Investigating novel *cis*-acting regulatory elements involved in the regulation of heat shock response in cardiomyocytes

Ira Fortuin

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Supervisor: Dr. Mervin Meyer

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KEYWORDS

Stress

Promoter modules

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Regulatory elements

Electrophoretic mobility shift assay

Promoter activity

ABSTRACT

Investigating novel *cis*-acting regulatory elements involved in the regulation of heat shock response in cardiomyocytes.

Ischemic heart disease is a disease which is characterized by the reduced blood supply to the heart. According to WHO 2013, ischemic heart disease is one of the major causes of death globally. For this reason, it is imperative to search for methods whereby heart cells can be protected from cell death. The upregulation of heat shock proteins (Hsps) is one of the major techniques which can be used to protect the heart cells from Hsps cell death and improve the tolerance to ischemic stresses in various models. The increased expression of Hsps during heat shock pre-conditioning is regulated by heat shock transcription factors (HSFs). HSFs orchestrate the initiation of gene expression by binding to sequence motifs, known as *cis*-acting regulatory elements (CAREs). Since gene expression is regulated at a transcriptional level, it is expected that functionally related genes (e.g. heat shock response genes) might also be regulated by the same transcription factors (TFs).

In this study an *in silico* approach was performed to identify the promoter sequences of 50 known heat shock responsive genes using Genomatix Software. This software was also used to identify transcription factor binding sites that are statistically over represented in the promoter sequences of these genes. The use of the Electrophoretic Mobility Shift Assay was included to confirm that protein cell lysates of stressed cells contain proteins (TFs) that bind to this sequence (SP1F_KLFS_01). Luciferase promoter reporter assay were also used to

investigate the transcriptional activity of mutant promoter constructs in which the SP1F_KLFS_01 was mutated.

SP1F_KLFS_01 is a ±25 base pair sequence that was identified in the promoter sequences of 19 heat shock responsive genes, including the well-known Hsp70 and Hsp90. This sequence is a potential binding site for two TFs, Specificity Protein-1 and Krueppel like TFs. Consequently, the aim of this study is to identify CAREs that are statistically over-represented in the promoter regions of heat shock response genes.

In conclusion, *in vitro* experiments of this study did not support the findings of the *in silico* experiments, therefore additional methods should be implemented to expand the investigation for the involvement of *cis*-acting regulatory elements in the regulation of heat shock proteins in cardiomyocytes, prior to heat shock.

DECLARATION

I declare that **"Investigating novel *cis*-acting regulatory elements involved in the regulation of heat shock response in cardiomyocytes"** is my own work that has not been submitted for any degree or examination in any other university and that all sources I have used or quoted have been indicated and acknowledged by complete references.

Ira Fortuin November 2013

Signed: .....

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First and foremost I would like to thank God for giving me the strength and ability to complete this project.

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LIST OF ABBREVIATIONS

Apaf1	Apoptosis protease activating factor 1
ASK1	Apoptosis signal-regulating kinase 1
CARE	*cis*-acting regulatory elements
DNA	Deoxyribonucleic acid
FADD	Fas Associated Death domain
ER	Endoplasmic reticulum
HS	Heat shock
HSE	Heat shock element
HSF	Heat shock factor
Hsp	Heat shock protein
Hsps	Heat shock proteins
I/R	Ischemia-reperfusion
JNK	c-Jun-N-terminal kinase
MI	Myocardial Infarction
mRNA	Messenger RNA
NHS	Non heat shock
TFBS	Transcription factor binding site
TRADD	TNF receptor-associated death domain
UPS	Ubiquitin Proteasomal system

CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Cardiovascular disease is a serious global public health dilemma. Globally, almost 20% of deaths are due to cardiovascular diseases, primarily due to coronary artery disease and stroke (Thygesen *et al.*, 2007). The five main manifestations of coronary heart disease include stable angina pectoris, unstable angina pectoris, heart failure, myocardial infarction (MI) and sudden death (Mendis *et al.*, 2010). MI is the main manifestation of coronary heart disease (Mendis *et al.*, 2010).

MI, commonly known as a heart attack, is associated with cardiomyocyte (heart muscle cells) cell death as a result of prolonged ischemia. Ischemia results from rapid disruption of blood supply to the heart, causing cardiomyocytes to be deprived of oxygen and nutrients, and eventually causing the death of these cells (Sahelian, 2012). The disruption of the blood supply can result from blockage of the coronary artery (blood vessels which supply the heart tissue with oxygen-rich blood and nutrients) by atherosclerotic plaques or blood clots forming in the coronary artery (Beltrami *et al.*, 1994). If blood flow to the heart tissue is not restored within 20 to 40 minutes after the blockage occurred, the cardiomyocytes will die (Thygesen *et al*., 2007). If cardiomyocytes continue to die for approximately 6 to 8 hours, this can be considered as a "complete" heart attack. The areas in the heart muscle where cardiomyocytes died is eventually replaced by scar tissue. The death of cardiomyocytes results from both necrosis and apoptosis (Mendis *et al*., 2010).

Six primary risk factors of MI have been identified in relation to the development of atherosclerotic coronary artery disease (Sahelian, 2012). These risk factors include, hypertension, diabetes mellitus, hyperlipidemia (elevated levels of blood cholesterol and triglycerides in the blood due to unhealthy food consumption), smoking, gender (generally males are more prone to suffer a MI), and familial history of atherosclerotic arterial disease (Nabel and Braunwald, 2012; Sahelian, 2012).

There are various treatments for MI. One of which is surgery; however there are limitations to this treatment method as there will be a chance of thrombosis, limited durability and the likelihood for further surgery in the future (Lange *et al.*, 2001). Tissue engineering and drug delivery approaches have been exploited in recent years as a treatment method for patients with MI. Approaches which effectively protected myocardial cells include stem cell therapy, cardiac TFs (Pu and Izumo, 2001) and heat shock proteins (Hsps) (Lee *et al.*, 2009).

The cytoprotective effects Hsps in cardiomyocytes have been demonstrated in several studies (Latchman, 2001). Mestril *et al.*, demonstrated that the exposure of embryonal rat heart derived cells (H9c2 (2-1)) to non-lethal heat stress resulted in the increased expression of Hsps, specifically inducible Hsp70 isoforms leading to cytoprotection (Mestril *et al.*, 1994). It was shown that cells stably overexpress the inducible form of Hsp70 protein, were much more resistant to ischemic-like stress, demonstrating that inducible Hsp70 plays a vital role in the protection of cardiac cells against ischemia. The heart was the first organ to present a protective effect with the over-expression of a single Hsp in intact animal models (Radford *et al.*, 1996). A number of research groups used transgenic mice models to

demonstrate that the over-expression of Hsp70 protects the heart against the harmful effects of ischemia (Latchman, 2003). The employment of heat shock proteins to protect cardiomyocytes against cell death during cardiac stress such as ischemia is a viable therapeutic strategy for some cardiovascular disorders (Gill *et al.*, 2006).

1.2 Heat shock protein family

Hsps (or stress proteins) are part of a protein family. The molecular weights of the proteins range from 8kDa to 150 kDa. Hsps are generally named according to their molecular weight, for example, the 70-kDa protein is referred to as Hsp70, and the encoding gene for this protein is *hsp70* (Pagel, 2008). The expression of Hsps can be induced rapidly in response to stress stimuli through transcription and translation. Hsp gene transcription is regulated by HSFs (Boshoff *et al.*, 2004). HSFs bind to heat shock elements within the promoter region of the heat shock gene and activate transcription of the heat shock gene (Hietakangas and Sistonen, 2005).

1.2.1 Heat shock proteins as molecular chaperones

The structure and function of a protein is dependent on its three-dimensional structure. An unstable environment within the cell can affect this three-dimensional structure. The three-dimensional structure of proteins which are arranged into spirals, loops and sheets will begin to unravel in response to stress allowing the interior of these proteins to be exposed (Sanders, 1994). Once the interior of proteins are exposed it may lead to adherence and globular formation of the protein causing the protein to become dysfunctional (Sanders, 1994).

Hsps are vitally important as they are needed to ensure that newly synthesized polypeptides are correctly folded (Figure 1.1A) (Latchman, 2001). There are two groups of proteins in the heat shock family, namely, chaperones and cytoprotective proteins. Chaperones are constitutively expressed in the cell and play a vital role in the repair (or refolding) of denatured proteins, or promote the degradation of denatured or damaged proteins (Calderwood *et al.,* 2006). The expression of these proteins is not affected by stressful stimuli (Vabulas *et al.*, 2010). Some well-known chaperone proteins include αB-crystallin, Hsp27, Hsp70, Hsp40, and Hsp90, as well as class I and class II chaperonins (Neef *et al.*, 2011). Table 1.1, lists some of the Hsps and the functional role they play in the cell.

Hsps responsible for cytoprotectivity in the cell may be constitutively expressed, however, their expression is significantly upregulated in response to non-lethal stressful conditions (as shown in Figure 1.1B) (Concannon *et al.,* 2003). This was demonstrated in studies where cells were pre-exposed to low levels of stress.

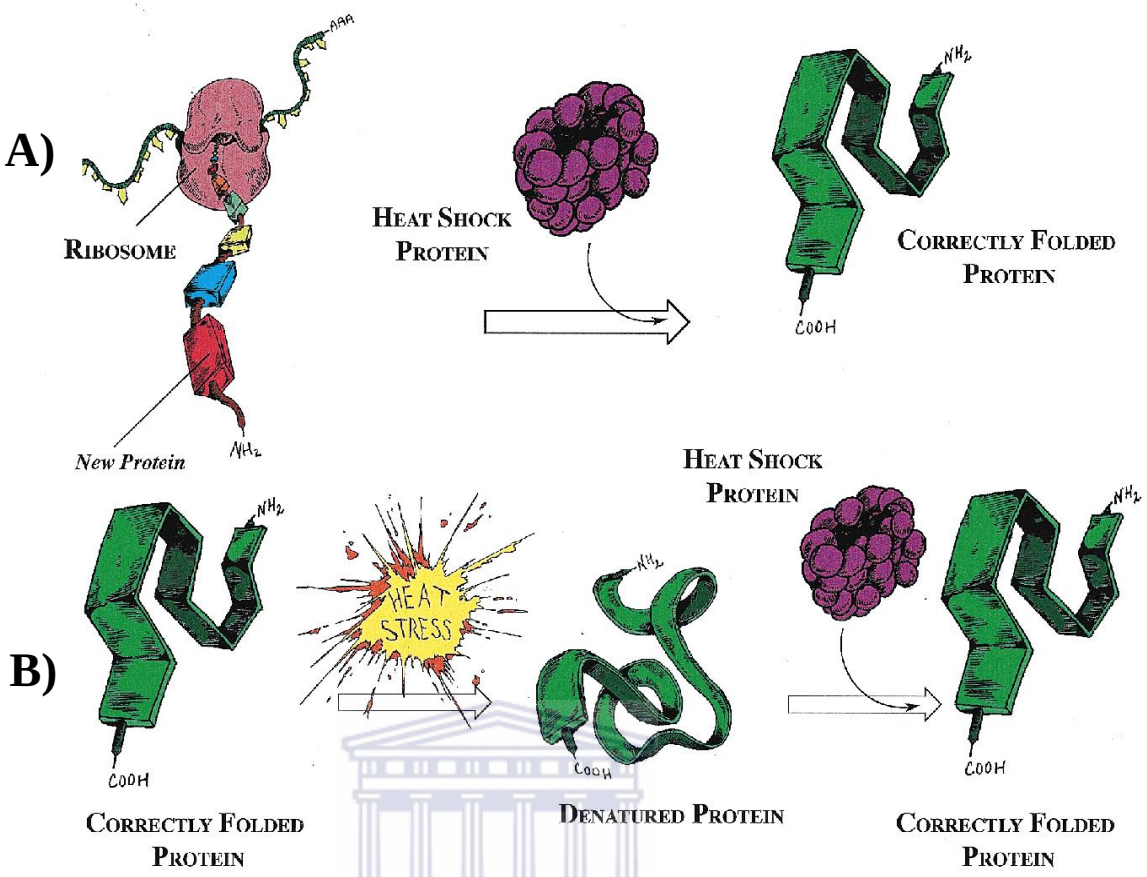


Figure 1.1: Heat shock proteins as molecular chaperones and as cytoprotective proteins in cells. A) Represents Hsps functioning as molecular chaperones, to aid in facilitating the correct folding and refolding of newly synthesized polypeptide chains which will assume its functional 3D protein configuration. B) Illustrates the ability of Hsps to repair denatured proteins. Upon a stressful insult, Hsps are expressed and assist in the refolding or degradation of the denatured protein (Whitley *et al.*, 1999).

Table 1.1 Heat shock proteins and their various functions (Adapted from Latchman, 2001).

Family	Members	Functional Role
Hsp90	Hsp100, Hsp90 Grp94	Maintenance of proteins such as steroid receptor. Src. in an inactive form until appropriate
Hsp70	Grp78 (=Bip) Hsp72, Hsp73 Hsx70	Protein folding and unfolding: assembly of multimeric complexes
Hsp60	Hsp60	Protein folding and unfolding: organelle translocation
Hsp56	Hsp56	Protein folding, component of steroid receptor complex
Hsp32	Hsp32	Cleaves heme to yield carbon monoxide and the protective anti-oxidant molecule, biliverdin
Hsp27	Hsp27, Hsp26, etc.	Unclear
Ubiquitin	Ubiquitin	Protein degradation

For example, the transient exposure of cells to heat stress, also known as "heat shock preconditioning", have been shown to lead to a heat shock response which cause the cells to be protected to subsequent stresses, such as hypoxia or ischemia (Whitley *et al.*, 1999). Heat shock preconditioning was also shown to protect cells against heavy metal poisoning, arterial injury, heat or ischemic injury (Marber *et al.*, 1993); and used in experimental models for cardiac ischemia, endotoxic

shock, arterial injury ethanol-induced gastric ulcerations and many more (Whitley *et al.,* 1999).

It has been observed that Hsps can prevent the demise of a cell by inhibiting specific points in the apoptotic pathway (FitzGerald *et al.*, 2005).

1.2.2 Heat shock proteins in disease

Increased levels of stress proteins has been linked to a number of disorders, including, congestive heart failure, aging, atherosclerosis, neurodegenerative disorders, malignant diseases, and autoimmune disorders (Luo *et al.,* 2010). Neurodegenerative disorders, such as Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD) and various other polyglutamine expansion disorders are associated with the deregulation of apoptosis leading to the death of certain neuronal cell populations. When investigating the involvement of Hsps in the progression of diseases, such as cancer, the anti-apoptotic properties of Hsps become relevant (Luo *et al.*, 2010).

Hsps provide a defence mechanism against misfolded proteins and mutated proteins, and therefore could respond in early stages of diseases with misfolding proteins. In patients with AD, HD and PD, early abnormalities in the axons can be partially reversed with the aid of Hsps (Wyttenbach and Arrigo, 2000). It is known that Hsps are capable of interacting with toxic oligomeric precursors of protein deposits and also with misfolded or mutated conformations of monomers (Muchowski and Wacker, 2005). The mechanisms whereby Hsps improve and

protect neuronal cells are still poorly understood; therefore more research is needed to exploit the full therapeutic potential of Hsps during neurodegeneration.

1.2.2.1 Parkinsons disease

PD is a neurodegenerative disease which is characterized by the loss of dopamine-producing neurons in the *substantia nigra*, a region in the midbrain responsible for eye movement, motor planning and addiction (Velíšková and Moshé, 2006). The common hallmark of PD is the formation of intracellular Lewy bodies, which are abnormal aggregates of misfolded α-synuclein protein that develops within neurons (Engelender, 2008). α-Synuclein is a presynaptic protein of unknown function which is mutated in PD and is the main component of Lewy body formation (Polymeropoulos *et al.*, 1997). This suggests that abnormal protein homeostasis may be involved in the pathogenesis of the disease. Several studies suggest that the ubiquitin proteasomal system (UPS) and Hsps are implicated in PD (Luo *et al.*, 2007). Ubiquitin molecules attach to damaged protein molecules and signal the degradation of ubiquitin-protein complexes via the protease, 26S proteasome (Samaii *et al.*, 2004). Studies that identified mutations in α-synuclein, strongly suggests that the UPS will not be able to degrade these mutant forms of α-synuclein, leading to the accumulation of this protein (Engelender, 2008). Dedmon *et al.*, demonstrated that Hsp70 could potentially inhibit α-synuclein fibril formation by binding to prefibrillar species, which will alter the toxic characteristics of α-synuclein aggregates (Dedmon *et al.*, 2005). Therefore, this study demonstrated that Hsp70 had a beneficial role in the pathogenesis of PD,

and that the chaperone activity is a vital characteristic in protecting neuronal cells against the damaging effects of protein misfolding (Dedmon *et al.*, 2005).

The upregulation of Hsp70 and Hsp25 in response to prior heat shock protected PC12 cells (a cell line derived from a neuroendocrine tumour of the rat adrenal medulla) against the neurotoxin 6-hydroxydopamine (6-OHDA) by lagging the release of cytochrome *c*, caspase activation and leading to a decreased level of apoptosis in cells exposed to 6-OHDA (Gorman *et al.*, 2005). Therefore, it was noted that the induction of Hsp25 protected the cells from 6-OHDA-induced apoptosis. 6-OHDA is generally used to mimic PD effects and lead to the demise of dopaminergic neurons (Gill *et al.*, 2006). Zourlidou *et al.*, also found Hsp27 to have protective properties against α-synuclein. It was shown that mammalian neuronal cells which over-express wild-type and the mutant forms (disease-associated) of α-synuclein are protected from several death stimuli by the exogenous Hsp27, therefore presenting a neuroprotective role for Hsp27 against α-synuclein- induced neurotoxicity (Zoulidou *et al.*, 2004).

1.2.2.2 Alzheimer's and Huntington disease

In patients with Alzheimer's disease, the accumulation of extracellular amyloid beta plaques, in the brain, has been noted (Ballard *et al.*, 2011). HD is a neurodegenerative disease caused by a CAG repeat expansion of the huntingtin (Htt) gene. HD destroys neuronal cells in the basal ganglia (part of the brain which controls movement, cognitive ability, and emotion) (Eidelberg and Surmeier, 2011).

The cytoprotective roles of Hsps in diseases such as HD and AD are not clearly defined as yet. However, it was noted that ethanol preconditioning (refers to a phenomenon whereby tissues are protected from the harmful effects of ischemia/reperfusion (I/R), by the prior ingestion of ethanol at very moderate levels) prevented amyloid beta-induced neurotoxicity as well as apoptosis in the hippocampal-entorhinal complex (situated in the brain) of neonatal rats (Belmadani *et al.,* 2004). It was shown that ethanol preconditioning induces Hsp70 expression which protects neurons from the toxic effects of amyloid beta accumulation in AD. In HD models, it was shown that cells treated with extracellular Hsp70/Hsc70 protein, reduces poly-glutamine aggregation as well as apoptosis (Novoselova *et al.,* 2005). This was done by using a purified Hsp70/Hsc70 sample from bovine muscle and using it in a model of HD. Once biotinylated Hsp70/Hsc70 was introduced to the cells a vast reduction (40-50%) in apoptotic cells was observed. It was then discovered that biotinylated Hsp70/Hsc70 co-localized alongside the polyglutamine inclusions, and when measuring the quantity and size of these inclusions, it was revealed that Hsp70/Hsc70 was capable of reducing both these parameters (Novoselova *et al.*, 2005).

The overexpression of Hsps was also observed to have protective effects in both animal models and cell culture models of stroke. In a Hsp70 knockout experiment in mice it was shown that loss of hsp70.1 lead to an impaired apoptotic pathway characterised by the rapid release of cytochrome *c* into the cytoplasm and the activation of caspase 3 following focal cerebral ischemia (Gill *et al.,* 2006).

