Preliminary Investigation of the C-terminal Mutations that cause Malignant Hyperthermia

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ABSTRACT

Malignant hyperthermia (MH) is a genetic disorder characterised by abnormal muscle contractures, hypermetabolism and hyperthermia. It is referred to as 'malignant' as it can lead to death when under anaesthetic if not recognised and treated immediately. The molecular basis of MH is an abnormality in the calcium release mechanism of the sarcoplasmic reticulum. Abnormal calcium release causes the physiological symptoms of an MH crisis. Genetic linkage studies have lead to the identification of the ryanodine receptor/Ca^{2+}-release channel as a causative factor in MH. The ryanodine receptor is a large protein which is regulated by a number of ligands including Ca^{2+}, Mg^{2+}, ATP, ryanodine and calmodulin.

Most mutations in the ryanodine receptor gene that cause MH are located near two main regulatory regions on the receptor. Three mutations have recently been identified that are located in the regulatory region of the C-terminal domain. The biochemical properties of one of these mutations have been studied. The current research project began to investigate the biochemical characteristics of the other two mutations in the C-terminal domain in relation to their ryanodine binding and calcium release properties.

Sarcoplasmic reticulum vesicles were isolated from skeletal muscle samples, and an attempt to identify ryanodine receptors by ^{3}H-ryanodine binding was made. RT-PCR using RNA extracted from a skeletal muscle sample was used to construct the cDNA for the C-terminal transmembrane domain of the ryanodine receptor. This cDNA was cloned into a mammalian expression vector and introduced into COS cells. RT-PCR was also used to produce the cDNA encoding a small polypeptide to an antigenic region in the C-terminal domain of the ryanodine receptor for the preparation of antibodies.

Although it appeared that there may have been ryanodine receptors in the SR vesicle preparation as determined by immunoblotting, ^{3}H-Ry binding to the ryanodine receptors was unable to confirm the presence of the receptors in the SR vesicles. Initial expression studies of the C-terminal domain in COS cells were inconclusive. Partial cleavage of a small antigenic polypeptide was obtained which could be used to produce antibodies to the C-terminal domain of the ryanodine receptor.

ABBREVIATIONS

^{3}H-Ry	tritiated ryanodine
amp	ampicillin
AMP-PCP	adenyl-(β,γ-methylene)-diphosphonate tetrathium salt
AMV	avian myeloblastosis virus
bp	base pair
BSA	bovine serum albumin
CaM	calmodulin
CHAPS	3-(3-cholamido-propyl-dimethylamino)-1-propanesulfonate
CHO	Chinese hamster ovary cells
C-terminal	carboxy terminal
cDNA	complementary deoxyribonucleic acid
cpm	counts per minute
DEPC	diethylpyrocarbonate
DHPR	dihydropyridine receptor
DMSO	dimethyl sulfoxide
DNase I	deoxyribonuclease one
dNTPs	dinucleotide triphosphates
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetra-acetic acid
EtBr	ethidium bromide
FCS	fetal calf serum
FKBP12	FK506 binding protein 12 kDa
GCG	genetics computing group
GuHCl	guanidium hydrochloride
GST	glutathione S-transferase
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethane sulfuric acid
HRP	horse radish peroxidase
IPTG	isopropyl-β-D-thiogalactoside
kan	kanamycin

kb	kilobase
kDa	kilo Dalton
LB	luria broth
MEM	minimum essential medium
Mes	4-morpholino ethane sulfonic acid
MH	malignant hyperthermia
MHS	malignant hyperthermia susceptible
M-MLV	Moloney mouse leukaemia virus
MOPS	4-morpholine propane sulfonic acid
MWM	molecular weight marker
N-terminal	amino terminal
oligo	oligonucleotide
oligo(dT)	oligodeoxythymidine
PAGE	polyacrylamide gel electrophoresis
Pipes	1,4-piperazinediethane sulfonic acid
Pfu	*Pyrococcus furiosus*
RNase	ribonuclease
RT	reverse transcriptase
RT-PCR	reverse transcriptase polymerase chain reaction
RyR1	skeletal muscle ryanodine receptor
RYR1	skeletal muscle ryanodine receptor gene
RyR-C	ryanodine receptor antibodies to the C-terminal domain
RyR-N	ryanodine receptor antibodies to the N-terminal domain
SDS	sodium dodecyl sulfate
spec	spectinomycin
SR	sarcoplasmic reticulum
Taq	*Thermus aquaticus*
TCA	trichloroacetic acid
TEMED	N,N,N′,N′-tetramethylethylenediamine
T_m	melting temperature
Tris	tris (hydroxymethyl) aminomethane
UV	ultraviolet light

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CHAPTER ONE – INTRODUCTION

1.1 Malignant Hyperthermia

1.1.1 Introduction

Malignant Hyperthermia (MH) is a dominantly inherited, autosomal disorder of skeletal muscle, which is primarily triggered by exposure to volatile anaesthetic agents or depolarising muscle relaxants. This can lead to a sudden hyperpyrexia due to hypermetabolism and muscle breakdown. If not recognised and treated it progresses to a worsening hyperpyrexic state, muscle contractures, muscle rigidity, cardiac arrhythmias and other systemic effects or death (MacLennan and Phillips, 1992).

MH is considered the most common cause of anaesthetic death in otherwise fit and healthy people. However, with early recognition and intervention with the correct treatment, the fatality rate has decreased from ~70% to ~5% (Denborough, 1998).

1.1.2 Genetics of Malignant Hyperthermia

Extensive genetic analysis has lead to the identification of 25 point mutations in the skeletal muscle ryanodine receptor (*RYR1*) gene. In biochemical and physiological studies at least 21 of them have been shown to have a causal role in MH (Tong *et al.*, 1997; Jurkat-Rott *et al.*, 2000; McCarthy *et al.*, 2000). A mutation in a second gene encoding the α1-subunit of the dihydropyridine receptor (Monnier *et al.*, 1997) has been shown to be responsible for MH in some families. These mutations account for only ~50% of MH cases (McCarthy *et al.*, 2000; Richter *et al.*, 1997).

While the main MH locus is found on chromosome 19q13.1 and encodes the *RYR1* gene, genetic linkage studies have indicated the location of several other loci. Other genes that may be involved in MH susceptibility have been mapped to chromosome 1q31-q32,

3q13.1, 5p, 7q21-22, 7q21.1, 17q and 17q11.2-q24 (reviewed in Jurkat-Rott *et al.*, 2000; Loke and MacLennan, 1998; Mickelson and Louis, 1996).

1.1.3 Biochemistry of Malignant Hyperthermia

Initially, skeletal muscle was implicated in MH after observation of muscle contractures and an increase in serum creatine kinase levels after an MH reaction (Denborough *et al.*, 1970a; Denborough *et al.*, 1970b). Creatine kinase is a protein released from muscle cells, which accumulates in the blood and is an indicator of the level of damage that has occurred to muscle cells.

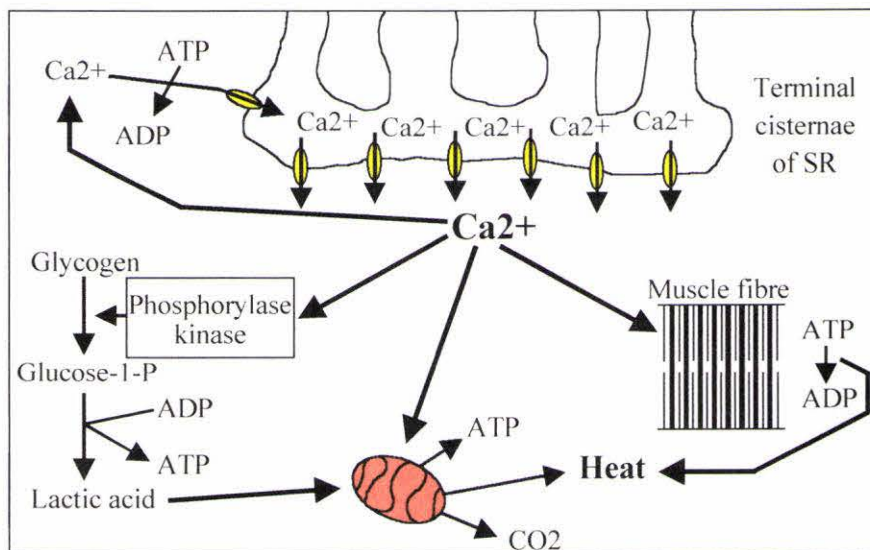

Figure 1.1: The molecular effects of calcium on muscle contraction and metabolism.

An increase in myoplasmic free calcium levels in skeletal muscle cells causes prolonged muscle contraction and enhanced metabolic processes. These actions lead to the biochemical events that manifest as Malignant Hyperthermia (Adapted from MacLennan and Phillips, 1992).

Indeed, the main molecular defect in MH appears to be an abnormality in the Ca^{2+}-release mechanism in the sarcoplasmic reticulum (SR) of skeletal muscle (Mickelson *et al.*, 1986). This causes a sustained release of calcium from the SR causing an increase in the myoplasmic calcium concentration and it is this calcium level that regulates muscle contraction and metabolic activity. In normal muscle cells calcium is released from the SR via Ca^{2+}- release channels and pumped back in via Ca^{2+}-ATPases. The released calcium is used to initiate muscle contraction by binding to troponin in the thin filaments, and activating glycolysis and aerobic metabolism by binding to and activating phosphorylase kinase. In MH muscle, calcium release is increased and consequently muscle contraction and metabolism is enhanced which accounts for the symptoms of MH (Figure 1.1).

The Ca^{2+}-release channel provides the only cellular site for the binding of the plant alkaloid ryanodine (Phillips *et al.*, 1996) and hence allows for the isolation, purification and biochemical study of the channel (Inui *et al.*, 1987). The properties of ryanodine binding to the Ca^{2+}-release channel led to it becoming known as the ryanodine receptor (RyR1).

Ryanodine binding is an indirect means of measuring calcium release as it binds to the receptor with high affinity, in a calcium-dependent manner, when in the open state (Mickelson and Louis, 1996; Valdivia *et al.*, 1991). Specific ligands that modulate calcium release also affect ryanodine binding in the same way. Therefore ryanodine binding reflects the functional state of the channel in that channel activators or inhibitors have an enhancing or suppressing effect on ryanodine binding (Palnitkar *et al.*, 1997; Richter *et al.*, 1997).

Calcium release can be modulated by a number of physiological ligands. These include endogenous (Ca^{2+}, ATP, and Mg^{2+}) and exogenous (caffeine, halothane, ryanodine, dantrolene, and 4-Chloro-*m*-cresol (4-CmC)) ligands. The Ca^{2+}-release channel is activated by μM Ca^{2+}, mM ATP, nM ryanodine, caffeine, 4-CmC (a preservative added to some intravenous medications (Herrmann-Frank *et al.*, 1996)), halothane and alkaline pH. Inhibitors of the channel are mM Ca^{2+}, mM Mg^{2+}, μM ryanodine, dantrolene and acidic pH (Loke and MacLennan, 1998; Palnitkar *et al.*, 1997; Samso and Wagenknecht, 1998;

Valdivia *et al.*, 1991; Zorzato *et al.*, 1990). According to Chen *et al* (1998) Ca^{2+} is the essential regulator of the ryanodine receptor and any other modulators exert their effects by influencing the calcium sensitivity of the receptor.

1.2 The Ryanodine Receptor

The ryanodine receptor functions as the Ca^{2+}-release channel in the SR and is the main regulator of calcium concentration in skeletal muscle cells. It is composed of four identical subunits of ~5038 amino acids each with a molecular weight of ~565 kDa (Phillips *et al.*, 1996). At a total molecular weight of ~2260 kDa, the ryanodine receptor is one of the largest proteins and the largest ion channel known. The entire gene is ~160 kb long and contains 106 exons ranging in size from 15 base pairs to 813 base pairs (Phillips *et al.*, 1996).

Three-dimensional studies show that the receptor consists of two main parts – a large cytoplasmic segment comprising ~80% of the structure, and a smaller (~20%) transmembrane domain at the C-terminal end of the protein. Hydropathy studies and sequence analysis have shown that the C-terminal region contains a number of transmembrane domains which are located in the membrane of the sarcoplasmic reticulum (Takeshima *et al.*, 1989; Zorzato *et al.*, 1990). The cytoplasmic domain spans the gap between the SR and the transverse tubule (t-tubule) of the muscle cell membrane (Figure 1.2).

There are three known isoforms of the ryanodine receptor – RyR1, RyR2, RyR3 – which are encoded on separate genes and exhibit widespread expression patterns in mammalian tissue (Giannini *et al.*, 1995). RyR1 is expressed mainly in skeletal muscle, but also brain and smooth muscle; RyR2 in cardiac muscle and also brain and endothelial cells; RyR3 in brain, and smooth muscle and epithelial cells (Samso and Wagenknecht, 1998). This widespread expression pattern may also suggest that ryanodine receptors have a role in calcium regulation in a rather large range of tissues (Giannini *et al.*, 1995).

1.3 The Excitation-Contraction Coupling Process

1.3.1 Excitation-Contraction Coupling

Calcium release is caused by depolarisation of the muscle cell membrane. This signal from a neuronal impulse is sensed by the voltage-sensing dihydropyridine receptor (DHPR) in the t-tubule membrane, and passed to the ryanodine receptor in the terminal cisternae of the SR membrane (Figure 1.2). This occurs by a mechanism that is not fully known.

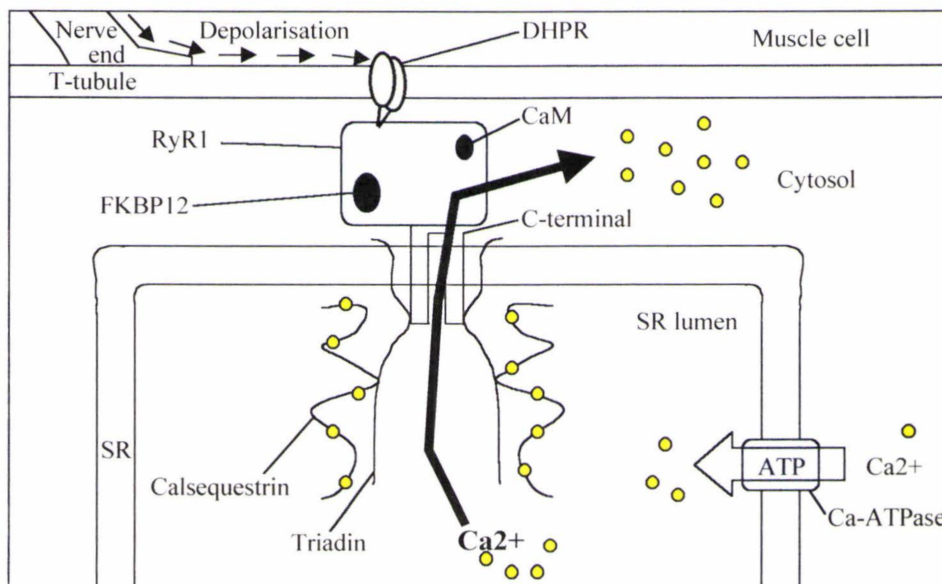

Figure 1.2: The excitation-contraction pathway of calcium release.

A depolarising signal is received by the DHPR and transmitted to the ryanodine receptor causing a release of calcium from the sarcoplasmic reticulum. One subunit of the ryanodine receptor is shown along with the relative position of other proteins that are involved in this process and their relation to the receptor (Adapted from Pessah et al., 1996).

1.3.2 The Ryanodine Receptor and the Dihydropyridine Receptor

The ryanodine receptor is the main component of the Ca^{2+}-release pathway. It is closely associated with the DHPR such that one DHPR associates with every alternate ryanodine receptor structure (Flucher and Franzini-Armstrong, 1996).

The DHPR is a multisubunit complex with each one consisting of four domains. The α1-subunit has a cytoplasmic loop between domain II and III. This has been shown to form a functional interaction with the ryanodine receptor (reviewed in McPherson and Campbell, 1993), but McPherson and Campbell (1993) suggested that there was no direct contact existing between the two proteins. Since then, it has been shown by cross-linking analysis that there is a direct linkage between the ryanodine receptor and the α1-subunit of the DHPR (Murray and Ohlendieck, 1997). Loke and MacLennan (1998) have identified a DHPR binding site near the N-terminal end of the ryanodine receptor.

A more recent study has used NMR spectroscopy to show that the helical structural formation of two peptides, A1 and A2, within the II – III loop of the DHPR is important for interaction with the ryanodine receptor (Casarotto *et al.*, 2000). This group suggest that activation of the ryanodine receptor during excitation-contraction coupling is dependent on the conformational changes within the II – III loop which causes the A1 and A2 peptides to interact strongly with the ryanodine receptor.

There also appear to be other proteins that may interact in some way with the ryanodine receptor and that are involved with the regulation of calcium release. These include triadin and calsequestrin on the luminal side, and FKBP12 and calmodulin (CaM) on the cytoplasmic side of the receptor.

1.3.3 Triadin

Initial investigators thought that triadin bound to both the ryanodine receptor and the DHPR (Caswell *et al.,* 1991). Later analysis indicated that only a short region of triadin was cytoplasmic while the majority of the protein resided in the lumen of the SR (Knudson *et*

al., 1993). This proved that it was highly unlikely to associate with the DHPR although association with the ryanodine receptor was still possible. It was also proposed that triadin serves as an anchor to keep calsequestrin near the ryanodine receptor in the terminal cisternae of the SR (reviewed in McPherson and Campbell, 1993).

Murray and Ohlendieck (1997) demonstrated an association between triadin and the ryanodine receptor, and more recently, ryanodine binding sites have been found at various points on the luminal region of triadin (Caswell *et al.*, 1999). These may bind to a position on the luminal loops in the C-terminal domain of the ryanodine receptor. The precise functional role of triadin in the excitation-contraction coupling process was not well defined in early 1998 (Protasi *et al.*, 1998), although it was suggested that it may be involved in transmitting the Ca^{2+}-release signal to calsequestrin (Pessah *et al.*, 1996).

Later in 1998, another study suggested that triadin was a negative regulator of the ryanodine receptor in that when triadin was present, ^{3}H-ryanodine binding was inhibited and channel opening was decreased (Ohkura *et al.*, 1998). When triadin was removed the ^{3}H-ryanodine binding was increased. These authors suggested that the ryanodine receptor was regulated by triadin in cooperation with calsequestrin in that, while triadin inhibited the channel, calsequestrin was an activator of the channel.

1.3.4 Calsequestrin

Calsequestrin molecules are concentrated in the lumen of the SR. They have a high capacity for binding calcium, which in effect lowers the free calcium concentration within the SR and is one form of storage of calcium in the SR. This binding is not tight and signals can cause release of calcium in response to opening of the ryanodine receptor channel (Murray and Ohlendieck, 1997; Pessah *et al.*, 1996; Szegedi *et al.*, 1999). Murray and Ohlendieck (1997) showed in their linkage studies, that although calsequestrin was present in the terminal cisternae, it was not found in a complex of the ryanodine receptor and the DHPR.

Later in the same year, it was shown that calsequestrin could form a complex with the ryanodine receptor and also the DHPR and triadin (Zhang *et al.*, 1997). This group found that junctin, another membrane protein, played a part in stabilising the complex and anchoring calsequestrin to the ryanodine receptor. More recently it has been found that when microsomal preparations are treated with halothane, a complex forms that contains the ryanodine receptor, DHPR as well as calsequestrin (Froemming *et al.*, 1999). Although these authors suggested that calsequestrin does complex with the ryanodine receptor, it is still unknown whether it is a direct or indirect association.

Szegedi *et al* (1999) have recently discovered that calsequestrin, when dephosphorylated, can regulate the ryanodine receptor and stimulate the subsequent release of calcium. In addition Herzog *et al* (2000) suggested that both phosphorylated and dephosphorylated calsequestrin could bind to the ryanodine receptor. This could prove to be a mechanism by which calcium is released from the SR via the alternate ryanodine receptors that are not associated with the voltage-sensing DHPRs. The signal or mechanism of phosphorylation and dephosphorylation is not known. Therefore, it remains to be seen whether it is still a depolarisation signal that directs the change via some other intermediate.

1.3.5 FKBP12

FKBP12 is a 12 kDa protein of the immunophilin family that binds the immunosuppressant drug FK-506 (Jayaraman *et al.*, 1992). This protein has been identified as part of the three-dimensional structure of the ryanodine receptor. One FKBP12 molecule has been found to associate with each of the cytoplasmic subunits of the ryanodine receptor and modulate its activity by stabilising the closed conformation of the receptor (Samso and Wagenknecht, 1998).

Although FKBP12 may associate tightly with the ryanodine receptor and modulate channel function, it can be exchanged with a soluble form or an isoform, or removed by drug treatment (Qi *et al.*, 1998) with subsequent changes in channel function. The initial tight binding of FKBP12 to the ryanodine receptor was discovered to be a weaker interaction as FKBP12 could be dissociated with CHAPS detergent (Ogawa *et al.*, 1999). Its close

association with the ryanodine receptor indicates that it has a role in the excitation-contraction coupling process, which was confirmed in the study by Qi *et al* (1998).

1.3.6 Calmodulin

Calmodulin is the other protein that interacts with, and regulates the ryanodine receptor in a calcium dependent manner. CaM inhibits the channel in the absence of calcium or in the presence of higher than μM calcium. (Ogawa *et al.*, 1999). When the channel has been activated by μM calcium, CaM will bind to the ryanodine receptor and inhibits the opening of the channel (Samso and Wagenknecht, 1998). When the intracellular concentration is in the nM range, more CaM binds and the receptor is activated. CaM binding sites on the ryanodine receptor have been identified in the central region (O'Driscoll *et al.*, 1996) and also in the C-terminal domain (Takeshima *et al.*, 1989).

1.4 Important Regions on the Ryanodine Receptor

1.4.1 Regulatory Regions

Calcium release and ryanodine binding are modulated by various ligands – Ca^{2+}, Mg^{2+}, ATP, CaM and ryanodine. Binding sites for these modulators have been predicted from primary sequence analysis and binding studies, and have been localised to three main regions on the receptor: the N-terminal domain, the centre and the C-terminal domain (Figure 1.3).

The conformation of the ryanodine receptor, mediated by the N-terminal region, has been shown to be important for ryanodine binding. When portions of the skeletal muscle N-terminal domain were replaced by the identical cardiac isoform segments of the ryanodine receptor, the high affinity binding site was lost (Nakai *et al.*, 1999). This is because the skeletal muscle and cardiac muscle ryanodine receptors behave differently in their ryanodine binding properties.

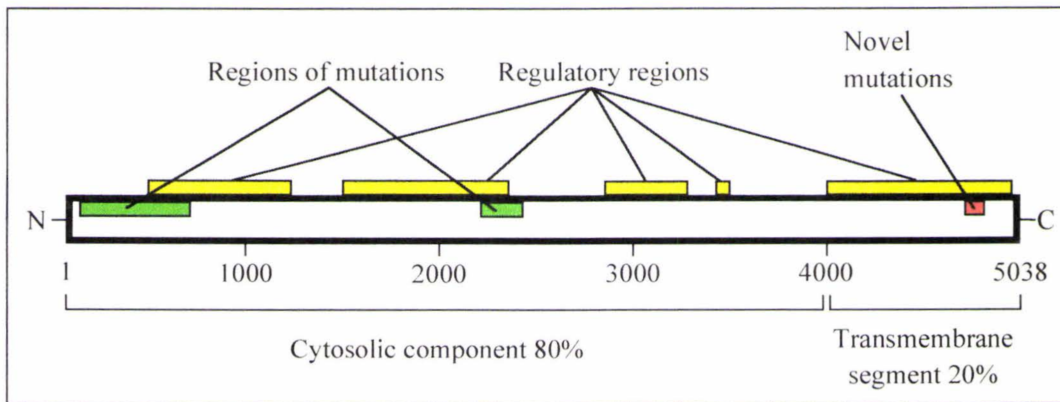

Figure 1.3: A linear representation of the location of the regulatory and mutation sites on the ryanodine receptor.

Regulatory regions are found over widespread areas of the receptor. The mutations are clustered in more compact regions. The transmembrane region of the C-terminal domain is almost entirely composed of regulator binding sites and contains the recently identified novel mutations.

Sites on the N-terminal region include a high affinity ryanodine binding site between amino acid residues 500 and 1300 (Chen *et al.*, 1993b). ATP binds between amino acids 1194 and 1199 (Zorzato *et al.*, 1990) and calcium binds between residues 1641 and 2437 (Samso and Wagenknecht, 1998).

The central region contains two main blocks of modulator binding sites. A low affinity binding site for calcium exists between amino acid residues 1872 and 1923 (Zorzato *et al.*, 1990). Calmodulin binds at several sites including between residues 2807 – 2840, 2909 – 2930, 2937 – 3225 and 3614 – 3637 (O'Driscoll *et al.*, 1996; Takeshima *et al.*, 1989; Zorzato *et al.*, 1990).

The C-terminal region contains binding sites for ATP, Ca^{2+}, CaM and ryanodine. ATP binds at residues 4447 – 4457 (Takeshima *et al.*, 1989; Zorzato *et al.*, 1990). Calcium binds between amino acids 4253 – 4264, 4407 – 4416 and 4489 – 4499 (Chen *et al.*, 1992;

Takeshima *et al.*, 1989). CaM binds between residues 4295 and 4325 (Takeshima *et al.*, 1989). Ryanodine has a low and high affinity binding site between residue 4475 and the end of the C-terminal domain (Callaway *et al.*, 1994).

No binding sites have been found for Mg^{2+} (Zorzato *et al.*, 1990).

The C-terminal domain has also been found to have a major role in calcium activation and inactivation in skeletal muscle ryanodine receptors (Nakai *et al.*, 1999). When the C-terminal domain of the skeletal muscle receptor was exchanged with the same part in the cardiac muscle receptor, the channel inactivation was decreased at high calcium concentrations. Another group has also shown that the C-terminal domain contains low affinity calcium binding sites (Du and MacLennan, 1999) which have an effect on inactivation of the channel. Cardiac muscle receptors are not inactivated by mM calcium like skeletal muscle receptors, but both are activated by μM calcium (Du and MacLennan, 1999), therefore the skeletal muscle sequence can be exchanged for cardiac receptor sequence as was done in these two studies to examine the effects of calcium activation and inactivation.

1.4.2 Location of Mutations associated with Malignant Hyperthermia

The known mutations are located in clusters near or within the identified regulatory regions. There are two main sites: one in the N-terminal domain (residues 35 – 614) and one in the central portion of the receptor (residues 2163 – 2458) (Figure 1.3).

A mutation in one of the modulator binding sequences would be expected to cause an alteration or reduction in the binding of that modulator leading to an alteration in the calcium release or ryanodine binding properties of the receptor. This occurs in malignant hyperthermia susceptible (MHS) muscle – calcium release and ryanodine binding is greatly enhanced. Seventeen mutations in the N-terminal domain and central region have been biochemically characterised and they have been shown to be more sensitive to caffeine and halothane (the *in vitro* diagnostic indicators) by exhibiting an increase in calcium release

and/or ryanodine binding (Censier *et al.*, 1998; Richter *et al.*, 1997; Tong *et al.*, 1997). A recent study suggests that there is an interaction between the N-terminal and central domains, where most mutations are clustered (Yamamoto *et al.*, 2000). In wild type channels an interaction occurs which closes the channel and regulates it accordingly. When a mutation exists, the interaction between the two domains prevents closure of the channel so that it remains open and increases the sensitivity of the channel to various modulators. This would account for the great exit of calcium ions during an MH crisis.

Mutations which have been identified include: C35R, R163C, G248R, G341R, I403M, Y522S, R552W, R614C, R614L, R2163C, R2163H, V2168M, T2206M, T2206R, G2434R, R2435L, R2435H (R2436H), R2454C, R2454H, R2458C and R2458H (Jurkat-Rott *et al.*, 2000; McCarthy *et al.*, 2000). Another mutation has recently been identified, R2452W, which occurs in the central region of the receptor (Chamley *et al.*, 2000)

Two mutations have recently been identified in a different region of the receptor. They are located in the C-terminal domain near the third main regulatory site T4826I and H4833Y (Brown *et al.*, 2000; Stowell *et al.*, 1999). A third mutation has also been identified in this region, I4898T, but it is linked to another myopathy called central core disease (CDD), and not to MH (Lynch *et al.*, 1999). This brings the total number of MH- or CCD-associated mutations to 25.

1.5 The C-terminal Region

1.5.1 Models of Transmembrane Domains

The C-terminal domain is an important region of the ryanodine receptor in that it contains a large regulatory region in which three MH- or CCD-associated mutations have been identified, two of which have not been characterised. It is also the region that contains the transmembrane (TM) domains.

Sequence analysis has revealed the presence of four (Takeshima *et al.*, 1989) or ten (Zorzato *et al.*, 1990) TM domains in the C-terminal fifth of the receptor. There are still

discrepancies with the accuracy of the models with some groups supporting either one or the other. Antibodies to selected regions were used to identify luminal sites in the C-terminal region (Grunwald and Meissner, 1995) which supported the 4-TM model. Balshaw *et al* (1999) also reviewed that the 4-TM model is favoured based on some studies with tryptic digestion and deletion mutations. Other research groups deduced that their studies of the three-dimensional structure of the receptor support the 10-TM region model (reviewed in Samso and Wagenknecht, 1998). Chen *et al* (1993b) also support this model after their tryptic digestion studies of ryanodine receptors in SR membrane vesicles which revealed that three fragments associate with the membrane. Four TM regions would be found in only one fragment whereas all three fragments would be needed to support the 10-TM model.

Other support for the 10-TM model has arisen from the study of the effects of mutations in a highly conserved hydrophobic region corresponding to the TM-9 domain in the Zorzato *et al* (1990) model (Du *et al.*, 1998a; Zhao *et al.*, 1999). Bhat *et al* (1997) found that the entire C-terminal domain (~20% of the receptor) could form a fully functional channel, which would suggest that the 10-TM model is supported as all the 10 transmembrane domains were in the segment they studied. On the other hand, a shortened version of the ryanodine receptor was isolated in brain tissue (Takeshima *et al.*, 1993), and was highly homologous to the last ~2.4 kb of the C-terminal end in skeletal muscle containing the four transmembrane domains of the Takeshima *et al* (1989) model. However, this shortened C-terminal region did not function as a full-length channel would.

In the Zorzato et *al* (1990) model, the TM domains are found between amino acids 3982 – 4003, 4021 – 4040, 4277 – 4301, 4342 – 4362, 4559 – 4581, 4648 – 4672, 4789 – 4821, 4837 – 4857, 4879 – 4899 and 4914 – 4938. The Takeshima *et al* (1989) model places the domains between residues 4564 – 4581, 4640 – 4665, 4835 – 4860 and 4917 – 4937.

Although it is still unclear whether the 4-TM model is more accurate than the ten, both models support an even number of TM domains. This is consistent with the overall

structure of the ryanodine receptor which has an ~80% cytoplasmic N-terminal portion and a small cytoplasmic C-terminal tail with the TM region in between.

1.5.2 The Calcium Channel

Initially it was assumed from sequence analysis that the TM segments formed the membrane-bound region of the calcium channel. It was also found that cDNA encoding the whole ryanodine receptor could be expressed in cells to form a functionally active calcium release channel (Chen *et al.*, 1993a).

Earlier in the same year, Takeshima *et al* (1993) identified a 2.4 kb mRNA species in brain tissue that hybridised to the C-terminal 656 amino acid region of the ryanodine receptor. The brain does express an isoform of the full-length receptor but this shortened form appeared to be derived from the C-terminal domain of the skeletal muscle ryanodine receptor using Met^{4382} as the initiation codon. Expression studies showed that this shortened form could produce a membrane protein with only four TM regions, but they could not obtain measurable ryanodine binding or calcium release results comparable with full-length ryanodine receptors.

More recent studies (Bhat *et al.*, 1997) have shown that the C-terminal domain alone is sufficient for channel activity. Expression of the last 1377 residues of the ryanodine receptor in CHO cells could produce a functionally active calcium release channel, which showed similar properties to the full-length version. The C-terminal domain contains calcium and ryanodine binding sites (Callaway *et al.*, 1994; Chen *et al.*, 1992). The main difference with the truncated version was an inability to be inactivated by calcium, suggesting that the calcium binding sites in the cytoplasmic domain have a different function than sites in the C-terminal domain.

A highly conserved region identified in the C-terminal domain of all ryanodine receptors can reduce or totally abolish ^{3}H-ryanodine binding depending on the site of the point mutation within the region (Zhao *et al.*, 1999). This region relates to amino acids 4891 – 4900 in human skeletal muscle ryanodine receptor (refer to appendix 5), and this group

suggests that it is probably the pore forming segment corresponding to the TM-9 domain in the Zorzato *et al* (1990) model. This is the location of the CCD mutation I4898T (Lynch *et al.*, 1999) which abolishes ryanodine binding.

1.5.3 Mutations

Lynch *et al* (1999) have identified a mutation, I4898T, in the C-terminal domain of the ryanodine receptor in a large family that are all affected by a severe form of central core disease. CCD is also an autosomal, dominantly inherited disorder characterised by hypotonia and muscle weakness that presents early in life. It is closely associated with MH in that the same gene is involved and five mutations that are linked to MH are also linked to CCD (Tong *et al.*, 1997). Both conditions result from an abnormality in the calcium release mechanism in skeletal muscle.

The ryanodine receptor with the I4898T mutation appears to be in a closed state as ryanodine binding was greatly reduced. The maximum Ca^{2+}-release level was greatly decreased and the intracellular concentration of calcium in resting cells was significantly increased from normal. This suggests that the mutant channel may be 'leaky' as calcium stores in the SR were also greatly reduced in resting muscle cells (Lynch *et al.*, 1999).

The I4898T mutation appeared to behave differently to other mutations in the ryanodine receptor in that it reduced ryanodine binding and seemed to inactivate the channel (Balshaw *et al.*, 1999). The ryanodine receptor with this mutation also had almost no response to caffeine or halothane, which strongly activate channel activity in MHS muscle. Zhao *et al* (1999) have also confirmed the effect of the I4898T mutation which is located in the TM-9 highly conserved region of the ryanodine receptor.

The Takeshima *et al* (1989) model places the I4898T mutation in a luminal loop of the receptor whereas in the Zorzato *et al* (1990) model it is the second to last amino acid in the ninth transmembrane domain heading into the luminal side (see Figure 1.4). Grunwald and Meissner's (1995) antibody studies also suggest that it is found in a luminal loop. The mutation has its greatest effect on calcium sensitivity (Lynch *et al.*, 1999), therefore it may

disrupt a luminal calcium binding site, or prevent triadin binding which prevents anchoring of the calsequestrin/Ca^{2+} complexes at the terminal cisternae of the SR.

The observation of low calcium stores in the lumen of the SR and high resting calcium concentration in the cytosol is consistent with this family having no MH episodes on exposure to anaesthetic agents (Lynch *et al.*, 1999). This may mean that this particular mutation is the first to be discovered on the ryanodine receptor that is solely responsible for CCD and not MH.

In light of this, and because other mutations in the N-terminal and central domains altered caffeine and halothane sensitivity, a study was carried out to determine the effects of the C-terminal domain mutations on the ryanodine receptor (Du *et al.*, 2000). This group replaced amino acids 4187 – 4628, the most divergent region in the C-terminal domain, with cardiac muscle receptor sequence and observed an altered sensitivity of the channel to caffeine and calcium. This would suggest that mutations in this, and other regions of the C-terminal domain could have a predictable effect on the ryanodine receptor.

The other two mutations identified in the C-terminal domain, T4826I and H4833Y (Brown *et al.*, 2000; Stowell *et al.*, 1999), occur in families that are susceptible to MH. There is no evidence of CCD in either of these families.

Biochemical analysis of these mutations has yet to be carried out to determine the effects of ryanodine binding and calcium release. It would be assumed that as they are both from MH families, as opposed to the Lynch *et al* (1999) CCD family, that both ryanodine binding and calcium release would be increased.

These two mutations are located on different sides of the SR membrane depending on which model is employed (Figure 1.4). The Takeshima *et al* (1989) model places the location of the mutations on the cytosolic side whereas in the Zorzato *et al* (1990) model the mutations would be on the luminal side of the SR. This is in contrast to the I4898T mutation. Regulation of the channel with either of these mutations would be expected to be

altered. But until the structure of the transmembrane domains is confirmed, the precise molecular effects of modulators on channel activity can not be defined. Some modulators may have more of an effect than others depending on which side of the SR membrane the mutation is on in relation to the TM model and binding site positions. Specific antibody binding studies could confirm the cellular location of the mutation and provide a clearer view as to which TM model is more accurate.

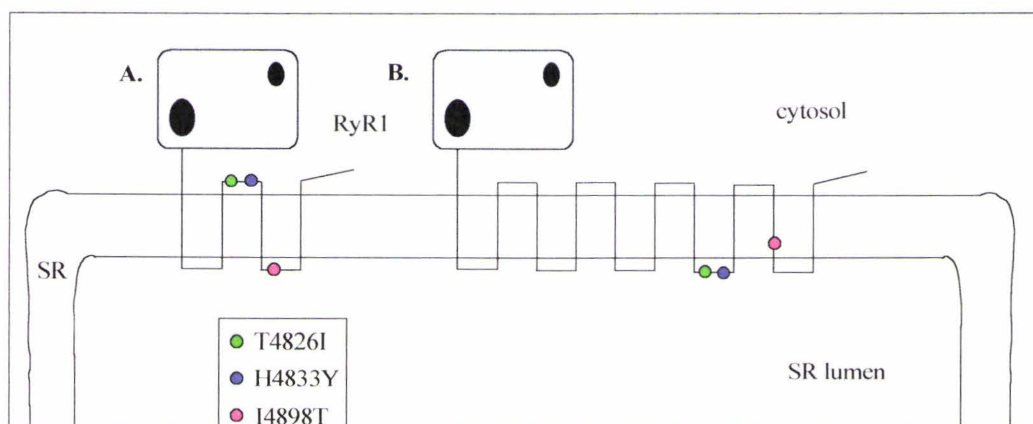

Figure 1.4: Schematic diagram of the location of the C-terminal domain mutations in relation to the transmembrane domain models.

