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DEVELOPMENTAL EXPRESSION OF VOLTAGE-GATED CALCIUM CHANNELS IN EMBRYONAL CARCINOMA CELLS

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Thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science

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ABSTRACT

Increases in intracellular calcium are responsible for a wide array of cellular responses in various cell types, including muscle contraction and neurosecretion. P19 embryonal carcinoma cells may be induced to differentiate into muscle cells (smooth, skeletal, and cardiac) or neurons, upon administration of dimethyl sulfoxide (DMSO) or retinoic acid (RA), respectively. Since development in this system follows kinetics similar to those observed in vivo in the embryo, I have examined the expression of calcium channels in P19 cells to determine how their expression is altered during differentiation into cardiac muscle cells.

Colonies of beating muscle cells formed from DMSO-induced differentiation of P19 cells were treated with low concentrations of specific L-type calcium channel blockers and all contractions ceased, implying the existence of functional L-type calcium channels in DMSO-differentiated P19 cells.

The L-type calcium channel is a multi-subunit complex composed of four subunits designated α1, α2, ß, and γ . The α1 subunit forms the calcium pore and contains ligand binding sites for the calcium channel blockers, while the other subunits serve functional roles in activation and inactivation of the channel.

The expression of the various subunits of the L-type calcium channel was examined by PCR (Polymerase Chain Reaction) and Northern blot analysis. The adult skeletal muscle isoform of the α1 subunit was undetectable by PCR; however, Northern blot analysis using the skeletal muscle α1 subunit probe revealed the presence of two possible isoforms in P19 cells, with mRNA sizes of 6.5 kb and 13 kb. The cardiac isoform of the α1 subunit was found to be expressed in DMSO-treated cells, and the appearance of the cardiac α1 subunit mRNA on Northern blots corresponded with the on-set of contractile activity. The α1 subunit transcript observed was found to correspond to a 9 kb message which was similar to that identified in adult cardiac muscle.

The expression of the other subunits of the L-type calcium channel was also investigated. The skeletal forms of the ß and gamma subunits were undetectable in P19 cells, whereas the α2 subunit was present in DMSO-treated P19 cells as determined by PCR but was undetectable by Northern blot analysis. Northern blot analysis using the ß subunit probe indicated the presence of a weakly related transcript in P19 cells. These results indicate that unique subunits of the L-type calcium channel may be expressed in P19 cells.

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LIST OF ABBREVIATIONS

αMEM	alpha minimal essential medium
AchR	acetylcholine receptor
ATP	adenosine triphosphate
Cam	calmodulin
cAMP	cyclic adenosine monophosphate
CTP	cytidine triphosphate
DEPC	diethyl pyrocarbonate
DHP	dihydropyridine
dNTP	deoxynucleotide triphosphate
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
E-C	excitation-contraction
EC	embryonal carcinoma
EDTA	ethylenediaminetetraacetic acid
GTP	guanosine triphosphate
IP3	inositol trisphosphate
IPTG	isopropylthio-ß-D-galactoside
kb	kilobases
kDa	kilodaltons
LB	Luria Bertani medium
MOPS	(3-(N-morpholino)propanesulfonic acid

mRNA	messenger ribonucleic acid
PCR	polymerase chain reaction
PKA	protein kinase A
PKC	protein kinase C
pmol	picomoles
RA	retinoic acid
RNase	ribonuclease
ROCC	receptor-operated calcium channel
RT	reverse transcriptase
SDS	sodium dodecyl sulfate
SR	sarcoplasmic reticulum
SSPE	salt buffer (150 mM NaCl, 10 mM NaH2PO4, 1 mM EDTA (pH 7.4))
STX	saxitoxin
TAE	Tris-acetate/EDTA
TCA	trichloroacetic acid
Tris	Tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid
TTX	tetrodotoxin
ul	microliter
UTP	uridine triphosphate
UV	ultra-violet
x-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside

CHAPTER 1. GENERAL INTRODUCTION

Increases in intracellular calcium levels are responsible for a wide array of cellular responses in various cell types, ranging from regulation of enzyme activities, hormone and neurotransmitter secretion to muscle contraction (Catterall, 1988; Carafoli, 1987). Upon appropriate stimulation, increases in intracellular calcium occur either as a result of calcium influx across the plasma membrane, calcium mobilization from intracellular stores, or both. In each case, either receptor-operated (Rink, 1988; Gallacher, 1988) or voltage-gated (Catterall, 1988; Miller, 1992) calcium channels are responsible for the rapid increase of intracellular calcium observed upon cellular stimulation.

One of the better characterized pathways for generating a calcium signal is the opening of voltage-gated calcium channels in the plasma membranes of electrically excitable cells such as muscle cells and neurons. However, despite the important role that calcium channels play in normal muscle function, very little is known about calcium channel regulation during early development of embryonic skeletal muscle; still less is known about their early development in cardiac cells.

The P19 cell line is an embryonal carcinoma cell line which, upon treatment with retinoic acid (RA) or DMSO, will undergo differentiation to form a variety of different cell types (McBurney et al., 1982). Treatment of these pluripotent embryonal cells with DMSO results in the production of both cardiac and skeletal striated muscle cells. The P19 cell line is one of the only known permanent cell lines which produces beating cardiac muscle.

The aim of this study is to investigate the types of calcium channels expressed in DMSO-differentiated P19 embryonal carcinoma cells and to determine how this expression is altered during the process of cell commitment and differentiaton.

In the introduction to follow, several topics will be discussed. A general overview of excitation-contraction coupling and cellular calcium homeostasis as well as the major ion transport proteins involved will be presented, followed by a more detailed review of calcium channels. The current knowledge about the developmental regulation of other types of ion channels in electrically excitable cells will then be addressed. Finally, a brief summary of some of the unique properties of embryonal carcinoma cells which make them suitable for this type of study will be presented.

1.1 Excitation-Contraction Coupling and Calcium Homeostasis

Excitation-contraction coupling is the term used to describe the coupling of an electrical impulse on the plasma membrane of a muscle cell with the intracellular increase of calcium ultimately resulting in muscle contraction. The electrical impulse (or action potential) which causes depolarization of the sarcolemmal membrane consists of three phases (Catterall, 1988). The initial phase is mediated by voltage-sensitive sodium channels which open and allow influx of sodium ions, thus causing rapid depolarization of the membrane. During the plateau phase, voltage-sensitive calcium channels open to mediate the entry of calcium ions which maintain the cell in a depolarized state. The opening of these calcium channels provides a signal for the release of more calcium ions from the sarcoplasmic reticulum, which then associate with the actin-myosin filaments of the contractile apparatus of the muscle cell and effect the onset of contraction. The last phase of the action potential occurs upon activation of voltage-sensitive potassium channels which repolarize the cell membrane by allowing the outward flow of potassium ions. The resting membrane potential and action potential threshold of the cell are determined primarily by the voltage-sensitive potassium channels (Catterall, 1988).

Excitation-contraction (E-C) coupling operates by fundamentally different mechanisms in cardiac and skeletal muscle. In cardiac muscle, E-C coupling occurs via a process of "calcium-induced calcium release" in which calcium entering the cell through voltage-gated L-type calcium channels of the plasma membrane triggers release of calcium by the calcium release channels (ryanodine receptors), rsulting in a larger intracellular accumulation of calcium. The ryanodine receptors are located at the junctional region where the sarcoplasmic reticulum (SR) comes in close proximity to invaginations of the plasma membrane. The necessity of extracellular calcium entry has been demonstrated by several elegant experiments in which addition of cadmium or removal of extracellular calcium from the bathing medium abolished all contractions (Ebashi, 1976). Presumably, in cardiac muscle the entering calcium interacts directly with the calcium-release channel of the SR, causing it to open.

It should be noted that several lines of evidence indicate that the relative contribution of extracellular calcium to heart contraction is greater in the newborn than in more developed adult hearts (Fabiato and Fabiato, 1978). Morphological studies also demonstrate poorly developed intracellular membrane systems such as t-tubules and SR in newborn hearts (Maylie, 1982). This indicates that, in contrast to adult cardiac muscle, calcium entry through

required for contraction in neonatal hearts. It should also be noted that in adult cardiac muscle, calcium channels are thought to be localized in the T-tubules, while in neonatal heart the calcium channels are localized in the cell plasma membrane. Whether the nature of the calcium channels is also different in neonatal versus adult hearts remains to be elucidated.

In contrast, the removal of extracellular calcium has no effect on the contraction of skeletal muscle (Ebashi, 1976). Calcium entry through the L-type calcium channels of the plasma membrane is thus not a requirement for the initiation of E-C coupling. Nevertheless, the L-type calcium channel appears to play a role in E-C coupling in skeletal muscle. Treatment of skeletal muscle preparations with specific L-type calcium channel blocking drugs such as the dihydropyridines (DHP) results in blockade of both charge movement across the sarcolemma and calcium release from the SR (Rios et al., 1987). It has been proposed that in skeletal muscle the L-type calcium channel may serve a dual role as both a calcium channel (minor function) and as a voltage sensor (major function) which interacts with the calcium-release channel via protein-protein associations, causing the latter to open (Catterall, 1991). Molecular studies have lent weight to this hypothesis (Tanabe et al., 1990).

In order to achieve relaxation, calcium ions must be removed from the cytosol of the muscle cell and brought back to resting levels. This is accomplished by several different ion transport mechanisms.

The sodium-calcium exchanger is a low affinity, high capacity calcium exporting system found in the plasma membrane. It exploits the energy in the sodium gradient (created by the sodium-potassium ATPase) to extrude calcium from the cell, transporting 3 Na+ ions in for every Ca++ ion transported out (Nicoll et al., 1990). Molecular cloning of the cardiac sarcolemmal exchanger has disclosed a predicted secondary structure of 12 transmembrane regions and a very large cytoplasmic loop between transmembrane segments 6 and 7 (Nicoll et al., 1990). The model also predicts a phosphorylation site (calmodulin-dependent kinase or cAMP-dependent kinase) and a calmodulin binding site within the large cytoplasmic domain. There are also three potential glycosylation sites on the extracellular surface of the membrane (Nicoll et al., 1990). In cardiac muscle, the exchanger provides the major mechanism of calcium removal during the course of a contraction cycle.

The Ca-ATPase or calcium pump, located in the plasma membrane as well as in the SR, is the main mechanism of calcium ion removal from the cytoplasm of skeletal muscle. In contrast to

exchangers and channels, calcium pumps are high affinity, low capacity systems which utilize the energy from ATP hydrolysis to move calcium against its concentration and electrochemical gradient. The pumps of the plasma membrane are very distinct from those found in the SR and are encoded by separate genes. The stoichiometry of transported calcium:hydrolyzed ATP is also different, being 1:1 for the pump of the plasma membrane and 2:1 for the pump found in the SR (Carafoli, 1992). To date, four genes (PMCA 1, PMCA 2, PMCA 3, and PMCA 4) have been identified which encode the calcium pumps of the plasma membrane (Carafoli, 1992); studies have unearthed three more (SERCA 1, SERCA 2, and SERCA 3) which encode the Ca-ATPases of the SR (Brandl et al., 1986; Korczak et al., 1988; Lytton et al., 1989; Burk et al., 1989).

The SERCA calcium pumps of the SR are no more homologous to the PCMA pumps than to any of the other ion pumps cloned to date (Carafoli, 1992; Brandl et al., 1986; Burk et al., 1989; Korczak et al., 1988; Lytton et al., 1989). The SERCA 1 gene product is found mainly in fast twitch skeletal muscle, whereas the SERCA 2 gene is expressed in slow twitch skeletal muscle, heart, brain, and several other tissues (Brandl et al., 1986; Gunteski-Hamblin et al., 1988; Korczak et al., 1988). Like PCMA 1 (Strehler et al., 1989), the SERCA 2 transcript is subjected to alternative splicing which produces two isoforms; these are designated SERCA2a and SERCA2b

(Gunteski-Hamblin et al., 1988; Lytton et al., 1988). Heart and slow twitch muscle express mainly the SERCA 2a isoform whereas most other tissues expressing SERCA 2 generate the 2b isoform (Gunteski-Hamblin et al., 1988). Alternative splicing of the C-terminal region of the SERCA 1 transcript may provide a means of developmental regulation for this gene in neonate vs. adult fast twitch skeletal muscle (Brandl et al., 1986; Brandl et al., 1987).

Although mitochondria play a relatively minor role in normal calcium homeostasis, they do possess sodium-calcium exchangers and electrophoretic uniporters which can act to provide uptake of excess calcium from the cytosol in pathological states where cytosolic calcium levels become dangerously high (Carafoli, 1987). Inorganic phosphate stores in the mitochondria are believed to complex with incoming calcium ions, leading to the formation of an insoluble calcium phosphate precipitate in the mitochondrial matrix (Carafoli, 1987). The mitochondria thus play an important role in protecting the cell from calcium overload in the diseased state (Carafoli, 1987).

1.2 Calcium Channels

1.2.1 Receptor-operated Calcium Channels

Receptor-operated calcium channels (ROCCs) may be broadly

defined as a class of calcium channels insensitive to voltage. Calcium currents through channels which show no dependence on voltage have been detected in several different cell types lacking action potentials such as platelets (Zschauer et al., 1988), T lymphocytes (Gardner, 1989), osteoclasts and parathyroid cells (Nemeth et al., 1990). In addition, smooth muscle contraction may be elicited by calcium entry which occurs under conditions when membrane potential does not change (Rink, 1988), suggesting the presence of ROCCs. However, no direct connection between agonist-receptor activation and channel opening in the plasma membrane has yet been demonstrated and there is some controversy surrounding the true nature of these elusive ROCCs (Rink, 1988; Petersen, 1988).

Release of calcium from channels located on the membranes of intracellular stores is well documented (Ferris et al., 1989; Ehrlich et al., 1988; Gallacher, 1988; Gardner, 1989; Taylor, 1990), and agonist-receptor interaction has been clearly shown to result in the opening of these voltage-insensitive calcium channels. One classic example is the inositol 1,4,5-trisphosphate (IP3) receptor, studied extensively in both brain and smooth muscle. Reconstitution assays employing purified IP3 receptor from brain have indicated that the IP3 binding protein mediates calcium release (Ferris et al., 1989); experiments with vesicles from smooth muscle SR

yielded similar results (Ehrlich et al., 1988). The subsequent cloning of the IP3 receptor from brain (Furuichi et al., 1989) demonstrated that it contains a certain degree of homology, in its putative transmembrane regions, to the calcium-release channel of skeletal and cardiac muscle sarcoplasmic reticulum (Furuichi et al., 1989; Mignery et al., 1989). The IP3 receptor and the calcium-release channel, two related intracellular calcium channels, may hence perform similar yet distinct functions of calcium mobilization.

As previously mentioned, the calcium-release channel, or ryanodine receptor, is located on the surface of internal cell membranes (e.g.,the SR). In muscle cells, its activation allows calcium to flood into the cytosol, thus providing the major source of calcium required for contraction of skeletal and cardiac muscle. Ryanodine receptors are so named because of their sensitivity to the plant alkaloid ryanodine. They may be induced to open in response to artificial stimulants such as ryanodine and caffeine. However, ryanodine receptors are most often activated physiologically by extracellular calcium via a mechanism of "calcium-induced calcium release". This has been shown to occur in cardiac muscle (Nabauer et al., 1989), in neurons (Ellisman et al., 1990), and in eggs which display internal calcium oscillations (Galione et al., 1991). In skeletal muscle, the ryanodine receptor opens in response to an as-yet poorly defined signal emanating from the

L-type calcium channel of the plasma membrane (see above). To date, different ryanodine receptors have been cloned from heart (Otsu et al., 1990) and skeletal muscle (Takeshima et al., 1989; Zorzato et al., 1990). A novel type of ryanodine receptor has recently been identified which displays a more ubiquitous distribution pattern (Giannini et al., 1992).

1.2.2 Voltage-gated Calcium Channels

To date, four distinct types of voltage-gated Ca channels have been identified (L,T,P, and N types) on the basis of their electrophysiological properties as well as their sensitivities to pharmacological agents (Miller, 1992).

The L-type Ca channels are characterized by longer channel open times and higher conductances (25 pS) than the other channel types; they also require strong depolarization of the membrane (up to 0 mV) in order to open (Kostyuk, 1989). L-type channels are blocked by three different classes of pharmacological agents: the benzothiazepines (e.g., diltiazem), the phenylalkylamines (e.g., verapamil), and the dihydropyridines (e.g., nifedipine). Due to their high affinity and slow rate of dissociation, the dihydropyridines (DHPs) have proven the most useful in studies on these channels (Janis et al., 1984). L-type channels are involved in excitation-contraction coupling (muscle) and excitation-

secretion (endocrine cells and some neurons) (Catterall, 1988).

The T-type (or Transient) channel has a much lower conductance (8 pS) than the L-type and stays open for a shorter period of time. In contrast to the L-type, the T-type channel is a low threshold channel, being activated at membrane potentials between -60 and -40 mV (Kostyuk, 1989). It has been postulated that low threshold, or T-type channels, may be involved in pacemaker activity since they are abundant in both atrial and Purkinje (i.e., impulse-conducting) cells of the heart (Hirano et al., 1989). The T-type channel is not blocked to any significant extent by the DHP drugs.

The N-type and P-type channels have been identified only in neurons and Purkinje cells, respectively. Although both are insensitive to dihydropyridines and both are activated at high thresholds like L-type channels, the N-type has a higher conductance (12-20 pS) than the P-type (10 pS) and is blocked by w-conotoxin (Miller, 1992). The P-type is insensitive to w-conotoxin but is blocked by funnel web spider toxin (FTX) (Miller, 1992). The N-type Ca channel is involved in neurotransmitter release (Miller, 1992); the function of the P-type remains unknown. Both the P-type (Mori et al., 1991) and more recently the N-type (Williams et al., 1992) channel have been cloned.

1.2.3 The Dihydropyridine-Sensitive L-type Calcium Channels

Skeletal muscle T-tubules were found to contain the highest density of DHP receptors and as a consequence have been the main source for purification and molecular characterization of these channels (Tuana et al., 1988).