Therefore, it can be deduced that the administration of Hsp70 to whole animal experiments, may have putative applications in the treatment of several diseases.

1.2.2.3 Heat shock proteins in cardiovascular diseases

Marber *et al.,* showed that Hsp70 is induced by ischemia, and an inverse relation between the expression of Hsp70 induced by either ischemic or heat shock preconditioning and infarct size in animal models (Marber *et al.,* 1993). Additionally, the increased expression of Hsp70 in response to simulated ischemia produces a cytoprotective effect in cultured cardiomyocytes (Mestril *et al.,* 1994). The overexpression of Hsp70 in transgenic mice enabled the improved recovery of myocardial function, the preservation of metabolic recovery, and the reduction in infarct size following I/R (Latchman, 2001). Similar to Hsp70, 78-kDa glucose-regulated protein (grp78) was also found to be upregulated during periods of ischemia. Studies revealed that the endoplasmic reticulum grp78 was rapidly upregulated during ischemic preconditioning (IP) (phenomenon whereby a tissue becomes resistant to sub lethal ischemic injury by prior exposure to brief periods of I/R) (Murry *et al.,* 1986). This upregulation translated into cardio protection, as it was associated with a decrease in the number of necrotic cardiomyocytes in rat hearts, after coronary ligation (Shintani-Ishida *et al.,* 2006).

Hsp27 and αβ-crystallin have also been shown to protect primary cardiomyocytes against ischemia (Radford *et al.,* 1996). The overexpression of Hsp27 and αβ-crystallin, in cultured cardiomyocytes, has been shown to decrease the release of

creatine kinase. It is thought that Hsp27 and αβ-crystallin stabilizes the cell membrane through the involvement with the cytoskeleton (Martin *et al.,* 1997).

Though the mechanisms by which Hsps protect cardiomyocytes are poorly understood, it is thought that Hsps are capable of facilitating cardio protection via their biological function as molecular chaperones (Benjamin and McMillan, 1998). For this reason it is suggested that Hsps are beneficial proteins which can be exploited in cardio protection.

1.2.3 Advances in the therapeutic exploitation of Heat shock proteins

Hsps are important regulators of apoptosis in cardiomyocytes. Hsps expressed in cardiomyocytes include, Hsp90, -70, -60, -27, and -10 (Hoefling, 2007). The induction of these proteins can occur in response to various agents, such as, cardiotrophin-1, heat shock, herbamycin A, and ethanol, conferring protection to the cardiomyocytes against stresses such as ischemia (Stephanou *et al.,* 1998). Members of the Hsp70 and -27 families are the most widely studied and abundant groups of the myocardial Hsps, and the overexpression of these proteins can protect cells against apoptotic inducers such as serum withdrawal, ceramide, and lethal hypoxia (Sreedhar and Csermely, 2004). Some Hsps have been shown to be critical regulators of apoptosis in cardiomyocytes during exposure to stressful conditions, such as ischemia. However, the failure to induce the expression of these proteins during these stressful conditions may be a pathogenic factor in the development of cardiovascular diseases (Gill *et al.,* 2002). The mechanism by which Hsps regulate the process of apoptosis is still poorly understood, however,

several studies on the mechanism of action of Hsp70 and Hsp27 are beginning to shed some insight on how Hsps control apoptosis (Brar *et al.*, 1999). The overexpression of Hsp60 and Hsp10 has also been demonstrated to protect cardiomyocytes from I/R-induced apoptosis (Lin *et al.*, 2001). These Hsps not only possess a cardioprotective effect in *in vitro* and animal studies, they also represent a potentially valuable treatment for cardiac disease (Radford *et al.*, 1996).

Increasing the expression of Hsps, by ischemia, heat stress or various chemical substances, are vital for the protection of the heart. Numerous approaches aiding in increasing the rate of physiological recovery after infarct stunning and ventricular dysfunction are becoming significant goals in the management of acute MI (Benjamin and McMillan, 1998). Using tissue or whole body hyperthermia strategies to increase the expression of Hsps can be very difficult and extremely unrealistic in conscious humans. For this reason, pharmacological strategies have been implemented to increase the expression of stress proteins for cytoprotection, therefore ensuring a potential advantage against ischemic damage to the heart (Benjamin and McMillan, 1998). A potential approach is the use of protease inhibitors, which increases the level of unfolded proteins within cells. Alternate approaches encompass the development of small molecules and peptides that simulate *in vivo* actions of chaperones with therapeutic benefits (Benjamin and McMillan, 1998). The cardioprotective effect of Hsp induction in *in vitro* and animal experiments can be a possible treatment for cardiac disorders (Gill *et al.*, 2002). Gene therapy may also be an alternative strategy, for example,

specifically targeting heart cells without inducing functional cellular alterations related with stress induced Hsp-induction (Brar *et al.,* 1999).

1.3 Cell survival in response to stressful insults

The ways in which cells respond to stress vary from the activation of pathways that promotes the survival of cells to the activation of programmed cell death which destroys injured cells. The cell's primary response to a stressful stimulus is to defend itself against the stressful insult and recover from the effects of the harmful stimulus (Fulda *et al.,* 2010).

The type as well as the level of the insult depends greatly on the type of stress and how effectively the cell will respond to deal with these conditions. For instance, protective responses (such as the unfolded protein response or the heat shock response) facilitate an increase in chaperone protein activity and enable the enhancement of the protein folding capacity within the cell, therefore, stabilizing the stress and in turn, promoting cell survival (Tamatani *et al.,* 2001). The adaptive capacity of a cell also primarily determines its fate, this means that, the level as well as the mode of stress depends on, various defence mechanisms and pro-survival strategies; however, if these mechanisms are ineffective, then the cell will signal for the activation of cell death programs to eradicate the injured cells (Hori *et al.,* 2006). The mechanism by which the cell dies (apoptosis, necrosis, pyroptosis, or autophagic cell death) frequently relies on its ability to manage the conditions it is exposed to (Fulda *et al.,* 2010).

1.4 Heat shock proteins and apoptosis

There are generally two responses by which cell death can occur, namely, accidental death (necrosis) and instructional death (apoptosis) (Fadeel and Orrenius, 2005). Necrosis can be defined as a passive form of cell death which generally occurs under pathological conditions, resulting in the swift loss of ion-flux control which leads to swelling and rupturing of the cell and its cellular contents (Fadeel and Orrenius, 2005). Apoptosis, on the other hand, is a controlled, energy-dependent form of cell death encompassing a complex network of signal transduction pathways in the initiation and execution phases (Krijnen *et al.,* 2002). Typical traits of apoptosis are condensation of nuclear chromatin, membrane blebbing, changes to the cell membrane, cell shrinkage, formation of apoptotic bodies and nuclear fragmentation (Krijnen *et al.,* 2002; FitzGerald *et al.,* 2005).

Apoptosis can be activated in response to a variety of stimuli for example, cytokines, cytotoxic drugs and oxidative stress (Fadeel and Orrenius, 2005). These various stimuli prompts the activation or initiation of apoptosis, by activating one or more signal transduction pathways, which then in turn activates a conserved family of aspartic-acid specific cysteine proteases, known as caspases (Gill *et al.,* 2006). Caspases (Cysteine aspartic acid-specific proteases) are inactive precursor zymogens which are constitutively expressed within cells, these zymogens can be activated in response to apoptotic stimuli by alterations in the three dimensional structure of the protein (Donepudi and Grütter, 2002). Once activated, these caspases orchestrate cell death by the cleavage of specific subsets

of cellular substrates, and this leads to distinctive biochemical and morphological changes coupled in apoptotic cells (FitzGerald *et al.*, 2005). Caspases can be classified as initiator caspases (upon apoptotic stimuli the caspases will initiate disassembly) or effector caspases (caspases which take part in the dissembly process of the cell) (MacFarlane and Williams, 2004).

1.4.1 Apoptotic pathways leading to the activation of caspase-3

The activation process of caspases can lead to three major pathways in apoptosis, namely, the intrinsic pathway (Figure 1.2), the extrinsic pathway (Figure 1.3), and the Endoplasmic reticulum (ER) stress signalling pathway (Figure 1.4). These pathways all have a similar purpose, to initiate apoptosis and destroy the cell (FitzGerald *et al.*, 2005; Gill *et al.*, 2006).

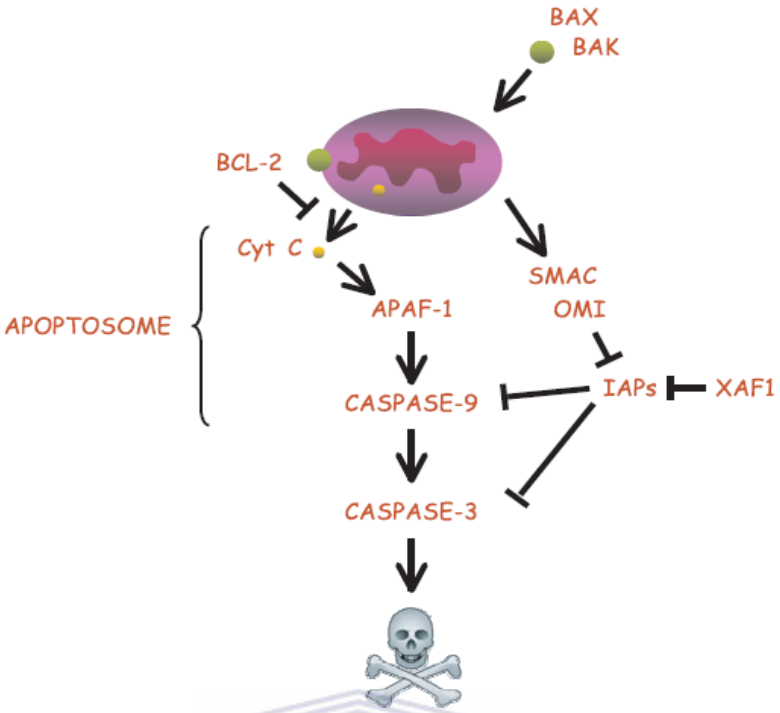


Figure 1.2: The intrinsic apoptotic pathway (Fadeel and Orrenius, 2005).

Pro-apoptotic proteins; BCL-2-associated X protein (Bax), BH3-interacting domain death agonist (Bid) and BCL-2 antagonist killer (Bak) aid in the release of mitochondrial factors which facilitates apoptosis (Saelens *et al.*, 2004). Mitochondria play a vital role in the regulation of apoptosis. Mitochondrial damage can occur in various modes via cytotoxic agents, for example, agents causing oxidative stress, resulting in the permeabilisation of the outer mitochondrial membrane and this causes the release of pro-apoptotic proteins (cytochrome *c*, SMAC/Diablo, apoptosis inducing factor and pro-caspases) into the cytosol (Elmore, 2007). Once cytochrome *c* is released, it forms a complex with adaptor protein (APAF-1) and pro-caspase 9, and these proteins form the apoptosome complex (Li *et al.,* 1997). Activated caspase 9 will be released from the complex and activate downstream caspases, such as caspases 3, 6, and 7. The

activation of pro-caspase 3 will result in apoptosis (Budihardjo *et al.*, 1999; Fadeel and Orrenius, 2005).

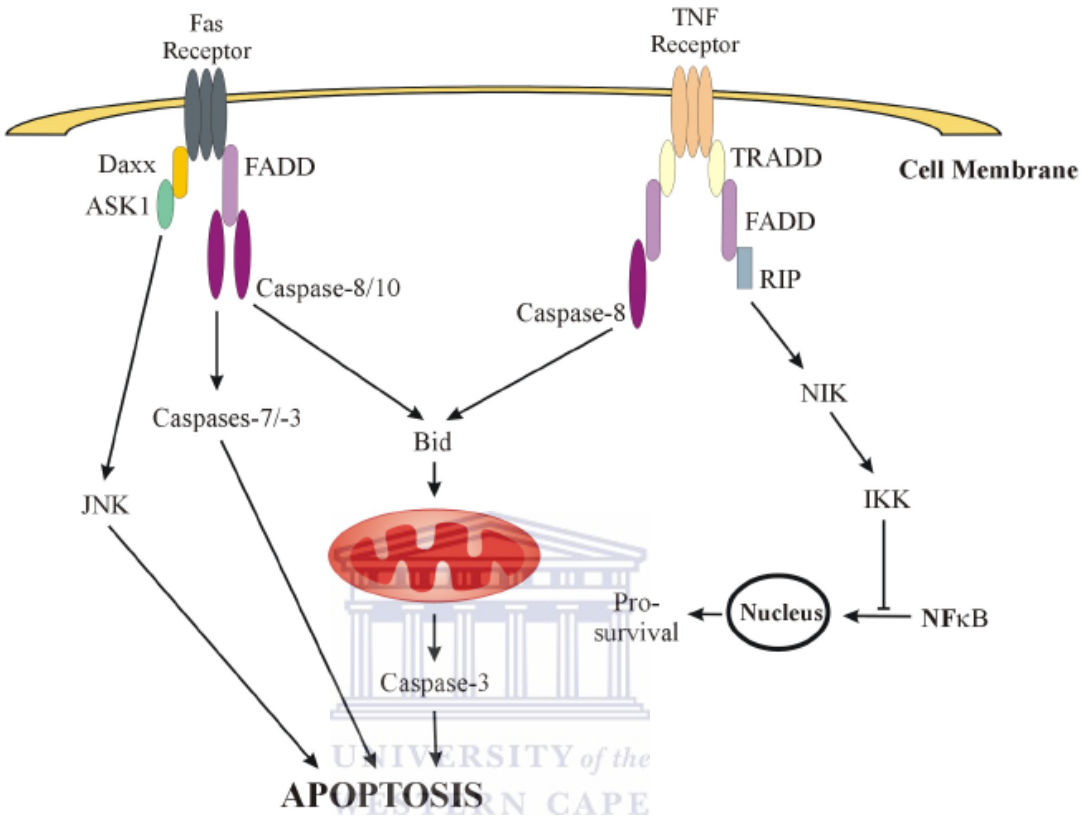


Figure 1.3: The extrinsic pathway in the apoptotic pathway (Gill *et al.*, 2006).

The extrinsic pathway or death receptor pathway is initiated by the binding of specific ligands to cell surface receptors (Elmore, 2007). Fas/CD95 ligands will bind to the Fas receptor and tumour necrosis factor (TNF) ligand will bind to the TNF receptor (TNFR) (Adams, 2003). The Fas and TNF receptors contain a similar region of homology at the cytoplasmic face (68 amino acids) and this is known as the death domain (DD) (Adams, 2003). The binding of the Fas ligand (FasL) to the Fas receptor, recruits an adapter protein Fas Associated Death domain (FADD) and the binding of TNF ligand to TNF receptor, recruits an

adapter protein (TNF receptor-associated death domain) TRADD (Wajant, 2002). FADD will then activate pro-caspase 8/10 with the aid of death effector domains (DED), and pro-caspase 8/10 will form a DISC (Death Inducing Signalling Complex) (Elmore, 2007). Oligmerization and activation of pro-caspase 8/10 will cleave Bid, resulting in the translocation of truncated BID (with caspase 8/10) to the mitochondria and setting off the intrinsic pathway (Kischkel *et al.,* 1995). Additionally, caspase 8/10 can activate caspase 7/3, leading to apoptosis. Another method of activating apoptosis through the Fas receptor is by the binding of an alternative adapter protein (Daxx) to Apoptosis Signal-regulating Kinase 1 (ASK1), which is a MAP kinase. This leads to the activation of the c-Jun-N-terminal kinase (JNK) pathway and this pathway activates apoptosis (FitzGerald *et al.*, 2005). Also, TNF is capable of inhibiting the pro-survival signals of nuclear factor-κB (NFκB) by the recruitment and activation of receptor interacting protein (RIP), NFκB inducing kinase (NIK) and inhibitor of κB kinase complex (IKK), resulting in TNF-induced apoptosis (Malinin *et al.,* 1997).

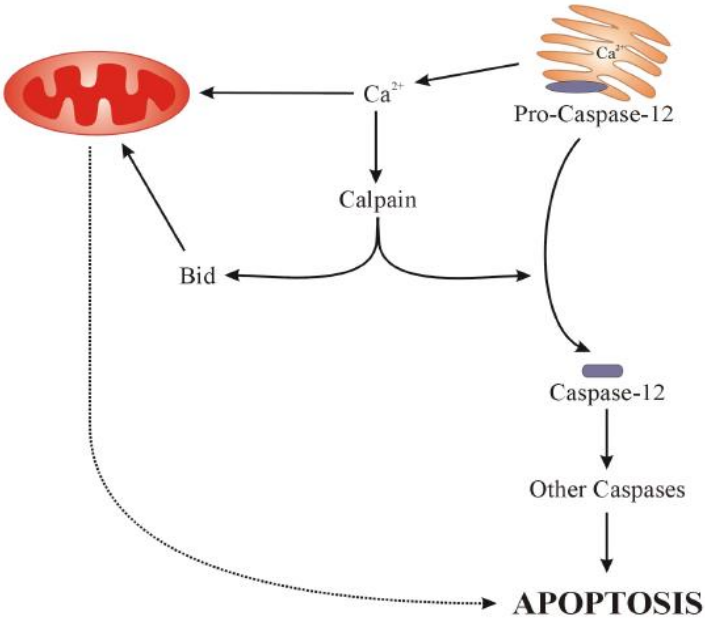


Figure 1.4: The ER stress signalling apoptotic pathway (Gill *et al.*, 2006).

The ER stress signalling pathway was discovered as a third mechanism for the activation caspases and the regulation of apoptosis (Nakagawa and Yuan, 2000). The ER is an important part of this pathway. It is known that post-translational modifications, protein folding, calcium storage and homeostasis all takes place in the lumen of the ER. Once the calcium homeostasis is interrupted, it can cause the accumulation of misfolded proteins (which is the case in diseases such as stroke and diabetes) and in turn activate the ER stress pathway (Nakagawa *et al.*, 2000). Figure 1.4 shows the activation of apoptosis through the ER stress signalling pathway. Once there is a disruption of cystolic calcium levels in the cell, calpain will be activated (Harriman *et al.*, 2002). The activation of calpain, results in the activation of Bid, which translocates to the mitochondria where it will trigger the intrinsic apoptotic pathway. On the other hand, calpain can activate pro-caspase 12 resulting in the activation of downstream caspases and eventually the activation of apoptosis (Rao *et al.*, 2004).

1.4.2 The control of apoptosis by stress proteins

Changes in the intracellular redox balance and the hasty production of reactive oxygen species can initiate the apoptotic pathway; this in turn results in alterations in the mitochondria and the release of pro-apoptotic factors (Sreedhar and Csermeley, 2004). A method of maintaining redox homeostasis and mitochondrial stability in the cell relies on Hsp70 and Hsp27. Figure 1.5 demonstrates how stress proteins can control the process of apoptosis. After its release from the mitochondria, Hsp27 can cleave cytochrome *c*, and pro-caspase 3, therefore preventing the formation of the apoptosome complex and eventually preventing the activation of downstream caspases and apoptosis. Hsp70 has also been shown to inhibit apoptosome formation, in cell-free systems. Overexpression of Hsp70 allows for the protection of cells from stress-induced apoptosis (upstream and downstream of the caspase cascade activation) (Garrido *et al.,* 2001). Furthermore, Hsp70 and Hsp27, have the ability to inhibit apoptosis and thus increase the cells survival to a wide range of lethal stimuli. Aside from the caspase-dependent apoptosis, Hsp27 has the ability to interfere directly with death-receptor inducing signals (extrinsic pathway). This is achieved through phosphorylated Hsp27 dimers capable of interacting with and inhibiting Daxx, which is linked with the Fas receptor and Ask 1 and downstream JNK pathway which in turn prevents apoptosis (Charette *et al.,* 2000). Charette *et al*, noted that no inhibition of Daxx was observed with an HSP27 phosphorylation mutant (only expressed as a oligomer) or when apoptosis was induced by the transfection of a Daxx mutant with a deleted HSP27 binding domain.