(A) The Takeshima et al (1989) 4-transmembrane domain model places the T4826I and the H4833Y mutations on the cytosolic side of the SR membrane and the I4898T mutation on the luminal side of the SR membrane. (B) The Zorzato et al (1990) 10-transmembrane domain model places the T4826I and the H4833Y mutations on the luminal side of the SR membrane and the I4898T mutation at the second to last position on the ninth transmembrane domain, closest to the luminal side of the SR membrane.

1.6 Significance of this Project

A total of 25 published mutations have now been identified in the ryanodine receptor of MHS individuals. Most of these occur within one of two groups in either the N-terminal

domain or the central region of the receptor. Eighteen of these mutations have been biochemically characterised and have been shown to be causative of the abnormal calcium release observed in MH muscle. Sixteen have been linked to only MH in humans, six have been linked to both MH and CCD (Jurkat-Rott *et al.*, 2000; McCarthy *et al.*, 2000; Tong *et al.*, 1997) and one has been linked to CCD but not MH (Lynch *et al.*, 1999).

Three mutations are located in the regulatory region within the C-terminal domain. One of these mutations appears to be linked only to CCD and exhibits reduced calcium release and ryanodine binding, indicative of an inactive or closed channel. The other two mutations have not yet been biochemically characterised although they are associated with MH.

1.7 Aims of the Project

This research project focuses on the C-terminal domain that includes a large regulatory region containing these novel mutations. The overall objective was to biochemically characterise the T4826I and H4833Y mutations in relation to their calcium release and ryanodine binding properties.

Sarcoplasmic reticulum vesicles containing ryanodine receptors from normal skeletal muscle samples were isolated and the presence of receptors was determined by ^{3}H-ryanodine binding or by immunoblotting.

The C-terminal domain of the ryanodine receptor was constructed by RT-PCR and expression studies were started. This would enable the introduction of point mutations into the C-terminal domain regulatory region so that the calcium release and ryanodine binding effects could be studied using recombinant protein.

CHAPTER TWO – MATERIALS AND METHODS

2.1 Materials

Tergitol, ryanodine and the 6H molecular weight marker were from Sigma-Aldrich, St. Louis, MO, USA. CHAPS, AMP-PCP, Complete™ and Complete™ mini protease inhibitor mixes were from Roche Molecular Biochemicals, Mannheim, Germany. The silver stain plus reagents, low molecular weight markers and the Bradford protein determination reagent were from Bio-Rad Laboratories, Hercules, CA, USA. ^{3}H-ryanodine was from NEN™ Life Science Products Inc., Boston, MA, USA. The scintillation fluid was from Wallac Scintillation Products, UK.

Trizol™ LS reagent was from Life Technologies™ Inc., Gaithersburg, MD, USA. The Ready-to-go DNA labelling beads (d-CTP), ProbeQuant™ G-50 micro columns and nylon transfer membrane for nucleic acids were from Amersham Pharmacia Biotechnology UK Ltd., Buckinghamshire, England. [α^{32}-P]dCTP was from NEN™ Life Science Products Inc., Boston, MA, USA.

C. therm. polymerase one-step RT-PCR system, *C. therm.* polymerase for reverse transcription in two-step RT-PCR, the Titan™ one tube RT-PCR system, and the GC-rich PCR system were from Roche Molecular Biochemicals, Mannheim, Germany. *REDTaq*™ DNA polymerase and RNase were from Sigma-Aldrich, St. Louis, MO, USA. PCRx enhancer system, Platinum® *Taq* DNA polymerase high fidelity, the eLONGase enzyme mix, the SuperScript™ preamplification system for first strand cDNA synthesis, M-MLV reverse transcriptase, oligo dT primer and random hexamers were from Life Technologies™ Inc., Gaithersburg, MD, USA. RQ1 RNase-free DNase was from Promega Corporation, Madison, WI, USA. PCR and sequencing primers were from Sigma-Aldrich, St. Louis, MO, USA or Life Technologies™ Inc., Gaithersburg, MD, USA.

pProEX-HTb was from Life Technologies™ Inc., Gaithersburg, MD, USA. pGEX-6P-3, pSVK3, Glutathione Sepharose 4B and PreScission™ were from Amersham Pharmacia Biotechnology UK Ltd., Buckinghamshire, England. pGEM®-T and pGEM®-T Easy Vector Systems were from Promega Corporation, Madison, WI, USA. Ampicillin, spectinomycin and kanamycin were from Sigma-Aldrich, St. Louis, MO, USA.

Proteinase K, calf alkaline phosphatase, restriction enzymes and buffers were from Roche Molecular Biochemicals, Mannheim, Germany, Life Technologies™ Inc., Gaithersburg, MD, USA or New England Biolabs Inc., Beverly, MA, USA. T4 DNA ligase and buffer was from Life Technologies™ Inc., Gaithersburg, MD, USA.

The High Pure PCR Product purification kit was from Roche Molecular Biochemicals, Mannheim, Germany. The Concert™ High Purity Plasmid Purification Systems, Concert™ Rapid Plasmid Miniprep System, Concert™ Rapid PCR Purification System, and the Concert™ Gel Extraction Systems were from Life Technologies™ Inc., Gaithersburg, MD, USA. The Quantum Prep® Plasmid Miniprep Kit was from Bio-Rad Laboratories, Hercules, CA, USA.

The GST, RyR-C-terminal and RyR-N-terminal antibodies were from Santa Cruz Biotechnology Inc., California, USA. Anti-rabbit and anti-goat immunoglobulins were from Sigma-Aldrich, St. Louis, MO, USA. The chemiluminescence blotting substrate (POD) kit was from Roche Molecular Biochemicals, Mannheim, Germany. Nitrocellulose blotting membrane was from Sartorius AG, Gottingen, Germany.

All Nunc products (T80 vented tissue culture flasks, cryotubes and 150 mm plates), penicillin, streptomycin and trypsin were from Life Technologies™ Inc., Gaithersburg, MD, USA. MEM was from Sigma-Aldrich, St. Louis, MO, USA. The FuGENE™ 6 transfection reagent was from Roche Molecular Biochemicals, Mannheim, Germany.

All other reagents and materials were of analytical grade or higher.

2.2 Methods

2.2.1 General Methods

Protein Content

The Bradford protein reagent (Bradford, 1976) was used to determine the protein concentration in each sample. This method is based on the binding of Coomassie Brilliant Blue G-250 dye to protein. When the dye is bound to protein it causes a shift in the absorption maximum of the Coomassie dye from 465 nm to 595 nm. This increase in absorption at 595 nm can be measured using a spectrophotometer.

1 mL of a 1/5 dilution of Bradford protein reagent was added to 20 μL of sample or BSA standard protein (in the range of 0 μg to 100 μg of protein) and mixed in a test tube. For smaller samples, 200 μL of a 1/5 dilution of the Bradford protein reagent was added to 5 μL of an appropriated dilution of the protein sample in a 96 well plate. In this instance, BSA standards were in the range of 0 μg to 2.5 μg. All analyses were carried out in triplicate. The absorbance at 595 nm was read after 15 minutes using a spectrophotometer or an automatic plate reader. The BSA standards were used to construct a standard curve from which the protein concentration in each sample was determined.

Protein Gels and Staining

Proteins in the samples were analysed by polyacrylamide gel electrophoresis under denaturing conditions using SDS (Laemmli, 1970) and a discontinuous buffer system as described by Ornstein (Ornstein, 1964) and Davis (Davis, 1964). The gel was composed of two layers – the resolving gel and stacking gel. The resolving gel composition varies depending on the percentage of acrylamide and was made with 2.5 mL resolving gel buffer (2.26 M Tris pH 8.8), x ml 40% acrylamide:bis stock (depending on final percentage), 50 μL 20% ammonium persulfate, 5 μL TEMED, 100 μL 10% SDS and H_2O to a final volume of 10 mL. The stacking gel was constant and consists of 2.5 mL stacking gel buffer (0.495 M Tris pH 6.8), 6.7 ml H_2O, 0.8 mL 40% acrylamide:bis stock, 0.1 mL 20% ammonium persulfate, 10 μL TEMED and 100 μL 10% SDS. The gels were electrophoresed in a 1 x

buffer diluted from a 5 x buffer containing 15 g/L Tris, 72 g/L glycine, 0.5% SDS in water, pH 8.3.

SDS-PAGE aims to separate proteins in a mixture based on their molecular weight. SDS binds to the proteins, which are denatured by heat in the presence of the reducing agent β-mercaptoethanol. This gives the proteins a net negative charge proportional to length. When the SDS-protein complex sample is subject to electrophoresis on an SDS polyacrylamide gel the proteins are separated by their charge. Small proteins with a low charge travel faster through the gel matrix than larger proteins with a greater charge.

A 1:1 mixture of protein sample : protein loading dye was boiled for 3 – 5 minutes before either a 7.5 μL or 15 μL sample was loaded on the gel. After electrophoresis in a Bio-Rad mini-protean® II cell apparatus for 40 minutes at 200 V, the gel was removed from between the glass plates and stained using Coomassie Blue (0.125% Coomassie Blue R-250, 50% methanol, 10% acetic acid) for 30 minutes and destained with destain I (50% methanol, 10% acetic acid) and destain II (5% methanol, 7% acetic acid). Alternatively, the gels were stained using the Bio-Rad silver stain plus kit.

Western Blot Analysis

Proteins in the samples were separated using an SDS-polyacrylamide gel at 200 V for 40 minutes as described above. The percentage of acrylamide in the gel depended on the size of the proteins being separated. For the experiments described, a 12% polyacrylamide gel was used to separate proteins in the range of ~14 – 100 kDa. and an 8% or 10% polyacrylamide gel was used to separate proteins greater than 100 kDa. The proteins were transferred to a nitrocellulose membrane by electroblotting with transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3) for 1 hour at 450 mA. The gel was stained with Coomassie Blue and destained as described previously to determine the efficiency of transfer. The membrane was blocked for 1 hour at room temperature with gentle shaking, or overnight at 4°C without shaking, in 1% (w/v) of blocking reagent in TBS (50 mM Tris, 150 mM NaCl, pH 7.5). A 1/1000 dilution of primary antibody was added to the membrane and incubated for 60 minutes prior to washing twice for 10 minutes with TBST (0.1% (w/v) of Tween

20 in TBS) and twice for 10 minutes each with 0.5% (w/v) blocking reagent. The membrane was then incubated with a 1/10000 dilution of the secondary antibody for 30 minutes then washed four times with TBST for 15 minutes each. The membrane was visualised according to the manufacturer's instructions in the BM Chemiluminescence Blotting Substrate (POD) kit (Roche Molecular Biochemicals).

This method is based on detection of proteins using chemiluminescence substrates which are safer and more sensitive than other methods which use radioactivity or rely on the production of coloured products. Detection occurs by the addition of a luminol substrate to the membrane which contains peroxidase-labelled secondary antibody bound to a specific primary antibody and the protein of interest. In the presence of hydrogen peroxide (in the detection solution), peroxidase catalyses the oxidation of the substrate luminol. This results in an excited luminol state which on decay down to the ground state, emits light which is detected on X-ray film. The luminescence reaction which is at its maximum after one to two minutes, remains constant for 20 to 30 minutes, and decreases to about 60% to 70% of maximum after 1 hour. (BM Chemiluminescence Kit protocol). If the membrane required reprobing with the same or different antibodies it was incubated in stripping reagent (100 mM β-mercaptoethanol, 2% SDS in TBS) for 30 minutes at 50°C, washed twice for 15 minutes each time in TBST then blocked in 1% blocking reagent and processed as described above.

Preparation of Competent *E. coli* cells

Competent *E. coli* XL-1 blue cells were prepared by Carole Flyger, IMBS, Massey University as follows. The *E. coli* XL-1 cells were streaked onto LB-tetracycline plates and incubated overnight at 37°C. The next day, 300 mL of SOB broth (20 g/L tryptone, 5 g/L yeast extract, 0.5 g/L NaCl, 2.5 mL of 1 M KCl, with 5 mL of 2 M $MgCl_2$ added prior to use) was inoculated with 10 – 12 colonies and grown at 22°C or 18°C until an A_{600} of 0.6 was reached. The cells were placed on ice for 10 minutes then centrifuged at 4000 rpm in a Sorvall GSA rotor for 10 minutes. The cells were resuspended in 12 mL of ice cold TB (10 mM PIPES, 15 mM $CaCl_2$, 250 mM KCl, 55 mM $MnCl_2.4H_2O$, pH 6.7) and centrifuged at 4000 rpm in a Sorvall GSA rotor for 10 minutes. This wash was repeated once then 1 – 2

mL of cold DMSO was added to the cells which were dispensed into 500 μL aliquots and snap-frozen using liquid N_2 prior to storage at -70°C.

Transformations

Transformation of *E. coli* XL-1 blue cells was carried out to introduce plasmids into bacterial cells for amplification and preparation of DNA vectors (and other DNA constructs later on) based on the method of Pope and Kent (1996). 100μL of competent *E. coli* XL-1 blue cells were added to 50 – 100 ng of vector DNA, mixed and incubated on ice for 5 minutes. 400 μL of LB was added and 100 μL of the mixture was spread onto prewarmed LB-amp plates. The plates were incubated overnight at 37°C.

Isolation and Analysis of Plasmid DNA

Isolation of plasmid DNA from bacterial cells was carried out by the rapid-boil plasmid preparation procedure (Holmes and Quigley, 1981). This method is a rather crude preparation which contains RNA, salts and other contaminants, but is suitable for analysing DNA by restriction digest or PCR. Single colonies of the ampicillin-resistant bacteria were picked off the LB-amp plates and used for the inoculation of 5 mL LB cultures containing ampicillin. The LB-amp cultures were incubated overnight at 37°C with vigorous shaking. The next day, 1.5 mL of the culture was pelleted at 12000 g in a microcentrifuge for 1 minute. The supernatant was removed and the pellet resuspended by pipetting up and down in 350 μL of STET (8% sucrose, 50 mM EDTA, 50 mM Tris pH 8, 0.5% triton X-100 added after autoclaving). 25 μL of freshly prepared lysozyme (10 mg/mL) was added and mixed well. The mixture was boiled for 40 seconds in a boiling water bath then immediately centrifuged at 12000 g in a microcentrifuge for 10 minutes. The gelatinous precipitate was removed and an equal volume of isopropanol was added to precipitate the DNA. The mixture was placed in the -70°C freezer for 30 minutes then centrifuged at 12000 g for 15 minutes at 4°C. The pellet was washed with 500 μL of ice cold 95% ethanol to minimise contamination by salts and centrifuged for 1 minute. The dried pellet was resuspended in 50 μL of TE (10 mM Tris, 1 mM EDTA, pH 8).

An aliquot of the DNA was digested with an appropriate restriction enzyme for 1 hour at 37°C. 2 μL of RNase (10 mg/mL) was added and the mixture incubated for a further 2 minutes at 37°C. The digests were analysed on a 1% agarose gel at 80 V for 1 hour and visualised with the UV transilluminator.

Large Scale Plasmid Preparation

A loop-full of the LB culture containing the correctly identified plasmid was streaked onto an LB-amp plate and incubated overnight at 37°C. A 5 mL aliquot of LB-amp was inoculated with a single colony and incubated at 37°C overnight with vigorous shaking. The entire contents of the 5 mL culture was used to inoculate a 500 mL LB culture containing ampicillin which was incubated overnight at 37°C with vigorous shaking. The Concert™ High Purity Maxiprep Plasmid Purification System (Life Technologies) was used to produce high quality pure DNA from the 500 mL culture. This system is based on a modified alkaline lysis method to break open the cells and neutralisation to precipitate the genomic DNA and separate it from plasmid DNA. The plasmid DNA is bound to an anion exchange resin under moderate salt conditions. RNA, protein and other contaminants are washed off the column under these conditions. The plasmid DNA is eluted from the column under high salt conditions, desalted and purified by isopropanol precipitation and ethanol washing. The dried pellet is resuspended in TE buffer.

Quantification of DNA

The concentration and quality of plasmid DNA was determined using the Pharmacia Biotech Ultraspec 300 UV/visible spectrophotometer. The nucleic acid scan (200 - 350 nm) was used to assess the purity of the DNA by calculating the $A_{260/280}$ absorbance ratio. Pure DNA gives a ratio of 1.8. Ratios >1.8 indicate RNA contamination whereas ratios <1.8 indicate protein contamination.

Preparation of Vectors for Ligation Reactions

The plasmids were digested with the appropriate restriction enzymes to produce vectors with compatible ends for subsequent ligation reactions prior to the cloning of specific

vector constructs. Restriction enzyme digests were generally prepared in 30 μL or 50 μL total volumes with ~1 μg DNA, 1 x compatible buffer, and 8 – 10 units (1 μL) of enzyme. More enzyme was required on occasion, but always less than 10% of the total volume as glycerol in the enzyme could inhibit cleavage activity. The reaction was incubated at 37°C for 1 hour then analysed for completeness of digestion on a 1% agarose gel.

In a DNA ligation reaction, a phosphate ester bond forms between the 3′ hydroxyl group and the 5′ phosphate group on the ends of DNA molecules. Prior to being used in a ligation reaction, the 5′ phosphate groups are removed off the ends of the vectors to prevent recircularisation without an insert during a ligation reaction. This ensures that the conditions are more suitable for the ligation of the appropriate vector with the insert of interest which contains the only 5′ phosphate groups necessary for the joining reaction.

After digestion, 1 μL of calf alkaline phosphatase (1 U/mL) was added to the mixture and incubated at 37°C for 15 minutes to remove the 5′ phosphate groups. 2 μL of proteinase K (10 mg/mL) and 4 μL of 10% SDS was added and incubated for a further 1 hour to remove the phosphatase. The vector DNA was purified by phenol/chloroform extraction, which also removes the proteinase K, and ethanol precipitation followed by quantification on a 1% agarose gel against known DNA standards.

Phenol/Chloroform Extraction

This method is used to purify DNA away from enzymes or other proteins in solution. An equal volume of Tris-equilibrated-phenol (pH 8)/chloroform (1:1) was added to the pooled digests and mixed well using a vortex. The mixture was centrifuged at 12000 g in a microcentrifuge for 5 minutes and the clear upper aqueous phase was removed into a new tube. This step was repeated once more then an equal volume of chloroform was added to the upper aqueous phase, mixed by vortexing and centrifuged at 12000 g in a microcentrifuge for 2 minutes. The DNA contained in the upper aqueous phase was removed into a new tube for ethanol precipitation.

Ethanol Precipitation

This method was used to concentrate or desalt DNA. 1/10 volume of 3 M sodium acetate pH 5.5 and 2.5 volumes of 95% ethanol was added to the sample containing the DNA, mixed well and left to stand for 30 minutes at -70°C. The mixture was then centrifuged for 15 – 30 minutes at 12000 g in a microcentrifuge at 4°C. The pellet was washed with 500 μL of ice cold 70% ethanol and centrifuged at 12000 g for 5 minutes. The dried pellet was resuspended in 20 – 50 μL of water or TE buffer. The concentration of the resuspended DNA was determined by analysis on a 1% agarose gel against known DNA standards.

Ligation of Vector and Insert

A molar ratio of 3 insert to 1 vector was used in ligation reactions with T4 DNA ligase to increase the probability of vector to insert ligations, rather than vector to vector ligations. All ligation reactions carried out involved the joining of cohesive ends with 5′ overhangs.

A typical ligation reaction involved adding 100 – 150 ng of DNA (3 insert to 1 vector ratio) to 2 μL of 5 x T4 DNA ligase buffer (250 mM Tris-HCl pH 7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% (w/v) polyethylene glycol-8000), 1 μL of T4 DNA ligase (1 U/μL) and water to a total volume of 10 μL. The reaction mixture was mixed by pipetting up and down and incubated at 25°C for 1 hour.

Half of the reaction mixture (5 μL) was used in a transformation reaction as described previously. The resultant transformants from LB-amp plates were analysed by the rapid-boil plasmid preparation method as described. Large scale preparations of positive transformants were carried out and analysed as described previously.

Glycerol Stocks of Recombinant Plasmids

Positive transformants were preserved by freezing an aliquot of cells in 20% glycerol. A single colony was picked off an LB-amp plate and incubated in 5 mL of an LB-amp culture at 37°C overnight with vigorous shaking. A 500 μL aliquot of the overnight culture was

mixed with 500 μL of 40% glycerol and snap-frozen using liquid air or nitrogen and stored at -70°C.

DNA Sequencing of Recombinant Plasmids

An insert that had been produced by PCR was always sequenced to confirm that it was the correct DNA sequence. Therefore all plasmid constructs were quantified, diluted to the required concentration and submitted for sequencing, with the appropriate primers, on the ABI337-36 (or 64) automated DNA sequencer. Most sequence reactions were carried out using BigDye version 1.0 dideoxy-terminator chemistry. All sequences were analysed using the Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wisconsin, USA, data analysis software package.

2.2.2 Isolation and Identification of Sarcoplasmic Reticulum Vesicles

Preparation of SR Vesicles

Sarcoplasmic reticulum vesicles were prepared following the protocol of Lunde and Sejersted (1997) with modifications, and as outlined in figure 2.1. All procedures were carried out a 4°C or cooler. Frozen skeletal muscle biopsy samples (50 mg to 100 mg) were removed from storage under liquid nitrogen. They were pulverised using a steel mortar and pestle which was cooled in liquid air. The pulverised tissue was homogenised at full speed using an ultra-turax T25 homogeniser in 10 volumes of a buffer containing 10 mM Hepes pH 7.4, 20 mM KCl, Complete™ protease inhibitor mix, and either 8 mM CHAPS or 1% (w/v) Tergitol for 30 seconds. The homogenate was centrifuged at 2500 g in a microcentrifuge for 10 minutes. The supernatant (S1) was collected and the pellet resuspended in the same buffer, homogenised at full speed for 15 seconds and centrifuged in a microcentrifuge at 2500 g for 10 minutes. This step was repeated once more. The final pellet (P1) was resuspended in 1 mL of the same buffer. The three supernatants were pooled and centrifuged in a Beckman TLA-100.2 rotor at 92000 g for 180 minutes. This supernatant (S4) was collected and the pellet (P2) was resuspended in 1 mL of the buffer without detergent. SR vesicle fractions were stored at -20°C.

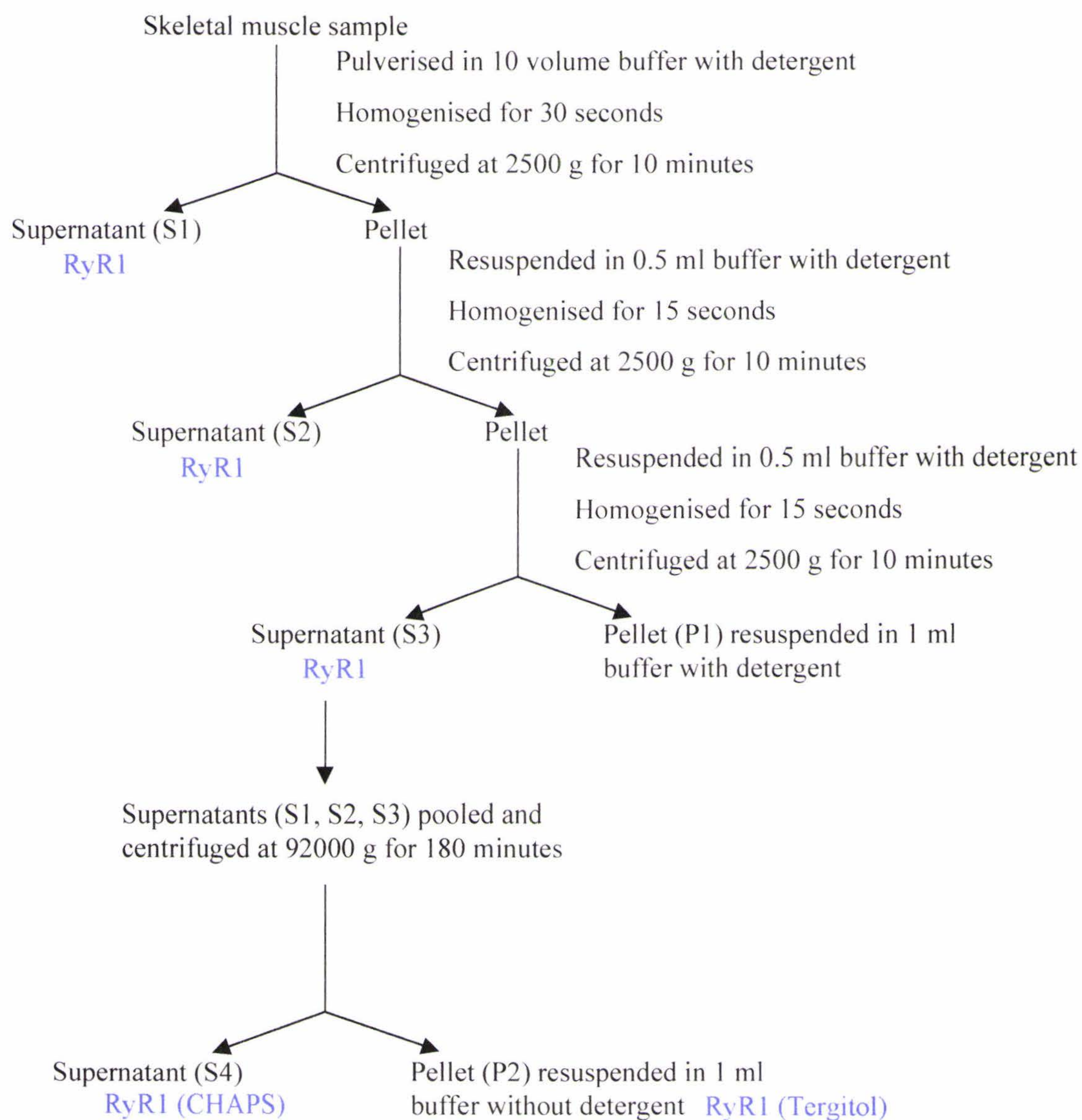

Figure 2.1: Flow diagram of SR vesicle isolation.

The expected location of the SR vesicles containing ryanodine receptors are indicated as RyR1. *CHAPS detergent was expected to solubilise ryanodine receptors more than the Tergitol detergent. Therefore ryanodine receptors would be expected to be in the S4 fraction for CHAPS solubilised receptors and in the P2 fraction for the Tergitol solubilised receptors.*

Tritiated Ryanodine Binding

^{3}H-Ryanodine (^{3}H-Ry) was used to determine the presence of ryanodine receptors in the SR vesicle preparations as specific binding of ryanodine to ryanodine receptors should occur. This method was based on that of Lunde and Sejersted (1997). SR vesicle samples containing 10 μg to 50 μg of total protein were incubated in a buffer containing 10 mM Tris pH 8, 150 mM KCl, 3 mM Na_2ATP, 2.5 μM $CaCl_2$, 20 nM ^{3}H-Ry and Complete™ protease inhibitor mix for 180 minutes at 37°C. The samples were diluted with 5 mL of ice-cold 10% ethanol. Unbound ^{3}H-Ry was removed by filtering the samples through a Whatman GC/F glass fibre filter using a Millipore sampling manifold. The filters were washed with 5 mL of ice-cold 10% ethanol, air dried and placed in a scintillation vial. 3 mL of scintillation fluid was added and the degree of radioactivity was measured by liquid scintillation counting. A control containing buffer only was used in each experiment to determine the level of background binding of ^{3}H-Ry to the filters. Where able, each experiment was carried out in triplicate.

Sucrose Gradient Purification of Sarcoplasmic Reticulum Vesicles

Partial purification of KCl extracted SR vesicles on a discontinuous (22%/34%/45%) sucrose gradient was carried out as described by O'Driscoll *et al* (1996). A frozen muscle sample was minced and homogenised in 5 volumes of a buffer containing 5 mM Tris-Maleate pH 6.8, 0.1 M NaCl and Complete™ protease inhibitor mix for 60 seconds using an ultra-turax T25 homogeniser. It was then centrifuged for 30 minutes at 2600 g in a Sorvall SS34 rotor. The supernatant was centrifuged at 10000 g for 30 minutes in a Sorvall SS34 rotor. The pellet was resuspended in 60 mL of buffer containing 5 mM Tris-Mes pH 6.8, 0.6 M KCl, and Complete™ protease inhibitor mix and centrifuged at 130000 g for 60 minutes in a Beckman T865 fixed angle rotor. The pellets were resuspended in 5 mL of buffer containing 5 mM Tris-Mes pH 6.8, 10% sucrose, 0.4 M KCl, 20 μM $CaCl_2$ and Complete™ protease inhibitor mix. The resuspended pellets were layered on top of a 22%/34%/45% sucrose gradient and centrifuged at 87000 g overnight in a Beckman TV50 rotor. The heavy SR (HSR) vesicles containing ryanodine receptors were recovered from the 34%/45% interface and centrifuged at 124000 g for 60 minutes in a Beckman Ti50 rotor. The pellets were resuspended in 50 μL of a buffer containing 10 mM K-Pipes pH 7,

0.3 M sucrose, 0.1 M KCl and Complete™ protease inhibitor mix and stored at -20°C. All steps in the procedure were carried out at 4°C.

2.2.3 Isolation of RNA from Skeletal Muscle

Isolation of RNA

RNA was isolated from skeletal muscle samples using Trizol™ LS reagent according to the manufacturer's instructions (Life Technologies). The Trizol™ LS reagent contains phenol and guanidine thiocyanate which maintain the integrity of RNA during homogenisation while disrupting cells and cellular components. By adding chloroform and after centrifugation, the solution separates into two phases, an aqueous phase containing RNA and an organic phase. The RNA was removed from the aqueous phase by precipitation with isopropanol. The RNA pellet was washed with ice-cold 75% ethanol. The pellet was then allowed to air-dry before being resuspended in RNase-free water and stored at -20°C.

Quantification and Analysis of RNA

RNA was quantified on a spectrophotometer using the $A_{260/280}$ ratio. Pure RNA has an $A_{260/280}$ ratio of 2.0. Following quantification, the RNA was concentrated by ethanol precipitation for loading a small volume on a gel. 0.1 volume of 2 M potassium acetate and 2.5 volumes of 100% ethanol was added to the RNA, mixed well and allowed to stand for 30 minutes at -70°C. The RNA was pelleted by centrifugation at 12000 g in a microfuge for 15 minutes at 4°C, allowed to dry and resuspended in 20 – 50 μL of RNase-free water. 5 μg to 15 μg of RNA was analysed on a 1.5% agarose gel containing 17.6% formaldehyde in a buffer containing 1 x MOPS (1 M MOPS, 250 mM sodium acetate, 5 mM EDTA, pH 7) at 100 mA for 3 – 4 hours.

2.2.4 Northern Blot Analysis

Preparation of Northern Blot

After electrophoresis and visualisation with a UV transilluminator to confirm the presence of RNA on the gel, the gel was washed in 20 x SSC (3 M NaCl, 0.3 M trisodium citrate) while the blotting apparatus was assembled.

Northern blotting uses capillary action to transfer the RNA from an agarose gel onto the membrane. A shallow container was filled to a depth of ~2 cm with 20 x SSC, and a glass plate was placed across the top. Two sheets of Whatman 3MM paper were wet in 20 x SSC and placed across the glass plate to form wicks into the SSC. The gel was placed upside down on the Whatman 3MM paper so the RNA is closer to the membrane for transfer, and a corner was cut off the gel for identification. A piece of nitrocellulose membrane on a nylon base was cut the same size as the gel, wet in 20 x SSC and placed on top of the gel. Two sheets of Whatman 3MM paper wet in 20 x SSC followed by two dry sheets were placed on top of the membrane. The gel was masked with saran wrap to prevent bypassing of the gel. Finally a stack of paper towels and an approximately 500 g weight was placed on top of the Whatman 3MM paper to facilitate the transfer of RNA by capillary action.

The apparatus was left for 24 – 48 hours to allow for sufficient transfer of RNA onto the membrane. Once disassembled, the membrane was dried between blotting paper and exposed to UV radiation on the transilluminator for 30 seconds to strengthen the non-covalent but irreversible attachment of RNA to the membrane by the formation of a small number of cross-links between the bases in the RNA and the membrane (Sambrook *et al.*, 1989). The dried blot was stored desiccated in an air-tight container until used for hybridisation.

Preparation of DNA Probe

A DNA probe was made from a genomic clone (R2543W) containing a 225 bp portion of the ryanodine receptor gene within the pGEM3Z plasmid. The multiple cloning site in pGEM3Z vectors is flanked by the T7 and Sp6 primer binding sites, therefore these primers

were used in a PCR reaction to amplify the portion of the ryanodine receptor gene for purification.

A PCR reaction was prepared in a 0.5 mL tube containing 5 μL of 10x buffer, 0.5 – 3.5 mM $MgCl_2$, 5 μL of 2.5 mM dNTPs, 5 μL of 50 ng/μL of each primer, 2 μL of plasmid DNA (1 ng/μL), 1.5 μL of *REDTaq™* DNA polymerase (1 U/μL) and H_2O to 50 μL. The reagents were mixed well, a drop of mineral oil placed on top to prevent evaporation at high temperatures and the tubes placed in a thermal cycler. The reaction was heated to 95°C for 5 minutes then the PCR was carried out for 30 cycles of 95°C denaturing for 1 minute, 50°C primer annealing to single-stranded DNA template for 1 minute, and 72°C DNA synthesis and elongation for 1 minute.

The PCR reaction was analysed on a 1% agarose gel at 100 V for 1 hour and purified using the High Pure PCR Purification Kit (Roche) according to the manufacturer's instructions. This method is based on the binding of DNA molecules to the surface of pre-treated glass fibre fleece columns. Binding of DNA occurs instantaneously and impurities are washed away. The DNA is eluted from the column with a low salt buffer or water.

The purified product was quantified on a 1% agarose gel against a set of known DNA concentration standards. The gel was stained in an ethidium bromide bath containing 5 μg/mL of ethidium bromide for 15 minutes then visualised using a UV transilluminator. The quantified DNA was stored at -20°C prior to labelling for hybridisation to the northern blot.

Labelling a DNA Probe

The DNA probe was labelled according to the Ready-To-Go™ DNA Labelling Beads (-dCTP) instructions (Amersham Pharmacia). Approximately 35 ng of DNA (1 μL) was added to 44 μL of TE (10 mM Tris-HCl, pH 8, 1 mM EDTA) buffer. This mixture was denatured in a boiling water bath for 3 minutes then plunged into ice for 2 minutes to prevent reannealing of the DNA. The denatured DNA and 5 μL of [α-^{32}P]dCTP (10 μCi/μL) were added to the reaction mix bead (buffer, dATP, dGTP, dTTP, FPLC*pure*™

Klenow Fragment (7 – 12 units) and random 9-mer oligodeoxyribonucleotides). The mixture was pipetted up and down to mix, pulse centrifuged and incubated at 37°C for 20 minutes.

Unincorporated labelled nucleotides were removed using ProbeQuant™ G-50 Micro Columns (Amersham Pharmacia). The unincorporated nucleotides were retained on the column and the purified label was collected in the bottom of the collection tube after centrifugation.

Monitoring the Progress of the Reaction

A 2 μL aliquot of labelled reaction mix (B) and a 2 μL aliquot of purified label mix (A) were diluted in 500 μL of water. 5 μL of each mix was spotted onto a Whatman GF/C filter and allowed to dry. The filters were placed in scintillation vials and 3 mL of scintillation fluid was added. The amount of radioactivity was determined using a ^{32}P window on a Beckman liquid scintillation counter.

The radioactivity on filter B represented the total amount in 5 μL of diluted reaction mix. The radioactivity on filter A represented the amount that was incorporated into the DNA.

$$\text{Incorporation of radioactivity} = \frac{\text{cpm on A} \times 100\%}{\text{cpm on B}}$$

$$\text{Specific activity of the labelled probe} = \frac{\text{cpm on A} \times 250\ (\text{DF}) \times 1000}{\text{ng of DNA}}$$

The probe was ready for hybridisation once the success of labelling had been confirmed and the specific activity of the labelled probe had been calculated.

Prehybridisation

The dried nitrocellulose membrane was wet by capillary action with 2 x SSC (0.3 M NaCl, 0.03 M trisodium citrate) then prehybridised for 3 hours at 68°C in ~20 mL of prehybridisation solution (6 x SSC (0.9 M NaCl, 0.09 M trisodium citrate)) with 1 x Denhardts reagent (0.02% Ficoll, 0.02% polyvinylpyrolidone, 0.02% BSA) in a rotary oven.

Hybridisation

Hybridisation was carried out in glass tubes in a rotary oven. The prehybridisation solution was removed and 20 mL of hybridisation solution (1 M NaCl, 50 mM phosphate, 2 mM EDTA, 0.5% SDS, 1 x Denhardts reagent, pH 6.5) was added. The labelled probe was boiled for 5 minutes to denature it then plunged into ice for 5 minutes to ensure it remained single-stranded before being added directly to the hybridisation solution. The hybridisation reaction was left to occur at 68°C overnight.

Washings

The labelled probe in the hybridisation solution was poured off and discarded appropriately. The first wash was carried out in glass tubes with subsequent washes carried out in plastic containers in the hybridisation oven. The membranes were washed twice in 6 x SSC with 1% SDS for 1 hour, then washed twice in 6 x SSC with 0.5% SDS for 30 minutes at 68°C. The final high stringency wash was carried out in preheated 1 x SSC (0.15 M NaCl, 0.015 M trisodium citrate) for exactly 30 minutes at 68°C. The 1 x SSC was poured off and the membrane left to air dry. The membrane was wrapped in saran wrap and exposed to X-ray film for 48 – 72 hours in an autoradiography cassette with two intensifying screens at -70°C before being developed in an automated developer.

2.2.5 Preparation for Production of Recombinant cDNA Constructs

Reverse Transcriptase Polymerase Chain Reaction

Moloney murine leukemia virus reverse transcriptase (M-MLV RT) contains a DNA polymerase capable of synthesising first strand cDNA from total RNA. It lacks DNA endonuclease activity and therefore does not degrade the DNA once it is made but contains RNase H activity which removes the RNA from RNA:cDNA hybrids once the DNA is synthesised.