The L-type calcium channel from skeletal muscle is composed of 5 subunits designated α1, α2, ß, δ, and γ . The α1 subunit, in both cardiac and skeletal muscle, is a large transmembrane protein which forms the functional pore of the channel through which Ca enters the cell. In skeletal muscle, the purified α1 subunit has a molecular weight of 170 kDa; purified α1 from cardiac muscle is 220 kDa (Catterall, 1988). Both isoforms of the α1 subunit have been cloned; the mRNA coding for the skeletal isoform is 6.5 kb in length; there are two (9 kb and 16 kb) mRNA transcripts detectable in cardiac muscle (Tanabe et al., 1987; Mikami et al., 1989). Other isoforms of L-type calcium channel α1 subunits have been identified in smooth muscle and brain (Biel et al., 1990; Koch et al., 1989; Hui et al., 1991; Huang et al., 1990). The use of heterologous expression systems has demonstrated that the cardiac α1 subunit alone is capable of serving as the main functional component of the L-type calcium channel (Bosse et al., 1992; Nargeot et al., 1992). Present models propose that the α1

subunit consists of four repeating domains of six transmembrane segments, the 4th transmembrane segment (S4) in each domain consisting of highly charged amino acid residues which allow the S4 segment to serve as a voltage sensor (Catterall, 1988). The suggested transmembrane arrangements of this model are consistent with the phosphorylation sites of the α1 subunit being located on the intracellular side of the membrane (Tanabe et al., 1987; Lai et al., 1990). The α1 subunit contains the DHP binding sites as well as consensus sites for phosphorylation by cAMP-dependent kinase (PKA), protein kinase C, and Ca-calmodulin dependent kinase (Lai et al., 1990; Catterall, 1988; Tanabe et al., 1987; Mikami et al., 1989).

Experiments employing chimaeric constructs of the α1 cardiac/skeletal L-type channels have determined that the cytoplasmic loop located between transmembrane repeat domains II and III of the α1 skeletal isoform confers skeletal-type E-C coupling (Tanabe et al., 1990). Presumably it is this portion of the α1 polypeptide which interacts with the calcium-release channel of the SR.

The ß subunit plays a role in modulating the kinetics of the L-type calcium channel and increases the number of DHP binding sites of the channel (Varadi et al., 1991). Isoforms of the ß subunit have been cloned from skeletal muscle (Ruth et al.,

1989), heart (Perez-Reyes et al., 1992; Hullin et al., 1992), brain (Perez-Reyes et al., 1992; Hullin et al., 1992), and smooth muscle (Hullin et al., 1992). The ß subunit is believed to be an extrinsic membrane protein closely associated with the cytoplasmic domains of the α1 subunit. Its coexpression with the α1 subunit in heterologous systems results in an increase in the rate of activation/ inactivation kinetics of the channel as well as an increase in the magnitude of measurable calcium current (Perez-Reyes et al., 1992; Hullin et al., 1992; Varadi et al., 1991). Recombinant expression of brain DHP-sensitive Ca current is dependent on coexpression of a ß subunit (Williams et al., 1992). There also seems to be a synergistic action between the α2 and ß subunits on channel properties as well as on channel expression in heterologous systems; it thus seems probable that these two subunits interact with each other, either directly or via the α1 subunit (Nargeot et al., 1992; Williams et al., 1992). Like the α1, the ß subunit has several consensus sites for phosphorylation by PKA, PKC and Cam kinase (Ruth et al., 1989; Perez-Reyes et al., 1992).

The α2 subunit is a highly glycosylated, hydrophobic transmembrane protein with a molecular weight of 175 kDa which shifts to 150 kDa under reducing conditions which disrupt disulfide bonds (Catterall, 1988; Miller, 1992). Upon reduction, three smaller peptides (25 kDa, 22 kDa, and 17 kDa)

are released from the α2 subunit; these have been identified as different forms of the δ subunit (Jay et al., 1991). The δ subunit is thus associated with α2 via disulfide bonds. The δ polypeptides are hydrophobic and highly glycosylated (Jay et al., 1991). While it has been postulated that the δ subunits may serve to firmly anchor the α2 subunit in the membrane (Miller, 1992), their function remains a mystery.

The α2 subunit from skeletal muscle has been cloned; the mRNA coding for the protein is 8 kb in size (Ellis et al., 1988). The mRNA for this subunit appears to have a very wide distribution in excitable tissues, being present in ileum, aorta, brain, heart, and hippocampus (Ellis et al., 1988). Another isoform of the α2 subunit has been cloned from brain (Williams et al., 1992). The function of the α2 subunit remains largely unknown, although it has been proposed that the α2 may somehow render the channel more efficient since coexpression of the cardiac α1 with the α2 in Xenopus oocytes results in a doubling in the magnitude of measurable calcium current (Nargeot et al., 1992).

The gamma subunit, purified and cloned from skeletal muscle, is a small hydrophobic, highly glycosylated protein with a molecular mass of 32 kDa and mRNA size of 1.2 kb (Jay et al., 1988). This subunit does not appear to be expressed in any tissues studied to date except for skeletal muscle and to a

lesser extent, in lung (Jay et al., 1988). There is some indication from coexpression studies in heterologous systems that the gamma subunit accelerates channel inactivation and renders the inactivation process more sensitive to voltage (Nargeot et al., 1992).

1.3 Developmental Regulation of Ion Channels

1.3.1 Acetylcholine Receptor

The acetylcholine receptor (AchR) is an ion channel important in neuromuscular transmission. It is a pentameric protein composed of four types of subunits ($\alpha, \beta, \gamma, \delta$). During the period following birth, the characteristics of the Ach-R change from the fetal (slow) to the adult (fast) form resulting in differences in conductances and gating properties (i.e., channel is open for shorter periods of time and conductance is 50% larger in the adult form). The mature or adult form of the AchR is found only at neuromuscular junctions in innervated muscle, whereas the fetal form is found scattered over the surface of embryonic muscle and is also expressed in denervated adult muscle (Brehm, 1989). It has been shown independently by several groups (Brehm, 1989) that a switch in AChR subunit expression of the fetal gamma subunit for the adult epsilon subunit is responsible for the change in electrophysiological properties observed with this

channel during the initial weeks of post-natal development. Both the gamma and epsilon subunits have been cloned; their primary sequences are similar (53% homologous) but not identical (Mishina et al., 1986).

The rationale behind the developmental switch in AchR subunit expression remains obscure, but it may have to do with calcium entry. It has been postulated that long channel open times, characteristic of the fetal form of the receptor, may be required to allow extracellular calcium to enter the cell (Brehm, 1989). This calcium is believed to be important in allowing AchR clustering under nerve endings at the neuromuscular junction; it could also play a role in causing spontaneous electrical firing of the muscle cell, which may be necessary for proper muscle development. The developmental switch to fast (adult) channels may then be required to prevent calcium levels entering the adult cell from reaching toxic levels (Brehm, 1989). In other words, once the synapse has matured, the high calcium levels required for synapse formation would no longer be necessary and might even be detrimental to the muscle cell. However, this theory - although attractive - remains largely speculative at this point.

1.3.2 Sodium Channels

Sodium channels are voltage-gated ion channels found in all excitable tissues. The α subunit forms the functional pore of the channel and contains the binding sites for the neurotoxins tetrodotoxin (TTX) and saxitoxin (STX). Its structure is thought to closely resemble that of the L-type calcium channel α1 subunit (Catterall, 1988). Several isoforms of the α subunit have been cloned from brain (Noda et al., 1986; Kayano et al., 1988) as well as from adult skeletal muscle (Trimmer et al., 1989), heart (Rogart et al., 1989), and electric eel (Noda et al., 1984). The purified sodium channel from brain contains an additional two subunits, ß1 (36 kDa) and ß2 (33 kDa) (Catterall, 1988). Dissociation of brain ß1 causes loss of ion flux, channel conductance, and voltage dependence of the purified sodium channels (Catterall, 1988).

There is a wealth of evidence to indicate that sodium channels undergo developmental changes in muscle (Harris et al., 1973; Kallen et al., 1990; Haimovich et al., 1986; Sherman et al., 1983; Gonoi et al., 1985), the central nervous system (Beckh et al., 1989), and in several cell lines which may be induced to differentiate into excitable cell types (Baumgold et al., 1987; Rendt et al., 1989; Caviedes et al., 1986). In skeletal muscle, sodium channels appearing early on in development are sensitive to higher (micromolar, as opposed to nanomolar) concentrations of TTX than channels found in the adult; these channels are thus termed "TTX-insensitive" due to their low

affinity for TTX. Ion flux and TTX binding studies have revealed that rat skeletal muscle cells in culture initially possess a single population of TTX-insensitive channels, and a second TTX-sensitive population of sodium channels evolves with time after the first week postnatal (Haimovich et al., 1986).

It is interesting to note that TTX-sensitive sodium channel levels may be affected by alterations not only in electrical activity, but also in cAMP and cytosolic calcium levels in skeletal muscle cells (Offord et al., 1989). Treatment of skeletal muscle cells in culture with 8-BrcAMP (cAMP analogue) results in an increase in both sodium channel mRNA levels and STX binding, whereas treatment with the calcium ionophore A23187 causes a decrease in mRNA and STX binding, as compared to controls (Offord et al., 1989). Denervation (Cooperman et al., 1987) and increases in cytosolic calcium thus appear to exert effects on sodium channel levels which directly oppose those observed with cAMP in skeletal muscle cells.

In adult heart, as well as in denervated skeletal muscle, there is a population of TTX-resistant sodium channels with properties similar to the TTX-insensitive channels found in early muscle development. The ever-widening use of molecular biology techniques has made it possible to clone TTX-insensitive sodium channels from adult heart (Rogart et al.,

1989) and from denervated adult skeletal muscle (Kallen et al., 1990). This sodium channel isoform was found to be expressed in denervated skeletal muscle, newborn and adult heart; its expression was undetectable in either brain or innervated adult skeletal muscle (Rogart et al., 1989). The TTX-insensitive channel cloned from denervated skeletal muscle showed a similar pattern of expression (Kallen et al., 1990).

The expression of this sodium channel cloned from denervated muscle was examined during skeletal muscle development; its levels were found to progressively decrease with time, being highest at day 1 following birth and undetectable at day 35 (Kallen et al., 1990). In contrast, the same study found that expression of a previously cloned (Trimmer et al., 1989) adult skeletal muscle TTX-sensitive sodium channel increased roughly 10-fold from birth to day 35. The molecular data is thus consistent with the electrophysiological and TTX binding affinity studies of earlier years which demonstrated that adult skeletal muscle does not possess the TTX-insensitive isoform of the sodium channel (Catterall, 1988). Interestingly, denervation of adult skeletal muscle results in reappearance of the TTX-insensitive sodium channel with time (Cooperman et al., 1987).

Developmental regulation of sodium channels also occurs in the central nervous system. In the brain and spinal cord, there

appear to be three distinct sodium channels expressed from early fetal to late postnatal stages of development in the rat (Beckh et al., 1989). These isoforms undergo both temporal and regional regulation during CNS development.

1.3.3 Calcium Channels

There is some evidence that rabbit skeletal muscle may express a neonatal isoform of the L-type calcium channel which is undetectable in adult rabbit skeletal muscle (Malouf et al., 1992). This neonatal form of the channel appears to possess an internal deletion of 2 kb which is in frame, preserving the reading frame of the $\alpha 1$ subunit and resulting in a polypeptide that contains the first repeat domain and a fusion of the second and fourth domains; the third domain is completely deleted (Malouf et al., 1992). Interestingly, this isoform lacks the region previously determined to bestow skeletal-type E-C coupling properties upon adult skeletal muscle (Tanabe et al., 1990). The significance of this remains unclear, however, since the adult (undeleted) mRNA of the $\alpha 1$ subunit is also expressed in newborn muscle (Malouf et al., 1992). Electrophysiological studies have shown that two distinct calcium currents are detectable in newborn rat muscle, I fast and I slow (Beam et al., 1988a; Beam et al., 1988b). While it seems clear that the I slow current corresponds to the slow L-type calcium current found in adult skeletal muscle, it is

possible that the neonate α1 isoform detected using molecular techniques corresponds to the I fast calcium current, shown electrophysiologically to disappear within few weeks after birth (Beam et al., 1988b).

The adult isoform of the L-type calcium channel from skeletal muscle has already been shown to undergo developmental regulation of some of its subunits. In the murine myogenic cell line C2C12, Northern blot analysis established that mRNA for the α1 subunit is undetectable in myoblasts but its expression is induced 20-fold upon formation of myotubes (Varadi et al., 1989). Similarly, DHP binding studies have revealed that nitrendipine (DHP) binding sites in primary cultures of embryonic skeletal muscle appear shortly after the onset of fusion (Morton et al., 1989). In contrast, the α2 subunit is expressed at low levels in myoblasts and is only weakly induced after fusion into myotubes, as shown by Northern hybridizations (Varadi et al., 1989). Western blot analysis and DHP binding experiments using rat skeletal muscle have shown that the α1 subunit is expressed initially in low amounts and then at increasingly higher levels with time after birth, whereas the α2 is expressed at relatively high levels throughout development from birth (Morton et al., 1989). The developmental appearance of the α1 polypeptide coincides with the time course of appearance of slow calcium current in rat skeletal muscle (Beam et al., 1988b). The developmental

expression and regulation of the ß and gamma subunits in developing skeletal muscle have not yet been fully investigated.

While it is clear from functional and morphological studies that early cardiac muscle has a different requirement for calcium entry, nothing is known about developmental aspects of the L-type calcium channel in the myocardium.

1.4 Embryonal Carcinoma Cells

Embryonal carcinoma (EC) cells are the stem cells of teratocarcinomas, which are malignant tumors of embryonic origin. Teratocarcinomas may be induced in mice; these tumors contain EC cells and some differentiated cell types derived from the pluripotent EC stem cells.

Although information on ion channel expression in EC cells is very limited, patch clamp experiments on the embryonal carcinoma cell line NT2/D1 have demonstrated that sodium channel expression is subject to developmental regulation. These EC cells, induced to form neurons upon treatment with retinoic acid, exhibit a neuronal morphology and possess electrically active sodium channels whose activity may be blocked by TTX (Rendt et al., 1989). In contrast, there is no evidence of sodium channels in the untreated, undifferentiated

embryonal carcinoma cells (Rendt et al., 1989). Undifferentiated NT2/D1 cells display only outward currents which are carried by potassium; measurements of single channel currents in the EC cell lines 1003 and 1009 have shown that undifferentiated cells possess two types of potassium channels: a calcium-activated potassium channel, and a voltage-dependent potassium channel (Simmoneau et al., 1985b; Simmoneau et al., 1985a). Neither sodium nor calcium currents have been detected in any of these EC cell lines in their undifferentiated state (Rendt et al., 1989; Simmoneau et al., 1985b).

The P19 cell line was developed from a teratocarcinoma induced by grafting a 7.5 day old mouse embryo onto the kidney capsule of an adult mouse (McBurney et al., 1982). This EC cell line possesses some rather unique properties. If maintained in culture in exponential growth phase, the pluripotential properties of P19 cells are preserved (Rudnicki et al., 1989). Upon treatment with retinoic acid (RA) or dimethyl sulfoxide (DMSO), these pluripotent embryonal cells undergo differentiation to form a variety of different cell types. Treatment with DMSO results in the production of striated muscle cells, both skeletal and cardiac (Rudnicki et al., 1990; Edwards et al., 1983). Use of RA results in the formation of neurons and glial cells (McBurney et al., 1988; Jones-Villeneuve et al., 1982). Fibroblast-like cell types

also can be seen in these cultures (Rudnicki et al., 1990). The neurons and muscle cells formed in differentiated P19 cultures express cell-type specific markers as demonstrated by their immunohistochemical staining with antibodies to neurofilaments and glial fibrillar protein in the case of neurons and glial cells (Jones-Villeneuve et al., 1982), and with antibodies to embryonic forms of cardiac myosin heavy chains (ßMHC) in cardiac cells (Rudnicki et al., 1990). Furthermore, P19 cells are able to contribute to a variety of normal tissues in vivo in chimaeric mice (Rossant et al., 1982). It is also interesting to note that in DMSO-treated P19 cultures, cardiac muscle develops with the same kinetics as is seen in vivo in the embryo (Rudnicki et al., 1990). For these reasons and since P19 cultures can be induced to differentiate into a wide variety of excitable cell types, this system was chosen to examine developmental expression of Ca channels in muscle cells.

1.5 Thesis Project

Excitation-contraction coupling in cardiac muscle undergoes developmental regulation in that muscle cells in early stages of development depend entirely on calcium entry through what may be a unique L-type calcium channel (Fabiato and Fabiato, 1978). The L-type calcium channels appear to undergo a change during development and maturation such that less calcium

enters the adult cardiac cell and the majority of contractile calcium is released from the SR (Langer, 1992; Ebashi, 1976).

Given what is presently known about the regulation and expression of calcium channels during early development of cardiac muscle, the specific aims of this project were as follows.

1) to develop an experimental system for studying the developmental expression of cardiac L-type calcium channels
2) to characterize in molecular terms the type of calcium channels expressed in early embryonic cardiac muscle

Chapter 2. MATERIALS AND METHODS

2.1 Tissue Culture

Passaging the cells:

P19 cells were grown as monolayer cultures in αMEM containing 2.5% fetal bovine serum and 7.5% calf serum. Cells were passaged every 48 hours or less, when reaching approximately 80% confluence. After every three weeks of passaging, cells were discarded and a new vial of fresh cells was thawed for use. To passage the cells, they were first washed with a phosphate-buffered saline solution, then treated with trypsin-EDTA for 3 minutes at 37 °C. The trypsin solution was inactivated by the addition of serum-containing αMEM, and the cells were vigorously dispersed by repeated pipetting. Cell density was determined using a Coulter counter, and cells were plated out at 10^5 cells/ml.

Differentiation with DMSO:

On "day 0", P19 cells were grown in aggregates in 100 mm petri dishes at a density of 10exp5 cells/ml and 0.5% DMSO was added. On "day 1", the cells in the petri dish were divided into 3 petri dishes; medium was added to bring the volume of each dish to 15 ml. DMSO was added to keep the drug concentration at 0.5%. Cells were left until "day 3", when the contents of each of the three 100 mm petris were

transferred to larger, 150 mm petri dishes, the medium was changed and more DMSO and αMEM was added to keep the drug concentration at 0.5% and the volume at 45 ml. On "day 5", DMSO was removed from the medium, fresh medium was added and cells were transferred to 150 mm tissue culture-grade dishes. Medium was changed every second day following plating on day 5 (i.e., changed on days 7, 9, etc.).