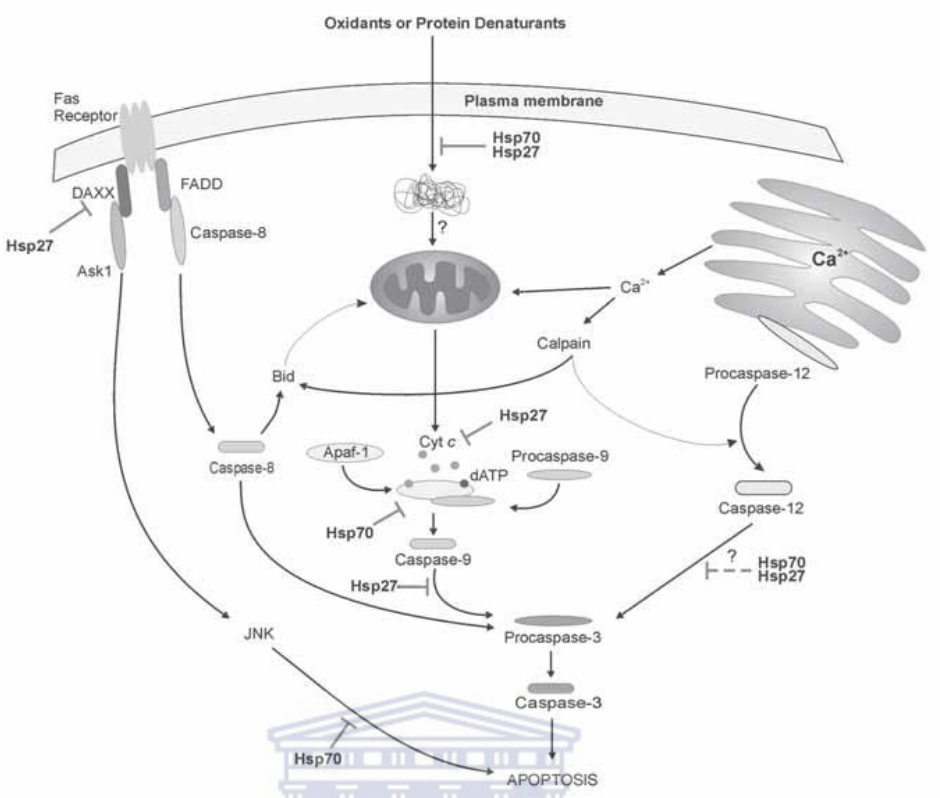


Figure 1.5: The interactions of Hsp27and Hsp70 in the apoptotic pathway (FitzGerald et al., 2005).

1.5 Transcriptional regulation of heat shock proteins

Cellular stress (increased temperature, mechanical damage and exposure to toxins) causes the interruption of various metabolic processes and cellular structures, resulting in the death of the cell when a critical threshold is surpassed (Welch, 1993). Heat stress (temperatures above 42°C) as well as ischemia are able to cause a great deal of damage to the cytoskeleton, such as re-organization of the cytoplasmic network, disintegration of threadlike intermediate filament networks into bulky perinuclear aggregates, relocalizaton of actin-containing fibres, and interruption of microtubules and mitotic spindles (Welch *et al.*, 1991).

Similar shared characteristics of heat stress and early reversible ischemic injury include mitochondrial swelling, loss of mitochondria, and uncoupling of oxidative phosphorylation (Neely and Grotyohann, 1984).

In 1962, Ritossa observed a pattern of chromosomal puffs in *Drosophila* cells when exposed to heat stress (Hietakangas and Sistonen, 2005). It was later demonstrated that the formation of these puffs are associated with the increased expression of Hsps (Hietakangas and Sistonen, 2005). Subsequent studies showed that numerous stress stimuli, for example heat stress, oxidative stress, exposure to protease inhibitors and heavy metals, can trigger the increased expression of Hsps (Ruis and Schüller, 1995). This increased expression of Hsps in response to stress stimuli is called the heat shock response. It is also an important physiological defence mechanism for the protection of cells from various harmful stimuli (Lee and Goldberg, 1998).

The expression of Hsps during the heat shock response is regulated by a set of HSFs. These TFs control the transcription of various heat shock genes which may protect the cellular proteins against harmful environmental stresses (Trinklein *et al.*, 2003). The mammalian HSF family contains four members, namely HSF 1, HSF 2, HSF 3 and HSF 4 (Rabindran *et al.*, 1991). Two of these mammalian TFs, HSF 1 and HSF 2, play a role in interacting with particular sites in the promoter region of heat shock-induced genes during stressful or differentiation conditions, resulting in an increase in the transcriptional activity in these genes (Trinklein *et al.*, 2003).

The activation of the heat shock response involves the trimerization and translocation of HSF1 from the cytosol to the nucleus in response to stressful stimuli (most commonly elevated temperatures) (Holmberg *et al.,* 2001). When in the nucleus, HSF1 attaches to heat shock elements (found in the TATA-box-proximal 5´-flanking regions of heat shock inducible genes) in promoter region of heat shock genes (Figure 1.6). Once the trimer binds to the heat shock element in promoter region of the Hsp gene it will result in the production of heat shock protein messenger RNA (mRNA) and several post-translational regulatory modifications of the protein will occur (Whitley *et al*., 1999). Eventually the mRNA will move to the cytosol where successive translation and production of heat shock proteins will occur (Figure 1.6) (Whitley *et al*., 1999).

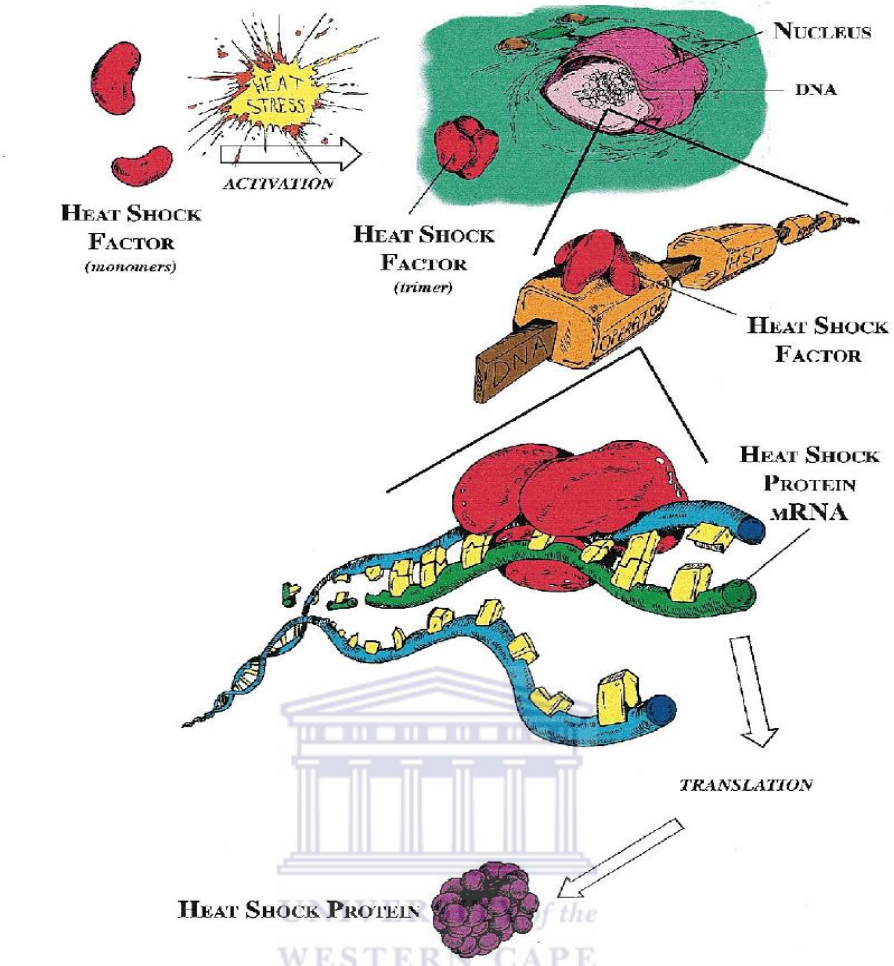


Figure 1.6: The expression of the heat shock protein (Whitley *et al.*, 1999).

However, in an unstimulated state, HSF1 will remain inactive and in the monomeric form. The heat shock response will therefore not be activated. The initiation, execution and termination of the heat shock response is therefore tightly regulated, through post-translational modifications and protein interactions (Trinklein *et al.*, 2003).

1.6 Transcriptional regulation of gene expression by transcription factors

TFs are regulatory proteins which are involved in regulating gene expression (Zaret and Carroll, 2011). The DNA-binding domains of TFs have a 10^6 fold higher affinity for their target sequences than the rest of the DNA strand, and these highly conserved sequences are used to classify TFs into “families”, for example, SOX proteins (TFs which bind to minor grooves in DNA), MADS box-containing proteins (TFs which bind to the motif CC[A/T]GG also known as the CAG-box sequences) and POU factors (TFs containing a bipartite DNA binding domain) (Remenyi *et al.*, 2004, Phillips, 2008). TFs also have the ability to function in groups or complexes, and form numerous interactions which will enable the binding of RNA polymerase to the complex and activate the transcription process (Phillips, 2008). In eukaryotes, genes are normally turned "*off*", therefore, TFs function to turn gene expression "*on*" (Garrett and Grisham, 2005). However, in bacteria, the genes are expressed constitutively until TF turns it "*off*". TFs operate by recognizing specific nucleotide sequences (motifs), which are situated before or after the gene on the chromosome (up or downstream) (Phillips, 2008).

Eukaryotic genes consist of promoter regions situated upstream of the gene, or enhancer regions which are situated up- or downstream of the gene, additionally eukaryotic genes contain certain motifs which can be recognized by several TFs (Garrett and Grisham, 2005).

The mechanism by which TFs regulate gene expression is of vital importance in the functioning of a gene. TFs will bind to either enhancer or promoter regions of

DNA. The transcription of the adjacent gene will then be up- or down-regulated. Mechanisms by which gene expression can be regulated via TFs include, stabilize or inhibiting the binding of RNA polymerase to the DNA sequence, acetylation or deacetylation of specific histone proteins, histone acetyltransferase activity (acetylates histone proteins, weakening DNA to Histone binding and accessing DNA to transcription, the employment of co-activator or co-repressor proteins to the DNA transcription factor complex (Srinivas and Swamynathan, 1996; Garrett and Grisham, 2005).

Research has shown that there is a uniform manner in which different organisms, respond to the same stressful stimulus. This process involves shutting off the general transcription mechanism and activating the transcription of a specific set of genes encoding Hsps or stress proteins. The stress proteins aid in maintaining correct protein conformation and prevent misfolding, as explained in section 3 (Srinivas and Swamynathan, 1996). The induction of heat shock proteins in response to stressful stimuli (such as increased temperatures or ischemia) can be facilitated by the heat shock transcription factor (HSF-1) which binds to heat shock elements (HSEs).

1.6.1 Regulation of the heat shock response by promoters of heat shock genes

The heat shock response mechanism is known to be highly regulated at the level of transcription; however, there are some cases where translational regulation will take place (Ananthan *et al.* 1986). The heat shock response mechanism is

generated through conserved *cis*-acting regulatory promoter elements (or HSEs) found in the TATA-box-proximal 5´-flanking regions of heat shock inducible genes (Morimoto, 1998). *Cis*-acting sequences are regulatory sequences that form part of the gene in question, therefore these *cis*-acting sequences will only influence the expression of the gene that contains them (Morimoto, 1998).

These sequences are mostly found upstream of the transcription start site, however, they can also be found further upstream, or even within the introns and exons of the gene, or on the 3' end of the gene. The presence of multiple HSEs within approximately one hundred base pairs has been shown to be a signature of the majority of eukaryotic heat shock inducible genes (Schöffl *et al.*, 1998).

Approximately all heat shock responsive promoters contain heat shock elements with a conserved 5 base pairs modular unit 5´-nGAAn-3´, and can be present in the promoters of Hsps as inverted repeats (Morimoto, 1998). Once a HSE is deleted from a promoter, the stress-response mechanism will automatically be tuned off or become non-responsive. However, if the HSE is engineered, they will convey stress inducibility to promoters (Pelham and Bienz, 1982). HSEs bind to *trans*-active HSF (under stressful conditions) to induce the expression of heat shock responsive genes. Efficient binding of HSF to HSE involves at least three units, this essentially means the following sequence must be present, 5´-nGAAnnTTCnnGAAn- 3´. It has been shown that in the HSF1 from the Arabidopsis plant is capable of binding consensus tripartite HSE sequences and

HSE-containing regions of the *D. melanogaster* HSP70 promoter (Hübel and Schöffl, 1994).

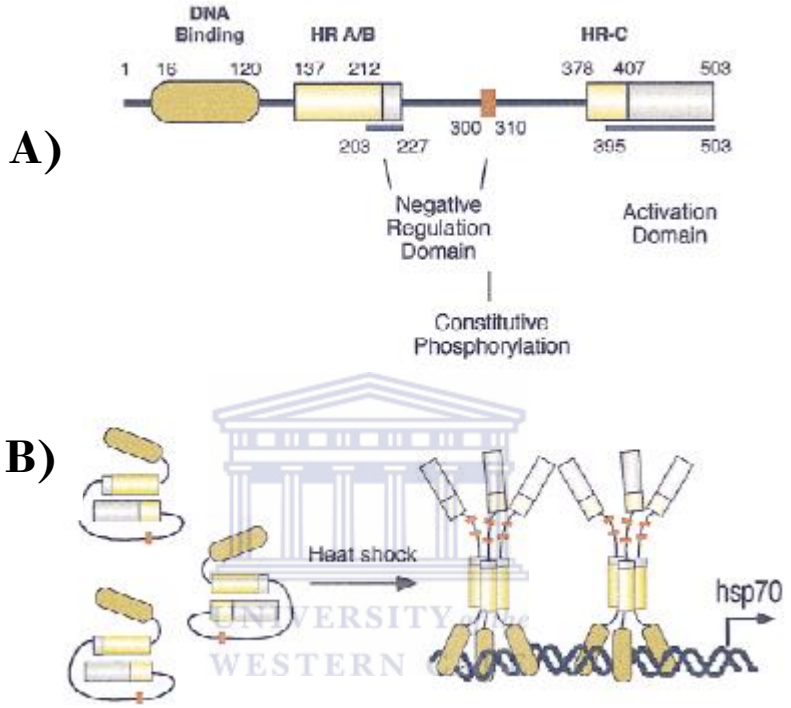


Figure 1.7: The general structure and regulatory features of HSFs (adapted from Morimoto, 1998). A) Demonstrates the structural motifs of HSF1, as well as the DNA-binding domain, hydrophobic heptad repeats (HR-A/B and HR-C), the carboxy-terminal transcription activation domain, and the negative regulatory domains which eventually influence the activity of HSF1. Amino acid residues also indicate the various positions of these domains in HSF1 (Morimoto, 1998). B) Illustrates the intramolecular negatively regulated monomer, which can be activated upon stressful stimuli to form homotrimers with DNA-binding activity (Morimoto, 1998).

In the binding of HSF to HSEs one mismatch of one nucleotide in the consensus sequence can be tolerated. The HSEs of all the heat shock promoters in *Drosophila* are shown to possess repeats of the dinucleotide GA (GA or CT) (Gilmour *et al.,* 1989). The binding of a GAGA factor protein to these GA repeats

have been shown to be of vital importance in the process of ATP dependent nucleosome disruption in heat shock inducible promoters. This aids in the open configuration of promoters and allow for transcription activation (Tsukiyama *et al.,* 1994; Srinivas and Swamynathan, 1996).

1.6.2 *In silico* promoter analysis for the determination of conserved motifs

The regulation of transcription is a very significant, and rate limiting step in gene expression. There are various types of *cis*-acting DNA sequence elements which play a role in contributing to this regulation, however, promoters are the easiest elements to locate as they are situated upstream of the transcription start sites. In earlier years, studies relating to functional studies of promoters were performed on a gene-by-gene basis and generating data sets as Eukaryotic Promoter Database (EPD), containing numerous human promoters (Trinklein *et al.,* 2003). Recent attempts in identifying promoters on a large scale have also been endeavoured with computational approaches (Davuluri *et al.,* 2001). Since the "draft" sequence of the human genome and the full-length cDNA sequence library are known, researchers speculate that strategies in identifying promoters would be to align full-cDNA sequences to human genome sequences, then predicting the transcription start sites (TSS), and identify sequences upstream from these sites. In this way the promoters will be identified as well as the sequence of interest. An example of this application was done by Suzuki and Sugano, who used an oligo-capping approach enabling the enrichment of full-length cDNAs (Suzuki and Sugano, 2001). Suzuki and Sugano then proceeded to map these cDNAs onto

genomic sequences to predict the TSS, these TSS were then used to identify potential promoter regions of 1031 human genes (Trinklein *et al.*, 2003).

Promoter analysis software tools for the prediction of various binding sites include, Genomatix MatInspector, Genomatic ModelInspector, MATRIX SEARCH, Signal Scan. For this study, Promoter analysis was done with Genomatix software (Munich/Germany) (Werner *et al.*, 2003).

Modern developments in bioinformatics, with regard to promoter detection, now allows for the detection of promoters in megabase sequences. Promoter function is largely governed by the binding of TFs to their cognitive binding sites in promoter DNA sequences (Scherf *et al.*, 2000). These binding sites act in a modular fashion to support protein complexes for transcriptional activation (Arnone and Davidson, 1997). Modular organization of promoters will also be reflected in the DNA sequence by specific groups of transcription factor binding sites which represents functional modules within promoters, also known as promoter modules. Promoters of genes from similar gene classes often have different nucleotide sequences (Werner, 2001). Yet, co-expressed genes can still be identified because it is believed that they share promoter modules even if they belong to different gene classes (Werner, 1999). Furthermore, promoters of related genes will share a more common framework of TF binding sites. Therefore, it can be well established that genes which are expressed in the same tissue, will share a mutual organisation of regulatory binding elements. Thus, promoter motifs can represent "footprinting" of transcriptional regulatory mechanisms and provide valuable information about various signals and tissue specific control of expression in

novel genes. Also, analysing promoters for organizational features may provide important links between constant nucleotide sequences of a genome and vital characteristics of gene regulation and expression (Werner *et al.*, 2003).

Hypothesis

The hypothesis of this study was that genes encoding proteins of the same group, for example heat shock responsive proteins, are related in a functional way, and that these genes are considered to share the same TFs and promoter motifs. Identifying these motifs will be beneficial in determining various characteristics of novel heat shock responsive genes on the basis of *in silico* techniques. This in turn, will provide information about the cytoprotective roles of genes and their various functions in certain pathways when exposed to stressful insults.

Objectives

The objectives of this study were to:

a) Identify common promoter sequences of Hsps in *Rattus norvegicus* species.

b) Identify shared *cis*-acting regulatory motifs in the promoter regions.

c) Investigate in a molecular approach, if shared motifs are involved in the regulation of Hsps.