All procedures involving RNA were carried out using pipette tips, microfuge tubes, glassware and water which had been treated with 0.01% DEPC by soaking overnight and then autoclaving to remove traces of DEPC. All solutions and equipment were reserved specifically for RNA work to prevent contamination by RNases.

Reactions were prepared as follows in a total volume of 20 µL: 1 µg total RNA, 1 x M-MLV RT buffer, 2 µL of 0.1 M DTT, 2 µL of 10 mM dNTPs, 0.25 µL of 500 ng/µL oligo(dT) primer, 0.1 µL of 200 U/µL M-MLV RT enzyme and DEPC treated water. The RNA and water were combined, heated to 65°C for 10 minutes to denature the RNA, pulse centrifuged and placed on ice. The rest of the components were added to the RNA and water, mixed and incubated at 37°C for 1 hour for synthesis of cDNA. The reaction was terminated by heat deactivation of the RT enzyme at 95°C for 5 minutes. The reaction was then placed on ice and used in a PCR reaction or stored at -20°C.

A standard PCR reaction using *REDtaq™* DNA polymerase was prepared and placed in a thermal cycler at: 96°C for 2 minutes, 30 cycles of 96°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute, with an extra elongation time of 2 minutes at 72°C. The PCR products were analysed on a 1% agarose gel. A diagnostic digest using unique restriction enzyme sites within the fragment was carried out to confirm that the PCR product was correct. This was also analysed on a 1% agarose gel.

After confirming that the PCR product was the correct size, it was digested with the appropriate cloning enzymes and purified using the High Pure PCR Purification kit as

described previously. The purified product was the 'insert of interest' used in subsequent ligation reactions with the expression vectors.

2.2.6 Expression of the cDNA Vector Constructs in E. Coli Cells

Induction of Cells Containing the Plasmid Constructs

E. coli BL21-DE3 cells were transformed with the plasmid constructs as described previously for *E. coli* XL-1 blue cells except that 200 – 300 ng of DNA was used as *E. coli* BL21-DE3 cells are not as competent to take up plasmid DNA as *E. coli* XL-1 blue cells.

E. coli BL21-DE3 cells are used for high level expression of cloned genes in vectors containing the bacteriophage T7 promoter. The bacteriophage T7 RNA promoter is integrated into the chromosome of BL21. T7 RNA polymerase is very selective for its own promoters which don't occur naturally in *E. coli.* T7 RNA polymerase also produces RNA chains about five times faster than *E. coli* RNA polymerase (Studier and Moffat, 1986). However, this feature of *E. coli* BL21-DE3 cells was not required for expression using pGEX-6P-3 or pProEX-HTb systems as they do not contain a T7 promoter. But high level expression should still be attainable with the *E. coli* BL21-DE3 cell line. (Studier and Moffat, 1986).

GST fusion proteins were produced in the *E. coli* BL21-DE3 cells that were transformed with recombinant pGEX plasmids. The expression of protein from the pGEX plasmid was under the control of the *tac* promoter. This promoter is chemically inducible by IPTG to give high level expression of the fusion protein.

A colony from a plate of *E. coli* BL21-DE3 transformed cells was used to inoculate a 5 mL LB-amp culture and incubated overnight at 37°C with vigorous shaking. The optical density (OD) at A_{600} was determined. An aliquot was added to fresh LB-amp cultures to an OD of 0.1 in each 5 mL culture. These tubes were incubated for approximately 2 hours at 37°C with vigorous shaking until an OD of between 0.6 and 0.8 at A_{600} was reached. The cultures were induced with IPTG to a concentration of 0.2 mM and incubated at various

temperatures with vigorous shaking. A 1 mL aliquot was taken after 3 hours and after overnight (~16 hours) incubation to assess the degree of induction.

Cell extracts were prepared as follows. The 1 mL aliquots were centrifuged at 12000 g in a microcentrifuge for 1 minute to pellet cellular material. The pellets from the 3 hour inductions were stored at -70°C until the next day when they were processed with the overnight inductions. The pellets were resuspended in 100 μL or 200 μL of 1 x PBS (8 g/L NaCl, 0.2 g/L KCl, 0.38 g/L $Na_2HPO_4.2H_2O$, 0.2 g/L KH_2PO_4, pH 7.2). The cells were broken open by sonication on ice for 10 seconds. The sonicate was centrifuged at 12000 g in a microcentrifuge for 5 minutes at 4°C to separate the insoluble proteins in the total cellular fraction. The supernatants were transferred to a new tube and the pellets were resuspended in the same amount of 1 x PBS as previously. A 1:1 ratio of sample solution to 2 x transfer dye for protein gels (2.5 mL stacking gel buffer (6g Tris in 100 mL H_2O, pH 8.8), 4 mL 10% SDS, 2 mL glycerol, 1 mL β-mercaptoethanol, 0.5 mL water and a few grains of bromophenol blue indicator dye) was combined and boiled in a boiling water bath for 3 – 5 minutes. 7.5 μL was loaded along with molecular weight markers and analysed on a 12% SDS polyacrylamide gel at 200 V for ~40 minutes. The gels were stained with Coomassie Blue dye and destained as described previously.

Cleavage of the GST-fusion Protein

The GST fusion protein was purified from bacterial lysates by using the affinity matrix Glutathione Sepharose 4B. The fusion protein binds tightly to the Glutathione Sepharose via the binding site of glutathione S-transferase (GST). Once the fusion proteins were bound to the matrix the protein of interest was able to be cleaved away from GST and analysed.

The protease specific to pGEX-6P-3 vectors is PreScission™ Protease. It specifically recognises the amino acid sequence Leu-Glu-Val-Leu-Phe-Gln↓Gly-Pro which is located between the GST domain and the multiple cloning site. This enables the expressed protein of interest to be totally cleaved away from GST.

After sonication and centrifugation of the cell extracts, the pellet was resuspended in 1 x PBS. A 20 μL or 40 μL volume of Glutathione Sepharose4B resin equilibrated in 1 x PBS was added to 100 μL of the resuspended pellet. This was mixed gently on an orbital shaker for 10 minutes. 100 μL of 1 x PBS was added, mixed by vortexing briefly and centrifuged in a microcentrifuge at 12000 g for 5 seconds. An aliquot of supernatant was reserved as the 'unbound' sample to compare with the 'bound' sample. The rest of the supernatant was discarded. The washing step was repeated twice more. After the final spin, the resin was resuspended in an equal volume of 1 x PBS to the resin used initially. 15 μL of each sample was analysed on a 12% SDS polyacrylamide gel against a 7.5 μL sample of molecular weight markers and a GST extract control.

To cleave the protein of interest, 0.5 – 1.5 μL of PreScission™ protease (2 U/μL) and 1 mM DTT was added to the bound fusion protein. The digest was carried out at 4°C on a shaker for 24 – 48 hours. Aliquots of appropriate samples were analysed on a 16.5% tricine gel against molecular weight markers.

Tricine SDS-PAGE is a discontinuous system based on the glycine SDS-PAGE of the Laemmli (1970) method. It uses tricine as the trailing ion and a different composition of gel and buffers to effectively separate proteins in the low molecular weight range of 1 – 100 kDa. (Schagger and Von Jagow, 1987). There were three layers in the gel. A separating gel (16.5 mL 40% acrylamide:bis stock, 13.2 mL buffer A (3 M Tris, 0.3% SDS, pH 8.45), 8.4 mL 50% glycerol, 1.9 mL water, 13.2 μL TEMED, 132 μL 20% ammonium persulfate), spacer gel (3.125 mL 40% acrylamide:bis stock, 4.2 mL buffer A, 5.175 mL water, 6.25 μL TEMED, 62.5 μL 20% ammonium persulfate), and a stacking gel (1.25 mL 40% acrylamide:bis stock, 3.1 mL buffer A, 8.15 mL water, 6.2 μL TEMED, 63 μL 20% ammonium persulfate). The gel was electrophoresed using 1 x cathode buffer prepared as 10 x stock (1 M Tris, 1 M tricine, 1% SDS, pH 8.25) and 1 x anode buffer prepared as a 5 x stock (1 M Tris, pH 8.9)

2.2.7 Expression of cDNA Vector Constructs in Mammalian Cells

Maintenance of mammalian cell lines requires the use of sterile equipment and techniques, therefore all procedures for tissue culture were carried out in a laminar flow workstation.

Preparation of Tissue Culture Media

Cells were grown in T80 vented tissue culture flasks using Eagles minimum essential media (MEM). The media was prepared according to the manufacturer's instructions. 2.2 g/L sodium bicarbonate was added and the pH adjusted to 6.8 to give a final pH of 7.1 after filter sterilisation using a 0.22 micron filter. The media was supplemented prior to use by the addition of 10% fetal calf serum and 1% penicillin (5000 U/mL) – streptomycin (5 mg/mL). The media was stored at 4°C but equilibrated to room temperature prior to use.

Beginning Cell Cultures from Frozen Stocks

COS cells were started from frozen stocks stored in 10% DMSO in FCS under liquid nitrogen. The frozen cells were thawed quickly, added to 5 mL of media and centrifuged for 2 minutes at low speed in a bench-top clinical centrifuge. The supernatant was discarded and the pellet was resuspended in 2 mL of complete media. Half of the suspension was added to each of two T80 vented flasks containing 12 mL of complete media. The flasks were incubated in a 37°C, 5% CO_2 incubator in a humidified atmosphere.

Maintenance of COS Cells

COS cells were grown to an 80 – 100% confluent monolayer in T80 flasks containing 12 mL of complete media. The media was changed every 2 – 3 days prior to the attainment of confluence. Once the required confluence was reached, the cells were passed into new flasks to maintain stocks of cells, or onto plates for transfections, by the following method.

The media was removed and the cells were washed twice with 2 mL of 1 x trypsin in PBSE (140 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.2H_2O$, 0.5 mM EDTA). The trypsin solution was removed and the flasks left flat for 5 – 10 minutes. The flasks were strongly

knocked to dislodge the cells from the bottom of the flask, as determined by microscopy. The free cells were resuspended in 5 mL of complete media. Half a mL of cell suspension was added to fresh T80 flasks containing 12 mL of complete media for maintenance of the COS cell line. 2 mL was added to 150 mm plates containing 15 mL of complete media for transfection reactions. The flasks and plates were placed in the 37°C, 5% CO_2 incubator.

Freezing Cells for Storage

COS cells were frozen for storage under liquid N_2 to ensure supply for future use. The cells were grown to 100% confluence and passed as described but were resuspended in 2 mL of FCS containing 10% DMSO. The resuspended cells from one T80 flask were aliquotted into two 1 mL cryotubes. They were slowly frozen at -70°C overnight prior to transferring to liquid N_2 for long term storage.

Transient Transfections

The non-liposomal transfection reagent FuGENE™ 6 was used to transfect the COS cells. This reagent was formulated to produce high levels of transfection while minimising toxicity to the cells. The procedure followed was essentially that which was detailed in the manufacturer's brochure.

The day before transfection, cells were passed into 150 mm plates containing 15 mL of complete media and incubated overnight in the 37°C, 5% CO_2 incubator. This would give cell growth of a monolayer of 50 – 80% confluency. The maximum recommended ratio of 2 μg DNA : 3 μL FuGENE™ 6 reagent was used.

85 μL of serum free (uncomplete) media was added to sterile microfuge tubes. Then 15 μL of FuGENE™ 6 reagent was added directly to the media without touching the sides of the tube as FuGENE™ will adsorb to the plastic. This mixture was incubated at room temperature for 5 minutes. 10 μg of DNA was placed in a second tube. The diluted FuGENE™ 6 reagent was added drop by drop to the DNA, tapped gently to mix and incubated at room temperature for 15 minutes.

COS cells were transfected by adding the DNA/FuGENE™ complex dropwise to the 150 mm plates containing 50 – 80% confluent COS cells. The plates were swirled to mix the DNA/FuGENE™ evenly over the cells. The plates were incubated in the 37°C, 5% CO_2 incubator for 48 hours.

Harvesting

Cells were harvested as described by Chen *et al* (1993a) with modifications as described below. This method preserves the microsomal fractions while disrupting cell membranes. All steps in the procedure were carried out on ice or at 4°C. Three 150 mm plates were used for each sample.

The media was removed from each plate and the cells washed twice with 3 mL of 1 x PBS (140 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.2H_2O$). The cells were harvested in 1 mL PBSE with 1 x Complete™ protease inhibitor by scraping. Cells were collected in microfuge tubes and centrifuged in a microcentrifuge at 13000 rpm at 4°C for 10 minutes. The pellet was washed in 1 mL of 1 x PBS with 1 x Complete™ protease inhibitor and recentrifuged for 10 minutes. The pellets were resuspended in 100 μL of a buffer containing 25 mM Tris-HEPES pH 7.4, 0.5 mM $MgCl_2$, 1 x Complete™ protease inhibitor. Each triplicate was combined and the cell membrane was disrupted by homogenising with 50 strokes in a glass homogeniser or by 2 cycles of freeze-thawing using liquid N_2. An equal volume (300 μL) of a buffer containing 25 mM Tris-HEPES pH 7.4, 0.5 M sucrose, 0.3 M KCl, 40 μM $CaCl_2$, 1 x Complete™ protease inhibitor was added to the cells. They were then homogenised with 25 strokes in a glass homogeniser, or subjected to two further cycles of freeze-thawing using liquid N_2. The cell suspension was centrifuged at 4340 g in a Beckman TLA-100 rotor for 30 minutes. The supernatants were removed and centrifuged at 80000 g in a Beckman TLA-100 rotor for 90 minutes. The pellets were resuspended in 100 μL of a buffer containing 25 mM Tris-HEPES pH 7.4, 0.3 M sucrose, 0.15 M KCl, 20 μM $CaCl_2$, 1 x Complete™ protease inhibitor. The proteins in the cell extract were analysed on an 8% polyacrylamide gel as described previously. The cell extracts were stored at -70°C.

CHAPTER THREE – ISOLATION OF SARCOPLASMIC RETICULUM VESICLES

3.1 Introduction

SR vesicles were isolated from skeletal muscles to allow the study of ryanodine binding and calcium release from whole, intact ryanodine receptors. By using the whole receptor, the effects of modulators of the channel could be more effectively studied between wild type and mutant channels.

The work described in this chapter is primarily based on the methods of Lunde and Sejersted (1997). These authors devised a method for isolating sarcoplasmic reticulum (SR) vesicles containing ryanodine receptors from small skeletal muscle biopsies. This would have been an ideal method to use for the small amount of muscle biopsy tissue available.

The C-terminal region of the ryanodine receptor is anchored in the membrane of the terminal cisternae of the SR. The cytoplasmic domain spans the region between the SR and the transverse tubule of the muscle cell plasma membrane. When a preparation of skeletal muscle cells is homogenised, portions of the SR reform into vesicles. These vesicles should contain ryanodine receptors and can be isolated by a series of centrifugation steps (Lunde and Sejersted, 1997). Alternatively a crudely prepared SR membrane fraction can be partially purified on a discontinuous sucrose gradient (Meissner, 1984; Mickelson *et al.*, 1986; O'Driscoll *et al.*, 1996). Both these methods were employed in an attempt to isolate SR vesicles containing ryanodine receptors that could be identified by the binding of ^{3}H-Ry.

3.2 Isolation of Sarcoplasmic Reticulum Vesicles by Homogenisation and Centrifugation

50 – 100 mg samples of rat skeletal muscle tissue were used in the isolation of SR vesicles. Two different detergents, tergitol and CHAPS, were used to compare their solubilisation

properties on the ryanodine receptors. CHAPS has been reported to cause more complete solubilisation of the receptors with no receptors detected in the P1 fraction and a significant amount in the S4 fraction (refer to Figure 2.1). Tergitol does not completely solubilise the receptors as most can be found in the P2 fraction (refer to Figure 2.1) (Lunde and Sejersted, 1997).

Each fraction was analysed on a 10% SDS polyacrylamide gel to confirm that a range of proteins were present in each fraction. Coomassie staining can detect 40 – 50 ng of protein per band on a gel (Promega Protein Guide Tips and Techniques Manual). Silver staining is more sensitive and can detect as low as 1 – 5 ng of protein per band on an SDS polyacrylamide gel (Promega Protein Guide Tips and Techniques Manual). Both staining techniques were used to observe the clear separation of proteins in each fraction.

The presence of ryanodine receptors in each fraction was determined by the binding of ^{3}H-Ry. Ryanodine (a plant alkaloid) binds specifically to the ryanodine receptor. The degree to which ^{3}H-Ry binds can be determined by measuring the level of radioactivity in each fraction by counting in a liquid scintillation counter.

In the first ^{3}H-Ry binding assay, 10 μg of total protein was used in each sample. The control with no vesicles, and hence no ryanodine receptors, showed a small amount of non-specific binding of ^{3}H-Ry to the filter. After correcting for the control, no binding above background was observed in any of the samples. As the amount of protein in the sample may have been limiting, a second isolation was carried out using twice as much starting muscle but keeping the sample volumes the same as in the first isolation. The amount of total protein was increased to 50 μg in each sample in the second ^{3}H-Ry binding assay. Again no significant ryanodine binding was observed. The unfractionated sample would be expected to give the highest cpm, as any ryanodine receptors should be present in this fraction before starting the isolation. But this fraction was very similar to the control with no vesicles (Figure 3.1). All results were $<1\%$ of the total ^{3}H-Ry control, lower than reported values (calculations not shown. Raw data in appendix 1). Lunde and Sejersted (1997) obtained $12.9 \pm 1.9\%$ ^{3}H-Ry binding in P1, $87.5 \pm 2.1\%$ in P2 and no ^{3}H-Ry binding

in S4 in their samples isolated in the buffer containing Tergitol. They did not show this data for the samples isolated in the buffer containing CHAPS.

All experiments thus far were carried out using rat skeletal muscle tissue samples. The ultimate aim was to analyse ryanodine receptors in human muscle, therefore an assay using a human skeletal muscle biopsy sample was carried out to determine whether the difficulties with ^{3}H-Ry binding were primarily due to a problem with rat muscle. A 50 mg sample of human skeletal muscle tissue was homogenised with the buffer containing tergitol detergent. Fresh buffer containing ATP was used for the ^{3}H-Ry binding assay. There was also no significant difference between the human sample and the control containing no vesicles, as there was with rat muscle (Figure 3.1).

Ryanodine has been shown to bind preferentially to ryanodine receptors when the receptors are in their open state (Coronado *et al.*, 1994; Meissner, 1986). Receptors in the SR vesicles may not have been in their open state as no ^{3}H-Ry appeared to bind to them, as observed by the sample fractions that had a very similar cpm as the control. ATP promotes the opening of the channels so that ryanodine can bind. The ATP used may not have been stable in the buffer solution at the temperature used as hydrolysis occurs at 0°C. ATP may have been depleted in the time between when the first and second ^{3}H-Ry binding assays were carried out, therefore there may have been insufficient ATP available to promote opening of the channels for ryanodine to bind. The calcium concentration can also affect the activity of the receptors in inhibiting or activating the binding of ryanodine if the receptor is in the open or closed state (reviewed in (Mickelson and Louis, 1996). Therefore, if the ryanodine receptor-calcium channels were in the open conformation, there should have been differences in the ^{3}H-Ry binding (cpm) between the control and fractionated samples. As no significant difference was observed, it would suggest that the ryanodine receptor channels were either closed or present in insufficient numbers to detect.

^{3}H-Ry binding to ryanodine receptors is dependent on the calcium concentration (Mickelson and Louis, 1996). According to these authors, the optimum calcium concentration for ryanodine binding is 6 μM, or in the range of 3 – 10 μM which covers the peak of a bell-shaped curve of calcium concentration against bound ryanodine. Therefore

the calcium concentration in the ^{3}H-Ry binding buffer was altered to cover a range around the 'optimum' concentration and included 0.25, 1, 2.5, 10 and 25 μM of calcium. The binding assay was carried out using the unfractionated tergitol-solubilised sample. All ryanodine receptors should initially be in the unfractionated sample and as tergitol does not completely solubilise the receptors (Lunde and Sejersted, 1997) they should be in their native state. Again there was no significant difference in the amount of ^{3}H-Ry bound to the receptors when different calcium concentrations were used (Figure 3.2).

An assay carried out with increasing amounts of total protein in the samples for ^{3}H-Ry binding also demonstrated no significant differences (graph not shown).

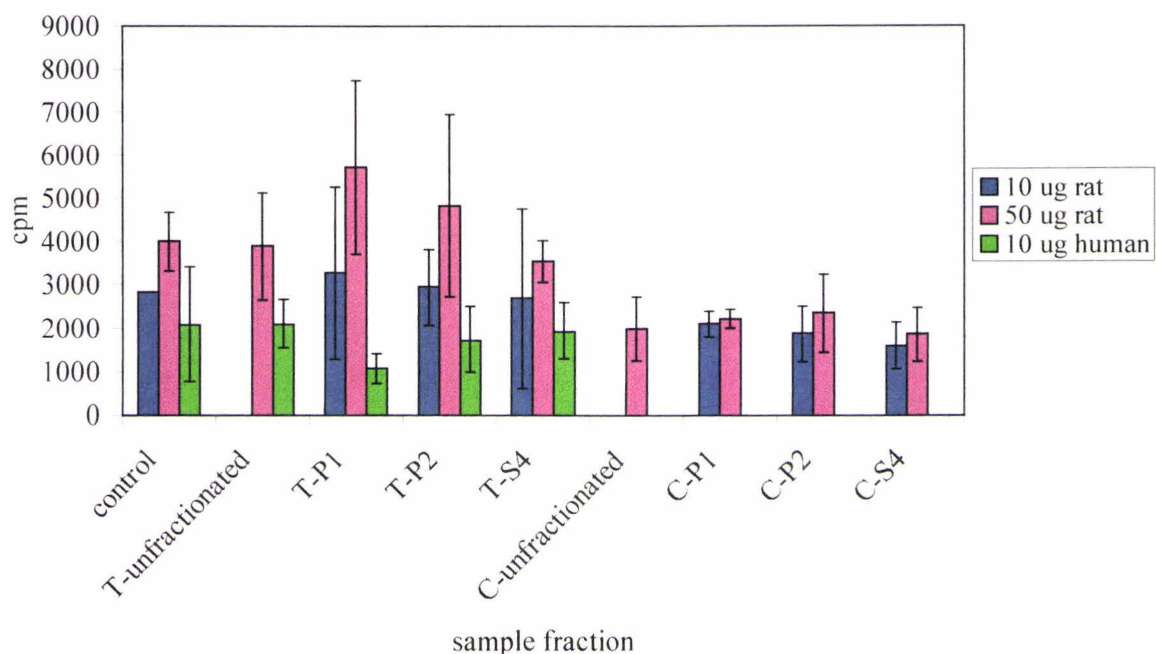

Figure 3.1: The initial ^{3}H-Ry binding assays.

Graph of three separate experiments using 10 μg or 50 μg protein from rat muscle or 10 μg protein from human muscle as indicated in the legend. Each experiment was carried out in triplicate. 'T' indicates the samples solubilised with tergitol detergent, 'C' for those solubilised with CHAPS. P1, P2, and S4 are the respective fractions obtained at specific steps in the isolation procedure as outlined in section 2.2.2. T- and C-unfractionated samples were omitted during the preparation. The total ^{3}H-Ry control was excluded from the graph as it was too large a value for the entire graph to be read clearly.

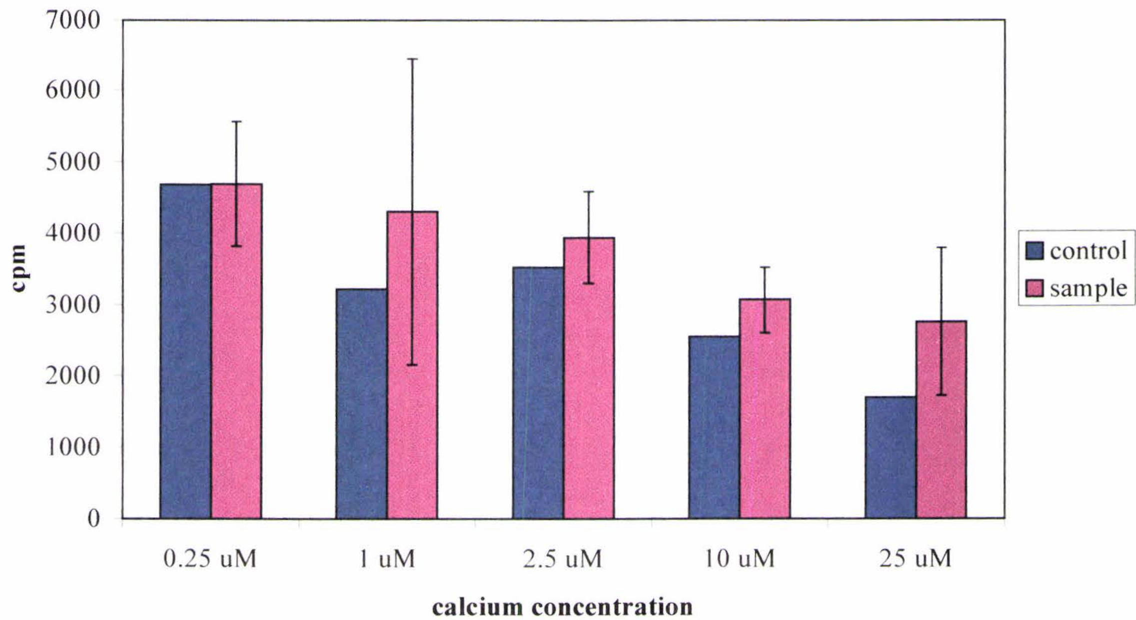

Figure 3.2: A change in calcium concentration in the ^{3}H-Ry binding assays.

The different calcium concentrations used in the ^{3}H-Ry binding buffer are as indicated. These are the results of one experiment carried out in triplicate. The total ^{3}H-Ry control was omitted as it made the rest of the graph unreadable due to its large value.

3.3 Isolation of Sarcoplasmic Reticulum Vesicles by Discontinuous Sucrose Gradient

The previous method did not appear to be effective using small skeletal muscle biopsy samples. A partial purification of KCl extracted SR vesicles on a discontinuous sucrose gradient was used (Meissner, 1984; Mickelson *et al.*, 1986; O'Driscoll *et al.*, 1996) to determine if a larger amount of muscle tissue was required to produce sufficient numbers of ryanodine receptors to demonstrate specific binding of ryanodine.

This method produces a more purified sample of ryanodine receptors out of a mixture of proteins. 12 g of rat muscle tissue was used as starting material and only one fraction was obtained off the sucrose gradient which concentrated all of the ryanodine receptors. A 10 μg and 50 μg protein sample was used in the ^{3}H-Ry binding assay. Using this amount of protein would mean that each sample should contain more ryanodine receptors than in the equivalent samples of the previous method, as there were fewer other proteins contributing to the total protein concentration due to the partial purification step.

The results of the ryanodine binding assay using 10 μg and 50 μg of total protein were similar to those obtained earlier. A binding assay using 135 μg of total protein demonstrated marginally improved ^{3}H-Ry binding than previously as it was approximately twice the value of the control (Figure 3.3). There was slightly better ^{3}H-Ry binding with increasing amounts of total protein, but it was still not significant compared to the total ^{3}H-Ry control. The samples were all $<2\%$ of the total ^{3}H-Ry, very much lower than values reported in the literature. Lunde and Sejersted (1997) obtained $12.9 \pm 1.9\%$ ^{3}H-Ry binding in P1, $87.5 \pm 2.1\%$ in P2 and no ^{3}H-Ry binding in S4.

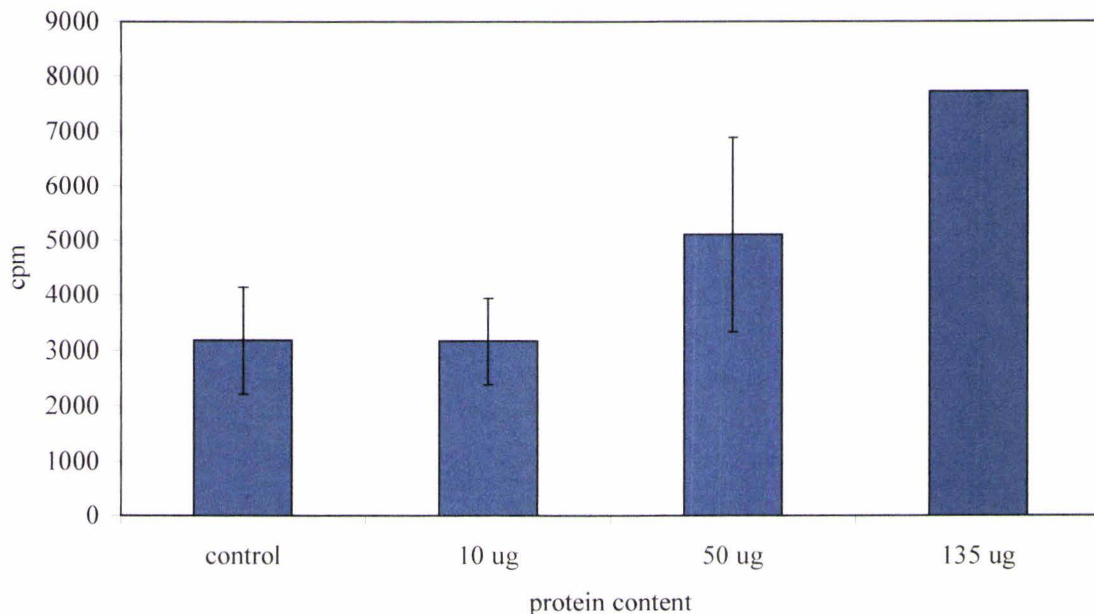

Figure 3.3: The effect of increasing amounts of total protein on ^{3}H-Ry binding.

Increasing amounts of total protein in the samples partially purified by a discontinuous sucrose gradient were assayed. These are the results of one experiment carried out in triplicate except the sample containing 135 μg of protein. This is a single experiment as there was insufficient material available. ^{3}H-Ry control values were not included.

Although these results from the partial purification of ryanodine receptors were slightly improved on the previous method, ~12 g of starting muscle tissue was used. This was not feasible for human muscle as 50 – 100 mg of tissue would be available.

The method used here was very similar to other published methods. A personal communication from Dr. A. Herrmann-Frank (University of Ulm, Germany) suggested to ensure that the purification procedures were carried out at 4°C and that protease inhibitors were used, as high-affinity ryanodine binding was highly proteolysis sensitive. All procedures had been carried out at 4°C or lower, and a Complete™ protease inhibitor cocktail had been used in all the buffers.

3.4 Improvements on Isolation by Homogenisation and Centrifugation

A personal communication with Tom Nelson, Department of Anesthesia, The Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, North Carolina, USA., disclosed that his group had been carrying out similar experiments. They had discovered that ATP was hydrolysed very quickly so that the ryanodine receptor channels may not have been in an open state for ryanodine binding. An ATP-regenerating system or a non-hydrolysable analogue of ATP instead of ATP, should maintain the ryanodine receptors in an open conformation to enable the binding of ^{3}H-Ry.

The experiments previously described were repeated using AMP-PCP, a non-hydrolysable analogue of ATP, in the ^{3}H-Ry binding buffer to encourage the ryanodine receptors to remain in their open conformations. Fresh preparations of SR vesicles were made, using tergitol in the buffers as the previous binding assays with tergitol preparations gave slightly better results than with CHAPS (refer to Figure 3.1). Only an unfractionated sample was used as the ryanodine receptors should be present in this sample prior to fractionation.

The usual ethanol dilution and washing steps were carried out initially, but again there was no significant difference between the control and the sample. It was possible that protein receptors with ryanodine bound were not being retained on the GF/C filters. There was no practical or suitable way of collecting the filtrates for each separate sample from the Millipore manifold vacuum filtration system that was used. Therefore it was decided to try and precipitate the proteins with trichloroacetic acid (TCA). This would determine if the proteins could be precipitated prior to filtration, in the hope that more of the receptor would remain bound to the filter.

The ^{3}H-Ry binding assay using TCA precipitation gave approximately three times the cpm for the unfractionated sample as in the previous assay, but this increase was also observed in the control containing no protein extract (Figure 3.4). This suggested that there was still non-specific binding of ^{3}H-Ry to the filters that could not be distinguished from specific binding in this assay. Repeats of these experiments also gave similar results between the control and the unfractionated sample in each assay.

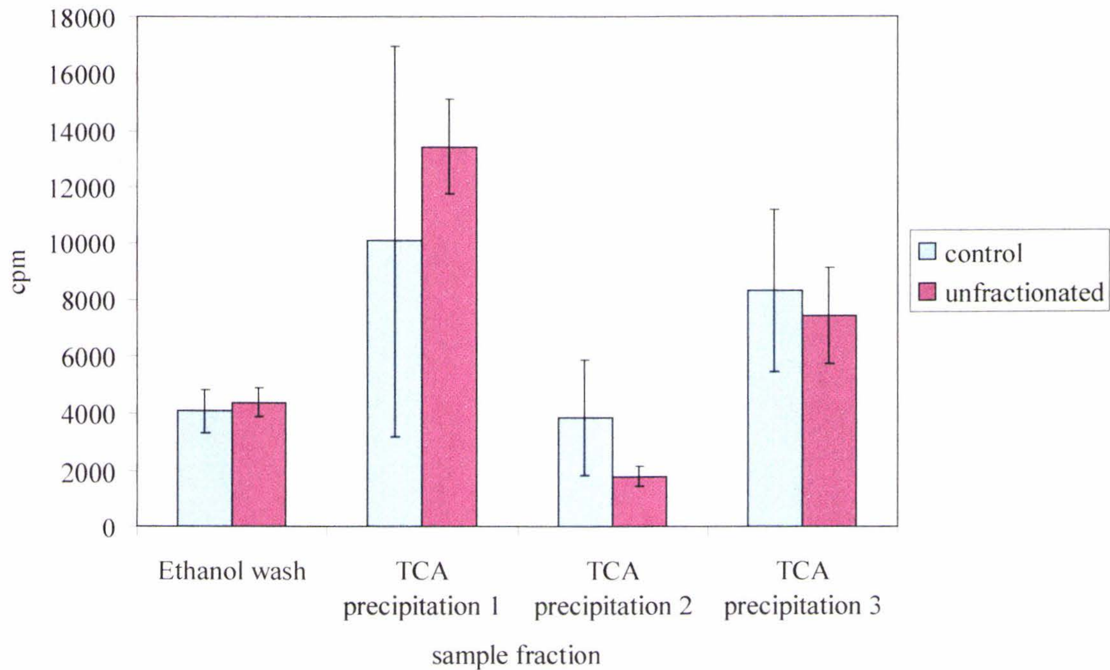

Figure 3.4: The effect of the substitution of ATP with AMP-PCP in the ^{3}H-Ry binding buffer.

These are the results of four separate experiments using the same SR vesicle preparation. The first was carried out using ethanol washed samples prior to filtration as in the previous assays. The other three used a TCA precipitation step to precipitate the proteins prior to filtration. There was a large variation between these three experiments therefore they are shown individually. Again the ^{3}H-Ry control was not shown as the value was too large for the graph.

3.5 Western Blot Analysis

Late in this project, commercial antibodies to a portion of both the N- and C-terminal domains of the ryanodine receptor became available. Western blot analysis can detect picogram quantities of protein on a nitrocellulose membrane (Promega Protein Guide Tips and Techniques Manual). Therefore if there were any ryanodine receptors present in the SR vesicles they could be detectable by immunoblotting.

Tergitol solubilised ryanodine receptors isolated by the homogenisation and centrifugation method of Lunde and Sejersted (1997) were separated on an 8% SDS polyacrylamide gel including high molecular weight markers. The proteins were transferred to a nitrocellulose membrane at 450 mA for 60 minutes. A 1/1000 dilution of the primary RyR-N or RyR-C antibodies, and a 1/10000 dilution of the secondary antigoat antibody were used.

The highest band in the high molecular weight markers is 205 kDa. Ryanodine receptor subunits are 565 kDa, therefore a band greater than the 205 kDa band detected by the antibodies could be the ryanodine receptor. Incubation with the RyR-C antibody revealed a band at ~205 kDa in the unfractionated sample and P1 fraction and a band >205 kDa in both the unfractionated sample and the S4 fraction (Figure 3.5 A). These same bands were detected by the RyR-N antibody after stripping the RyR-C antibody off the membrane. As completeness of the stripping could not be determined, fresh gels were prepared and the immunoblotting repeated. After incubation of the new membrane with RyR-N, bands at ~205 kDa in the unfractionated sample and in the P1 fraction and bands >205 kDa in the P2 and S4 fractions, but not in the unfractionated sample could be detected. (Figure 3.5 B). When comparing the Coomassie stained gel (Figure 3.5 C), it would appear that very little protein had been transferred to the membrane and been detected by the antibodies. On this gel, $\leq$ 4 μg of protein was loaded on the gel, whereas O'Driscoll *et al.* (1996) had used 20 μg of protein for a similar experiment.

Tergitol solubilised receptors should be abundant in the P2 fraction (Lunde and Sejersted, 1997). This is not consistant with the results of western blot analysis. Although a band was possibly detected in the P2 fraction by RyR-N, most of the ryanodine receptors appeared to be in the S4 fractions as detected by both RyR-C and RyR-N. This would suggest that tergitol caused a more complete solubilisation of the ryanodine receptor than what was expected or insufficient protein was loaded on the gel to be detected by the antibodies. This result is not reflected in the ^{3}H-Ry binding studies as there was no significant difference between the P2 and the S4 fractions.

