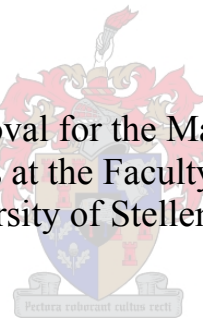


# **Identifying ligands of the C-terminal domain of cardiac expressed connexin 40 and assessing its involvement in cardiac conduction disease**

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Biomedical Sciences at the Faculty of Health Sciences,  
University of Stellenbosch



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## *Declaration*

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

Signature.....Date.....

## *Abstract*

Connexins (Cx) are major proteins of gap junctions, dynamic pores mediating the relay of ions and metabolites between cells. Cxs 40, 43 and 45 are the predominant cardiac isoforms and their distinct distribution raises questions about their functional differences. Their cytoplasmic (C)-terminal domains are involved in protein-protein interactions. Furthermore, mutations in the myotonic dystrophy protein kinase (*DMPK*)-causative gene are associated with disruptions in cardiac conduction similar to that described for Cx knock-out mice. *DMPK* is a Cx43 ligand, raising the possibility that defects in Cx40 ligands may be involved in the development of cardiac conduction disturbances. We hypothesised that delineation of the protein ligands of the C-termini of Cx40 and of Cx45 (parallel study conducted by N Nxumalo) would help elucidate their functional roles.

Yeast-two-hybrid methodology was used to identify putative Cx40 ligands. Primers were designed to amplify the C-terminus-encoding domain of the human Cx40 gene (*Cx40*), the DNA product was cloned into the pGBKT7 vector which was used to screen a cardiac cDNA library in *Saccharomyces cerevisiae*. Successive selection stages reduced the number of putative Cx40 ligand-containing colonies (preys) from 324 to 33. The DNA sequences of the 33 ligands were subjected to BLAST-searches and internet database literature searches to assign identity and function and to exclude false positive ligands based on subcellular location and function. Eleven plausible ligands were identified: cysteine-rich protein 2 (CRP2), beta-actin (ACTB), creatine kinase, muscle type (CKM), myosin, heavy polypeptide 7 (MYH7), mucolipin1 (MCOLN1), voltage-dependent anion channel 2 (VDAC2), aldehyde dehydrogenase 2 (ALDH2), DEAH box polypeptide 30 (DHX30), NADH dehydrogenase, 6, (NDUFA6), prosaposin (PSAP) and filamin A (FLNA). Cxs 40 and 45 showed differences in the classes of proteins with which they interacted; the majority of putative Cx40 interactors were cytoplasmic proteins, while Cx45 interactors were mitochondrial proteins. These results suggest that Cxs 40 and 45 are not only functionally different, but may also have different cellular distributions. Further analyses of these protein interactions will shed light on the independent roles of Cxs 40 and 45.

## *Opsomming*

Connexins (Cx) is die hoof proteïne van gaping verbindings, dinamiese poriëe wat die aflos van ione en metaboliete tussen selle bemiddel. Cxs 40, 43 en 45 is die hoofsaaklike hart iso-forme en hul afsonderlike distribusies rig vroeë omtrent hul funksionele verskille. Hul sitoplasmiese (C)-terminale domeine is betrokke by proteïen-proteïen interaksies. Verder, mutasies in die miotoniese distrofie proteïen kinase (*DMPK*)-verantwoordelike geen is geassosieer met versteerings in hart impuls geleiding soortgelyk aan die gevind in Cx uit-klop muis. *DMPK* is 'n Cx43 ligand, wat die moontlikheid lig dat defekte in Cx40 ligande mag verantwoordelik wees vir die ontwikkeling van hart impuls geleiding abnormaliteite.

Gis-twee-hibried tegnologie was gebruik om Cx40 ligande te identifiseer. 'Primers' was ontwerp om die C-terminus-kodeerende domein van die mens Cx40 geen (*Cx40*) te amplifiseer. Die DNS produk was in die pGBKT7 vector in cloneer wat gebruik was om a hart cDNA biblioteek in *Saccharomyces cerevisiae* te sif. Agtereenvolgende seleksie stappe het die getal moontlike Cx40 ligand-bevattende kolonies (prooi) van 324 na 33 verminder. Die DNS volgordes van die 33 ligande was onderwerp aan BLAST-soektogte en internet databasis literatuur soektogte om identiteit en funksie te bepaal, sowel as om vals positiewe ligande uit te sluit op grond van hul subsellulêre lokasie en funksie. Elf moontlike ligande was identifiseer: cysteine-ryk proteïen 2 (CRP2), beta-actin (ACTB), creatine kinase, muscle type (CKM), myosin, heavy polypeptide 7 (MYH7), mucolin1 (MCOLN1), voltage-dependent anion channel 2 (VDAC2), aldehyde dehydrogenase 2 (ALDH2), DEAH box polypeptide 30 (DHX30), NADH dehydrogenase, 6, (NDUFA6), prosaposin (PSAP) and filamin A (FLNA). Cxs 40 en 45 het verskille getoon in die klasse van proteïne waarmee hul interaksies gehad het; die moontlike Cx40 ligande was hoofsaaklik sitoplasmiese proteïne, terwyl Cx45 ligande hoofsaaklik mitokondriale proteïne was. Die resultate stel voor dat Cxs 40 en 45 nie net funksioneel van mekaar verskil nie, maar dat hulle moontlik ook verskillende sellulêre distribusies het. Verderre analiese van die proteïen interaksies sal die individuele rolle van Cxs 40 en 45 openbaar.

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*“Ask the Lord to bless your plans and you will be successful in carrying them out”  
Prov. 16:3*

“Work when you must work, play when you must play”

Corr Beyers

*List of abbreviations*

3'-UTR	: 3 prime untranslated region
5'-UTR	: 5 prime untranslated region
µg	: Micorgrams
µl	: Microlitre
A	: Adenosine
Ade	: Adenine
ACTB	: Beta-actin
ALDH2	: Aldehyde dehydrogenase 2 family
Amp	: Ampicillin
APOC2	: Apolipoprotein C2
ATCC	: American Type Culture Collection
ATP	: Adenosine triphosphate
AV	: Atrioventricular node
BLAST	: Basic local alignment search tool
BLASTN	: Basic local alignment search tool (nucleotide)
BLASTP	: Basic local alignment search tool (protein)
BLASTX	: Basic local alignment search tool (translated)
bp	: Base pair
BB	: Bundle branch
BBB	: Bundle branch block
C	: Carboxyl
°C	: Degree Celsius
Ca <sup>2+</sup>	: Calcium
cAMP	: Cyclic adenosine monophosphate
cDNA	: Complementary DNA
CIP	: Calf intestinal alkaline phophatase
CK1	: Casein kinase 1
CKM	: Creatine kinase, muscle type
CL	: Cytoplasmic loop



cM	: Centimorgan
CMTX	: Charcot-Marie-Tooth X-linked inherited peripheral neuropathy
CRP2	: Cysteine-rich proteins 2
CSPD	: Chemiluminescent substrate
Cx	: Connexin
dATP	: Deoxy-adenosine triphosphate
dCTP	: Deoxy-cytosine triphosphate
dGTP	: Deoxy-guanosine triphosphate
DHX30	: DEAH (Asp-Glu-Ala-His) box polypeptide 30
DM	: Myotonic dystrophy
DMPK	: Myotonic dystrophy protein kinase
DMSO	: Dimethyl sulphoxide
DNA	: Deoxyribonucleic acid
dNTP	: Deoxy-nucleotide triphosphate
E1,2	: Extracellular loops
ECG	: Electrocardiography
<i>E.coli</i>	: <i>Escherichia coli</i>
EDTA	: Ethylene-diamine-tetra-acetic acid
ERK	: Extracellular signal-regulated kinases
FLNA	: Filamin A, alpha (actin binding protein 280)
G	: Guanine
GJA5	: Gap junction alpha 5
GST	: Glutathione-S-transfers-pull down assays
H <sub>2</sub> O	: Water
HCM	: Familial hypertrophic cardiomyopathy
HHAT	: Hedgehog acyltransferase
His	: Histidine
ICCD	: Isolated cardiac conduction disease
IP <sub>3</sub>	: Inositol-1,4,5-triphosphate
Kan	: Kanamycin
KCNH1	: Potassium voltage-gated channel H

KLK1	: Kallikrein
DA	: Dalton
LAMB3	: Laminin $\beta$ 3
LEF	: Lymphoid enhancer-binding factor
Leu	: Leucine
LB	: Luria-Bertani broth
LBBB	: Left bundle branch block
LOD	: Logarithm of odds
LPGAT1	: Lysophosphatidylglycerol acyltransferase 1
LTD	: Limited
M	: Molar
M1,2,3,4	: Membrane-spanning domains
M2H	: Mammalian-two-hybrid
MAPK	: Mitogen-activated protein kinase
MCOLN1	: Mucolipin 1
MCS	: Multiple cloning site
MgCl <sub>2</sub>	: Magnesium chloride
ml	: Millilitre
mM	: Millimolar
MRC	: Medical Research Council
mRNA	: Messenger ribonucleic acid
MYH7	: Myosin, heavy polypeptide 7
N	: Amino
NCBI	: National Centre for Biotechnological Information
NDUFA6	: NADH dehydrogenase 1 alpha subcomplex, 6
ng	: Nanograms
NH <sub>4</sub> Ac	: Ammonium acetate
NO	: Nitric oxide
ODDD	: Oculodentodigital dysplasia
OMIM	: Online Mendelian Inheritance in Man
PBS	: Phosphate buffered saline

PCCD	: Progressive familial cardiac conduction disease
PCI	: Phenol Chloroform Isoamyl
PCR	: Polymerase chain reaction
PEG	: Polyethylene glycol
PF	: Purkinje fibers
PFHB	: Progressive familial heart block
pH <sub>i</sub>	: Intracellular pH
PKC	: Protein kinase C
PPP2R5A	: Protein phosphatase 2A b56alpha
PSAP	: Prosaposin
RAMP	: Retinoic acid regulated nuclear matrix associated protein
RCOR3	: REST corepressor 3
RRAS	: Related RAS viral (r-ras) oncogene homolog
RBBB	: Right bundle branch block
RNA	: Ribonucleic acid
SA	: Sinoatrial node
<i>S.cerevisiae</i>	: <i>Saccharomyces cerevisiae</i>
SD	: Synthetic dropout
SEAP	: Secreted alkaline phosphatase
SLC30A1	: Zinc transporter 1
SMC	: Vascular smooth muscle cells
SRF	: Serum response factor
SYT14	: Synaptotagmin XIV
Ta	: Annealing temperature
TCF	: B-catenin-T-cell factor
TRAF3IP3	: Tumor necrosis factor receptor Jun N-terminal kinase-activating modulator
TRP	: Transient receptor potential channels
Trp	: Tryptophan
UK	: United Kingdom
Ura	: Uracil

US	: United States
UV	: Ultraviolet
V	: Volts
VASP	: Vasodilator-stimulated phosphoprotein
VDAC2	: Voltage dependent anion channel 2
W	: Watts
www	: World Wide Web
Y2H	: Yeast-two-hybrid

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# *Chapter 1*

## *Introduction*

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## Preface

The Heart. A source of life. The work horse of the person called Man. The house that stores emotions, that drives thoughts and actions, and most importantly, that on which life depends. The study to unravel its deepest workings is in its youth and has shone light on many avenues of which its electrical core strikes with intrigue. Many find the emotional heart intriguing, while we, the scientists, are enticed by the electrical heart. We, with all our science, try to paint a portrait of the electrical heart to understand its deepest thoughts and workings. To, in the end, be able to say without doubt - yes, we understand - and find that the smallest seemingly insignificant is the most significant which brings to a close the questions asked. This is a study to find the seemingly insignificant, to analyse and, to this end, answer the questions that will help us understand the deeper workings of the heart we know as the electrical work horse of the person called Man (Keyser, 2007).

### 1.1 The heart

Electrical impulse generation and propagation thereof in the specialised conduction system of the heart are essential for adequate heart function. A factor influencing the spread of electrical impulse is the cell-to-cell communication between cardiomyocytes in the working myocardium (Severs, 2000). The cell-to-cell communication is brought about by specialised membranous intercellular channels, namely, gap junctions (Herve *et al.*, 2005; van der Velden *et al.*, 2002).

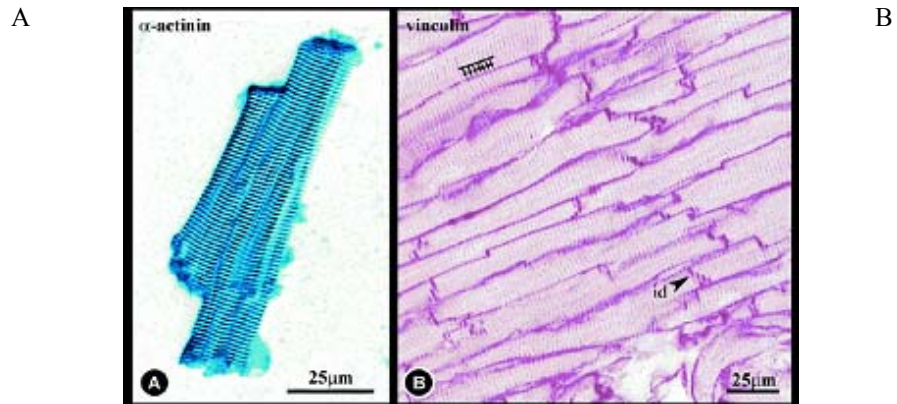
The gap junction channels function in connecting adjacent cells and provide pathways for intercellular current flow which results in the coordinated spread of impulse through the specialised conduction system (Lampe *et al.*, 2000; Wei *et al.*, 2004). Connexin (Cx) protein subunits form the building blocks of gap junction channels, of which Cx40, 43 and 45 are the predominant cardiac forms (Lampe *et al.*, 2004). Interestingly, each has been shown to have distinct developmental and regional distribution patterns which raise questions about their functional similarities (Söhl *et al.*, 2004).

The Cx-encoding genes have been candidate causative genes for cardiac conduction system diseases because of their predominant role in electrophysiological function (Söhl *et al.*, 2004). Interestingly, it has been shown that mutations in the myotonic dystrophy protein kinase (*DMPK*)-causative gene are associated with disruptions in cardiac conduction similar to that described for Cx knock-out mice (Berul *et al.*, 1999). *DMPK* is a ligand of Cx43 (Mussini *et al.*, 1999; Schiavon *et al.*, 2002) which raises the possibility that defects in other unidentified Cx ligands are involved in the development of conduction disturbances.

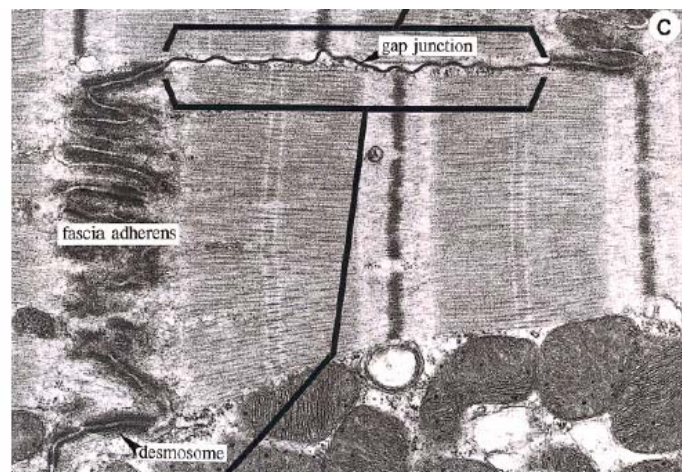
It was therefore the focus of the present study to identify ligands interacting with the cytoplasmic carboxyl terminus of Cx40, which is a region involved in regulation of channel properties and protein-protein interactions (Goodenough *et al.*, 1996; Sosinsky *et al.*, 2005; Duffy *et al.*, 2002). Identification of Cx40 ligands will shed light on the molecular and cellular function of Cx40 in the heart and will aid in the investigation of its possible involvement in conduction disturbances. A comparison with other studies will help define the specific individual roles of cardiac Cxs. For these reasons, the following sections will describe the cardiac conduction system, the gap junction channels, the cardiac Cx isoforms with focus on Cx40, and the South African cardiac conduction diseases for which Cxs, or their ligands, are novel candidates.

### **1.1.1 The cardiac muscle cell**

A typical cardiac muscle cell, i.e. cardiomyocyte, is an elongated cell with a length of 100 to 150  $\mu\text{m}$  and a width of 20 to 35  $\mu\text{m}$ . The contractile myofilaments, namely, actin, myosin and associated proteins, are packed together to form the striated myofibrils that fill most of the cardiomyocyte cell as shown in figure 1.1A. Intercalated disks occur at the blunt ends of each myocyte thereby joining it with multiple neighbouring myocytes (figure 1.1B) (Severs, 2000). The gap junctions, fascia adherens and desmosomes are the three types of cell junctions that physically connect the disk membranes. They act in concert to integrate cardiac electromechanical function. Shown in figure 1.2 are the three types of junctions at the intercalated disks.



**Figure 1.1 Confocal microscopy of cardiac muscle cells.** (A) A single cardiomyocyte. The myofibrils are seen as striations. (B) Section of cardiac muscle. Numerous cells like that in (A) joined together at intercalated disks (id) (Severs, 2000).



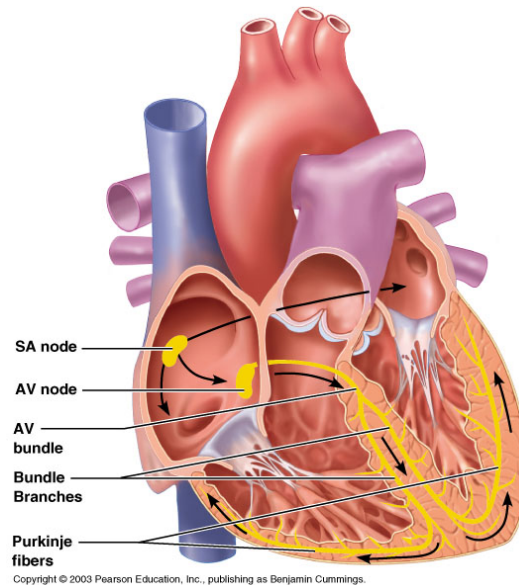
**Figure 1.2 Thin-section electron micrograph illustrating the three types of cell junction of the intercalated disk.** Gap junctions are recognised where the adjacent plasma membrane profiles run in close contact. The fascia adherens and the desmosomes are characterised by a much wider intermembrane space and by prominent electron-dense membrane-associated proteins (Severs, 2000).

### **1.1.2 Impulse propagation throughout the heart**

Contraction of the cardiac chambers is brought about by the orderly spread of action potentials throughout the heart. Each wave of electrical excitation spreads rapidly along the plasma membranes of adjoining cardiac muscle cells, which triggers release of calcium from an intracellular membrane-bound compartment, namely, the sarcoplasmic reticulum, which in turn stimulates contraction of the myofibrils (Gaussin *et al.*, 2004). Integration of the electrical and mechanical properties of each and every myocyte within the heart is achieved by the intercalated disks (Severs, 2000).

Each cardiac cycle is initiated in the sinoatrial (SA) node, which is fast autorhythmic tissue located in the right atrium (Gaussin *et al.*, 2004). The spread of electrical excitation through the heart and the important components of the conduction system are illustrated in figure 1.3. After generation in the SA node, the action potential spreads through the atrial myocardium from right to left and superior to inferior. This causes a wave of contraction to extend down toward the ventricles. The impulse is prevented from passing to the ventricular myocardium by a thick non-conductive connective tissue septum, and is delayed as it passes slowly through the atrioventricular (AV) node located at the junction of the atria and ventricles.

The impulse is then rapidly conducted along the His-bundle (or AV bundle), which separates into a left and right bundle branch (LBB and RBB), in order to excite the ventricular myocardium at the bottom part of the heart. From here, Purkinje fibres spread off to left and right to carry the action potential throughout all the ventricular tissue (Gaussin *et al.*, 2004). The net result is a wave of contraction that travels upward back through the ventricles, expelling their contents into the pulmonary artery and aorta (White *et al.*, 1999). The spread of the action potential throughout the heart, and anomalies there of, can be monitored by means of electrocardiography (ECG) (Severs, 2000).



**Figure 1.3 Illustration of the spread of electrical excitation throughout the heart and the important components of the conduction system.** Arrows indicate spread of electrical excitation. Abbreviations; SA: sinoatrial, AV:atrioventricular (<http://www.bmb.psu.edu>).

### 1.1.3 Cardiac conduction disturbances - heart block and arrhythmias

Disturbances in the conduction system disrupt the normal spread of the electrical impulse throughout the heart and thereby lead to impaired functioning of the heart by causing conditions to develop such as heart block (atrioventricular and intraventricular block) or arrhythmias. Disturbances in cardiac conduction can occur due to a variety of factors, such as developmental and congenital defects, acquired injury or ischemia of portions of the conduction system, or by inherited diseases that alter cardiac conduction system function (Wolf *et al.*, 2006).

Heart block can occur anywhere in the specialised conduction system beginning with the SA connections, the AV junction, the bundle branches and their fascicles, and ending in the distal ventricular Purkinje fibres (figure 1.3). Atrioventricular heart block (or AV block) is a condition in which the electrical signals that stimulate heart muscle contraction are partially or totally blocked between the atria and ventricles. It has been classified according to the level of impairment - slowed conduction (1<sup>st</sup> degree heart block), intermittent conduction failure (2<sup>nd</sup> degree heart block), or complete conduction

failure (3<sup>rd</sup> degree heart block) (Otomo *et al.*, 2005; Benson *et al.*, 2004). Intraventricular heart block occurs when the electrical impulse is slowed or blocked as it spreads through the two divisions of the His bundle branches in the ventricles; the right bundle branch (RBB) and left bundle branch (LBB). Pathologies such as hypertensive heart disease, cardiomyopathy and coronary artery disease have been shown to have association with bundle branch block (Mueller *et al.*, 2006; Go *et al.*, 1998).

Irregular heart rhythms (or arrhythmias) can be caused by damage to the electrical pathways of the heart and can be detected by looking for abnormal ECG readings. For example, a heart block at the AV node occurs because of tissue damage at this region. This severs the signal from the atria to the ventricles which causes the autorhythmic cells of the ventricles to go at their own pace and subsequently cause the atria and ventricles to contract out of synchrony, causing cardiac arrhythmia (Seferovic *et al.*, 2006).

Atrial fibrillation occurs when the electrical signals do not spread out evenly from the SA node when it generates an action potential. Cell death or other heart tissue damage can slow down the action potential in some parts and let it spread rapidly in other parts. This causes a circular pattern to the spread of action potential such that the signals keep occurring over and over. Rather than having a uniform cardiac contraction from top to bottom, an array of different parts of the atria are trying to contract. Contractions eventually cease when the atria are not contracting uniformly. Heart efficiency is reduced since the ventricles are no longer completely full when they contract.

Ventricular fibrillation has the same cause and effect as atrial fibrillation but with more severe consequences. When the atria are functioning poorly, the ventricles still pump blood but with less efficiency. When the ventricles cease to function, no blood is pumped and death rapidly ensues (<http://www.gpnotebook.co.uk>) (<http://www.americanheart.org>) (<http://www.bmb.psu.edu>).



## 1.2 The Gap Junction Channel

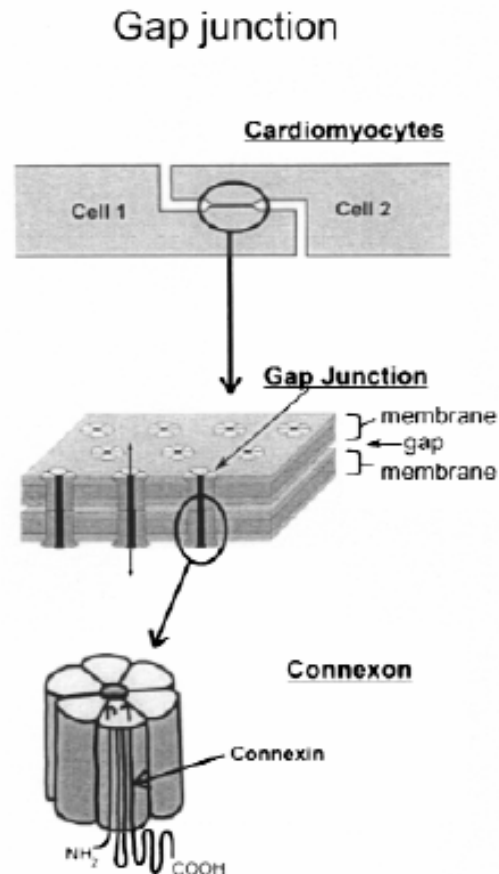
### 1.2.1 The membrane channel: Gap junction

Gap junctions form part of the class of cell contact-mediating protein complexes and other members of this class include tight junctions, desmosomes and cell adhesion molecules (Herve *et al.*, 2005). Gap junctions are recognised by electron microscopy as being regions where the plasma membranes of adjacent cells closely approach each other but are separated by a small gap of 2-3nm as indicated in figure 1.2 and figure 1.4 (Severs, 2000). The following sections will describe the protein subunits that fill the space between the plasma membranes in order to form the gap junction channels.

Gap junctions function in connecting the cytoplasm of adjacent cells and thereby enable direct chemical communication to occur (Lampe *et al.*, 2000; van Veen *et al.*, 2001). Various compounds up to a molecular mass of 1000 Dalton (Da), such as metabolites, ions, secondary messengers (e.g. calcium [ $\text{Ca}^{2+}$ ], inositol-1,4,5-triphosphate [ $\text{IP}_3$ ], cyclic adenosine monophosphate [cAMP]) and water, can be exchanged through gap junction channels. The passage of charged molecules allows for electrical impulses to be conducted through the channels (Söhl *et al.*, 2004, Lampe *et al.*, 2000, 2004; van der Velden *et al.*, 2002). Abnormal cell-to-cell communication through gap junctions is believed to play a role in the pathogenesis of diverse diseases such as cardiac arrhythmias and uterine malfunction at birth, X-linked Charcot-Marie-Tooth demyelinating disease, cardiac malformation and defects of laterality, epileptic seizures, spreading depression and Chagas' disease (Peracchia *et al.*, 1997).

Gap junctions have been identified in all tissues except in striated muscle where the cells have fused during development. Cells not using the gap junction mode of cell-to-cell communication are erythrocytes, platelets and sperm (Severs, 2000). In the heart, the cardiomyocytes synchronise their contractions by communicating electrically across the gap junctions in the intercalated disks (Yeager *et al.*, 1998; Wei *et al.*, 2004). The summation of the synchronous beating of the individual myocytes accounts for the

rhythmic pumping of the heart (Kanno *et al.*, 2001). The signaling pathways produced by the gap junctions not only permit rapidly coordinated activities such as contraction of cardiac and smooth muscle cells, but also transmission of neuronal signals at electrical synapses (Severs *et al.*, 2004; Bennett *et al.*, 1997). Gap junctional communication has also been shown to function in slower physiological processes, such as cell growth and development (Loewenstein *et al.*, 1992).



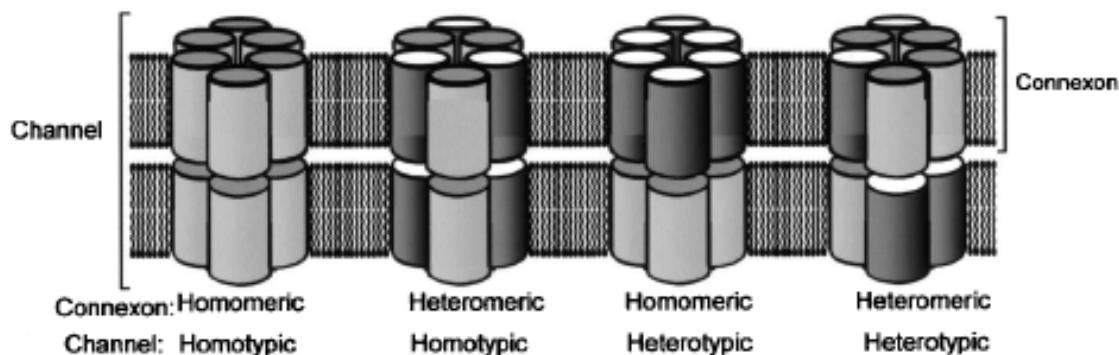
**Figure 1.4 Diagram of gap junction joining two adjacent cells.** Two connexon (hemi-channels) from adjacent cells dock to each other to form a full gap junction channel. A connexon is formed by six protein subunits, called connexins (Suzuki *et al.*, 2001).

### 1.2.2 The gap junction channel unit

Gap junctions are composed of connexin (Cx) protein subunits that are encoded by a family of closely related genes. Twenty Cx genes have been identified in the mouse genome and 21 in the human genome (table 1.1), as will be discussed in following

sections (Söhl *et al.*, 2004). A hemi-channel (connexon) is formed when six Cx subunits converge and a complete gap junction channel is assembled when two hemi-channels from adjacent cells dock to each other (figure 1.4 and figure 1.5) (Segretain *et al.*, 2004). Connexons can be composed of six identical Cx subunits to form a homomere or they can contain more than one Cx isoform to form a heteromere. Homotypic channels are composed of two identical connexons and heterotypic channels of two connexons having different Cx isoforms (Evans *et al.*, 2002; Krutovskikh *et al.*, 2000).

The formation of these different types of channels offers greater complexity in the regulation of communication through gap junctions (Sosinsky *et al.*, 2005). The formation of different types of channels is possible because most cell types express more than one Cx isoform (Evans *et al.*, 2002; Goodenough *et al.*, 1996). The large number of different Cx isoforms expressed makes it important for precise regulation of the biosynthesis of gap junctions, their structural composition, and their degradation for proper gap junctional functioning (Segretain *et al.*, 2004; Krutovskikh *et al.*, 2000).



**Figure 1.5 Possible arrangements of connexins in a gap junction channel unit (Evans *et al.*, 2002).**

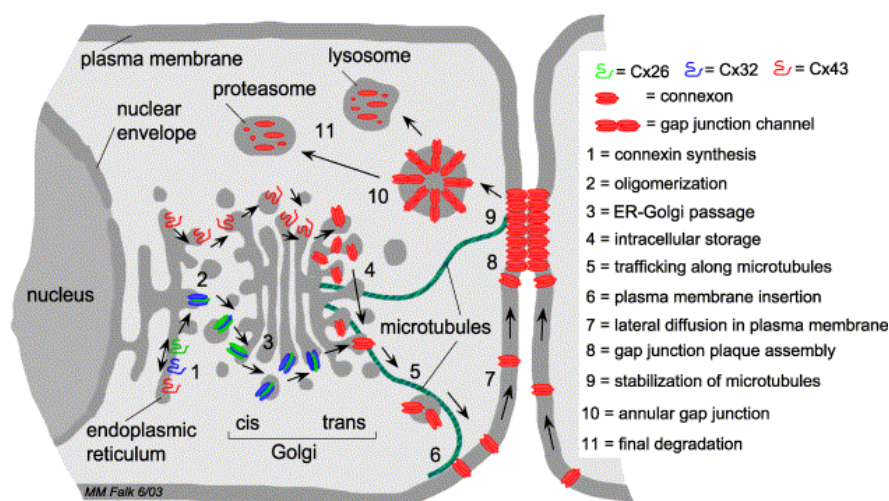
### 1.2.3 Synthesis, assembly and degradation of gap junction channels

The process of synthesis, assembly and degradation of gap junction channels can be divided into eleven steps according to current literature (figure 1.6). The process starts with synthesis of Cx polypeptides at endoplasmic reticulum membranes after which the Cxs oligomerise into homo- and heteromeric gap junction connexons (step 1, 2) (Falk *et*

*al.*, 1994, 1998). They then pass through the golgi stacks (step 3), where some are intracellularly stored in trans-golgi membranes (step 4) (Zhang *et al.*, 1996). Trafficking of the connexons occurs along the microtubules (step 5), after which they are inserted into the plasma membrane (step 6) (Ahmad *et al.*, 2001).

Lateral diffusion of connexons (step 7) in the plasma membrane occurs for aggregation of individual gap junction channels into plaques (step 8) (Ahmad *et al.*, 1999; Falk *et al.*, 1997; Evans *et al.*, 1999; Martin *et al.*, 2001). Peripheral microtubule plus-ends are then stabilised by binding to the gap junctions (step 9). The channel plaques are then internalised (step 10) which leads to cytoplasmic annular junctions. The half-life of Cxs is about 1 to 5h. Complete degradation of the gap junction channels (step 11) occurs by lysosomal and proteasomal pathways (Segretain *et al.*, 2004; Evans *et al.*, 2002; Jordan *et al.*, 2001; Larsen *et al.*, 1978; Laing *et al.*, 1995; Spray *et al.*, 1998).

The Cxs come into contact with an array of cellular elements, each affecting the Cxs' life cycle during the process of synthesis, assembly and degradation of gap junction channels. Changes in these interactions could have deleterious consequences for normal gap junction functioning, which may contribute to the pathogenesis of diseases.



**Figure 1.6 Representation of the steps that lead to synthesis, assembly, and degradation of gap junction channels based on current literature (Segretain *et al.*, 2004).**

## 1.3 The Connexins

### 1.3.1 The connexin gene family

Twenty *Cx* genes have been identified in the mouse genome and 21 in the human genome (table 1.1) with the genes generally showing 40% nucleotide sequence identity (Willecke *et al.*, 2002). Nineteen of the genes can be grouped as orthologous pairs, with some differing in their tissue or cellular expression (Söhl *et al.*, 2002). There are *Cx* genes that occur only in the mouse (mCx33) or the human genome (hCx25 and hCx59) and the biological reasons for this are unknown. Another family of gap junction proteins is the innexins, which are only expressed in invertebrates such as *Drosophila melanogaster* (fruit fly) or *Caenorhabditis elegans* (nematode worm), and do not show any sequence similarity to *Cxs* (Phelan *et al.*, 2001; White *et al.*, 1999). Genes that show some sequence identity to innexins were discovered in the genomes of higher vertebrates and are called pannexins (Söhl *et al.*, 2002; Panchin *et al.*, 2000).

*Cxs* have been divided into major  $\alpha$ - and  $\beta$ -classes and a minor  $\gamma$ -class according to their extent of sequence identity and the length of their cytoplasmic loop (Eiberger *et al.*, 2001; Krutovskikh *et al.*, 2000; White *et al.*, 1999). They are abbreviated as “GJ” for ‘gap junction’ and numbered according to the order of discovery. Connexin 40, with the number referring to the molecular mass of the protein in kDa, is therefore also known as ‘GJA5’ because it is the fifth connexin of the  $\alpha$  group (Söhl *et al.*, 2004; Wei *et al.*, 2004).

*Cxs* are differentially expressed throughout the human body with morphologically complex tissues having the broadest *Cx* profile. The three major *Cxs* associated with cardiomyocytes are *Cxs40*, *43* and *45*, which all exhibit different channel properties (Gros *et al.*, 1996; Söhl *et al.*, 2004). *Cx37* is a minor cardiac *Cx* and is synthesised in the endocardial cells. Unlike *Cx40*, *43* and *45*, which will be discussed below, *Cx37* is not associated with human heart defects and no heart defects have to date been detected in studies with *Cx37*-deficient mice (Gros *et al.*, 2004). Each of the cardiac *Cxs* has distinct

Table 1.1 The connexin gene family and chromosomal distribution of its members in mouse and human (Söhl *et al.*, 2004).

Mouse connexin			Human connexin		
Cx	GJ	chr	chr	GJ	Cx
mCx23		10	6		hCx23
			6		hCx25
mCx26	Gjb2	14	13	GJB2	hCx26
mCx29	Gjb1	5	7	GJE1	hCx30.2 (hCx31.3)
mCx30	Gjb6	14	13	GJB6	hCx30
mCx30.2	Gjb11	11	17	GJA11	hCx31.9
mCx30.3	Gjb4	4	1	GJB4	hCx30.3
mCx31	Gjb3	4	1	GJB3	hCx31
mCx31.1	Gjb5	4	1	GJB5	hCx31.1
mCx32	Gjb1	X	X	GJB1	hCx32
mCx33	Gjb6	X			
mCx36	Gjb9	2	15	GJA9	hCx36
mCx37	Gjb4	4	1	GJA4	hCx37
mCx39		18	10		hCx40.1
mCx40	Gjb5	3	1	GJA5	hCx40
mCx43	Gjb1	10	6	GJA1	hCx43
mCx45	Gjb7	11	17	GJA7	hCx45
mCx46	Gjb3	14	13	GJA3	hCx46
mCx47	Gjb12	11	1	GJA12	hCx47
mCx50	Gjb8	3	1	GJA8	hCx50
			1	GJA10	hCx59
mCx57	Gjb10	4	6		hCx62
		$\Sigma 20$			$\Sigma 21$

This table reflects the current state of the sequence information available from the NCBI database (<http://www.ncbi.nlm.nih.gov>). Cx31.3 is the only transcript isoform known of the hCx30.2 gene. chr, chromosomal assignment (Söhl *et al.*, 2004).

Table 1.2 Different features of mouse and human cardiovascular connexin genes (Söhl *et al.*, 2004).

	Cx37	Cx40	Cx43	Cx45
Protein sequence identity Mouse vs. human	91%	85%	98%	98%
Transcript sizes	1.7kb	3.5kb	3.0kb	2.2kb
Cell types with major expression	Endothelial cells	Cardiomyocytes Endothelial cells	ubiquitous	Endothelial cells, neurons, smooth muscle
Cell-type specific expression in heart	Endothelial cells	Cardiomyocytes Endothelial cells	Cardiomyocytes	Cardiomyocytes
Unitary conductance	300pS	200pS	60-100pS	20-40pS
Phenotype(s) of Cx- deficient mice	Female sterility	Atrial arrhythmias	Heart malformations	Defective vascular development
Human hereditary disease(s)	Association with atherosclerosis	n.a.	Oculodentodigital dysplasia (ODDD) syndactyly type III	n.a.

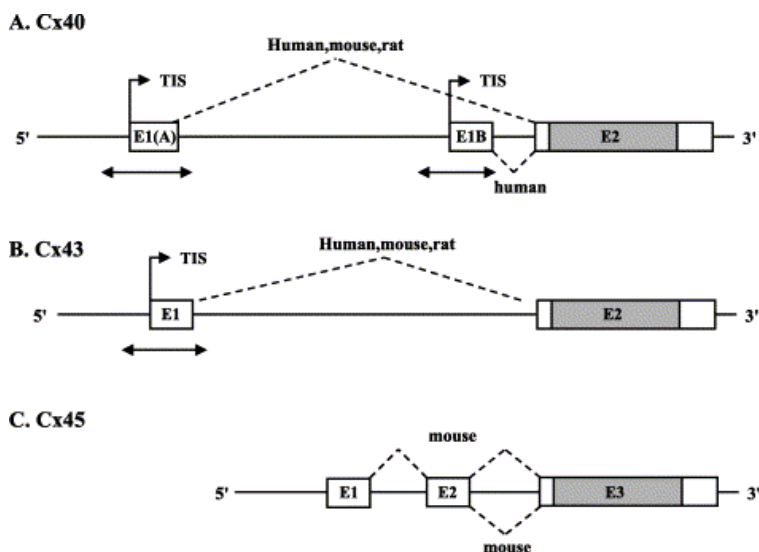
n.a. : not analyzed

developmental and regional distribution, which raises the question about the similarities and differences in their functions in the cardiovascular system, and if their functions are differentially affected by proteins, i.e. ligands, that interact with them. Table 1.2 indicates the distinctive features of mouse and human cardiovascular *Cx* genes.

### 1.3.2 Connexin gene structure

Shown in figure 1.7 are the gene structures of the three major cardiac *Cx* isoforms *Cx40*, *43* and *45*. Most of the *Cx* genes have a common structure starting with the first exon that contains the 5' untranslated region (5'-UTR) which is followed by an intron of varying length. Next is the second exon that contains the remaining 5'-UTR, the coding sequence, and the 3'-UTR (Wei *et al.*, 2004). Different splicing patterns exist which result in different gene products for most of the *Cx* genes, including *Cx40*. It is a tissue specific event for which the mechanism is not yet known (Söhl *et al.*, 2004).

The different splicing patterns that exist for *Cx40* result in the transcription of two variants, namely, variant A and B. Variant A represents the longer transcript and is expressed in endothelial cells. Variant B differs in the 5'-UTR compared to variant A because of the different splicing patterns (figure 1.7), and is expressed in placental cytotrophoblasts (Wei *et al.*, 2004; Söhl *et al.*, 2004; Teunissen *et al.*, 2004). The gene sequence of variant A which encodes the C-terminus of *Cx40* was used in the present study as a bait in the Y2H assay to identify ligands (section 1.8), because it is expressed in heart tissue.



**Figure 1.7 Gene structure of connexin 40 (A), 43 (B) and 45 (C).** Exon (E) sequences are indicated as boxes and the shaded parts represent protein-coding sequences. Positions of reported transcription initiation sites (TIS) and regions with promoter activity (double-headed horizontal arrows) are indicated. Different splicing patterns (dotted lines) result in the occurrence of RNA species derived from the following exons: Cx40-E1A/E2 (human, rat, mouse), E1B/E2 (human); Cx43-E1/E2 (human, mouse, rat); Cx45-E1/E2/E3, E2/E3 (mouse) (Teunissen *et al.*, 2004).

### 1.3.3 Connexin protein structure

The archetypal Cx protein is a four alpha-helical transmembrane spanning protein, as illustrated in figures 1.8 and 1.9 (Moreno *et al.*, 2002; Richard, 2000). It has two extracellular loops (E1 and E2), one cytoplasmic loop (CL), four membrane-spanning domains (1M, 2M, 3M and 4M) and one cytoplasmic amino (N) and one carboxyl (C) termini (Goodenough *et al.*, 1996; Bukauskas *et al.*, 2004; van Veen *et al.*, 2001). Various functional properties have been assigned to parts of the protein. The extracellular loops have three highly conserved cysteine residues, which function in forming disulfide-bridges that stabilise the loops during docking of the connexons to each other, in order to form a functional channel (Wei *et al.*, 2004; Richard, 2000).



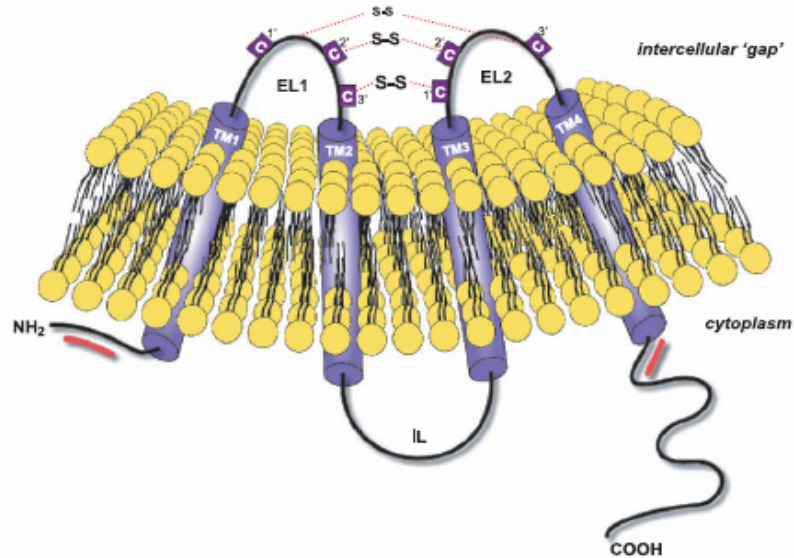


Figure 1.8 Illustration of the secondary structure of a single connexin protein in the membrane (Moreno *et al.*, 2002).

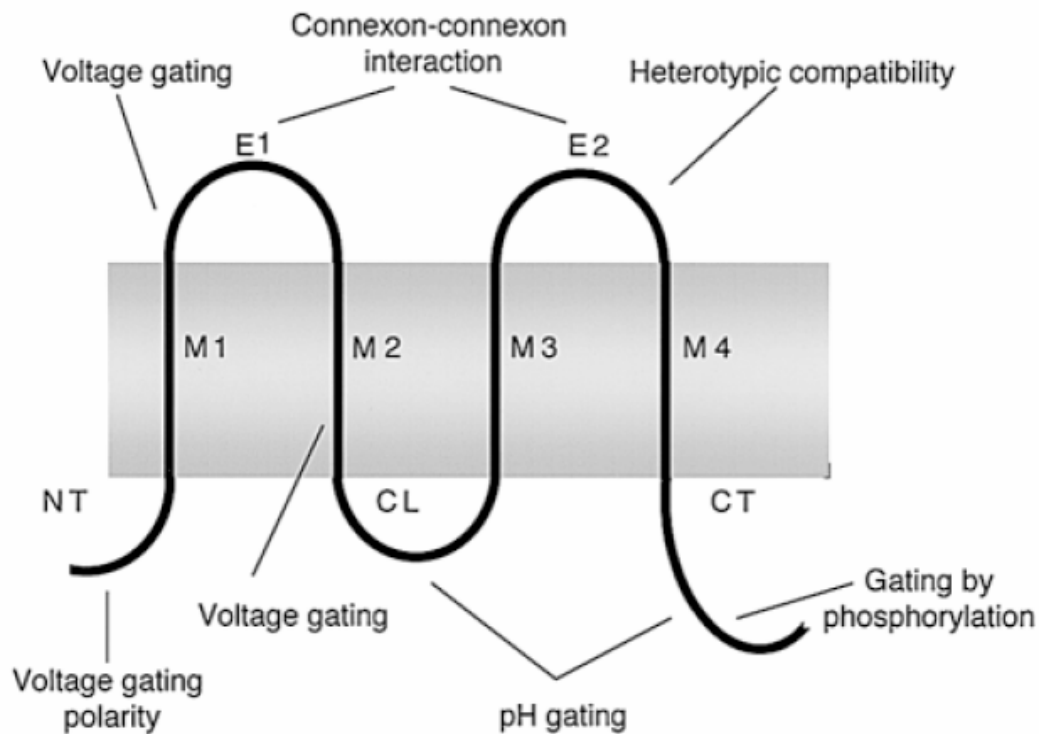


Figure 1.9 Diagram of a connexin protein with its structural motifs and their presumed function. M1-M4: transmembrane domains; E1/E2: extracellular domains 1 and 2; CL: cytoplasmic loop; NT: amino-terminus; CT: carboxyl-terminus (Richard, 2000).

Each of the transmembrane domains participates in oligomerisation into hexameric connexon hemichannels. They form the pore of the gap junction channel and are therefore important for channel permeability (Krutovskikh *et al.*, 2000; Sosinsky *et al.*, 2005). The N-terminus has been shown to play a role in voltage-gating of gap junction channels (Krutovskikh *et al.*, 2000) and it forms a voltage-gating mechanism with the M1/E1 boundary and the M2 (Richard, 2000). Studies done with Cx32 have shown that the highly conserved N-terminus incorporates a putative calmodulin binding motif and is necessary for the insertion of connexins into the membrane (Torok *et al.*, 1997). The calmodulin binding motif is also essential for Ca<sup>2+</sup>-mediated regulation of cell coupling (Krutovskikh *et al.*, 2000).

Most of the amino acid sequence variability, among the different Cxs, occurs in the intracellular domains, namely, the CL and C-terminus (Sosinsky *et al.*, 2005; Peracchia *et al.*, 1997). Different protein-protein interactions with these domains have been found among the different Cxs and the functions and regulatory aspects of gap junctions may be influenced by these different protein-protein interactions (Duffy *et al.*, 2002). The CL and C-terminus are also not independent subdomains but have been shown to interact with each other under physiological stimuli such as pH, in a particle and receptor model fashion, during channel gating which will be discussed in section 1.6 (Sosinsky *et al.*, 2005).

The C-terminus functions as a vital regulatory element of channel gating and has an influence on the rates of Cx trafficking and the synthesis, assembly and degradation of gap junction channels (Lampe *et al.*, 2000). The C-terminus contributes to defining channel properties, as truncation of this domain in *Cx43* has been shown to modify channel conductance (Sosinsky *et al.*, 2005). The C-terminus and the CL are the only domains that contain phosphorylation sites for different kinases (Krutovskikh *et al.*, 2000). Phosphorylation (a form of post-translational modification) of the C-terminus is a general mechanism for setting thresholds in the regulation of protein-protein interactions and also plays roles in channel gating (Fishman *et al.*, 1991). The mechanism of channel gating by means of phosphorylation will be discussed in section 1.6.4.

### 1.3.4 Connexins of the cardiovascular system

Gap junctions are important in cardiac function because they mediate the spread of the electrical impulses throughout the heart which allows for the synchronous contraction of the cardiac chambers (Severs *et al.*, 2001). As discussed in previous sections, Cx proteins are the building blocks of gap junction channels and Cx40, 43 and 45 are the predominantly expressed cardiac isoforms with each having different conductive properties and distribution patterns (Gaussin *et al.*, 2004). Cx40 channels have the largest unitary conductance (160 pS), Cx43 channels have unitary conductance of 100-120 pS and Cx45 channels have the smallest unitary conductance (30-40 pS) (Veenstra *et al.*, 1992; Elenes *et al.*, 2001).

The different parts of the specialised conduction system have different conductive properties; the SA and AV nodes are pacemaking and slow conducting, whereas the bundle of His and Purkinje fibres are fast-conducting pathways. Therefore, the distribution patterns of the cardiac Cxs are determined in part by their individual conductive properties and functions (Gaussin *et al.*, 2004; van Veen *et al.*, 2001). They also have different expression patterns during the stages of development. Figure 1.10 shows a diagram representing their distribution patterns in the heart and table 1.2 shows the different features of mouse and human cardiovascular Cx genes.

#### Cx43

Cx43 is principally responsible for electrical synchrony and electrical impulse propagation in the ventricles. It is abundantly expressed in all the cardiac compartments, regardless the stage of development, except for the SA and AV nodes, the His-bundle and the proximal parts of the bundle branches (figure 1.10) (Gros *et al.*, 2004; van Rijen *et al.*, 2001; van Veen *et al.*, 2001).

#### Cx45

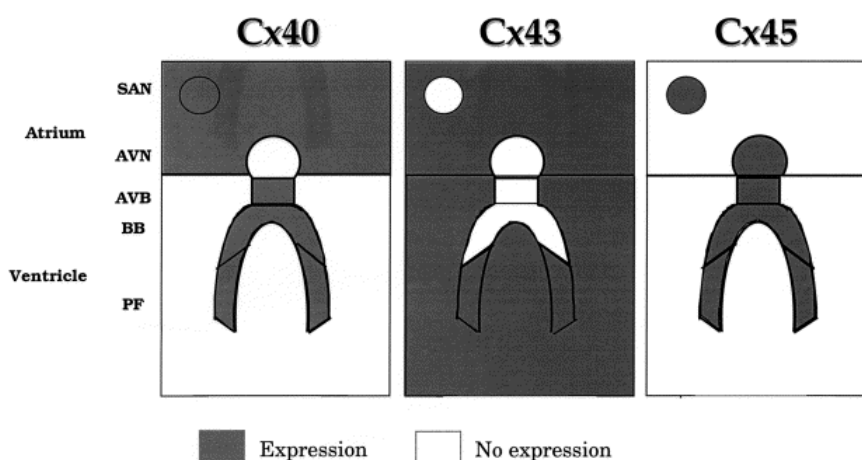
Cx45 is essential for embryonic heart development and is at this stage expressed in all heart compartments, after which it is downregulated in the adult heart (Gaussin *et al.*,

2004, van Veen *et al.*, 2001). In the adult myocardium, its expressed in the SA and AV nodes, the various parts of the ventricular conduction system, the most peripheral regions of the interventricular septum and at low levels in the surrounding working myocardium of atria and ventricles (figure 1.10) (Gros *et al.*, 2004; Coppen *et al.*, 1999).

### Cx40

Cx40 is a major determinant of electrical impulse propagation in the atria and the specialised conduction system (Gu *et al.*, 2003). *Cx40* is strongly expressed in both the atria and ventricle in early stages of development, after which it is downregulated causing it to be absent in the ventricular working myocardium (Veen *et al.*, 2001). In the adult heart, it is mainly expressed in the atrium, coronary vascular endothelium, and the fast conducting tissue of the His-Purkinje system, which is a network of cells specialised for rapid conduction of excitation to the apical ventricular myocardium (figure 1.10) (Saffitz *et al.*, 2000; Gourdie *et al.*, 1993; Veenstra *et al.*, 1992).