Aims

The aims of this study were to find novel *cis*-acting regulatory sequences within the promoter sequences of Hsp70 promoters in cardiomyocytes (*Rattus norvegicus* species) when exposed to a non-lethal stress e.g. heat shock. This was done by using two experimental approaches, namely, *In silico* and molecular biology. It is noted that conserved regions in these *cis*-acting regulatory sequences are an essential part for the regulation of Hsp70. Once Hsp70 is expressed in increased amounts, it could protect the cardiomyocytes from apoptosis. However, it is proposed that when mutating these conserved bases in the *cis*-acting regulatory sequences of Hsp70, it could turn off the regulation of Hsp70. In this study conserved regions were found and mutated, and following molecular experimental procedures, EMSA and Luciferase promoter reporter assay. Electrophoretic mobility shift assay was used to determine protein:DNA complexes, together with Promoter reporter assays to confirm that protein cell lysates of stressed cells contain proteins (transcription factors) that bind to the conserved sequence. These studies were done to evaluate the effects that these conserved regions have on the upregulation of Hsp70 in cardiomyocytes when exposed to heat shock, and in turn protect the cardiomyocyte from degradation (apoptosis).

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials used and Suppliers

Materials	Suppliers
Acrylamide-BIS (37:5:1)	Bio-Rad
Agarose	Whitehead Scientific
Ammoniun persulphate (APS)	Promega
Bovine serum albumin (BSA)	Roche
Bromophenol blue	Merck
Coomassie Brilliant Blue R 250	Sigma-Aldrich
Cytobuster ™ Protein Extraction Reagent	Novagen
Dimethyl dicarbonate	Sigma
Dithiothreitol (DTT)	Roche
Ethylene Diamine Tetra-acetic acid) EDTA	Sigma
6X Gel Loading Buffer	Fermentas
Glycerol	Merck
Glycine	Merck
Glacial acetic acid	Merck

Hybond- N^+ Positively charged nylon transfer membrane	Amersham Pharmacia biotech
Isoamyl alcohol	Merck
Kanamycin	Roche
Methanol	Merck
Nuclease free water	Fermentas
Penicillin	Sigma-Aldrich
Ponceau S Stain	Sigma
Puromycin	Life Technologies
RNase A	Roche
Sodium dodecyl sulphate (SDS)	Promega
Sodium Chloride (NaCl)	Merck Chemicals
Sodium hydroxide	Merck
Sybr® Safe DNA Gel stain	Life Technologies
Tris[hydroxymethyl] aminoethane (Tris)	Merck
Tryptone	Merck
N, N, N´, N´-Tetra methylethylene-diamine (TEMED)	Sigma-Aldrich
Yeast extract	Merck

2.2 Cell culture

2X Trypsin-Versene® (EDTA)	Lonza
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich
Dulbecco's Modified Eagle medium (DMEM)	GIBCO-Life Technologies
Foetal Bovine Serum (FBS)	GIBCO-Life Technologies
Metafectene- PRO™ Transfection Reagent	Biontex Laboratories
Phosphate buffered saline (PBS)	Lonza

2.3 Kits used

LightShift® Chemiluminescent EMSA kit	Thermo Scientific
NucleoSpin® TriPrep kit	Macheret-Nagel
Reverse Transcription System Kit	Promega
Secrete-Pair ™ Dual Luminescence Assay Kit	GeneCopoeia
Supersignal ® West Pico Chemiluminescent Subtrate	Thermo Scientific
Wizard ® Plus SV Minipreps DNA Purification system kit	Promega

2.4 Solutions and Buffers used

2X Sample buffer: 100 mM Tris-HCl (pH 6.8), 25 % glycerol, 0.01 % BPB, 50 mM DTT.

6X Glycerol BPB Gel Loading Buffer: 30 % Glucose and 0.3 % Bromophenol blue.

10X Running buffer: 25 mM Tris-HCl, 192 mM glycine, 0.1 % SDS.

10X TE: 0.1 M Tris, 0.01 M EDTA.

10X TBE: 0.9 M Tris, 0.89 M Boric Acid, 25 mM EDTA.

Ammonium persulphate (APS): A 10 % stock solution was prepared in distilled water and stored at -20°C.

Blocking solution: 5 % fat free milk powder dissolved in TBST**.**

Bovine Serum Albumin (BSA): 3 % BSA dissolved in TBST and stored at 4°C.

Coommassie staining solution: 0.02 % Coommassie Brilliant Blue R 250, 40 % methanol, 10 % acetic acid.

Destaining solution: 10 % Methanol and 10 % Acetic acid.

DEPC treated water: 0.1 % diethylpyrocarbonate dissolved in 2 litres of distilled water. Incubated at 22 °C overnight and autoclaved.

Kanamycin: 100 mg/ml stock prepared in distilled water and stored at -20°C.

Luria agar: 10 g/l Tryptone, 5 g/l Yeast Extract Powder, 5 g/l NaCl, 14 g/l Bacteriological Agar.

Luria Broth: 10 g/l Tryptone, 5 g/l Yeast Extract Powder, 5 g/l NaCl.

Ponceau S staining solution: 0.1 % Ponceau S and 5 % Glatial acetic acid.

Separating buffer: 1.5 M Tris-HCl, pH 8.8**.**

Stacking buffer: 0.5 M Tris-HCl, pH 6.8.

Sodium hydroxide/Sodium dodecyl sulphate (NaOH/SDS): 0.2 M NaOH and 1 % SDS

Transfer buffer: 25 mM Tris-HCl, 192 mM glycine, 20 % methanol. After preparation the buffer was kept at 4°C.

TBST: 0.1 % Tween-20, 150 mM NaCl, 20 mM Tris. After the preparation the solution was kept at 4°C.

2.5 BIOINFORMATICS

2.5.1 *In silico* analysis of heat shock responsive genes

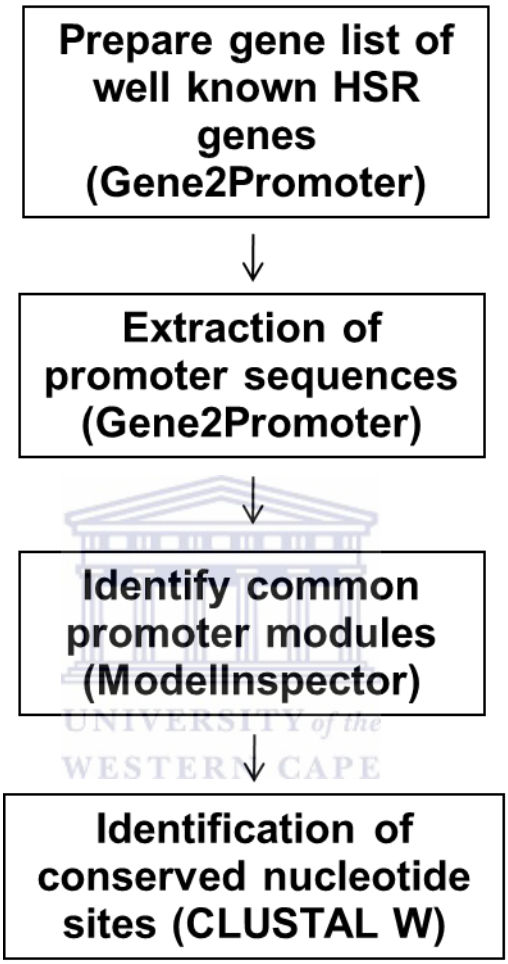


Figure 2.1 Strategy devised for *in silico* promoter analysis.

A gene list of 50 well-known heat shock responsive proteins was generated using Genomatix software suite, Gene2Promoter (Genomatix, Germany). Using the search term "heat shock" (for *Rattus norvegicus* species), all genes that are heat shock responsive was identified. Subsequently, promoter sequences for these genes were identified, within these heat shock responsive proteins using Gene2Promoter. Only promoters which were experimentally verified were

considered. PromoterInspector was used to identify the experimentally verified promoters Promoters that were *in silico* predicted were excluded from the study.

2.5.2 Identification common promoter motifs

Common transcription factor binding sites were identified using MatInspector and ModelInspector was used to identify conserved promoter modules. CLUSTALW was used to identify highly conserved nucleotide sites in the promoter modules.

2.6 PROMOTER REPORTER CONSTRUCTS

2.6.1 Cloning vectors

Two plasmid DNA vectors pEZX-pG04 and pEZX-pG04-Mut were acquired from Genecopeia™, USA. Both of these vectors are based on the GLuc-ON™ promoter reporter systems from Genecopeia™.

2.6.2 pEZX-pG04 / GLuc-ON Promoter Reporter Clone

The GLuc-ON™ promoter, from Genecopeia, is a dual-reporter system which uses GLuc as the promoter reporter and Secreted Alkaline Phosphatase (SEAP) as the internal control for signal normalization. The plasmid contains a Puromycin resistance gene, used in the selection of stable cell lines. The pEZX-pG04 plasmid contained the Hsp70 promoter sequence and can be used as a reporter for Hsp70

promoter activity. pEZX-pG04-Mut also contained Hsp70 promoter, but a mutation was introduced in the conserved region of the promoter module that is shared by heat shock responsive genes and was identified by *in silico* promoter analysis.

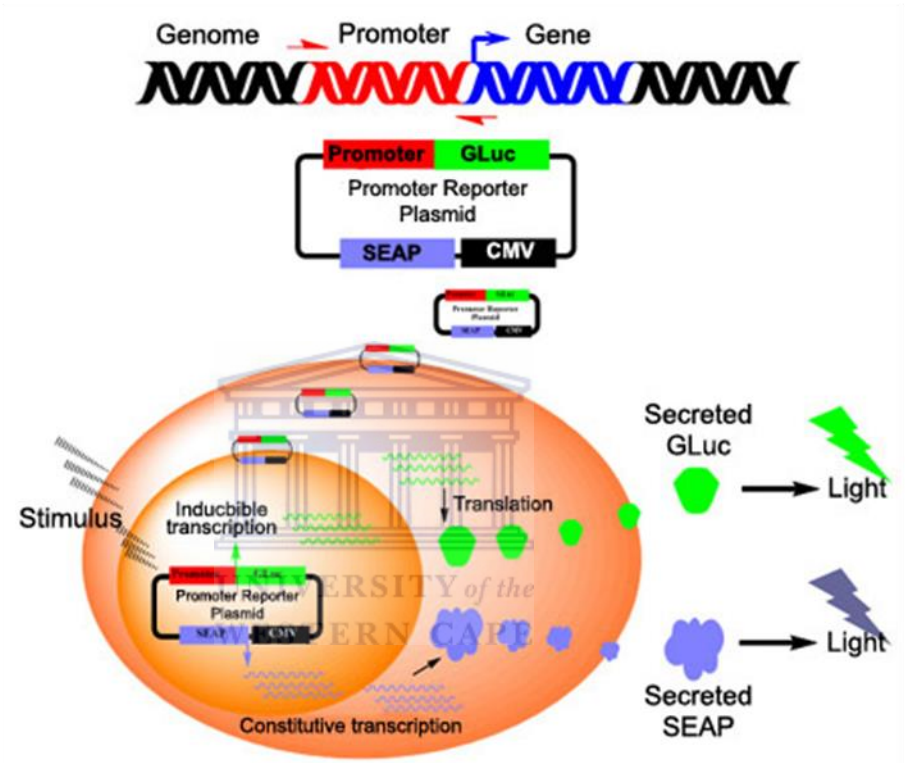


Figure 2.2 Expression-ready pre-designed dual reporter pEZX-pG04 system created by Genecopoeia, representing the SEAP expression system as well as the GLuc-ON™ Promoter system. The dual GLuc-ON promoter reporter system contains the SEAP expression and the luciferase expression. SEAP is constitutively expressed in the plasmid and can be detected with the appropriate wavelength after the addition of a substrate. However, upon a stimulus (such as heat shock) the promoter will be activated and induce GLuc (luciferase), this can be measured with the appropriate wavelength after the addition of a substrate.

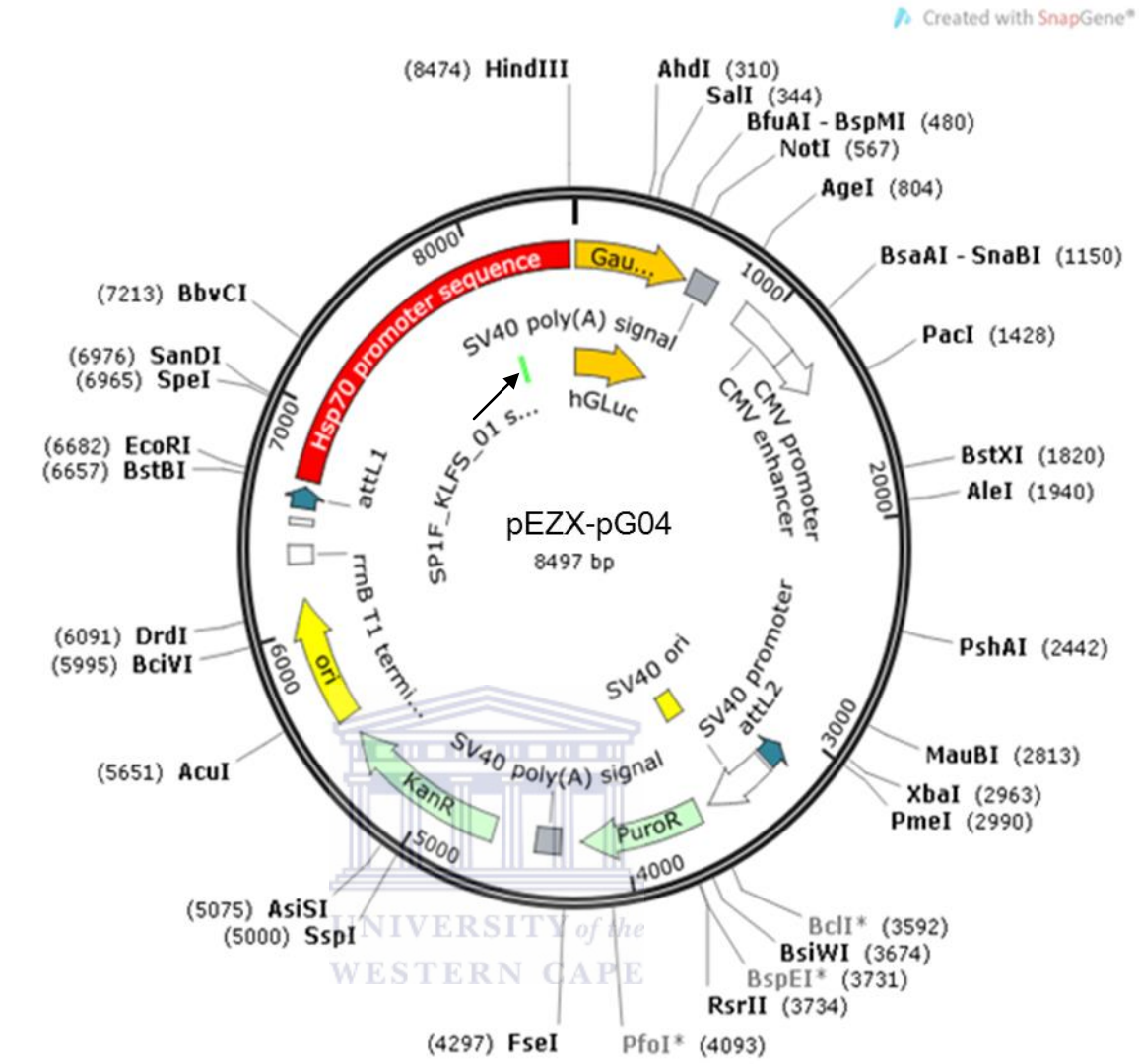


Figure 2.3 A circular map of the pEZX-pG04 Vector System, created by SnapGene Viewer. The map indicates the multiple cloning site for various restriction enzymes as well as antibiotic restsiance genes. Hsp70 promoter region (shown in red, in figure 2.3), located 6688 bp downstream of the *Gaussia* luciferase (Gau), contained the conserved promoter module SP1F_KLFS_01 (as shown in green, in figure 2.3). The pEZX-pG04-Mut contained point mutations within the SP1F_KLFS_01 sequence, located 1410 bp downstream of the transcription start site (TSS) of the Hsp70 promoter region. The pEZX-pG04 contained no point mutations. Both constructs were generated by the Genecopeia™ company.

2.7 Transformation of Escherichia coli (E.coli) mc1061 cells.

Bacterial strain *E.coli* mc1061 was used for all transformation procedures. Competent cells of the bacterial strain were kindly donated from Ms. C Kimar, (University of Stellenbosch, South Africa). The cells were thawed on ice and 100 µl of the cells were added to 200-800 ng of the pEZX-pG04 plasmid DNA or 200-800ng of pEZX-pG04-Mut plasmid DNA. The samples were incubated on ice for 20 minutes. The cells were heat shocked at 42 °C for 1 minute and then incubated on ice for 5 minutes. Pre-warmed Luria broth (900 µl) was added and the tubes were incubated on a rotating wheel for 1 hour at 37 °C. After incubation 100 µl of culture was plated on Luria broth (LB) agar plates which contained 100 mg/ml kanamycin. The plates were incubated at 37 °C overnight. Colonies were selected and used to inoculate Luria broth containing 100 mg/ml kanamycin. The cultures were allowed to grow overnight at 37 °C.

2.8 Storage of bacterial strains

Glycerol stocks were prepared directly from overnight cultures, by adding 400µl 80% sterile glycerol to 600µl of the overnight culture, which was then stored at -80°C.

2.9 Plasmid DNA Isolation

Plasmid DNA was isolated from bacterial cells with the Wizard ® Plus SV Minipreps DNA Purification system kit from Promega.

2.9.1 Large-scale preparation and isolation of plasmid DNA

A volume of 5 µl of the glycerol stock was streaked onto a LB agar plate (with appropriate antibiotic) and incubated at 37 °C overnight. This plate was then used for the isolation of plasmid DNA.

Luria broth (100 ml) containing 100 mg/ml kanamycin was inoculated with a single colony of transformed *E. coli* and cultured overnight at 37°C with shaking. The culture (100 ml) was removed from the incubator and centrifuged at 3,000 x g for 15 minutes using a Beckman J2-21centrifuge. The supernatant was discarded. Plasmid isolation was carried out with the use of the SV MiniPreps DNA purification kit. Cell Resuspension Solution (300 µl) was used to resuspend the bacterial cell pellet, followed by the addition of Cell Lysis Solution (500 µl). The solution was inverted 4 times followed by the addition of Alkaline Protease Solution (20 µl). The solution was inverted 4 times, incubated at room temperature for 5 minutes. Neutralization solution (500 µl) was added to the solution and inverted 4 times to mix and centrifuged at 13, 500 x *g* for 10 minutes using the Eppendorf 5415D Benchtop Centrifuge. Columns and collection tubes were set up and the clear lysate was transferred to the column, left on the bench for 1 min and centrifuged at 12,000 x *g* for 1 minute. The flow through was discarded, and the column re-inserted into the collection tube. Wash Solution (750

µl) was added to the column and centrifuged at 12,000 x *g* for 1 minute. The flow through was discarded and the column re-inserted into the collection tube. Wash Solution (250 µl) was added to the column and centrifuged at 13, 500 x *g* for 1 minute. The column was transferred to a sterile 1.5 ml centrifuge tube, 50 µl of nuclease-free water was added and the tube was left at room temperature for 1 minute. The tube was centrifuged for 2 minutes at 13, 500 x *g*. The eluted DNA was stored at –20 °C. The plasmid DNA was quantified with the use of the NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies).

2.10 Agarose gel electrophoresis of plasmid DNA

The plasmid DNA was electrophoresed on a 1 % agarose gel. The agarose was made by the addition of the appropriate amount of agarose and 1X TBE. The mixture was boiled until the agarose was dissolved. The mixture was cooled and Sybr Safe was added to a final concentration of 0.8X. The agarose was poured into gel casting trays and allowed to solidify. DNA was mixed with an appropriate amount of 6x gel loading buffer. The gels were electrophoresed at 100 V in 1X TBE electrophoresis buffer. After electrophoresis, the gels were viewed with the use of UV trans-illuminator. Gel images were captured with an image capturing system (ChemiDoc UVP System).