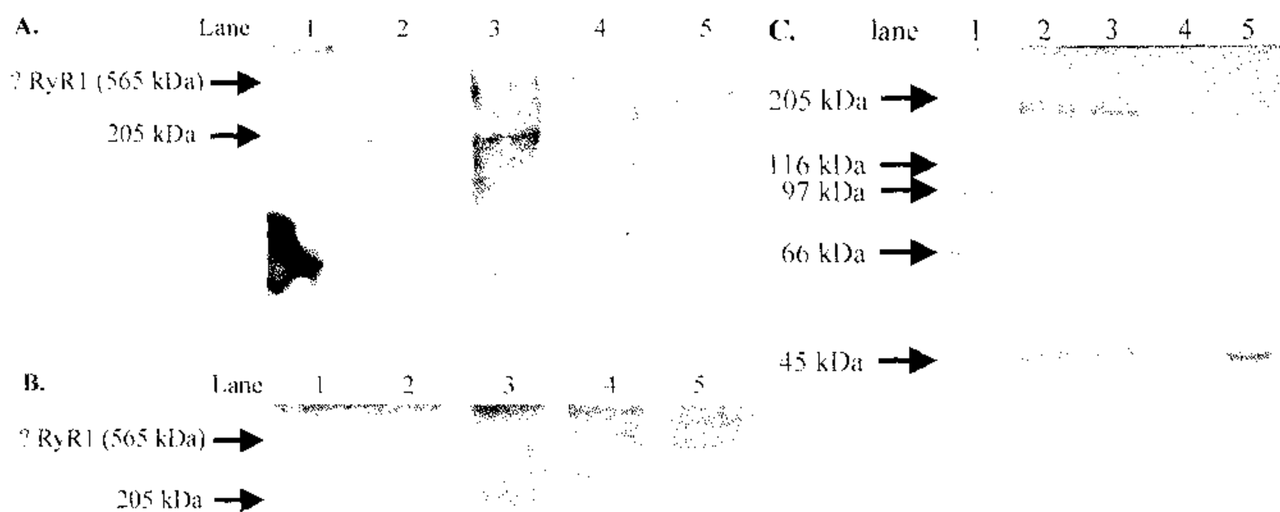

Figure 3.5: Western blot of SR vesicle using (A) RyR-C and (B) RyR-N antibodies.

Proteins were separated on an 8% SDS polyacrylamide gel and transferred to a nitrocellulose membrane by electroblotting at 450 mA for 60 minutes. The primary antibody, (A) RyR-C or (B) RyR-N, was incubated with the membrane for 1 hour and the secondary antibody (antigoat) for 30 minutes after a washing step. The bands were detected using the chemiluminescence western blotting kit (Roche). Lane 1 – high molecular weight marker, lane 2 – unfractionated tergitol (T) sample, lane 3 – T-P1, lane 4 – T-P2, lane 5 – T-S4. The top arrow in (A) indicates a band in lanes 2 and 5 that could be ryanodine receptors. The top arrow in (B) indicates a band in lanes 4 and 5 that could be ryanodine receptors. The bottom arrow in both figures indicates a band at approximately the level of the 205 kDa MWM band. (C) The Coomassie stained gel of the proteins prior to transfer to the membrane for immunoblotting.

3.6 Chapter Summary

The methods used in this chapter were not very successful in the isolation and identification of sarcoplasmic reticulum vesicles containing ryanodine receptors. The binding of ^{3}H-Ry was not successful as the receptors may not have been in their open conformation as ryanodine only binds to open ryanodine receptors; or the receptors may have been present in insufficient numbers to be detectable by ^{3}H-Ry binding and liquid scintillation counting.

Partial purification of SR vesicles on a discontinuous sucrose gradient gave slightly better results but a 120 – 240 fold increase in the amount of skeletal muscle tissue was required. This would be impractical for use with human muscle as only small amounts of tissue were available.

Western blot analysis possibly showed the presence of RyR1 immunoreactive protein in the SR vesicle preparations, although not entirely in the expected fractions. There were only small quantities as indicated by the faintness in the intensities of the observed bands.

However, there was a more prominent band in the unfractionated sample and the P1 fraction at the ~205 kDa level. This could be a degradation product although it does not equate to one of the tryptic digest fragments (Chen *et al.*, 1993b). If degradation had occurred then the ryanodine receptor would not be a complete and functioning channel. This would possibly explain why ryanodine binding was not observed in the SR vesicle preparations.

It is not known where exactly the RyR-C or RyR-N antibodies map to on the receptor, and they were raised against relatively short polypeptides of 18 and 19 amino acids respectively. Therefore they could be binding non-specifically to something else in the vesicle preparation which could explain the inconsistencies between what was seen on the immunoblot (Figure 3.5) and what was expected, that is, strong bands in the unfractionated and the P2 samples. The faintness of the bands could also be due to not having enough protein on the gel, and subsequently the membrane, to be detected by the antibodies as <4 μg was used when other groups had used 20 μg. There was insufficient protein sample available to be able to load 20 μg.

It may have been possible that SR vesicles were not actually isolated. This could be checked by immunoblotting with one of the other proteins that associate with the ryanodine receptor in the SR, assuming that they are still present after the homogenisation and solubilisation procedure. Antibodies to SR-proteins were not available at the time that immunoblotting with the RyR-C and RyR-N antibodies was carried out.

Therefore, since the SR vesicle isolation could not be optimised for small amounts of skeletal muscle tissue, and because only limited amounts of human muscle tissue was available to use, it was decided that it was not viable to pursue this approach. The focus of the research changed to concentrating on making the C-terminal domain of the ryanodine receptor by using a recombinant DNA approach. This is described in a following chapter.

CHAPTER FOUR – ISOLATION OF RNA

4.1 Introduction

As the isolation of SR vesicles containing ryanodine receptors did not appear to be successful, a recombinant DNA approach was undertaken to produce an antigenic peptide which could be used to raise antibodies against the ryanodine receptor. This would allow analysis of the SR vesicle preparations for the presence of ryanodine receptors. Since the SR vesicles could not be used to characterise the two C-terminal mutations, a recombinant DNA approach was also used to reconstruct the transmembrane domain of the ryanodine receptor, into which the two mutations could be introduced. To do this, RNA was isolated to be used to produce the cDNA for both the C-terminal transmembrane domain and the antigenic peptide by RT-PCR

4.2 Isolation of RNA

All equipment and water in solutions were treated with 0.01% DEPC to prevent contamination and degradation of RNA by RNases. The isolation of RNA from skeletal muscle tissue was carried out as outlined in section 2.2.3. 100 mg of muscle tissue was used in each isolation procedure. A total of four isolation procedures were carried out which yielded 20 – 85% of the expected concentration of 1 – 1.5 μg RNA/mg of starting muscle tissue. The $A_{260/280}$ ratios were all less than that of pure RNA indicating that there may have been DNA and/or proteins still present in the samples which were not totally purified away in the Trizol™ procedure (Table 4.1)

skeletal muscle source	concentration (ng/μL)	yield (μg RNA/mg tissue)	% of expected yield	$A_{260/280}$
#1 rat	401	0.401	27 – 40	1.90
#2 rat	853	0.853	57 – 85	1.99
#1 human	296	0.296	20 – 30	1.92
#2 human	439	0.439	29 – 44	1.96

Table 4.1: Concentration and $A_{260/280}$ ratio for each RNA islation procedure.

Although most of the yields were a little low, the procedure produced sufficient RNA to analyse on a gel (Figure 4.1) for northern blot analysis. The presence of the discrete 28S and 18S rRNA bands with no obvious degradation products indicated that the RNA was of a good quality. The continuous smear in each lane is indicative of the total amounts of mRNA within the preparation.

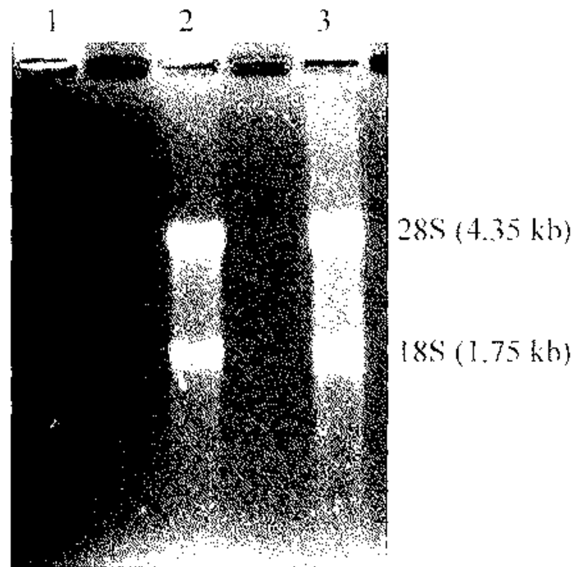

Figure 4.1: RNA gel.

This figure shows the 28S (~4.35 kb) and 18S (~1.75 kb) rRNA bands characteristic of RNA. A 1.5% agarose gel containing 17.6% formaldehyde in a 1 x MOPS buffer was used to analyse the RNA. Electrophoresis was carried out at 100 mA for 3 - 4 hours. A 15 μg sample of RNA with 2 μL of 10 mg/mL ethidium bromide in the sample buffer was loaded in each lane. Lane 1 - blank, lane 2 - rat muscle, lane 3 - human muscle. RNA markers were not available.

4.3 Preparation and Labelling of Probe

A genomic clone, R2543W, previously prepared by Danielle James, IMBS, Massey University, was used to prepare a probe for northern blotting. This clone consisted of a 225 bp portion of the ryanodine receptor gene which had been inserted into the pGEM3Z vector. The 225 bp portion of the ryanodine receptor was amplified by PCR to use as a probe. A magnesium titration consisting of 0.5, 1.5, 2.5 and 3.5 mM of magnesium was required to successfully amplify the piece of DNA, of which all four concentrations appeared to work equally well (data not shown).

The amplified DNA was purified using a PCR purification kit as described in section 2.2.4 Approximately 40 ng/μL of DNA with a total yield of 8 μg, was recovered by this method and used in a labelling reaction.

The DNA probe was labelled with [α-^{32}P]dCTP and purified as described in section 2.2.4. Two labelling reactions were carried out with the percentage incorporation and specific activity of the labelled probe calculated for each reaction (Table 4.2).

probe reaction	cpm on 'purified'	cpm on 'total'	% incorporation	specific activity (cpm/μg DNA)
#1	12498	47019	22	1.78×10^7
#2	3080	36147	8.5	4.4×10^6

Table 4.2: The monitoring progress of the [α-^{32}P]dCTP labelling reaction.

The percentage incorporation of radioactivity in the labelled probe was very low. This may have been because the double-stranded DNA was not adequately reduced to single-stranded DNA by boiling prior to the labelling reaction. If the DNA had remained mostly double-stranded, the random primers would have had limited access to single strands and therefore the incorporation of [α-^{32}P]dCTP into the double-stranded DNA would have been low. Successful detection of the desired RNA band on a northern blot relies on a probe with a high specific activity.

A specific activity of $\geq 1 \times 10^9$ cpm/µg of DNA is desirable (Ready-to-go DNA labelling beads Technical Manual). The specific activity obtained in the labelling reaction was low but satisfactory, therefore these minimally labelled probes were used to probe a northern blot.

4.4 Northern Blot

Northern blot analysis was carried out as described in section 2.2.4. Three blots were carried out before a positive result was obtained.

The RNA for the first blot was transferred to the nitrocellulose membrane for 42 hours to ensure a sufficient amount of large RNA molecules would transfer across as the ryanodine receptor mRNA is ~15,000 bases. The probe with 22% incorporation of [α-^{32}P]dCTP (probe #1) was used in the hybridisation process. After 48 hours exposure the film was developed. The probe bound weakly to mRNA at the expected level of the ryanodine receptor mRNA in the human muscle sample but not the rat muscle sample (data not shown). The human *RYR1* should be highly homologous to rat *RYR1*. Only a small portion of rat *RYR1* mRNA has been deposited in the GenBank database (~747 bp) which is 88% identical to the human ryanodine receptor (data not shown). There was also a large amount of non-specific background binding to the membrane. A negative staining pattern could be seen which outlined the total RNA in both the rat and human muscle lanes, and the 28S and 18S bands in the human sample were prominent.

Denhardts reagent was used in the prehybridisation solution to block the membrane to reduce non-specific background binding of the probe to the membrane. This amount was increased to 2 x Denhardts reagent in the prehybridisation of blot #2 and 100 µg/mL of herring sperm DNA was added to the prehybridisation solution. Herring sperm DNA is a carrier DNA that is used to block sites on the membrane by binding to the membrane, therefore the DNA probe cannot bind as it is not complementary to the herring DNA. The first labelled probe was also used for this hybridisation although it had passed through one half-life.

The autoradiograph of blot #2 after a 67 hour exposure at -70°C between two intensifying screens showed greatly reduced background with the increase in Denharts reagent and the addition of herring sperm DNA. But no bands at the level expected for the ryanodine receptor mRNA were detected. The gel used for transfer of RNA was made with formaldehyde that had started to precipitate out of solution. Some of the precipitate had transferred to the membrane as observed by visualisation under the UV transilluminator, thereby reducing the amount of RNA that could have been transferred. This would result in less RNA available for the DNA probe to bind to. Also as the α-^{32}P had passed through one half-life, it may have contributed to the reduced signal.

Fresh labelled probe was made (probe #2) and used to hybridise to a fresh membrane (blot #3) after prehybridisation with 2 x Denhardts reagent and herring sperm as before. After a 66 hour exposure at -70°C with two intensifying screens, the autoradiograph was developed and a faint band at the expected level of the ryanodine receptors was detected along with a smear of RNA (Figure 4.2). The position of the mRNA *RYR1* band in relation to the 28S and 18S bands is at relatively the same position as that found by other groups on a northern blot (Jayaraman *et al.*, 1992; Nakashima *et al.*, 1997). The smear may be due to degradation of the RNA sample. This could be due to the integrity of the RNA not being maintained during the Trizol™ preparation or RNases may have been introduced at some point. The faintness of the band may have been due to the low specific activity of the probe DNA. A longer exposure time may also have improved the quality of the band on the autoradiograph.

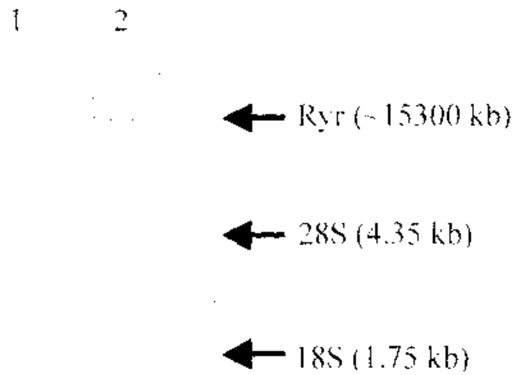

Figure 4.2: Northern blot of Skeletal Muscle Ryanodine Receptor mRNA.

This figure shows the detection of skeletal muscle ryanodine receptor mRNA by hybridisation with an α-^{32}P labelled DNA probe of genomic origin. The autoradiograph was developed after a 66 hour exposure between two intensifying screens at -70°C. Lane 1 - rat muscle, lane 2 human muscle. RNA markers were not available but the relative position of the 28S and 18S marker bands are indicated.

4.5 Chapter Summary

RNA was successfully isolated from skeletal muscle tissue by the Trizol™ method as indicated by the clear separation of the 28S and 18S rRNA bands on a 1.5% agarose gel containing formaldehyde. An α-^{32}P labelled DNA probe prepared from a genomic clone hybridised to a band of mRNA at the level of the ryanodine receptor mRNA in a northern blot analysis. This confirms that ryanodine receptor mRNA was present in the total RNA isolation. Therefore this RNA was suitable for use in a reverse transcriptase reaction to make a cDNA template for use in PCR to amplify portions of the ryanodine receptor coding region. The RT-PCR procedure will be described in the following two chapters.

CHAPTER FIVE – PREPARATION FOR ANTIBODY PRODUCTION

5.1 Introduction

At the commencement of this project there were no commercially available antibodies against the ryanodine receptor. Antibodies were required to be able to confirm the expression of the C-terminal domain after it had been constructed and also to determine whether ryanodine receptors were present in the SR vesicle preparation. An attempt was made to prepare a polypeptide of the ryanodine receptor for the eventual production of antibodies. Antigenic regions within the primary sequence of the ryanodine receptor had previously been identified (Fill *et al.*, 1991). The position of the antigenic regions in relation to the SR membrane depended on which transmembrane model was employed. The four transmembrane model (Takeshima *et al.*, 1989) has more of the antigenic region on the cytosolic side than does the 10 transmembrane model (Zorzato *et al.*, 1990). In both models there is a portion of the antigenic region facing into the luminal side of the membrane. Specific primers were designed to amplify a small antigenic region within the C-terminal domain of the ryanodine receptor (Figure 5.1).

Two vector systems were chosen to clone the amplified piece of DNA. Both pGEX-6P-3 and pProEX-HTb vectors are suitable for high level expression and purification of recombinant proteins in *E. Coli* cells. pGEX-6P-3 is a glutathione S-transferase (GST) fusion vector that contains the *tac* promoter for inducible expression by IPTG. This produces a fusion protein of the recombinant protein attached to GST. The recombinant protein can be purified using Glutathione Sepharose 4B affinity resin and cleaved from GST by using a site-specific protease called PreScission™ protease. This protease cleaves between the GST sequence and the multiple cloning site thus releasing the recombinant protein. The pProEX-HTb vector is a histidine tagged expression system that contains the *Trc* promoter for high level expression also inducible by IPTG. The recombinant protein can be purified by the attachment of the 6-histidine tag to a nickel nitrilo-tri-acetic acid resin. The protein can then be cleaved off using rTEV (recombinant tobacco etch virus)

protease which is a site-specific protease useful for the removal of affinity tags from fusion proteins (Parks *et al.*, 1994). It does this by recognising and cleaving at the TEV site located between the 6-histidine residues and the multiple cloning site.

Both vector systems (Appendix 2) were initially used in experiments designed to express the recombinant protein. It was not known which vector would express sufficient recombinant protein for use in producing antibodies, or whether one system would exhibit more efficient expression than the other.

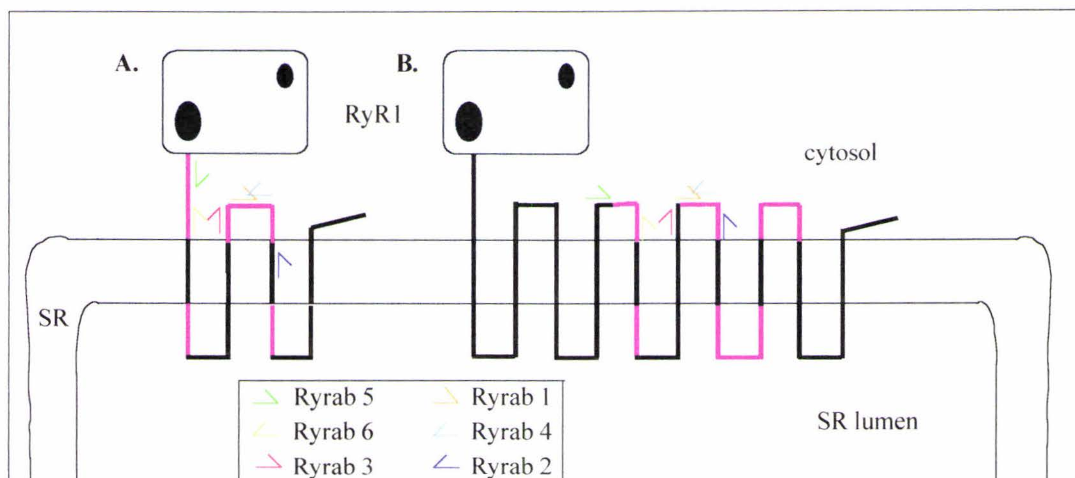

Figure 5.1: Schematic diagram of the location of the antigenic regions on the ryanodine receptor and primer positions.

The relative position of the antigenic regions of the ryanodine receptor are shown in purple in (A) the Takeshima (1989) 4-transmembrane model and (B) the Zorzato (1990) 10-transmembrane model. (Adapted from Fill et al (1991)). The relative locations of the primers used to generate an antigenic polypeptide are shown in colour. Diagram not to scale.

5.2 Reverse Transcription and PCR Amplification of a 400 bp Product

M-MLV reverse transcriptase was used to synthesise cDNA from RNA as described in section 2.2.5. *REDTaq*™ DNA polymerase was used to amplify a portion in the antigenic region of the ryanodine receptor using ryrab 1 and ryrab 2 primers (Appendix 3). The primers were designed to amplify a 400 bp segment of the ryanodine receptor (hereafter called Ryr-400) within the antigenic region. The forward primer contained a *Bam* HI restriction site and the reverse primer an *Eco* RI restriction site to facilitate insertion into the multiple cloning site of the respective vectors.

The first attempt at RT-PCR using the above mentioned enzymes was successful in producing a product of ~400 bp in size. A diagnostic digest using *Cla* I, a restriction enzyme that cuts once in this region to give a 300 bp and a 100 bp product, was carried out and confirmed that the PCR product was correct (Figure 5.2).

Multiple PCR reactions were carried out to produce sufficient quantities of the 400 bp product to purify. The PCR products were end digested with *Bam* HI and *Eco* RI as described in the section 2.2.5 to produce compatible ends for ligating into the expression vectors.

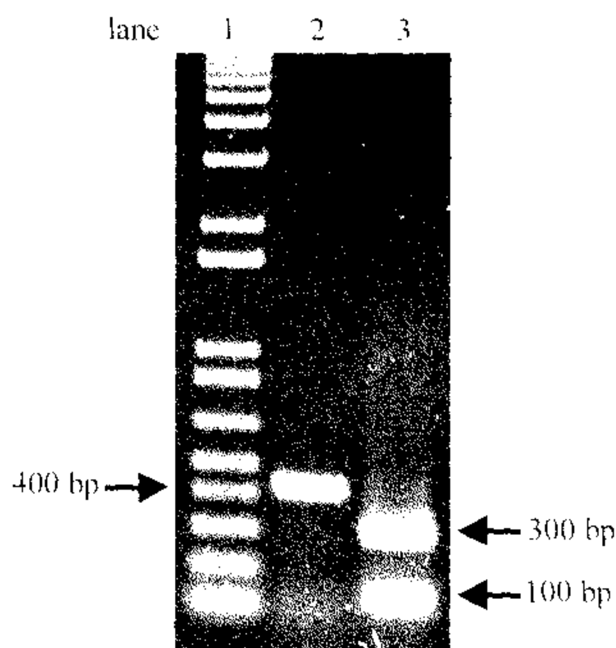

Figure 5.2: Ryr-400 digest.

Gel photograph confirming the size of Ryr-400 by digestion with the Cla I restriction enzyme. The products were analysed on a 1% agarose gel at 80 V for 90 minutes and stained in a solution of 5 μg/mL of ethidium bromide for 10 minutes. Lane 1 - 1 kb plus ladder, lane 2 - Ryr-400, lane 3 - Ryr-400 after digestion with Cla I.

5.3 Generating Vector-Ryr-400 Constructs

Both vectors were initially used for ligation of Ryr-400 to produce pGEX-400 and pProEX-400, but several attempts were required before successful ligation and transformation was achieved. The recommended ratio of three inserts to one vector, with a total of 100 ng of DNA, was used in a ligation reaction as described in section 2.2.1, but *E. Coli* XL-1 blue cells (genotype in table 5.1) were unable to be transformed under these conditions. The ratio of vector to insert was altered and the time and temperature of the reaction was changed from 25°C for 1 hour to 16°C overnight, on separate occasions, but still no colonies grew on LB-amp plates grown at 37°C overnight.

E. coli Cell Line	Genotype	Reference
XL-1 blue	*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46 *thi relA*1 lac$^-$ F'(*proAB*$^+$ *lacI*^{q} △M15 Tn *10* (*tet*^{r}))	Sambrook *et al.*, 1989
BL21-DE3	*hsdS gal* (*λ*cI*ts* 856 *ind*1 *Sam* 7 *nin*5 *lac*UV5-T7 *gene1*)	Sambrook *et al.*, 1989
SG13009[pREP4]	Nal^s, Str^s, Rif^s, Lac$^-$, Ara$^-$, Gal$^-$, Mtl$^-$, F$^-$, RecA$^+$, Uvr$^+$, Lon$^+$	The QIAexpress System Technical Manual

Table 5.1: Genotypes of the *E. coli* cell lines used for cloning and expression.

The cells grew abundantly on LB plates which indicated that they were viable but uncut vectors only gave 1 x 10^3 – 1 x 10^4 colonies/µg of DNA. Competent cells should produce colonies in the vicinity of 1 x 10^6 – 1 x 10^7 colonies/µg DNA. These lower amounts would indicate that the cells were not taking up the constructs very efficiently. The reason for this is unknown as the results were not consistent for each transformation reaction although the techniques and procedures were identical for all experiments. Vector only transformations were carried out using uncut pProEX-HTb, pGEX-6P-3 and pGEM3Z (as the R2543W clone) to check the methods used. This produced 8 x 10^4, 3.5 x 10^4 and 2.5 x 10^5 colonies/µg DNA respectively which is a low but acceptable transformation efficiency. These control experiments showed that the cold shock method of transformation being used was adequate.

The procedure was repeated with no success. Even fewer colonies were produced with the uncut vector suggesting that the cells may not have been as competent as previously. It was also possible that ligation between vector and Ryr-400 was not occurring, therefore the ligation was analysed on a 1% agarose gel. The linear vector band was present and also Ryr-400 bands at the 400 bp and 800 bp level suggesting that the insert bands were ligating to each other (data not shown). It was possible that only one enzyme had cut properly, or the enzymes may not have been removed from the ends of the PCR product using the High Pure PCR Purification kit. The PCR reaction and digestion procedure was repeated and a phenol/chloroform method used to extract the DNA as this should remove the enzymes

from the ends more efficiently. A ligation reaction was set up and analysed on a 1% agarose gel. There were linear vector bands and Ryr-400 bands at their expected sizes indicating that ligation had not occurred. There was a faint band just above the linear vector band which suggested that ligation may have occurred via one end of each molecule to give a linear fragment (data not shown).

Single digests with *Eco* RI- or *Bam* HI-cut Ryr-400 were carried out and ligated separately to determine which, if either, enzyme was not digesting the DNA properly. *Eco* RI-cut, but not phosphatased, pGEX-6P-3 was ligated to determine if the ligase was active. The *Eco* RI-cut vector did religate which demonstrated that the T4 DNA ligase was functional. Both *Eco* RI- and *Bam* HI-cut Ryr-400 with ligase added gave smears between the 400 – 800 bp level as compared to the 'no ligase' lane which had clear bands at the 400 bp level (data not shown). This suggested that some ligation events were occurring, possibly blunt ended ligations to each other or recircularisation if both ends were blunt, but no definite band at the 800 bp level to suggest that the same cut ends were ligating properly. These results suggested that the restriction enzymes were not fully digesting the ends of the PCR product to enable ligation with the vectors. Although the primers, and therefore the fragment, were designed with four additional bases after the restriction site, the enzymes should have been able to effectively digest the DNA. But it has been documented that the number of nucleotides added onto the end of an oligonucleotide after the restriction site is important for how efficiently the enzyme will cleave at its recognition site (The New England Biolabs catalogue 1998/1999). Since complete cleavage was possibly not occurring, an alternative cloning strategy was devised using the pGEM®-T vector system (Figure 5.3)

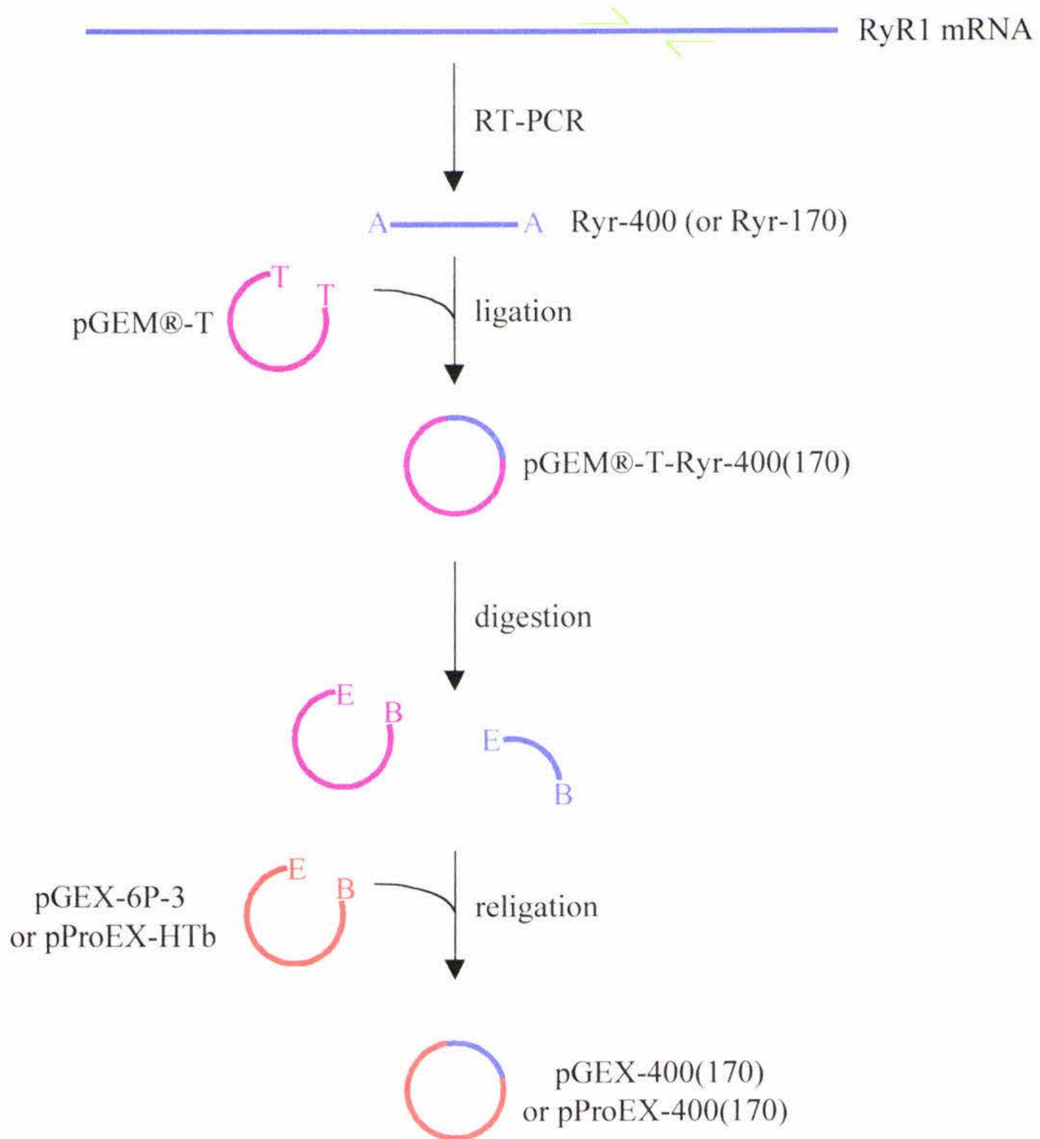

Figure 5.3: Schematic diagram of the cloning strategy for pGEX-400(170) and pProEX-400(170).

Ligation of Ryr-400 or Ryr-170 to pGEX-6P-3 or pProEX-HTb was carried out via the pGEM®-T vector system. 'A' is the adenosine which is added to the end of PCR products by REDTaq™ DNA polymerase. 'T' is the thymidine on the ends of the linearised pGEM®-T vector. 'E' indicates the Eco RI compatible ends and 'B' indicates the Bam HI compatible ends on the DNA fragments.

pGEM®-T is a precut linear vector with a 3′-T overhang. *Taq* DNA polymerase adds a 3′-A to the PCR product during chain extension (Promega pGEM®-T and pGEM®-T Easy Vector Systems Technical Manual) so that the PCR product can be directly ligated into pGEM®-T, then subcloned into the desired vector. The multiple cloning site of pGEM®-T vectors is located within the *lacZ* gene therefore interrupting the coding sequence of β-galactosidase. Recombinant clones can generally be identified by blue/white selection on LB-amp plates containing Xgal and IPTG. Vectors that contain the PCR product will usually produce white colonies although blue colonies will be produced if the PCR product is ligated in-frame with the *lacZ* gene (pGEM®-T and pGEM®-T Easy Vector Systems Technical Manual, Promega).

A ligation reaction was carried out with pGEM®-T and the Ryr-400 PCR product and used to transform *E. coli* XL-1 blue cells. Three colonies were produced of which two proved to contain the Ryr-400 insert. This fragment was digested out of pGEM®-T, purified, quantified and successfully ligated into both pProEX-HTb and pGEX-6P-3. *E. coli* XL-1 blue cells were transformed with pGEX-400 or pProEX-400 and colonies were produced which contained the correct constructs (data not shown). The constructs (Appendix 2) were confirmed by sequencing with the GEX3 and M13 reverse primer respectively using the ABI337-36 automated sequencer (Appendix 4). They both contained the correct sequences apart from a single base change of A14372G which was possibly due to an error in the PCR procedure. The base change also caused an amino acid change from asparagine to serine (both polar amino acids) within a hydrophobic patch. Although this is unlikely to have an effect on the structure of the recombinant protein, it may or may not have an effect on the expression and stability of the recombinant protein. It may also have been a problem if used as an antigen to raise antibodies in rabbits. Although the protein was ~133 amino acids long initially, it would be processed to a size of ~9 to 13 amino acids for the antigen presentation pathway in rabbits. The eventual antibodies that would be raised in the rabbits may recognise a mutated epitope but as the intention was to produce polyclonal antibodies, this should not have been a problem with recognition of the ryanodine receptor.

5.4 Expression of the Ryr-400 cDNA

The 400 bp product should produce a protein of ~15 – 16 kDa. GST is a 26 kDa protein therefore the pGEX-400 fusion protein should be ~41 – 42 kDa while the pProEX-400 protein will be ~15 – 16 kDa. A prominent band at these expected sizes should be seen on an SDS polyacrylamide gel after induction of the respective constructs if expression was successful.

E. coli BL21-DE3 cells were transformed with the constructs and a small number of colonies were produced after overnight incubation. Induction was carried out as described in section 2.2.6 but no expression was observed under any induction conditions after analysis on a 12% SDS polyacrylamide gel. There were no obvious differences between the induced and uninduced cultures and a similar banding pattern was observed with both vectors (data not shown). A change in IPTG concentration or in the length of time of induction had no effect on expression patterns.

A control experiment using the empty pGEX-6P-3 vector was conducted. This system expressed a strong band at the expected size of GST (Figure 5.4). This indicated that the GST expression system was functional. The GST fusion protein with Ryr-400 may be unstable in the experiments conducted, or may be expressed in very small amounts such that it is masked by other cellular proteins, or it may not be produced at all.

Two other cell lines were used to determine whether the fusion proteins were expressed in small quantities. *E. Coli* BL21-ptRNA cells contain a plasmid that expresses rare tRNAs (Kim *et al.*, 1998) and can be useful for proteins with low expression patterns. These cells were grown on LB plates containing 100 μg/mL of ampicillin and 100 μg/mL of spectinomycin to maintain the ptRNA plasmid in the cells. Again no obvious fusion protein could be detected upon induction (data not shown).

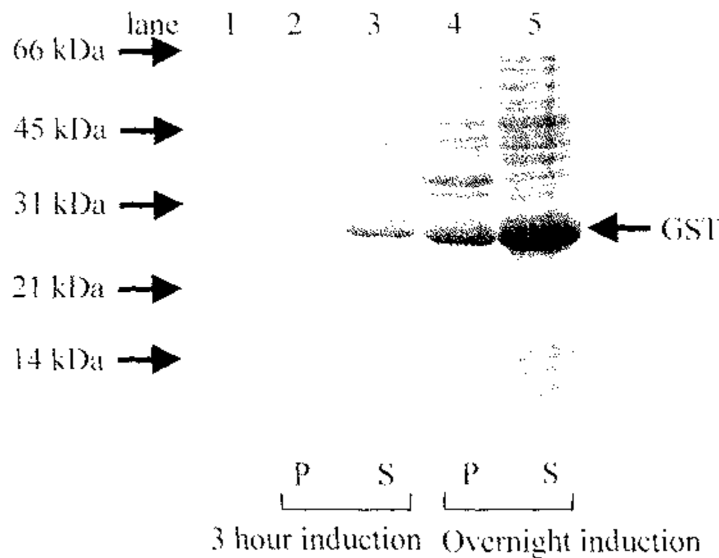

Figure 5.4: Gel photograph of a GST-expression system.

The pGEX-6P-3 expression vector was induced with 0.2 mM IPTG at 26°C for 3 hours or overnight as indicated in the figure. The proteins were extracted as described in section 2.2.6, separated on a 12% SDS polyacrylamide gel for 40 minutes at 200 V, stained with Coomassie Blue dye and destained with destain I and II. A strong band at ~26 kDa is indicative of the GST protein. 'P' is the pellet fraction and 'S' is the supernatant fraction. Lane 1 - molecular weight marker, lanes 2 and 3 - 26°C 3 hour induction, lanes 4 and 5 - 26°C overnight induction.

E. coli SG13009 cells are useful for producing poorly expressible proteins (The QIAexpress System Technical Manual). They contain the pREP4 repressor plasmid, a low copy plasmid which expresses the *lac* repressor protein which is encoded by the *lac* I gene. To maintain the pREP4 plasmid, the cells need to be grown on LB-amp plates containing 25 μg/mL of kanamycin. In this expression system, the *lac* repressor protein made by pREP4 binds to the operator sequences on the vector to regulate recombinant protein expression. When IPTG is added, it binds to and inactivates the *lac* repressor protein. This enables the recombinant protein to be transcribed as there is now no repressor protein binding to the transcription elements (The QIAexpress System Technical Manual).

The frequency of transformation was much better with SG13009 cells than with the other cells, producing greater than 2.5 x 10^3 colonies/μg DNA on the uncut vector plates. Transformation of the constructs was low although pGEX-400 was better than pProEX-400, producing 1.1 x 10^3 and 165 colonies/μg DNA respectively. Both constructs were induced as described in section 2.2.6. The pGEX-400 fusion produced a band in the induced sample which was possibly not in the uninduced sample, although it is slightly larger (~50 kDa) than expected. pProEX-400 did not appear to be expressed as there was no alteration between the banding patterns in the uninduced and induced samples at the expected size of ~15 – 16 kDa (Figure 5.5).

