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.

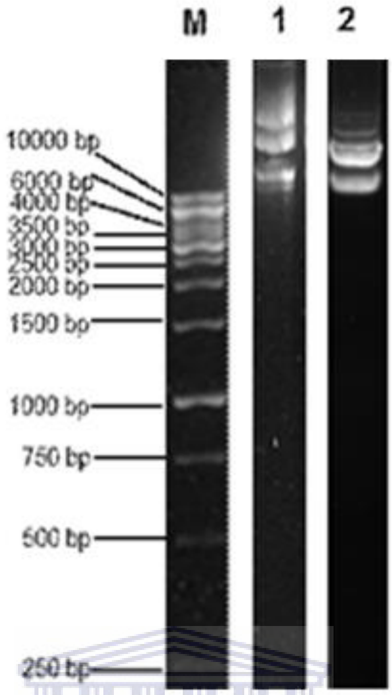


Figure 3.4 Large-scale plasmid DNA preparation of pEZX-pG04 and pEZX-pG04-Mut. Lane M is the GeneRuler 1kb DNA ladder, Lane 1 is the plasmid preparation of pEZX-pG04 and Lane 2 is the plasmid preparation of pEZX-pG04-Mut.

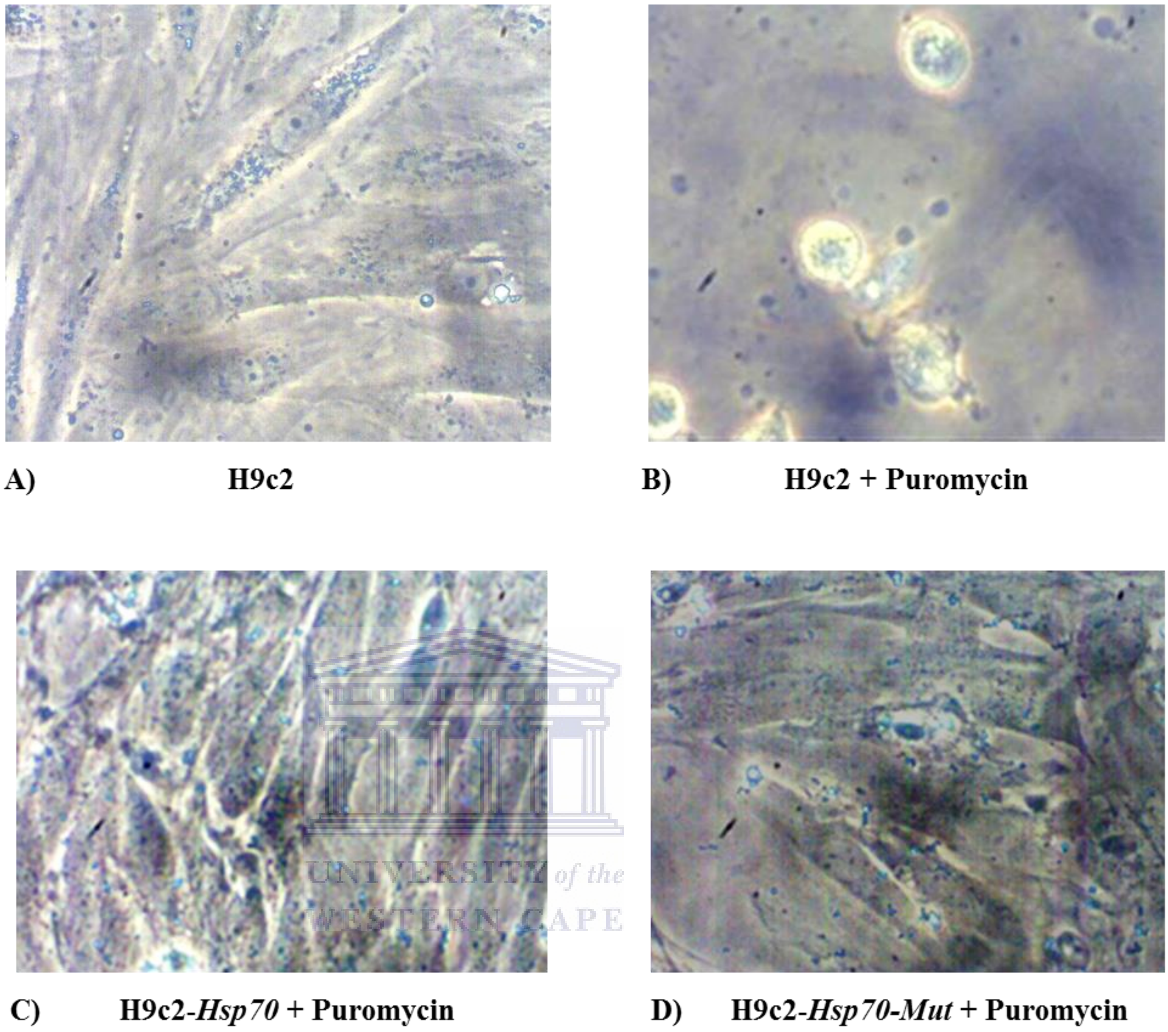


Figure 3.5 H9c2 cells transfected with pEZX-pG04 and pEZX-pG04-Mut. **A** shows the untransfected cells, **B** shows cells untransfected treated for 12 days with 0.5 µg/ml puromycin, **C** shows cells