2.11 CELL CULTURE AND THE MAINTENANCE OF H9c2 CELLS

2.11.1 Thawing of H9c2 cells

H9c2 cells (kindly donated from Prof. Afshin Samali, National University of Ireland, Galway), derived from the rat heart, were used for the purpose of this study. The frozen vials of H9c2 cells were removed from the -150 °C freezer and thawed in a 37 °C water bath. The cells were transferred to a 15 ml tube which contained 3 ml Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % Foetal Bovine Serum (FBS) and 10 % penicillin and streptomycin (complete media). The latter was transferred into a 25 cm^2 tissue culture flask. The flask was incubated at 37 °C in 5 % CO_2 and media was changed every 48 hours until 60 - 90 % confluency was reached.

2.11.2 Trypsinization of cells

The cells were trypsinized once confluency was reached. The DMEM media in the flask was discarded and cells were washed with phosphate buffered saline (PBS). The PBS was discarded and the cells were trypsinized with the addition of 2 X trypsin-versene® (EDTA). The cells were incubated for 2 – 3 minutes at 37 °C, 5 % CO_2. DMEM media was added to the flask to stop trysinization. The cells were collected by centrifugation at 3 000 *g*.

2.11.3 Freezing of cells

For long term storage the cells were trypsinized and centrifuged. The cell pellet was resuspended in DMEM media containing 10 % DMSO. The latter was aliquoted into 2 ml cryo-vials and stored at -150 °C.

2.11.4 Maintenance of H9c2 cells

H9c2 cells were cultured in a 25 cm^2 flask and incubated at 37 °C, 5 % CO_2, until 60 – 90 % confluency was reached. DMEM media was decanted from the culture flask. The cells were washed with PBS and the PBS was decanted. Cells were trypsinized as described in section 2.11.2. Cells were then counted with the use of the Countess automated cell counter (Invitrogen). Cells were seeded in a 6-well plate at a density of 1 x 10^6 cells per well and allowed to grow till 50 – 60% confluency in a 37°C incubator with 5% CO_2.

2.11.5 Transfection of pEZX-pG04 and pEZX-pG04-Mut constructs into H9c2 cells

The pEZX-pG04 plasmid DNA and the pEZX-pG04-Mut plasmid DNA (transfection-ready clones) were prepared as described section 2.9.1. Four tubes were set up for the preparation of Metafectene^{TM} PRO (as shown in table 2.1). DNA concentrations of 2 μg were set up for pEZX-pG04 and pEZX-pG04-Mut plasmid DNA reactions.

Table 2.1 Contents of tubes used for the transfection of pEZX-pG04 wild type and mutant constructs into H9c2 cells.

Tube	Contents
A	2 µg of pEZX-pG04 plasmid consisted of 2.7 µl of the plasmid DNA and 47.3 µl of DMEM media (serum-free)
B	2 µg of pEZX-pG04-Mut plasmid consisted of 20 µl of the plasmid DNA and 30 µl of DMEM media (serum-free)
C	6 µl Metafectene™ PRO and 44 µl of DMEM media (serum free)
D	6 µl Metafectene™ PRO and 44 µl of DMEM media (serum free)

Tubes A and C, and tubes B and D were mixed and incubated at room temperature for 20 minutes.

H9c2 cells were seeded in 6 well culture plates the day before. An untransfected control was also included in the experiment. Media was decanted and the cells were washed with PBS. Serum-free DMEM media (1 ml) was added onto the cells, followed by the addition of the DNA/metafectene mixtures (tubes A and C, and tubes B and D) and the plate was gently swirled. The plate was placed in the incubator at 37 °C and 5% CO_2 for 3 hours. The plate was swirled gently every 30 min. After 3 hours, 1 ml complete DMEM media was added to the wells and incubated at 37 °C and 5% CO_2. After 48 hours the cells were treated with

DMEM media which contained 1 µg/ ml of puromycin. The cells were maintained in this media for 3 weeks until the cells in the control (no DNA added) were all dead.

2.12 Heat shock preconditioning assay

H9c2 cells were cultured in cell culture flasks (either 6 cell well culture plates or 25 cm^2 flasks) till confluency was reached. Once confluent, fresh culture media was placed on the cells. The cells were heat shocked at 42°C for 1 hour and allowed to recover for 0, 1, 4, 6 and 24 hours at 37°C, 5% CO_2. A non-heat shocked control was always included.

2.13 Extraction of cellular proteins from H9c2 cells

Total cellular proteins were isolated using Cytobuster™ Protein Extraction Reagent. Culture media was removed and the cells were washed once with PBS for 5 minutes on a shaker at room temperature. Once this was completed, the PBS was decanted and replaced with 500µl Cytobuster™ Protein Extraction Reagent. The flask was placed on a shaker at room temperature for 5 minutes. The cells were scraped off in the Cytobuster™ Protein Extraction Reagent, using a cell scraper. The extract was transferred into a 1.5ml sterile Eppendorf tube and centrifuged for 5 minutes at 16 000 x *g*. The supernatant contained the proteins and was transferred to a sterile 1.5ml Eppendorf tube. Extracted proteins were quantified using the Qubit® 2.0 Fluorometer by Invitrogen. The proteins were

separated on a 12 % protein gel (see section 2.15.1) at 120 V, and stained with coomassie (as described in section 2.14), and protein size as well as equal loading for Western blot analysis was determined. The protein lysates were stored at -20 °C.

2.14 Coomassie Staining and De-staining of polyacrylamide gels

Gels were placed in Coomasie staining solution, and was allowed to shake gently overnight. Then it was de-stained using de-staining solution, for 2 hours with gentle shaking. After the bands were clearly visible, with exposure to white light, the de-staining solution was decanted and the gel was washed extensively with distilled water and stored in plastic wrap at room temperature, or imaged using the ChemiDoc UVP system.

2.15 Western Blot Analysis of Hsp70 expression

2.15.1 Polyacrylamide gel electrophoresis separation of protein samples and Western Blotting

Protein separation was achieved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). A 12 % SDS- polyacrylamide gel was prepared as follows:

Table 2.2 Materials used for the preparation of sodium dodecyl sulphate polyacrylamide gel

Solutions	12% Separating gel	5% Stacking gel
Distilled water	4.28 ml	2.67 ml
40% Acrylamide (37:5:1)	3 ml	0.5 ml
1.5 M Tris pH8.8	2.5 ml	-
0.5 M Tris pH6.8	-	0.63 ml
10 % sodium dodecyl sulphate (SDS)	0.1 ml	0.05 ml
10 % Ammonium persulphate (APS)	0.1 ml	0.05 ml
TEMED	0.02 ml	0.02 ml

A 12 % separating gel was prepared (shown in table 2.2) and poured into a gel casting apparatus and covered with isopropanol to even out the gel. The gel was allowed to polymerize for approximately 20 minutes at room temperature. Once the gel has solidified, the isopropanol was poured off and washed extensively with distilled water to remove all traces of isopropanol. The stacking gel was then prepared as in table 2.2, and was poured on the separating gel, followed by the insertion of a 1 mm comb, and allowed to polymerise for approximately 20 minutes at room temperature. Protein extracts (20 μg) were then mixed with an

appropriate amount of 2 x sample buffer and boiled at 95° C for 5 minutes. The protein samples were then centrifuged for 5 minutes at 13 200 x *g,* and 20 µl of sample was loaded onto a pre-electrophoresed 12 % polyacrylamide gel. Samples were electrophoresed until the bromophenol blue band was electrophoresed out of the separating gel which was approximately 60 min of applying a voltage of 120 V in a Mini-Protean tetra cell chamber (BIO-RAD). After the proteins have been separated, it was transferred onto a polyvinylidene fluoride (PVDF) membrane and electroblotted in transfer buffer for 90 minutes in a 4°C cold room with continuous stirring to maintain a cooling temperature. For the confirmation of protein transfer to the membrane, the membrane was stained for 5 minutes in Ponceau S Staining solution and once confirmed, the stain was rinsed off with distilled water.

Once protein transfer and confirmation process was completed the membrane was blocked in 5% fat-free milk powder dissolved in TBST for 60 minutes at room temperature.

2.15.2 Primary and secondary probing with antibodies and detection

After blocking the membrane, it was washed four times with TBST for 5 minutes each at room temperature with continuous shaking. The Hsp70 (3A3) (Santa Cruz Biotechnology Inc., Europe) primary antibody (diluted 1:500 in 3% BSA-TBST solution) was added onto the membrane and placed on a shaker overnight at 4°C. The membrane was washed 3 times with TBST for 5 minutes each, then incubated with a horse raddish peroxidase (HRP)-conjugated goat anti-mouse secondary

antibody (Santa Cruz Biotechnology Inc., Europe) diluted at 1:2000 in 3% BSA-TBST solution, for 60 minutes at room temperature on a shaker. Following the incubation, the membrane was then washed three times in TBST, for 5 minutes each wash. The detection of the HRP conjugated antibodies on the membrane was done with the aid of the Supersignal® West Pico Chemiluminescent Subtrate (Thermo Scientific), and the membrane was imaged using the ChemiDoc UVP system.

After detecting the expression patterns of Hsp70 on the membrane, in the protein lysates, the antibodies were stripped from the membrane by incubating the membrane in 0.2 M NaOH for 5 minutes at room temperature. The membrane was then washed in distilled water for 10 minutes on a shaker, and the membrane was blocked in 5% fat-free milk powder dissolved in TBST. The membrane was washed thrice in TBST, and incubated with β-Actin (C-2) mouse monoclonal antibody (Santa Cruz Biotechnology Inc., Europe) (diluted 1:1000 in 3% BSA-TBST solution) overnight at 4°C. Following the primary antibody incubation, the membrane was washed three times with TBST for 5 minutes each and incubated with a HRP-conjugated goat antimouse secondary antibody for 60 minutes at room temperature. The detection of the HRP conjugated antibodies on the membrane was done with the aid of the Supersignal® West Pico Chemiluminescent Subtrate (Thermo Scientific), as described above.

2.16 Promoter Reporter assay

H9c2 cells that were stably transfected with either pEZX-pG04 or pEZX-pG04-Mut were cultured in 6 well cell culture plates. The cells were then heat shocked by incubating the culture plates at 42°C for 1 hour. Cells were then allowed to recover for various time periods (0, 1, 4, 6, and 24 hours) at 37°C, 5% CO_2. For comparison studies in cytoprotection experiments, a non-heat shocked control was included. The cell culture medium was collected (100 µl) at the end of every recovery period and stored at -20°C.

The Secrete-Pair ™ Dual Luminescence Assay Kit (supplied by GeneCopoeia) was used for the detection and analysis of secreted reporter proteins, *Gaussia* Luciferase (GLuc) and Secreted Alkaline Phosphatase (SEAP) activities, in a cell culture medium.

2.16.1 Gaussia Luciferase Assay for high sensitivity using GL-H buffer

A volume of 100 µl of cell culture medium of the relevant time period was placed at room temperature till completely thawed. Buffer GL-H (10 X) was thawed at room temperature, inverted a few times and vortexed for approximately 4 seconds. Once the Buffer GL-H was thawed a 1 X solution was made. Once the GL-H buffer was prepared a GLuc assay working solution was prepared by adding 10 µl of Substrate GL to 1 ml of 1 X buffer GL-H. The solution was mixed well by inverting a few times, and incubated at room temperature in the dark for 25 minutes. A volume of 10 µl of cell culture medium was added to a 96 well

black-base plate and 100 µl of the GLuc assay working solution was added to the cell culture medium samples and gently mixed several times. The plate was then incubated for 30 seconds (in the dark) and measured directly using GloMax®-Multi^{+} Microplate Multimode Reader. The luminometer was set to 1 second integration. All experiments were done in duplicate.

2.17 Electrophoretic mobility shift assay (EMSA)

The electrophoretic mobility shift assay (supplied by Thermo™ Scientific) was used to study DNA-protein interactions. The principle is based on the fact that protein-nucleic acid complexes migrate more slowly than the corresponding free nucleic acid, in a polyacrylamide or agarose gel. This in turn results in a "shift" in the migration of labelled DNA. The Thermo Scientific Light Shift® Chemiluminescent EMSA kit was used, as it uses a non-isotopic approach to detect DNA-protein interactions. For the detection of biotinylated labelled DNA, the Streptavidin-Horseradish Peroxidase conjugate as well as the Chemiluminescent substrates was used.

2.17.1 Annealing of complementary pairs of oligonucleotides

A *Rattus norvegicus* Hsp70 sequence (Gene accession: NM_031971) was used for the design of the oligonucleotides. Promoter sequences were extracted as described in section 2.5.1, followed by the identification of conserved sequences (promoter modules) in the promoter region of Hsp70. CLUSTALW was used to

identify highly conserved nucleotide sites in the promoter modules, and these highly conserved nucleotide sites were used for the creation of mutant oligonucleotides. Biotinylated labelled oligonucleotides (mutant) were generated by Inqaba Biotec™.

A concentration of 60 fmol of consensus oligonucleotides (labelled and unlabelled as shown in table 2.3) were incubated in a sterile thin walled PCR tube. The same was done for the mutant oligonucleotides (labelled and unlabelled). The annealing reaction was performed in a PCR machine by incubating the tube at 80°C for 10 minutes and then dropping the temperature every 10 minutes until it reached room temperature.

Table 2.3 Mutant DNA sequences for SP1F_KLFS_01

	Forward Primer	**Reverse Primer**
Labelled Mutant oligonucleotide	5'-Biotin- GAGGGTATACTGGGCCGG- 3'	5'-Biotin- CCGGCCCAGTATACCCTC- 3'

2.17.2 Control and experimental binding reactions with annealed oligonucleotides

Electrophoretic mobility gel shift experiments were carried out according to the protocol supplied by the ThermoScientific LightShift® Chemiluminescent EMSA kit.

Total cellular protein extracts were prepared (section 2.13) from p11 H9c2 cardiomyocytes (1 hour heat shock at 42 °C, and 24 hour recovery at 37 °C) and used for all gel shift experiments. Polyacrylamide gels (4%) were prepared according to table 2.2. The assembly of the PAGE was similarly prepared as described in section 2.15.1

Table 2.4 Materials used for the preparation of two 4% SDS- polyacrylamide gel

Solutions	**5% Resolving gel**
Distilled water	10.1 ml
40% Acrylamide (37:5:1)	1.6 ml
1.5 M Tris pH8.8	4 ml
10 % sodium dodecyl sulphate (SDS)	0.160 ml
10 % Ammonium persulphate (APS)	0.160 ml
TEMED	0.016 ml

Once gels were poured it was pre-electrophoresed at 120V for 30 minutes in a Mini-Protean tetra cell chamber (BIO-RAD).

Control (C1, C2 and C3) and experimental (E1, E2 and E3) binding reactions were prepared in 1.5 ml sterile Eppendorf tubes according to tables 2.5 and 2.6, respectively. The binding reactions were then incubated for 20 minutes at room

temperature, followed by the addition of 5 µl of 5X loading buffer to each binding reaction. Gels were electrophoresed for approximately 40 minutes at 100V until the bromophenol blue dye has migrated half way down the gel. Once the gel was completed, proteins and DNA were transferred to a positively charged nylon transfer membrane (Hybond- N+) in a clean electrophoresis unit. Proteins were transferred in a Mini-Protean tetra cell chamber (BIO-RAD) for 30 minutes at a voltage of 100 V in cooled 0.5X TBE. The transferred DNA was crosslinked to the membrane with the use of a UV Stratalinker 2400 (Stratagene) crosslinking machine, equipped with 254nm bulbs. Crosslinking was done by using the auto-crosslinking function for 1 minute.

The detection of biotin-labeled DNA, on the nylon membrane, was done with the aid of the Supersignal® West Pico Chemiluminescent Subtrate (Thermo Scientific), as well as a ChemiDoc UVP system (membrane exposure of 10 seconds – 4 minutes).

Table 2.5 The Control binding reactions for the EMSA system.

Reagents	Final concentration	C1	C2	C3
Ultrapure water	-	12 µl	11 µl	9 µl
10X binding buffer	1X	2 µl	2 µl	2 µl
50% Glycerol	2.5%	1 µl	1 µl	1 µl
100mM $MgCl_2$	5mM	1 µl	1 µl	1 µl
1 µg/µl Poly (dI•dC)	50 ng/µl	1 µl	1 µl	1 µl
1% NP-40	0.05%	1 µl	1 µl	1 µl
Unlabeled EBNA DNA (from Genecopeia™ kit)	4 pmol	-	-	2 µl
EBNA Extract (from Genecopeia™ kit)	1 Unit	-	1 µl	1 µl
Biotin-EBNA Control (from Genecopeia™ kit) DNA	20 fmol	2 µl	2 µl	2 µl

Table 2.6 The Experimental binding reactions for the heat shocked test system

Reagents	**Final concentration**	**E1**	**E2**	**E3**
Ultrapure water	-	5 µl	4 µl	-
10X binding buffer	1X	2 µl	2 µl	2 µl
1 µg/µl Poly (dI•dC)	50 ng/µl	1 µl	1 µl	1 µl
50% Glycerol	2.5%	-	-	-
100mM $MgCl_2$	5mM	-	-	-
1% NP-40	0.05%	-	-	-
Protein Extract (heat shocked and non-heat shocked lysates)	1 Unit	-	1 µl	1 µl
Biotin-labeled SP1F_KLFS_01 oligonucleotides (consensus)	60 fmol	12 µl	12 µl	12 µl

CHAPTER 3: RESULTS AND DISCUSSION

The aim of this study was to identify novel CAREs that are involved in the regulation of the expression of heat shock response genes. Genes that are functionally related (e.g. heat shock responsiveness) will be regulated in the same way (Werner, 2001). These genes may therefore also be regulated by the same TFs and consequently these genes may have the similar CAREs in their promoter regions. It is thus possible that genes encoding heat shock response proteins share a sequence signature in their promoter region, which regulates the expression of these genes in response to heat stress. Identifying these CAREs will give us insights on gene expression of heat shock responsive genes and their functional characteristics. These novel CAREs can also be beneficial in therapeutic advances, by turning gene expression "on" where it was previously turned "off" (Garrett and Grisham, 2005). TFs will recognize specific nucleotide sequences (motifs/CAREs), which are located before or after the gene and turn "on" gene expression (Phillips, 2008).

An *in silico* approach was used to identify CAREs that are shared by known heat shock response genes. This was followed by an *in vitro* approach to evaluate the involvement of these CAREs in the regulation of gene expression in response to heat stress.

3.1 The identification of promoter sequences of known heat shock responsive genes.

The promoter sequences for well-known heat shock responsive genes were identified with the use of the Gene2Promoter tool in the Genomatix software (http://www.genomatix.de). Gene2Promoter is an online software tool used to identify promoter sequences in all query gene sequences. This is done by identifying the locus in a query gene sequence and alternatively listing all transcripts and promoters specific for that locus (http://www.genomatix.de).

Using the search term "heat shock", 50 genes (from *Rattus norvegicus* species) were identified (Table 3.1). These genes included the well-known heat shock proteins Hsp70, Hsp90 and αβ-crystalline (Wyttenbach and Arrigo, 2000). The Gene2Promoter tool identifies three categories of promoter sequences, namely (1) experimentally verified promoter sequences, (2) *in silico* predicted promoter sequences that were verified using the PromoterInspector tool (http://www.genomatix.de), and (3) *in silico* predicted promoter sequences that were not verified in any way. Experimentally verified promoter sequences are promoters that were confirmed using *in vitro* assays such as promoter mapping as the functional promoter for a particular gene (http://www.genomatix.de). PromoterInspector is an online software tool which predicts promoter regions with high specificity in mammalian genomic sequences (http://www.genomatix.de). In this study, only promoter sequences from categories (1) and (2) were considered.