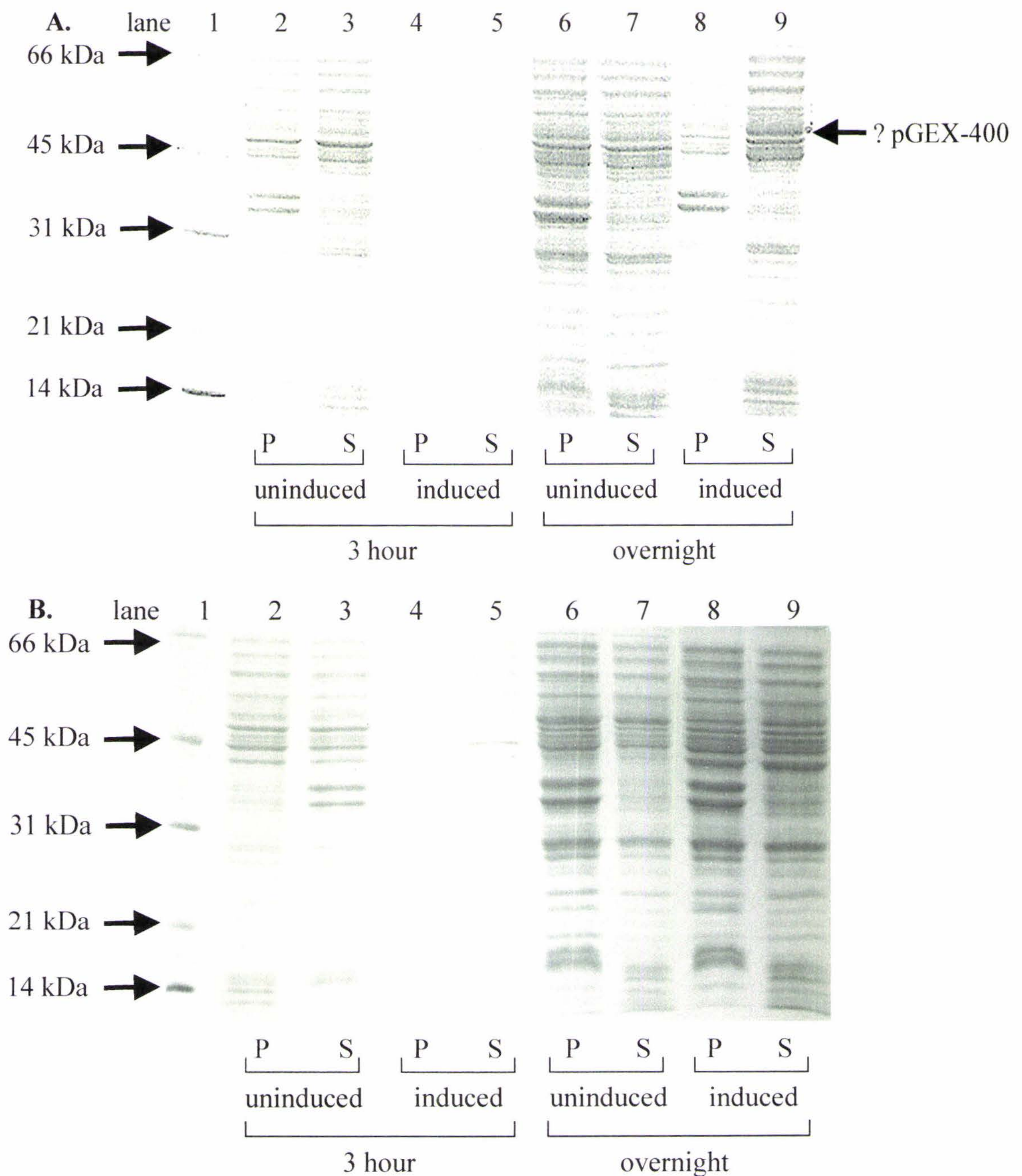

Figure 5.5: Expression of (A) pGEX-400 and (B) pProEX-400.

pGEX-400 and pProEX-400 were induced with 0.2 mM IPTG at 37°C for 3 hours or overnight as indicated in the figure. The proteins were extracted as described in section 2.2.6. 'P' is the pellet fraction and 'S' is the supernatant fraction. The arrow in (A) is indicating a possible band in lane 9. The proteins were separated on a 12% SDS polyacrylamide gel for 40 minutes at 200 V, stained with Coomassie Blue dye and destained with destain I and II.

A western blot using anti-GST antibodies was carried out on the sample that possibly contained the GST fusion, using a GST extract from pGEX-6P-3 as the control (Figure 5.6). Although there is a lot of background with many non-specific bands in this figure, they are mostly the same between the pellets or supernatants of both the uninduced and induced samples for each induction. The lower band (arrow (b)), present to different intensities, could be GST which appears to have a baseline expression level in all samples. The bands indicated by arrow (a) could be the fusion protein. In the SG13009 cells this band occurs in the induced pellet but not the uninduced in the overnight inductions. It is approximately 40 kDa which is the expected size of the pGEX-400 fusion protein. This band appears to be in the four-hour induction with the BL21-ptRNA cells but occurs in both the induced and uninduced samples, and is in both the pellet and supernatants, which suggests that it may be a non-specific protein that the anti-GST antibody has bound to.

The background binding might have been reduced by optimising the western blotting conditions especially if it was due to non-specific binding of the secondary antibody. But as the expression was not very high, it was decided to try the quicker method of binding the samples to GST resin to see if a fusion protein was present. The pellet fractions would need to be solubilised prior to binding to Glutathione Sepharose 4B resin. The same band appeared in the soluble fractions of the BL21-ptRNA induction, therefore the supernatant fractions were used to bind to GST resin.

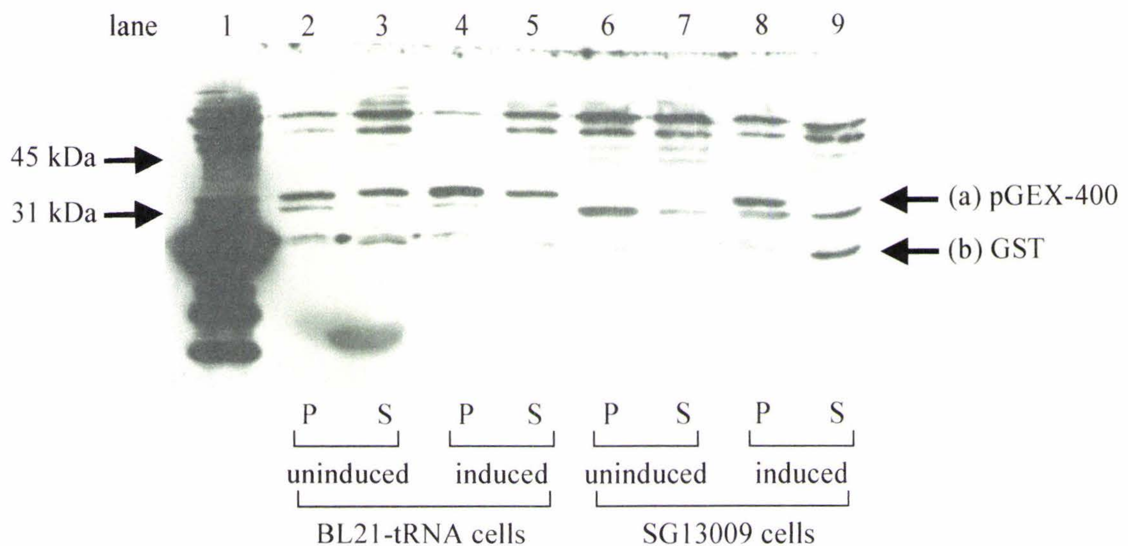

Figure 5.6: Western blot of extracts from a protein induction of pGEX-400 with anti-GST antibody.

Proteins were separated on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane by electroblotting at 450 mA for 45 minutes. A 1/500 dilution of the primary antibody (anti-GST) was incubated with the membrane for 1 hour and a 1/5000 dilution of the secondary antibody for 30 minutes after washing. The bands were detected using the chemiluminescence western blotting kit (Roche). 'P' is the pellet fraction, 'S' is the supernatant fraction. Induction was carried out using 0.2 mM IPTG. Lane 1 – GST extract from a 26℃, 3 hour induction of pGEX-6P-3, lanes 2 to 5 – 4 hour induction at 37℃, lanes 6 to 9 – overnight induction at 37℃.

The induction procedure using BL21-ptRNA cells and pGEX-400 was scaled up in a 50 mL culture and induced at 37°C for four hours to obtain sufficient quantity of fusion protein to bind to the GST resin. A 10 μL sample of pGEX-400 bound to Glutathione Sepharose 4B resin was analysed on a 12% SDS polyacrylamide gel. A very faint band was observed in the induced sample (Figure 5.7, lane 2) but it was at the same level as GST (Figure 5.7, lane 4). This would indicate that Ryr-400 was not stably expressed, degraded or was not expressed at all although a very small amount of GST was produced.

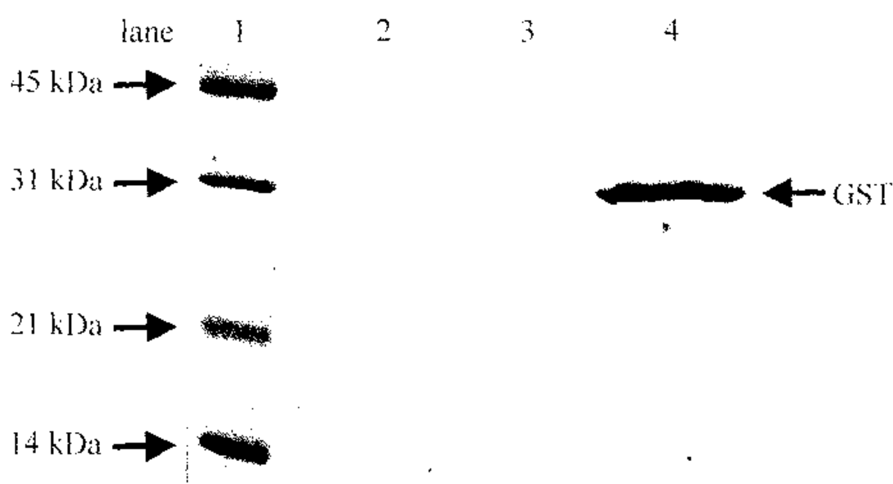

Figure 5.7: Binding of pGEX-400 to GST-resin.

pGEX-400 was induced at 37 °C for 4 hours then bound to Glutathione Sepharose 4B resin. A 10 µL aliquot was analysed on a 12% SDS polyacrylamide gel at 200 V for 30 minutes then stained with Coomassie Blue and destained with destain I and II. Lane 1 – low molecular weight marker, lane 2 – pGEX-400 induced with 0.2 mM IPTG, lane 3 – pGEX-400 uninduced, lane 4 – GST from induction of pGEX-6P-3 for 3 hours at 26 °C.

5.5 Modification of the Fusion Protein Constructs

On closer inspection of the antigenic region represented in both transmembrane models, Ryr-400 was in a different position in each model. In the 10 transmembrane model most of Ryr-400 is within a transmembrane region, whereas in the four transmembrane model it only just overlaps with a transmembrane region. This could have an effect on the expression pattern if the transmembrane region of Ryr-400 is not stable or adopts a tertiary structure that is degraded very quickly by the cellular machinery. Therefore new primers to a different part of the antigenic region of the ryanodine receptor were designed that did not include any of the transmembrane regions according to both models. That is, the new primers would amplify a region that was entirely in the cytosol as opposed to being a transmembrane region, as a cytosolic region may be more stable and not affected by hydrophobic interactions as a transmembrane domain (see Figure 5.1).

5.5.1 RT-PCR of the 170 bp Product and Generation of the Constructs

RT-PCR using M-MLV reverse transcriptase and combinations of the new primers should produce the following size PCR products: ryrab 3 and ryrab 4 – 290 bp; ryrab 5 and ryrab 6 – 490 bp. A product of the expected size was produced with ryrab 3 and ryrab 4 but there was also a smaller amount of product in the 'no RT' lane confirming that genomic DNA had been amplified. No product was produced when the ryrab 5 and ryrab 6 primers were used.

The genomic DNA could be removed by treating the RNA sample with DNase. This was carried out and the DNase treated RNA was used in an RT reaction using *C. therm* polymerase. A subsequent PCR reaction using *REDTaq*™ DNA polymerase with the ryrab 3 and ryrab 4 primers did not yield any products (data not shown). Therefore ryrab 1 and ryrab 4 primers representing a cytosolic portion of the C-terminal domain were used to amplify a 170 bp region (Ryr-170) out of the 3.5 kb DNA product (see section 6.2). This product was digested with *Cla* I to confirm that it was correct (Figure 5.8).

Ryr-170 was successfully ligated to pGEX-6P-3 and pProEX-HTb to produce constructs named pGEX-170 and pProEX-170.

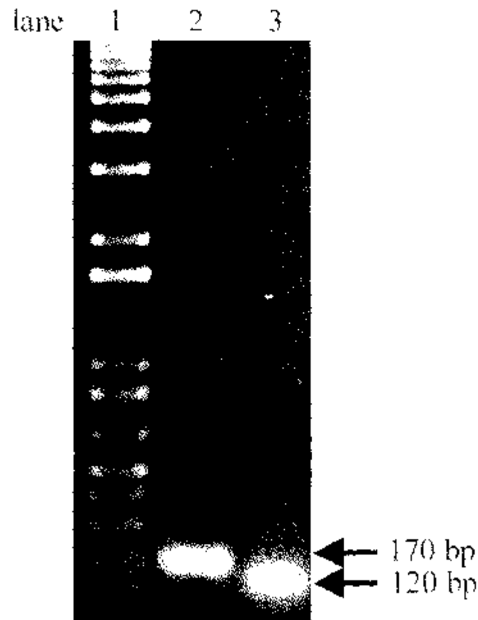

Figure 5.8: Ryr-170 digest.

Confirmation of the size of Ryr-170 by digestion with Cla I restriction enzyme. The 50 bp segment is not resolved on this gel although a decrease in size can be seen. Fragments were analysed on a 1% agarose gel at 80 V for 90 minutes, stained in 5 μg/mL of ethidium bromide solution and visualised on a UV transilluminator. Lane 1 – 1 kb plus ladder, lane 2 – Ryr-170, lane 3 – Ryr-170 after digestion with Cla I.

5.5.2 Expression of the Ryr-170 cDNA

The 170 bp cDNA should produce a protein ~6.8 kDa in size and therefore a prominent band at this level should be observed after induction of pProEX-HTb. pProEX-170 was sequenced on the ABI337-36 automated sequencer from the M13 reverse primer but did not produce a useful sequence. Although sequence was not obtained, an attempt to express pProEX-170 in *E. coli* BL21-DE3 cells was carried out but expression was unable to be obtained (data not shown). Therefore this construct was not analysed any further.

pGEX-170 was sequenced (appendix 4) from the GEX3 primer (appendix 3). The sequence contained four base changes, T14234C, C14238T, A14239T and A14265C,

which caused two amino acid changes from leucine to proline (L4742P) and methionine to leucine (M4744L). These amino acids are all hydrophobic amino acids which shouldn't alter the overall charge on the expressed protein but may change the tertiary structure due to the addition of the ring structure of proline.

However, expression studies were continued because pGEX-170 expressed well in *E. coli* BL21-DE3 cells to produce a strong band at about the level of the 31 kDa molecular weight marker band, although it is slightly smaller than 32.8 kDa, the expected size for pGEX-170 (Figure 5.9 A). Western blot analysis was carried out to ascertain whether the band observed in the 37°C induction was the GST fusion protein or if it was just GST. The anti-GST primary antibody was used. As can be seen in figure 5.9 B, the band produced in the 37°C induction (lanes 6 – 9) is slightly larger than the band produced in the GST alone induction (lane 10). This indicates that the pGEX-170 fusion protein appears to have been expressed in *E. coli* BL21-DE3 cells.

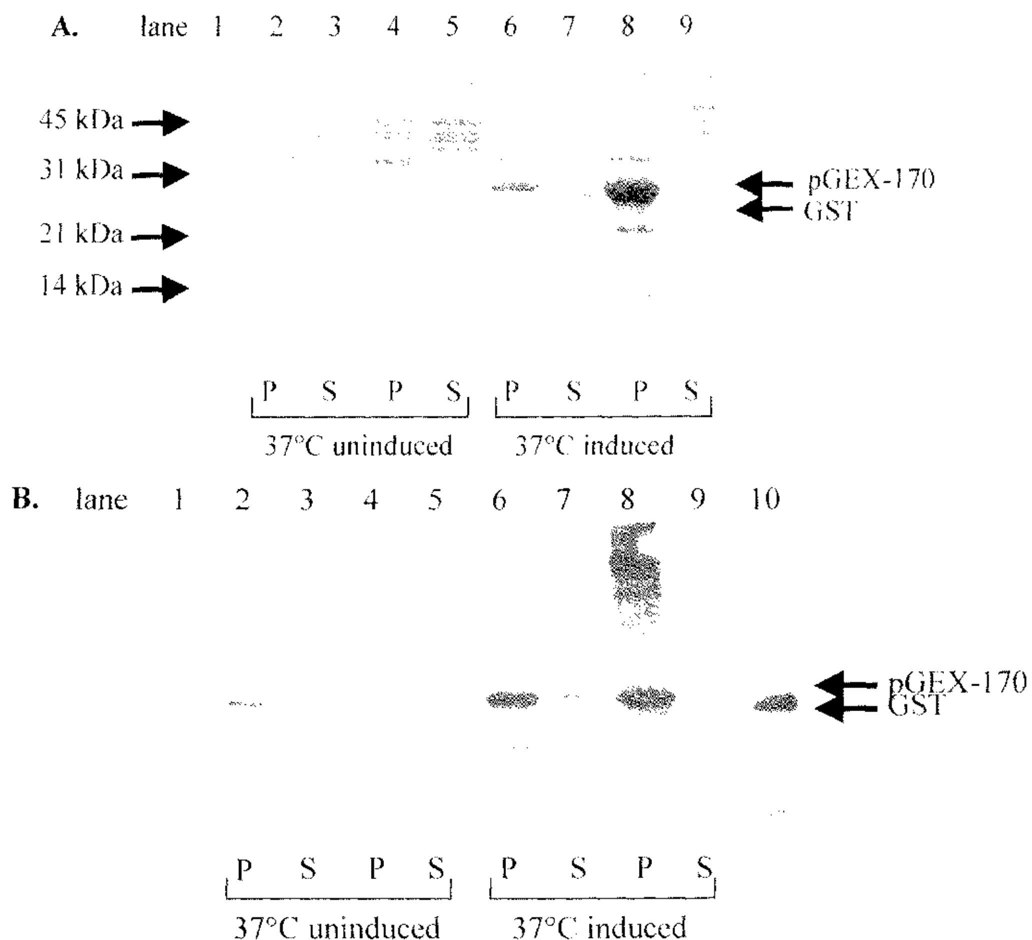

Figure 5.9: (A) Expression and (B) Western blot of pGEX-170.

(A) pGEX-170 was expressed in E. coli BL21-DE3 cells for a 3 hour or overnight incubation. The induction was carried out using 0.2 mM IPTG. The proteins were analysed on a 12% SDS polyacrylamide gel for 40 minutes at 200 V, stained with Coomassie Blue dye and destained with destain I and II. 'P' is the pellet and 'S' is the supernatant fraction. Lane 1 – low molecular weight marker, lanes 2 and 3 – 3 hour incubation, lanes 4 and 5 – overnight incubation, lanes 6 and 7 – 3 hour incubation, lane 8 and 9 – overnight incubation.

(B) The proteins were separated on a 12% SDS polyacrylamide gel and transferred to a nitrocellulose membrane by electroblotting for 45 minutes at 450 mA. A 1/1000 dilution of anti-GST antibody and a 1/10000 dilution of the secondary antibody were used. The bands were detected using the chemiluminescence western blotting kit from Roche. 'P' is the pellet and 'S' is the supernatant fraction. Lanes 1 – 9 as in (A), lane 10 – a GST extract from pGEX-6P-3.

5.5.3 Binding of pGEX-170 to Glutathione Sepharose 4B and Cleavage by PreScission™ Protease

The pGEX-170 fusion protein was found mostly in the pellet fractions as seen on both the gel (figure 5.9 A) and the western blot (figure 5.9 B). pGEX-170 needed to be solubilised before it could be bound to GST-resin for purification. This was achieved with guanidium hydrochloride (GuHCl) which reversibly denatures and solubilises the protein.

The post-sonication pellet was resuspended in 6 M GuHCl in 20 mM Tris buffer. Once the protein was solubilised the GuHCl needed to be diluted out to a concentration that would maintain the fusion protein in a soluble state that would bind to the Glutathione Sepharose 4B resin. A two-fold dilution of the GuHCl was sufficient to achieve this result (data not shown).

The induction of pGEX-170 was scaled up to 50 mL to obtain enough protein to bind to the resin and subsequently cleave for the production of antibodies. The fusion protein was located in the pellet fraction. After solubilising in 6 M GuHCl, the GuHCl could not be diluted out sufficiently to retain the fusion protein in the supernatant and therefore very little pGEX-170 was able to bind to the GST-resin (Figure 5.10 A).

Ten 5 mL cultures of pGEX-170 were grown as solubility of the fusion protein could not be maintained in a 50 mL culture. The 5 mL cultures were induced to produce the equivalent amount of fusion protein as would have been obtained in one 50 mL culture and induction. In the first attempt, solubilisation of pGEX-170 was maintained in a two-fold dilution of 6 M GuHCl. The samples were combined prior to pelleting the cells and disrupting the membranes by sonication but only a small amount of pGEX-170 bound to the GST-resin (data not shown). 1.5 µL of PreScission™ protease was incubated with the bound fusion protein for 36 hours on a shaking tray at 4°C. Partial cleavage may have occurred as a very faint band at ~6.7 kDa was observed after silver staining of the 16.5% tricine gel which was not seen by Coomassie staining (Figure 5.11 A).

The possible cleavage product was in the mixed fraction before centrifugation to pellet the resin and insoluble proteins, but not in the supernatant after centrifugation. This could

indicate that the cleavage product may not have folded properly and had become insoluble as soon as it was cleaved off GST. The pellet (resin with GST bound and the cleaved product) was resuspended in 6 M GuHCl to see if the small cleavage product was soluble at all. The supernatant and pellet fractions were analysed on a 16.5% tricine gel with silver staining. A cleavage product was not detected in either fraction (Figure 5.11 B).

The experiment was repeated using 5 ml cultures, but this time the samples were treated individually and not combined at any stage. Solubility was confirmed in the two-fold dilution of 6M GuHCl by the analysis of a Coomassie stained 12% SDS polyacrylamide gel (Figure 5.10 B). A larger amount of protein was present in the two-fold dilution supernatant than in the pellet, whereas the three-fold dilution contained more protein in the pellet. Each sample bound well to the GST-resin (Figure 5.10 C) and partial cleavage of one sample occurred after a 48 hour incubation with 1 µL of PreScission™ protease on a shaking tray at 4°C (Figure 5.12).

The Ryr-170 cleavage product was not soluble as it was present in the pelleted fraction as was observed with Ryr-400. Therefore it would need to be solubilised before it could be used in the production of antibodies. Also only a small amount could be cleaved away from GST as indicated by the large amount of pGEX-170 still present compared to the small amount of cleaved product (Figure 5.12, lane 3). This procedure would need to be greatly scaled up to produce enough soluble protein suitable for introduction into rabbits for producing antibodies. But at this time, antibodies to a portion of the N- and C-terminal domains of the ryanodine receptor became available from Santa Cruz Biotechnology.

A western blot analysis using the antibody to the C-terminal domain (RyR-C) was carried out to confirm the presence of the Ryr-170 cleavage product. A faint negative image band at the level of GST and pGEX-170 bands was observed, but the Ryr-170 was not detected (data not shown). The peptide antigen used to raise the RyR-C antibody was only 18 amino acids long and there was no information available as to which portion of the C-terminal domain that it represented. Therefore it may not have been able to detect Ryr-170.

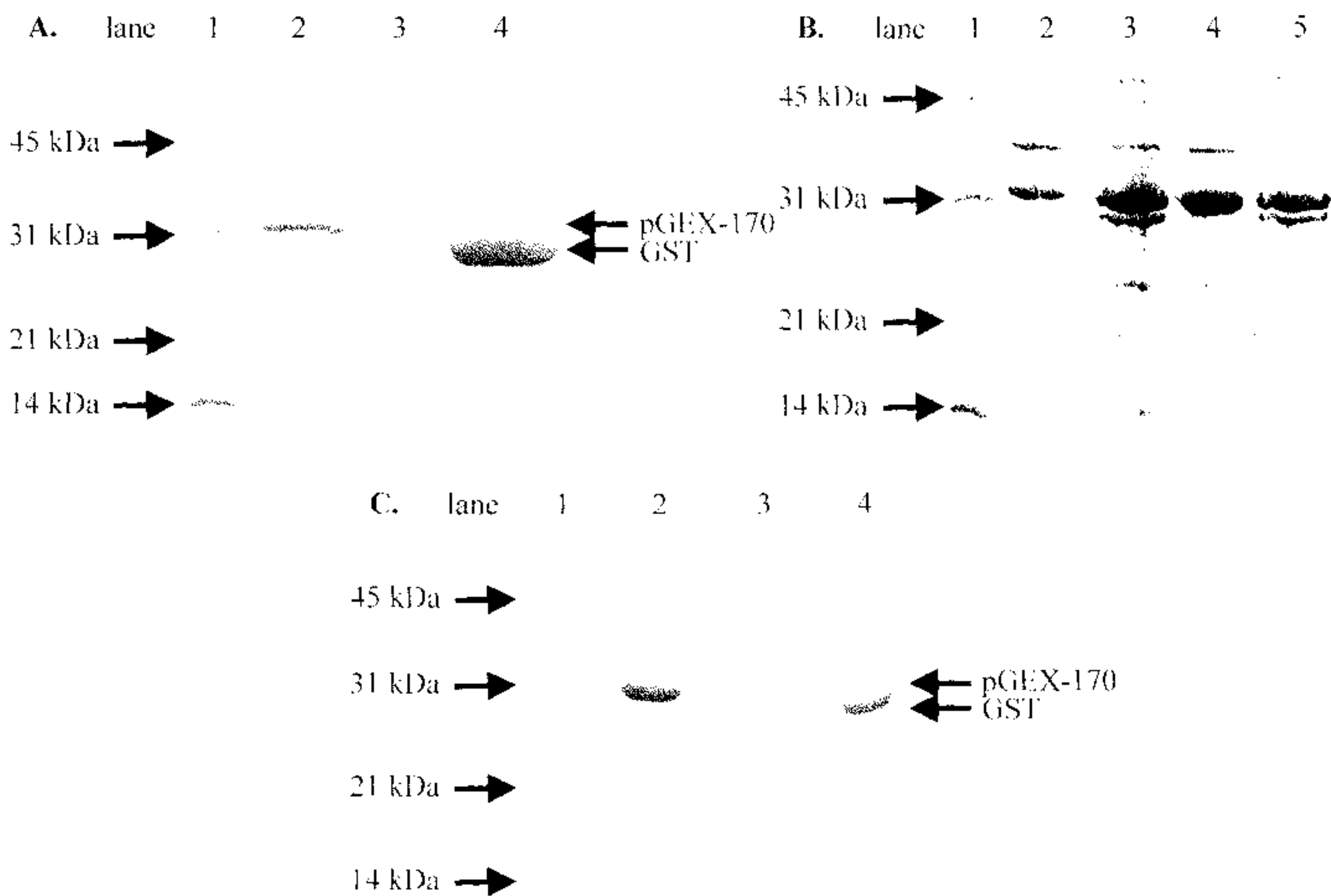

Figure 5.10: Solubilisation properties of pGEX-170.

All proteins were analysed on a 12% SDS polyacrylamide gel for 40 minutes at 200 V, stained with Coomassie blue dye and destained with destain I and II. Lane 1 in all figures is the molecular weight marker. (A) Insoluble Ryr-170 solubilised with 6 M GuHCl and diluted out to a 2 fold (lane 2) or 3 fold (lane 3) dilution and bound to GST resin. Lane 4 is a GST sample from pGEX-6P-3 also bound to GST resin. (B) A representative gel photograph demonstrating the solubility of a 2 fold (lane 2 - pellet, lane 3 - supernatant) and 3 fold (lane 4 - pellet, lane 5 - supernatant) dilution of the 10 five ml cultures after solubilisation with 6 M GuHCl. (C) A representative gel photograph demonstrating the binding of the 2 fold dilution of the 6 M GuHCl solubilisation of Ryr-170 to GST-resin. Lane 2 - bound sample, lane 3 - unbound, lane 4 - a GST sample from pGEX-6P-3 bound to GST resin.

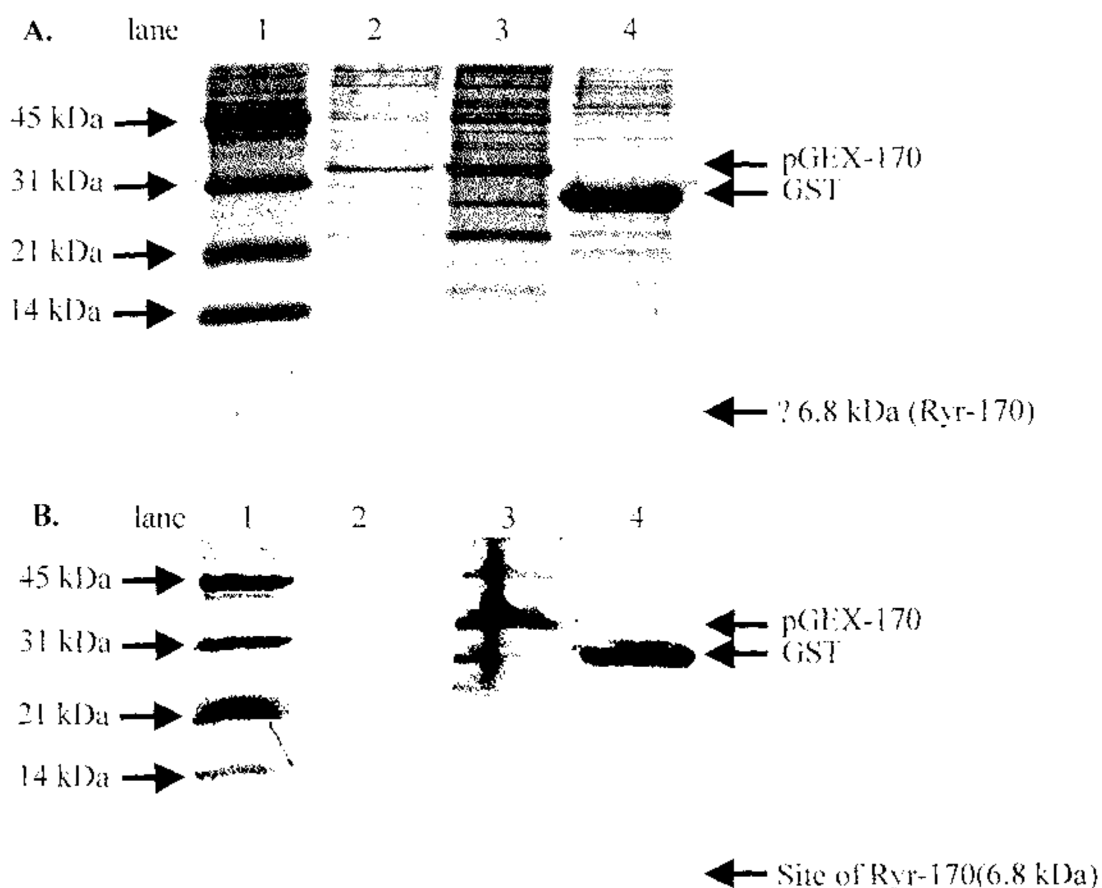

Figure 5.11: (A) Cleavage of pGEX-170 and (B) Solubilisation of Ryr-170 cleavage product.

(A) The Ryr-170 protein from a 50 ml culture induced with 0.2 mM IPTG was cleaved off GST by incubation with 1.5 μl of PreScission™ protease for 36 hours. The proteins were analysed on a 16.5% tricine SDS polyacrylamide gel at 30 mA for 90 minutes and visualised using the silver stain plus kit from Biorad. Lane 1 – low molecular weight marker, lane 2 – cleaved sample (supernatant), lane 3 – mixed sample after cleavage but prior to centrifugation to pellet the GST resin, lane 4 – GST from a pGEX-6P-3 induction.

(B) The pellet fraction from (A) lane 3 was solubilised in 6 M GuHCl in 20 mM Tris. The proteins were analysed on a 16.5% tricine SDS polyacrylamide gel for 90 minutes at 30 mA and the bands were detected using the silver stain plus kit (Biorad). Lane 1 - low molecular weight marker, lane 2 – solubilised sample (supernatant), lane 3 - solubilised sample (pellet), lane 4 – GST from a pGEX-6P-3 induction.

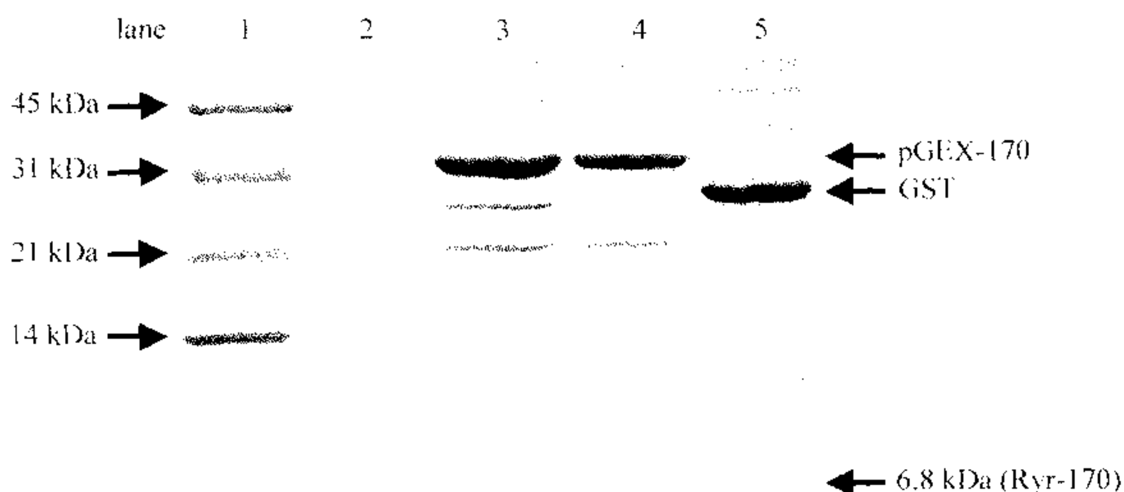

Figure 5.12: Partial cleavage of pGEX-170 with PreScission™ protease.

The Ryr-170 protein from a 5 mL culture was induced with 0.2 mM IPTG and cleaved off GST by incubation with 1 μL of PreScission™ protease for 48 hours. The proteins were analysed on a 16.5% tricine SDS polyacrylamide gel for 120 minutes at 20 mA and visualised by staining with Coomassie Blue stain and destained with destain I and II. Lane 1 - low molecular weight marker, lane 2 - cleaved sample (supernatant), lane 3 - cleaved sample (mix), lane 4 - uncleaved sample, lane 5 - GST.

5.6 Chapter Summary

Due to time constraints, the preparation of antibodies was not pursued. Limited cleavage of the GST-Ryr-170 fusion protein was achieved but two ryanodine receptor antibodies had become available commercially which could be used instead to confirm the presence of ryanodine receptors in other experiments. Expression of Ryr-170 but not Ryr-400 was obtained. This may have been because Ryr-400 included a large portion of a transmembrane domain which may have caused it to be unstable as a fusion protein. Ryr-170 was made entirely of a cytosolic portion of an antigenic region of the ryanodine receptor. Partial cleavage of Ryr-170 was clearly seen on a tricine gel stained with Coomassie Blue dye. Although this could not be confirmed by immunoblotting, the gel clearly showed the presence of a GST band in the cleaved sample (Figure 5.12, lane 3) which is not in the uncleaved sample (Figure 5.12, lane 4), indicating that the smallest band is probably Ryr-170. More complete cleavage may have occurred if more PreScission™ protease and/or a longer incubation time had been used.

CHAPTER SIX – PREPARATION OF THE RECOMBINANT C-TERMINAL DOMAIN OF THE RYANODINE RECEPTOR

6.1 Introduction

Sarcoplasmic reticulum vesicles containing ryanodine receptors were unable to be identified by ^{3}H-Ry binding and therefore could not be analysed for their calcium release properties. As vesicles containing normal ryanodine receptors could not be identified, no attempt was made to isolate vesicles with ryanodine receptors from muscle samples known to carry the mutations of interest. Therefore recombinant DNA techniques were employed to construct the C-terminal domain of the ryanodine receptor.

The full-length ryanodine receptor cDNA of normal and mutated channels had previously been cloned and expressed in mammalian cells by several groups (Chen *et al.*, 1993a; Tong *et al.*, 1999; Treves *et al.*, 1994) and reported to form a functionally active calcium-release channel. The C-terminal domain had also been cloned and expressed in mammalian cells and was found to function fully as a calcium-release channel in a similar way to the full-length receptor (Bhat *et al.*, 1997). Other groups had introduced mutations into the regulatory regions of the C-terminal domain and found that they could alter the sensitivity of the channel to calcium and ryanodine (Du *et al.*, 2000; Du and MacLennan, 1999; Nakai *et al.*, 1999; Zhao *et al.*, 1999).

The ultimate aim of this project was to introduce two novel mutations, located in the C-terminal domain, into the ryanodine receptor. Since it was found that the C-terminal domain alone could function as the calcium-release channel, it was decided to make the C-terminal domain and express it in a mammalian cell line to be able to study calcium release and ryanodine binding.

