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POSTDOCTORAL STUDIES

Maryam Kamkar

AUTEUR DE LA THÈSE / AUTHOR OF THESIS

Ph.D. (Biology)

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FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

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Promoter Region of the Zebrafish *hsp10/60* Genes

TITRE DE LA THÈSE / TITLE OF THESIS

Marc Ekker

DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

Marie-Andrée Akimenko

Patrick Walsh

John Heikkila

William Willmore

Douglas Johnson

Gary W. Slater

Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

Characterization and Functional Analysis of
the Bidirectional Promoter Region of the
Zebrafish *hsp10/60* Genes

by
Maryam Kamkar

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Abstract

Protein folding *in vivo*, often requires a set of proteins called chaperones. Chaperones assist the folding of newly synthesized proteins, to obtain their active tri-dimensional structures. They may also provide adaptive response against adverse conditions such as heat shock and stress. Chaperonins 10 and 60 (Hsp10 and Hsp60) are present in the mitochondria of all cells. The Hsp10/60 machinery occurs as a complex with a 2:1 stoichiometry of Hsp10 to Hsp60. *hsp10/60* genes are linked in a head-to-head manner with a short intergenic region. To investigate the bidirectionality of the *hsp10/60* promoter region, we made a number of reporter constructs and tested them by microinjection into fertilized zebrafish eggs and by transfection of mammalian 293 and zebrafish ZF-4 cells. Our results showed that the promoter region is comprised of a ~1-kb fragment that encompasses the *hsp10* transcription start site, the intergenic region, and the transcription start site of *hsp60*. A larger fragment of about 4 kb, covering the start codon of both genes, which includes the first exon of *hsp10*, the first intron of *hsp60*, and the first two exons of *hsp60*, directs twice as much expression of reporter genes in the *hsp60* direction as compared to the *hsp10* direction. This fragment also causes strong expression in the yolk syncytial layer, particularly during the gastrula stage. Deletion of the first intron of *hsp60* results in the loss of the 2:1 ratio in expression and the loss of expression in the yolk, suggesting the existence of a *cis*-acting element within the intronic region. This element may be responsible for maintaining protein concentrations suitable for maintaining normal stoichiometry. A 86-bp region within the first intron of *hsp60* seems to include a site that is linked to the expression in the yolk. However, the region responsible for the stronger expression in the *hsp60* direction could not be more precisely localized. The *hsp10/60* genes show a detectable expression under normal conditions and are inducible by at least two-fold under heat shock conditions. The *hsp10/60* promoter could be used as an inducible promoter in transgenic studies especially for simultaneous co-expressions.

Résumé

Le repliement des protéines *in vivo* requiert souvent une catégorie de protéines appelées les chaperons. Le rôle des protéines chaperon est d'assister le repliement des protéines nouvellement synthétisées afin d'obtenir leurs structures actives tridimensionnelles. Elles pourraient aussi donner des réponses adaptatives contre des conditions défavorables, notamment le stress et le choc thermique. Les chaperons 10 et 60 (Hsp10 et Hsp60) sont présents dans les mitochondries de toutes les cellules. La machinerie Hsp 10/60 forme un complexe de stœchiométrie 2 :1 de Hsp10 et Hsp60. Les gènes *hsp10/60* sont reliés tête-à-tête par une courte région intergénique. Afin de comprendre la bidirectionalité de la région promotrice de *hsp10/60*, plusieurs constructions rapporteurs ont été générées et testées par microinjection dans des embryons de poissons zèbres et par transfection de cellules ZF-4 et de cellules mammifères 293. Ceci a démontré que la région promotrice contient un fragment d'environ 1-kp comprenant le site d'initiation de la transcription de *hsp10*, la région intergénique, et le site d'initiation de la transcription de *hsp60*. Un fragment plus grand d'environ 4kpb incluant le premier exon de *hsp10*, le premier intron de *hsp60*, et les deux premiers exons de *hsp60*, dirige une expression deux fois plus élevée des gènes rapporteurs dans la direction *hsp60* comparativement à la direction *hsp10*. Ce fragment cause une forte expression dans la couche syncytiale du vitellus, tout particulièrement au stade de la gastrula. La délétion du premier intron de *hsp60* provoque une perte du rapport 2 :1 et de l'expression du vitellus, suggérant la présence d'un élément qui agit en *cis* à l'intérieur de l'intron. Cet élément pourrait être responsable du maintien des concentrations protéiques appropriées pour une conservation d'une stœchiométrie normale. Une région de 86-pb dans le premier intron de *hsp60* semble inclure un site lié à l'expression dans le vitellus. Cependant, la région responsable pour la forte expression dans la direction de *hsp60* n'a pas pu être précisément localisée. Les gènes *hsp10/60* montrent une expression facilement décelée sous