The promoter sequences for only 19 (Table 3.2) out of the 50 heat shock responsive genes (38 %) were either previously experimentally verified or verified using the PromoterInspector tool. The table in Appendix 1 shows the promoter sequences for these genes. The promoter sequences for genes that were *in silico* predictions, and not verified, were not considered in this study.

Table 3.1 List of genes encoding heat shock proteins

GENE NAME	GENE ID	GENE NAME	GENE ID
Heat shock 70kDa protein 1B (mapped)	294254/ GXL_187112	**Heat shock protein, α- crystalline-related, 9**	363681/ GXL_734563
Heat shock protein 1	24471/GXL_126867	**Heat shock transcription factor 2 binding protein**	499413/ GXL_626779
Heat shock protein 5	25617/ GXL_246319		
Heat shock protein 3	78951/ GXL_242292	**Heat shock protein family, member 7 (cardiovascular)**	50565/ GXL_738767
Heat shock protein 4	266759/ GXL_123650		
Heat shock protein 9	291671/GXL_322308	**Heat shock protein 90, α (cystolic), class A member 1**	299331/ GXL_168670
Heat shock protein 14	307133/ GXL_113340		
Heat shock protein B8	113906/ GXL_50908	**Heat shock protein 90, α (cystolic), class B member 1**	301252/ GXL_201364
Heat shock protein A8	24468/GXL_60677		
Heat shock protein 12B	311427/ GXL_324465	**AHA, activator of Heat shock protein ATPase homolog 2 (yeast)**	305577/ GXL_735349
Heat repeat containing 6	497972/ GXL_621766		
Heat repeat containing 2	304332/ GXL_622470	**Hspb associated protein 1**	171460/ GXL_60652
Heat repeat containing 1	361262/ GXL_322134	**PDGFA associated protein 1**	64527/ GXL_468142
Heat shock protein beta 2	161476/ GXL_166395	**TNF receptor-associated protein 1**	287069/ GXL_467987
Heat repeat containing 5A	362737/ GXL_630201	**DnaJ (Hsp40) homolog, subfamily B, member 6**	362293/ GXL_324763
Heat shock protein 4 like	294993/ GXL_627335		
Heat shock Protein 2	60460/ GXL_468860	**DnaJ (Hsp40) homolog, subfamily A, member 1**	65028/ GXL_1468
Heat-responsive protein 12	65151/ GXL_42893		
Heat repeat containing 7B1	301596/ GXL_632079	**Eukaryotic translation initiation factor 4E binding protein 1**	116636/ GXL_90244
Heat shock transcription factor 2	64441/GXL_23166		
Heat shock protein 1 (chaperonin)	63868/ GXL_201339	**Heat shock protein B8**	113906/ GXL_50908
Heat shock transcription factor 4	291960/ GXL_736263	**Crystalline, alpha B**	25420/ GXL_188635
Heat shock 105kDa/ 110kDa protein 1	288444/ GXL_126693	**Rho family GTPase 3**	295588/ GXL_37575
Heat shock factor binding protein 1	286899/ GXL_207601	**Annexin A4**	79124/ GXL_211980
Heat shock protein 1 (chaperonin 10)	25462/ GXL_233373	**Glucose-6-phosphate isomerase**	292804/ GXL_254197
Calcium regulated heat stable protein 1	260416/ GXL_97624	**Gene of unknown function on chromosome 6 of rat**	299331/ GXL_168670
Heat shock factor binding protein 1-like	100192205/ GXL_822705		
Heat shock protein 13	29734/ GXL_60748	**Gene of unknown function (on chromosome 11 of rat)**	24468/ GXL_60677
Heat shock transcription factor, Y linked 2	316409/ GXL_201358		
Heat shock protein 90, beta, member 1	362862/ GXL_42957	**Heat shock protein 12A**	307997/GXL_737061

Table 3.2 List of genes with promoter sequences that were experimentally verified using either PromoterInspector or *in vitro* assays.

GENE NAME	GENE ID
Heat shock 70kDa protein 1B (mapped)	294254/ GXL_187112
Heat shock protein 5	25617/ GXL_246319
Heat repeat containing 2	304332/ GXL_622470
Heat repeat containing 1	361262/ GXL_322134
Heat shock protein 4 like	294993/ GXL_627335
Heat shock protein 4 like_2	294993/ GXL_627335
Heat shock Protein 2	60460/ GXL_468860
Heat shock transcription factor 4	291960/ GXL_736263
Heat shock transcription factor 4_2	291960/ GXL_736263
Heat shock protein 1 (chaperonin 10)	25462/ GXL_233373
Heat shock protein 90, beta, member 1	362862/ GXL_42957
Heat shock protein 90, α (cystolic), class A member 1	299331/ GXL_168670
Heat shock protein 90, α (cystolic), class A member 1_2	299331/ GXL_168670
Heat shock protein 90, α (cystolic), class A member 1_3	299331/ GXL_168670
Heat shock protein 90, α (cystolic), class B member 1	301252/ GXL_201364
AHA, activator of Heat shock protein ATPase homolog 2 (yeast)	305577/ GXL_735349
PDGFA associated protein 1	64527/ GXL_468142
DnaJ (Hsp40) homolog, subfamily B, member 6	362293/ GXL_324763
DnaJ (Hsp40) homolog, subfamily A, member 1	65028/ GXL_1468

3.2 The identification of common promoter modules in the promoter sequences of known heat shock genes.

The promoter sequences for the 19 known heat shock responsive genes were analysed using the ModelInspector tool (www.genomatix.de) to identify all the promoter modules (i.e. potential TF binding sites) the promoter regions of these genes. ModelInspector is an online software tool which uses a library of predefined models to search DNA sequences for matches to these models. A

model would consist of various individual sequence elements (e.g. TF binding sites, hairpins, repeats etc.). (http://www.genomatix.de). Table 3.2 shows a summary of the results.

CAREs that are statistically over represented in the promoter sequences of genes that share a common function may also regulate the expression of these genes. The most common promoter module in the promoter regions of the19 heat shock responsive genes was SP1F_KLFS_01 (a total of 15 promoters contained SP1F_KLFS_01 sequence). SP1F_KLFS_01 is a promoter module that consists of two overlapping TF binding sites, which are SP1F (Specificity protein 1 or GC box factor) and KLF (Kruppel-like factor), these binding sites enable the binding of SP1F and KLFs TFs (McConnell and Yang, 2010). According to Augustin *et al,* GC box factor, plays a vital role in chromatin silencing and embryonic development, whereas KLF assists in the development and stress response mechanism to external stress (Augustin *et al.*, 2011). KLFs have been studied extensively for their role in cell proliferation, differentiation and cell survival in the context of cancer (McConnell and Yang, 2010). KLFs also share a high degree of homology with the SP1 family of zinc-finger TFs. It has been shown that the multiple SP1 TFs are highly responsive to the stressful stimuli such as ethanol exposure (Wilke *et al.,* 2000). KLF4 and KLF5 (two closely related members of the KLFs family) respond to numerous external stressful stimuli and are involved in restoring cellular homeostasis following exposure to stressors (McConnell *et al.*, 2007). It is therefore possible that SP1F and KLF work together to regulate the expression of heat shock responsive genes following heat stress.

Table 3.3 Promoter modules in the promoter regions of heat shock responsive genes.

Model Name	Total matches in (+) and (-) strands	# of matches in (+) strand	# of matches in (-) strand	# of promoter sequences containing the model
SP1F_KLFS_01	24	12	12	15
SMAD_E2FF_01	13	7	6	10
E2FF_SP1F_01	12	7	5	7
SP1F_SP1F_01	10	7	3	5
SP1F_SP1F_06	9	5	4	7
NFKB_SP1F_04	8	4	4	6
SP1F_AP2F_01	6	2	4	6
NFKB_SP1F_03	5	5	0	5
SP1F_E2FF_01	5	2	3	5
KLFS_CREB_KLFS_01	5	0	5	4
KLFS_SP1F_01	5	4	1	4
SP1F_SP1F_02	5	1	4	4
SP1F_ETSF_04	4	0	4	4
STAT_NFKB_05	4	1	3	4
AP1R_PAX6_01	3	2	1	3
CAAT_CAAT_01	3	0	3	2
CP2F_SP1F_01	3	2	1	2
CREB_EBOX_02	3	0	3	3
NF1F_EBOX_01	3	1	2	2
SMAD_EBOX_02	3	2	1	3
SP1F_EGRF_02	3	1	2	3
SP1F_ETSF_01	3	2	1	3
SP1F_ETSF_02	3	0	3	3
SP1F_SP1F_05	3	0	3	3
SP1F_SREB_01	3	1	2	3
NBRE_STAT_01	2	2	0	2
NFKB_CREB_01	2	0	2	2
NKXH_NKXH_01	2	1	1	2
NKXH_SRFF_01	2	1	1	2
NR2F_EREF_01	2	1	1	2
SMAD_AP1F_01	2	1	1	1
SP1F_ETSF_03	2	1	1	2
SP1F_STAT_01	2	2	0	2
SP1F_YY1F_01	2	2	0	2
SREB_CAAT_01	2	0	2	2
STAT_GREF_01	2	1	1	2
STAT_SP1F_02	2	1	1	2
AP1F_CEBP_03	1	0	1	1

Model Name	Total matches in (+) and (-) strands	# of matches in (+) strand	# of matches in (-) strand	# of promoter sequences containing the model
CAAT_CAAT_02	1	0	1	1
CAAT_EBOX_01	1	1	0	1
CAAT_SP1F_02	1	1	0	1
CAAT_SREB_02	1	0	1	1
CAAT_SREB_03	1	1	0	1
CDEF_CHRF_03	1	0	1	1
CEBP_CEBP_01	1	0	1	1
CEBP_CREB_01	1	0	1	1
CEBP_MYBL_04	1	1	0	1
CEBP_SP1F_01	1	0	1	1
CREB_CEBP_01	1	1	0	1
CREB_EBOX_01	1	0	1	1
CREB_HNF1_01	1	0	1	1
CREB_NFAT_SP1F_01	1	1	0	1
CREB_SP1F_01	1	1	0	1
E2FF_E2FF_03	1	0	1	1
IRFF_ETSF_02	1	0	1	1
KLFS_KLFS_NFKB_01	1	0	1	1
KLFS_NR2F_KLFS_01	1	1	0	1
MAZF_SP1F_01	1	1	0	1
NEUR_SP1F_01	1	0	1	1
NF1F_NR2F_01	1	0	1	1
NFAT_GATA_01	1	1	0	1
NFKB_NKXH_01	1	0	1	1
NFKB_RBPF_01	1	1	0	1
NFKB_SORY_01	1	1	0	1
NFKB_SREB_NFKB_01	1	1	0	1
NKXH_CEBP_01	1	1	0	1
P53F_AP1F_01	1	0	1	1
P53F_SP1F_01	1	0	1	1
SP1F_CREB_01	1	1	0	1
SP1F_EGRF_01	1	1	0	1
SORY_SORY_EGRF_01	1	1	0	1
SP1F_CAAT_01	1	0	1	1
SP1F_MYOD_01	1	1	0	1
SP1F_NFKB_02	1	1	0	1
SP1F_SP1F_03	1	0	1	1
SP1F_SP1F_08	1	1	0	1
SREB_RXRF_01	1	0	1	1

3.3 Sequence analysis of the SP1F_KLFS_01 modules in the promoter sequences of heat shock responsive genes.

A sequence alignment (Table 3.4) of the SP1F_KLFS_01 modules identified in the promoter sequences of the 15 heat shock response genes shows a high degree of conservation of 5 nucleotides (5′-CGCCC-3′) in the SP1F_KLFS_01 modules.

Table 3.4 Sequence alignment of SP1F_KLFS_01 sequences of genes encoding heat shock responsive proteins. The sequence alignment was performed using CLUSTALW2.1 (Chenna *et al.*, 2003). A total of 24 SP1F_KLFS_01 sequences from the 15 genes were aligned. The asterisk under the sequence alignment indicate the conserved nucleotides (5′-CGCCC-3′).

Promoter Name	Sequence Alignment
Heat shock protein 4-like_2	-----CCCCCCCCGCCCGT---GACA- 18
Heat shock protein 90, beta, member 1_1	-----CGTCCCCCGCCCGC---AAGC- 18
Heat repeat containing 2_1	-----CACGCCCCGCCCAT---TTGG- 18
Heat shock protein 4-like_1	-----GCCGCCCCGCCCTG---CCCT- 18
Heat shock protein 90, beta, member 1_2	-----TCCACCCCGCCCAA---CCCG- 18
Heat shock transcription factor 4_1	TGCCGCCCGCCCCGCCCTA---CTCC- 23
Heat shock transcription factor 4_1a	TGCCGCCCGCCCCGCCCTA---CTCC- 23
Heat shock 70kD protein 1B (mapped) (Hspa1b)	-----CCGGCCCCGCCCAC---CCTC- 18
Heat shock protein 4-like_1	-----TCCGGGACGCCCCC---CCCC- 18
Heat shock protein 90, alpha (cytosolic), class B member 1	-----TCTTCCCCGCCCCC---TCCC- 18
Heat repeat containing 2_2	-----CTGGTCCCGCCCTTTACCCCTT 22
Heat shock protein 1 (chaperonin 10)_2	-----GCGGCCCCGCCCT----CCTTA 18
PDGFA associated protein 1	-----GGGGCCCCGCCCA----CCTCG 18
Heat shock protein 90, alpha (cytosolic), class A member 1	-----CGGGCCCCGCCCCT---CCAC- 18
Heat repeat containing 1_1	-----GGAGCCCCGCCCCC---T-CCA 18
Heat shock protein 2_2	-----TGAGCCCCGCCCAG---TGCC- 18
Heat shock protein 2_1	-----CGCGCCCCGCCCCG---CGCC- 18
Heat shock protein 1 (chaperonin 10)_1	-----CCGGCCCCGCCCCG---CGGC- 18
AHA1, activator of heat shock protein ATPase homolog 2 (yeast)_2	-----CCTGCCCCGCCCCG---CTGC- 18
Heat repeat containing 1_2	-----AAAGCCCCGCCCTC---CGCT- 18
DnaJ (Hsp40) homolog, subfamily A, member 1_1	-----GAAGTCCCGCCCCT---CGCTT 19
Heat shock protein 90 alpha (cytosolic), class B member 1_2	-----CTAGCCCCGCCCTT---GGAT- 18
AHA1, activator of heat shock protein ATPase homolog 2 (yeast)_1	-----AAGACCCCGCCCTA---TGCG- 18
AHA1, activator of heat shock protein ATPase homolog 2 (yeast)_3	-----AAGTCCCCGCCCCG---GGCC- 18 .*****

3.4 The generation of promoter reporter constructs for Hsp70

To investigate whether the SP1F_KLFS_01 module is involved in the regulation of the expression of heat shock responsive genes, the GLuc-ON™ promoter constructs from GeneCopoeia Inc. was used. The GLuc-ON™ promoter constructs from GeneCopoeia Inc. are designed to perform promoter analysis in real-time (http://www.genecopoeia.com). The dual-reporter system uses *Gaussia luciferase* (GLuc) as the promoter reporter and Secrete Alkaline Phosphatase (SEAP) as the internal control for signal normalization. The promoter sequence (in this case the rat *Hsp70* promoter) was cloned upstream (on the 5´ end) of the GLuc gene. Hsp70 was used since it is a well-known heat shock responsive gene and since it was shown in this study to contain one SP1F_KLFS_01 module (Figure 3.1) in its promoter sequence.

Two constructs were generated: the one construct (pEZX-pG04) contained the wild type rat *Hsp70* promoter, while the second construct (pEZX-pG04-Mut) contained a mutated version of the rat *Hsp70* promoter. Both constructs were purchased from GeneCopoeia Inc. In the mutated *Hsp70* promoter (pEZX-pG04-Mut) the conserved nucleotides (5′-CGCCC-3′) in the SP1F_KLFS_01 module was replaced with a mutated (5′-ATACT-3′) sequence. DNA sequence data for pEZX-pG04 and pEZX-pG04-Mut was supplied by GeneCopoeia Inc. Figure 3.2 shows a sequence alignment between the *Hsp70* promoter sequence (>GXP_2964156) and the promoter region of pEZX-pG04, while Figure 3.3 shows an alignment between the *Hsp70* promoter sequence (>GXP_2964156) and the promoter region of pEZX-pG04-Mut. A 100% match between *Hsp70*

promoter and the promoter region of pEZX-pG04 was obtained, while a 99 % match was obtained for the *Hsp70* promoter and the promoter region of pEZX-pG04-Mut. Four mismatches were observed between the *Hsp70* promoter and the promoter region of pEZX-pG04-Mut. These mismatches correspond to the four-nucleotide replacements (5′-ATACT-3′) in the SP1F_KLFS_01 module of the promoter region of pEZX-pG04-Mut.