2.2 Isolation of Total RNA

Total cellular RNA was extracted from cells and tissue using the LiCl-urea method of Auffray and Rougeon (Auffray et al., 1980). For isolation of RNA from tissue, the muscle was first removed from the animal and quick-frozen in liquid nitrogen. The tissue was then pulverized in liquid nitrogen using a mortar and pestle. The powdered tissue was then added to an ice cold solution of 8M urea/3M LiCl and subjected to homogenization for 2 minutes at high speed (setting 7 on a Polytron homogenizer).

For cultured cells, the solution of 8M urea/3M LiCl was added directly to the plates to lyse the cells; the cells were then scraped off the plates into a polypropylene tube using a rubber policeman. The solution was homogenized as stated for tissue samples.

Lysates were left overnight at 4 °C. Total RNA was pelleted

the following day by centrifugation at 8000 rpm for 30 minutes at 4 °C. Pellets were washed twice with 3M LiCl, then resuspended in DEPC-treated water. If the RNA preparation contained large amounts of impurities (as determined by each sample's optical density ratio of 260 nm/280 nm), it was subjected to several phenol-chloroform extractions to improve its quality. All RNA samples were stored at -80 °C until use.

2.3 RNA Formaldehyde Gels

RNA was electrophoresed on 0.8% denaturing formaldehyde gels at 30 volts overnight or at 100 volts for 4 hours. Agarose gels were composed of 0.8% agarose, 7% formaldehyde, 20 mM MOPS, 5 mM sodium acetate, and 1 mM EDTA. Gels were pre-run for 30 minutes at 40 volts in 20 mM MOPS, 5 mM sodium acetate and 1 mM EDTA; a circulator pump was used to keep the buffer well mixed during electrophoresis. Samples (10 to 20 ug of RNA) were resuspended in 30 ul of sample buffer (50% deionized formamide, 3.7% formaldehyde, 20 mM MOPS, 5 mM sodium acetate, and 1 mM EDTA), then heated at 65 °C for 20 minutes. Samples were quickly cooled on ice for 10 minutes, loading buffer containing ethidium bromide was added and samples were loaded on the gel.

2.4 Northern Transfer

MSI MAGNA nylon membrane filters were used for RNA transfer procedures, according to manufacturer's instructions. A pyrex

dish was filled with 500 ml of 10X SSPE; a piece of 3M Whatman paper was soaked in this solution and hung over a glass plate propped on the pyrex dish so that both ends of the Whatman paper rested in the 10X SSPE. The paper was smoothed out to remove air bubbles, and 2 more pieces of Whatman paper saturated with 10X SSPE were placed on top of the first piece. The gel was then positioned on the top piece of Whatman paper, strips of Parafilm were placed around the sides of the gel, and a piece of MAGNA nylon membrane cut to the size of the gel was placed on top of the gel. Air bubbles were smoothed out using a glass pipet. Three SSPE-saturated pieces of Whatman paper cut to size were then layered over the gel assembly, followed by a 2-inch stack of paper towels and a glass plate. The whole gel assembly was secured with a heavy weight and left to transfer by capillary action overnight. The next day, wet paper towels were replaced with fresh dry ones, and the transfer was left for another 8 to 24 hours, depending on the efficiency of transfer (determined using a UV lamp on the filter and the gel). After transfer, blots were baked in a vacuum oven at 80 C for 1 hour, then UV-crosslinked for 1 min. 45 seconds. Blots were stored in plastic bags at 4 C until use.

2.5 Northern Hybridization

MAGNA nylon membranes were pre-hybridized in heat-sealable bags at 56 C for a minimum of 4 to 8 hours. Pre-hybridization

hybridization buffer consisted of 50% deionized formamide, 5X SSPE, 0.1% SDS, 2.5X Denhardt's solution, 1-2% denatured carrier DNA, and 50 ug/ml tRNA. Carrier DNA was boiled for 5 minutes, then cooled on ice before being added to the pre-hybridization mixture. For each membrane (measuring 12 cm x 14 cm), 10 ml of filtered pre-hybridization solution was used. Probe was added at a concentration of 20 ng/ml (1-5 x 10exp6 cpm/ml) to each blot; hybridization was at 56 °C for 18-22 hours. Membranes were washed in 0.1% SDS, 0.1X SSPE for 2 x 15 minutes at 65 °C, then exposed to XAR X-ray film between regular intensifying screens at -80 °C for 24-72 hours.

If deemed necessary, filters were treated with RNase A at a concentration of 1 ug/ml for 15 minutes in 2X SSPE to remove non-specific binding of the probe. Filters were then washed at 45 °C for 15-30 minutes in 0.1% SDS, 0.1% SSPE, and subsequently exposed to XAR X-ray film between intensifying screens at -80 °C for 5-10 days.

2.6 Riboprobe Synthesis

Riboprobes were synthesized using the Riboprobe Gemini System II in vitro transcription kit by Promega under RNase-free conditions and according to the manufacturer's instructions. All plasmid DNA to serve as template was prepared using the cesium chloride gradient method (see below); the plasmid was linearized with the appropriate restriction enzyme to yield an

anti-sense probe (complementary to the messenger RNA of interest); 1 ug of DNA was used for each transcription reaction. The reactions were performed in a total volume of 20 ul consisting of transcription 5X buffer (200 mM Tris-HCl pH 7.5, 30 mM MgCl2, 10 mM spermidine, 50 mM NaCl), 500 uM of ATP, GTP, and UTP, 18 uM final of cold CTP, 50 uCi radioactive 32P CTP, RNasin, 1 mM DTT, and 10 units of RNA polymerase. Incubation was at 37 °C for 1 hour. DNA template was removed from the mixture by incubation with an RNase-free DNase at 37 °C for 15-30 minutes. Probes were extracted with phenol/chloroform, then ethanol-salt precipitated to remove unincorporated nucleotides. All probes were either used immediately or stored at -20 °C for no more than 48 hours before use.

Incorporation efficiency was determined by using a 1 ul aliquot (diluted to 100 ul) and performing a 10% TCA precipitation over glass filters on half of this aliquot i.e., glass filter in scintillant/ other half of aliquot in scintillant (total cpm) x 100 yields % incorporation. Only probes with greater than 30% incorporation were used for Northern hybridizations.

2.7 Reverse Transcriptase-Polymerase Chain Reaction

Oligonucleotide primers were designed based on the published DNA sequences of the α1, α2, ß and gamma subunits of the L-

type channel from adult rabbit skeletal muscle, and the α1 subunit from adult rabbit cardiac muscle (Tanabe et al., 1987; Ellis et al., 1988; Ruth et al., 1989; Jay et al., 1988; Mikami et al., 1989). Primers were 22-24 base pairs in length; their sequences, as well as the expected size of the amplified DNA, are shown below:

α1 skeletal:	3707 bp to 4349 bp	P1-TCATCTTCACACTGGAGATGATCC
	(642 bp fragment)	P2-TCACACTGGAGATGATCCTCAAGC
α1 cardiac:	3695 bp to 4244 bp	P5-CCGAGTGGAGATCTCCATCTTCTT
	(549 bp fragment)	P6-GGAGTTCTCCTCTGCGTTCATAGA
α2 skeletal:	2675 bp to 3180 bp	P10-GGCCTATGAGTCAGGCATTAGAGT
	(505 bp fragment)	P11-GGAGCCGTGGAAAAGTCAAACTCA
ß skeletal:	from 153 to 685 bp	B1-TCCATGGTCCAGAAGACCAGCA
	(532 bp fragment)	B2-CGCAGCTTCTGTTCCTGCAGCA
gamma skeletal:	from 22 to 579 bp	G1-TGGAGCACCCACCCCTCTGCAG
	(557 bp fragment)	G2-ATAGTACTCGATCCAGACGGTG

(NOTE: See RESULTS section for schematic diagrams of amplified regions)

The reverse transcriptase reaction was performed on 1 ug of total RNA using 1 mM of each dNTP (Pharmacia), 100 pmoles of random hexamers (Pharmacia), and 200 units of MuLV reverse transcriptase (BRL) in a 20 ul reaction volume. Samples were heated to 95 °C for 5 minutes to break up secondary structure of the RNA, then cooled on ice for 5 minutes before addition of the enzyme. Incubation was at 23 °C for 10 minutes, followed by 42 °C for one hour. To arrest the reaction, tubes were heated to 95 °C for 10 minutes, then quickly cooled on ice.

The polymerase chain reaction was performed on the above RT reaction, using 100 pmoles of PCR primers and either 1-2 units of Taq DNA polymerase (BRL) (for cardiac and skeletal α1) or 2 units of Vent DNA polymerase in a MgSO4 buffer (New England Biolabs) (for the other subunits, gamma, α2, ß). The program used was as follows: 94 °C denaturation for 40 seconds, annealing of primers at 55 °C for 60 seconds, and extension of primers at 72 °C for 90 seconds (for 30 cycles), plus an additional 15 minute extension step (72 °C) at the end of the reaction. A 10 ul aliquot of each sample was loaded onto a 1% agarose TAE mini-gel for analysis.

More detailed information on the RT-PCR techniques described here may be found elsewhere (Kawasaki et al., 1989).

2.8 Southern Transfer

See above protocol for Northern transfer (DNA instead of RNA).

2.9 Southern Hybridization

See above protocol for Northern hybridization (DNA instead of RNA samples).

2.10 DNA Restriction Digests

Most restriction digests were performed using 1-20 ug of DNA and 1-2 units of enzyme, in a final volume of 20-40 ul. Digests were carried out at the temperature specified as being optimal for the enzyme being employed (usually 37 °C); incubation times were 1.5 to 2 hours. Restriction enzymes were purchased either from BRL (Bethesda Research Laboratories), Pharmacia, or NEB (New England Biolabs). Digested samples were analyzed on 1% agarose TAE mini-gels containing 0.1 ul of ethidium bromide and electrophoresed at 100 volts until a satisfactory separation (as determined visually by UV light) was achieved. When necessary, restriction enzymes were inactivated either by heat or with phenol-chloroform extractions.

2.11 DNA Ligations

PCR fragments to be subcloned were electrophoresed out on 1% SEAKEM GTG agarose (FMC BioProducts) TAE mini-gels and excised from the gel. The gel sections were then treated with

the GENECLEAN (Bio/Can Scientific, Inc.) glass milk purification procedure.

For "sticky end" ligations (e.g., skeletal α1), the purified PCR fragments were cut with the appropriate restriction enzyme, electrophoresed on a SeaKem gel and Genecleaned as above, then resuspended in a final volume of 15 ul. The plasmid vector PTZ19 was cut with the same restriction enzymes to yield compatible ends, then dephosphorylated with calf alkaline phosphatase. Ligation using T4 ligase (BRL) was at 15 °C overnight, in a total volume of 15 ul (ratio of vector:PCR fragment was roughly 1:3).

Blunt-ended ligations (e.g., cardiac α1) were performed essentially as follows: Purified PCR fragments were rendered blunt using either a Klenow fill-in reaction or a mung bean exonuclease reaction. The fragments were then phosphorylated using T7 polynucleotide kinase, and re-purified with a phenol-chloroform extraction and ethanol-salt precipitation. The plasmid KS Bluescript was cut with the restriction enzyme sma 1 to render it blunt, purified using Geneclean, and dephosphorylated with calf alkaline phosphatase. Ligation was essentially the same as described above, except a smaller volume (10 ul) was used and the ligation was performed overnight at room temperature.

2.12 Transformation of Competent Bacteria

Subcloning efficiency DH5α competent E. coli (BRL) were transformed according to the manufacturer's instructions. Briefly, 1-3 ul of ligation reaction were used to transform 50 ul of competent cells. The ligation mixture was added to cells on ice, left to incubate for 30 minutes, and the cells were heat-shocked for 20 seconds at 37 °C. After being placed on ice for an additional 5 minutes, 750 ul of LB broth (no antibiotics) was added and the cells were incubated with shaking at 37 °C for one hour. Cells were then plated out on LB-agar plates containing IPTG, x-gal, and 75 ug/ml ampicillin, and incubated at 37 °C overnight. The following morning, those colonies which appeared white instead of blue were used to innoculate 3 ml of LB (with 75 ug/ml ampicillin) for mini-preps (see below).

2.13 Miniprep Plasmid Purification

Transformed E. coli were innoculated into 3 ml of LB broth containing 75ug/ml ampicillin overnight with vigorous shaking at 37 °C. The cells were harvested in a microfuge at 4 °C, 14 000 rpm for 10 minutes, then resuspended in 200 ul of solution 1 (0.5% glycerol, 10 mM EDTA, 25 mM TRIS pH 8.0). The bacteria were kept on ice for 10 minutes, then 400 ul of solution 2 (1% SDS, 0.2N NaOH) was added to lyse the cells, followed by gentle mixing. The cells were left on ice for another 10 minutes before the addition of 300 ul of solution 3 (3M

potassium acetate, 2M acetic acid) to precipitate chromosomal DNA. Tubes were vortexed, left on ice for 10 minutes, then centrifuged at 14 000 rpm for 10 minutes at 4 °C to pellet all cellular debris and chromosomal DNA. The supernatent was transferred to clean eppendorf tubes and an equal volume of isopropanol was added. The samples were mixed, placed at -80 °C for one hour, then centrifuged at 14 000 rpm for 10 minutes at 4 °C. The supernatents were discarded, and the pellets containing the plasmid DNA were washed twice with 1 ml of 70% ethanol. Pellets were dried in a speed vac, then resuspended in 100 ul of distilled, deionized water. Samples were stored at -20 °C until use.

From each 100 ul of sample, 10 ul was used for restriction digest analysis. The remaining 90 ul, to be used for sequencing, was subjected to a LiCl precipitation step to remove RNA from the sample: 200 ul of a 25% LiCl solution was added to the 90 ul of mini-prep and placed on ice for 10 minutes. Samples were centrifuged at 14 000 rpm for 10 minutes at 4 °C and the supernatent was collected. The plasmid DNA was precipitated by the addition of 0.1 volume of 3M sodium acetate and 2.5 volume of 95% ethanol; samples were left at -80 °C for one hour then centrifuged for 10 minutes in a microfuge (see conditions above). Supernatents were discarded, pellets were washed twice with 1 ml of 70% ethanol to remove excess salt and then dried in a speed vac. Each

sample was treated as described in one of the protocols listed under "DNA Sequencing" (see below).

2.14 Purification of Plasmid DNA by Equilibrium Centrifugation CsCl-Ethidium Bromide Gradients

This procedure is essentially a scale-up of the mini-prep protocol above, followed by an extra CsCl gradient purification step.

Transformed E.coli (DH5α) were innoculated into 200-300 ml of LB containing 75 ug/ml ampicillin and grown up overnight with vigorous shaking at 37 °C. The cells were harvested by centrifugation at 6000 rpm for 10 minutes at 4 °C. The pellet was resuspended in 16 ml of solution 1 (see above), then incubated on ice for 10 minutes. Solution 2 (see above) was then added (32 ml) and mixed gently. After incubating samples on ice for 10 minutes, 24 ml of solution 3 was added and mixed, followed by another 10 minute incubation on ice. Samples were centrifuged at 6000 rpm for 10 minutes at 4 °C, then the supernatent was decanted through cheese cloth into a clean oakridge tube. An equal volume of isopropanol was added and mixed; samples were left to incubate on ice for 10 minutes. Tubes were centrifuged at 6000 rpm for 10 minutes at 4 °C, and the supernatents were discarded. The pellets were dried and resuspended in 2 ml of TE (10 mM Tris, 1 mM EDTA). Exactly 2.30 g of CsCl were added and dissolved, followed by

the addition of 200 ul of ethidium bromide. The mixture was briefly centrifuged to remove any large particulate matter from the sample, and the clear supernatent was loaded into Beckman Quickseal tubes. Samples were centrifuged in a vertical rotor at 60 000 rpm for 12-16 hours. The band of plasmid DNA was collected off each gradient using a 3 ml syringe and a 25G needle, and placed in a polypropylene tube. Each sample was washed repeatedly with an equal volume of water-saturated butanol to remove the ethidium bromide from the plasmid DNA (4-6 washings). Four volumes of Tris-EDTA were added to each sample, followed by 2.5 volumes of 95% ethanol. Samples were left at -80 °C for one hour, then centrifuged at 8000 rpm at 4 °C for 10 minutes. Supernatents were discarded; pellets were washed twice with 70% ethanol, dried, and resuspended in sterile water. The concentration and purity of the DNA were determined by reading the optical density of each sample at 260 nm and 280 nm in a Beckman spectrophotometer.

2.15 DNA Sequencing

NOTE - All sequencing was performed using the dideoxy method of Sanger (see (Sambrook et al., 1989)).

- The sequences of all probes used for Northern blot analysis were verified before use

Double-stranded sequencing using Sequenase:

DNA was resuspended in 20 ul distilled, deionized water, and 0.1 volume of a solution of 2M NaOH/2 mM EDTA was added to denature the double-stranded DNA. Incubation for 5 minutes at room temperature was followed by neutralization with 3 ul of 3M sodium acetate (pH 5.0) and 7 ul of water. Samples were vortexed, 75 ul of 95% ethanol were added and after freezing at -70 °C for one hour, samples were centrifuged at 14 000 rpm at 4 °C for 10 minutes. Supernatents were discarded, pellets were washed twice with 70% ethanol, dried, and resuspended in 7 ul water. Sequencing was performed using S-35 labelled ATP and the United States Biochemical sequencing kit Sequenase version 2.0. Samples were loaded (3 ul per well) on a 6% denaturing polyacrylamide gel and electrophoresed at 1800-2000 volts for 6-8 hours. Gels were fixed in 10% methanol/ 10% acetic acid for 20 minutes, then dried in a Bio-Rad gel dryer at 80 °C for 45 minutes. Gels were exposed directly to Kodak XAR x-ray film at room temperature for 16-24 hours.

Double-stranded sequencing using Vent (exo-) polymerase:

Sequencing was performed essentially as mentioned above, except that the denaturation step was omitted and the Circumvent sequencing kit from New England Biolabs was used.

