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Molecular cloning and characterization of a putative voltage-insensitive calcium channel from non-excitable cells

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MOLECULAR CLONING AND CHARACTERIZATION OF A PUTATIVE VOLTAGE-INSENSITIVE CALCIUM CHANNEL FROM NON-EXCITABLE CELLS

by

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Abstract

MOLECULAR CLONING AND CHARACTERIZATION OF A PUTATIVE VOLTAGE-INSENSITIVE CALCIUM CHANNEL FROM NON-EXCITABLE CELLS

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Yongsheng Ma

Adviser: Dr. Andrew R. Marks

Voltage-gated Ca^{2+} channels have been cloned and characterized from excitable cells including nerve and muscle. Analysis of the primary structures of these channels have shown that they share a common structural motif consisting of 24 putative transmembrane segments organized in four repeats. Voltage sensors are proposed to reside at the fourth transmembrane segment of each repeat. These channels play important roles in many cellular functions including propagation of action potentials, muscle excitation-contraction coupling, maintenance of electrical activity, excitation-secretion coupling, and neurotransmitter regulation. In non-excitable cells, a Ca^{2+} channel has been postulated to exist based on electrophysiological studies [Gardner, 1990]. The primary structure of this Ca^{2+} channel in a non-excitable cell is, however, not known. In this study, murine erythroleukemia cells (MELC) were used as a model for non-excitable cells and the calcium dependence of HMBA-induced MELC differentiation was demonstrated [Gillo et al.,

1993]. Whole-cell patch clamp did not detect voltage-gated Ca^{2+} channels in MELC cells [Gillo et al., 1993]. However, MELC did exhibit a dihydropyridine (DHP)-sensitive calcium influx [Gillo et al., 1993]. This finding stimulated us to clone and characterize a putative Ca^{2+} channel from MELC and the resulting clone was termed the MELC Ca^{2+} channel (MELC-CC). Analysis of the MELC-CC cDNA sequence revealed that it is a novel isoform of the cardiac voltage-gated Ca^{2+} channel. The MELC-CC is truncated at its amino terminus, lacking the four putative transmembrane segments designated IS1 through IS4. This finding suggests that the MELC-CC could have physiological properties that differ from those in the voltage-dependent Ca^{2+} channel of excitable cells.

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CHAPTER I: INTRODUCTION

SIGNIFICANCE

MELC is a virally transformed erythrocyte cell line [Friend 1971, 1977]. It is arrested in erythroid development at a stage comparable to the erythroid colony-forming unit (CFU-e) and can grow indefinitely under appropriate culture conditions. MELC can be induced to terminal differentiation when cultured with a number of chemicals, including the polar/apolar compound hexamethylene bisacetamide (HMBA) [Marks, 1978, 1989]. It is known that changes in the intracellular levels of Ca^{2+} play a crucial role in MELC differentiation and we have recently reported that Ca^{2+} is required during HMBA-induced differentiation [Gillo et al., 1993]. However, the mechanism by which Ca^{2+} enters MELC during induced differentiation has not been determined. The MELC-CC is a candidate molecule whose function may be to mediate Ca^{2+} influx and regulate intracellular Ca^{2+} levels.

The putative channel mediating Ca^{2+} influx in MELC is dihydropyridine (DHP)-sensitive [Gillo et al., 1993]. DHP belongs to a group of structurally unrelated molecules that block voltage-dependent Ca^{2+} channels [Catterall, 1988]. These drugs are widely used clinically for the treatment of angina and hypertension. The molecular cloning and characterization of the primary structure of the MELC-CC and the pharmacology of Ca^{2+} entry into MELC should provide a basis for a better understanding of the role of Ca^{2+} in cellular differentiation and of possible mechanisms by which intracellular Ca^{2+} concentration can be modulated in non-excitable cells. Furthermore, such studies may help to elucidate how cyto-differentiation agents can overcome maturation defects commonly

associated with cellular transformation and induce loss of proliferative capacity. Moreover, examination of the primary structure of Ca^{2+} channels from cells which do not express the L-type currents characteristic of voltage-dependent Ca^{2+} channels (VDCC) should provide insights into the structural components responsible for voltage activation of the VDCC.

OVERVIEW

In 1958 Fatt and Ginsberg first described the inflow of Ca^{2+} ions across the plasma membrane of crayfish muscle fibers [Fatt and Ginsberg, 1958]. At that time, it was not known whether other cells had the same influx of Ca^{2+} ions since the methods available for recording currents in living cells typically had high background noise. Moreover, no direct evidence for the existence of channels was available from biological preparations. In 1976, a patch-clamp technique was developed for the study of ion channels [Neher and Sakmann, 1976]. A Ca^{2+} channel mediating the influx of Ca^{2+} ions was recorded in muscle cells, neurons, eggs, hybridomas and other cell types by Hagiwara and his co-workers [Hagiwara et al., 1983]. A group of compounds termed "calcium antagonists" were identified that block ion flux through these Ca^{2+} channels [Lee and Tsien, 1983]. These compounds block ion flux by binding to two distinct sites located on the Ca^{2+} antagonist receptor [Triggle, 1983]. At the same time evidence accumulated that Ca^{2+} ions could act as intracellular messengers that control cellular functions in many cellular systems. With the realization of the importance of Ca^{2+} as a second messenger in cellular processes, attention was directed towards the molecules

mediating Ca^{2+} fluxes, and the mechanisms by which Ca^{2+} fluxes were regulated.

In 1984, using [^{3}H] nitrendipine (a Ca^{2+} channel antagonist), the dihydropyridine-receptor (DHP-R) was isolated from skeletal muscle transverse tubules [Curtis and Catterall, 1984]. Since 1987, cDNAs encoding the α_1 subunits of Ca^{2+} channels have been cloned by several laboratories [Tanabe, 1987, Mikami, 1989, Koch et al., 1990, Hui et al., 1991, Snutch et al., 1991]. Currently it is known that Ca^{2+} channels, together with other Ca^{2+} regulatory proteins including Ca^{2+} pumps (both plasma membrane and sarcoplasmic reticulum forms) and Ca^{2+}/Na^{+} exchangers, are distributed on the plasma membrane as well as the membranes of intracellular organelles. Table 1 illustrates different pathways in which Ca^{2+} fluxes are mediated.

Table 1

Ca Transporting Systems of Cell Membranes

Transporting mode	Membrane	Ca affinity
Channels	Plasma membranes Endo(sarco)plasmic reticulum	Low
ATPases	Plasma membranes Endo(sarco)plasmic reticulum	High
Exchangers(Na/Ca)	Plasma membranes Inner mitochondrial membrane	Low
Electrophoretic uniporters	Inner mitochondrial membrane	Low

The development of experimental techniques allowing Ca^{2+} measurements using fluorescent Ca^{2+} indicators, quin2, fura2 [Grynkyewicz et al., 1985] and fluo-3 [Minta, 1989], the refinement of biochemical studies, and the application of recombinant DNA techniques, has led to the elucidation of the structure and function of a variety of Ca^{2+} channels from nerve, muscle and other excitable cells [DeCoursey et al., 1984, Tanabe, 1987, Mikami, 1989, Matteson and Deutsch, 1984, Koch et al., 1990, Gerasimenko et al., 1991, Hui et al., 1991, Preston et al., 1991, Snutch et al., 1991, Hoth and Penner, 1992, Lechleiter and Clapham, 1992].

Calcium and calcium regulators

Calcium acts as a ubiquitous second messenger in diverse signaling systems. At steady state, Ca^{2+} is distributed unevenly throughout the cell with the lowest concentration in the cytoplasm. In normal resting cells the cytoplasmic free Ca^{2+} concentration is low (10^{-7} M), but extracellular and organellar (e.g. ER) $[Ca^{2+}]$ are approximately four orders of magnitude higher [Janis and Triggle, 1983]. The steady-state intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) may be altered by changes in membrane potential or by agonist stimulation that results in increased Ca^{2+} flux into the cytoplasm.

In excitable cells, cytoplasmic free Ca^{2+} concentration is regulated by Ca^{2+} influx through the voltage-dependent Ca^{2+} channels (VDCC) on the plasma membrane and Ca^{2+} release into the cytoplasm via the inositol 1,4,5-trisphosphate (IP_3) receptor, located on the ER, and via the ryanodine receptor on the sarcoplasmic reticulum. In the cytoplasm, Ca^{2+} ions bind to Ca^{2+} binding proteins which contain

multiple EF hand structures [Moews and Kretsinger, 1975], including troponin and calmodulin. They, in turn, activate enzymes participating in downstream cellular events. A 1 μM rise in free cytosolic Ca^{2+} triggers cellular responses, including muscle contraction and hormone secretion and T-cell activation. In response to increasing $[Ca^{2+}]_i$, Ca^{2+}-ATPases are activated to pump Ca^{2+} ions back across the plasma membrane to the extracellular space or into the lumen of endoplasmic reticulum (ER) or other intracellular vesicles that store Ca^{2+} thus maintaining normal $[Ca^{2+}]_i$.

Other mechanisms for regulating Ca^{2+} influx in both excitable and non excitable cells have also been proposed; 1) a receptor-operated Ca^{2+} channel (ROCC) coupled to second messengers; 2) a Ca^{2+} leak channel dependent on the electrochemical gradient for Ca^{2+}; 3) a stretch-activated non selective cation channel; 4) internal Na^+-dependent Ca^{2+} entry (Na^+-Ca^{2+} exchange); and 5) the currently proposed voltage-independent Ca^{2+} channel (VICC).

Ca^{2+} channels

Ca^{2+} channels are macromolecular structures defining pores in both the plasma membrane and the membranes of intracellular organelles. They have the important property of selective permeability that allows some restricted class of ions to flow passively down their electrochemical gradients at rates exceeding 10^6 ions per second [Mason et al., 1986]. Based on tissue localization and activation mechanisms, three main groups of Ca^{2+} channels have been described: 1) VDCCs, 2) intracellular calcium release channels (CRC), and 3) receptor mediated Ca^{2+} channels.

1) *Voltage-dependent Ca^{2+} channel*

VDCC are multi-subunit complexes mediating Ca^{2+} influx. The best characterized VDCC is the L-type Ca^{2+} channel from skeletal muscle. It is a complex of five subunits: α_1, β, γ, α_2 and δ [Catterall, 1988]. The primary structure of each of these five subunits has been determined by cDNA cloning [Tanabe et al., 1987, Jay et al., 1990, Ruth et al., 1989]. Co-immunoprecipitation with specific antibodies against the subunits and co-sedimentation on sucrose gradients has demonstrated the association of the five subunits forming the Ca^{2+} channel complex [Catterall, 1988, Vaghy et al., 1988]. However, the α_1 subunit itself can form a functional Ca^{2+} channel as demonstrated by expression of the α_1 subunit alone in L-cells [Perez-Reyes et al., 1989, Lacerda, 1991]. The proteins comprising the VDCCs include transmembrane domains, Ca^{2+} flux pores, electrical sensing domains [Vaghy et al., 1988; Naito et al., 1989]. The VDCCs also serve as pharmacological receptors, with specific sites for activator and antagonist ligands linked to channel function.

On the basis of distinct biophysical and pharmacological properties, VDCCs have been further classified into L type, T type, N type and P type [Hess et al., 1985, Nowycky et al., 1985, McCleskey et al., 1986, Llinas et al., 1989, Miller, 1992]. Together they form a VDCCs super family. Included in this family of VDCCs is the L-type cardiac DHP-R, the primary structure of which has been determined by cDNA cloning [Mikami, 1989]. It is an integral membrane protein. It is evolutionarily related to other ion channels by virtue of a highly conserved primary structure consisting of four internally repeated motifs comprising 24 putative transmembrane segments. Each motif

has six putative transmembrane segments. The fourth transmembrane segment in each motif contains positively charged amino acids, arginine or lysine, at every 3rd or 4th residue. Mutational studies have demonstrated that these regions contribute to the voltage-sensor function of the channels [Stuhmer, 1989]. Sequence comparison of the cardiac DHP-R α_1 subunit to other α_1 subunits cloned to date, reveal a high degree of sequence diversity in the intracellular and extracellular regions of the molecule and a high degree of homology in the transmembrane regions. The intracellular regions also contain putative protein kinase phosphorylation sites, suggesting the involvement of the channel in a regulatory function. There is no evidence for glycosylation on the α_1 subunit of the DHP-R from skeletal muscle [Takahashi et al., 1987]. Whether the α_1 subunit of the cardiac DHP-R is glycosylated is not known.

It is a common feature for many membrane proteins to have a stretch of 16-30 amino acid residues on their N-terminals serving as signals for translocating to the ER. The hydrophobicity of this stretch of residues and one or more positively charged amino acids near its N-terminus are the characteristics [Walter, 1984]. However, the NH_2-terminal region of the α_1 subunits of L-type Ca^{2+} channels shows no hydrophobic segment when analyzed by the hydropathy plots, thus a leader signal sequence has not been identified.

2) *Intracellular Ca^{2+} release channel*

CRCs are the proteins located on the organelle and plasma membranes mediating Ca^{2+} release. The current known intracellular

calcium release channels include IP_3 receptors and ryanodine receptors.

IP_3 receptors have been found on the endoplasmic (sarcoplasmic) reticulum of many cell types and on the plasma membrane of T lymphocytes [Khan et al., 1992]. The primary structure and function of IP_3 receptors have also been studied [Ferris et al., 1989; Chadwick et al., 1990; Furuichi et al., 1989]. This phosphoinositol pathway is initiated by the G-protein coupled activation of cell surface receptors that in turn activates phospholipase C which converts phosphoinositides to diacylglycerol (DAG) and IP_3. IP_3 binds to the IP_3 receptor on the ER resulting in release of intracellular Ca^{2+} and Ca^{2+} influx [Khan et al., 1992].

The ryanodine receptor is a protein with high affinity for the plant alkaloid ryanodine. The primary structure and functional expression from cDNA of the cardiac ryanodine receptor has been determined [Nakai et al., 1990]. It is a tetramer composed of four 565 kD subunits with a total molecular mass of 2.3 million Daltons. It is localized to the terminal cisternae of sarcoplasmic reticulum (SR). Hydropathy plot analysis of the deduced amino acid sequence of a single subunit predicts four possible transmembrane regions near the carboxyl terminus of the polypeptide thought to form the "baseplate". The amino terminal nine tenths of the molecule is thought to form the cytoplasmic foot structure. The cDNA of the skeletal muscle ryanodine receptor has been cloned and characterized [Takahashi et al., 1989, Marks et al., 1989 and Otsu et al., 1990].

3) *Receptor mediated Ca^{2+} channels*

Ca^{2+} influx through receptor mediated Ca^{2+} channels is resulted from the receptor occupation and not dependent on membrane depolarization. A receptor mediated Ca^{2+} channel was first suggested in smooth muscle cells [Bolton, 1979]. In smooth muscle, ligands stimulated a rise in $[Ca^{2+}]_i$ via Ca^{2+} influx while the membrane potential was held constant and VDCCs were blocked with specific Ca^{2+} channel antagonists [Banham and Tsien, 1987]. The receptor mediated Ca^{2+} channels have also been postulated to explain Ca^{2+} entry into cells that are not electrically excitable. One example is thrombin-stimulated platelets [Zschauer, 1988]. Evidence favoring the existence of receptor mediated Ca^{2+} channel includes the observation that activation of a variety of cell surface receptors results in a biphasic increase in $[Ca^{2+}]_i$ due to the release or mobilization of intracellular Ca^{2+} stores and to the entry of Ca^{2+} from the extracellular space [Hughes, 1988, Keller et al., 1992]. It is proposed that receptor mediated Ca^{2+} channel may participate in signaling during cell growth, differentiation, T cell activation and possibly in modulating contraction and secretion [Benham and Tsien, 1987; Clementi et al., 1992]. However, the existence of a calcium-specific receptor mediated Ca^{2+} channel on non excitable cell membranes has not yet been directly proven and their structure and function remain unknown. A receptor mediated Ca^{2+} channel reported by Zschauer showed properties in common with VDCCs such as a high degree of selectivity for divalent cations, a single channel conductance of ~10 pS (in 150 mM Ba^{2+}), and sensitivity to blockade by inorganic Ca^{2+} channel blockers such as Ni^{2+}. However, there are

major differences between the two classes, for example, receptor mediated Ca^{2+} channels are not voltage-dependent and are not blocked by DHP sensitive Ca^{2+} channel antagonists.

Cyclic nucleotide-gated ion channels from sensory neurons, such as photoreceptor cells and olfactory sensory neurons, have been cloned and characterized [Kaupp et al., 1989, Goulding et al., 1992]. These channels directly respond to the elevations of cyclic nucleotide and mediate ion flux leading to changes in membrane potential. Receptor and stretch-activated channels that promote Ca^{2+} entry into endothelial cells appear to be non selective cation channels [Lansman et al., 1987].

Isoforms of the Ca^{2+} channels

Ca^{2+} channels are a diverse class of molecules found in all excitable cells. They share an overall structural similarity but exhibit numerous isoforms. Multiple isoforms can be expressed within a single cell.

Diversity within the Ca^{2+} channel gene family is due in part to the expression of distinct α_1 subunit genes as well as to alternative splicing [Perez-Reyes et al., 1990, 1991]. Tissue-specific variability also accounts for some of the differences [McKenna et al., 1990]. cDNA sequence comparison between the cardiac and smooth muscle α_1 subunits reveals that they are encoded by a single gene [Perez-Reyes et al., 1989; Biel et al., 1990] that is distinct from the gene encoding the skeletal muscle α_1 subunit. In rat brain, at least four different Ca^{2+} channels are expressed, each encoded by a distinct gene as demonstrated by Southern blot and DNA sequencing analysis

[Snutch et al., 1990]. These molecules have been named class A, B, C and D. The molecule of the class C type shares 90-97% identity with that found in cardiac muscle, rabbit lung, and rat aorta [Snutch et al., 1991, Mikami et al., 1989, Biel et al., 1990, Koch, 1990]. Polymerase chain reaction studies have provided evidence that the diversity is derived from the alternate use of equal sized exons encoding the IVS3 transmembrane region [Perez-Reyes et al., 1990]. Using genomic sequence analysis and S1 nuclease protection assays, two groups have demonstrated that the rat brain class C Ca^{2+} channel variability arises from developmentally regulated, mutually exclusive splicing of a single primary transcript [Snutch et al., 1991; Diebold et al., 1992]. Human fibroblasts contain only the cardiac Ca^{2+} channel subtype, which exhibits at least four sites of molecular diversity due to alternative splicing at the IIS6, IIIS2, IVS3 and C-terminal regions. Interestingly, the IVS3 transmembrane segment, which is the product of mutually exclusive expression of one of two exons in rabbit lung and aorta, and rat brain, is produced by mutually permissive expression of the same two exons in human fibroblasts [Nikolai, 1992].

The properties and modulation of the L-type Ca^{2+} channel

L-type Ca^{2+} channels are activated by membrane depolarization and transduce electrical signals into chemical signals. Functional similarities among L-type Ca^{2+} channels include: (1) slow, long-lasting currents and unitary conductance with mono and divalent charge carriers; (2) high affinity for dihydropyridines and sensitivities to both inorganic and organic Ca^{2+} channel blockers and a stereotypic

response to organic Ca^{2+} channel agonists; (3) regulation by cyclic AMP-dependent events; (4) slow inactivation when Ca^{2+} is not the charge carrier; (5) steady-state inactivation only at positive holding potentials; (6) lability of channel function in a cell-free environment [Bean, 1989, Hosey et al., 1988, Hess, 1990].

Whole-cell clamping and inside-out excised patch recording are techniques well suited to studying channel modulation [Neher, 1983]. These techniques monitor channel function under conditions where the molecular composition of the cytoplasmic and extracellular solutions can be controlled. The best-established mechanism of Ca^{2+} channel modulation is that of the L-type Ca^{2+} channels mediated by cAMP-dependent kinase. Both intracellular and extracellular stimulation can alter the function of these channels and modulate them [Hess, 1990, Trautwein and Hescheler, 1990]. Ca^{2+} current is activated by: 1) cAMP; 2) protein kinase A in a pre-activated form that requires no cAMP; 3) by activation of endogenous protein kinase C by the tumor-promoting phorbol ester TPA (Tetradecanoylphorbol-Acetate); 4) intracellular injection of the purified enzyme [DeRiemer, 1985]. An increasing number of channels are now known to be modulated by these molecules. In addition, the activation of protein kinase C [Lacerda et al., 1988], internal GTP binding proteins, specifically G_S and its subunits, [Yatani et al., 1987, 1988] and internal inositol trisphosphate [Vilven and Coronado, 1988] have also been postulated to modulate DHP-sensitive Ca^{2+} channels. This modulation may involve phosphorylation of the channel itself as the last step of the signaling cascade. Calcineurin is a Ca^{2+} sensitive protein phosphatase that removes the phosphate from the activated

channels and results in channel inactivation [Eckert, 1984, Armstrong, 1989].

Biochemical studies using intact muscle cells demonstrated that the effects of cAMP-elevating agents on channel function *in vivo* was due to the phosphorylation on the α_1 subunit of the Ca^{2+} channel [Cecilia et al., 1991]. It was shown that cAMP-dependent phosphorylation with β-adrenergic stimulation increases current through cardiac L-type Ca^{2+} channels. Single channel recording showed that the increase of the current was due to a shift in the gating mode rather than causing more channels to be inserted in the membrane [Yue, 1990].

Divalent ions, including Ni^{2+}, Cd^{2+}, Co^{2+}, Mn^{2+} and La^{3+}, can block Ca^{2+} channels [Hille, 1992]. Because these ions move so slowly in the pore they interfere with more permeant ions, which then must wait their turn. Many pharmacological agents can modulate the Ca^{2+} channel properties and block or activate the channels. Because they are not particularly specific to Ca^{2+} channels, high concentrations can depress Na^+ and K^+ channel currents as well [Hille, 1992].

Model of Ca^{2+} channel structure/function relationships

It is thought that the Ca^{2+} channel pore is formed by four transmembrane domains, each of which contributes one fourth of the pore wall. H5 (or SS1 and SS2 or S7 and S8) was first proposed by Guy and Seetharamulu (1986) and Greenblatt et al. (1985). Later H5 was proposed to be membrane associated and to contribute to pore formation by Guy (1990) (Figure 1).

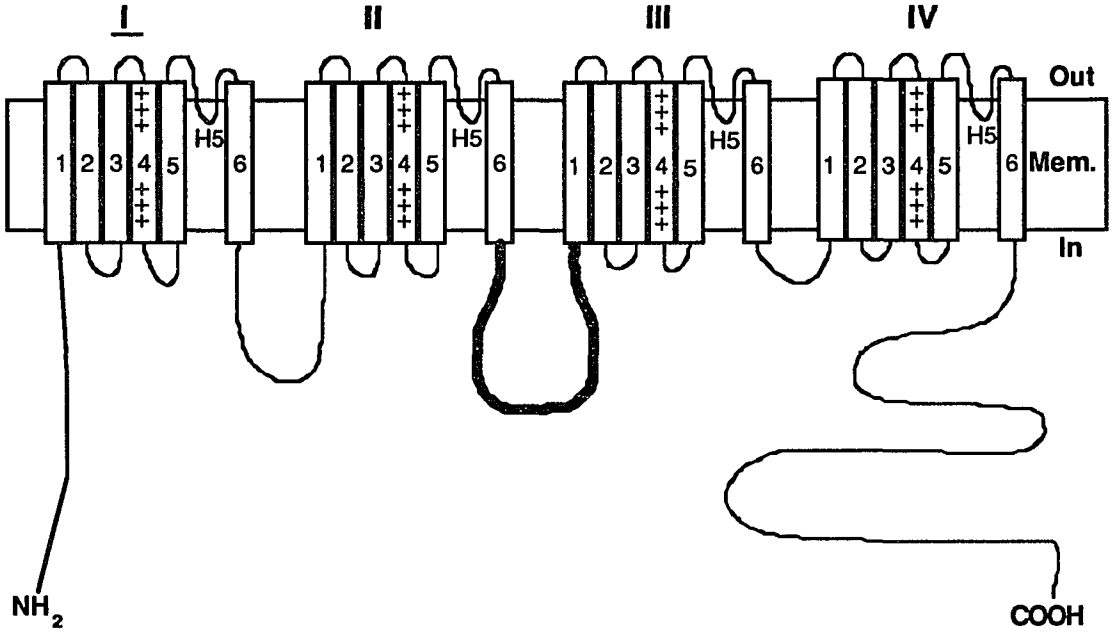


Figure 1. Schematic representation of voltage-dependent calcium channel membrane topography. Primary sequence analysis of VDCC predicts four motifs (I to IV), each containing six transmembrane regions (rectangles labeled 1-6). A positively charged S4 region(+) exists within each motif that has been suggested to be responsible for the voltage sensing. Based on studies from the K^+ channel, it has been proposed that the H5 region encodes putative channel lining sequences. The bold cytoplasmic loop between the repeat II and III shows the region critical for excitation-contraction coupling [Tanabe et al., 1990]. Repeat I is underlined to indicate its critical role in determining calcium channel activation kinetics [Tanabe et al., 1991]. The linker region between S5 and S6 in motif IV of the L-type Ca^{2+} channel has been suggested as a major site for DHP action [Tang et al., 1993].

It has been postulated that ion selectivity is due to the structural differences of the ion-conducting pores. Therefore, mutation or replacement of amino acids in the putative pore have been used as a way to study its structure. It has been found that the sodium channel differs from other members of the voltage-gated ion channels in the sequence within the H5 segment. Negatively charged glutamic acids, found in all four H5 segments in K^+ and Ca^{2+} channels were used to replace the positively and uncharged residues in the equivalent positions in the sodium channel and changed the ion selectivity form sodium to calcium [Stefan, 1992].

Sensitivity and high affinity to DHP is a distinct characteristic that differentiates L-type Ca^{2+} channels from other type channels. The DHP-binding site has been proposed to comprise a combination of the extracellular ends of the S6 helices of the third and fourth repeating domains as well as the loop linking S5 and S6 of the third domain, using a photo affinity labeling technique [Nakayama et al., 1991]. Recently, chimeric channels were created in which portions of motif III or motif IV of a DHP-insensitive brain Ca^{2+} channel were introduced into the DHP-sensitive cardiac L-type Ca^{2+} channel. These chimeric channels were expressed in oocytes and the effects of a DHP agonist and antagonist showed that the linker region between S5 and S6 in motif IV of the L-type Ca^{2+} channel is a major site for DHP action [Tang et al., 1993]. However, deletion of the putative DHP-binding sites in motifs III and IV failed to eliminate DHP binding indicating that these sequences are not required for formation of the ligand binding site as predicted by photo affinity labeling experiments. Based on the above information, Ca^{2+} channels can be

viewed as a cylindrically symmetric aggregate of multiple subunits [or of tandem-repeated homologous domains within a single polypeptide] with the ion conduction pore located on the axis of symmetry. However, elucidating the three dimensional structure will have to rely on biophysical studies using crystallography with atomic resolution [Ponder, 1987, Bowie, 1990].

The functional significance of each motif, and the putative cytoplasmic regions linking the repeats and the amino and carboxyl-terminal regions have also been studied. Repeat I of the DHP-R is critical in determining Ca^{2+} channel activation kinetics [Tanabe et al., 1991]. The current produced with the chimeric constructs carrying the cardiac repeat I resembles the current produced by the native cardiac muscle DHP-R. Whereas the current produced with the chimeric constructs carrying the skeletal repeat I resembles the current produced by the native skeletal muscle DHP-R. The region of the skeletal muscle DHP-R required for excitation-contraction coupling has been localized to the cytoplasmic loop between repeats II and III [Tanabe et al., 1990]. The chimeric construct containing skeletal II-III cytoplasmic loop expressed in dysgenic myotubes shows skeletal-type E-C coupling while the loop of I-II is more functionally interchangeable between the cardiac and skeletal muscle DHP-R. Single amino-acid substitutions in the regions encompassing the short segment SS2 of the third and/or fourth repeats of sodium channel (such as replacing lysine at position 1422 in repeat III and /or alanine at position 1714 in repeat IV of rat sodium channel II with the glutamic acid) can alter the ion-selectivity of the sodium channel to resemble those of Ca^{2+} channels, suggesting that these

residues constitute part of the selectivity filter of the channel [Heinemann et al., 1992]. Other regions of the channel appear to be less sensitive to structural alterations. For example, a 211 amino-acid deletion at the carboxyl-terminus of the skeletal muscle DHP-R does not affect its function [Beam et al., 1992].

STATEMENT OF THE THESIS

Calcium influx plays a critical role in cell signal transduction and differentiation. While in excitable cells Ca^{2+} influx is effected through a voltage-dependent channel, in non excitable cells Ca^{2+} influx may involve a voltage-insensitive Ca^{2+} channel. MELC were chosen as a model system of non-excitable cells to study voltage insensitive Ca^{2+} channels for the following reasons: 1) MELC are non-excitable erythroid precursor cells; 2) inward Ca^{2+} fluxes are important for MELC growth and differentiation; 3) voltage-activated Ca^{2+} current has not been detected in these cells using the whole cell patch-clamp technique; 4) MELC have been studied extensively as a model for understanding the molecular control of cellular differentiation; and 5) MELC differentiation closely resembles the process of normal murine erythropoiesis [Harrison, 1976].

MELC can be maintained in an undifferentiated state indefinitely in an α-MEM medium. The polar/apolar compound hexamethylene bisacetamide (HMBA) induces MELC to enter the differentiation process leading to a mature red blood cell phenotype. MELC committed to terminal differentiation can be identified using a "commitment assay". Cells that are irreversibly committed to terminal differentiation exhibit limited cell division manifested by small colonies that do not express hemoglobin after culture in semisolid media. Terminal differentiation can be unequivocally assayed due to the expression of hemoglobin and the loss of the ability to proliferate [Marks et al., 1989]. It has been observed that several events leading to terminal differentiation are closely related

to Ca^{2+} influx in MELC [Smith et al., 1982; Bridges et al., 1981; Levenson et al., 1980 and 1982]. In addition to the versatility of this system to clone genes specific for erythroid differentiation, MELC provide a model for studying the role of Ca^{2+} influx in early steps of commitment to differentiation. Also, MELC offer the opportunity to study a single cell type instead of a mixture of cell types, as in lung or brain.