>GXP_2963156(Hspa1b/rat) loc=GXL_187112|sym=Hspa1b|geneid=294254| acc =GXP_2963156| taxid=10116|spec=Rattusnorvegicus|chr=20 |ctg=NC_005 11 9| str=(+)|start=3955107|end=3955707|len=601|tss=501|descr=heat shock 70kD protein 1B (mapped)| comm=GXT_23117308/ ENSRNOT0000004966 7/501 /bronze

5´- GCCAAGCGTTATCCCTCCCGTTTTGAGAAACTTTCTGCGTCCGCCATCCTGTAGGA
AGAATTTGTACACCTTAAACTCCCTCCCTGGTCTGATTCCCAAATGTCTCTCACCGCCC
AGCACTTTCAGGAGCTGACCCTTCTCAGCTTCACATACAGAGACCGCTACCTTGCGTC
GCCATGGCAACACTGTCACAACCGGAACAAGCACTTCCTACCACCCCCCGCCTCAGG
AATCCAATCTGTCCAGCGAAGCCCAGATCCGTCTGGAGAGTTCTGGACAAGGGCGGT
ACCCTCAACATGGATTACTCATGGGAGGCGGAGAAGCTCTAACAGACCCGAAACTGC
TGGAAGATTCCTGGCCCCAAGGCCTCCTCCCGCTCGCTGATTGGCCCATGGGAGGGTG
GGCGGGGCCGGAGGAGGCTCCTTAAAGGCGCAGGGCGGCGCGCAGGACACCAGATT
CCTCCTCCTAATCTGACAGAACCAGTTTCTGGTTCCACTCGCAGAGAAGCAGAGAAGC
GGAGCAAGCGGCGCGTTCCAGAACCTCGGGCAAGACCAGCCTCTCCCAGAGCATCCC
CACCGCGAAGCGCAACCTTCTCCAGAGC- 3´

Figure 3.1 The promoter sequence of *Hsp70*. The promoter sequence of the rat *Hsp70* gene as identified by Gene2Promoter is shown. The SP1F_KLFS_01 sequence is highlighted in yellow.

```
Query  1     GCCAAGCGTTATCCCTCCCGTTTTGAGAAACTTTCTGCGTCCGCCATCCTGTAGGAAGAA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1015  GCCAAGCGTTATCCCTCCCGTTTTGAGAAACTTTCTGCGTCCGCCATCCTGTAGGAAGAA  1074

Query  61    TTTGTACACCTTAAACTCCCTCCCTGGTCTGATTCCCAAATGTCTCTCACCGCCCAGCAC  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1075  TTTGTACACCTTAAACTCCCTCCCTGGTCTGATTCCCAAATGTCTCTCACCGCCCAGCAC  1134

Query  121   TTTCAGGAGCTGACCCTTCTCAGCTTCACATACAGAGACCGCTACCTTGCGTCGCCATGG  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1135  TTTCAGGAGCTGACCCTTCTCAGCTTCACATACAGAGACCGCTACCTTGCGTCGCCATGG  1194

Query  181   CAACACTGTCACAACCGGAACAAGCACTTCCTACCACCCCCCGCCTCAGGAATCCAATCT  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1195  CAACACTGTCACAACCGGAACAAGCACTTCCTACCACCCCCCGCCTCAGGAATCCAATCT  1254

Query  241   GTCCAGCGAAGCCCAGATCCGTCTGGAGAGTTCTGGACAAGGGCGGTACCCTCAACATGG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1255  GTCCAGCGAAGCCCAGATCCGTCTGGAGAGTTCTGGACAAGGGCGGTACCCTCAACATGG  1314

Query  301   ATTACTCATGGGAGGCGGAGAAGCTCTAACAGACCCGAAACTGCTGGAAGATTCCTGGCC  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1315  ATTACTCATGGGAGGCGGAGAAGCTCTAACAGACCCGAAACTGCTGGAAGATTCCTGGCC  1374

Query  361   CCAAGGCCTCCTCCCGCTCGCTGATTGGCCCATGGGAGGGTGGGCGGGGCCGGAGGAGGC  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1375  CCAAGGCCTCCTCCCGCTCGCTGATTGGCCCATGGGAGGGTGGGCGGGGCCGGAGGAGGC  1434

Query  421   TCCTTAAAGGCGCAGGGCGGCGCGCAGGACACCAGATTCCTCCTCCTAATCTGACAGAAC  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1435  TCCTTAAAGGCGCAGGGCGGCGCGCAGGACACCAGATTCCTCCTCCTAATCTGACAGAAC  1494

Query  481   CAGTTTCTGGTTCCACTCGCAGAGAAGCAGAGAAGCGGAGCAAGCGGCGCGTTCCAGAAC  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1495  CAGTTTCTGGTTCCACTCGCAGAGAAGCAGAGAAGCGGAGCAAGCGGCGCGTTCCAGAAC  1554

Query  541   CTCGGGCAAGACCAGCCTCTCCCAGAGCATCCCCACCGCGAAGCGCAACCTTCTCCAGAG  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1555  CTCGGGCAAGACCAGCCTCTCCCAGAGCATCCCCACCGCGAAGCGCAACCTTCTCCAGAG  1614

Query  601   C  601
             |
Sbjct  1615  C  1615
```

Figure 3.2 Sequence alignment between the *Hsp70* promoter and the promoter region of pEZX-pG04. BLAST (Altschul *et al.*, 1990) was used to align the *Hsp70* promoter sequence (Query sequence) and the pEZX-pG04 (Sbjct sequence). The position of the SP1F_KLFS_01 sequence in both the *Hsp70* promoter sequence and the promoter region of pEZX-pG04 are highlighted in yellow.

```
Query  1     GCCAAGCGTTATCCCTCCCGTTTTGAGAAACTTTCTGCGTCCGCCATCCTGTAGGAAGAA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7707  GCCAAGCGTTATCCCTCCCGTTTTGAGAAACTTTCTGCGTCCGCCATCCTGTAGGAAGAA  7766

Query  61    TTTGTACACCTTAAACTCCCTCCCTGGTCTGATTCCCAAATGTCTCTCACCGCCCAGCAC  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7767  TTTGTACACCTTAAACTCCCTCCCTGGTCTGATTCCCAAATGTCTCTCACCGCCCAGCAC  7826

Query  121   TTTCAGGAGCTGACCCTTCTCAGCTTCACATACAGAGACCGCTACCTTGCGTCGCCATGG  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7827  CTTCAGGAGCTGACCCTTCTCAGCTTCACATACAGAGACCGCTACCTTGCGTCGCCATGG  7886

Query  181   CAACACTGTCACAACCGGAACAAGCACTTCCTACCACCCCCCGCCTCAGGAATCCAATCT  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7887  CAACACTGTCACAACCGGAACAAGCACTTCCTACCACCCCCCGCCTCAGGAATCCAATCT  7946

Query  241   GTCCAGCGAAGCCCAGATCCGTCTGGAGAGTTCTGGACAAGGGCGGTACCCTCAACATGG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7947  GTCCAGCGAAGCCCAGATCCGTCTGGAGAGTTCTGGACAAGGGCGGTACCCTCAACATGG  8006

Query  301   ATTACTCATGGGAGGCGGAGAAGCTCTAACAGACCCGAAACTGCTGGAAGATTCCTGGCC  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  8007  ATTACTCATGGGAGGCGGAGAAGCTCTAACAGACCCGAAACTGCTGGAAGATTCCTGGCC  8066

Query  361   CCAAGGCCTCCTCCCGCTCGCTGATTGGCCCATGGGAGGGTGGGCGGGGCCGGAGGAGGC  420
             |||||||||||||||||||||||||||||||||||    | ||||||||||||||||||
Sbjct  8067  CCAAGGCCTCCTCCCGCTCGCTGATTGGCCCATGGGAGGGTATACTGGGCCGGAGGAGGC  8126

Query  421   TCCTTAAAGGCGCAGGGCGGCGCGCAGGACACCAGATTCCTCCTCCTAATCTGACAGAAC  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  8127  TCCTTAAAGGCGCAGGGCGGCGCGCAGGACACCAGATTCCTCCTCCTAATCTGACAGAAC  8186

Query  481   CAGTTTCTGGTTCCACTCGCAGAGAAGCAGAGAAGCGGAGCAAGCGGCGCGTTCCAGAAC  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  8187  CAGTTTCTGGTTCCACTCGCAGAGAAGCAGAGAAGCGGAGCAAGCGGCGCGTTCCAGAAC  8246

Query  541   CTCGGGCAAGACCAGCCTCTCCCAGAGCATCCCCACCGCGAAGCGCAACCTTCTCCAGAG  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  8247  CTCGGGCAAGACCAGCCTCTCCCAGAGCATCCCCACCGCGAAGCGCAACCTTCTCCAGAG  8306

Query  601   C  601
             |
Sbjct  8307  C  8307
```


Figure 3.3 Sequence alignment between the *Hsp70* promoter and the promoter region of pEZX-pG04-Mut. BLAST (Altschul *et al.,* 1990) was used to align the *Hsp70* promoter sequence (Query sequence) and the pEZX-pG04-Mut (Sbjct sequence). The position of the SP1F_KLFS_01 sequence in both the *Hsp70* promoter sequence and the promoter region of pEZX-pG04-Mut are highlighted in yellow.

3.5 The transfection of H9c2 cells with pEZX-pG04 and pEZX-pG04-Mut.

Large-scale plasmid DNA preparations of pEZX-pG04 and pEZX-pG04-Mut were generated as described in Section 2.9.1 (Figure 3.4) Lane 2 and 3 shows the 3 forms of plasmid DNA (supercoiled, nicked and linear).

H9c2 cells were transfected with 2 μg of the two plasmids as described in Section 2.11.5 using MetafectenePro™. Figure 3.5 shows the results of the transfection after 12 days of selection in 0.5 μg/ml puromycin. Cells that are successfully transfected with either pEZX-pG04 or pEZX-pG04-Mut should be resistant to puromycin treatment since the plasmid carries the puromycin resistance gene. Treatment with 0.5 μg/ml puromycin will kill all the cells that are not transfected with the plasmid. Figure 3.5 (**B)** shows that untransfected cells that were treated with 0.5 μg/ml puromycin did not survive the selection process. No viable cells were present in the well. Cells that were stably transfected with pEZX-pG04 (**C**) and pEZX-pG04-Mut (**D**) survived the selection process, suggesting that the cells were successfully transfected with the plasmid DNA. H9c2 cells successfully transfected with pEZX-pG04 and pEZX-pG04-Mut were renamed H9c2-*Hsp70* and H9c2-*Hsp70-Mut*, respectively. H9c2-*Hsp70* cells can therefore be used as a reporter for wild type *Hsp70* promoter, while H9c2-*Hsp70-Mut* cells can be used as a reporter for the mutant *Hsp70* promoter.

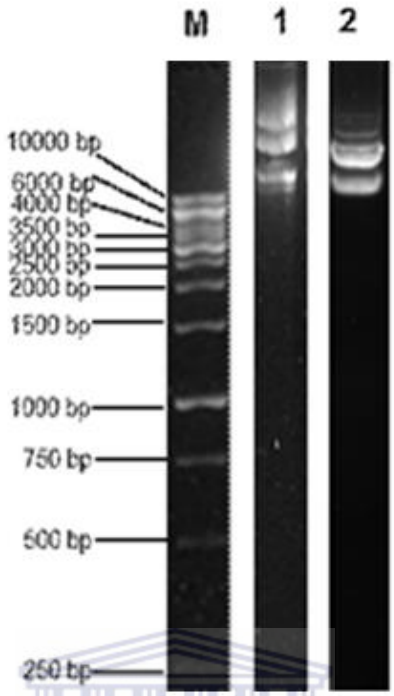


Figure 3.4 Large-scale plasmid DNA preparation of pEZX-pG04 and pEZX-pG04-Mut. Lane M is the GeneRuler 1kb DNA ladder, Lane 1 is the plasmid preparation of pEZX-pG04 and Lane 2 is the plasmid preparation of pEZX-pG04-Mut.

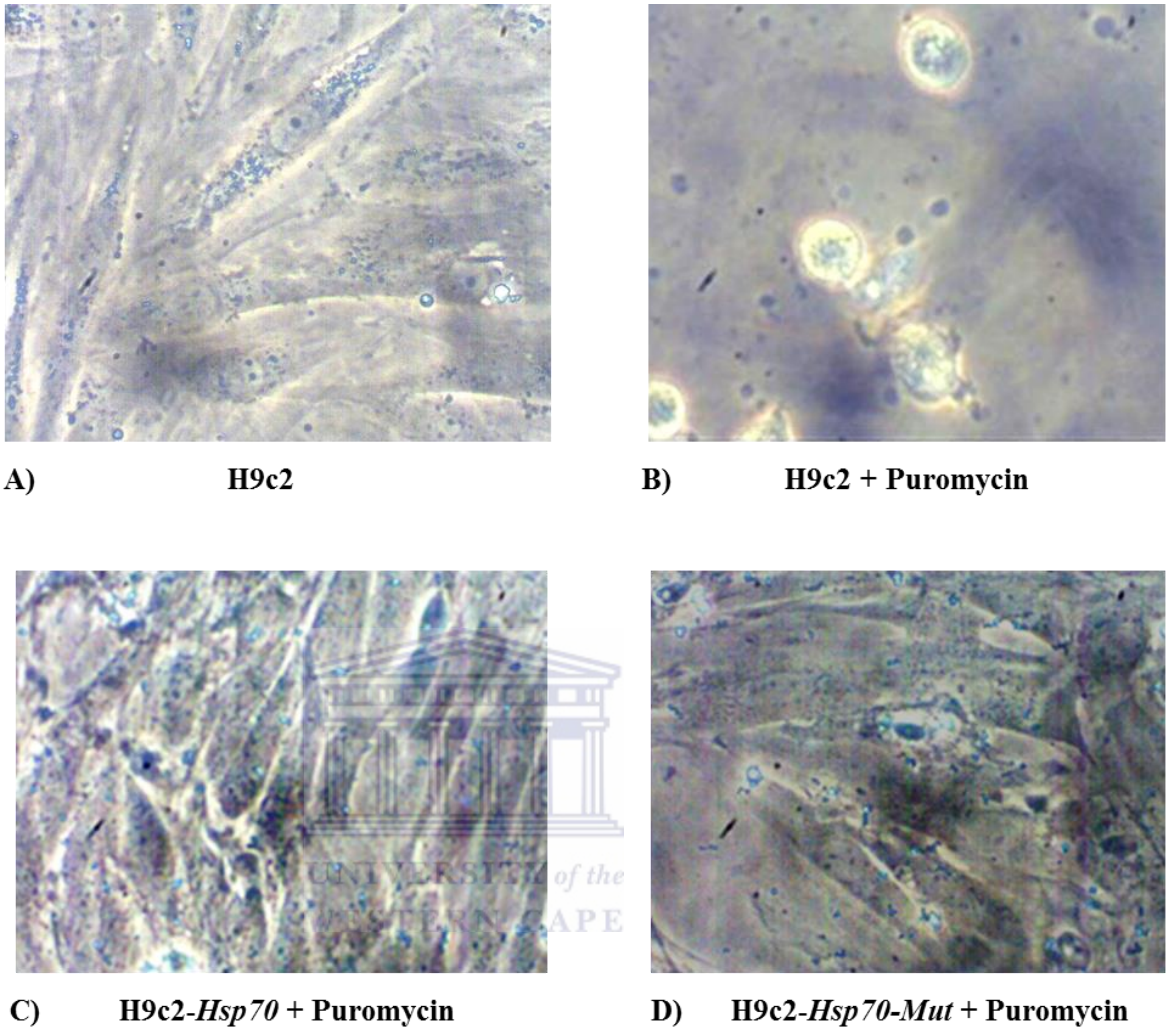


Figure 3.5 H9c2 cells transfected with pEZX-pG04 and pEZX-pG04-Mut. **A** shows the untransfected cells, **B** shows cells untransfected treated for 12 days with 0.5 µg/ml puromycin, **C** shows cells that were transfected with 2 µg pEZX-pG04 and treated with 0.5 µg/ml puromycin (generating H9c2-*Hsp70* cells) and **D** shows cells that were transfected with 2 µg pEZX-pG04-Mut and treated with 0.5 µg/ml puromycin (generating H9c2-*Hsp70-Mut* cells). The images were taken at 20 times magnification using the Nikon microscope fitted with a Leica camera.

3.6 The expression of Hsp70 in response to heat stress.

It is known that heat stress induces the expression of Hsp70 (Mosser *et al.*, 1997). Heat stress can therefore be used as a stimulus to evaluate *Hsp70* promoter activity. To confirm that Hsp70 is induced by heat stress, H9c2 cells were exposed to 42 °C for 1 hour and the expression of Hsp70 was evaluated by Western blot analysis 1, 4, 6 and 24 hours after exposure to heat stress. Total protein lysates were prepared using the Cytobuster™ Protein Extraction Reagent (as described in Section 2.13). Figure 3.6 **A** shows the total protein lysates isolated from the cells and Figure 3.6 **B** shows the Western blot analysis. Figure 3.6 **A** demonstrates that equal quantities of the protein samples were loaded on the gel. The Western blot (Figure 3.6 **B**) shows that the expression levels of Hsp70 increase significantly 6 hours after exposure to heat stress and remains elevated at 24 hours.

This suggests that the activity of the *Hsp70* promoter will also increase 6 hours after exposure to heat stress and that this would be the appropriate time point to assess *Hsp70* promoter activity.

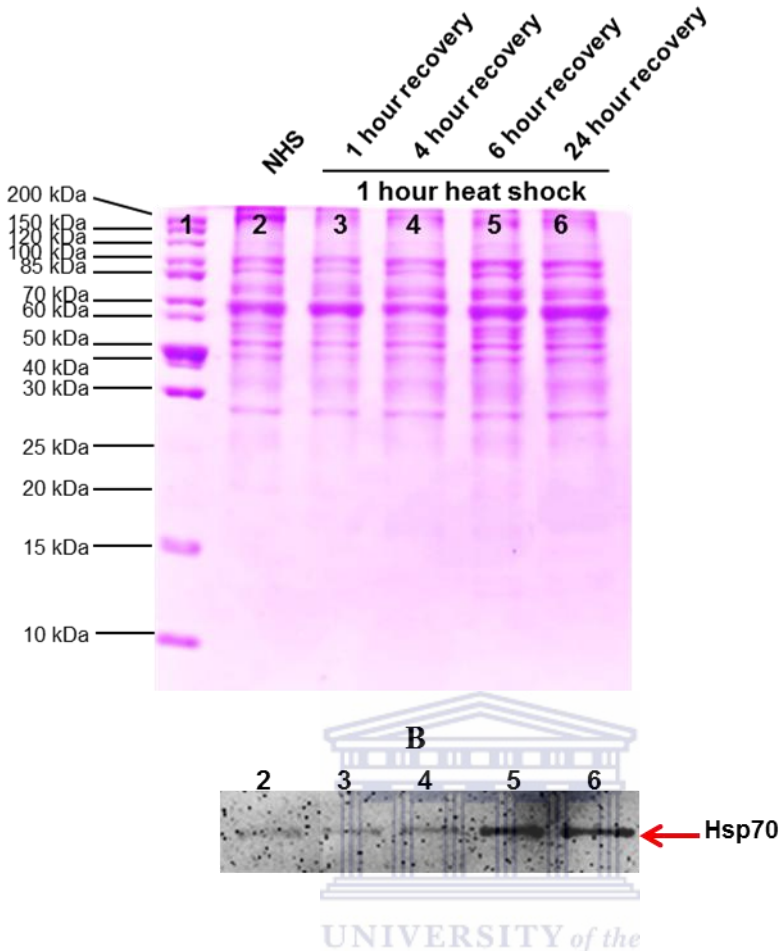


Figure 3.6 Analysis of Hsp70 expression in response to heat stress. H9c2 cells were exposed 42 °C for 1 hour and allowed to recover for 1, 4, 6, 24 hours. Total protein lysates were prepared and analysed by SDS-PAGE (**A**). Lane 1 is the Spectra Multi-colour Broad Range Protein Ladder (Fermentas) and Lane 2 (Non-heat shocked or NHS) is a lysate prepared from H9c2 cells that were not subjected to heat stress. Lanes 3, 4, 5 and 6 are lysates prepared from H9c2 cells that were subjected to heat stress and allowed to recover for 1, 4, 6 and 24 hours, respectively. A Western blot analysis was performed on the same samples using an anti-Hsp70 antibody (**B**).

3.7 The *Hsp70* promoter reporter assay

Based on the *in silico* analysis of the promoter sequences of heat shock response genes, it was suspected that the SP1F_KLFS_01 promoter module is involved in the regulation of the gene expression of heat shock responsive genes such as *Hsp70.* To evaluate the involvement of the SP1F_KLFS_01 promoter module in regulating the expression of heat shock responsive genes, two cells lines H9c2-*Hsp70* and H9c2-*Hsp70-Mut* were generated. H9c2-*Hsp70* can be used in reporter assays for the *Hsp70* promoter, while the H9c2-*Hsp70-Mut* can be used in the reporter assays for the mutant *Hsp70* promoter. Hsp70 promoter activity was evaluated in H9c2, H9c2-*Hsp70* and H9c2-*Hsp70-Mut* cells following exposure to heat stress. The cells were cultured in 6 well plates and exposed to 42 °C for 1 hour as described in Section 2.12. Hsp70 promoter activity was assessed in these cells by measuring the luciferase activity 1, 4, 6, and 24 hours after heat stress as described in Section 2.16 (Figure 3.7). Since H9c2 cells were not transfected (Figure 3.5), no luciferase activity was observed in these cells. Both H9c2-*Hsp70* and H9c2-*Hsp70-Mut* cells demonstrated a time dependent increase in luciferase activity between 4 and 24 hours after exposure to heat stress. This is in line with the Western blot result, which showed that the expression levels of Hsp70 increase 6 hours after exposure to heat stress. However, Figure 3.7 also shows that there is no significant difference in the Hsp70 promoter activity in H9c2-*Hsp70* and H9c2-*Hsp70-Mut* cells. This suggests that the mutation that was introduced into the *Hsp70* promoter in H9c2-*Hsp70-Mut* cells did not affect the activity of the promoter. Furthermore, it also suggests that the SP1F_KLFS_01 promoter

module may not be involved in the regulation of the expression of heat shock response genes.