Therefore, this compartmentalised expression pattern of *Cx40*, *43* and *45* controls the orderly sequential spread of activation from the atrial to ventricular chambers which is essential for normal heart function (Gourdie *et al.*, 1999).



**Figure 1.10 Generalised expression pattern of Cx40, Cx43 and Cx45 in the different regions of the mammalian heart.** SAN: sinoatrial node; AVN: atrioventricular node; AVB: atrioventricular bundle or His-bundle; BB: bundle branches; PF: Purkinje fibers (van Veen *et al.*, 2001).

### 1.3.5 Knockout mouse studies

Knockout mouse studies have been done with the cardiac *Cxs*, in order to broaden the knowledge of their functions in the cardiovascular system. The studies showed the importance of them in heart morphogenesis; *Cx40*-deficient mice had AV septation defects and outflow tract malformations (Kirchhoff *et al.*, 2000; Gu *et al.*, 2003), *Cx45*-deficient mice had endocardial cushion defects and *Cx43*-deficient mice had heart outflow tract defects and defects in their coronary arteries (Reaume *et al.*, 1995; Ya *et al.*, 1998).

Knockout studies with *Cx43* showed that mouse embryos in which both alleles of the *Cx43* gene had been disrupted survived until birth but died of asphyxiation shortly after delivery. Their death was caused by an obstruction of the right ventricular outflow tract, which prevented the blood flow from reaching the lungs. The heterozygous mutants were viable and able to breed (Gros *et al.*, 2004; Kirchhoff *et al.*, 2000; Reaume *et al.*, 1995). *Cx45* homozygous-null mice died early in gestation, at embryonic day 10.5, with conduction block and endocardial cushion defects. Studies with *Cx40* showed slowed conduction and partial AV block in the *Cx40* knockout mice (Kumai *et al.*, 2000; Kirchhoff *et al.*, 1998, 2000; van Veen *et al.*, 2001). Studies in which the *Cxs* were substituted with each other indicated that they could only partially fulfill the functions of the *Cxs* for which they were substituted (Gros *et al.*, 2004). Knockin gene replacement studies, in which *Cx40* were substituted for *Cx43*, showed no conduction abnormalities. This indicated that heart conduction is independent of the unitary conductance of the gap junction channel (Plum *et al.*, 2000).

### 1.3.6 Human diseases associated with connexin mutations

Deficient or improper gap junction channel function caused by mutations in the genes encoding Cx proteins has recently been associated with a variety of diseases such as peripheral neuropathy, oculodentodigital dysplasia (ODDD), sensorineural deafness, skin disorders, cataracts and visceratrial heterotaxia (Lampe *et al.*, 2004). These different

phenotypes not only show the diversity of the expression patterns of *Cxs*, but they also illustrate that gap junctions play different roles in different tissues (Goodenough *et al.*, 1996). Following is a discussion from current literature of the diseases associated with *Cx* mutations. Interestingly, *Cx43* and *40*, which will be discussed in section 1.4.2, are the only ones in which mutations have been found to have association with cardiac diseases.

### **1.3.6.1 Peripheral neuropathy**

The first human disease to be associated with the impairment of *Cx* function was Charcot-Marie-Tooth X-linked inherited peripheral neuropathy (CMTX). CMTX is a demyelinating syndrome with progressive degeneration of peripheral nerves brought on by a defect in Schwann cells. Over 200 mutations have been identified in *Cx32*, that is located on the X chromosome, in CMTX patients (Nelis *et al.*, 1999; Bergoffen *et al.*, 1993). Most of the mutations are sited in the coding region of *Cx32* and span the entire length of the protein. The mutations differ in their ability to affect the function of the *Cx32* protein and also the clinical phenotype of the disease (Ionasescu *et al.*, 1996)

### **1.3.6.2 Sensorineural deafness**

Five *Cxs* have been found to be involved in deafness (syndromic and nonsyndromic), namely, *Cx26*, *Cx30*, *Cx31*, *Cx32* and *Cx43*, with *Cx26* being the most frequently causative one out of the five (Kelsell *et al.*, 2001; Richard *et al.*, 2003). The mutations are not restricted to any functional domains of the proteins and are distributed throughout the length of the proteins. Autosomal-recessive and autosomal-dominant forms of hearing impairment have been associated with 50 mutations in the coding region of the *Cx26* gene. The most common mutation is a recessive frame shift mutation (35delG), which causes premature translation termination (Zelante *et al.*, 1997). Studies have shown that *Cx26* is essential for cochlear function and cell survival in the sensory epithelium of the inner ear (Cohen-Salmon *et al.*, 2002).

### 1.3.6.3 Skin disorders

Studies in mice revealed that at least nine different *Cx* genes are coexpressed in the epidermis. Gap junctions of the epidermis play important roles in regulating keratinocyte growth and differentiation (Choudhry *et al.*, 1997). Some of the mutations in *Cx26*, *Cx30* and *Cx31* have been found to associate not only with deafness but also with skin disorders (Richard *et al.*, 2003). Mutations in *Cx26* have been shown to be linked to Vohwinkel syndrome, which is an autosomal-dominant condition with mutilating keratoderma accompanied by deafness (Maestrini *et al.*, 1999). Mutations in *Cx30.3* and *Cx31* are associated with erythrokeratoderma variabilis (a skin disease) (Richard *et al.*, 1998, 2003). The mutations associated with skin disorders have also been found not to be restricted to any particular *Cx* protein domain, just as in the case with the *Cx* mutations in CMTX and deafness.

### 1.3.6.4 Cataracts

Three *Cxs* are expressed in the lens of the eyes, namely, *Cx43*, *Cx46* and *Cx50*. The fibre cells of the lens are interconnected by an extensive network of gap junctions containing *Cx46* and *Cx50*. These *Cxs* play roles in maintaining homeostasis and supporting cell growth (White *et al.*, 2002). Mutations in *Cx46* and *Cx50* have been identified in patients with inherited zonular pulverulent cataracts. Different from the mutations associated with CMTX, deafness and skin disorder, these mutations associated with cataracts, are largely restricted to the extracellular loop or transmembrane domains of the *Cx* proteins (Berry *et al.*, 1999; Berthoud *et al.*, 2003; Jiang *et al.*, 2003).

### 1.3.6.5 Oculodentodigital dysplasia (ODDD)

ODDD is a human disease that has recently been shown to have association with *Cx* mutations. It is a congenital disorder characterized by developmental abnormalities of the face, eyes, limbs and dentition and is associated with dominant mutations in *Cx43* (Paznekas *et al.*, 2003). The mutations are not restricted to any functional domains and

are distributed throughout the length of the proteins, except for the C-terminus (Wei *et al.*, 2004).

#### **1.3.6.6 Visceroatrial heterotaxia**

Mutations of *Cx43* have been reported in patients with heart malformations and defects of laterality (heterotaxia) such as viscerotaxial heterotaxia (Krutovskikh *et al.*, 2000; Wei *et al.*, 2004). Some of these mutations occur in the C-terminus, which is a region containing multiple consensus motifs for phosphorylation by several intracellular protein kinases (van Veen *et al.*, 2001). It is proposed that these motifs are disrupted by the mutations and thereby impair cell-cell communication during the stages of development. This, in turn, causes malformations of the heart and defects of laterality (Britz-Cunningham *et al.*, 1995).

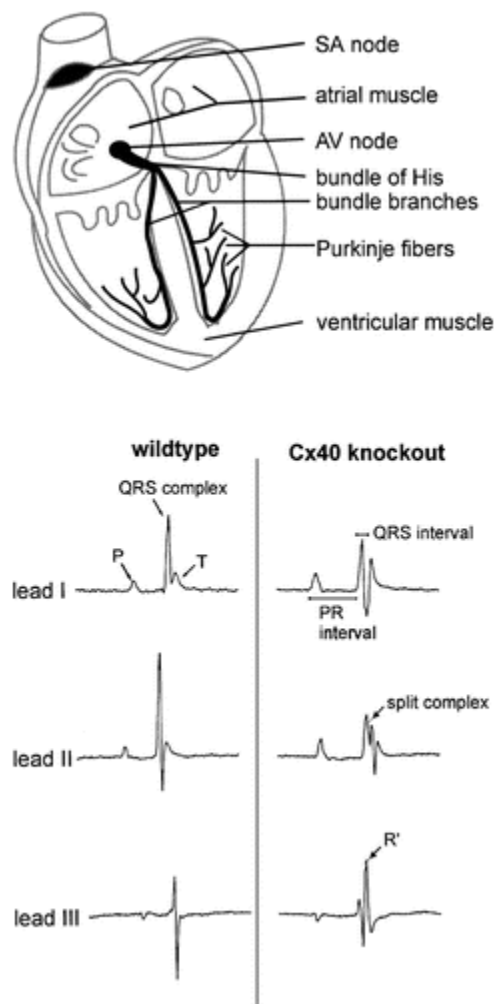
### **1.4 Connexin 40**

The following section will focus on *Cx40* because this cardiac isoform is the focus of the present study. Abnormal cardiac conduction in *Cx40*-deficient mice, polymorphisms in the *Cx40* gene and association of *Cx40* polymorphisms with hypertension are the aspects that will be addressed in this section.

#### **1.4.1 Abnormal cardiac conduction in *Cx40*-deficient mice**

Knockout mice studies have been done in order to delineate the function of *Cx40* in the cardiac conduction system. In these studies, AV and intraventricular conduction abnormalities were observed in the *Cx40* knockout mice. Figure 1.11 shows the slowed conduction in *Cx40* homozygous knockouts compared with wild-type mice. The decreased AV conduction can be seen on the ECG in the form of the PR interval being ~20% longer in the knockouts than in the wild-type or heterozygote control mice. The PR interval reflects the time required for excitation to traverse the atrium, AV node, and His-Purkinje system (White *et al.*, 1999; van Rijen *et al.*, 2001).





**Figure 1.11 ECG taken from a wild-type and a Cx40 knockout mouse.** The first deflection, the P wave, represents the depolarisation of the SA node and both atria. This is followed by a long PR interval, which corresponds to the slow conduction through the AV node and rapid conduction through the His-Purkinje system. The QRS complex represents the ventricular depolarisation and atrial repolarisation that occurs simultaneously. The T wave, which is the last deflection on the ECG, is caused by the ventricular repolarisation (White *et al.*, 1999; <http://www.bmb.psu.edu>)]

The delay in intraventricular conduction can also be seen on the ECG in the form of the QRS complex being ~33% longer than wild-type control mice (White *et al.*, 1999; van Rijen *et al.*, 2001). The QRS complexes of the Cx40 knockout mice are also more notched than the wild-type mice, which suggests altered ventricular activation sequences. These abnormalities observed by ECG indicate bundle branch block in one of the two major divisions of the His-Purkinje system, namely, the RBB (Saffitz *et al.*, 2000).

In a study done by Tamaddon and colleagues (Tamaddon *et al.*, 2000), they mapped the activation sequence of the mouse RBB. They observed that absence of *Cx40*, in the knockouts, reduced propagation velocity in the RBB by ~40% without apparent delay in the left bundle branch (LBB) or slowing in ventricular propagation velocities (Saffitz *et al.*, 2000). This is a characteristic that has been observed in patients with cardiovascular disease (Gros *et al.*, 2004). Clinical studies have shown that the RBB is more vulnerable to conduction block than the LBB, possibly because of its smaller diameter in comparison with the LBB. This causes minor discontinuities to be more prominent in causing activation block by means of load mismatching (van Rijen *et al.*, 2000).

In a study done by Gu and colleagues (Gu *et al.*, 2003), it was shown that both *Cx40* heterozygous and homozygous-null mice exhibited a variety of complex cardiac malformations such as conotruncal defects and endocardial cushion defects, which suggested the involvement of *Cx40* expression in cardiogenesis. The molecular mechanisms in which the *Cx40*-deficiency leads to these cardiac malformations are unknown. These animal studies revealed that *Cx40* is an important determinant of impulse propagation in the atria and the specialised conduction system and that it has a role in cardiogenesis (Gros *et al.*, 2004; Gu *et al.*, 2003).

#### **1.4.2 Polymorphisms in the *Cx40* gene**

Two closely linked polymorphisms were identified by Groenewegen and colleagues (Groenewegen *et al.*, 2003) within the promoter region of *Cx40*, at nucleotides -44 (G→A) and +71 (A→G). The *Cx40* haplotype (-44AA/+71GG) was shown to have strong association with familial atrial standstill, which is primarily an electrical excitability and conduction disorder (Makita *et al.*, 2005). Functional characterisation revealed that this *Cx40* haplotype lead to a >50% reduction in promoter activity (Firouzi *et al.*, 2004). They therefore suggested that the *Cx40* polymorphisms may possibly cause changes in expression levels and distribution patterns of the protein, which could modulate atrial electrophysiological properties that favour susceptibility for atrial arrhythmia (Gollob *et al.*, 2006).

The distance between the two polymorphisms is very small and the following will therefore be a report of the position of the -44 polymorphism in the *Cx40* promoter region. The -44 position in the promoter is located a few nucleotides from two major regulatory sites, an Ets-1/NK-box/T-box/SP1 site and a GATA site (Dupays *et al.*, 2003). The transcription factors functional at these sites are important regulators of *Cx40* expression. The SP1 factor maintains basal promoter activity. The T-box transcription factors such as T-box2, T-box3, T-box5 and T-box20 may activate or repress transcription and NK and GATA factors such as Nkx2-5, GATA4 and GATA5 confer tissue specificity (Linhares *et al.*, 2004).

Recent work has demonstrated the complexity of transcriptional regulatory mechanisms governing selective promoter usage, alternative 5'-UTR splicing, tissue-specific expression and translational efficiency of *Cxs* (Anderson *et al.*, 2005). It is thus possible that a small change in the regulatory region of *Cx40* may have an impact on the level of its expression; for example, through modification to the initiation or stabilisation of the transcriptional complex (Schiavi *et al.*, 1999; Pfeifer *et al.*, 2004).

### **1.4.3 Association of *Cx40* polymorphisms with hypertension**

Hypertension is a risk factor for a multitude of potentially life-threatening complications, such as myocardial infarction, congestive heart failure, renal failure and stroke. The pathogenesis of high blood pressure is multifactorial and involves both genetic and environmental factors (August *et al.*, 2003). It was recently suggested that gap junctions may play key roles in the pathogenesis and eventual clinical manifestations of cardiovascular disease, including hypertension (Haefliger *et al.*, 2004). They have been shown to play critical roles in the coordination of vasomotor responses and the regulation of vascular tone in the cells of the vascular wall (Hill *et al.*, 2002; Christ *et al.*, 1996; Rummery *et al.*, 2004).

A study by Firouzi and colleagues (Firouzi *et al.*, 2006) showed a significant association between the *Cx40* polymorphisms (-44A/+71G) and hypertension in men, but not in

women. As the male sex is a risk factor for cardiovascular disease and as men have higher blood pressure than women, it has been proposed that female sex hormones might offer protection against a genotypic predisposition in women (Fisher *et al.*, 1997; Mendelsohn *et al.*, 1999).

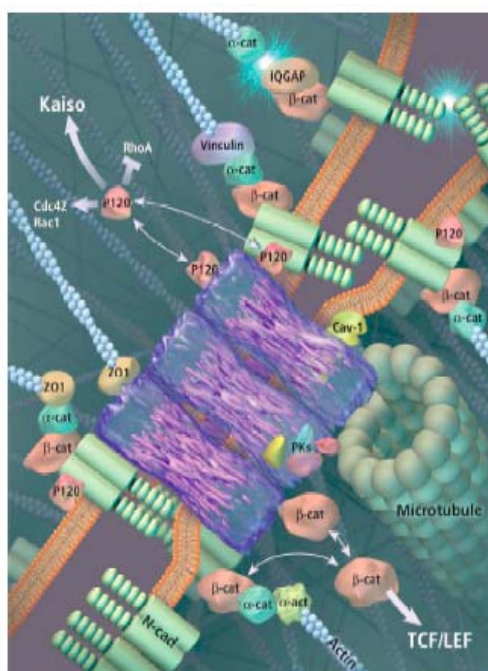
The *Cx40* polymorphisms may enhance the risk of hypertension through the impaired control and coordination of vasomotor responses along the vessel wall as supported by studies in *Cx40*-deficient mice (Figuroa *et al.*, 2003). Recent studies in *Cx40*-deficient mice revealed a key role for this protein in the control and regulation of blood pressure. *Cx40*-deficient mice had significantly higher blood pressure levels, compared with the wild-type mice, and also displayed irregular arteriolar vasomotion and impaired conduction of vasodilatory signals along their arterioles (de Wit *et al.*, 2000, 2003).

### **1.5 Speculative model of protein-protein interactions**

Recent work has been done by means of yeast-two-hybrid (Y2H) screens, glutathione-S-transferase (GST)-pull down assays, antibody arrays, and proteomic analysis in order to identify potential Cx binding proteins of which the functions are being elucidated (Segretain *et al.*, 2004). Wei and colleagues proposed a speculative model of protein-protein interactions based on studies done with Cx43, the most studied isoform (Wei *et al.*, 2004). This model could also apply to the other cardiac Cx isoforms, in particular Cx40, because of biochemical studies that have shown that Cx43 and Cx40 co-localise in order to form heteromeric connexons (Valiunas *et al.*, 2001; Evans *et al.*, 2002). Figure 1.12 is an artist's impression of this speculative model. Wei and colleagues proposed a diverse array of protein binding partners such as signaling proteins ( $\alpha/\beta$ -catenin, p120ctn), structural proteins (ZO-1, caveolin-1), membrane proteins (cadherins), and proteins that interact with, or are part of, the cell cytoskeleton ( $\alpha$ -actinin, microtubule) (Wei *et al.*, 2004).

Several protein kinases such as Src, protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) can phosphorylate the C-terminus of Cx43 or Cx40. This could

alter channel gating or protein-protein interactions that may be important in cell signaling. Transcriptional effects may be elicited by means of p120ctn/Kaiso or  $\beta$ -catenin-T-cell factor (TCF)/lymphoid enhancer-binding factor (LEF), which could result in long-term effects through gene expression changes. The proposed protein-protein interactions may cross talk with cell signaling pathways, which regulate cell adhesion, cell motility and the actin cytoskeleton. Protein-protein interactions identified in the present study with similar functions to those proposed for Cx43 will make it possible to draw a speculative model of the Cx40 interactome, in order to better understanding the molecular and cellular functions of Cx40 in the heart.



**Figure 1.12 Artist's impression of the speculative model of protein-protein interactions made with Cx43.** The following are ligands of Cx43: signaling proteins ( $\alpha/\beta$ -catenin, p120ctn), structural proteins (ZO-1, caveolin-1), membrane proteins (cadherins), proteins that interact with or are part of the cytoskeleton ( $\alpha$ -actinin, microtubule) and protein kinases (Src, PKC, MAPK) (Wei *et al.*, 2004).

## 1.6 Differential gating of gap junction channels

Proper gating of the gap junction channel is essential for normal cell-to-cell communication, and subsequently for adequate impulse propagation throughout the heart. Abnormal gating may account for certain defects of the conduction system (Söhl *et al.*,

2004, Lampe *et al.*, 2004). Functional channels are mostly in an open state, but can close in response to certain changes in the ionic composition of the cytosol. As a result of the channel closure, neighbouring cells uncouple from each other electrically and metabolically (Peracchia *et al.*, 1997).

Channel closure may be affected by a change in conformation of the channel protein or by specific domains in the C-terminus which may flip over like a “ball and chain” to block the pore (Moreno *et al.*, 2002; Ahmad *et al.*, 2001). Gating via these mechanisms is regulated by cellular calcium concentrations, intracellular pH ( $\text{pH}_i$ ), transjunctional or transmembrane voltage, and protein phosphorylation (Severs, 2000). The Cx40 ligands identified in the present study may function in the gating mechanisms of channels composed of this Cx protein subunit and therefore influence gap junctional conductivity.

### **1.6.1 Cellular calcium concentrations**

Closure of the channels occurs in the presence of high concentrations of calcium ions and this suggests that the channels can be modulated by a variety of calcium-dependent cellular events. It has been proposed that calmodulin serves as a mediator for the calcium effect because it has been found to bind to Cx32 (Ahmad *et al.*, 2001; Hertzberg *et al.*, 1987). The depletion of calmodulin has been shown to reduce the sensitivity of gap junctional communication to elevated calcium levels in *Xenopus* oocytes (Peracchia *et al.*, 1996; Bukauskas *et al.*, 2004).

### **1.6.2 Intracellular pH ( $\text{pH}_i$ )**

The regulatory sites for pH gating are located at the CL and C-terminus of the Cx proteins (figure 1.9). These are regions with low homology across the family of Cx genes, which may explain why different homotypic and heterotypic channels exhibit a variable degree of sensitivity to intracellular acidification (Yahuaca *et al.*, 2000). A particle-receptor (“ball and chain”) model has been described for the mechanism of Cx pH gating (van Veen *et al.*, 2001; Moreno *et al.*, 2002). This model has been suggested to function as follows, according to biochemical studies done with Cx43: Intracellular acidification

leads to the binding of Cx43 C-terminus (acts as a particle) to a region in the CL domain which includes a histidine 95 residue (acts as a receptor). This action causes the closure of the channel. It is not known whether all of the Cxs make use of this mechanism (Ek-Vitorin *et al.*, 1996; Eckert *et al.*, 2002; Bukauskas *et al.*, 2004).

### **1.6.3 Transjunctional or transmembrane voltage**

Cxs are sensitive to transjunctional or transmembrane voltage and form closed channels when large voltages are applied (Dahl *et al.*, 1996). Different homotypic and heterotypic channels exhibit voltage gating to different degrees and the voltage-gating properties of a Cx may be species-specific (White *et al.*, 1995). It has been shown that the C-terminus, N-terminus, M1/E1 boundary, and a conserved proline residue in the M2 participate in the voltage-gating properties of the channels, probably in a similar way to pH gating; according to a particle-receptor model (figure 1.9) (Suchyna *et al.*, 1993; Verselis *et al.*, 1994; Revilla *et al.*, 1999). Voltage-gating might also be influenced by the interaction of a connexon with its opposing connexon (van Veen *et al.*, 2001).

### **1.6.4 Protein phosphorylation**

Sequence analysis of the CL and the C-terminus has revealed multiple consensus motifs for phosphorylation by several intracellular protein kinases, with the C-terminus being the primary region to be phosphorylated (van Veen *et al.*, 2001; Lampe *et al.*, 2000). Once the C-terminus of a Cx is phosphorylated, it may interact with either the pore-forming region of the channel or a intermediary molecule, i.e. ligand, to form a complex resulting in closure of the channel, which may cause the neighbouring cells to uncouple from each other electrically and metabolically (Herve *et al.*, 2005; Peracchia *et al.*, 1997). It has been shown that gap junctional communication and phosphorylation of Cx proteins are linked and highly regulated during the cell cycle (Lampe *et al.*, 2004). Phosphorylation is a common form of post-translational modification, which has not been reported for the N-terminus. Cx26 is the only isoform that is not phosphorylated (Goodenough *et al.*, 1996) and this could be due to the fact that it is the shortest Cx and only has few C-

terminus amino acids that could interact with cytoplasmic signaling elements (Segretain *et al.*, 2004).

Phosphorylation has also been shown to play important roles in the synthesis, trafficking and removal of Cxs from the plasma membranes (Goodenough *et al.*, 1996; Lampe *et al.*, 2004). Some kinases act in inhibiting the formation of channels and others function as important conduits of channel formation. Phosphorylation of Cx43 by several different kinases such as protein kinase C (PKC), extracellular signal-regulated kinases (ERK), and casein kinase 1 (CK1) has been shown to stimulate gap junction removal of this Cx from the plasma membrane (Segretain *et al.*, 2004). Also, many reagents, growth factors and viral oncogenes influence gap junction gating and their effects are often associated with changes in basal patterns of connexin phosphorylation (Goodenough *et al.*, 1996).

Phosphorylation may also serve to create a specific binding site that promotes interaction with a domain that governs protein-protein interactions (Wei *et al.*, 2004). These domain-driven interactions may represent novel means of regulating Cx processing or function that has so far been inadequately investigated (Lampe *et al.*, 2000).

## **1.7 Conduction system diseases for which connexins are novel candidates**

Inherited cardiac conduction diseases of many different etiologies have been identified such as Holt-Oram syndrome, Atrial septal defect, Progressive familial cardiac conduction disease (PCCD), Isolated cardiac conduction disease (ICCD), and Progressive familial heart block type I and type II (PFHBI and PFHBII) (Kleber *et al.*, 1997; Zipes *et al.*, 1998; Smits *et al.*, 2005). The following discussion will focus on the two South African diseases which apply to the present study, namely, PFHBI and PFHBII, for which originally Cxs were novel candidate genes because of their dominant role in electrophysiological function (Söhl *et al.*, 2004). However, they were subsequently excluded as candidate genes because they did not map to the disease target loci (Arieff, 2004; Fernandez *et al.*, 2005). Also included in this section is a discussion on Myotonic



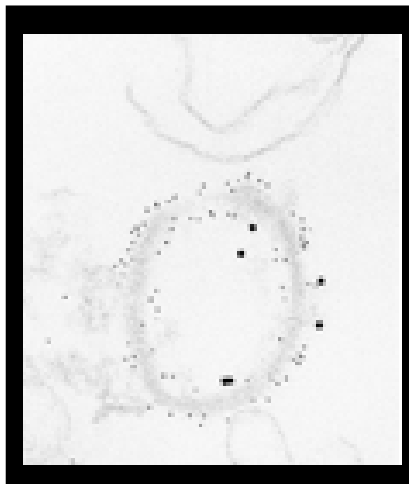
dystrophy (DM), in which cardiac expressed Cx43 has been reported as a ligand of the myotonic dystrophy protein kinase (*DMPK*)-causative gene product (Mussini *et al.*, 1999; Schiavon *et al.*, 2002). This finding raised the question of whether other functionally related interactions may occur with the C-terminus of Cx40 in a similar way to that identified for Cx43 and *DMPK*, and whether these interactions contribute to development of PFHBI or PFHBII when defective ligands are involved.

### **1.7.1 Myotonic dystrophy - co-localisation of *DMPK* with Cx43**

Myotonic dystrophy is a dominantly inherited disease characterised by myotonia and progressive muscle wasting, arrhythmia and cardiac conduction defects, mental retardation, cataracts, and disorders of the endocrine system (O'Brien *et al.*, 1984; Perloff *et al.*, 1984; Harper, 1989). It is one of the most prevalent muscular diseases in adults and is caused by expansion of a trinucleotide (CTG) repeat in the 3'untranslated region of *DMPK* (Brook *et al.*, 1992; Berul *et al.*, 1999). Studies to identify the localisation of *DMPK* in the heart found that *DMPK* localises to the cytoplasmic surface of junctional and extended junctional sarcoplasmic reticulum, which suggested that *DMPK* is involved in the regulation of excitation-contraction coupling (Salvatori *et al.*, 1994, 1997; Shimokawa *et al.*, 1997). *DMPK* was also found associated with gap junctions along the intercalated disks, whereas it was absent in the two other kinds of junctional complexes, namely, fascia adherens and desmosomes (Mussini *et al.*, 1999; Schiavon *et al.*, 2002).

Immunogold labeling of gap junction purified fractions showed that *DMPK* co-localised with Cx43 (Mussini *et al.*, 1999) (figure 1.13). Phosphorylation of Cxs is important in several physiological events such as assembly, docking and gating of gap junction channels (Lampe *et al.*, 2004; Evans *et al.*, 2002; Segretain *et al.*, 2004). It is therefore suggested that *DMPK* plays a regulatory role in the transmission of signals between cardiomyocytes through its interaction with gap junctions and, when defective, might cause the development of cardiac conduction defects associated with DM (Mussini *et al.*, 1999; Schiavon *et al.*, 2002; Berul *et al.*, 1999). Therefore, it has been proposed that it is the Cx-associated role of *DMPK* that is responsible for the cardiac phenotype in

individuals affected by DM. The possibility exists that other functionally related interactions may occur with the C-terminus of Cx40 in a similar way to that identified for Cx43 and DMPK.

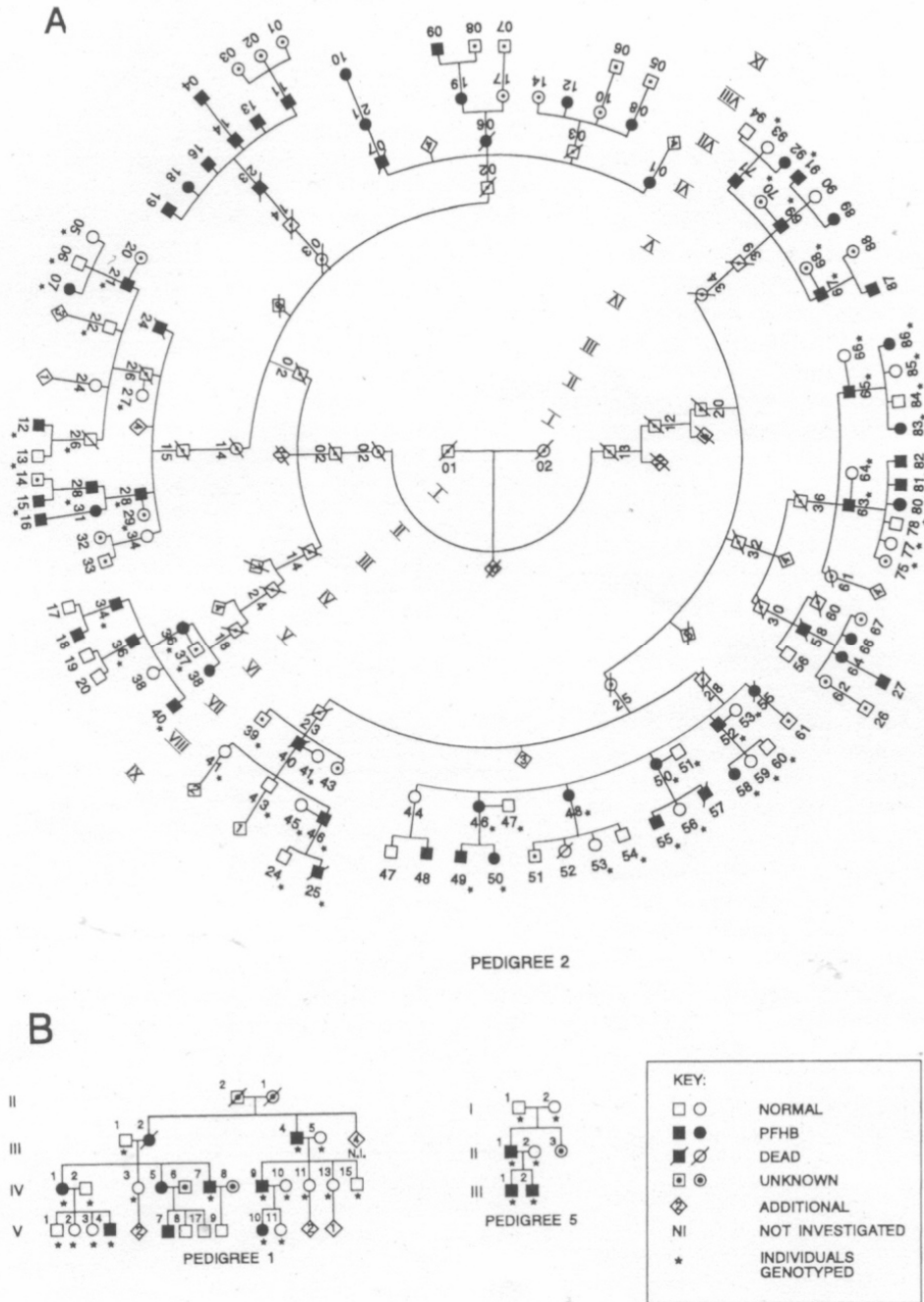


**Figure 1.13 Immunogold labeling of cardiac gap junctions demonstrating the co-localisation of DMPK and connexin 43.** DMPK (large gold particles) and connexin 43 (small particles) (Mussini *et al.* 1999).

## 1.7.2 Progressive familial heart block type I (PFHBI)

### Clinical description

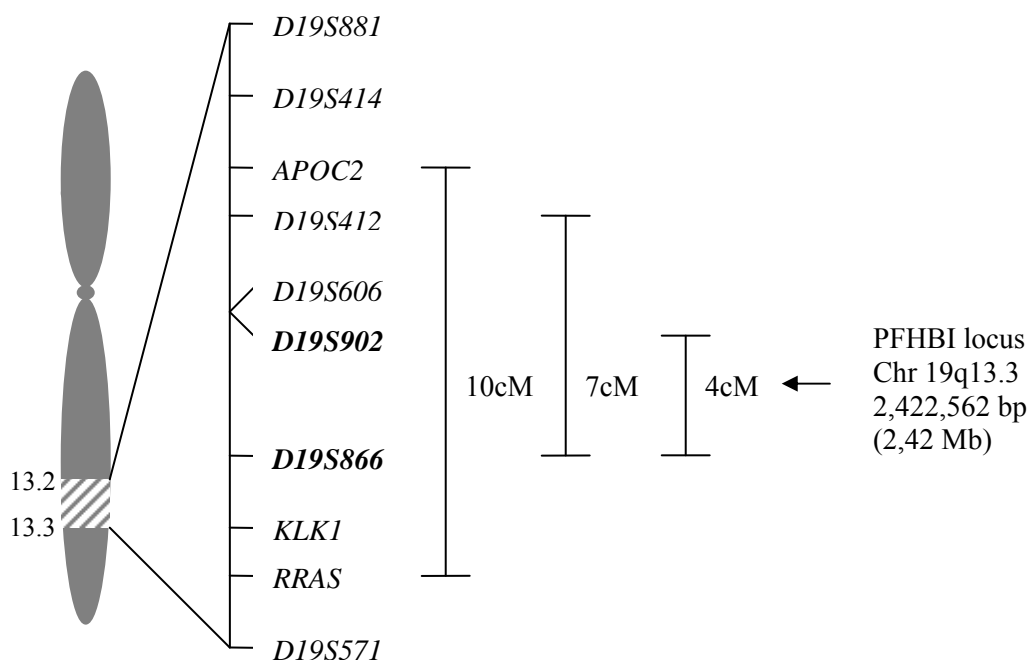
Progressive familial heart block type I (PFHBI) (OMIM 113900) was first described by Brink and Torrington in 1977 in several branches of a large South African pedigree (Brink and Torrington 1977). It is an autosomal dominantly inherited, progressive BB conduction disorder which may progress to complete heart block (Brink *et al.*, 1995). It is defined on ECG by evidence of BB disease, namely, RBBB, left anterior or posterior hemiblock, or complete heart block with broad QRS complexes (Van der Merwe *et al.*, 1988, 1986). Figure 1.14 shows the three apparently unrelated pedigrees 1, 2, and 5 in which PFHBI has been shown to segregate (Brink *et al.*, 1995). Members of pedigree 2, consisting of 9 generations, are descendents from one ancestor who emigrated from Lisbon, Portugal in 1696. Pedigree 1 and 5 are smaller pedigrees, in which the affected members display the same phenotype and disease-associated haplotype as individuals in pedigree 2. There is, however, at this stage, no family data that links pedigree 1 and 5 to the same ancestor of pedigree 2 (personal communication with Brink).



**Figure 1.14 Representation of the pedigrees in which PFHBI segregates.** A) Pedigree 2, a nine-generation kindred. B) Pedigree 1 and 5, two smaller families that could not be linked to the main pedigree. Affected individuals had either right bundle-branch block (RBBB), complicated right bundle-branch block (RBBB), or complete heart block (Brink *et al.*, 1995).

### Refining the target locus

Initially, Brink and colleagues mapped the gene for PFHBI, by means of genetic linkage analysis, to a 10cM region on chromosome 19q13.2-q13.3 (figure 1.15) (Brink *et al.*, 1995). At this time, a French group mapped a second conduction defect, namely, ICCD, by linkage analysis to the same region on chromosome 19 as PFHBI (De Meeus *et al.*, 1995). It was shown that these two diseases shared common clinical features and was subsequently decided that they are likely the same disease. Subsequent studies used genetic fine mapping to reduce the target locus to a 7cM region (personal communication with Brink).



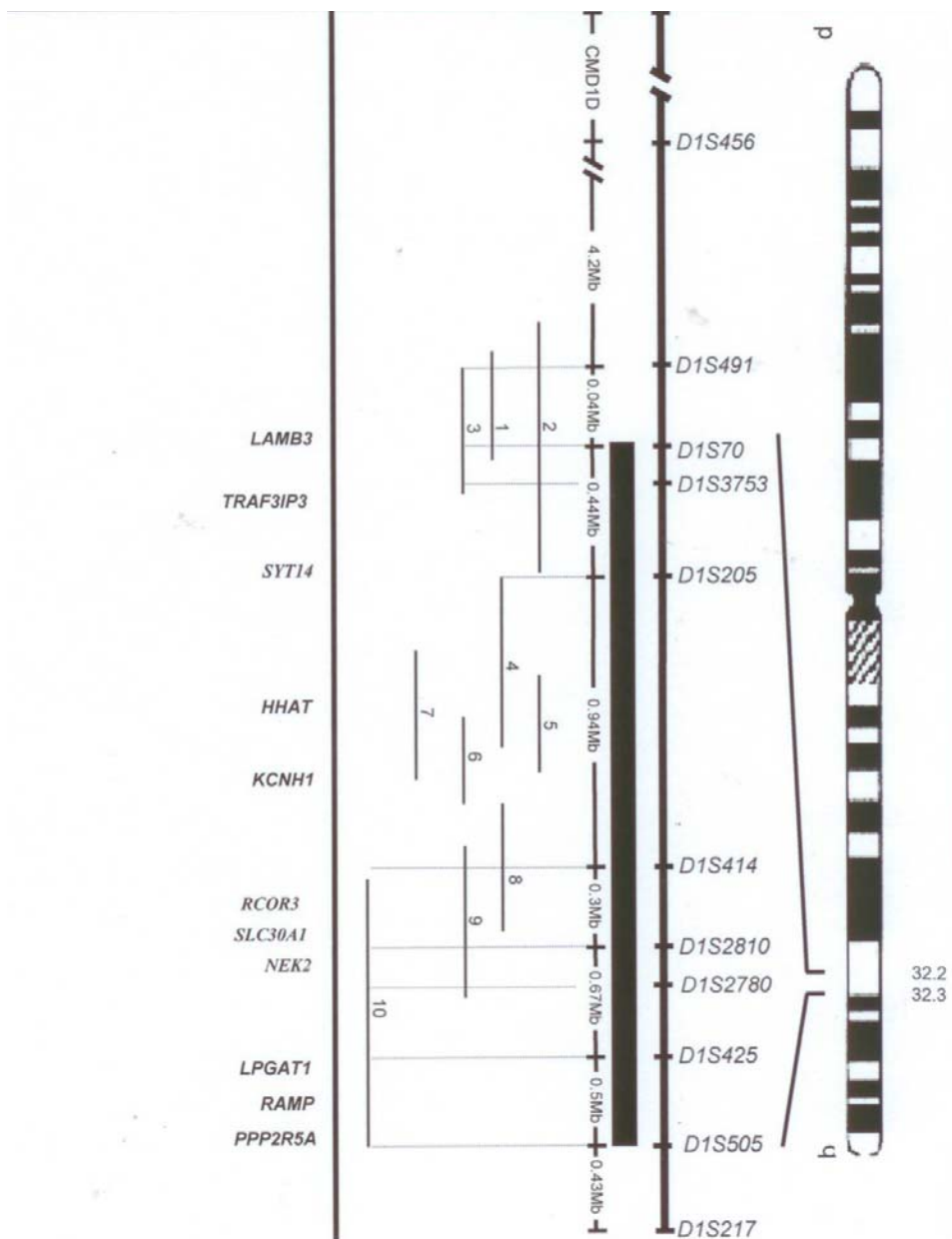
**Figure 1.15 Representation of progressive refining of the PFHBI locus**

Abbreviations: apolipoprotein C2 (*APOC2*), kallikrein (*KLK1*), related RAS viral oncogene homolog (*RRAS*), Chr: chromosome (accumulated data from Arieff, 2004).

The *DMPK* gene was originally an attractive candidate gene for PFHBI because of the cardiac complications shared between DM and PFHBI. However, the direct involvement of *DMPK* in PFHBI was excluded because of genetic recombination events which placed it outside the target locus (Brink, 1997). Subsequent studies reduced the PFHBI locus to a 4cM region on chromosome 19q13.3 (between markers *D19S606* and *D19S866*) (figure 1.15). This was translated to a physical distance of 3,743,618bp (3,74Mb), by using sequence information available from the Human Genome Project. This physical distance was subsequently reduced to 2,422,562bp (between markers *D19S902* and *D19S866*) (figure 1.15) by means of genetic fine mapping (Arieff, 2004). Chromosome 19 is one of the most gene-rich chromosomes and it is predicted that 62 genes lie at the PFHBI locus, of which some might be genes encoding ligands of Cxs.

### **1.7.3 Progressive familial heart block type II (PFHBII)**

Progressive familial heart block type II (PFHBII) (OMIM 140400) is an autosomal dominantly inherited cardiac conduction disorder which segregates in a South African family which was first described by Brink and Torrington in 1977. It has been defined on ECG as a sinus bradycardia with a left posterior hemiblock and narrow QRS complexes (Brink *et al.*, 1995). PFHBII presents clinically with syncopal episodes and Stoke-Adams seizures when complete heart block supervenes and the conduction abnormalities are accompanied by dilated cardiomyopathy (Fernandez *et al.*, 2004). Recently, the PFHBII locus has been placed in a 3.9cM region on chromosome 1q32.2-q32.3 (between markers *DIS70* and *DIS505*) by means of linkage studies (figure 1.16) (Fernandez *et al.*, 2005). The locus was found to lack Cx-encoding genes and their direct involvement in PFHBII was therefore excluded. However, it is possible that some of the genes that lie at the locus might be genes encoding ligands of Cxs.



**Figure 1.16 Representation of refining the PFHBII locus**

Abbreviations: laminin  $\beta$ 3 (*LAMB3*), tumor necrosis factor receptor Jun N-terminal kinase-activating modulator (*TRAF3IP3*), synaptotagmin XIV (*SYT14*), hedgehog acyltransferase (*HHAT*), potassium voltage-gated channel H (*KCNH1*), REST corepressor 3 (*RCOR3*), zinc transporter 1 (*SLC30A1*), never in mitosis gene A-related kinase 2 (*NEK2*), lysophosphatidylglycerol acyltransferase 1 (*LPGAT1*), retinoic acid regulated nuclear matrix associated protein (*RAMP*), protein phosphatase 2A b56alpha (*PPP2R5A*).

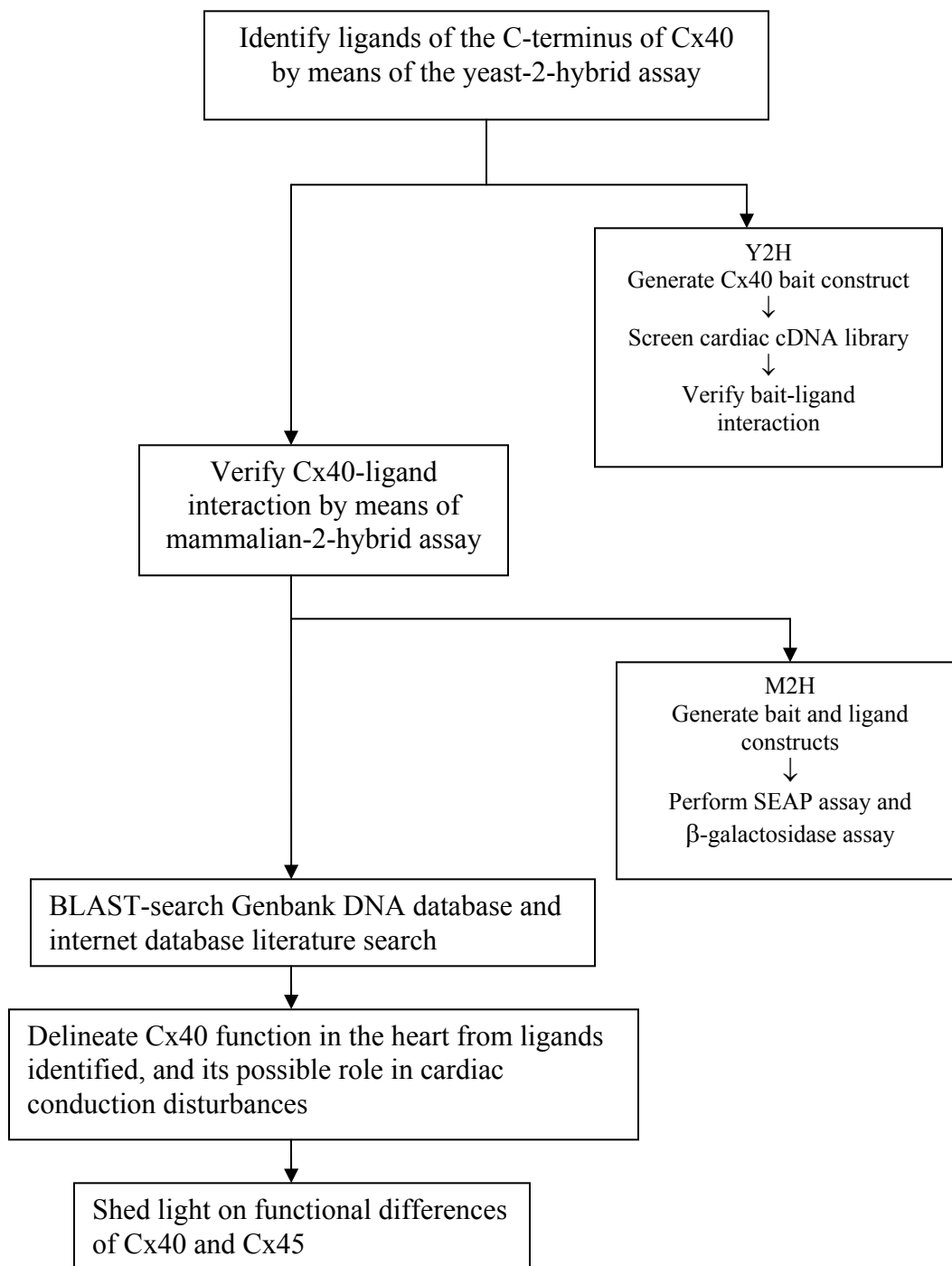
■ = target locus between markers *D1S70* and *D1S505* (Fernandez, 2004).

## 1.8 The present study

The aim of the present study was to identify ligands of the C-terminus of the human cardiac expressed Cx40, in order to understand the role of the interactions at a molecular and cellular level and to investigate the possible involvement of specific ligands in cardiac conduction disturbances. Figure 1.17 shows an outline of the present study. In order to identify Cx40 ligands, the C-terminus encoding domain, an important region involved in regulation of channel properties and protein-protein interactions, was used as a bait construct, to screen a commercially available cardiac cDNA library to identify prey ligands as part of the Y2H analysis. The sequenced inserts of the putative Cx40 ligands retrieved from the library screen were then subjected to BLAST-search and internet database literature searches, in order to assign identity and to determine plausibility of them being true Cx40 ligands, on the bases of their function and their subcellular localisation.

The putative Cx40 ligands were subjected to M2H analysis, in order to verify the interactions identified between the Cx40 bait and the library of putative ligands. To this end, a speculative model of the Cx40 interactome was drawn in order to speculate about the functions of Cx40 in the heart through its interactions with the ligands identified in the present study. In addition, Y2H and M2H results obtained from a parallel study done with Cx45, were compared, in order to shed light on the functional differences of Cx40 and 45 in the heart. Furthermore, the sequences of the putative Cx40 ligands were BLAST-searched against DNA databases covering cardiac conduction disorders, such as PFHBI and PFHBII, in order to identify genes encoding newly identified Cx40 ligands as candidate disease causative genes

### *Outline of the present study*



**Figure 1.17** Flowchart showing the outline of the present study



## *Chapter 2*

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## *Materials and methods*

### **2.1 Summary of methodology**

The yeast-2-hybrid (Y2H) method was used in the present study to screen a library of cardiac cDNA in order to identify the ligands of the C-terminus of human cardiac expressed *Cx40* (i.e. *GJA5*). Figure 2.1 shows an outline of the methodology. Initially, the bait-insert fragment for the assay was generated by PCR-amplification of the C-terminus encoding domain of *Cx40* from genomic DNA using specially designed primers with engineered restriction enzyme sites. The bait-insert fragment was subsequently cloned into a Clontech pGBKT7 shuttle vector. The pGBKT7-*GJA5* bait construct was then transformed into *E.coli* and its integrity verified by sequencing, after which it was cloned into *S.cerevisiae* yeast strain AH109 for the library screening. Preliminary assays were conducted to ensure that the pGBKT7-*GJA5* bait construct was not associated with activation of endogenous reporter genes (*ADE2*, *HIS3*, *MEL1*), toxicity or diminished mating efficiency in the *S.cerevisiae* host.

Thereafter, the bait-containing strain of *S.cerevisiae* was mated to a Clontech, commercially available, *S.cerevisiae* yeast strain Y187 pretransformed with a cardiac cDNA library. This allowed identification of interactions between the pGBKT7-*GJA5* bait construct and the prey clones (encoding putative ligands) contained within the library. To this end, the *S.cerevisiae* mating mixture was plated onto primary selection media which selected for diploid His<sup>+</sup> colonies. These colonies were subjected to more stringent selection media that selected for diploid Ade<sup>+</sup>, His<sup>+</sup> colonies which were subsequently tested for expression of the *MEL1* reporter gene by means of X- $\alpha$ -galactosidase assay. Positive colonies that were selected from this round of screening indicated the presence of strong bait and prey interactions and were subjected to another round of selection. An interaction specificity test (i.e. heterologous mating) was conducted in order to verify the pGBKT7-*GJA5* bait construct and putative prey clone interactions and to exclude non-specific interactions. The inserts of putative prey clones were sequenced and BLAST-searched against the Genbank DNA database in order to

assign identity and function. Furthermore, the sequenced inserts were also BLAST-searched against Genbank databases covering the PFHBI and PFHBII loci in order to determine whether they were candidate causative genes for these South African cardiac conduction diseases. Results showed that the genes encoding the putative preys were not candidates for these diseases. Internet database literature searches were done with each of the putative prey clones in order to prioritise them according to function and their subcellular localisation in order to assess their candidature for being true Cx40 ligands. Those identified as good candidates were subjected to mammalian-2-hybrid (M2H) analysis, in order to assess the validity of the pGBKT7-*GJA5* bait construct and prey clone interactions and to subsequently shed light on the function of Cx40 in the cardiac conduction system.

The M2H analysis was performed using the Matchmaker™ Mammalian Assay Kit 2 which included the pM, pVP16 and pG5SEAP vectors. Initially, the pM-*GJA5* bait and pVP16-prey constructs was generated in the same way as the pGBKT7-*GJA5* bait construct was, i.e. PCR-amplification of a bait and prey insert fragments with specially designed gene specific primers with engineered restriction enzyme sites and subsequent cloning of the inserts into the designated vectors. The pM-*GJA5* and pVP16-prey constructs were subsequently co-transfected with the pG5SEAP reporter vector and a pSV- $\beta$ -Gal reporter vector, which was used for a  $\beta$ -Galactosidase enzyme assay, into cultured H9C2 mammalian cells. The Great EscAPe™ chemiluminescent Detection Kit was used, in order to determine whether the *SEAP* (secreted alkaline phosphatase) reporter gene was activated by an interaction of the bait and prey proteins. The *SEAP* activity in the culture medium of the transfected cells was determined by means of a chemiluminescent substrate. The level of *SEAP* activity is directly proportional to the intensity of the bait and prey interaction and can therefore be used in determining the validity and specificity of their interaction. Several control assays were included as comparisons for each experiment, in order to assess the efficiency of the system. Furthermore, the chemiluminescence values obtained from the *SEAP* assay were normalised with the absorbance values obtained from the  $\beta$ -Gal assay, in order to determine transfection efficiency of the mammalian cells.