These features of MELC provide an excellent model to study the molecules involved in Ca^{2+} influx required for differentiation. Given the absence of a VDCC in MELC and the presence of a DHP-sensitive Ca^{2+} influx [Gillo, 1993], I propose to determine the structure of a putative voltage-insensitive Ca^{2+} channel that may play a critical role during MELC differentiation. My hypothesis is that this putative Ca^{2+} channel is structurally related to, but distinct from, the DHP-R of excitable cells and has unique functional properties that define a novel class of VICCs.

CHAPTER II: MATERIALS AND METHODS

MELC cell lines and cell culture

MELC [Friend et al., 1971] strain SC9 (a gift from Dr. Victoria Richon, Memorial Sloan-Kettering Cancer Center, New York, NY), was maintained in alpha minimal essential medium (α-MEM) containing 10% fetal calf serum [Melloni et al., 1988]. The cells were grown in medium without antibiotic supplement whenever possible in order to minimize side effects. Cell cultures for all experiments were initiated with cells in logarithmic growth phase at a density of 10^5 cells/ml and the cell density was always kept below 1.6 X 10^6 cells/ml to permit continuous proliferation. The cultures were grown in loosely capped 200 ml flasks incubated at 37° C in a humidified 5% CO_2 atmosphere. HMBA (Sigma) was prepared as 40X concentrated stock in α-MEM media without fetal calf serum and added to the cultures at a final concentration of 5 mM. For routine passage of cultures, cells were pelleted at 500 X g for 10 min and resuspended in fresh α-MEM every 2-3 days. To start a new culture, a vial was removed from liquid nitrogen and quickly thawed in a 37° C water bath while agitating. The contents were transferred to a 15 ml tube and an equal volume of medium with 10% fetal calf serum prewarmed to 37° C was added to dilute and disperse the cells gently. The cells were maintained for 5 minutes at room temperature and the dilution and dispersion was repeated two more times. Then the cells were pelleted at 500 X g for 5 minutes and subsequently plated in culture at approximately 2 X 10^5 cells/ml [Friend et al., 1966]. Benzidine staining was used to assay for the cell differentiation based on the detection of hemoglobin which stains blue with benzidine [Fibach et al., 1977].

Commitment assay

Commitment to differentiation, characterized by irreversible induction of differentiation and limited cell division (small colony size), was assayed by subcloning aliquots of cells removed from suspension culture onto semi-solid medium (as described below) that lacked inducers. The phenotype of the resulting colonies was determined after 4 days of growth [Gusella, 1976, Levenson et al., 1980]. To assess commitment to differentiation, MELC were removed from induced suspension cultures, washed twice with phosphate-buffered saline to remove HMBA and other chemicals. Approximately 500 cells/ml were added to α-MEM media containing 2.2% methylcellulose and mixed in a syringe by gently inverting 50 times. The mixture was then plated on culture dishes and incubated at 37^0 C with humidified 5% CO_2. The proportion of cells committed to differentiate was determined 4 days later by scoring the number of benzidine reactive colonies with <64 cells [Fibach et al., 1977]. A minimum of 200 colonies was scored for each experiment.

Characterization of changes in intracellular Ca^{2+} with Fluo3

Changes in cytosolic Ca^{2+} in single MELC were monitored by measuring the fluorescence of cells loaded with the Ca^{2+} sensitive dye Fluo 3 [Minta et al., 1989]. The medium used in the assay was EBSS-H, a modification of Earle's balanced salts (EBSS) in which sodium bicarbonate is replaced with 26 mmol/L HEPES pH 7.4 and 0.1% bovine serum albumin (Sigma). MELC cell suspensions washed with EBSS-H were added to coverslips and incubated for 1 hour at 37^0 C in a humidified incubator. Cells were loaded with EBSS-H solution

containing 2μM Fluo 3 (Molecular Probes) and 0.09% of pluronic F-127 with a subsequent incubation at 37° C for 30 min. Cells were washed once with EBSS-H and incubated in EBSS-H for 30 min at 37° C. Coverslips were mounted on a microscope tissue chamber (Biophisica Technologies). Cells were constantly perfused using experimental solutions, with or without drugs, at a rate of 2 to 3 ml/min. Fluorescence was measured using a Zeiss inverted microscope (IM 35) equipped with a 40X oil-immersion objective, a photometer head (PMQII), and MAC 1000 controller which had two filter wheels for dual excitation and emission fluorescent dyes (Ludl Electronic Products, Scarsdale NY). Data analysis was with SCOPE software (Kinetek, Yonkers, NY). The following filters were used: Fluo-3, excitation filter 485DF22, emission filter 530DF30, dichroic beam splitter 505DRLP (all from Omega Optics Inc., Brattleboro, VT).

Probes

1. cDNA probes

Fifty ng of plasmid DNA or PCR products in 10 μl of nuclease free H_2O were boiled in a 1.5 ml eppendorf tube for 5 minutes to denature the DNA followed by submersion of the tube in ice water for 2 minutes. Following the denaturation, random priming labeling reaction was performed in 25 μl of reaction volume by adding 10 μl of labeling buffer (which consists of: 6 μg/ml of hexadeoxy-ribonucleotides, 440 mM of HEPES pH 6.6, 110 mM Tris pH 8.0, 11 mM of $MgCl_2$, 22 mM β-mecaptoethanol, 44 mM of each of dATP, dGTP and dTTP), 5 μl of α ^{32}P dCTP (NEN), and 5 units of DNA polymerase I-Klenow fragment. After one hour incubation at 37° C

the probe was purified as follows. The reaction mixture was loaded on a Sephadex G-50 column in a 1 cc syringe and washed with 150 µl of TE buffer pH 8.0 by centrifugation at 500X g for 30 seconds. A 1 µl aliquot of the probe was taken and mixed with 5 ml liquid scintillation solution and measured in the scintillation counter. The probe was denatured in a boiling H_2O bath for 5 minutes and then transferred directly to ice cold hybridization buffer with minimum final concentration of 1 million cpm/ml solution.

2. Oligo probes

Five hundred ng of a synthetic oligonucleotide (18-25 bases in length) was mixed with 50 mM Tris-HCl pH 7.8; 10mM $MgCl_2$, 5mM DTT, 0.1 mM spermidine, 100 µCi γ-^{32}P ATP (3000 Ci/mM) (NEN), and 20 U of T4 polynucleotide kinase in 50 µl reaction volume. The reaction was incubated at 37^0 C for one hour and the probe purification was carried out as described for the cDNA probe. For primer extension assay, the labeled oligo probes were electrophoretically separated in an 8% denaturing acrylamide gel. The radioactive bands on the gel were excised, transferred to an eppendorf tube, and eluted in 300 µl elution buffer containing 0.5M NH_4OAc, 1mM EDTA, and 0.2% SDS. A one microliter aliquot of the probe was taken for the measurement of cpm using a scintillation counter.

3. cRNA probes

Five to ten µg of the plasmid of interest were linearized with an appropriate restriction enzyme such that the bacteriophage T7, T3 or

SP6 promoter on the vector could be used to direct the synthesis of a radio-labeled antisense cRNA probe with a defined length. The linearized DNA was purified with phenol, phenol/chloroform, followed by chloroform and precipitated with 75% ETOH. The DNA pellet was then dissolved in DEPC treated H_2O. One μg of this template DNA was mixed with: 20mM DTT, 1mM of ATP, 1m of GTP, 1mM of CTP, 40 mM Tris-HCl (pH 7.5), 10mM $MgCl_2$, 50 ng/ml BSA, 25 U ribonuclease inhibitor, 5 μl of α-^{32}P CTP (800 Ci/mmol), and 10 U of a proper RNA polymerase, then incubated for 50 minutes in a 25^0 C water bath. Two units of DNAase I were added to the reaction to remove template DNA. Probes were electrophoretically separated on a 6% denaturing acrylamide gel at 200 volts for 1.5 hour. Radioactive bands of expected length localized by autoradiography were excised and transferred into an eppendorf tube containing 300 μls of elution buffer (0.5M NH_4OAc, 1mM EDTA and 0.2% SDS). The cRNA probe was eluted for 30 min at room temperature with gentle shaking (Vortex, speed 4). A 1 μl aliquot of the probe was taken to measure the cpm in a scintillation counter and the remainder of the probe was stored at -20^0 C until use within one week.

RNA Purification

1. Total RNA isolation

Total cellular RNA was purified from MELC using the standard guanidinium isothiocyanate/cesium chloride centrifugation method with minor modifications [Marks et al., 1989]. The exact protocol is as follows. A one ml cell pellet was resuspended and pelleted at 1000 X g for 5 minutes twice with 10 volumes of ice-cold PBS lacking

calcium and magnesium ions. The pellet was then resuspended in 10 volumes of the lysis buffer containing: 4M Guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% N-lauryl sarcosyl and 0.7% β-mercaptoethanol. This mixture was passed through a 22 gauge needle for three forceful rounds to sheer the DNA. The suspension was layered on top of a 4 ml 5.7M cesium chloride cushion in a Beckman 16 x 76 mm quickseal tube. Taking care not to mix the two layers during loading, the sample was spun in a Beckman 75Ti rotor at 40K rpm at 18° C for 18 hours. The top of the tube was opened using a razor blade and the supernatant was sucked off with a Pasture pipette under gentle vacuum. The pellet was gently rinsed once with 500 ml of 75% ice cold ETOH and resuspended in 400 μl of DEPC-treated H_2O. The RNA was precipitated with 3 volumes of 100% ethanol in the presence of 0.3 M of sodium acetate. The resulting pellet was dried briefly under vacuum and resuspended in 300 μl of DEPC-treated H_2O. Spectrophotometric readings at 260 nm were used to quantitate the RNA. If the ratio of absorbency at 260 nm to that at 280 nm was greater than 1.6, the RNA was considered pure. If it was less than 1.6, repurification was performed.

2. mRNA isolation

One μg of total RNA (derived from 3.3 X 10^8 MEL cells), suspended in 500 μl of H_2O, was denatured in a water bath at 65° C for 5 minutes. Poly A+ RNA was isolated using two successive passages of total RNA through an one ml oligo(dT)-cellulose column (Pharmacia) prepared in a 3 cc syringe and equilibrated with 5 ml of buffer containing: 40 mM Tris pH 7.4, 1M NaCl, 1mM EDTA, and 0.1%

of SDS. The column was then washed with 5 ml of elution buffer (10 mM Tris, pH 7.4, 1 mM EDTA, and 0.05% of SDS) and 10 drop fractions were collected. A 2 µl sample from each fraction was spotted on the top of an ethidium bromide agarose (1%) plate in order to identify the RNA-containing fractions. Positive samples were then pooled and precipitated with 2.5 volumes of ethanol in the presence of 0.3 M sodium acetate pH 6.0 [Davis et al., 1986].

DNA preparation

1. Plasmid isolation

a. Micro plasmid prep ("Zippy Prep"): This method was used to select plasmids that were the correct size for sequence analysis following an Exo III digestion. An individual bacteria colony was removed from the transformation plate (Exo III digestion generated plasmid transformants) using the tip of a toothpick and resuspended in 20 µl of lysis buffer containing: 1 mg/ml lysozyme, 1 mg/ml RNAase A, 50 mM Tris-HCl pH 8.0, and 50 mM EDTA in a 1.5 ml eppendorf tube and incubated for 5 minutes at room temperature. An equal volume of TE-equilibrated phenol was added, the tube was vortexed for 30 seconds and then centrifuged for 5 minutes at 14,000 rpm. A 10 µl of the supernatant was carefully removed from the top layer and run on a 1% agarose gel. The plasmids that migrated in between of the full length construct and the vector alone were chosen for further purification and sequence analysis.

b. One step miniprep: One half ml of overnight bacteria culture was transferred into a 1.5 ml eppendorf tube and an equal volume of phenol:chloroform:iso-amylalcohol (25:24:1) was added.

The tube was vortexed for one minute at maximum speed and then centrifuged at 14,000 rpm for 5 minutes. Approximately 0.45 ml of the upper aqueous phase was transferred to a new eppendorf tube and mixed with 0.5 ml of isopropanol, vortexed and centrifuged immediately thereafter at 14,000 rpm for 5 minutes. The supernatant was discarded and the pellet washed twice with 70% ethanol. The pellet was dried and resuspended in 100 µl of TER buffer containing 10 mM Tris, pH 7.5, 1 mM EDTA, and 20 mg/ml of RNAase A. Five to ten µls of that DNA was digested with the appropriate restriction enzyme for analysis [Chowdhury, 1991].

c. Midiprep: A bacterial colony, or 5 µl of frozen bacteria stock, was inoculated into 50 ml of the proper medium supplemented with the proper antibiotic in a 250 ml flask and incubated overnight at 37° C with vigorous shaking (250 rpm). The cells were pelleted in a 50 ml tube and resuspended in 3.6 ml of solution I containing: 5 mg/ml lysozyme, 50 mM glucose, 25 mM Tris-Cl pH 8.0, and 10 mM EDTA. The cell suspension was incubated at room temperature for 5 minutes and 7.2 ml of solution II containing 0.2 N NaOH and 1% SDS was added. The tube was inverted 20 times and then chilled on ice for 5 minutes. 5.4 ml of solution III containing 3M KOAc and 11.5% of glacial acetic acid was added, tube was inverted for 20 times and then chilled on ice for another 5 minutes. Following a spin at 4K for 15 minutes at 4° C, the supernatant was transferred to a 30 ml Corex tube, mixed with 0.6 volumes of isopropanol, and incubated at room temperature for 15 minutes. The DNA was pelleted at 10K for 15 minutes at 4° C. The supernatant was discarded and the pellet was dissolved in 4.4 ml of TE (pH 7.4). 4.6 grams of CsCl and 100 µls of

ethidium bromide (10 mg/ml) was added and mixed well. The sample was loaded into a 13X51 mm polyallomer quick-seal centrifuge tube and centrifuged in a VTi 65.1 rotor at 65K for 4 hours or 54K 15 hours at 20^0 C. The lower band was removed (~0.6 ml) with a 18-gauge needle under long wave length UV and transferred to an eppendorf tube. The sample was extracted 4 times with an equal volume of TE (pH 8.0)-saturated butanol to remove all ethidium bromide. The aqueous phase was transferred into a 15 ml Corex tube and 3 volumes of TE (pH 8.0) and 2.5 volumes of 100% ethanol were added. After 20 minute incubation on ice, the plasmid was pelleted at 10K for 15 minutes at 4^0 C. The supernatant was discarded and the pellet was redissolved in 400 µls of TE (pH 8.0) and transferred to an eppendorf tube. The DNA was extracted once with phenol/chloroform, chloroform and then precipitated with 2.5 volumes of ethanol in the presence of 0.3 M sodium acetate. The DNA pellet was then washed with 75% ethanol, dried in Speedvac and dissolved in 200 µls of H_2O.

d. Superminiprep: This modified miniprecipitation method was used to prepare plasmid DNA for sequence analysis. Three ml of overnight bacterial culture was pelleted in an eppendorf tube. The pellet was resuspended in 100 µls of solution I containing: 50 mM Glucose, 25 mM Tris pH 8.0, 10mM EDTA, and 10 mg/ml lysozyme and vortexed briefly. Following a 10 minute incubation at room temperature, solution II (200 µl) containing 1% SDS and 0.2 N NaOH was added to the bacterial suspension and mixed by multiple inversions. This mixture was incubated for 5 minutes on ice. Two hundred microliters of solution III containing: 3M KOAc and 11.5% of

glacial acetic acid was then added, mixed briefly by vortexing and incubated for 5 min on ice. The samples were centrifuged at full speed for 5 min , the supernatant was transferred to a new tube, and recentrifuged for 5 min to obtain particle free lysate. The supernatant was precipitated with two volumes of 100% ethanol, and centrifuged for 2 minutes (12,000g at room temperature). The pellet was dissolved in 200 µl of H_2O, RNAase A was added to a final concentration of 50 µg/ml, and the reaction was incubated for 30 minutes at 37° C. Then the reaction was digested with proteinase K at a final concentration of 0.5 µg/ml, and incubated for 30 additional minutes at 42° C. The reaction mixture was then extracted 2 times with 200 µl of phenol/chloroform (70:30), and once with 200 µl of pure chloroform. DNA was precipitated with 2.5 volume of 100% ethanol without adding any extra-salt, mixed by inversion and centrifuged for 3 minutes (12,000g at room temperature). The pellet was washed with 75% ethanol, dried in Speedvac and redissolved in 20-50 µl H_2O. DNA concentration was determined spectrophotometrically at 280/260 nM. Typically 50-100 µg of plasmid DNA was isolated by this method and 10 µg was used for each sequencing reaction.

2. Phage DNA isolation

Ten µl of an overnight culture of host bacterial cells were grown in a 50 ml conical tube with antibiotics (e.g. ampicillin) in the appropriate media. This culture was added to 500 ml of fresh media without antibiotics in a 2 liter flask and incubated at 37° C, shaking at 250 rpm. After two hours, when the bacteria was in its

logarithmic growth phase, $1x10^{10}$ pfu of phage was added. The culture was incubated at 37^0 C with shaking for additional 6 to 8 hours. When lysis occurred, 10 ml of chloroform was added to the culture and incubated for an additional 15 minutes. Subsequently, the culture was transferred to a centrifuge bottle and spun at 5000X g at 20^0 C for 20 minutes. DNAase I and RNAase (each to a final concentration of 1 μg/ml) were added to the supernatant. The nuclease reaction was incubated for 30 minutes at room temperature. Solid polyethylene glycol (PEG 8000) was added to a final concentration of 10% and crystal sodium chloride was added to a final concentration of 1M with slow stirring on a magnetic stirrer at room temperature. The solution was placed on ice for one hour and then centrifuged at 10,000X g for 15 minutes at 4^0 C. The pellet was resuspended in 3 ml of buffer L3 from the Qiagen phage precipitation kit (Qiagen Inc.). The manufacturer's instructions (after step 4) for the Qiagen kit were then followed. The resulting phage was dissolved in 200 μl of H_2O.

3. Genomic DNA isolation

A total of 10^9 MELC were collected by centrifugation at 1000 X g for 5 minutes. The pellet was washed once in 10 ml of PBS and resuspended in 10 volumes of lysis buffer containing 10 mM Tris pH 7.4; 10 mM EDTA; 150 mM NaCl; 0.4% SDS and 1 mg/ml of proteinase K. The reaction was incubated for 3 hours at 50^0 C with periodical swirling. An equal volume of TE-saturated phenol (pH 8.0) was added and the tube was gently inverted for 50 times to extract the DNA followed by a quick spin at 200 X g for 2 minutes. The organic

phase, including the interface, was discarded. The phenol extraction was repeated two more times. The aqueous phase DNA was dialyzed overnight at 4° C against 4 liters of a solution (50 mM Tris-HCl pH 8.0; 10 mM EDTA and 10 mM NaCl) with 4 changes. The sample was then incubated with 100 µg/ml of DNAase-free RNAase at 37° C for 2 hours. The DNA was gently extracted twice with phenol/chloroform and then dialyzed extensively against TE pH 8.0 with frequent change of the solution at 4° C for two days.

Solution hybridization assays

1. Northern analysis

Twenty to fifty µg of total cellular RNA were vacuum dried, resuspended in 5 µl of H_2O and mixed with 15 µl of RNA loading buffer containing 8% of formaldehyde; 67% formamide; 1X Mops; 2mM EDTA; 5.7% glycerol; 0.1% SDS and 0.01% of the dyes bromophenol blue and xylene cyanol. The RNA was electrophoretically size separated on a 1% agarose gel containing 20 mM Mops pH 7.4; 1mM EDTA and 3% formaldehyde. The electrophoresis was performed for 15 hours at 30 mA in 1X Mops buffer with circulation to provide resolution of high molecular weight mRNAs. The gel was denatured in solution of 0.05N NaOH and 0.15M NaCl for 30 minutes and neutralized in 0.1 M Tris pH 7.5 and 0.15M NaCl for 30 minutes. RNA was transferred onto nitrocellulose filters overnight in 10X SSC buffer (1X = 0.15M NaCl/0.015M sodium citrate, pH 7.0). Following the transfer, the filter was photographed, UV crosslinked with 120 mJoules and baked at 80° C in the vacuum for 2 hours. The filter was then prehybridized in a solution containing 5X

SSC; 0.266 mg/ml; calf-thymus DNA; 25 mM sodium phosphate pH 6.4; 50% of formamide and 0.1% each of the following: SDS, BSA, Ficoll and PVP in a 42° C water bath overnight. The prehybridization solution was removed and the filter was then hybridized in a solution containing 5X SSC; 0.1 mg/ml of calf-thymus DNA; 50 mM of sodium phosphate pH 6.4; 0.1% of SDS; 50% of formamide and 0.02% of the following: BSA, Ficoll and PVP at 42° C overnight. The filter was washed twice with 2X SSC and 0.1% SDS at room temperature for 10 minutes, twice with 1X SSC and 0.1% SDS at 42° C for 20 minutes and once in 0.2X SSC and 0.1% SDS for 15 to 20 minutes at 50-65° C. The filter was air dried, wrapped with Saran wrap and exposed to film (X-OMAT, AR, Eastman KODAK, Rochester, NY) with a single intensifying screen at -80° C for the desired time period and developed in M35AX-Omat Kodak machine.

2. Southern analysis

Ten µg of genomic DNA was digested with 100 units of restriction enzyme in a 100 µl reaction volume at 37° C overnight. The reactions were phenol/chloroform extracted and ethanol precipitated. The pellet was dissolved in H_2O and mixed with loading buffer containing 0.25% of bromophenol blue; 0.25% of xylene cyanol and 40% (W/V) sucrose. The sample was run on a 0.7% agarose gel at 100 V for 5 hours. Capillary DNA transfer onto nitrocellulose filters was conducted overnight in 10X SSC buffer. Prehybridization and hybridization were performed as described for Northern analysis. Final washing was in 0.2 X SSC and 0.1% SDS solution at 60°

C for 15 min. Films were autoradiographed using a single intensifying screen at -80° C for the desired time period.

3. Western analysis

A 30-50 μg of protein sample was separated on 6-8% of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [Laemmli, 1970]. Prestained molecular weight standards (Bethesda Research Laboratories) were used in the assay. The protein was transferred to a PVDF (polyvinylidene difluoride) membrane (Du Pont) overnight at 4° C and 0.3 A in a buffer containing 25 mM Tris; 200 mM glycine and 15% methanol. The membrane was then air-dried, wet with methanol and rinsed with water. PBST solution pH 7.5 (80 mM of di-sodium hydrogen orthophosphate anhydrous, 20 mM sodium dihydrogen orthophosphate, 100 mM sodium chloride and 0.1% Tween-20) supplemented with 5% of dry milk was used to block the membrane at room temperature for 1 hour. Primary antiserum were diluted 100-2000 time with PBST buffer and incubated with the membrane for 1 hr at room temperature with gentle shaking. After extensive washing with PBST buffer, the membrane was incubated in horseradish peroxidase labeled anti-rabbit IgG solution at room temperature for 1 hr. The membrane was washed thoroughly with PBST 6 times, and then incubated in the detection solution (supplied by the ECL kit, Amersham) for 1 min and exposed to a Hyperfilm-MP for the desired period of time.

MELC-CC cDNA Cloning

Constructing cDNA library

Poly A+ RNA was purified (as mentioned under "mRNA purification") from HMBA induced MELC. Random hexamers were used to synthesize the library to increase the representation of 5' ends of mRNAs which might have significant secondary structures [Stratagene]. In short, 5 μg of polyadenylated RNA was denatured at 65° C for 10 minutes, then chilled on ice followed by adding 0.037 μg random hexamers for the first strand cDNA synthesis with Moloney Murine Leukemia Virus (MMLV) reverse transcriptase. The reaction was incubated at 37° C for 1 hour. The second strand cDNA synthesis was performed with the reagents from a cDNA cloning kit [Stratagene] at 12° C for 1 hour and then at 22° C for another hour. Klenow fragment was used to generate blunt ends for the cDNAs. The cDNAs were then ligated to the EcoR I/Not I adapters (which has a phosphorylated blunt end and non-phosphorylated EcoRI overhang) with the T4 DNA ligase in the presence of ATP at 12° C overnight. The above reaction mixture was then phosphorylated by the T4 polynucleotide kinase and inserted into the λ vector (1 μg) which was dephosphorylated on their EcoR I overhangs. Five sets with different amount of λ phages (0.1-5 μg) were then packaged following the instruction as described in the manufacturers protocol (Stratagene Gigapack II packaging extract). The library had an estimated titer of 3.75 x 10^4 PFU/μl. One million plaques were screened and positive clones were selected. The average insert size was 2.0 kb. Oligo dT primed MELC cDNA library was a gift from Dr.

Victoria Richon, Memorial Sloan-Kettering Cancer Center, New York, NY.

Isolation of cDNA Clones

A MELC specific cDNA probe (MELC-CC549) was used to screen the library. The oligo dT primed cDNA library was first screened with this cDNA fragment randomly labeled to a specific activity of 1 X 10^9 cpm/mg. One million plaques were transferred to the nitrocellulose filters by lifting followed by immersing the filters in denaturing buffer and neutralization buffer sequentially for 5 min each step. Filters were then air dried and baked at 80^0 C in a vacuum oven for 2 hr and then soaked in prehybridization and hybridization solution as mentioned in the section of "Southern analysis". A final washing of the filter was done at 60^0 C for 20 minutes in buffer containing 0.2X SSC and 0.1% SDS. These positive clones were excised from the phage using the helper phage for *in vitro* excision and sequenced with M13 forward and reverse primers on ABI Model 373 automated sequencer.

Polymerase Chain Reaction

All the PCR was performed in the Twinblock PCR thermal cycler at 100 µl reaction volumes. The Taq polymerase was purchased from Promega company. The magnesium was used at 1.5 mM unless otherwise stated in the text, otherwise, PCR amplification was performed as described previously [Marks et al., 1990].

Isolation of MELC genomic clones

MELC genomic DNA library was a gift from Dr. Victoria Richon (Memorial Sloan-Kettering Cancer Center, New York, NY). It was constructed by digesting the MELC genomic DNA with Sau3A and cloned into BamHI site of EMBL 3. A total of $2X10^5$ recombinants, grown in Le392 bacterial host, were screened using MELC-CC cDNA probe derived from 2MEL (BamHI to HindIII). Similar procedure as described in the section for "Southern analysis" was used for the screening.

RNAase Protection Assay

All the RNA samples used in the RNAase protection assay were total RNA from either the normal MELC or HMBA induced MELC. The enzymes and all the reagents used were from Erase-a-base kit (Ambion) unless otherwise stated. For riboprobe synthesis, plasmids containing the fragments of interest were constructed such that the bacteriophage T7 and T3 promoters could be used to direct the synthesis of radiolabeled cRNA probes. The proper enzymes were used to cut the DNA leaving either a 5' overhang or blunt ends followed by proteinase treatment. The DNA was then extracted with phenol/chloroform and ethanol precipitated under RNAase free condition. The RNA transcription was carried out in 20 µl of the buffer containing 1 µg of template DNA, 10 mM DTT, 0.5 mM of each of the ATP, GTP and TTP, 1X transcription buffer (Ambion), 25 units RNAase inhibitor, 50 µCi[α-^{32}P] CTP and 10 units of the proper RNA polymerase. The reaction was incubated at 25^0 C for one hour and the DNA template was removed with 2 units of DNAse I at 37^0 C for

15 minutes. The reaction was then mixed with equal volume of loading buffer and run on a 5% of denaturing acrylamide gel for one hour at 200 volts. After electrophoresis, the gel was covered with plastic wrap and exposed to a X-ray film for 1-5 minutes at room temp in the dark. One corner of the film and the gel were aligned so as to precisely localize of the probe. The area of the gel indicating the probe was excised and eluted in 350 µl of buffer containing 0.5 M NH_4OAc; 1mM EDTA and 0.1% SDS at room temperature with gentle shaking on the vortex at dial 3 for 25 minutes. Hybridization of 30 to 100 µg of total RNA with this radiolabeled cRNA (2 x 10^4 cpm) was performed in solution containing 80% deionized formamide; 40 mM PIPES pH 6.4; 400 mM NaOAc pH 6.4 and 1 mM EDTA at 37-42^0 C overnight. The reaction was digested with RNAase A (0.33 units/ml)/RNAase T1 (100 units/ml) at 37^0 C for 30 minutes. The protected RNA fragment was precipitated and resuspended in loading buffer and size fractionated on 6-8% (depend on the size of the interest product) polyacrylamide gels under denaturing conditions. The gel was run at 200 volts for four to five hours in 1X Tris-borate EDTA buffer. The gel was dried on Whatman paper and exposed to X-ray film at -80^0 C with one intensifying screen for the desired time period.

Primer Extension

Synthetic nucleotide primer (Genset) was 5' end-labeled with T4 polynucleotide kinase and $\gamma^{32}P$ ATP, and then gel purified as described under "probe" section. The condition for hybridization of the primer to target RNA was as follows: $5x10^5$ cpm of labeled

primers; 0.4 M NaOAc pH 6.4; 40 mM PIPES pH 6.4; 1 mM EDTA and 80% formamide in 30 ml reaction volume at 85° C for 10 minutes and then 37° C for 12 hours. After ETOH precipitation, the pellet was dried at room temperature on the bench with the lid open. The reverse transcription was performed in the solution containing 20mM Tris-HCl (pH 8.4); 50 mM KCl; 2.5 mM $MgCl_2$ and 0.1 mg/ml BSA. The SuperScript™ reverse transcriptase (BRL) was added and the reaction was incubated at 42° C for 1 hour in the presence of 0.5 mM each of dTTP, dCTP, dGTP, dATP and 10mM DTT followed by RNAase H digestion at 55° C for 10 minutes. The reaction was extracted once with phenol/chloroform (1:1) and the products were precipitated with the reagents from the RNAase protection kits (Ambion Bx and Dx buffer). The pellet was resuspended in 8 ml loading buffer containing 80% formamide; 0.1% xylene cyanol; 0.1% bromophenol blue and 2mM EDTA. The sample was denatured at 100° C for 3 minutes, size fractionated by electrophoresis in 8% denaturing 8M urea, and then PAGE run at 200V for 5 hours. The gel was dried on Whatman paper and exposed to X-ray film at -80° C with one intensifying screen for up to 48 hours.