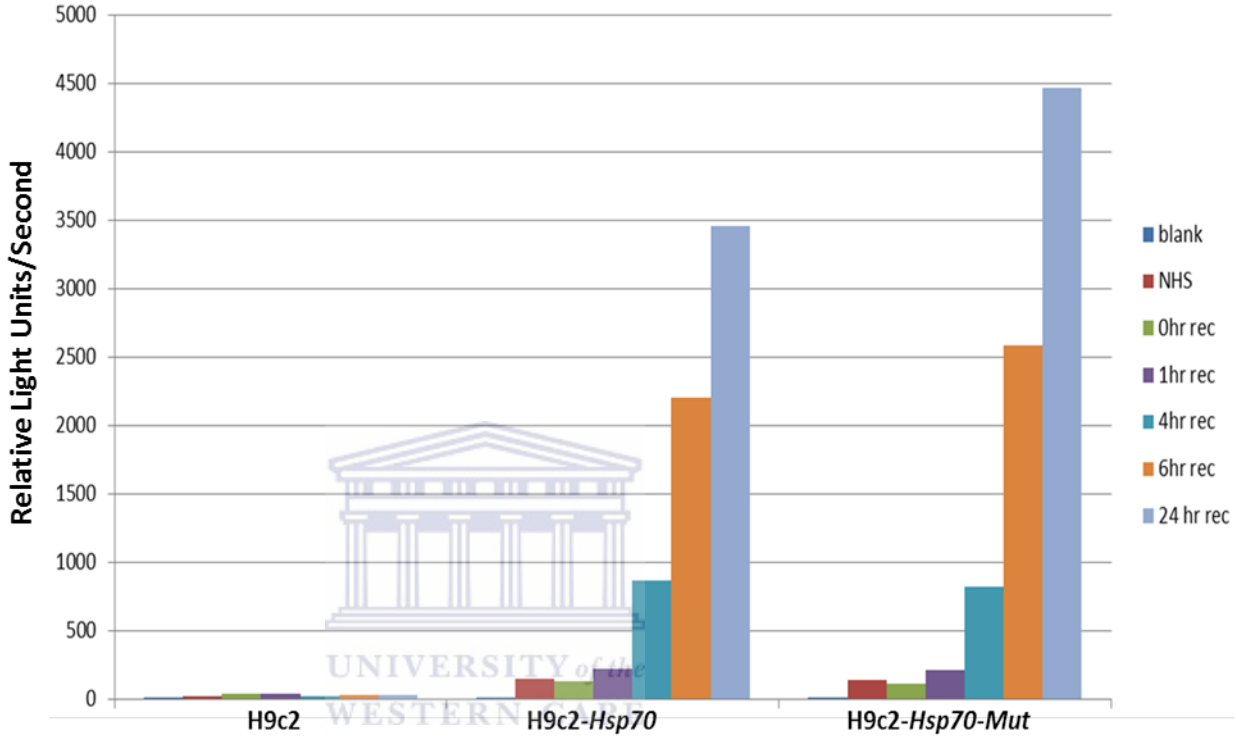
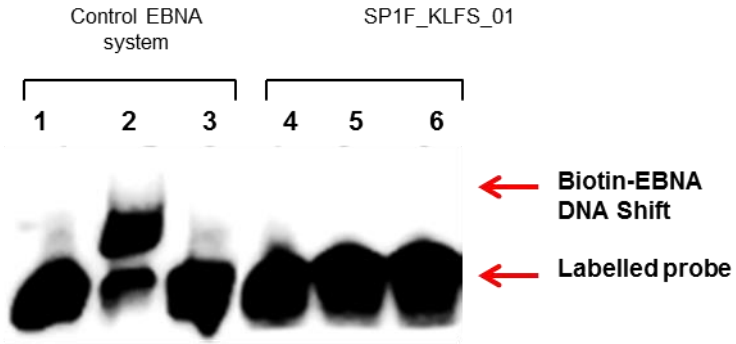


Figure 3.7 *Hsp70* promoter activity in H9c2-*Hsp70* and H9c2-*Hsp70-Mut* cells. A) *Gaussia* Luciferase (GLuc) assay for high sensitivity, using GL-H buffer. Indicating increased luciferase promoter expression after 1, 4, 6 and 24 hour recovery in both the H9c2-*Hsp70* and H9c2-*Hsp70-Mut* cells.

3.8 Evaluating protein binding to SP1F_KLFS_01 sequence following heat shock

The promoter reporter assay could not confirm the involvement of SP1F_KLFS_01 promoter module in the control of Hsp70 expression. Therefore, a second experimental approach was used to evaluate the involvement of SP1F_KLFS_01. The Electrophoretic mobility shift assay (EMSA) can be used to study the possible interaction between DNA-binding proteins and DNA. The principle of the assay is based on the fact that DNA-protein complexes migrates slower on a gel compared to unbound DNA (Holden and Tacon, 2011). EMSA analysis is often used to show the binding of the TFs to their respective sites in the *cis*-regulatory elements (Werner, 2001). As shown in Figure 3.7, *Hsp70* promoter activity incresed after a recovery period of 4 hours, and continues until 24 hours. It is therefore expected that TFs that regulate the expression of *Hsp70* and other heat shock responsive proteins, will be present in the proteome of the cells at this time point. If SP1F_KLFS_01 is involved in the regulation of *Hsp70* expression then proteins present in a cell lysate, prepared at this time point, should bind to a synthetic copy of the SP1F_KLFS_01 sequence. H9c2 cells were therefore subjected to heat shock, allowed to recover for 1, 4, 6 and 24 hours and total cellular proteins were extracted using Cytobuster ™ Extraction Reagent (Section 2.13). For this experiment the 24 hour recovery sample was used as it showed the highest expression in the Western blot analysis. The EMSA was performed as described in Section 2.17. A biotin-labelled probe representing the SP1F_KLFS_01 sequence was used as the target. Figure 3.8 shows a pronounced shift for the Biotin-EBNA control DNA binding to the proteins present in the

EBNA protein extract (Lane 2). However, no shift was observed when the Biotin-SP1F_KLFS_01 probe was incubated with the protein lysate prepared from heat shocked cells (Lane 5).



Non- HS lysate	-	-	-	-	+	-
HS lysate	-	-	-	-	-	+
Biotin-SP1F_KLFS probe	-	-	-	+	+	+
Unlabelled EBNA DNA	-	-	+	-	-	-
EBNA Extract	-	+	+	-	-	-
Biotin-EBNA Control DNA	+	+	+	-	-	-

Figure 3.8 Electrophoretic Mobility Shift Assay (EMSA) investigating the binding of SP1F_KLFS_01 to protein lysate and showing no binding of protein (heat shocked and non-heat shocked lysates) to DNA.

The EMSA results clearly validated the promoter reporter assay data by showing that SP1F_KLFS_01 is not a target for TFs expressed in H9c2 cells after heat shock, and that this promoter module is probably not involved in the regulation of heat shock response in these cells. However, it is possible that binding of the

proteins to SP1F_KLFS_01 was affected by the fact that total cellular protein lysate as opposed to nuclear lysates was used.

Future directions

Seeing that *in vitro* experiments of this study did not support the findings of the *in silico* experiments, there are various steps which could be taken to further investigate the involvement of *cis*-acting regulatory elements in the regulation of heat shock.

Other promoter modules could be investigated, such as SMAD_E2FF_01 and E2FF_SP1F_01 to verify the regulation of the heat shock response in cardiomyocytes.

Nuclear extracts should be extracted from protein lysates opposed to total cellular extract to ensure that the correct experimental procedure for the EMSA experiment in followed.

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APPENDIX 1

Promoter sequences for heat shock responsive genes

>GXP_2963156(Hspa1b/rat)
loc=GXL_187112|sym=Hspa1b|geneid=294254|acc=GXP_2963156|taxid=10116|spec=Rattus norvegicus|chr=20|ctg=NC_005119|str=(+)|start=3955107|end=3955707|len=601|tss=501|descr=heat shock 70kD protein 1B (mapped)|comm=GXT_23117308/ENSRNOT00000049667/501/bronze

GCCAAGCGTTATCCCTCCCGTTTTGAGAAACTTTCTGCGTCCGCCATCCTGTAGGAAGAATTTGTACACCTTAA
ACTCCCTCCCTGGTCTGATTCCCAAATGTCTCTCACCGCCCAGCACTTTCAGGAGCTGACCCTTCTCAGCTTCAC
ATACAGAGACCGCTACCTTGCGTCGCCATGGCAACACTGTCACAACCGGAACAAGCACTTCCTACCACCCCCC
GCCTCAGGAATCCAATCTGTCCAGCGAAGCCCAGATCCGTCTGGAGAGTTCTGGACAAGGGCGGTACCCTCA
ACATGGATTACTCATGGGAGGCGGAGAAGCTCTAACAGACCCGAAACTGCTGGAAGATTCCTGGCCCCAAGG
CCTCCTCCCGCTCGCTGATTGGCCCATGGGAGGGTGGGCGGGGCCGGAGGAGGCTCCTTAAAGGCGCAGGG
CGGCGCGCAGGACACCAGATTCCTCCTCCTAATCTGACAGAACCAGTTTCTGGTTCCACTCGCAGAGAAGCAG
AGAAGCGGAGCAAGCGGCGCGTTCCAGAACCTCGGGCAAGACCAGCCTCTCCCAGAGCATCCCCACCGCGAA
GCGCAACCTTCTCCAGAGC

>GXP_3419496(Hspa5/rat) loc=GXL_246319 |sym=Hspa5|geneid=25617 |acc=GXP_3419496| taxid=10116|spec=Rattus norvegicus| chr=3|ctg=NC_005102|str=(+)| start=13783636| end=13784237|len=602|tss=501|descr=heat shock protein 5|comm=GXT_24675410/ trans_ENSMUST00000145466/501/silver

GCGCGCTCGATACTGGCTGTGACTACACTGACTTGGACACTTGGCCTTTTGCGGGTTTGAGAGGTAAGCGTCG
CGGCCTGCTTCCAGGCCTACCCTGATTTTGGTTCGTGGCTCCTCCTGACCCTGAGACCTCTGTCGCCCTCAGAT
CAGAACCGTCGTCGCGTTTCGGGGCTACAGCCTGTTGCTGGACTCTGTGAGACACCTGACCGACCGCTGAGCG
ACTGACTGGTCCACAGCGCCGGCAAGATGAAGTTCACTGTGGTGGCGGCGGCGCTGCTGCTGCTGTGTGCGG
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TTCCTGGTAAGTGGCAATCGTCGGAGGAGGTTGGGGGGGGGGAGTGGGGCGTGGCCTCCGGGGCTACCGT
GAGAAAGTGCGGTGCTGATTCCTTTTCTGTGGGGTTTTCCCATCAGCGTCGGTGTATTCAAGAACGGCCGCGT
GGAGATCATAGCCAACGATCAGGGCAACCGCATCACACCGTCGTATGTGGCCTTCACTCCTGAAGGGGAGCG
TCTGATTGGCGATGCGGCCAAGAAC

>GXP_1149850(Heatr2/rat) loc=GXL_622470|sym=Heatr2|geneid=304332| acc=GXP_1149850| taxid=10116|spec=Rattus norvegicus|chr=12|ctg=NC_005111|str=(-)|start=15994871| end=15995471|len=601|tss=501|descr=HEAT repeat containing 2|comm=GXT_23485096/ NM_001134857/501/silver;GXT_24168388/ENSRNOT00000035545/501/silver

TCGCCCTGCCTACAAAAGACAACGCTCCGCCTGCCACCTCCTGCAACGCTCCCAAAGTCCACGCCCCGCCCATT
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AGCACCACTCCTAAGACGCGCACACGCTGCCTCGAGCAACCGAGTCAGCCGGAAGTCTTGCCGTAAAGCGTG
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CGGAGGTGGCCGCGGGCTTCGCCGAGGCGGATGAAGCAGCGGAACTGACCCGCGCGCTAGGTCGTCTGTTA
CCGGGACTGGA

>GXP_3994204(Heatr1/rat) loc=GXL_322134|sym=Heatr1|geneid=361262|acc=GXP_3994204 | taxid=10116|spec=Rattus norvegicus|chr=17|ctg=NC_005116|str=(-)|start=68655147| end=68655810|len=664|tss=504|descr=HEAT repeat containing 1|comm=GXT_25266975/ trans_NM_144835/504/silver

GTGGAAGTTTCCCGTGAATGAACCATCCCTGCTACACACCCATAAAGCTCCAAAAGACGACTGCAGAAACTGA
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CGGGAGGCCAGGGTGGGCCTT

>GXP_2964569(Hspa4l/rat) loc=GXL_627335|sym=Hspa4l|geneid=294993| acc=GXP_2964569| taxid=10116|spec=Rattus norvegicus|chr=2|ctg=NC_005101| str=(+)|start=127697753| end=127699004|len=1252|tss=786|descr=heat shock protein 4-like|comm=GXT_24198951/ trans_XM_002808421/786/silver

AGAACACAAGGAATTGAATGAGAAGGATCAGCAGATACATGAAGAACCAGATAAGGGTCAGCATATACATG
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CTAGGTAAAAGCCAGGAGATACATGAAGAACTGGACCCTAAGGAACGGGGGACAAAGGGGCTGGATAAGG
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TCAGGGCCTAGAAAACCAGTTTTCATGGGGAAAGCACCGGAAAGTGACAGGTTGGGACCGGGCAGCAGCGG
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>GXP_3418059(Hspa4l/rat) loc=GXL_627335|sym=Hspa4l|geneid=294993| acc=GXP_3418059| taxid=10116|spec=Rattus norvegicus|chr=2|ctg=NC_005101|str=(+)|start=127698574| end=127699176|len=603|tss=501|descr=heat shock protein 4-like|comm=GXT_24680446/trans_ENSMUST00000108086/501/silver

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>GXP_862885(Hspa2/rat) loc=GXL_468860|sym=Hspa2|geneid=60460|acc=GXP_862885| taxid=10116|spec=Rattus norvegicus|chr=6|ctg=NC_005105|str=(+)| start=99000041| end=99000641|len=601|tss=501|descr=heat shock protein 2|comm=GXT_22198962/NM_021863/501/silver

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>GXP_1731603(Hsf4/rat) loc=GXL_736263|sym=Hsf4|geneid=291960| acc=GXP_1731603| taxid=10116|spec=Rattus norvegicus|chr=19|ctg=NC_005118|str=(+)| start=35085032| end=35085632|len=601|tss=501|descr=heat shock transcription factor 4|comm=GXT_23138720/NM_001106177/501/silver;GXT_24179864/ENSRNOT00000020682/501/silver

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>GXP_3995149(Hsf4/rat) loc=GXL_736263|sym=Hsf4|geneid=291960|acc=GXP_3995149|
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trans_ENST00000517729/1091/silver

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>GXP_278484(Hspe1/rat) loc=GXL_233373|sym=Hspe1|geneid=25462|acc=GXP_278484|
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end=53895625|len=793|tss=501|descr=heat shock protein 1 (chaperonin
10)|comm=GXT_21772313/ NM_012966/501/silver

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>GXP_52220(Hsp90b1/rat) loc=GXL_42957|sym=Hsp90b1|geneid=362862|acc=GXP_52220| taxid=10116|spec=Rattus norvegicus|chr=7|ctg=NC_005106|str=(-)|start=23347425| end=23348025|len=601|tss=501|descr=heat shock protein 90, beta, member 1|comm=GXT_2310 7638/ENSRNOT00000059555/501/silver;GXT_23485332/NM_001012197/501/silver;GXT_24170419 /ENSRNOT00000059554/501/silver

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>GXP_202074(Hsp90aa1, LOC499735, LOC691091/rat) loc=GXL_168670|sym=Hsp90aa1, LOC499735, LOC691091|geneid=299331, 499735, 691091|acc=GXP_202074| taxid=10116| spec=Rattus norvegicus|chr=6|ctg=NC_005105|str=(-)|start=135418994| end=135419647| len=654|tss=501,552|descr=heat shock protein 90, alpha (cytosolic), class A member 1; similar to heat shock protein 1, alpha; similar to heat shock protein 1, alpha|comm=GXT_21799309/ NM_175761/501/silver;GXT_25267617/trans_ENSMUST00000124156/552/silver

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>GXP_2972579(Hsp90aa1, LOC499735, LOC691091/rat) loc=GXL_168670|sym=Hsp90aa1, LOC499735, LOC691091|geneid=299331, 499735, 691091|acc=GXP_2972579| taxid=10116| spec=Rattus norvegicus|chr=6|ctg=NC_005105|str=(-)|start=135418406| end=135419006| len=601|tss=501|descr=heat shock protein 90, alpha (cytosolic), class A member 1; similar to heat shock protein 1, alpha; similar to heat shock protein 1, alpha|comm=GXT_24190764/ XR_085622/ 501/silver;GXT_24190787/XR_007019/501/silver

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>GXP_4005779(Hsp90aa1, LOC499735, LOC691091/rat) loc=GXL_168670|sym=Hsp90aa1, LOC499735, LOC691091|geneid=299331, 499735, 691091|acc=GXP_4005779|taxid=10116 |spec=Rattus norvegicus|chr=6|ctg=NC_005105|str=(-)|start=135418680| end=135419280| len=601|tss=501|descr=heat shock protein 90, alpha (cytosolic), class A member 1; similar to heat shock protein 1, alpha; similar to heat shock protein 1, alpha|comm=GXT_25267618/ trans_ENSMUST00000149189/501/silver

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>GXP_241620(Hsp90ab1/rat) loc=GXL_201364|sym=Hsp90ab1|geneid=301252| acc=GXP_241620| taxid=10116|spec=Rattus norvegicus|chr=9|ctg=NC_005108| str=(+)|start=11032807| end=11033596|len=790|tss=664,690|descr=heat shock protein 90 alpha (cytosolic), class B member 1|comm=GXT_21772405/NM_001004082/690/silver;GXT_24183722/ENSRNOT00000026920/664/silver

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>GXP_1727035(Ahsa2/rat) loc=GXL_735349|sym=Ahsa2|geneid=305577|acc=GXP_1727035| taxid=10116|spec=Rattus norvegicus|chr=14|ctg=NC_005113|str=(-)|start=104435411| end=104436011|len=601|tss=501|descr=AHA1, activator of heat shock protein ATPase homolog 2 (yeast)|comm=GXT_23136523/NM_001107241/501/silver;GXT_24174123/ENSRNOT00000056855/501/silver;GXT_24174124/ENSRNOT00000007297/501/silver

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>GXP_853081(Pdap1/rat) loc=GXL_468142|sym=Pdap1|geneid=64527| acc=GXP_853081| taxid=10116|spec=Rattus norvegicus|chr=12|ctg=NC_005111| str=(+)|start=9784230| end=9784844|len=615|tss=515|descr=PDGFA associated protein 1|comm=GXT_22199040/ NM_022595/515/silver

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>GXP_514212(Dnajb6/rat) loc=GXL_324763|sym=Dnajb6|geneid=362293|acc=GXP_514212| taxid=10116|spec=Rattus norvegicus|chr=4|ctg=NC_005103|str=(-)|start=796137| end=796737| len=601|tss=501|descr=DnaJ (Hsp40) homolog, subfamily B, member 6|comm=GXT_21925644/ NM_001013209/501/silver;GXT_24169497/ENSRNOT00000014194/501/silver

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>GXP_1742(Dnaja1/rat) loc=GXL_1468|sym=Dnaja1|geneid=65028|acc=GXP_1742| taxid=10116|spec=Rattus norvegicus|chr=5|ctg=NC_005104|str=(+)| start=58100294| end=58100894|len=601|tss=501|descr=DnaJ (Hsp40) homolog, subfamily A, member 1|comm=GXT_21769964/NM_022934/501/silver

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