des conditions normales et sont induits par au moins un facteur deux sous des conditions de choc thermique. Le promoteur *hsp60* peut donc être utilisé comme promoteur inductible dans des études transgéniques surtout pour les expériences de co-expression simultanée.

Dedication

In loving memory of,

My true inspiration, Dr. Mohammad Kamkar

A caring dad

*Whose immense passion for science
Came to its final rest during the course of this study.*

For,

My dear mom Safieh Kamkar

Who dedicated her life to us.

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Abbreviations

ATP	Adenosine tri-phosphate
bp	Base pair
CPN	Chaperonin
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
eGFP	Enhanced green fluorescent protein
Ef1- α	Elongation factor 1 alpha
Fluc	Firefly luciferase
FP	Fluorescent protein
GFP	Green fluorescent protein
hpf	Hour(s) post fertilization
HS	Heat shock
HSE	Heat shock element
HSF	Heat shock factor
Hsp	Heat shock protein
HSR	Heat shock response
IGR	Intergenic region
IRES	Internal ribosomal entry site
kb	Kilobase

kDa	Kilodalton
LU	Light unit
Luc	Luciferase
mRNA	Messenger ribonucleic acid
PCR	polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RLU	Relative light unit
Rluc	<i>Renilla</i> luciferase
RNA	Ribonucleic acid
RT-PCR	Real-time polymerase chain reaction
SP1	Zinc finger containing DNA binding transcription factor
TIS	Transcription initiation site
UTR	Untranslated region
YSL	Yolk syncytial layer

Introduction

1.1 Protein folding

“The native conformation [of a protein] is determined by the totality of interatomic interactions and hence by the amino acid sequence, in a given environment.” The preceding is the statement presented by Anfinsen in early 70s. It earned him the chemistry Nobel Prize in 1972 for showing that proteins fold autonomously without requiring any additional factors or energy input (Anfinsen, 1973). Protein folding is a process in which linear amino-acid sequences gain their unique, well-defined, tridimensional conformation to form a functional protein (Hartl, 1996). While Anfinsen had shown the spontaneous formation of native proteins is determined by global free energy minimum, the question is then why do cells require chaperones? In fact, the thermodynamics rule works for small single-domained globular proteins, only in low concentrations (Fink, 1999, Hartl and Hayer-Hartl, 2002). Such small proteins bury their hydrophobic residues within milliseconds of folding initiation (Dobson and Karplus, 1999). Longer exposure of hydrophobic residues, which are natively buried inside the core, makes the protein prone to interacting with other similar proteins or cellular macromolecules resulting in aggregation (Agashe and Hartl, 2000). For large proteins with multiple domains, successful folding is only possible at very low protein concentration with long incubation time at lower temperatures. Both in cells and test tube there is always competition between folding and aggregation and the latter is more amenable *in vivo*. At physiological conditions, requiring higher temperature the hydrophobic effect is stronger compared to *in vitro*. Moreover, protein folding inside the cells happens in a crowded (Fulton, 1982, Goodsell, 1991) viscous (Elowitz et al., 1999) environment with low or no free water (Fink, 1999). The "crowd effect" resulting from the high density of proteins, nucleic acids and other macromolecules (Young et al., 2004) makes them even

