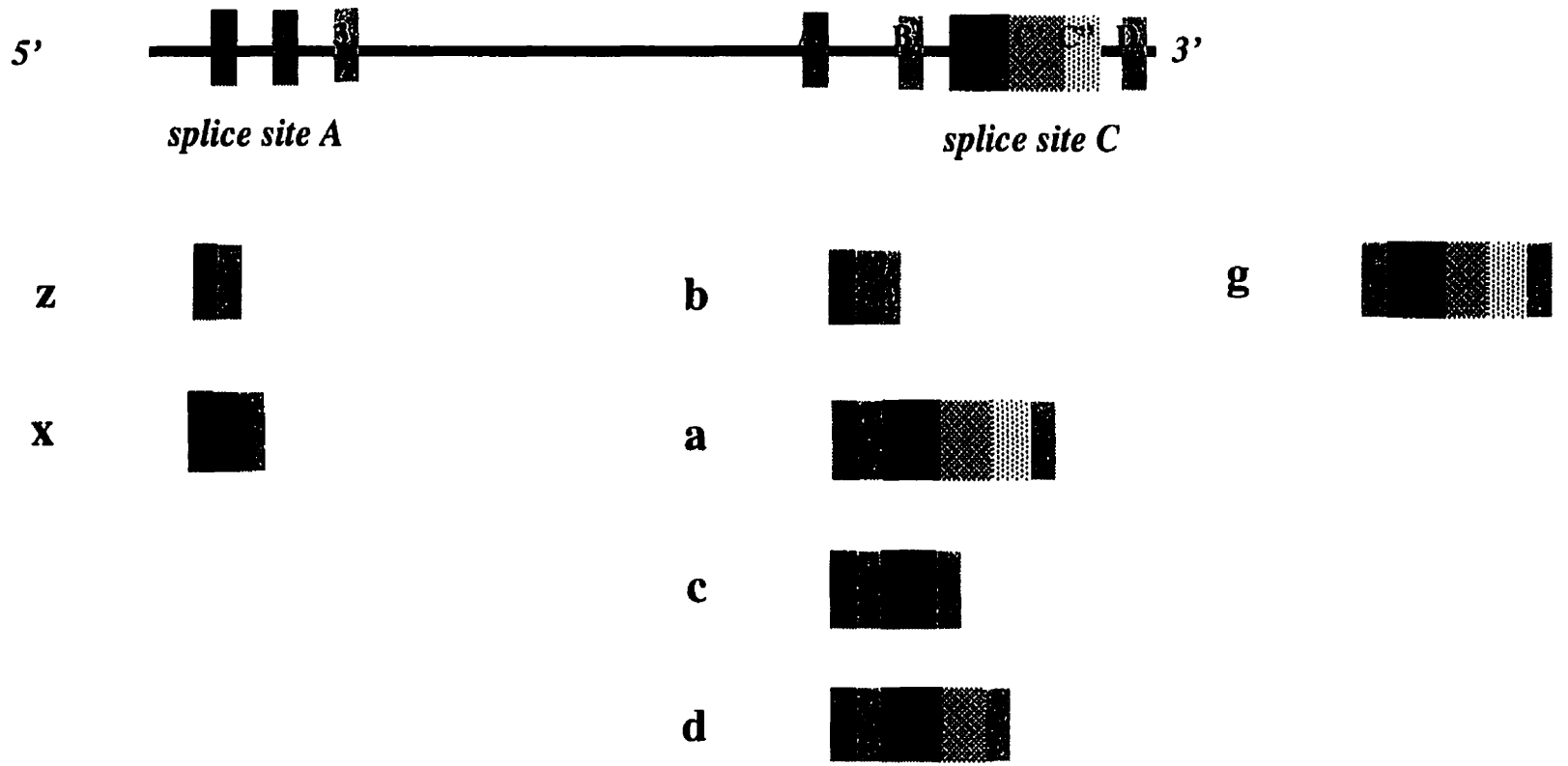

Figure 7. Schematic representation of the splice products at site "A" and "C" of the PMCA1 and PMCA4 transcripts. The *upper line* represents the exon/intron structure of the 3' region of the PMCA gene. Exons are "*boxed*". The sites for splicing at site "A" and "C" are indicated. The mRNA products for the isoforms at site A are indicated as **z** and **x**. The mRNA products for the isoforms at site "C" are indicated as **b, a, c, d** and **g**.

2.1 Myogenesis: Skeletal Muscle Differentiation.

It is generally believed that skeletal muscle originates from the mesoderm during vertebrate embryogenesis and determination of the skeletal muscle lineage probably occurs during gastrulation (37). Morphology of skeletal muscle cells is dependent upon the developmental stage and extent of cell differentiation. Myoblasts are the mononucleated cell precursors of the mature skeletal muscle cells, which develop from mesodermal stem cells and become restricted to a myogenic fate. Although undifferentiated, myoblasts are committed to the myogenic lineage and finally differentiate when they receive the appropriate environmental signals. Once myoblasts enter the differentiation pathway, they withdraw from the cell cycle and fuse with other myoblasts to form multinucleated myotubes. The change of phenotype involves the activation of muscle-specific genes whose products are characteristic for the contractile and metabolic properties of the muscle fiber (38) (see **Figure 8**).

2.2 The structural features of the basic helix-loop-helix (bHLH) protein transcription factors.

Original work in the late '70s and early '80s led to the novel proposal that one or a few regulatory genes had the potential to activate a cascade of events that induce muscle differentiation. In 1979, S. M. Taylor and P. A. Jones observed that the mesodermal stem cell line C3H10T1/2 could be converted to myoblasts after brief treatment with the demethylating agent 5-azacytidine (39). The frequency of conversion suggesting that one or a few regulatory *loci* were altered following hypomethylation and were responsible for establishing the myogenic lineage (40). This idea was supported by genomic transfection of 10T1/2 with DNA from azacytidine-induced myoblast that results in myogenic conversion. The results indicated that an azacytidine-induced demethylation in the DNA of a single locus is sufficient to convert 10T1/2 cells into myoblasts (41). The myd gene is possibly the upstream gene and may activate MyoD gene (50) (see **Figure 8**).

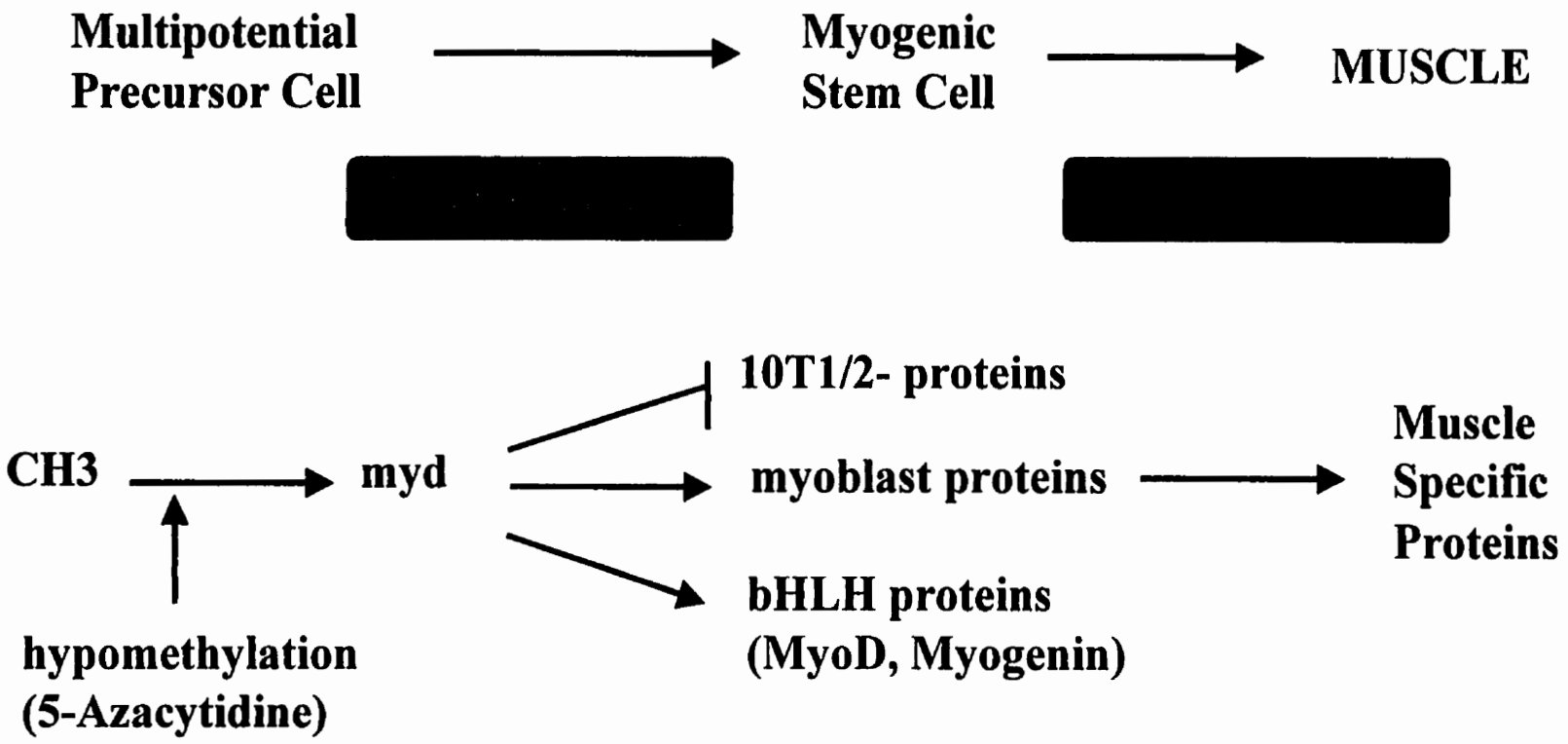

Figure 8 . Schematic representation of skeletal muscle development *in vitro* pathway. Cytosine methylation (indicated by CH3) is inhibited by azacytidine. The resultant hypomethylation of the myd gene leads to activation of the key myogenic genes.

In 1987 Davis et al, by using a subtraction-hybridization procedure to screen a cDNA library, isolated a new cDNA which is expressed in myoblasts but not in 10T1/2 cells. They named this gene MyoD (or MyoD1) for myoblast determination gene number 1 (42). MyoD was the first myogenic regulatory gene to be identified. The discovery of MyoD (or MyoD1) led to the identification of other members of the MyoD family. This work can be regarded as the most important event in the field of development research, because it was the first time researchers had shown that a single muscle-specific gene was sufficient to generate the complete muscle phenotype in undifferentiated embryonic cells. MyoD is expressed only in myoblasts and skeletal muscle tissue but not in smooth or cardiac muscle, or in non-muscle tissue. When the MyoD cDNA is expressed in 10T1/2 fibroblast, it gives rise to stable myogenic clones with the potential to undergo myogenesis. Another important myogenic regulatory gene, named myogenin, was identified two years later (43). The isolation of myogenin cDNA was also achieved by subtraction-hybridization of cDNA from BUdR-resistant myoblasts. Shortly after, the cDNAs of myf-5 and MRF-4 were isolated independently (44,45). All of these factors are expressed exclusively in skeletal muscle and have the ability to activate myogenesis in a variety of non-myogenic cell types.

The similar function of these four myogenic regulatory factors is mainly dependent on their secondary structure identity. High homology (~ 80%) exists within a 70 amino acid segment in each member of the MyoD family. This region contains a basic residue-rich fragment and a sequence which adopts a helix-loop-helix (bHLH) configuration (46). The HLH structure forms an interface for the dimerization of two protein factors. The basic HLH domain binds the DNA sequence CANNTG (N is any nucleotide), known as "E-box"(47). The "E-box" sequence is usually in the promoter region of most muscle-specific genes, including α-cardiac actin, myosin heavy chain and acetylcholine transferase subunit (48,49). The "E-box" serves as a binding site for heterodimers of myogenic bHLH proteins and other "E-box" binding proteins (50). Mutation of the "E-box" DNA sequence in the control region of AchR-δ gene prevents binding of the MyoD-E2A and myogenin-E2A heterodimers, thus decreasing AchR-δ gene expression in myotubes and increasing its expression in myoblasts and non-muscle

cells (51). Although the "E-Box" is important for transcriptional activation of many muscle-specific genes, it is not sufficient for high-level transcription by itself and other muscle transcription factors such as members of the MEF (muscle enhancer factor) and GATA families are also involved.

2.3 *In vivo* functional studies of the bHLH proteins.

Functional analysis of the MyoD family members was achieved by mutating the proteins and examining the consequences *in vivo*. In order to understand the role of the myogenin gene in myogenesis, it was inactivated in myogenin-negative mice (52). Homozygous mutations were lethal perinatally due to the resulting major defects in skeletal muscles. These findings indicated that myogenin was essential for muscle development and demonstrated that other members of the myogenic gene family cannot compensate for the defect in its expression. In another study, in MyoD mutant mice, a high level of myf-5 expression was maintained and skeletal muscle was formed (53). In mice defective in both MyoD and myf5, the development of skeletal muscle did not occur, and the myoblast population was absent because such cells do not appear in the embryo or do not survive (54). Mice with a homozygous germline mutation in the MRF4 gene only showed a minimal reduction in the expression of muscle-specific genes. however, they showed a dramatic increase in the expression of myogenin. These results suggest that myogenin may compensate for the deficiency of MRF4 and demonstrate that MRF4 is in need of the down-regulation of myogenin expression that normally occurs in postnatal skeletal muscle (55).

2.4 Regulation of the genes encoding bHLH proteins.

Questions concerning which factor is the "upstream" regulator in muscle differentiation or what mechanisms control the MyoD family gene expression have naturally drawn much attention. The principal regulatory mechanism for these family members involves autoregulation where each of these gene products can regulate its own gene. In addition, MyoD regulates the expression of other factors that are active

downstream in the cascade of events during myogenesis. There have been considerable efforts to elucidate the control regions of the genes coding for members of the MyoD family. The enhancer sequences of the MyoD gene that have been identified include two upstream enhancers at -20 kb and -6 kb of the murine MyoD gene. Both enhancers have "E-box" motifs, therefore, they are potentially regulated by other members of the MyoD family or by other bHLH proteins. Both enhancers can direct muscle expression in transgenic mice to some extent (56). The MyoD gene promoter is also active in avian species. The chick MyoD promoter has several "E box" and MEF2 (muscle enhancer factor 2) binding elements, however, even without these motifs, transcriptional activity is still detected (57). The myogenin and MRF4 genes promoter regions also have "E-box" and MEF2 binding sites. A sequence in the myogenin promoter (-133 bp) can control muscle-specific expression (58). Besides autoregulation among the MyoD family members and interaction with MEF2, other types of activators (eg. meso and Notch) of the MyoD family may be implicated as well. The meso-1 and meso-2 genes also encode proteins containing the bHLH motif which form heterodimers with the E2A proteins, and probably interact with the "E-box" (59). It has been shown that ectopic skeletal muscle was formed after injection of meso-1 gene into Xenopus embryos (60). For the Notch gene, its products can interact with MyoD through the bHLH domain to suppress its activity (61).

2.5 Other muscle regulatory transcription factors.

Apart from the myogenic regulatory factors, which have been reviewed above, there are other factors involved in the regulation of myogenesis in skeletal muscle. It has been observed that certain HLH proteins lack a DNA-binding domain (i.e. the basic region) and they can form nonfunctional heterodimers with normal bHLH proteins. A factor named Id (for inhibitor of differentiation) is an inhibitory HLH protein present at a high level in proliferating skeletal myoblasts and is down regulated in the absence of serum (62). During myogenic differentiation, a decrease in Id expression has been assumed to release E2A products and MyoD, and the released proteins can then heterodimerize to activate muscle-specific genes. M. Kurabayashi reported that

Doxorubicin (Adriamycin), a potent chemotherapeutic agent with negative effect on cardiomyopathy, completely inhibited myoblast fusion, resulting in the accumulation of muscle-specific transcripts in the mouse C2 skeletal muscle cell line through an enhancement of Id mRNA levels. The observation that E2-5, which forms inactive heterodimers with Id, can overcome the Doxorubicin-induced suppression of MyoD function in 10T1/2 cells, suggests that Doxorubicin inhibits MyoD-dependent muscle-specific gene expression by maintaining high levels of Id gene expression (63).

2.6 Growth factors and the myogenic programs.

A variety of growth factors can regulate the proliferation and differentiation of muscle cells, this discussion will only concentrate on three growth factors: insulin-like growth factors, transforming growth factor-β and fibroblast growth factor.

A. Insulin-like growth factors.

The insulin-like growth factors (IGFs) differ from other growth factors in stimulating, rather than inhibiting myogenic differentiation. The order of their capability in stimulating myogenic differentiation is IGF-I, IFG-II and insulin. Physiological levels of IGFs can stimulate myogenic cell line and chick embryo muscle cells differentiation (64). The most likely molecular basis for IGF-I action is the induction of myogenin, this idea resulted from the observation that treatment of muscle cells with IGF-I induced a 60-fold increase in myogenin mRNA content, and that a 15 nucleotide antisense oligomer of myogenin mRNA could block the stimulation of differentiation (65). The role of the IGF binding proteins (IGFBPs) was also investigated. It was found that IGF binding proteins inhibit IGF action. An IGF-I analog that has very low affinity for IGF binding proteins showed a 10- to 100-fold increase in ability to stimulate both proliferation and differentiation of L6A1c cells compared with the effect of native IGF-I (66). IGF-II is also the principal IGF peptide that is expressed in differentiating muscle cells. IGF-II is involved in autocrine and paracrine actions in muscle cells, which include the stimulation of spontaneous myogenic differentiation *in vitro* and down-regulation of the IGF-I

receptor (67,68). Growth medium contains a high percentage of serum which can negatively control IGF-II gene expression. IGF-II gene expression in L6A1 myoblasts was also regulated by a feedback pathway primarily via the type1 IGF receptor and seems to be inhibited by IGFBPs secreted by myoblasts in low serum differentiation medium (69). In IGF-I receptor down-regulation studies, IGF-II pretreatment significantly decreased binding of IGF-I to the IGF-I receptor and decreased IGF-I receptor mRNA (67). Quinn et al demonstrated that IGF-1 receptors not only amplify signals of myogenic differentiation, but also mediate mitogenic signals triggered by TGF-beta (70). In that study, a cell line over-expressing the IGF-1 receptor (C2-LISN) was developed. When this cell line was grown in low serum medium with both TGF-β and high concentration of IGF-I, C2-LISN failed to differentiate and grew to high density multilayer of cells. If TGF-β was removed from low serum medium, multilayered C2-LISN myoblasts differentiated rapidly. It was demonstrated that the IGF-1 receptor produces dual effects on myoblast proliferation and differentiation (70).

B. Transforming Growth Factor-β.

Large amounts of evidence from various muscle cell line such as L6A1, L6E9, C2C12 and BC3H1 have indicated that transforming growth factor β (TGF-β) is an inhibitor of myogenic differentiation. (71-73). These studies showed that TGF-β blocks all aspects of myogenic differentiation. Intracellularly, oncogenes might mediate the action of TGF-β. In BC3H1 cells, a considerable increase in *jun*B and a moderate increase in c-*jun* mRNA were observed in response to TGF-β (74). Besides inhibition of differentiation, TGF-β was also shown to induce myoblast differentiation, depending on cell culture conditions. Two types of TGF-β receptors are present in myoblasts, however, their exact function is not clear. To study which type of receptor is implicated in myogenesis, a truncated type II TGF-β receptor was over-expressed in C2C12 myoblasts. The non-functional receptor was a negative inhibitor of type II receptor signaling (75). The cells with a truncated receptor did not form myotubes, and MyoD and myogenin were not expressed after the onset of differentiation. Retrovirus-mediated expression of

MyoD in these cells rescued their ability to form myotubes *in vitro*, indicating that blockade of myoblast differentiation by the truncated TGF-β receptor may relate to decreased MyoD expression. This consequence suggests that type II receptor-mediated TGF-β signaling is required for myogenic differentiation (75).

C. Fibroblast growth factors.

Acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF) are the two major forms of FGF. bFGF is more widely distributed than aFGF (76). FGFs are effective stimulators of myoblast proliferation and inhibitors of myoblast differentiation *in vitro*. In terms of the potency of inhibiting differentiation, bFGF is much stronger than aFGF (77). J.C. Fox and his colleagues have observed that exogenously FGFs stimulate the amount of mRNAs encoding acidic and basic FGF in Sol 8 myoblasts by a paracrine mechanism, and both types of FGF suppress myogenin and MyoD expression in myoblasts (78). It is known that the abundance of endogenous aFGF and bFGF are decreased when cultured muscle cells undergo differentiation. To explore this mechanism in detail, Fox et al used antisense RNA to inhibit the endogenous production of FGF in Sol8 cell line. It was observed that the immature myogenic differentiation could be rescued by acidic FGF (79). These data indicated that endogenously produced FGF plays a crucial role in regulating the myogenic process. In a study examining the signal transduction system responsible for inhibiting myogenesis, FGF was found to activate phosphorylation of a conserved sequence in the DNA-binding domain of myogenin. It was indicated that PKC phosphorylates this motif both *in vivo* and *in vitro* (80). In a myogenin mutant lacking the PKC phosphorylation site, myogenesis was not repressed by FGF, establishing that this site is a target for FGF-dependent repression of skeletal muscle differentiation. The effect of MRF-4 phosphorylation status on its regulatory activity was also studied (81). MRF-4 can be phosphorylated by PKC and by PKA. Over-expression of either kinase inhibits MRF-4 activity and blocks differentiation. Phosphorylation of a specific threonine residue within the DNA-binding domain prevents MRF-4 from binding to specific DNA targets *in vitro*. However, *in vivo* studies showed that the phosphorylation status of threonine residues

does not play a major role in regulating MRF-4 activity. The direct modification of the MRF-4 protein by PKC or PKA may not contribute to the negative regulation of MRF-4 by FGF, indicating that negative regulation of MRF-4 by bFGF may not involve a direct modification of the protein at the identified PKA and PKC sites, but instead may involve the modification of specific co-regulators that interact with MRF-4 (81).