Primer concentration was 4 pmol per labelling reaction and 20 ng of DNA was used. Gels were exposed to x-ray film for 24-72 hours.

Chapter 3. RESULTS

3.1 Differentiation of P19 Embryonal Carcinoma Cells by exposure to DMSO

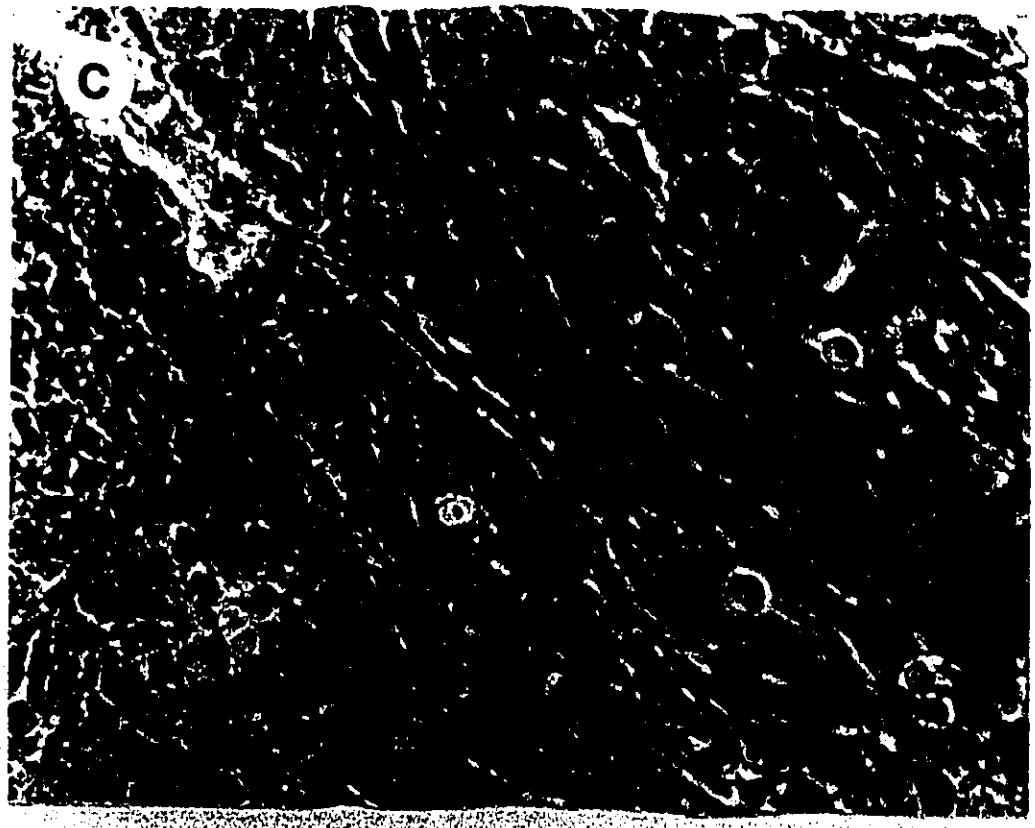
Differentiation of P19 cells with DMSO resulted in the production of several different cell types with different morphologies, as illustrated in figure 3.1. Morphology of undifferentiated P19 cells is displayed in panel A. Aggregation of P19 cells with exclusion of DMSO from the medium results in the formation of differentiated fibroblast-like cells (panel B). This indicates that aggregation alone is sufficient to induce some degree of differentiation in P19 cells. High concentrations (1%) of DMSO resulted in the production of predominantly neuronal cells with extensive processes (panel C). Lower concentrations (0.3-0.5%) of DMSO yielded cardiac muscle cells which began to spontaneously contract on day 7 (panel D, strip of cells extending across the top of the field) as well as skeletal myotubes which became morphologically evident shortly thereafter, around day 9-10 (panels E and F). Since we wished to investigate channel expression during muscle development, the presence of neurons in these cultures could potentially confuse the interpretation of our results (i.e., neurons also possess L-type calcium channels). All experiments were therefore performed with 0.3-0.5% DMSO in the medium to ensure that other excitable cell

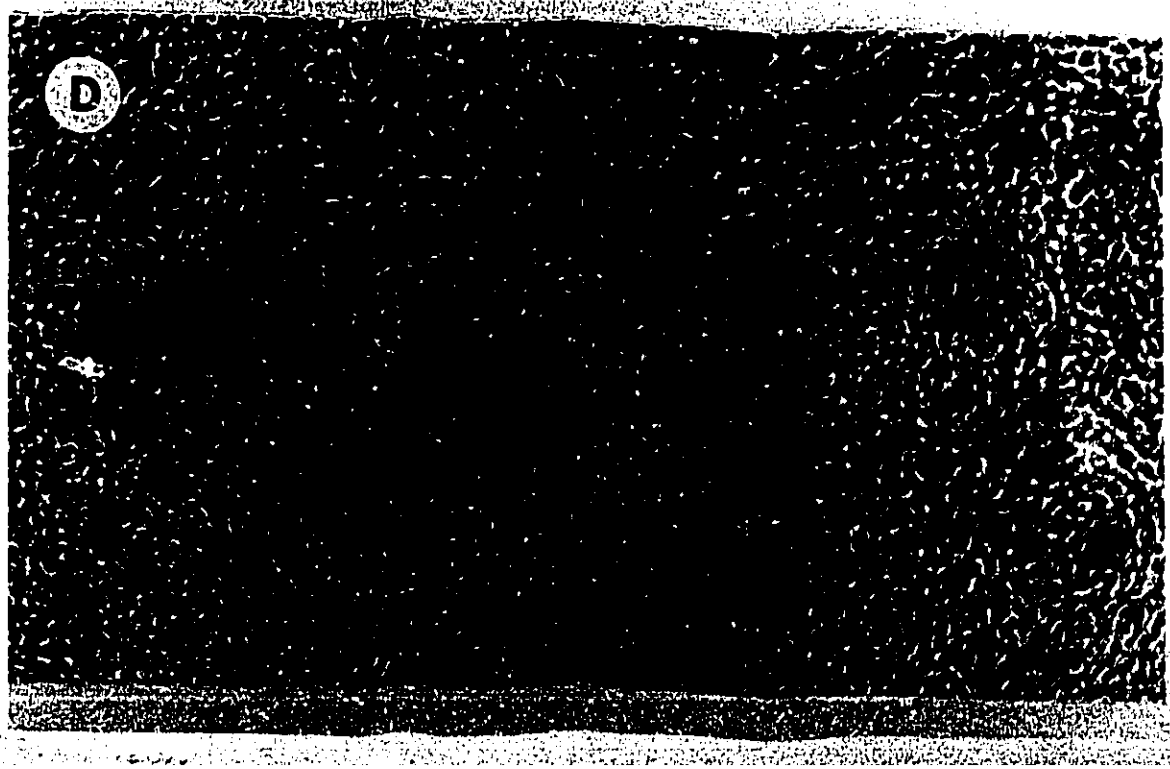
FIGURE 3.1

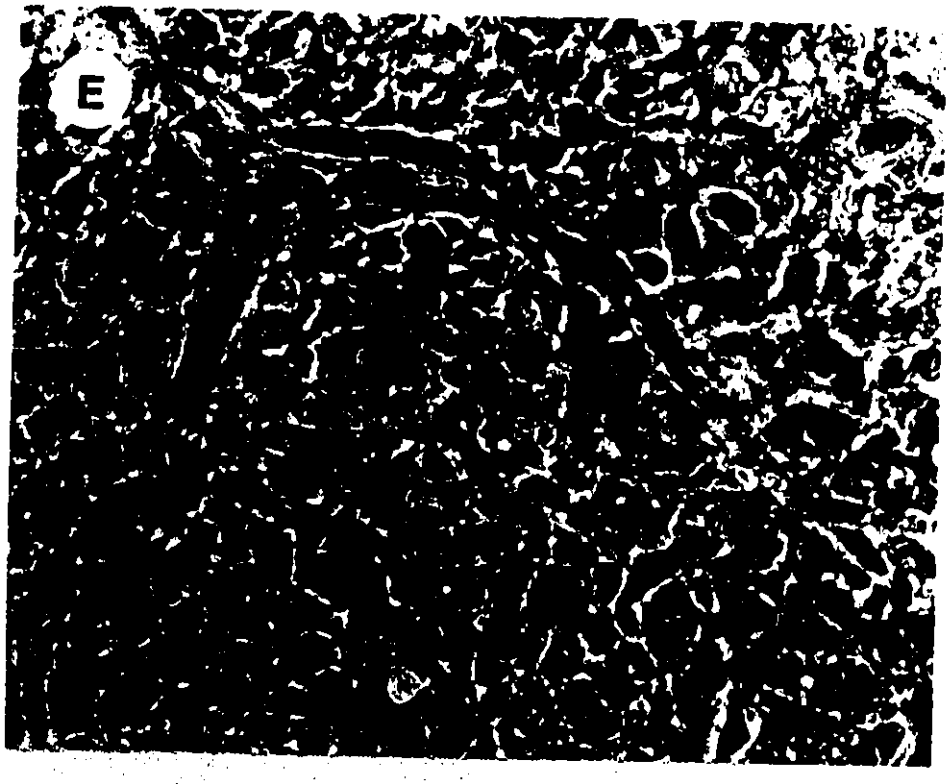
Morphology of undifferentiated and DMSO-differentiated P19 cells. Undifferentiated P19 cells (panel A); fibroblast-like cells resulting from aggregation of P19 cells in the absence of DMSO (panel B); neuronal cells on day-10, produced by treatment with 1% DMSO (panel C); cardiac cells formed on day-10 after 0.5% DMSO differentiation (panel D); skeletal myotubes fusing together after day-10, 0.5% DMSO treatment (panels E and F).

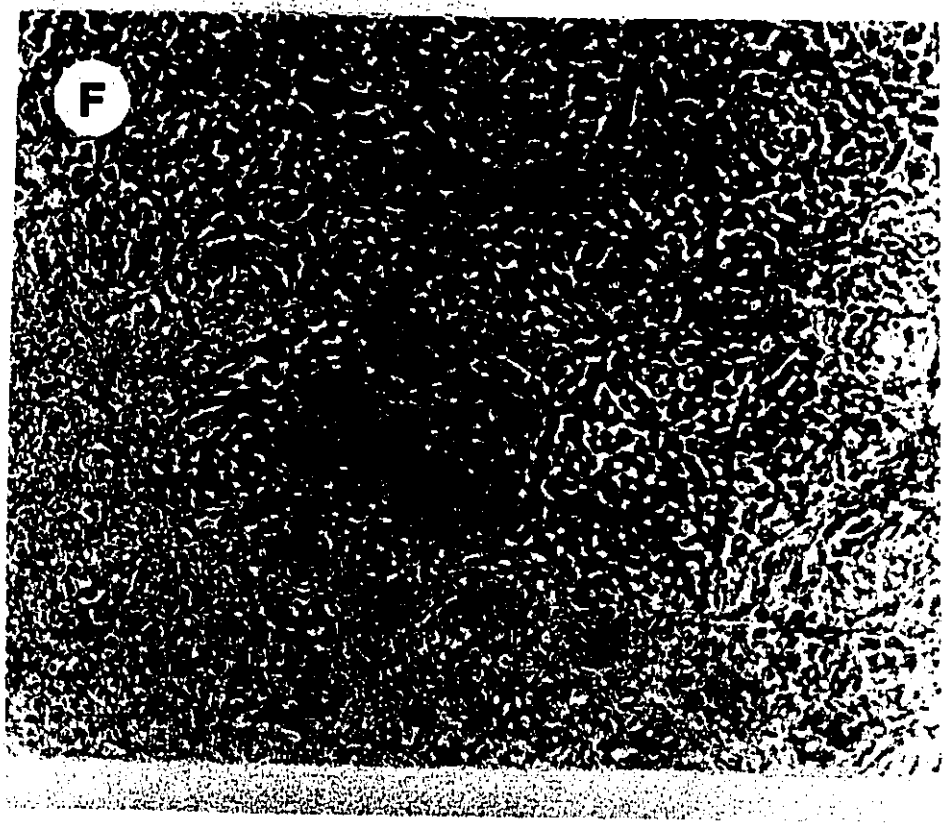

A


B


C


D


E


F

types such as neuronal cells would not be present in the differentiated cultures of muscle cells.

Since DMSO-differentiated P19 cells result in contracting cardiac muscle, they must contain functional calcium channels. When beating cells were treated with low concentrations (10-100 uM) of calcium channel blocking drugs (such as nifedipine) which block L-type calcium channels, all contractions ceased, suggesting the presence of L-type calcium channels in these cells (data not shown). To more closely examine Ca++ channel expression, the cDNA clones for the various subunits of the L-type calcium channel were required. These cDNA clones were unavailable to us; therefore, the subunits were cloned using information from published sequences and employing the technique of PCR to amplify fragments of the clones for use as probes.

3.2 Expression of the Cardiac α1 Subunit of the L-type Calcium Channel in P19 Cells

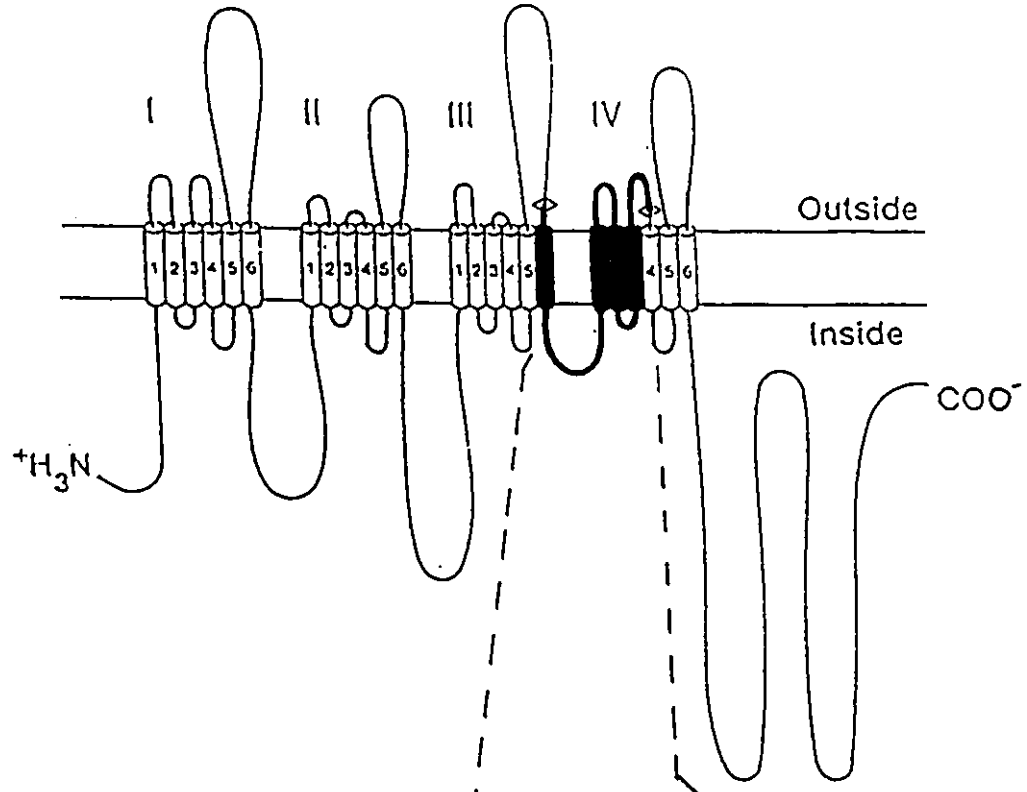
In order to determine whether the cardiac calcium channel α1 subunit was expressed in P19 cells, a reverse transcriptase-polymerase chain (RT-PCR) reaction using primers specific to the α1 subunit from adult rabbit cardiac muscle was performed. PCR is a powerful technique which allows the generation of

large amounts of a chosen target sequence of DNA from very little starting material. Using the enzyme reverse transcriptase to copy cellular RNA into DNA templates, PCR can then be used to detect the expression of rare mRNAs. PCR oligonucleotide primers were designed to amplify a 549bp fragment of the rabbit cardiac α1 subunit (see figure 3.2). From the time course in figure 3.3 it can be seen that a band of the appropriate size is visible in DMSO-differentiated P19 cells from day 0 to day 12, as well as in the adult rabbit heart positive control. This indicates that the cardiac α1 subunit is expressed at the mRNA level in undifferentiated, as well as DMSO-differentiated P19 cells. In order to determine whether the ubiquitous presence of the PCR product was due to cross-over contamination from sample to sample, a negative water control was inserted between treatment of each sample (see figure 3.3). The negative controls remained blank, thus ruling out the possibility of sample contamination.