that were transfected with 2 µg pEZX-pG04 and treated with 0.5 µg/ml puromycin (generating H9c2-*Hsp70* 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.

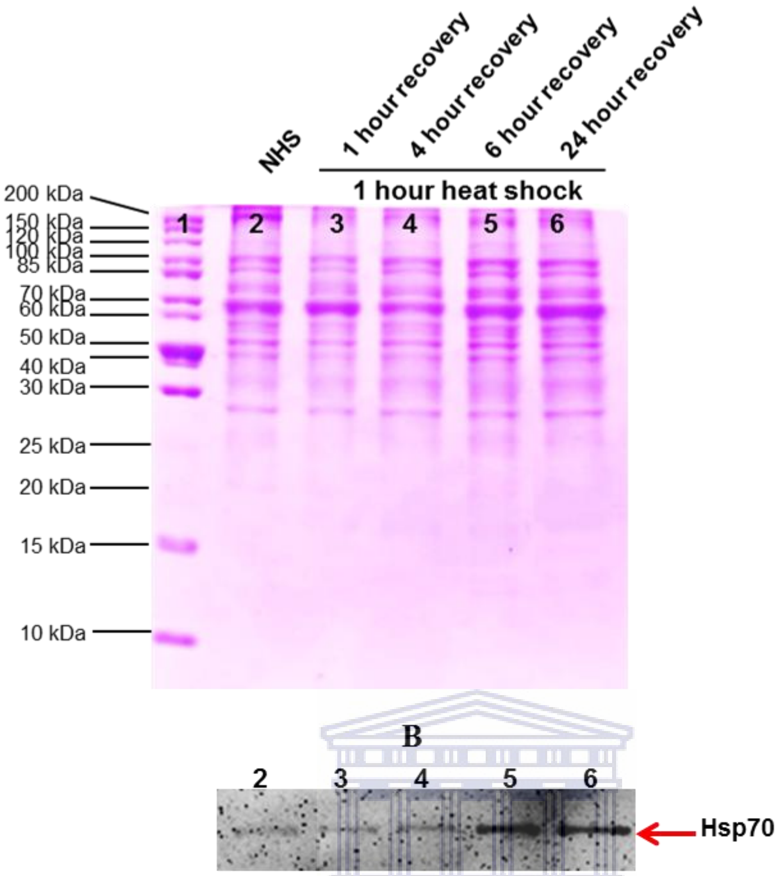


Figure 3.6 Analysis of Hsp70 expression in response to heat stress. H9c2 cells were exposed 42 °C for 1 hour and allowed to recover for 1, 4, 6, 24 hours. Total protein lysates were prepared and analysed by SDS-PAGE (**A**). Lane 1 is the Spectra Multi-colour Broad Range Protein Ladder (Fermentas) and 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.