3.1 Basic mechanisms of alternative pre-mRNA splicing.

In the first section of this chapter, the diversity of Ca^{2+}-ATPases generated by multigene and pre-mRNAs processing was discussed. In this section, the mechanism of alternative pre-mRNA splicing and its implications in muscle development will be discussed. Before a primary mRNA becomes a mature RNA, it must undergo a series of processing events in the nucleus. These events usually comprise the addition of a 7-methyl guanosine to the 5' end of the pre-mRNA, as well as the addition of a poly(A) tail to the 3' end of the primary transcript. In addition, the introns of transcripts must be precisely cut out and the exons linked together to form the open reading frame. Generally, there are two basic patterns of pre-mRNA splicing: constitutive splicing and alternative splicing (82). In constitutive splicing, every exon of a gene primary product is spliced to generate a single mature mRNA. In alternative splicing, certain exons may be excluded from the mature mRNA transcript to generate various alternative mRNA products (therefore distinct protein isoforms) from a single gene. Different forms of alternative splicing have been observed and/or proposed. These include cassette exons, mutually exclusive exons, internal donor and acceptor sites and retained exons, among others. (1) Cassette exon: certain exons can be included or excluded dependent on different needs. High diversity can be produced when several cassette exons exist in the same gene, as in PMCA1 and PMCA4 (27, 32). (2) Internal donor and acceptor site: this machinery results in removal of introns of different length with variation in exon size, causing insertion of small alternative peptide segments, and shifting the reading frame. An example of this process can be found in Drosophila's transformer gene (83). (3) Retained intron: the introns can be cut or retained in the mature mRNA. The retained intron results in the insertion of a peptide segment if the open reading frame is

maintained (84). (4) Internal mutual exclusion of exons: one of two exon pairs is always join into the mature RNA, but never both; one of them is always cut out. Contractile proteins in skeletal muscle such as myosin light chain 1/3 are processed in this manner (85) (see **Figure 5**).

In mammalian cell-free systems, exogenous pre-mRNAs can be accurately spliced *in vitro*. This system provides a model to analyze the splicing reactions (86). Usually, pre-mRNA splicing involves two-steps: cleavage and ligation. Briefly, the pre-mRNA is cleaved at the 5' splice site to generate 5' donor splice site, and the guanosine residue at the 5' end of the intron is joined to an adenine residue near the 3' splice site to form a lariat configuration. The second step involves cleavage at the 3' splice site to form 3' splice acceptor site and is followed by ligation of the exons. The splicing reaction occurs within spliceosomes which consist of a pre-mRNA and a small nuclear ribonucleoprotein (snRNP) complex (87); spliceosomes also contain a number of non-snRNP splicing factors. SnRNPs, found in the nuclei of all eukaroytic cells, consist of one snRNA molecule, ranging in size from 56 to 217 nucleotides, and a set of about 10 different proteins (88). Their functions in the spliceosome have been partially identified. The U1 snRNP binds to the 5' splice site, while the U2 snRNP binds to 3' splice site (89).

3.2 Splice site selection.

In the study of constitutive splicing mechanisms and regulated alternative splicing, an essential feature that remains unresolved is how specific pairs of 5' and 3' splice sites are selected for the splicing reaction. Most pre-mRNAs contain multiple introns and exons, and there are numerous cryptic 5' and 3' splice sites throughout both introns and exons. When splicing proceeds, it is prerequisite that the cell must distinguish between the normal splice site and the cryptic sites. Characterization of both general splicing factor 2 (SF2) and the alternative splicing factor (ASF) established the mechanism for this process (90,91). SF2, which binds nonspecifically to RNA, has an activity required for the first step (cleavage) in the splicing reaction. It is necessary for the assembly and stabilization of spliceosomes and promotes the annealing of complementary RNA (92). It has been reported that changes in the concentration of this

general splicing factor strongly influence 5' splice site selection. Thus, SF2 may function not only in the cleavage-ligation reactions, but also in directing or modulating splice site selection.

3.3 Alternative splicing of pre-mRNAs for Ca^{2+} -ATPases.

In recent years, a large body of evidence on development-dependent splicing of Ca^{2+}-ATPases has accumulated. This information may provide mechanistic insights of how alternative splicing may modulate the functional switch between distinct isoforms of Ca^{2+} pumps (see **Figures 6** and **7**). R. Casteels and coworkers have completed a comprehensive study in this area. First, they found undifferentiated BC3H1 myoblasts only express the SERCA2b isoform, while differentiated myocytes primarily contain the SERCA2a isoform. The isoform switch was detected by immunoblot analysis with isoform-specific antibodies. Functionally, the induction of SERCA2a after differentiation resulted in the decreased sensitivity of the Ca^{2+} uptake in permeabilized cells to the Ca^{2+}pump inhibitor thapsigargin, which indicated that the transport capability of the pump is enhanced after myogenesis *in vivo* (96). They also noticed that the change from non-muscle to muscle-specific RNA processing for both the PMCA1 and SERCA2 genes was linked to myogenic conversion of the BC3H1 cells. Their results showed that myogenic RNA processing could be reversed for both types of Ca^{2+}pumps. The protein synthesis inhibitor cycloheximide rapidly down-regulated the expression of the PMCA1 and SERCA2 muscle-specific messenger (97). More recently, they made a minigene (pCMβSERCA2) containing the 3' end of the SERCA2 gene to define the cis elements involved in differential processing. When this chimeric plasmid was stably transfected into BC3H1 cells, the pre-mRNA from this minigene was differentially processed depending on the differentiation state of the cells. The pCMβSERCA2 minigene and a myogenin expression vector were co-transfected into C3H/10T1/2 cell line to induce muscle-specific splicing of transcripts from pCMβSERCA2. The results indicated that trans-acting factors responsible for muscle-specific processing could be induced by one or more of the myogenic regulatory factors such as myogenin (98).

Hammes et al used two different muscle-derived clonal cell lines, L6 and H9c2

(2-1), which originated from skeletal and cardiac muscle, respectively, and one neuronal cell line PC-12, to investigate the expression of isoform-specific mRNAs of PMCA in muscle and neuronal cells during differentiation (99). They found, in addition to the PMCA1b and PMCA4b house-keeping isoforms, that myogenic differentiation of these two cells could induce the novel expression of the splicing variants 1c, 1d and 4a. Differentiation of the neuronal PC-12 cells could induce a comparable isoform subtype switch. The isoform-specific mRNAs 1c, 1d and 4a were only expressed in excitable cells. To resolve whether myogenic determination factors could direct the expression of a differentiation-specific PMCA mRNA pattern, they transferred a myogenin expression vector into rat fibroblast. They found the fibroblast was converted into multinucleated myotubes, meanwhile, isoform-specific mRNAs 1c, 1d, and 4a were expressed in the newly generated muscle cells.

In Dr. A. Zarain-Herzberg's laboratory, RT-PCR and cDNA cloning techniques were used to study the expression of the various PMCA and SERCA mRNAs in fetal and adult human heart (26). PMCA1 and PMCA4 were expressed at very early stages of fetal heart formation as well in the adult heart. PMCA2 and PMCA3 mRNA were expressed at low levels or not detected in fetal heart. SERCA2a, SERCA2b and SERCA3 mRNA expression could be detected at all cardiac developmental stages. Furthermore, our group confirmed a novel tissue specific variant of the PMCA4 gene expressed in both fetal and adult human heart but not in placenta. This novel variant corresponds to PMCA1d in term of DNA sequence, therefore, it was named PMCA4d (26).

Neyses et al studied the effect of increased PMCA function on differentiation process (100). In that study, the human PMCA4b isoform under the control of a CMV promoter was stably over-expressed in L6 cells to generate a new cell line L6-PMCA. Enhanced expression and localization of the pump was demonstrated by Western blotting and immunofluorescence. Over-expression of PMCA promoted differentiation assessed by measuring creatine kinase activity, the number of nuclei in myotubes and a panel of differentiation-specific genes. In the highly expressing clone, the differentiation rate could be accelerated 5-fold. Differentiation was not altered in clones where PMCA4b gene expression was silenced. In a functional assay, Ca^{2+} concentration in the cytosol was

markedly reduced in L6-PMCA and the ionophore A23187 partly reversed the effect on differentiation.

Combining these data with other observations, it is reasonable to assume that differential expression or preferentially pre-mRNA processing of Ca^{2+}-ATPase can be activated during differentiation. Consequently, promoting the expression of certain PMCA isoforms may increase the rate of muscle cell differentiation, leading to the formation of a positive feedback pathway that contributes to the irreversible process of differentiation during *in vitro* myogenesis.

Taken together the accumulated scientific evidence obtained in other laboratories as well as in ours, led us to speculate that Ca^{2+}-ATPase isoform induction and /or switching is likely under the control of myogenic determination factors such as MyoD and myogenin. To examine this hypothesis, we first quantitatively assessed the changes of mRNA expression pattern of SR and SL Ca^{2+}-ATPase during skeletal muscle differentiation *in vitro*. Then, myogenesis *in vitro* was promoted or suppressed by addition of 5-azacytidine and BUdR, reagents which can affect endogenous MyoD expression. Finally, a MyoD-transfected cell line, 10RMD, was used to further understand the relationship between this regulatory factor and the mRNA expression of the SR and SL Ca^{2+}-ATPase isoforms.

II. MATERIALS AND METHODS

1. Cell lines.

The following cell lines were used for this study: C2C12 mouse fast-twitch skeletal muscle myoblast (101), Sol8 mouse slow-twitch skeletal muscle myoblast (102), C3H /10T1/2 mouse embryo fibroblast (103), purchased from the American Type Culture Collection, ATCC, Rockville, MD. The L6 rat skeletal muscle myoblast cell line (104) was kindly provided by Dr. Theodore Y.C. Lo, Department of Biochemistry, University of Western Ontario, London. The 10 RMD cell line (C3H/10T1/2 stably transfected with MyoD) was a generous gift from Dr. Andrew Lassar, Harvard Medical School, U.S.A.

2. Induction of muscle differentiation *in vitro*.

The cell lines were grown on 90 mm culture dishes in Dulbecco's modified Eagle's medium (Gibco BRL, Canada) with 15 % (v/v) heat-inactivated (56°C for 30 min) fetal bovine serum (HyClone, Logan, Utah or Gibco BRL, Canada).

To induce differentiation of the C2C12, Sol8 and L6 cells, the myoblasts were grown until approximately 70-80% confluent, then the cells were rinsed twice with DMEM, and the media was replaced with 15 ml of DMEM supplemented with 3 % heat-inactivated horse serum. Under these conditions, about 80 % to 90 % of the cells differentiated into multinucleated myotubes within 2-7 days. C2C12 and Sol8 cells usually begin to fuse into multinucleated myotubes after switching to the differentiation medium in 1-2 days, and become mature myotubes in 5-7 days. L6 cells take relative longer time to complete the phenotypic change (forming myotubes after 2-4 days). For

the 10T1/2 and stably transfected MyoD cell line (10RMD) the conditions were the same as for the C2C12 and Sol8 cells.

3. Induction of myogenesis with 5-azacytidine.

The drug 5-azacytidine (AZA) is a S-triazine nucleotide analog of cytidine with a broad spectrum of biological effects. It is useful for the treatment of leukemia and is capable of inducing the oncogenic transformation of embryonic cell lines (105). Furthermore, it has been shown that AZA can induce the differentiation of mouse embryonic C3H/10T1/2CL8 fibroblasts to myoblasts or myotubes, adipocytes and chondrocytes (106). The phenotypic conversion aroused by AZA was attributed to demethylation or hypomethylation of specific gene loci that activate regulatory genes such as the MyoD gene (41). To examine the effect of AZA on Ca^{2+}-ATPase gene expression during L6 cell differentiation, the cells were plated in growth media until they reached 70 to 80% confluence, then switched to differentiation media (DMEM+3% HS) containing 3 μM AZA. After 24 h of exposure to AZA, the cultures were washed with DMEM and maintained in differentiation media without AZA for 10 days, with media changes every 2 days. In a parallel experiment, another group of L6 cells were plated under the same conditions without AZA treatment and used as control.

4. Inhibition of myogenesis with 5-bromo-2'-deoxyuridine.

Previous experimental work has provided evidence that the pyrimidine analog 5-bromo-2'-deoxyuridine (BUdR) can be incorporated into DNA by competing with deoxy-thymidine, consequently decreasing the expression of MyoD, myogenin and blocking muscle differentiation (107,108). To study the effect of BUdR-dependent suppression of

the expression of the mRNA transcripts for the myogenic determination factors MyoD and myogenin as well as Ca^{2+}-transport APTases, C2C12 cells were seeded at low cell density (~20,000 cells per 90 mm petri dish) in growth medium (DMEM) supplemented with 15 % FBS and 10 μM deoxycytidine with or without the addition of 5 or 50 μM BUdR. Deoxycytidine was used to promote the incorporation of BUdR into DNA. After 2 or 4 days, a time sufficient for most of the cells to have incorporated BUdR into their DNA, parallel plates of cells were harvested for RNA isolation and analysis or were switched to differentiation medium (DMEM supplemented with 3% horse serum and 10 μM deoxycytidine) for an additional 4 days. The concentration of BUdR supplemented was maintained by adding the reagent every two days.

5. Isolation of total cellular RNA.

Total cellular RNA was isolated using the guanidine thiocyanate procedure of Chomczynski and Sacchi (109). Briefly, culture medium was removed from the plates by aspiration, the monolayers were washed twice with 5 ml of ice-cold phosphate-buffered saline (PBS) (100 mM NaCl, 50 mM Tris-HCl and 1 mM EDTA), the dishes were placed on ice and 5 ml of cold working solution D (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M β-mercaptoethanol) was added. Then 0.5 ml of 2 M sodium acetate (pH = 4.5) was added per 90 mm petri dish. The cells were lysed by pipeting up and down three times and the viscous lysate was transferred to a 15 ml sterile tube. Five milliliter of DEPC-treated water-saturated phenol and 1 ml of chloroform:isoamyl alcohol mixture (48:1) were then added and the sample was vortexed for 30 sec. The emulsion was centrifuged at 4,000 rpm for 20 min at 4°C to separate the aqueous and

The upper, aqueous phase containing total RNA was transferred to a new sterile tube without taking any lower phase and the above procedure was repeated once. The aqueous phase was mixed with 1.0 volume of cold chloroform:isoamyl mixture, vortexed 30 sec and centrifuged at 4,000 rpm for 10 min at 4°C. The total RNA in the aqueous phase was precipitated with isopropyl alcohol (1:1 volume) for 2 hours at 0°C and recovered by centrifuging at 7,000 rpm for 30 min. The total RNA pellet was washed twice with 75 % ethanol. The RNA pellet was resuspended in 75 mM sodium acetate pH 7.0. To quantify RNA concentration, the absorbance of the sample was measured at 260 nm and 280 nm using a Model Spectronic 1001 Plus spectrophotometer (Milton Roy, U.S.A.). The ratio between the absorbance at 260 and 280 nm provides the estimate of the purity of total RNA, with pure preparations of RNA having an 260/280 ratio between 1.7 and 2.0. The amount of RNA was calculated by a formula derived from the extinction coefficient of RNA where one absorbance unit at 260 nm equals 40 µg /ml RNA. The integrity and purity of the total RNA was verified by electrophoresis on 1% agarose-formadehyde gels (agarose dissolved in 20 mM MOPS, 1mM EDTA, 2.2 M formaldehyde). Aliquots of RNA were mixed with 20 µl loading buffer (50% formamide, 6% formaldehyde, 2 mM EDTA, 6% glycerol, 0.1% SDS, 0.25% bromophenol blue and 0.25% xylene cyanol FF), incubated at 65°C for 15 min and allowed to cool before loading. The composition of the running buffer was 1x MOPS/EDTA (20 mM MOPS, 1mM EDTA) buffer and 1.8% formaldehyde. The conditions for electrophoresis were 3-5 V/cm, current 10 mA at 4°C for 12 to 15 hours. At the end of run, the agarose gel was immersed in water containing ethidium bromide (0.5 µg/ml) to stain for 30-45 minutes.

A Polaroid photograph of the gel was taken and the densitometric data of the 28S and 18S rRNA were collected using a Hewlett-Packard ScanJet II scanner, DeskScan MacIntosh software and quantified using the SoftScan densitometric MacIntosh analysis program (BioSoft, U.K.). The densitometric values of the rRNAs were used to calculate the 28S/18S as an index of integrity and quality of the RNA.

6. Reverse transcription of total RNA.

First-strand cDNA synthesis was performed using reverse transcriptase as follows: two micrograms of total RNA (in a volume of 4 µl) were annealed to 40 pmol of random hexamer synthetic oligonucleotides (Gibco BRL, Canada) in 1 µl of water at 65°C for 5 min and then slowly allowed to cool to room temperature. The reverse transcription reaction was carried out in a final volume of 20 µl in the presence of 1 X PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 200 units of MMLV-reverse transcriptase (Gibco BRL, Canada), 10 mM each of dATP, dCTP, dTTP and dGTP (Boehringer Mannheim, Germany), 10 units of RNase inhibitor (Gibco BRL, Canada), and 5 mM $MgCl_2$. The reaction was carried out at 42°C for 30 min, then the samples were stored at -20 °C until used for PCR.

7. Polymerase chain reaction protocol

A titration curve was prepared for each sample of reverse transcribed RNA with all PCR primer sets. The concentration of cDNA that fit the linear range for amplification of the polymerase chain reaction product was used to perform a semi-quantitative analysis of expression of the genes of interest.

Briefly, aliquots of the first strand cDNA samples were subjected to a PCR amplification in a final volume of 45 µl of a reaction mixture containing the following: 1 X PCR buffer (Gibco BRL, Canada), 0.2 mM of each dNTP (Boehringer Mannheim, Germany), 2 mM $MgCl_2$ and 1.25 units of Taq DNA polymerase (Gibco BRL, Canada). The reaction mixture was covered with two drops of silicon oil (about 40 µl), heated at 82°C for 1 minute to perform a "hot-start" reaction and 5 µl of specific PCR primer pairs (forward/reverse) were added (see **Table1**). The amplification reaction was performed over 35 cycles of incubations at 94 °C for 45 sec, 55 °C for 45 sec, and 72 °C for 2 min. The last amplification cycle was followed by a final extension step at 72 °C for 10 min. The PCR was carried out in a thermal cycler Model SCS-2 (Stratagene, CA, U.S.A.). The PCR products were fractionated by electrophoresis in 2.0 % agarose gels containing 40 mM Tris-acetate, 1 mM EDTA and 0.5 µg/ml ethidium bromide. A 100 bp DNA ladder (Gibco BRL, Canada) was used to determine the sizes of PCR products after electrophresis.

A Polaroid photograph of the gel was taken and densitometric data of the DNA amplification was collected using a Hewlett-Packard ScanJet II scanner and DeskScan MacIntosh software, and quantified using the SoftScan densitometric MacIntosh analysis program (BioSoft, U.K.). The densitometric values obtained for GAPDH message were used to normalize the values for each specific transcript and correct for reaction loading errors.

Table 1. Information of primers used for PCR experiments

Primer sequence	Primer position (nt)	Message		PCR product size (bp)		Ref.
5'-CCTGCCGATGATCTTCAAGCTC -3'	3021-3042	(SERCA1a,b	F)	SERCA1a	345	12
5'-TTACAAGGTTCAGAGGGAGAGCAG -3'	3365-3342	(SERCA1a,b	R)	SERCA1b	303	
5 -CCATAGAGATGTGCAATGCCCCTC-3'	2921-2943	(SERCA2a,b	F)	SERCA2a		
5- TTCTGCAATGTTTAGGAAGCGG-3'	3223- 3202	(SERCA2a	R)	SERCA2a	303	119
5'-ACCTCCATCACCAGCCAGTATG-3'	3408-3387	(SERCA2b	R)	SERCA2b	530	110
5'-GAGATCCCTGAGGAGGAATTGG-3'	3502-3523	(PMCA1b/c	F)			
5'-TGTGCGGCTCTGAATCTTCTATC-3'	3893-3871	(PMCA1b/c	R)	PMCA1a	392	34
				PMCA1b	238	
				PMCA1c	325	
5'-TGAGATTGACCATGGAGATG-3'	629-650	(PMCA4b/c	F)			
5'-AACAGTTTCAGCATCCGACAGG-3'	1083-1062	(PMCA4b/c	R)	PMCA4a	455	38
				PMCA4b	277	
				PMCA4d	385	
5'-GCTCTGATGGCATGATGGATTAC-3'	773-795	(MyoD	F)			
5'-CTGTTCTGTGTCGCTTAGGGATG-3'	1035-1013	(MyoD	R)	MyoD	262	61
5'-TGGTGCCCAGTGAATGCAAC-3'	513-532	(Myogenin	F)			
5'-AAAGCCCCCTGCTACAGAAGTG-3'	796-775	(Myogenin	R)	Myogenin	283	62
5'-TGAAGGTCGGAGTCAACGGATTTGGT-3'	71-96	(GAPDH	F)			
5'-CATGTGGGCCATGAGGTCCACCAC-3'	1030-1053	(GAPDH	R)	GAPDH	982	37

III. RESULTS

The main goal of this thesis is to examine the expression of the sarcoplasmic reticulum (SR) and sarcolemmal (SL) Ca^{2+}-ATPase genes during skeletal muscle cell differentiation *in vitro*. We examined the relationship between the mRNA expression of SR and SL Ca^{2+}-ATPases with those of the myogenic regulatory factors MyoD and myogenin in order to assess whether MyoD or myogenin could influence Ca^{2+}-pump gene transcription and/or post-transcriptional processing (splicing) of the primary transcripts. Three different myogenic cell lines, C2C12, Sol8 and L6, and a fibroblast cell line (10T1/2), stably transfected with MyoD cDNA (10RMD) and over-expressing this protein, were used for this study. We decided to compare the temporal pattern of mRNA expression for the SR and SL Ca^{2+}-ATPase isoforms in the various skeletal muscle cell lines to elucidate which Ca^{2+}-pumps are expressed at different stages of muscle differentiation. We also decided to study the possible role of the myogenic regulatory factors MyoD and myogenin on the expression of the Ca^{2+}-pump genes. The conditions for growth and differentiation of the cell lines in culture are described in the "Materials and Methods" section. In order to analyze the mRNA expression of the above mentioned genes, the total RNA of cells in culture was isolated and first-strand cDNA was prepared from these three cell lines at different stages of myogenic differentiation. We developed a semi-quantitative RT-PCR technique to analyze the mRNA expression of the SERCA and the PMCA isoforms, as well as of the myogenin and MyoD muscle-specific transcription factors and performed the procedure as described under "Materials and Methods". Densitometric analysis of the amplified PCR products was used to quantify the relative level of these transcripts. The densitometric values for the Ca^{2+} pumps and myogenic factors were normalized using the corresponding values of the constitutively expressed enzyme GAPDH. The time course plots presented in **Figures 15,16, 20, 21, 25 and 26** were from at least two independent experiments conducted in duplicate. The

values were expressed as the mean +/- S.E. We selected the DNA sequence for the primer pairs for PCR amplification from published cDNA sequences using the MacVector software DNA and protein analysis package version 5.0.2 (Oxford Molecular Group). The selected primer pairs were analyzed for specificity by comparing the primer sequences to the entire GeneBank data using the BLAST software (NCBI). The DNA primers were synthesized using a Beckman 1000 oligonucleotide synthesizer. **Table 1** lists the primers used for this work.