In vitro Synthesis of MELC mRNA

In vitro synthesis of MELC 5' cap mRNA was performed following the manufacturer's protocol [mCAP Stratagene]. This method was used because the 5' cap structure of *in vitro* mRNA transcripts increases the yield and stability of the synthesized RNA and it also enhances the translation efficiency both by rabbit reticulocyte lysate and of micro-injected *Xenopus* Oocytes [mCAP

Stratagene]. Plasmid DNA was banded twice in CsCl gradient centrifugation. The DNA was linearized by restriction enzyme, treated with proteinase K, and pelleted under the RNAase free condition. One μg of this DNA template was transcribed in a solution containing 200 mM Tris pH 7.5; 250 mM NaCl; 40 mM $MgCl_2$ and 10 mM spermidine in the presence of rNTP's and cap analog. T3, T7 or SP6 RNA polymerase was then added to the reaction and incubated at 37° C for 30 min. DNAase I was used to remove the DNA template. The reaction was then extracted with phenol/chloroform and ethanol precipitated in the presence of the 0.3 M sodium acetate pH 5.0. The resulting pellet was then washed with 80% ethanol and dried. The concentration of the RNA sample was measured with a spectrophotometer at 260 nM UV.

Microinjection of Xenopus *oocytes and oocyte membrane isolation*

Oocytes were isolated by standard techniques [Dumont, 1972 Marcus-Sekura and Hitchcock, 1987] from *Xenopus* laevis. In short, the oocytes were defolliculated during 2 hr incubation in 2 mg/ml collagenase at room temperature in Ca^{2+}-free ND96 solution (96 mM NaCl; 2mM KCl; 1 mM $MgCl_2$ and 5 mM HEPES, pH 7.5). Oocytes were examined under a dissecting microscope and healthy-looking stage V and stage VI cells [Dumont, 1972] were selected and maintained at 21° C in enriched ND96 solution for one day prior to injection with *in vitro* transcribed RNA. Oocytes were microinjected according to the methods described by Gurdon [Gurdon et al., 1971]. Approximately 20 ng of *in vitro* synthesized capped RNA in 50 nl volume was

injected into each oocyte followed by incubation for 5 days at 21° C in Barth's medium. Five hundred injected oocytes were homogenized in 1 ml of ice-cold assay buffer (75 mM Tris, pH 7.4; 12.5 mM $MgCl_2$; 1 mM EDTA and 15% (W/V) sucrose) with 30 strokes in a Dounce homogenizer. The homogenate was centrifuged at 3000X g for 10 minutes to dispose pigment granules. The supernatant fraction was further centrifuged at 10,000X g for 10 minutes. The resulting supernatant was applied to the ultracentrifuge at 400,000X g for 45 minutes. The pellet was washed with cold assay buffer, then resuspended in 11.4 mM Tris/HCl buffer at volume of 500 ml per 1,000 homogenized oocytes [Kobilka et al., 1987].

Binding of ^3H PN-200-110 to the MELC membrane protein

Fifty μg of MELC membrane preparation was added to the 5 ml glass tubes with 0.5 ml of buffered salt solution (BSS) of the following composition (mM): NaCl 145, KCl 5, $MgCl_2$ 1.25, $CaCl_2$ 1.25 and Tris 20, pH 7.5. The radioligand ^3H PN-200 110 was diluted with BSS solution and added to the reaction tubes. The reaction was incubated at 37° C for 45 min in the dark. The reaction was then diluted with 5 ml ice cold BSS solution and filtered on Whatman GF/F filters [Whatman International Ltd]. The filters were washed with 5 ml of ice cold BSS solution three times, dried and then immersed in 5 ml of Scinti Verse I solution [Fisher Scientific]. Radioactive counts from the filters were determined with a Beckman scintillation counter.

CHAPTER III: RESULTS

CELL BIOLOGY and PHYSIOLOGY STUDIES

HMBA-induced MELC differentiation requires Ca^{2+} influx

Based on the hypothesis that Ca^{2+} can trigger non excitable cell growth and differentiation [Gardner, 1989, Bridges et al., 1981], two questions were addressed with MELC as a model system: 1) is Ca^{2+} required for the growth and differentiation in MELC; and, if so, 2) is the source of Ca^{2+} intracellular or extracellular? The following experiments were designed to answer these two questions. In order to answer the first question, MELC were cultured in the presence of the differentiation inducer HMBA at a concentration of 5 mM (Figure 2). HMBA induced 80% of the cells to terminally differentiate within 48 hours (Figure 2). In contrast, when free Ca^{2+} was removed from the extracellular media by addition of the Ca^{2+} chelator, EGTA (2.7 mM/L sufficient to lower the extracellular Ca^{2+} concentration to ~10 μM) the cells exhibited a slower rate of growth and inhibited differentiation (Figure 2). The pharmacologic regulation of intracellular free Ca^{2+} concentration was studied using the fluorescent Ca^{2+}-sensitive dye, fluo-3. Fluo-3 loaded MELCs demonstrated an increase in fluorescence intensity that peaked approximately 3 to 6 minutes after addition of HMBA (5mmol/L) to the perfusate (Figure 3A1). When the experiment was repeated using Ca^{2+} free medium the HMBA-induced rise in intracellular Ca^{2+} was blocked (Figure 3A2). This indicated that the rise in cytoplasmic Ca^{2+} induced by HMBA was due to the influx of extracellular Ca^{2+} rather than intracellular Ca^{2+} release. These experiments suggested

that MELC require Ca^{2+} for growth and that Ca^{2+} influx is required for HMBA-induced MELC differentiation.

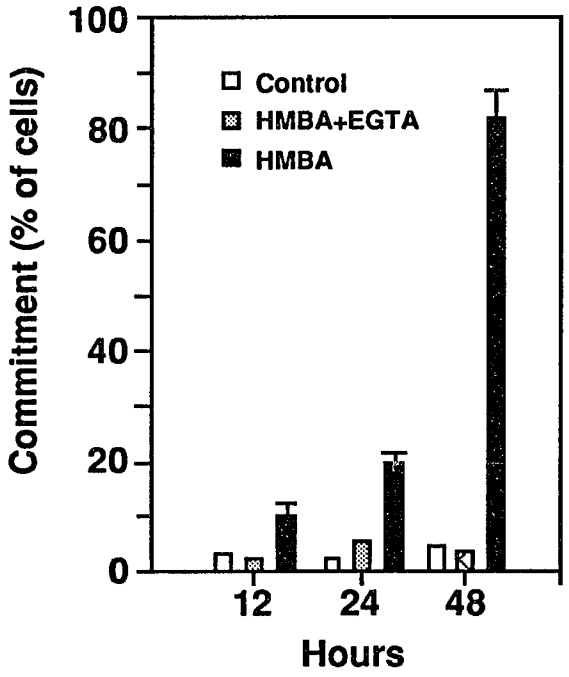


Figure 2. HMBA-induced MELC differentiation requires extracellular calcium. HMBA (5 mmol/L) induced 80% of the cell population to commit to terminal differentiation within 48 hours (solid box). EGTA (2.7 mM/L), a calcium chelator, inhibited a fraction of the MELC population from differentiating (hatched box). In the control experiment, when cells were grown in the absence of drug, no effects on cell growth and differentiation were seen (open box).

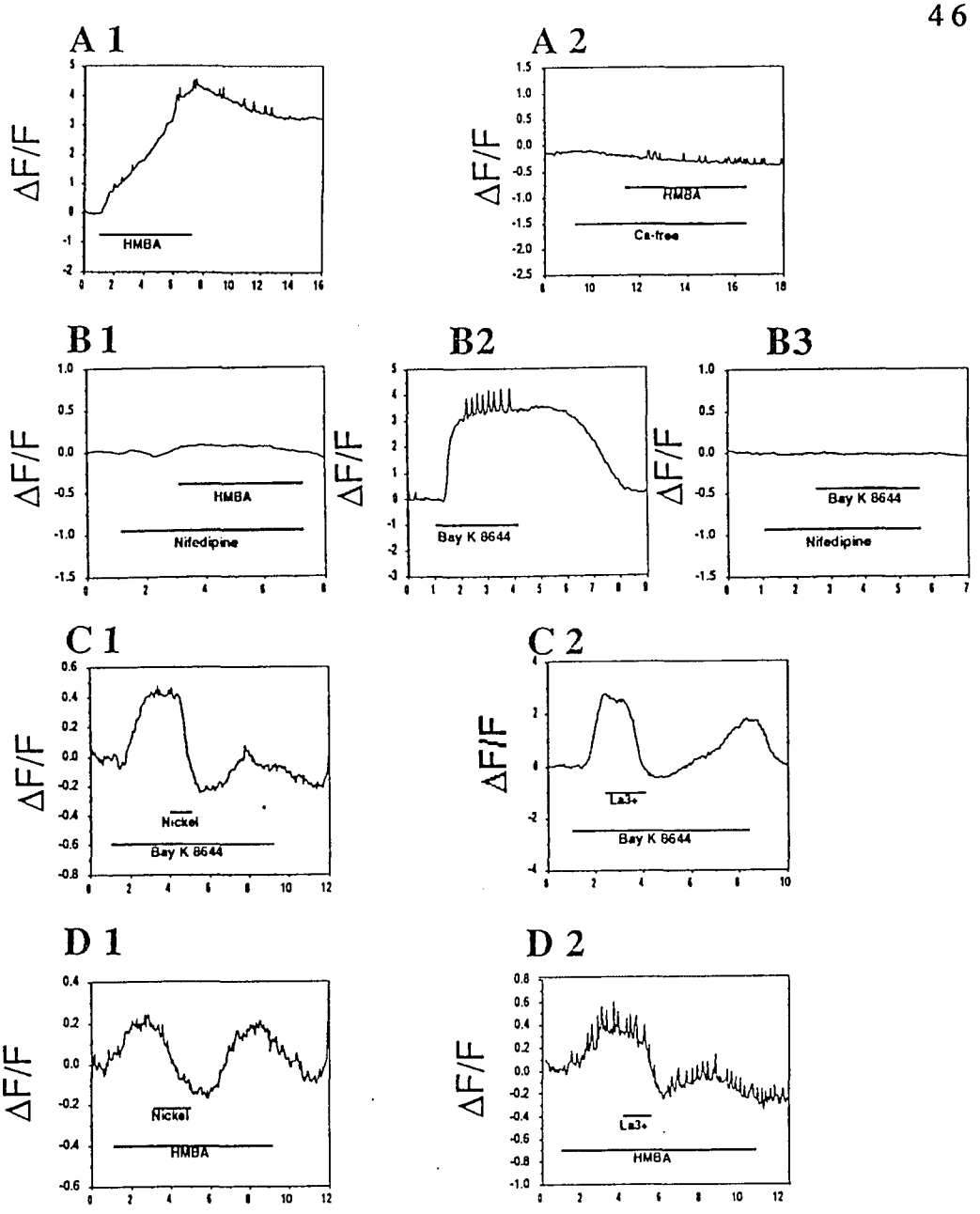


Figure 3. MELC intracellular free Ca^{2+} concentration was studied using fluo-3. Changes in fluorescence (F/F, arbitrary units) is plotted versus time (minutes). 3A) HMBA (5 mmol/L) stimulates Ca^{2+} influx within 6 minutes (3Al), which was not observed in Ca^{2+} free medium (3A2). 3B) MELC cultured with HMBA plus Nifedipine (10 μmol/L) which blocked the HMBA-induced increase in Fluo-3 signal (3B1). BAY K 8644 (10 μmol/L) stimulates intracellular Ca^{2+} increase comparable to that seen using HMBA (3B2), which was blocked by Nifedipine (10 μmol/L) (3B3). 3C) Bay K 8644 induced Ca^{2+} influx was reversibly blocked using the inorganic cations nickel (1 mmol/L) and lanthanum (1 mmol/L) (3C1 and 3C2). 3D) HMBA-induced Ca^{2+} influx was reversibly blocked using the inorganic cations nickel (1 mmol/L) and lanthanum (1 mmol/L) (3D1 and 3D2).

Ca^{2+} entry through a DHP sensitive pathway

Once it was established that Ca^{2+} enters MELC by influx from extracellular space, the next step was to elucidate the mechanism governing this process. The first pathway tested was a DHP-sensitive pathway. Using fluo-3 loaded cells it was found that nifedipine (10 μM/L), a specific DHP-sensitive Ca^{2+} channel blocker, inhibited HMBA-induced Ca^{2+} influx in MELC (Figure 3B1). Similarly, the DHP-sensitive Ca^{2+} channel agonist Bay K 8644 (1μM/L) induced a rise in intracellular Ca^{2+} comparable to that seen using HMBA (5 mM) (Figure 3B2) and nifedipine (10 μM/L) inhibited Bay K 8644 induced Ca^{2+} increase in MELC (Figure 3B3). Both Bay K 8644 and the HMBA-induced Ca^{2+} influx was reversibly blocked using the inorganic cations Nickel (1 mmol/L) and Lanthanum (1 mmol/L) (Figure 3C and 3D respectively). Furthermore, when MELC were cultured with 10μM/L nifedipine together with the HMBA (5mM/L), HMBA-induced differentiation was inhibited by ~40% (Figure 4). These data indicated that the Ca^{2+} influx required for HMBA-induced MELC differentiation occurs, at least in part, via a DHP-sensitive pathway.

Voltage-gated Ca^{2+} channels are not detected

From these pharmacological studies, it was not possible to discern whether Ca^{2+} influx occurred via a voltage-gated Ca^{2+} channel or a receptor operated channel. The possibility that the voltage-gated channel would be involved was tested using whole cell patch clamp experiment on MELC. This experiment was done in collaboration with Dr. Gillo [Gillo et al., 1990]. The results of this

experiment are compatible with the absence of a voltage-gated Ca^{2+} channel in MELC [Gillo et al., 1990].

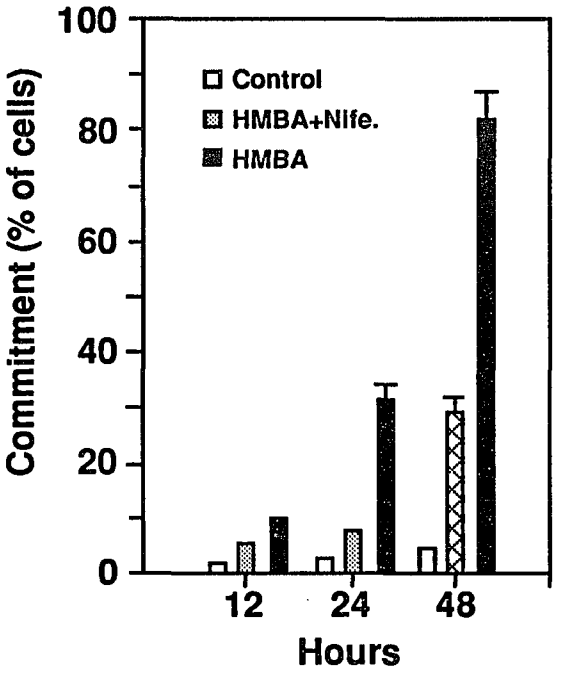


Figure 4. Nifedipine inhibited HMBA-induced MELC differentiation. When MELC were cultured with 10 μM/L nifedipine, a voltage-gated Ca^{2+} channel blocker, together with the HMBA (5 mM/L) (hatched box), HMBA-induced differentiation was inhibited by more than 60% at 48 hours when compared with the HMBA induction without nifedipine treatment (solid bar). In the control experiment, when cells were grown in the absence of drug, no effects on cell growth and differentiation were seen (open box). This experiment suggests that nifedipine inhibited MELC differentiation by blocking Ca^{2+} influx through a VDCC homologous structure.

STRUCTURAL STUDIES

PCR demonstrates a DHP-R like mRNA in MELC

The effects of nifedipine and Bay K 8644 on MELC suggested that a DHP-R homologue was expressed in MELC. Taking advantage of the similarities of the transmembrane regions of the VDCCs, sense and antisense oligonucleotides were designed and polymerase chain reaction (PCR) was employed to detect DHP-R like mRNA MELC.

Two oligonucleotide primers were synthesized based on the sequence of the skeletal muscle DHP-R [Tanabe, 1987]: antisense (5'-AGATGGTGTCGACCATGATGAGGGCGAACA-3', corresponding to nucleotides 3595-3625 except that two conservative changes in the nucleotide sequence were made: a T to C switch at nucleotide 3616 and a G to C switch at nucleotide 3613 to create a SalI site without changing the deduced amino acid sequence) and sense (5'-CCACGCTCCTGCAGTTCA-3', nucleotides 3029-3046 containing a PstI site). The antisense primer was used to synthesize first strand cDNA from 1 μg of MELC total RNA with avian myeloblastosis virus reverse transcriptase (Life Sciences, Fl). Both primers were then used to amplify a cDNA under stringent conditions (94^0 C for 1 min, 50^0 C for 2 min, 74^0 C for 1 min for 30 cycles). As a control, MELC total RNA without reverse transcription was used in a similar PCR experiment resulting in no amplification. PCR products were size fractionated on an agarose gel, and the band corresponding to ~600 bases was excised and purified. The purified cDNAs were digested using SalI and PstI and subcloned into a pBluescript SK$^-$ to create pMELC-CC549. The cDNA was sequenced using the dideoxy chain

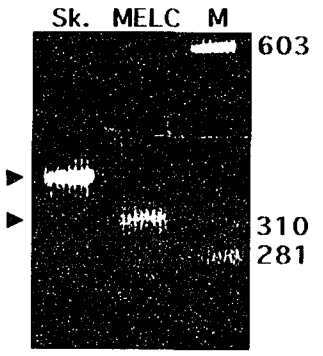
termination method [Sambrook, 1989] and an ABI Model 373 sequencer. Upon nucleotide sequence analysis, this cDNA fragment revealed 75% homology to the skeletal muscle DHP-R α_1 subunit [Tanabe, 1987] (Figure 5). The degree of homology confirmed the original hypothesis that MELC has a DHP-R homologue.

Further PCR analysis was performed to determine whether the voltage-sensor (S4) regions were present in the MELC-CC. Primers 376 bps apart from each other surrounding the IIIS2 to IIIS4 region were designed: sense (5'-CTGGCTGCAGAGGACC-3', nucleotides 2671-2686 except that two changes in the nucleotide sequence were made: a C to T switch at nucleotide 2676 and a C to A switch at nucleotide 2679 to create a PstI site) and antisense (5'-TGAACTGCAGGAGCGTGG-3', nucleotides 3029-3046 containing a PstI site). MELC produced a 316 bp fragment (named MELC-CC316) as opposed to the 376 bp fragment produced by the skeletal muscle (Figure 6A). The deduced amino acid sequence of this PCR product was identical at IIIS4 region to skeletal muscle DHP-R indicating that the S4 region was present. However, a 60 base pair deletion corresponding to the IIIS2 region was detected in the MELC-CC (Figure 6B). This deletion, unique among all the α_1 subunits cloned [Tanabe 1987, Mikami, 1989, Penner et al., 1989, Takeshima et al., 1989, Adams et al., 1990, Nakai et al., 1990, Tanabe et al., 1990, Mori et al., 1991, Tanabe et al., 1991, Biel et al., 1990], corresponds to the entire putative IIIS2 transmembrane segment.

```
MELC-CC549                                TGTTCGCCTGCATTGGGGTCCAGCTCTTCAAGGGAAA
                                                          *                   *
Rabbit Skeletal  ccacgctcctgcagttcaTGTTCGCCTGCATTGGTGTCCAGCTCTTCAAGGGCAA
                                                                 IIIS5
MELC-CC549       GCTCTATACCTGTTCGGATAGTTCTAAACAGGCGGAGGCAGAATGCAAGGGTAAC
                  *   ** *   **** **** *  *** * *  * ** *    *   **
Rabbit Skeletal  GTTCTTCAGCTGCAACGACCTATCCAAGATGACAGAAGAGGAGTGCAGGGGCTAC

MELC-CC549       TATATCACATACAAAGATGGAGAGGTCGATCACCCCATTATCCAGCCTCGAAGCT
                   *******     * * * *** *** ******** * **  *  ****
Rabbit Skeletal  TACTATGTGTACAAGGACGGGGACCCCACGCAGATGGAGCTGCGCCCCCGCCAGT

MELC-CC549       GGGAGAACAGCAAGTTTGACTTTGACAATGTTTTGGCAGCCATGATGGCTCTCTT
                   ****   *** *  **          *  **  * *          * *
Rabbit Skeletal  GGATACACAATGACTTCCACTTTGACAACGTGCTGTCGGCCATGATGTCGCTCTT

MELC-CC549       CACCGTCTCCACCTTCGAAGGGTGGCCAGAGCTGCTGTACCGCTCCATTGACTCC
                    *  *           *  *     **           * **    *
Rabbit Skeletal  CACGGTGTCCACCTTCGAGGGATGGCCCCAGCTGCTGTACAGGGCCATAGACTCC

MELC-CC549       CACACAGAAGACAAGGGCCCCATCTACAACTACCGTGTGGAGATCTCCATCTTCT
                 *  ***  *    *       * *      *    *        **
Rabbit Skeletal  AACGAGGAGGACATGGGCCCCGTTTACAACAACCGAGTGGAGATGGCCATCTTCT
                                                                 IIIS6
MELC-CC549       TCATCATCTATATCATCATCATTGCCTTCTTCATGATGAACATCTTCGTGGGTTT
                           *      *                              *     *
Rabbit Skeletal  TCATCATCTACATCATCCTCATTGCCTTCTTCATGATGAACATCTTTGTGGGCTT

MELC-CC549       CGTCATTGTCACCTTCCAGGAGCAGGGGGAACAAGAGTACAAGAACTGTGAGCTG
                 *     *                          ***                *
Rabbit Skeletal  TGTCATCGTCACCTTCCAGGAGCAGGGGGAGACAGAGTACAAGAACTGCGAGCTG

MELC-CC549       GACAAGAACCAGAGACAATGTGTGGAATATGCCCTCAAGGCCCGACCCTTGCGAA
                             * *  *      * *        *        *  ** *  **
Rabbit Skeletal  GACAAGAACCAGCGCCAGTGTGTGCAGTATGCCCTGAAGGCCCGCCCACTTCGGT

MELC-CC549       GGTACATCCCCAAGAACCAGCACCAGTACAAAGTGTGGTACGTGGTCAACTCTAC
                  *                 ***        * *           *    *   **
Rabbit Skeletal  GCTACATCCCCAAGAACCCATACCAGTACCAGGTGTGGTACGTCGTCACCTCCTC

MELC-CC549       CTACTTCGAGTATCTGA
                       *  *  *
Rabbit Skeletal  CTACTTTGAATACCTGAtgttcgccctcatcatgctcaacaccatct
```

Figure 5. Nucleotide sequence comparison of MELC-CC549 (see text) with the skeletal muscle DHP-R α_1 subunit. The overall homology (75%) of MELC-CC549 to the skeletal muscle DHP-R α_1 subunit demonstrated that a VDCC homologous message is expressed in the MELC. However, the sequences between IIIS5 and IIIS6 diverge from each other suggesting that MELC-CC549 might represent a novel message. The lowercase letters represent the primers used in the PCR assay. The DNA fragments encoding the putative transmembrane regions, IIIS5 and IIIS6, for the skeletal muscle DHP-R are underlined and labeled. The symbol "*" indicates the nucleotide mismatches.

A



B

```
MELC-CC316                                  CCGTCCAGCACACCTCCTTCAGGAACCAT.....
                                              *   * ******   * *     *  *
Rabbit Skeletal  ctggctgcagaggaccCCATCCGGGCGGAGTCCGTGAGGAATCAGATCCTTGGAT
                  IIIS1
MELC-CC316       ..............................................ATGAC

Rabbit Skeletal  ATTTTGATATTGCCTTCACCTCTGTCTTCACTGTGGAGATTGTCCTCAAGATGAC
                                          IIIS2
MELC-CC316       TGCTTACGGGGCTTTCCTGCACAAGGGCTCTTTCTGCCGAAACTACTTCAATATC
                 ** *     *  *                    *        *           *
Rabbit Skeletal  GACCTACGGCGCCTTCCTGCACAAGGGCTCCTTCTGCCGCAACTACTTCAACATC

MELC-CC316       CTGGACCTGCTGGTGTTGTGCGTGTCCCTCATCTCCTTTGGCATCCAGTCCAGCG
                               *  ***     *         * *  **  *        *
Rabbit Skeletal  CTGGACCTGCTGGTGGTGGCTGTGTCTCTCATCTCCATGGGTCTCGAGTCCAGCA
                            IIIS3
MELC-CC316       CGATCAACGTTGTGAAGATTTTACGAGTGCTGCGAGTCCTCAGACCCCTGAGGGC
                  *   **   *  *     ** **       ** *  *   * *      * *
Rabbit Skeletal  CCATCTCCGTGGTAAAGATCCTGAGAGTGCTAAGGGTGCTCCGGCCCCTGCGAGC
                                              IIIS4
MELC-CC316       CATCAACAGGGCCAAGGGGCTAAAGCATGTGGTTCAGTGCGTGTTTGTGGCCATC
                           *     *   * *     *     *           **
Rabbit Skeletal  CATCAACAGAGCCAAAGGGTTGAAGCACGTGGTCCAGTGCGTGTTCGTGGCCATC

MELC-CC316       CGGACCATCGGGAACATCGTAATTGTCA
                   *                 ** *
Rabbit Skeletal  CGCACCATCGGGAACATCGTCCTGGTCAccacgctcctgcagttca
                                              IIIS5
```

Figure 6. Detection of a partial deletion of the MELC transmembrane region at IIIS2. A) PCR analysis shows the predicted 376 bp product in skeletal muscle, but a smaller product, ~316 bps, was amplified from MELC RNA (MELC-CC316, indicated by arrows). A DNA size marker was included and labeled. B) Nucleotide sequence comparison of the MELC-CC316 and the skeletal muscle DHP-R. A 60 bp deletion in MELC corresponding to part of the IIIS2 region was detected (represented by dots within the MELC-CC316 sequence). Interestingly, the amino acid sequences deduced from both IIIS4 regions are identical. The lowercase letters at both ends of skeletal muscle DHP-R sequence represent the primers used in the PCR. The putative transmembrane segments IIIS1 to IIIS5 are underlined and labeled. The symbol "*" indicates the nucleotide mismatches.

Northern Analysis

Northern analysis was used to study the regulation of MELC-CC mRNA during induced differentiation. MELC were cultured with 5 mM/ml HMBA or grown without HMBA as a control, cell density was maintained constant at 1 X 10^5/ml by splitting cells (previously shown to induce 80% of the cells to commit to terminal differentiation, see Figure 2) for up to 100 hrs. Approximately 1 X 10^8 cells from each time point were harvested for total RNA purification. The Northern blot was probed with the MELC-CC549 cDNA labeled to a specific activity of 1 X 10^9 cpm/mg at 42^0 C for 12 hours. Final washing was performed in 0.1X SSC, 0.1% SDS at 65^0 C for 15 minutes. Autoradiography was carried out at -80^0 C for 12 days with two intensifying screens. As shown in figure 7, MELC-CC expression increased in response to HMBA-induced differentiation and peaked after 36 hours of HMBA treatment (Figure 7).

Molecular cloning and sequence analysis

PCR and Northern hybridization demonstrated that an mRNA was expressed in MELC that is homologous to the skeletal muscle α_1 subunit. Therefore, the MELC-CC549 PCR derived cDNA was used to screen an oligo-dT primed MELC cDNA library. A total of 4 X 10^6 recombinants were screened and one positive clone, 2MEL, was isolated after three rounds of consecutive screening. Partial nucleotide sequence analysis of this clone showed that the sequence covered by 2MEL had striking homology to the rabbit cardiac DHP-R [Mikami, 1989] with 95% identity at the cDNA level and 90% identity at the amino acid level except that the IIIS2 fragment was deleted in

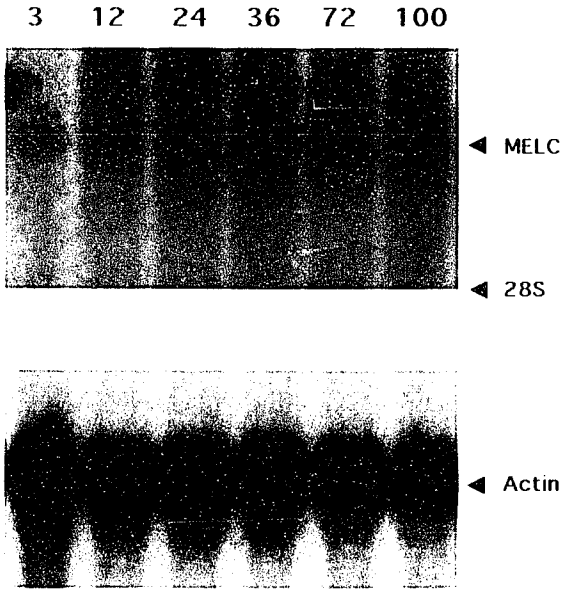


Figure 7. Northern analysis of MELC-CC mRNA expression during HMBA-induced MELC differentiation. Total RNA was obtained from MELC after 3, 12, 24, 36, 72 and 100 hours of HMBA treatment. The MELC-CC expression peaked after 36 hours of HMBA treatment. 20 µg total RNA from each sample was size fractionated on a 1% formaldehyde agarose gel. The blot was hybridized with the entire 2MEL cDNA probe. The position of the 28S rRNA is indicated. Levels of actin mRNA expression were determined to normalize differences in RNA loading in each lane.

2MEL. The cDNA fragment of 2MEL between BamHI-HindIII (located close to the 5' end of 2MEL), and the one between SacI-FspI (close to the 3' end of 2MEL) were radioactively labeled and used to rescreen

the library in order to completely clone the putative MELC-CC. Two additional clones, 5MEL and 3MEL (their names being based on their relative orientation to the 2MEL, i.e.. 5' and 3' respectively) were isolated from 4 X 10^6 recombinants. These three clones were partially sequenced showing that the overlapping regions of the clones were identical and that the clones cover ~8 Kb. However, sequence analysis of the first one kb of 5MEL showed that it was in fact the 28S ribosomal RNA (therefore a cloning artifact). Neither of these clones encoded the first four transmembrane regions of the cardiac DHP-R (Figure 8).