## *Outline of the methodology*

### **CONSTRUCTION OF GAL4 DNA-BD-TARGET FUSION PROTEIN (Bait construct)**

Design primers to amplify cytoplasmic C-terminus-encoding domain of *Cx40*  
Clone product into CLONTECH pGBKT7 shuttle vector



### **VERIFICATION OF FRAME AND INTEGRITY OF INSERT**

Automated DNA sequencing



### **EXPRESSION OF FUSION PROTEIN & AUTOACTIVATION ASSAY**

Transform bait construct into yeast strain AH109 (Ade-, His-, Leu-, Trp-, MATa)  
Test for autonomous reporter (*ADE2*, *HIS3*, *MEL1*) gene activation and cell toxicity



### **YEAST-2-HYBRID ASSAY**

Identify putative ligands using yeast-2-hybrid methodology  
[mate bait strain with the prey strain (yeast pretransformed with a cardiac cDNA library)]



### **IDENTIFY PUTATIVE LIGANDS OF CX40**

BLAST-search sequenced inserts of interacting prey clones  
against Genbank DNA databases to identify genes, Internet database  
literature searches



### **VERIFICATION OF TRUE PROTEIN:PROTEIN INTERACTORS OF CX40 C-TERMINUS**

Mammalian-2-hybrid assay

**Figure 2.1 Outline of the methodology**

## 2.2 DNA extraction

In order to amplify the C-terminus-encoding domain of *Cx40* from genomic DNA, a previously banked sample was used. The DNA had been extracted from 5ml of an anonymous blood sample of a subject enrolled as control in a project approved by the University of Stellenbosch ethics committee, following a method modified in the Molecules and Genes in Inherited Conditions (MAGIC) laboratory, of the Department of Biomedical Sciences (Corfield *et al.*, 1993).

## 2.3 Polymerase Chain Reaction (PCR) Amplification

### 2.3.1 Oligonucleotide primer design

Oligonucleotide primers were designed using sequence data available from the Genbank DNA database (<http://www.ncbi.nlm.nih.gov/Entrez>). Before synthesis, the primer sequences was analysed for complimentarity (self-complimentarity and primer-primer complimentarity) and compatibility of melting temperatures using DNAMAN<sup>TM</sup> version 4 software (Lynnion Biosoft Corp©). The oligonucleotide primers were synthesised according to standard phosphoramidite methodology at the Department of Molecular and Cell Biology, University of Cape Town (UCT), Cape Town, South Africa.

#### 2.3.1.1 Design of primers for the *GJA5* bait-insert for Y2H analysis

Primers were designed to PCR-amplify the cytoplasmic C-terminus-encoding domain of *Cx40* (i.e. *GJA5*) from genomic DNA (section 2.2), to obtain the bait-insert fragment to be cloned into a Clontech pGBKT7 cloning vector (figure 1, Appendix II) (BD Bioscience, Clontech, Paulo Alto, CA, USA). The DNA sequence of *GJA5* was obtained from the Genbank DNA database (<http://www.ncbi.nlm.nih.gov/Entrez>), and is shown in figure 2.2A. with the C-terminus encoding sequence indicated with a shaded box. The forward primer was designed to the start of the C-terminus at nucleotide nr.682 and the

reverse primer at nucleotide nr.1077. Amplification with these primers produced a *GJA5* bait-insert fragment of 426 nucleotides to be used in generating the Y2H construct. The C-terminus with the primers designed for Y2H and M2H (section 2.3.1.2 and 2.3.1.3) are indicated in figure 2.2B

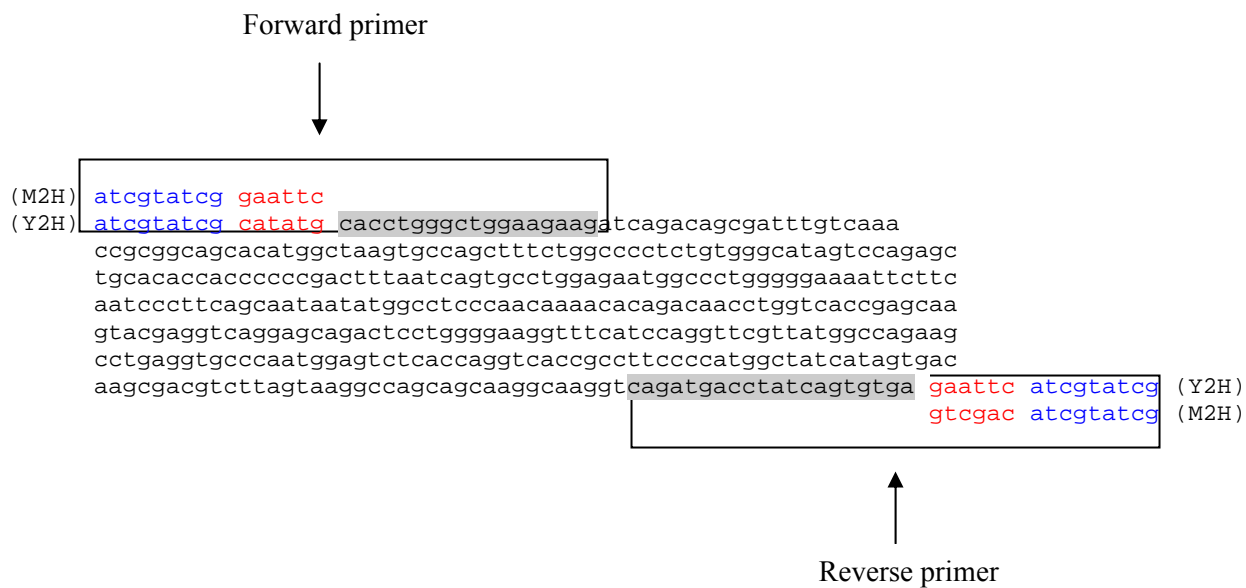
The primers were designed to consist of a universal enzyme seat sequence, a restriction enzyme sequence and the *GJA5* bait-insert specific sequence. The universal enzyme seat is a 5' overhang to facilitate the restriction enzyme digestion of the recognition sequence. The restriction enzymes *NdeI* and *EcoRI* were chosen for the forward and reverse primers, respectively. These enzymes have recognition sites in the multiple cloning site of the pGBKT7 vector and not in the *GJA5* bait-insert. The test enzyme *SfiI*, which does not have recognition sites in the bait-insert, was also selected in order to perform verification tests for successful cloning (section 2.8.3). The sequences of the *GJA5* bait-insert primers are shown in table 2.1.

```

1 atgggcgatt ggagcttcct gggaaatttc ctggaggaag tacacaagca ctcgaccgtg
61 gtaggcaagg tctggctcac tgcctcttc atattccgta tgctcgtgct gggcacagct
121 gctgagtctt cctgggggga tgagcaggct gatttccggt gtgatacgat tcagcctggc
181 tgccagaatg tctgctacga ccaggctttc cccatctccc acattcgcta ctgggtgctg
241 cagatcatct tctgtccac gccctctctg gtgtacatgg gccacgccat gcacactgtg
301 cgcattgcagg agaagcgcaa gctacgggag gccgagaggg ccaaagaggt ccggggctct
361 ggctcttacg agtaccgggt ggagagaaag gcagaactgt cctgctggga ggaagggaat
421 ggaaggattg ccctccaggg cactctgctc aacacctatg tgtgcagcat cctgatccgc
481 accaccatgg aggtgggctt cattgtgggc cagtacttca tctacggaat cttcctgacc
541 accctgcatg tctgccgcag gagtccctgt cccaccggtg tcaactgtta cgtatcccgg
601 cccacagaga agaatgtctt cattgtcttt atgctggctg tggctgcact gtccctcctc
661 cttagcctgg ctgaactcta ccacctgggc tggagaaga tcagacagcg atttgtcaaa
721 ccgcggcagc acatggctaa gtgccagctt tctggcccct ctgtgggcat agtccagagc
781 tgcacaccac cccccgactt taatcagtgct ctggagaatg gccctggggg aaaattcttc
841 aatcccttca gcaataatat ggctcccaa caaacacag acaacctggt caccgagcaa
901 gtacgaggtc aggagcagac tcctggggaa ggtttcatcc aggttcgtta tggccagaag
961 cctgagggtc ccaatggagt ctcaccaggc caccgccttc ccatggcta tcatagtgac
1021 aagcgacgtc ttagtaaggc cagcagcaag gcaaggtcag atgacctatc agtgtga

```

**Figure 2.2A Representation of *Cx40* coding sequence (NM\_005266) from the Genbank DNA database (<http://www.ncbi.nlm.nih.gov/Entrez>). Shaded box indicates the C-terminus encoding domain (nucleotides 682-1077) that was used for Y2H and M2H analysis.**



**Figure 2.2B Representation of the primers used for PCR-amplification of the C-terminus encoding domain of *Cx40*.** Line boxes indicate the forward and reverse primers for the Y2H and M2H analysis. The different enzyme seats and digestion sites are indicated for each. Shaded boxes indicate the 5' and 3' sequences used to design the primers. **Blue: Universal enzyme seat; Red: restriction enzyme site.**

Table 2.1 Primers for the *GJA5* bait-insert for Y2H analysis

Name	Sequence	Ta (°C)
GJA5 F	5' -atcgtatcg catag CACCTGGGCTGGAAGAAG-3'	51
GJA5 R	5' -atcgtatcg gaattc TCACACTGATAGGTCATCTG-3'	48

Blue: Universal enzyme seat; Red: restriction enzyme site; Black: Gene specific sequence  
Abbreviations: °C, degrees Celsius; Ta, Annealing temperature



### 2.3.1.2 Design of vector-specific primers for insert screening

In order to amplify the prey inserts cloned into the pGAD library vector, and for subsequent sequence analysis, primers were designed to vector-specific sequences flanking the multiple cloning site of the pGAD vector (figure 2, Appendix II) (BD Bioscience, Clontech, Paulo Alto, CA, USA). The vector sequences used in the design of the primers were obtained from the Clontech<sup>TM</sup> Matchmaker<sup>TM</sup> vector handbook ([www.clontech.com](http://www.clontech.com)). Primers were provided by Clontech (BD Bioscience, Clontech, Paulo Alto, CA, USA) for pGBKT7, pM and pVP16 (figures 1, 3 and 4, Appendix II). Vector-specific primers for the pGBKT7 cloning vector (BD Bioscience, Clontech, Paulo Alto, CA, USA) were used in order to amplify the *GJA5* bait-insert from the pGBKT7-*GJA5* construct (section 2.7.1) used in Y2H. Vector-specific primers for the pVP16 and pM cloning vectors, used in M2H, were used to amplify the prey- and bait-inserts from the vectors, respectively. Table 2.2 shows the sequences for the different vector-specific primers.

### 2.3.1.3 Design of primers for the M2H analysis

New primers were designed in order to amplify the *GJA5* bait-insert from the pGBKT7-*GJA5* construct used in Y2H, to clone it into the pM GAL4 DNA-BD cloning vector for M2H (section 2.21). Primers were designed for each of the putative prey clones in order to amplify them from pGAD for cloning into the pVP16 GAL4 DNA-AD vector. The forward primers for the preys were gene specific whereas the reverse primers were specific for the pGAD vector. A second set of reverse primers, which were gene specific, shown in table 2.4., were also designed for some of the preys because of difficulty encountered with cloning experiments. The second set of reverse primers produced a gene specific PCR product lacking the pGAD vector sequence which was initially included in the product when using the first set of primers (table 2.3). Exclusion of the vector sequence improved cloning efficiency. A universal enzyme seat sequence and restriction enzyme sequence were included into the design of the primers. The sequences for the primers for M2H are shown in table 2.3, 2.4 and 2.5.

Table 2.2. Primers for the pGBKT7, pGAD, pM and pVP16 vectors used in Y2H or M2H.

	Vector name	Primer sequence	Ta (°C)
<b>Y2H vectors</b>	<b>pGBKT7</b> (specific for the bait-insert)		
	F	TCATCGGAAGAGAGTAG	50
	R	TCACTTTAAAATTTGTATACA	51
	<b>pGAD</b> (specific for the prey-insert)		
	Outer F	CGATGATGAAGATACCCACCAAA	57
	Outer R	TCAAGTGAAGTTGACACGTAGCAC	57
	Inner F	TAATACGACTCACTATAGGGCGAGC	59
	Inner R	CGACGTCTACTTAGCATCTATGACTTT	59
<b>M2H vectors</b>	<b>pM</b> (specific for the bait-insert)		
	F	TCATCGGAAGAGAGTAG	50
	<b>pVP16</b> (specific for the prey-insert)		
	F	CTGGATATGGCCGACTTCGAG	54
	R	CTAGAAGCTTCTGCAGACGCG	54

Abbreviations: °C, degrees Celsius; Ta, Annealing temperature

**Table 2.3 Primers for the bait and primary putative prey clones for M2H**

Name	Forward Primer	Ta(°C)	Reverse Primer	Ta(°C)
GJA5 Bait	5'-atcgtatcg gaattc CACCTGGGCTGGAAGAAG-3'	51	5'-atcgtatcg gtcgac TCACACTGATAGGTCATCTG-3'	48
29	5'-atcgtatcg ggatcc GCGTCCGCCAAGCTACTT-3'	54	5'-actgcagaa acgcgt cta GCACAGTTGAAGTGAACCTGC-3'	50
35	5'-atcgtatcg gaattc AGCGTTGCCCGGGCCAAG-3'	56	5'-atcgtatcg acgcgt cta GCACAGTTGAAGTGAACCTGC-3'	50
62	5'-atcgtatcg gaattc GGGTCACCCACACTGTGC-3'	56	5'-atcgtatcg aagctt cta TCATCTGCAGCTCGAGCTCG-3'	54
153	5'-atcgtatcg gaattc ATGTGTATTCCATCATATGC-3'	50	5'-atcgtatcg tctaga cta GCACAGTTGAAGTGAACCTGC-3'	50
163	5'-atcgtatcg gtcgac GAGGAGTCCCTGGAACATCT-3'	52	5'-atcgtatcg tctaga cta CACAGTTGAAGTGAACCTGC-3'	48
164	5'-actctggat gaattc CCTCCGCCCCCAGCGACGATC-3'	64	5'-gctagatta aagctt cta AGTATCTACGATTCATCTGC-3'	46
204	5'-atcgtatcg acgcgt CACGGGCAGCTGCTTGCGC-3'	58	5'-atcgtatcg tctaga cta CACAGTTGAAGTGAACCTGC-3'	48
219	5'-atcgtatcg gaattc GGAGGCGGTTGTGGAGGCTG-3'	58	5'-gtagtaagc acgcgt cta CACAGTTGAAGTGAACCTGC-3'	48
222	5'-acgtagtca gaattc ATGCCATTCCGTAACACCC-3'	50	5'-acttagacg aagctt cta AGTATCTACGATTCATCTGC-3'	46
306	5'-atcgtatcg gaattc ATGGCCTCCAAATGCC-3'	48	5'-atcgtatcg aagctt cta CACAGTTGAAGTGAACCTGC-3'	48

**Table 2.4 Second set of reverse primers for the primary putative prey clones for M2H**

Name	Second set of reverse primers	Ta(°C)
29	5'-actgcagaa acgcgt TCATGGATCGTGGCCAAC-3'	49
35	5'-atcgtatcg acgcgt TTATGAGTCTTCTGAGGC-3'	47
62	5'-tagcttagc tctaga CTAGAAGCATTGCGGTGGA-3'	50
163	5'-atcgtatcg tctaga CTACTCCTCATCAAGCCCTTCG-3'	55
204	5'-atcgtatcg tctaga TCAGTCGTCAGCTGTCTTGC-3'	52
306	5'-atcgtatcg aagctt CTAGGGCTGGACCTTGCC-3'	53

**Table 2.5 Primers for the secondary putative prey clones for M2H**

Name	Forward Primer	Ta(°C)	Reverse Primer	Ta(°C)
31	5'-attctatcg gtcgac GAGCCCATTAAGAAGCACG -3'	50	5'-atcgtatcg tctaga CTAGTTCCACACATGGCG-3'	50
99	5'-atcttatgc ggatcc GCCGAATTCAGTATCTGG-3'	47	5'-atcgtatcg aagctt TCAGGGCACCACAACGC-3'	51

Blue: Universal enzyme seat; Red: restriction enzyme site; Black: Gene specific sequence  
 Abbreviations: °C, degrees Celsius; Ta, Annealing temperature

### **2.3.2 PCR-amplification to generate the *GJA5* bait-insert fragment for Y2H analysis**

One microlitre (100ng) of genomic DNA (section 2.2) was used as template for the PCR-amplification of the C-terminus of *GJA5* in order to generate the bait-insert fragment to be used in Y2H analysis. PCR reactions were performed in 50 $\mu$ l reactions and consisted of 150 ng/ $\mu$ l of each primer (table 2.1), 5u/ $\mu$ l Taq DNA polymerase enzyme (Bioline UK Ltd, London, UK) 2.5mM of each dNTP (dGTP, dCTP, dATP, dTTP) (Promega Corp, Madison Wisconsin USA), 50 $\mu$ l 10x buffer (Bioline UK Ltd, London, UK), 25mM Mg<sub>2</sub>Cl (Bioline UK Ltd, London, UK) and ddH<sub>2</sub>O to a final volume of 50 $\mu$ l. Amplification was performed on a GeneAmp® PCR System 2720 thermal cycler (Applied Biosystems) using the following cycling parameters: an initial denaturation step at 94°C for 5min, followed by 25 cycles of denaturation at 94°C for 30sec, annealing at 53°C for 30sec and extension at 72°C for 30sec. A final extension step followed at 72°C for 7min, followed by storage at 4°C. PCR amplified products was subsequently resolved by electrophoresis on a 2% agarose gel for verification.

### **2.3.3 PCR-amplification of the *GJA5* bait-insert and putative prey-inserts for M2H analysis**

In order to generate the constructs for M2H, the *GJA5* bait-insert needed to be amplified from the pGBKT7-*GJA5* construct to be cloned into the pM vector. In the same way, the prey inserts had to be amplified from the pGAD vector to be cloned into the pVP16 vector. Amplification was performed in 50 $\mu$ l reaction volumes containing 0.5 $\mu$ l plasmid preparation (100ng), 150 ng/ $\mu$ l of each primer (table 2.3, 2.4, or 2.5), 5u/ $\mu$ l Taq DNA polymerase enzyme (Bioline UK Ltd, London, UK), 2.5mM of each dNTP (dGTP, dCTP, dATP, dTTP) (Promega Corp, Madison Wisconsin USA), 50 $\mu$ l 10x buffer (Bioline UK Ltd, London, UK), 25mM Mg<sub>2</sub>Cl (Bioline UK Ltd, London, UK) and ddH<sub>2</sub>O to a final volume of 50 $\mu$ l. Amplification was performed on a GeneAmp® PCR System 2720 thermal cycler (Applied Biosystems) using the following cycling parameters: an initial

denaturation step at 94°C for 5min, followed by 25 cycles of denaturation at 94°C for 30sec, annealing at 55°C for 30sec and extension at 72°C for 30sec. A final extension step followed at 72°C for 7min, with subsequent storage at 4°C. The annealing temperature was 55°C for all but one prey (nr.99), which was 57°C. PCR amplified products were subsequently resolved by electrophoresis on a 1% agarose gel for verification. Product sizes of the *GJA5* bait and the different preys are shown in table 2.6.

Table 2.6 PCR-amplification product sizes of the *GJA5* bait-insert and prey clones used for the M2H analysis.

Name		PCR-amplification product size (bp)
	GJA5 Bait	426*
<b>Prey clone Nr.</b>		
29	NDUFA6	400
31	PSAP	750-800
35	ALDH2	550-600
62	ACTB	650-700
99	FLNA	1159-1700
153	VDAC2	1000-1500
163	MYH7	1500
164	MCOLN1	1000-1500
204	DHX30	80-90
222	CK	1500
306	CRP2	600-700

\* = The same product size was produced when using the Y2H primers to generate the bait insert fragment.

### 2.3.4 Bacterial colony PCR amplification

Bacterial colony PCR was performed to identify bacterial colonies harbouring the desired recombinant plasmid to be used in Y2H or M2H analysis. This was done because the vectors used in Y2H and M2H do not support blue-white screening as a method of

identifying the desired recombinant plasmid. Instead of using 100ng of genomic DNA as template in the PCR reactions, an individual bacterial colony (section 2.14.1) was picked from an agar plate supplemented with the appropriate antibiotic (Appendix I), and used as template.

Amplifications was performed in 50 $\mu$ l reactions and consisted of 150 ng/ $\mu$ l of each primer (table 2.2 or table 2.3, 2.4, 2.5), 5u/ $\mu$ l Taq DNA polymerase enzyme (Bioline UK Ltd, London, UK), 2.5mM of each dNTP (dGTP, dCTP, dATP, dTTP) (Promega Corp, Madison Wisconsin USA), 50 $\mu$ l 10x buffer (Bioline UK Ltd, London, UK), 25mM Mg<sub>2</sub>Cl (Bioline UK Ltd, London, UK) and ddH<sub>2</sub>O to a final volume of 50 $\mu$ l. Amplification was performed on a GeneAmp® PCR System 2720 thermal cycler (Applied Biosystems) using the following cycling parameters: an initial denaturation step at 94°C for 3min, followed by 30 cycles of denaturation at 94°C for 30sec, annealing at 55°C for 30sec (50°C when using the pGBKT7 vector primers) and extension at 72°C for 30sec. A final extension step followed at 72°C for 2min, and subsequent storage at 4°C. PCR amplified products were subsequently resolved by electrophoresis on a 1% agarose gel for verification.

## **2.4 Gel Electrophoresis**

### **2.4.1 Agarose gel electrophoresis for the visualisation of PCR-amplified products**

In order to verify whether the PCR-amplification (section 2.3) was successful, the amplification products were subjected to electrophoresis as follows: 8 $\mu$ l PCR product was mixed with 2 $\mu$ l bromophenol blue (Appendix I) loading dye. Each sample was loaded into a separate well of a 1-2% (depending on the size of the amplified fragment) horizontal agarose gel of 7x9x1 cm dimensions, containing SB buffer and 1 $\mu$ g/ml ethidium bromide (Appendix I). A molecular size marker of either bacteriophage  $\lambda$  DNA digested with *Pst*I ( $\lambda$ *Pst*) (Promega, Madison WI, USA) or a 100bp ladder DNA marker

(Promega, Madison WI, USA) was co-electrophoresed with all the PCR amplified products. Electrophoresis was performed at 200V/cm for 10min in SB running buffer. The DNA fragments were visualized on a long wave 3UV trans-illuminator (UVP, Inc. Upland, CA, USA) following electrophoresis. Photographs were obtained using an ITC Polaroid camera and Sony videographic printer (Sony Corporation, Shinagawa-ku, Tokyo, Japan). Presence of fragment of expected size indicated successful amplification.

#### **2.4.2 Agarose gel electrophoresis for the visualisation of plasmids purified from *E.coli***

Purified plasmids from *E.coli* (section 2.15.1) were subjected to electrophoresis in order to verify their successful purification. Two to three microlitres of purified plasmid was mixed with 2µl of bromophenol blue loading dye (Appendix I) and subjected to electrophoresis on a 1% horizontal agarose gel of 7x9x1 cm dimensions, containing SB buffer and 1µg/ml ethidium bromide (Appendix I). The bacteriophage λ Pst marker was co-electrophoresed with all the purified plasmids. Electrophoresis occurred at 180V/cm for 15min in SB running buffer (Appendix I). The plasmids were visualised on a long wave 3UV trans-illuminator and photographs were obtained using an ITC Polaroid camera and Sony video-graphic printer (Sony Corporation, Shinagawa-ku, Tokyo, Japan).

### **2.5 Automated Sequencing**

The primers used to sequence PCR-amplified products were the same as the ones used in the initial PCR reactions (section 2.3). The primers for sequencing of cloned inserts were vector specific primers. Automated sequencing of PCR-amplified products and cloned inserts was performed at the Core Sequencing Facility of the Department of Genetics at the University of Stellenbosch, RSA on a ABI Prism™ 377 or a ABI Prism™ 3100 automated sequencer (PE. Applied Biosystems, Forster City, CA, USA).

## 2.6 Analysis of DNA sequences

The ChromasPro computer program (Techelysium Pty Lmt, Helensvale, Queensland, Australia) and DNAMAN<sup>TM</sup> version 4 software (Lynnion Biosoft Corp©) were used in order to analyse the frame and integrity of the bait-insert fragment, as well as to assign identity to the putative ligand-positive prey clones obtained through the Y2H assay. The sequence of the bait-insert was compared to the mRNA sequences in the Genbank DNA database (<http://www.ncbi.nlm.nih.gov/Entrez>). The sequences for the prey clones were BLAST-searched against the Genbank DNA database using BLASTN, TBLASTX and BLASTP (<http://www.ncbi.nlm.nih.gov/Entrez>), in order to assign their identity.

## 2.7 Generation of constructs

### 2.7.1 Generation of Y2H constructs

In order to generate the pGBKT7-*GJA5* bait construct for Y2H, the *GJA5* bait-insert was cloned into the pGBKT7 cloning vector (figure 1, Appendix II). This was accomplished by sequential double-digestion reactions (section 2.8.1) and ligation reactions (section 2.10) of the PCR-amplified *GJA5* bait-insert (section 2.3.2) and the pGBKT7 cloning vector. Following transformation of the pGBKT7-*GJA5* bait construct into *E.coli* DH5 $\alpha$  strain (section 2.14.1), bacterial colony PCR-amplification reactions (section 2.3.4) were performed in order to select for colonies harbouring the recombinant plasmids. Colonies with the correct PCR product sizes were subjected to restriction enzyme digestion tests (section 2.8.3), in order to verify successful cloning.

Thereafter, the pGBKT7-*GJA5* bait construct was subjected to DNA sequence analysis in order to verify the frame and integrity of the bait-insert sequence and the conservation of the GAL4 DNA-BD reading frame (sections 2.5 and 2.6). The pGBKT7-*GJA5* bait construct was then transformed into the *S.cerevisiae* yeast strain AH109 (section 2.14.2), in order to generate the bait yeast strain, and subsequently to screen a Clontech



MATCHMAKER pre-transformed cardiac cDNA library (section 2.17.4) (BD Bioscience, Clontech, Paulo Alto, CA, USA). The library (section 2.17.1) consisted of cardiac cDNAs which were cloned into the pGADT7-Rec (figure 2, Appendix II) prey vector and transformed into the *S.cerevisiae* yeast strain Y187.

## 2.7.2 Generation of M2H constructs

In order to generate the constructs for M2H, the *GJA5* bait-insert was PCR-amplified from the purified pGBKT7-*GJA5* bait construct (section 2.15.1 and 2.3.3) and cloned into the pM GAL4 DNA-BD cloning vector (figure 3, Appendix II). In addition, the prey inserts were PCR-amplified (section 2.3.3) from the pGAD library cloning vector (figure 2, Appendix II) and cloned into the pVP16 GAL4 DNA-AD cloning vector (figure 4, Appendix II). This was accomplished by sequential double-digestion reactions with selected restriction enzymes (section 2.8.2, table 2.7) of the PCR-amplified *GJA5* bait-insert fragment, the PCR-amplified prey-insert fragments and the respective cloning vectors.

Following ligation reactions (section 2.10), the pM-*GJA5* bait and pVP16-prey constructs were transformed into *E.coli* DH5 $\alpha$  strain in separate reactions (section 2.14.1). Bacterial colony PCR-amplification reactions (section 2.3.4) were performed, in order to select for colonies harbouring the recombinant plasmids. Colonies with the correct PCR product sizes (table 2.6) were subjected to restriction enzyme digestion tests (section 2.8.3) in order to verify successful cloning.

Thereafter, the pM-*GJA5* bait and pVP16-prey constructs were subjected to DNA sequence analysis in order to verify the frame and integrity of the insert sequences and the conservation of the GAL4 DNA-BD and GAL4 DNA-AD reading frames (section 2.5 and 2.6). The constructs were subsequently transfected into mammalian cells (section 2.14.3) for M2H analysis (section 2.21).

## **2.8 Restriction enzyme digestion**

### **2.8.1 Restriction enzyme digestion for cloning the *GJA5* bait-insert into pGBKT7 for Y2H**

In order to clone the PCR generated *GJA5* bait-insert (section 2.3.2) into the pGBKT7 vector (figure 1, Appendix II), for Y2H analysis, both the insert and vector were sequentially double-digested with *NdeI* (5' enzyme) and *EcoRI* (3' enzyme) (Promega, Madison WI, USA). Restriction enzyme digestions were performed in 100ul reactions and consisted of 50μl insert DNA (1ug) or 10μl pGBKT7 vector DNA (1ug), 10μl enzyme buffer D (Promega, Madison WI, USA), 5ul *NdeI* (10u/μl), and the appropriate volume of ddH<sub>2</sub>O (insert: 35μl, vector: 75μl). The reactions were incubated at 37°C for 2hr, after which they were purified using the GFX® DNA purification kit (section 2.15.3) (Amersham Pharmacia Biotech, New Jersey, USA) and subsequently eluted in 50μl ddH<sub>2</sub>O in preparation for digestion with the 3' enzyme.

To the 50μl sample was added 5μl *EcoRI* (10u/μl), 10 μl enzyme buffer H (Promega, Madison WI, USA) and 35μl ddH<sub>2</sub>O. The reactions were incubated at 37°C for 2hr, after which they were again purified using the GFX® DNA purification kit (Amersham Pharmacia Biotech, New Jersey, USA). The digested insert was eluted in 20μl ddH<sub>2</sub>O, while the digested vector was eluted in 50μl ddH<sub>2</sub>O for subsequent treatment with calf intestinal alkaline phosphatase (CIP) (section 2.9), prior to being used in ligation reactions (section 2.10).

### **2.8.2 Restriction enzyme digestion for cloning inserts into pM and pVP16 for M2H**

In order to clone the PCR-amplified *GJA5* bait-insert (section 2.3.3) from the pGBKT7-*GJA5* construct used in Y2H, into the pM vector (figure 3, Appendix II) for M2H, the bait-insert and pM vector were sequentially double-digested with the 5' and 3' enzymes shown in table 2.7. In addition, the PCR-amplified prey-inserts (section 2.3.3), from the

library pGAD vector used in Y2H, were also sequentially double-digested with 5' and 3' enzymes shown table 2.7, in order to clone the prey-inserts into the pVP16 vector (figure 4, Appendix II) for M2H. The same protocol was followed as described in section 2.8.1 for digestion of the inserts and vectors.

### 2.8.3 Restriction enzyme digestion test

Restriction enzyme digestion tests were conducted in order to test for successful cloning of the inserts into the vectors. Initially, bacteria colony PCR-amplification (section 2.3.4) was performed on *E.coli* transformed with the bait or prey constructs (recombinant plasmids) generated as described in sections 2.7.1 and 2.7.2. Colonies that produced the correct PCR product size (section 2.3.2 and 2.3.3) were identified as colonies which harbour putative recombinant plasmids and were selected for the enzyme digestion test. This entailed digesting the recombinant plasmid with the 5', 3' and test enzymes (section 2.3.1.1 and table 2.7) in separate, parallel reactions.

The tests were performed in 20µl reactions and consisted of 2µl purified plasmid DNA, 2µl of the appropriate enzyme, 2µl enzyme buffer and 14µl ddH<sub>2</sub>O. The reactions were incubated at 37°C over night, after which they were resolved by electrophoresis on a 1% agarose gel for visualisation of digestion products. Successful cloning was indicated by linearisation of the recombinant plasmid by digestion with the 5' and 3' enzymes and an absence of digestion by the test enzyme causing the plasmid to retain a circular configuration.

The test enzyme has recognition sites in the non-recombinant plasmid but not in the *GJA5* bait-insert or prey-inserts; therefore, samples in which the plasmids were linearised by the test enzyme were identified as the results of unsuccessful cloning experiments. Different restriction enzymes were selected as indicated in table 2.7 because of the multiple cloning sites of the different cloning vectors (Appendix II).

Table 2.7 Restriction enzymes used for the bait and each of the preys

Name	5' enzyme	3' enzyme	Test enzyme
GJA5 Bait	<i>EcoRI</i>	<i>Sall</i>	<i>BamHI</i>
<b>Prey clone Nr.</b>			
29	<i>BamHI</i>	<i>MluI</i>	<i>Sall</i>
31	<i>Sall</i>	<i>XbaI</i>	<i>HindIII</i>
35	<i>EcoRI</i>	<i>MluI</i>	<i>BamHI</i>
62	<i>EcoRI</i>	<i>HindIII</i>	<i>BamHI</i>
99	<i>BamHI</i>	<i>HindIII</i>	<i>MluI</i>
153	<i>EcoRI</i>	<i>XbaI</i>	<i>Sall</i>
163	<i>Sall</i>	<i>XbaI</i>	<i>HindIII</i>
164	<i>EcoRI</i>	<i>HindIII</i>	<i>Sall</i>
204	<i>MluI</i>	<i>HindIII</i>	<i>PstI</i>
222	<i>EcoRI</i>	<i>HindIII</i>	<i>Sall</i>
306	<i>EcoRI</i>	<i>HindIII</i>	<i>Sall</i>

## 2.9 Calf intestinal alkaline phosphatase (CIP) treatment of double-digested vectors

Double-digested vectors (section 2.8.1 and 2.8.2) were treated with calf intestinal alkaline phosphatase (CIP), in order to prevent self-circularising of the vector. The reaction removes phosphates at the 5' overhang enzyme digestion sites and thereby increases ligation efficiency of the inserts and vectors. The CIP treatment was prepared in 100µl reaction volumes as follows: 50µl vector DNA (1µg), 10µl CIP buffer (Promega, Madison WI, USA), 2µl CIP enzyme (Promega, Madison WI, USA) and 38µl ddH<sub>2</sub>O. The reactions were incubated at 37°C for 15min, followed by a 15min incubation at 56°C. Another 2µl of CIP enzyme was added to the reaction and the incubation steps repeated. The samples were then purified by using the GFX® DNA purification kit (Amersham Pharmacia Biotech, New Jersey, USA) and eluted in 20µl ddH<sub>2</sub>O, prior to being used in ligation reactions.

## 2.10 Ligation reaction

DNA ligation was performed, in order to generate the Y2H bait construct (section 2.7.1) to be used in Y2H analysis and M2H bait and prey constructs (section 2.7.2) for M2H analysis. The ligations were performed in 10 $\mu$ l reaction volumes as follows: 1 $\mu$ l double-digested vector (CIP treated) (section 2.9), 3 $\mu$ l double-digested insert (section 2.8), 1 $\mu$ l T4 DNA ligase buffer (Promega, Madison WI, USA), 5U T4 DNA ligase (Promega, Madison WI, USA) and 4 $\mu$ l ddH<sub>2</sub>O. The sample was incubated for 16 hours at 4°C, following which 5 $\mu$ l of the sample was transformed into *E.coli* DH5 $\alpha$  (section 2.11.1 and Appendix IV) and plated onto LB agar plates containing the appropriate antibiotic (Appendix I). Successful ligation reactions were confirmed by bacterial colony PCR of the transformed bacteria (section 2.3.4).

## 2.11 Bacterial strain, yeast strains and cell line

### 2.11.1 Bacterial strain

In order to facilitate the selection and purification of Y2H and M2H constructs, ligation reactions (section 2.10) were transformed into *E.coli* DH5 $\alpha$  (section 2.14.1). Transformed bacterial colonies were selected on the basis of their ability to grow on LB agar plates (Appendix I) which contained selection antibiotics; kanamycin was used when selecting for pGBKT7 recombinant plasmids, and ampicillin when selecting for pGAD, pVP16 and pM recombinant plasmids. The recombinant plasmids were identified by means of colony PCR-amplification (section 2.3.4).

### 2.11.2 *S.cerevisiae* strains

The pGBKT7-*GJA5* bait construct was transformed into *S.cerevisiae* yeast strain AH109 (section 2.14.2 and Appendix IV), while the clones present in the pre-transformed

Clontech cDNA library, which was used in Y2H, had been transformed into *S.cerevisiae* yeast strain Y187 (Appendix IV) by the manufacturer.

### **2.11.3 Cell line**

A H9C2 cell line was provided by the American Type Culture Collection (ATCC). The pM-*GJA5* and pVP16-prey constructs (section 2.7.2) were co-transfected with the pG5SEAP and pSV- $\beta$ -Galactosidase reporter vectors (figures 5 and 6, Appendix II) into the cells for M2H analysis.

## **2.12 Preparation of *E.coli* DH5 $\alpha$ competent cells for bacterial transformation**

A scrape of an *E.coli* DH5 $\alpha$  frozen (-70°C) glycerol stock was inoculated into 10ml LB media (Appendix I). The culture was incubated overnight at 37°C in a YIH DER model LM-530 shaking incubator (SCILAB instrument CO. Ltd, Taipei, Taiwan) at about 200rpm. Following the incubation, a 1ml aliquot of this culture was inoculated into a 2L Ehrlemeyer flask (with cotton-wool bung and tin foil lid) containing 200ml LB media. The bacteria was given time to grow at room temperature with slow shaking at 85rpm on a Labcon orbital shaker (Labcon Pty, Ltd, Maraisburg, RSA) until mid log phase ( $OD_{600nm} = 0.4-0.6$ ). The broth was aliquoted into 4 x 50ml sterile polypropylene tubes and centrifuged at 3000rpm for 15min at 4°C in a Multitex centrifuge (MSE instruments, England). From this step onwards, everything was kept cold. The supernatant was discarded and the pellet gently resuspended in one-third volume (~16ml) of ice cold CAP buffer (Appendix I). The cells were re-pelleted by centrifugation at 3000rpm for 15min at 4°C in a Multitex centrifuge (MSE instruments, England) and the supernatant discarded. The pellet was resuspended in one-twelfth volume (~4ml) of ice cold CAP buffer and 200 $\mu$ l aliquots prepared in 2ml microfuge tubes. It was left overnight at 4°C and stored at -70°C until use.

## **2.13 Culturing of H9C2 cell line**

### **2.13.1 Culture of H9C2 cells from frozen stocks**

#### **2.13.1.1 Thawing the cells**

Frozen H9C2 cells were thawed rapidly by immersion in a 37°C waterbath (Memmert<sup>®</sup>, Schwabach, Germany) for 10min. Once the cells were thawed, the outside of the vial was immediately sterilized with 70% ethanol.

#### **2.13.1.2 Removing DMSO from stocks and culturing cells**

In order to ensure maximum viability of the cells upon plating, DMSO was removed from the thawed stocks as follows: One millilitre of growth media (Appendix I), prewarmed to 37°C was added to the thawed stock and mixed by gentle pipetting. The mixture was transferred to a 12ml Greiner tube (Greiner Bio-one, Frickenhausen, Germany) and another 5ml growth media was added. The cells were then pelleted by centrifugation at 10000rpm for 1min using a Sorval<sup>®</sup> GLC-4 General Laboratory centrifuge (Separations Scientific, Johannesburg, South Africa) followed by removal of the supernatant. The pellet was resuspended in another 5ml growth media and the cells were once again centrifuged at 10000rpm for 1 min using a Sorval<sup>®</sup> GLC-4 General Laboratory centrifuge (Separations Scientific, Johannesburg, South Africa). Following this, the cells were resuspended in 10ml growth media and transferred into a T25 culture flask. The flask was gently swirled in order to distribute the cells evenly over the growth surface of the flask. The flask was then incubated at 37°C in a Farma termostretri-cycle 5% carbon dioxide humidified incubator (Farma International, Miami, Florida, U.S.A).

#### **2.13.2 Subculturing of H9C2 cells**

H9C2 cells were subcultured every 2-4 days when they reached approximately 80%-90% confluency. Briefly, the growth media was removed from the flask as the cells were washed with sterile phosphate buffered saline (PBS) containing no calcium or

magnesium. Two millilitres of trypsin (Highveld Biological, Lyndhurst, South Africa) was added to this, in order to facilitate the detachment of the cells from the growth surface of the flask. After 3min, 5ml growth media was added and the cells were gently resuspended. The cells were then transferred into 4 flasks each containing 10ml of growth media.

## **2.14 Transformation and transfection of plasmids into *E.coli*, *S.cerevisiae*, and mammalian cells**

### **2.14.1 Bacterial plasmid transformation**

Bacterial transformation commenced by thawing competent *E.coli* DH5 $\alpha$  cell tubes (stored at -70°C) (section 2.12) undisturbed on ice for about 15-30min. One tube containing 200 $\mu$ l competent cells was used per transformation. One microlitre (1 $\mu$ g) of plasmid preparation or 3-5 $\mu$ l of ligation reaction was added to 200 $\mu$ l cells and gently mixed. The mixture was incubated on ice for 20-30min, after which it was placed in a Lasec 102 circulating water-bath (Lasec Laboratory and Scientific Company Pty Ltd, Cape Town, RSA) at 42°C for exactly 45s. The tubes were then removed from the water-bath and left at room temperature for 2min. Following this, 1ml of LB medium was added to the mixture, which was incubated for 1hr at 37°C, while shaking at 200rpm in a YIH DER model LM-530 shaking incubator (SCILAB instruments CO. Ltd, Taipei, Taiwan).

Plating of 200 $\mu$ l of the culture was done onto LB agar plates containing the appropriate antibiotic (kanamycin for pGBKT7 constructs; ampicillin for pM and pVP16 constructs). The remaining 800 $\mu$ l was centrifuged at 13rpm for 2min in a Beckman Microfuge Lite (Beckman Instruments Inc, CA, USA), the supernatant discarded and the pellet resuspended in 200 $\mu$ l LB media. This concentrated mixture was then plated onto the appropriate LB agar plates. The plates were incubated, inverted, for 16hr at 37°C in a model 329 stationary CO<sub>2</sub> incubator (Former Scientific, Marieta, Ohio, USA), after which



colonies were clearly visible and were numbered and picked for colony PCR (section 2.3.4).

### **2.14.2 *S.cerevisiae* plasmid transformations**

The appropriate *S.cerevisiae* strain (AH109 or Y187) (section 2.11.2) to be transformed was streaked, from frozen stocks at  $-70^{\circ}\text{C}$ , onto YPDA agar plates (Appendix I). The plates were incubated at  $30^{\circ}\text{C}$  for 2-3 days in a Sanyo MIR262 stationary ventilated incubator (Sanyo, Electronic Company Ltd, Ora-Gun, Japan). Next, a scrape of a single yeast colony, representing a volume of about 20-50 $\mu\text{l}$ , was resuspended in 1ml of sterile ddH<sub>2</sub>O in a sterile 2ml tube and vortexed well. The cells were repelleted by centrifugation at 13000rpm for 30 sec in a Beckman Microfuge Lite (Beckman Instruments Inc, CA, USA). The supernatant was removed and the pellet resuspended in 1ml 100mM lithium acetate (LiAc) (Appendix I) and incubated at  $30^{\circ}\text{C}$  for 5min in a Sanyo MIR262 stationary ventilated incubator (Sanyo, Electronic Company Ltd, Ora-Gun, Japan) without shaking.

Thereafter, the cells were repelleted by centrifugation at 13000rpm for 20sec, after which all the LiAc was removed from the pellet. The following was added on top of the pellet in this order: 240 $\mu\text{l}$  of 50% polyethylene glycol (PEG), 36 $\mu\text{l}$  1M LiAc, 25 $\mu\text{l}$  of 2mg/ml heat-denatured and snap-cooled sonicated herring sperm DNA (Promega, Madison WI, USA), 10-20 $\mu\text{l}$  plasmid preparation, and ddH<sub>2</sub>O to a final volume of 350 $\mu\text{l}$ . The sample was then mixed by vortexing using a Snijders model 34524 press-to-mix vortex (Snijders Scientific, Tilburg, Holland) for 1min and incubated at  $42^{\circ}\text{C}$  for 20-30min in a Lasec 102 circulating waterbath (Lasec Laboratory and Scientific Company Pty Ltd, Cape Town, RSA). After the incubation, the cells were pelleted by centrifugation at 13000rpm and all the supernatant removed. The cells were then resuspended in 250 $\mu\text{l}$  ddH<sub>2</sub>O, of which about 150 $\mu\text{l}$  was plated, by means of sterile glass beads, onto the appropriate selection plates (Appendix I). The plates were incubated, inverted, at  $30^{\circ}\text{C}$  for 2-5 days in a Sanyo MIR262 stationary ventilated incubator (Sanyo, Electronic Company Ltd, Ora-Gun, Japan).

### 2.14.3 Transfection of H9C2 cells

Approximately  $1-3 \times 10^4$  H9C2 cells per well in a 24-well tissue culture plate (Whitehead Scientific (Pty) Ltd.) were plated in complete growth media (Appendix I) forty-eight hours before transfecting the cells, and allowed to incubated at 37°C in a 5% Farma-thermosteri-cycle carbon dioxide humidified incubator (Farma, International, Miami, Florida, U.S.A). Two days later, the cells were visualised under a Nikon TMS light microscope (Nikon, Tokyo, Japan) to determine the level of confluence. Cells were only transfected once they reached approximately 80% confluence. For each transfection performed, 100µl of serum-free medium was aliquoted into a sterile 1.5ml eppendorf microfuge tube. Three microlitres GeneJuice® (EMD Biosciences, Darmstadt, Germany) were added to each tube. This mixture was thoroughly vortexed using a Snijders model 34524 press-to-mix vortex (Snijders Scientific, Tilburg, Holland) and incubated at room temperature for 5 minutes.

A total of 1µg of the four plasmids combined (the bait, prey and two reporter vectors) was added to the mixture and mixed gently by pipetting. These plasmids were the pM-GJA5 bait construct and one of the pVP16-prey constructs, which were generated for M2H (section 2.7.2), and two reporter vectors, namely, pG5SEAP and pSV-β-Gal (figures 5 and 6, Appendix IV). Shown in table 2.8 is the protocol followed in assessing the bait and prey interactions. A number of controls, which will be described in section 2.21, were included, in order to verify the efficiency of the reporter assay.

The GeneJuice/DNA/medium was incubated at room temperature for 15min. The entire volume of the mixture was then added drop-wise to the cells in the growth media. The culture plates were gently rocked back and forth in order to evenly distribute the drops across the surface of the plate. The cells were incubated at 37°C, in a 5% carbon dioxide humidified incubator for 48 hours. The cells were subsequently pelleted by centrifugation at 13000rpm, thereafter, 1ml of the supernatant were transferred into a sterile microfuge tube. The supernatants were stored at -20°C until needed for reporter assays (section 2.21.1).

Table 2.8 Protocol for assessing the *GJA5* bait and prey interactions

Category	Transfection (5:5:1:1)	GAL4 DNA-BD pM ( $\mu$ l)	GAL4 DNA-AD pVP16 ( $\mu$ l)	Reporter plasmid pG5SEAP ( $\mu$ l)	Reporter plasmid pSV $\beta$ -Gal ( $\mu$ l)
A	Experimental 1	pM-GJA5 (1.38)	pVP16-NDUFA6 (8.33)	pG5SEAP	pSV- $\beta$ -Gal
	Experimental 2	pM-GJA5	pVP16-PSAP (0.9)	pG5SEAP	pSV- $\beta$ -Gal
	Experimental 3	pM-GJA5	pVP16-ALDH2 (8.33)	pG5SEAP	pSV- $\beta$ -Gal
	Experimental 4	pM-GJA5	pVP16-ACTB (8.33)	pG5SEAP	pSV- $\beta$ -Gal
	Experimental 5	pM-GJA5	pVP16-FLNA (2.1)	pG5SEAP	pSV- $\beta$ -Gal
	Experimental 6	pM-GJA5	pVP16-VDAC2 (1.38)	pG5SEAP	pSV- $\beta$ -Gal
	Experimental 7	pM-GJA5	pVP16-MYH7 (1.38)	pG5SEAP	pSV- $\beta$ -Gal
	Experimental 8	pM-GJA5	pVP16-MCOLN1 (2.1)	pG5SEAP	pSV- $\beta$ -Gal
	Experimental 9	pM-GJA5	pVP16-DHX30 (2.1)	pG5SEAP	pSV- $\beta$ -Gal
	Experimental 10	pM-GJA5	pVP16-CK (1.38)	pG5SEAP	pSV- $\beta$ -Gal
	Experimental 11	pM-GJA5	pVP16-CRP2 (2.1)	pG5SEAP	pSV- $\beta$ -Gal
B1	Untransfected control Negative control (1)	None	None	None	None
B2	GeneJuice control Negative control (2)	None	None	None	None
C	Basal Control	pM (8.33)	pVP16 (1.04)	pG5SEAP	pSV- $\beta$ -Gal
D	GAL4 BD control	pM-GJA5	pVP16	pG5SEAP	pSV- $\beta$ -Gal
E	VP16 AD control (1)	pM	pVP16-NDUFA6	pG5SEAP	pSV- $\beta$ -Gal
	VP16 AD control (2)	pM	pVP16-PSAP	pG5SEAP	pSV- $\beta$ -Gal
	VP16 AD control (3)	pM	pVP16-ALDH2	pG5SEAP	pSV- $\beta$ -Gal
	VP16 AD control (4)	pM	pVP16-ACTB	pG5SEAP	pSV- $\beta$ -Gal
	VP16 AD control (5)	pM	pVP16-FLNA	pG5SEAP	pSV- $\beta$ -Gal
	VP16 AD control (6)	pM	pVP16-VDAC2	pG5SEAP	pSV- $\beta$ -Gal
	VP16 AD control (7)	pM	pVP16-MYH7	pG5SEAP	pSV- $\beta$ -Gal
	VP16 AD control (8)	pM	pVP16-MCOLN1	pG5SEAP	pSV- $\beta$ -Gal
	VP16 AD control (9)	pM	pVP16-DHX30	pG5SEAP	pSV- $\beta$ -Gal
	VP16 AD control (10)	pM	pVP16-CK	pG5SEAP	pSV- $\beta$ -Gal
	VP16 AD control (11)	pM	pVP16-CRP2	pG5SEAP	pSV- $\beta$ -Gal
F1	Positive control (1)	pM3-VP16 (1.66)	None	pG5SEAP	pSV- $\beta$ -Gal
F2	Positive control (2)	pM53 (2.1)	pVP16-T (2.1)	pG5SEAP	pSV- $\beta$ -Gal
G1	Negative control (3)	None	None	pG5SEAP	None
G2	Negative control (4)	None	None	None	pSV- $\beta$ -Gal

The ratio of pM:pVP16:pG5SEAP:pSV $\beta$ -Gal used in the transfections were 5:5:1:1. Abbreviations: DNA-BD: DNA binding domain, DNA-AD: DNA activation domain. Category A: bait and prey interaction experiment; B1/2: negative controls 1 and 2; C: basal control; D: binding domain control; E: prey control; F1/2: positive controls; G1/2: negative controls 3 and 4.

## **2.15 DNA and plasmid purification**

The standard methods of purifying plasmid DNA from *E.coli* (section 2.15.1) and *S.cerevisiae* (section 2.15.2) produced samples that needed further purification for subsequent specific uses. The GFX cleanup system (section 2.15.3) was employed for the purification of both PCR products and of plasmids; however, this system were insufficient for cleaning samples for downstream cloning purposes. Therefore, the ammonium acetate precipitation method (section 2.15.4) was applied when purifying DNA for cloning purposes. The purification of DNA by means of the PureYield™ plasmid midiprep system (section 2.15.5) was employed for the purification of plasmid DNA from *E.coli* for use in the transfection (section 2.13.3) of H9C2 cells for M2H. This method removed endotoxins which are harmful for mammalian cells.

### **2.15.1 Bacterial plasmid purification**

One colony of *E.coli* containing the plasmid of interest was picked from an appropriate selection plate and inoculated into 10ml LB media, supplemented with the correct antibiotic, in a 50ml polypropylene tube. The culture was incubated at 37C overnight, while shaking at 250rpm in a YIH DER model LM-530 shaking incubator (SCILAB Instruments co. Ltd, Taipei, Taiwan). The following day, freezer cultures were prepared by adding an equal volume of 50% autoclaved glycerol (Appendix I) to ~ 1ml of overnight culture and freezing them at -70C. The remaining overnight culture was centrifuged in a Beckman model TJ-6 centrifuge (Beckman Coulter, Scotland, UK) for 10min at 3000rpm after which the supernatant was discarded and the pellet resuspended in 1ml cell resuspension solution (Appendix I). Two millilitres of cell lysis buffer (Appendix I) was added and mixed by gentle inversion, followed by incubation at room temperature for 5min. Thereafter, 2ml of neutralisation solution (Appendix I) was added to the mixture, which was gently inverted, and incubated at room temperature for another 5min.