1. Sarcoplasmic Reticulum Ca $^{2+}$-ATPases.

Previous reports demonstrated that myogenic differentiation of BC3H1 and C2C12 cells in culture induced a pronounced change in the expression of the SERCA2 mRNA (96,110). It has also been reported that the pattern of SERCA2 mRNA expression was altered during *in vitro* differentiation of the C2C12 cell line. In C2C12 myoblasts only the SERCA2b (non-muscle isoform) mRNA was detected. However, after 36 hrs in differentiation media the early myotubes began to express the SERCA2a (cardiac/slow isoform) which increased 4-5 fold during myotube maturation. In contrast, the SERCA2b continued to be expressed at low levels both in undifferentiated and differentiated cells (110). In this thesis work, we investigated the mRNA expression for the SERCA2a and SERCA2b isoforms by semi-quantitative RT-PCR. It should be noted that the only difference between the nucleotide sequence of SERCA2a and SERCA2b mRNAs occurs in the 3' region of the message as result of alternative splicing (see **Figure 6**). The translation of these two mRNAs results in two proteins that differ exclusively in their carboxyl terminal region where the last four amino acids of SERCA2a are replaced by a tail of 49 extra amino acids present SERCA2b isoform (see **Figure 3A** and **B**)(119,120). The forward primer for SERCA2a and 2b is located at position 2921 bp to 2943 bp of the coding cDNA strand (see **Table 1**) (119). The reverse primer for SERCA2a, was

complementary to the sequence at position 3202 bp to 3223 bp and yields an expected product of 303 bp (119). The SERCA2b reverse primer was designed using the sequence derived from the rabbit SERCA2b cDNA between position 3408 bp to 3387 bp (110). The expected size of the SERCA2b product is 530 bp.

The results of the PCR analysis to determine the temporal mRNA levels of SERCA2 in C2C12 cells is shown in **Figure 9.** For the SERCA2a isoform (303 nt product), the mRNA expression in myoblasts was low and according to densitometric data SERCA2a mRNA level was only about 20% of SERCA2b level. In contrast, the level of SERCA2a mRNA increased steadily each day during myotube differentiation, reaching five-fold higher levels in mature myotubes. For the SERCA2b isoform (530 bp product), the mRNA level decreased over 2 days of differentiation, then increased slightly at late stages of differentiation. **Figure 10** shows the SERCA2a and SERCA2b mRNA expression level in Sol8 cells. As seen in C2C12 cells, the SERCA2a mRNA was increased 249% from day 0 to day 4 after *in vitro* differentiation in Sol8 cells, whereas the SERCA2b mRNA showed the constant expression. It was evident that the total level of SERCA2 mRNAs (SERCA2a + SERCA2b) was increased 52%. In **Figure 11** the SERCA2a and 2b mRNA expression is shown for L6 cells. The mRNA for isoform SERCA2a was not detected in myoblasts and at early stages of differentiation (day 0 and day 1) but was increased after day 3 of differentiation; whereas SERCA2b mRNA was expressed in myoblasts and was decreased by 55% at day 3 and 27% at day 5, respectively.

The SERCA1 gene encodes two Ca^{2+}-ATPase isoforms expressed in the sarcoplasmic reticulum of fast-twitch skeletal muscle cells (6). Brandl et al first discovered that fast-twitch Ca^{2+}-ATPase transcripts are developmentally regulated by alternative splicing, where a 42-base pair exon is retained in the adult transcript (SERCA1a) but is excised in the neonatal transcript (SERCA1b) (6).

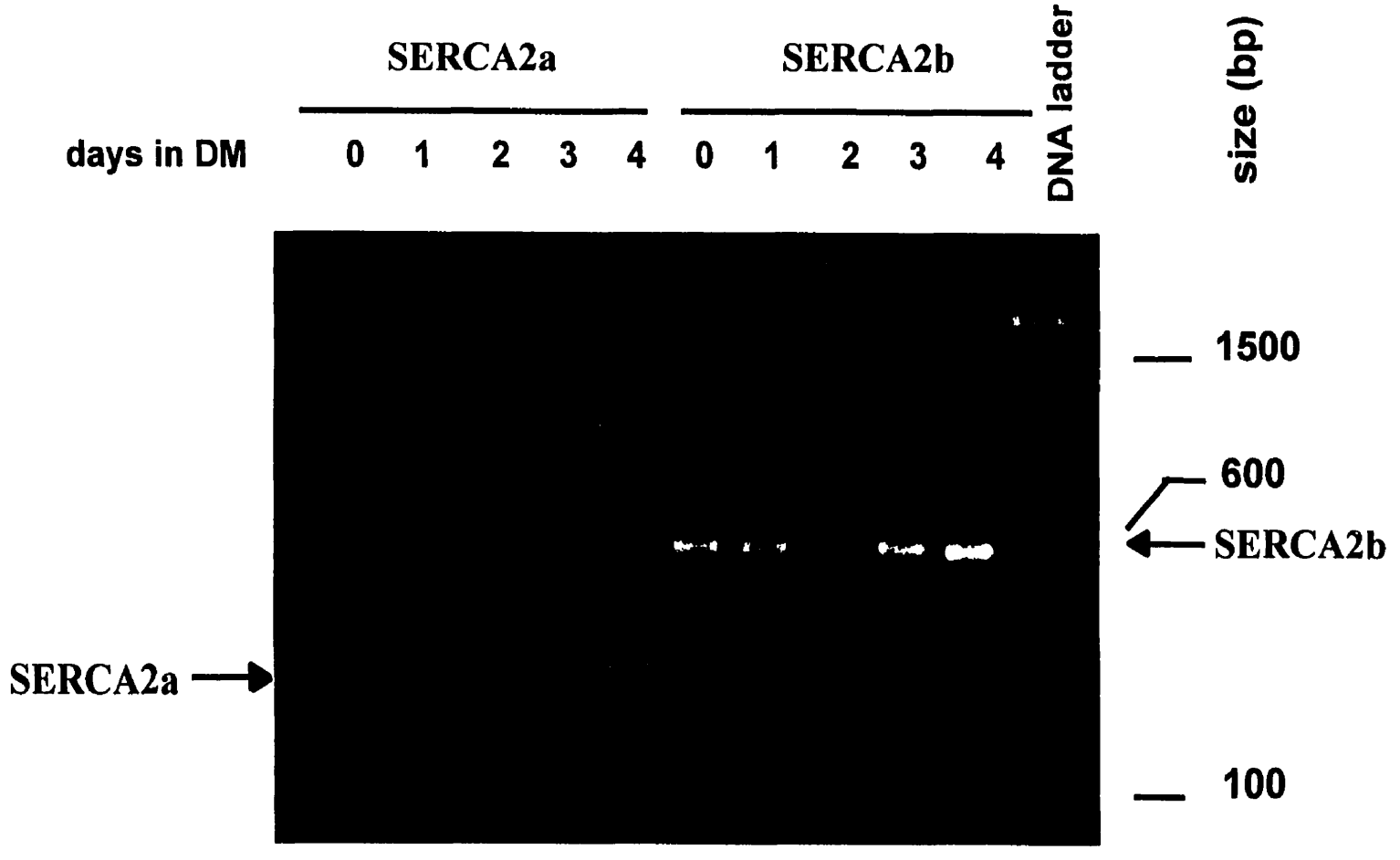

Figure 9. RT-PCR analysis of the expression pattern of SERCA2a and SERCA2b isoforms during myogenic differentiation in the C2C12 cell line. Semi-confluent C2C12 myoblasts were switched to 3% horse serum/DMEM and total RNA isolated at the indicated times, random hexamer-primed cDNA prepared from RNA, PCR amplification was performed using specific primers listed in Table 1. DM: differentiation medium.

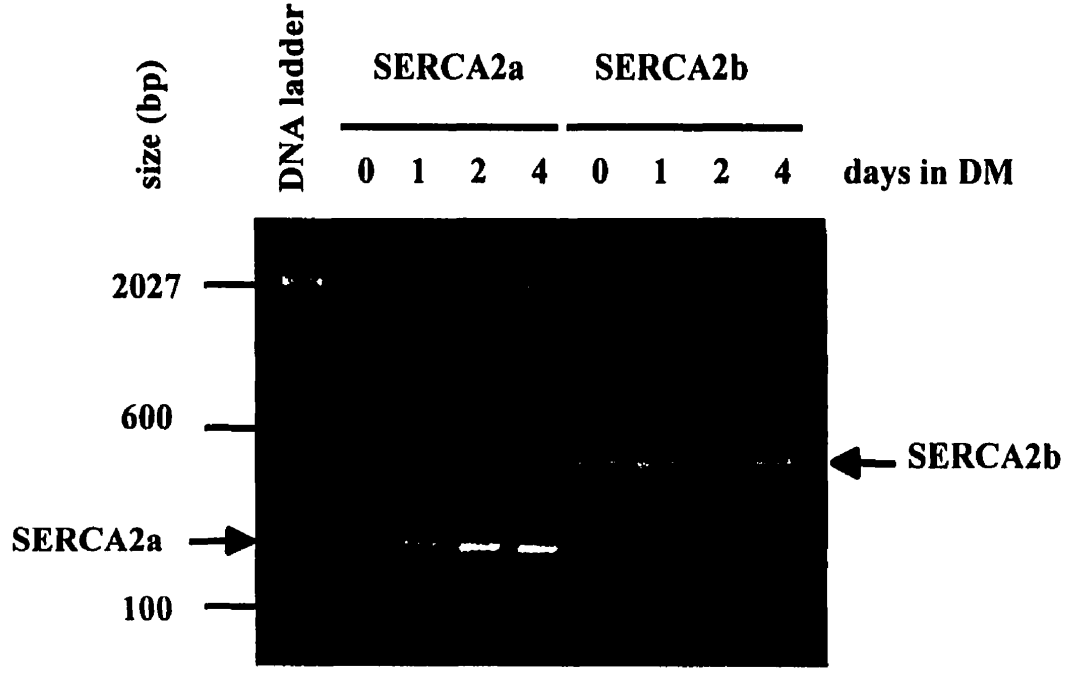

Figure 10. RT-PCR analysis of the expression pattern of SERCA2a and SERCA2b isoforms during myogenic differentiation in the Sol8 cells. The myoblasts were proliferated to 70% confluence, then switched to 3% horse serum/DMEM and total RNA was isolated at the indicated times, random hexamer-primed cDNA was prepared from RNA and PCR amplification was performed using specific primers listed in Table 1. DM: differentiation medium.

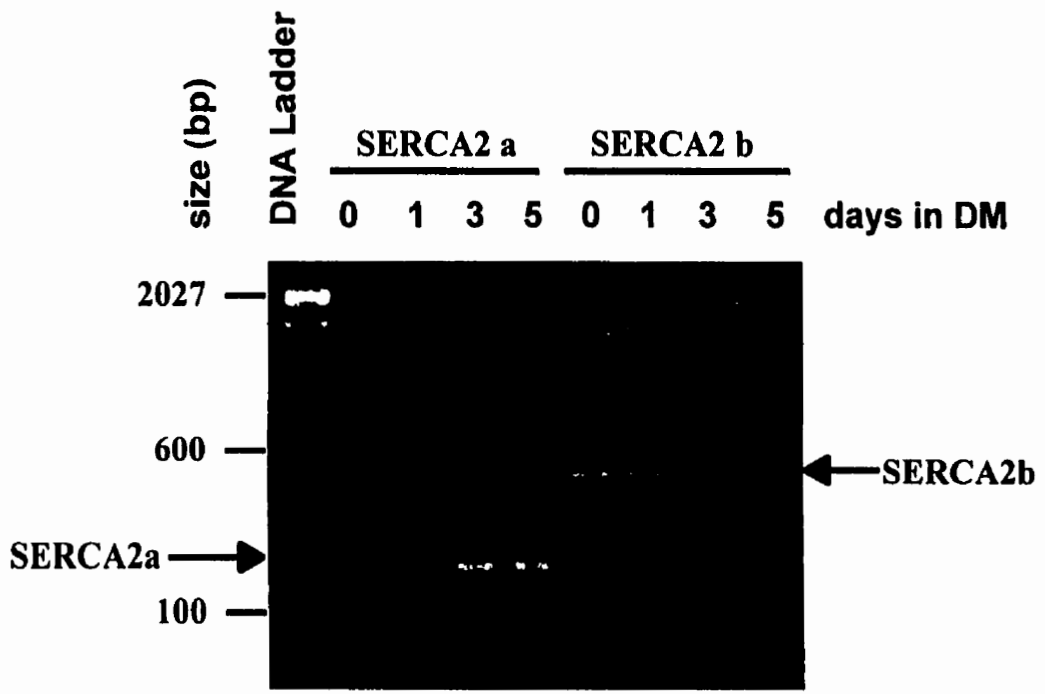

Figure 11. RT-PCR analysis of the expression pattern of SERCA2a and SERCA2b isoforms in L6 cell line. The cells were cultured in growth and differentiation medium. RT-PCR amplification was performed using specific primers listed in Table 1.
DM: differentiation medium.

Later on, Korczak et al isolated and characterized the genomic DNA encoding the fast-twitch isoform of the Ca^{2+}-ATPase of rabbit sarcoplasmic reticulum and discovered an alternative spliced exon responsible for the developmental expression of the SERCA1a and b isoforms (7). In order to specifically detect SERCA1a and SERCA1b mRNA isoform expression, we designed a forward primer for PCR analysis complementary to position 3021 bp to 3042 bp of the SERCA1 mRNA transcripts. The backward primer was complementary to the 3342 bp to 3365 bp region of the SERCA1a cDNA (6). Therefore, this primer pair covers the alternatively spliced exon (3146-3187 bp) which is excised in the neonatal isoform SERCA1b. **Figure 12** shows the SERCA1 mRNA isoform mRNA expression during differentiation of the C2C12 cell line. Isoform SERCA1b (303 bp product) was barely detected in the myoblast stage and was increased in later stages of differentiation (day 5). Isoform SERCA1a (345 bp product) expression was not detected in myoblasts and only slightly detected in mature myotubes. SERCA1 mRNA expression in the L6 cell line was also measured (see **Figure 13**); the neonatal isoform SERCA1b (303 bp product) appeared after 3 days of myogenic differentiation, whereas the SERCA1a mRNA (345 bp product) was detected only at day 9 of myotube maturation. These results demonstrate that the SERCA1 gene is exclusively expressed in differentiated skeletal muscle cells and that the splicing machinery of mature myotubes shifts the equilibrium towards the processing of the adult SERCA1a isoform. **Figures 14 and 15** show the time course after densitometric analysis of SERCA2a and SERCA2b expression in C2C12, Sol8 and L6 cell lines. From these results it was concluded that SERCA2a mRNA expression was increased more than 2-fold in all of the cell lines studied, although the time-dependent expression of SERCA2a varies depending of the cell line. For comparative purposes, the expression of GAPDH mRNA during C2C12 and Sol8 cells differentiation was quantified (see **Figure 16**). The densitometric data showed that GAPDH mRNA was expressed at constant levels during the different stages of myogenic differentiation.

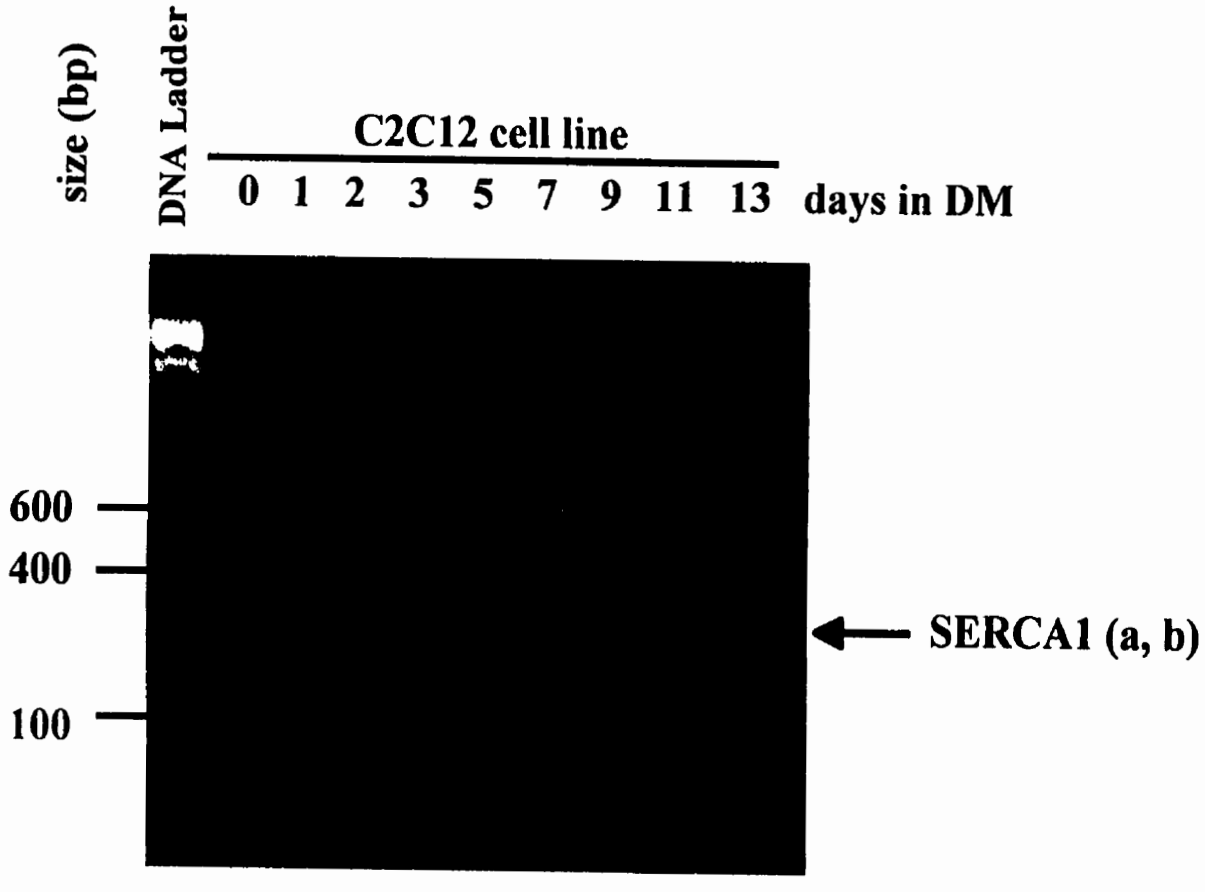

Figure 12. RT-PCR analysis of SERCA1 gene expression in C2C12 cells Total RNA from cultured cells was isolated and RT-PCR was conducted as described in "Materials and Methods". DM: differentiation medium.

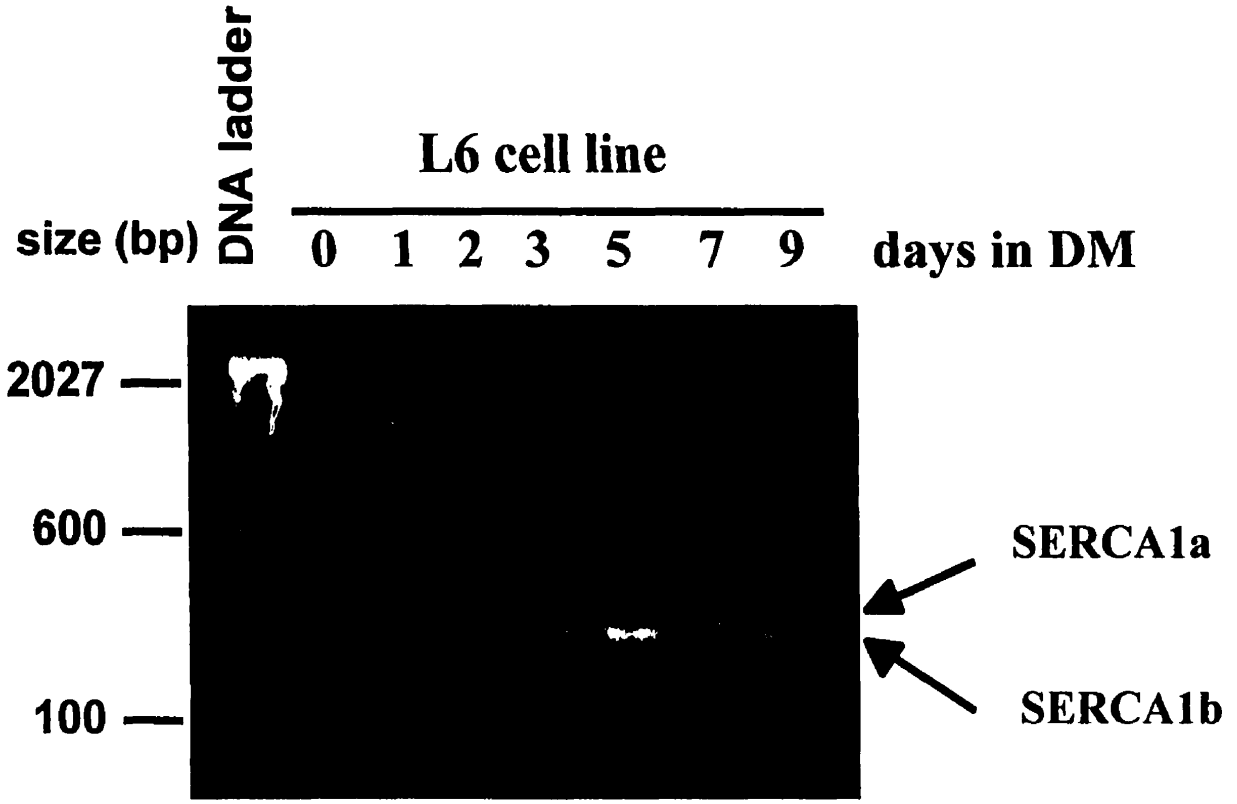

Figure 13. RT-PCR analysis of SERCA1 mRNA expression in L6 cell line. Total RNA from cultured cells was isolated and RT-PCR was conducted as described in the text, DM: differentiation medium.