In order to clone the first four transmembrane regions of the DHP-R, a new cDNA library was constructed using HMBA-induced MELC mRNA. As previously mentioned, HMBA causes differentiation of MELC induces the MELC-CC message (Figure 7). A random primed MELC cDNA library was constructed to increase representative of the 5' end of the MELC-CC cDNA (as mentioned in the Method section). Screening 1 X 10^6 recombinants from this second library with a 2MEL cDNA probe (BamHI-HindIII, spanning the region from the nucleotide 545 to 880 of the MELC-CC cDNA) yielded three additional cDNA clones (designated: 5M613, 5M623, and 5M713) (Figure 8). However, they also failed to contain the 5' end of cardiac DHP-R, as demonstrated by partial sequence analysis. Furthermore, 5' partial sequence analysis revealed that these three clones contained identical sequences (Figure 8). Importantly, the 5' ends of all five clones ended within 100 bp of each other suggesting that this might be the actual 5' end of the MELC-CC mRNA.

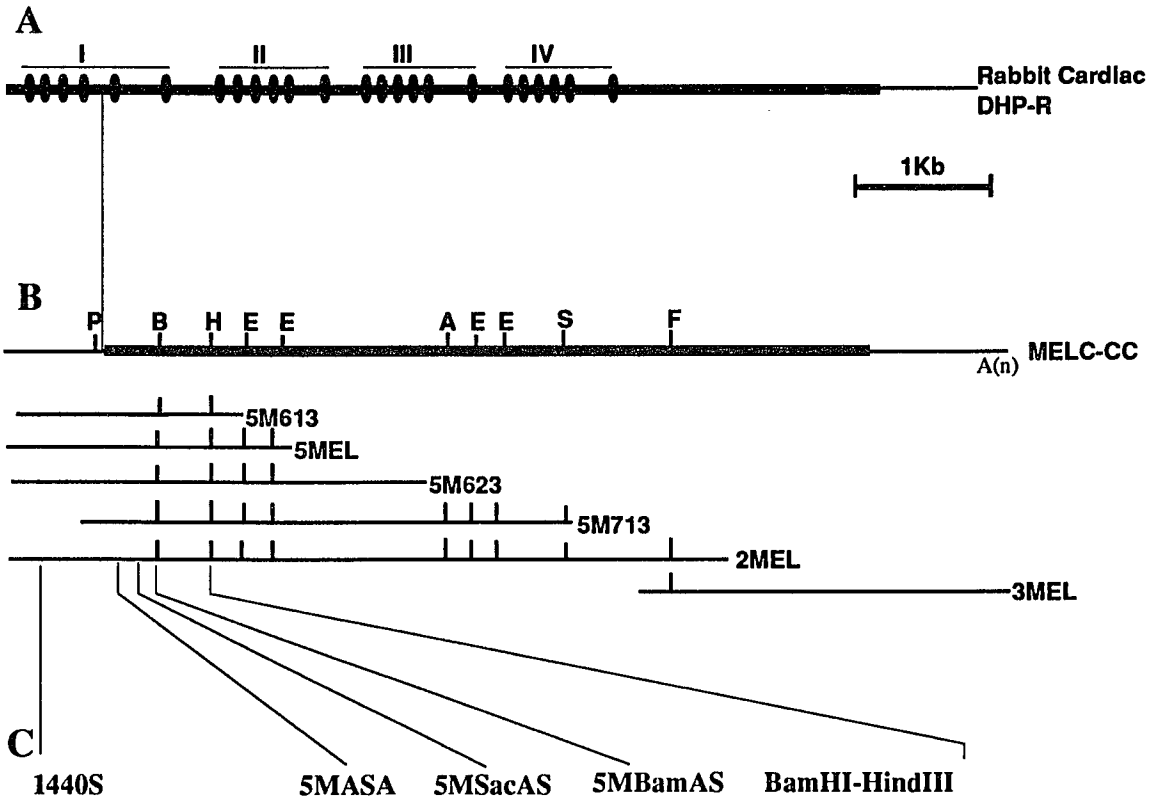


Figure 8. Schematic representation of the locations of individual clones and the probes used in cloning for MELC-CC cDNA. A) The rabbit cardiac DHP-R was aligned with MELC-CC. The solid bar represents the open reading frame. Each of the predicted transmembrane repeat is indicated by solid ovals and four motifs are labeled I, II, III and IV. B) The MELC-CC cDNA sequence is represented. The solid bar represents the protein coding region of MELC-CC cDNA. The 5' and 3' untranslated regions are indicated by thin lines. The restriction sites used in preparing probes and in making the full length constructs are indicated by letters. P-PstI; B-BamHI; H-HindIII; E-EcoRI; A-AccIII; S-SacII; F-FspI. 5MEL, 2MEL and 3MEL were cloned from an uninduced MELC cDNA library. 5M613, 5M623 and 5M713 were cloned from the HMBA-induced MELC cDNA library. C) The relative positions of the important oligonucleotides and the cDNA fragment used for later experiments (as probes and primers) are indicated.

Strategy for Nucleotide Sequencing

In order to reveal the primary structure of the putative MELC-CC, clones 5MEL, 2MEL and 3MEL were chosen for complete (both strands) sequence analysis. Three approaches were used to sequence these MELC-CC clones. They are as follows:

1. Sequencing of Restriction Fragments

MELC-CC overlapping clones, 2MEL, 5MEL and 3MEL, were digested with restriction enzymes to produce smaller fragments, which were then subcloned into pBluescript and sequenced from both ends with the pBluescript primers M13 forward and M13 reverse. This allowed rapid sequencing of the areas adjoining the known restriction sites. The fragments sequenced and their relative positions are shown in Figure 9A.

2. Exonuclease III digestion

In order to generate another series of constructs for sequencing analysis, subclones 2MR11, 2MR12, 3MSalI, and 3MSal/F were digested with Exonuclease III (Figure 9B). Plasmids derived using this method were named by a specific code that summarized the source of the DNA, the number of the clone, the enzyme used in the restriction digestion, the band representing the DNA fragment on a gel, the time point of the Exo III digestion and the colony from a plate corresponding to the same time point. For example, M2RI2X5A denotes 2MEL cDNA clone cut with the EcoRI enzyme, the second band from the top of a gel of the exo-digestion at the fifth time point, and colony A from that plate.

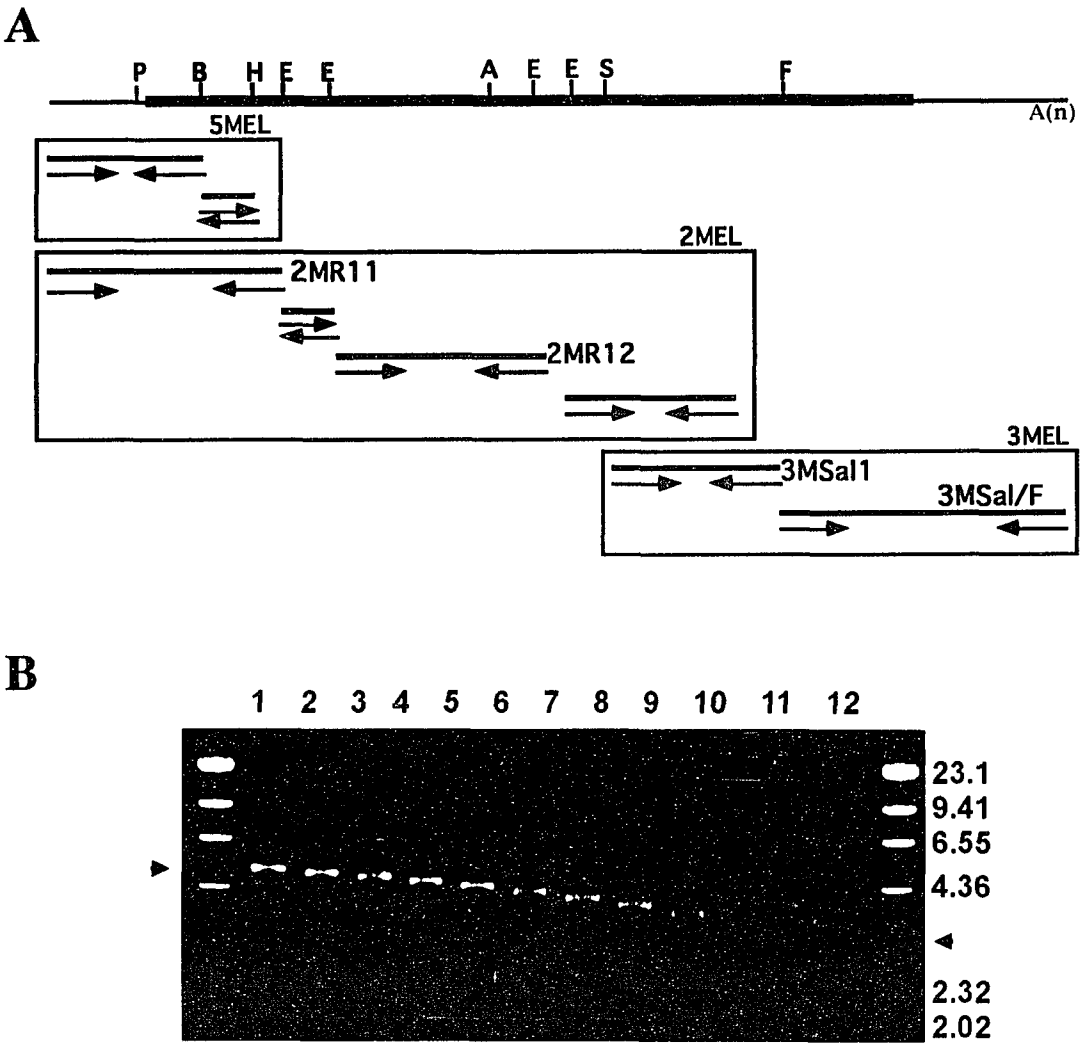


Figure 9. A schematic representation of the strategies used for sequencing the MELC-CC cDNA. A) The nucleotide sequence of the protein-coding region of MELC-CC is indicated by a thick solid bar. The restriction enzyme sites on MELC-CC cDNA are represented by single letters, P-PstI, B-BamHI, H-HindIII, E-EcoRI, A-ApaI, S-SacI and F-FspI. Three subclones, 5MEL, 2MEL and 3MEL, used for sequencing are boxed and labeled. The restriction fragments of each individual clone is shown by lines. The ends of each restriction fragment sequenced are indicated by arrows. The subclones sequenced by exonuclease deletion are named along each fragment within the boxes. B) ExoIII deletion products run on an agarose gel. Numbers on the top of the figure stand for the time points for the digestion. The arrows indicate the position of the linearized plasmid before the deletion assay (left side) and the position of the pBluescript vector (right side). HindIII digested λ phage was run on both sides of the gel as size markers.

3. Oligo walking

Small regions that were not detected using either restriction fragment analysis or ExoIII deletions were sequenced using oligo walking. This technique involves designing sense and antisense synthetic oligonucleotides based on information from the MELC cDNA sequence and the α_1 subunits of skeletal muscle and cardiac muscle. The names of the oligonucleotides and their respective sites appear in Table 2. Note that "S" and "AS" stands for sense and antisense, respectively, and both are relative to the MELC-CC cDNA sequence.

A summary of the cloning and sequencing strategy is shown in Figure 9. The full length MELC-CC sequence as derived from the consensus sequence of 2MEL, 5MEL and 3MEL, with the deduced amino-acid sequence is shown in figure 10. The comparison of the MELC-CC with the α-1 subunit of the rabbit cardiac DHP-R is shown in figure 11.

Primary structure Analysis of the MELC Ca^{2+} channel

1. Analysis of Nucleotide Sequence

These three overlapping clones were sequenced on both strands and assembled into a consensus sequence using the MacVector program to produce a 7629 bp cDNA (Figure 10). This open reading frame is preceded by an in-frame stop codon at nucleotide 345-347 (TGA). Although there is an in-frame ATG translation initiation site at nucleotides 405-407, the nucleotide sequence upstream does not reveal the eukaryotic consensus initiation site (GCCACCAUGG) [Kozak, 1991]. The 3' untranslated region is comprised of 1618 nucleotides with two conventional

Table 2

Oligonucleotides used in the MELC-CC study

Name	Location (relative to MELC)	sequence (5′ to 3′)
1440 AS	45-64	TACCCACGGCCACAGAGGAC
5M AS AS	378-402	CCTTGATGATGGAGTTCAGGACCAC
5M Sac AS	467-487	CCCATGAAGAGCTCCAGGCCG
5M BAM AS	536-556	GGGGAAGGATCCTCTTTTGCC
5M AS 0	579-598	CCGTTCTGACACTGTCGCCC
2MR4 AS 2	1125-1144	CCCTCGATGTCACCTCCAGC
2MR4 AS 1	1388-1407	TCAGGAGCATTTCTGCAGTG
MAS2421	1822-1841	CCATAAGCCATGATCCCATC
3S1(IIIS1)AS	2442-2460	CAGCACACCTCCTTCAGGA
MAS3810	3147-3165	GTCCAGCTCACAGTTCTTG
MAS5000	3591-3609	TGGTGAAGCTGCTGAGCCG
MAS5566	4867-4885	TGATGTTGGCATTGGAGCC
3M AS 4	6542-6559	GAGCACCAGGCAGCAGCC
3M AS 3	6913-6930	GNNAGCCTGCTGATTGTG
3M AS 2	7188-7207	CAGAAGCAGAAGCAGAGACA
3M AS 1	7482-7501	GCTTGGCACAATTGCAATTC
1440S	45-64	GTCCTCTGTGGCCGTGGGTA
MS2302	1704-1721	GAGGGAAGTTCAATTTCG
MS2700	2101-2117	CTGAAATCCATTACAGC
MS3640	2970-2991	GCTGCTGTACCGCTCCATTGAC
MS5135	4491-4508	GCAGGGGCTGGTGGGCA
MS6108	5407-5424	CATTCTCCTACCACATTC
3M S0 1	5850-5867	CAACGGCACCCTCTTACC
3M S0 1 1	6095-6112	TGGAGGTAACCGGAACAG
3M S0 2	6445-6462	CAGGGACTCTTTCCCTGC
3M S0 3	6665-6682	GAGCGGTTACAACTGCGC
3M S0 4	6983-7000	TGTGCTGGGCATATGGAC

Table 2. The names of the oligonucleotides and their location with respect to the MELC-CC cDNA and their sequences (the first base stands for the 5' end of that oligo). Note that in most cases, "S" stands for the sense and "A or AS" stands for antisense, relative to the MELC-CC cDNA sequence.

polyadenylation signals (AAUAAA) at nucleotide 7600 and 7609 (Figure 10). The longest open reading frame encodes an 1869 amino acid sequence that shows a 93% identity with the previously published rabbit cardiac α_1 subunits [Mikami et al., 1989] (Figure 11). The cDNA encodes a protein with a predicted molecular mass of 210,769 daltons.

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 GGTAGGCTCTGGTAATCCACCACGGAGAAGCCTGAGATGACTAAGTCCTCTGTGGCCGTGGGTAG   65
TCACAGTGAGTTCTCGTGCTGAGCTCTGCTCTGGAAGGAGACACACTAGGGTGTCTCTGAGATGGG  131
CTATTTAAAAATCTTAATAGTTACGGATGAAAACAACAACATGTTCTCCCCCTCCGAAGCACAGCT  197
CTGCTGCCGATCCTTTTATTTGGATGTGCCTCCAGGAGAGATTTGGAGTCAAGCCAACCCGAACTT  263
AGATTCTAGCCCTGTCACTCATTGGCTGTGACCTTGAGCCTCTTTTTGCTCATCTAAAGCCTGGGA  329
CTAGGGACTCCCACTTGAGCTGCTAAGAAGACTGCAGGAGGTCTCCAGGTGGTCCTGAACTCCATC  395
ATCAAGGCCATGGTGCCTCTGCTGCACATTGCCCTTCTTGTGCTCTTCGTCATCATCATTTATGCT  461
          M  V  P  L  L  H  I  A  L  L  V  L  F  V  I  I  I  Y  A
ATTATCGGCCTGGAGCTCTTCATGGGAAAGATGCACAAGACCTGCTACAACCAGGAGGGCATAATA  527
 I  I  G  L  E  L  F  M  G  K  M  H  K  T  C  Y  N  Q  E  G  I  I
GATGTTCCGGCAAAAGAGGATCCTTCCCCTTGTGCTTTGGAGACAGGCCATGGGCGACAGTGTCAG  593
 D  V  P  A  K  E  D  P  S  P  C  A  L  E  T  G  H  G  R  Q  C  Q
AACGGGACCGTGTGCAAACCCGGGTGGGATGGGCCCAAGCACGGCATCACCAACTTCGACAACTTC  659
 N  G  T  V  C  K  P  G  W  D  G  P  K  H  G  I  T  N  F  D  N  F
GCCTTCGCCATGCTGACGGTGTTCCAGTGTATCACCATGGAGGGCTGGACAGACGTGCTGTACTGG  725
 A  F  A  M  L  T  V  F  Q  C  I  T  M  E  G  W  T  D  V  L  Y  W
GTCAATGATGCCGTAGGAAGGGACTGGCCCTGGATCTATTTTGTAACACTAATCATCATAGGGTCA  791
 V  N  D  A  V  G  R  D  W  P  W  I  Y  F  V  T  L  I  I  I  G  S
TTTTTTGTACTTAACTTGGTTCTCGGTGTTCTTAGCGGGGAGTTTTCCAAAGAGAGGGAGAAAGCC  857
 F  F  V  L  N  L  V  L  G  V  L  S  G  E  F  S  K  E  R  E  K  A
AAAGCCCGAGGAGATTTCCAGAAGCTTCGAGAGAAGCAGCAACTAGAAGAAGATCTCAAAGGCTAC  923
 K  A  R  G  D  F  Q  K  L  R  E  K  Q  Q  L  E  E  D  L  K  G  Y
CTGGACTGGATCACCCAGGCAGAAGACATTGACCCCGAGAACGAAGACGAGGGCATGGATGAAGAC  989
 L  D  W  I  T  Q  A  E  D  I  D  P  E  N  E  D  E  G  M  D  E  D
AAGCCTCGAAACAGAGGCGCTCCAGCGGGCTTGCATGATCAGAAGAAAGGGAAGTTTGCTTGGTTT 1055
 K  P  R  N  R  G  A  P  A  G  L  H  D  Q  K  K  G  K  F  A  W  F
AGTCACTCTACAGAAACCCATGTGAGCATGCCCACAAGTGAGACTGAGTCTGTCAACACTGACAAC 1121
 S  H  S  T  E  T  H  V  S  M  P  T  S  E  T  E  S  V  N  T  D  N
GTGGCTGGAGGTGACATCGAGGGAGAAAACTGTGGAGCCAGGCTTGCCCATCGGATCTCCAAATCC 1187
 V  A  G  G  D  I  E  G  E  N  C  G  A  R  L  A  H  R  I  S  K  S
AAATTCAGCCGCTACTGGCGCAGGTGGAATCGATTCTGCAGAAGAAAATGCCGTGCAGCAGTTAAG 1253
 K  F  S  R  Y  W  R  R  W  N  R  F  C  R  R  K  C  R  A  A  V  K
TCCAACGTCTTCTACTGGCTCGTGATCTTCCTGGTGTTCCTCAACACCCTCACCATTGCCTCCGAA 1319
 S  N  V  F  Y  W  L  V  I  F  L  V  F  L  N  T  L  T  I  A  S  E
CATTACAACCAGCCTCACTGGCTCACAGAAGACCAAGACACAGCCAATAAAGCCCTCCTGGCCCTT 1385
 H  Y  N  Q  P  H  W  L  T  E  D  Q  D  T  A  N  K  A  L  L  A  L
TTCACTGCAGAAATGCTCCTGAAGATGTACAGCCTGGGTCTGCAGGCCTATTTTGTGTCCCTCTTC 1451
 F  T  A  E  M  L  L  K  M  Y  S  L  G  L  Q  A  Y  F  V  S  L  F
AACCGCTTTGACTGTTTCATTGTGTGTGGGGGCATCCTGGAGACCATCCTGGTGGAGACGAAGATC 1517
 N  R  F  D  C  F  I  V  C  G  G  I  L  E  T  I  L  V  E  T  K  I
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ATGTCTCCCCTGGGCATCTCTGTGCTGAGATGTGTGCGGTTGCTCAGGATCTTCAAGATCACCAGG 1583
 M  S  P  L  G  I  S  V  L  R  C  V  R  L  L  R  I  F  K  I  T  R
TACTGGAATTCCTTGAGCAACCTTGTGGCATCCTTGCTGAACTCAGTGCGCTCCATTGCCTCCCTG 1649
 Y  W  N  S  L  S  N  L  V  A  S  L  L  N  S  V  R  S  I  A  S  L
CTGCTGCTCCTCTTCCTCTTCATCATCATCTTCTCCCTCCTGGGGATGCAGCTCTTTGGAGGGAAG 1715
 L  L  L  L  F  L  F  I  I  I  F  S  L  L  G  M  Q  L  F  G  G  K
TTCAATTTCGATGAGATGCAGACCCGTAGGAGCACGTTCGATAACTTCCCGCAGTCTCTCCTCACT 1781
 F  N  F  D  E  M  Q  T  R  R  S  T  F  D  N  F  P  Q  S  L  L  T
GTGTTTCAGATCCTGACCGGGGAGGACTGGAATTCGGTGATGTATGATGGGATCATGGCTTATGGC 1847
 V  F  Q  I  L  T  G  E  D  W  N  S  V  M  Y  D  G  I  M  A  Y  G
GGCCCCTCTTTTCCAGGGATGTTAGTCTGTATTTACTTCATCATCCTCTTCATCTGTGGAAATTAT 1913
 G  P  S  F  P  G  M  L  V  C  I  Y  F  I  I  L  F  I  C  G  N  Y
ATCCTACTGAATGTGTTCTTGGCCATTGCGGTGGACAACCTGGCTGATGCGGAGAGCCTGACCTCA 1979
 I  L  L  N  V  F  L  A  I  A  V  D  N  L  A  D  A  E  S  L  T  S
GCCCAAAAGGAGGAGGAAGAAGAGAAGGAGAGGAAGAAGCTGGCCAGGACTGCCAGCCCAGAAAAG 2045
 A  Q  K  E  E  E  E  E  K  E  R  K  K  L  A  R  T  A  S  P  E  K
AAACAGGAGGTGATGGAGAAGCCAGCCGTGGAGGAGAGCAAAGAGGAGAAAATTGAACTGAAATCC 2111
 K  Q  E  V  M  E  K  P  A  V  E  E  S  K  E  E  K  I  E  L  K  S
ATTACAGGCAGTGGAGAATCCCCACCCACTACCAAGATACACATGGATGCACTCCAGCCCAGTGAA 2177
 I  T  G  S  G  E  S  P  P  T  T  K  I  H  M  D  A  L  Q  P  S  E
CCCGAGGATAAGAGTCCCCACTCCAACCCAGACACTGCAGGGGAAGAGGATGAAGAGGAGCCAGAG 2243
 P  E  D  K  S  P  H  S  N  P  D  T  A  G  E  E  D  E  E  E  P  E
ATGCCTGTGGGGCCACGCCCCCGGCCCCTGTCTGAGCTGCACCTTAAGGAAAAGGCAGTTCCCATG 2309
 M  P  V  G  P  R  P  R  P  L  S  E  L  H  L  K  E  K  A  V  P  M
CCGGAAGCCAGTGCATTTTTCATCTTCAGCCCAAACAACAGGTTCCGCCTGCAGTGCCACCGTATT 2375
 P  E  A  S  A  F  F  I  F  S  P  N  N  R  F  R  L  Q  C  H  R  I
GTCAATGACACGATCTTCACCAACCTCATCCTCTTCTTCATTCTGCTCAGCAGCATCTCTCTGGCT 2441
 V  N  D  T  I  F  T  N  L  I  L  F  F  I  L  L  S  S  I  S  L  A
GCTGAGGACCCCGTCCAGCACACCTCCTTCAGGAACCATATGACTGCTTACGGGGCTTTCCTGCAC 2507
 A  E  D  P  V  Q  H  T  S  F  R  N  H  M  T  A  Y  G  A  F  L  H
AAGGGCTCTTTCTGCCGAAACTACTTCAATATCCTGGACCTGCTGGTGTTGTGCGTGTCCCTCATC 2573
 K  G  S  F  C  R  N  Y  F  N  I  L  D  L  L  V  L  C  V  S  L  I
TCCTTTGGCATCCAGTCCAGCGCGATCAACGTTGTGAAGATTTTACGAGTGCTGCGAGTCCTCAGA 2639
 S  F  G  I  Q  S  S  A  I  N  V  V  K  I  L  R  V  L  R  V  L  R
CCCCTGAGGGCCATCAACAGGGCCAAGGGGCTAAAGCATGTGGTTCAGTGCGTGTTTGTGGCCATC 2705
 P  L  R  A  I  N  R  A  K  G  L  K  H  V  V  Q  C  V  F  V  A  I
CGGACCATCGGGAACATCGTAATTGTCACCACTCTGCTGCAGTTCATGTTCGCCTGCATTGGGGTC 2771
 R  T  I  G  N  I  V  I  V  T  T  L  L  Q  F  M  F  A  C  I  G  V
CAGCTCTTCAAGGGAAAGCTCTATACCTGTTCGGATAGTTCTAAACAGGCGGAGGCAGAATGCAAG 2837
 Q  L  F  K  G  K  L  Y  T  C  S  D  S  S  K  Q  A  E  A  E  C  K
GGTAACTATATCACATACAAAGATGGAGAGGTCGATCACCCCATTATCCAGCCTCGAAGCTGGGAG 2903
 G  N  Y  I  T  Y  K  D  G  E  V  D  H  P  I  I  Q  P  R  S  W  E
AACAGCAAGTTTGACTTTGACAATGTTTTGGCAGCCATGATGGCTCTCTTCACCGTCTCCACCTTC 2969
 N  S  K  F  D  F  D  N  V  L  A  A  M  M  A  L  F  T  V  S  T  F
GAAGGGTGGCCAGAGCTGCTGTACCGCTCCATTGACTCCCACACAGAAGACAAGGGCCCCATCTAC 3035
 E  G  W  P  E  L  L  Y  R  S  I  D  S  H  T  E  D  K  G  P  I  Y
AACTACCGTGTGGAGATCTCCATCTTCTTCATCATCTATATCATCATCATTGCCTTCTTCATGATG 3101
 N  Y  R  V  E  I  S  I  F  F  I  I  Y  I  I  I  I  A  F  F  M  M
AACATCTTCGTGGGTTTCGTCATTGTCACCTTCCAGGAGCAGGGGGAACAAGAGTACAAGAACTGT 3167
 N  I  F  V  G  F  V  I  V  T  F  Q  E  Q  G  E  Q  E  Y  K  N  C
GAGCTGGACAAGAACCAGAGACAATGTGTGGAATATGCCCTCAAGGCCCGACCCTTGCGAAGGTAC 3233
 E  L  D  K  N  Q  R  Q  C  V  E  Y  A  L  K  A  R  P  L  R  R  Y
ATCCCCAAGAACCAGCACCAGTACAAAGTGTGGTACGTGGTCAACTCTACCTACTTCGAGTATCTG 3299
 I  P  K  N  Q  H  Q  Y  K  V  W  Y  V  V  N  S  T  Y  F  E  Y  L
ATGTTCGTTCTCATCCTGCTCAACACCATCTGCCTGGCCATGCAGCACTATGGCCAGAGCTGCCTC 3365
 M  F  V  L  I  L  L  N  T  I  C  L  A  M  Q  H  Y  G  Q  S  C  L
TTCAAAATCGCCATGAATATACTCAACATGCTTTTCACCGGCCTCTTCACAGTGGAGATGATCCTG 3431
 F  K  I  A  M  N  I  L  N  M  L  F  T  G  L  F  T  V  E  M  I  L
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AAGCTCATTGCCTTCAAACCCAAGGGTTACTTTAGTGATCCCTGGAATGTTTTTGACTTCCTCATC 3497
 K  L  I  A  F  K  P  K  G  Y  F  S  D  P  W  N  V  F  D  F  L  I
GTCATTGGGAGCATAATTGATGTCATTCTCAGTGAGACTAATAGTGCAGAGGAGAACTCCCGCATC 3563
 V  I  G  S  I  I  D  V  I  L  S  E  T  N  S  A  E  E  N  S  R  I
TCCATCACTTTCTTCCGCCTCTTCCGGGTCATGCGCCTGGTGAAGCTGCTGAGCCGCGGGGAAGGC 3629
 S  I  T  F  F  R  L  F  R  V  M  R  L  V  K  L  L  S  R  G  E  G
ATCCGAACCCTGCTGTGGACCTTCATCAAGTCCTTCCAGGCTCTGCCCTATGTGGCTCTTTTGATT 3695
 I  R  T  L  L  W  T  F  I  K  S  F  Q  A  L  P  Y  V  A  L  L  I
GTGATGCTGTTCTTTATCTATGCAGTGATTGGGATGCAGGTGTTTGGGAAGATTGCCCTGAATGAC 3761
 V  M  L  F  F  I  Y  A  V  I  G  M  Q  V  F  G  K  I  A  L  N  D
ACCACAGAGATCAATCGGAACAACAACTTCCAGACGTTCCCCCAGGCTGTGTTACTGCTGTTCAGG 3827
 T  T  E  I  N  R  N  N  N  F  Q  T  F  P  Q  A  V  L  L  L  F  R
TGTGCCACCGGAGAGGCCTGGCAGGACATCATGCTGGCCTGCATGCCAGGCAAGAAGTGTGCCCCA 3893
 C  A  T  G  E  A  W  Q  D  I  M  L  A  C  M  P  G  K  K  C  A  P
GAGTCTGAGCCCAGCAACAGCACGGAAGGGGAGACCCCCTGTGGCAGCAGCTTTGCTGTCTTCTAC 3959
 E  S  E  P  S  N  S  T  E  G  E  T  P  C  G  S  S  F  A  V  F  Y
TTCATCAGCTTCTACATGCTCTGTGCCTTCCTGATCATCAACCTCTTTGTAGCTGTTATCATGGAC 4025
 F  I  S  F  Y  M  L  C  A  F  L  I  I  N  L  F  V  A  V  I  M  D
AACTTTGACTACCTGACTAGGGATTGGTCTATCCTCGGTCCCCATCACCTGGATGAATTCAAGAGA 4091
 N  F  D  Y  L  T  R  D  W  S  I  L  G  P  H  H  L  D  E  F  K  R
ATCTGGGCCGAGTATGACCCTAAAGCCAAGGGTCGGATCAAACACTTGGATGTGGTGACCCTCCTC 4157
 I  W  A  E  Y  D  P  K  A  K  G  R  I  K  H  L  D  V  V  T  L  L
CGTCGAATTCATCCCCCACTGGGTTTTGGGAAATTGTGTCCTCACCGTGTGGCCTGCAAACGACTG 4223
 R  R  I  H  P  P  L  G  F  G  K  L  C  P  H  R  V  A  C  K  R  L
GTGTCCATGAACATGCCTCTGAACAGCGATGGCACAGTCATGTTCAATGCTACCCTGTTTGCCCTC 4289
 V  S  M  N  M  P  L  N  S  D  G  T  V  M  F  N  A  T  L  F  A  L
GTCAGGACAGCCCTGAGGATCAAAACAGAAGGGAACCTAGAGCAAGCCAATGAGGAGCTTCGGGCC 4355
 V  R  T  A  L  R  I  K  T  E  G  N  L  E  Q  A  N  E  E  L  R  A
ATCATCAAGAAAATCTGGAAGAGGACTAGCATGAAGCTGTTGGACCAGGTGGTGCCCCCTGCAGGC 4421
 I  I  K  K  I  W  K  R  T  S  M  K  L  L  D  Q  V  V  P  P  A  G
GATGACGAGGTCACAGTGGGCAAGTTCTATGCCACCTTCCTGATCCAAGAGTACTTCAGGAAATTC 4487
 D  D  E  V  T  V  G  K  F  Y  A  T  F  L  I  Q  E  Y  F  R  K  F
AAAGAGCGAAAAGAGCAGGGGCTGGTGGGCAAGCCCTCACAAAGGAATGCACTGTCCCTCCAGGCT 4553
 K  E  R  K  E  Q  G  L  V  G  K  P  S  Q  R  N  A  L  S  L  Q  A
GGCTTGCGCACCTTGCATGACATTGGGCCTGAGATCCGGCGGGCCATCTCTGGGGATCTGACTGCT 4619
 G  L  R  T  L  H  D  I  G  P  E  I  R  R  A  I  S  G  D  L  T  A
GAGGAGGAGTTGGACAAGGCTATGAAGGAGGCGGTGTCTGCTGCCTCCGAAGATGACATCTTCAGG 4685
 E  E  E  L  D  K  A  M  K  E  A  V  S  A  A  S  E  D  D  I  F  R
AGGGCTGGAGGCCTGTTCGGCAACCATGTCACCTACTATCAGAGTGACAGCAGGGGCAACTTTCCT 4751
 R  A  G  G  L  F  G  N  H  V  T  Y  Y  Q  S  D  S  R  G  N  F  P
CAGACGTTCGCCACCCAGCGCCCACTGCACATCAACAAGACAGGGAACAACCAAGCTGACACTGAG 4817
 Q  T  F  A  T  Q  R  P  L  H  I  N  K  T  G  N  N  Q  A  D  T  E
TCACCGTCCCATGAGAAGCTGGTGGACTCCACGTTCACCCCCAGCAGCTACTCATCCACGGGCTCC 4883
 S  P  S  H  E  K  L  V  D  S  T  F  T  P  S  S  Y  S  S  T  G  S
AATGCCAACATCAACAATGCCAACAACACTGCCCTGGGCCGCTTCCCCCATCCCGCTGGCTACTCC 4949
 N  A  N  I  N  N  A  N  N  T  A  L  G  R  F  P  H  P  A  G  Y  S
AGCACGGTCAGCACTGTGGAGGGCCATGGGCCTCCCTTGTCCCCTGCTGTCCGAGTACAGGAGGCA 5015
 S  T  V  S  T  V  E  G  H  G  P  P  L  S  P  A  V  R  V  Q  E  A
GCATGGAAGCTCAGCTCTAAGAGGTGCCACTCCCGAGAGAGCCAGGGAGCCACGGTGAATCAGGAG 5081
 A  W  K  L  S  S  K  R  C  H  S  R  E  S  Q  G  A  T  V  N  Q  E
ATATTTCCAGATGAGACCCGCAGCGTAAGGATGAGTGAAGAAGCCGAGTACTGCAGTGAGCCCAGC 5147
 I  F  P  D  E  T  R  S  V  R  M  S  E  E  A  E  Y  C  S  E  P  S
CTGCTCTCCACAGATATGTTCTCCTACCAGGAAGATGAACACCGACAACTGACCTGCCCAGAGGAG 5213
 L  L  S  T  D  M  F  S  Y  Q  E  D  E  H  R  Q  L  T  C  P  E  E
GACAAGAGGGAGATCCAGCCATCTCCAAAGAGGAGTTTCCTTCGCTCTGCCTCTCTAGGTCGAAGG 5279
 D  K  R  E  I  Q  P  S  P  K  R  S  F  L  R  S  A  S  L  G  R  R
GCCTCCTTCCATCTGGAATGTCTAAAGCGACAAAAGGATCAAGGGGGAGACATCTCTCAGAAGACA 5345
 A  S  F  H  L  E  C  L  K  R  Q  K  D  Q  G  G  D  I  S  Q  K  T
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GCCTTGCCCTTGCATCTGGTTCATCATCAGGCATTGGCAGTGGCAGGCTTGAGCCCCCTCCTGCAG 5411
 A  L  P  L  H  L  V  H  H  Q  A  L  A  V  A  G  L  S  P  L  L  Q
AGAAGCCATTCTCCTACCACATTCCCCAGGCCGTGCCCCACACCCCCTGTCACTCCAGCCAGCCGG 5477
 R  S  H  S  P  T  T  F  P  R  P  C  P  T  P  P  V  T  P  A  S  R
GGCAGCACCCTACGGCCCATCCCTCACCTACGGCTGAAGGGGGCAGAGTCCAGCGAGAAACTCAAC 5543
 G  S  T  L  R  P  I  P  H  L  R  L  K  G  A  E  S  S  E  K  L  N
AGCAGCTTCCCATCCATCCACTGCAGCTCCTGGTCTGAGGAGACGACAGCCTGTAGTGGGAGCAGC 5609
 S  S  F  P  S  I  H  C  S  S  W  S  E  E  T  T  A  C  S  G  S  S
AGCATGGCCCGGAGAGCCCGGCCCGTCTCCCTCACCGTGCCCAGCCAGGCTGGAGCTCCAGGGAGA 5675
 S  M  A  R  R  A  R  P  V  S  L  T  V  P  S  Q  A  G  A  P  G  R
CAGTTCCATGGCAGTGCCAGCAGCCTGGTGGAAGCGGTCTTGATTTCAGAAGGACTGGGACAGTTT 5741
 Q  F  H  G  S  A  S  S  L  V  E  A  V  L  I  S  E  G  L  G  Q  F
GCTCAAGATCCCAAGTTCATCGAGGTCACCACCCAGGAGCTGGCTGACGCCTGCGACATGACAATA 5807
 A  Q  D  P  K  F  I  E  V  T  T  Q  E  L  A  D  A  C  D  M  T  I
GAGGAGATGGAGAACGCCGCAGACAACATCCTCAGTGGGGGCCGCCAGCAGAGCCCCAACGGCACC 5873
 E  E  M  E  N  A  A  D  N  I  L  S  G  G  R  Q  Q  S  P  N  G  T
CTCTTACCTTTGGTGAACTGTAGGGACCCGGGGCAGGACAGGGCTGTGGTCCCAGAGGACGAGAGC 5939
 L  L  P  L  V  N  C  R  D  P  G  Q  D  R  A  V  V  P  E  D  E  S
TGCGCATATGCCCTGGGGCGAGGCCGGAGCGAGGAGGCGCTCGCGGACAGCAGGTCCTACGTCAGC 6005
 C  A  Y  A  L  G  R  G  R  S  E  E  A  L  A  D  S  R  S  Y  V  S
AACCTGTAGTCCTCAGGGCTGGCGAGACGCGGGTGGTTTTTTTATTCGTTTCAATGTTCCTAATGG 6071
 N  L
GTTCGTTTCAGAAGTGCCTCACTGTTCTCGTGACCTGGAGGTAACCGGAACACGGTCTTCATTCAC 6137
TGCTGTCGGGATAAGCCTCAGAGCTGGGCGGTGTACGGAGTCGGCTTTTCAGGGGAGAAGGCCAAG 6203
ACCGTGGTGCGGGGGCTCCAGCACCTTCCCGCGGCAGCACCGCCCCAAGGACCGCCACCCCCACCC 6269
CTGAGCAAAAGGGTGTTTTCCCCTTGCTTGTATAAACAGTCATTTGCACATGTTCTGTCTGAGCCT 6335
GGCCGTCTCTATGGAGCAGGCCCCCAGGGATCTATGGCAGGAATGGGCCAGCGCCCCCAGTAGGAG 6401
CCGGGAGGTGGCTGCGAGGTTCCCAGCAGTGCAGGTCTGGTCCCTATGGTCCCTTCAGGGACTCTT 6467
TCCCTGCAAGGAGCTGAGATGCAGGTGGCAGGAGCCAGTGCAGATCACACCACCCGCCCTCAGCTA 6533
GCCAGGCCAGGGGGGCGGCAGGCTGCTGCCTGGTGCTCGGGGTTTCATGGTTTTGAGGGTTCTTGT 6599
CAGCATGTTGCGACTTTCTGGGGTTTGGTTTCTTTATTACTATTTGTTGTGTTTTCCCACGGGGAG 6665
GGGAGGAATAAGAGCGGTTACAACTGCGCGGCCTCACTTCACTGTTTCCACATTTGCATTTGCGTA 6731
TTTAAGTCGGATTTGTTATGATTGTATTCTTTAAATGGTGCGGTCCACCCCCACCGCCACCCCCAC 6797
CCCCACTGGAGCAAGGGTTCAATATCACCAGAGAAAGGTTTTACCTGCTCTGTGTCTGCCCAGTAA 6863
CTTGTTCCAATTTCCTTAAGTAAAAGCAACTTTTTTCTTTCTTTCGAGTTTGGTTGAGCATCACAA 6929
TCAGCAGGCTAACAGGCAGTTAGATCAGGCGGTGTGCGCCTGGGCGATTGAGCTGGGCTCCTTTCT 6995
GTGCTGGGCATATGGACTGGTTCAAGAGAGAAGAAATATGGGCATCTTTGTGTCACACTTGTGTCC 7061
ATAGTATGTGCGTATGTGCACCCACGTGGTATGTGTGCGCCCCACCCCACCCCCGCACAAAAGCCT 7127
GTAGAACCCCGTTTGGGTTTGCCTGCAGGGAGTTCTAAATCTGGGGCTATTTGAAAGCAAGAACAA 7193
ACCACTGTCTCTGCTTCTGCTTCTGAAACGAGAATCGGTAACTGCATTTTTCTGTCCCACGAGATA 7259
TGCAAAAGCAATGCAATAATATCCATTTTAAAATATGGGTGTGAGTTGTGTCAGCATTAAAATTCT 7325
ATTTTAAAAAAAAAAAACCACGAAATTTAAGGGAAAAACTCAAGAAGACATTTTGCTTCGATATAT 7391
TCTGTGTAATGTTTTATTGCATTGATAATGTTTCTGTTGAAGAAACTGTTATACTTGAATTCAGGT 7457
CAGTTTCAGTATTTTTCAAATATTTTTTTAAAACTGAATTGCAATTGTGCCAAGCAAATACAATGA 7523
CTTAAGTTTGTTTTAAAATTCACTTCTTGTATATTTTGCTGCATGTAAAGTAAATCGTTTTGTATT 7589
TGGAAAAAAAAATAAAAAAAAATAAAAAAAAAAAAAAAA                             7629
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Figure 10. The MELC-CC cDNA sequence with the deduced amino-acid sequence. The single-lettered amino-acid is aligned below the cDNA at the center of each codon. The first ATG for the open reading frame and two conventional polyadenylation signals are underlined. An in-frame stop codon preceding the first ATG is dotted underlined. The numbers on the right side of each line denote the position of the last nucleotide in that row.