To this was added 5ml of Phenol:Chloroform:Isoamylalcohol (25:24:1 [PCI]) (Sigma, St Louis, MO, USA) and mixed well by inversion, after which it was centrifuged in a Multex centrifuge (MSE instrumentation, England, UK) for 15min at 3000rpm, in order to allow for phase separation. The upper clear plasmid-containing (aqueous) phase was transferred into a new 50ml polypropylene tube. Approximately 0.6-0.7x volume 100% isopropanol (Merck, Darmstadt Germany) was added to the tube, which was mixed well by gentle inversion. This was then centrifuged in a Multex centrifuge (MSE instrumentation, England, UK) for 45min at 3000rpm, after which the isopropanol was poured off the white pellet at the bottom of the tubes. The pellet was then carefully washed twice with 2ml ice cold 70% ethanol and allowed to air dry. The pellet was resuspended in 100-200 $\mu$ l ddH<sub>2</sub>O and subsequently 3 $\mu$ l of the plasmid preparation was resolved by electrophoresis on a 1% agarose gel for verification of integrity and purity of the plasmid.

### **2.15.2 *S.cerevisiae* plasmid purification**

The ‘Smash and Grab’ plasmid isolation method was used, in order to purify *S.cerevisiae* plasmids. A *S.cerevisiae* plate culture containing the plasmid of interest was inoculated into 1ml synthetic dropout (SD) medium containing the appropriate dropout supplement (Appendix I) (BD Bioscience, Clontech, Paulo Alto, CA, USA) in a 15ml polypropylene tube, and incubated overnight at 30°C, while shaking at 250rpm in a YIH DER model LM-530 shaking incubator (SCILAB instruments CO. Ltd, Taipei, Taiwan). Four millilitres YPDA (Appendix I) was added the next day and the *S.cerevisiae* allowed to grow for another 4 hours at 30°C with shaking. Following this, it was centrifuged at 14000rpm for 5min in a Beckman model TJ-6 centrifuge (Beckman Coulter, Scotland, UK), and the supernatant discarded. The pellet was resuspended in the remaining supernatant and then transferred to 2ml Eppendorf microfuge tubes.

The following was added to the suspension: 200 $\mu$ l ‘Smash-and-Grab’ buffer (Appendix I), 200 $\mu$ l PCI, and 0.3g sterile 450-600 $\mu$ m glass beads. This mixture was vortexed by using a Snijders model 34524 press-to-mix vortex (Snijders Scientific, Tilburg, Holland)

for 2.5min and centrifuged for 5min at top speed for phase separation in a Beckman Microfuge Lite (Beckman Instruments Inc, Ca, USA) centrifuge. Thereafter, the aqueous layer was transferred to new 1.5ml microfuge tubes. The plasmids were cleaned by adding 400µl 100% isopropanol and centrifuging for 30-45min, after which the supernatant was decanted and 400µl of ice cold 70% ethanol added. The mixture was centrifuged for 10min at top speed, after which the ethanol was decanted and the wash repeated. The plasmids were resuspended in 100 µl ddH<sub>2</sub>O.

### **2.15.3 Purification of DNA by means of GFX cleanup**

The GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech, New Jersey, USA) was used to purify samples such as PCR products and plasmids. The method involved adding 500µl of capture buffer (Amersham Pharmacia Biotech, New Jersey, USA) to a GFX column that have been placed in a collection tube. The product to be purified was then transferred to the GFX column and mixed thoroughly with the buffer. It was then centrifuged for 30s at full speed in a Beckman Microfuge Lite (Beckman Instruments Inc, Ca, USA) centrifuge. The flow-through was discarded and 500ul of wash buffer (Amersham Pharmacia Biotech, New Jersey, USA) added to the GFX column. It was again centrifuged for 30s at full speed. The collection tube was discarded and the GFX column transferred to a fresh 1.5ml microfuge tube. Fifty microlitres of ddH<sub>2</sub>O was directly added to the top of the glass fibre matrix in the GFX column and left to stand at room temperature for 1min. It was then centrifuged for 1min at full speed in order to recover the purified DNA.

### **2.15.4 Purification of DNA by means of Ammonium Acetate (NH<sub>4</sub>Ac) precipitation**

The ammonium acetate (NH<sub>4</sub>Ac) precipitation method was used, in order to purify PCR products and plasmid preparations. To the initial volume of 100µl of DNA was added the following: 50µl 7,5M NH<sub>4</sub>Ac (Appendix I), 300µl 100% ethanol and 1µl glycogen

(20mg/ml), after which the mixture was centrifuged for 1 min at full speed in a Beckman Microfuge Lite (Beckman Instruments Inc, Ca, USA) centrifuge. Next, the supernatant was discarded and 1ml of 70% ethanol added to the pellet. This was centrifuged for 10 min at full speed, after which the supernatant was discarded and the pellet allowed to air dry, in order to remove remaining ethanol. The pellet was then resuspended in 50µl of ddH<sub>2</sub>O.

### **2.15.5 Purification of DNA by means of the PureYield™ plasmid midiprep system**

An overnight culture was prepared by inoculating 100µl of the *E.coli* freezer culture containing a prey plasmid of interest (section 2.7.2) into 20ml of LB medium (Appendix I) supplemented with ampicillin antibiotic, in a 50ml polypropylene tube. The culture was incubated at 37°C overnight, while shaking at 250rpm in a YIH DER model LM-530 shaking incubator (SCILAB instruments CO. Ltd, Taipei, Taiwan). The overnight culture was centrifuged the following day, in a Beckman model TJ-6 centrifuge (Beckman Coulter, Scotland, UK) for 10min at 3000rpm, after which the supernatant was discarded and the pellet resuspended in 2ml cell resuspension solution (Promega, Madison WI, USA). Two millilitres of cell lysis buffer (Promega, Madison WI, USA) was added, mixed by gentle inversion, and incubated at room temperature for 3min. Following this step, 3.3ml of neutralisation solution (Promega, Madison WI, USA) was added to the mixture which was mixed by gentle inversion, after which it was incubated at room temperature for 2-3min.

Next, a blue PureYield™ clearing column (Promega, Madison WI, USA) was placed into a new 50ml polypropylene tube. The lysate was poured into the clearing column and incubated for 2min to allow the debris to rise, after which it was centrifuged at 1,500 x g for 5min. Following, a white PureYield™ binding column (Promega, Madison WI, USA) was placed into a new 50ml polypropylene tube and the lysate was poured into this column. It was centrifuged at 1,500 x g for 3min, the flow-through discarded and 5ml of endotoxin removal wash (Promega, Madison WI, USA) added to the binding column.

This was again centrifuged for 3min at 1,500 x g. The flow-through was discarded and 20ml of column wash solution (Promega, Madison WI, USA) added to the binding column, which was centrifuged for 5min at 1,500 x g. Another centrifugation step followed, in order to ensure the removal of ethanol. Next, 600ul of nuclease-free water (Promega, Madison WI, USA) was added to the binding column in a new 50ml tube and centrifuged at 1,500 x g for 5min to recover the purified DNA. The flow-through was transferred to a 1.5ml microfuge tube and 5µl was resolved by electrophoresis on a 1% agarose gel for verification of integrity and purity of the plasmid preparation.

## 2.16 Verification of integrity of the Y2H construct

### 2.16.1 Phenotypic assessment of the *S.cerevisiae* strains

Each of the *S.cerevisiae* strains, AH109 and Y187, used in the Y2H analysis was assessed phenotypically before being used for transformations. *S.cerevisiae* strains, AH109 and Y187, have deficiencies for the adenine (*Ade*), histidine (*His*), leucine (*Leu*) and tryptophan (*Trp*) genes, except for the uracil (*Ura*) gene, as indicated in appendix IV. Therefore, it was possible to assess the phenotype of the strains by plating them onto agar plates lacking essential amino acids as shown in table 2.9, namely, agar plates SD<sup>-Ade</sup>, SD<sup>-Trp</sup> SD<sup>-His</sup>, SD<sup>-Leu</sup> and SD<sup>-Ura</sup>. *S.cerevisiae* strains, AH109 and Y187, that were unable to grown on SD<sup>-Ade</sup>, SD<sup>-Trp</sup>, SD<sup>-His</sup>, SD<sup>-Leu</sup> and able to grow on SD<sup>-Ura</sup> were used for transformation and subsequent Y2H analysis.

Table 2.9 Phenotypic assessment of *S.cerevisiae* strains on specific SD selection media

Strain	Growth on agar plates				
	SD/-Ade	SD/-His	SD/-Leu	SD/-Trp	SD/-Ura
AH109	-	-	-	-	+
Y187	-	-	-	-	+

SD: synthetic dropout; - = no growth; + = growth

### 2.16.2 Test for autonomous reporter gene activation

*S.cerevisiae* AH109 transformed with the pGBKT7-*GJA5* bait construct (section 2.7.1) was tested for the ability of this plasmid to autonomously activate the *ADE2* and *HIS3* reporter genes. The reporter genes are used in selection for bait and prey interactions and



false interactions would have been detected in Y2H if the reporter genes were autonomously activated by the pGBKT7-*GJA5* bait construct. The test was done by streaking the transformed and un-transformed *S.cerevisiae* AH109 onto agar plates lacking essential amino acids as shown in table 2.10, namely, agar plates SD<sup>-Ade</sup>, SD<sup>-Trp</sup>, SD<sup>-His</sup>, SD<sup>-Leu</sup> and SD<sup>-Ura</sup>. Autoactivation of the reporter genes by pGBKT7-*GJA5* would have been indicated by growth on the SD<sup>-Ade</sup> and SD<sup>-His</sup> plates. Therefore, the desired result is lack of growth of the transformed *S.cerevisiae* AH109 on these plates (table 2.10). Growth on SD<sup>-Trp</sup> indicates the presence of the selection gene in the pGBKT7-*GJA5* bait construct and growth on SD<sup>-Ura</sup> and lack thereof on SD<sup>-Leu</sup> confirms the phenotype of *S.cerevisiae* AH109 as assessed in section 2.16.1.

Table 2.10 Test for autonomous reporter gene activation by the Y2H bait construct

Strain	Growth on agar plates				
	SD/-Ade	SD/-His	SD/-Leu	SD/-Trp	SD/-Ura
AH109	-	-	-	-	+
AH109 transformed with pGBKT7- <i>GJA5</i>	-	-	-	+	+

### 2.16.3 Test for toxicity of the bait protein for the transformed *S.cerevisiae*

The test for toxicity of the pGBKT7-*GJA5* bait construct (section 2.7.1) on the host *S.cerevisiae* AH109 was done by comparing the growth rate in liquid culture of three categories of *S.cerevisiae* AH109: *S.cerevisiae* AH109 (pGBKT7-*GJA5*), *S.cerevisiae* AH109 (pGBKT7 {a non-recombinant vector control}) and un-transformed *S.cerevisiae* AH109. Diminished growth of *S.cerevisiae* (pGBKT7-*GJA5*), in comparison with the un-transformed *S.cerevisiae* AH109, would indicate that the bait protein negatively influences the growth of host *S.cerevisiae* AH109 and that it is toxic to host *S.cerevisiae* AH109.

The toxicity test commenced by plating the three above mentioned categories of *S.cerevisiae* AH109 onto SD<sup>-Trp</sup> plates and allowing them to grow at 30°C in a Sanyo MIR262 stationary ventilated incubator (Sanyo, Electronic Company Ltd, Ora-Gun,

Japan) for about 4 days. Thereafter, overnight cultures were prepared by inoculating a yeast colony of each into separate 50ml polypropylene tubes containing 10ml of SD<sup>-Trp</sup> medium (Appendix I) and thereafter incubation at 30°C for 24hr in a YIH DER model LM-530 shaking incubator (SCILAB instrument CO. Ltd, Taipei, Taiwan) with shaking at 200rpm. The following day, 1ml of the overnight culture was inoculated into 50ml SD<sup>-Trp</sup> medium and 50ml YPDA medium in autoclaved Erlenmeyer flasks and incubated overnight at 30°C while shaking at 200rpm. Every 2hrs, over a period of 8 hrs, a 1ml aliquot of the culture was taken and its OD<sub>600</sub> was measured. An overnight (24hr) reading was also taken. A linearised graph of the log of these OD<sub>600</sub> readings versus time was constructed using the PRISM computer program, and the slopes of the graphs generated for each of the three categories of *S.cerevisiae* AH109 were compared, in order to determine whether the bait protein was toxic for *S.cerevisiae* AH109.

#### **2.16.4 Testing the mating efficiency**

In order to test whether the pGBKT7-*GJA5* bait construct (section 2.7.1) decreases the mating efficiency of the host *S.cerevisiae* AH109, a mating efficiency test was conducted. Decreased mating efficiency would cause a reduction in the number of pretransformed library clones screened in screening the Y2H library. Two categories of matings, namely, bait mating and compatible control mating (table 2.11) were performed, in order to test the mating efficiency.

The bait mating involved mating *S.cerevisiae* AH109 (pGBKT7-*GJA5*) with the prey host *S.cerevisiae* Y187 (pTD1.1 control prey vector), supplied by the manufacturer (BD Bioscience, Clontech, Paulo Alto, CA, USA). An efficiency of equal to or higher than the minimum of 2% recommended by the manufacturer of the MATCHMAKER Y2H system would indicate that the bait construct did not negatively influence the mating efficiency of *S.cerevisiae* AH109 and that it would result in screening an appropriate number of about 10<sup>6</sup> library clones in Y2H. Compatible control matings were also performed in which the *S.cerevisiae* AH109 (pGBKT7-53 control bait vector), supplied by the

manufacturer (BD Bioscience, Clontech, Paulo Alto, CA, USA), was mated with the prey host *S.cerevisiae* Y187 (pTD1.1 control prey vector).

To this end, each of the *S.cerevisiae* strains was plated onto the appropriate SD plates (pGBKT7-*GJA5* and pGBKT7-53 on SD<sup>-Trp</sup> plates; pTD1.1 on SD<sup>-leu</sup> plates) and incubated at 30°C for 2-5 days in a Sanyo MIR262 stationary ventilated incubator (Sanyo, Electronic Company Ltd, Ora-Gun, Japan). Thereafter, the mating experiment was performed by inoculating a scrape of a colony of the pTD1.1 prey into 1ml YPDA medium (Appendix I) in two 2ml microfuge tubes. In addition, a scrape of a single colony of the pGBKT7-*GJA5* bait was inoculated into one of these tubes and a single colony of pGBKT7-53 into the remaining tube. This procedure was to produce a bait mating and a compatible control mating as indicated in table 2.11.

The matings were incubated overnight at 30°C while shaking at 200rpm. Thereafter, serial dilutions (1:10; 1:100 and 1:1000) of the mating cultures were plated onto SD<sup>-Trp</sup>, SD<sup>-Leu</sup>, SD<sup>-Trp/-Leu</sup> agar plates and incubated for 4-5 days at 30°C in a Sanyo MIR262 stationary ventilated incubator (Sanyo, Electronic Company Ltd, Ora-Gun, Japan). Following the incubation period, the colonies on each plate was counted and used to calculate the mating efficiency (Appendix III).

Table 2.11 Mating efficiency test of *S.cerevisiae* AH109 (pGBKT7-*GJA5*)

Category	Mating
Bait mating	pGBKT7- <i>GJA5</i> (bait) X pTD1.1 (prey)
Compatible control mating	pGBKT7-53 (bait) X pTD1.1 (prey)

## 2.17 Yeast-Two-Hybrid analysis

### 2.17.1 Principle of the Y2H method

The principle of the Y2H method involves the manipulation of the GAL4 transcription activator complex. Normal transcription, illustrated in figure 2.3A, involves the joining together of the GAL4 DNA-binding domain (BD) and the GAL4 DNA-activation domain

(AD) to form a transcription activator complex. This complex then binds to the promoter region in order to activate transcription of the genes downstream of the promoter.

In the Y2H system, illustrated in figure 2.3B, the two domains do not interact directly in order to activate transcription; rather, the complex interacts indirectly via other proteins fused to their domains (i.e. bait fusion protein or prey fusion protein), which functions in interactions in order to form the active GAL4 transcription complex. Therefore, transcription of the genes downstream of the promoter will only occur when the fusion proteins interact in order to form the active transcription complex. The genes transcribed are used as indicators of protein-protein interactions and are made use of in the screening and selection assays in order to select for true protein-protein interactions. In the Y2H system the *ADE2*, *HIS3*, and *MEL1* reporter genes are used as indicators of protein-protein interactions.

In the present study, the C-terminus of *Cx40* was used as the bait fragment to be fused with the GAL4 DNA-BD in generating the bait construct which was subsequently transformed into *S.cerevisiae* AH109. The GAL4 DNA-AD fusion proteins were part of the Clontech, commercially available pretransformed cardiac cDNA library which was transformed into *S.cerevisiae* Y187. During the Y2H analysis, these two *S.cerevisiae* strains were mated with each other in order to produce a progeny of diploid *S.cerevisiae* cells in which the two fusion proteins occur in order to form the transcription complex. The progeny of *S.cerevisiae* colonies were subsequently subjected to screening and selection assays, such as nutritional selection stages, X- $\alpha$ -galactosidase assay and heterologous mating, in order to select for colonies (i.e. prey clones) in which a true protein-protein interaction occurred between the *GJA5* bait protein and the library prey protein (i.e. ligand). After selecting the prey clones, their inserts were sequenced and subjected to BLAST-search and internet database literature searches in order to identify the prey and to determine the plausibility of the bait and prey protein interactions. Following, the selected prey clones were subjected to M2H analysis in order to verify their interactions with the C-terminus of *Cx40*.

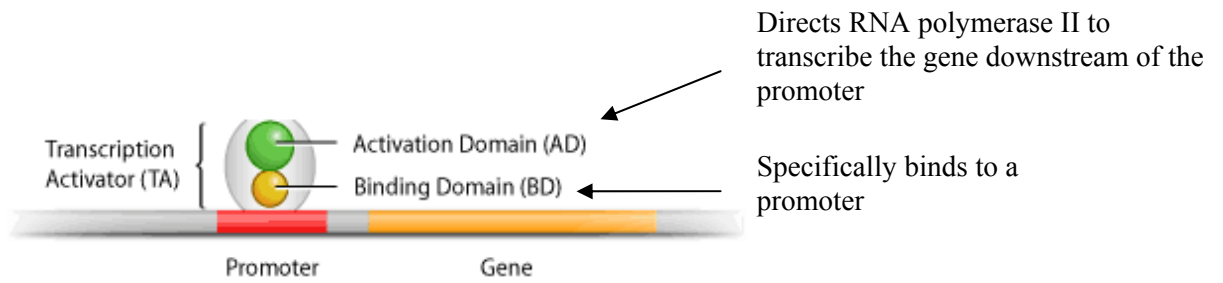


Figure 2.3A Mechanism of normal transcription (Sobhanifar, 2003)

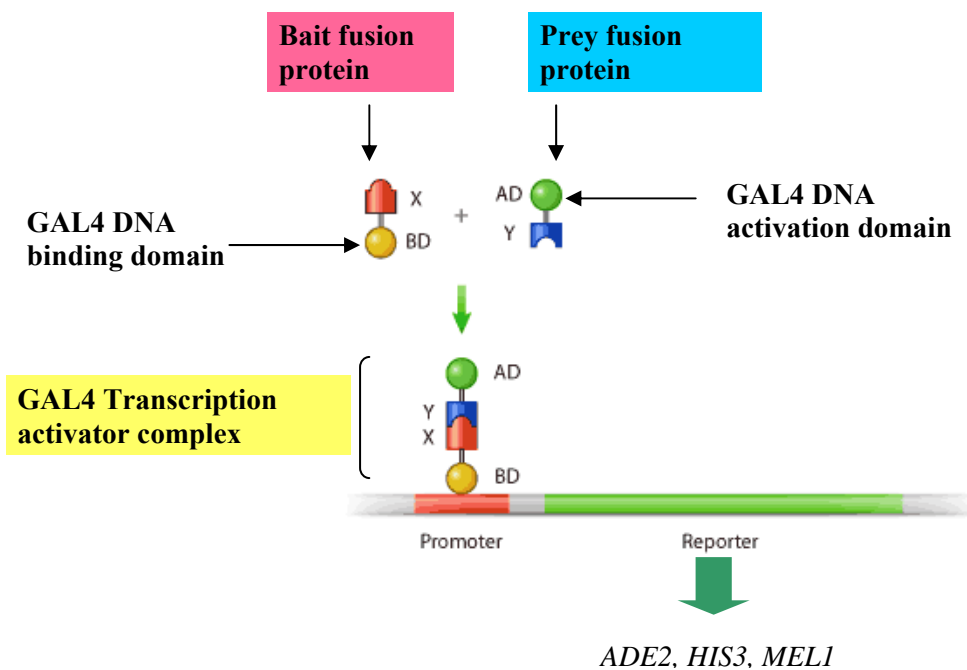
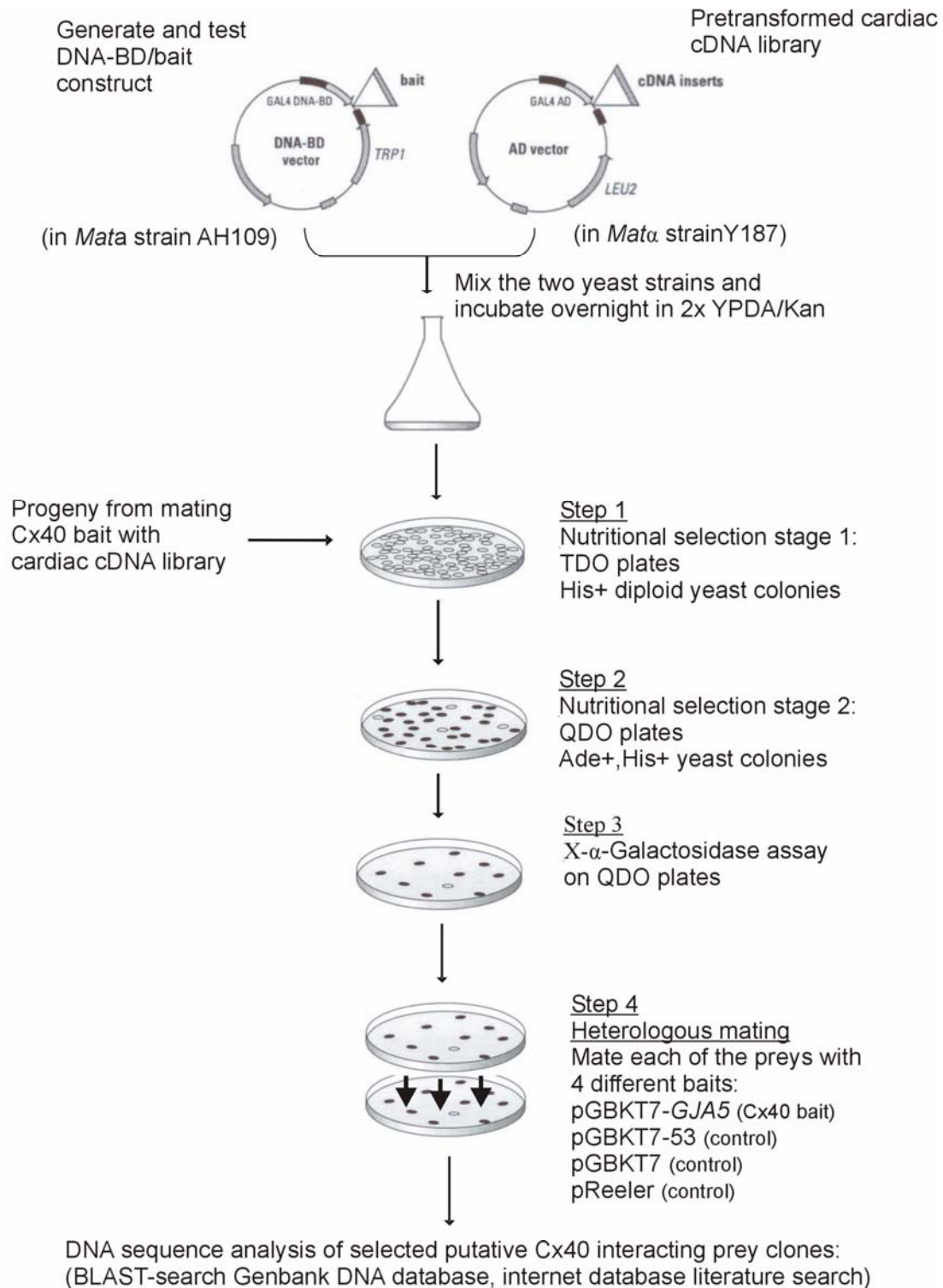


Figure 2.3B Principle of the Y2H method (Sobhanifar, 2003)



**Figure 2.4 Illustration of the screening and selection methods used in identifying true protein-protein interactions.** Abbreviations: TDO: solid media lacking Leu, Trp and His; QDO: solid media lacking Leu, Trp, His and Ade; YPDA: a blend of yeast extract, peptone and dextrose supplemented with adenine; Kan: kanamycin (Adapted from the BD Matchmaker™ Pretransformed libraries user manual).

### 2.17.2 The cardiac cDNA library

A pre-transformed human MATCHMAKER cardiac cDNA library (BD Bioscience, Clontech, Paulo Alto, CA, USA) consisting of *S.cerevisiae* Y187 transformed with a cardiac cDNA library, constructed in pGADT7-Rec (figure 2, Appendix II), was used in the Y2H analysis. The library was constructed from a pool of 3 male caucasians; ages 33, 55 and 55 years. Their cause of death was trauma (BD Bioscience, Clontech, Paulo Alto, CA, USA). The library contains approximately  $1.15 \times 10^7$  independent clones cloned into the pGADT7-Rec vector through *EcoRI* and *XhoI* sites. The average insert size for this library is 2.0kb, which ranges between 0.5 to  $\geq 3.0$ kb.

### 2.17.3 Establishment of the bait culture

*S.cerevisiae* AH109 (pGBKT7-GJA5) (section 2.7.1) was streaked onto SD<sup>-Trp</sup> plates and allowed to grow at 30°C for 4 days. Following the incubation, overnight cultures were prepared by inoculating 1ml of SD<sup>-Trp(+Ade)</sup> medium (Appendix I) with the resultant *S.cerevisiae* colonies, which was incubated at 30°C while shaking at 200rpm in a YIH DER model LM-530 shaking incubator (SCILAB instrument CO. Ltd, Taipei, Taiwan). The next day, 250µl of the overnight culture was inoculated into four separate 500ml Ehrlenmeyer flasks, each containing 50ml SD<sup>-Trp(+Ade)</sup> and incubated overnight at 30°C while shaking at 200rpm. Four separate bait cultures were produced, in order to facilitate the pooling of the initial cultures, thereby allowing the generation of a final bait culture with a titre of about  $1 \times 10^{10}$ , i.e., 100-fold excess of bait to prey to facilitate high mating efficiency. The next day, the cultures were transferred into individual 50ml polypropylene tubes and the cells pelleted by centrifugation at 3000rpm for 10min, at room temperature, in a Beckman Microfuge Lite (Beckman Instruments Inc., CA, USA). Most of the supernatants were discarded with the remaining being used to resuspend the pellets. The four suspensions (bait cultures) were combined and transferred to a new 50ml polypropylene tube after which OD<sub>600</sub> readings were taken.

### 2.17.4 Haemocytometric cell count

Haemocytometric cell count was done using a Neubauer Haemocytometer, in order to determine the titre of bait culture used in the library mating experiment. One microliter of the bait culture (section 2.17.3) was used to prepare a 1:1000 dilution. Before using the haemocytometer, a glass coverslip was placed over the counting surface (figure 2.5A). About 2 $\mu$ l of the dilution of bait culture was pipetted into one of the V-shaped wells. This allowed for the area under the coverslip to be filled with the sample through capillary action. The counting chamber was then placed on a microscope (Nippon kogaku K.K, Nikon, Japan) stage and the counting area brought into focus in order to do a cell count. The organisation of the counting area is shown in figure 2.5B and C. The number of cells per milliliter was determined as follows: The number of cells was counted in the squares marked with the shaded boxes as indicated in figure 2.5C. The number of cells was then multiplied with the number of blocks (17) to give an average number of cells within the large central quadrant. The following formula was used to determine the number of cells per millilitre:

Number of cells/ml = number of cells x dilution factor x  $10^4$  (the constant is used because the depth of the haemocytometer is 0.1mm).

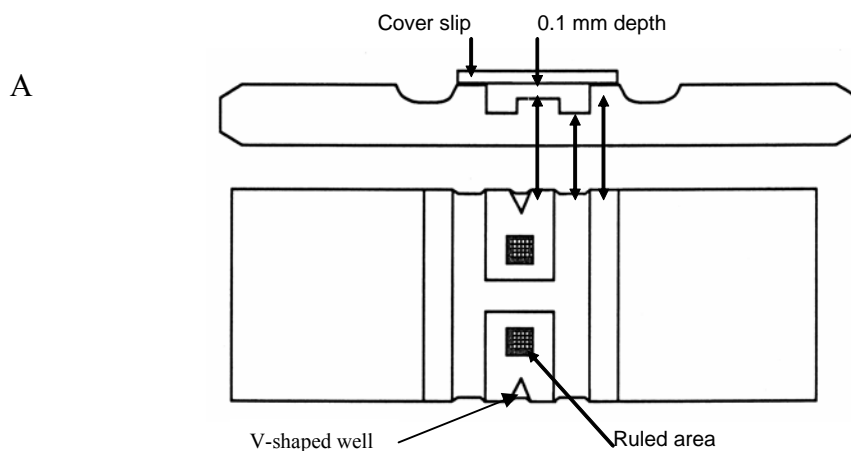
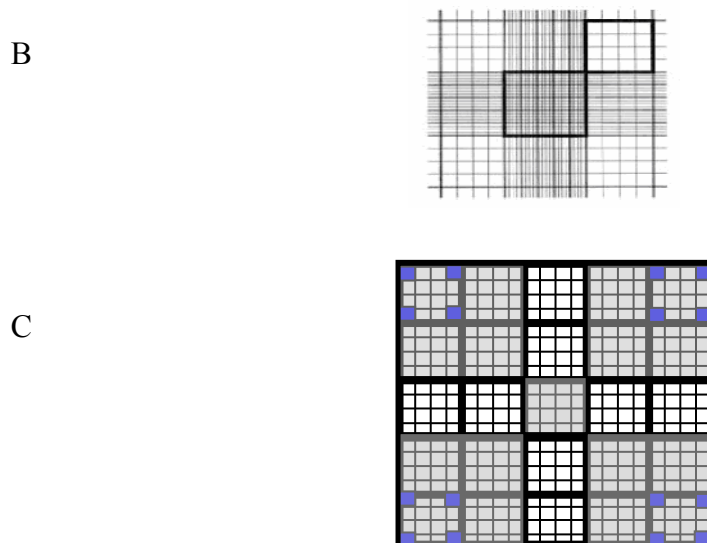


Figure 2.5 Continues on next page





**Figure 2.5 Representation of the Neubauer Haemocytometer.** (A) Neubauer haemocytometer side and top view. The central platform contains the ruled counting area and is 0.1mm under the cover slip, which is suspended on the raised ridges. (B) Magnified view of the ruled counting area. (C) View of the central quadrant of the haemocytometer that was used to determine the number of cells per millilitre (taken from McNeel and Brown, 1992).

### 2.17.5 The library mating

An aliquot (1ml) of the pre-transformed cardiac cDNA library culture was removed from the  $-70^{\circ}\text{C}$  freezer and thawed at room temperature (BD Bioscience, Clontech, Paulo Alto, CA, USA), after which it was vortexed using a Snijders model 34524 press-to-mix vortex (Snijders Scientific, Tilburg, Holland). Ten microliters of the culture was aliquoted into a  $1.5\mu\text{l}$  microfuge tube for library titering. The bait culture (section 2.17.3) was resuspended in 45ml 2x YPDA medium (Appendix I) supplemented with  $10\mu\text{g/ml}$  kanamycin in a 2L Ehrlenmeyer flask. The remaining  $990\mu\text{l}$  of library culture were added to the bait culture in the Ehrlenmeyer flask. This mating culture was then incubated at  $30^{\circ}\text{C}$  overnight, while shaking at 40rpm in a YIH DER model LM-530 shaking incubator (SCILAB instrument CO. Ltd, Taipei, Taiwan).

Following the overnight incubation, the mating culture was transferred to a sterile 50ml polypropylene tube and the cells pelleted by centrifugation at 3000rpm for 5min in a Multex centrifuge (MSE instrumentation, England, UK). Thereafter, the supernatant was removed. The Ehrlenmeyer flask, in which the mating was performed, was rinsed twice

with 40ml 2x YPDA containing 10µg/ml kanamycin. Each time the flask was rinsed, the 2x YPDA medium was used to resuspend the cell pellet and the cells then re-pelleted by centrifugation at 3000rpm for 10min at room temperature. Following the final centrifugation step, the supernatant was removed and the pellet resuspended in 10ml 0.5x YPDA containing 10µg/ml kanamycin (Appendix I).

Serial dilutions of 50µl aliquots (1:10, 1:100, 1:1000, 1:10 000) of the cell suspension were plated onto 90mm SD<sup>-Trp</sup>, SD<sup>-Leu</sup> and SD<sup>-Trp/-Leu</sup> agar plates to determine the mating efficiency and the number of clones screened. These plates were incubated, inverted, at 30°C for 4 days. Two hundred microlitre aliquots of the remaining mating culture were plated onto 140mm TDO plates (Appendix I) [200µl culture/plate for 13ml culture = 65 plates]. The TDO plates were incubated, inverted, at 30°C for 2 weeks in a Sanyo MIR262 stationary ventilated incubator (Sanyo, Electronic Company Ltd, Ora-Gun, Japan).

### **2.17.6 Establishing a library titre**

The 10µl aliquot of library culture was mixed with 1ml of 0.5x YPDA containing 10µg/ml kanamycin, in a sterile 1.5ml microfuge tube and vortexed using a Snijders model 34524 press-to-mix vortex (Snijders Scientific, Tilburg, Holland). This mixture (A) was used to prepare mixtures B and C. To prepare B, 10µl of A was added to 1ml of 0.5x YPDA containing 10µg/ml kanamycin, in a sterile 1.5ml microfuge tube and vortexed. To prepare C, another 10µl of A was added to 50µl of 0.5x YPDA containing 10µg/ml kanamycin, in a sterile 1.5ml microfuge tube and vortexed. The total volume of C was then plated onto SD<sup>-Leu</sup> agar plates. Fifty microliters and 100µl of B were also plated onto separate SD<sup>-Leu</sup> agar plates and incubated, inverted, at 30°C for 4 days in a Sanyo MIR262 stationary ventilated incubator (Sanyo, Electronic Company Ltd, Ora-Gun, Japan).

### **2.17.7 Selection of diploid yeast colonies containing putative interactor peptides**

In order to select for diploid yeast colonies in which an interaction between the *GJA5* bait protein and prey protein had taken place, the colonies were plated onto TDO and QDO plates (Appendix I). Growth of *S.cerevisiae* on the TDO plates (figure 2.4, step 1: nutritional selection stage 1), indicated the transcriptional activation of the *HIS3* nutritional reporter gene. *S.cerevisiae* growth on the QDO plates (figure 2.4, step 2: nutritional selection stage 2), indicated transcriptional activation of the *HIS3* and *ADE2* nutritional reporter gene. The activation of these genes in these diploid *S.cerevisiae* cells is indicative of an interaction between the *GJA5* bait and prey proteins.

The library mating culture which was plated directly onto 65 140mm TDO agar plates (Appendix I, section 2.17.5) was subsequently incubated in a Sanyo MIR262 stationary ventilated incubator (Sanyo, Electronic Company Ltd, Ora-Gun, Japan) for 2 weeks. Freezer cultures were prepared of each of the subsequent colonies by inoculating each one into a 2ml microfuge tube containing an equal volume of 50% autoclaved glycerol and ~ 1ml of liquid QDO medium (Appendix I) and freezing them at -70°C for storage. The growth of the colonies on the TDO plates were monitored every 4 days and colonies were picked and restreaked onto new TDO and QDO plates, in order to test for the activation of *HIS3* and *ADE2* nutritional reporter genes. These plates were incubated for 3-6 days at 30°C. Colonies growing on QDO plates after incubation were picked and plated onto new QDO plates containing X- $\alpha$ -galactose, to assess activation of *MEL1*, and incubated at 30°C for a further 3-5 days.

### **2.18 X- $\alpha$ -Galactosidase assay**

The *MEL1* gene is a colourimetric reporter gene that encodes  $\alpha$ -galactosidase, a secreted enzyme, that can be assayed directly on X- $\alpha$ -Gal indicator plates which employ blue screening. When the *GJA5* bait protein and library prey protein interact, *MEL1* is

expressed and the product ( $\alpha$ -galactosidase) is secreted into the culture medium where it catalyses the hydrolysis of melibiose and subsequently produces a blue end product. The selected diploid *S.cerevisiae* colonies containing putative interactor peptides (section 2.17.7) were assessed for their ability to activate the *MEL1* reporter gene (figure 2.4, step 3), by assessing their ability to produce the blue end product.

*S.cerevisiae* colonies in which the *HIS3* and *ADE2* reporter genes have been activated, as determined by their growth on QDO agar plates (section 2.17.7), were replicated from QDO plates onto Hybond N+ nylon membranes (Amersham pharmacia biotech Ltd, England). These membranes were subsequently placed colony-side up onto a QDO plate covered with 20mg/ml X- $\alpha$ -Gal solution (BD Bioscience, Clontech, Palo Alto, CA, USA) and the plates incubated at 30°C for 16-48hr in a Sanyo MIR262 stationary ventilated incubator (Sanyo, Electronic Company Ltd, Ora-Gun, Japan).

Following the incubation, the intensity of the blue colour of *S.cerevisiae* colonies that had activated the *MEL1* reporter gene, was assessed. Blue color intensity of the *S.cerevisiae* colonies was directly proportional to the strength of the *GJA5* bait and prey interaction. Therefore, strong interactions had intense blue color and were assigned scores ranging from three (darker-blue) to four (bright-blue) and were classified as primary prey clones. The weak interactions had less blue color and were assigned scores ranging from one (pink-blue) to two (blue-green) and were classified as secondary prey clones.

## **2.19 Rescuing prey plasmids from diploid colonies**

Each of the individual putative prey plasmids needed to be isolated from the diploid *S.cerevisiae* colonies (containing both pGBKT7-*GJA5* and prey plasmid), in order to perform subsequent experiments with the prey clones, such as heterologous mating and sequencing, to identify the putative prey protein. In order to do this, the plasmid DNA was isolated from each of the diploid cells following the protocol described in section 2.15.2 and transformed into *E.coli* DH5 $\alpha$  as described in section 2.14.1. The

transformants were inoculated into LB/amp liquid medium (Appendix I), which only allowed for the growth of transformants containing the prey plasmids. These prey plasmids were subsequently transformed into the *S.cerevisiae* Y187, following the protocol discussed in section 2.14.2.

## **2.20 Exclusion of non-specific bait and prey interactions i.e. heterologous mating**

In order to exclude non-specific bait and prey interactions, the heterologous mating experiment (figure 2.4, step 4) was performed. Each of the prey clones in *S.cerevisiae* Y187 was streaked onto 20mm SD<sup>-Leu</sup> plates and incubated at 30°C for 4 days in a Sanyo MIR262 stationary ventilated incubator (Sanyo, Electronic Company Ltd, Ora-Gun, Japan). Additionally, *S.cerevisiae* AH109 (pGBKT7-*GJA5*) and 3 heterologous baits, i.e. *S.cerevisiae* AH109 (pGBKT7), *S.cerevisiae* AH109 (pGBKT7-53) and *S.cerevisiae* AH109 (pGBKT7-Reeler), was streaked onto 20mm SD<sup>-Trp</sup> plates and incubated at 30°C for 4 days in a Sanyo MIR262 stationary ventilated incubator (Sanyo, Electronic Company Ltd, Ora-Gun, Japan).

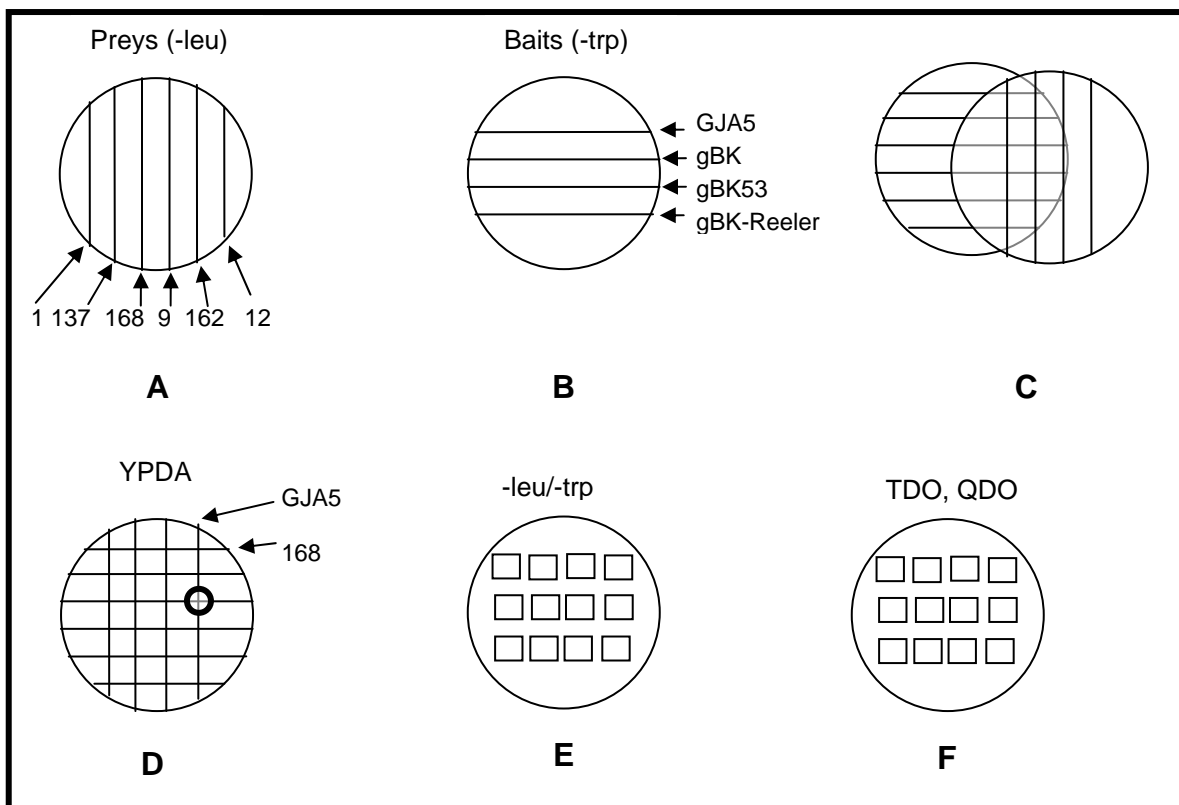
Following the incubation, the different plasmid containing *S.cerevisiae* were restreaked onto 140mm plates in parallel lines (6 preys/plate; 4 baits/plate) (figure 2.6 A&B) and incubated at 30°C for 3 days in a Sanyo MIR262 stationary ventilated incubator (Sanyo, Electronic Company Ltd, Ora-Gun, Japan). Following the incubation, replica plating was done in order to mate the Y187 preys with the AH109 baits.

A 2L glass beaker, covered with fresh sterile paper towels after each plating, was used for replica plating. The bait plates were first turned over onto the glass beaker to imprint the *S.cerevisiae* lines on the paper towels covering the glass beaker, after which the same was done with the prey plates, perpendicular to the bait *S.cerevisiae* lines (figure 2.6C). Following this, an YPDA plate was placed over the beaker in order to replica plate the bait and prey *S.cerevisiae* lines (figure 2.6D) and thereafter the plate was incubated at

30°C for 24 hrs in a Sanyo MIR262 stationary ventilated incubator (Sanyo, Electronic Company Ltd, Ora-Gun, Japan). Heterologous mating will therefore only occur where the bait and prey lines cross over each other on the YPDA plates. Following the incubation, a scrape of the matings at the cross over regions for each of the preys were streaked onto SD<sup>-Trp/-Leu</sup> plates (figure 2.6E) and incubated at 30°C for 4 days in a Sanyo MIR262 stationary ventilated incubator (Sanyo, Electronic Company Ltd, Ora-Gun, Japan), after which the colonies were restreaked onto TDO (figure 2.6F) and QDO plates (Appendix I) and incubated at 30°C for 7 days. During this incubation period, the growth of *S.cerevisiae* was scored at days 4 and 7. Scores ranging from zero (no growth) to four (very good growth) were assigned.

Growth is indicative of strength of bait and prey interaction. Therefore, prey clones with the highest growth scores were considered putative true interactors with the *GJA5* bait and were selected for further analysis (i.e. M2H). However, preys that show interaction with the pGBKT7-Reeler bait and pGBKT7 bait, in addition to interacting with the *GJA5* bait, were identified as preys which form non-specific interactions and were excluded from further analysis. The pGBKT7-Reeler bait is specific for ligands expressed in the brain. Thus, preys from the cardiac cDNA library that show interaction were considered to have non-specific activation domains and were excluded from the study.

The pGBKT7 bait is a vector which does not contain a GAL4 domain to function in an interaction with the preys; therefore, interactions between pGBKT7 and the preys are non-specific interactions and were excluded. The pGBKT7-53 bait is a control and contains a GAL4 DNA-BD which has the possibility to interact with the prey GAL4 DNA-AD. Preys that interacted with this control and with the *GJA5* bait were considered for further analysis. Therefore, the ideal prey obtained from the Y2H library screening was one that interacts only with the *GJA5* bait with a growth score of three or four in the heterologous mating experiment.



**Figure 2.6 Illustration of the exclusion of non-specific bait and prey interactions i.e. heterologous mating.** For letters, refer to text (section 2.20)

## 2.21 Mammalian-2-Hybrid analysis

The M2H analysis was performed using the Matchmaker™ Mammalian Assay Kit 2 (BD Biosciences, Palo Alto, U.S.A.), which included the pM, pVP16 and pG5SEAP vectors (Appendix II). The H9C2 cells used for the M2H analysis were cultured and transfected as described in sections 2.13 and 2.14.3 respectively. Expression of the secreted alkaline phosphatase (*SEAP*) reporter gene on the pG5SEAP reporter vector (Appendix II) only occurred when the fusion proteins generated by the pM and pVP16 constructs, that were co-transfected with the reporter vectors, interacted.

The Great EscAPe™ chemiluminescent Detection Kit (BD Biosciences, Clontech, Palo Alto, CA, U.S.A) was used, in order to determine whether the *SEAP* reporter gene was activated. This was done by measuring the *SEAP* activity in the culture medium (since

this is a secreted alkaline phosphatase), using the CSPD chemiluminescent substrate. In addition, a  $\beta$ -Galactosidase enzyme assay (Promega, Madison WI, USA), was performed using the pSV- $\beta$ -Gal vector (Appendix II). Luminescence values from the SEAP assay were normalised with the  $\beta$ -Gal assay values, in order to determine transfection efficiency of the mammalian cells.

### **2.21.1 Secreted alkaline phosphatase (SEAP) reporter gene assay**

Fifteen microlitres of each culture medium supernatant from transfected H9C2 cells (section 2.14.3) to be assayed was aliquoted into separate wells of a white opaque 96-well flat-bottom microtitre plate (PerkinElmer Life And Analytical Sciences, Inc Boston, MA, U.S.A.). To this, 45 $\mu$ l of 1X dilution buffer (BD Biosciences, Clontech, Palo Alto, CA, U.S.A.) was added and the plate incubated at 65°C for 30 min in a waterbath (Mettler<sup>®</sup>, Schwabach, Germany). Following the incubation, the plate was left on ice for 3min to cool the samples, following this, the samples were allowed to equilibrate to room temperature before 60 $\mu$ l assay buffer (BD Biosciences, Clontech, Palo Alto, CA, U.S.A.) was added to each well.

The chemiluminescent substrate (CSPD) was then prepared by making a 1:20 dilution of the CSPD (BD Biosciences, Clontech, Palo Alto, CA, U.S.A ) in chemiluminescent enhancer (BD Biosciences, Clontech, Palo Alto, CA, U.S.A). The diluted substrate was then added to each well and the plate was incubated for 10 min at room temperature. The SEAP activity was determined by reading the chemiluminescent signal every 10 minutes for a period of 2 ½ hours using a Bio-Tek<sup>®</sup> Synergy HT plate luminometer (Winooski, Vermont, U.S.A.).

Several control assays were included as comparisons for each experiment as shown in table 2.8. The reading obtained for the untransfected control (category B1) and the GeneJuice control (category B2), represents the background SEAP signal of the H9C2 cells used in the experiments, while the basal control (category C) reading corresponds to basal level (endogenous phosphatase) of SEAP activity in the experiments. The GAL4



DNA-BD control (category D) and the pVP16 AD controls (category E) shown in table 2.8 were included, in order to determine whether the pGBKT7-*GJA5* construct, or each of the putative ligands used in the experiments, function autonomously as *SEAP* reporter gene transcription activators. Two additional negative control assays were also included; one containing only pG5SEAP (category G1) and one containing only pSV- $\beta$ -Gal (category G2).

Positive control assays were also included in the experiment. The pM3-VP16 positive control plasmid (category F1) encodes a fusion of the GAL4 DNA-BD and VP16 activation domain and therefore gives very strong SEAP expression when co-transfected with pG5SEAP. The co-transfection of the pM53, pVP16T and pG5SEAP was also included in the experiment as a positive control (category F2). The pM53 expresses a fusion of the GAL4 DNA-BD to the mouse p53 antigen, while the pVP16T expresses a fusion of the VP16 activation domain to the Simian Virus 40 large T-antigen which is known to interact with p53 and therefore produces strong SEAP expression.

### **2.21.2 $\beta$ -Galactosidase enzyme assay**

Thirty microlitres of cell lysates of each culture medium from transfected H9C2 cells (section 2.14.3) to be assayed was aliquoted into separate wells of a colourless, see-through 96-well flat-bottom microtitre plate (PerkinElmer Life And Analytical Sciences, Inc Boston, MA, U.S.A.). To this, 20 $\mu$ l of 1x reporter lysis buffer (Promega, Madison WI, USA) and 50 $\mu$ l of assay buffer (Promega, Madison WI, USA) was added and the plate incubated at 37°C for 3hr in a model 329 stationary CO<sub>2</sub> incubator (Former Scientific, Marieta, Ohio, USA). Next, the reaction was stopped by adding 150 $\mu$ l of 1M sodium carbonate (Promega, Madison WI, USA), after which the absorbance was read at 420nm using a Bio-Tek<sup>®</sup> Synergy HT plate luminometer (Winooski, Vermont, U.S.A.).

## *Chapter 3*

### *Results*

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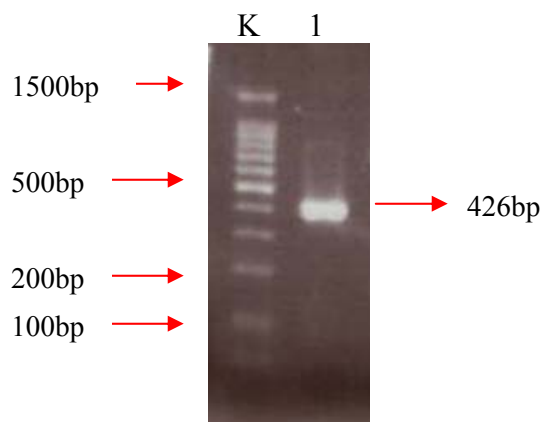
## Identifying ligands of the C-terminal domain of cardiac expressed Cx40

### 3.1 Yeast-2-hybrid analysis

#### 3.1.1 Generation of the Y2H construct

##### 3.1.1.1 PCR-amplification to generate the bait-insert fragment

The *Cx40* (i.e. *GJA5*) bait-insert fragment, for generating the Y2H construct, was obtained by PCR-amplification of the C-terminus encoding domain of Cx40 from genomic DNA using specially designed primers with engineered restriction enzyme sites (section 2.3.1.1, table 2.1). Amplification with these primers generated a *GJA5* bait-insert fragment of 426 nucleotides as shown in figure 3.1.