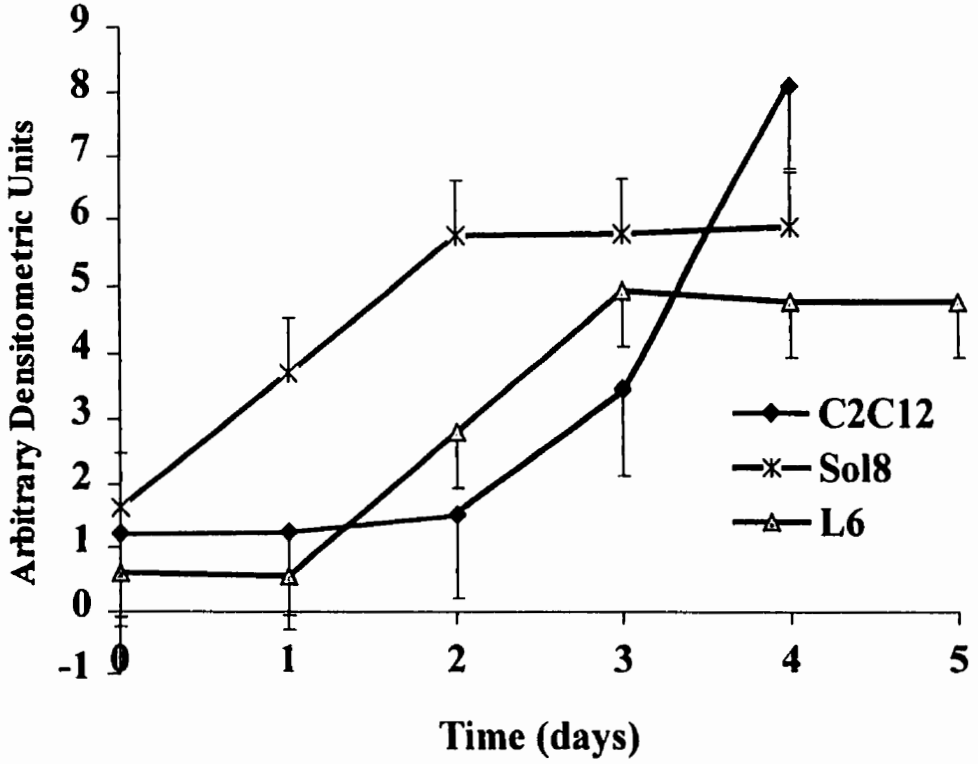

Figure 14. Time course of SERCA2a mRNA expression during C2C12, Sol8 and L6 cells myogenic differentiation. The myoblasts were induced to differentiate in DMEM containing 3% horse serum. Cells were harvested for RNA at the indicated times. Specific mRNAs were amplified by RT-PCR as described under "Materials and Methods". Each amplification products was quantified by densitometric scanning relative to a GAPDH control. Values represent the mean ± S.E. of three experiments with assays conducted in duplicate.

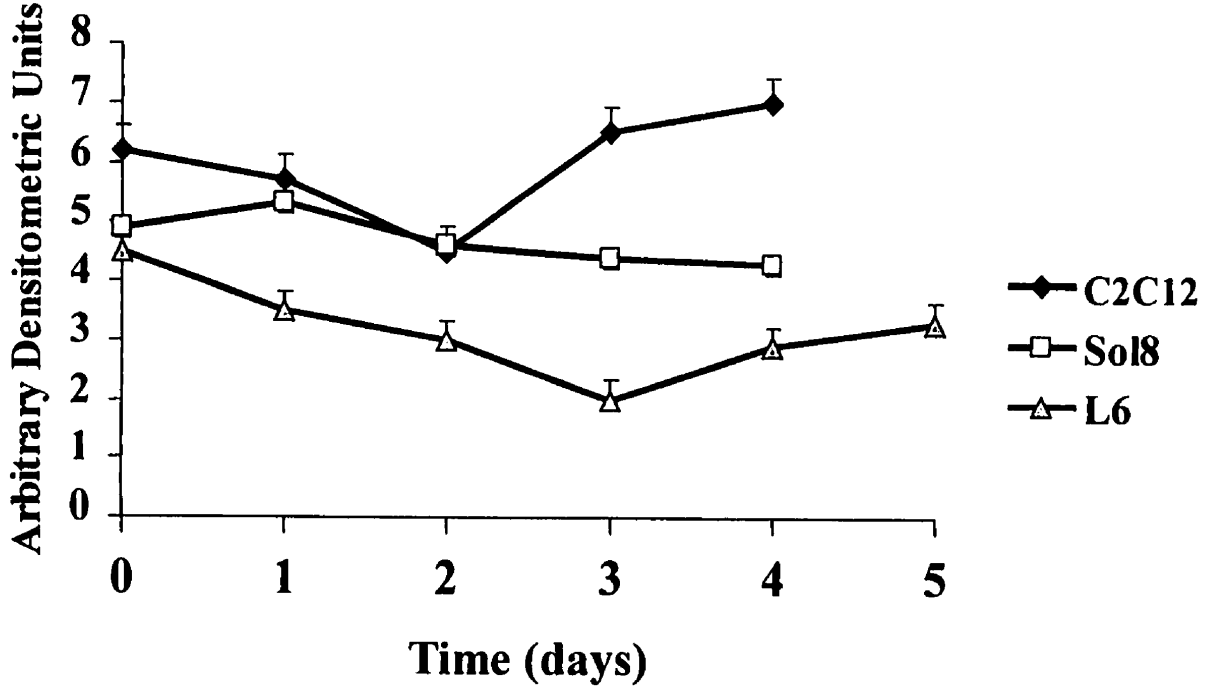

Figure 15. Time course of SERCA2b mRNA expression during C2C12, Sol8 and L6 cells myogenic differentiation. The myoblasts were induced to differentiate in DMEM containing 3% horse serum. Cells were harvested for RNA at the indicated times. Specific mRNAs were amplified by RT-PCR as described under "Materials and Methods". Each amplification products was quantified by densitometric scanning relative to a GAPDH control. Values represent the mean ± S.E. of three experiments with assays conducted in duplicate.

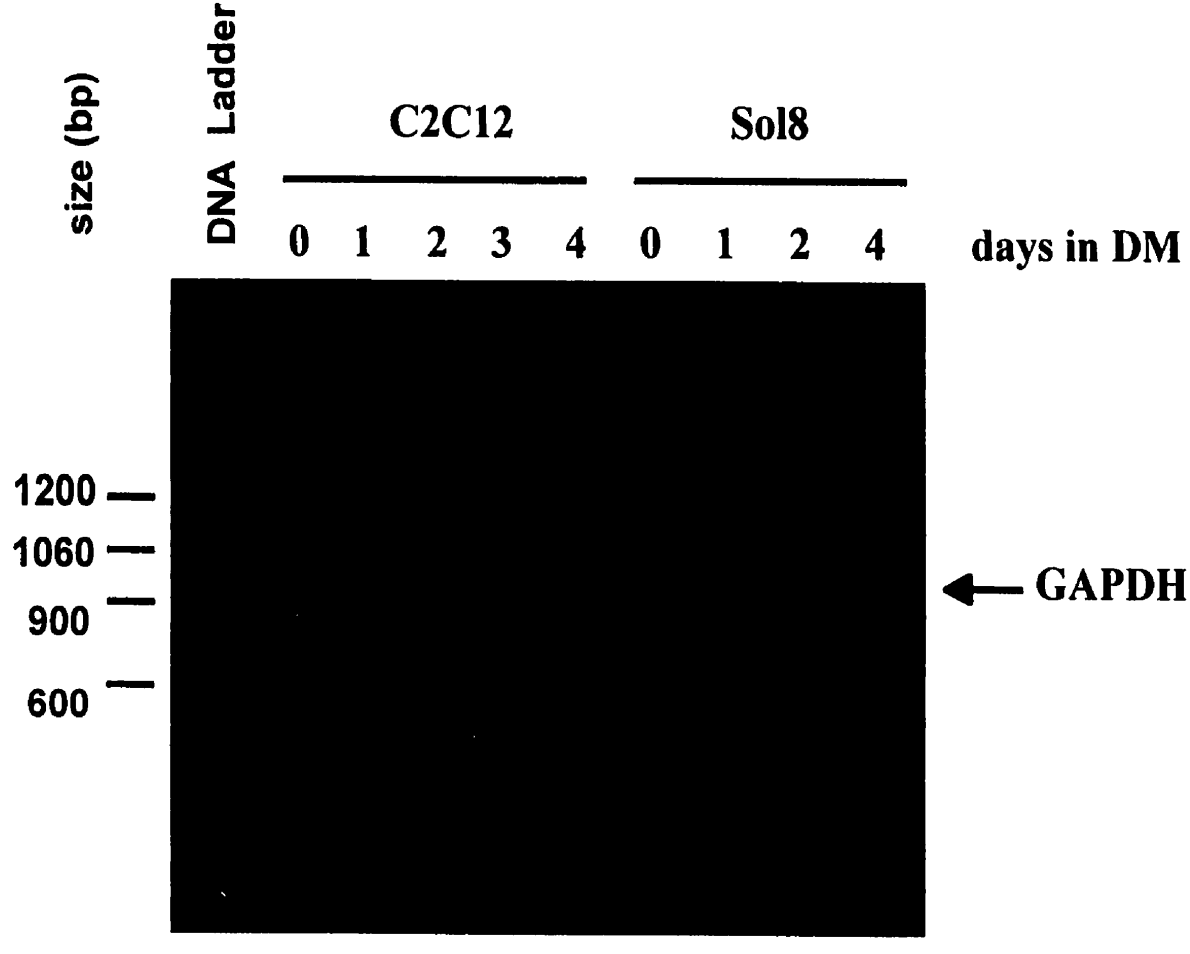

Figure 16. **RT-PCR analysis of mRNA expression of GAPDH during C2C12 and Sol8 cell differentiation.** PCR amplification was performed using the primers listed in Table 1. DM: differentiation medium.

2. Sarcolemmal Ca $^{2+}$-ATPases.

There is increasing evidence to suggest that the isoform diversity of plasma membrane Ca^{2+}-ATPases (PMCA) is regulated by splicing of the primary mRNA transcripts (see **"Literature Review"** and **Figure** 7). However, little is known regarding splicing of PMCA mRNAs at splice site A in striated muscles (26) and other tissues (24-27). The only isoforms described are those produced by splicing at site B/C of the pre-mRNA transcripts; therefore, in this thesis work we limited the analysis of PMCA isoform expression to the splicing at site B/C of the PMCA mRNAs. **Figure 17** shows the PMCA1 and PMCA4 isoform-specific mRNA expression pattern in C2C12 myoblasts and myotubes. For PMCA1, the specific pair of primers for the rat PMCA1 yielded products for subtypes 1b (238 bp) and 1c (325 bp). The mRNA for PMCA1b was expressed in myoblasts and decreased after two days in differentiation media to a 4-5 fold lower level in mature myotubes. In contrast, the mRNA for PMCA1c was not detected in myoblasts and appeared after 3 days of myogenic differentiation. The ratio of PMCA1c to PMCA1b at day 0 (8:92) was reversed at day 5 (89:11). For the PMCA4 transcripts in C2C12 cells, the expression of subtype 4b (277 bp) was the main transcript detected in myoblasts and the relative amount of this transcript was decreased by 31% in 5 day myotubes. **Figure 18** shows the PMCA1 and PMCA4 mRNA expression in Sol8 myoblasts and myotubes. The ratio of PMCA1c to PMCA1b from (21:79) in myoblasts (day 0 in differentiation medium) to (76:24) in differentiated myotubes (day 4 in differentiation medium) confirming the results obtained in other cell lines. Compared to C2C12 cells, Sol8 cells showed a similar pattern of expression for PMCA1b and 1c. For PMCA4, the expression of subtype 4b (277 bp) was decreased significantly by 42 % when Sol8 cells were induced to differentiate, while, PMCA4a (455 bp) and PMCA4d (385 bp) mRNA levels remained constant.

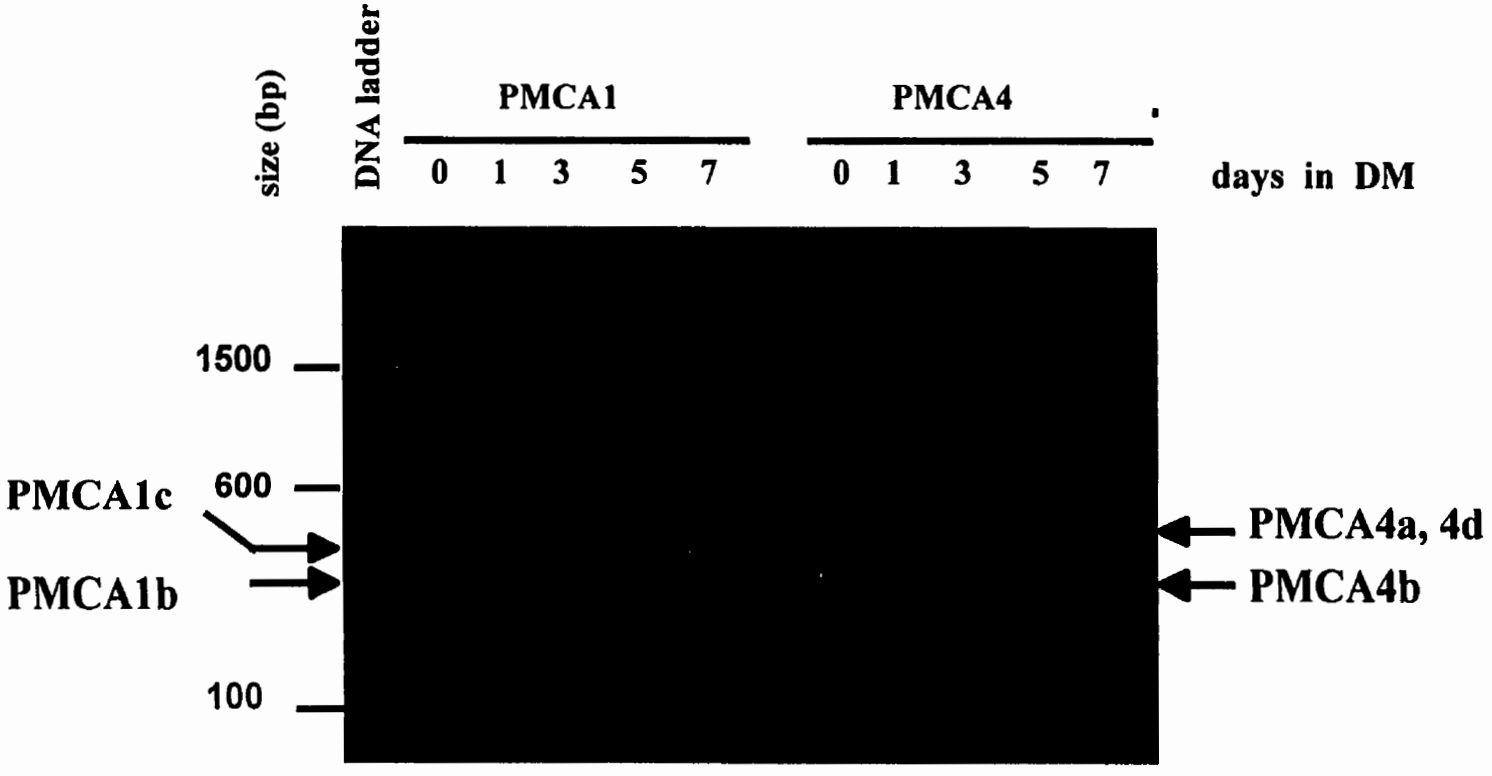

Figure 17. RT-PCR analysis of the expression pattern of PMCA1 and PMCA4 isoforms in C2C12 cell line. The cells were cultured in growth and differentiation medium, RT-PCR amplification was performed using specific primers listed in Table 1. DM: differentiation medium.

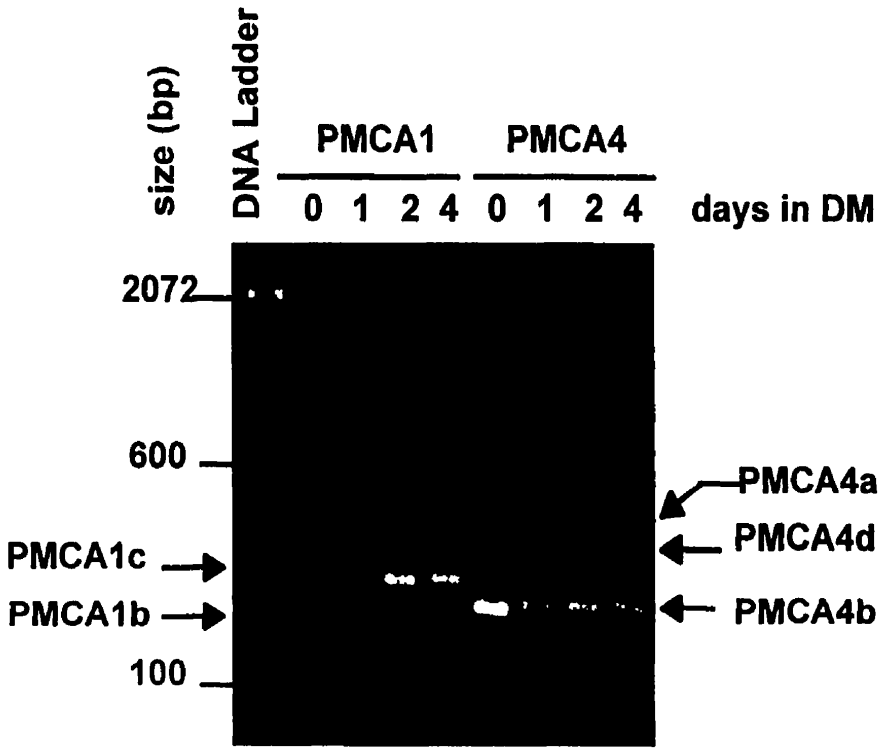

Figure 18. RT-PCR analysis of the expression pattern of PMCA1 and PMCA4 isoforms during myogenic differentiation in Sol8 cell line. The myoblasts were proliferated to 70% confluence, then switched to 3% horse serum/DMEM and total RNA isolated at the indicated times, RT-PCR was performed using specific primers listed in Table 1. DM: differentiation medium.

Figure 19 shows the PMCA isoform-specific expression in the L6 cell line. L6 myoblasts only expressed the mRNAs for isoforms PMCA1b and PMCA4b. After 3 days, expression of PMCA1b was reduced while PMCA1c mRNA became evident. PMCA4a and PMCA 4d mRNAs were not detected in myoblasts and at early stages of differentiation (day 0 and day 1), but were induced after 3 days of myogenic differentiation. PMCA1b and 4b mRNA levels were decreased by 37% and 46% respectively after 5 days in differentiation medium. **Figure 20** and **21** shows the time course of the densitometric data for mRNA expression of PMCA1b and PMCA1c mRNAs during differentiation.

Taken together, the results obtained from three different skeletal muscle cell lines strongly suggests a switch in PMCA isoform expression from PMCA1b to PMCA1c, although the total amount of the transcripts (PMCA1b and 1c) does not seem to increase significantly. The above observations suggest that there was no transcriptional activation of the PMCA1 gene during C2C12 differentiation. These results also suggest that the switch in mRNA levels for PMCA1 and PMCA4 isoforms may arise from changes in the mRNA splicing machinery in differentiated muscle cells which favors the production of one type of transcript over the others.