To ascertain whether the PCR product was actually the correct amplification product and not merely a non-specific amplification product of the same size as the positive control, a PCR-Southern blot was performed. This involves transfer of the PCR products to nylon membranes, which are subsequently probed with the cardiac specific α1 probe. To construct the cardiac α1 subunit probe, the adult rabbit heart

FIGURE 3.2

PCR-amplified region of the cardiac α1 subunit of the L-type calcium channel. Oligonucleotide primers were designed to amplify a 549 bp fragment, from 3695 bp to 4244 bp, of the cardiac α1 subunit from adult rabbit heart. A schematic representation of the α1 polypeptide secondary structure is shown, and the region corresponding to the amplified nucleotide sequence is indicated. Primer sequences are shown in boxed areas.




```
3661 CCCACACGGAAGACAAGGGCCCTATCTACAACTACCGAGTGGAGATCTCCATCTTCTTCA
3721 TCATCTACATCATCATCATCGCCTTCTTCATGATGAACATCTTCGTGGGTTTCGTCATTG
3781 TCACCTTCCAGGAGCAGGGGGAGCAGGAGTACAAGAACTGTGAGCTGGACAAGAACCAGC
3841 GGCAGTGCGTGGAATATGCCCTCAAGGCCCGGCCCCTGCGGAGGTACATCCCCAAGAACC
3901 AGCACCAGTACAAAGTGTGGTACGTGGTCAACTCCACCTACTTTGAGTACCTGATGTTCG
3961 TCCTCATCCTGCTCAACACCATCTGCTTGGCCATGCAGCACTACGGCCAGAGCTGCCTGT
4021 TCAAAATCGCCATGAACATCCTCAACATGCTCTTCACCGGCCTCTTCACCGTGGAAATGA
4081 TCCTGAAGCTCATTGCCTTCAAACCCAAGGGTTACTTTAGTGATCCCTGGAATGTTTTTG
4141 ACTTCCTCATCGTAATTGGCAGCATAATTGACGTCATTCTCAGTGAGACTAATCCAGCTG
4201 AACATACCCAATGCTCTCCCTCTATGAACGCAGAGGAGAACTCCGCATCTCCATCACCT
```

FIGURE 3.3

Time course of cardiac α1 subunit expression as determined by Reverse Transcriptase-Polymerase Chain Reaction using primers specific to the α1 subunit of the L-type Ca channel from adult rabbit cardiac muscle. Amplification products in DMSO-differentiated P19 cells at days indicated by numbers above each lane; alternating lanes containing primers with no template (negative controls). Lambda HindIII marker in lane "M", and adult cardiac muscle (positive control) are indicated.

positive control PCR product was subcloned into a bacterial expression vector. The PCR product was then sequenced to ensure that it coded for the correct DNA. (NOTE: All subunit probes employed in these studies were constructed and verified in this manner.) If the PCR products are non-specific then they should not hybridize to the radioactive cardiac α1 probe under high-stringency conditions. The results of this experiment are depicted in figure 3.4. It can be seen that the α1 cardiac-specific probe does indeed recognize the P19 cell PCR amplification products, confirming that they are specific (bottom panel). In addition, PCR products of larger sizes were detected on the PCR-Southern blot in figure 3.4, both in the P19 samples and in the adult cardiac positive control lane, which were not visible by ethidium bromide staining of the gel (top panel).

To further study the nature of the transcript that encodes the α1 cardiac subunit in P19 cells, the RNA was analyzed by Northern blot analysis (figure 3.5). Northern blot analysis indicated the presence of a transcript of 9 kb and 16 kb in the adult rabbit heart positive control (lane "C"). In P19 cells, a 9kb transcript is present in day-7 DMSO-treated cells; this corresponds to the day when P19 cells begin to spontaneously contract. Before day 7, differentiated P19 cells do not appear to contain enough α1 cardiac mRNA to yield a detectable signal on a Northern blot. Adult rabbit cardiac

muscle has been shown to contain two transcript sizes for the α1: a major 9kb transcript and a more minor 16kb transcript (Mikami et al., 1989). The 16kb transcript was not detected in P19 cells using Northern blot analysis, even after day 7 (lane "D-7"). The cardiac α1 probe also recognizes two transcripts in adult rabbit brain which are of slightly different size from those detected in cardiac muscle (see lane "Br"). Interestingly the cardiac probe also hybridizes with a 6.5kb transcript in adult rabbit skeletal muscle (lane "Sk"). Despite the fact that P19 cells also form skeletal myotubes when treated with DMSO ((Rudnicki et al., 1990; Edwards et al., 1983), also see figure 3.1 panels E and F), this 6.5 kb transcript is undetectable in P19 cells.

3.3 Expression of the Skeletal α1 Subunit of the L-type Calcium Channel

Since P19 cells also differentiate into skeletal muscle, we wanted to investigate whether the α1 subunit corresponding to the skeletal muscle isoform was expressed. RT-PCR using primers specific to the skeletal α1 subunit (see figure 3.6) was thus performed. Figure 3.7 shows the results of an RT-PCR experiment using primers specific to the α1 subunit from adult rabbit skeletal muscle. The targeted sequence of 642 bp (see figure 3.6) was amplified in the skeletal muscle positive

FIGURE 3.4

PCR-Southern blot analysis of cardiac α1 subunit expression in P19 cells. PCR products were electrophoresed on an agarose gel (top panel) and then transferred to a nylon membrane, which was probed with a riboprobe (cRNA) to the adult α1 subunit (bottom panel). Exposure was for 30 minutes at -80 C between regular intensifying screens. P19 samples differentiated with DMSO at days indicated above each lane are shown. Markers (lane "m") and positive controls (adult rabbit cardiac muscle, lane "Card."; embryonic mouse heart, lane "Emb.") are also shown.

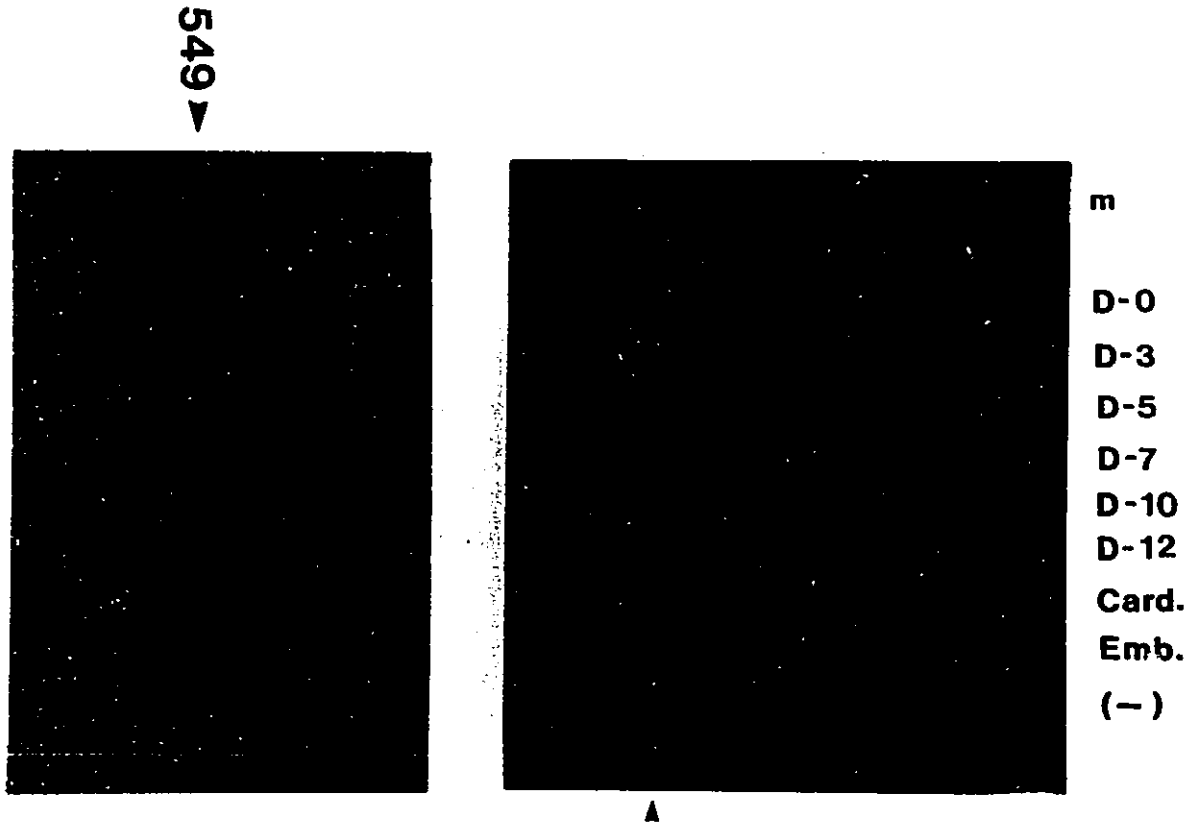

549
m
D-0
D-3
D-5
D-7
D-10
D-12
Card.
Emb.
(–)

FIGURE 3.5

Northern blot analysis of cardiac α1 mRNA in P19 cells. Hybridization of α1 subunit cRNA (riboprobe) to 20 ug total RNA from adult tissues (cardiac and skeletal muscle, brain) and DMSO-differentiated P19 cells at days indicated by numbers above each lane. The inset panel (bottom panel) demonstrates the comparative hybridization of tubulin cDNA to each lane. The 28S ribosomal RNA is indicated.

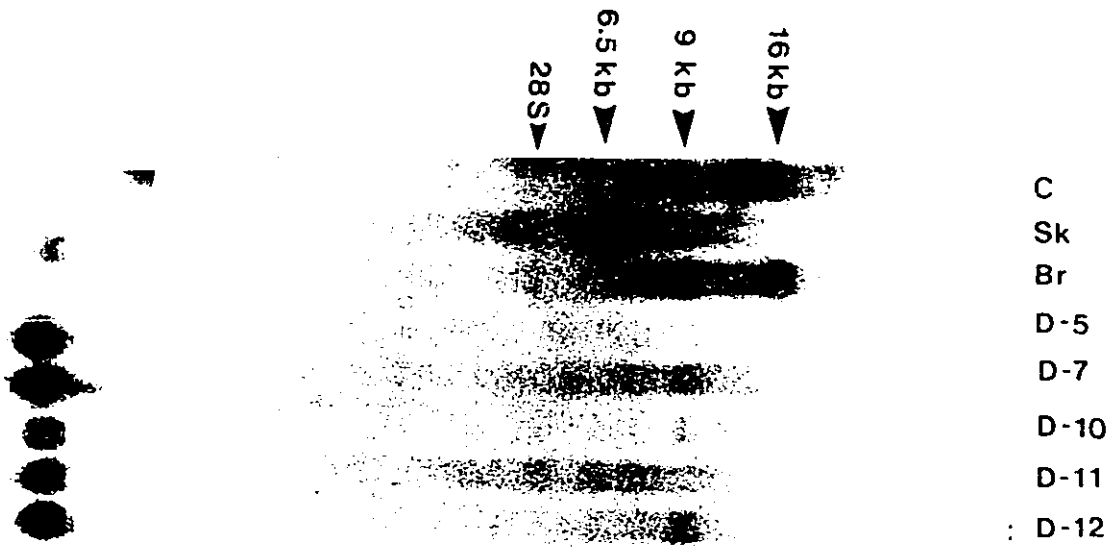
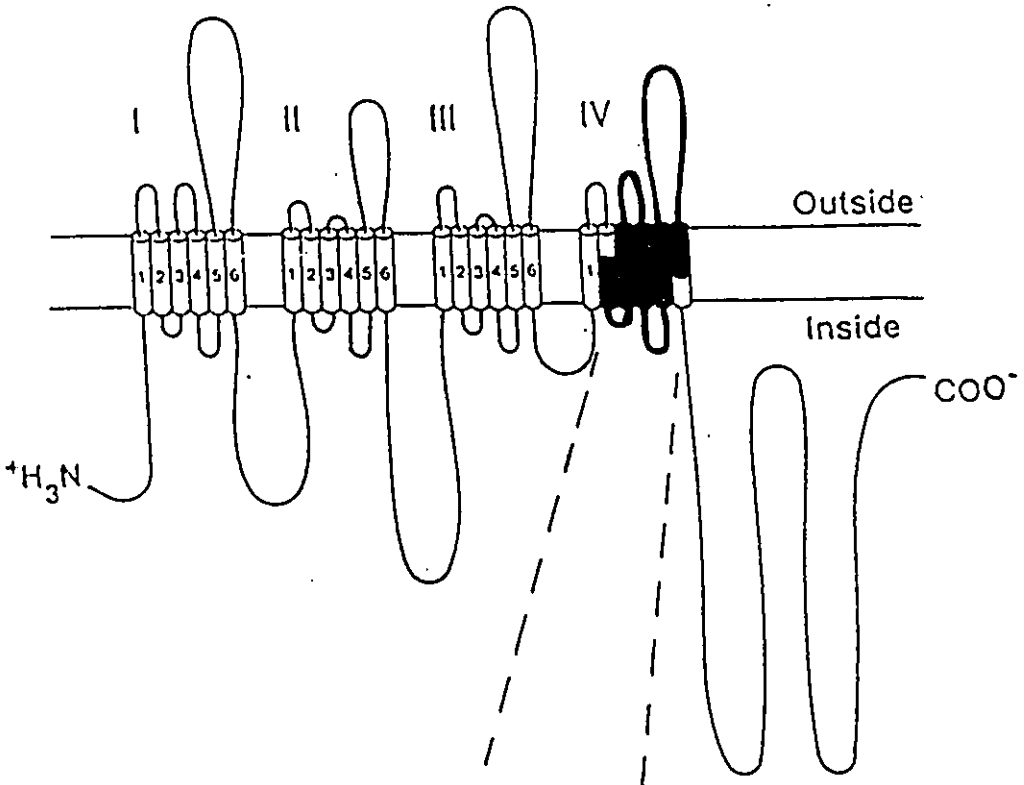

28S
6.5 kb
9 kb
16 kb
C
Sk
Br
D-5
D-7
D-10
D-11
D-12

FIGURE 3.6

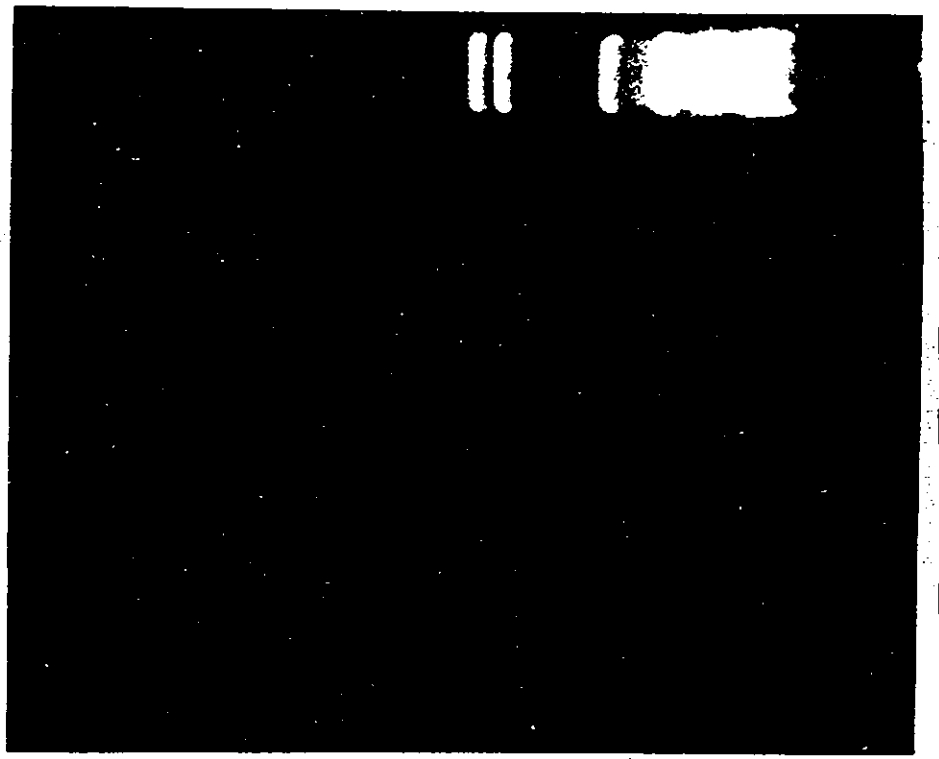
PCR-amplified region of the skeletal α1 subunit of the L-type calcium channel. Oligonucleotide primers were designed to amplify a 642 bp fragment, from 3707 bp to 4349 bp, of the skeletal α1 subunit from adult rabbit skeletal muscle. A schematic representation of the α1 polypeptide secondary structure is shown, and the region corresponding to the amplified nucleotide sequence is indicated. Primer sequences are shown in boxed areas.




```
3701 TCATCTTCACACTGGAGATGATCCTCAAGCTCTTGGCGTTCAAGGCCAGG
3751 GGCTATTTCGGAGACCCCTGGAATGTGTTCGACTTCCTGATCGTCATCGG
3801 CAGCATCATTGACGTCATCCTCAGCGAGATCGACACTTTCCTGGCCTCCA
3851 GCGGGGGACTGTATTGCCTGGGTGGCGGCTGCGGGAACGTTGACCCAGAC
3901 GAGAGCGCCCGCATCTCCAGTGCCTTCTTCCGCCTGTTCCGGGTCATGAG
3951 GCTGATCAAGCTGCTGAGTCGGGCCGAGGGCGTGCGCACGCTGCTGTGGA
4001 CGTTCATCAAGTCCTTCCAGGCCCTGCCCTACGTGGCCCTGCTCATCGTC
4051 ATGCTGTTCTTCATCTACGCCGTCATCGGCATGCAGATGTTTGGAAAGAT
4101 CGCCCTGGTGGACGGGACCCAGATCAACCGCAACAACAACTTCCAGACCT
4151 TCCCGCAGGCCGTGCTGCTGCTCTTCAGGTGTGCGACAGGGGAGGCGTGG
4201 CAAGAGATCCTGCTGGCCTGCAGCTACGGGAAGTTGTGCGACCCAGAGTC
4251 AGACTACGCCCCGGGCGAGGAGTACACGTGTGGCACCAACTTCGCCTACT
4301 ACTACTTCATCAGCTTCTACATGCTCTGCGCCTTCCTGATCATCAACCTC
```

FIGURE 3.7

Reverse Transcriptase-Polymerase Chain Reaction using primers specific to the α1 subunit of the L-type Ca channel from adult rabbit skeletal muscle. Lane 1: lambda HindIII marker, lane 2: adult rabbit cardiac muscle, lane 3: adult rabbit skeletal muscle (positive control), lane 4: day 10 DMSO-differentiated P19 cells, lane 5: D310 cells (P19 derivative that does not form muscle), lane 6: day 0 (undifferentiated) P19 cells, lane 7: F9 embryonal carcinoma cells, lane 8: primers with no template (negative control). Expected size of α1 adult skeletal muscle PCR product is indicated by the arrow.


Cardiac
Skeletal
D-10
D310
D-0
F9
(–)

control but not in cardiac muscle, nor in the embryonal carcinoma cell lines D310, F9, nor in differentiated P19 cells (lane D-10). The P19 and other cell lines, however, did contain amplified products of different sizes. To investigate whether these products represent some novel type of calcium channel homologous to the skeletal L-type calcium channel, the adult rabbit skeletal muscle α1 subunit fragment was subcloned and used as a probe on a PCR-Southern blot. It can be seen in figure 3.8 that the skeletal α1 subunit probe hybridizes to the skeletal muscle positive control but no signal is visible in any of the other lanes, P19 or otherwise. This suggests that not only is the adult skeletal isoform of the α1 subunit undetectable in differentiated P19 cells, but the other PCR products are probably due to nonspecific amplification.