**Figure 3.1** Representative 2% agarose gel showing the PCR-amplification product of the *GJA5* bait-insert fragment for Y2H. Lane K: 100bp DNA ladder. Lane 1: *GJA5* bait-insert fragment.

##### 3.1.1.2 Restriction enzyme digestion and ligation for cloning

The PCR-amplified *GJA5* bait-insert fragment and the pGBKT7 cloning vector were successfully double-digested with *NdeI* and *EcoRI* (section 2.8.1). A ligation reaction (section 2.10) followed the CIP-treatment of the vector in order to generate the pGBKT7-

*GJA5* bait construct (section 2.9). Ligation reactions were transformed into *E.coli* DH5 $\alpha$  in order to select for recombinant plasmids by means of bacterial colony PCR-amplification (section 2.3.4).

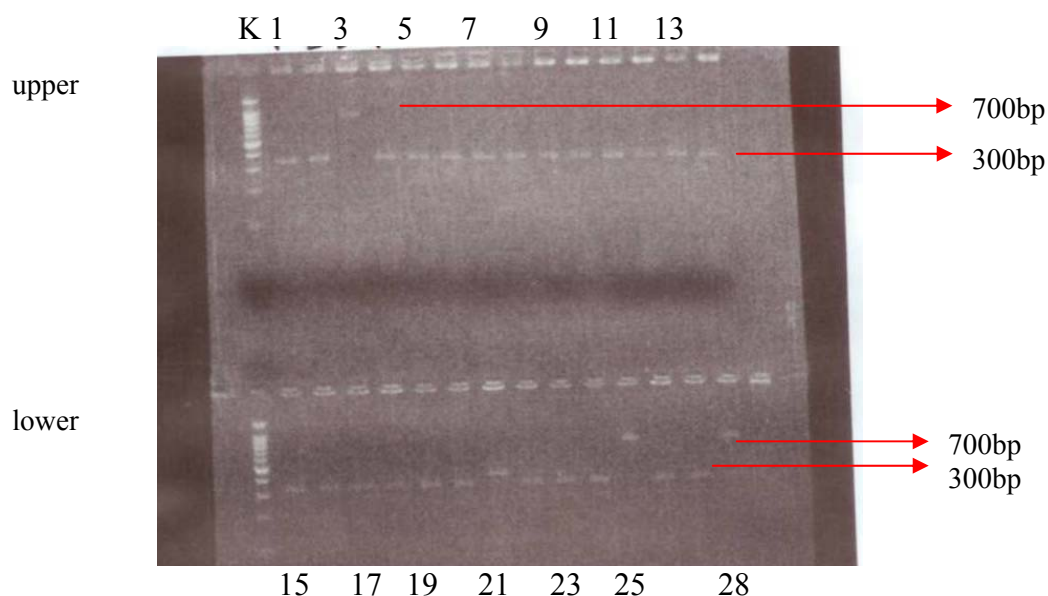
### 3.1.1.3 Selection for successful cloning

#### Bacterial colony PCR-amplification

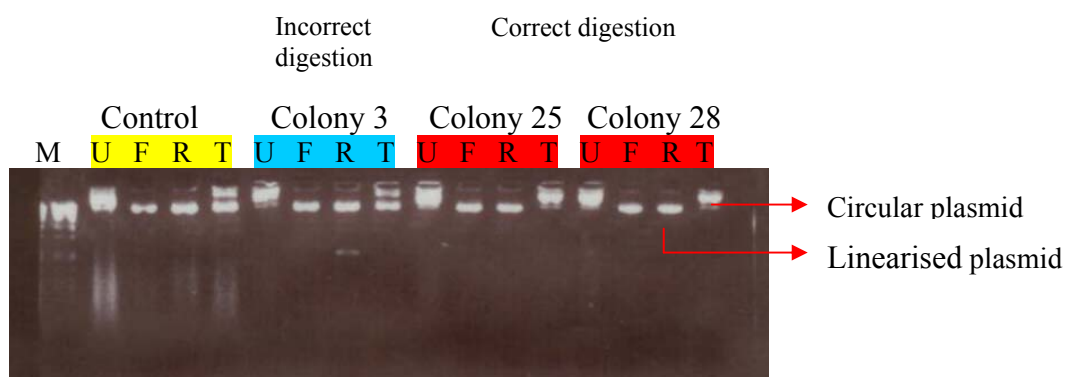
Bacterial colony PCR-amplification reactions were done in order to select for recombinant pGBKT7 plasmids which were subsequently subjected to a restriction enzyme digestion test in order to verify successful cloning (section 2.3.4 and section 2.8.3). PCRs were done using primers flanking the multiple cloning site of the pGBKT7 cloning vector (section 2.3.1.2, table 2.2). Products were produced of either the non-recombinant pGBKT7 plasmid (+300bp) or the recombinant pGBKT7 plasmid (+700bp), which contains the bait-insert fragment as shown in figure 3.2. Plasmid DNA of three out of twenty-nine colonies (numbers 3, 25 and 28) that produced the 700bp PCR product were isolated (section 2.15.1) and subjected to the restriction enzyme digestion test (section 2.8.3)

#### Restriction enzyme digestion test

The restriction enzyme digestion test showed that colony 25 and 28 contained the correct pGBKT7 recombinant plasmid as shown in figure 3.3. Undigested plasmids retain their circular configuration and migrate through the gel at a slower speed in comparison with the linearised plasmids which has been digested as shown in figure 3.3. Successful cloning of the *GJA5* bait-insert into pGBKT7 was verified by digestion of the recombinant plasmid by the 5' (*NdeI*) and 3' (*EcoRI*) enzymes and an absence of digestion by the *SfiI* test enzyme. The test enzyme did not digest the recombinant plasmid because the *GJA5* bait-insert did not contain a recognition site for *SfiI*. Colony 3 was therefore identified as a unsuccessful cloning experiment because it was digested by the test enzyme. A non-recombinant pGBKT7 plasmid that contains a recognition site for *SfiI* was included in the experiment as a control.



**Figure 3.2 Representative 1% agarose gel showing colony PCR-amplification products.** Lane K: 100bp DNA ladder. Lane 1: PCR product (300bp) of the non-recombinant pGBKT7 cloning plasmid. Lane 3, 25, 28: PCR product (700bp) of the recombinant pGBKT7 cloning plasmid containing the *GJA5* bait-insert fragment.



**Figure 3.3 Representative 1% agarose gel showing the restriction enzyme test of the pGBKT7-*GJA5* bait construct for Y2H.** Lane M:  $\lambda Pst$  molecular weight marker. Lane U: Uncut plasmid. Lane F: 5' (*NdeI*) enzyme digestion of plasmid. Lane R: 3' (*EcoRI*) enzyme digestion of plasmid. Lane T: *SfiI* test enzyme digestion of plasmid. Yellow label: Digestion of non-recombinant pGBKT7 control. Blue label: Incorrect digestion of recombinant pGBKT7. Red labels: Correct digestion of recombinant pGBKT7.

### 3.1.1.4 Sequence analysis of the bait construct

The purified plasmid DNA of *E.coli* colonies number 25 and number 28 were subjected to sequence analysis (section 2.6). The results of the sequence analysis shown in figure 3.4A and figure 3.4B showed that the pGBKT7-*GJA5* construct was in the correct reading frame and that the integrity of the nucleotide sequence of the *GJA5* bait-insert had been preserved. It was subsequently decided to continue using colony number 25 for Y2H because number 25 and number 28 gave similar sequence results. The generated Y2H construct was subsequently transformed (section 2.14.2) into *S.cerevisiae* AH109 to be used in screening the cardiac cDNA library.

#### Multiple Sequence Alignment

Colony nr.25 : *GJA5* cyto tail : pGBKT7

A

	(forward primer)	
Colony nr.25	CATATGCACCTGGGCTGGAAGAAGATCAGACAGCGATTTG	144
<i>GJA5</i> cyto tail	.....CACCTGGGCTGGAAGAAGATCAGACAGCGATTTG	34
pGBKT7	CATATG.....	1298
Consensus		
Colony nr.25	TCAAACCGCGGCAGCACATGGCTAAGTGCCAGCTTTCTGG	184
<i>GJA5</i> cyto tail	TCAAACCGCGGCAGCACATGGCTAAGTGCCAGCTTTCTGG	74
pGBKT7	.....	1298
Consensus		
Colony nr.25	CCCCTCTGTGGGCATAGTCCAGAGCTGCACACCACCCCC	224
<i>GJA5</i> cyto tail	CCCCTCTGTGGGCATAGTCCAGAGCTGCACACCACCCCC	114
pGBKT7	.....	1298
Consensus		
Colony nr.25	GACTTTAATCAGTGCCTGGAGAATGGCCCTGGGGGAAAAT	264
<i>GJA5</i> cyto tail	GACTTTAATCAGTGCCTGGAGAATGGCCCTGGGGGAAAAT	154
pGBKT7	.....	1298
Consensus		
Colony nr.25	TCTTCAATCCCTTCAGCAATAATATGGCCTCCCAACAAAA	304
<i>GJA5</i> cyto tail	TCTTCAATCCCTTCAGCAATAATATGGCCTCCCAACAAAA	194
pGBKT7	.....	1298
Consensus		
Colony nr.25	CACAGACAACCTGGTCACCGAGCAAGTACGAGGTCAGGAG	344
<i>GJA5</i> cyto tail	CACAGACAACCTGGTCACCGAGCAAGTACGAGGTCAGGAG	234
pGBKT7	.....	1298
Consensus		
Colony nr.25	CAGACTCCTGGGGAAGGTTTCATCCAGGTTTCGTTATGGCC	384
<i>GJA5</i> cyto tail	CAGACTCCTGGGGAAGGTTTCATCCAGGTTTCGTTATGGCC	274

pGBKT7	.....	1298
Consensus		
Colony nr.25	AGAAGCCTGAGGTGCCCAATGGAGTCTCACCAGGTCACCG	424
GJA5 cyto tail	AGAAGCCTGAGGTGCCCAATGGAGTCTCACCAGGTCACCG	314
pGBKT7	.....	1298
Consensus		
Colony nr.25	CCTTCCCCATGGCTATCATAGTGACAAGCGACGTCTTAGT	464
GJA5 cyto tail	CCTTCCCCATGGCTATCATAGTGACAAGCGACGTCTTAGT	354
pGBKT7	.....	1298
Consensus		
	(reverse primer)	
Colony nr.25	AAGGCCAGCAGCAAGGCAAGGTCAGATGACCTATCAGTGT	504
GJA5 cyto tail	AAGGCCAGCAGCAAGGCAAGGTCAGATGACCTATCAGTGT	394
pGBKT7	.....	1298
Consensus		
Colony nr.25	<b>GAGAATTC</b> <b>CCGGGGATCCGTCGACCTGCAGCGGCCGATA</b>	544
GJA5 cyto tail	GA.....	396
pGBKT7	..GAATTCCCGGGGATCCGTCGACCTGCAGCGGCCGATA	1336

Figure 3.4A Sequence homology alignment of the sequence of colony nr.25 with the C-terminus encoding sequence of *GJA5* (NM\_005266) from the Genbank DNA database (<http://www.ncbi.nlm.nih.gov/Entrez>) and the pGBKT7 vector. Shaded boxes – grey: gene specific primer sequence; red: restriction enzyme recognition sequence; yellow: vector sequence.

#### Multiple\_Sequence\_Alignment

Colony nr.28 : GJA5 cyto tail : pGBKT7

**B**

	(forward primer)	
Colony nr.28	<b>CATATG</b> CACCTGGGCTGGAAGAAGATCAGACAGCGATTTG	141
GJA5 cyto tail	.....CACCTGGGCTGGAAGAAGATCAGACAGCGATTTG	34
pGBKT7	CATATG.....	1298
Consensus		
Colony nr.28	TCAAACCGCGGCAGCACATGGCTAAGTGCCAGCTTTCTGG	181
GJA5 cyto tail	TCAAACCGCGGCAGCACATGGCTAAGTGCCAGCTTTCTGG	74
pGBKT7	.....	1298
Consensus		
Colony nr.28	CCCCTCTGTGGGCATAGTCCAGAGCTGCACACCACCCCC	221
GJA5 cyto tail	CCCCTCTGTGGGCATAGTCCAGAGCTGCACACCACCCCC	114
pGBKT7	.....	1298
Consensus		
Colony nr.28	GACTTTAATCAGTGCCTGGAGAATGGCCCTGGGGGAAAAT	261
GJA5 cyto tail	GACTTTAATCAGTGCCTGGAGAATGGCCCTGGGGGAAAAT	154
pGBKT7	.....	1298
Consensus		
Colony nr.28	TCTTCAATCCCTTCAGCAATAATATGGCCTCCCAACAAAA	301
GJA5 cyto tail	TCTTCAATCCCTTCAGCAATAATATGGCCTCCCAACAAAA	194

pGBKT7	.....	1298
Consensus		
Colony nr.28	CACAGACAACCTGGTCACCGAGCAAGTACGAGGTCAGGAG	341
GJA5 cyto tail	CACAGACAACCTGGTCACCGAGCAAGTACGAGGTCAGGAG	234
pGBKT7	.....	1298
Consensus		
Colony nr.28	CAGACTCCTGGGGAAGGTTTTATCCAGGTTTCGTTATGGCC	381
GJA5 cyto tail	CAGACTCCTGGGGAAGGTTTTATCCAGGTTTCGTTATGGCC	274
pGBKT7	.....	1298
Consensus		
Colony nr.28	AGAAGCCTGAGGTGCCCAATGGAGTCTCACCAGGTCACCG	421
GJA5 cyto tail	AGAAGCCTGAGGTGCCCAATGGAGTCTCACCAGGTCACCG	314
pGBKT7	.....	1298
Consensus		
Colony nr.28	CCTTCCCCATGGCTATCATAGTGACAAGCGACGTCTTAGT	461
GJA5 cyto tail	CCTTCCCCATGGCTATCATAGTGACAAGCGACGTCTTAGT	354
pGBKT7	.....	1298
Consensus		
	(reverse primer)	
Colony nr.28	AAGGCCAGCAGCAAGGCAAGGTCAGATGACCTATCAGTGT	501
GJA5 cyto tail	AAGGCCAGCAGCAAGGCAAGGTCAGATGACCTATCAGTGT	394
pGBKT7	.....	1298
Consensus		
Colony nr.28	<b>GAGAATTC</b> CCGGGGATCCGTCGACCTGCAGCGGCCGCATA	541
GJA5 cyto tail	GA.....	396
pGBKT7	..GAATTC	1336
Consensus		

Figure 3.4B Sequence homology alignment of the sequence of colony nr.28 with the C-terminus encoding sequence of *GJA5* (NM\_005266) from the Genbank DNA database (<http://www.ncbi.nlm.nih.gov/Entrez>) and the pGBKT7 vector. Shaded boxes – grey: gene specific primer sequence; red: restriction enzyme recognition sequence; yellow: vector sequence.

### 3.1.2 Verification of integrity of the Y2H construct

#### 3.1.2.1 Auto-activation test

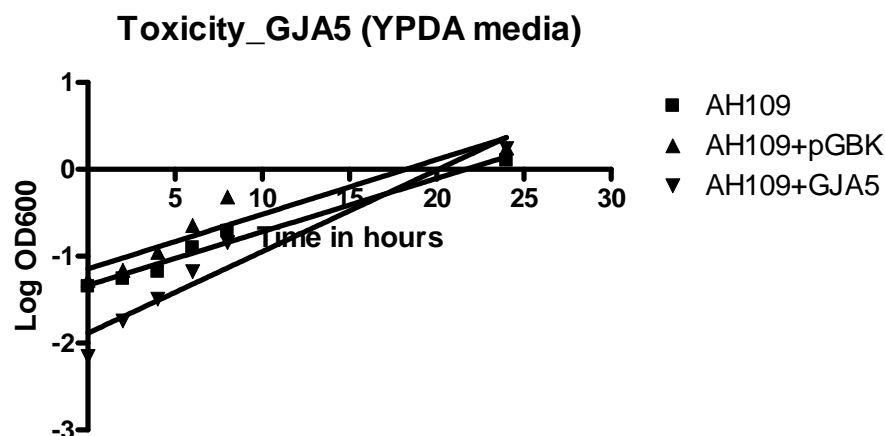
A lack of growth was observed on the SD<sup>-Ade</sup>, SD<sup>-His</sup>, and SD<sup>-Leu</sup> growth media plates (Appendix I) which indicated that the pGBKT7-*GJA5* bait construct could not autonomously activate expression of the endogenous reporter genes (section 2.16.2) and



that the phenotype of *S.cerevisiae* AH109 was retained after transformation with the pGBKT7-*GJA5* bait construct.

### 3.1.2.2 Toxicity test

Results of the toxicity test are shown in figure 3.5 in the form of a linear growth curve representing the three categories of *S.cerevisiae* AH109 (section 2.16.3). The slope of *S.cerevisiae* AH109 (pGBKT7-*GJA5*) was similar to the *S.cerevisiae* AH109 (pGBKT7) positive control and greater than the slope of the un-transformed *S.cerevisiae* AH109. This indicated that the *GJA5* bait protein did not cause a decrease in the growth of the host *S.cerevisiae* AH109, and that it was not toxic to *S.cerevisiae* AH109, and so could be used in Y2H analysis.



**Figure 3.5** Linear growth curve of un-transformed *S.cerevisiae* AH109, *S.cerevisiae* AH109 (pGBKT7) and *S.cerevisiae* AH109 (pGBKT7-*GJA5*). The *GJA5* protein did not diminish the growth of the host *S.cerevisiae* AH109 and was therefore not toxic to the host.

### 3.1.2.3 Mating efficiency of *S.cerevisiae* AH109 transformed with the Y2H construct

In order to test whether pGBKT7-*GJA5* decreased the mating efficiency of the host *S.cerevisiae* AH109, a mating efficiency test was conducted as described in section

2.16.4. The growth on SD agar plates of the progeny *S.cerevisiae* from the two categories of matings (i.e. bait mating and compatible control mating) are shown in table 3.1 and were used to calculate the mating efficiency using the calculations shown in appendix III. Calculations showed that the mating efficiency of the bait mating was 4.3% which was higher than the recommended minimum value of 2%. This showed that the pGBKT7-*GJA5* construct did not decrease the mating efficiency of *S.cerevisiae* AH109 and would result in screening of  $10^6$  individual prey clones if mated at a 100-fold excess against the commercial pretransformed library (titre= $3 \times 10^8$ ).

Table 3.1 Growth of progeny *S.cerevisiae* colonies on growth selection media

	SD <sup>-Trp</sup>			SD <sup>-Leu</sup>			SD <sup>-Trp/-Leu</sup> (diploid yeast colonies)			Mating efficiency (%)
	1:10	1:100	1:1000	1:10	1:100	1:1000	1:10	1:100	1:1000	
Mating culture dilution	1:10	1:100	1:1000	1:10	1:100	1:1000	1:10	1:100	1:1000	
Compatible control mating	-	*	1072	-	*	300	-	528	72	24
Bait mating	*	*	556	*	*	1380	*	1120	24	4.3

\*: uncountable number of colonies, -: no growth on plates

### 3.1.3 Y2H screening of pretransformed cardiac cDNA library

The pGBKT7-*GJA5* bait construct was approved for use in the Y2H analysis following the verification tests performed in section 3.1.2 and work was set about in screening the cardiac cDNA library as described in section 2.17.

#### 3.1.3.1 Library mating efficiency and number of clones screened

Library mating efficiency calculations were done using the results of growth of the library mating progeny *S.cerevisiae* colonies on SD agar plates (section 2.17.5) as shown in table 3.2. Calculations indicated that the library mating efficiency was 4.12%, which was above the recommended minimum of 2% and showed that the library mating

efficiency was within acceptable limits to continue the Y2H analysis. Furthermore, calculations showed that  $10.4 \times 10^4$  pretransformed cardiac cDNA library clones were screened using the pGBKT7-*GJA5* construct (final resuspension volume = 13ml).

Table 3.2 Growth of library mating progeny *S.cerevisiae* colonies on selection media for calculation of mating efficiency and number of clones screened.

	SD <sup>-Trp</sup>				SD <sup>-Leu</sup>				SD <sup>-Trp/-Leu</sup> (diploid yeast colonies)			
Mating culture dilution	1:10	1:100	1:1000	1:10000	1:10	1:100	1:1000	1:10000	1:10	1:100	1:1000	1:10000
Number of colonies	*	*	*	3200	922	*	*	970	38	8	-	-

\*: uncountable number of colonies, -: no growth on plates

### 3.1.3.2 Library titre and bait culture titre

A library titre was established as described in section 2.17.6. Calculations (Appendix III) were done using the results of *S.cerevisiae* growth on SD<sup>-leu</sup> agar plates as shown in table 3.3.

Table 3.3 Growth of *S.cerevisiae* on SD<sup>-Leu</sup> agar plates for calculating the library titer

50µl of dilution B	100µl of dilution B	Complete volume of dilution C	Dilution factor	Library titre (cfu/ml)
706	1362	*	10 <sup>4</sup>	1.412 x 10 <sup>8</sup>

\*: uncountable number of colonies, cfu/ml: colony forming units per milliliter

Haemocytometric cell count was done in order to determine the titre of bait culture used in the library mating experiment. The number of cells was 61 and, according to calculations in section 2.17.3, the titre of the bait culture was  $1.037 \times 10^{10}$  cfu/ml.

### 3.1.3.3 Selection of diploid *S.cerevisiae* colonies containing putative interactor peptides

As detailed below, successive nutritional selection stages (section 2.17.7) reduced the number of diploid colonies from an initial number of 324 to 233 colonies containing

putative interactor peptides. The 233 colonies was subsequently further reduced to 186 colonies by means of the X- $\alpha$ -galactosidase assay (section 2.18), which also classified them as primary or secondary colonies (section 3.1.3.4). Thereafter, the heterologous mating experiment (sections 2.20 and 3.1.3.5) selected 33 from the 186 colonies to be subjected to sequence analysis (sections 2.6 and 3.1.3.6). The results for the 33 selected colonies are shown in tables 3.4, 3.5A, 3.5B, 3.6A, and 3.6B.

### **Nutritional selection stage 1**

The first nutritional selection stage (section 2.17.7, figure 2.4), which made use of TDO plates, selected for diploid *S.cerevisiae* colonies with the ability to activate expression of the *HIS3* reporter gene. From this selection stage, 324 colonies were shown to activate *HIS3* expression and are indicated in table 3.4, column A.

### **Nutritional selection stage 2**

The second nutritional selection stage (section 2.17.7, figure 2.4) made us of QDO plates in order to select for diploid *S.cerevisiae* colonies with the ability to activate expression of the *ADE2* reporter gene in addition with the *HIS3* reporter gene. This selection stage selected 233 colonies with this ability and are indicated in table 3.4, column B.

### **3.1.3.4 X- $\alpha$ -Galactosidase assay**

The 233 *S.cerevisiae* colonies that were selected in section 3.1.3.3 were assessed for their ability to activate the colourimetric *MEL1* reporter gene by assessing their ability to produce the blue end product in the X- $\alpha$ -Galactosidase assay (section 2.18). A number of 186 colonies were found to activate the *MEL1* reporter gene of which 142 were classified as primary colonies and 44 as secondary colonies. Shown in table 3.4, column C, are the X- $\alpha$ -Galactosidase assay scores for the primary and secondary colonies.

Table 3.4 Activation of nutritional and colourimetric reporter genes by the *GJA5* bait construct and prey clone interactions

Colony Nr.	Identical clones	A Growth on TDO ( <i>HIS3</i> activation)	B Growth on QDO ( <i>ADE2</i> activation)	C X- $\alpha$ -Galactosidase assay ( <i>MEL1</i> activation)
<b>Primary colonies</b>				
<b>29</b>	<b>none</b>	++++	++	+++
<b>35</b>	<b>none</b>	++++	++++	+++
37	none	++++	+++	+++
55	none	++++	++++	+++
<b>62</b>	<b>none</b>	++++	++++	+++
133	none	++++	++	+++
136	none	++++	+++	+++
<b>153</b>	<b>none</b>	++++	+++	++++
154	none	++++	++++	++++
<b>163</b>	<b>230</b>	++++	++++	++++
<b>164</b>	<b>none</b>	++++	++++	++++
<b>204</b>	<b>none</b>	++++	++++	++++
219	none	++++	++++	+++
<b>222</b>	<b>none</b>	++++	++++	++++
230	163	++++	++++	++++
248	none	++++	++	++++
250	none	++++	+++	++++
257	none	++++	+++	++++
271	306	++++	+	++++
273	306	++++	+++	++++
275	306	++++	++++	+++
278	306	++++	++	+++
295	306	++++	++++	++++
299	306	++++	++++	++++
<b>306</b>	<b>271,273,275,278, 295,299,313,314</b>	++++	++++	++++
313	306	++++	++++	++++
314	306	++++	++++	++++
<b>Secondary colonies</b>				
23	none	++++	++++	++
<b>31</b>	<b>none</b>	++++	++++	++
79	none	++++	+++	++
95	none	++++	++++	+
<b>99</b>	<b>none</b>	++++	++++	+
285	none	++++	+++	++

Bold: Clones used in M2H; X- $\alpha$ -Galactosidase assay = Primary clones: ++++ (bright-blue) and +++ (darker-blue); Secondary clones: ++ (blue-green) and + (pink-blue)

TDO: solid media lacking Leu, Trp and His; QDO: solid media lacking Leu, Trp, His and Ade. Growth of colonies on solid media: ++++ = very good; +++ = good; ++ = weak; + = very weak; - = no growth

Identical clones = prey clones with the same identity as determined by sequence analysis

### 3.1.3.5 Heterologous mating

The heterologous mating experiment was conducted, as described in section 2.20, with the 186 diploid *S.cerevisiae* colonies (prey clones) that were shown to have the ability to activate expression of the *ADE2*, *HIS3* and *MEL1* reporter genes. The results of the heterologous mating experiment are shown in table 3.5A (primary colonies) and table 3.5B (secondary colonies). The majority grew well on the TDO plates because this medium selected for activation of only one of the reporter genes, namely, *HIS3*. The QDO medium selected for activation of *ADE2* and *HIS3* and was therefore more stringent in growth selection. Therefore, the growth of *S.cerevisiae* on QDO plates (shaded columns) was given preference when analysing the results of the heterologous mating. It was found that out of 186 prey clones, 33 showed binding specificity for the *GJA5* bait protein and were subsequently subjected to sequence analysis. Binding specificity of these preys were indicated by growth of *S.cerevisiae* colonies on QDO plates as a result of prey matings with the pGBK-*GJA5* bait and absence of growth in matings of the preys with the other three heterologous baits (pGBKT, pGBKT-53 and pGBK-reeler) as shown in table 3.5A and B.

### 3.1.3.6 Sequence analysis

The inserts of the 33 putative prey clones (i.e. ligands), selected by means of heterologous mating, were sequenced and subjected to analysis, in order to assign identity and function and to exclude false positive ligands based on subcellular location and function. The frame and integrity of the prey sequences was analysed using the ChromasPro computer program and DNAMAN<sup>TM</sup> version 4 software and subsequently subjected to BLAST-searches against the Genbank DNA database using BLASTN (genomic hit) and BLASTP (in-frame protein hit) (<http://www.ncbi.nlm.nih.gov/Entrez>). The identities of each of the preys are indicated in table 3.6A (primary clones) and 3.6B (secondary clones).

Table 3.5A Interaction of preys with heterologous baits in the specificity tests as assessed by *ADE2* and *HIS3* activation - Primary clones

Clone#	x pGBK- <i>GJA5</i> TDO	x pGBK- <i>GJA5</i> QDO	x pGBKT7 TDO	x pGBKT7 QDO	x pGBK-53 TDO	x pGBK-53 QDO	x pGBK-reeler TDO	x pGBK-reeler QDO
<b>29</b>	+++	+++	+++	-	++	-	++	-
<b>35</b>	+++	+	+++	-	++	-	+++	-
37	+++	+	+++	-	++	-	+++	-
55	+++	+	+++	-	++	-	+++	-
<b>62</b>	+++	++++	+++	-	++	-	++	-
133	+++	+	+++	-	++	-	++	-
136	++	+	+++	-	++	-	++	-
<b>153</b>	++	+	+++	-	++	-	++	-
154	++	+	+++	-	++	-	++	-
<b>163</b>	+++	+	+++	-	++	-	+++	-
<b>164</b>	+++	+	+++	-	++	-	+++	-
<b>204</b>	$\theta$	$\theta$	$\theta$	$\theta$	$\theta$	$\theta$	$\theta$	$\theta$
219	+++	+	+++	-	++	-	++	-
<b>222</b>	+++	+	+++	-	++	-	++	-
230	$\theta$	$\theta$	$\theta$	$\theta$	$\theta$	$\theta$	$\theta$	$\theta$
248	+++	+	+++	-	++	-	+++	-
250	+++	+	+++	-	++	-	+++	-
257	+++	+	++	-	++	-	+++	-
271	+++	+	++	-	++	-	+++	-
273	+++	+	++	-	++	-	+++	-
275	+++	+	++	-	++	-	+++	-
278	+++	+	++	-	++	-	+++	-
295	+++	+	++	-	++	-	+++	-
299	+++	+	+++	-	++	-	+++	-
<b>306</b>	+++	+	+++	-	++	-	++	-
313	+++	+	+++	-	++	-	++	-
314	+++	+	+++	-	++	-	++	-

Table 3.5B Interaction of preys with heterologous baits in the specificity tests as assessed by *ADE2* and *HIS3* activation - Secondary clones

Clone#	x pGBK- <i>GJA5</i>	x pGBK- <i>GJA5</i>	x pGBKT7	x pGBKT7	x pGBK-53	x pGBK-53	x pGBK-reeler	x pGBK-reeler
	TDO	QDO	TDO	QDO	TDO	QDO	TDO	QDO
23	++++	+++	++++	-	++++	-	+++	-
<b>31</b>	++++	+++	++++	-	+++	-	+++	-
79	++++	++	++++	-	+++	-	+++	-
95	++++	+++	++++	-	++++	-	++++	-
<b>99</b>	++++	+++	++++	-	++++	-	++++	-
285	++++	++	++++	-	+++	-	+++	-

θ : Did not transform into *S.cerevisiae* Y187; Bold: Clones used in M2H. TDO: solid media lacking Leu, Trp and His; QDO: solid media lacking Leu, Trp, His and Ade. Growth of clones on solid media: ++++ = very good; +++ = good; ++ = weak; + = very weak; - = no growth



Included in the tables are the X- $\alpha$ -gal assay results which indicate the strength and specificity of the bait and prey interactions. Amongst the 33 prey clones, some were identified by BLAST-search as containing partial cloning vector sequences and partial mitochondrial sequences (clone# 133, 37, 55, 23, 79, 95), and were therefore excluded. Internet database literature searches were done for each of the remaining ligands, in order to prioritise them according to function and subcellular localisation. From this, 11 ligands (section 3.2) were identified as having good candidature for being true Cx40 ligands and were subjected to M2H analysis (section 3.3), in order to assess the validity of the bait protein and prey protein interactions.

The identities of the 11 ligands are as follows: Cysteine-rich protein 2 (CRP2) (clone# 306, 313, 314, 273, 271, 275, 278, 295, 299), Beta-actin (ACTB) (clone# 62), Creatine kinase (CK) (clone# 222), Myosin, heavy polypeptide 7 (MYH7) (clone# 163,230), Mucolin 1 (MCOLN1) (clone# 164), Voltage-dependent anion channel 2 (VDAC2) (clone# 153), Aldehyde dehydrogenase 2 (ALDH2) (clone# 35), DEAH (Asp-Glu-Ala-His) box polypeptide 30 (DHX30) (clone# 204), NADH dehydrogenase, alpha subcomplex, 6 (NDUFA6) (clone# 29), Prosaposin (PSAP) (clone# 31) and Filamin A (actin binding protein 280) (FLNA) (clone# 99).

### **3.1.3.7 Screening the PFHBI and PFHBII loci**

The sequenced inserts of the 33 putative prey clones were BLAST-searched against Genbank databases covering the PFHBI locus, chromosome 19q13.3 (between markers *D19S902* and *D19S866*), and the PFHBII locus, chromosome 1q32.2-32.3 (between markers *DIS70* and *DIS505*). None of the preys were found to map to these disease loci. This excluded the genes encoding the putative Cx40 ligands as potential candidate causative genes for PFHBI and II.

Table 3.6A Identification of putative *GJA5* interacting prey clones – **Primary clones**

Clone Nr.	GENOMIC HIT		IN-FRAME PROTEIN HIT		Subcellular location	X- $\alpha$ -Gal assay
	Blastn Acc# (e-value)	Identity	Blastp Acc# (e-value)	Identity		
29	NM_002490.3 (1e-168)	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6 (NDUFA6)	NP_002481.2 (2e-85)	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6 (NDUFA6)	This amino acid sequence is of a SOLUBLE PROTEIN Mitochondrial inner membrane; matrix side	+++
35	NM_000690.2 (0.0)	Aldehyde dehydrogenase 2 (ALDH2)	NP_000681.2 (4e-98)	Aldehyde dehydrogenase 2 (ALDH2)	Mitochondrial matrix. This amino acid sequence is of a SOLUBLE PROTEIN	+++
37	AY714050.1 (0.0)	Mitochondrial isolate	No significant similarity	None	-	+++
55	AY13995.1 (0.0)	Mitochondrial isolate	No significant similarity	None	-	+++
62	AK223055.1 (0.0)	Beta-actin (ACTB)	AAH12854.1 (2e-71)	Beta-actin (ACTB)	This amino acid sequence is of a SOLUBLE PROTEIN	+++
133	AF033313 (0.0)	Cloning vector pGAD42.1	CAA99098.1 (2e-09)	Alcohol dehydrogenase 1A (class I), alpha polypeptide (ADH1A)	Cytoplasmic. This amino acid sequence is of a SOLUBLE PROTEIN	+++
136	NM_015313.1 (0.0)	Rho guanine nucleotide exchange factor 12 (LARG)	No significant similarity	none	This amino acid sequence is of a SOLUBLE PROTEIN	+++
153	NM_003375.2 (0.0)	Voltage-dependent anion channel 2 (VDAC2)	CAI40914.1 (3e-08)	Voltage-dependent anion channel 2 (VDAC2)	Outer mitochondrial membrane. This amino acid sequence is of a SOLUBLE PROTEIN.	++++
154	AC007878.2 (3e-116)	FLJ25369 Hypothetical protein	No significant similarity	none	-	++++
163,230	NM_000257.1 (0.0)	Myosin, heavy polypeptide 7, cardiac muscle, beta (MYH7)	BAD92945.1 (1e-124)	Myosin, heavy polypeptide 7, cardiac muscle, beta (MYH7)	Thick filaments of the myofibrils. This amino acid sequence is of a SOLUBLE PROTEIN	++++
164	BC005149.2 (0.0)	Mucolipin 1 (MCOLN1)	AAH05149.1 (1e-133)	Mucolipin 1 (MCOLN1)	This amino acid sequence is of a	++++

					<b>MEMBRANE PROTEIN with 6 transmembrane helices.</b>	
<b>204</b>	<b>NM_138615.1 (2e-90)</b>	<b>DEAH (Asp-Glu-Ala-His) box polypeptide 30 (DHX30)</b>	<b>AAH14237.1 (0.021)</b>	<b>DEAH (Asp-Glu-Ala-His) box polypeptide 30 (DHX30)</b>	<b>This amino acid sequence is of a membrane protein which have 1 transmembrane helix.</b>	<b>++++</b>
219	NM_014712.1 (0.0)	KIAA0339 SET-domain-containing protein 1 (SET1)	BAA20797.2 (0.0)	KIAA0339 SET-domain-containing protein 1 (SET1)	This amino acid sequence is of a SOLUBLE PROTEIN	+++
<b>222</b>	<b>NM_001824.2 (0.0)</b>	<b>Creatine kinase, muscle (CKM)</b>	<b>AAS79321.1 (7e-147)</b>	<b>Creatine kinase, muscle (CKM)</b>	<b>Cytoplasmic.</b>	<b>++++</b>
248	No significant similarity	none	No significant similarity	none	-	++++
250	AL161779.32 (0.0)	SMC1 structural maintenance of chromosomes 1-like 1	BAC86840.1 (5e-05)	SMC1 structural maintenance of chromosomes 1-like 1	Nuclear protein This amino acid sequence is of a SOLUBLE PROTEIN.	++++
257	AC010299.8 (0.0)	LOC90288 Hypothetical protein	No significant similarity	none	-	++++
<b>306,313, 314,273, 271,275, 278,295, 299</b>	<b>NM_001312.2 (0.0)</b>	<b>Cysteine-rich protein 2 (CRP2)</b>	<b>AAV38714.1 (6e-127)</b>	<b>Cysteine-rich protein 2 (CRP2)</b>	<b>This amino acid sequence is of a SOLUBLE PROTEIN</b>	<b>+++/+</b>

**Bold:** Clones used in M2H

X- $\alpha$ -Galactosidase assay = Primary clones: ++++ (bright-blue) and +++ (darker-blue);

- = no result

Table 3.6B Identification of putative *GJA5* interacting prey clones – **Secondary clones**

Clone Nr.	GENOMIC HIT		IN-FRAME PROTEIN HIT		Subcellular location	X- $\alpha$ -Gal assay
	Blastn Acc# (e-value)	Identity	Blastp Acc# (e-value)	Identity		
23	DQ658411.1 (0.0)	Mitochondrial isolate	No significant similarity	None	-	++
<b>31</b>	<b>NM_001042466.1 (0.0)</b>	<b>Prosaposin (PSAP)</b>	<b>AAA36596.1 (1e-115)</b>	<b>Prosaposin (PSAP)</b>	<b>This amino acid sequence is of a membrane protein which have 1 transmembrane helix.</b>	<b>++</b>
79	DQ658411.1 (0.0)	Mitochondrial isolate	No significant similarity	None	-	++
95	DQ658411.1 (0.0)	Mitochondrial isolate	No significant similarity	None	-	+

99	NM_001456.1 (5e-142)	<b>Filamin A, alpha (actin binding protein 280) (FLNA)</b>	AAI09290.1 (5e-66)	<b>Filamin A, alpha (actin binding protein 280) (FLNA)</b>	<b>Cytoplasmic; peripheral. This amino acid sequence is of a SOLUBLE PROTEIN</b>	+
285	NM_212471.1 (2e-150)	Protein kinase, cAMP-dependent, regulatory, type I alpha (PRKAR1A)	No significant similarity	none	-	++

**Bold:** Clones used in M2H

X- $\alpha$ -Galactosidase assay = Secondary clones: ++ (blue-green) and + (pink-blue)

- = no results

### 3.2 Ligands chosen for further analysis

Out of the 33 putative ligand-positive prey clones that were prioritised according to function and subcellular localization, by means of internet database literature searches, 11 were identified as having strong candidature for being true Cx40 ligands. They were subsequently subjected to M2H analysis in order to assess the validity of their interaction with the *GJA5* bait. The following is a brief description of each of these ligands, which will be discussed in detail in the following chapter.

#### Cysteine-rich protein 2

Cysteine-rich protein 2 (CRP2) functions both as a cytoplasmic scaffold protein and as a nuclear transcription factor (Chang *et al.*, 2003; Dawid *et al.*, 1995). Its cytoplasmic subcellular location makes it a plausible interactor of the C-terminus of Cx40. Nine of the 33 prey clones were CRP2, which increased the probability of it being a true Cx40 ligand.

#### Beta actin

This is a cytoplasmic cytoskeletal actin and functions in intracellular trafficking, as well as in driving changes in cellular shape associated with cell motility, cytokinesis,

endocytosis and cell adhesion (Hu *et al.*, 2004; Peckham *et al.*, 2001). Its interaction with Cx40 might aid in these functions.

### **Creatine kinase, muscle type**

Creatine kinase (CK) reversibly catalyzes the transfer of phosphate between ATP and various phosphogens (e.g. creatine phosphate) and therefore has a central role in energy transduction (Kammermeier, 1987; Steeghs *et al.*, 1997). Heart failure of many different etiologies has been shown to have association with decreased total CK activity (Liao *et al.*, 1996). Animal studies showed that CK-deficient mice developed left ventricular hypertrophy (Nahrendorf *et al.*, 2005). The fact that this isoform is located in the cytoplasm makes an interaction with the C-terminus of Cx40 possible.

### **Myosin heavy polypeptide 7**

One of the most important components of the sarcomere thick filament is myosin heavy polypeptide 7 (MYH7) (Perrot *et al.*, 2005). Whether an interaction with Cx40 is possible is under question, because it is unknown whether Cx proteins reside in the sarcomere. It was however included in the study because 2 (clone# 163 and 230) of the 33 prey clones had this identity. Mutations in MYH7 are one of the causes of familial hypertrophic cardiomyopathy and defects in MYH7 have also been shown to be associated with dilated cardiomyopathy (Perrot *et al.*, 2005; Maron *et al.*, 1981).

### **Mucolipin 1**

This is a transmembrane protein which places it in the proximity of the subcellular location of Cx40 (Pedersen *et al.*, 2005; Slaugenhaupt *et al.*, 2002). It has functions in  $\text{Ca}^{2+}$  homeostasis which is important for cardiac muscle contraction.

## **Voltage dependent anion channel 2**

Voltage dependent anion channel 2 (VDAC2) forms a channel through the mitochondrial outer membrane that allows diffusion of small hydrophilic molecules such as ATP and ADP, which are important components for cardiac muscle contraction (Sampson *et al.*, 1997, 1996; Sardiello *et al.*, 2003). It has also been identified in the plasma membrane of cell (Elinder *et al.*, 2005). The fact that VDAC2 is a membrane bound protein makes an interaction with the C-terminus of Cx40 possible. Interestingly, Cxs have been identified in the mitochondrial membranes (Boengler *et al.*, 2005) and VDAC2 was therefore not excluded from the study.

## **Aldehyde dehydrogenase 2**

This protein functions in alcohol metabolism and it plays a role in vasoactivity (Hempel *et al.*, 1993; Chen *et al.*, 2005). As discussed in the introduction (section 1.4.3) animal studies have shown the importance of Cx40 in vasoactivity (de Wit *et al.*, 2000, 2003). The possibility therefore exists that ALDH2 could function in combination with Cx40 in regulating vasoactivity. Interestingly, ALDH2 has been shown to be a risk factor for hypertension and myocardial infarction in Japanese men.

## **DEAH (Asp-Glu-Ala-His) box polypeptide 30**

A large number of the DEAH proteins, including this protein, have not been fully characterised (Jankowsky *et al.*, 2000). It is, however, known to be a membrane bound protein that might interact with the C-terminus of Cx40 based on its cellular location.

## **NADH dehydrogenase (ubiquinone)**

This protein is part of the first and largest complex in the mitochondrial respiratory electron transport chain which functions in the production of ATP (Chretien *et al.*, 2003). Defects in the transport chain mostly affect organs with high energy demands such as the

heart, muscle and brain (Ton *et al.*, 1997). With it being a membrane bound protein, it is possible that an interaction could occur with the C-terminus of Cx40.

### **Prosaposin**

This protein functions in signal transduction and cell-to-cell communication and exists both as a secretory protein and as an integral membrane protein (Sun *et al.*, 2002). Its functions and subcellular location therefore makes it a strong candidate for being a true Cx40 ligand

### **Filamin A (actin binding protein 280)**

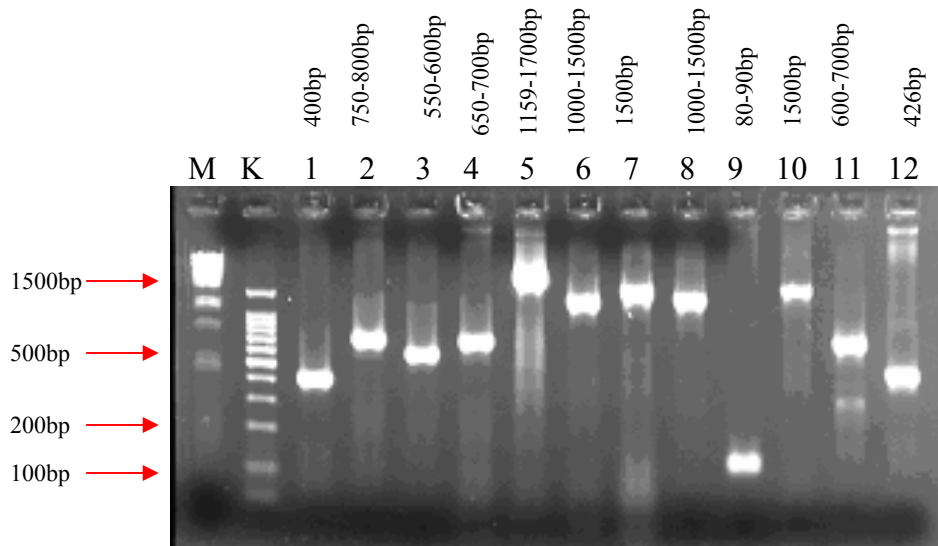
Filamin A (FLNA) functions in regulating reorganisation of the actin cytoskeleton by interacting with integrins, transmembrane receptor complexes and second messengers (Gorlin *et al.*, 1990). It also serves as a scaffold for a wide range of cytoplasmic signaling proteins (Loo *et al.*, 1998; Stossel *et al.*, 1985). These functions of FLNA and its subcellular location makes a interaction with the C-terminus of Cx40 possible which might be a significant interaction that facilitates the functions of FLNA in the cell.

## **3.3 Mammalian-2-Hybrid analysis**

### **3.3.1 Generation of M2H constructs**

#### **3.3.1.1 PCR-amplification to generate the bait-insert fragment and the prey-insert fragments**

PCR-amplification was performed as described in section 2.3.3 with specially designed primers with engineered restriction enzyme sites (table 2.3, 2.4 and 2.5), in order to obtain the *GJA5* bait-insert and prey clone insert fragments. Figure 3.6 shows the PCR-amplification products of each of the prey-insert fragments and the *GJA5* bait-insert fragment.



**Figure 3.6 Representative 1% agarose gel showing the PCR-amplification products of the putative prey-inserts and the *GJA5* bait-insert used for the M2H analysis.** Lane M:  $\lambda Pst$  molecular weight marker; Lane K: 100bp DNA ladder; Lane 1: NADH dehydrogenase 1 alpha subcomplex, 6; Lane 2: Prosaposin; Lane 3: Aldehyde dehydrogenase 2; Lane 4:  $\beta$ -actin; Lane 5: Filamin A; Lane 6: Voltage dependent anion channel 2; Lane 7: Myosin heavy polypeptide 7; Lane 8: Mucolipin 1; Lane 9: DEAH box polypeptide 30; Lane 10: Creatine kinase; Lane 11: Cysteine-rich protein 2; Lane 12: *GJA5* bait-insert.

### 3.3.1.2 Restriction enzyme digestion and ligation for cloning

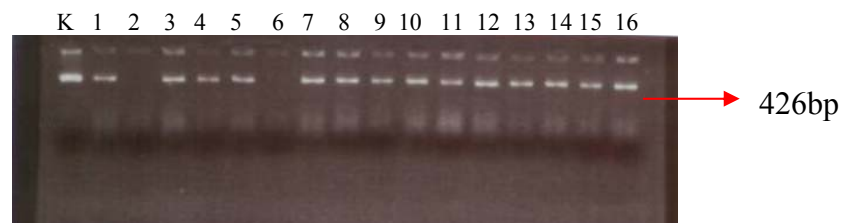
Each of the prey-insert fragments, the bait-insert fragment and the cloning vectors was sequentially double-digested with restriction enzymes specific to each as indicated in table 2.6, section 2.8.3. Ligation reactions (section 2.10) followed the CIP-treatment (section 2.9) of the vectors in order to generate the pM-*GJA5* bait construct and the pVP16-prey constructs. Ligation reactions were transformed into *E.coli* DH5 $\alpha$  in order to select for recombinant plasmids by means of bacterial colony PCR-amplification (section 2.3.4).



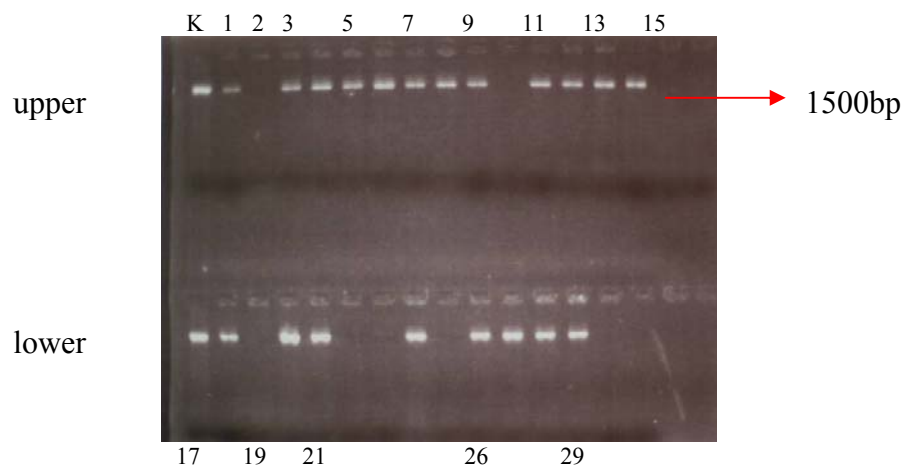
### 3.3.1.3 Selection for successful cloning

#### Bacterial colony PCR-amplification

Bacterial colony PCR-amplification reactions were done in order to select for recombinant pM and pVP16 plasmids which were subsequently subjected to restriction enzyme digestion tests in order to verify successful cloning (section 2.3.4 and section 2.8.3). Colony PCRs were done using the same primers used for generating the insert fragments as described in section 3.3.1.1. PCR products were produced of the recombinant cloning vectors that contained either the *GJA5* bait-insert or the prey-insert fragments that were verified by agarose gel electrophoreses and size comparisons with known size markers. Shown in figure 3.7 are the individual colony PCR products of the *GJA5* bait-insert fragment that was cloned into the pM vector. Shown in figure 3.8 are the individual colony PCR products of a prey-insert fragment that was cloned into the pVP16 vector. The results of only one representative prey (clone# 163) are shown due to the number of prey constructs that were generated. Plasmid DNA of the *E.coli* colonies that produced the correct PCR product size were isolated (section 2.15.1) and subjected to the restriction enzyme digestion test (section 2.8.3).



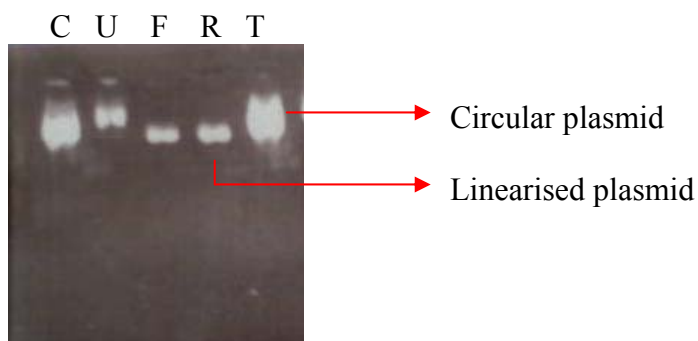
**Figure 3.7 Representative 1% agarose gel of colony PCR-amplification of the pM-*GJA5* bait construct for M2H.** Lane K: Bait-insert fragment PCR product of the correct size. Lane 1: Colony PCR-amplification product of the correct size. Lane 2: Sample in which amplification did not occur.



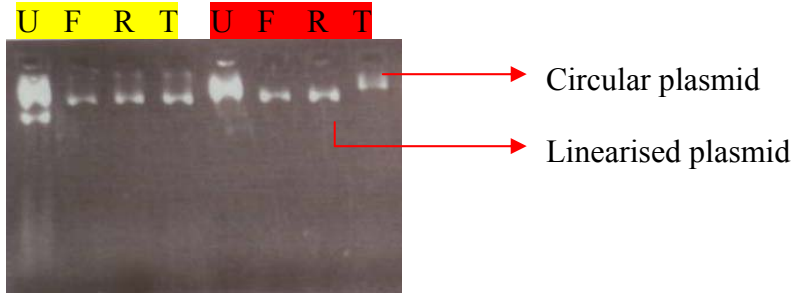
**Figure 3.8 Representative 1% agarose gel of colony PCR-amplification of prey clone# 163 for M2H.** Lane K: Prey-insert fragment PCR product of the correct size. Lane 1: Colony PCR-amplification product of the correct size. Lane 2: Sample in which amplification did not occur.