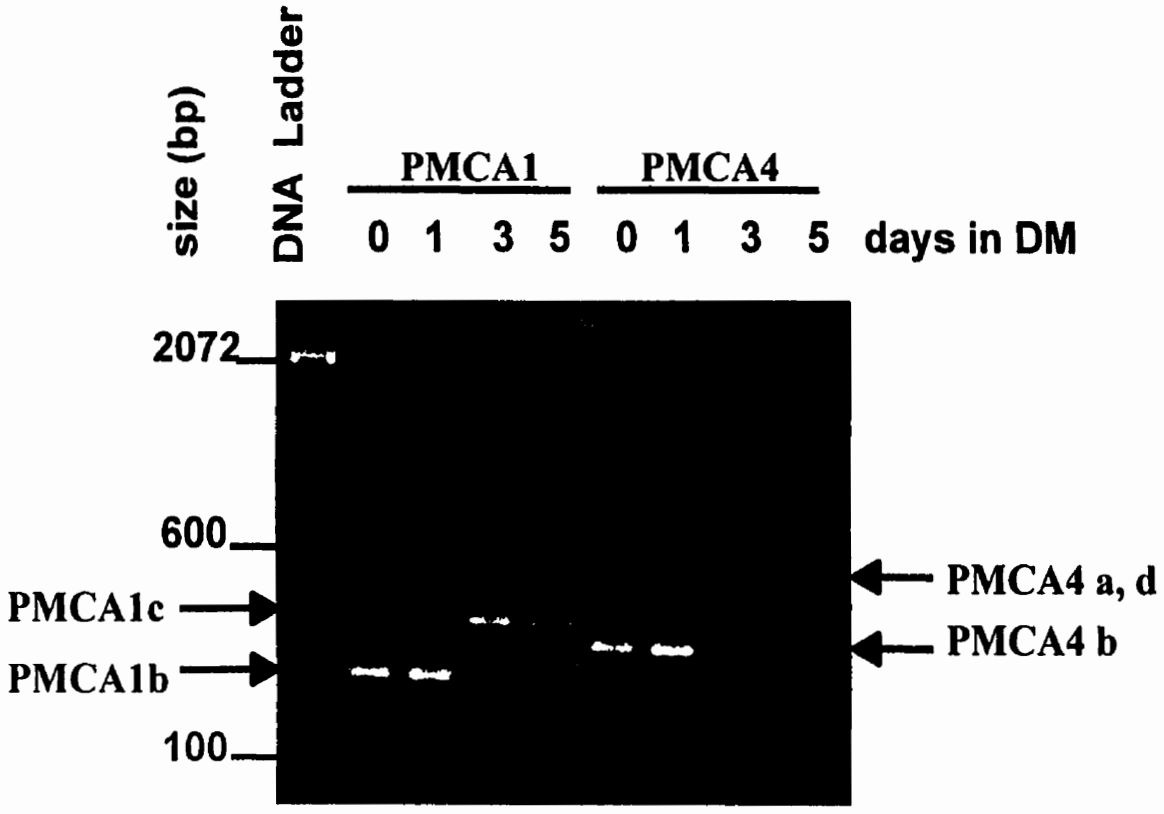

Figure 19. RT-PCR analysis of PMCA1 and PMCA4 isoforms expression in L6 cell line. Total RNA obtained at the indicated times, PCR amplification was performed using specific primers listed in Table 1. DM: Differentiation medium.

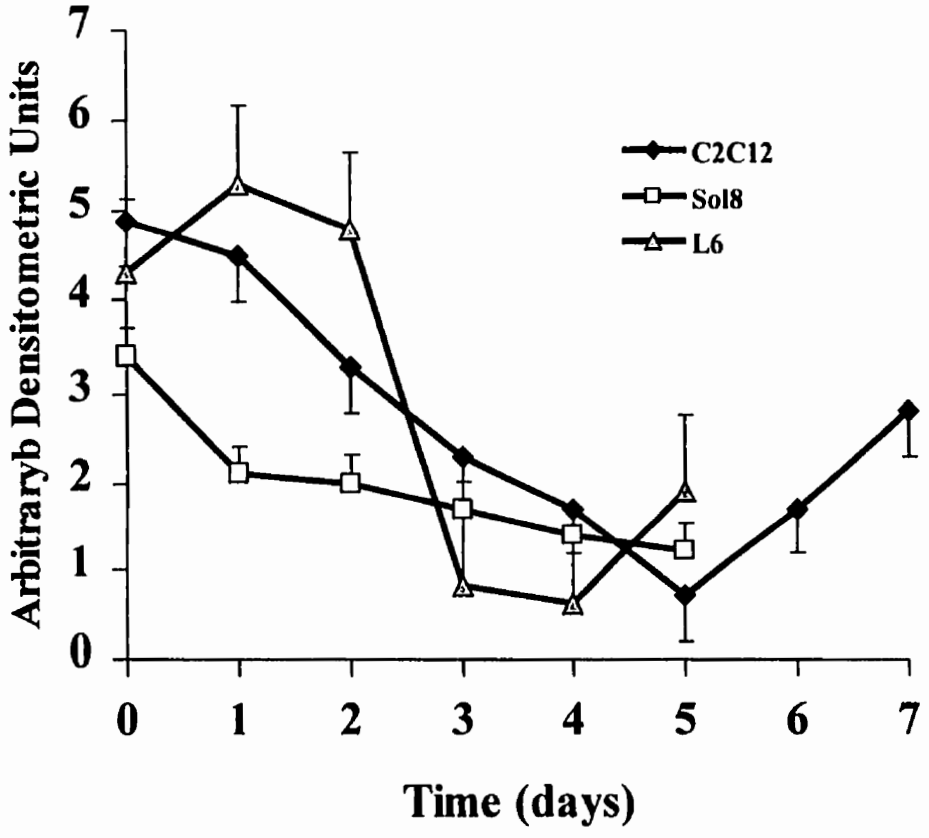

Figure 20. Time course of PMCA1b mRNA expression during C2C12, Sol8 and L6 cells myogenic differentiation. The myoblasts were induced to differentiate in DMEM containing 3% horse serum. Cells were harvested for RNA at the indicated times. Specific mRNAs were amplified by RT-PCR as described under "Materials and Methods". Each amplification products was quantified by densitometric scanning relative to a GAPDH control. Values represent the mean ± S.E. of three experiments with assays conducted in duplicate.

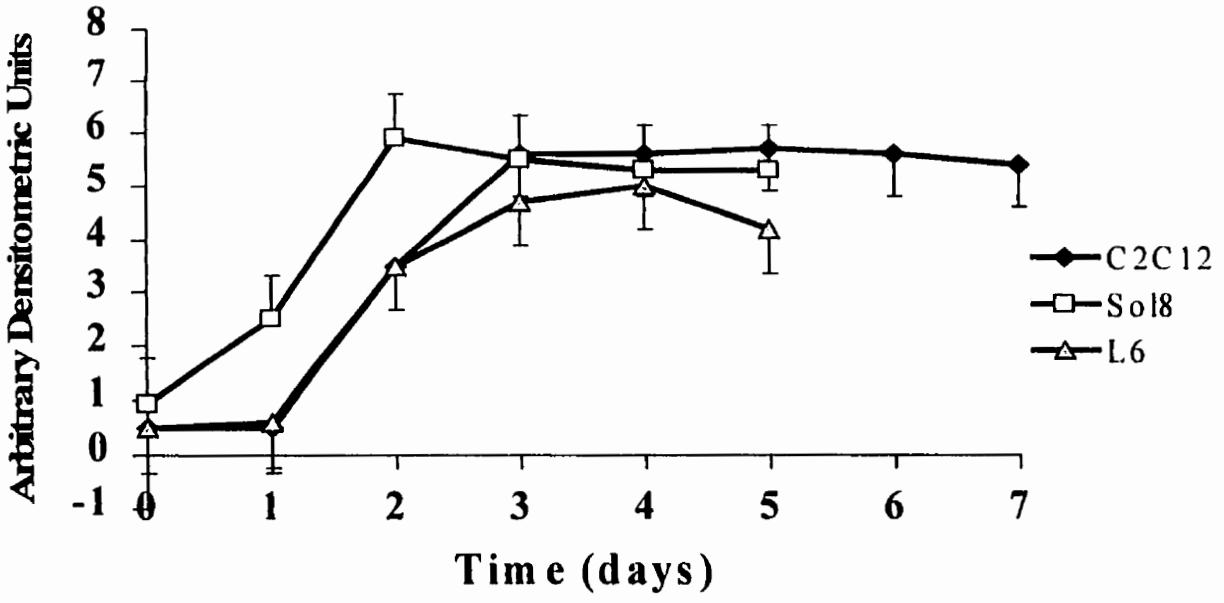

Figure 21. Time course of PMCA1c mRNA expression during C2C12, Sol8 and L6 cells myogenic differentiation. The myoblasts were induced to differentiate in DMEM containing 3% horse serum. Cells were harvested for RNA at the indicated times. Specific mRNAs were amplified by RT-PCR as described under "Materials and Methods". Each amplification products was quantified by densitometric scanning relative to a GAPDH control. Values represent the mean ± S.E. of three experiments with assays conducted in duplicate.

3. MyoD and myogenin mRNA expression.

The expression pattern of the mRNA for the SL and SR Ca^{2+}-ATPases in different skeletal muscle cell lines obtained during the course of these studies, led us to hypothesize that the expression pattern of the Ca^{2+}-ATPases may be at least in part under the control of some myogenic transcription factors during skeletal myogenesis *in vitro*. In order to study the relationship between myogenic regulatory factors and expression of Ca^{2+}-ATPases genes, we decided to analyze the expression of myogenin and MyoD mRNAs in the three skeletal muscle cell lines that we studied. **Figure 22** shows the expression pattern of MyoD and myogenin mRNA in C2C12 differentiating cells. The results were similar with those obtained for the L6 cell line. In myoblasts, myogenin was only expressed at very low level, whereas in myotubes, myogenin gene expression was increased 84% over the first day after switching to differentiation medium and remained at high levels throughout the differentiation process. Interestingly, MyoD mRNA was detected in C2C12 myoblasts but not in L6 myoblasts. We currently do not know if the MyoD mRNA is being translated in myoblasts to a functional transcription factor. In C2C12 cells, MyoD mRNA expression increased gradually from day 1 to day 7 compared to myoblasts. **Figure 23** shows the results obtained in L6 and Sol8 cells. In L6 cells, the MyoD mRNA was detected in 1 day myotubes but not in myoblasts. The MyoD mRNA increased 13-fold by day 3, then decreased by day 5 to level equivalent to day 1 of differentiation. In Sol8 cells MyoD mRNA was detected in myoblasts and was not significantly changed in myotubes. **Figure 24** shows the mRNA levels of the myogenic determination factor myogenin during L6 differentiation. In the myoblast stage, only a very low level of myogenin mRNA could be detected, however, after

induction of differentiation (DMEM + 3% HS) myogenin mRNA levels were increased 6-fold at day 1 and 13-fold in 7 day myotubes. The time course for MyoD mRNA expression in C2C12, Sol8 and L6 cell lines during differentiation is shown in **Figure 25.** **Figure 26** shows the time course of myogenin mRNA expression in C2C12 and L6 cell lines during differentiation. C2C12 and L6 cells only expressed myogenin mRNA in myotubes, not in myoblasts, confirming the notion that myogenin gene is activated during the differentiation process.

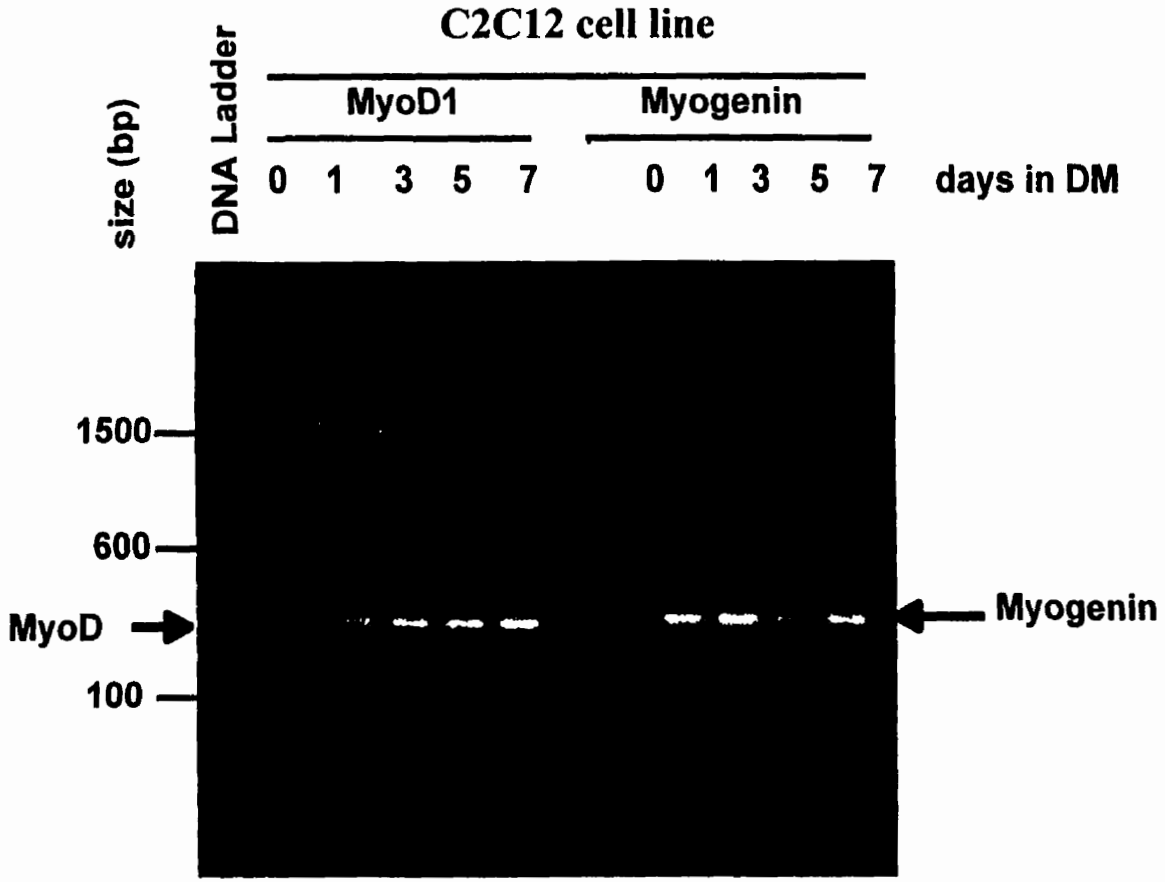

Figure 22. The mRNA expression of MyoD and myogenin during C2C12 cell differentiation. RT-PCR amplification was performed as described in "Materials and Methods". PCR primers for myogenin and MyoD1 are listed in Table 1, and products for myogenin and MyoD1 are 284bp and 263 bp respectively.

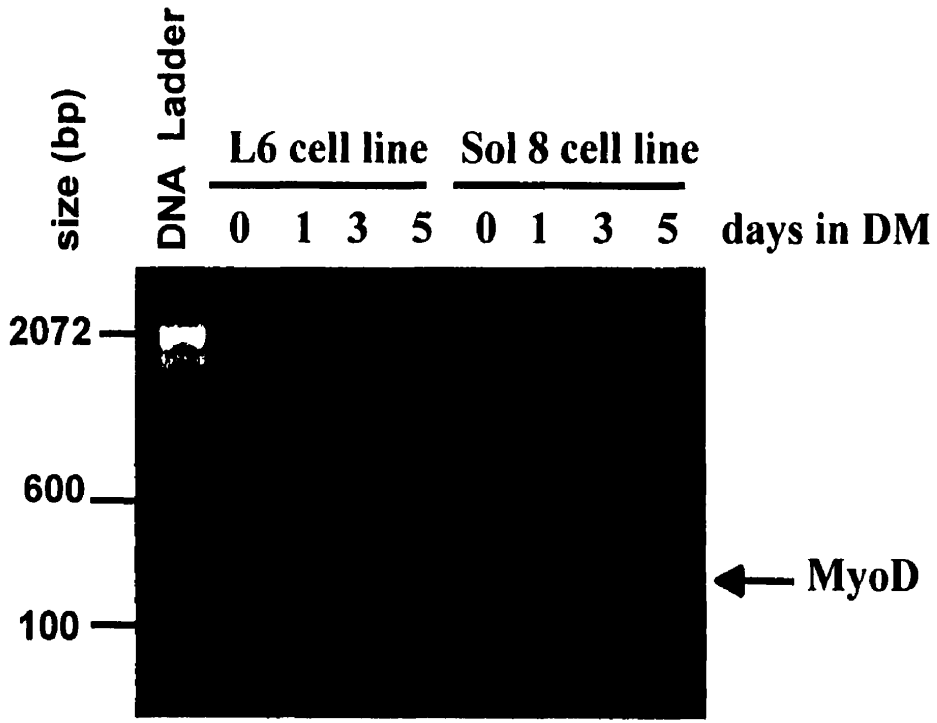

Figure 23. RT-PCR analysis of mRNA expression of myogenic regulatory factor MyoD during L6 and Sol8 cells differentiation. When L6 and Sol8 reached semi-confluence, the myoblasts were switched to differentiation medium (3% horse serum in DMEM medium).

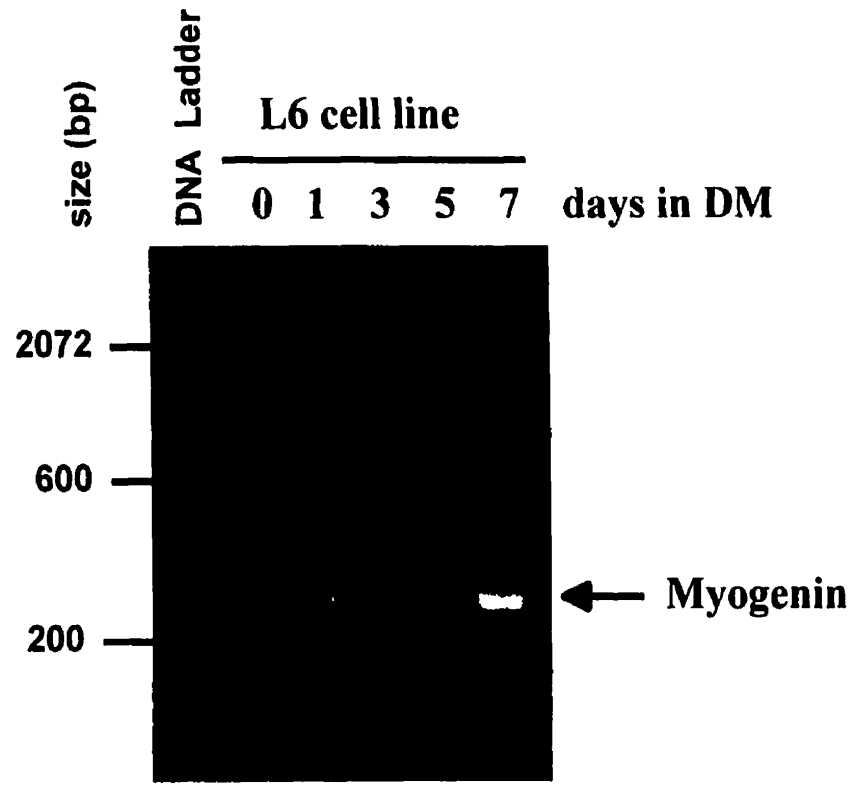

Figure 24. RT-PCR analysis of mRNA expression of myogenic regulatory factor myogenin during L6 cell differentiation. RT-PCR amplification was performed by using the primers listed in Table 1. DM: differentiation medium.

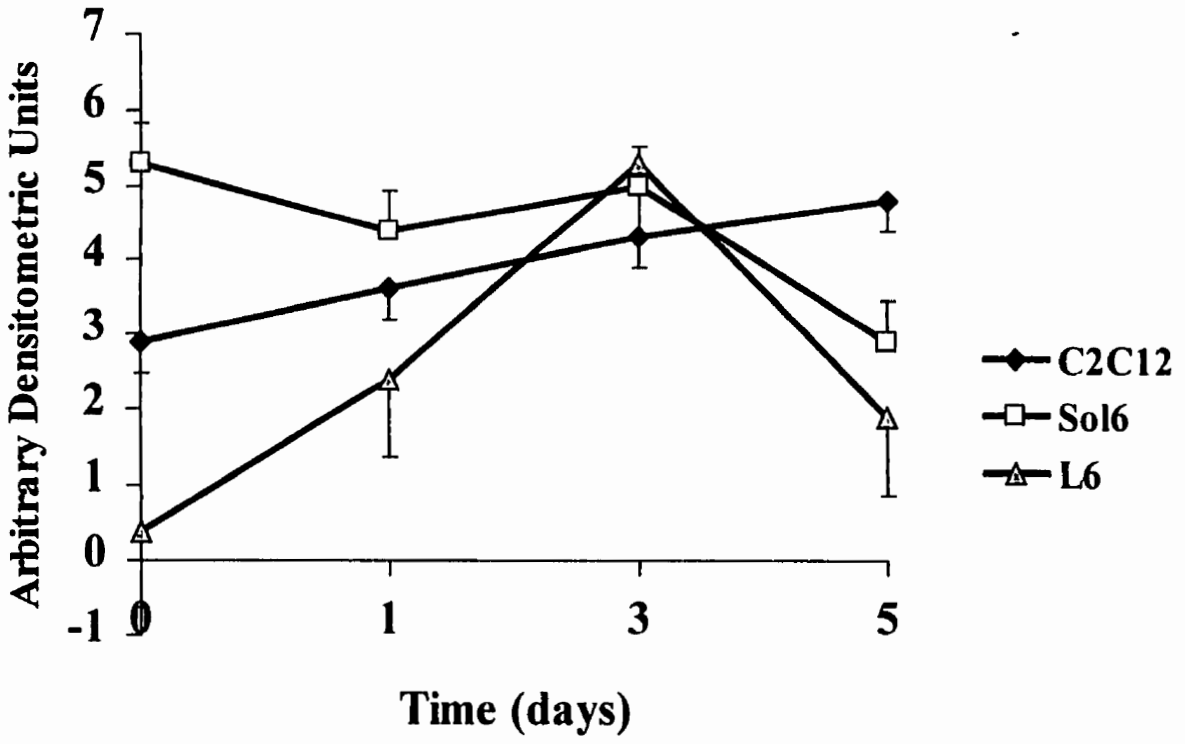

Figure 25. Time course of MyoD mRNA expression during C2C12, Sol8 and L6 cells myogenic differentiation. The myoblasts were induced to differentiate in DMEM containing 3% horse serum. Cells were harvested for RNA at the indicated times. Specific mRNAs were amplified by RT-PCR as described under "Materials and Methods". Each amplification products was quantified by densitometric scanning relative to a GAPDH control. Values represent the mean ± S.E. of three experiments with assays conducted in duplicate.