2. Analysis of the Primary Amino Acid Structure

Analysis of the primary amino acid sequence revealed several differences upon comparison to the α_1 subunit of the rabbit cardiac DHP-R. First, 20 out of the 24 putative transmembrane regions, as predicted by comparison to the α_1 subunit of the rabbit cardiac DHP-R, were conserved. The missing 4 (designated IS1 to IS4 in the DHP-R) were the most N-terminal regions which include the first S4 region. Second, the rest of the putative transmembrane regions of C/DHP-R are highly conserved between the two sequences (except for the IIIS2) exhibiting ~99% homology including three S4 regions that are identical to each other [Mikami et al., 1989] (Figure 11). A 25 amino-acid fragment is inserted in the MELC-CC between IS6 and IIS1 (Figure 11). The S2 and S3 segments within each motif contain conserved basic and acidic amino acids, respectively. Montal has suggested that these residues may play a role in voltage sensing and/or pore formation [Montal, 1990]. Third, comparison of the MELC-CC sequence with the consensus sequence for protein phosphorylation sites, reveals a threonine residue and 17 serine residues located in the predicted intracellular domains of the MELC-CC that are potential cyclic AMP dependent phosphorylation sites. Since the affinity of a protein phosphorylation site depends not only on the primary sequence, but also on higher order structures, it will be necessary to perform supplemental experiments such as site directed mutagenesis to confirm the actual phosphorylation sites in the MELC-CC. In addition to the 18 phosphorylation sites found intracellularly, three N-glycosylation sites were identified in this molecule at amino acid residues 64, 1118 and 1169 in the

extracellular domain. Lastly, the rabbit cardiac and mouse MELC proteins exhibited differences at the carboxyl termini probably due to species differences and differential splicing (Figure 11).

```
C/DHP-R  MLRALVQPATPAYQPLPSHLSAETESTCKGTVVHEAQLNHFYISPGGSNYGSPRPAHANM   60

C/DHP-R  NANAAAGLAPEHIPTPGAALSWQAAIDAARQAKLMGSAGNATISTVSSTQRKRQQYGKPK  120
                                                          IS1
C/DHP-R  KQGSTTATRPPRALLCLTLKNPIRSACISIVEWKPFEIIILLTIFANCVALAIYIPFPED  180
                                 IS2                              IS3
C/DHP-R  DSNATNSNLERVEYLFLIIFTVEAFLKVIAYGLLFHPNAYLRNGWNLLDFIIVVVGLFSA  240
                                          IS4
C/DHP-R  ILEQATKADGANALGGKGAGFDVKALSAFRVLRPLRLVSGVPSLQVVLNSIIKAMVPLLH  300
                                                                  !!!!!!
MELC-CC                                                           MVPLLH    6
                IS5
C/DHP-R  IALLVLFVIIIYAIIGLELFMGKMHKTCYNQEGVADVPAEDDPSPCALETGHGRQCQNGT  360
         !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!  !!!!  !!!!!!!!!!!!!!!!!!
MELC-CC  IALLVLFVIIIYAIIGLELFMGKMHKTCYNQEGIIDVPAKEDPSPCALETGHGRQCQNGT   66
                                                                     *
C/DHP-R  VCKPGWDGPKHGITNFDNFAFAMLTVFQCITMEGWTDVLYWMQDAMGYELPWVYFVSLVI  420
         !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!  !! !   !! !!! ! !
MELC-CC  VCKPGWDGPKHGITNFDNFAFAMLTVFQCITMEGWTDVLYWVNDAVGRDWPWIYFVTLII  126
            IS6
C/DHP-R  FGSFFVLNLVLGVLSGEFSKEREKAKARGDFQKLREKQQLEEDLKGYLDWITQAEDIDPE  480
          !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
MELC-CC  IGSFFVLNLVLGVLSGEFSKEREKAKARGDFQKLREKQQLEEDLKGYLDWITQAEDIDPE  186

C/DHP-R NEDEGMDEEKPRN MSMPTSETESVNTENVAGGDIEGENCGARLAHRISKSKFSRYWRRWN  540
        !!!!!!!! !!!!  !!!!!!!!!!!! !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
MELC-CC NEDEGMDEDKPRN VSMPTSETESVNTDNVAGGDIEGENCGARLAHRISKSKFSRYWRRWN  271
                      ^
          (RGAPAGLHDQKKGKFAWFSHSTETH)
                                IIS1                              IIS2
C/DHP-R  RFCRRKCRAAVKSNVFYWLVIFLVFLNTLTIASEHYNQPHWLTEVQDTANKALLALFTAE  600
         !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
MELC-CC  RFCRRKCRAAVKSNVFYWLVIFLVFLNTLTIASEHYNQPHWLTEDQDTANKALLALFTAE  331
                                     IIS3                         IIS4
C/DHP-R  MLLKMYSLGLQAYFVSLFNRFDCFIVCGGILETILVETKVMSPLGISVLRCVRLLRIFKI  660
         !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
MELC-CC  MLLKMYSLGLQAYFVSLFNRFDCFIVCGGILETILVETKIMSPLGISVLRCVRLLRIFKI  391
                                          IIS5
C/DHP-R  TRYWNSLSNLVASLLNSVRSIASLLLLLFLFIIIFSLLGMQLFGGKFNFDEMQTRRSTFD  720
         !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
MELC-CC  TRYWNSLSNLVASLLNSVRSIASLLLLLFLFIIIFSLLGMQLFGGKFNFDEMQTRRSTFD  451
                                                              IIS6
C/DHP-R  NFPQSLLTVFQILTGEDWNSVMYDGIMAYGGPSFPGMLVCIYFIILFICGNYILLNVFLA  780
         !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
MELC-CC  NFPQSLLTVFQILTGEDWNSVMYDGIMAYGGPSFPGMLVCIYFIILFICGNYILLNVFLA  511
```

```
C/DHP-R  IAVDNLADAESLTSAQKEEEEEKERKKLARTASPEKKQEVVGKPALEEAKEEKIELKSIT  840
         !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!  !!! !! !!!!!!!!!!!
MELC-CC  IAVDNLADAESLTSAQKEEEEEKERKKLARTASPEKKQEVMEKPAVEESKEEKIELKSIT  571

C/DHP-R  ADGESPPTTKINMDDLQPNESEDKSPYPNPETTGEEDEEEPEMPVGPRPRPLSELHLKEK  900
           !!!!!!!!! !! !!! ! !!!!!  !! ! !!!!!!!!!!!!!!!!!!!!!!!!!!!!
MELC-CC  GSGESPPTTKIHMDALQPSEPEDKSPHSNPDTAGEEDEEEPEMPVGPRPRPLSELHLKEK  631
                                                  IIIS1
C/DHP-R  AVPMPEASAFFIFSPNNRFRLQCHRIVNDTIFTNLILFFILLSSISLAAEDPVQHTSFRN  960
         !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
MELC-CC  AVPMPEASAFFIFSPNNRFRLQCHRIVNDTIFTNLILFFILLSSISLAAEDPVQHTSFRN  691
                      IIIS2                                IIIS3
C/DHP-R  HILFYFDIVFTTIFTIEIALKMTAYGAFLHKGSFCRNYFNILDLLVVSVSLISFGIQSSA 1020
                              !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
MELC-CC  ....................HMTAYGAFLHKGSFCRNYFNILDLLVLCVSLISFGIQSSA  731
                    IIIS4                                      IIIS5
C/DHP-R  INVVKILRVLRVLRPLRAINRAKGLKHVVQCVFVAIRTIGNIVIVTTLLQFMFACIGVQL 1080
         !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
MELC-CC  INVVKILRVLRVLRPLRAINRAKGLKHVVQCVFVAIRTIGNIVIVTTLLQFMFACIGVQL  791

C/DHP-R  EKGKLYTCSDSSKQTEAECKGNYITYKDGEVDHPIIQPRSWENSKFDFDNVLAAMMALFT 1140
         !!!!!!!!!!!!!! !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
MELC-CC  FKGKLYTCSDSSKQAEAECKGNYITYKDGEVDHPIIQPRSWENSKFDFDNVLAAMMALFT  851
                                                  IIIS6
C/DHP-R  VSTFEGWPELLYRSIDSHTEDKGPIYNYRVEISIFFIIYIIIIAFFMMNIFVGFVIVTFQ 1200
         !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
MELC-CC  VSTFEGWPELLYRSIDSHTEDKGPIYNYRVEISIFFIIYIIIIAFFMMNIFVGFVIVTFQ  911
                                                                  IVS1
C/DHP-R  EQGEQEYKNCELDKNQRQCVEYALKARPLRRYIPKNQHQYKVWYVVNSTYFEYLMFVLIL 1260
         !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
MELC-CC  EQGEQEYKNCELDKNQRQCVEYALKARPLRRYIPKNQHQYKVWYVVNSTYFEYLMFVLIL  971
                                        IVS2
C/DHP-R  LNTICLAMQHYGQSCLFKIAMNILNMLFTGLFTVEMILKLIAFKPKGYFSDPWNVFDFLI 1320
         !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
MELC-CC  LNTICLAMQHYGQSCLFKIAMNILNMLFTGLFTVEMILKLIAFKPKGYFSDPWNVFDFLI 1031
           IVS3                                    IVS4
C/DHP-R  VIGSIIDVILSETNPAEHTQCSPSMNAEENSRISITFFRLFRVMRLVKLLSRGEGIRTLL 1380
         !!!!!!!!!!!!!!           !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
MELC-CC  VIGSIIDVILSETN..........SAEENSRISITFFRLFRVMRLVKLLSRGEGIRTLL 1080
                                IVS5
C/DHP-R  WTFIKSFQALPYVALLIVMLFFIYAVIGMQVFGKIALNDTTEINRNNNFQTFPQAVLLLF 1440
         !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
MELC-CC  WTFIKSFQALPYVALLIVMLFFIYAVIGMQVFGKIALNDTTEINRNNNFQTFPQAVLLLF 1140
                                                  *          IVS6
C/DHP-R  RCATGEAWQDIMLACMPGKKCAPESEPHNSTEGETPCGSSFAVFYFISFYMLCAFLIINL 1500
         !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
MELC-CC  RCATGEAWQDIMLACMPGKKCAPESEPSNSTEGETPCGSSFAVFYFISFYMLCAFLIINL 1200
                                     *
C/DHP-R  FVAVIMDNFDYLTRDWSILGPHHLDEFKRIWAEYDPEAKGRIKHLDVVTLLRRIQPPLGF 1560
         !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
MELC-CC  FVAVIMDNFDYLTRDWSILGPHHLDEFKRIWAEYDPKAKGRIKHLDVVTLLRRIHPPLGF 1260

C/DHP-R  GKLCPHRVACKRLVSMNMPLNSDGTVMFNATLFALVRTALRIKTEGNLEQANEELRAIIK 1620
         !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
MELC-CC  GKLCPHRVACKRLVSMNMPLNSDGTVMFNATLFALVRTALRIKTEGNLEQANEELRAIIK 1320
```

```
C/DHP-R  KIWKRTSMKLLDQVVPPAGDDEVTVGKFYATFLIQEYFRKFKKRKEQGLVGKPSQRNALS 1680
         !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
MELC-CC  KIWKRTSMKLLDQVVPPAGDDEVTVGKFYATFLIQEYFRKFKERKEQGLVGKPSQRNALS 1380

C/DHP-R  LQAGLRTLHDIGPEIRRAISGDLTAEEELDKAMKEAVSAASEDDIFRRAGGLFGNHVSYY 1740
         !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!! !!
MELC-CC  LQAGLRTLHDIGPEIRRAISGDLTAEEELDKAMKEAVSAASEDDIFRRAGGLFGNHVTYY 1440

C/DHP-R  QSDSRSAFPQTFTTQRPLHISKAGNNQGDTESPSHEKLVDSTFTPSSYSSTGSNANINNA 1800
         !!!!!  !!!!! !!!!!!! ! !!!! !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
MELC-CC  QSDSRGNFPQTFATQRPLHINKTGNNQADTESPSHEKLVDSTFTPSSYSSTGSNANINNA 1500

C/DHP-R  NNTALGRLPRPAGYPSTVSTVEGHGSPLSPAVRAQEAAWKLSSKRCHSQESQIAMACQEG 1860
         !!!!!!! ! !!!! !!!!!!!!!! !!!!!!! !!!!!!!!!!!!!! !!! !   !!
MELC-CC  NNTALGRFPHPAGYSSTVSTVEGHGPPLSPAVRVQEAAWKLSSKRCHSRESQGATVNQEI 1560

C/DHP-R  ASQDDNYDVRIGEDAECCSEPSLLSTEMLSYQDDENRQLAPPEEEKRDIRLSPKKGFLRS 1920
                  ! !! !!!!!!!!! ! !!! !! !!!  !!! !! !  !!!  !!!!
MELC-CC  FPDETRSVRMSEE.AEYCSEPSLLSTDMFSYQEDEHRQLTCPEEDKREIQPSPKRSFLRS 1619

C/DHP-R  ASLGRRASFHLECLKRQKNQGGDISQKTVLPLHLVHHQALAVAGLSPLLQRSHSPTSLPR 1980
         !!!!!!!!!!!!!!!!!! !!!!!!!!! !!!!!!!!!!!!!!!!!!!!!!!!!!!!  !!
MELC-CC  ASLGRRASFHLECLKRQKDQGGDISQKTALPLHLVHHQALAVAGLSPLLQRSHSPTTFPR 1679

C/DHP-R  PCATPPATPGSRGWPPQPIPTLRLEGADSSEKLNSSFPSIHCGSWSGENSPCRGDSSAAR 2040
         !! !!! !! !!!    !!! !!! !! !!!!!!!!!!!!!! !!! !   ! ! !! !!
MELC-CC  PCPTPPVTPASRGSTLRPIPHLRLKGAESSEKLNSSFPSIHCSSWSEETTACSGSSSMAR 1739

C/DHP-R  RARPVSLTVPSQAGAQGRQFHGSASSLVEAVLISEGLGQFAQDPKFIEVTTQELADACDL 2100
         !!!!!!!!!!!!!!! !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
MELC-CC  RARPVSLTVPSQAGAPGRQFHGSASSLVEAVLISEGLGQFAQDPKFIEVTTQELADACDM 1799

C/DHP-R  TIEEMENAADDILSGGARQSPNGTLLPFVNRRDPGRDRAGQNEQDASGACAPGCGQSEEA 2160
         !!!!!!!!!! !!!!!  !!!!!!!!! !! !!!! !!!   ! ! ! ! ! ! ! !!!!
MELC-CC  TIEEMENAADNILSGGRQQSPNGTLLPLVNCRDPGQDRAVVPE.DESCAYALGRGRSEEA 1858

C/DHP-R  LADRRAGVSSL                                                   2171
         !!! !  !! !
MELC-CC  LADSRSYVSNL                                                   1869
```

Figure 11. Alignment of the deduced MELC-CC amino-acid sequence derived from 5MEL, 2MEL and 3MEL with that of the rabbit cardiac DHP-R (C/DHP-R). A 93% overall amino acid homology to the rabbit cardiac $\alpha 1$ subunits suggest that they are from the same gene. Only 20 out of 24 putative transmembrane regions are conserved in MELC-CC making this channel unique. The missing 4 transmembrane segments (designated IS1 to IS4 in the C/DHP-R) were the most N-terminal regions. The conservation between the C/DHP-R and the MELC-CC was over 90%. In addition, deletions (indicated by dots) and alternative splicing sites (double underlined) were found within the MELC-CC sequence. The bold letters located between IS6 and IIS1 represent the deduced amino-acid sequence inserted in the MELC-CC. Potential cAMP dependent protein kinase phosphorylation sites are underlined with dots. Potential N-glycosylation sites are indicated with a (*) below the amino-acid residue. The putative cardiac DHP-R transmembrane regions are underlined and labeled over the top of the fragment with letters ISI to IVS6. The numbers on the right side of the figure indicate the position of the last amino acid in the row.

Analysis of the 5' end of the MELC cDNA

In order to demonstrate that the lack of the first four transmembrane regions of the MELC-CC sequence was not due to a cloning artifact but the expression of a truncated message, five strategies were utilized. They are as follows:

1. Northern Analysis

A Northern analysis was performed to study mRNA from MELC in order to determine if the first four transmembrane regions were expressed *in vivo*. Two identical Northern blots with MELC total RNA (30 μg) and mouse heart total RNA (15 μg) were probed with two separate probes. One was probed with a PCR derived cDNA probe corresponding to the first four transmembrane segments IS1-IS4 of the mouse cardiac DHP-R (corresponding to the nucleotide 532-843) [Mikami, 1989] and the other was probed with a cDNA probe corresponding to nucleotides 1-6235 of the MELC-CC cDNA.