### Restriction enzyme digestion test

The recombinant pM-*GJA5* and pVP16-prey plasmids were digested with the 5', 3' and test enzymes (section 2.8.3) in separate, parallel reactions. Figure 3.9 shows the restriction enzyme test of the pM recombinant plasmid. The successful cloning of the *GJA5* insert into the vector was indicated by digestion of the plasmid by the 5' and 3' enzymes causing it to be linearised, and an absence of digestion by the test enzyme. Figure 3.10 shows successful cloning of one representative prey-insert (clone# 163) into the pVP16 vector. A non-recombinant pVP16 plasmid was included in the test.



**Figure 3.9 Representative 1% agarose gel showing the restriction enzyme test of the pM-*GJA5* bait construct for M2H.** Lane C: Uncut non-recombinant pGBKT7 control. Lane U: Uncut bait plasmid. Lane F: 5' enzyme digestion of bait plasmid. Lane R: 3' enzyme digestion of bait plasmid. Lane T: Test enzyme digestion of bait plasmid.



**Figure 3.10 Representative 1% agarose gel showing the restriction enzyme test of the pVP16-prey construct of clone# 163 for M2H.** Lane U: Uncut plasmid. Lane F: 5' enzyme digestion of plasmid. Lane R: 3' enzyme digestion of plasmid. Lane T: Test enzyme digestion of plasmid. Yellow labels: Digestion of non-recombinant pVP16 control. Red labels: Correct digestion of prey plasmid.

### 3.3.1.4 Sequence analysis

The plasmid DNA of the selected *E.coli* colonies, which were identified as containing the correct recombinant pM or pVP16 plasmids, were subjected to sequence analysis. The results of the sequence analysis showed that the nucleotide sequence of the *GJA5* bait-insert and 11 prey-inserts were maintained after generating the M2H constructs. It also showed that the inserts were in frame with their respective GAL4 domains. Figure 3.11 A to L shows the sequence analysis results of each of the 11 prey-inserts and the *GJA5* bait-insert.

#### Multiple\_Sequence\_Alignment 29\_pVPinsert 29.cds pVP16\_2

A

	(forward primer)	
29_pVPinsert	TTCCCGGGGATCCGGCGTCCGCCAAGCTACTTCTACCGCC	79
29.cds	aTggCGGGGAg.CGGCGTCCGCCAAGCTACTTCTACCGCC	117
pVP16_2	TTCCCGGGGATCC.....	89
Consensus		
29_pVPinsert	AGCACCTTCGTGAAGCCCATTTTCAGTCGGGACATGAACG	119
29.cds	AGCACCTTCGTGAAGCCCATTTTCAGTCGGGACATGAACG	157
pVP16_2	.....	89
Consensus		
29_pVPinsert	AGGCCAAGCGGAGGGTGCGCGAGCTCTACCGCGCCTGGTA	159
29.cds	AGGCCAAGCGGAGGGTGCGCGAGCTCTACCGCGCCTGGTA	197
pVP16_2	.....	89

## Consensus

29_pVPinsert	TCGGGAGGTGCCGAACACTGTGCACCAATTCCAGCTGGAC	199
29.cds	TCGGGAGGTGCCGAACACTGTGCACCAATTCCAGCTGGAC	237
pVP16_2	.....	89
Consensus		
29_pVPinsert	ATCACTGTGAAAATGGGACGGGATAAAGTCCGAGAAATGT	239
29.cds	ATCACTGTGAAAATGGGACGGGATAAAGTCCGAGAAATGT	277
pVP16_2	.....	89
Consensus		
29_pVPinsert	TTATGAAGAATGCCCATGTGCACAGACCCAGGGTGGTTGA	279
29.cds	TTATGAAGAATGCCCATGTGCACAGACCCAGGGTGGTTGA	317
pVP16_2	.....	89
Consensus		
29_pVPinsert	TCTTCTGGTCATTAAGGGAAAGATCGAACTGGAAGAAACA	319
29.cds	TCTTCTGGTCATTAAGGGAAAGATCGAACTGGAAGAAACA	357
pVP16_2	.....	89
Consensus		
29_pVPinsert	ATTAAAGTATGGAAGCAGCGGACACATGTTATGCGGTTCT	359
29.cds	ATTAAAGTATGGAAGCAGCGGACACATGTTATGCGGTTCT	397
pVP16_2	.....	89
Consensus		
29_pVPinsert	TCCATGAAACAGAAGCGCCAAGGCCAAAGGATTTCTATC	399
29.cds	TCCATGAAACAGAAGCGCCAAGGCCAAAGGATTTCTATC	437
pVP16_2	.....	89
Consensus		
	(reverse primer)	
29_pVPinsert	CAAGTTCTATGTTGGCCACGATCCATGAACGCGTCTGCAG	439
29.cds	CAAGTTCTATGTTGGCCACGATCCATGA.....	465
pVP16_2	.....ACGCGTCTGCAG	101
Consensus		

**Multiple Sequence Alignment****B****31\_pVPinsert (nr10) 31.cds pVP16\_2**

		(forward primer)	
31_pVPinsert (nr10)	GGAATTCCCAGGGATCCGTCGACGAGCCCATTAAGAAGCA		69
31.cds	tcatccctgcccctggaactggtgGAGCCCATTAAGAAGCA		920
pVP16_2	GGAATTCCCAGGGATCCGTCGAC.....		106
Consensus			
31_pVPinsert (nr10)	CGASGTCCCARCMAAGTCWGATGTTTACTGTGAGGTGTGT		109
31.cds	CGAgGTCCCAgCaAAGTcTGATGTTTACTGTGAGGTGTGT		960
pVP16_2	.....		106
Consensus			
31_pVPinsert (nr10)	GAATTCCTGGTGAAGGARGTGACCMASCTGAWTGACMACM		149
31.cds	GAATTCCTGGTGAAGGAgGTGACCaAgCTGAtTGACaACa		1000
pVP16_2	.....		106
Consensus			

31_pVPinsert (nr10)	ACAASACTGAAAAASAAATACTCKACGCTTTTGACAAAAT	189
31.cds	ACAAGACTGAgAAAgAAATACTCgACGCTTTTGACAAAAT	1040
pVP16_2	.....	106
31_pVPinsert (nr10)	GTGCTCGAAGCTGCCSAARTCCCTRTCGGAAGAGTGCCAG	229
31.cds	GTGCTCGAAGCTGCCgAAgTCCCTgTCGGAAGAGTGCCAG	1080
pVP16_2	.....	106
Consensus		
31_pVPinsert (nr10)	GAGGTGGTGGACACGTACSGCAGCTCCATCCTGTCCATCC	269
31.cds	GAGGTGGTGGACACGTACgGCAGCTCCATCCTGTCCATCC	1120
pVP16_2	.....	106
Consensus		
31_pVPinsert (nr10)	TGCTGGASGAGGTGAGCYCTGARCTGGYGWGCASCAYGCT	309
31.cds	TGCTGGAgGAGGTGAGCcCTGAgCTGGtGtGCgCAtGCT	1160
pVP16_2	.....	106
Consensus		
31_pVPinsert (nr10)	GCACCTCTGCTCTGGCACSCSGCTGCCTGCACTGACCKYT	349
31.cds	GCACCTCTGCTCTGGCACgCgGCTGCCTGCACTGACCgtT	1200
pVP16_2	.....	106
Consensus		
31_pVPinsert (nr10)	CMCSTGACTCAGMCAAAGGACGGTGGCTTCWGCKAASTGT	389
31.cds	CaCgTGACTCAGcCAAAGGACGGTGGCTTctGCgAAgTGT	1240
pVP16_2	.....	106
Consensus		
31_pVPinsert (nr10)	GCAWKAARCTGGYGGGTTATTTGGATCKCAMCCTGRWSAA	429
31.cds	GCAagAAgCTGGtGGGTTATTTGGATCgCAaCCTGgagAA	1280
pVP16_2	.....	106
Consensus		
31_pVPinsert (nr10)	AAACARCACCAASYMKGARATCCTGSCTGCTCTTGASAAA	469
31.cds	AAACAgCACCAAgcagGAgATCCTGgCTGCTCTTGAgAAA	1320
pVP16_2	.....	106
Consensus		
31_pVPinsert (nr10)	GGCTGCAGCTTCTGCCMSACCCTTACCAKAARCARTGTG	509
31.cds	GGCTGCAGCTTCTGCCAgACCCTTACCAgAAgCAgTGTG	1360
pVP16_2	.....	106
Consensus		
31_pVPinsert (nr10)	ATCMRTTYGTGGCASAGWAMRAGCCCGTGCTKATCSAGAT	549
31.cds	ATCagTTtGTGGCAGAGtAcgAGCCCGTGCTgATCgAGAT	1400
pVP16_2	.....	106
Consensus		
31_pVPinsert (nr10)	CCTGGTGGAGGTGATGGATCCTTCCTTCSTGTGCTTGAAA	589
31.cds	CCTGGTGGAGGTGATGGATCCTTCCTTCgTGTGCTTGAAA	1440
pVP16_2	.....	106
Consensus		
31_pVPinsert (nr10)	ATTGGAGCCTGCCCCCTCGGCCATAAGCCCTTGTTGGSAA	629
31.cds	ATTGGAGCCTGCCCCCTCGGCCATAAGCCCTTGTTGGgAA	1480
pVP16_2	.....	106
Consensus		

31_pVPinsert (nr10)	CTGAGAARTGTATATGGSGYCCAAGCTACTGGTGCCARAA	669
31.cds	CTGAGAAgTGTATATGGgGcCCAAGCTACTGGTGCCAgAA	1520
pVP16_2	.....	106
Consensus		
31_pVPinsert (nr10)	CACAKASACAGCAKCCCASTGCAATGCTGTGCGAGCATTGC	709
31.cds	CACAgAgACAGCAgCCCAGTGCAATGCTGTGCGAGCATTGC	1560
pVP16_2	.....	106
Consensus		
	(reverse primer)	
31_pVPinsert (nr10)	AAACKYCATGTGTGGAAGCTAGTCTAGATAASTAATGATCA	749
31.cds	AAACgCgCATGTGTGGAAGCTAG.....	1581
pVP16_2	.....TCTAGATAAgtAATGATCA	125
Consensus		

### Multiple Sequence Alignment

#### 35\_pVPinsert 35.cds pVP16\_2

C

	(forward primer)	
35_pVPinsert	ACGGTGGGGAATTCAGCGTTGCCCGGGCCAAGTCTCGGGT	68
35.cds	agtttgtGGAgcggAGCGTTGCCCGGGCCAAGTCTCGGGT	1040
pVP16_2	ACGGTGGGGAATTC.....	0
Consensus		
35_pVPinsert	GGTCGGGAACCCCTTTGATAKCAAGACCGAGCAGGGGCCG	108
35.cds	GGTCGGGAACCCCTTTGATAgCAAGACCGAGCAGGGGCCG	1080
pVP16_2	.....	0
Consensus		
35_pVPinsert	CAGGTGGATGAAACTCAGTTTAAGAAGATCCTCGGCTACA	148
35.cds	CAGGTGGATGAAACTCAGTTTAAGAAGATCCTCGGCTACA	1120
pVP16_2	.....	0
Consensus		
35_pVPinsert	TCAACACGGGGAAGCAAGAGGGGGCGAAGCTGCTGTGTGG	188
35.cds	TCAACACGGGGAAGCAAGAGGGGGCGAAGCTGCTGTGTGG	1160
pVP16_2	.....	0
Consensus		
35_pVPinsert	TGGGGGCATTGCTGCTGACCGTGGTT.ACTTCATCCAGCC	227
35.cds	TGGGGGCATTGCTGCTGACCGTGGTT.ACTTCATCCAGCC	1199
pVP16_2	.....	26
Consensus		
35_pVPinsert	CACTGTGTTTGGAGATGTGCAGGATGGCATGACCATCGCC	267
35.cds	CACTGTGTTTGGAGATGTGCAGGATGGCATGACCATCGCC	1239
pVP16_2	.....	63
Consensus		
35_pVPinsert	AAGGAGGAGATCTTCGGGCCAGTGATGCAGATCCTGAAGT	307
35.cds	AAGGAGGAGATCTTCGGGCCAGTGATGCAGATCCTGAAGT	1279
pVP16_2	.....	89
Consensus		
35_pVPinsert	TCAAGACCATAGAGGAGGTTGTTGGGAGAGCCAACAATTC	347
35.cds	TCAAGACCATAGAGGAGGTTGTTGGGAGAGCCAACAATTC	1319

pVP16_2	.....	89
Consensus		
35_pVPinsert	CACGTACGGGCTGGCCGCAGCTGTCTTCACAAAGGATTTG	387
35.cds	CACGTACGGGCTGGCCGCAGCTGTCTTCACAAAGGATTTG	1359
pVP16_2	.....	89
Consensus		
35_pVPinsert	GACAAGGCCAATTACCTGTCCCAGGCCCTCCAGGCGGGCA	427
35.cds	GACAAGGCCAATTACCTGTCCCAGGCCCTCCAGGCGGGCA	1399
pVP16_2	.....	89
Consensus		
35_pVPinsert	CTGTGTGGGTCAACTGCTATGATGTGTTTGGAGCCCAGTC	467
35.cds	CTGTGTGGGTCAACTGCTATGATGTGTTTGGAGCCCAGTC	1439
pVP16_2	.....	89
Consensus		
35_pVPinsert	ACCCTTTGGTGGCTACAAGATGTCGGGGAGTGGCCGGGAG	507
35.cds	ACCCTTTGGTGGCTACAAGATGTCGGGGAGTGGCCGGGAG	1479
pVP16_2	.....	89
Consensus		
35_pVPinsert	TTGGGCGAGTACGGGCTGCAGGCATACACTGAAGTGAAAA	547
35.cds	TTGGGCGAGTACGGGCTGCAGGCATACACTGAAGTGAAAA	1519
pVP16_2	.....	89
Consensus		
	(reverse primer)	
35_pVPinsert	CTGTCACAGTCAAAGTGCCTCAGAAGAACTCATAAACGCG	587
35.cds	CTGTCACAGTCAAAGTGCCTCAGAAGAACTCATAA.....	1554
pVP16_2	.....ACGCG	94
Consensus		
35_pVPinsert	TCTGCAGAAGCTTCTAGATAAGTAATGATCATAATCAGCC	627
35.cds	.....	1554
pVP16_2	TCTGCAGAAGCTTCTAGATAAGTAATGATCATAATCAGCC	134
Consensus		

### Multiple Sequence Alignment

#### 62\_pVPinsert 62cds pVP16\_2

**D**

	(forward primer)	
62_pVPinsert	GCCTTGGATTGACGAGTACGGTGGGGAATTCGGGGTCACC	51
62cds	taccactggcatcgtgatggactccggtgaCGGGTCACC	480
pVP16_2	GCCTTGGATTGACGAGTACGGTGGGGAATTC.....	0
Consensus		
62_pVPinsert	CACRCTGTGCCCATCTACGAGGGGTATGCCCTCCCCATG	91
62cds	CACaCTGTGCCCATCTACGAGGGGTATGCCCTCCCCATG	520
pVP16_2	.....	0
Consensus		
62_pVPinsert	CCATCCTGCGTCTGGACCTGGCTGGCSGGGACCTGACTGA	131
62cds	CCATCCTGCGTCTGGACCTGGCTGGCcGGGACCTGACTGA	560
pVP16_2	.....	0
Consensus		
62_pVPinsert	CTACCTCATGAAGATCCTCACCGAGCGGGCTACRGCTTC	171

62cds	CTACCTCATGAAGATCCTCACCGAGCGCGGCTACaGCTTC	600
pVP16_2	.....	0
Consensus		
62_pVPinsert	ACCACCACGGCCGAGCGGGAAATCGTGCGTGACATTARGG	211
62cds	ACCACCACGGCCGAGCGGGAAATCGTGCGTGACATTaAGG	640
pVP16_2	.....	0
Consensus		
62_pVPinsert	AGAAGCTGTGCTACGTCGCCCTGGACTTCGAGCAAGAGAT	251
62cds	AGAAGCTGTGCTACGTCGCCCTGGACTTCGAGCAAGAGAT	680
pVP16_2	.....	0
Consensus		
62_pVPinsert	GGCCACGGCTGCTTCCAGCTCCTCCCTGGAGAAGAGCTAC	291
62cds	GGCCACGGCTGCTTCCAGCTCCTCCCTGGAGAAGAGCTAC	720
pVP16_2	.....	0
Consensus		
62_pVPinsert	GAGCTGCCTGACGGCCAGGTCATCACCATTGGCAATGAGC	331
62cds	GAGCTGCCTGACGGCCAGGTCATCACCATTGGCAATGAGC	760
pVP16_2	.....	0
Consensus		
62_pVPinsert	GGTTCGGCTGCCCTGAGGCACTCTTCCAGCCTTCCTTCCT	371
62cds	GGTTCGGCTGCCCTGAGGCACTCTTCCAGCCTTCCTTCCT	800
pVP16_2	.....	0
Consensus		
62_pVPinsert	GGGCATGGAGTCCTGTGGCATCCACGAAACTACCTTCAAC	411
62cds	GGGCATGGAGTCCTGTGGCATCCACGAAACTACCTTCAAC	840
pVP16_2	.....	0
Consensus		
62_pVPinsert	TCCATCATGAAGTGTGACGTGGACATCCGCAAAGACCTGT	451
62cds	TCCATCATGAAGTGTGACGTGGACATCCGCAAAGACCTGT	880
pVP16_2	.....	0
Consensus		
62_pVPinsert	ACGCCAACACAGTGCTGTCTGGCGGCACCACCATGTACCC	491
62cds	ACGCCAACACAGTGCTGTCTGGCGGCACCACCATGTACCC	920
pVP16_2	.....	0
Consensus		
62_pVPinsert	TGGCATTGCCGACAGGATGCAGAAGGAGATCACTGCCCTG	531
62cds	TGGCATTGCCGACAGGATGCAGAAGGAGATCACTGCCCTG	960
pVP16_2	.....	0
Consensus		
62_pVPinsert	GCACCCAGCACAATGAAGATCAAGATCATTGCTCCTCCTG	571
62cds	GCACCCAGCACAATGAAGATCAAGATCATTGCTCCTCCTG	1000
pVP16_2	.....	0
Consensus		
62_pVPinsert	AGCGCAAGTACTCCGTGTGGATCGGCGGCTCCATCCTGGC	611
62cds	AGCGCAAGTACTCCGTGTGGATCGGCGGCTCCgTCCTGGC	1040
pVP16_2	.....	22



## Consensus

62_pVPinsert	CTCGCTGTCCACCTTCCAGCAGATGTGGATCAGCAAGCAG	651
62cds	CTCGCTGTCCACCTTCCAGCAGATGTGGATCAGCAAGCAG	1080
pVP16_2	.....	62
62_pVPinsert	GAGTATGACGAGTCCGGCCCCCTCCATCGTCCACCGCA...	688
62cds	GAGTATGACGAGTCCGGCCCCCTCCATCGTCCACCGCA...	1117
pVP16_2	.....	101
Consensus	(reverse primer)	
62_pVPinsert	AA.GCTTCTAGATAAGTAATGATCATAATCAGCCATATCA	727
62cds	AAtGCTTCTAG.....	1128
pVP16_2	.....TCTAGAATAAGTAATGATCATAATCAGCCATAcC	140
Consensus		

**Multiple Sequence Alignment****E****99\_pVP16insert 99\_cds pVP16**

	(forward primer)	
99_pVP16insert	GATTGACGAGTACGGTGGGGAATTCCCGGGGATCCGCCGA	55
99_cds	GccctggcctggaGagaGctgAagCtgGaGtgcCaGCCGA	6773
pVP16	GATTGACGAGTACGGTGGGGAATTCCCGGGGATCC.....	2819
Consensus		
99_pVP16insert	ATTCAGTAYCTKGACCCGGGAAGCTGGTGCTGGAGGCCTG	95
99_cds	ATTCAGTAtCTgGACCCGGGAAGCTGGTGCTGGAGGCCTG	6813
pVP16	.....	2859
Consensus		
99_pVP16insert	GCCATTGCTGTTCGAGGGCCCCAGCAAGGCTGAGATCTCTT	135
99_cds	GCCATTGCTGTTCGAGGGCCCCAGCAAGGCTGAGATCTCTT	6853
pVP16	.....	2899
Consensus		
99_pVP16insert	TTGAGGACCGCAAGGACGGCTCCTGTGGTGTGGCTTATGT	175
99_cds	TTGAGGACCGCAAGGACGGCTCCTGTGGTGTGGCTTATGT	6893
pVP16	.....	2939
Consensus		
99_pVP16insert	GGTCCAGGAGCCAGGTGACTACGAAGTCTCAGTCAAGTTC	215
99_cds	GGTCCAGGAGCCAGGTGACTACGAAGTCTCAGTCAAGTTC	6933
pVP16	.....	2979
Consensus		
99_pVP16insert	AACGAG...GAACACATTCCCGACAGCCCCTTCGTGGTGC	252
99_cds	AACGAG...GAACACATTCCCGACAGCCCCTTCGTGGTGC	6970
pVP16	.....	3019
Consensus		
99_pVP16insert	CTGTGGCTTCTCCGTCTGGCGACGCCCGCCGC.CTCACTG	291
99_cds	CTGTGGCTTCTCCGTCTGGCGACGCCCGCCGC.CTCACTG	7009
pVP16	.....	3055
Consensus		
99_pVP16insert	TTTCTAGCCTTCAGGTGAGGCACCGAGAGAAACCGCCAC	331
99_cds	TTTCTAGCCTT.....	7020

pVP16 ..... 3095  
 Consensus

### Multiple Sequence Alignment

163\_pVPinsert 163cds pVP16\_2

**F**

(forward primer)

163_pVPinsert	ATTCCCGGGGATCCGTCGA	GAGGAGTCCCTGGAACATCTG	80
163cds	caaactcaagaacgcctat	GAGGAGTCCCTGGAACATCTG	4485
pVP16_2	ATTCCCGGGGATCCGTCGA.....		1822
Consensus			
163_pVPinsert	GAGACCTTCAAGCGGGAGAACA	AAAAACCTGCAGGAGGAGA	120
163cds	GAGACCTTCAAGCGGGAGAACA	AAAAACCTGCAGGAGGAGA	4525
pVP16_2	.....		1822
Consensus			
163_pVPinsert	TCTCCGACTTGACTGAGCAGTT	GGGTTCCAGCGGAAAGAC	160
163cds	TCTCCGACTTGACTGAGCAGTT	GGGTTCCAGCGGAAAGAC	4565
pVP16_2	.....		1822
Consensus			
163_pVPinsert	TATCCATGAGCTGGAGAAGGT	CCGAAAGCAGCTGGAGGCC	200
163cds	TATCCATGAGCTGGAGAAGGT	CCGAAAGCAGCTGGAGGCC	4605
pVP16_2	.....		1822
Consensus			
163_pVPinsert	GAGAAGATGGAGCTGCAGTCA	GCCCTGGAGGAGGCCGAGG	240
163cds	GAGAAGATGGAGCTGCAGTCA	GCCCTGGAGGAGGCCGAGG	4645
pVP16_2	.....		1859
Consensus			
163_pVPinsert	CCTCCC..TGGAGCACGAGGAG	GGGCAAGATCCTCCGGGCC	278
163cds	CCTCCC..TGGAGCACGAGGAG	GGGCAAGATCCTCCGGGCC	4683
pVP16_2	.....		1899
Consensus			
163_pVPinsert	CAGCTGGAGTTCAACCAGATCA	AAGGCAGAGATCGAGCGGA	318
163cds	CAGCTGGAGTTCAACCAGATCA	AAGGCAGAGATCGAGCGGA	4723
pVP16_2	.....		1938
Consensus			
163_pVPinsert	AGCTGGCAGAGAAGGACGAGG	AGATGGAACAGGCCAAGCG	358
163cds	AGCTGGCAGAGAAGGACGAGG	AGATGGAACAGGCCAAGCG	4763
pVP16_2	.....		1978
Consensus			
163_pVPinsert	CAACCACCTGCGGGTGGTGGAC	TGCTGCAGACCTCCCTG	398
163cds	CAACCACCTGCGGGTGGTGGAC	TGCTGCAGACCTCCCTG	4803
pVP16_2	.....		2018
Consensus			
163_pVPinsert	GACGCAGAGACACGCAGCCGCA	ACGAGGCCCTGAGGGTGA	438
163cds	GACGCAGAGACACGCAGCCGCA	ACGAGGCCCTGAGGGTGA	4843
pVP16_2	.....		2058
Consensus			

163_pVPinsert	AGAAGAAGATGGAAGGAGACCTCAATGAGATGGAGATCCA	478
163cds	AGAAGAAGATGGAAGGAGACCTCAATGAGATGGAGATCCA	4883
pVP16_2	.....	2098
Consensus		
163_pVPinsert	GCTCAGCCACGCCAACCGCATGGCCGCCGAGGCCCAGAAG	518
163cds	GCTCAGCCACGCCAACCGCATGGCCGCCGAGGCCCAGAAG	4923
pVP16_2	.....	2138
163_pVPinsert	CAAGTCAAGAGCCTCCAGAGCTTGTGAAGGACACCCAGA	558
163cds	CAAGTCAAGAGCCTCCAGAGCTTGTGAAGGACACCCAGA	4963
pVP16_2	.....	2178
Consensus		
163_pVPinsert	TTCCGCTGGACGATGCAGTCCGTGCCAACGACGACCTGAA	598
163cds	TTCaGCTGGACGATGCAGTCCGTGCCAACGACGACCTGAA	5003
pVP16_2	.....	2218
Consensus		
163_pVPinsert	GGAGAACATCGCCATCGTGGAGCGGCGCAACAACCTGCTG	638
163cds	GGAGAACATCGCCATCGTGGAGCGGCGCAACAACCTGCTG	5043
pVP16_2	.....	2258
Consensus		
163_pVPinsert	CAGGCTGAGCTGGAGGAGTTGCGTGCCGTGGTGGAGCAGA	678
163cds	CAGGCTGAGCTGGAGGAGTTGCGTGCCGTGGTGGAGCAGA	5083
pVP16_2	.....	2298
Consensus		
163_pVPinsert	CAGAGCGGTCCCGGAAGCTG..GCGGAGCAGGAGCTGATT	716
163cds	CAGAGCGGTCCCGGAAGCTG..GCGGAGCAGGAGCTGATT	5121
pVP16_2	.....	2338
Consensus		
163_pVPinsert	GAGACTAGTGAGCGGGTGCAGCTGCTGCATTCCCAGAACA	756
163cds	GAGACTAGTGAGCGGGTGCAGCTGCTGCATTCCCAGAACA	5161
pVP16_2	.....	2376
Consensus		
163_pVPinsert	CCAGCCTCATCAACCAGAAGAAGAAGATGGATGCTGACCT	796
163cds	CCAGCCTCATCAACCAGAAGAAGAAGATGGATGCTGACCT	5201
pVP16_2	.....	2416
Consensus		
163_pVPinsert	GTCCCAGCTCCAGACTGAAGTGGAGGAGGCAGTGCAGGAG	836
163cds	GTCCCAGCTCCAGACTGAAGTGGAGGAGGCAGTGCAGGAG	5241
pVP16_2	.....	2456
Consensus		
163_pVPinsert	TGCAGGAATGCTGAGGAGAAGGCCAAGAAAGCCATCACGG	876
163cds	TGCAGGAATGCTGAGGAGAAGGCCAAGAAgGCCATCACGG	5281
pVP16_2	.....	2496
Consensus		
163_pVPinsert	ATGCCGCCATGATGGCAGAGGAGCTGAAGAAGGAGCAGGA	916
163cds	ATGCCGCCATGATGGCAGAGGAGCTGAAGAAGGAGCAGGA	5321
pVP16_2	.....	2536
Consensus		

163_pVPinsert	CACCAGCGCCCACCTGGAGCGCATGAAGAAGAACATGGAA	956
163cnds	CACCAGCGCCCACCTGGAGCGCATGAAGAAGAACATGGAA	5361
pVP16_2	.....	2576
Consensus		
163_pVPinsert	CAGACCATTAAGGACCTGCAGCACCGGCTGGACGAAGCCG	996
163cnds	CAGACCATTAAGGACCTGCAGCACCGGCTGGACGAAGCCG	5401
pVP16_2	.....	2616
Consensus		
163_pVPinsert	AGCAGATCGCCCTCAA.GGCGGCAAGAAGCAGCTGCAGAA	1035
163cnds	AGCAGATCGCCCTCAA <sub>g</sub> GGCGGCAAGAAGCAGCTGCAGAA	5441
pVP16_2	.....	2627
Consensus		
163_pVPinsert	GCTGGAAGCGCGGGTGC GGGAGCTGGAG.ATGARCTGAGC	1074
163cnds	GCTGGAAGCGCGGGTGC GGGAGCTGGAG <sub>a</sub> ATGAgCTGgag	5481
pVP16_2	.....	2627
Consensus		
163_pVPinsert	GAGCAGAGCGCACGCAAGTCGTGATGTCATGAAGAAAGCT	1114
163cnds	GccgAGcagaagCGCAA <sub>c</sub> gCagagTcggt....GAAgGgc	5517
pVP16_2	.....gc	2629
Consensus		
163_pVPinsert	AGCGTCCATCAGGAAGCTCACTACYRAAMCGCA.....	1147
163cnds	AtgaggaAgagcGAgcggCgCatCaaggagctcacctacc	5557
pVP16_2	AGatTgtActgaGAgTgcacCatatggAcatattgtcggt	2669
Consensus	a a ga c	

### Multiple Sequence Alignment

#### 153\_pVPinsert CDS153 pVP16

**G**

	(forward primer)	
153_pVPinsert	AGTACGGTGGGGAATTCATGTGTATTCCCTCCATCATATGC	64
CDS153	AGacttGcGcGcgtccaATGTGTATTCCCTCCATCATATGC	56
pVP16	AGTACGGTGGGGAATTC.....	678
Consensus		
153_pVPinsert	TGACCTTGGCAAAGCTGCCAGAGATATTTTCAACAAAGGA	104
CDS153	TGACCTTGGCAAAGCTGCCAGAGATATTTTCAACAAAGGA	96
pVP16	.....	718
Consensus		
153_pVPinsert	TTTGGTTTTGGGTTGGTGA <sub>A</sub> ACTGGATGTGAAAACAAAGT	144
CDS153	TTTGGTTTTGGGTTGGTGA <sub>A</sub> ACTGGATGTGAAAACAAAGT	136
pVP16	.....	757
Consensus		
153_pVPinsert	CTTGCAGTGGCGTGGAAATTTTCAACGTCCGGCTCATCTAA	184
CDS153	CTTGCAGTGGCGTGGAAATTTTCAACGTCCGGtTCATCTAA	176
pVP16	.....	797
Consensus		
153_pVPinsert	TACAGACACTGGTAAAGTTACTGGGACCTTGGAGACCAAA	224

CDS153	TACAGACACTGGTAAAGTTACTGGGACCTTGGAGACCAAA	216
pVP16	.....	831
Consensus		
153_pVPinsert	TACAAGTGGTGTGAGTATGGTCTGACTTTTCACAGAAAAGT	264
CDS153	TACAAGTGGTGTGAGTATGGTCTGACTTTTCACAGAAAAGT	256
pVP16	.....	831
Consensus		
153_pVPinsert	GGAACACTGATAAACTCTGGGAACAGAAATCGCAATTGA	304
CDS153	GGAACACTGATAAACTCTGGGAACAGAAATCGCAATTGA	296
pVP16	.....	853
Consensus		
153_pVPinsert	AGACCAGATTTGTCAAGGTTTGAACTGACATTTGATACT	344
CDS153	AGACCAGATTTGTCAAGGTTTGAACTGACATTTGATACT	336
pVP16	.....	893
Consensus		
153_pVPinsert	ACCTTCTCACCAAACACAGGAAAGAAAAGTGGTAAAATCA	384
CDS153	ACCTTCTCACCAAACACAGGAAAGAAAAGTGGTAAAATCA	376
pVP16	.....	933
Consensus		
153_pVPinsert	AGTCTTCTTACAAGAGGGAGTGTATAAACCTTGGTTGTGA	424
CDS153	AGTCTTCTTACAAGAGGGAGTGTATAAACCTTGGTTGTGA	416
pVP16	.....	973
Consensus		
153_pVPinsert	TGTTGACTTTGATTTTGCTGGACCTGCAATCCATGGTTCA	464
CDS153	TGTTGACTTTGATTTTGCTGGACCTGCAATCCATGGTTCA	456
pVP16	.....	1009
Consensus		
153_pVPinsert	GCTGTCTTTGGTTATGAGGGCTGGC.....TTGCTGGCTA	499
CDS153	GCTGTCTTTGGTTATGAGGGCTGGC.....TTGCTGGCTA	491
pVP16	.....CgcgcGg...GAGGcggtttgcgtaTTGggcGCTc	1049
Consensus	c      g      gagg                  ttg      gct	
153_pVPinsert	CCAGATGACCTTTGACAGTGCCAAATCAAAGCTGACAAGG	539
CDS153	CCAGATGACCTTTGACAGTGCCAAATCAAAGCTGACAAGG	531
pVP16	.....	1089
Consensus		
153_pVPinsert	AATAACTTTGCAGTGGGCTACAGGACTGGGGACTTCCAGC	579
CDS153	AATAACTTTGCAGTGGGCTACAGGACTGGGGACTTCCAGC	571
pVP16	.....	1129
Consensus		
153_pVPinsert	TACACACTAATGTCAATGATGGGACAGAATTTGGAGGATC	619
CDS153	TACACACTAATGTCAAcGATGGGACAGAATTTGGAGGATC	611
pVP16	.....	1169
Consensus		
153_pVPinsert	AATTTATCAGAAAGTTTGTGAAGATCTTGACACTTCAGTA	659
CDS153	AATTTATCAGAAAGTTTGTGAAGATCTTGACACTTCAGTA	651
pVP16	.....	1209

## Consensus

153\_pVPinsert AACCTTGC.TTGGACATCAGGTACCAACTGCACTCGTTTT 698  
 CDS153 AACCTTGC.TTGGACATCAGGTACCAACTGCACTCGTTTT 690  
 pVP16 ..... 1247  
 Consensus

153\_pVPinsert GGCATTGCAGCT....AAATATCAGTTGGATCCCCTGCT 734  
 CDS153 GGCATTGCAGCT....AAATATCAGTTGGATCCCCTGCT 726  
 pVP16 ..... 1287  
 Consensus

153\_pVPinsert TCCATTTCTGCAAAAAGTCAACAACCTCTAGCTTAA..... 768  
 CDS153 TCCATTTCTGCAAAAAGTCAACAACCTCTAGCTTAA..... 760  
 pVP16 ..... 1320  
 Consensus

**Multiple Sequence Alignment****H****164\_pVPinsert CDS164 pVP16\_3**

164\_pVPinsert .TRCTTACGATGCCCTTGG.ATTGACGAGTACGGTGGGGA 38  
 CDS164 ggtggTtactgaCtgcattc.cagGtgGatccCcccGaGcg 599  
 pVP16\_3 tgttTacCGATGCCCTTGGaATTGACGAGTACGGTGGGGA 71  
 Consensus c g ga c g g  
 (forward primer)

164\_pVPinsert ATTCCTCCGCCCCCAGCGACGATCTCACCTCTTGGAA 78  
 CDS164 gccCCCTCCGCCCCCAGCGACGATCTCACCTCTTGGAA 639  
 pVP16\_3 ATTC..... 88  
 Consensus c

164\_pVPinsert AGCAGCTCCAGTTACAAGAACCTCACGCTCAAATTCCACA 118  
 CDS164 AGCAGCTCCAGTTACAAGAACCTCACGCTCAAATTCCACA 679  
 pVP16\_3 ..... 88  
 Consensus

164\_pVPinsert AGCTGGTCAATGTCAACCATCCACTTCCGGCTGAAGACCAT 158  
 CDS164 AGCTGGTCAATGTCAACCATCCACTTCCGGCTGAAGACCAT 719  
 pVP16\_3 ..... 88  
 Consensus

164\_pVPinsert TAACCTCCAGAGCCTCATCAATAATGAGATCCCGGACTGC 198  
 CDS164 TAACCTCCAGAGCCTCATCAATAATGAGATCCCGGACTGC 759  
 pVP16\_3 ..... 88  
 Consensus

164\_pVPinsert TATACCTTCAGCGTCCTGATCACGTTTGGACAACAAAGCAC 238  
 CDS164 TATACCTTCAGCGTCCTGATCACGTTTGGACAACAAAGCAC 799  
 pVP16\_3 ..... 88  
 Consensus

164\_pVPinsert ACAGTGGGCGGATCCCCATCAGCCTGGAGACCCAGGCCCA 278  
 CDS164 ACAGTGGGCGGATCCCCATCAGCCTGGAGACCCAGGCCCA 839  
 pVP16\_3 ..... 88  
 Consensus

164\_pVPinsert CATCCAGGAGTGTAAAGCACCCAGTGTCTTCCAGCACGGA 318

CDS164	CATCCAGGAGTGTAAAGCACCCCAGTGTCTTCCAGCACGGA	879
pVP16_3	.....	88
Consensus		
164_pVPinsert	GACAACAGCTTCCGGCTCCTGTTTGACGTGGTGGTCATCC	358
CDS164	GACAACAGCTTCCGGCTCCTGTTTGACGTGGTGGTCATCC	919
pVP16_3	.....	88
Consensus		
164_pVPinsert	TCACCTGCTCCCTGTCTTCCCTCCTCTGCGCCCGCTCACT	398
CDS164	TCACCTGCTCCCTGTCTTCCCTCCTCTGCGCCCGCTCACT	959
pVP16_3	.....	88
Consensus		
164_pVPinsert	CCTTCGAGGCTTCCCTGCTGCAGAACGAGTTTGTGGGGTTC	438
CDS164	CCTTCGAGGCTTCCCTGCTGCAGAACGAGTTTGTGGGGTTC	999
pVP16_3	.....	88
Consensus		
164_pVPinsert	ATGTGGCGGCAGCGGGGACGGGTATCAGCCTGTGGGAGC	478
CDS164	ATGTGGCGGCAGCGGGGACGGGTATCAGCCTGTGGGAGC	1039
pVP16_3	.....	112
Consensus		
164_pVPinsert	GGCTGGAATTTGTCAATGGCTGGTACATCCTGCTCGTCAC	518
CDS164	GGCTGGAATTTGTCAATGGCTGGTACATCCTGCTCGTCAC	1079
pVP16_3	.....	119
Consensus		
164_pVPinsert	CAGCGATGTGCTCACCATCTCGGGCACCATCATGAAGATC	558
CDS164	CAGCGATGTGCTCACCATCTCGGGCACCATCATGAAGATC	1119
pVP16_3	.....	119
Consensus		
164_pVPinsert	GGCATCGAGGCCAAGAAGCTTGGCGAGCTACGACGTCTGCA	598
CDS164	GGCATCGAGGCCAAGAAGCTTGGCGAGCTACGACGTCTGCA	1159
pVP16_3	.....	119
Consensus		
164_pVPinsert	GCATCCTCCTGGGCACCTCGACGCTGCTGGTGTGGGTGGG	638
CDS164	GCATCCTCCTGGGCACCTCGACGCTGCTGGTGTGGGTGGG	1199
pVP16_3	.....	119
Consensus		
164_pVPinsert	CGTGATCCGCTACCTGACCTTCTTCCACAACACTACAATATC	678
CDS164	CGTGATCCGCTACCTGACCTTCTTCCACAACACTACAATATC	1239
pVP16_3	.....	119
Consensus		
164_pVPinsert	CTCATCGCCACACTGCGGGTGGCCCTGCCCAGCGTCATGC	718
CDS164	CTCATCGCCACACTGCGGGTGGCCCTGCCCAGCGTCATGC	1279
pVP16_3	.....	119
Consensus		
164_pVPinsert	GCTTCTGCTGCTGCGTGGCTGTCATCTACCTGGGCTACTG	758
CDS164	GCTTCTGCTGCTGCGTGGCTGTCATCTACCTGGGCTACTG	1319
pVP16_3	.....	119

## Consensus

164_pVPinsert	CTTCTGTGGCCGGATCGTGCTGGGGCCCTATCATGTGGAG	796
CDS164	CTTCTGTGGctGGATCGTGCTGGGGCCCTATCATGTGGAG	1359
pVP16_3	.....	119
Consensus		
164_pVPinsert	TCCCAGCTCTACCTTTACTCCTTCATCAGCCTCTTCATCT	809
CDS164	TCCCAGCTCTACCTTTACTCCTTCATCAGCCTCTTCATCT	1519
pVP16_3	.....	119
Consensus		
164_pVPinsert	ACATGGTGCTCAGCCTCTTCATCGCGCTCATCACCGGCGC	849
CDS164	ACATGGTGCTCAGCCTCTTCATCGCGCTCATCACCGGCGC	1559
pVP16_3	.....	119
Consensus		
164_pVPinsert	CTACGACACCATCAAGCATCCCGGGCGGCACGCGCAGAG	889
CDS164	CTACGACACCATCAAGCATCCCGGGCGCagGCGCAGAG	1599
pVP16_3	.....	119
Consensus		
164_pVPinsert	GAGAGCGAGCTGCACGCCTACATCGCACAGTGCCAGGACA	929
CDS164	GAGAGCGAGCTGCagGCCTACATCGCACAGTGCCAGGACA	1639
pVP16_3	.....	119
Consensus		
164_pVPinsert	GCCCCACCTCCCGGCAAGTTCCGCCGCGGGAGCGGCTCGG	969
CDS164	GCCCCACCT.CCGGCAAGTTCCGCCGCGGGAGCGGCTCGG	1678
pVP16_3	.....	119
Consensus		
164_pVPinsert	CCTGCAGCCTTCTCTGCTGCTGCGGAAGGGACCCCCTCGG	1009
CDS164	CCTGCAGCCTTCTCTGCTGCTGCGGAAGGGACCCCCTCGG	1717
pVP16_3	.....	119
Consensus		
164_pVPinsert	AGGAGCATTGCTGCTGGTGATGATTGACCTGACTGCCG	1049
CDS164	AGGAGCATTGCTGCTGGTGAattga.....	1743
pVP16_3	.....	119
Consensus		

**FILE: Multiple\_Sequence\_Alignment****NAMES: 204\_pVPinsert 204.cds pVP16\_3****I**

(forward primer)

204_pVP	ACGCGT CACGGGCAGCTGCTTGCCTACTGGCAGAGCTGCTGCGAGGACCCTGTGGCA	48
cds204	.....CACGGGCAGCTGCTTGCCTACTGGCAGAGCTGCTGCGAGGACCCTGTGGCA	9
pVP16_3	ACGCGT.....	50
Consensus		

(reverse primer)

204_pVP	GCTTTGATGTGCGCAAGACAGCTGACGACTGATCTAGATAAGTAATGATCATGCTGGC	88
cds204	GCTTTGATGTGCGCAAGACAGCTGACGACTGA.....	49
pVP16_3	.....TCTAGATAAGTAATGATCATGCTGGC	90
Consensus		



## Multiple\_Sequence\_Alignment

## 222\_pVP16insert 222cds pVP16\_3

(forward primer)

222_pVP16insert	ATGCCTTGG..ATTGACGAGTACGGTGGGGAATTCATGCC	48
222cds	.....ATGCC	5
pVP16_3	ATGCCcTtGgaATTGACGAGTACGGTGGGGAATTC.....	75
Consensus		
222_pVP16insert	ATTCGGTAACACCCACAACAAGTTCAAGCTGAATTACAAG	88
222cds	ATTCGGTAACACCCACAACAAGTTCAAGCTGAATTACAAG	45
pVP16_3	.....	75
Consensus		
222_pVP16insert	CCTGAGGAGGAGTACCCCGACCTCAGCAAACATAACAACC	128
222cds	CCTGAGGAGGAGTACCCCGACCTCAGCAAACATAACAACC	85
pVP16_3	.....	75
Consensus		
222_pVP16insert	ACATGGCCAAGGTACTGACCCTTGAAGCTCTACAAGAAGCT	168
222cds	ACATGGCCAAGGTACTGACCCTTGAAGCTCTACAAGAAGCT	125
pVP16_3	.....	75
Consensus		
222_pVP16insert	GCGGGACAAGGAGACTCCATCTGGCTTCACTGTAGACGAT	208
222cds	GCGGGACAAGGAGACTCCATCTGGCTTCACTGTAGACGAT	165
pVP16_3	.....	75
Consensus		
222_pVP16insert	GTCATCCAGACAGGAGTGGACAACCCAGGTCACCCCTTCA	248
222cds	GTCATCCAGACAGGAGTGGACAACCCAGGTCACCCCTTCA	205
pVP16_3	.....	75
Consensus		
222_pVP16insert	TCATGACCGTGGGCTGCGTGGCTGGTGATGAGGAGTCCTA	288
222cds	TCATGACCGTGGGCTGCGTGGCTGGTGATGAGGAGTCCTA	245
pVP16_3	.....	75
Consensus		
222_pVP16insert	CGAAGTTTTCAAGGAAGCTTTTGACCCCATCATCTCGGAT	328
222cds	CGAAGTTTTCAAGGAAGCTTTTGACCCCATCATCTCGGAT	285
pVP16_3	.....	75
Consensus		
222_pVP16insert	CGCCACGGGGGCTACAAACCCACTGACAAGCACAAGACTG	368
222cds	CGCCACGGGGGCTACAAACCCACTGACAAGCACAAGACTG	325
pVP16_3	.....	75
Consensus		
222_pVP16insert	ACCTCAACCATGAAAACCTCAAGGGTGGAGACGACCTGGA	408
222cds	ACCTCAACCATGAAAACCTCAAGGGTGGAGACGACCTGGA	365
pVP16_3	.....	75
Consensus		
222_pVP16insert	CCCCAACTACGTGCTCAGCAGCCGCGTCCGCACTGGCCGC	425
222cds	CCctAACTACGTGCTCAGCAGCCGCGTCCGCACTGGCCGC	405
pVP16_3	.....	75

## Consensus

222_pVP16insert	GCGAGCGCCGGGCGGTGGAGAAGCTCTCTGTGGAAGCTCT	465
222cds	GCGAGCGCCGGGCGGTGGAGAAGCTCTCTGTGGAAGCTCT	485
pVP16_3	.....	108
Consensus		
222_pVP16insert	CAACAGCCTGACGGGCGAGTTCAAAGGGAAGTACTACCCT	505
222cds	CAACAGCCTGACGGGCGAGTTCAAAGGGAAGTACTACCCT	525
pVP16_3	.....	119
Consensus		
222_pVP16insert	CTGAAGAGCATGACGGAGAAGGAGCAGCAGTAGCTCATCG	545
222cds	CTGAAGAGCATGACGGAGAAGGAGCAGCAGcAGCTCATCG	565
pVP16_3	.....	119
Consensus		
222_pVP16insert	ATGACCACTTCCTGTTTCGACAAGCCCGTGTCCCCGCTGCT	585
222cds	ATGACCACTTCCTGTTTCGACAAGCCCGTGTCCCCGCTGCT	605
pVP16_3	.....	119
Consensus		
222_pVP16insert	GCTGGTCTCAGGCATGGCCCGCGACTGGCCCGACGCCCGT	625
222cds	GCTGGcCTCAGGCATGGCCCGCGACTGGCCCGACGCCCGT	645
pVP16_3	.....	119
Consensus		
222_pVP16insert	GGCATCTGGCACAATGACAACAAGAGCTTCCTGGTGTGGG	665
222cds	GGCATCTGGCACAATGACAACAAGAGCTTCCTGGTGTGGG	685
pVP16_3	.....	119
Consensus		
222_pVP16insert	TGAACGAGGAGGATCACCTCCGGGTCATCTCCATGGAGAA	705
222cds	TGAACGAGGAGGATCACCTCCGGGTCATCTCCATGGAGAA	725
pVP16_3	.....	119
Consensus		
222_pVP16insert	GGGGGGCAACATGAAGGAGGTTTTCCGCCGCTTCTGCGTA	745
222cds	GGGGGGCAACATGAAGGAGGTTTTCCGCCGCTTCTGCGTA	765
pVP16_3	.....	119
Consensus		
222_pVP16insert	GGGCTGCAGAAGATTGAGGAGATCTTTAAGAAAGCTGGCC	785
222cds	GGGCTGCAGAAGATTGAGGAGATCTTTAAGAAAGCTGGCC	805
pVP16_3	.....	119
Consensus		
222_pVP16insert	ACCCCTTCATGTGGAACCAGCACCTGGGCTACGTGCTCAC	825
222cds	ACCCCTTCATGTGGAACCAGCACCTGGGCTACGTGCTCAC	845
pVP16_3	.....	119
Consensus		
222_pVP16insert	CTGCCCATCCAACCTGGGCACTGGGCTGCGTGGAGGCGTG	865
222cds	CTGCCCATCCAACCTGGGCACTGGGCTGCGTGGAGGCGTG	885
pVP16_3	.....	119
Consensus		

222_pVP16insert	CATGTGAAGCTGGCGCACCTGAGCAAGCACCCCAAGTTCG	905
222cds	CATGTGAAGCTGGCGCACCTGAGCAAGCACCCCAAGTTCG	925
pVP16_3	.....	119
Consensus		
222_pVP16insert	AGGAGATCCTCACCCGCCTGCGTCTGCAGAAGAGGGGTAC	945
222cds	AGGAGATCCTCACCCGCCTGCGTCTGCAGAAGAGGGGTAC	965
pVP16_3	.....	119
Consensus		
222_pVP16insert	AGGTGGCGTGGACACAGCTGCCGTGGGCTCAGTATTTGAC	985
222cds	AGGTGGCGTGGACACAGCTGCCGTGGGCTCAGTATTTGAC	1005
pVP16_3	.....	119
Consensus		
222_pVP16insert	GTGTCCCAACGCTGATCGGCTGGGCTCGTCCGAAGTAG.A	1024
222cds	GTGT.CCAACGCTGATCGGCTGGGCTCGTCCGAAGTAGaA	1044
pVP16_3	.....	119
Consensus		
222_pVP16insert	CAGGTGCAGCTGGTGGTGGATGGTGTGAAGCTCATGTTGA	1064
222cds	CAGGTGCAGCTGGTGGTGGATGGTGTGAAGCTCATG <sub>g</sub> TG <sub>g</sub>	1084
pVP16_3	.....	119
Consensus		
222_pVP16insert	ATGGAGAGAAGAAGTTGGAGAAAGGCCAGTCCATCGACGA	1100
222cds	AaatgGAGAAGAAGTTGGAGAAAGGCCAGTCCATtGACGA	1124
pVP16_3	.....	119
Consensus		
222_pVP16insert	CCATGATCCCGCCAGAGATAGCGCCTGCCCCATCTAKC	1138
222cds	.CATGATCCcGcCccAGAagtaG.....	1146
pVP16_3	.....	119
Consensus		

### Multiple Sequence Alignment

#### 306\_pVPinsert 306cds pVP16\_3

**K**

		(forward primer)	
306_pVPinsert	GRRKGCCKTGGATTGASGAGTACGGTGGGGAATTCATGGC		49
306cds	.....ATGGC		5
pVP16_3	atgccCttgGaATTGAcGAGTACGGTGGGGAATTC.....		80
Consensus			
306_pVPinsert	CTCCAAATGCCCCAAGTGCGACAAGACCGTGTACTTCGCC		89
306cds	CTCCAAATGCCCCAAGTGCGACAAGACCGTGTACTTCGCC		45
pVP16_3	.....		99
Consensus			
306_pVPinsert	GAGAAGGTGAGCTCCCTGGGGAARGACTGGCACAAGTTCT		129
306cds	GAGAAGGTGAGCTCCCTGGGGAAGACTGGCACAAGTTCT		85
pVP16_3	.....		99
Consensus			
306_pVPinsert	GCCTCAAGTGCGAGCGCTGCAGCAAGACGCTGACGCCCGG		169
306cds	GCCTCAAGTGCGAGCGCTGCAGCAAGACGCTGACGCCCGG		125
pVP16_3	.....		99