Using the same skeletal α1 probe as for the Southern, Northern blots of P19 cells differentiated with DMSO show the presence of two transcripts, a large 13.5kb and a smaller 6.5kb transcript, as early as day 3 (figure 3.9). The 13.5kb transcript is absent in adult skeletal muscle and appears to be a novel transcript as it is not present in any of the other excitable cell types shown (adult heart, skeletal muscle, or brain). Furthermore, relative intensities of the two transcripts seem to indicate that the expression of the large transcript decreases with time in culture as compared to the smaller 6.5kb transcript. The 6.5 kb transcripts are found in

FIGURE 3.8

PCR-Southern blot analysis of skeletal α1 subunit expression in P19 cells. PCR products were electrophoresed on an agarose gel (see figure 3.7) and then transferred to a nylon membrane, which was probed with a riboprobe (cRNA) to the adult α1 subunit. Exposure was for 30 minutes at -80 C between regular intensifying screens. Lane 1: adult rabbit cardiac muscle, lane 2: adult rabbit skeletal muscle (positive control), lane 3: day 10 DMSO-differentiated P19 cells, lane 4: D310 cells (P19 derivative that does not form muscle), lane 5: day 0 (undifferentiated) P19 cells, lane 6: F9 embryonal carcinoma cells, lane 7: primers with no template (negative control)

C
Sk
d-10
D_{310}
d-0
F9
(−)

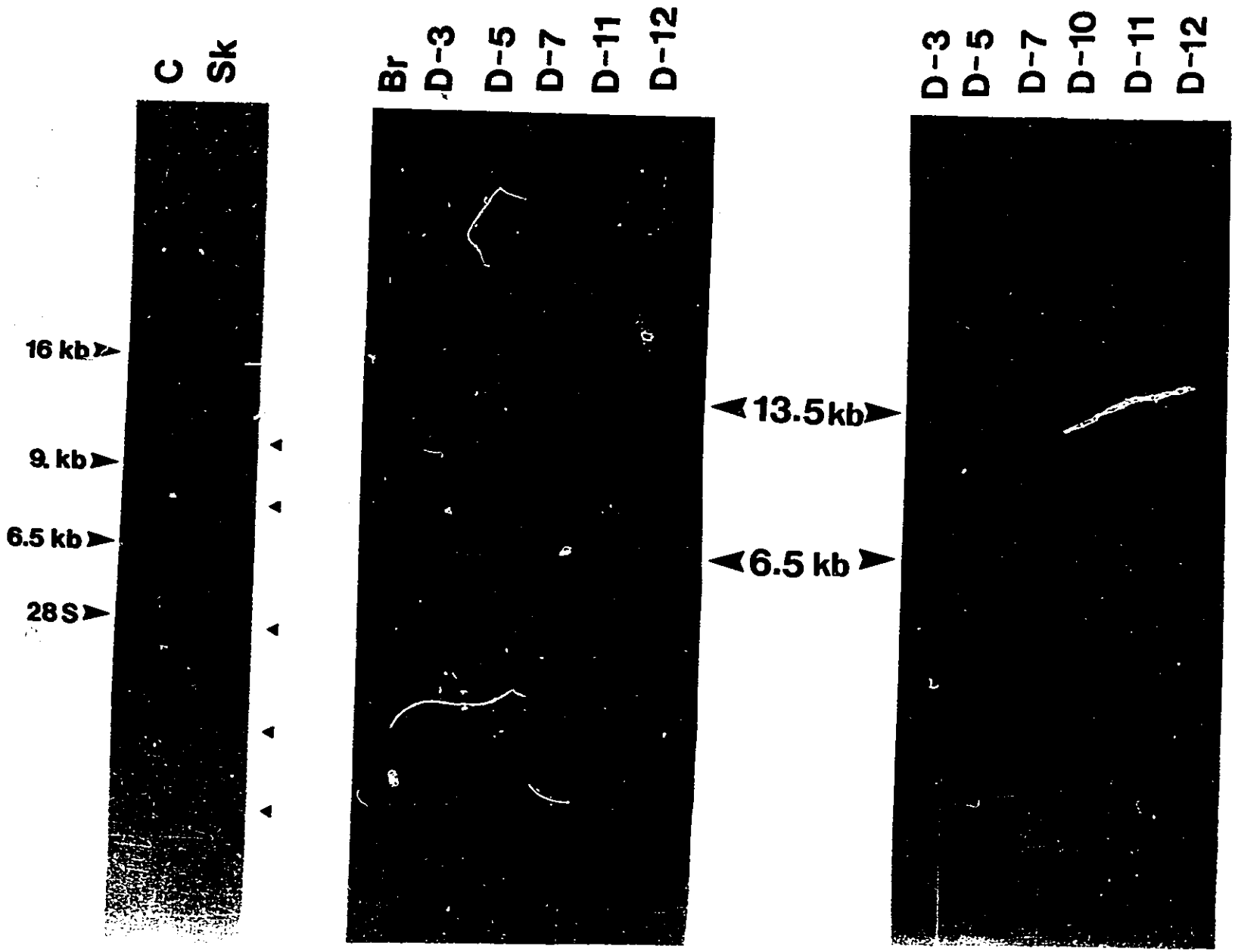
642 bp ➤

FIGURE 3.9

Northern blot analysis of skeletal α1 mRNA in P19 cells. Northern blots using 20 ug total RNA/lane and a riboprobe constructed from the skeletal Ca channel alpha-1 subunit-amplified PCR product. * Lane 1: adult rabbit cardiac muscle, lane 2: adult rabbit skeletal muscle, ** lane 3: adult rabbit brain, lanes 4 & 9: day 3 DMSO-differentiated P19 cells, lanes 5 & 10: day 5 DMSO-differentiated P19 cells, lanes 6 & 11: day 7 DMSO-differentiated P19 cells, lanes 7 & 13: day 11 DMSO-differentiated P19 cells, lanes 8 & 14: day 12 DMSO-differentiated P19 cells, lane 12: day 10 DMSO-differentiated P19 cells.

* 2-day exposure, lanes 1 & 2

** 5-day exposure, lanes 3 through 14


C
Sk
16 kb
9. kb
6.5 kb
28S
Br
D-3
D-5
D-7
D-11
D-12
13.5kb
6.5 kb
D-3
D-5
D-7
D-10
D-11
D-12

much lower abundance in P19 cells than those seen in adult skeletal and cardiac muscle; the adult tissue samples were exposed for less than 48 hours (left panel), whereas the P19 samples (middle and right panel) were exposed to x-ray film for over a week. In contrast to the cardiac α1 riboprobe (figure 3.5), the skeletal α1 riboprobe appears to have very little homology to the brain isoform of the L-type calcium channel since the probe does not recognize the brain α1 subunit transcripts (see lane "Br", middle panel of figure 3.9). The skeletal α1 probe strongly hybridizes to the 6.5 kb α1 subunit mRNA in adult skeletal muscle and weakly recognizes the 9 kb and 16 kb cardiac α1 subunit transcripts (left panel). It is interesting to note that the smaller 6.5 kb transcript detected in P19 cells is the same size as the adult skeletal transcript for the α1 subunit of the L-type calcium channel, yet its absence from the PCR reaction using adult skeletal α1-specific primers would lead us to conclude that it is different from the α1 subunit found in adult skeletal muscle.

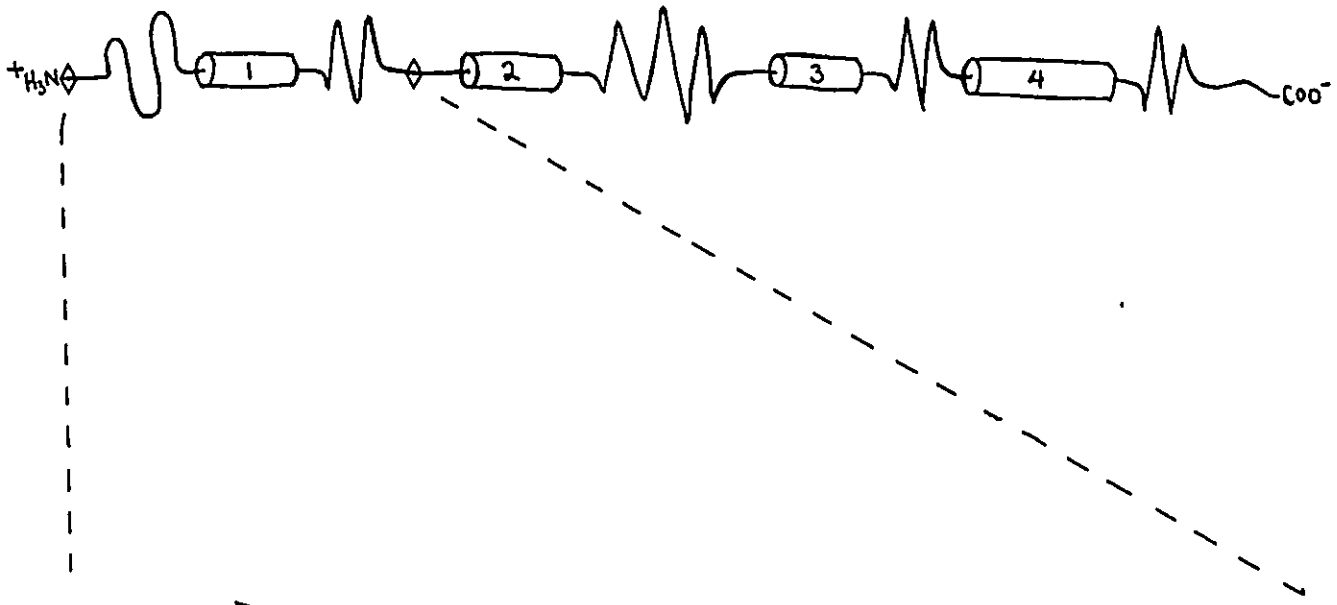
3.4 Expression of the ß Subunit of the L-type Calcium Channel in P19 Cells

Since the ß subunit of the L-type channel is believed to play an important role in the function of the α1 subunit, its

expression was investigated in P19 cells treated with DMSO. Like the skeletal isoform of the α1 subunit, the skeletal ß subunit was undetectable in differentiated P19 cells even by PCR (figure 3.11). The primers in this case were designed to amplify a 532 bp fragment of the ß subunit from skeletal muscle (figure 3.10). Northern blots probed with a riboprobe to the ß subunit yield a signal at approximately 13 kb in the P19 samples, while hybridization at approximately 1.8 kb and 6.5 kb was observed in adult skeletal muscle and a 3 kb transcript was detected in brain (figure 3.12 panel A). Riboprobes, or RNA probes, have the advantage of being more sensitive than nick-translated DNA probes. However, background noise (i.e., non-specific hybridization) tends to be higher when riboprobes are used since RNA:RNA hybrids are much more stable than are DNA:RNA hybrids and riboprobes have more tendency to bind to, for example, the 18S and 28S ribosomal RNAs (Wahl et al., 1987). Consequently, it is sometimes useful to treat Northern blots with RNase A, a ribonuclease, after hybridization to remove all nonspecific background. RNase A preferentially attacks single stranded RNA when used in low amounts, degrading excess probe and all RNA on a Northern blot which is not specifically bound to the riboprobe (Wahl et al., 1987). Any mRNA which has a high degree of homology to the riboprobe of interest will form a very tightly bound double-stranded RNA:RNA hybrid which is resistant to degradation by RNase A.

FIGURE 3.10

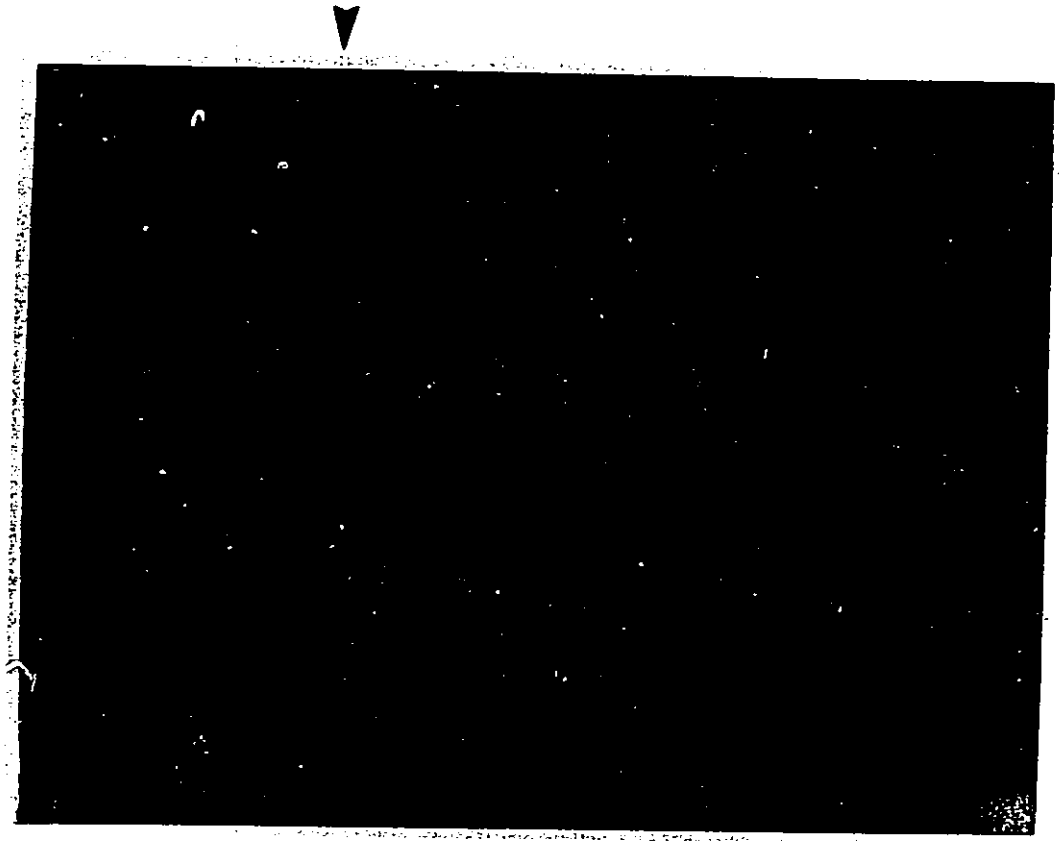
PCR-amplified region of the skeletal ß subunit of the L-type calcium channel. Oligonucleotide primers were designed to amplify a 532 bp fragment, from 153 bp to 685 bp, of the skeletal ß subunit from adult rabbit skeletal muscle. A schematic representation of the ß polypeptide secondary structure is shown, and the region corresponding to the amplified nucleotide sequence is indicated. Primer sequences are shown in boxed areas.




```
151 CCTCCATGGTCCAGAAGACCAGCATGTCCCGGGGTCCTTACCCACCCTCC
201 CAGGAGATCCCCATGGAGGTCTTCGACCCCAGCCCGCAGGGCAAATACAG
251 CAAGAGAAAAGGGCGCTTCAAACGGTCCGACGGGAGCACCTCCTCAGATA
301 CAACATCCAACAGCTTTGTGCGCCAGGGCTCTGCCGAGTCCTACACCAGC
351 CGTCCGTCGGACTCTGATGTCTCCCTGGAGGAGGACCGGGAAGCCTTAAG
401 GAAGGAGGCAGAGCGCCAGGCATTAGCCCAGCTTGAGAAAGCCAAGACCA
451 AGCCAGTAGCATTTGCCGTGCGGACAAATGTCGGCTACAATCCATCTCCA
501 GGGGATGAGGTGCCTGTGGAGGGAGTGGCCATCACCTTTGAGCCCAAGGA
551 CTTCCTGCACATCAAGGAGAAATACAACAATGACTGGTGGATCGGGCGGC
601 TGGTGAAGGAGGGCTGCGAGGTTGGCTTCATCCCCAGCCCCGTCAAACTG
651 GACAGCCTGCGCCTGCTGCAGGAACAGAAGCTGCGTCAGAGCCGCCTCAG
```

FIGURE 3.11

Reverse Transcriptase-Polymerase Chain Reaction using primers specific to the ß subunit of the L-type Ca channel from adult rabbit skeletal muscle. Lane 1: lambda HindIII marker, lane 2: adult rabbit skeletal muscle (positive control), lanes 3 & 4: adult rabbit cardiac muscle, lanes 5 & 6: day 10 DMSO-differentiated P19 cells, lanes 7 & 8: day 0 (undifferentiated) P19 cells, lane 9: day 10 RA-differentiated P19 cells, lane 10: primers with no template (negative control)



Skeletal

Card.

Card.