As shown in figure 12, no detectable message was seen in MELC RNA probed with the cardiac DHP-R probe while the two predicted bands and one additional band were detected in mouse heart RNA. When probed with the cDNA corresponding to 1-6235 of the MELC Ca^{2+} channel mRNA, the band pattern in mouse heart was identical to its counterpart in the Northern probed with the cDNA encoding the first four transmembrane segments. The MELC exhibited similar patterns to the mouse heart, but the bands in MELC were slightly smaller corresponding to the difference between mRNAs containing all 24 transmembrane segments (cardiac) versus those containing only 20 transmembrane segments (MELC). For

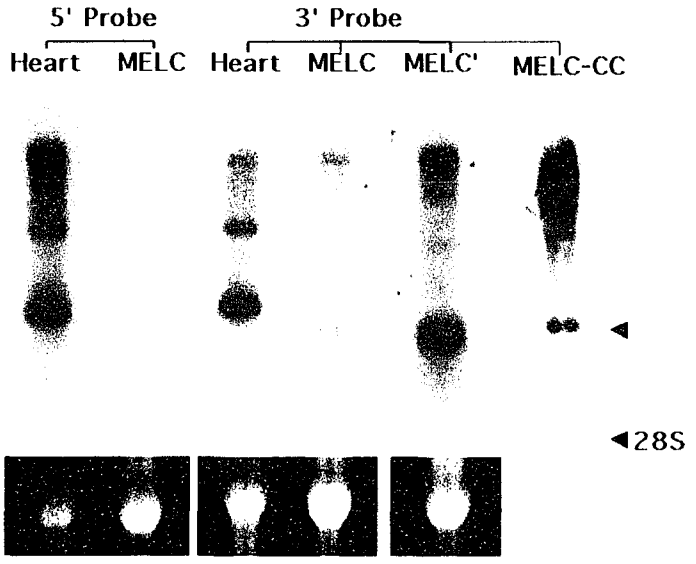


Figure 12. Northern analysis of the expression of MELC-CC and the cardiac calcium channel. Total RNA from mouse heart (15 μg) and MELC (30 μg) were run on formaldehyde agarose gels. A 5' cDNA probe spanning IS1-IS4 regions of the cardiac DHP-R and a 3' cDNA probe corresponding to 1-6235 of the MELC-CC were used. No detectable message was seen in MELC RNA probed with the 5' cardiac DHP-R probe while three bands were detected in mouse heart RNA, corresponding to mRNAs of 7.5, 9.5 and 22 kb. Thus the 5' portion of the C-DHP-R mRNA is not expressed in MELC. Using the 3' MELC-CC cDNA probe, the same three bands were also detected in mouse heart RNA, however three slightly smaller bands were now detected in MELC RNA. This finding indicates that three DHP-R mRNAs are expressed in MELC. The difference in size between the mouse heart mRNAs and the MELC mRNAs can be explained by deletion of the 5' sequences encoding the first four transmembrane regions. In the labne labelled MELC-CC *in vitro* transcribed full length MELC-CC RNA was loaded and hybridized with the 3' MELC-CC probe as a size marker. Northerns were exposed 24 hours, except for the lane labelled MELC' which represents a 7 day exposure. The position of the 28S rRNA is also indicated as a size marker. The lower panel represent the ethidium bromide stain of the 28S RNA used to control for loading of the RNA samples.

comparison, *in vitro* transcribed MELC-CC mRNA is shown at the far right in figure 12. The band from the *in vitro* transcribed mRNA is the same size as the ~7,600 band detected from MELC. This finding is consistent with the hypothesis that the α_1 subunit in MELC is truncated. To control for the amount of RNA loaded in each lane, ribosomal RNA was stained with ethidium bromide as shown in figure 12.

2. Additional Library Screening

Upon complete sequence analysis of the five aforementioned 5' MELC-CC clones, we determined that a region upstream of base 370 diverged completely from the cardiac DHP-R and did not contain an open reading frame any longer than 20 amino acids. A GenBank search using these 369 bases failed to produce any matching sequences. In addition, it was found that the non homologous region terminates with the base pairs "AG" indicating that an RNA splice site could be present. These findings strengthen the likelihood that these MELC-CC cDNAs encode mRNAs lacking the first four putative transmembrane regions of motif I. However, if the mRNA secondary structure prevents the reverse transcriptase from progressing during first strand cDNA synthesis, the 5' end of the cDNA would not be detected using the BamHI-HindIII probe. The random primed MELC cDNA library was rescreened using a PCR derived from mouse cardiac DHP-R cDNA corresponding to the IS1-IS4 region (located between 5'- CCGGAAGACGACTCCAACG-3' to 5'-GACTCCGGACACTTG CCG-3' and corresponding to the nucleotide 532-843). Nevertheless, no positive clones were isolated from 5 X 10^6 recombinants screened.

3. RACE

It is possible that the MELC-CC expresses some form of the first four transmembrane regions that diverges sufficiently from those of the mouse heart and could not be recognized by the cardiac DHP-R IS1-IS4 probes. RACE (rapid amplification of cDNA ends) [Frohman, 1988] was used to clone those regions. RACE is a procedure for amplification of nucleic acid sequences from a messenger RNA template between a defined internal site and unknown sequence at the 5' end of the mRNA. The following antisense primers were used: 5MBamAS located at 536 to 556 and with a sequence of 5'GGGGAAGGATCCTCTTTTGCC3'; 5MSacAS located at 467-487 and with a sequence of 5'CCCATGAAGAGCTCCAGGCCG3'; 5MASA located at 387-402 and with a sequence of 5'CCTTGATGATGGAGTTCAGGACCAC3'; and 1440AS located at 45-64 and with a sequence of 5'TACCCACGGCCACAGAGGAC3'; and the anchor primer 5'CUACUA CUACUAGGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG3'. In order to construct the first strand cDNA reverse transcription was performed at 42^0 C using SuperScript RT (Gibco BRL) on MELC mRNA with the primer 5MBamAS. Mouse heart mRNA was amplified using the same primer as a control. Aliquots of the above cDNAs were used as templates in PCR reactions which used the 5MSacAS and anchor primers. A 10 µl aliquot of each PCR product was electrophoresed on a 1.6% agarose gel and transferred to nitrocellulose. Southern hybridization was used to probe with 5MASA (5MASA is 3' to MELC nucleotide 370 and is identical to both MELC and cardiac DHP-R). The MELC sample exhibited smeared signals (at about 350-450 base pairs), but no signals of desired molecular weight were found that

could have corresponded to messages encoding the first four transmembrane repeats (Figure 13). A predicted ~1 kb band was observed in the control mouse heart sample. When probed with the primer 1440AS (5' to MELC nucleotide 370 and it is non homologous to cardiac DHP-R) an identical signal pattern was identified in the MELC sample as when probed with 5MASA, and no signal was detected in heart (Figure 13). When the blot was probed with the mouse heart cDNA probe IS1-IS4, a band was seen at ~ 1 kb in the control, as predicted, but no bands were seen in the MELC (Figure 13). To control for DNA contamination, MELC mRNA alone was used as the template and no product was obtained. Consistent with previous results, this experiment also failed to detect any new sequence upstream of base pair 370.

4. Primer extension

To determine the base pair from which the transcription of the MELC-CC mRNA starts, the primer extension assay was used. The antisense primer, 1440AS, (a synthetic 20-base oligonucleotide complementary to the MELC-CC cDNA at nucleotide 45-64) was used to start the extension reaction. Fifty micrograms of MELC total RNA were extended using AMV reverse transcriptase and a heterogeneous mixture of extended products was generated (Figure 14). Two major bands were located at 75 and 105 bases. These results indicate that there may be two transcription initiation sites exist for the MELC-CC mRNA synthesis.

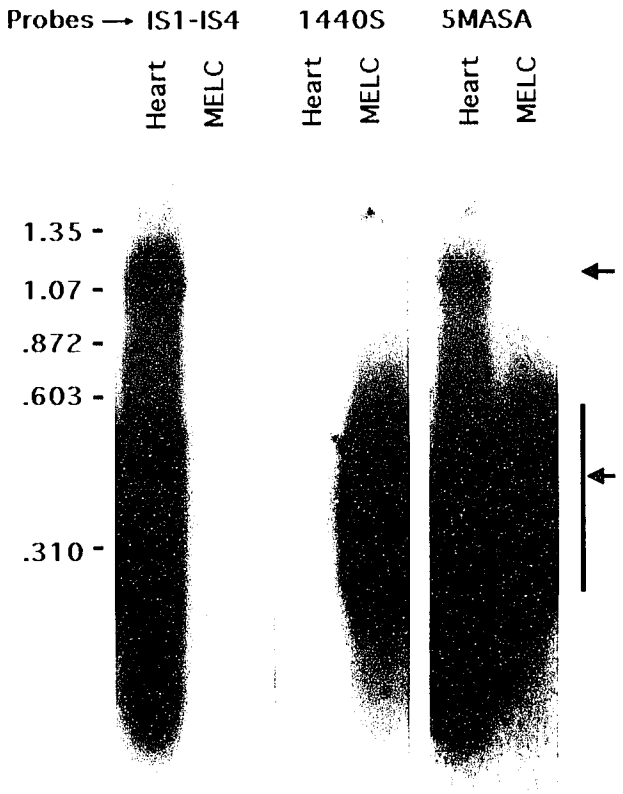


Figure 13. RACE (Rapid amplification of cDNA ends) analysis was used in an attempt to isolate additional 5'sequence from the MELC-CC. A 5' anchor primer and a 3' 5MSacAS primer were used in the RACE protocol and the PCR products were size fractionated on a 1.6% agarose gel. Three internal probes (mouse heart cDNA IS1-IS4, MELC-CC 5' oligo 1440S and oligo 5MASA (sequence homologous to both cardiac and MELC)) were used for Southern hybridization. Probe IS1-IS4 detected a ~1 kb band in heart as predicted but not in MELC suggesting that IS1-IS4 is not expressed. Probe 1440S detected products in MELC in the 300-500 bp size range but did not detect any signal from heart at this short exposure suggesting that regions corresponding to 1440S are not expressed in heart. This result also suggests that the end of the MELC-CC message is ~300-500 base pair away from the 5MSacAS which correspondes to nucleotide 467-487 of MELC-CC). Probe 5MASA detected signals from both MELC and heart. The band patterns were the same as those detected using the probe 1440S in MELC and the same as those detected using probe IS1-IS4 in heart. Hae III digested Ø X174 DNA was used as a size marker (in kilobases).

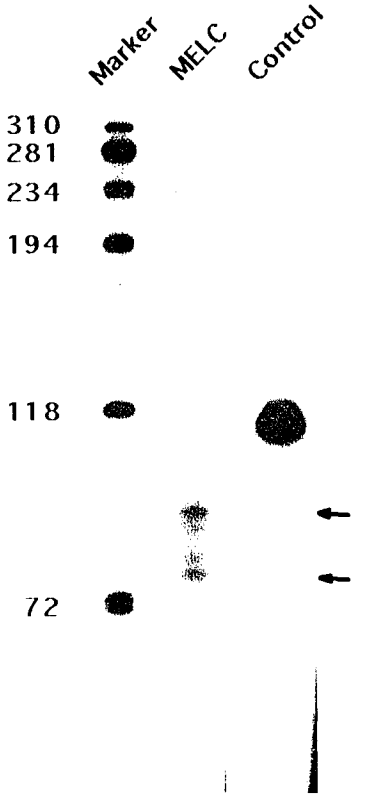


Figure 14. Primer extension analysis of the 5' termini of MELC-CC RNA. 1440AS was used as an extension primer. MELC total RNA was used in the extension assay and two major extended bands at size ~75 and 105 bp were identified (indicated by arrows). This finding is consistent with MELC-CC having two major transcription initiation sites. An *in vitro* transcribed RNA derived from clone 5M623 from the MELC-CC with a predicted extension product of 115 bp (including 83 bp from pBluescript) was used as control. Size markers were Hae III digested ØX174.

5. RNAase Protection Assay

In order to confirm the putative transcription start sites suggested by primer extension, a MELC genomic library was screened using a 2MEL BamHI-HindIII fragment to generate a probe that extended upstream of the 5' end of the MELC-CC mRNA. A positive

clone, designated clone-13, was isolated and sequenced. A subclone of clone-13, named pP2ApaI/SacI (located between -674 and +97 of the MELC-CC), overlapped with the 5' terminal region of the MELC-CC cDNA. pP2ApaI/SacI was then cut into two probes of different lengths using BanI or EcoRI on the 5' end (which extend from +97 to -41 and -135 respectively). These probes were used in RNAase protection assays (Figure 15) and produced fragments that were heterogeneous in size, however there was a cluster of fragments at 100-130 bases in length (corresponding to -3 to -33 respect with the MELC-CC first nucleotide). This data supports the results of the primer extension assay, for example the 75 bp and 105 bp bands seen in the primer extension assay correspond to the 100-130 bp bands of the RNAase protection assay, respectively. These combined results strongly suggest that transcription of MELC-CC mRNAs is initiated from at least two sites.

In addition, when probed with mouse cardiac α_1 subunit cDNA corresponding to the first four transmembrane segments, a specific mRNA was detected in heart, lung and NIH3T3 cells but not in MELC (Figure 16). Taken together these data indicated that the full length MELC-CC mRNA encodes a unique truncated Ca^{2+} channel.

Comparison of Nucleotide Sequence to that of DHP-Sensitive Ca^{2+} Channels

After confirming that the entire MELC-CC clone had been isolated, a detailed sequence comparison of this clone to the known DHP-R was conducted in order to gain information on similarities and differences between the structure of the VICC and VDCC, and what

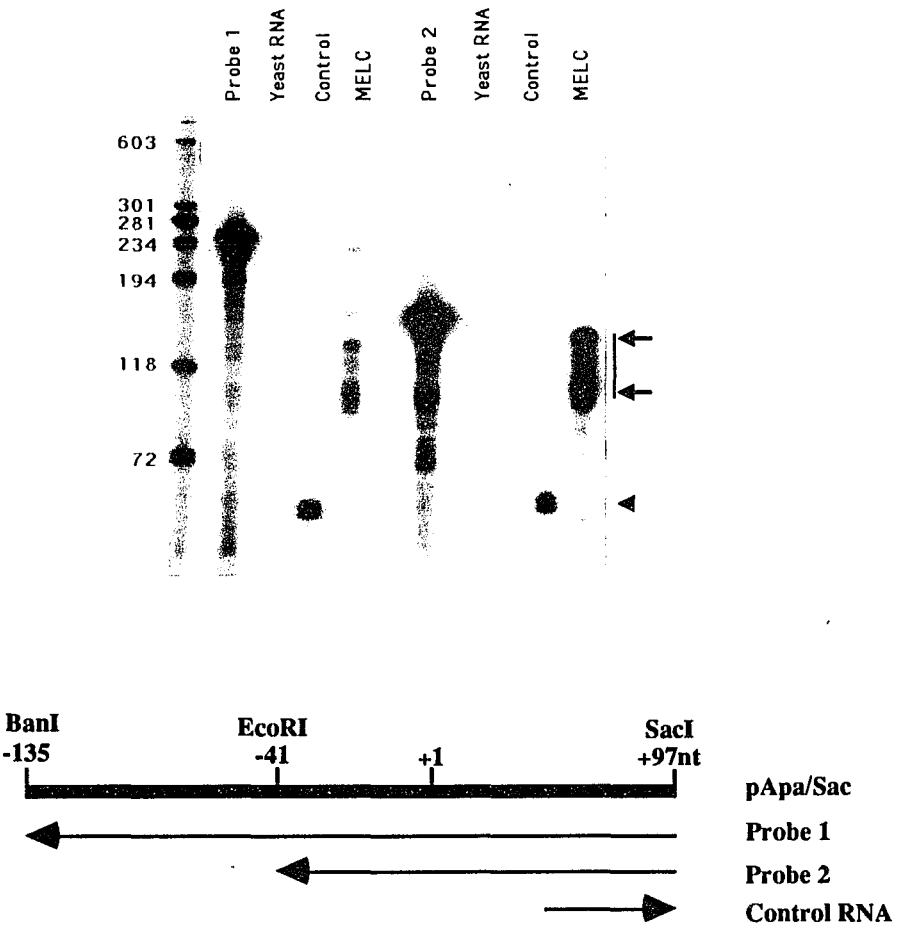


Figure 15. The 5' terminus of MELC mRNA was mapped using RNAase protection analysis with MELC genomic DNA (M/G DNA) probes. Two major bands at ~100 and 130 nucleotides are protected with both probes as indicated by the verticle bar and arrow. An *in vitro* transcribed MELC-CC RNA was used as control and the predicted 66 bp band was generated as indicated by the arrowhead. The schematic shows the probes derived from the MELC genomic DNA. Probe 1 was generated with a BanI/SacI digestion and probe 2 was generated by a EcoRI/SacI digestion. The numbers below each enzyme indicate the distance from the end of that probe relative to the first nucleotide of the MELC-CC cDNA. Size markers are Hae III-digested ØX174 DNA.

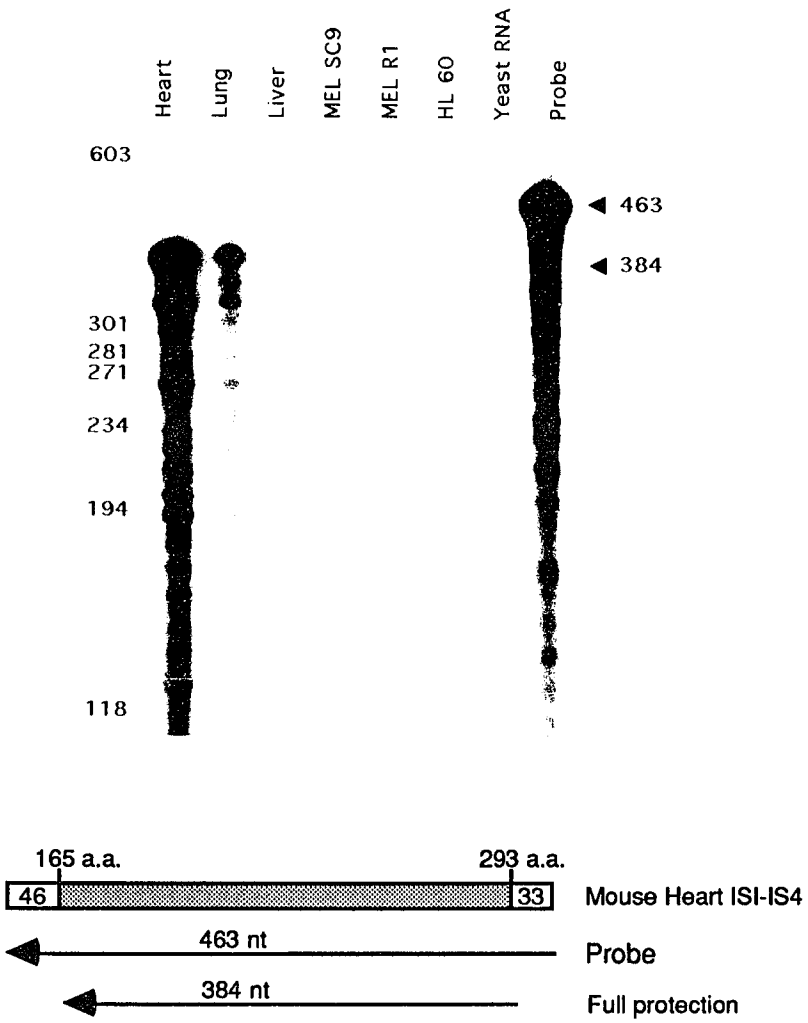


Figure 16. Tissue specific expression of the mouse cardiac Ca^{2+} channel. Using RNAase protection with a probe spanning IS1-IS4 (corresponding to the amino acids 165 to 293). Expression of the mouse cardiac Ca^{2+} channel was detected in mouse heart and lung RNAs. These RNAs protected a 384 bp fragment (indicated with arrows) using a full length probe (463 bp). No protected products were detected in liver, MELC, HL 60. (MELC SC9 is a wild type cell line and MELC R1 is a vincristine resistant cell line). Yeast RNA was used as a control for RNAase digestion. The schematic diagram shows the size of the full length probe as well as the size of the fully protected product. The hatched box represents the ISI to IS4 transmembrane region of the cardiac CC that are not expressed in MELC. The numbers in the open boxes represent the plasmid nucleotides.

physiological implications these difference might have. The analysis revealed five major differences between the MELC-CC and the α_1 subunit of DHP-R channels:

1. 5' truncation

The primer extension and the RNAase protection analysis indicated that the MELC-CC does not contain the transmembrane segments designated IS1 through IS4 making it a truncated version of the DHP-R.

2. Insertion between motif I and II

An insertion of 75 nucleotides, located between motif I and II in MELC-CC (corresponding to nucleotides 1003-1077 of the MELC-CC), has been identified in the 2MEL and 5MEL. Inclusion of this exon seems to be regulated during HMBA-induced MELC differentiation as the presence or absence of this insertion in a clone depends upon whether the library was constructed using undifferentiated or differentiated MELC. Splicing could account for this difference, but interestingly this inserted fragment (GAGGC GCTCCAGCGGGCTTGCATGATCAGAAGAAAGGGAAGTTTGCTTGGTTTAGTC ACTCTACAGAAACCCATG) does not show any conventional splicing signals [Sharp, 1985, Reed and Maniatis, 1986] at either end.

3. Deletion of IIIS2 transmembrane segment

When compared with the rabbit cardiac DHP-R cDNA sequence, there was a sixty base pair segment deletion at nucleotide 2477 in the MELC-CC cDNA. Interestingly, this deleted segment appeared in

the clone 713 which was isolated from the HMBA-induced MELC cDNA library. As suggested by the PCR assay (Figure 6A), the MELC-CC form with the IIIS2 deletion was present only in the undifferentiated MELC RNA. These findings provide further evidence that alternative splicing is regulated during differentiation in MELC.

4. Alternative exon of the IVS3

The insertion between motif I and II and the deletion of the IIIS2 in the MELC-CC are regulated during MELC differentiation by alternative splicing. Based on these observations we were interested in determining whether the expression of the IVS3 exon was also regulated during differentiation (alternative usage of two exons at IVS3 had been reported during rat heart development [Diebold, 1992]) in MELC-CC IVS3 corresponds to 1018-1045. The clone 5M713 isolated from the HMBA-induced MELC cDNA library was compared to MELC-CC. Exon S3A (as labeled by Diebold, 1992) was expressed. In addition, the D1 exon (as denoted by Diebold 1992) encoding the extracellular region adjoining IVS3 just 3' of S3A is not expressed in MELC-CC (Figure 17).

5. C-terminal variation

The C-terminal sequence of MELC-CC encoding amino acid 1552 to 1619 shows a dramatically different structure from that of the rabbit cardiac DHP-R with only 56% identity [Mikami, 1989] (Figure 18). In contrast, this part of the sequence exhibits an 81% identity with that of the rat aorta VSMα_1 [Koch et al., 1990] (Figure 18). The

rat aortic VSMα_1 exhibits only 55% identity with rabbit cardiac DHP-R, probably due to alternative splicing.

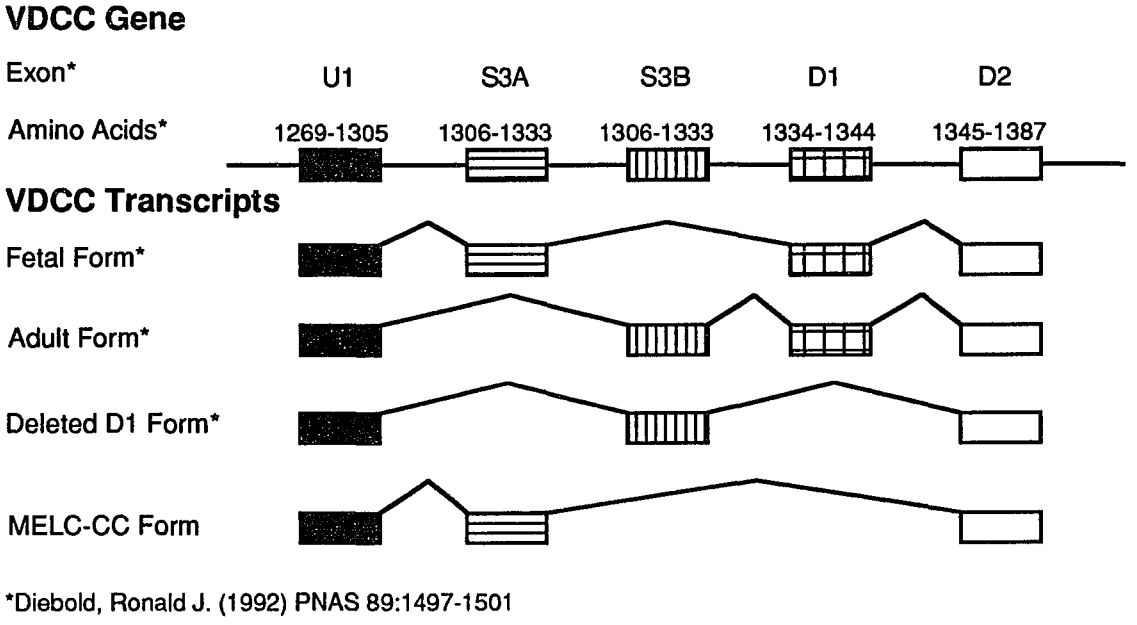


Figure 17. Comparison of the alternative splicing pattern of MELC-CC $\alpha 1$ subunits with that of the rat cardiac $\alpha 1$ subunits. The genomic structure of exons for the IVS3 transmembrane region and its adjacent regions are shown. The amino acid positions corresponding to the rat cardiac $\alpha 1$ subunits are numbered. The S3A or S3B equivalent in the MELC-CC amino acid sequence is 1018-1045. D1 equivalent in the MELC-CC amino acid sequence is at amino acid 1046. A unique splicing pattern is exhibited in MELC-CC.

```
Rabbit cardiac  QIAMACQEGASQDDNYDVRIGEDAECCSEPSLLSTEMLSYDDDENRQLAPPEEEKRDIRLSPKKGFLRS
                 ! !!!  !!!!!!!!!  !! !  !         ! !  !!  !   !!   !  ! !!   !!
MELC-CC         QGATVNQEIFPDETRS.VRMSEEAEYCSEPSLLSTDMFSYQEDEHRQLTCPEEDKREIQPSPKRSFLRS
                      ^ ^^       ^  ^   ^           ^^  ^^  ^     ^         ^
Rat aorta       QGATVSQDMFPDETRSSVRLSEEVEYCSEPSLLSTDILSYD.DENRQLTCLEEDKREIQPCPKRSFSLR
```

Figure 18. Comparison of the C-terminal sequences of the MELC-CC with that of the rabbit cardiac and rat aorta VDCCs (VSMα1). The non homologous amino acids between MELC-CC and rabbit cardiac VDCC are labeled with (!). The non homologous amino acids between MELC and rat aorta VDCC are labeled with (^). There is 56% homology between the MELC-CC and the rabbit cardiac suggesting an alternative splice in this region. The 81% homology between the MELC and the rat aorta VDCC may be due to species differences. Dots were inserted to maximize the sequence alignment.

Binding studies

To address whether or not MELC have any structural moieties that bind organic Ca^{2+} channel modulators we conducted radioligand binding assays using protocols established for excitable tissue preparations [Godfraind et al., 1985]. Two different binding studies were performed. The first was performed in MELC membranes. The second binding study was performed on oocytes injected with *in vitro* transcribed MELC-CC mRNA.

1. Dihydropyridine Binding in MELC Membranes

MELC membrane preparations were used to determine [^{3}H](+)-PN200-110 binding [New England Nuclear, 87.0 Ci/mmol] PN200-110 is high affinity ligand for the L-type Ca^{2+} channel. The MELC-CC structure possesses all the domains necessary for DHP binding (S6

helices of the third and fourth repeating domains as well as the loop linking S5 and S6 of the third domain [Nakayama et al., 1991, Striessnig et al., 1991]). In addition, physiological data indicated that a DHP-sensitive molecule resided on the MELC plasma membrane (Figure 3). MELC membranes (50 μg) showed only very weak binding activity as compared to mouse heart membrane (30 μg) used as a control (Figure 19). The specificity of the MELC binding was tested by adding excess unlabeled ligand (cold PN200-110 [Sandoz Research Institute]) to determine whether [^{3}H](+)-PN200-110 binding could be competed. No competition was found with unlabeled ligand concentrations of up to 1000 fold molar excess indicating that the weak binding on MELC membrane was non-specific. Notably, the binding of [^{3}H](+)-PN200-110 was competed by excess cold PN200-110 when mouse heart cell membrane were used as the positive control binding substrate.

2. DHP Binding to Oocytes Injected with MELC-CC mRNA

In an attempt to determine whether the MELC-CC cDNA clone could express a protein that would bind DHP, oocytes were injected with *in vitro* transcribed MELC-CC mRNA. The binding assay was performed as described for the MELC membrane, however, no specific binding activity was observed. Taken together these data were consistent with: 1) a low level of MELC-CC protein in MELC plasma membrane; 2) absence of a high affinity binding site for DHP binding in MELC and in the MELC-CC; 3) failure of the MELC-CC cDNA to express in oocytes.

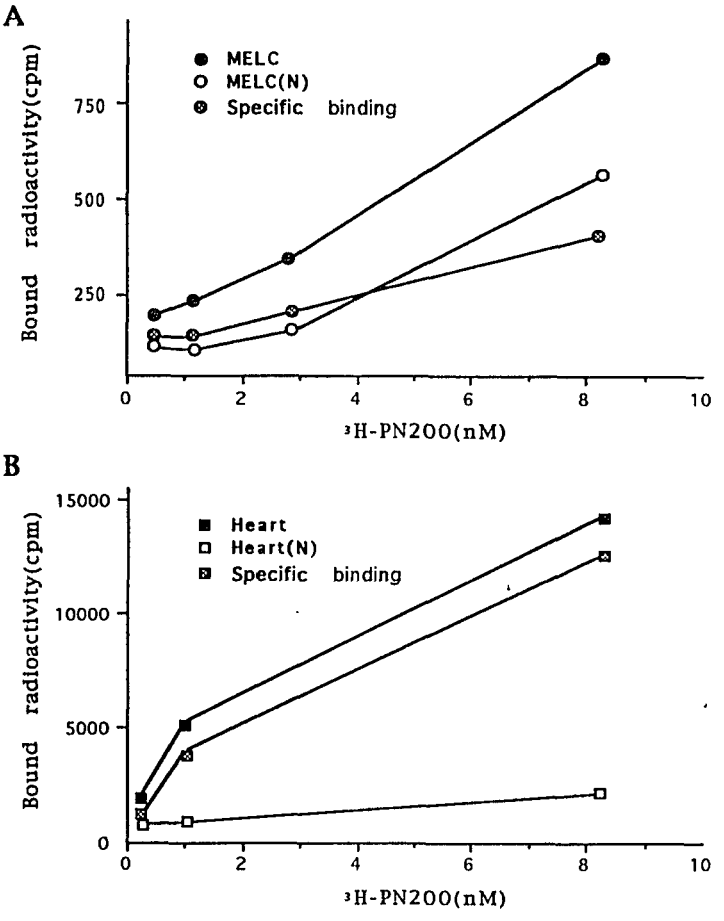


Figure 19. [^{3}H] PN-200-110 binding as a function of increasing concentrations of radioligand. A) Samples of MELC membrane fraction (circles) were incubated with [^{3}H] PN-200-110 as described under Methods. Solid and open symbols refer to specific and non-specific binding, respectively. A relatively weak specific binding activity was suggested in MELC samples. B) Samples of mouse heart light microsomal fraction (squares) were incubated with [^{3}H] PN-200-110 as described under Methods. Solid and open symbols refer to specific and non-specific binding, respectively. Hatched symbols refer to specific binding.