6.2 Reverse Transcription and PCR Amplification of the 3.5 kb C-Terminal Domain

The C-terminal domain of the coding region of the ryanodine receptor contains an approximately 800 bp region that has a very high GC content of ~76%. GC pairs are stronger than AT pairs due to the extra hydrogen bond between the pairs of nucleotides. This can make it difficult to denature the double-stranded DNA molecules sufficiently for DNA polymerases to bind and synthesise new strands of DNA in a PCR reaction. Several RT and PCR enzymes were available which were specifically designed to amplify long transcripts and through regions of high GC content. Many combinations of reverse transcriptases and DNA polymerases were employed to produce the 3.5 kb C-terminal domain region of the ryanodine receptor.

The following RT-PCR systems were used:

The *C. therm.* one-step system (Roche) uses *C. therm.* as the RT and the Klenow fragment of the DNA polymerase from *Carboxydothermus hydrogenoformans* and *Taq* DNA polymerase in the PCR step. This system is reported to reduce secondary structure problems by using high temperatures in the RT reaction and DMSO in the RT-PCR buffer to reduce the stability of RNA hairpin structures. It has high fidelity due to the use of magnesium ions in the RT-PCR buffer and can amplify fragments up to 3 kb long. The RT reaction is not inhibited by high amounts of nonspecific RNA in the reaction. (*C. therm.* Polymerase One-Step RT-PCR System instruction manual).

C. therm. two-step system (Roche) uses *C. therm.* as the RT and any DNA polymerase can be used for the PCR step. The system has the same properties as the one-step system except it can amplify up to 4 kb products. It also has higher fidelity if a proofreading enzyme is used in the PCR step. It can be useful for GC rich sequences due to the addition of 5% DMSO in the RT reaction and 1 – 2 M betain in the PCR step. (*C. therm.* Polymerase for Reverse Transcription in Two-Step RT-PCR instruction manual).

Titan™ one step system (Roche) uses AMV (avian myeloblastosis virus) as the RT and the Expand™ high fidelity enzyme mix of *Taq* and *Pwo* DNA polymerase for PCR. Titan™ is reported to reduce secondary structure by carrying out the RT step at high temperature and

adding DMSO to the RT-PCR buffer. It reduces the error rate due to the proofreading activity of *Pwo* polymerase, which has increased fidelity compared to using *Taq* DNA polymerase alone, and has increased sensitivity with the efficiency of these enzymes. This system can amplify up to 6 kb of DNA. (Titan™ One Tube RT-PCR System information sheet).

The PCRx enhancer system (Life Technologies) is reported to be useful for amplifying high GC and problematic templates, and for use with primers with large T_m differences. It reduces the T_m to enable a wider range of annealing temperatures to be used and broadens the range of magnesium concentrations that can be used. Platinum® *Taq* polymerase has been recommended for the amplification of GC rich targets up to 10 kb long. (PCRx Enhancer System information sheet).

Platinum® *Taq* DNA polymerase high fidelity (Life Technologies) is an enzyme mix consisting of recombinant *Taq* DNA polymerase, *Pyrococcus species* GB-D polymerase and Platinum® *Taq* antibody. It has an increased fidelity and reduced error rate due to the proofreading activity of *Pyrococcus species* GB-D polymerase and can amplify up to 12 – 20 kb products. The anti-*Taq* polymerase binds to and inhibits polymerase activity providing an automatic hot start to reduce nonspecific amplification of DNA in the PCR step. (Platinum® *Taq* DNA Polymerase High Fidelity information sheet).

The GC-rich PCR system (Roche) is specifically for amplifying GC rich targets up to 5 kb long. It uses *Taq* DNA polymerase with the proofreading enzyme *Tgo* DNA polymerase to increase the fidelity and reduce error rates in the amplification of long targets. It also contains DMSO in the reaction buffer to reduce secondary structures. (GC-Rich PCR System information sheet).

All RT-PCR reactions were carried out according to the appropriate manufacturer's instructions. The 5′ PCR primer, ryrsvk 1 (appendix 3), was designed to use Met^{3874} as the initiation codon. It contained the Kozak ribosome binding sequence necessary for translation in mammalian cells and an *Xba* I restriction site to facilitate cloning. The 3′

PCR primer, ryrsvk 2 (appendix 3), incorporated the stop codon after amino acid 5038 and a *Sal* I restriction site to facilitate cloning. These two primers were designed to generate a PCR product of ~3500 bp long.

Many experiments were carried out in attempts to amplify the 3.5 kb fragment. (Data is not shown for the unsuccessful attempts at RT-PCR). These included using the *C. therm.* one-step system, *C. therm.* RT with Platinum® *Taq* DNA polymerase ± the PCRx enhancer, M-MLV or *C. therm.* RT with *Taq* DNA polymerase, or the Titan™ one-step system. These reactions were also carried out with a reduction in the amount of primer used, a reduction in the amount of RNA or cDNA used, changes in the annealing temperature, changes in the magnesium concentrations, and/or the addition of 5 seconds to the elongation time in each PCR cycle for 20 cycles. The combination of enzymes and procedures used yielded no significant results. RT-PCR reactions produced either smears of DNA, many bands with none at the expected size, or no banding pattern at all.

Although these RT-PCR systems were supposed to amplify a 3.5 kb fragment, with the exception of the *C. therm.* one step system, it was decided to amplify the fragment in two smaller pieces which could be rejoined later. Therefore two primers, ryrsvk 3 and ryrsvk 4 (appendix 3) were designed around a unique restriction enzyme site. They would be used in two separate PCR reactions, ryrsvk 1 with ryrsvk 3 and ryrsvk 2 with ryrsvk 4, to generate products of 1.2 kb and 2.3 kb respectively. This would enable the two fragments to be digested with *Xho* I (or *Not* I) and ligated together to construct the 3.5 kb fragment (Figure 6.1). These two primers were located in the GC-rich region as other unique restriction enzyme sites within the 3.5 kb fragment would not have shortened the product size significantly.

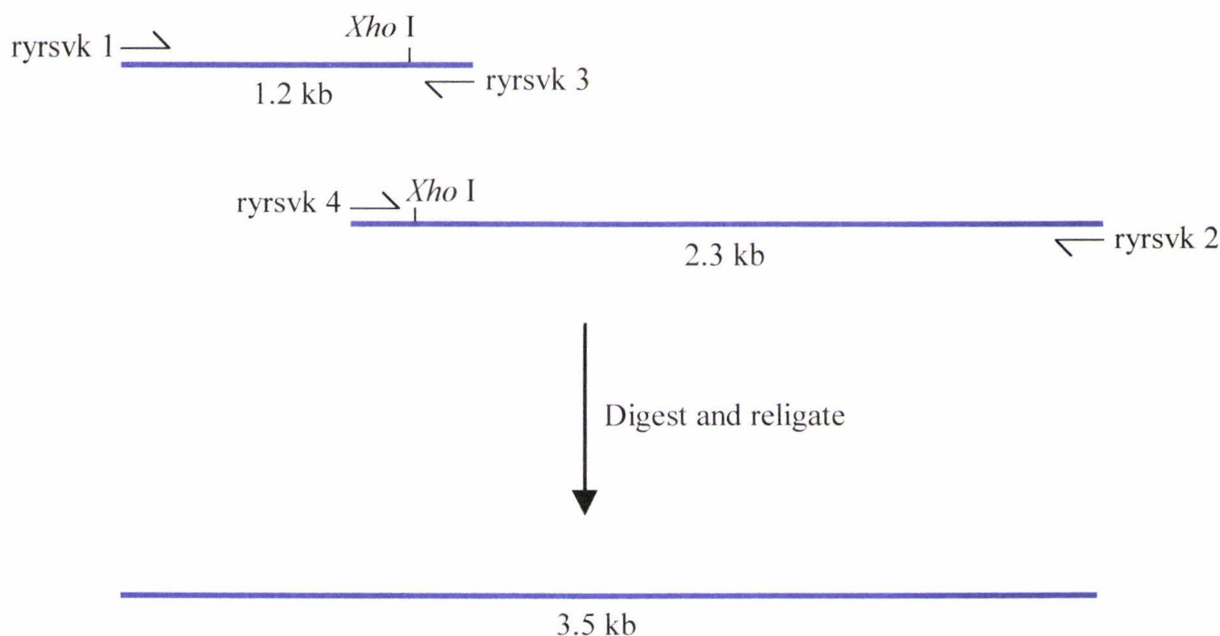

Figure 6.1: Schematic diagram showing the design of ryrsvk 3 and ryrsvk 4 primers.

Both ryrsvk 3 and ryrsvk 4 primers for PCR were designed around the Xho I site. This would enable the ends of the two fragments to be digested with Xho I and ligated together to reform Ryr-3.5. (Diagram not to scale).

Again many combinations of enzyme systems were used which included M-MLV with *REDTaq*™ DNA polymerase, *C. therm.* one-step, *C. therm.* RT with *REDTaq*™ DNA polymerase and betain, *C. therm.* RT with the GC-rich PCR system, and the Titan™ one-step system. A touch-down PCR programme was used for most of these reactions as the T_m of the primer pairs were 10°C - 12°C different. This type of PCR programme uses a range of annealing temperatures to cover the variation in T_m in an attempt to get the primers to bind at some point in the process to be able to amplify sufficient product to analyse. A typical programme was set up as follows. 94°C for 2 minutes; 2 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 2.5 minutes; 2 cycles of 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 2.5 minutes; 2 cycles of 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 2.5 minutes; 2 cycles of 94°C for 30 seconds, 54°C for 30 seconds, 72°C for 2.5 minutes; 30 cycles of 94°C for 30 seconds, 52°C for 30 seconds, 72°C for 2.5 minutes; and 72°C for 7 minutes. Alternatively the programme was set up as follows.

94°C for 5 minutes; 3 cycles of 94°C for 45 seconds, 58°C for 30 seconds, 72°C for 4 minutes; 3 cycles of 94°C for 45 seconds, 56°C for 30 seconds, 72°C for 4 minutes; 5 cycles of 94°C for 45 seconds, 54°C for 30 seconds, 72°C for 2.5 minutes; 30 cycles of 94°C for 30 seconds, 52°C for 30 seconds, 72°C for 4 minutes; and 72°C for 5 minutes. The exact programme was varied depending on the T_m of each primer pair and the polymerase enzyme being used in the PCR reaction. No products at the expected size were produced for any of the enzymes that were used.

It was possible that amplification was unsuccessful because the primers may not have annealed to the DNA or amplified through the GC-rich region. Also the RT step with the oligo(dT) primer, or in the one-step systems, may not have produced long enough transcripts. Therefore the PCR was carried out using each primer separately in a reaction with primers provided by T. M. McCarthy, University of Cork, Ireland, to see whether the ryrsvk primers were in fact annealing to the DNA or if DNA of a sufficient length was being produced in the RT step. Combinations of the TM primers were also used together in PCR to determine if long enough transcripts were produced. The primers that were used were TM12575, TM12418, TM12198, TM13356, TM 13731, TM 13377 and TM13973 (appendix 3). These primers had been used successfully for RT-PCR or sequencing of RyR1 by Rosemary L. Brown, IMBS, Massey University (personal communication). *C. therm.* RT was used with the GC-rich PCR enzyme or *REDTaq*™ DNA polymerase in a touch-down PCR programme. Again amplification products were not produced.

An RT reaction using *C. therm.* RT and priming with random hexamers, which may result in first strand cDNA long enough to accommodate both PCR primers, was carried out. This was followed by touch-down PCR using *REDTaq*™ DNA polymerase or the GC-rich PCR system, neither of which produced any products. An RT step at 70°C was carried out to try and reduce possible secondary structures in the RNA, and a hot start PCR programme where the enzyme is not added until the reaction reaches 95°C was used. Both of these approaches were unsuccessful.

Rosemary Brown had previously amplified regions of the ryanodine receptor using SuperScript™ (Life Technologies) RT and eLONGase® (Life Technologies) in the PCR reaction. The SuperScript™ preamplification system uses the SuperScript II RNase H⁻ RT to synthesise the first strand cDNA from a total RNA preparation. The enzyme does not contain RNase H activity, therefore RNA is not degraded during the synthesis of the first strand of cDNA, and it has been suggested that longer cDNA transcripts are often obtained (SuperScript™ Preamplification System for First Strand Synthesis instruction manual). The eLONGase® enzyme is a mixture of *Taq* DNA polymerase and *Pyrococcus species* GB-D polymerase which can amplify DNA templates up to 12 kb long and it contains a proofreading enzyme (3′ – 5′ exonuclease activity) which corrects nucleotide misincorporation (eLONGase® Enzyme Mix product information sheet).

The SuperScript™ RT reaction was carried out according to the manufacturer's instructions. The PCR reaction using eLONGase® was carried out using Rosemary Brown's protocol as follows. In a total volume of 50 µL, 5 µL of buffer A, buffer B, and each primer (20 ng/µL), 1 µL of 10 M dNTPs (0.2 M), 8.75 µL of 40% glycerol (7%), 4 µL of 100% DMSO (8%) and 2 µL of eLONGase® was added. A touch-down PCR using 94°C denaturation for 45 seconds, 54°C down to 48°C annealing for 30 seconds and 72°C elongation for 3 minutes was carried out for a total of 39 cycles. An initial RT-PCR carried out did not produce a convincing positive result. It was possible that the RNA sample used contained degradation products as seen on a northern blot (see figure 4.2) which may have inhibited the production of full-length cDNA. A repeat PCR experiment using the above conditions and a sample of cDNA prepared previously and stored by Rosemary Brown produced a product of ~3.5 kb (Ryr-3.5). This was confirmed with a diagnostic digest using *Eco* RI and *Xho* I restriction enzymes which should give 2 kb and 1.5 kb, and 2.3 kb and 1.2 kb bands respectively. Bands of these expected sizes were observed (Figure 6.2).

The reaction was repeated using a cDNA sample prepared by using the superscript RT on an RNA sample prepared as described in section 2.2.3, and using the eLONGase® enzyme in the PCR step. This reaction did produce a 3.5 kb size band but there were also other bands present in the sample and a large number of bands in the 'no RT' sample indicating

that genomic DNA had been amplified. The genomic DNA was unable to be removed sufficiently by DNase treatment and it was not possible to reduce the other bands present in the 3.5 kb sample. Therefore the cDNA prepared by Rosemary Brown was used in all subsequent reactions as it produced a much cleaner product.

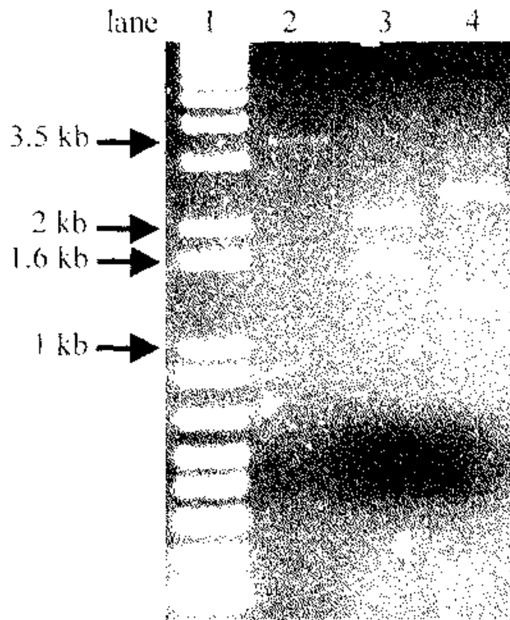

Figure 6.2: Gel photograph confirming the size of Ryr-3.5.

The Ryr-3.5 PCR product was digested with Eco RI or Xho I for 1 hour at 37 ℃. 5 μL of each sample was analysed on a 1% agarose gel for 90 minutes at 80 V. The gel was stained in a 5 μg/μL solution of EtBr and the bands visualised using a UV transilluminator. Lane 1 - 1 kb plus ladder, lane 2 - Ryr-3.5 PCR product, lane 3 - Eco RI digest (2 kb and 1.5 kb bands), lane 4 - Xho I digest (2.3 kb and 1.2 kb bands).

6.3 Generating pSVK3-Ryr-3.5 Constructs

The initial ligation of gel purified Ryr-3.5 to pSVK3 and transformation of *E. coli* XL-1 blue cells produced two possible clones. Diagnostic digests of the clones was inconclusive

although a PCR check of the miniprep DNA produced a possible band at the 3.5 kb level (data not shown).

One of these clones was submitted for sequencing from the T7 primer on the ABI337-36 automated DNA sequencer, but sequencing was not successful. A digest using the maxiprep plasmid DNA with the cloning enzymes, *Xba* I and *Sal* I, was carried out. This gave 1.9 kb and 3 kb sized products when analysed on a 1% agarose gel. These products were smaller than the expected sizes of 3.5 kb (Ryr-3.5) and 3.9 kb (pSVK3) (data not shown). A deletion may have occurred during the transformation to account for the shortened products.

The second clone was redigested with *Xba* I and *Sal* I and analysed on a 1% agarose gel. This gave products of the expected size of 3.9 kb and 3 – 3.5 kb, suggesting that it might contain the insert. Diagnostic digests were again inconclusive and sequencing from the T7 primer or ryrsvk 1 were also unsuccessful. All digests were again repeated and it would appear that although the vector band was the correct size (3.9 kb), the insert band at ~3 kb was too small to be the C-terminal cDNA (data not shown).

Several PCR reactions with eLONGase® were carried out to produce more of the 3.5 kb product. The ends were digested with the cloning enzymes *Xba* I and *Sal* I, and the DNA was extracted using phenol and chloroform instead of gel purification as a better recovery of DNA might be expected. Although a smaller band was present in the PCR reaction, it should have been possible to screen for this when the transformants were analysed by the rapid-boil method followed by digestion with the appropriate restriction endonucleases.

The 3.5 kb fragment could not be consistently reproduced and only a very small yield (~100 ng) was obtained by the phenol/chloroform extraction. This was enough for one ligation reaction, however the transformation reaction was unsuccessful.

There was a small amount of Ryr-3.5 PCR product remaining which had been amplified using eLONGase®. The eLONGase® enzyme mix contains *Taq* DNA polymerase which adds a 3′-A to the PCR product. This would enable the PCR product to be directly ligated

to the pGEM®-T Easy vector as for the production of the Ryr-400 and Ryr-170 constructs described in Chapter Five.

A ligation reaction between Ryr-3.5 and pGEM®-T Easy was carried out and *E. coli* XL-1 blue cells were transformed. Many colonies were produced ($3 \times 10^3 - 5 \times 10^3$ colonies/µg DNA). Thirty-six colonies were analysed by the rapid-boil plasmid preparation method as described in section 2.2.1 before three possible clones were identified. Large scale preparations of the three clones were carried out. A digest of the maxiprep plasmid DNA with *Xba* I and *Sal* I was analysed on a 0.7% agarose gel for clearer resolution of the 3.5 kb and 3.9 kb bands (Figure 6.3).

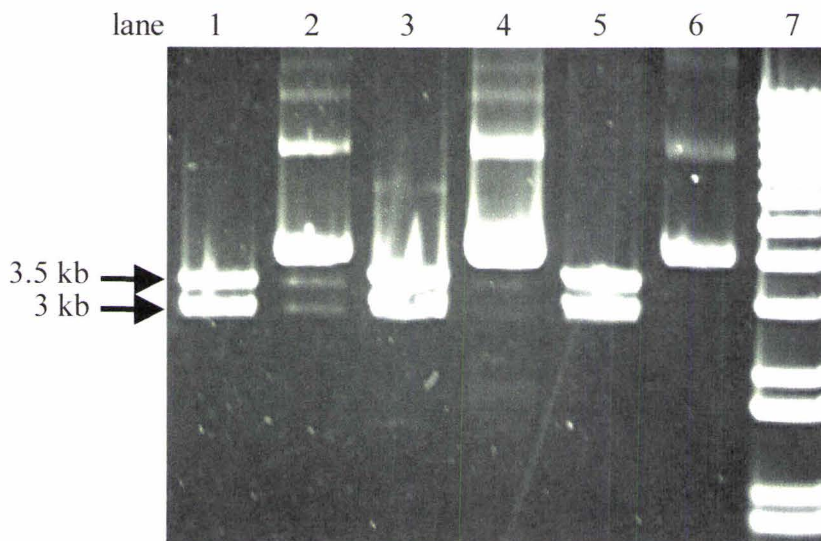

Figure 6.3: Gel photograph of Ryr-3.5 digested out of the pGEM®-T Easy vector.

A maxiprep plasmid DNA consisting of the pGEM®-T Easy vector and Ryr-3.5 was digested with the cloning enzymes Xba I and Sal I for 1 hour at 37°C. 5 µL of each digest and 1 µL of each uncut sample was analysed on a 0.7% agarose gel for 90 minutes at 80 V. The gel was stained in a solution of 5 µg/µL EtBr and the bands visualised with a UV transilluminator. Lanes 1,3,5 – digested samples of each of the three possible clones, lanes 2,4,6 – an uncut sample of each of the respective clones, lane 7 – 1 kb plus ladder.

All three clones were submitted for sequencing from the T7 and Sp6 universal primers on the ABI337-36 automated sequencer to determine if the ends of each clone were correct. One clone did not sequence from the T7 primer and was not analysed further. The other two clones both sequenced well with both primers. These clones will eventually be known as pSVK-3.5a and pSVK-3.5b as they were subsequently subcloned in the vector pSVK3. The sequences were analysed using the GCG (genetics computing group) package of data analysis software and each sequence was compared to the relevant section of the human skeletal muscle ryanodine receptor sequence. The sequence of pSVK-3.5a obtained using the Sp6 primer was ~420 bases long and contained one nucleotide change, G14846A, which caused an amino acid change (E4956K) at the 3′ end. The 5′ end was sequenced from the T7 primer and produced a sequence of ~360 nucleotides long which did not contain any base changes. The 5′ end of pSVK-3.5b, sequenced from Sp6 and was ~420 nucleotides long, had one nucleotide change, G11802A, which resulted in a stop codon (W3934STOP) but had a perfect 3′ end of ~360 bases sequenced from the T7 primer. (The full sequence of pSVK-3.5a with the primers used is in appendix 5).

While the sequencing process was being carried out, Ryr-3.5a and Ryr-3.5b were digested out of pGEM®-T Easy and subcloned into the pSVK3 vector. Approximately 1 μg of each clone was digested with *Xba* I and *Sal* I in a total volume of 50 μL, and a 5 μL aliquot was analysed on a 0.7% agarose gel to confirm that complete digestion had occurred. The remaining digest (~4 μg of each clone in total) was separated on a 0.7% agarose gel at 30 V for ~4 hours to ensure good separation of the 3 kb pGEM®-T Easy vector band from the 3.5 kb Ryr-3.5 band. The gel was stained in a fresh ethidium bromide bath containing 5 μg/mL of EtBr. The 3.5 kb bands were excised out of the gel after visualisation under UV light and purified using the Concert™ gel extraction system (Life Technologies).

The gel-purified Ryr-3.5a and Ryr-3.5b fragments were ligated to pSVK3 (constructs shown in appendix 2) and used to transform *E. coli* XL-1 blue cells. pSVK-3.5a produced 83 colonies (8.3×10^3 colonies/μg DNA) and pSVK-3.5b produced 177 colonies (1.77×10^4 colonies/μg DNA). Blue/white selection is not available for pSVK3, therefore all of the colonies could potentially be screened. Rapid-boil analyses of the plasmid DNA was

used to screen eight and 32 colonies of pSVK-3.5a and pSVK-3.5b respectively before two of each clone were identified. The other colonies analysed contained either vector only or a smaller product that was probably the smaller band present in the PCR reaction. It was difficult to analyse the digestion of the clones with the cloning enzymes due to the similar sizes of the products (3.5 kb and 3.9 kb), therefore *Xba* I and *Cla* I were used. *Xba* I is one of the cloning enzymes and *Cla* I is an enzyme with a unique restriction site at nucleotide position 14294, ~800 bases from the 3' end of the Ryr-3.5 sequence. These enzymes should digest pSVK-3.5 to produce 4.7 kb and 2.7 kb products as analysed on a 1% agarose gel. Bands of these sizes were observed (Figure 6.4).

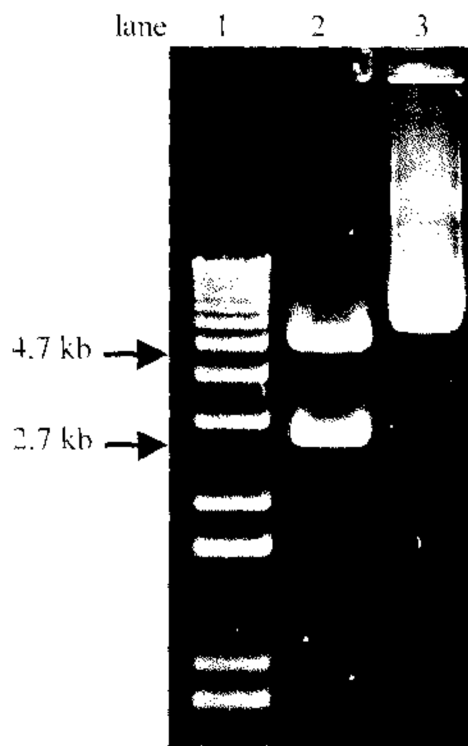

Figure 6.4: Representative gel photograph confirming the size of pSVK-3.5 clones.

Each pSVK-3.5 clone was digested with Xba I and Cla I for 1 hour at 37°C to produce a 4.7 kb and a 2.7 kb band. 5 μL of the digest and 1 μL of the uncut construct were analysed on a 1 % agarose gel for 60 minutes at 100 V. The gel was stained in 5 μg/μL EtBr and the bands visualised under a UV transilluminator. Lane 1 1 kb plus ladder, lane 2 Xba I/Cla I cut pSVK-3.5, lane 3 uncut pSVK-3.5.

pSVK-3.5a (within the pGEM®-T Easy vector) was sequenced on the ABI337-36 (or 64) automated sequencer from the following primers: Sp6, 12084F, TM12277, 12443F, TM13066, TM12985, ryrab 5, TM13356, 13592F, TM13731, TM13973, ryrab 4, 13870R, 14663F and T7 (appendix 3). Each sequence was analysed using the GCG package of data analysis software and initially 12 possible amino acid changes were identified. These mutations were either sequenced in the opposite direction or compared to overlapping sequences in the same direction. Most of the nucleotide changes were sequencing errors and were eventually resolved but a total of four were found to possibly be PCR-induced errors. The nucleotide changes were G11679A, G12328A, A13461G, G13478A, T13696G and G14846A which caused the following amino acid changes: E4494G, E4500K, F4572L and E4956K. (See appendix 5 for location of nucleotide and amino acid changes). pSVK-3.5b (in the pGEM®-T Easy vector) was not totally sequenced initially as it contained a stop codon near the start. It was sequenced across the region that contained three of the amino acid changes in pSVK-3.5a using the TM13973 primer, and the mutations that were present in pSVK-3.5a were absent from this second clone. Therefore to obtain a complete sequence of Ryr-3.5 without mutations, the mutated portions of each clone were digested out, the correct portions of sequence religated and used to transform *E. coli* XL-1 blue cells (Figure 6.5).

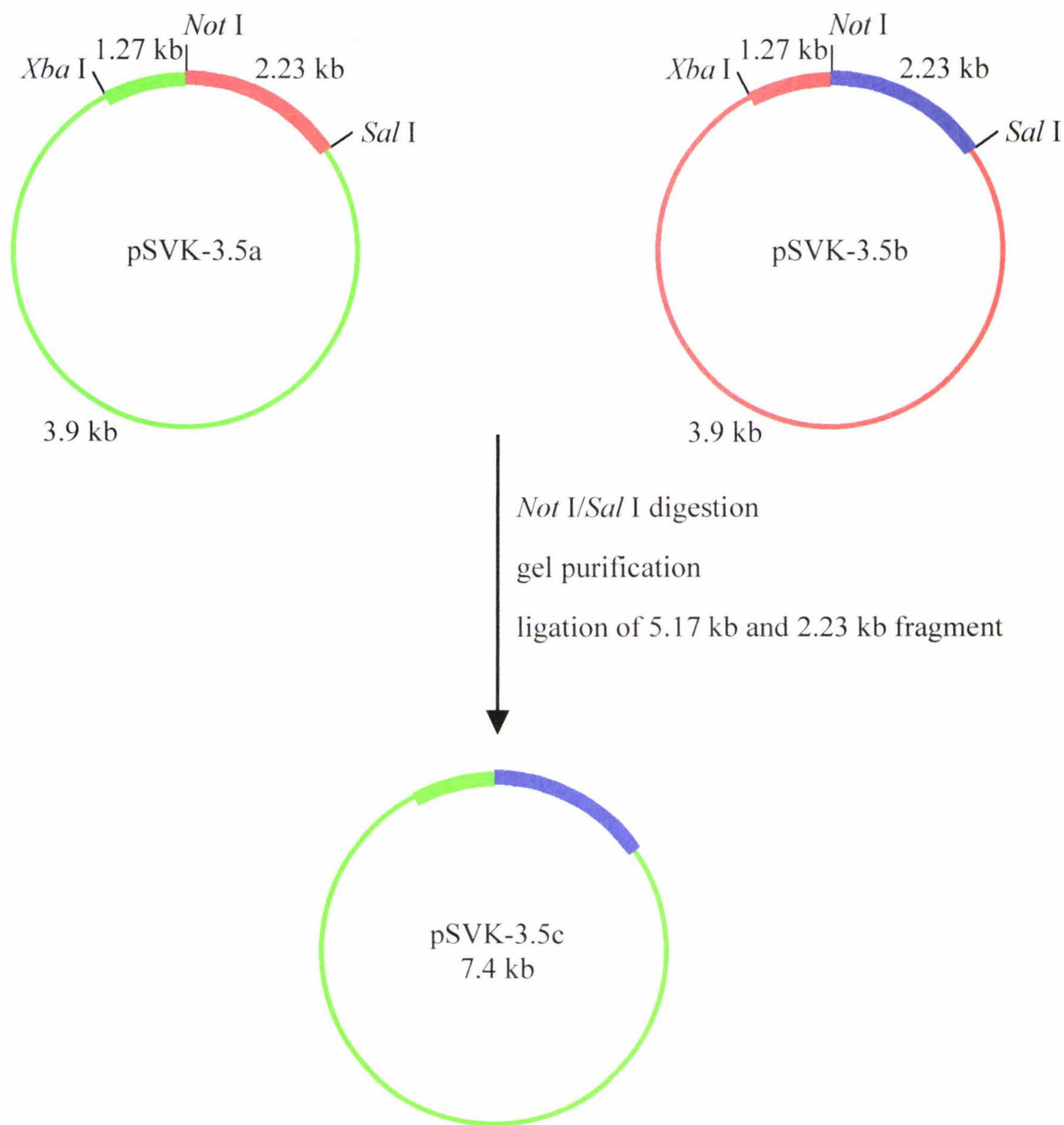

Figure 6.5: Schematic diagram showing the strategy employed to generate pSVK-3.5c.

pSVK-3.5a and pSVK-3.5b were digested with Not I and Sal I restriction enzymes to produce a 5.17 kb and a 2.23 kb fragment. The fragments were separated on a 1% agarose gel for 3 hours at 40 V. The appropriate fragments were excised and gel purified using the Concert™ gel extraction system (Life Technologies). The 5.17 kb pSVK-3.5a fragment was ligated to the 2.23 kb fragment of pSVK-3.5b. The resultant pSVK-3.5c plasmid was used to transform E. coli XL-1 blue cells for large scale preparation, and subsequent transfection of COS cells. (Diagram not to scale).

The rest of the 3′ end of pSVK-3.5b (in the pGEM®-T Easy vector) up to the *Not* I site was sequenced on the ABI337-64 automated sequencer. The sequence obtained from the 14870R primer contained two base changes G14521A and T14534C which caused one amino acid change Y4852H. An overlapping sequence was not obtained but both of these nucleotide changes were definite on the sequencing electropherogram. A small portion of the sequence (~170 nucleotides) was not obtained even after many attempts using plasmid DNA and also PCR products of a smaller portion which included the segment that required sequencing. This region was part of the high GC region but it had sequenced without difficulty in the pSVK-3.5a construct. The TM13066 primer did not produce clear sequence and it contained several discrepancies. Alternative sequencing chemistries were used, including BigDye version 2.0, Dyenamic-ET, the dGTP kit and the dRhodamine kit, all of which were specific for templates with high GC content, but all were unsuccessful. A PCR product using primers TM12985 and TM13973 was prepared but several attempts at producing overlapping sequence with TM12668 were not successful. (Figure 6.6).

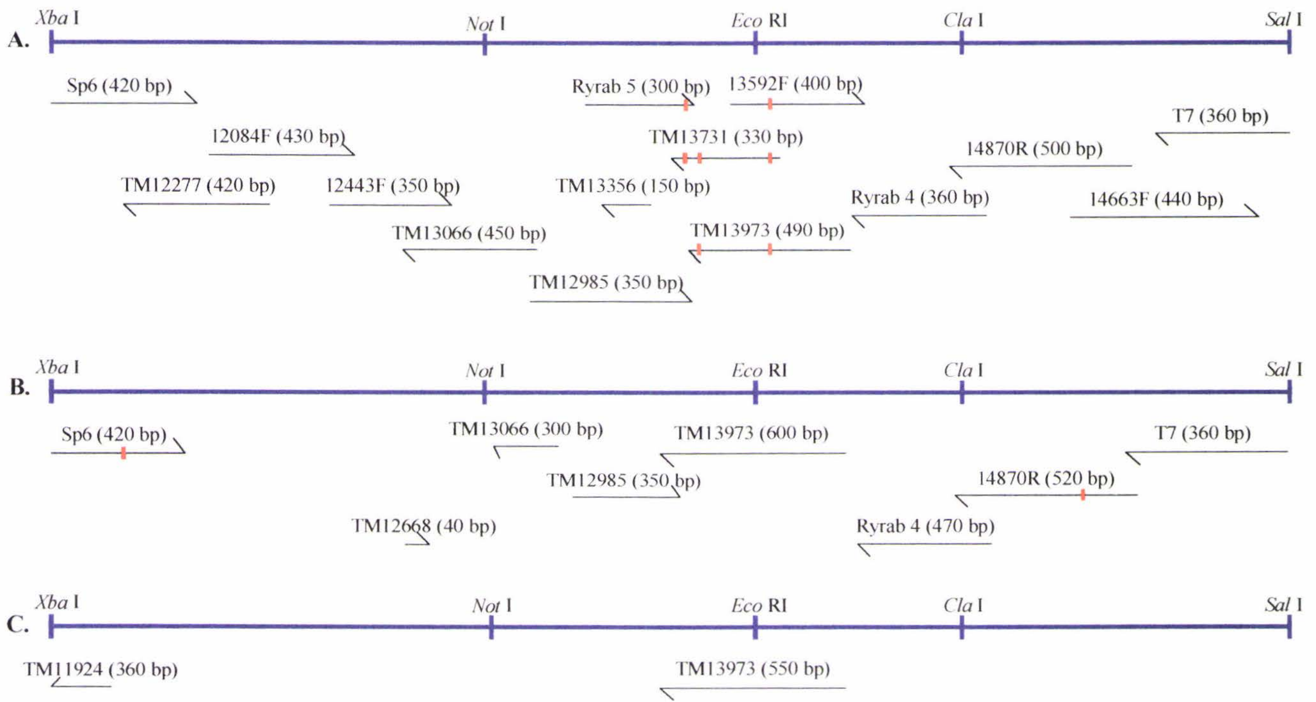

Figure 6.6: Schematic diagram of the sequencing strategy.

The primers used to sequence each construct are shown along with the length of the sequenced product in brackets. The relative position of the mutations produced in the PCR process are indicated in red. The relative sites of the restriction enzymes that were used are also shown. (A) pSVK-3.5a sequenced as the pGEM®-T construct. (B) pSVK-3.5b sequenced as the pGEM®-T construct. (C) pSVK-3.5c sequenced as the pSVK3 construct.

The resulting rejoined clone, pSVK-3.5c (Figure 6.5), was sequenced from the TM13973 and the T7 primer to confirm the absence of the mutations. Initial sequences using plasmid DNA of pSVK-3.5c were unsuccessful, therefore PCR was used to amplify a smaller portion of the clone around the mutation sites. The T7 and TM12277 primers were used to amplify the 5′ end of pSVK-3.5c. Sequencing from the T7 primer was unsuccessful. Resequencing from TM11924 was successful and confirmed the absence of a stop codon. The middle sequence containing three mutations was amplified by PCR using TM12985 and TM13973, and sequenced from TM13973 which confirmed that the correct sequence had been obtained. Although the complete sequence of pSVK-3.5b, used to produce pSVK-3.5c, up to the *Not* I site was not obtained, expression studies were still attempted.

6.4 Expression of pSVK-3.5c

6.4.1 Introduction

Transient transfection is used to introduce cloned DNA into mammalian cells to enable the study of the expression levels of the cDNA. Transient transfection occurs when there is no integration of the cDNA into the chromosomal DNA of the cells. There are four main methods of transfection: calcium phosphate-mediated transfection, DEAE-dextran-mediated transfection, electroporation and liposome-mediated transfection (Sambrook *et al.*, 1989). In the calcium phosphate- and DEAE-dextran-mediated transfection methods, the DNA enters the cytoplasm of the cell by endocytosis and is then transferred to the nucleus. The exact mechanism of uptake of DNA is not well known. These methods have a high efficiency of transfection but require a large amount of DNA and cells. Electroporation requires the application of an electric pulse to the cells which causes nanometer sized pores in the membrane through which the DNA enters. This method is efficient and only a small amount of DNA is integrated into the cells. The liposome-mediated transfection method involves the incorporation of the DNA into cationic liposomes. These liposomes are targeted to the cell surface where they fuse with the cell membrane and release the DNA into the cell. This is also a fairly efficient method of transfection. Although all these methods are reliable methods for introducing DNA into cells, the overall efficiency of transfection is largely dependent on the type of cell that is used (Sambrook *et al.*, 1989).