D−10

D−10

D−0

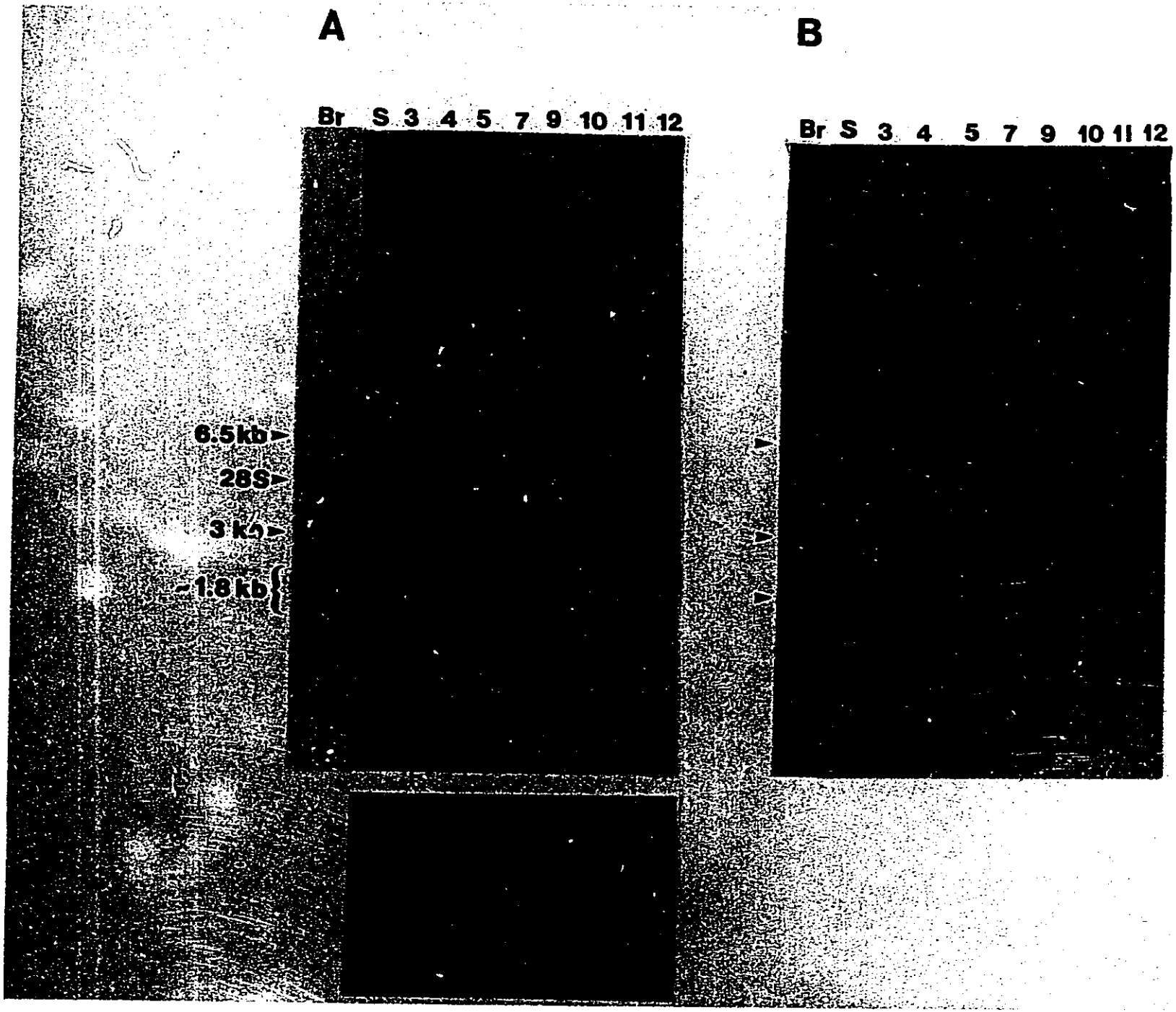
D−0

R−10

(—)

FIGURE 3.12

Northern blot analysis of ß subunit expression in DMSO-treated P19 cells. Hybridization of ß subunit cRNA (riboprobe) to 20 ug total RNA from adult tissues (skeletal muscle, lane "S" and brain, lane "Br") and DMSO-differentiated P19 cells at days indicated by numbers above each lane. Panel A indicates probe hybridization before RNase treatment; panel B illustrates hybridization after treatment with RNase. The inset panel (bottom panel) demonstrates the comparative hybridization of tubulin cDNA to each lane.


A
Br S 3 4 5 7 9 10 11 12
6.5kb
28S
3 kb
~1.8 kb
B
Br S 3 4 5 7 9 10 11 12

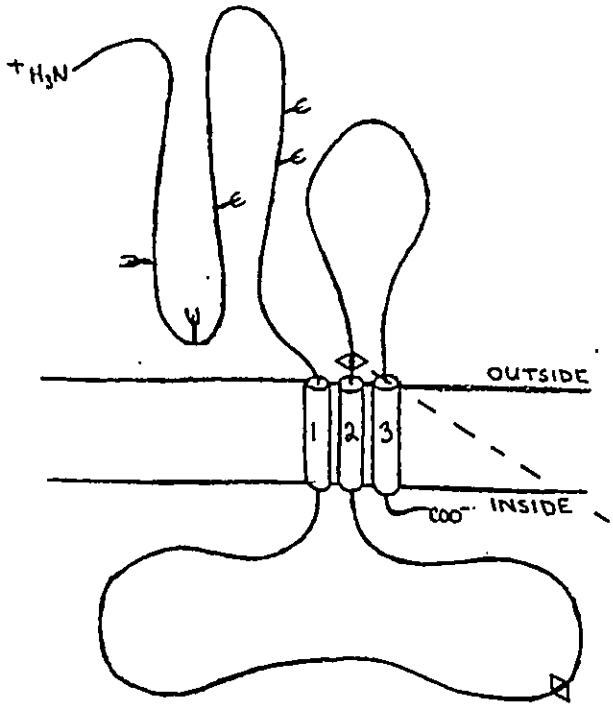
When the Northern blot in figure 3.12 is treated with RNase the hybridization signal visible at 13 kb is removed but the signals seen in adult tissues i.e., the 1.8 kb and 6.5 kb transcripts in skeletal muscle and the 3 kb transcript in brain remain unaffected (figure 3.12 panel B). This implies that the binding seen in the P19 lanes (figure 3.12, panel A) is non-specific. However, when a ß subunit riboprobe of the incorrect orientation was used as a probe under identical conditions on a duplicate Northern blot it did not bind to the same regions of the blot (data not shown). Presumably, if the binding seen in figure 3.12A were truly due to spurious, nonspecific hybridization, one would expect a probe of the opposite orientation to bind nonspecifically to this region, too. The signal seen at 13 kb may thus represent an mRNA species possessing a very low degree of homology to the skeletal ß subunit transcript.

3.5 Expression of the α2 subunit of the L-type Calcium Channel in P19 Cells

Since the α2 subunit of the L-type channel is thought to serve a functional role, its expression in P19 cells was examined. Primers specific to the α2 subunit sequence from adult skeletal and cardiac muscle were designed to amplify a 505 bp piece of DNA using PCR (see figure 3.13). The results from

FIGURE 3.13

PCR-amplified region of the α2 subunit of the L-type calcium channel. Oligonucleotide primers were designed to amplify a 505 bp fragment, from 2675 bp to 3180 bp, of the skeletal α2 subunit from adult rabbit skeletal muscle. A schematic representation of the α2 polypeptide secondary structure is shown, and the region corresponding to the amplified nucleotide sequence is indicated. Primer sequences are shown in boxed areas.




```
2651 CTACTTTAACAAAAGTGGACCTGGGGCCTATGAGTCAGGCATTATGGTAA
2701 GCAAAGCTGTAGAAATATATATCCAAGGAAAACTTCTTAAACCTGCAGTT
2751 GTTGGAATTAAAATTGATGTAAATTCTTGGATAGAGAATTTCACCAAAAC
2801 TTCAATCAGGGATCCGTGTGCTGGTCCAGTTTGTGACTGCAAACGAAACA
2851 GTGATGTAATGGATTGTGTGATTCTAGATGACGGTGGGTTTCTTTTGATG
2901 GCCAACCATGATGATTATACCAATCAGATTGGAAGATTCTTTGGAGAGAT
2951 TGATCCAAGCTTGATGAGACACCTGGTCAATATATCAGTTTATGCCTTTA
3001 ACAAATCTTATGATTATCAGTCGGTGTGTGAACCTGGTGCTGCGCCAAAG
3051 CAGGGAGCAGGGCACCGCTCGGCTTATGTGCCATCAATAGCAGACATACT
3101 GCAGATTGGATGGTGGGCCACTGCTGCTGCCTGGTCTATTCTTCAGCAGT
3151 TTCTGTTGAGTTTGACTTTTCCACGGCTCCTTGAGGCAGCTGATATGGAG
```

FIGURE 3.14

Time course of α2 subunit expression as determined by Reverse Transcriptase-Polymerase Chain Reaction using primers specific to the α2 subunit of the L-type Ca channel from adult rabbit skeletal muscle. Amplification products in DMSO-differentiated P19 cells at days indicated by numbers above each lane; alternating lanes containing primers with no template (negative controls). Lambda HindIII marker and adult cardiac muscle (lane "C", positive control) is shown.

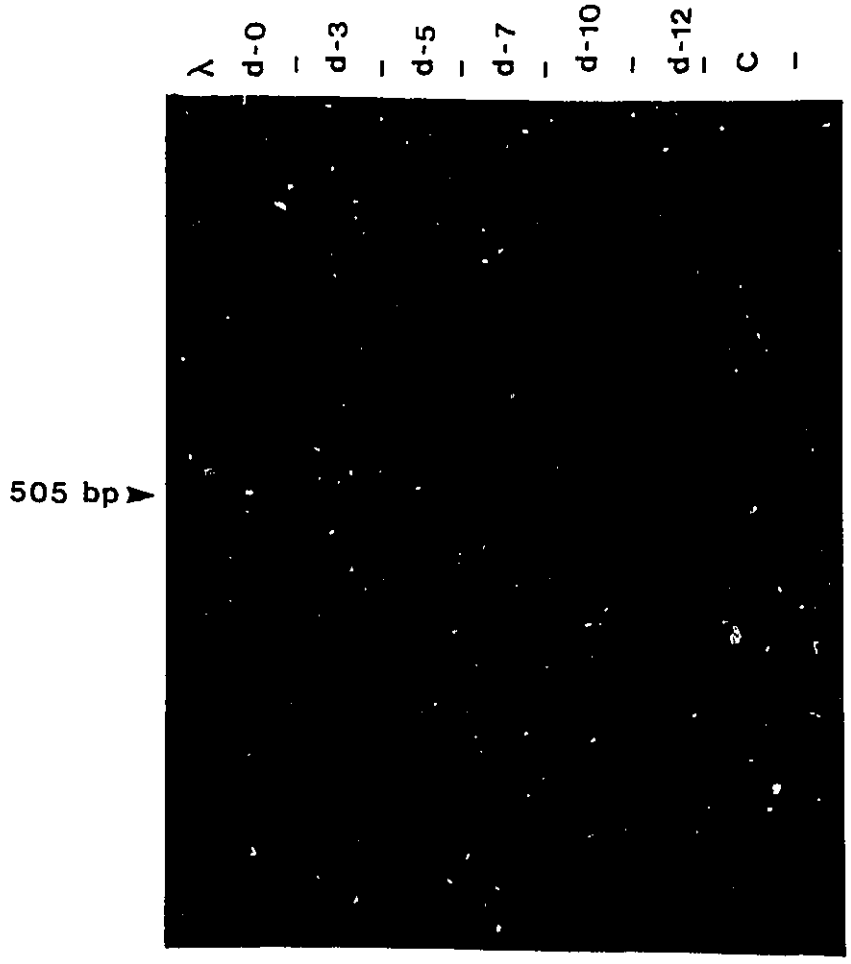
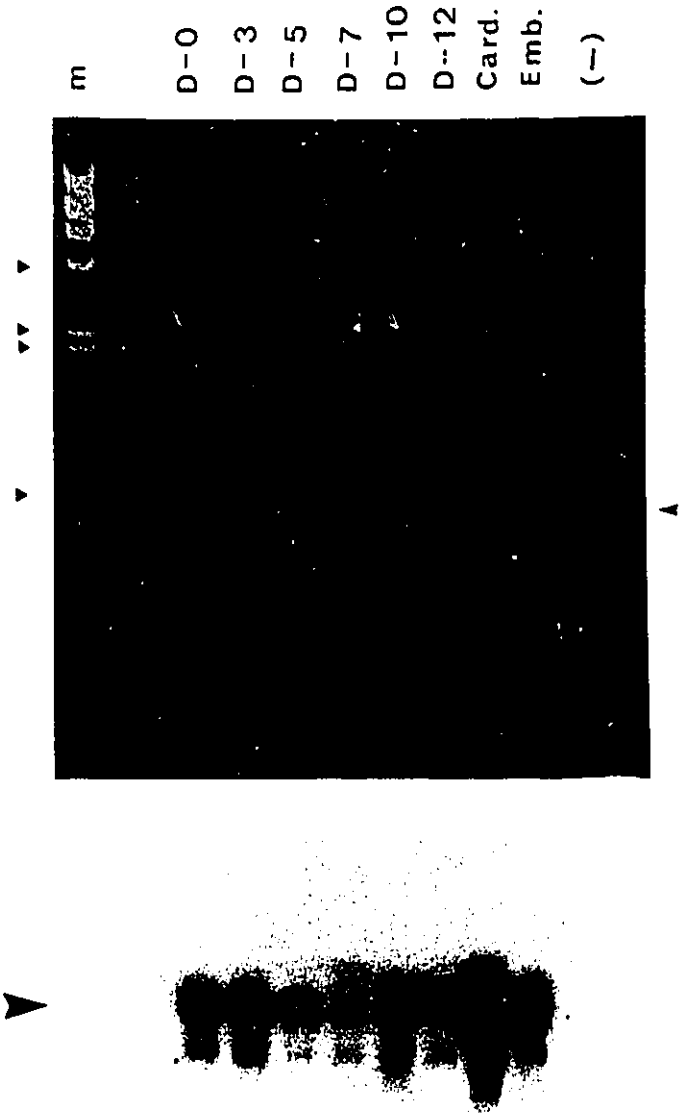

λ
d-0
d-3
d-5
d-7
d-10
d-12
C
505 bp

FIGURE 3.15

PCR-Southern blot analysis of cardiac α2 subunit expression in P19 cells. PCR products were electrophoresed on an agarose gel (top panel) and then transferred to a nylon membrane, which was probed with a riboprobe (cRNA) to the adult α2 subunit (bottom panel). Exposure was for 2 hours at -80 C between regular intensifying screens. P19 samples differentiated with DMSO at days indicated above each lane are shown. Markers (lane "m") and positive controls (adult rabbit cardiac muscle, lane "Card."; embryonic mouse heart, lane "Emb.") are also shown.


m
D-0
D-3
D-5
D-7
D-10
D-12
Card.
Emb.
(-)
505

these experiments, illustrated in figure 3.14, demonstrate that the α2 subunit is present from day 0 to day 12 in DMSO-differentiated P19 cells, as well as in adult rabbit cardiac muscle (lane "C"). A PCR-Southern blot, shown in figure 3.15, affirms the specificity of the amplified product; the radioactive probe of the adult α2 subunit hybridizes to the 505 bp DNA fragments amplified from P19 cells. An additional band of smaller size also hybridizes to the α2 radioactive probe, both in P19 cells, in embryonic mouse heart (bottom panel, lane "Emb.") and in adult cardiac muscle (bottom panel, lane "Card."). However, Northern blot analysis using the same riboprobe yields no detectable signal in P19 cells after the nylon membrane has been treated with RNase to remove non-specific binding of the probe (figure 3.16). The positive control lanes containing adult mRNA samples from rabbit heart and skeletal muscle still display the appropriate sized transcripts (9 kb) after RNase treatment (see lanes "SK" and "C", figure 3.16) This suggests that P19 embryonic muscle cells contain very low mRNA levels of the (adult) α2 subunit of the L-type calcium channel which are below detectable levels on a Northern blot.

3.6 Expression of the Skeletal Gamma Subunit of the L-type Calcium Channel

The fourth subunit of the L-type calcium channel has also been shown to play a significant role in channel function. The

FIGURE 3.16

Northern blot analysis of α2 subunit mRNA in P19 cells. Hybridization of α2 subunit cRNA to 20 ug total RNA from adult tissues and DMSO-differentiated P19 cells at days indicated by numbers above each lane after RNase treatment. Positive controls (adult skeletal muscle, lane "SK"; adult heart, lane "C"; neonate heart, lane "N") are indicated. The inset (bottom) panel illustrates the comparative hybridization of tubulin cDNA to each lane.

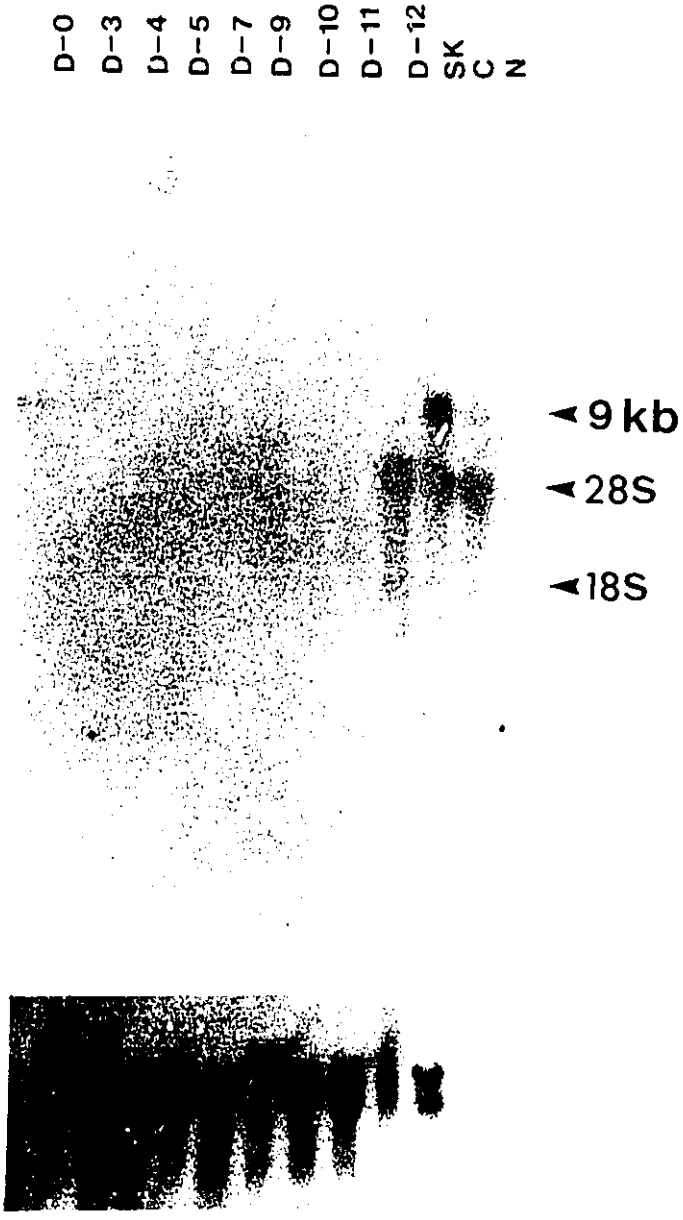
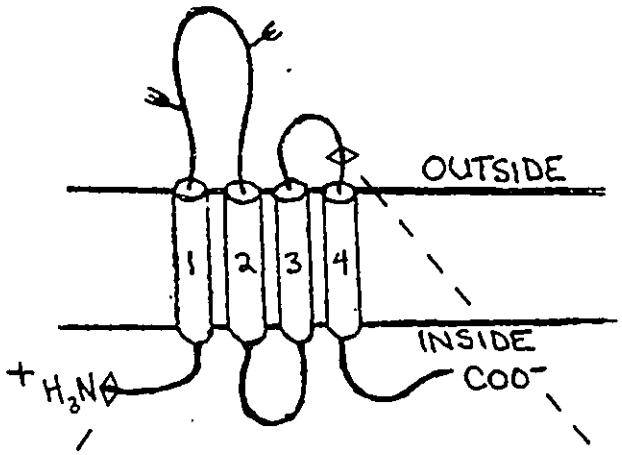