α_2 subunit Northern Analysis

In many tissues the α_2 subunit is co-expressed with the α_1 subunit of the DHP-R. Since α_2 subunit probes can cross hybridize to message RNA from a variety of tissues [Ellis et al., 1988 Hayakawa et al., 1990], MELC α_2 mRNA expression was investigated using an α_2 cDNA probe cloned from rabbit skeletal muscle (corresponding to the rabbit skeletal muscle α_2 subunit nucleotide 2301-2804). Northern analysis of total MELC RNA (30 μg) failed to detect a specific message. This result indicated that α_2 is not expressed in the MELC at high levels. However, it is possible that the α_2 subunit in MELC may not be sufficiently homologous to the rabbit skeletal muscle form to be detected using Northern hybridization, or the MELC-CC may not include an α_2 subunit.

Tissue distribution of the MELC-CC

The possibility that the MELC-CC is a channel common to non excitable cells was tested by investigating the expression of its message in a variety of tissues and cells. Northern analysis was performed using RNA from T-cell, liver, kidney and NIH3T3 fibroblasts probed with 2MEL cDNA. No signals were detected. When the same samples were analyzed using RNAase protection with probes corresponding to nucleotide 1-550 of the MELC-CC cDNA two protected bands of 550 and 178 base pair each were seen in mouse cardiac muscle while a fully protected product was seen in MELC (Figure 20). Notably, two protected bands of 550 and 178 base pair each were also seen in NIH3T3 fibroblasts and the weak signal indicated the low abundance of the message. The low abundance

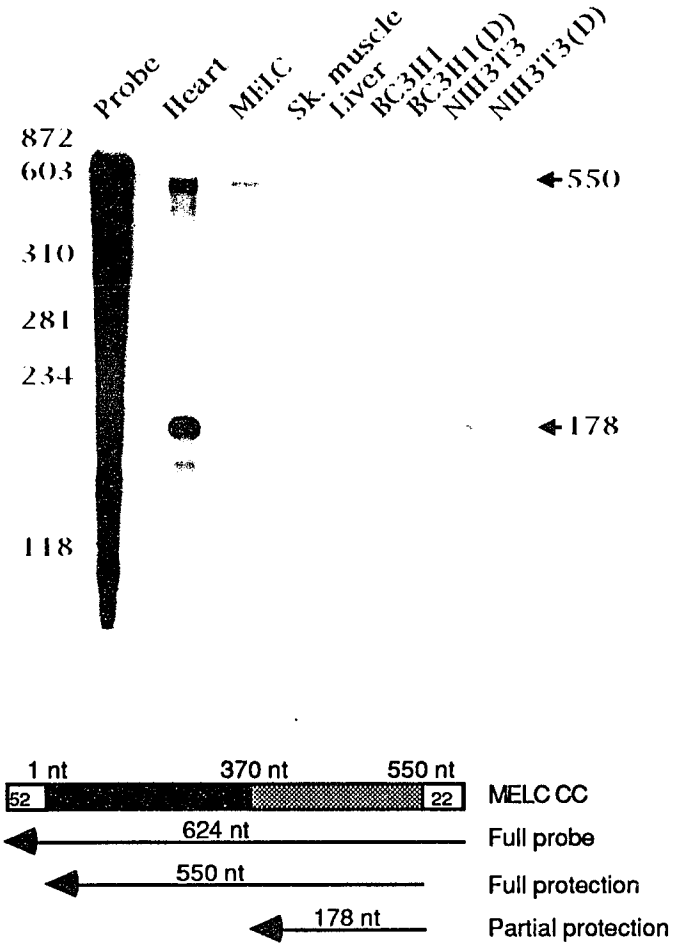


Figure 20. Tissue-specific expression of MELC-CC RNA in MELC and mouse heart, differentiated (D) and undifferentiated cells including the following: BC3H1 (murine muscle cells), NIH3T3 (murine fibroblasts), and MELC was analyzed using RNAase protection. MELC-CC mRNA was detected in mouse heart and MELC. Partial and fully (possibly due to the unprocessed RNA contamination) protected products were detected in the heart while the partially protected product accounts for the majority of the signal. Only the fully protected product was seen in MELC suggesting that this is the only form of the mRNA expressed in these cells. The sizes of the fully and partially protected products are indicated with arrows. Full protection was seen in heart, NIH3T3 and MELC. Partial protection was seen in heart, skeletal muscle and NIH3T3 cells. The schematic shows that a MELC-CC probe spanning the nucleotide 1 to 550 was used. The solid box represents the MELC-CC specific region where the sequence diverges from the cardiac DHP-R. The hatched box represents the homologous region between the cardiac and MELC-CC $\alpha 1$ subunits. The numbers in the open boxes represent the plasmid. Size markers are Hae III-digested ØX174 DNA.

may explain the failure of the Northern detection of the message from NIH3T3 cells.

MELC-CC GENE STRUCTURE ANALYSIS

PCR assay

The nucleotide sequence comparison of MELC-CC α_1 subunit to that of the mouse cardiac α_1 subunit revealed a sudden divergence starting at nucleotide 370 on the 5' end. Two possibilities could explain this divergence. Either a gene rearrangement had occurred in the MELC-CC gene or transcription of the MELC-CC had been initiated from an internal intron. To investigate these possibilities, two PCR assays using MELC genomic DNA as template were performed. Primers 1440S (complementary to MELC-CC nucleotide 45-64) and 5MASA (complementary to MELC-CC nucleotide 378-402) were used in the first PCR assay. The reaction in MELC yielded two bands (Figure 21), one at 357 bp as predicted and a second larger band with the same intensity. As a control DBA/2 (the murine strain from which the MELC was generated) genomic DNA was used as template in the PCR assay, only a single predicted 357 bp PCR product was generated (Figure 21). This indicated that there were two different alleles of this gene in MELC genome and that a gene rearrangement had occurred with a definite insertion in between primer of 1440S and 5MASA in one of the alleles as shown by the appearance of the larger band. When primers 1440S and 5MBamAS (complementary to MELC-CC nucleotide 536-556) were used in the second PCR assay, the amplified products from both templates were

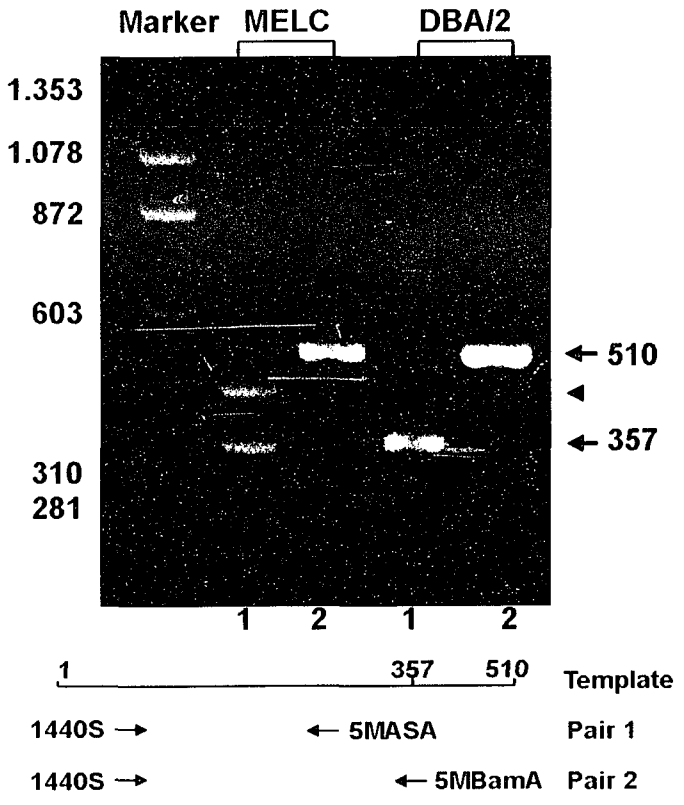


Figure 21. Detection of MELC-CC gene rearrangement using PCR amplification of MELC and DBA/2 mouse DNA. Primers used in PCR were 1440S (sense), 5MASA and 5MBamAS (antisense). PCR products were electrophoresed on a 1% agarose TAE gel and stained with ethidium bromide. Using the first pair of PCR primers, two amplified products, 446 and 357 bp were obtained from MELC, whereas in DBA/2, only the 357 bp product was present. The extra band seen in MELC is due to an 89 base pair insertion in the MELC-CC gene. This was determined by excising the bands and sequencing them. Using the second pair of primers, a 510 bp product was seen both in MELC and DBA/2. The absence of a second band in MELC suggests that a large insertion could have prevented amplification from occurring using the primers of 5MASA and 5MBamAS. The positions of the primers, 1440S, 5MASA and 5MBamAS are indicated. HaeIII digested ØX 174 was used as size marker.

the same. The absence of a second band in MELC suggested that either a large enough fragment inserted between the primers of 5MASA and 5MBamAS prevented PCR amplification or mutation(s) occurred within the sequence of the primer of 5MBamAS. Therefore the PCR product could only be derived from the normal allele. This result suggests that another rearrangement could exist within this region of the MELC genome.

PCR clone sequence analysis

To analyze the nucleotide sequences of these PCR products (Figure 21), two bands amplified from the MELC genomic DNA with 1440S and 5MASA and one from DBA/2) were excised from the gel, subcloned into pBluescript plasmids and sequenced. The sequence data confirmed that the larger band was due to an 89 bp insertion at nucleotide 370 of MELC-CC cDNA. The sequences of the predicted bands from both the MELC and DBA/2 showed an identical sequence as that found in the MELC-CC mRNA.

First of all, these results indicated that gene rearrangement with a 89 bp insertion in one of the alleles in MELC-CC gene has occurred. Secondly, the divergent sequences on 5' end of the 370 nucleotides in the MELC-CC cDNA are derived from an "intron".

Analysis of MELC Genomic Sequence

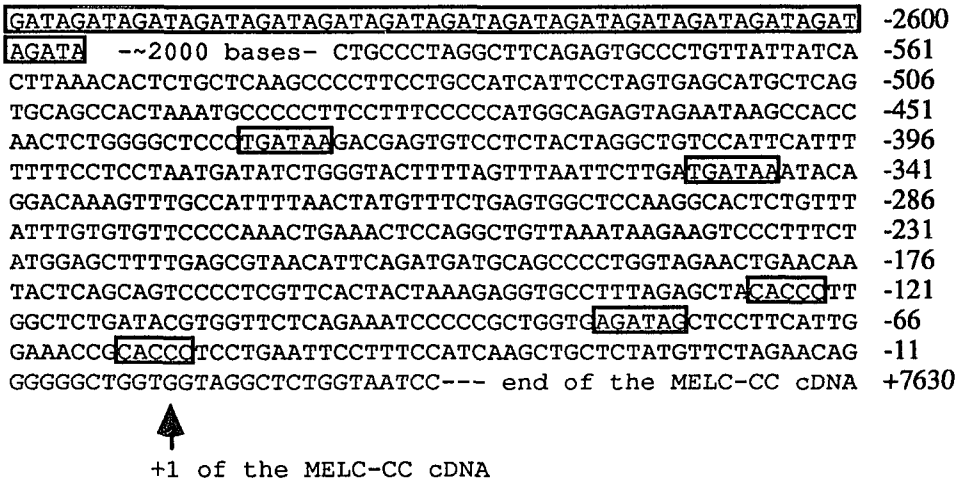
Having found that the 5' end of MELC-CC mRNA was structurally different from that of the mouse cardiac DHP-R, putative transcriptional promoter elements near the 5' terminus of MELC-CC mRNA were examined by cloning and sequencing MELC genomic

DNA. The 2MEL cDNA probe, BamHI-HindIII, was used to screen 2 X 10^5 recombinants from a MELC genomic library. Clone 13 (15 Kb) was isolated and restriction fragments were selected by Southern hybridization using two synthetic oligonucleotides 5MASA and 1440AS as probes. The fragment which hybridized to both oligos was subcloned into pBluescript and named pP2. Restriction mapping and nucleotide sequencing data indicated that pP2 extends 3.6 kb upstream to the 5' cap site of the MELC-CC cDNA sequence. Three transcription factor recognition elements were identified: three GATA boxes with consensus sequence (A/T)GATA(G/A) was localized at -81, -350 and -434 bp upstream from the putative cap site of MELC-CC and two CACCC boxes were localized at -58 and -127 bp upstream from the putative cap site of the MELC-CC. No TATA or CAAT motifs were present (Figure 22). Moreover, there was a stretch of 15 GATA repeats located 2.8 kb upstream of the putative transcription start site. This clone represents only one of the two alleles and whether the same elements exist in its counterpart on the other allele remains unclear.

MELC-CC gene mapping with Southern hybridization

Southern hybridization was performed on genomic DNAs from MELC and DBA/2 to provide a physical map of restriction sites within the MELC-CC gene. Four restriction enzymes (NdeI, PstI, XhoI and BglII) were used to digest MELC and DBA/2 genomic DNA separately at 37^0 C overnight for complete digestion. The cDNA probe from 2MEL, BamHI-HindIII, was hybridized to the Southern blot (Figure 23). The hybridization patterns from NdeI, XhoI and BglII digestions

A



B

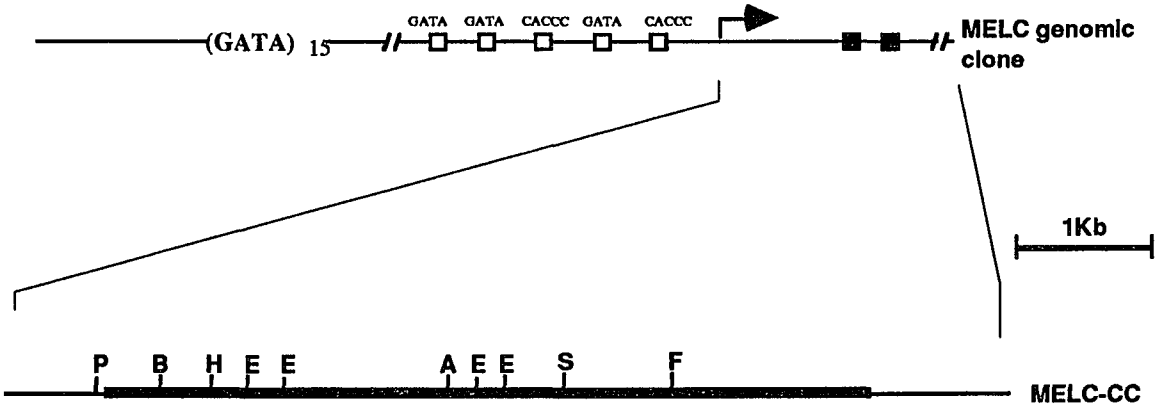


Figure 22. Upstream flanking sequence of MELC-CC revealed GATA and CACCC transcription elements. A) Partial sequence of MELC genomic clone, P2, is shown. This clone extends ~3.5 Kb upstream of the MELC-CC cDNA. The GATA repeats in the distal region and GATA and CACCC elements in the proximal regions are boxed. These elements could represent promoter regions regulating MELC-CC transcription. B) A diagram of the relationship of the MELC genomic clone and the MELC-CC is shown. The putative transcription initiation site is indicated by an arrow.

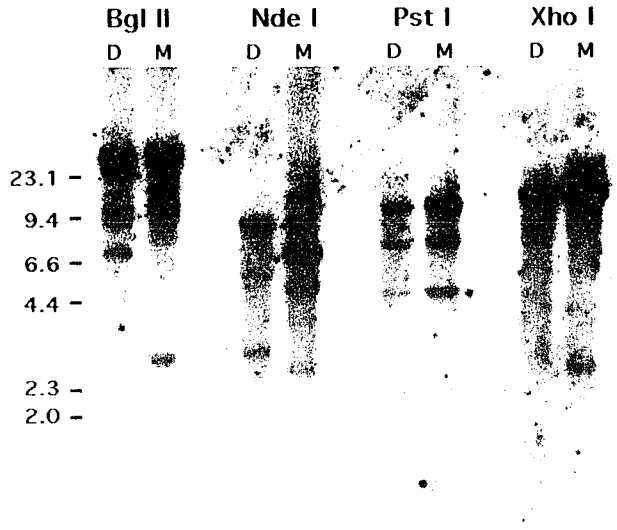


Figure 23. Southern analysis of the MELC (M) and DBA/2 (D) mouse Ca^{2+} channel genomic DNA structure. Four pairs of DNA samples were digested with four restriction enzymes, Bgl II, Nde I, Pst I and Xho I, respectively. DNAs were size fractionated on a 0.8% of agarose TAE gel. The blot was probed with the 2MEL cDNA, BamHI-HindIII. The restriction fragment patterns in the Bgl II, Nde I and Xho I digests differ between MELC and DBA/2. The band patterns from the Pst I digests are similar. This result suggests that gene rearrangement has occurred within the MELC-CC gene. These results are also consistent with a rearrangement because only one allele is detected in the MELC DNA and it differs from the DBA/2 allele. Hind III digested λ phage DNA was used as size marker.

were different between MELC and DBA/2. The band patterns from the PstI digestion were similar for both MELC and DBA/2. This Southern analysis suggested that gene rearrangement had occurred within the MELC-CC gene. It also demonstrated that the

rearrangement had occurred on both alleles since otherwise there would be one set of identical bands in both DNA samples within each pair plus the extra (or lacking) bands from MELC.

PROPOSED FUNCTIONAL STUDIES OF THE MELC-CC

Synthesis of Ca^{2+} Channel Chimera and In Vitro translation

Based on the molecular cloning analysis it appears that as differentiation of MELC occurs, two isoforms of Ca^{2+} channels are differentially expressed. These two species of channels are generated by alternative splicing of a single gene. The Ca^{2+} channel mRNA expressed during the undifferentiated state of MELC cells (clone 2MEL) contains an insertion of 75 bp between motif I and motif II. It differs from the mRNA of the differentiated form of Ca^{2+} channel (5M713) in that this insertion is lacking. Likewise, a deletion at the putative transmembrane region IIIS2 is found in 2MEL. This deletion is absent in 5M713.

In order to characterize the function of these channels, chimeric constructs of different fragments of cDNA were created and their mRNA synthesized and translated. These constructs were obtained by subcloning into the pSP64T vector [Melton, 1984, Krieg, 1984]] containing the bacteriophage SP6 promoter and subsequently transcribed *in vitro* using SP6 polymerase. The constructs synthesized in this way were: pSP64T/MEL, pSP64T/MEL/713 and pSP64T/M/H2C/713 (Figure 24A-C).

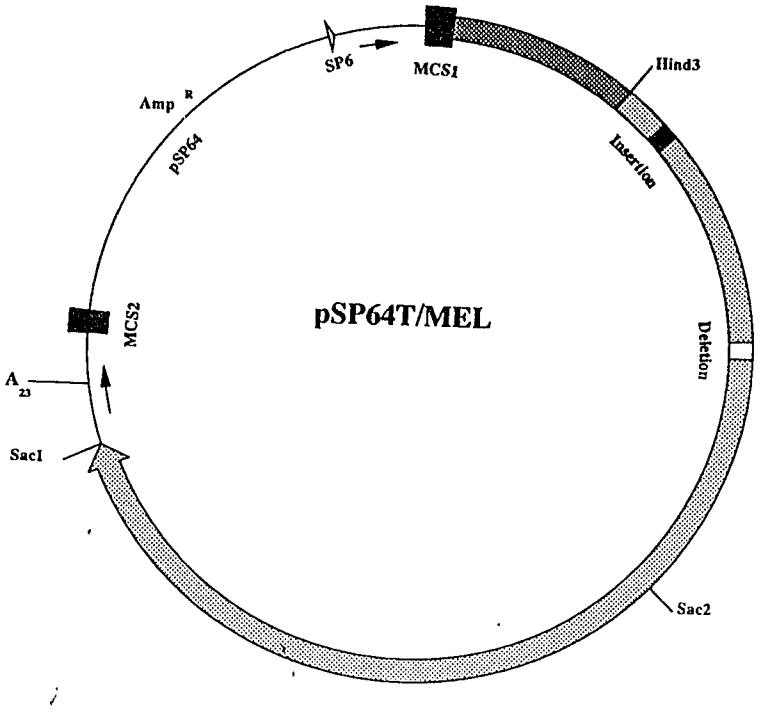


Figure 24A. Schematic diagram of the expression plasmid pSP64T/MEL. The insert of this plasmid was derived from clones 5M613 (dark hatched area), 2MEL (lightly hatched area from Hind3 to Sac2) and 3MEL (lightly hatched area from Sac2 to Sac1). This construct represents the MELC-CC expressed in the undifferentiated MELC. Positions of the MELC-CC insertion and deletion are labeled. Plasmid, pSP64T (Promega), was used as vector. A SP6 promoter is indicated by a hollow arrow head. The restriction sites important for the construction of the plasmid are indicated. Two multi-cloning sites (MCS1 and MCS2) are indicated by thicker black boxes. A poly A (A_{23}) is also indicated.

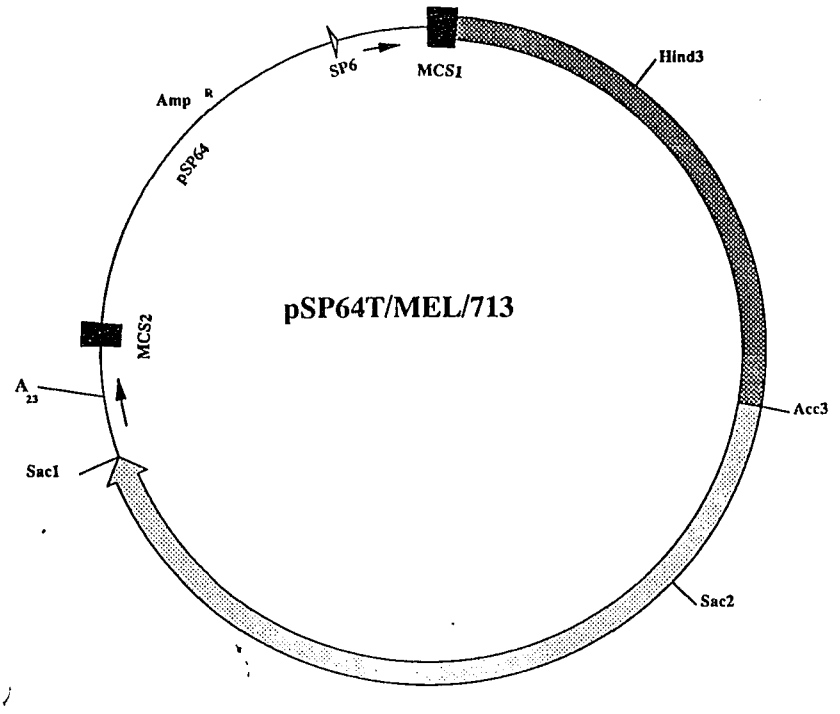


Figure 24B. Schematic diagram of the expression plasmid pSP64T/MEL/713. The insert of this plasmid was derived from clones 5M613 (dark hatched area from the begining to Hind3), 5M713 (dark hatched area from Hind3 to Acc3), 2MEL (lightly hatched area from Acc3 to Sac2) and 3MEL (lightly hatched area from Sac2 to Sac1). This construct represents the MELC-CC expressed in the differentiated MELC. Plasmid, pSP64T (Promega), was used as vector. A SP6 promoter is indicated by a hollow arrow head. The restriction sites important for the construction of the plasmid are indicated. Two multi-cloning sites (MCS1 and MCS2) are indicated by thicker black boxes. A poly A (A_{23}) is also indicated.

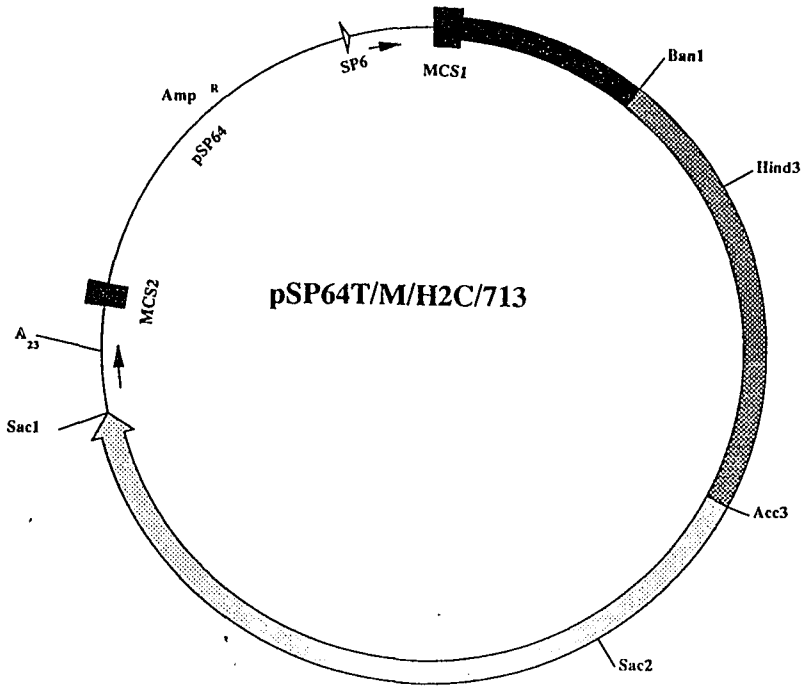


Figure 24C. Schematic diagram of the expression plasmid pSP64T/M/H2C/713. The insert of this plasmid was derived from mouse cardiac α_1 subunit (black area from the begining to Ban1), 5M613 (dark hatched area from Ban1 to Hind3), 5M713 (dark hatched area from Hind3 to Acc3), 2MEL (lightly hatched area from Acc3 to Sac2) and 3MEL (lightly hatched area from Acc3 to Sac1). This construct represents a putative chimeric α_1 subunit construct. Plasmid, pSP64T (Promega), was used as vector. A SP6 promoter is indicated by a hollow arrow head. The restriction sites important for the construction of the plasmid are indicated. Two multi-cloning sites (MCS1 and MCS2) are indicated by thicker black boxes. A poly A (A_{23}) is also indicated.

Plasmid pSP64T/M/H2C/713 was constructed using segments from pM/H2C (isolated from a mouse heart cDNA library) and pSP64T/MEL/713. It has the N-terminal region of the cardiac Ca^{2+} channel. This segment includes the first four transmembrane domains, extends toward the carboxyl end until nucleotide 407 of MELC-CC where the BanI site is located. After this Ban I site, the rest of the Ca^{2+} channel sequence comes from 5M713, the clone isolated from the differentiated state of MELC. The C-terminal region of this construct is from 3MEL. When these constructs are translated *in vitro*, no predicted products were generated. In the control experiment, mRNA of luciferase was used and predicted product was produced.

The MELC cDNA shows no conventional translation initiation element at the 5' end of the coding sequence. In order to facilitate the *in vitro* translation of the message, a Kozak consensus sequence was created into constructs, pSP64T/MEL, pSP64T/MEL/713, at the nucleotide 400-404 of MELC-CC mRNAs. The strategy used to carry this out entails the use of PCR. Primers were made based on the sequence of an area of pSP64/MEL that included a BamHI site and an area in the pBluescript vector 5' of the start of the Ca^{2+} channel insert. Two more primers were synthesized. These primers have sequences corresponding to the 5' end of the channel insert and sequences encoding either the sense or the antisense strand of the Kozak consensus sequence. Using the pSP64T/MEL as template, Kozak (antisense) primer and the primer containing the XhoI site was used in a PCR reaction to amplify the region immediately 5' of the channel sequence. This PCR reaction also places the Kozak consensus

at the end of the amplified segment. Using a similar method, the primer encoding the sense strand of the Kozak consensus and the primer encoding the channel sequences in the BamHI region was used in a PCR reaction with pSP64T/MEL as template. The products of the two PCR reactions were then combined and used as template in a subsequent round of PCR using the primers corresponding to the XhoI region on the vector and the BamHI site region in the channel sequence. The resulting PCR product spanned from XhoI to BamHI with an incorporated Kozak sequence at the first ATG. A schematic representation of this strategy to add a Kozak consensus to the pSP64T/MEL sequence is depicted in figure 25.

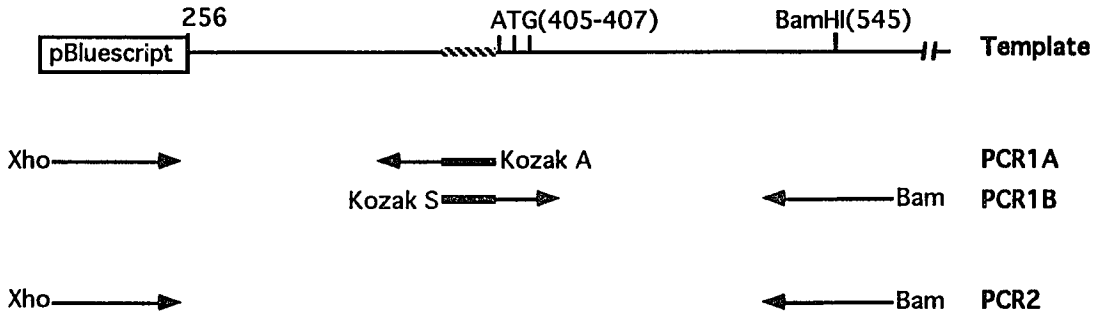


Figure 25. PCR strategy for engineering a Kozak sequence into the expression plasmids. Oligonucleotide primers are represented by names with the arrows. Primer Xho and Bam are complimentary to the template at the corresponding positions. Kozak A and Kozak S were designed so that solid bars are the consensus Kozak sequence mismatching the corresponding sequence on the MELC-CC template and the thin lines are complimentary to the template. First round PCR reactions used pSP64T/MEL as template. Primers Xho and Kozak A were used in PCR1A, primers Kozak S and Bam were used in PCR1B. Mixture of the first round PCR products (PCR1A and PCR1B) was used as template for the second round PCR (PCR2), the Xho and Bam were used as primers. The resulting PCR product spanned from XhoI to BamHI with an incorporated Kozak sequence at the first ATG. The first ATG in the MELC-CC open reading frame is indicated in the template and the BamHI site is also indicated.