The FuGENE™ 6 transfection reagent (Roche) is another reagent that is used for transient transfection. The reagent consists of a 'proprietary blend of lipids (non-liposomal formulation)' (FuGENE™ 6 Transfection Reagent information sheet) which introduces DNA into cells in a liposomal-mediated manner. This reagent is reported to produce a high level of transfection efficiency and has a low toxic effect on the cells (FuGENE™ 6 Transfection Reagent information sheet).

COS cells were used in this study as they had been used previously to transiently express ryanodine receptor cDNA (Chen *et al.*, 1993a; Treves *et al.*, 1994). Also plasmids carrying the SV40 regulatory regions as pSVK3 does, which generally have low to moderate

expression levels in other transfected mammalian cells, have high levels of expression in COS cells (Sambrook *et al.*, 1989).

6.4.2 Transient Transfections

pSVK-3.5c was prepared using the Quantum Prep® plasmid maxiprep kit (Bio-Rad) for use in transfection reactions which were carried out as outlined in section 2.2.7.

The FuGENE™ 6 transfection reagent (Roche) was used for the transient transfection of ryanodine receptor DNA into COS cells. This reagent has been used successfully for transient transfection of COS cells by other members of the laboratory.

Due to time constraints on this project, only two transfection reactions were carried out. In the first experiment transient transfection was carried out and the cell extracts were prepared as outlined in section 2.2.7 by disrupting cell membranes using homogenisation. For the second experiment, the transfection reaction was the same but the cell extracts were prepared using the freeze-thaw method to disrupt the cell membranes whilst keeping microsomal membranes intact.

Cell extracts were prepared, and after determining the protein concentration, the proteins were separated on an 8% SDS polyacrylamide gel and analysed by immunoblotting (Figure 6.7). Both methods of disrupting the cells produced similar results. The results show the same banding pattern in both transfected and untransfected cells, with bands occurring at ~35 kDa, ~50 kDa, ~60 kDa, ~97 – 116 kDa, and a band at ~140 kDa. If the C-terminal domain of the ryanodine receptor had been produced, a band of ~140 kDa would be expected. Although there was a band at the expected size of the C-terminal domain (~140 kDa), it appeared in both lanes, suggesting that expression had not occurred in sufficient amounts to be detected by immunoblotting or that it was not a part of the ryanodine receptor, but non-specific binding of the Ryr-C antibody.

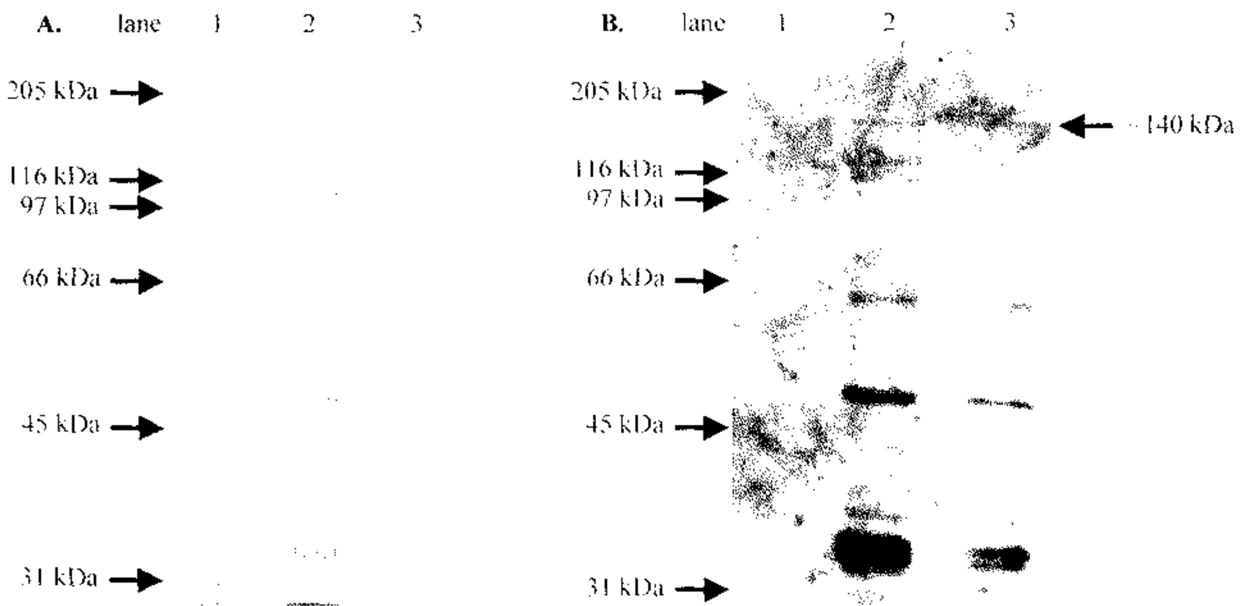

Figure 6.7: Immunoblot of COS cell extracts transfected with pSVK-3.5.

(A) The proteins in the COS cell extracts were separated on an 8% SDS polyacrylamide gel for 30 minutes at 200 V, stained with Coomassie Blue and destained with destain I and II. (B) The separated proteins were transferred to a nitrocellulose membrane by electroblotting for 45 minutes at 450 mA. The membrane was incubated with a 1/1000 dilution of the RyR-C primary antibody and a 1/10000 dilution of the antigoat secondary antibody. The bands were detected using the chemiluminescence western blotting kit from Roche. For both figures, lane 1 – molecular weight marker, lane 2 – extract from untransfected cells, lane 3 – extract from cells transfected with pSVK-3.5c DNA.

6.5 Chapter Summary

Although it took many attempts to amplify and clone the C-terminal domain of the ryanodine receptor, successful cloning was eventually achieved. This was demonstrated by a diagnostic digest producing the correct sized bands (see figure 6.4) and by sequencing of the entire 3.5 kb fragment (Appendix 5). Sequencing was carried out with Ryr-3.5 in the pGEM®-T Easy vector and not in the pSVK3 vector. In the production of pSVK-3.5c, gel slices were purified out of an agarose gel prior to ligation and subsequent cloning of the construct. The cDNA was exposed to UV irradiation as the gel slice was being excised out

of the gel. UV irradiation can introduce mutations into DNA. Mutations may have occurred in the construct which prevented the C-terminal domain from being expressed. Therefore, in retrospect, it would have been more expedient to have sequenced the entire Ryr-3.5 cDNA in the pSVK-3.5c construct to ensure that no untoward mutations had occurred before carrying out the expression studies.

It was also not always possible to sequence the DNA from a plasmid preparation, especially when trying to sequence the pSVK-3.5c construct, which suggests that possibly either some of the vector DNA or the Ryr-3.5 cDNA was not correct. However, diagnostic digests of the pSVK-3.5c construct resulted in DNA fragments of the correct size. It may have been useful to sequence over the ends of the Ryr-3.5 and into the vector to ensure that the joins were correct. This was done with the TM11924 primer which produced a sequence of ~360 bases long and sequenced over the multiple cloning site of pSVK3 from the *Xba* I site to the T7 primer site. Although sequencing of the 3′ end was not carried out, it would seem that the vector and cDNA were probably correct.

Expression studies were commenced but did not appear to be successful at this stage. Due to time constraints, the transient transfection protocol was not optimised. The parts of the pSVK-3.5c construct that were sequenced were shown to contain a single amino acid change, Y4852H, which is in a transmembrane domain in both the Zorzato *et al* (1990) and the Takeshima *et al* (1989) models. The two amino acids, tyrosine and histidine, are both polar amino acids and therefore may not affect the tertiary structure in such a way that expression and folding would not occur. Also, a complete sequence of the portion of pSVK-3.5b that was used to construct pSVK-3.5c was not obtained. Therefore the construct could possibly contain a stop codon or other amino acid changes that may hinder expression studies. Possible optimisation conditions will be discussed in the next chapter. Ryanodine binding and calcium release properties were also unable to be studied due to time constraints. A discussion on this will follow in Chapter Seven.

CHAPTER SEVEN – SUMMARY AND FUTURE DIRECTIVES

7.1 Overall Summary

The initial aim of this project was to biochemically characterise the novel mutations in the C-terminal domain of the ryanodine receptor with respect to the ryanodine binding and calcium release properties. Although this was not achieved, considerable preparative work towards this goal has been achieved.

In the sarcoplasmic reticulum isolation, no significant ryanodine binding was observed. Western blot analysis identified possible bands at ~205 kDa and >205 kDa (see figure 3.5 A and B). If the ~205 kDa band was a degradation product of the ryanodine receptor this could explain why ryanodine binding was not observed if ryanodine binding sites were not maintained. If the band >205 kDa was a band indicative of ryanodine receptors, then absence of ryanodine binding may have been due to the closed conformational state of the receptor. Ryanodine only binds to the ryanodine receptor when the receptor is in the open conformation (Coronado *et al.*, 1994; Meissner, 1986), and various conditions promote this. If ryanodine receptors were present but only in small amounts, or were present in insufficient amounts to be detected by ^{3}H-Ry, then this could partly account for the faint detection that was observed on immunoblotting.

RNA was isolated from skeletal muscle tissue. The northern blot analysis showed the presence of ryanodine receptor mRNA (see figure 4.2). The mRNA appeared to contain some degradation products as indicated by the smeary pattern detected on the membrane. Although the Trizol™ LS reagent information sheet claimed that the ingredients of the reagent maintain the integrity of the RNA during the homogenisation step, the method of homogenisation that was used (15 second bursts with an ultra-turax T25 homogeniser) may have disrupted the integrity of the RNA as well as of the other cellular components. This might have accounted for the difficulty that was encountered in trying to amplify the 3.5 kb C-terminal domain of the ryanodine receptor by RT-PCR.

The muscle tissue that was used for both the SR vesicle and RNA isolation procedures had been stored at -20°C post biopsy before being stored under liquid nitrogen for long term storage. The short-term storage at -20°C may have contributed to the degradation of proteins and/or RNA. This could account for the inefficient binding of ^{3}H-Ry to the ryanodine receptors that was observed, if the ryanodine receptors were not intact but were partially degraded. The ~205 kDa band that was observed on the western blot of the SR vesicle preparation could have been a degradation product of this sort. Also the inability to obtain consistently clean cDNA products from RNA might be due to these storage conditions in that long transcripts were not produced initially as the RNA might not have been totally intact.

Expression of pGEX-400 and pProEX-400 containing the recombinant Ryr-400 cDNA was unable to be obtained. This was possibly because Ryr-400 contained part of a transmembrane domain which may have been unstable in the expression system used. pGEX-170 was successfully expressed in *E. coli* BL21-DE3 cells. The 6.8 kDa polypeptide of ~56 amino acids long could be partially cleaved away from GST as indicated in figure 5.12. It was deemed unnecessary to obtain full cleavage of the fusion protein or to pursue the production of antibodies as commercial antibodies had become available by this time.

After many months, the C-terminal domain of the ryanodine receptor was amplified and cloned. Two possible clones were identified. One of these clones, pSVK-3.5a, was confirmed by sequencing the entire 3.5 kb segment (see appendix 5). The sequence of pSVK-3.5a, in the pGEM®-T vector, contained 6 nucleotide changes causing four amino acid changes. These amino acid changes were not seen in the equivalent portion of the second clone, pSVK-3.5b, although this clone contained a stop codon at the 5′ end and a single amino acid change at the 3′ end. Therefore to obtain a complete 3.5 kb C-terminal domain with no PCR-induced mutations, it was necessary to digest pSVK-3.5a and pSVK-3.5b with the appropriate restriction enzymes and rejoin the correct portions without the mutations to form pSVK-3.5c. Two attempts at obtaining expression of pSVK-3.5c in COS cells was carried out but expression was not detected by immunoblotting. Time constraints prevented the optimisation of expression studies.

7.2 Future Directives

Preparative work in constructing the C-terminal domain of the ryanodine receptor to be able to study ryanodine binding and calcium release effects on normal and mutant channels was almost complete. Work would need to be done to optimise the conditions for expressing pSVK-3.5c in mammalian cells. Once expression was confirmed, introducing the mutations into the C-terminal domain could be carried out so that ryanodine binding and calcium release could be studied.

7.2.1 Expression of the C-terminal Domain

Several parameters using the FuGENE™ 6 reagent could be altered. The FuGENE™ 6 Transfection Reagent information sheet recommends that the maximum ratio of 2 µg DNA:3 µL FuGENE™ 6 reagent be used in a transfection reaction. This ratio was used without success, therefore it could be altered to include a variety of ratios. The cells were harvested after ~48 hours of transfection incubation. The information sheet states that the length of transfection depends on the vector construct and the type of protein being expressed, and that expression can be measured after 4 – 72 hours incubation. A series of transfection reactions could be carried out and the cells harvested after 4, 8, 12, 24, 36 and 72 hours. The level of expression could be detected by immunoblotting with RyR-C antibodies until a clear positive result with no background was obtained.

Transient transfection has been achieved for the whole ryanodine receptor or for just the C-terminal domain by a number of groups using different methods. COS cells were transfected by a DEAE-dextran method (Chen *et al.*, 1993a) or by a 'scrape loading' technique (Treves *et al.*, 1994). HEK293 cells have been transfected using a calcium phosphate precipitation method (Chen *et al.*, 1997; Tong *et al.*, 1999; Tong *et al.*, 1997), and CHO cells have been transfected with ryanodine receptor DNA using electroporation (Bhat *et al.*, 1997). If expression studies could not be optimised using FuGENE™ 6 reagent and COS cells, then a different cell line and/or transfection technique could be used.

To determine whether the recombinant ryanodine receptor C-terminal domain had associated with the endoplasmic reticulum in COS cells, *in situ* detection using fluorescently labelled antibodies could be attempted. This would involve labelling the primary antibody, RyR-C, with a fluorescent tag and incubating the labelled antibody with the COS cells. By staining the cells and observing the presence of the fluorescent antibody, it would confirm that the cells did contain the expressed Ryr-3.5 C-terminal domain.

The efficiency of the antibodies is not known. Only short antigenic peptides of 18 –19 amino acids long were used to raise antibodies which may or may not recognise and bind to exposed portions of the ryanodine receptor. The effectiveness of the antibodies could be tested by *in situ* staining of the antibody in skeletal muscle slices obtained from a muscle biopsy. This could be carried out using slices of muscle biopsy tissue embedded in parafilm on a microscope slide. The primary antibody, RyR-C or RyR-N, could be introduced to the muscle slice before the biotin-labelled secondary antibody, antigoat, is added. With the addition of streptavidin, horseradish peroxidase (HRP) and the appropriate substrate, it could be possible to detect the antibody. Ryanodine receptors should be present in the muscle slice, and if the antibodies are detected by this staining procedure it would indicate that they are suitable for use in immunoblotting procedures to detect RyR1.

It is also not known exactly where on the ryanodine receptor that the RyR-C and RyR-N antibodies were. Therefore, it may be useful to pursue antibody production using the 6.8 kDa polypeptide to obtain antibodies to a portion of the receptor that is known.

7.2.2 Mutagenesis of the C-terminal Domain

Site-directed mutagenesis using PCR could be used to introduce each of the novel mutations into the C-terminal domain. A 377 base pair segment of the ryanodine receptor around the mutation site could be generated in a PCR reaction which would include either the H4833Y or the T4826I mutation. The primers that would be used are indicated in table 7.1. This segment includes the *Cla* I and *Acc* I restriction enzyme sites. These sites are unique within the ryanodine receptor and therefore these enzymes could be used to remove the normal segment of ryanodine receptor and insert the sequence carrying the mutation.

Pfu DNA polymerase (Stratagene) could be used in the PCR reactions as it has high fidelity and a very low error rate due to the 3′ – 5′ exonuclease proofreading activity that allows it to correct nucleotide mismatches. The PCR products are blunt ended and do not contain a 3′-A overhang (*Pfu* DNA polymerase information sheet) therefore it is a useful polymerase for site-directed mutagenesis. A diagram outlining the process that would be used to generate the C-terminal domain with the mutations is shown in figure 7.1. The *Acc* I restriction enzyme recognises the same site as *Sal* I which is in the multiple cloning site of the vector. Therefore it would be necessary to introduce the mutations into Ryr-3.5 before cloning into the pSVK3 vector possibly via an alternative vector system to the pGEM®-T vector system as this vector also has a *Sal* I restriction site in the multiple cloning site. Sequencing over the area of the mutations would be necessary to confirm the incorporation of the mutation into the C-terminal domain sequence.

Primer	sequence 5′ – 3′
mut F	TGC TGA CCT GGG TCA TGT CC
mut R	CCA CGT ACA TGT GAA ACA GG
4826F	ACG CTG CGG A**T**C ATC CTG TCC
4826R	GGA CAG GAT G**A**T GCG CAG CGT C
4833F	CTC TGT CAC C**T**A CAA TGG GAA AC
4833R	TTC CCA TTG TAG GTG ACA GAG

Table 7.1: Table of primers to be used for site-directed mutagenesis.

The bases indicating the mutations are shown in bold.

Rabbit *RYR1* is ~91% identical to human *RYR1*. An ~800 bp portion of the C-terminal domain of the rabbit ryanodine receptor is available which includes the sites of the T4826I and H4833Y mutations. Attempts could be made to introduce these mutations into the rabbit clone in a similar manner to that described above. Once each mutation is incorporated into the rabbit clone, the smaller portion could be subcloned back into the full-length rabbit *RYR1* cDNA. This would enable the calcium release and ryanodine binding properties of the human mutations to be studied within the context of the rabbit RyR1 protein to examine the effects that the mutations may have on channel function.

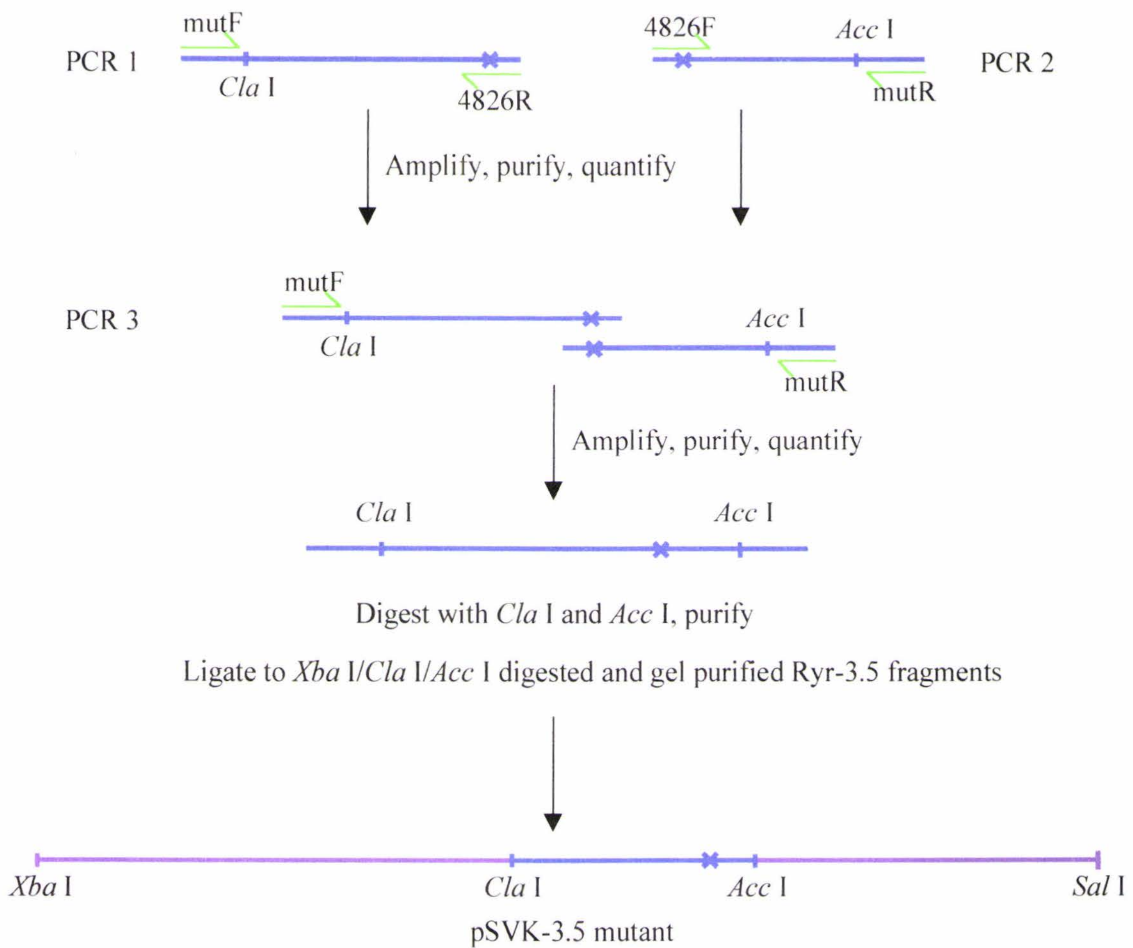

Figure 7.1: Schematic diagram of the process of site-directed mutagenesis.

This diagram shows the process for introducing the T4826I mutation into the ryanodine receptor C-terminal domain. The same process would apply for the H4833Y mutation. The 'X' indicates the relative position of the mutation. Diagram is not drawn to scale.

7.2.3 Ryanodine Binding and Calcium Release Studies

When the wild type pSVK-3.5c and mutated pSVK-3.5 are cloned and expression in mammalian cells is confirmed by immunoblotting with RyR-C antibodies, further studies

could be carried out to determine the effect that the mutations have on ryanodine binding and calcium release.

Ryanodine binding is an indirect measure of calcium release and could be used to study the recombinant C-terminal domain of the ryanodine receptor expressed in COS cells. ^{3}H-Ry binding using AMP-PCP to keep the channels in an open conformation, could be studied as described in section 2.2.2. Any non-specific binding of ryanodine could be determined by adding at least a 1000 times excess of unlabelled ryanodine to the buffer with the ^{3}H-Ry (Chen *et al.*, 1997; Lunde and Sejersted, 1997). Unlabelled ryanodine would be expected to bind to any non-specific sites because of the excess and ^{3}H-Ry would bind competitively to specific sites and therefore a decrease in radioactivity would be detected.

Calcium release could be studied in primary muscle cells. Primary muscle cells can be isolated from muscle biopsy tissue and grown in tissue culture medium. Calcium release from the sarcoplasmic reticulum could be studied using the calcium-fluorescent indicator dye Fura-2/AM (Roche) and a fluorescence microscope. Fura-2/AM is hydrolysed to Fura-2 by esterases in the cytosol. The excitation ratio of Fura-2 at 340/380 nm can be used to calculate the concentration of intracellular calcium because the intensity and wavelength of the maximum Fura-2 emission fluorescence is independent of the calcium concentration (Fura-2/AM information sheet).

It may be possible to study the effect of calcium release on the mutant ryanodine receptor if muscle biopsy tissue containing the T4826I and H4833Y mutations is obtained. This would be useful, particularly for the family carrying the H4833Y mutation, as this is a small family where linkage analysis has not been possible.

Several groups have used Fura-2 for examining calcium release in tissue culture cells (Du *et al.*, 1998b; Du *et al.*, 2000; Tong *et al.*, 1999; Tong *et al.*, 1997). Cells were transfected and loaded with Fura-2 after 30 or 45 minutes. A sample of intact cells was excited at 340 nm and 380 nm and the emitted fluorescence was fed into a photomultiplier tube. Digital images or light intensities over a time period were analysed by software packages to calculate the 340/380 nm ratio and concentration of calcium. This method could be used to

study the effects of calcium release on the C-terminal domain expressed in COS cells. The calcium release properties of the C-terminal domain mutations that would be introduced into the C-terminal cDNA by site-directed mutagenesis, could also be studied in this way.

Once a normal measure of calcium release and ryanodine binding is obtained in the respective systems, the experiments could be repeated with various modulators as discussed in Chapter One. This would give an indication of how the mutant channel is affected in relation to the wild-type channel: or how the modulators regulate just the C-terminal domain. It would be useful in determining the effects that the C-terminal domain mutations have on channel activity.

Although the overall aim to biochemically characterise the two novel C-terminal domain mutations was not achieved, the C-terminal domain was produced by RT-PCR and early attempts to express it in COS cells were carried out. A considerable amount of additional work will need to be carried out in order to determine the effects of the two mutations on the ryanodine binding and calcium release properties of skeletal muscle ryanodine receptors.

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APPENDIX ONE – ^{3}H-Ry binding raw data

Experiment 1 – 10 μg protein (rat) used for ^{3}H-Ry binding assay.

	cpm	Average cpm	Standard Deviation
Total ^{3}H-Ry	534990.00		
Control	2829.09		
T-P1	1738.62	3285.06	1991.38
	2584.52		
	5532.05		
T-P2	2398.81	2949.01	864.63
	3942.14		
	2506.09		
T-S4	1884.88	2689.10	2069.97
	1141.93		
	5040.50		
C-P1	2394.76	2086.38	293.48
	1810.50		
	2053.88		
C-P2	2327.44	1867.03	628.56
	2122.74		
	1150.92		
C-S4	2059.28	1587.47	526.11
	1683.03		
	1020.15		

Experiment 2 – Amount of protein increased to 50 μg protein (rat) in binding assay.

	cpm	Average cpm	Standard Deviation
Total ^{3}H-Ry	500386.66		
Control	3249.35	4003.50	686.29
	4591.36		
	4169.80		
T-unfractionated	5104.50	3896.89	1252.22
	2604.39		
	3981.78		
T-P1	7202.86	5734.87	2018.36
	3433.22		
	6568.52		
T-P2	6342.15	4836.49	2133.11
	5746.48		
	2420.84		
T-S4	4094.14	3541.61	483.80
	3193.97		
	3336.72		
C-unfractionated	2835.21	1994.53	728.17
	1560.93		
	1587.46		
C-P1	1967.84	2201.04	217.84
	2399.29		
	2236.00		
C-P2	2075.03	2351.37	892.47
	3349.33		
	1629.76		
C-S4	1271.39	1858.94	611.37
	1656.46		
	2468.96		

Experiment 3 – 10 μg protein (rat) used and a change in [Ca^{2+}] in binding assay buffer.

	cpm	Average cpm	Standard Deviation
0.25μM	4570.77	4696.5	874.00
	5626.56		
	3892.17		
Control (0.25μM)	4688.68		
1μM	2637.20	4310.80	2146.75
	6731.21		
	3564.00		
Control (1μM)	3211.89		
2.5μM	4185.73	3946.38	653.93
	4446.91		
	3206.49		
Control (2.5μM)	3522.56		
10μM	2547.76	3069.64	458.58
	3408.19		
	3252.97		
Control (10μM)	2558.42		
25μM	3914.73	2769.59	1040.74
	2512.68		
	1881.37		
Control (25μM)	1702.49		

Experiment 4 – 10 μg protein (human) for assay.

	cpm	Average cpm	Standard Deviation
Total ^{3}H-Ry	498026.66		
Control	1171.64	2100.33	1315.16
	3605.25		
	1524.10		
Unfractionated	2055.79	2110.79	553.85
	2690.08		
	1586.49		
P1	1472.46	1081.62	345.99
	957.90		
	814.50		
P2	2619.12	1749.08	753.48
	1315.71		
	1312.41		
S4	2134.61	1939.98	638.87
	2458.90		
	1226.44		

Experiment 5 – SR vesicles partially purified on a sucrose gradient were used in assay.

	cpm	Average cpm	Standard Deviation
Total ^{3}H-Ry	485984.00		
Control	3490.78	3177.20	962.57
	3943.87		
	2096.94		
10μg protein	2427.35	3160.44	780.54
	3156.64		
	3987.33		
50μg protein	5567.50	5110.09	1788.95
	6625.93		
	3136.84		

Experiment 6 – An increased amount of total protein was used in the assay.

	cpm	Average cpm
Total ^{3}H-Ry	522006.66	
Control	3066.90	3727.23
	4387.56	
#2.T-P1 (500μg)	1400.79	
#2.T-P2 (250μg)	1307.94	
#5 (135μg)	7735.48	

Experiment 7 – 10 μg protein (human), using AMP-PCP in the binding buffer.

	cpm	Average cpm	Standard Deviation
Total ^{3}H-Ry	437479.97		
Control	4940.00	4086.41	743.86
	3576.79		
	3742.43		
Unfractionated	4305.53	4384.06	505.17
	3922.75		
	4923.90		

Experiment 8 – 10 μg protein (human), AMP-PCP, TCA precipitation prior to filtering.

	cpm	Average cpm	Standard Deviation
Total ^{3}H-Ry	403799.97		
Control	3286.45	10082.94	6879.90
	17043.33		
	9919.05		
Unfractionated	12930.00	13429.34	1673.81
	12061.71		
	15296.30		

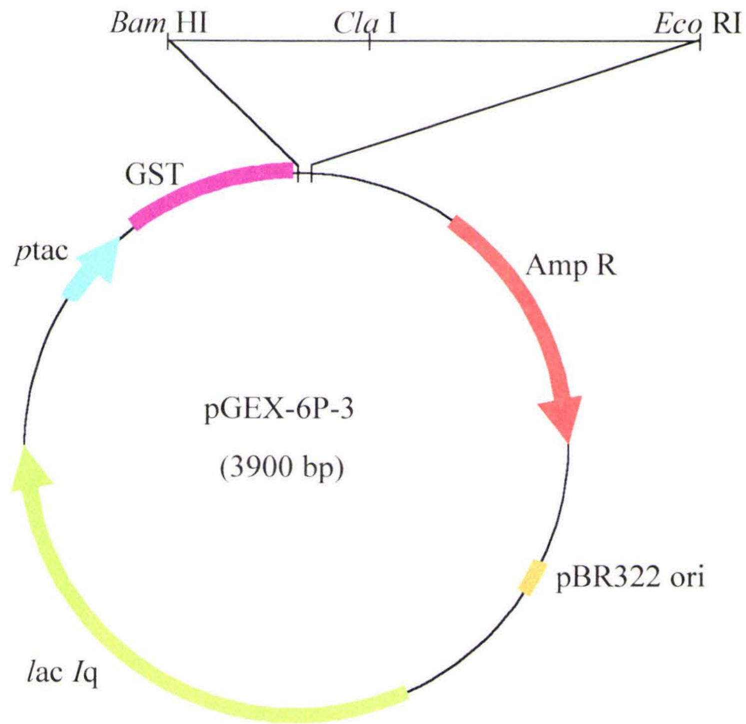
Experiment 9 – 10 μg protein (human), AMP-PCP, TCA precipitation.

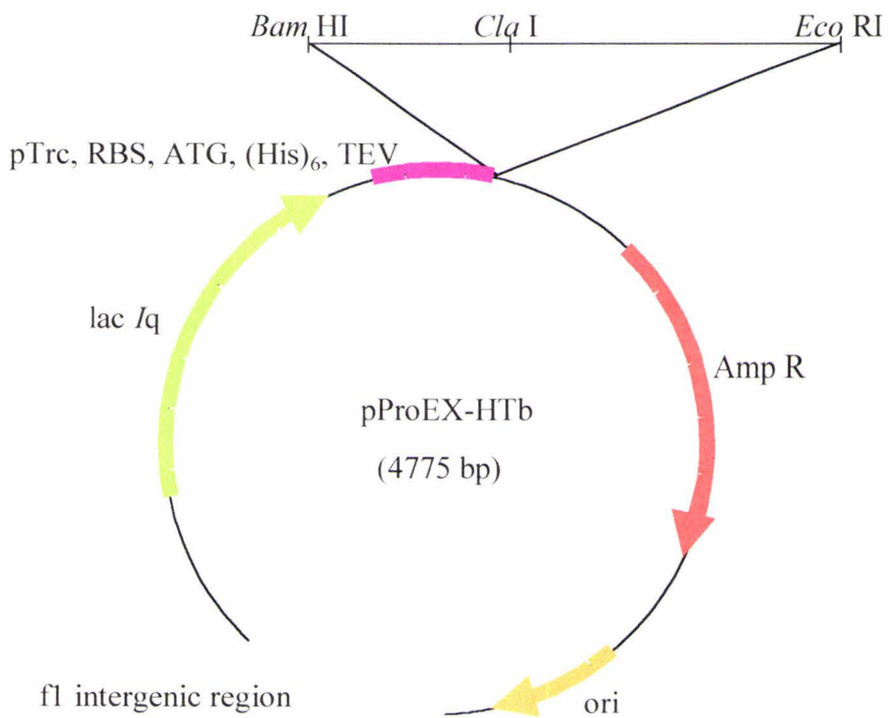
	cpm	Average cpm	Standard Deviation
Total ^{3}H-Ry	254712.00		
Control	6113.43	3834.20	2023.51
	3140.00		
	2249.16		
Unfractionated	2058.98	1801.94	347.59
	1940.38		
	1406.46		

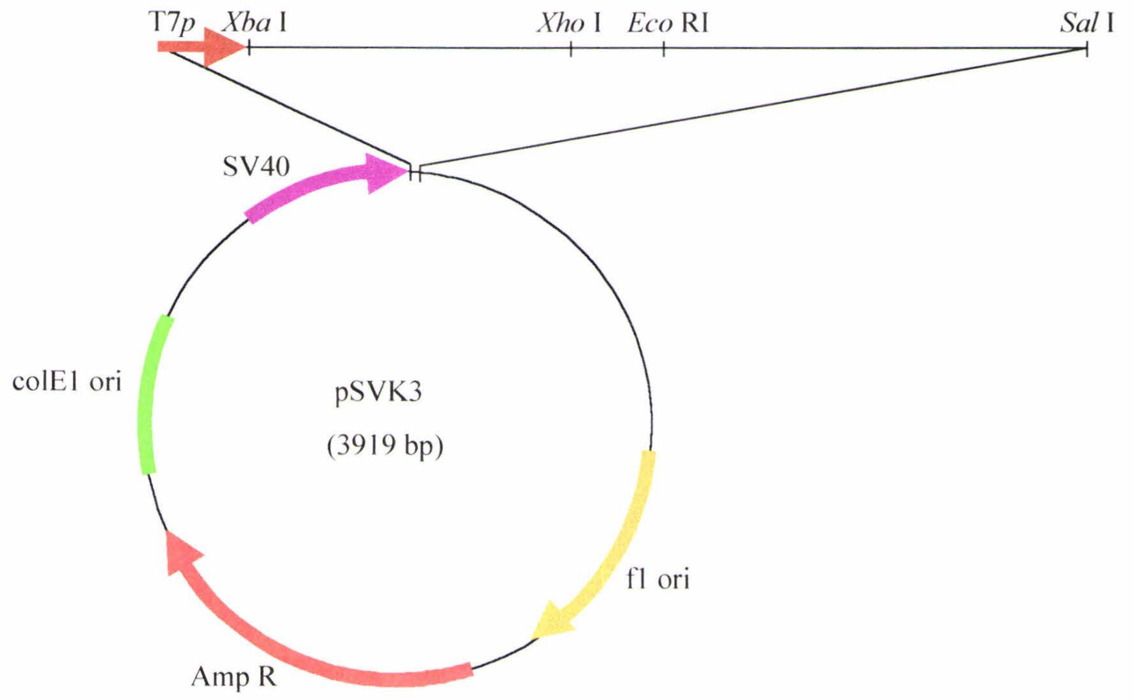
Experiment 10 – 10 μg protein (human), AMP-PCP, TCA precipitation.

	cpm	Average cpm	Standard Deviation
Total ^{3}H-Ry	407566.66		
Control	11567.78	8317.78	2884.57
	7324.36		
	6061.21		
Unfractionated	7052.41	7421.89	1701.91
	5935.07		
	9278.18		

APPENDIX TWO – vector constructs



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Sal I
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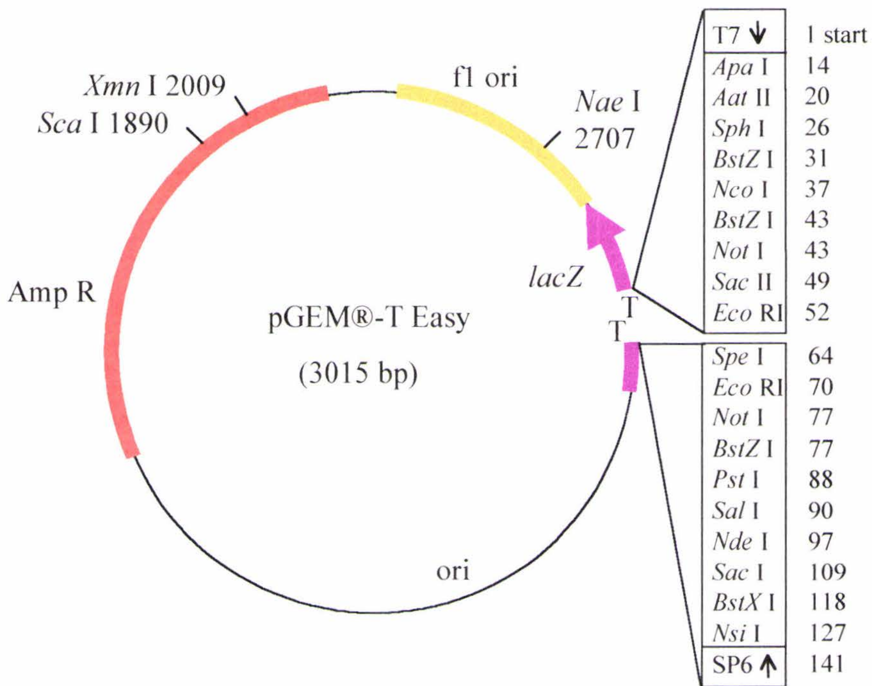
colE1 ori
pSVK3
(3919 bp)
f1 ori
Amp R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T
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Aat II 20
Sph I 26
BstZ I 31
Nco I 37
BstZ I 43
Not I 43
Sac II 49
Eco RI 52
Spe I 64
Eco RI 70
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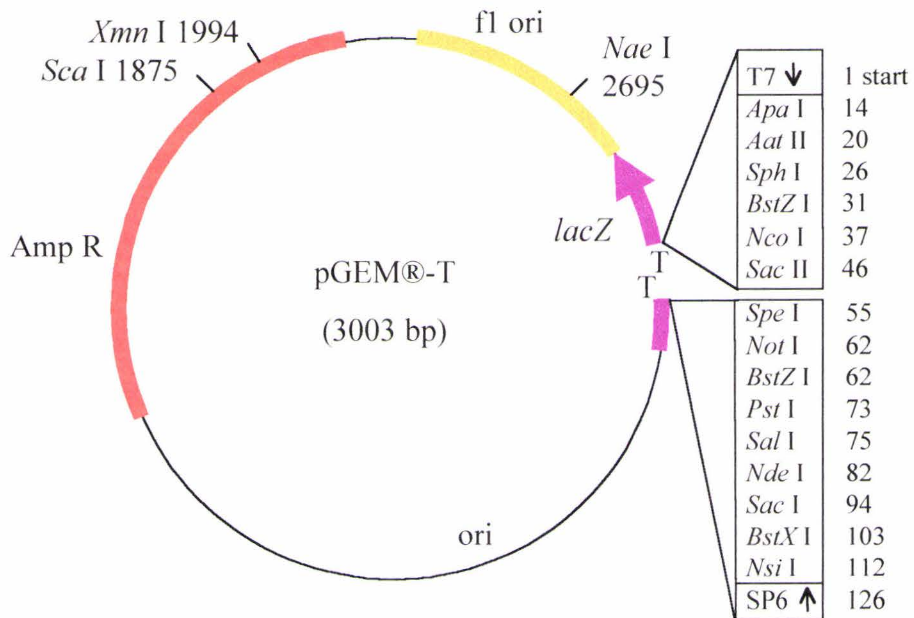
Sal I 90
Nde I 97
Sac I 109
BstX I 118
Nsi I 127
SP6 141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Sca I 1875
f1 ori
Nae I 2695
Amp R
lacZ
pGEM®-T
(3003 bp)
T
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ori
T7 1 start
Apa I 14
Aat II 20
Sph I 26
BstZ I 31
Nco I 37
Sac II 46
Spe I 55
Not I 62
BstZ I 62
Pst I 73
Sal I 75
Nde I 82
Sac I 94
BstX I 103
Nsi I 112
SP6 126