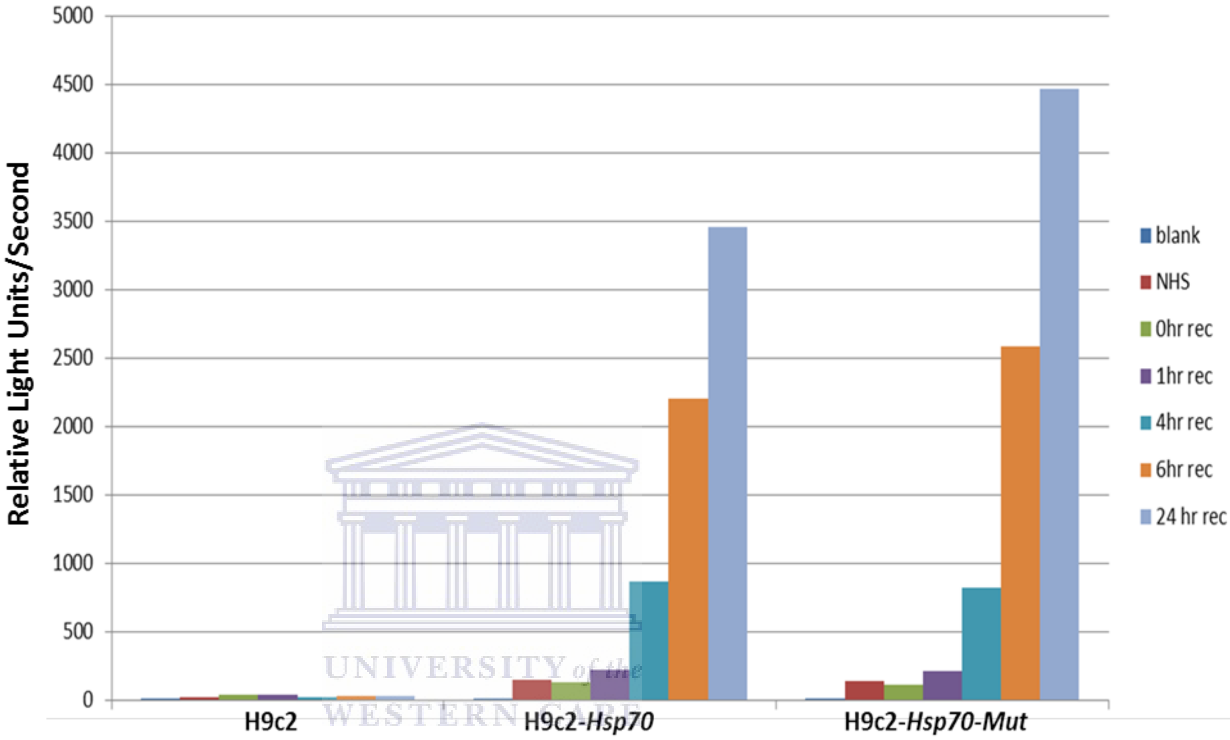
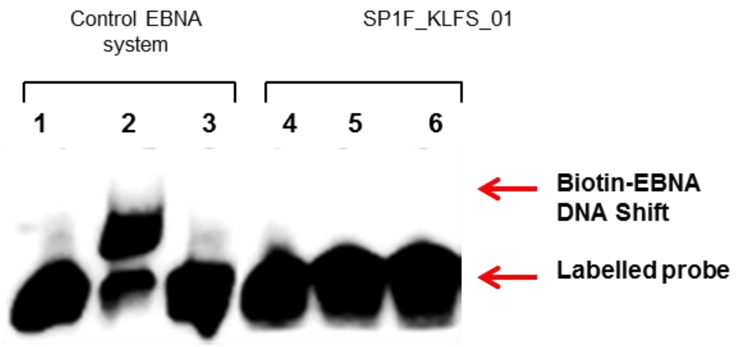


Figure 3.7 *Hsp70* promoter activity in H9c2-*Hsp70* and H9c2-*Hsp70-Mut* cells. A) *Gaussia* Luciferase (GLuc) assay for high sensitivity, using GL-H buffer. Indicating increased luciferase promoter expression after 1, 4, 6 and 24 hour recovery in both the H9c2-*Hsp70* and H9c2-*Hsp70-Mut* cells.

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).



	1	2	3	4	5	6
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

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heat shock 70kD protein 1B (mapped)|comm=GXT_23117308/ENSRNOT00000049667/501/bronze

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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|
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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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