Consensus		
306_pVPinsert	GGGCCACGCCGAGCATGACGGGAAGCCGTTCTGCCACAAG	209
306cds	GGGCCACGCCGAGCATGACGGGAAGCCGTTCTGCCACAAG	165
pVP16_3	.....	99
Consensus		
306_pVPinsert	CCGTGCTACGCCACCCTGTTCTGGACCCAAAGGCGTGAACA	249
306cds	CCGTGCTACGCCACCCTGTTCTGGACCCAAAGGCGTGAACA	205
pVP16_3	.....	99
306_pVPinsert	TCGGGGGCGCGGGCTCCTACATCTACGAGAAGCCCCTGGC	289
306cds	TCGGGGGCGCGGGCTCCTACATCTACGAGAAGCCCCTGGC	245
pVP16_3	.....	99
Consensus		
306_pVPinsert	GGAGGGGCGCAGGTCACCGGCCCCATCGAGGTCCCCGCG	329
306cds	GGAGGGGCGCAGGTCACCGGCCCCATCGAGGTCCCCGCG	285
pVP16_3	.....	99
Consensus		
306_pVPinsert	GCCCGAGCAGAGGAGCGGAAGGCGAGCGGCCCCCGAAGG	369
306cds	GCCCGAGCAGAGGAGCGGAAGGCGAGCGGCCCCCGAAGG	325
pVP16_3	.....	99
Consensus		
306_pVPinsert	GGCCCAGCAGAGCCTCCAGTGTCACTTTTACCGGGGA	409
306cds	GGCCCAGCAGAGCCTCCAGTGTCACTTTTACCGGGGA	365
pVP16_3	.....	99
Consensus		
306_pVPinsert	GCCCAACACGTGCCCGCGCTGCGGCAAGAAGGTGTACTTC	449
306cds	GCCCAACACGTGCCCGCGCTGCaGCAAGAAGGTGTACTTC	405
pVP16_3	.....	99
Consensus		
306_pVPinsert	GCTGAGAAGGTGATGTCTCTGGGCAAGGATTGGCACCGGC	489
306cds	GCTGAGAAGGTGAcGTCTCTGGGCAAGGATTGGCACCGGC	445
pVP16_3	.....	99
Consensus		
306_pVPinsert	CCTGCCTGCGCTGCGAGCGCTGCGGGAAGACTGACCCC	529
306cds	CCTGCCTGCGCTGCGAGCGCTGCGGGAAGACTGACCCC	485
pVP16_3	.....	99
Consensus		
306_pVPinsert	CGGCGGGCACGCGGAGCACGACGGCCAGCCCTACTGCCAC	569
306cds	CGGCGGGCACGCGGAGCACGACGGCCAGCCCTACTGCCAC	525
pVP16_3	.....	99
Consensus		
306_pVPinsert	AAGCCCTGCTATGGAATCCTCTTCGGACCCAAGGGAGTGA	609
306cds	AAGCCCTGCTATGGAATCCTCTTCGGACCCAAGGGAGTGA	565
pVP16_3	.....	99
Consensus		
306_pVPinsert	ACACCGGTGCGGTGGGCAGCTACATCTATGACCGGGACCC	649
306cds	ACACCGGTGCGGTGGGCAGCTACATCTATGACCGGGACCC	605

pVP16_3	.....	99
Consensus		
	(reverse primer)	
306_pVPinsert	CGAAGGCAAGGTCCAGCCCTAGAAAGCTTCTAGATAAGTAA	689
306cnds	CGAAGGCAAGGTCCAGCCCTAG.....	627
pVP16_3	.....AGAAGCTTCTAGATAAGTAA	119
Consensus	ag	

### Multiple Sequence Alignment

#### Bait F (nr4) GJA5 cyto tail pM\_2

**L**

	(forward primer)	
Bait F (nr4)	MTGTATCGCCGGATTCCACCTGGGCTGGAAGAAGATCAGA	49
GJA5 cyto tail	.....CACCTGGGCTGGAAGAAGATCAGA	24
pM_2	cTGTATCGCCGGAaTCCcCggGGatcc.....	67
Consensus	c c gg	
Bait F (nr4)	CAGCGATTTGTCAAACCGCGGCAGCACATGGCTAAGTGCC	89
GJA5 cyto tail	CAGCGATTTGTCAAACCGCGGCAGCACATGGCTAAGTGCC	64
pM_2	.....	67
Consensus		
Bait F (nr4)	AGCTTTCTGGCCCCTCTGTGGGCATAGTCCAGAGCTGCAC	129
GJA5 cyto tail	AGCTTTCTGGCCCCTCTGTGGGCATAGTCCAGAGCTGCAC	104
pM_2	.....	67
Consensus		
Bait F (nr4)	ACCACCCCCGACTTTAATCAGTGCCTGGAGAATGGCCCT	169
GJA5 cyto tail	ACCACCCCCGACTTTAATCAGTGCCTGGAGAATGGCCCT	144
pM_2	.....	67
Consensus		
Bait F (nr4)	GGGGGAAAATTCTTCAATCCCTTCAGCAATAATATGGCCT	209
GJA5 cyto tail	GGGGGAAAATTCTTCAATCCCTTCAGCAATAATATGGCCT	184
pM_2	.....	67
Consensus		
Bait F (nr4)	CCCAACAAAACACAGACAACCTGGTCACCGAGCAAGTACG	249
GJA5 cyto tail	CCCAACAAAACACAGACAACCTGGTCACCGAGCAAGTACG	224
pM_2	.....	67
Consensus		
Bait F (nr4)	AGGTCAGGAGCAGACTCCTGGGGAAGGTTTCATCCAGGTT	289
GJA5 cyto tail	AGGTCAGGAGCAGACTCCTGGGGAAGGTTTCATCCAGGTT	264
pM_2	.....	67
Consensus		
Bait F (nr4)	CGTTATGGCCAGAAGCCTGAGGTGCCCAATGGAGTCTCAC	329
GJA5 cyto tail	CGTTATGGCCAGAAGCCTGAGGTGCCCAATGGAGTCTCAC	304
pM_2	.....	67
Consensus		
Bait F (nr4)	CAGGTCACCGCCTTCCCCATGGCTATCATAGTGACAAGCG	369
GJA5 cyto tail	CAGGTCACCGCCTTCCCCATGGCTATCATAGTGACAAGCG	344
pM_2	.....	67
Consensus		

Bait F (nr4)	ACGTCTTAGTAAGGCCAGCAGCAAGGCAAGGTCAGATGAC	409
GJA5 cyto tail	ACGTCTTAGTAAGGCCAGCAGCAAGGCAAGGTCAGATGAC	384
pM_2	.....	67
Consensus	(reverse primer)	
Bait F (nr4)	CTATCAGTGTGAGTCGACGCGTCTGCAGAAGCTTCTAGAT	449
GJA5 cyto tail	CTATCAGTGTGA.....	396
pM_2	.....GTCGACGCGTCTGCAGAAGCTTCTAGAT	95
Consensus		

### Multiple Sequence Alignment

Rev\_Com\_Bait GJA5 cyto tail pM\_2

**M**

	(forward primer)	
Rev_Com_Bait	GTTGACTGTATCGCCGGAATTCACCTGGGCTGGAAGAAG	800
GJA5 cyto tail	.....CACCTGGGCTGGAAGAAG	18
pM_2	GTTGACTGTATCGCCGGAATTC.....	67
Consensus		
Rev_Com_Bait	ATCAGACAGCGATTTGTCAAACCGCGGCAGCACATGGCTA	840
GJA5 cyto tail	ATCAGACAGCGATTTGTCAAACCGCGGCAGCACATGGCTA	58
pM_2	.....	67
Consensus		
Rev_Com_Bait	AGTGCCAGCTTTCTGGCCCCTCTGTGGGCATAGTCCAGAG	880
GJA5 cyto tail	AGTGCCAGCTTTCTGGCCCCTCTGTGGGCATAGTCCAGAG	98
pM_2	.....	67
Consensus		
Rev_Com_Bait	CTGCACACCACCCCCSMCYTAATCAGTGCCTGGAGAAT	920
GJA5 cyto tail	CTGCACACCACCCCCgaCttTAATCAGTGCCTGGAGAAT	138
pM_2	.....	67
Consensus		
Rev_Com_Bait	GGCCCTGGGGGAAAATTCTTCAATCCCTTCAGCAATAATA	960
GJA5 cyto tail	GGCCCTGGGGGAAAATTCTTCAATCCCTTCAGCAATAATA	178
pM_2	.....	67
Consensus		
Rev_Com_Bait	TGGCCTCCCAACAAAACACAGACAACCTGGTCACCGAGCA	1000
GJA5 cyto tail	TGGCCTCCCAACAAAACACAGACAACCTGGTCACCGAGCA	218
pM_2	.....	67
Consensus		
Rev_Com_Bait	AGTACGAGGTCAGGAGCAGACTCCTGGGGAAGGTTTCATC	1040
GJA5 cyto tail	AGTACGAGGTCAGGAGCAGACTCCTGGGGAAGGTTTCATC	258
pM_2	.....	67
Consensus		
Rev_Com_Bait	CAGGTTCGTTATGGCCAGAAGCCTGAGGTGCCCAATGGAG	1080
GJA5 cyto tail	CAGGTTCGTTATGGCCAGAAGCCTGAGGTGCCCAATGGAG	298
pM_2	.....	67
Consensus		
Rev_Com_Bait	TCTCACCAGGTCWCCGCTTCCCCATGGCTATCATAGTGA	1120

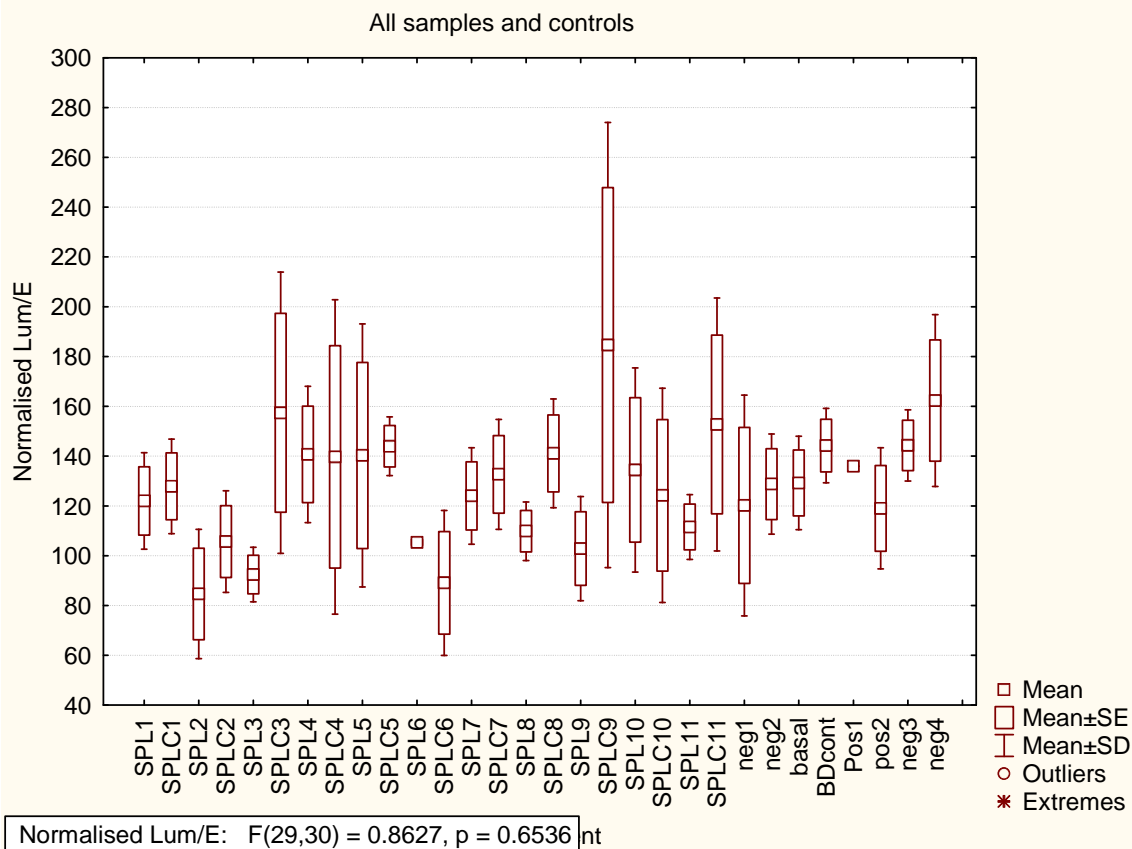
GJA5 cyto tail	TCTCACCAGGTCaCCGCCTTCCCCATGGCTATCATAGTGA	338
pM_2	.....GTCgaCGCgTctgCagaaGCTtctAgAtaag	98
Consensus	gtc cgc t c gct a a	
Rev_Com_Bait	CAAGCGACGTCTTAGTAAGGCCAGCAGCAAGGCAACGMG.	1159
GJA5 cyto tail	CAAGCGACGTCTTAGTAAGGCCAGCAGCAAGGCAAgGtca	378
pM_2	tAA.....	101
Consensus	aa	

**Figure 3.11 Sequence homology alignments of each of the 11 prey-inserts and the GJA5 bait-insert with their reference sequences from the Genbank DNA database (<http://www.ncbi.nlm.nih.gov/Entrez>) and their respective cloning vectors.** Shaded boxes – grey: gene specific primer sequence; red: restriction enzyme recognition sequence; yellow: vector sequence. A: NADH dehydrogenase, subcomplex, 6 (*NDUFA6*), B: Prosaposin (*PSAP*), C: Aldehyde dehydrogenase 2 (*ALDH2*), D: Beta-actin (*ACTB*), E: Filamin A (actin binding protein 280) (*FLNA*), F: Myosin, heavy polypeptide 7 (*MYH7*), G: Voltage-dependent anion channel 2 (*VDAC2*), H: Mucolipin 1 (*MCOLN1*), I: DEAH box polypeptide 30 (*DHX30*), J: Creatine kinase, muscle type (*CKM*), K: Cysteine-rich protein 2 (*CRP2*), L: *GJA5* bait-insert forward sequence, M: *GJA5* bait-insert reverse sequence.

### 3.3.1.5 SEAP and $\beta$ -Galactosidase enzyme assay

The SEAP and  $\beta$ -Galactosidase enzyme assays were performed with the pM-*GJA5* and pVP16-prey constructs, as described in section 2.21 and table 2.8, and the assay results are shown in figure 3.12. The assay results for the controls were incorrect, which indicated that the M2H system performed inefficiently. The SEAP activity for the negative controls was higher than the activity of the positive and basal controls. Therefore it was not possible to interpret the results for the experimental samples (category A, table 2.8).

The SEAP activity for most of the experiments was in the same range as that observed for the basal control. This indicated that no interactions occurred for the bait and prey experiments or the controls that could have activated the expression of *SEAP*. The  $\beta$ -Gal assay indicated that the results of the SEAP assay were due to poor transfection efficiency of the H9C2 cells. Furthermore, statistical evaluation showed no significant p-values for the bait and prey interaction experiments.



**Figure 3.12** Box plot results of SEAP assay with values normalised to the  $\beta$ -Gal absorbance values. Abbreviations: SPL: sample; SPLC: sample control. For categories, see table 2.8, sections 2.14.3 and 2.21.1

SPL1= bait and prey interaction experiment1 (category A)

SPLC1= prey control for experiment1 (category E)

Neg1= untransfected control (category B1)

Neg2= GeneJuice control (category B2)

Basal= basal control (category C)

BD control= GAL4 DNA-BD control (category D)

Pos1= positive control (category F1)

Pos2= positive control (category F2)

Neg3= negative control containing only pG5SEAP (category G1)

Neg4= negative control containing only pSV- $\beta$ -Gal (category G2)



## *Chapter 4*

### *Discussion*

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## 4.1 Interactome analysis of Cx40

### 4.1.1 Yeast-2-Hybrid analysis

#### 4.1.1.1 Library mating efficiency and number of independent clones screened

Mating efficiency calculations showed that approximately  $10.4 \times 10^4$  pretransformed cardiac cDNA library clones were screened using the pGBKT7-*GJA5* bait construct (section 3.1.3.1). This was less than the library titre which was  $1.412 \times 10^8$  cfu/ml. This indicated that  $10^3$  library clones were not screened and that it is possible that important ligands might have been missed. Even though less than the desired number of one million clones was screened, the number of independent clones screened was still sufficient to yield significant Y2H library screening results.

#### 4.1.1.2 Preys excluded from further analysis

Following the Y2H library screening, 33 prey clones shown in tables 3.6A and 3.6B, were identified as putative *GJA5* ligands (section 3.1.3.6). BLAST-search and internet database literature searches were done for each of the ligands which made use of websites such as <http://harvester.embl.de>, (bioinformatic harvester) <http://www.ncbi.nlm.nih.gov> (national centre for biotechnological information) and <http://www.hprd.org> (human protein reference database). This made it possible to prioritise the 33 prey clones according to their function and subcellular location as shown in table 4.1, in order to facilitate the selection of the most plausible *GJA5* ligands for further analysis. Following is a discussion of the preys that were excluded from further analysis based on their BLAST-search and internet database literature search results.

##### 4.1.1.2.1 Insignificant BLAST-search results

From the 33 prey clones that were sequenced (section 3.1.3.6, tables 3.6A and 3.6B), five (clones # 133, 37, 55, 23, 79, 95) were identified as containing partial cloning vector

sequences and partial mitochondrial sequences and were subsequently excluded from the study. Four of the prey clone inserts (clone# 136, 154, 257, 285) gave no significant protein matches in the NCBI Genbank protein databases (<http://www.ncbi.nlm.nih.gov/BLASTp>), even though they had significant DNA matches in the NCBI DNA database (<http://www.ncbi.nlm.nih.gov/BLASTn>). A reason for the lack of protein matches for these prey inserts could be that in classical two-hybrid library constructions, the cDNA library is constructed from the 3'-UTR and not the 5'-UTR. This results in only one out of six of the library cloned inserts being in frame with the transcription factor activation domain (Van Crielinge and Beyaert, 1999). These four prey clones were subsequently excluded from further analysis. In addition, the prey-insert of clone# 219 was found to be in the reverse orientation in the library vector, for which the reason is unknown. This prey insert would have produced the wrong protein product if it were to be cloned into a new vector and transfected into the mammalian cells for M2H.

#### **4.1.1.2.2 Subcellular localisation vs. function**

According to the Y2H method, insignificant ligands are excluded from further analysis based on their function and subcellular localisation. Internet database literature searches showed that most of the ligands in the present study were localised either in the cytoplasm, mitochondria, nucleus or in plasma membranes. Ligands with these subcellular locations were not excluded due to reasons which will be discussed below. Only one ligand, namely, clone# 250 (structural maintenance of chromosome 1), to be discussed below, was excluded due to functions which did not apply to the present study.

Ligands that had their subcellular location in the cytoplasm, or that were membrane bound proteins, were given preference when selecting the most plausible *GJA5* ligands. The reason for this was that the Cx40 protein is a membrane-bound protein and that its C-terminus, which interacts with the ligands, extends into the cytoplasm. Membrane-bound proteins had the possibility to interact with Cx40 based on their proximity to it and were therefore not excluded from the study.

Recent studies by Boengler and colleagues (Boengler *et al.*, 2005) showed that Cx43 has its subcellular location not only in the cytoplasm, but interestingly, also in the nuclei and mitochondria of cardiomyocytes. They identified this by means of immuno-electron microscopy, confocal microscopy and Western blot analysis. Therefore, clones that were found to localise to the nucleus or mitochondria were not excluded from the study. The prey-insert of clone# 250 (structural maintenance of chromosome 1) was found to be localised in the nucleus; however, its function proved to be insignificant for the present study and it was therefore excluded from further analysis. Another prey clone (clones #163 and 230; Myosin heavy polypeptide 7) is located in the sarcomere. Cxs have not been reported to reside in the sarcomere. Therefore, based on its localisation, it should have been excluded, but because it was present in more than one copy amongst the prey clones, and because the present study aims to add to the knowledge about Cxs, it was decided to include it for further analysis.

#### **4.1.1.3 Preys chosen as putative Cx40 ligands**

From the Y2H analysis, 11 preys were identified as having strong candidature for being Cx40 interacting ligands based on their function and subcellular location. These preys were subsequently subjected to the M2H analysis, in order to verify this. The following sections will discuss the rationale, based on current literature, for selecting these proteins as putative Cx40 interacting ligands. In addition, table 4.1 shows a list of the prioritised ligands and figure 4.1 shows a speculative model of the Cx40 interactome developed during the present study.

##### **4.1.1.3.1 Cysteine-rich protein 2**

The family of cysteine-rich proteins (CRPs) includes three members, namely, CRP1, CRP2 and CRP3 (Grubinger *et al.*, 2004; Chang *et al.*, 2003). They share high sequence homologies but differ in their expression patterns. Interestingly, similar to the cardiac Cx isoforms, the CRP proteins have been shown to have distinct patterns of tissue

distribution during cardiovascular development (Jain *et al.*, 1998), for which CRP2 will be discussed in following sections. This in turn also raises the question about their individual functions and the roles that they play in the development of the heart (Jain *et al.*, 1998). It has been shown that the CRP proteins associate with the actin cytoskeleton in the nucleus and cytoplasm. Their presence in these different cellular compartments indicates possible differential functions of the CRP proteins that still have to be elucidated (Grubinger *et al.*, 2004).

Table 4.1 Prioritised list of putative Cx40-interacting ligands

Clone number	Identification of putative ligands	Subcellular localisation	Reference
306, 313, 314, 273, 271, 275, 278, 295, 299	Cysteine-rich protein 2 (CRP2)	Cytoplasmic (Cell cortex, actin-rich structures) Nucleus	Van Ham <i>et al.</i> , 2003
62	Beta-actin (ACTB)	Cytoplasmic (Cell cortex)	Welch <i>et al.</i> , 2002
222	Creatine kinase, muscle type (CKM)	Cytoplasmic	Park <i>et al.</i> , 2002
163, 230	Myosin, heavy polypeptide 7 (MYH7)	Thick filaments of the myofibrils	Perrot <i>et al.</i> , 2005
164	Mucolipin 1 (MCOLN1)	Transmembrane protein	Pedersen <i>et al.</i> , 2005
153	Voltage dependent anion channel 2 (VDAC2)	Outer mitochondrial membrane	Mannella <i>et al.</i> , 1992
35	Aldehyde dehydrogenase 2 family (ALDH2)	Mitochondrial matrix	Perozich <i>et al.</i> , 1999
204	DEAH (Asp-Glu-Ala-His) box polypeptide 30 (DHX30)	Transmembrane protein	Silverman <i>et al.</i> , 003
29	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6 (NDUFA6)	Mitochondrial inner membrane, matrix side	Chretien <i>et al.</i> , 2003
31	Prosaposin (PSAP)	Transmembrane protein	Hiraiwa <i>et al.</i> , 1992
99	Filamin A, alpha (actin binding protein 280) (FLNA)	Cytoplasmic (peripheral cytoplasm)	Gorlin <i>et al.</i> , 1990

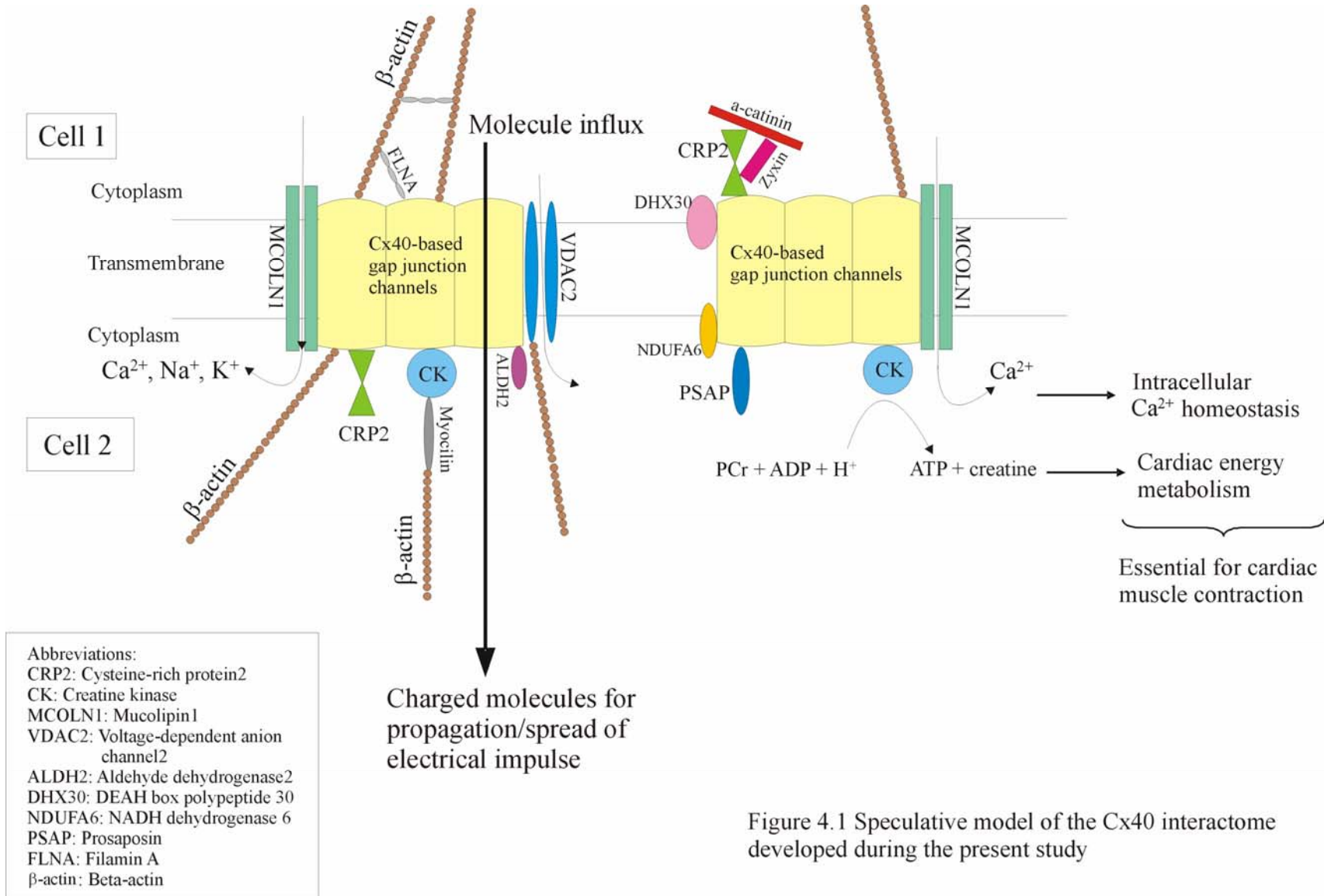


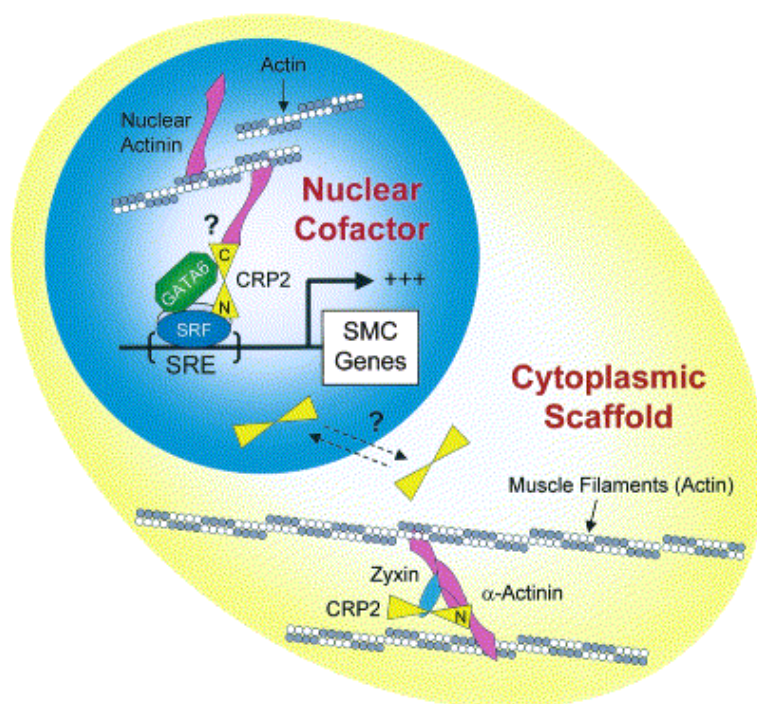
Figure 4.1 Speculative model of the Cx40 interactome developed during the present study

Functions such as cell differentiation, transcriptional regulation and organisation of the actin cytoskeleton have been assigned to the CRP family (Grubinger *et al.*, 2004). They have been proposed to serve as scaffolds that link protein partners, because of the presence of two LIM domains which are double-zinc finger-like structures that mediate protein-protein interactions (Konrat *et al.*, 1997). It may target proteins to distinct subcellular locations and mediate assembly of multimeric protein complexes (Chang *et al.*, 2003; Dawid *et al.*, 1995). Structural similarities have been shown for the N-terminal LIM domains among the CRP proteins, but not for the C-terminal LIM domains (Louis *et al.*, 1997; Konrat *et al.*, 1997).

The CRP2 protein is expressed mainly in vascular smooth muscle cells (SMC) (Jain *et al.*, 1996; Henderson *et al.*, 2002) and was chosen for further analysis in the present study based on its proposed functions and cellular localisation. It has been shown to function both as a cytoplasmic scaffold protein and as a nuclear transcription cofactor as illustrated in figure 4.2 (Van Ham *et al.*, 2003). The nuclear localisation of CRP2 allows for formation of transcription complexes that might associate with an actin-based nuclear matrix. This activates transcription of a serum response factor (SRF)-dependent target gene (Chang *et al.*, 2003).

CRP2 may work as a molecular adaptor to assemble a transcriptosome consisting of SRF, GATA6 and other essential factors on SMC-restricted promoters (Nishida *et al.*, 2002). This function of CRP2 as transcription cofactor might lead to an up-regulation of smooth muscle-specified genes, which is important for cardiovascular SMC differentiation. Therefore, CRP2 might play a scaffold role for the assembly and maintenance of a SMC cytoskeleton, in which Cx40 might also be involved, which is involved in cell substrate adhesion and contractile responses to vasoactive stimulation (Chang *et al.*, 2003; Louis *et al.*, 1997; Pomies *et al.*, 1997). The cytoplasmic localisation of CRP2 with mature muscle filaments may, in turn, be due to the strong binding of the N-terminal LIM domain to the cytoskeleton-associated proteins  $\alpha$ -actinin and zyxin. Zyxin and  $\alpha$ -actinin have been shown to be important regulators of actin cytoskeletal organisation (Chang *et al.*, 2003).

Different expression patterns have been shown, by means of mouse animal studies, for CRP2 in the cardiovascular system. At early stages of development, CRP2 is expressed throughout the developing heart. As development progresses into adulthood, its expression decreases in the ventricles while being maintained in the atrium (Jain *et al.*, 1998). Interestingly, similar expression patterns have been shown for Cx40 in the developing heart which was reviewed in section 1.3.4 of the introduction. The similar expression patterns of Cx40 and CRP2 indicate that they might function together, in a synergistic fashion, to facilitate the normal development of the heart. It is proposed that there might be an underlying function shared or regulated by an interaction between CRP2 and Cx40 and when defective this might contribute to the development of cardiac diseases affecting the conduction system of the heart.



**Figure 4.2 Representation of the dual functions of cysteine-rich protein 2** (Chang *et al.*, 2003).



#### 4.1.1.3.2 Beta-actin

This protein was selected for further analysis in the present study because of its cellular location and function, to be discussed below, which makes an interaction with the C-terminus of Cx40 possible. According to current literature, six actin isoforms have been identified, namely, four muscle types (skeletal, cardiac, aorto-type smooth muscle, and stomach-type smooth muscle actins) and two non-muscle types (cytoplasmic  $\beta$ - and  $\gamma$ -actins) (Schmitt-Ney *et al.*, 2004; Nakajima-Iijima *et al.*, 1985). The muscle types are tissue specific and are functionally involved in muscle contractions whereas the cytoplasmic actins are expressed in all kinds of cells where they may play roles in a variety of cell functions. The cytoskeleton has been shown to regulate cell shape, transport, motility and integrity and is composed of three protein filament systems, namely, microfilaments, intermediate filaments and microtubules. A large number of proteins have been shown to associate with each system for regulation purposes, of which some might include members of the Cx family (Peckham *et al.*, 2001; Schmitt-Ney *et al.*, 2004; <http://anatomy.med.unsw.edu.au>).

The cytoplasmic cytoskeletal protein,  $\beta$ -actin (a microfilament), has been shown to have functions in intracellular trafficking and in driving changes in cellular shape that is associated with cell motility, cytokinesis, endocytosis and cell adhesion (Hu *et al.*, 2004; Peckham *et al.*, 2001). Interactome analyses have indicated an array of  $\beta$ -actin interactors involved in different cellular processes. Interestingly, some of these are LIM domain containing proteins through which an interaction with Cx40 might be facilitated, based on the section discussed about CRP2 (a LIM-domain-containing protein). Following sections will discuss the  $\beta$ -actin interacting filamin A protein (a cytoskeletal anchoring protein), which might also function in facilitating an interaction between  $\beta$ -actin and Cx40 (Brakebusch *et al.*, 2003). In addition,  $\beta$ -actin has been shown to have functions in the nucleoskeleton, in RNA processing and export, and in transcription and its association with RNA polymerase III is important for transcription processes (Hu *et al.*, 2004). The role played by Cx40 in the nucleus has still to be elucidated, however, one might

speculate that it might be involved in the nucleolar machinery through an interaction with nucleoskeletal  $\beta$ -actin.

#### 4.1.1.3.3 Creatine kinase

The creatine kinase (CK) isoenzyme family consists of five isoenzymes, which are dimers formed from four distinct polypeptide chains that are each encoded by separate genes (Saupe *et al.*, 1998). Two of the isoenzymes are located in the mitochondrion (ubiquitous Mito-CK and sarcomeric Mito-CK) and three are located in the cytoplasm: - brain enzyme consisting of two identical B subunits (BBCK), muscle enzyme consisting of two identical M subunits (MMCK) and a hybrid enzyme (MBCK) (Saupe *et al.*, 1998; Steeghs *et al.*, 1997). The isoenzyme identified in the present study as a putative Cx40 ligand is MMCK (table 3.6A) that is, together with Mito-CK, a predominant cardiac isoenzyme.

The CK isoenzymes function in catalysing the transfer of a phosphoryl group between PCr and ATP by the following reaction: phosphorylated creatine + ADP +  $H^+$   $\leftrightarrow$  ATP + creatine. By catalyzing this reaction, CK functions to maintain a high concentration of ATP and low concentrations of the products of ATP hydrolysis, namely, ADP,  $P_i$ ,  $H^+$ , in the cells (Kammermeier, 1987; Steeghs *et al.*, 1997). This ensures that free energy released from ATP hydrolysis will be efficient to maintain ion gradients and to perform the mechanical work of the cell. Therefore, CK plays an important central role in energy transduction which is vital for the normal functioning of tissues with large fluctuating energy demands, such as skeletal muscle, heart and brain (Saupe *et al.*, 1998; Spindler *et al.*, 2004). Heart failure of many different etiologies has been shown to be associated with decreased total CK activity and with large changes in the relative numbers of the isoenzymes in the myocardium (Ingwall *et al.*, 1993; Liao *et al.*, 1996). The result of the changes in the enzyme system is a decreased ability of the failing heart to synthesis ATP from PCr.

Animal studies have been performed, in order to shed light on the functioning of the CK family of isoenzymes. These studies showed that, when CK activity is lowered below a certain level, increases in cardiac work become more energetically costly. This is in terms of high-energy phosphate use, accumulation of ADP, and decreases in free energy released from ATP hydrolysis but not in terms of myocardial oxygen consumption (Saupe *et al.*, 1998). The net effect of these energetic changes is the development of “substantial” left ventricular (LV) hypertrophy (Nahrendorf *et al.*, 2005). It was found that Mito-CK<sup>-/-</sup> - and M/Mito-CK<sup>-/-</sup> - deficient mice had approximately 70% increase in LV weight compared to age and body weight-matched wild-type animals. It was suggested that CK ‘ablation’ could only be tolerated in the heart if major adaptational changes occurred which consequently lead to massive LV hypertrophy. Studying the signaling mechanisms involved in initiating hypertrophic responses in CK-deficient mice might give some understanding of why it occurs (Nahrendorf *et al.*, 2005; Spindler *et al.*, 2002; Saupe *et al.*, 2000).

It has been shown that cardiac energy metabolism is closely linked to intracellular calcium homeostasis (Steeghs *et al.*, 1997) and animal studies with CK-deficient mice have shown significant disturbances in calcium homeostasis (Boehm *et al.*, 2000; Crozatier *et al.*, 2002). In addition, calcium is one of the many molecules that pass through the gap junction channels from one cardiomyocyte to another (Söhl *et al.*, 2004, Lampe *et al.*, 2000, 2004). An interaction of the C-terminus of Cx40 with MMCK might occur which could possibly form part of the cardiac energy metabolism machinery. A defective interaction might influence the passage of Ca<sup>2+</sup> through the gap junction channel, which leads to a disturbance in Ca<sup>2+</sup> homeostasis, which consequently affects the system of cardiac energy metabolism.

Interestingly, interactome analyses showed that MMCK interacts with a LIM-domain-containing protein. Therefore, one could speculate that a interaction between MMCK and the C-terminus of Cx40 might be facilitated by the interaction of MMCK with the LIM domain containing protein, based on the section discussed about CRP2 (a LIM-domain-containing protein). It was also shown that  $\beta$ -actin (a cytoplasmic cytoskeletal actin) is a

downstream interactore of MMCK, through an interaction with myocilin, which might play an important role in incorporating Cx40 into the MMCK interactome. In addition, the gene encoding MMCK is located at marker *D19S1105* which is 2.5Mb outside the PFHBI locus as indicated in figure 4.5. It is also located near the DM locus. Defects in *MMCK* such as deletions or expansion repeat mutations, could have a negative effect on the downstream DM and PFHBI-causative genes.

#### **4.1.1.3.4 Myosin, heavy polypeptide 7, cardiac muscle, beta**

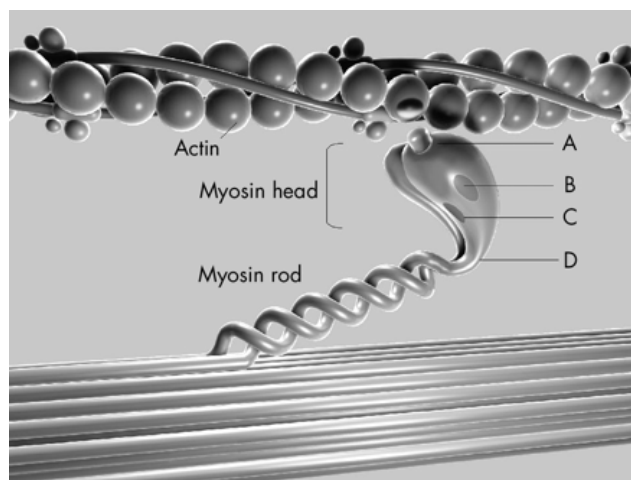
Familial hypertrophic cardiomyopathy (HCM) is an autosomal dominant cardiac disease characterised by left ventricular hypertrophy, which is usually asymmetric and often involves the interventricular septum (Perrot *et al.*, 2005; Maron *et al.*, 1981). The prevalence of the disease in the general population is estimated to be 1 in 500 persons (Garcia-Castro *et al.*, 2003; Ackerman *et al.*, 2002). The disease is clinically heterogeneous with inter- and intra-familial variations that range from benign to malignant forms with a high risk of cardiac failure and sudden cardiac death (Richard *et al.*, 1999). Defects in the gene encoding  $\beta$ -myosin heavy polypeptide 7 (*MYH7*) have been shown to be one of the causes of this cardiac disease (Marian *et al.*, 2001).

Muscle contraction and cell motility depend on myosin, which is the most important component of the sarcomere thick filament, and generation of force for the muscle contraction is mostly accomplished by the globular head of the  $\beta$ -myosin heavy polypeptide (Perrot *et al.*, 2005). Most of the mutations identified in *MYH7* were found in exons encoding the globular head of the molecule that posses the important enzymatic activities. The mutations were shown to cluster at specific functional domains, indicated in figure 4.3, associated with actin-binding, domains close to the ATP binding site or adjacent to the region that connects the two reactive cysteine residues, at the myosin light chain binding interface, or at the head-rod junction (Woo *et al.*, 2003; Rayment *et al.*, 1995). Mutations were also identified in the myosin neck domain, which may influence neck flexibility during contraction, and in the myosin rod (i.e. myosin tail) which may disturb thick filament assembly and binding of accessory proteins (Blair *et al.*, 2002).

Therefore, *MYH7* mutations might alter the myosin structure and, in turn, the functional properties of the molecule such as ATPase activity and sliding velocity (Perrot *et al.*, 2005). Interestingly, it has been shown that the type of *MYH7* functional domain affected by mutations is predictive of overall prognosis in HCM; mutations in the actin-binding site and the rod domain are independent predictors of an adverse outcome (Woo *et al.*, 2003).

It was shown through Y2H analysis in the present study that the C-terminus of Cx40 possibly interacts with the rod domain of  $\beta$ -myosin. As discussed above, the rod domain provides the structural backbone for the thick filament and it is therefore suggested that the interaction of this region with the C-terminus of Cx40 functions in anchoring or stabilising the myosin tail in the sarcomere. It is proposed that mutations in the rod domain could possibly hinder the interaction with Cx40 which may cause disruption in the functioning of  $\beta$ -myosin in muscle contraction and cell motility which consequently might lead to development of cardiac arrhythmias. Therefore, Cx40 might function as a modifier of HCM through its interaction with  $\beta$ -myosin. Whether  $\beta$ -myosin is a true Cx40 interacting ligand is under question, because Cx proteins have not been reported to localise in the sarcomere, neither are there any reports of them having roles in sarcomere functioning. Interestingly, 2 of the 33 prey clones that were sequenced (table 3.6A) were identified as  $\beta$ -myosin which strengthened its selection for further analysis.

In addition, it has been shown that defects in *MYH7* are also the cause of myosin storage myopathy, in which muscle biopsies show type 1 fibre predominance and increased interstitial fat and connective tissue (Tajsharghi *et al.*, 2003). Dilated cardiomyopathy is a disorder characterised by cardiac dilation and reduced systolic function and is also caused by defects in *MYH7* (Villard *et al.*, 2005). Defects in *MYH7* are also a cause of Laing early-onset distal myopathy in which there is a selective weakness of the anterior tibial muscles (Lamont *et al.*, 2006).



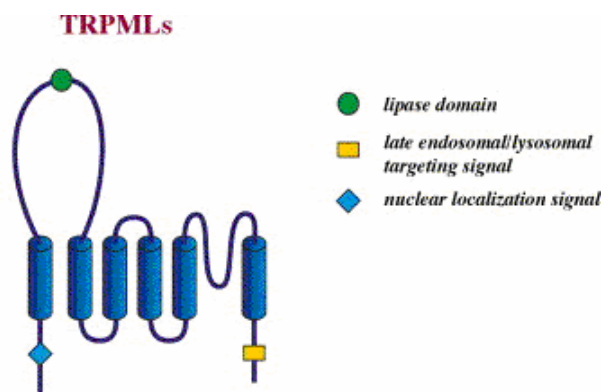
**Figure 4.3 Illustration of the functional domains of the  $\beta$ -myosin heavy chain and its relation to actin.** (A) actin binding site, (B) active site (ATP binding site), (C) essential light chain binding interface, (D) head-rod junction (Woo *et al.*, 2003).

#### 4.1.1.3.5 Mucolipin 1: Transient receptor potential channel

The family of transient receptor potential channels (TRP) function in contributing to changes in cytosolic free  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) by providing entry pathways for  $\text{Ca}^{2+}$ , by modulating the driving force for the  $\text{Ca}^{2+}$  entry, and by providing intracellular pathways for  $\text{Ca}^{2+}$  release from cellular organelles (Pedersen *et al.*, 2005). Changes in the  $[\text{Ca}^{2+}]_i$  play a central role in many fundamental cellular processes such as muscle contraction, transmitter release, cell proliferation, gene transcription and cell death (Berridge *et al.*, 2000). Seven families make up the TRP superfamily; TRPC (Canonical), TRPM (Melastatin), TRPV (Vanilloid), TRPA (Ankyrin), TRPP (Polycystin), TRPML (Mucolipin) and TRPN (no mechanoreceptor potential C; only found in *Caenorhabditis elegans*, *Drosophila* and zebra fish and not in the mammalian genome) (Montell *et al.*, 2002; Corey *et al.*, 2003; Clapham *et al.*, 2003).

The present study has indicated, by means of Y2H analysis, that an interaction might occur with the C-terminus of Cx40 and the transmembrane domains of the Mucolipin1 TRP channel (MCOLN1). MCOLN1 is one of the less-studied TRP channels, but it is known that TRP channels are activated by a wide range of stimuli including intra- and extracellular messengers, chemical, mechanical, and osmotic stress, and some by the

filling state of intracellular  $\text{Ca}^{2+}$  stores (Clapham *et al.*, 2003). It is known that gap junctions allow the passage of small molecules such as those mentioned here (Söhl *et al.*, 2004, Lampe *et al.*, 2000, 2004), and it is therefore suggested that gap junction channels might play a role in the activation of the MCOLN1 channel by influencing this wide range of stimuli.



**Figure 4.4 Representation of the Mucolipin 1 transmembrane protein and its functionally important sites** (lipase domain, late endosomal/lysosomal targeting signal and the nuclear localisation signal) (Pedersen *et al.*, 2005).

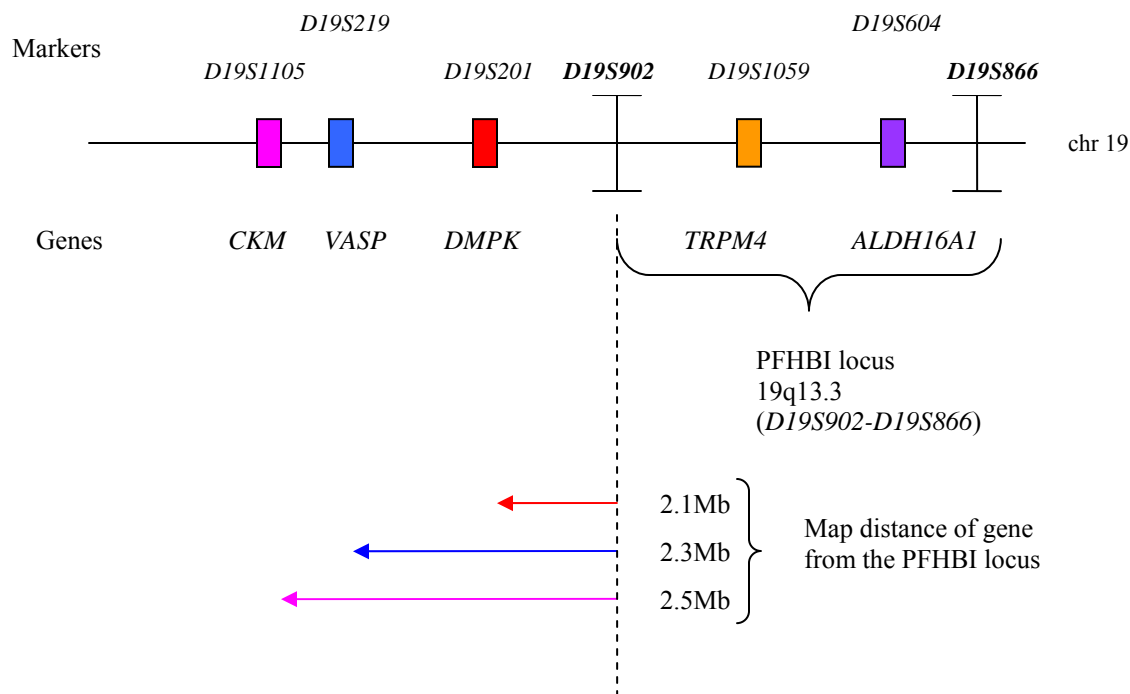
The MCOLN1 protein, which is indicated in figure 4.4, has six transmembrane domains with the N-terminus and C-terminus in the cytoplasm (Pedersen *et al.*, 2005). It has three functionally important sites, namely, the lipase domain, late endosomal/lysosomal targeting signal and the nuclear localisation signal (Pedersen *et al.*, 2005). The MCOLN1 protein is ubiquitously expressed and is suggested to reside in late endosomes/lysosomes (Slaugenhaupt *et al.*, 2002). However, it has been shown that MCOLN1 can function on the plasma membranes of oocytes injected with MCOLN1 cRNA (LaPlante *et al.*, 2002). It was shown that MCOLN1 can be induced to translocate to the plasma membrane during the process of lysosomal exocytosis which was shown to be a  $\text{Ca}^{2+}$ -dependent reaction. In addition, MCOLN1 is not only permeable to  $\text{Ca}^{2+}$ , but also  $\text{Na}^{+}$  and  $\text{K}^{+}$ , and the expression of MCOLN1 on the plasma membrane was associated with generation of measurable whole-cell currents (LaPlante *et al.*, 2004). The MCOLN1-mediated control of lysosomal  $\text{Ca}^{2+}$  levels plays an important role in proper lysosome formation and recycling and disturbances thereof has been linked to mutations in the gene encoding MCOLN1 (Slaugenhaupt *et al.*, 2002). The resulting lysosomal storage disorder is

Mucopolidosis IV and is caused by defects in the late endocytic pathways (Bargal *et al.*, 1997; Chen *et al.*, 1998; LaPlante *et al.*, 2004; 2002).

One could therefore speculate that an interaction between MCOLN1 and the C-terminus of Cx40 might be important in maintaining intra- and extracellular  $\text{Ca}^{2+}$  homeostasis because both channels function in the milieu of  $\text{Ca}^{2+}$  transport and trafficking in the cell (Söhl *et al.*, 2004; Pedersen *et al.*, 2005). A defect in their interaction might influence the trafficking of  $\text{Ca}^{2+}$ , and, in turn, cause a disturbance in the  $\text{Ca}^{2+}$  homeostasis. As mentioned above, changes in the  $[\text{Ca}^{2+}]_i$  play a central role in many fundamental cellular processes such as muscle contraction, transmitter release, cell proliferation, gene transcription and cell death. As in the rest of the human body, these are also important for normal heart functioning of which cardiac muscle contraction is most dependent on adequate  $\text{Ca}^{2+}$  homeostasis. In addition, during the late endocytic pathways, exocytosis is triggered which results in lysosomes fusing with the plasma membranes (LaPlante *et al.*, 2004). At this point, an interaction could occur between MCOLN1 (in the lysosome membranes) and Cx40 (in the plasma membranes) which might be important for completion of the exocytosis process. It is however not known whether Cx proteins have functions in processes involving exocytosis but it is something to speculate about.

Interestingly, one of the members of the TRP family, TRPM4, was shown to map to the PFHBI locus, as indicated in figure 4.5 (section 1.7.2, figure 1.15). From current literature, this channel protein is of interest because of its role as a calcium-activated nonselective cation channel, which might be involved in development of cardiac arrhythmias induced by calcium waves (Guinamard *et al.*, 2006; Nilius *et al.*, 2006). This channel might have similar domains to that of MCOLN1 which could enable an interaction with the C-terminus of Cx40. Furthermore, as discussed in section 4.1.1.1, TRPM4 might be a true Cx40 ligand which was not identified in the present study due to the number of independent clones screened.





**Figure 4.5 Representation of the position of the ALDH and TRP isoforms in the PFHBI locus.** The positions of *TRPM4* and *ALDH16A1* are shown in the PFHBI locus between markers *D19S902* and *D19S866*. The map distance of *CKM*, *VASP* and *DMPK* from the *D19S902* marker are shown with arrows. Abbreviations: creatine kinase, muscle type (*CKM*), vasodilator-stimulated phosphoprotein (*VASP*), myotonic dystrophy protein kinase (*DMPK*), transient receptor potential cation channel 4 (*TRPM4*), aldehyde dehydrogenase 16 (*ALDH16A1*), chr: chromosome (<http://www.ensembl.org>).