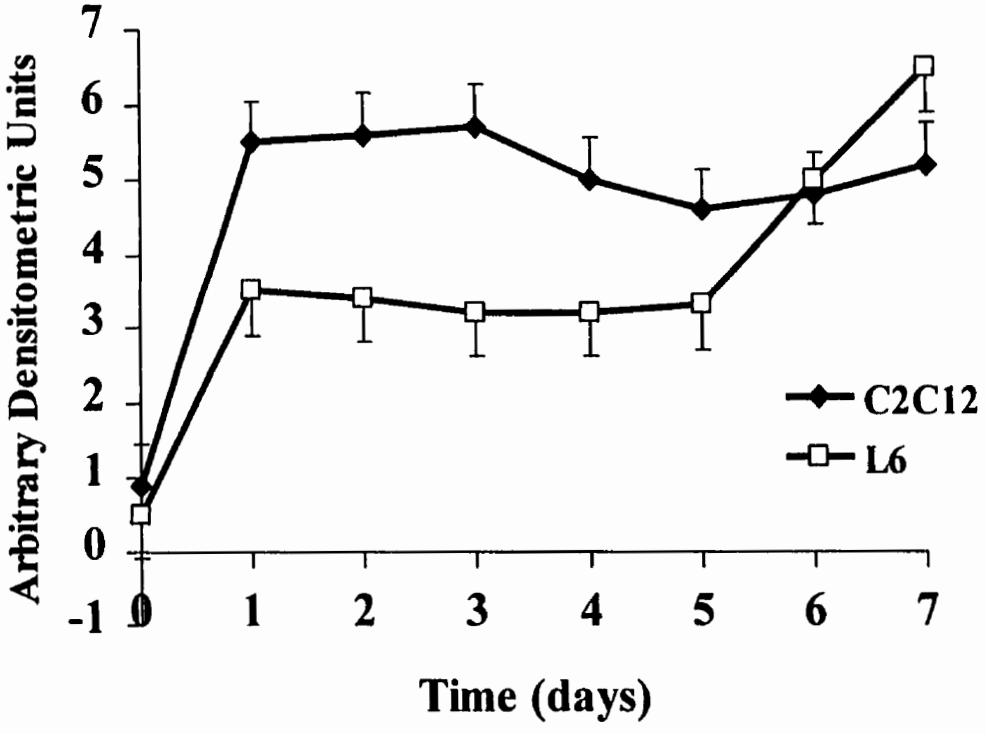

Figure 26. Time course of Myogenin mRNA expression during C2C12 and L6 cells myogenic differentiation. The myoblasts were induced to differentiate in DMEM containing 3% horse serum. Cells were harvested for RNA at the indicated times. Specific mRNAs were amplified by RT-PCR as described under "Materials and Methods". Each amplification products was quantified by densitometric scanning relative to a GAPDH control. Values represent the mean ± S.E. of three experiments with assays conducted in duplicate.

4. PMCA1 and PMCA4 isoform mRNA expression in 5-Azacytidine treated-L6 cells.

From previous reports, we know the following facts: (1) C3H10T1/2 fibroblasts could be converted to myoblasts by 5-azacytidine (39). (2) Hypomethylation of specific DNA sequences plays an important role in the myogenic conversion of C3H10T1/2 cells (40). (3) 10T1/2 myoblasts express MyoD after 5-azacytidine treatment (42). In order to observe the expression pattern of Ca^{2+}-ATPase gene during promoted myogenesis *in vitro*, L6 cells were treated by 5-azacytidine (AZA) to enhance myogenic differentiation and PMCA gene expression was determined. When L6 cells reached 70% confluence in growth medium, the cells were switched to differentiation media and exposed to 3 μM 5-azacytidine for 24 hours. The cells were then rinsed with media without serum, and maintained in differentiation medium for one week. After 1, 2, 4 and 6 days of differentiation, total RNA was isolated and subjected to RT-PCR analysis to detect PMCA transcripts. **Figure 27** and **28** show the expression pattern of PMCA1 and PMCA4 mRNA isoforms in L6 cells treated with AZA, respectively. We observed that in L6 cells treated with AZA the PMCA1c (325 bp), PMCA4a (455 bp) and PMCA4d (385 bp) mRNAs were present 24 hours earlier than that in L6 cells that were not treated with the drug. Densitometric analysis indicated that PMCA1c (325 bp) in the AZA treated group was increased from day 0 to day 1 by 69% in comparison with the control group. In L6 cells, no significant change in the time course of PMCA4a and 4d mRNA expression was observed between AZA treated and untreated groups, however, there was a more profound decrease in PMCA4b mRNA in the AZA treatment group. Taken together these results support the hypothesis that "acceleration" of the differentiation process by inducing the expression of MyoD might correlate with promotion of PMCA gene expression and that the mechanisms that process the primary mRNA transcripts might be regulated by myogenic factors.

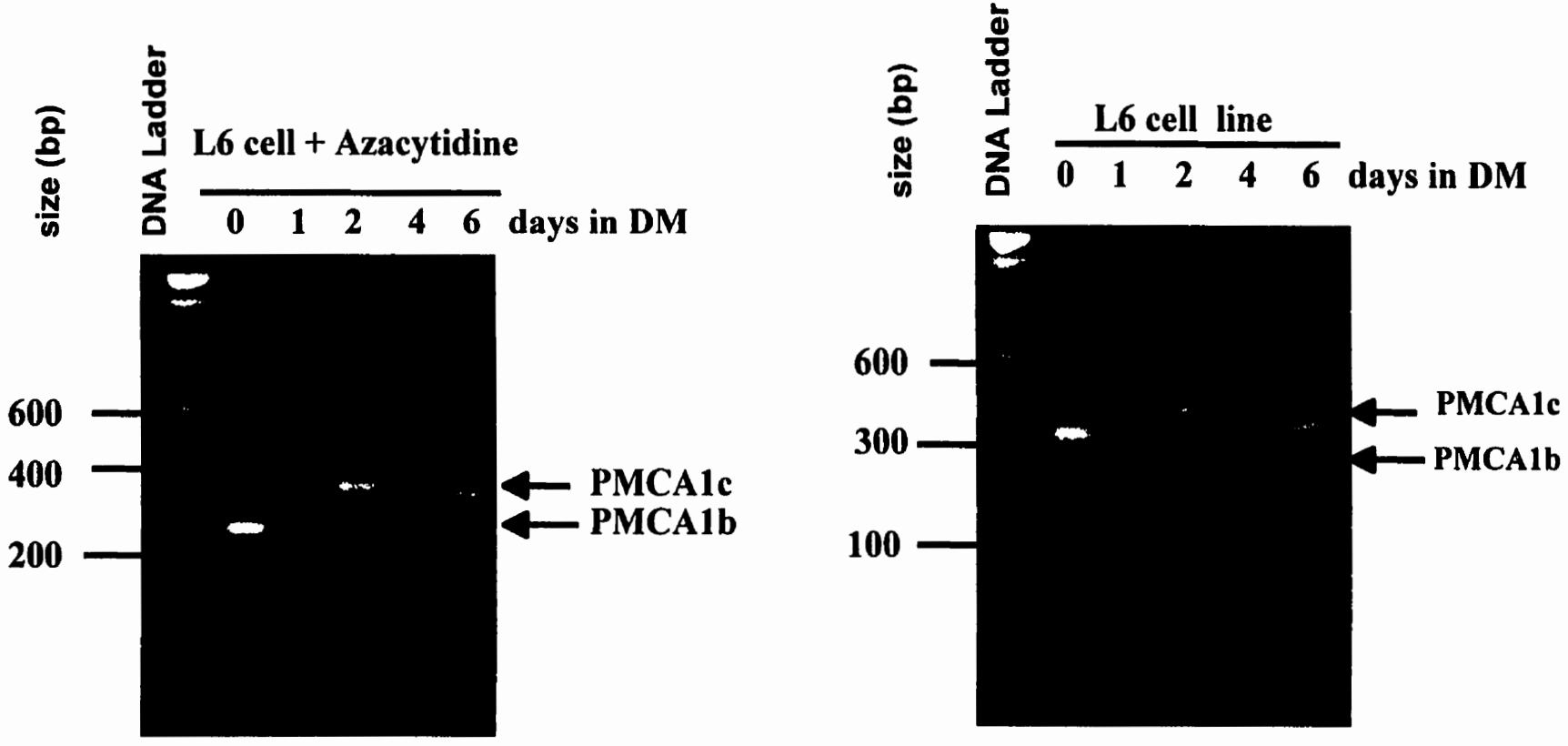

Figure 27. RT-PCR analysis of PMCA1 mRNA expression of 5-AZA treated L6 cells. L6 cells were switched to differentiation medium from growth medium when L6 myoblasts reached 70% confluence and treated with 3 μM AZA for 24 hours. RNA was isolated from AZA-treated cells grown in differentiation at indicated time and RT-PCR was performed as described in "Materials and Methods"

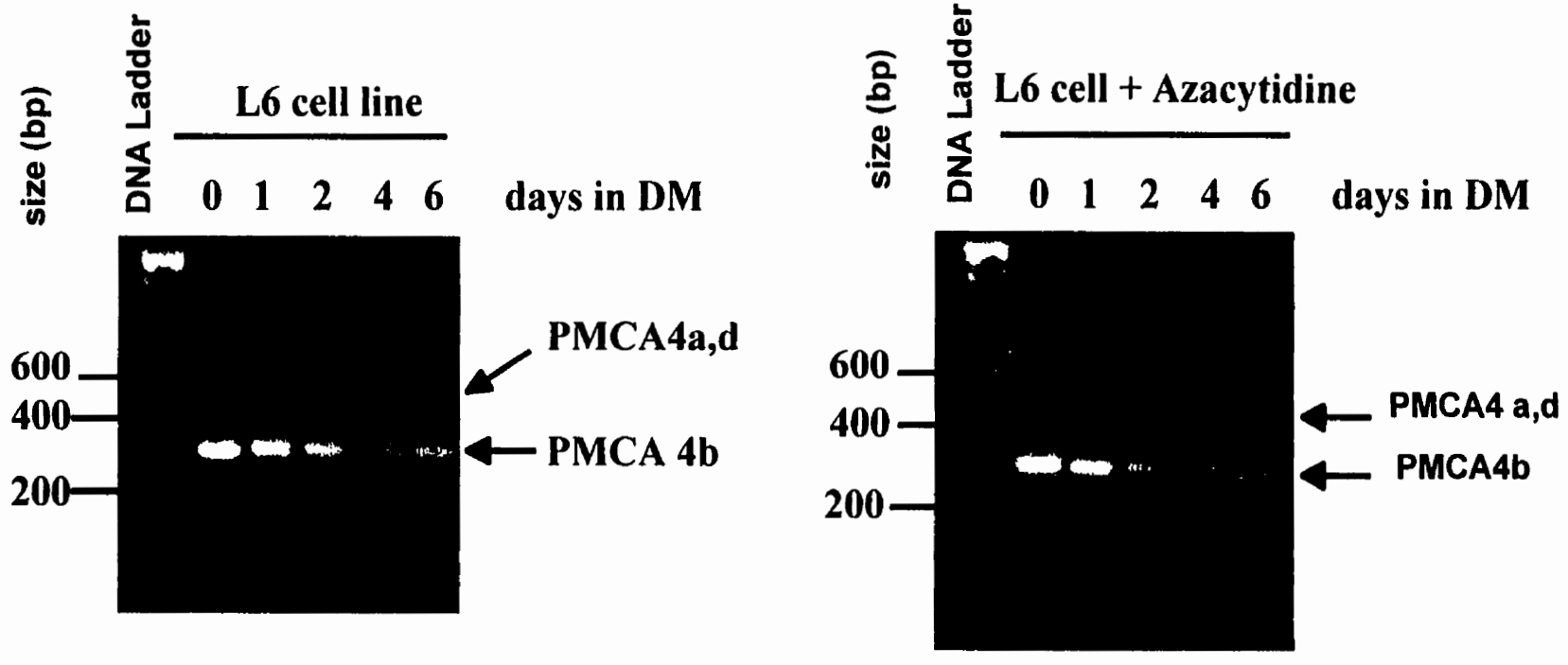

Figure 28. RT-PCR analysis of PMCA4 isoform of AZA-treated L6 cells. The cells were treated with 3 μM AZA for 24 hours, then washed the culture with DMEM medium and reefed with differentiation medium at different times, and PCR analysis was conducted as described in the text.

5. 5-Bromo-2'-Deoxyuridine inhibits SERCA2 gene expression by blocking C2C12 differentiation.

It is known that the pyrimidine analog 5-bromodeoxyuridine (BUdR) competes with thymidine for incorporation into DNA when added to cultured cells. Replacement of thymidine by BUdR in DNA has the effect of blocking the expression of the differentiated phenotype in many cell lineages without significantly altering either the general housekeeping functions of a cell or cell viability (108). Subsequent work showed that BUdR blocks myogenic differentiation by inhibiting MyoD gene expression (107). We speculated that down-regulation of MyoD and /or myogenin by BUdR should influence Ca^{2+}-ATPase gene expression, because muscle-specific isoform expression of Ca^{2+}-ATPase is highly dependent on myogenic differentiation.

To analyze the effect of BUdR treatment on the expression of SERCA2 mRNA, we plated C2C12 myoblasts at low density in growth medium supplemented with 10 μM deoxycytidine with or without the addition of various concentrations of BUdR. After 2 and 4 days, a time sufficient for most of the cells to have incorporated BUdR into their DNA, parallel plates of cells were harvested for RNA analysis. **Figures 29** and **30** show that in the presence of BUdR (50 μM), MyoD (263 bp) expression was decreased and myogenin (284 bp) expression was completely blocked. Even in the presence of low concentrations of BUdR (5 μM) over 2 or 4 days, when C2C12 cells continued to express MyoD, the myogenin mRNA levels were markedly reduced (by 72%) in day 2 and slightly decreased after 4 days. The possible reason for this deviation will be discussed in the next section. High concentrations of BUdR (50 μM) did not influence cell viability, but strongly suppressed myogenesis *in vitro* by inhibiting MyoD and myogenin. High cell confluence (eg. Day 4) could reverse this suppression. SERCA2a mRNA level was also decreased in dosage-dependent manner.

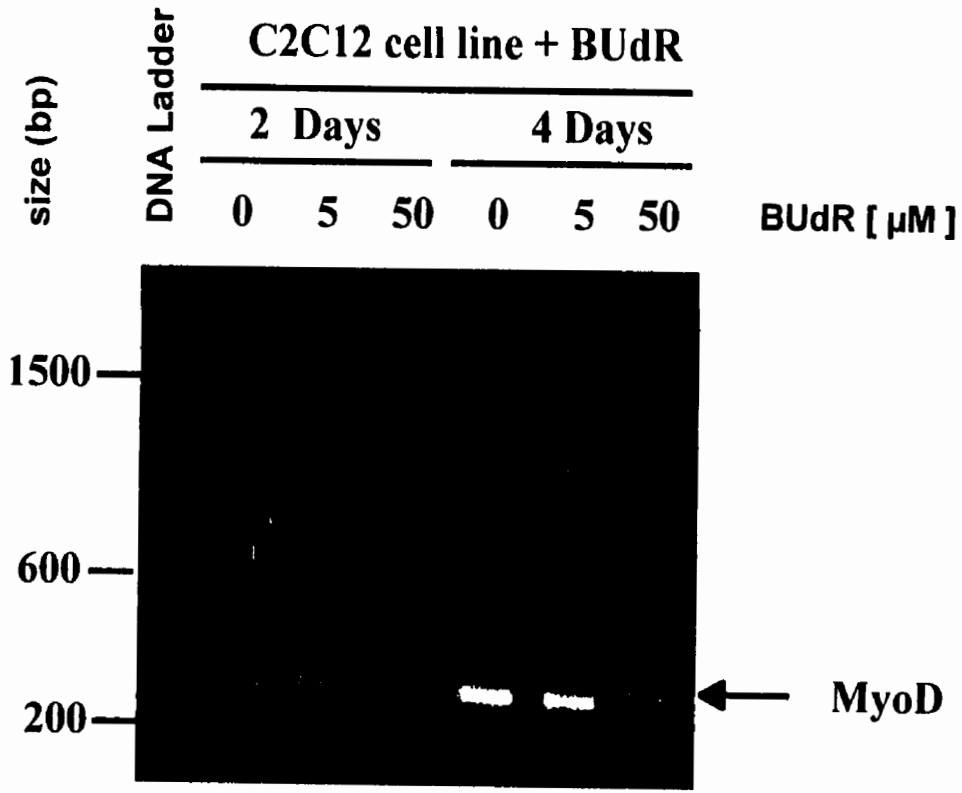

Figure 29. RT-PCR analysis of MyoD mRNA expression in BUdR-treated C2C12 cells. C2C12 myoblasts were seeded at low density in growth medium and added of different concentrations of BUdR to block *in vitro* myogenesis. RNA was harvested after treatment for 2 and 4 days in growth medium, then RT-PCR was performed as described in the text.

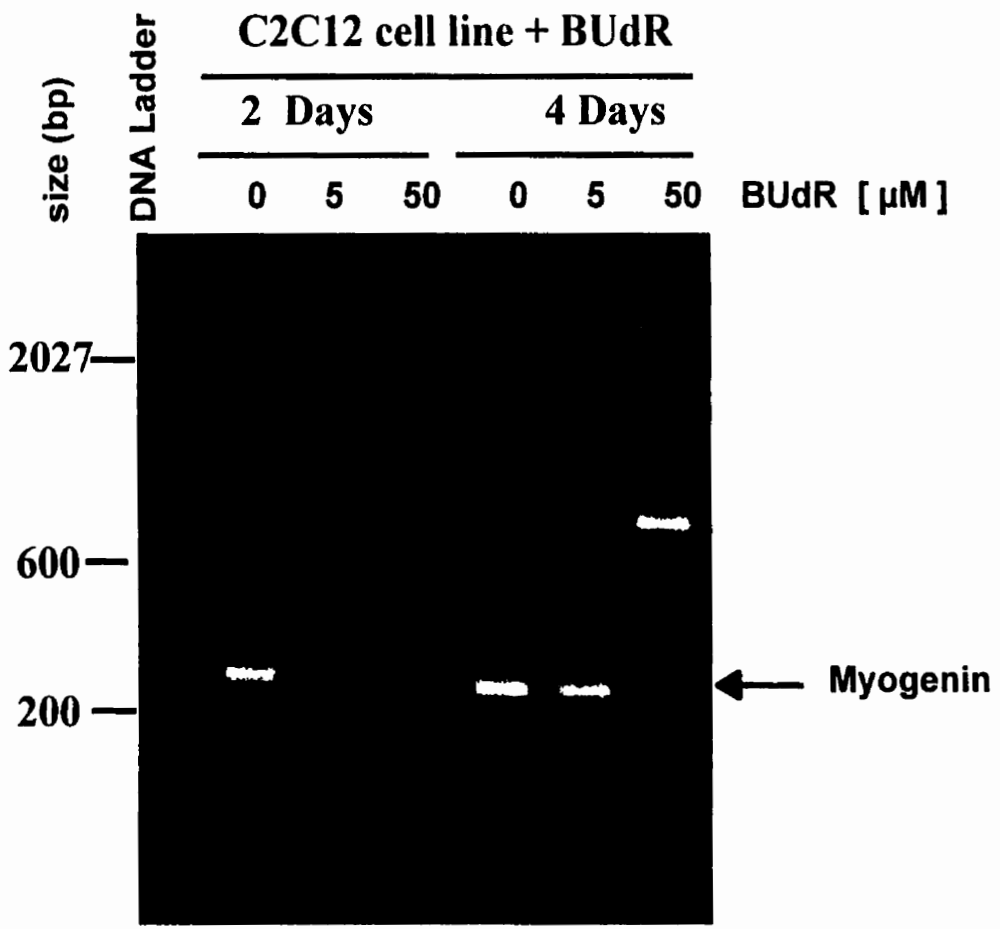

Figure 30. RT-PCR analysis of myogenin mRNA expression in BUdR-treated C2C12 cells. C2C12 myoblasts were seeded at low density in growth medium (GM), then treated with BUdR at the indicated concentration for 2 and 4 days in GM, then RT-PCR was performed as described in the text.

Figure 31 shows the SERCA2a mRNA expression pattern (303 bp product) in C2C12 cells treated with 5 and 50 μM BUdR over 2 and 4 day periods. The SERCA2a mRNA was not detectable in either control or treated groups after 2 days in differentiation medium, probably because of the low density of cells at 2 days. However, when the myoblasts reached 80% confluence (4 days), BUdR inhibited the SERCA2a mRNA expression in a concentration-dependent manner. The SERCA2a mRNA was inhibited by 46% in 5 μM and disappeared in 50 μM of BUdR, whereas the non-muscle isoform SERCA2b mRNA level was not changed by BUdR-treatment (see **Figure 32**). These results suggested that inhibition of myogenesis with BUdR prevented the appearance of the muscle-specific SERCA2a mRNA. We currently do not know if the effect of BUdR is exclusively at the level of mRNA processing (splicing) and/or decreased transcriptional activity of the SERCA2 gene.