APPENDIX THREE - primers

Primers used for PCR and Sequencing reactions

Primer	Sequence (5' to 3')	Use
ryrab 1	GGC CGG ATC CTA CGG GCG GGA GCG GAT TGC	*
ryrab 2	GCC GGA ATT CTA CTC GCT CTT GTT GTA GAA C	*
ryrab 3	GGC CGG ATC CGA GGA CGA TGA CGT GAA GG	*
ryrab 4	GCC GGA ATT CTA GTT GTC TGT GAA GAT GAC C	* †
ryrab 5	GGC CGG ATC CAA GAA GGT GAC GGT GAC C	* †
ryrab 6	GCC GGA ATT CTA CTC CTT CTT TGG AGG GGG	*
ryrsvk 1	GGC CTC TAG ACC ACC ATG GCG GAT GAT GAA TTC	*
ryrsvk 2	CCG GGT CGA CTC AGC TAA GCT GGT CCT C	*
ryrsvk 3	TAG CTG AGG CCT CGC AGG	*
ryrsvk 4	GAC CGA CGA GGA CGA GGG	*
12084F	TGA TGA AGC TCG CTC AG	†
12443F	CGA AAT GAT CAA CTG CG	†
13592F	AGA AAG CCG ATG CCG AG	†
14663F	TCT TCC GCA AGT TCT AC	†
14870R	TGA CGA TGA CGA AGA AG	†
GEX3	GGG CTG GCA AGC CAC GTT TGG TG	†
M13 reverse	AGC GGA TAA CAA TTT CAC ACA GG	†
Sp6	ATT TAG GTG ACA CTA TAG	†
T7	TAA TAC GAC TCA CTA TAG GG	† *
TM11924	TGC GTC CCA TAG GCG ACT GT	†
TM12198	CTC TGA AGC CTT CCA GGA CTA	*
TM12277	TGG ACC GCT GAA CTG CTT CTG	† *
TM12418	ATG CTC CGA CAG GTT GGT CAG	*
TM12575	GAG ACC AAC CGC GCC CAG TG	*
TM12668	AGA TGG AGC TCT TCG TGA GTT TCT G	† *
TM12985	CTG CTC TGG GCA GCA GTG AC	†
TM13066	ACC AGG CCG CCG CCG AAC AG	†
TM13356	GGT TCC GCA GGC GTC GTG TCC	†
TM13377	GCC CAC ACC CGA GGG CTC TC	*
TM13731	TCC CCT GGT GGA GAG TCT GAG	†
TM13973	TTA CCA GGG GCA CCT TGA GAC	† *

* primers used in the polymerase chain reaction

† primers used in sequencing reactions

APPENDIX FOUR – Ryr-400 and Ryr-170 sequences

Ryr-400 sequence

```
                  Bam HI          ryrab 1
        GGCCGGATCCTACGGGCGGGAGCGGATTGCTGAGCTACTGGGCATGGACCTGGCCACACT
14206             ---------+---------+---------+---------+---------- 14255
                  ATGCCCGCCCTCGCCTAACGACTCGATGACCCGTACCTGGACCGGTGTGA
 4733             Y  G  R  E  R  I  A  E  L  L  G  M  D  L  A  T  L  4749

        AGAGATCACAGCCCACAATGAGCGCAAGCCCAACCCGCCGCCAGGGCTGCTGACCTGGCT
14256   ---------+---------+---------+---------+---------+---------+ 14315
        TCTCTAGTGTCGGGTGTTACTCGCGTTCGGGTTGGGCGGCGGTCCCGACGACTGGACCGA
 4750   E  I  T  A  H  N  E  R  K  P  N  P  P  P  G  L  L  T  W  L  4769
             Cla I                                                G
        CATGTCCATCGATGTCAAGTACCAGATCTGGAAGTTCGGGGTCATCTTCACAGACAACTC
14316   ---------+---------+---------+---------+---------+---------+ 14375
        GTACAGGTAGCTACAGTTCATGGTCTAGACCTTCAAGCCCCAGTAGAAGTGTCTGTTGAG
 4770   M  S  I  D  V  K  Y  Q  I  W  K  F  G  V  I  F  T  D  N  S  4789
                                                               S
        CTTCCTGTACCTGGGCTGGTATATGGTGATGTCCCTCTTGGGACACTACAACAACTTCTT
14376   ---------+---------+---------+---------+---------+---------+ 14435
        GAAGGACATGGACCCGACCATATACCACTACAGGGAGAACCCTGTGATGTTGTTGAAGAA
 4790   F  L  Y  L  G  W  Y  M  V  M  S  L  L  G  H  Y  N  N  F  F  4809

        CTTTGCTGCCCATCTCCTGGACATCGCCATGGGGGTCAAGACGCTGCGCACCATCCTGTC
14436   ---------+---------+---------+---------+---------+---------+ 14495
        GAAACGACGGGTAGAGGACCTGTAGCGGTACCCCCAGTTCTGCGACGCGTGGTAGGACAG
 4810   F  A  A  H  L  L  D  I  A  M  G  V  K  T  L  R  T  I  L  S  4829

        CTCTGTCACCCACAATGGGAAACAGCTGGTGATGACCGTGGGCCTTCTGGCGGTGGTCGT
14496   ---------+---------+---------+---------+---------+---------+ 14555
        GAGACAGTGGGTGTTACCCTTTGTCGACCACTACTGGCACCCGGAAGACCGCCACCAGCA
 4830   S  V  T  H  N  G  K  Q  L  V  M  T  V  G  L  L  A  V  V  V  4849
                                                         ryrab 2
        CTACCTGTACACCGTGGTGGCCTTCAACTTCTTCCGCAAGTTCTACAACAAGAGCGAGTA
14556   ---------+---------+---------+---------+---------+---------+ 14615
        GATGGACATGTGGCACCACCGGAAGTTGAAGAAGGCGTTCAAGATGTTGTTCTCGCTCAT
 4850   Y  L  Y  T  V  V  A  F  N  F  F  R  K  F  Y  N  K  S  E  *  4869
         Eco RI
        G
14616   -
        CTTAAGGCCG
```

The sequence of Ryr-400 was aligned with the human ryanodine receptor mRNA using the 'bestfit' application of the GCG package of data analysis software. The position of the primers used to generate this portion of RYR1 cDNA are shown in bold with the position of the restriction enzymes used for cloning in blue. The Cla I restriction site used in diagnostic digests is indicated in blue. The protein translation is shown with the position of the nucleotide and amino acid change in bold.

Ryr-170 sequence

```
                 Bam HI       ryrab 1                      C    TT
          GGCCGGATCCTACGGGCGGGAGCGGATTGCTGAGCTACTGGGCATGGACCTGGCCACA
14206                       -------+---------+---------+---------+---------+ 14253
                            ATGCCCGCCCTCGCCTAACGACTCGATGACCCGTACCTGGACCGGTGT
 4733                       Y   G   R   E   R   I   A   E   L   L   G   M   D   L   A   T   4748
                          C                                       P   L
      CTAGAGATCACAGCCCACAATGAGCGCAAGCCCAACCCGCCGCCAGGGCTGCTGACCTGG
14254 ---------+---------+---------+---------+---------+---------+ 14313
      GATCTCTAGTGTCGGGTGTTACTCGCGTTCGGGTTGGGCGGCGGTCCCGACGACTGGACC
 4749 L   E   I   T   A   H   N   E   R   K   P   N   P   P   P   G   L   L   T   W   4768
               Cla I                                  ryrab 4
      CTCATGTCCATCGATGTCAAGTACCAGATCTGGAAGTTCGGGGTCATCTTCACAGACAAC
14314 ---------+---------+---------+---------+---------+---------+ 14373
      GAGTACAGGTAGCTACAGTTCATGGTCTAGACCTTCAAGCCCCAGTAGAAGTGTCTGTTG
 4769 L   M   S   I   D   V   K   Y   Q   I   W   K   F   G   V   I   F   T   D   N   4788
        Eco RI
      TAG
14374 ---          14376
      ATCTTAAGGCCG
 4789 *            4789
```

The sequence of Ryr-170 was aligned with the human ryanodine receptor mRNA using the 'bestfit' application of the GCG package of data analysis software. The protein translation is shown with the position of the primers used to generate this portion of RYR1 cDNA shown in bold. The position of the restriction enzymes used for cloning and the Cla I restriction site used in diagnostic digests are indicated in blue. The nucleotide and amino acid changes are in bold.

APPENDIX FIVE – Ryr-3.5 sequence

Ryr-3.5 (as pSVK-3.5a) sequence

```
             XbaI          ryrsvk 1
      GGCCTCTAGACCACCATGGCGGATGATGAATTCACACAAGACCTGTTCCGATTCCTACAA
11620                           ----+---------+---------+---------+---------+ 11664
                           TACCGCCTACTACTTAAGTGTGTTCTGGACAAGGCTAAGGATGTT
 3874                           M  A  D  D  E  F  T  Q  D  L  F  R  F  L  Q   3888
                           A
      TTGCTCTGTGAGGGGCACAATAATGATTTCCAGAACTACCTACGGACACAGACAGGGAAC
11665 ---------+---------+---------+---------+---------+---------+ 11724
      AACGAGACACTCCCCGTGTTATTACTAAAGGTCTTGATGGATGCCTGTGTCTGTCCCTTG
 3889 L  L  C  E  G  H  N  N  D  F  Q  N  Y  L  R  T  Q  T  G  N    3908

      ACGACCACTATTAACATCATCATTTGCACTGTGGACTACCTCCTGCGGCTGCAGGAATCC
11725 ---------+---------+---------+---------+---------+---------+ 11784
      TGCTGGTGATAATTGTAGTAGTAAACGTGACACCTGATGGAGGACGCCGACGTCCTTAGG
 3909 T  T  T  I  N  I  I  I  C  T  V  D  Y  L  L  R  L  Q  E  S    3928

      ATCAGCGACTTCTACTGGTACTACTCGGGCAAGGATGTCATTGAAGAGCAGGGCAAGAGG
11785 ---------+---------+---------+---------+---------+---------+ 11824
      TAGTCGCTGAAGATGACCATGATGAGCCCGTTCCTACAGTAACTTCTCGTCCCGTTCTCC
 3929 I  S  D  F  Y  W  Y  Y  S  G  K  D  V  I  E  E  Q  G  K  R    3948

      AACTTCTCCAAAGCCATGTCGGTGGCTAAGCAGGTGTTCAACAGCCTCACTGAGTACATC
11825 ---------+---------+---------+---------+---------+---------+ 11884
      TTGAAGAGGTTTCGGTACAGCCACCGATTCGTCCACAAGTTGTCGGAGTGACTCATGTAG
 3949 N  F  S  K  A  M  S  V  A  K  Q  V  F  N  S  L  T  E  Y  I    3968
                                                 TM11924
      CAGGGTCCCTGCACCGGGAACCAGCAGAGCCTGGCGCACAGTCGCCTATGGGACGCAGTG
11885 ---------+---------+---------+---------+---------+---------+ 11944
      GTCCCAGGGACGTGGCCCTTGGTCGTCTCGGACCGCGTGTCAGCGGATACCCTGCGTCAC
 3969 Q  G  P  C  T  G  N  Q  Q  S  L  A  H  S  R  L  W  D  A  V    3988
                                                12084F
      GTGGGATTCCTGCACGTGTTCGCCCACATGATGATGAAGCTCGCTCAGGACTCAAGCCAG
11945 ---------+---------+---------+---------+---------+---------+ 12004
      CACCCTAAGGACGTGCACAAGCGGGTGTACTACTACTTCGAGCGAGTCCTGAGTTCGGTC
 3989 V  G  F  L  H  V  F  A  H  M  M  M  K  L  A  Q  D  S  S  Q    4008

      ATCGAGCTGCTGAAGGAGCTGCTGGATCTGCAGAAGGACATGGTGGTGATGTTGCTGTCG
12005 ---------+---------+---------+---------+---------+---------+ 12064
      TAGCTCGACGACTTCCTCGACGACCTAGACGTCTTCCTGTACCACCACTACAACGACAGC
 4009 I  E  L  L  K  E  L  L  D  L  Q  K  D  M  V  V  M  L  L  S    4028

      CTACTAGAAGGGAACGTGGTGAACGGCATGATCGCCCGGCAGATGGTGGACATGCTCGTG
12065 ---------+---------+---------+---------+---------+---------+ 12124
      GATGATCTTCCCTTGCACCACTTGCCGTACTAGCGGGCCGTCTACCACCTGTACGAGCAC
 4029 L  L  E  G  N  V  V  N  G  M  I  A  R  Q  M  V  D  M  L  V    4048

      GAATCCTCATCCAATGTGGAGATGATCCTCAAGTTCTTCGACATGTTCCTGAAACTCAAG
12125 ---------+---------+---------+---------+---------+---------+ 12184
      CTTAGGAGTAGGTTACACCTCTACTAGGAGTTCAAGAAGCTGTACAAGGACTTTGAGTTC
 4049 E  S  S  S  N  V  E  M  I  L  K  F  F  D  M  F  L  K  L  K    4068
```

```
                     TM12198
      GACATTGTGGGCTCTGAAGCCTTCCAGGACTACGTAACGGATCCCCGTGGCCTCATCTCC
12185 ---------+---------+---------+---------+---------+---------+ 12244
      CTGTAACACCCGAGACTTCGGAAGGTCCTGATGCATTGCCTAGGGGCACCGGAGTAGAGG
 4069 D  I  V  G  S  E  A  F  Q  D  Y  V  T  D  P  R  G  L  I  S   4088
                                              TM12277
      AAGAAGGACTTCCAGAAGGCCATGGACAGCCAGAAGCAGTTCAGCGGTCCAGAAATCCAG
12245 ---------+---------+---------+---------+---------+---------+ 12304
      TTCTTCCTGAAGGTCTTCCGGTACCTGTCGGTCTTCGTCAAGTCGCCAGGTCTTTAGGTC
 4089 K  K  D  F  Q  K  A  M  D  S  Q  K  Q  F  S  G  P  E  I  Q   4108
                              A              12443F
      TTCCTGCTTTCGTGCTCCGAAGCGGATGAGAACGAAATGATCAACTGCGAAGAGTTCGCC
12305 ---------+---------+---------+---------+---------+---------+ 12364
      AAGGACGAAAGCACGAGGCTTCGCCTACTCTTGCTTTACTAGTTGACGCTTCTCAAGCGG
 4109 F  L  L  S  C  S  E  A  D  E  N  E  M  I  N  C  E  E  F  A   4128

      AACCGCTTCCAGGAGCCAGCACGCGACATCGGCTTCAACGTGGCGGTGCTGCTGACCAAC
12365 ---------+---------+---------+---------+---------+---------+ 12424
      TTGGCGAAGGTCCTCGGTCGTGCGCTGTAGCCGAAGTTGCACCGCCACGACGACTGGTTG
 4129 N  R  F  Q  E  P  A  R  D  I  G  F  N  V  A  V  L  L  T  N   4148
       TM12418
      CTGTCGGAGCATGTGCCGCATGACCCTCGCCTGCACAACTTCCTGGAGCTGGCCGAGAGC
12425 ---------+---------+---------+---------+---------+---------+ 12484
      GACAGCCTCGTACACGGCGTACTGGGAGCGGACGTGTTGAAGGACCTCGACCGGCTCTCG
 4149 L  S  E  H  V  P  H  D  P  R  L  H  N  F  L  E  L  A  E  S   4168

      ATCCTTGAGTACTTCCGCCCCTACCTGGGCCGCATCGAGATCATGGGCGCGTCACGCCGC
12485 ---------+---------+---------+---------+---------+---------+ 12544
      TAGGAACTCATGAAGGCGGGGATGGACCCGGCGTAGCTCTAGTACCCGCGCAGTGCGGCG
 4168 I  L  E  Y  F  R  P  Y  L  G  R  I  E  I  M  G  A  S  R  R   4188
                                                 TM12575
      ATCGAGCGCATCTACTTCGAGATCTCAGAGACCAACCGCGCCCAGTGGGAGATGCCCCAG
12545 ---------+---------+---------+---------+---------+---------+ 12604
      TAGCTCGCGTAGATGAAGGTCTAGAGTCTCTGGTTGGCGCGGGTCACCCTCTACGGGGTC
 4189 I  E  R  I  Y  F  E  I  S  E  T  N  R  A  Q  W  E  M  P  Q   4208

      GTGAAGGAGTCCAAGCGCCAGTTCATCTTCGACGTGGTGAACGAGGGCGGCGAGGCTGAG
12605 ---------+---------+---------+---------+---------+---------+ 12664
      CACTTCCTCAGGTTCGCGGTCAAGTAGAAGCTGCACCACTTGCTCCCGCCGCTCCGACTC
 4209 V  K  E  S  K  R  Q  F  I  F  D  V  V  N  E  G  G  E  A  E   4228
              TM12668
      AAGATGGAGCTCTTCGTGAGTTTCTGCGAGGACACCATCTTCGAGATGCAGATCGCCGCG
12665 ---------+---------+---------+---------+---------+---------+ 12724
      TTCTACCTCGAGAAGCACTCAAAGACGCTCCTGTGGTAGAAGCTCTACGTCTAGCGGCGC
 4229 K  M  E  L  F  V  S  F  C  E  D  T  I  F  E  M  Q  I  A  A   4248
                                              ryrsvk 4
      CAGATCTCGGAGCCCGAGGGCGAGCCGGAGACCGACGAGGACGAGGGCGCGGGCGCGGCG
12725 ---------+---------+---------+---------+---------+---------+ 12784
      GTCTAGAGCCTCGGGCTCCCGCTCGGCCTCTGGCTGCTCCTGCTCCCGCGCCCGCGCCGC
 4249 Q  I  S  E  P  E  G  E  P  E  T  D  E  D  E  G  A  G  A  A   4268
                                                Xho I
      GAGGCGGGCGCGGAAGGCGCGGAGGAGGGCGCGGCGGGGCTCGAGGGCACGGCGGCCACG
12785 ---------+---------+---------+---------+---------+---------+ 12844
      CTCCGCCCGCGCCTTCCGCGCCTCCTCCCGCGCCGCCCCGAGCTCCCGTGCCGCCGGTGC
 4269 E  A  G  A  E  G  A  E  E  G  A  A  G  L  E  G  T  A  A  T   4288
```

```
                                          Not I                    ryrsvk 3
      GCGGCGGCGGGGGCGACGGCGCGGGTTGTGGCGGCCGCAGGCCGGGCCCTGCGAGGCCTC
12845 ---------+---------+---------+---------+---------+---------+ 12904
      CGCCGCCGCCCCCGCTGCCGCGCCCAACACCGCCGGCGTCCGGCCCGGGACGCTCCGGAG
 4289 A  A  A  G  A  T  A  R  V  V  A  A  A  G  R  A  L  R  G  L   4308

      AGCTACCGCAGCCTGCGGCGGCGCGTGCGGCGGCTGCGGCGGCTTACGGCCCGCGAGGCG
12905 ---------+---------+---------+---------+---------+---------+ 12964
      TCGATGGCGTCGGACGCCGCCGCGCACGCCGCCGACGCCGCCGAATGCCGGGCGCTCCGC
 4309 S  Y  R  S  L  R  R  R  V  R  R  L  R  R  L  T  A  R  E  A   4328
                          TM12985
      GCCACCGCAGTGGCGGCGCTGCTCTGGGCAGCAGTGACGCGCGCTGGGGCCGCTGGCGCG
12965 ---------+---------+---------+---------+---------+---------+ 13024
      CGGTGGCGTCACCGCCGCGACGAGACCCGTCGTCACTGCGCGCGACCCCGGCGACCGCGC
 4329 A  T  A  V  A  A  L  L  W  A  A  V  T  R  A  G  A  A  G  A   4348
                                                  TM13066
      GGGGCGGCGGCGGGCGCGCTGGGCCTGCTCTGGGGCTCGCTGTTCGGCGGCGGCCTGGTG
13025 ---------+---------+---------+---------+---------+---------+ 13084
      CCCCGCCGCCGCCCGCGCGACCCGGACGAGACCCCGAGCGACAAGCCGCCGCCGGACCAC
 4349 G  A  A  A  G  A  L  G  L  L  W  G  S  L  F  G  G  G  L  V   4368
              ryrab 5
      GAGGGCGCCAAGAAGGTGACGGTGACCGAGCTCCTGGCAGGCATGCCCGACCCCACCAGC
13085 ---------+---------+---------+---------+---------+---------+ 13144
      CTCCCGCGGTTCTTCCACTGCCACTGGCTCGAGGACCGTCCGTACGGGCTGGGGTGGTCG
 4369 E  G  A  K  K  V  T  V  T  E  L  L  A  G  M  P  D  P  T  S   4388

      GACGAGGTGCACGGCGAGCAGCCGGCCGGGCCGGGCGGAGACGCAGACGGCGAGGGTGCC
13145 ---------+---------+---------+---------+---------+---------+ 13204
      CTGCTCCACGTGCCGCTCGTCGGCCGGCCCGGCCCGCCTCTGCGTCTGCCGCTCCCACGG
 4389 D  E  V  H  G  E  Q  P  A  G  P  G  G  D  A  D  G  E  G  A   4408

      AGCGAGGGCGCTGGAGACGCCGCGGAGGGCGCTGGAGACGAGGAGGAGGCGGTGCACGAG
13205 ---------+---------+---------+---------+---------+---------+ 13264
      TCGCTCCCGCGACCTCTGCGGCGCCTCCCGCGACCTCTGCTCCTCCTCCGCCACGTGCTC
 4409 S  E  G  A  G  D  A  A  E  G  A  G  D  E  E  E  A  V  H  E   4428

      GCCGGGCCGGGCGGTGCCGACGGGGCGGTGGCCGTGACCGATGGGGGCCCCTTCCGGCCG
13265 ---------+---------+---------+---------+---------+---------+ 13324
      CGGCCCGGCCCGCCACGGCTGCCCCGCCACCGGCACTGGCTACCCCCGGGGAAGGCCGGC
 4429 A  G  P  G  G  A  D  G  A  V  A  V  T  D  G  G  P  F  R  P   4448
                                               TM13356       TM13377
      GAAGGGGCTGGCGGTCTCGGGGACATGGGGGACACGACGCCTGCGGAACCGCCCACACCC
13325 ---------+---------+---------+---------+---------+---------+ 13384
      CTTCCCCGACCGCCAGAGCCCCTGTACCCCCTGTGCTGCGGACGCCTTGGCGGGTGTGGG
 4449 E  G  A  G  G  L  G  D  M  G  D  T  T  P  A  E  P  P  T  P   4468

      GAGGGCTCTCCCATCCTCAAGAGGAAATTGGGGGTGGATGGAGTGGAGGAGGAGCTCCCG
13385 ---------+---------+---------+---------+---------+---------+ 13444
      CTCCCGAGAGGGTAGGAGTTCTCCTTTAACCCCCACCTACCTCACCTCCTCCTCGAGGGC
 4469 E  G  S  P  I  L  K  R  K  L  G  V  D  G  V  E  E  E  L  P   4488
                      G                 A               13592F
      CCAGAGCCAGAGCCCGAGCCGGAACCAGAGCTGGAGCCGGAGAAAGCCGATGCCGAGAAT
13445 ---------+---------+---------+---------+---------+---------+ 13504
      GGTCTCGGTCTCGGGCTCGGCCTTGGTCTCGACCTCGGCCTCTTTCGGCTACGGCTCTTA
 4489 P  E  P  E  P  E  P  E  P  E  L  E  P  E  K  A  D  A  E  N   4508
                      G                 K
```

```
      GGGGAGAAGGAAGAAGTTCCCGAGCCCACACCAGAGCCCCCCAAGAAGCAAGCACCTCCC
13505 ---------+---------+---------+---------+---------+---------+ 13564
      CCCCTCTTCCTTCTTCAAGGGCTCGGGTGTGGTCTCGGGGGGTTCTTCGTTCGTGGAGGG
 4509 G  E  K  E  E  V  P  E  P  T  P  E  P  P  K  K  Q  A  P  P   4528
                ryrab 6
      TCACCCCCTCCAAAGAAGGAGGAAGCTGGAGGCGAATTCTGGGGAGAACTGGAGGTGCAG
13565 ---------+---------+---------+---------+---------+---------+ 13624
      AGTGGGGGAGGTTTCTTCCTCCTTCGACCTCCGCTTAAGACCCCTCTTGACCTCCACGTC
 4529 S  P  P  P  K  K  E  E  A  G  G  E  F  W  G  E  L  E  V  Q   4548

      AGGGTGAAGTTCCTGAACTACCTGTCCCGGAACTTTTACACCCTGCGGTTCCTTGCCCTC
13625 ---------+---------+---------+---------+---------+---------+ 13684
      TCCCACTTCAAGGACTTGATGGACAGGGCCTTGAAAATGTGGGACGCCAAGGAACGGGAG
 4549 R  V  K  F  L  N  Y  L  S  R  N  F  Y  T  L  R  F  L  A  L   4568
                 G                                           TM13731
      TTCTTGGCATTTGCCATCAACTTCATCTTGCTGTTTTATAAGGTCTCAGACTCTCCACCA
13685 ---------+---------+---------+---------+---------+---------+ 13744
      AAGAACCGTAAACGGTAGTTGAAGTAGAACGACAAAATATTCCAGAGTCTGAGAGGTGGT
 4569 F  L  A  F  A  I  N  F  I  L  L  F  Y  K  V  S  D  S  P  P   4588
               L
      GGGGAGGACGACATGGAAGGCTCAGCTGCTGGGGATGTGTCAGGTGCAGGCTCTGGTGGC
13745 ---------+---------+---------+---------+---------+---------+ 13804
      CCCCTCCTGCTGTACCTTCCGAGTCGACGACCCCTACACAGTCCACGTCCGAGACCACCG
 4589 G  E  D  D  M  E  G  S  A  A  G  D  V  S  G  A  G  S  G  G   4608

      AGCTCTGGCTGGGGCTTGGGGGCCGGAGAGGAGGCAGAGGGCGATGAGGATGAGAACATG
13805 ---------+---------+---------+---------+---------+---------+ 13864
      TCGAGACCGACCCCGAACCCCCGGCCTCTCCTCCGTCTCCCGCTACTCCTACTCTTGTAC
 4609 S  S  G  W  G  L  G  A  G  E  E  A  E  G  D  E  D  E  N  M   4628

      GTGTACTACTTCCTGGAGGAAAGCACAGGCTACATGGAACCCGCCCTGCGGTGTCTGAGC
13865 ---------+---------+---------+---------+---------+---------+ 13924
      CACATGATGAAGGACCTCCTTTCGTGTCCGATGTACCTTGGGCGGGACGCCACAGACTCG
 4629 V  Y  Y  F  L  E  E  S  T  G  Y  M  E  P  A  L  R  C  L  S   4648
                                                             TM13973
      CTCCTGCATACACTGGTGGCCTTTCTCTGCATCATTGGCTATAATTGTCTCAAGGTGCCC
13925 ---------+---------+---------+---------+---------+---------+ 13984
      GAGGACGTATGTGACCACCGGAAAGAGACGTAGTAACCGATATTAACAGAGTTCCACGGG
 4649 L  L  H  T  L  V  A  F  L  C  I  I  G  Y  N  C  L  K  V  P   4668

      CTGGTAATCTTTAAGCGGGAGAAGGAGCTGGCCCGGAAGCTGGAGTTTGATGGCCTGTAC
13985 ---------+---------+---------+---------+---------+---------+ 14044
      GACCATTAGAAATTCGCCCTCTTCCTCGACCGGGCCTTCGACCTCAAACTACCGGACATG
 4669 L  V  I  F  K  R  E  K  E  L  A  R  K  L  E  F  D  G  L  Y   4688
                          ryrab 3
      ATCACGGAGCAGCCTGAGGACGATGACGTGAAGGGGCAGTGGGACCGACTGGTGCTCAAC
14045 ---------+---------+---------+---------+---------+---------+ 14104
      TAGTGCCTCGTCGGACTCCTGCTACTGCACTTCCCCGTCACCCTGGCTGACCACGAGTTG
 4689 I  T  E  Q  P  E  D  D  D  V  K  G  Q  W  D  R  L  V  L  N   4708

      ACGCCGTCTTTCCCTAGCAACTACTGGGACAAGTTTGTCAAGCGCAAGGTCCTGGACAAA
14105 ---------+---------+---------+---------+---------+---------+ 14164
      TGCGGCAGAAAGGGATCGTTGATGACCCTGTTCAAACAGTTCGCGTTCCAGGACCTGTTT
 4709 T  P  S  F  P  S  N  Y  W  D  K  F  V  K  R  K  V  L  D  K   4728
```

```
                        ryrab 1
      CATGGGGACATCTACGGGCGGGAGCGGATTGCTGAGCTACTGGGCATGGACCTGGCCACA
14165 ---------+---------+---------+---------+---------+---------+ 14224
      GTACCCCTGTAGATGCCCGCCCTCGCCTAACGACTCGATGACCCGTACCTGGACCGGTGT
 4729 H  G  D  I  Y  G  R  E  R  I  A  E  L  L  G  M  D  L  A  T   4748

      CTAGAGATCACAGCCCACAATGAGCGCAAGCCCAACCCGCCGCCAGGGCTGCTGACCTGG
14225 ---------+---------+---------+---------+---------+---------+ 14284
      GATCTCTAGTGTCGGGTGTTACTCGCGTTCGGGTTGGGCGGCGGTCCCGACGACTGGACC
 4749 L  E  I  T  A  H  N  E  R  K  P  N  P  P  P  G  L  L  T  W   4768
                 Cla I                                  ryrab 4
      CTCATGTCCATCGATGTCAAGTACCAGATCTGGAAGTTCGGGGTCATGTTCACAGACAAC
14285 ---------+---------+---------+---------+---------+---------+ 14344
      GAGTACAGGTAGCTACAGTTCATGGTCTAGACCTTCAAGCCCCAGTACAAGTGTCTGTTG
 4769 L  M  S  I  D  V  K  Y  Q  I  W  K  F  G  V  I  F  T  D  N   4788

      TCCTTCCTGTACCTGGGCTGGTATATGGTGATGTCCCTCTTGGGACACTACAACAACTTC
14345 ---------+---------+---------+---------+---------+---------+ 14404
      AGGAAGGACATGGACCCGACCATATACCACTACAGGGAGAACCCTGTGATGTTGTTGAAG
 4789 S  F  L  Y  L  G  W  Y  M  V  M  S  L  L  G  H  Y  N  N  F   4808

      TTCTTTGCTGCCCATCTCCTGGACATCGCCATGGGGGTCAAGACGCTGCGCACCATCCTG
14405 ---------+---------+---------+---------+---------+---------+ 14464
      AAGAAACGACGGGTAGAGGACCTGTAGCGGTACCCCCAGTTCTGCGACGCGTGGTAGGAC
 4809 F  F  A  A  H  L  L  D  I  A  M  G  V  K  T  L  R  T  I  L   4828

      TCCTCTGTCACCCACAATGGGAAACAGCTGGTGATGACCGTGGGCCTTCTGGCGGTGGTC
14465 ---------+---------+---------+---------+---------+---------+ 14524
      AGGAGACAGTGGGTGTTACCCTTTGTCGACCACTACTGGCACCCGGAAGACCGCCACCAG
 4829 S  S  V  T  H  N  G  K  Q  L  V  M  T  V  G  L  L  A  V  V   4848
       Acc I                                14663 F       ryrab 2
      GTCTACCTGTACACCGTGGTGGCCTTCAACTTCTTCCGCAAGTTCTACAACAAGAGCGAG
14525 ---------+---------+---------+---------+---------+---------+ 14584
      CAGATGGACATGTGGCACCACCGGAAGTTGAAGAAGGCGTTCAAGATGTTGTTCTCGCTC
 4849 V  Y  L  Y  T  V  V  A  F  N  F  F  R  K  F  Y  N  K  S  E   4868

      GATGAGGATGAACCTGACATGAAGTGTGATGACATGATGACGTGTTACCTGTTTCACATG
14585 ---------+---------+---------+---------+---------+---------+ 14644
      CTACTCCTACTTGGACTGTACTTCACACTACTGTACTACTGCACAATGGACAAAGTGTAC
 4869 D  E  D  E  P  D  M  K  C  D  D  M  M  T  C  Y  L  F  H  M   4888

      TACGTGGGTGTCCGGGCTGGCGGAGGCATTGGGGACGAGATCGAGGACCCCGCGGGTGAC
14645 ---------+---------+---------+---------+---------+---------+ 14704
      ATGCACCCACAGGCCCGACCGCCTCCGTAACCCCTGCTCTAGCTCCTGGGGCGCCCACTG
 4889 Y  V  G  V  R  A  G  G  G  I  G  D  E  I  E  D  P  A  G  D   4908
                                                      14870R
      GAATACGAGCTCTACAGGGTGGTCTTCGACATCACCTTCTTCTTCTTCGTCATCGTCATC
14705 ---------+---------+---------+---------+---------+---------+ 14764
      CTTATGCTCGAGATGTCCCACCAGAAGCTGTAGTGGAAGAAGAAGAAGCAGTAGCAGTAG
 4909 E  Y  E  L  Y  R  V  V  F  D  I  T  F  F  F  F  V  I  V  I   4928

      CTGTTGGCCATCATCCAGGGTCTGATCATCGACGCTTTTGGTGAGCTCCGAGACCAACAA
14765 ---------+---------+---------+---------+---------+---------+ 14824
      GACAACCGGTAGTAGGTCCCAGACTAGTAGCTGCGAAAACCACTCGAGGCTCTGGTTGTT
 4929 L  L  A  I  I  Q  G  L  I  I  D  A  F  G  E  L  R  D  Q  Q   4948
```

```
                                A
       GAGCAAGTGAAGGAGGATATGGAGACCAAGTGCTTCATCTGTGGAATCGGCAGTGACTAC
14825  ---------+---------+---------+---------+---------+---------+ 14884
       CTCGTTCACTTCCTCCTATACCTCTGGTTCACGAAGTAGACACCTTAGCCGTCACTGATG
 4949  E  Q  V  K  E  D  M  E  T  K  C  F  I  C  G  I  G  S  D  Y   4968
                             K
       TTTGATACGACACCGCATGGCTTCGAGACTCACACGCTGGAGGAGCACAACCTGGCCAAT
14885  ---------+---------+---------+---------+---------+---------+ 14944
       AAACTATGCTGTGGCGTACCGAAGCTCTGAGTGTGCGACCTCCTCGTGTTGGACCGGTTA
 4969  F  D  T  T  P  H  G  F  E  T  H  T  L  E  E  H  N  L  A  N   4988

       TACATGTTTTTCCTGATGTATTTGATAAACAAGGATGAGACAGAACACACGGGTCAGGAG
14945  ---------+---------+---------+---------+---------+---------+ 15004
       ATGTACAAAAAGGACTACATAAACTATTTGTTCCTACTCTGTCTTGTGTGCCCAGTCCTC
 4989  Y  M  F  F  L  M  Y  L  I  N  K  D  E  T  E  H  T  G  Q  E   5008

       TCTTATGTCTGGAAGATGTACCAAGAGAGATGTTGGGATTTCTTCCCAGCTGGTGATTGT
15005  ---------+---------+---------+---------+---------+---------+ 15064
       AGAATACAGACCTTCTACATGGTTCTCTCTACAACCCTAAAGAAGGGTCGACCACTAACA
 5009  S  Y  V  W  K  M  Y  Q  E  R  C  W  D  F  F  P  A  G  D  C   5028
                         ryrsvk 2        Sal I
       TTCCGTAAGCAGTATGAGGACCAGCTTAGCTGA
15065  ---------+---------+---------+---                            15097
       AAGGCATTCGTCATACTCCTGGTCGAATCGACTCAGCTGGGCC
 5029  F  R  K  Q  Y  E  D  Q  L  S  *                              5038
```

The Ryr-3.5 sequence was aligned with the human ryanodine receptor mRNA using the 'bestfit' application of the GCG package of data analysis software. The protein translation is shown with the nucleotide and amino acid changes indicated in bold. The position of the restriction enzyme sites used for cloning and in diagnostic digests are shown in blue. The position of the primers used to generate this construct or in sequencing reactions are indicated in bold.