#### 4.1.1.3.6 Voltage-dependent anion channel 2

The voltage-dependent anion channel (VDAC) family contains three members, namely, VDAC1, VDAC2 and VDAC3 (Sampson *et al.*, 1997, 1996; Sardiello *et al.*, 2003), of which VDAC2 was identified as a putative Cx40 interacting ligand in the present study. It was selected for further analysis (M2H analysis), because of its functions and cellular location which will be discussed below.

The VDAC proteins are also known as mitochondrial porins and form the major pathways for metabolite flux across the outer (i.e. cytoplasmic facing) mitochondrial membrane (Manella *et al.*, 1992; Lee *et al.*, 1998; Xu *et al.*, 1999). Recently, it has been reported that VDACS also localise to the plasma membrane (Elinder *et al.*, 2005; Hinsch

*et al.*, 2004). These channel proteins behave as general diffusion pores for small hydrophilic molecules (Kamorov *et al.*, 2005). They have also been shown to have other functions such as binding to receptors or the cytoskeleton, in acting as a coordination point for the formation of large protein complexes and in regulation of the molecular cascade leading to apoptosis (McEnery *et al.*, 1992; Linden and Karlsson *et al.*, 1996). These functions and the fact that VDAC2 is a membrane protein make it possible for one to speculate about an interaction between the C-terminus of Cx40 and VDAC2.

An interaction might be an important regulator of the metabolite flux across the mitochondrial membrane of not only VDAC2 but also of the gap junctions in the mitochondrial membranes. As mentioned previously, the presence of Cx43 in the mitochondrion has recently been identified (Boengler *et al.*, 2005) and it is possible that Cx40 might also be located in this cellular organelle. Deficiencies in the VDAC proteins cause a disturbed transport of ATP and ADP across the mitochondrial membranes and an abnormal ion composition of the mitochondrial matrix can be detrimental to the process of oxidative phosphorylation (Huizing *et al.*, 1998, 1996a; Ruitenbeek *et al.*, 1995). It is proposed that disruptions in the interaction between VDAC2 and Cx40, caused by, e.g., VDAC deficiency, might consequently influence the transport of ATP and ADP across the mitochondrial membrane. This could in turn influence energy transduction which is important for cardiac muscle contraction. In addition, an interaction with the C-terminus of Cx40 might have functions in regulating voltage sensitivity of VDAC2 because the C-terminus has important channel gating properties (Moreno *et al.*, 2002; Ahmad *et al.*, 2001).

Interestingly, the present study also showed a possible interaction between Cx40 and  $\beta$ -actin (a cytoskeletal protein). As mentioned above, VDACS function in binding to the cytoskeleton (McEnery *et al.*, 1992). It is therefore possible that the interaction between VDAC2 and Cx40 might be facilitated by  $\beta$ -actin, which subsequently leads to the incorporation of them into the cytoskeleton. In addition, Cx40 might form part of a large complex of proteins because of the ability of VDAC to act as a coordination point for the

formation of large protein complexes (Linden and Karlsson *et al.*, 1996) and thereby function in signal transduction.

#### **4.1.1.3.7 Aldehyde dehydrogenase 2**

The present study identified aldehyde dehydrogenase 2 (ALDH2) as a possible Cx40 interacting ligand. Even though it was shown to be expressed in the mitochondrial matrix, it was selected for further analysis because of recent reports of the presence of Cx43 in cardiomyocyte mitochondria (Boengler *et al.*, 2005) and because of its possible function with Cx40 in vasoactivity, to be discussed below. The following discussion will shed light on the function of ALDH2 and its putative interaction with the C-terminus of Cx40.

Two major ALDH isoenzymes have been identified in the liver, namely, cytosolic ALDH1 and mitochondrial ALDH2 (Perozich *et al.*, 1999). The ALDH2 isoenzyme is an acetaldehyde-metabolising enzyme and is the second enzyme of the major oxidative pathway of alcohol metabolism (Hempel *et al.*, 1993). The enzyme contains an Aldehyd domain, which confers catalytic activity, and is expressed in several other human tissues, including the heart, but with highest level in the liver (Impraim *et al.*, 1982). Most Caucasians have the two isoenzymes, while approximately 50% of Orientals have only the cytosolic isoenzyme. Orientals show extremely high sensitivity to alcohol, which has been called “Oriental flushing”, and which has been shown to be caused by a genetic deficiency in the ALDH2 isoenzyme. This deficiency cause an elevation in blood acetaldehyde levels (Amamoto *et al.*, 2002; Harada *et al.*, 1981; Takeshita *et al.*, 1993).

Excessive alcohol consumption has been shown to be a strong risk factor for high blood pressure (MacMahon *et al.*, 1987; Marmot *et al.*, 1994; Tsuruta *et al.*, 2000). Takagi and colleagues (Takagi *et al.*, 2001) showed that the *ALDH2* gene is a risk factor for hypertension and myocardial infarction in Japanese men. They showed that the effects of alcohol consumption on blood pressure differed according to the *ALDH2* genotype. Individuals with a certain *ALDH2* genotype had increased high density lipoprotein (HDL) cholesterol levels and an increased risk for myocardial infarction. It has been reported

that alcohol consumption results in a dose-dependent increase in plasma concentrations of HDL through an increase in the HDL apolipoprotein transport rate (Takagi *et al.*, 2001; 2002). Functions of Cx40, or any of the other members of the Cx family, in alcohol metabolism has not been reported to date, but it is possible that the C-terminus of Cx40 might function in transduction of signals important in the process of alcohol metabolism.

Animal studies performed by Chen and colleagues (Chen *et al.*, 2005) showed an association of *ALDH2* with vasoactivity. They showed that mitochondrial bioconversion of glycerol trinitrate (GTN) to nitric oxide (NO) was absent in mitochondria of *ALDH2*-null mice. GTN stimulates NO-based signaling to dilate blood vessels. This indicated that *ALDH2* is necessary for normal vasoactivity (Chen *et al.*, 2005; Murphy *et al.*, 2005). As discussed in the introduction (section 1.4.3) animal studies have shown the importance of Cx40 in vasoactivity (de Wit *et al.*, 2000, 2003). It is proposed in the present study that the *ALDH2* enzyme interacts with the C-terminus of Cx40 which is located in the plasma membranes of the endothelium cells lining the blood vessels in the cardiovascular system. Their interaction could be an important regulator for vasoactivity and defects in their interaction might cause the hypertension reported in Cx40-deficient mice.

Interestingly, the gene encoding the isoenzyme *ALDH16A1* was shown to map to the *PFHBI* locus and is indicated in figure 4.5 (section 1.7.2, figure 1.15). This isoenzyme has not been fully characterised; however, it might have similar domains to *ALDH2* which could enable a interaction with the C-terminus of Cx40. It might also be necessary for *ALDH16A1* and *ALDH2* to interact or form part of a larger complex, which incorporates Cx40, in order to perform their functions in the cell. Furthermore, as discussed in section 4.1.1.1, *ALDH16A1* might be a true Cx40 ligand which was not identified in the present study due to the number of independent clones screened. Interactome analyses of *ALDH2* have identified the vasodilator-stimulated phosphoprotein (*VASP*) as a down stream interactor of *ALDH2*. The *VASP* gene was shown to map very close but outside of the *PFHBI* and *DM* loci, at marker *D19S219*, as indicated in figure 4.5. However, defects in *VASP* such as deletions or expansion repeat

mutations could have a negative effect on the downstream DM and PFHBI causative genes.

#### **4.1.1.3.8 DEAH (Asp-Glu-Ala-His) box polypeptide 30**

The DEAH (Asp-Glu-Ala-His) box polypeptide 30 (DHX30) was identified in the present study as a putative Cx40 interacting ligand. Unfortunately, a large number of the DEAH proteins, including DHX30, have not been fully characterised. It is, however, known to be a membrane-bound protein that might interact with the C-terminus of Cx40 based on its cellular location. This strengthened the grounds for selecting it for further analysis by M2H. The following is a brief discussion of nucleic acid helicases from current literature.

Nucleic acid helicases are classified into DNA and RNA helicases, which depends on their substrate specificity (Abdelhaleem *et al.*, 2003). RNA helicases have been divided into two families, namely, DEAD-box and DEAH-box proteins; also known as DExH/D proteins. The names originate from the conserved helicase domain motif Asp-Glu-Ala-Asp (DEAD) and Asp-Glu-Ala-His (DEAH) (Jankowsky *et al.*, 2000). They are a widely dispersed group of proteins and are found in almost all biological processes involving RNA. They have been shown to play roles in nuclear and mitochondrial splicing processes, RNA editing, rRNA processing, translation initiation, nuclear mRNA export and mRNA degradation (Tanner *et al.*, 2001). They are also essential factors in cell development and differentiation (Silverman *et al.*, 2003; Abdelhaleem *et al.*, 2003). As discussed in previous sections, the presence of Cx43 has been identified in cardiomyocyte mitochondria and nuclei (Boengler *et al.*, 2005). It is therefore possible that Cx40 might also be present in cardiomyocyte nuclei which in turn could interact with the membrane bound DHX30 protein. This could be important in signal transduction pathways for initiation of transcription processes. They might function in not only the import of molecules into the nucleus, but also the export thereof. The export could entail transcription products to be translated in the cytoplasm. In addition, it has been shown that DExH/D proteins contain accessory domains outside of the helicase domain that

offer specificity by possibly serving as sites for interaction with regulatory proteins (Silverman *et al.*, 2003). The Cx40 protein could possibly be one of these regulatory proteins.

#### **4.1.1.3.9 NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6**

NADH dehydrogenase oxidoreductase (complex I) is the first and largest complex in the mitochondrial respiratory electron transport chain (Chretien *et al.*, 2003). It is located in the mitochondrial inner membrane, on the matrix side, and is a membrane bound multi-subunit assembly, consisting of 45 different subunits (Brandt *et al.*, 2003). Most of the subunits have unknown functions. The subunit in complex I, namely, NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6 (NDUFA6) that was identified in the present study as a possible interacter with the C-terminus of Cx40, is a subunit that belongs to the complex I LYR family. This family has been named LYR because of a highly conserved tripeptide motif close to the N-terminus of the protein (Dunbar *et al.*, 1997; Emahazion *et al.*, 1998).

Studies have shown that *NDUFA6* is preferentially expressed in the heart, lungs and skeletal muscle (Chretien *et al.*, 2003; Ton *et al.*, 1997). This protein was selected for further analysis based on its membrane bound location which brings it in the proximity of the localisation of the Cx40 protein. It was also selected because of the role it plays in energy metabolism, as part of complex I, which is important for cardiac muscle contraction. Unfortunately, this protein has not been fully characterised and this is therefore a generalised discussion of the subunits of complex I. The function of complex I is to catalyse the transfer of two electrons from NADH to coenzyme Q, which leads to the production of ATP; most of the ATP in cells is produced in the mitochondrion. Therefore, defects in complex I predominantly affect organs such as the heart, muscle and brain, which have high demands for ATP (Beal *et al.*, 1996; Ton *et al.*, 1997). A variety of human diseases such as myopathy, hypertrophic cardiomyopathy, encephalopathy, lactic acidosis and strokelike episodes have been associated with defects

in the enzymes of the respiratory chain complex (Zeviani *et al.*, 1991; Silvestri *et al.*, 1994; Mariotti *et al.*, 1994; King *et al.*, 1992).

One could speculate about an interaction between the C-terminus of Cx40 and NDUFA6. Both are subunits of a larger complex involved in the transport of molecules important in an array of biological processes. An interaction between them could have regulatory purposes for the action of molecular transport. Cx40 might facilitate the transduction of signals in the respiratory electron transport chain through an interaction of its C-terminus with the NDUFA6 subunit. Defective interactions could negatively influence cardiac energy metabolism by decreasing the activity of complex I which could consequently lead to development of the above mentioned human diseases. In addition, the net effect of the interaction of these two proteins might be important in the fast conducting tissue which is the region where Cx40 is expressed in the cardiac conduction system. Functions of Cxs in the respiratory electron transport chain have not been reported and seem unlikely to occur. However, NDUFA6 has not been fully characterised which leaves room in the present study to speculate about its possible interaction with cardiac Cxs.

#### **4.1.1.3.10 Prosaposin**

The present study identified the prosaposin protein (PSAP) as a putative Cx40-interacting ligand. The PSAP protein has been shown to be a multifunctional protein with intra- and extra-cellular functions (Sun *et al.*, 2002). It has been shown that the PSAP protein is processed intracellularly into four related ~80 amino acid proteins, namely, saposins A, B, C and D (Hiraiwa *et al.*, 1992). They are necessary for the activity of specific lysosomal glycosphingolipids (GSLs) hydrolyses. It has been shown that deficiency of PSAP in human or mouse results in the storage of multiple GSLs in a variety of organs (Paton *et al.*, 1992; Hulkova *et al.*, 2001). Extracellularly, unprocessed PSAP has functions as a neurite outgrowth or nerve regeneration factor (Sun *et al.*, 2002).

The functions of PSAP in signal transduction and cell-to-cell communication are important functions that apply to the present study, because gap junction channels also

function in this regard. Therefore, an interaction of this protein with the C-terminus of Cx40 could be important in the transduction of certain cellular signals. Defects in the interaction could uncouple the chemical communication between adjacent cells which might be dependent on the interaction of PSAP and Cx40. It is however not known whether PSAP has functions in the spread of the electrical impulse in the cardiac conduction system.

The PSAP protein has been shown to be both a secretory protein and a integral membrane protein (Hiraiwa *et al.*, 1992). An interaction with the C-terminus of Cx40 might therefore occur through a interaction with one or all of the saposins in their membrane bound cellular location. With which of the saposins this interaction occurs, is not clear in the present study. Furthermore, an interaction between the unprocessed PSAP and Cx40 is unlikely to occur, because the unprocessed PSAP is an extracellular protein (Sun *et al.*, 2002) which places it outside of the intracellular location of the C-terminus of Cx40.

#### **4.1.1.3.11 Filamin A (actin binding protein 280)**

The actin binding protein, filamin A (FLNA), a putative Cx40 ligand according to the present study, crosslinks actin filaments into networks in the cytoplasm and participates in the anchoring of membrane proteins for the actin cytoskeleton (Gorlin *et al.*, 1990). It is proposed that FLNA might facilitate the trafficking and incorporation of Cx40 into the gap junction channel in the plasma membrane through its function as an anchoring protein. The FLNA protein has also been shown to function in regulating reorganisation of the actin cytoskeleton by interacting with integrins, transmembrane receptor complexes and second messengers (Loo *et al.*, 1998; Stossel *et al.*, 1985). The Cx40 protein could possibly be one of the membrane components that interact with FLNA to facilitate the regulation of the actin cytoskeleton.

Furthermore, FLNA has been shown to serve as a scaffold for a wide range of cytoplasmic signaling proteins (Gorlin *et al.*, 1990; Stossel *et al.*, 1985). It is possible that Cx40 could be incorporated into a larger complex of signaling proteins through its



interaction with FLNA. As previously discussed,  $\beta$ -actin (a cytoskeletal protein) is a possible Cx40 ligand. It is proposed that the interaction of these proteins with the C-terminus of Cx40 facilitates the organisation and stabilisation of  $\beta$ -actin and FLNA in the cytoplasmic cytoskeleton.

#### **4.1.1.4 Functional consequence of Cx40 interacting ligands**

The synthesis, assembly and degradation of gap junction channels (section 1.2.3), the channel gating mechanisms (section 1.6), the transport of molecules through the channel (section 1.2.1) and to this end, the conduction of an electrical impulse in the cardiac conduction system (section 1.1.2) can all be influenced by a ligand that interacts with the C-terminus of Cx40. None of the ligands identified for Cx40 in the present study have been shown to form protein-protein interactions with any of the other cardiac Cx isoforms. It is however possible to speculate, from current literature, about the functional consequences of these Cx40-interacting ligands.

##### **4.1.1.4.1 Signal transduction**

The ligands such as CRP2, PSAP, FLNA and VDAC2 (figure 4.1) which are involved in forming large protein complexes or act as scaffolds, play roles in signal transduction in the cell by recruiting signaling proteins. They might be important in Cx40-based gap junction formation and maintenance, and in electrical impulse conduction (Giepmans *et al.*, 1998; Herve *et al.*, 2004; Duffy *et al.*, 2002). The DHX30 protein, which is a membrane bound protein, might also be important in the signal transduction processes.

The cytoskeletal proteins, namely,  $\beta$ -actin and FLNA, might interact with Cx40, in order to facilitate the anchoring of membrane proteins, to regulate organisation of the cytoskeleton, and to maintain cellular shape (Herve *et al.*, 2004). Abnormal cellular shape disrupts the gap junction channel distribution between cardiomyocytes and consequently leads to defects in electrical impulse conduction, which is necessary for adequate heart

muscle contractions. Furthermore, cardiac malformations have been observed in *Cx40*-deficient mice as discussed in section 1.4.1 (Gu *et al.*, 2003). This could be due to disruption in the interactions between Cx40 and the cytoskeletal proteins, because of the *Cx40*-deficiency, which causes abnormal cardiomyocyte cellular shape and subsequently leads to development of cardiac malformations. The cytoskeletal proteins also function in recruiting other proteins to the membrane which play roles in signal transduction.

Therefore, the C-terminus of Cx40 could function in clustering of other proteins which consequently connects intracellular signaling pathways which might be important in Cx40-based gap junction formation and maintenance, and subsequently, in electrical impulse conduction.

#### **4.1.1.4.2 Cardiac muscle contraction**

Essential for cardiac muscle contraction is the maintenance of intracellular calcium homeostasis and cardiac energy metabolism (Boehm *et al.*, 2000; Crozatier *et al.*, 2002). The ligands identified in the present study with involvement in these mechanisms are MCOLN1 ( $\text{Ca}^{2+}$  homeostasis) and VDAC2, NDUFA6 and CK (energy metabolism) (figure 4.1). Their interaction with Cx40 might facilitate some of their cellular functions. All, except for CK, either form part of, or are, membrane channels, of which the gating might be influenced by the regulation properties of the C-terminus of Cx40.

This will influence the transport of molecules through these channels and, subsequently, other biological processes in the cell. Furthermore, an interaction might occur between the C-terminus of Cx40 and the rod domain of  $\beta$ -myosin. The rod domain provides the structural backbone for the sarcomere thick filament and it is suggested that the interaction of this domain with the C-terminus of Cx40 functions in anchoring or stabilising the myosin tail in the sarcomere. The functions of  $\beta$ -myosin in muscle contraction and cell motility might be influenced by this interaction.

#### **4.1.1.4.3 Vasoactivity**

Animal studies showed that *Cx40*-deficient mice had significantly higher blood pressure levels, compared with wild-type mice, and also displayed irregular arteriolar vasomotion and impaired conduction of vasodilatory signals along their arterioles (de Wit *et al.*, 2000, 2003). Interestingly, it has also been shown that ALDH2 is necessary for normal vasoactivity (Chen *et al.*, 2005). Therefore, an interaction between ALDH2 and the C-terminus of Cx40 might be an important regulator of vasoactivity and also possibly for blood pressure levels.

#### **4.1.1.4.4 Net effect of interactions**

Adequate spread of electrical impulse in the fast conducting tissue of the His-Purkinje system (section 4.1.1.4.1 and 1.1.2), which is a network of cells specialised for rapid conduction of excitation to the apical ventricular myocardium (Saffitz *et al.*, 2000), adequate cardiac muscle contraction of predominantly the atrium (sections 4.1.1.4.2 and 1.3.4), and adequate arteriolar vasomotion and conduction of vasodilatory signals along the arterioles, in order to regulate blood pressure levels (sections 4.1.1.4.3 and 1.4.3).

#### **4.1.1.5 Functional differences of Cx40 and Cx45**

A ligands search conducted with Cx45, by N Nxumalo (Nxumalo, 2007), in parallel with the present study, identified mostly mitochondrial proteins as Cx45 ligands whereas those for Cx40, in the present study, were mostly cytoplasmic. From the difference in cellular localisation of the ligands of each of these isoforms, we can deduce that Cx40 and Cx45 have different functions in the heart. It is possible that Cx45 might have more roles in energy metabolism than Cx40 because of its interaction with mitochondrial proteins. The predominant function of Cx40 might be in signal transduction and regulation of the cytoskeleton because most of its ligands function in this regard. It is possible that significant ligands, which would have shed more light on the functions of Cx40 and Cx45, might have been missed, due to limitations of the Y2H analysis discussed below,

in the process of screening the library of cardiac proteins. Therefore, one can only speculate to a point from where a margin of error must be drawn about the differences in functions of Cx40 and Cx45.

#### **4.1.1.6 Limitations of Y2H analysis**

The desired biotechnological approach to detect protein-protein interactions is that of a system without limitations. Unfortunately, such is hard to find. Even though the Y2H method is a frequently used technique to detect and study protein-protein interactions, it also has its limitations which will be discussed in the following section.

Some classes of proteins are not suitable for Y2H analysis, e.g., transcriptional activators that may activate transcription of reporter genes without any interactions with other proteins (auto-activation) (Sobhanifar *et al.*, 2003). Another limitation is the necessity of nuclear localisation of the interacting ligands, in order to activate transcription of the reporter genes. The nuclear recruitment of extracellular proteins or proteins with stronger targeting signals, such as membrane bound proteins, is at a disadvantage. The nucleus may not be the ideal cellular organelle for the investigation of certain interactions (Van Crielinge *et al.*, 1999).

The Y2H method makes use of artificially made fusion proteins which poses a potential risk when attempting to identify protein-protein interactions. It is possible that the fusion might alter the conformation of the bait and/or prey proteins and consequently may alter their functions and binding properties (Sobhanifar *et al.*, 2003). Another limitation is the use of *S.cerevisiae* as the host. Some protein-protein interactions depend on post-translational modifications, such as disulfide bridge formation, glycosylation or phosphorylation. These may not occur properly or at all in the *S.cerevisiae* system. In addition, the *S.cerevisiae* might not be able to correctly fold the constructed fusion protein (Fields *et al.*, 1994; Van Crielinge *et al.*, 1999).

Another limitation of the Y2H method is the possibility of identifying false positive binding partners by indicating reporter gene activity where no direct bait-prey interaction has taken place. This occurs when the bait construct acts as an auto-activator of transcription (Sobhanifar *et al.*, 2003). The ability of auto-activation of transcription was one of the aspects that were tested for in the present study when evaluating the Cx40 bait construct. The results showed that the bait construct was unable to activate transcription of the reporter genes autonomously. The present study employed a system that utilised multiple reporter genes, each under slightly altered GAL4-responsive upstream activation sequences, in order to minimize the number of false positives. In addition, heterologous mating experiments were performed, in order to exclude non-specific bait and prey interactions from the study. Another limitation of Y2H is the possible lack of detection of significant ligands due to insufficient screening of the cardiac cDNA library. This could be due to factors such as sub-standard growth medias, or inefficient library cloning vectors and bait constructs.

Protein-protein interactions detected by the Y2H method are, as in many other biotechnological approaches, putative and should be confirmed by means of other molecular biochemical techniques. The present study employed the M2H method, in order to confirm the bait and prey interactions identified.

#### **4.1.2 Mammalian-2-Hybrid analysis**

##### **4.1.2.1 SEAP assay and $\beta$ -Galactosidase enzyme assay**

The SEAP assay results obtained for the M2H analysis were incorrect for the controls, which indicated that the M2H system performed inefficiently. The  $\beta$ -Galactosidase enzyme assay verified this by showing poor transfection efficiency of the H9C2 mammalian cells with the four constructs. This can be seen in figure 3.12, section 3.3.2, in which the SEAP assay values were normalised for the  $\beta$ -Galactosidase assay values, in order to determine transfection efficiency.

A reason why the SEAP assay failed could be due to the type of mammalian cells used, which seemed to transfect poorly under standard conditions. It has been shown that transfection efficiency for different cell lines varies and that different cell lines require different transfection methods (Ausubel *et al.*, 1994). Correct SEAP assay results might have been obtained after the transfection efficiencies were compared of preliminary control matings, in order to select the appropriate protocol. Another reason for the incorrect SEAP assay results could be that the construct DNA contained impurities, such as endotoxins, that were not removed efficiently during DNA purification. Furthermore, the cell density, the concentration of DNA, growth media conditions and transfection time are parameters that could have influenced the SEAP assay results. Technical research errors are another factor that could have negatively influenced the assay's outcome.

#### **4.1.2.2 Limitations of M2H analysis**

The necessity for nuclear localisation of interacting ligands, in order to activate transcription of reporter genes, are a limitation of the M2H system, in the same way as it is for the Y2H system. Therefore, the nucleus may not be the ideal cellular organelle for the investigation of certain bait and prey protein interactions (Van Crielinge *et al.*, 1999). Furthermore, it may be possible for the bait or prey fusion constructs to autonomously activate transcription of reporter genes because M2H is a GAL4-based system. For this reason, several control experiments, as indicated in table 2.8, section 2.14.3, were included to determine whether any of the constructs were auto-activators of transcription. Another limitation is efficient transfection of multiple numbers of constructs into mammalian cells (Ausubel *et al.*, 1994). Not all transfection protocols work efficiently for different mammalian cell lines and it is therefore necessary to conduct preliminary control matings, in order to select the appropriate transfection protocol.

## 4.2 Future directions

Future directions will be to verify whether the ligands identified for the C-terminus of Cx40 in the present study are true interactors. The M2H assay will facilitate this following the use of appropriate transfection protocols for the selected cell line, in order to yield significant SEAP assay and, subsequently, statistical results. The co-immunoprecipitation assay and western blot analysis are methods that will also shed light on the bait and prey protein interaction under investigation. Another method, namely, immunofluorescence confocal microscopy could be employed, which will make it possible to determine whether the similar subcellular localisation of two proteins makes possible a physical interaction between them. The Cx40 protein has different expression patterns in the developing heart and the ligands can be investigated to determine whether their interaction with Cx40 is dependent on certain stages of heart development. Furthermore, the role that the ligands play in the functions of Cx40 in the conduction system can be investigated and whether disruptions in these interactions contribute to development of heart diseases such as PFHBI and PFHBII.

## 4.3 Conclusion

The present study identified 11 different putative Cx40-interacting ligands. The specific roles that each one plays in the milieu of Cx40 functioning is one on which to ponder. However, from the discussion it is possible to speculate that these ligands form parts of larger intracellular signaling pathways which are important in Cx40-based gap junction formation and maintenance. This influences the propagation and spread of the ever important electrical impulse through the regions of the conduction system where Cx40 is the predominant isoform. In light of the search for the PFHBI and PFHBII disease causative genes, it is not known whether these Cx40 ligands play roles in heart disease development; however, isoforms of the ALDH and TRP ligands, do map to the PFHBI target locus which, therefore, identifies them as novel disease causative candidates to be investigated.

*Appendix I****DNA extraction solutions*****Cell lysis buffer**

Sucrose	0.32M
Triton-X-100	1%
MgCL <sub>2</sub>	5mM
Tris-HCL	10mM
H <sub>2</sub> O	1L

**3M NaAc**

NaAc.3H <sub>2</sub> O	40.18g
ddH <sub>2</sub> O	50ml

Adjust pH to 5.2 with glacial acetic acid and adjust volume to 100mL with ddH<sub>2</sub>O

**DNA extraction buffer**

NaCl	0.1 M
Tris-HCL	0.01M
EDTA (pH8)	0.025M
SDS	0.5%
Proteinase K	0.1mg/ml

**TBE buffer (10x stock)**

Tris-HCl	0.89M
Boric Acid	0.89M
Na <sub>2</sub> EDTA (pH8)	20mM



### *Bacterial plasmid purification solutions*

#### **Cell resuspension solution**

50mM Tris-HCL, ph 7.5	2.5ml 1M Tris
10mM EDTA	1mL 0.5 EDTA
Make up to 50ml with dH <sub>2</sub> O	

#### **Cell lysis solution**

0.2M NaOH	2.5ml 4M NaOH
1% SDS	5ml
Make up to 50ml with dH <sub>2</sub> O	

#### **Neutralization solution**

1.32M KOAc, pH4.8	13.2ml 5M KOAc
Make upto 50ml with dH <sub>2</sub> O	

### *Yeast plasmid purification solutions*

#### **Yeast lysis buffer (Smash-and-Grab buffer)**

SDS	1%
Triton X-100	2%
NaCl	100mM
Tris (pH 8)	10mM
EDTA (pH 8)	1mM
Make up to 50ml with ddH <sub>2</sub> O	

## *Electrophoresis solutions, loading dyes & agarose gels*

### **SB-Buffer (20x stock)**

38.137 g/mol di-sodium tetraborate decahydrate

Add ddH<sub>2</sub>O up to 1L

### **Bromophenol blue loading dye**

Bromophenol blue 0.2% (w/v)

Glycerol 50%

Tris (pH 8) 10mM

### **Agarose gel solutions**

Concentration	1%	1.5%	2%
Agarose (g)	1	1.5	2
1x SB stock (ml)	100	100	100

Melt agarose, in SB solution, in microwave oven until completely dissolved.  
Let cool for 10min, add 7 $\mu$ l ethidium bromide and pour into gel tray.

### ***Molecular size marker***

#### **Lambda *Pst*I**

Bacteriophage Lambda DNA (250 $\mu$ g) 100 $\mu$ l

Buffer M 15 $\mu$ l

*Pst*I 11 $\mu$ l

H<sub>2</sub>O 32 $\mu$ l

Incubate at 37°C for 2 hours followed by heat inactivation at 65°C for 5 minutes.

### *PCR buffer*

#### **10x NH<sub>4</sub> PCR buffer**

Ammonium sulfate	160mM
Tris-HCl (pH8.8)	670mM
Tween-20	0.1%

### *Solutions for the preparation of bacterial competent cells*

#### **CAP buffer**

60mM CaCl <sub>2</sub>	2.21g
15% glycerol	37.5ml
10mM PIPES	0.76g

Make up to 250ml with sterile millipore H<sub>2</sub>O.  
pH to 7.0. Store in fridge.

### *Bacterial media*

#### **Luria-Bertani (LB) medium**

Bacto tryptone	5g
Yeast extract	2.5g
NaCl	5g

Make up to 500ml with ddH<sub>2</sub>O

Autoclave at 121°C for 15min. Let cool down to 55°C before adding appropriate antibiotic (ampicillin: 25mg/ml; kanamycin: 5mg/ml).

*GJA5* bait-construct for Y2H: Kanamycin

*GJA5* bait-construct for M2H: Ampicillin

Prey constructs for M2H: Ampicillin

**LB agar plates**

Bacto tryptone	5g
Yeast extract	2.5g
NaCl	5g
Agar	8g

Make up to 500ml with ddH<sub>2</sub>O

Autoclave at 121°C for 15min. Let cool down to 55°C before adding appropriate antibiotic (ampicillin: 25mg/ml; kanamycin: 5mg/ml), prior to pouring plates.

***Yeast media*****YPDA medium**

Glucose	12g
Yeast extract	6g
Difco peptone	12g
L-adenine hemisulphate (0.2% stock)	9ml

Make up to 600ml with ddH<sub>2</sub>O.

Autoclave at 121°C for 15min

**YPDA agar**

Glucose	12g
Yeast extract	6g
Difco peptone	12g
L-adenine hemisulphate (0.2% stock)	9ml
Bacto agar	12g

Make up to 600ml with ddH<sub>2</sub>O.

Autoclave at 121°C for 15min. Let cool down to 55°C prior to pouring.

**0.5x YPDA medium**

Glucose	3g
Yeast extract	1.5g
Difco peptone	3g
L-adenine hemisulphate (0.2% stock)	2.25ml
Make up to 300ml with ddH <sub>2</sub> O	

**2x YPDA medium**

Glucose	12g
Yeast extract	6g
Difco peptone	12g
L-adenine hemisulphate (0.2% stock)	9ml
Make up to 300ml with ddH <sub>2</sub> O	

**Synthetic Dropout (SD) medium**

Glucose	12g
SD dropout supplement	0.4g
Yeast nitrogen base without amino acids	4g
4M NaOH	160μl
Make up to 600ml with ddH <sub>2</sub> O	
Autoclave at 121°C for 15min.	

Single dropout media: SD<sup>-Ade</sup> or SD<sup>-Trp</sup> or SD<sup>-His</sup> or SD<sup>-Leu</sup> or SD<sup>-Ura</sup>

DDO: SD<sup>-Trp/-Leu</sup>

TDO: SD<sup>-Trp/-Leu/-His</sup>

QDO: SD<sup>-Trp/-Leu/-His/-Ade</sup>

**For SD agar plates:** add 12g agar. Autoclave at 121°C for 15min.

Let cool down to 55°C prior to pouring

### *Solutions used for yeast transformations*

#### **1M LiAc**

5.1g LiAc

Make up to 50ml with ddH<sub>2</sub>O

#### **100mM LiAc**

5ml 1M LiAc

Add 45ml sterile H<sub>2</sub>O

#### **50% PEG**

25g Polyethyleneglycol 4000

Add ddH<sub>2</sub>O up to 50ml

### *Eukaryotic cell culture media*

#### **Complete growth medium**

DMEM 90ml

Hams F12 90ml

Foetal calf serum 20ml

Penstrep

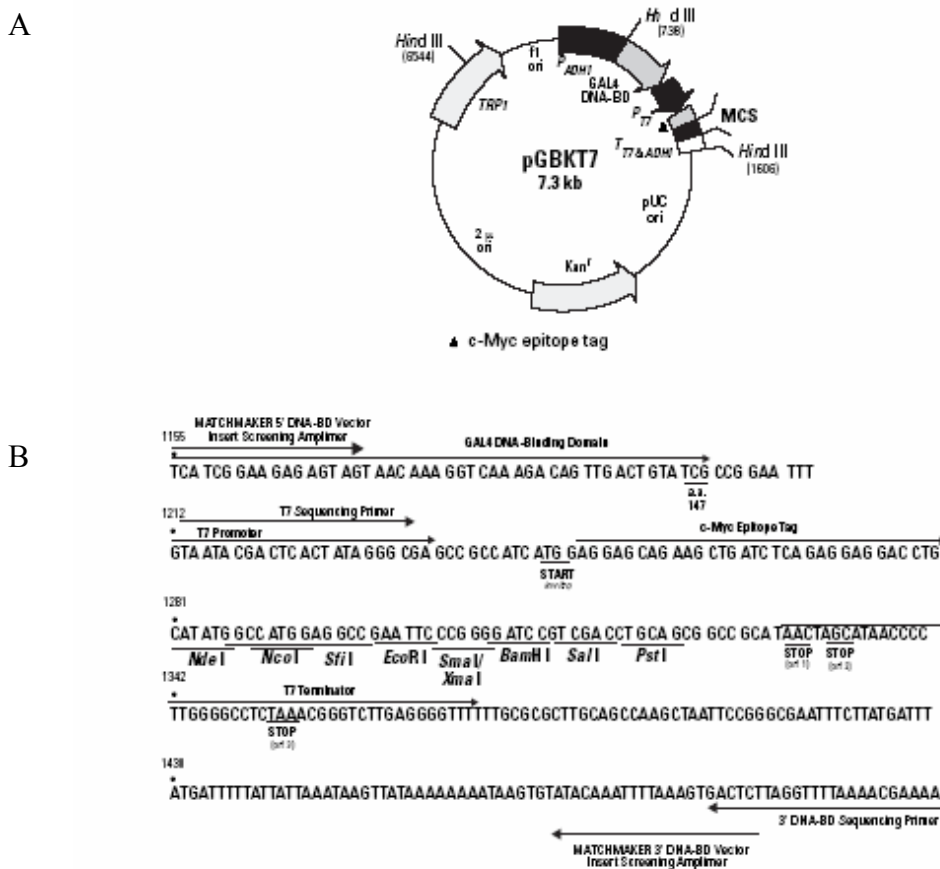
#### **Serum-free medium**

DMEM 100ml

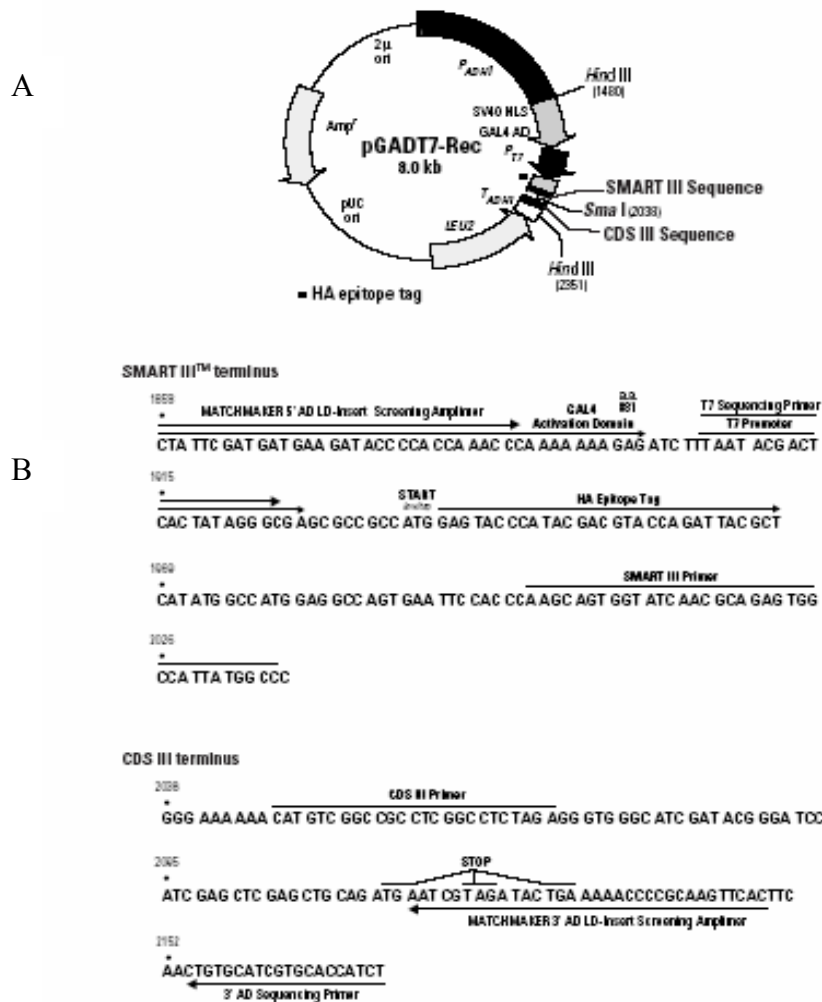
Hams F12 100ml

## Appendix II

### Vectors

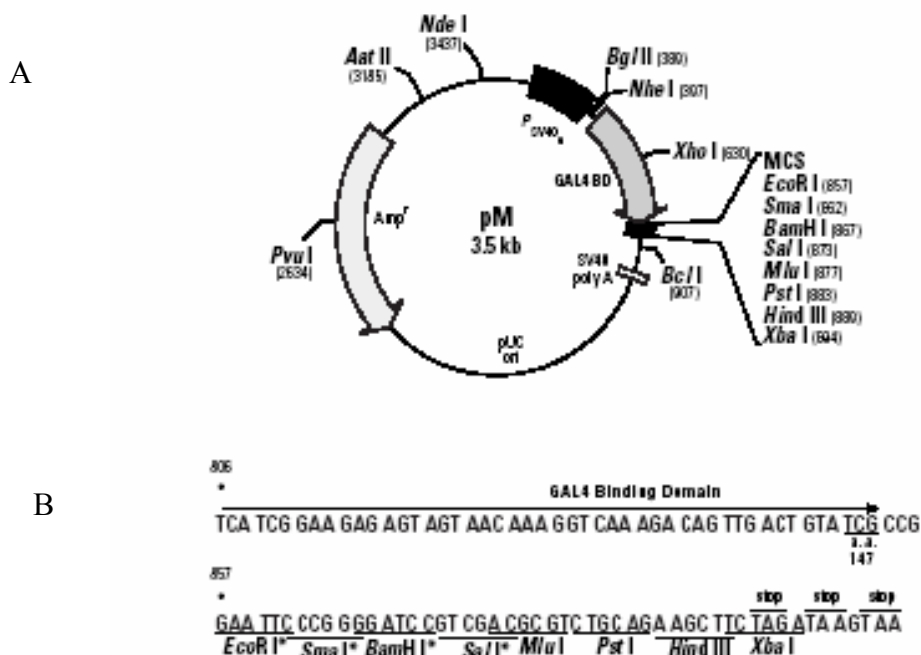


**Figure 1 Restriction map and multiple cloning site (MCS) of the Y2H bait vector - pGBKT7.** A) The positions of the kanamycin resistance gene (*Kan<sup>r</sup>*), TRP1 and GAL4-BD coding sequence, f1 bacteriophage and pUC plasmid origins of replication, the truncated *S.cerevisiae* ADH1 promoter sequence ( $P_{ADH1}$ ), the T7 RNA polymerase promoter and the T7 and c-Myc epitope tag are indicated on the map. B) Nucleotide sequence of the pGBKT7 MCS. The positions of all unique restriction enzyme recognition sequences, stop codons in the T7 terminator sequence, the GAL4-BD coding sequence, the T7 promoter sequence, c-Myc epitope tag and the positions of pGBKT7-F and pGBKT7-R primers are indicated on the sequence (taken from Clontech MATCHMAKERS vectors handbook).

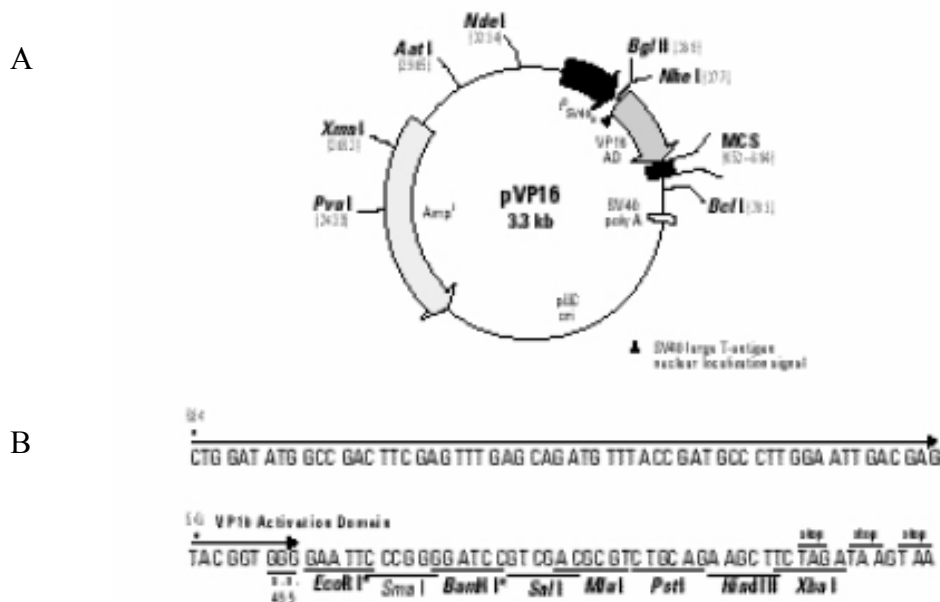


**Figure 2 Restriction map and multiple cloning site (MCS) of the Y2H prey vector – pGAD.** A) The position of the ampicillin resistance gene (*Amp<sup>r</sup>*), *LEU2* and *GAL4-AD* coding sequence, yeast 2  $\mu$  and pUC plasmid origins of replication, the truncated *S.cerevisiae* *ADH1* promoter sequence ( $P_{ADH1}$ ), the T7 RNA polymerase promoter, and the HA epitope tag are indicated on the map. B) Nucleotide sequence of the pGADT7-Rec MCS. The positions of all unique restriction enzyme recognition sequences, stop codons in the T7 terminator sequence, the *GAL4-AD* coding sequence, the T7 promoter sequence, HA epitope tag and the positions of pGADT7-F and pGADT7-R primers (outer and inner) are indicated on the sequence (taken from Clontech MATCHMAKERS vectors handbook).

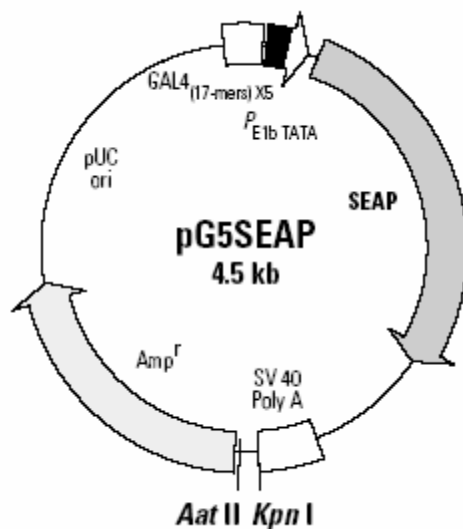




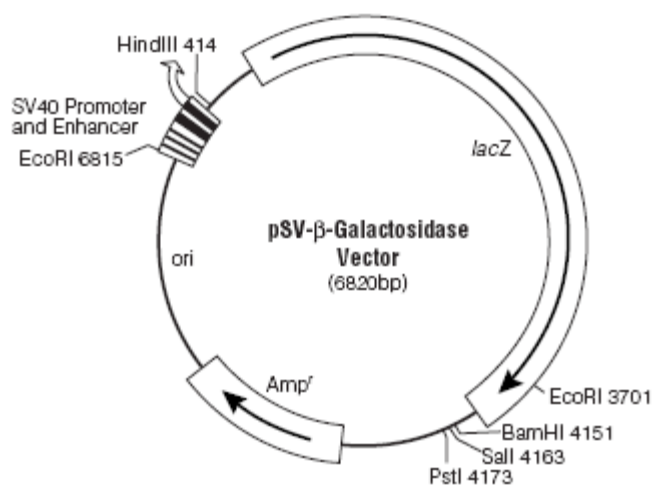
**Figure 3 Restriction map and multiple cloning site (MCS) of the M2H GAL4 DNA-BD vector – pM.** A) The positions of unique restriction sites are indicated in bold. The position of the ampicillin resistance gene (*Amp<sup>r</sup>*), GAL4-BD coding sequence, the SV40 promoter and SV40 polyA transcription termination sequence and the MCS are indicated on the map. B) Nucleotide sequence of the pM MCS. The positions of all unique restriction sites, stop codons, the position of the GAL4-BD coding sequence and the position of the pM sequencing primer are all indicated on the map (taken from Clontech MATCHMAKER Two-Hybrid Assay Kit User Manual).



**Figure 4 Restriction map and multiple cloning site (MCS) of the M2H GAL4 DNA-AD vector – pVP16.** A) The positions of unique restriction sites are indicated in bold. The position of the ampicillin resistance gene (*Amp<sup>r</sup>*), GAL4-AD coding sequence, the SV40 promoter and SV40 polyA transcription termination sequence and the MCS are indicated on the map. B) Nucleotide sequence of the pVP16 MCS. The positions of all unique restriction sites, stop codons, the position of the GAL4-AD coding sequence and the position of the pVP16-F and pVP16-R primers are indicated on the map (taken from Clontech MATCHMAKER Two-Hybrid Assay Kit User Manual).



**Figure 5 Map of pG5SEAP reporter vector for M2H assay.** This vector contains an ampicillin resistance gene (*Amp<sup>r</sup>*), five GAL4 binding sites and an adenovirus E1b minimal promoter sequence upstream from the secreted alkaline phosphatase (SEAP) gene, as indicated on the map (taken from Clontech MATCHMAKER Two-Hybrid Assay Kit User Manual).



**Figure 6 Map of pSV-β-Galactosidase reporter vector for M2H assay.** This vector contains SV40 promoter and enhancer sequences, an ampicillin resistance gene (*Amp<sup>r</sup>*) and the gene encoding LacZ, as indicated on the map (taken from Promega Technical Bulletin).

## *Appendix III*

### ***Calculating yeast mating efficiency***

Count number of colonies on all plates with 30-300 colonies after 4 days

$$\# \text{colony forming units (cfu)/ml} = \frac{\text{cfu} \times 1000 \mu\text{l/ml}}{\text{Volume plated } (\mu\text{l}) \times \text{dilution factor}}$$

1. Number of cfu/ml on SD-Leu plates = viability of prey partner
2. Number of cfu/ml on SD-Trp plates = viability of bait partner
3. Number of cfu/ml on SD-Leu/Trp plates = viability of diploids
4. Lowest number of cfu/ml of SD-Leu or SD-Trp plates indicate limiting partner
5. Mating efficiency =  $\frac{\# \text{cfu/ml of diploids} \times 100}{\# \text{cfu/ml of limiting partner}}$

#### *Library titre*

Count number of colonies on all plates with 30-300 colonies after 4 days

$$\# \text{cfu/ml} = \frac{\# \text{colonies}}{\text{Plating volume (ml)} \times \text{dilution factor}}$$

$$\# \text{colonies clones screened} = \# \text{cfu/ml} \times \text{final resuspension volume}$$

## *Appendix IV*

### **Bacterial strain phenotype**

*E. coli* strain DH5 $\alpha$

$\Phi$ 80d *lacZ* $\Delta$ *M15* *recA1*, *endA1*, *Gry* A96 *thi-1*, *hsdR17* *supE44*, *relA1*, *deoR* $\Delta$   
(*lacZYAargF*)u169

### **Yeast strain phenotype**

*S. cerevisiae* strain AH109

*MAT* $\alpha$ , *trp1-901*, *leu2-3*, *ura3-5*, *his3-200*, *gal4* $\Delta$ , *gal80* $\Delta$ , *LYS2::GAL1*<sub>uas</sub>-*GAL1*<sub>TATA</sub>-*HIS3*, *GAL2*<sub>UAS</sub>-*GAL2*<sub>TATA</sub>-*ADE2*, *URA3::MEL1*<sub>UAS</sub>-*MEL1*<sub>TATA</sub>-*lacZ* (James *et al.*, 1996)

*S. cerevisiae* strain Y187

*MAT* $\alpha$ , *ura3-52*, *his3-200*, *ade2-101*, *trp1-901*, *leu2-3*, 112, *gal4* $\Delta$ , *met*<sup>-</sup>, *gal80* $\Delta$ , *URA3::GAL1*<sub>UAS</sub>-*GAL1*<sub>TATA</sub>-*lacZ* (Harper *et al.*, 1993)

## *Appendix V*

### *List of suppliers*

Agar	Merck
Agarose	Whitehead Scientific
Ampicillin	Roche
BamHI	Promega
Bromophenol blue	Merck
Calf intestinal alkaline phosphatase	Promega
dATP	Boehringer Mannheim
dCTP	Boehringer Mannheim
dGTP	Boehringer Mannheim
dNTPmix	TaKaRa
dTTP	Boehringer Mannheim
EcoRI	Promega
Ethanol	Boehringer Mannheim
GFX® DNA purification kit	Amersham Pharmacia
Glucose	Kimix
Glycerol	FMC Promega
Herring sperm DNA	Promega
Isopropanol	Merck
K-acetate	Sigma
Kanamycin	Roche
Lambda DNA	Promega
LiAc	Sigma
Matchmaker™ Mammalian assay kit	BD Bioscience
MgCl <sub>2</sub>	Bioline
NaCl	BDH Chemicals
Na <sub>2</sub> HPO <sub>4</sub> ·7H <sub>2</sub> O	Merck
Na <sub>2</sub> HPO <sub>4</sub> ·H <sub>2</sub> O	Merck

NaOH	Sigma
NdeI	Promega
Oligonucleotide primers	Department of Molecular and Cell Biology, University of Cape Town (UCT), Cape Town, South Africa
PEG4000	Merck
Peptone	Difco
pG5LUC	BD Bioscience
pGBKT7	BD Bioscience
PCR buffer	Bioline U.K.
pM	BD Bioscience
Phenol/chloroform/isoamyl	Sigma
PureYield™ plasmid midiprep system	Promega
pVP16	BD Bioscience
QDO	BD Bioscience
SD <sup>-L</sup>	BD Bioscience
SD <sup>-T</sup>	BD Bioscience
SD <sup>-L/-T</sup>	BD Bioscience
T4 Ligase	Promega
Taq polymerase	Bioline
TDO	BD Bioscience
Tris	Merck
Tris-HCL	Merck
Trypsin	Highveld Biological
Tryptone	Fluka
Urea	BDH Chemicals
X- $\alpha$ -galactose	Southern Cross
Yeast extract	Difco
Yeast nitrogen base	BD Bioscience

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