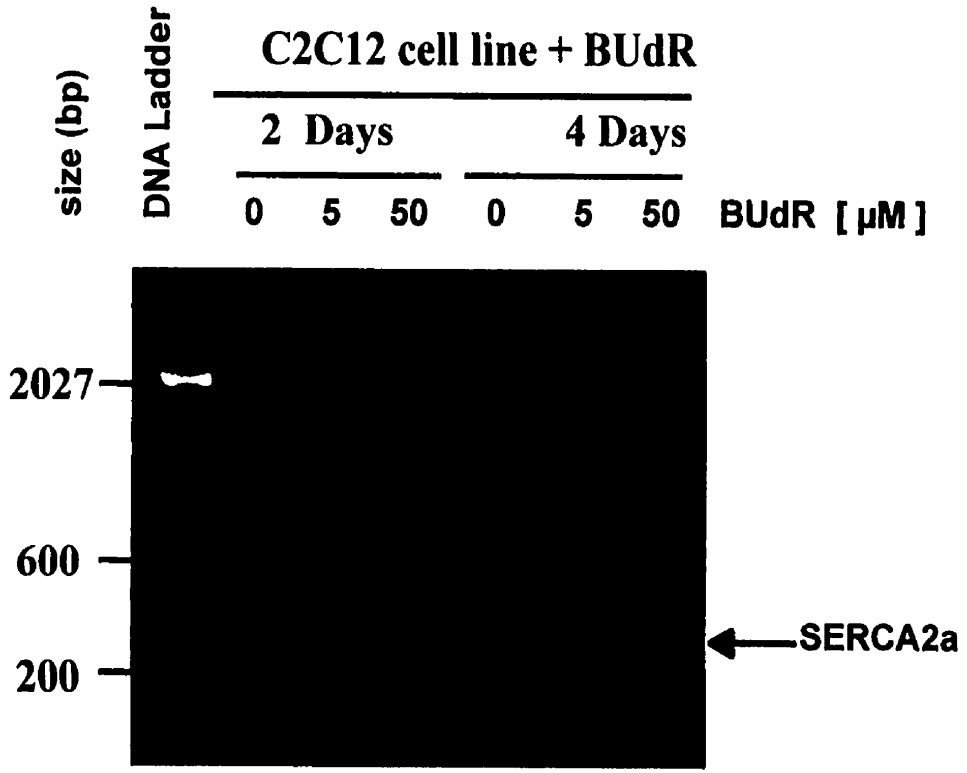

Figure 31. RT-PCR analysis of mRNA expression of SERCA2a in BUdR-treated C2C12 cells. C2C12 myoblasts were seeded at low density in growth medium (GM), then treated with BUdR at the indicated concentration for 2 and 4 days in GM, then RT-PCR was performed as described in the text. The amplified DNA fragment for SERCA2a is 303 bp in length.

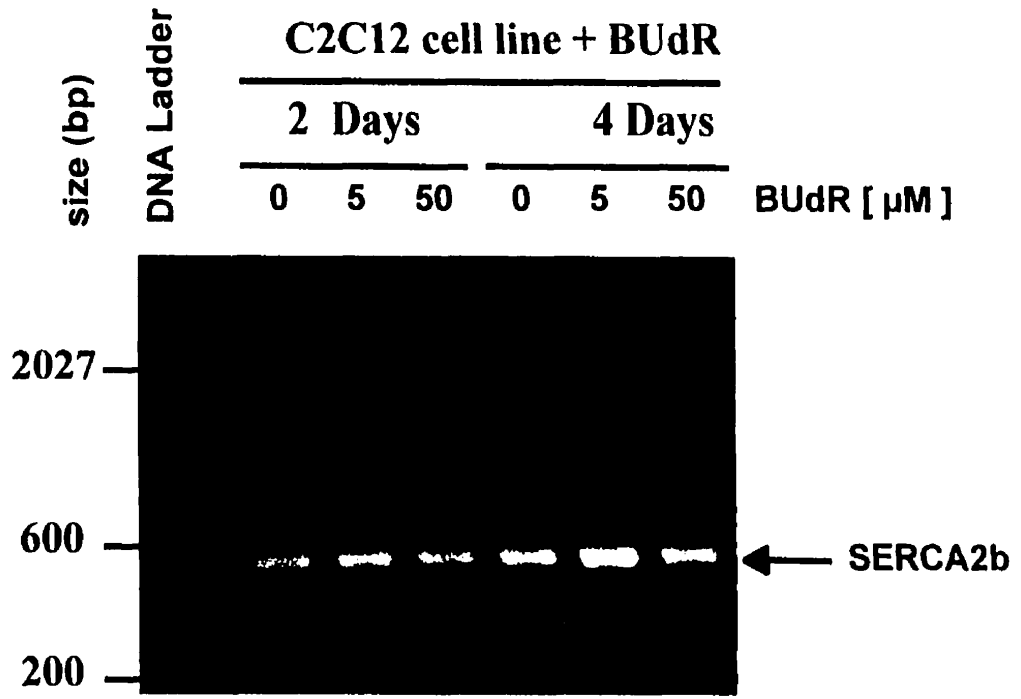

Figure 32. RT-PCR analysis of SERCA2b isoforms mRNA expression in BUdR-treated C2C12 cells. C2C12 myoblasts grown under non-differentiating conditions with low cell density, and BUdR was added to the concentration of 5 and 50 µM. Random -hexamer-primed cDNA was synthesized from RNA isolated from cells treated with BUdR for 2 and 4 days and PCR analysis was performed as described in " Materials and Methods". The PCR product for the SERCA2b cDNA is 530 bp in length.

6. The mRNA expression of SERCA2 and PMCA isoforms in MyoD transfected 10T1/2 cells.

To further elucidate whether MyoD directly influences the expression of mRNAs for SL and SR Ca^{2+}-pumps, we used the 10RMD cell line, a C3H10T1/2 cell line that stably over-expresses MyoD. The expression of the myogenic transcription factors MyoD and myogenin, as well as the Ca^{2+}-ATPases, was analyzed by RT-PCR. **Figures 33 A** and **B** show the results using the MyoD stable transfectant 10RMD. The 10T1/2 cell line, which is the parental cell line for 10RMD, and the C2C12 cell line were used for comparative purposes. The results show that myogenin mRNA (284 bp) was expressed both in myoblast and in myotube stages, but the MyoD (263 bp) expression level was much higher in 10RMD than the one observed in C2C12 cells and undetectable in 10T1/2 cells. **Figure 34** shows that SERCA2a (303 bp) and PMCA4 mRNAs were not expressed in 10T1/2 cells in growth or differentiation media. The over-expressing MyoD cell line 10RMD expressed very low levels of SERCA2a and PMCA4 mRNAs in growth media, but were increased several fold after 2 days in differentiation media, to levels comparable to the ones observed in C2C12 cells. **Figures 35** and **36** show the expression of SERCA2a (303 bp) and SERCA2b (530 bp) mRNA of 10RMD and 10T1/2 cells during the time course of myogenic differentiation. SERCA2a was not detected in 10RMD myoblasts and in 10T1/2 fibroblasts in either growth or differentiation medium. However, the expression of the SERCA2a mRNA isoform was dramatically increased in 10RMD cells during myogenic differentiation. For SERCA2b, the mRNA level was only slightly increased in 10RMD differentiated myotubes.

In conclusion, the MyoD transfected cell line 10RMD expressed high levels of SERCA2a and PMCA4 mRNA compared with their parental fibroblast cell line 10T1/2. The results obtained in the 10 RMD cell line are in agreement with the results obtained in

L6, C2C12 and Sol8, and indicates that myogenic factors, specifically MyoD and myogenin, may modulate the expression of the PMCA and SERCA pumps directly or by initiating the differentiation program. Currently we do not know if the effect of MyoD and myogenin is exclusively at the post-transcriptional level (splicing and mRNA stability) or whether is also modulates transcription these genes.

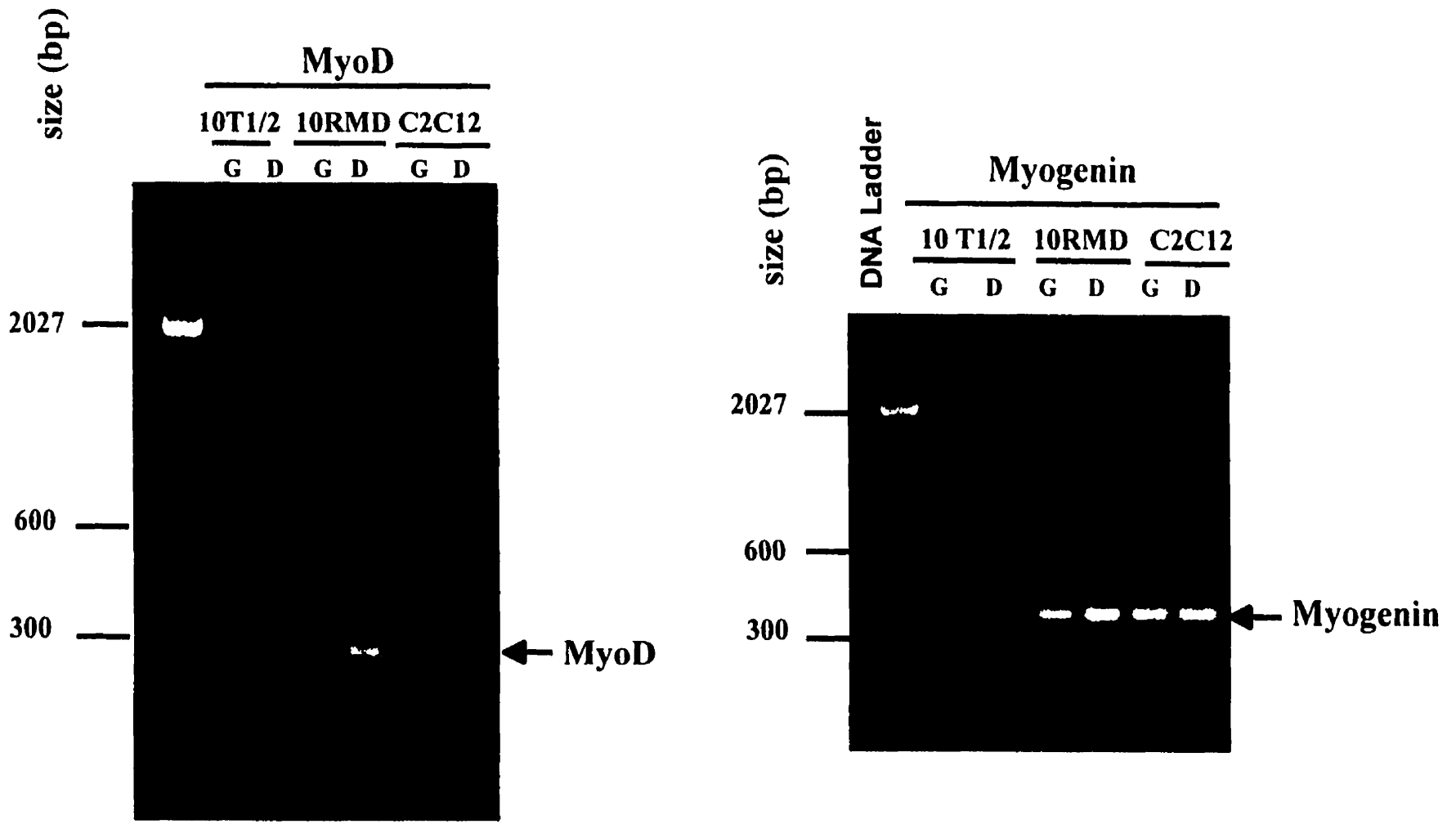

Figure 33. Panel A, B. PCR analysis of MyoD and myogenin mRNA expression in 10T1/2 over-expressing MyoD1 stable transfectants 10RMD and C2C12. Three cell lines were switched from growth medium to low serum medium when the cells reached semi-confluence. Total RNA was isolated from both growing cells and cells placed in DM for 3 days. RT-PCR analysis was performed as described in the text.

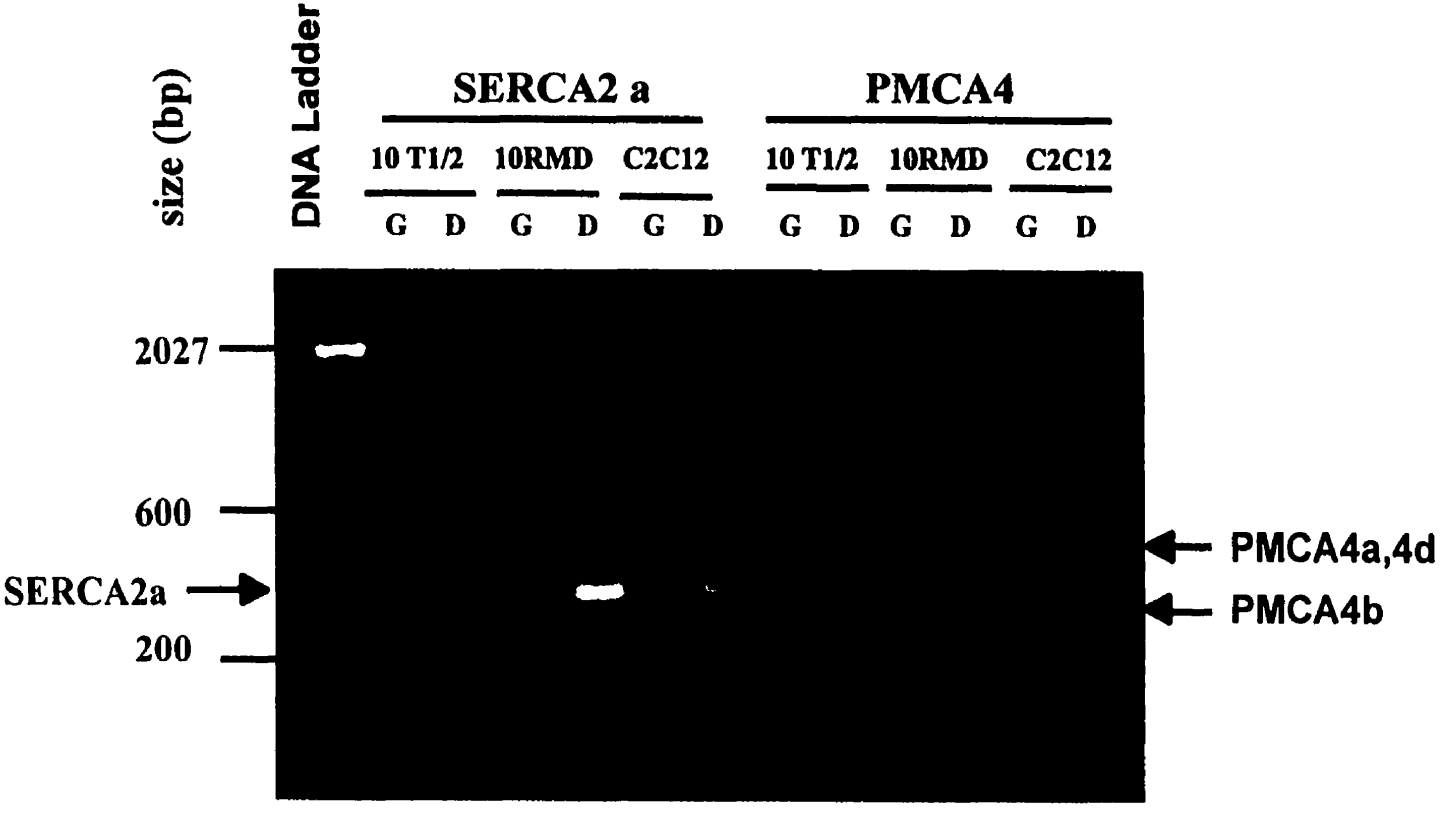

Figure 34. RT-PCR analysis of SERCA2a and PMCA4 mRNA expression in undifferentiated and differentiated state of 10T1/2, 10RMD and C2C12 cells. Total RNA was isolated from both growing cells and cells placed in DM for three days. RT-PCR was performed as described in "Materials and Methods". PCR products for SERCA2a (303 bp), PMCA4b (277 bp), PMCA4d (385 bp) and PMCA4a (455bp) were identified by their size.

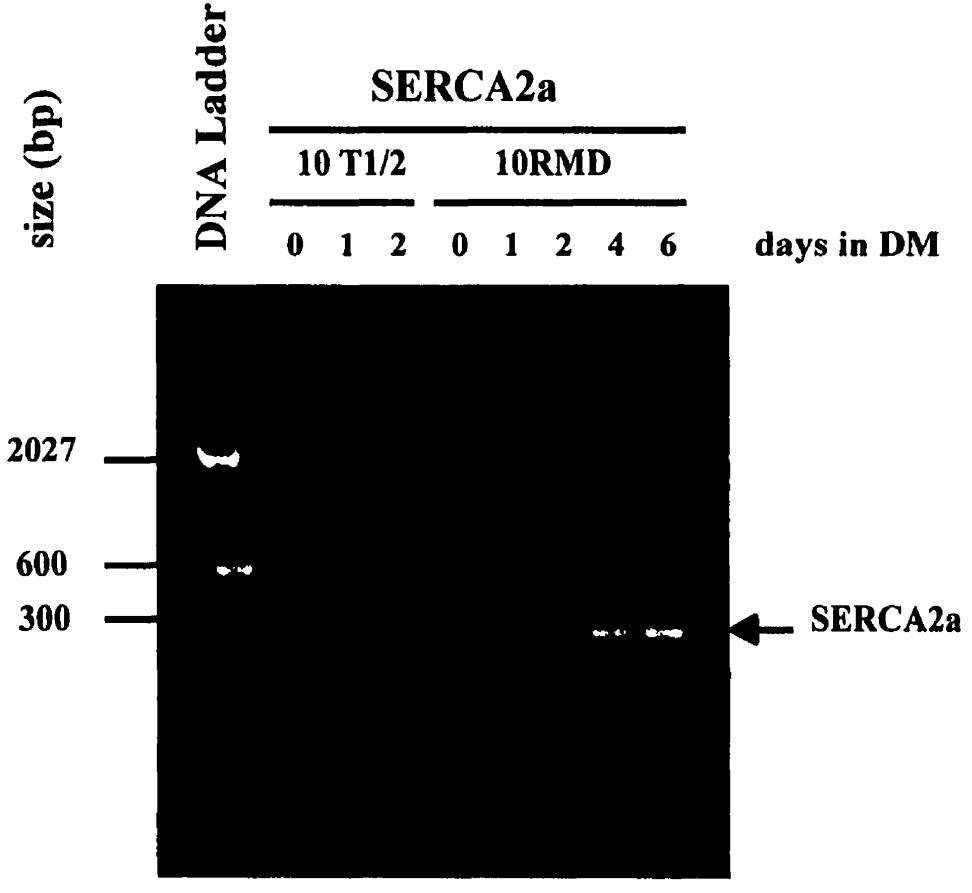

Figure 35. RT-PCR analysis of SERCA2a isoform expression in 10T1/2 and 10RMD cells.
Random hexamer primed cDNA prepared from total RNA isolated from cultured cells incubated in growth and differentiation medium for the indicated times and PCR was conducted as described in " Materials and Methods". The PCR product for SERCA2a is 303 bp in length.

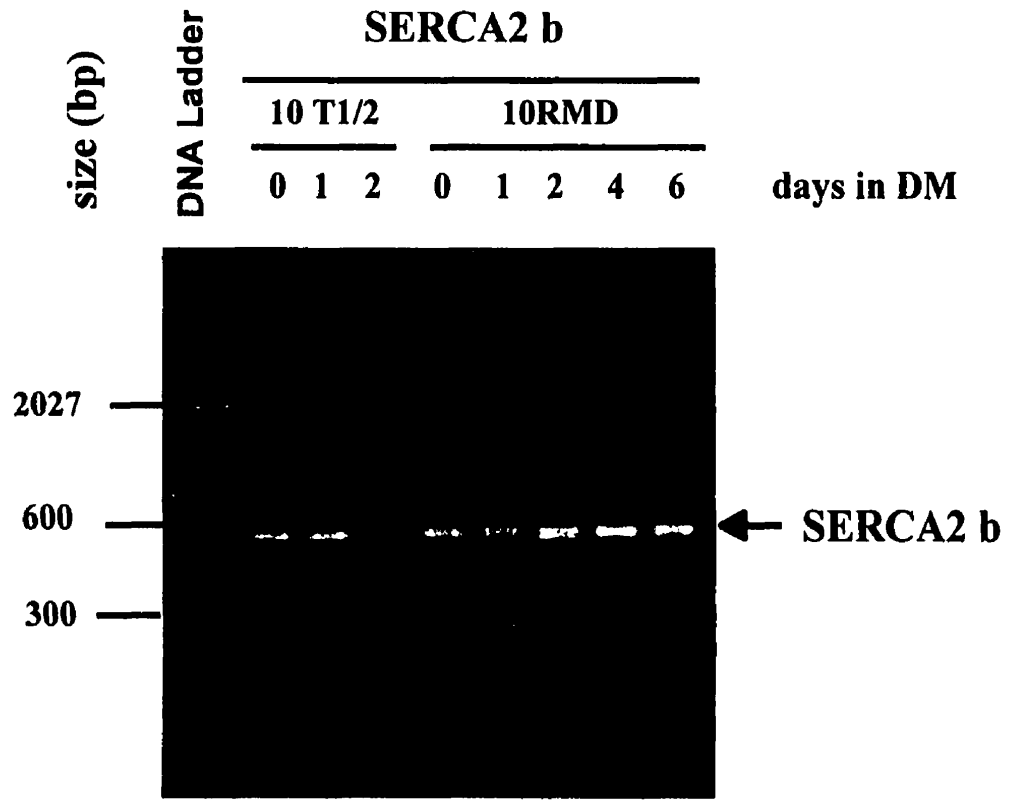

Figure 36. RT-PCR analysis of SERCA2b isoform expression in 10T1/2 and 10RMD cells. Random hexamer primed cDNA prepared from total RNA isolated from cultured cells incubated in growth and differentiation medium for the indicated times and PCR was conducted as described in " Materials and Methods". The PCR product for SERCA2b is 530 bp in length.

IV. DISCUSSION

The purpose of this study was to investigate the expression of Ca^{2+}-ATPase genes during myogenic differentiation in various immortal skeletal muscle cell lines. We were also interested in studying differential regulation of Ca^{2+}-ATPase expression by the myogenic determination factors MyoD and myogenin.

Three skeletal muscle cell lines C2C12, Sol8 and L6 were used. The C2C12 cell line was derived from murine fast-twitch skeletal muscle (101). This cell line differentiates rapidly and produces extensive contracting myotubes expressing characteristic muscle proteins. Therefore, the C2C12 cell line provides an excellent model to study skeletal myogenesis *in vitro*. The Sol8 cell line was isolated from primary cultures of mouse soleus muscle (slow-twitch skeletal muscle) (102), which have a phenotype similar to slow-twitch muscle fibers. It has been demonstrated that the Sol8 cell line expresses high levels of the SERCA2a pump. The L6 cell line was isolated originally from primary cultures of rat thigh muscle maintained for the first two passages in the presence of methyl cholanthrene (103). To date, at least three published reports have shown that L6, L6E9 and L6J1-C do not express MyoD either in undifferentiated or differentiated state (43,111,124). Morphologically, by our observations, there are some differences between the various myogenic cell lines: (1) Upon medium switching (serum withdrawal), C2C12 and Sol8 cells initiate myogenesis much faster, by forming multinucleated syncitia, than L6 cell does. (2) In the late stages of differentiation, C2C12 and Sol8 cells form a more extensive and typical myotube network compared with L6. The most likely reason for the different phenotypes of these muscle cell lines is that they were derived from different sources (fast twitch muscle for C2C12, slow twitch muscle for Sol8), and results from different cell types should be more convincing in demonstrating the mRNA expression pattern of focused genes.