In vitro transcribed mRNAs from this constructs will be injected into the oocyte system for functional study.

Expression of MELC-CC in *Xenopus* Oocyte

The mRNA of Ca^{2+} channels isolated from MELC were injected into *Xenopus* oocytes to detect functional expression of the putative Ca^{2+} channel. No calcium currents were detected in membrane patch clamp experiments although endogenous oocyte potassium currents were recorded.

CHAPTER IV: DISCUSSION

Calcium plays a critical role in the signal transduction pathway in many cell systems. Many pathways through which calcium enters the cytoplasm have been identified at a molecular level (Table 1). Detailed studies of Ca^{2+} entry into the cytoplasm have been performed in excitable cells, including muscles and nerves, where the increase in cytosolic Ca^{2+} is due in part to the influx of the Ca^{2+} through voltage-dependent Ca^{2+} channels [DeCoursey et al., 1984, Tanabe, 1987, Mikami, 1989, Matteson and Deutsch, 1984, Koch et al., 1990, Gerasimenko et al., 1991, Hui et al., 1991, Preston et al., 1991, Snutch et al., 1991, Hoth and Penner, 1992, Lechleiter and Clapham, 1992]. The purification and characterization of voltage-dependent ion channels from excitable cells has been facilitated by the use of specific high affinity ligands. In non excitable cells, such as platelets and mast cells, it has been demonstrated that Ca^{2+} influx is mainly via a voltage-insensitive pathway [Penner and Neher, 1988; Jacob, 1990] suggesting the presence of a non voltage activated plasma membrane Ca^{2+} channel. The activation of this putative channel has been proposed through second messengers rather than by voltage. IP_3, IP_4, cAMP and intracellular calcium have been thought to be potential activators [Kuro and Gardner, 1987, Irvine and Moor, 1986, Foreman et al., 1977, Von Tscharner et al., 1987]. To date, high affinity ligands to these channels have not been found. Thus, the purification and structural analysis of these channels have not been reported despite the fact that they have been suspected to play a role in regulating intracellular Ca^{2+} for almost three decades. Therefore we sought to purify and structurally characterize Ca^{2+}

channel that may participate in the rise in $[Ca^{2+}]_i$ observed during HMBA-induced differentiation in MELC.

Presence of a putative voltage insensitive calcium channel in MELC.

Evidence for the requirement of a plasma membrane channel was provided by investigating the calcium requirement during MELC differentiation. It was established that extracellular calcium is required for MELC growth and differentiation by demonstrating that when EGTA was used to remove calcium from the medium, MELC growth and differentiation was inhibited [Gillo, 1993]. These findings with EGTA are not the result of general toxicity to the cells as the cells were viable (assessed by trypan blue exclusion) even after 48 hours of culture with EGTA.

The calcium influx pathway in MELC could be specifically blocked by Ca^{2+} channel antagonists (nifedipine 10μM/L, La^{3+} and Ni^{2+}) and activated by Bay K 8644 (10μM/L) suggesting that the channel responsible for the influx of calcium was structurally similar to VDCCs. The concentrations of nifedipine and Bay K 8644 used in the experiments were higher than those used to study the VDCCs, where 5 μM/L for each drug was used [Hess, 1990]. This suggested that these channels could be slightly less sensitive to DHP than the VDCC.

It is well established that the VDCCs form a family of molecules sharing common structures [Bean, 1989, Hess, 1990]. Recent studies have revealed that the second-messenger-gated channel, the bovine rod photoreceptor cyclic GMP-gated channel [Kaupp et al., 1989], is

also a member of voltage-gated ion channel gene super family [Jan and Jan, 1990]. We extended our hypothesis to include the concept that sequences responsible for calcium flux through the plasma membrane and for DHP binding would be conserved in the MELC Ca^{2+} channel but that specific sequences required for voltage sensing would be absent from the VICC expressed in MELC.

In addition, our studies showed that only one phase of cytoplasmic calcium increase occurred in MELC following HMBA induction. The initial transient cytoplasmic calcium increase was not observed. This is unusual as the intracellular calcium increase in many other cell systems involves an initial rapid rise followed by a slower plateau phase [Berridge and Irvine, 1989]. The rapid rise is due to intracellular calcium release triggered by a diffusible messenger, IP_3, in response to agonist stimulation [Berridge, 1993]. The plateau phase was thought to be from calcium influx. Calcium influx of this nature is thought to involve a "capacitative" mechanism [Putney, 1986 and 1990] induced by the emptying of intracellular Ca^{2+} pools. Randriamampita and Tsien recently identified a diffusible messenger, calcium influx factor (CIF), that is released from intracellular compartments in activated Jurkat cells and causes calcium influx when applied to lymphocytes [Randriamampita and Tsien, 1993], but direct evidence of the primary structure and the function of such a Ca^{2+} channel has not been addressed experimentally in any of these reports. It has been proposed that the CIF is released in response to depletion of intracellular stores. Of interest CIF has been shown to activate calcium influx from the extracellular side of the plasma membrane suggesting that after its

release it passes out of the cell and activates a channel from its extracellular domain. It seems implausible that an intracellular factor would exit the cell and activate a channel extracellularly rather than activating the same channel from the cytoplasmic side. However, CIF would be a candidate for activating the VICC as well. It is less likely that MELC use the capacitative pathway as intracellular calcium stores do not exist in these cells.

To identify the presence of a member of the DHP-R family in MELC, PCR amplification of MELC mRNA using VDCC-based primers was performed. These experiments provided evidence for the presence of an mRNA in MELC that was highly homologous to the VDCC α_1 subunit. Northern analysis, using a cDNA probe derived from the MELC, detected a relatively high level of MELC-CC message in both undifferentiated and differentiated MELC. There are two major α_1 subunit RNA species of ~8,900 and ~15,000 nucleotides in rabbit heart [Mikami et al., 1989]. However, Northern analysis of MELC and mouse heart total RNA samples demonstrated an extra band at ~30,000 in both samples. These two high molecular weight molecules could represent unprocessed RNA because a third Northern loaded with MELC poly A+ RNA showed only a single band at ~8,900 bp.

We also determined that the expression of the MELC mRNA was regulated during induced differentiation. The hypothesis was that since we had shown that calcium influx was required for HMBA-induced differentiation, it was possible that the level of VICC expression would be induced by HMBA as well. This in fact turned out to be the case as MELC-CC mRNA levels were increased

approximately 5-10 fold during HMBA-induced differentiation with a peak level occurring approximately 36 hours after exposure to HMBA. Previous studies have demonstrated that mRNA encoding the α_1 subunit is upregulated in the myogenic tissue culture cell line C2C12 during differentiation [Varadi et al., 1989]. Studies of primary cultures of mouse skeletal muscle cells have shown similar observations in that the DHP binding sites are dramatically increased upon cessation of proliferation of the muscle cells [Romey et al., 1989, Morton et al., 1989].

In summary these studies demonstrated that a DHP-sensitive molecule was expressed on the MELC membrane. However, it remains possible that the nifedipine used in physiological studies could bind to a structure other than the VICC. Against this possibility is the fact that the effects of Bay K 8644 were observed at 1 μM concentrations which are thought to be highly specific for VDCC activation [Gillo et al., 1993]. Nevertheless the present data does not exclude the possibility that calcium influx during HMBA-induced differentiation could be via a molecule other than the VICC. One candidate for this alternative pathway for example would be a Na^+/Ca^{2+} exchanger.

It has been shown recently that HMBA inhibits a cell cycle kinase cdk4 and that this results in cessation of proliferation and allows the differentiation program to be initiated. None of the steps in this signaling pathway have been identified as calcium dependent yet, although much work remains to be done in this area. In yeast several of the check points in the cell cycle have been postulated to have a calcium dependence and calcium calmodulin kinase is

believed to play a role. The requirement for calcium influx to permit HMBA induced differentiation suggests that these cells also require calcium for cell cycle regulation.

HMBA induces MELC differentiation, however the exact mechanism of HMBA-induced MELC differentiation is not known. The possibility exists that HMBA could interact directly with MELC-CC or indirectly through other plasma membrane proteins for example by triggering IP_3 production. However, when extracellular calcium is eliminated, there is no increase of the intracellular calcium in respond to HMBA suggesting that there is no effect of HMBA on intracellular calcium stores. But if a Ca^{2+} channel sensitive to IP_3 is located on the plasma membrane and responds to HMBA (perhaps via IP_3) no increase of intracellular calcium in response to HMBA would be observed when the extracellular calcium is eliminated. HMBA stimulated calcium influx peaked at 3 to 6 minutes suggesting that HMBA itself is not the direct ligand to the channel. Nifedipine, a Ca^{2+} channel antagonist, inhibited only 60% of MELC from differentiating suggesting either that the block of the calcium channel was incomplete or that other non-DHP sensitive pathways exist in MELC for calcium influx.

A recent study on human erythroblasts treated with erythropoietin [Cheung et al., 1992] revealed similar results to those we obtained in MELC. Cheung et al. did not detect an initial IP_3 induced calcium transient increase and concluded that the erythropoietin-induced calcium increase was due to calcium influx rather than intracellular calcium release. They found that erythropoietin induced calcium influx could be blocked by Ni^{2+} and

by high doses of nifedipine (10-50 μM). Their results suggest that calcium influx is mediated via a voltage-insensitive ion channel permeable to calcium.

Unique MELC-CC structure

Of the multiple Ca^{2+} channels cloned to date from excitable tissues all have four sets of six highly conserved putative transmembrane regions with the exception of the potassium channels which have only a single set of six putative transmembrane regions [Catterall, 1988]. The MELC-CC represents the first example of a DHP-sensitive ion channel in non excitable cells and its primary structure resembles the VDCC DHP-R of excitable cells. The MELC-CC differs from the VDCC in that several putative transmembrane regions are lacking. The lack of the first four transmembrane regions was demonstrated by the Northern analysis and the RNAase protection assay. In both cases, probes corresponding to IS1 to IS4 of the mouse cardiac α_1 subunit failed to detect any message from MELC (Figure 7 and 16).

The amino acid homology between the MELC-CC and the cardiac DHP-R is most striking in the putative transmembrane regions where there is 84% for IS6, 95% for IIIS3 and 100% identity for all other transmembrane regions. This suggests that MELC-CC and cardiac DHP-R $\alpha 1$ subunit may be encoded by the same gene. In spite of the great similarity, the MELC-CC shows unique features, such as, a portion of the second transmembrane region in motif III is absent. In addition, there is significant divergence of sequence at both the carboxyl and amino termini in the untranslated regions. S4 segments

are considered to be the voltage sensor for the Ca^{2+} channels as demonstrated in mutational studies [Stuhmer, et al., 1989]. Three out of the four S4 segments are expressed in the MELC-CC and they are identical to those of the VDCC of the cardiac α_1 subunit. This finding would suggest that all four S4 segments are required to make a voltage-sensitive channel. Another possibility is that additional information encoded by the remaining sequence at the 5' end that is deleted from the MELC-CC is required for normal function of the VDCC. Of course it is also possible that the lack of the first four transmembrane segments renders the MELC-CC non-functional and eliminates the ability to comment on the role of these missing sequences in generating voltage-dependence of channel gating.

As a previous study has reported [Hui et al., 1991], the length and homology of the cytoplasmic loop between repeat I and II differs among α_1 sequences from heart, brain and skeletal muscle. However, the insertion in this region of the MELC-CC is unique. The insertion between motif I and II introduces 75 nucleotides. The predicted amino acid sequence, RGAPAGLHDQKKGKFAWFSHSTETH, is extremely hydrophilic consistent with its predicted cytoplasmic location. Biel et al. [Biel et al., 1990] isolated a Ca^{2+} channel from rabbit lung with a similar alternative splicing pattern as the MELC-CC in this region, but the clone is derived from a library of lung tissue that is a mixture of many cell types, rendering the tissue of origin of the clone uncertain. In contrast, the MELC-CC isolated from the MELC is derived from a pure cell type. So it is possible that the clone they have reported could actually be derived from cell types other than smooth muscle as they had assumed.

The transmembrane segment, IVS3, is encoded by one of two exons (3SA and 3SB) of the cardiac calcium channel gene expressed in a mutually exclusive fashion [Diebold, 1992]. It appears that MELC preferentially uses one of these (3SA) two exons regardless of the MELC differentiation stage. Moreover, the exon D1 adjacent to exon 3SB is not expressed in MELC-CC. The sequences of these two exons are highly conserved and the consequences of any structural differences between the products of these two exons are not known. The two forms of this segment are expressed in many different species, as well as in different tissues within the same species. No obvious tissue specific expression preference is observed (Figure 26). In Diebold's report, they have identified a subgroup of channel message that produce a form of the DHP-R α_1 subunit message lacking the D1 region. However, this deleted form differs from the MELC-CC form in that its message includes the S3B exon as opposed to the S3A exon (Figure 17). The primary structure at the C-terminal of the MELC-CC, which is located in the cytoplasm, shows another difference compared to other L-type channels.

The cDNA clones isolated from the HMBA-induced MELC cDNA library lack the characteristics discussed above in two major places: 1) insertion between motif I and II, 2) deletion of the IIIS2. Presumably MELC exhibit alternative splicing that is regulated during HMBA-induced MELC differentiation. In addition, the possibility of contamination with premature RNA during construction of the undifferentiated MELC cDNA library cannot be excluded.

These intracellular alternative domains might have a functional role in interacting with second messengers regulating MELC-CC function.

Amino acid sequence derived from exon S3A

1	Rat aorta VSMα1(Koch 1990)	HYFCDAWNTFDALIVVGSIVDIAITEVH
2	Rat adult heart(Diebold 1992)	HYFCDAWNTFDALIVVGSIVDIAITEVH
3	Rabbit heart α1(Slish 1989)	HYFCDAWNTFDALIVVGSIVDIAITEVH
4	Mouse ovary CaCh 2d	HYFCDAWNTFDALIVVGSIVDIAITEVH
5	Human cardiac CaCh 2d	HYFCDAWNTFDALIVVGSIVDIAITEVH

Amino acid sequence derived from exon S3B

1'	Rat aorta VSMα1.7(Koch 1990)	GYFSDPWNVFDFLIVIGSIIDVILSETN
2'	Rat fetal heart(Diebold 1992)	GYFSDPWNVFDFLIVIGSIIDVILSETN
3'	Rabbit heart α1(Mikami 1989)	GYFSDPWNVFDFLIVIGSIIDVILSETN
4'	Mouse ovary CaCh 2a	GYFSDPWNVFDFLIVIGSIIDVILSETN
5'	Human cardiac CaCh 2a	GYFSDPWNVFDFLIVIGSIIDVILSETN
*	MELC-CC	GYFSDPWNVFDFLIVIGSIIDVILSETN

Figure 26. The amino acid sequence alignments for two alternative exons encoding the IVS3 transmembrane region. The expression of these two exons is mutualy exclusive in a variety of species [Diebold, 1992, Koch et al., 1990]. A 53% homology exist at amino acid level for these two exons. The functional significance if any of this alternative splice has not been identified. However, only exon S3B was detected in MELC. The underlined areas show sequence homology between both the S3A and S3B exons.

Transmembrane topology of MELC-CC

The predicted ion channel structures have been postulated [Catterall, 1988] with the aid of modern molecular biology techniques. The hydrophobicity of each amino acid within a molecule depends in part on its neighbors. Twenty or more of the hydrophobic amino acids in an alpha-helical structure are needed to cross the cell membrane. Using a combination of hydropathy analyses, site-directed antibody binding, ligand binding and analogies to other more thoroughly studied ion channels (e.g. Na and K channels) predictions regarding the VDCC structure have been made.

The absence of several putative transmembrane regions in MELC brings into question the proposed models for the topology of ion channels. If in fact the S2 regions are membrane spanning segments then the absence of two such regions (IS2 and IIIS2) would alter the topology of the channel in a dramatic fashion for the MELC-CC. For example, the amino and carboxyl termini would no longer be on the same side of the membrane with an odd (19) rather than even (24) number of transmembrane segments. However, if one postulates that there are only four transmembrane segments for each motif and the S2 together with S3 transmembrane regions are membrane associated, rather than transmembrane associated by definition, then the absence of one such segment would not alter the basic topography of the MELC-CC protein structure on the plasma membrane. Thus, knowing the three-dimensional structure of ion channels including the MELC-CC is the key to understanding the real topography of ion channel on the membrane.

H5 segments are proposed to be the components lining the channel pore. The predicted primary amino acid structure of MELC-CC includes all four H5 segments necessary for the pore formation. The presence of all four H5 segments indicates that the channel pore of the MELC-CC could be intact. What would be missing would be four transmembrane segments that are thought to lie outside of the direct channel region. Thus the mature (differentiated) form of the MELC-CC mRNA could be expected to express a truncated Ca^{2+} channel without disturbing much of the overall proposed topography.

The voltage-dependent Ca^{2+} channel is comprised of 5 subunits, α_1, α_2, β, γ and δ [Catterall, 1988]. The α_1 subunit of the cardiac and skeletal Ca^{2+} channels have been expressed in Xenopus oocytes and shown to produce L-type Ca^{2+} channel currents [Mikami, 1989, Perez-Reyes, 1989]. This indicates that the α_1 subunit by itself is sufficient to form the Ca^{2+} channel. The other four subunits of the Ca^{2+} channel have uncertain roles in regulating Ca^{2+} channel function, although it has been observed that co-expression of the α_2 subunit with the α_1 subunit increases the magnitude of the calcium current in Xenopus oocytes [Mikami, 1989]. The MELC channel described in this thesis corresponds to the α_1 subunit of the cardiac Ca^{2+} channel, thus it is likely that this subunit alone is sufficient to conduct calcium current, although this current will not be voltage-dependent since the MELC lacks voltage-dependent calcium currents.

MELC-CC Gene rearrangement

PCR amplification of genomic MELC and DBA/2 DNA using primers 1440S and 5MASA generated two products from MELC as

compared to one predicted product from the DBA/2. Sequence analysis showed that the extra product from MELC is due to an 89 bp insertion on one of the alleles. Importantly, this 89 base fragment is absent from the MELC-CC cDNA and the sequence following the region in MELC-CC cDNA shows no indication of other abnormalities. The possibility that this 89 bp intron sequence is spliced out from the MELC-CC mRNA is unlikely as there are no conservative splicing sites on either end of the 89 base fragment. Therefore, it is concluded that the allele bearing the insertion may not be transcribed, and the allele without this 89 base insertion has been used for the MELC-CC mRNA transcription and functional studies. It is interesting to consider where this insertion comes from. Sequence comparison of this fragment with the Friend virus genomic DNA failed to show any homology [GenBank]. This fragment is not homologous to any other known sequences when searched in GenBank. It is unlikely to be an artifact of gene amplification as the nearby sequence in the genomic clone does not show any sequence similarity. Therefore, it is assumed that the insertion is from the translocation through an unknown piece of DNA fragment.

MELC is a virally transformed cell line. Rearrangements have been documented frequently in other genes in MELC [Peters, 1990]. DNA rearrangement is an obligatory requirement for the expression of some genes. Functional immunoglobulin genes are constructed from germline DNA by DNA rearrangement [Davis, 1980]. The expression of the variable surface glycoprotein genes in trypanosomes [Borst et al., 1982] involve the insertion of a structural gene, that is expressed, next to a relevant promoter. In the latter

case the rearrangement/duplication is such that the original structural gene is left intact. In MELC genomic Southern analysis revealed a hybridization pattern consistent with a rearrangement having occurred in the MELC-CC gene region upstream from the exon encoding the transmembrane region IS5 in the MELC-CC. The possibility of a gene rearrangement in the MELC-CC gene region is supported by the fact that the restriction fragment hybridization pattern on Southern analysis (Figure 23), using a probe derived from the IS5 region in MELC-CC demonstrates different patterns when MELC genomic DNA is compared to genomic DNA from the parent strain of mouse, DBA/2. The Southern analysis further suggests that both alleles in MELC contain rearrangements since only one pattern is seen and the wild type (DBA/2) pattern is not seen in this region in the MELC genome. In contrast, PCR data obtained by amplifying the region surrounding the junction between the homologous and non-homologous portions of the MELC-CC (Figure 21) revealed an 89 bp insertion found only in one allele (non-transcribed). This discrepancy indicates that while both alleles are the same in the IS5 region in MELC-CC there must be differences in the junction region further upstream.

Since we have been unable to detect MELC-CC protein several possibilities must be considered. The lack of DHP binding (Figure 19) is not surprising since it has recently been reported that DHP binds only when the α_1 is expressed with other subunits (including α_2 and β) [Puri et al., 1994]. However, we were also unable to detect MELC-CC protein using several different anti-MELC-CC antibodies. Among the possibilities to explain this finding are: 1) the antibodies might

not be capable of recognizing the protein on immunoblots (this was ruled out by demonstrating that the antibodies we used were capable of detecting the α_1 subunit of the cardiac DHP-R when it was overexpressed in insect (Sf9) cells; 2) the MELC-CC could be expressed at very low levels in MELC; 3) the cloned expressed protein might not be capable of making a functional channel in Xenopus oocytes; 4) there could be sequence alterations in the MELC-CC cDNA (either artifactual or otherwise) that would prevent the translation of the MELC-CC mRNA (we have ruled out this possibility by expression, in *Xenopus* oocytes, of a fusion protein that is a combination of the truncated MELC-CC and the first four transmembrane regions of the murine cardiac DHP-R, this fusion protein forms a functional L-type calcium channel with properties that are comparable to the cardiac DHP-R expressed in oocytes; 5) the MELC-CC gene could represent a pseudogene (however this is made less likely by the fact that such a long open reading frame would be unlikely to be faithfully preserved without the introduction of stop codons).

Recent study in normal cells (instead of transformed cell lines) on the rabbit brain ryanodine receptor gene has discovered that a partial skeletal muscle ryanodine receptor gene has expressed in brain [Takeshima et al., 1993]. Thus it appears that truncated gene expressions exist in normal tissue and cells as well.

Proposed model for the MELC-CC expression.

MELC genomic cloning located three GATA boxes, two CACCC boxes and a 15 GATA repeat close to the MELC-CC gene on its 5'

flanking region. Primer extension and RNAase protection analysis demonstrated the close relationship of the MELC-CC transcription initiation to these putative transcription elements.

In many cases, tissue specific gene expression is determined by cis elements and transcription factors. GATA box, (A/T)GATA(G/A), and CACCC box have been identified in promoter regions of many genes where they have enhancer activity and have been associated with transcriptional regulation during hematopoetic differentiation [Orkin, 1990, Miller and Bieker, 1993]. Transcription factors (GATA 1, 2 and 3) and transcription activator (e.g. EKLF) that bind to the GATA and CACCC sequences respectively are highly expressed in MELC during erythrocyte growth and differentiation process [Kim et al., 1990, Martin and Orkin, 1990, Miller and Bieker, 1993, Zon et al., 1991]. These factors bind to GATA consensus elements in regulatory regions of the alpha- and beta-globin gene clusters and other erythroid cell-specific genes during erythroid cell maturation [Pevny et al., 1991]. During cellular maturation the levels of GATA-1 RNA and protein increase progressively [Tsai et al., 1991]. These transcription factors regulate the expression of genes that possess GATA elements in their promoter regions and they play important roles in erythropoiesis. They all contain a zinc finger domain, bind to (A/T)GATA(G/A) motifs of many promoters and enhancers and drive a strong positive transcription. The functional significance of longer GATA repeats is currently not well understood. The expression pattern of MELC-CC is parallel to that of the GATA transcription factors. It is not known either if 89 bp insertion has activated the transcription. It can not be excluded that a possible distal region

gene rearrangement occurred and lead to the expression of the MELC-CC.

Therefore, a possible scheme for the expression of the MELC-CC gene is proposed (Figure 27). MELC-CC gene transcription initiates from an internal intron of the calcium channel gene due to the presence of GATA and CACCC promoter elements and the high level expression of the GATA and CACCC binding transcription factors in the MELC. A truncated message is produced in MELC when compared with the full length of the cardiac message at mRNA level. Four H5 segments for the MELC-CC are present, but MELC only contains 20 of the 24 transmembrane segments present in the cardiac Ca^{2+} channel.

As the structure of the channel is closely related to its function, it is thought that the functional differences between Ca^{2+} channel subtypes may be due to the selective expression of different primary structures as well as the expression of different structural subunits or modulator proteins, the existence of cell-specific post-translational processing pathways, and differences in the local lipid membrane environment. It is, therefore, expected that MELC-CC may have a totally different function with respect to its counterpart in other tissues.

Different Transcription initiation in MELC contributes to the diversity of the channel super family

The diversity of the α_1 Ca^{2+} channel super family is due to the expression of distinct α_1 subunit genes [Ellis, 1988]. For instance, the rat brain has at least four different Ca^{2+} channels expressed encoded by different genes [Snutch et al., 1990] named class A, B, C and D.

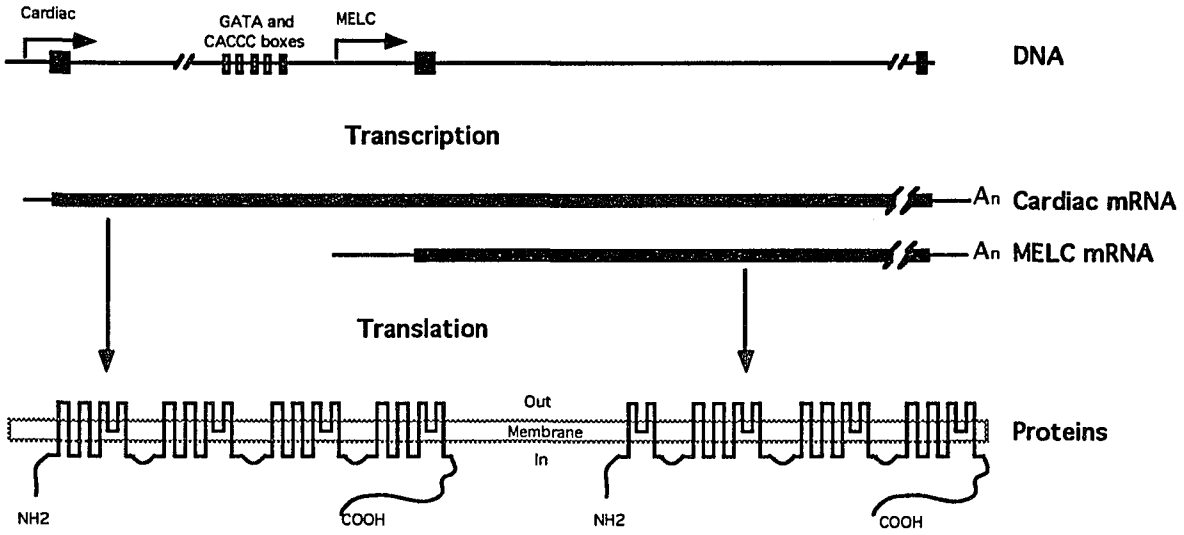


Figure 27. Proposed expression scheme for the MELC-CC gene and comparison to the cardiac calcium channel gene. MELC-CC transcription initiates from an internal intron (thin lines in between solid boxes) of the Ca^{2+} channel gene (possibly under the regulation of GATA and CACCC promotors and corresponding transcription factors expressed in MELC). A truncated message is expressed in MELC (the thick lines represent open reading frames). The predicted channel topology of the Ca^{2+} channel proteins within the cell membrane is indicated below. Four H5 segments for both MELC and cardiac are present, but MELC contains only 20 of the 24 transmembrane segments present in the cardiac Ca^{2+} channel.

The class C has two isoforms derived from alternative splicing [Snutch et al., 1991]. Alternative splicing process is a quite common phenomenon of gene regulation resulting in the diversity of the Ca^{2+} channel isoforms [Perez-Reyes et al., 1990 and 1991]. As for individual gene regulation, alternative splicing can be a tissue [Miller, 1987] and developmental specific processes [Diebold et al., 1992]. A similar molecule of the class C type was also found in cardiac muscle,

rabbit lung, and rat aorta [Mikami et al., 1989, Biel et al., 1990, Koch, 1990]. According to the results shown here MELC-CC is an isoform of the cardiac L-type Ca^{2+} channel that is derived from a truncated transcript at the 5' end due to the use of a different transcription initiation site. This information might contribute to the concept that regulation of transcription initiation sites is another mechanism through which diversity of Ca^{2+} channels can be generated and perhaps in a tissue-specific manner. The characterization of MELC-CC further establishes the existence of multiple subtypes of DHP-sensitive L-type Ca^{2+} channels.

Future study

The full length of MELC-CC will be expressed in MELC to both enhance and inhibit calcium fluxes during induced differentiation by transfection with full length sense and antisense cDNAs. Other subunits will be cloned and coexpressed together with the MELC-CC constructs in the oocyte system to study the function of this channel.

The baculovirus expression system will be employed to study MELC-CC. This system has the capability of expressing the authentic protein at very high levels. The baculovirus expression construct plasmid pSP64T/MEL has been constructed as follows: an insert from plasmid pSP64T/MEL was released with EagI and XbaI and cloned into the baculovirus in the compatible sites. They were ligated together to form a plasmid called pBacMEL. This plasmid will be used to infect the SF9 cell cultures. The function of this channel will be studied by expression in *Xenopus* oocytes. HMBA, Bay K,

erythropoietin and IP_4 will be examined for their ability to activate the MELC-CC.

It is established that the differentiation of the MELC requires calcium influx. A putative Ca^{2+} channel mediating the influx of the calcium has been cloned from MELC. However, a number of questions arise from the studies of this putative Ca^{2+} channels in cultured MEL cells: How does HMBA activate the MELC-CC? Is MELC-CC the only channel in the MELC mediating the calcium influx or other channels are also involved? What mechanism (or mechanisms) is involved in activation of channel opening and calcium influx?

Studies on MELC-CC mRNA demonstrated that all of the potential transcription start sites are located down stream of a GATA box identified in the MELC genomic clone. To test the function of the GATA box in the MELC-CC transcription process, reporter gene constructs (e.g. luciferase, CAT or growth hormone) could be used to examine the regulation of expression by this promoter region in transfected MELC. It would also be of interest to determine whether the VICC is expressed in the GATA-1 minus ES cells [Pevny et al., 1991].

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