D-0
D-3
D-4
D-5
D-7
D-9
D-10
D-11
D-12
SK
C
N
9kb
28S
18S

FIGURE 3.17

PCR-amplified region of the gamma subunit of the L-type calcium channel from skeletal muscle. Oligonucleotide primers were designed to amplify a 557 bp fragment, from 22 bp to 579 bp, of the gamma subunit from adult rabbit skeletal muscle. A schematic representation of the gamma polypeptide secondary structure is shown, and the region corresponding to the amplified nucleotide sequence is indicated. Primer sequences are shown in boxed areas.




```
  1 GCGCCGCCGCCAGACCCTACCTGGAGCACCCACCCCTCTGCAGCCGCCAT
 51 GTCCCCGACGGAAGCCCCAAAGGTCCGCGTGACCCTCTTCTGCATCCTGG
101 TGGGCATCGTGCTGGCCATGACGGCCGTGGTGAGCGACCACTGGGCCGTG
151 CTGAGCCCCCACATGGAGAACCACAACACCACCTGCGAGGCCGCCCACTT
201 CGGCCTGTGGCGGATTTGCACCAAGCGCATCGCCCTGGGCGAGGACAGGA
251 GCTGCGGACCCATCACCCTGCCTGGGGAGAAGAACTGCTCCTACTTCCGG
301 CATTTTAACCCAGGCGAGAGCTCGGAGATCTTCGAATTCACCACGCAGAA
351 GGAGTACAGCATCTCGGCGGCCGCCATCAGCGTCTTCAGCCTGGGCTTCC
401 TCATCATGGGCACCATCTGCGCGCTCATGGCCTTCAGGAAGAAGCGGGAT
451 TACCTGCTGCGGCCGGCGTCCATGTTCTACGTCTTTGCAGGCCTCTGCCT
501 CTTCGTGTCACTGGAGGTAATGCGGCAGTCGGTGAAACGCATGATCGACA
551 GCGAGGACACCGTCTGGATCGAGTACTATTACTCCTGGTCCTTTGCCTGC
```

presence of the gamma subunit was examined in DMSO-differentiated P19 cells using RT-PCR. Primers were chosen that would amplify a 557 bp fragment of the gamma DNA sequence using PCR (see figure 3.17). The results of this experiment are shown in figure 3.18. It can be seen that only the positive control lane containing the adult rabbit skeletal muscle sample yielded any signal, insinuating that the gamma subunit is absent in P19 cells.

FIGURE 3.18

Reverse Transcriptase-Polymerase Chain Reaction using primers specific to the gamma subunit of the L-type Ca channel from adult rabbit skeletal muscle. Lane 1: lambda HindIII marker, lane 2: primers with no template (negative control) lane 3: F9 embryonal carcinoma cells, lane 4: day 0 (undifferentiated) P19 cells, lane 5: day 9 DMSO-differentiated P19 cells, lane 6: day 10 RA-differentiated P19 cells, lane 7: adult rabbit cardiac muscle, lane 8: adult rabbit skeletal muscle (positive control)

(−)

F9

D-0

D-9

R-10

Card.

Sk.

Chapter 4. DISCUSSION

The L-type calcium channel is a major determinant of E-C coupling in cardiac muscle cells. It has been well documented that in immature cardiac muscle cells, the E-C coupling is entirely dependent on extracellular calcium, while as the cells age, intracellular calcium stores become the main determinant of the calcium required for contraction (Fabiato and Fabiato, 1978). We have been interested in the question of whether a unique calcium channel is expressed in embryonic cardiac muscle cells, and whether this channel exhibits developmental regulation. Since it is difficult to study cardiac muscle cell development in culture I first investigated whether the P19 system could be employed to study cardiac muscle cell development in culture.

Previous studies have shown that P19 cells induced to differentiate with DMSO display spontaneous contractile activity and express a number of muscle-specific gene products (Rudnicki et al., 1990; Edwards et al., 1983). In addition, DMSO-treated P19 cells possess Z-bands and junctional complexes morphologically similar to intercalated disks found in cardiac muscle, as determined by electron microscopy (Smith et al., 1987). Further studies into the nature of the cell types produced by DMSO treatment have revealed that P19 cells form predominantly embryonic cardiac muscle under these

conditions (Rudnicki et al., 1990). Immunofluorescence experiments have established that two isoforms of myosin normally present only in cardiac muscle, atrial myosin heavy chain (αMHC) and atrial myosin light chain 2 (A-MLC2), are expressed in DMSO-differentiated P19 cells (Rudnicki et al., 1990). Northern blot analysis has demonstrated that cardiac and skeletal muscle-specific actin mRNAs, are expressed after day 6 (Rudnicki et al., 1990). Northern hybridization using probes to the skeletal-specific troponin-T and fast myosin light chain (MLC) isoforms revealed that the majority of differentiated muscle cells were of the cardiac type (Rudnicki et al., 1990).

Differentiation of P19 cells using 1% DMSO in the literature normally results in the production of muscle cells, which is in direct contrast to what was found in this study (Edwards et al., 1983). The use of 1% DMSO resulted in the formation of neurons instead of muscle cells. It has been documented that high concentrations of DMSO (i.e., higher than 1%) may yield neurons in P19 cells under some circumstances (Edwards et al., 1983); in light of this fact, the DMSO concentration was accordingly lowered to 0.5%. At this concentration, muscle cells formed after DMSO treatment. Also of note, P19 cells differentiated with DMSO usually start to form cardiac cells and begin to spontaneously contract on day 5 or day 6 (Edwards et al., 1983; Rudnicki et al., 1990), whereas in this study

beating cells never appeared before day 7. The reason for these differences is not entirely clear, although differences in serum batches can often result in slight differences in P19 cell behaviour (Dr.M.McBurney, personal communication). The serum employed in these experiments was obtained from a different company than what was used in the original publications characterizing P19 differentiation by DMSO (McBurney et al., 1982; Edwards et al., 1983). The morphological characteristics of differentiated cardiac muscle cells are quite obvious in that the cells rhythmically contract, which is consistent with previous work (McBurney et al., 1982; Edwards et al., 1983; Rudnicki et al., 1990) that P19 cells differentiate into cardiac muscle.

The results presented in this thesis suggest that the cardiac α1 and α2 subunits are present in differentiated P19 cells, whereas the adult isoform of the α1 from adult skeletal muscle is not. Curiously, both the α2 and the cardiac α1 were detected at all stages of differentiation by PCR - even in undifferentiated cells. In addition, several isoforms of cardiac α1 and α2 appear to be present in both P19 cells and adult cardiac muscle, as illustrated by the multiple bands detected with the PCR-Southern blots (figures 3.4 and 3.15). These isoforms may be novel forms of the subunits, or they may represent isoforms which have been cloned previously by other groups from muscle or brain (Huang et al., 1990; Williams et

Williams et al., 1992; Hui et al., 1991; Biel et al., 1990; Koch et al., 1989; Malouf et al., 1992).

There is some evidence, as demonstrated by studies on sodium channels in primary muscle cultures, that differences in serum may cause fluctuations in ion channel expression (Brodie et al., 1991). To investigate this possibility, P19 cells from another laboratory which had been passaged and DMSO-differentiated with a different serum lot were analyzed by RT-PCR. The result was the same: both the cardiac α1 and α2 subunits appear to be expressed in P19 cells at all stages of development.

The cardiac α1 subunit is detectable in P19 cells by PCR and Northern blot analysis whereas the α2 subunit is detectable only by PCR. Riboprobes have been shown to be more sensitive than conventional DNA probes by at least 10-fold (Wahl et al., 1987), which is why they were employed in this study. Nevertheless, since all Northern blots were performed using total RNA, it is possible that a signal for α2 would be detectable if a Northern blot containing poly A+ purified RNA (i.e., RNA enriched for messenger RNA) from P19 cells were probed with the same α2 probe. This type of experiment would also help to elucidate whether the exact time at which α1 mRNA increases in abundance is concomitant with an increase in α2 subunit mRNA expression, or whether α2 is expressed at

relatively steady-state levels throughout differentiation as has been documented for the α2 subunit protein in developing skeletal muscle in newborn rats (Morton et al., 1989). The PCR experiments performed in this thesis do not provide this information, since these experiments are of a qualitative rather than quantitative nature.

The adult skeletal isoform of the α1 subunit is clearly absent in P19 cells, as demonstrated by the RT-PCR experiments and from the PCR-Southern blots it appears unlikely that any of the amplification products seen in the embryonal carcinoma cell lines code for embryonic forms of the skeletal α1 subunit (see figures 3.7 and 3.8). However, Northern blot analysis suggests that some novel transcripts may be expressed in P19 cells which are homologous to the skeletal α1 subunit (figure 3.9) Two transcripts, of 13 kb and 6.5 kb, appear to be expressed in P19 cells very early during differentiation (day 3) but are absent from undifferentiated cells. Since no PCR products of the appropriate size were amplified from P19 cells using skeletal α1 specific primers, the 6.5kb transcript detected in DMSO-differentiated P19 cells may thus represent an embryonic form of the α1 subunit which is related to, but distinct from, the adult skeletal isoform. The fact that it is expressed so early during differentiation means that it could be invaluable as an early marker of P19 cell commitment of differentiation down the muscle pathway, since no markers

are yet available which appear as early as day 3 during DMSO differentiation.

The other L-type Ca channel subunits present in adult skeletal muscle (ß, gamma) are undetectable in P19 cells, even using the highly sensitive technique of PCR. This may imply that there exist embryonic forms of these subunits which are not recognized by the primers we designed. This is supported in part by the fact that the ß subunit riboprobe weakly recognizes a large transcript of 13 kb which disappears upon treatment with RNase, yet the same ß probe synthesized in the opposite orientation does not bind nonspecifically to this region. The existence of several isoforms of the ß subunit in adult cardiac muscle and brain, which impart different kinetic properties on the channel, has already been well documented (Perez-Reyes et al., 1992; Hullin et al., 1992). The large 13 kb transcript may be only weakly homologous to the sequence of the adult ß subunit and treatment with RNase is thus sufficient to remove the signal. To further substantiate this claim, it would be necessary to perform more Northern hybridizations using conditions of varying stringency. The use of a ß subunit probe designed to recognize a different region of the adult skeletal muscle ß subunit sequence may also provide additional information about the nature of the large 13 kb transcript.

The positive control lanes seen on the ß subunit Northern blots contain, in addition to the expected size transcripts (1.8 kb in skeletal muscle and 3 kb in brain, see figure 3.12), a transcript which is 6.5 kb in size in the adult skeletal muscle lane. This transcript is a specific, real transcript since treatment with RNase does not destroy the signal on the Northern blot (compare panels A and B, figure 3.12). It may represent a novel isoform of the ß subunit, or it may simply represent the 1.8 kb transcript which still contains introns that have not been removed by splicing events (i.e., pre-spliced mRNA). Some RNA isolation methods have been shown to result in purification of unspliced as well as mature, correctly spliced mRNA species (Sambrook et al., 1989; Wahl et al., 1987). In order to determine whether the 6.5 kb transcript represents a pre-spliced RNA species or a novel ß subunit transcript, it would be necessary to isolate two separate fractions of RNA, a cytoplasmic and a nuclear fraction, and repeat the Northern blot analysis with these RNA fractions. A mature mRNA transcript would appear in the cytoplasmic fraction, whereas a pre-spliced RNA species containing introns would be present only in the nuclear fraction where splicing events are known to occur.

Since DMSO-differentiated P19 cells which form skeletal muscle myotubes do not contract in these cultures, it is also possible that the ß and gamma skeletal L-type calcium channel

subunits are simply absent from these cells and this may explain in part their inability to contract in culture the way their cardiac counterparts are able to do. In light of these results, determination of the presence or absence of these same subunits in embryonic skeletal muscle tissue at a time equivalent to day 10 DMSO-differentiated P19 cells would provide interesting information on subunit expression in an animal model.

One intriguing possibility which follows from these experiments is that, at least in the case of cardiac muscle, the α1 subunit may be the only subunit necessary for normal cardiac function in embryonic heart muscle and the other subunits may be expressed at significant levels only later during development. Since the α1 subunit is the functional pore-forming subunit of the L-type Ca channel, this is a plausible proposal. Furthermore, it has been demonstrated that the cardiac α1 expressed in Xenopus oocytes can function by itself as a Ca channel; the same experiments using the skeletal α1 subunit failed to give rise to a functional channel (Nargeot et al., 1992). However, it should be noted that microinjection of the skeletal α1 cDNA into mouse L cells and into dysgenic muscle cells, which lack the slow L-type Ca current and α1 subunit, has resulted in successful re-establishment of a functional L-type Ca channel (Nargeot et al., 1992). It is thus possible that the cardiac α1 is able

to function independently of the other subunits, whereas the skeletal α1 may require other subunits which would be present in cells more developed than the relatively simple and primitive oocyte.

This same type of scenario has also been noted in the case of the sodium channel. The sodium channel α subunit has been cloned from several different sources (Noda et al., 1986; Kayano et al., 1988; Trimmer et al., 1989; Rogart et al., 1989; Noda et al., 1984); its primary sequence shows a striking resemblance to the L-type calcium channel α1 subunit (Tanabe et al., 1987). Although the purified sodium channel from mammalian brain consists of three subunits (α, ß1, ß2), the sodium channel purified from electric eel seems to consist of only a large α subunit and no other auxiliary subunits (Catterall, 1988; Noda et al., 1984). The electric eel sodium channel thus provides an example of an α subunit, highly homologous to the calcium channel α1 subunit, which alone is capable of serving as the main functional component of the channel.

The proposal that the cardiac α1 subunit may function independently of the other subunits conforms to the results seen here on the Northern blots for the α1 cardiac subunit: mRNA levels are detectable by Northern analysis only around day 7, which is when the cells in these experiments start to

spontaneously contract. The α2 subunit, on the other hand, is conspicuously absent on Northern blots on day 7. Studies in adult tissues have shown the α2 mRNA to be quite abundant in both cardiac and skeletal muscle, even more abundant than the α1 subunit (Ellis et al., 1988). Using a tubulin (a housekeeping gene) probe as a control, it was determined that the Northern blots used in this thesis for α1 and α2 contain roughly equivalent amounts of RNA. The absence of the α2 subunit on Northern blots, which is in direct contrast to what is seen in adult tissue, leads one to suspect that it may play very little role in actual channel function in embryonic cardiac cells. The other possibility is that there exist embryonic forms of the α2 subunit which are undetectable with the α2 riboprobe employed in this study.

Now that the sequences are available (Perez-Reyes et al., 1992; Hullin et al., 1992), design of a probe specific to the cardiac ß subunit of the cardiac L-type channel would help test the hypothesis that the α1 cardiac subunit can function independently of the other subunits in embryonic muscle. (At the time that the ß subunit primers were being designed, the cardiac-specific sequence had not been published.) One would expect that if the above theory is correct and only the α1 subunit is required for function in embryonic heart, even the cardiac-specific ß subunit should be absent or only expressed in very low amounts in DMSO-treated P19 cells. In keeping

with this theory, it would be worth investigating whether the other subunits of the cardiac L-type channel (e.g., α2, ß) are present in real murine embryonic hearts by Northern analysis. However, technical difficulties may render this type of study an arduous task (e.g., embryonic mouse hearts of the appropriate age are about 3 mm in diameter, must be removed with the aid of a microscope, and do not yield more than miniscule amounts of RNA).

Although the cardiac α1 subunit is detectable in undifferentiated cells by PCR, it is undetectable on Northern blots in undifferentiated cells; the α1 levels seen with PCR may thus be so low before day 7 as to be functionally insignificant. Furthermore, one could argue that detection of the message in minute amounts does not guarantee that the protein is being expressed. Of course, this argument could apply equally to detection of the mRNA on the Northern blots, but past experiments on skeletal muscle and α1 subunit expression have shown that the appearance of α1 transcripts on Northern blots seem to correlate reasonably well with the appearance of the α1 subunit protein, as determined by either DHP-binding, Western blot analysis, or electrophysiological measurements (Morton et al., 1989; Beam et al., 1988). To determine whether the cardiac α1 subunit protein is actually being expressed in our P19 system, however, future experiments using some of the above techniques would be required.

It is interesting to note that a recent study on potassium channels has shown that two different types of voltage-sensitive potassium channels with differing pharmacological and biophysical properties are generated by different expression levels of the same mRNA species (Honore et al., 1992). It has currently been determined that undifferentiated P19 cells express a calcium channel which seems to possess some electrophysiological properties similar to L-type calcium channels (Dr. P. Pawson, personal communication). It is thus feasible that low levels of the α1 subunit could result in expression of an L-type calcium channel which would display properties different from the one detected in differentiated P19 cells, where the mRNA levels are expressed in higher amounts.

It would be of value in the future to further characterize the P19 cell PCR product amplified with the cardiac-specific α1 subunit primers. A neonatal isoform of the L-type calcium channel in skeletal muscle has already been identified (Malouf et al., 1992). To ascertain whether our α1 subunit is identical to the adult cardiac sequence or whether it is a novel, closely related embryonic heart isoform, it would be necessary - and well worth the effort - to determine its nucleotide sequence.

In summary, the findings of this study suggest that the P19 system may be useful for studying cardiac embryonic gene expression even though P19 cells differentiate into a heterogeneous population of cells when treated with DMSO (i.e., mixed population of skeletal and cardiac muscle cells). The results also indicate the existence of a unique expression pattern of the L-type calcium channel subunits in embryonic cardiac muscle derived from differentiation of P19 cells.

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