To precisely analyze mRNA expression for the genes of interest, we first optimized the RT-PCR process by amplifying the cDNA templates in different reaction cycles. These data provided the information necessary to identify the linear range of the

amplification. We also measured mRNA expression of GAPDH as an internal control during myogenic differentiation; previous data indicated that GAPDH did not display much variation over medium switching. Moreover, pictures of all electrophoretic gels were quantitatively scanned and relative band densities could be compared. By these efforts, the experimental results could be regarded as semi-quantitative.

Expression of SERCA2a and SERCA2b at the mRNA level were assessed by RT-PCR in the three myogenic cell lines. SERCA 2a expression was increased during myogenesis *in vitro*, while SERCA2b mRNA levels were slightly lower after differentiation (see **Figure 15** and **Figure 16**). A relevant and still unanswered question is whether the induction of SERCA2 gene expression or mRNA processing is directly regulated by myogenic regulatory factors, or whether activation of the SERCA2 gene concomitant with the induction of entire set of muscle specific genes during myogenesis is unrelated. It has been previously demonstrated that transcription of the SERCA2 gene was increased during differentiation of C2C12 cells (110). In that study, a C2C12 stable transfectant containing a chimeric SERCA2/CAT gene construct including -245 bp of SERCA2 5'-flanking region showed increased transcriptional activity in myotubes, suggesting that the regulatory elements necessary for expression during myogenic differentiation are present in this construct (110). In order to determine which transcription factors regulate the transcriptional activity of SERCA2a gene, Dr. Zarain-Herzberg's laboratory has examined the role of myogenic determinant factors on the transcriptional activity of the SERCA2 5'-regulatory regions during myogenic differentiation of the C2C12 and Sol8 muscle cell lines (112). Analysis by gel-shift assay revealed that MyoD and MEF-2 binding sites were present in the 5'-regulatory region of

SERCA2 gene. Accordingly, chimeric genomic constructs containing 284 and 658 bp of 5'-flanking region of the rabbit SERCA2 gene were co-transfected with the MyoD cDNA cloned in the expression vector pCDNA. Transient transfection assays showed that the construct carrying the first 284 bp of the SERCA2 gene promoter was trans-activated 3- to 4-fold by MyoD in C2C12 and Sol8 myotubes. However, the construct containing the large fragment of 5'-flanking region (658 bp) was trans-activated 4-fold in Sol8 myotubes but only 1.8 - fold in C2C12 myotubes. These results suggest that induction of the SERCA2 gene expression in differentiated skeletal muscle cells by MyoD occurred at the level of transcription. However, the changes in mRNA processing of the SERCA2 pre-mRNA may be due to other mechanisms, independent of this increase in gene transcription. Induction of total gene expression (SERCA2a + SERCA2b) possibly results from enhanced transcriptional activity of this gene, whereas increases in SERCA2a mRNA relative to SERCA2b might result from an increase in the activity of muscle-specific pre-mRNA splicing machinery.

Relatively less information is available about SERCA1 gene expression in muscle differentiation. Harrer et al reported recently that differentiation of C2C12 myoblasts to myotubes was associated with induction of SERCA1 expression as assessed by Western blot analysis using a Ca^{2+}-ATPase isoform specific antibody (13). The mRNA for SERCA1 has been shown to be expressed in L6 myotubes, as detected by Northern blot (113). By using RT-PCR analysis, we detected a shift in the expression of two isoforms of the SERCA1 gene (SERCA1a and SERCA1b).

For the sarcolemmal Ca^{2+}-ATPase isoforms coded by the gene for PMCA1, only subtype PMCA1b and low levels of PMCA1c were detected in Sol8 myoblasts (as shown

in **Figure 18**). After induction of myogenic differentiation, the expression pattern was gradually reversed, and the ratio of PMCA1c to PMCA1b, initially 21:79, was changed to 76:24. The mechanism for splicing of the PMCA1 transcript is represented schematically in **Figure 7.** Jaegere et al screened various pig tissues and identified the PMCA1c messenger as the predominant PMCA1 transcript in skeletal muscle (124). It was therefore designated as a "muscle-specific" variant. The PMCA1b messenger was the only one detected in non-muscle tissues, and was termed as the "non-muscle" splice variant. In addition, PMCA1b was found in every analyzed tissue. From previous data and the results in this work, we conclude that the PMCA1b is the "house-keeping" isoform of the PMCA1 gene, and its expression seems to be independent of developmental stage and tissue type. The PMCA1d splice variant was not detected in any of the analyzed tissues, whereas PMCA1a was found to be the predominant mRNA expressed in neural tissues (97,124).

In the case of PMCA4, the switch in the expression pattern between different isoforms was not as obvious as for the PMCA1 products. PMCA4b mRNA was decreased in response to exposure to mitogen deficient medium. In L6 cells, the mRNAs for PMCA4a and PMCA4d were increased, while the abundance of PMCA4b was decreased with differentiation (**Figure 19**). Apparently, the shift between these subtypes after differentiation results from changes in the splicing pattern (99).

Isoform switching can be explained by a shift in splice site recognition from the first 5' (distal to the 3' splice site) site to the second 5' site (proximal to the 3' splice site). As was discussed in the literature review, we know that control of site selection during splicing is usually under the control of splicing factor 2 (SF2)(108). The mechanisms

underlying splicing pattern changes during skeletal muscle cell differentiation remain unanswered. The question as to which mechanisms modulate the expression of alternative splicing factors during muscle differentiation is of great interest. In this respect we can speculate that there are two possibilities for these changes: (1) myogenic determination factors, such as MyoD and myogenin, directly control alternative splicing at the promoter level. It is known that the binding site for the myogenic determination factors is a DNA motif termed "E-box" (CANNTG). Whether an "E-box" in cis could control alternative splicing is not known. (2) MyoD or myogenin control the synthesis of trans-acting factors (SF2, ASF) directing the splicing pattern of the PMCA1 and PMCA4 primary transcripts during differentiation. However, there is no direct evidence of a change in SF2/ASF concentration or activity during myogenic cell line differentiation, and the effect of myogenin or MyoD on the expression pattern of ASF/SF2 in transfected C3H/10T1/2 cells has not been investigated. This may be the direction for further investigation.

In contrast to earlier reports (43, 111), we found that the L6 cell line expressed MyoD as well as myogenin in the differentiated stages of myogenic differentiation. We speculate that one of the reasons for the discrepancy with previous reports may arise from the detection method used. For example, the basis for concluding that MyoD could not be detected in L6 cells was a Northern blot analysis (43). In contrast, the results presented in this thesis were obtained by RT-PCR analysis, and we designed sets of primers that are highly specific for MyoD and myogenin and exclude the possibility of contamination with other unspecific transcripts. Another reason might result from not using identical cell lines. Muthuchamy et al used L6E9 cell which is derivative cell line from L6 cells (111).

Constantinides et al have found that the non-myoblastic cell line C3H/10T1/2CL8 formed functional myotubes following treatment with 3 μM 5-azacytidine (AZA). These myotubes arose from the fusion of mononucleated precursors (105). Later, using the restriction enzyme Hpa II which is sensitive to cytosine methylation, they demonstrated that the DNA synthesized in 5-azacytidine-treated cultures was maximally undermethylated 48 hr after treatment. The results suggest that the new phenotype may be caused by under-methylation of certain genomic loci. (106). Muthuchamy et al has shown that AZA treatment induces endogenous MyoD expression in L6E9 cells (111). This cell line, which fails to synthesize myosin light chain 1 (MLC-1) and cardiac α-actin, exhibits a deficiency in the expression of MyoD, yet it expresses myogenin, Myf-5 and MRF-4. L6E9 treated with AZA for 24 hr accumulated MLC1 transcripts in cells maintained in differentiation medium for 1 week, and cardiac α-actin transcripts after 3 days. Similarly, Miller et al has shown that myogenic cell lines generated from 10 T1/2 cells treated with AZA and stably transfected with MyoD or myogenin expressed the embryonic, slow skeletal (cardiac), and perinatal MHC isoforms (114). By analyzing our data (**Figures 27 and 28**), it has been shown that AZA can increase PMCA1 and PMCA4 isoform expression, which may account for the increased transcription and/or post-transcription processing of PMCA1 and PMCA4 genes. From the results in **Figure 27**, in which L6 cells were cultured in differentiation medium for 24 hours, we concluded that the increase of PMCA1c mRNA in the AZA-treated cells may arise from enhanced splicing of the primary transcripts. Confirmation of this phenomenon must still be obtained, and further work is indicated, and would include (1) determining the concentration and activity of splice factors SF2 and ASF under the influence of AZA;

(2) establishing whether MyoD or myogenin interact with the PMCA1 or PMCA4 gene promoters.

In this work, expression of the myogenic determinant genes was altered by the treatment of C2C12 myoblasts with the pyrimidine analog 5-bromodeoxyuridine (BUdR), a treatment previously reported to inhibit expression of the myotube phenotype (108). Tapscott et al first demonstrated that BUdR blocks myogenesis by extinguishing expression of MyoD (107). The above report suggested that incorporation of BUdR into the muscle structural genes may not play a significant role in blocking differentiation. Instead, BUdR blocks MyoD expression, either directly or indirectly, and this prevents the expression of the myogenic program in myoblasts. It seems that the level of myogenin mRNA was altered more significantly than that of MyoD by BUdR treatment (**Figure 29**). This fact can be explained by evidence that MyoD is not a "direct" activator of downstream myogenic genes (muscle-specific creatine kinase or cardiac α-actin gene). It has been reported that MyoD first activates myogenin gene expression, and myogenin in turn induces downstream muscle-specific genes (115). In other words, BUdR reduced the expression of the upstream factor MyoD, and the downstream factor myogenin was not produced. We also noticed the fact that genomic myogenin (810 bp product containing an intron) was detected when BUdR was present at 5 or 50 μM (**Figure 30**). It was speculated that trace amounts of genomic DNA in the total RNA samples resulted in a true "competition" of the PCR reaction between the genomic myogenin sequence and the mRNA of myogenin (284 bp product). A strong signal for genomic myogenin was observed when expression of myogenin was entirely abolished by treatment with 50 μM BUdR for 4 days. **Figure 31** displays the results of C2C12 cells grown at low density

(less than 50% confluence for 2 days) in absence of BUdR, revealing that the cells did not express the SERCA2a mRNA compared to cultures at high cell density (0 μM BUdR for 4 days). The mechanisms responsible for the high level of SERCA2a mRNA expression in confluent cultures is not completely understood. However, our observations and other reports (116) suggest that really confluent cultures of myoblasts will initiate myotube formation spontaneously even in the presence of high concentration fetal bovine serum. It is reasonable to assume that physical contact between myoblasts will trigger the release of extracellular signals, which in turn induce myogenic gene activation. The SERCA2b mRNA expression was kept at high levels indicating that cell viability was not damaged by high concentrations of BUdR (50 μM)(**Figure 32**).

Two laboratories have previously used transfection of myogenin cDNA into C3H/10T1/2 cells (98) and fetal rat skin fibroblasts (99) to study the regulation of the SERCA and PMCA gene expression by myogenin during myogenic differentiation. When myogenin expression vector and a minigene (pCMβSERCA2) which contains the 3' end of the SERCA2 gene were co-transfected to C3H/10T1/2 fibroblasts, the results showed that myogenin could induce the trans-acting factor(s) responsible for activation of splicing. Hammes et al found that myogenin-transfected FR fibroblasts displayed the PMCA muscle-specific mRNA variants PMCA1c, 1d and PMCA4a and 4d (99). To date there are no results available regarding the role of MyoD in modulating the isoform switch of Ca^{2+}-ATPase. We used the MyoD-transfected cell line 10RMD to assess the influence of this muscle regulatory factor on mRNA expression of Ca^{2+}-ATPase. It is of interest to point out that there is an apparent contradiction between the results presented in **Figure 33** and **Figure 22**. In **Figure 22**, myogenin mRNA was barely detectable in

C2C12 myoblasts while in **Figure 33** C2C12 myoblasts expressed myogenin mRNA at high levels. This inconsistency may arise because cells at high confluence were used for the experiment shown in **Figure 33**. It was postulated that myoblasts could be divided into two stages: proliferating myoblasts as myogenin-negative mononucleated cells and differentiating myoblasts as myogenin-positive mononucleated cells. Expression of myogenin in differentiating myoblasts is crucial for the exit from the proliferation state into the differentiation program. Another concern about this experiment comes from **Figures 33 and 34**. The results indicated that MyoD-transfected 10RMD myoblasts express MyoD and myogenin at high levels, but SERCA2a, PMCA1 and PMCA4 are expressed at low levels. Only after the medium was switched were the SERCA2a and PMCA4 genes expressed at high levels. A potential explanation for these results is that expression of muscle specific Ca^{2+}-ATPase has two prerequisite conditions: presence of a myogenic determination factor and low serum medium. In growth medium, muscle cell lines can express MyoD and myogenin, however, growth factors and mitogens present in the medium suppress muscle-specific SERCA2a and PMCA gene expression. Serum withdrawal serves to turn on the myogenic program of these cells, leading to the formation of mature myotubes and induction of the SERCA2a and PMCA genes.

It is interesting to note that no MyoD, myogenin or other myogenic regulatory factors have been found in cardiomyocytes, in which the muscle-specific alternative splicing of SERCA2, PMCA1 and PMCA4 also occurs (26). Therefore, expression of MyoD and myogenin is not an obligatory condition for muscle-specific expression and/or processing of Ca^{2+}-ATPases messages. Other transcription regulatory factors present in cardiomyocytes might direct the expression of the SR and SL Ca^{2+}-ATPases. One

candidate might be a member of the GATA family of zinc finger transcription factors, which bind a core GATA motif and whose expression is restricted to cardiac myocytes. GATA-4 has been demonstrated to activate transcription of the α-myosin heavy-chain (αMHC) gene, a cardiac muscle-specific contractile protein gene in which two putative GATA-binding sites were found within the proximal enhancer of this gene (121). Another potential factor is the homeodomain protein MHox that is expressed at high levels in cardiac, skeletal and smooth muscle cells and binds to an A+T-rich sequence associated with several muscle-specific genes (122). By analogy with other homeodomain proteins which regulate cell type-specific transcription in other tissues, it is tempting to speculate that MHox may participate in the control of muscle-specific transcription at specific stages of development.

Arai et al have measured the major sarcoplasmic reticulum proteins as well as contractile protein expression during *in vivo* cardiac and fast-twitch skeletal muscle development, and these include phospholamban, calsequestrin, SERCA1, SERCA2 and myosin heavy chain and α,β,γ-actins (126). In their observations of SR Ca^{2+}-ATPase gene expression, they found that SERCA2a transcripts were highly expressed in fetal cells, but were eventually replaced by SERCA1 mRNA, the principal isoform expressed in adult animals. In our study, SERCA2a mRNA expression was gradually increased during myogenesis *in vitro*. While the functional significance of isoform switch both *in vivo* and *in vitro* remains unclear, it would appear that an *in vitro* myogenesis model could reflect some aspects of muscle development *in vivo*. As discussed in the **Literature Review**, over-expression of PMCA4b could promote the process of differentiation (100). It is also reasonable to assume that Ca^{2+}-ATPase isoform switching

in SR and SL could accelerate muscle development *in vivo* by adjusting Ca^{2+}-dependent ATPase activities and thus influencing the sensitivity to Ca^{2+} concentration. However, it is also possible that isoform switching occurs as a result of cell differentiation and does not influence its progression.

In this work, we have used a semi-quantitative RT-PCR approach to assess muscle specific expression of the isoforms for SL and SR Ca^{2+}-ATPases at the mRNA level. We confirmed that the SERCA1 and SERCA2 pre-mRNAs experience isoform switching. Muscle isoform expression increased 5 to 8-fold; meanwhile the non-muscle isoforms decreased slightly during differentiation. Interestingly, we discovered that PMCA1c and PMCA1b undergo an inverse switch during differentiation, and we explored the factor(s) affecting alternative splicing during myogenesis. It was observed that BUdR suppressed SERCA2a gene expression in a dosage-dependent manner, and the change in this Ca^{2+}-ATPase isoform is presumably coupled to inhibition of MyoD and myogenin gene expression. We also found that L6 cells not only express myogenin, they also express MyoD, which is in contrast with previous reports.

Based on above the observations, it is reasonable to speculate that MyoD, myogenin or another myogenic regulatory factor can function directly or indirectly to modulate splice selection. In future experiments, it might be demonstrated that MyoD or myogenin interact with the spliceosomem complex. This interactioncould either involve the RNA-binding proteins SF2 or SF5 or a direct association between MyoD/myogenin with the 3'end of the pre-mRNA. Van Den Bosch et al recently reported that they have screened for the presence of specific *cis*-active sequences, either splice enhancers or splice suppressors, involved in tissue-specific regulation of SERCA2 splicing (125). They

provided evidence for the presence of sequence elements surrounding the acceptor site of splicing that inhibit both muscle-specific and the neuronal-specific splicing. Combined with our observations, I hypothesize that myogenic regulatory factors (MyoD/myogenin) might induce the positive *trans*-acting factors which act on this sequence element during muscle differentiation and suppress muscle-specific splicing.

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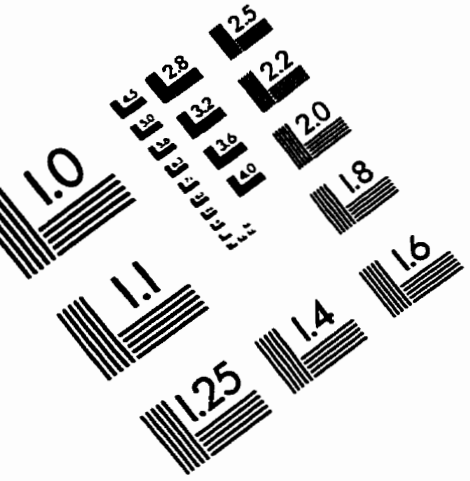
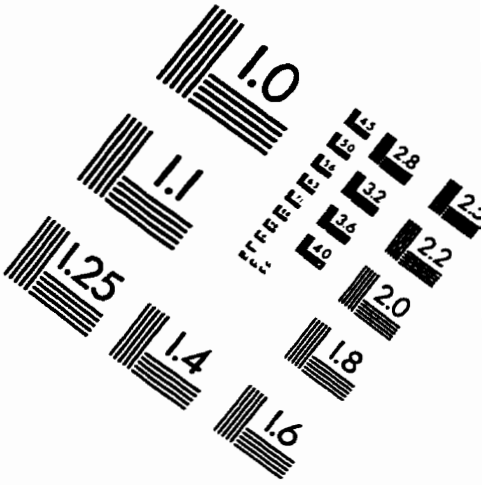
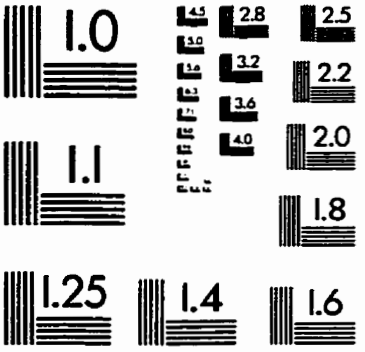
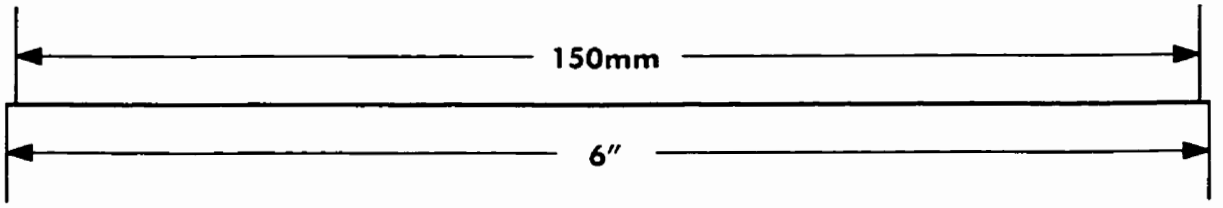
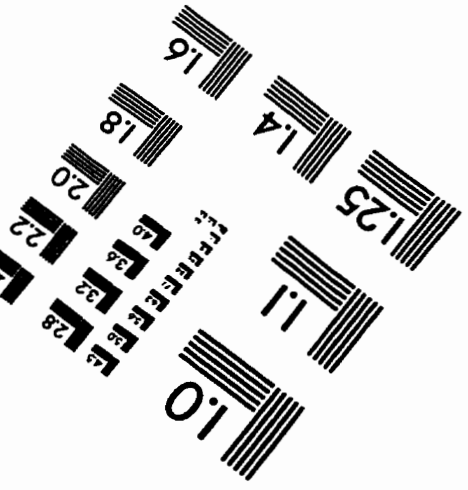
IMAGE EVALUATION
TEST TARGET (QA-3)

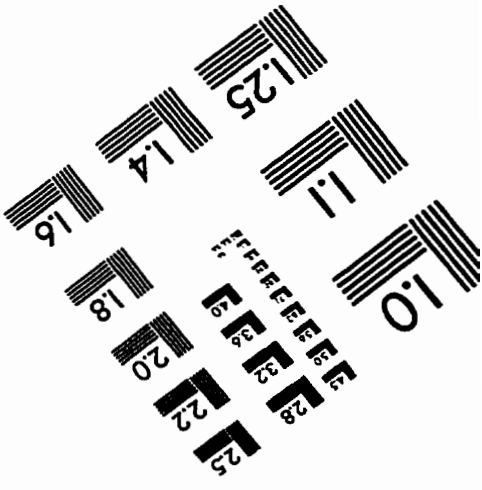












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