more hydrophobic (Fink, 1999). Since protein concentration and temperature are not controllable *in vivo*, aggregation would be unavoidable in cells (Gragerov et al., 1992). Assuming the presence of exact same conditions in both states, *in vitro* deals with fully synthesized chain as opposed to *in vivo*. Protein synthesis is an obligatory vectorial process (N-terminal to C-terminal). As a result, protein folding and biosynthesis are coupled. Therefore, the whole peptide chain does not exist at once and it becomes available sequentially. In the absence of chaperones, folding will happen simultaneous to biosynthesis resulting in misfolded and/or intermediate imperfect structures. This type of folding will expose the hydrophobic residues and cause aggregation (Agashe and Hartl, 2000).

Considering the amount of energy a cell has invested just for protein biosynthesis, cells have developed the chaperone machinery to fold a protein properly into a functional form (Walter and Buchner, 2002).

1.2 Chaperone machinery

For a nascent polypeptide chain, the first contact with the cellular environment is very critical. If it were not protected at this stage, the whole outcome would be altered (Feldman and Frydman, 2000). As a consequence, cells have developed a specific protein folding machinery (Ellis, 1987; Rothman, 1989; Gething and Sambrook, 1992; Hendrick and Hartl, 1993). This essential machinery is present in all cells and organisms. Molecular chaperones (heat shock proteins) are important elements in this machinery. Chaperones are specialized proteins that assist the folding of the newly synthesized and denatured proteins (Bukau and Horwich, 1998) by minimizing incorrect interactions between molecules (Agashe and Hartl, 2000). In most cases, this procedure takes place in an energy/ATP dependent manner (Bukau and Horwich, 1998). Laskey et al., (1978) originally used the term molecular chaperone to describe the function of nucleoplasmin. Nucleoplasmin is an acidic nuclear

protein that assists chromatin assembly by inhibiting improper interactions between DNA and histones. Ellis (1987) generalized this term to diverse proteins with the role to facilitate the folding of other proteins. Later, Hendrick and Hartl (1993) further developed this concept.

A newly synthesized protein chain needs to be protected against interactions at least at four stages (Houry, 2001)

- 1) as a nascent chain still attached to the ribosome
- 2) at the extension level while it is released from ribosome
- 3) as a folding intermediate with developed secondary structure
- 4) as a misfolded protein following stress

Chaperones prevent the formation of nonnative conformations due to irreversible aggregation. They also facilitate the folding and assembly (Hartl, 1996, Fink, 1999) of newly synthesized proteins. In addition, they allow misfolded structures to unfold and refold. Newly synthesized proteins also need to be conserved in unfolded conformation to facilitate their translocation across membrane into the organelles. Chaperones also have roles in controlling the folding of bio-regulatory proteins, disassembling multimeric protein structures, and proteolytic degradation or disposal of misfolded proteins (Hendrick and Hartl, 1993). This mechanism was initially discovered observing the sudden increase in chaperones following heat shock. However, it is not only related to elevated temperature and occurs in various stressful conditions. Exposure of hydrophobic surfaces as a result of denaturation makes the protein available to heat shock proteins. Noncovalent interactions between the hydrophobic domains and chaperones prevent multimeric aggregation between proteins. This allows the next healing steps to follow (Bukau and Horwich, 1998).

1.3 Hsp10 and Hsp60 proteins

Chaperones are found in all kingdoms of life. There are at least 5 main families and more than 20 different subfamilies of evolutionary conserved chaperones known to date (Agashe and Hartl, 2000). They are classified according to their molecular weights (Borges and Ramos, 2005). Table 1-1 summarizes the members of heat shock protein family (Kiang, 2004).

60-kDa chaperonin, Hsp60 (MW=57kDa) and 10-kDa co-chaperonin (MW=10 kDa), Hsp10 are present ubiquitously in generally the mitochondria and chloroplasts of all eukaryotic cells. They assist the proper folding of newly synthesized and newly translocated proteins in mitochondria and chloroplast (Bukau and Horwich, 1998). Chaperonins are also capable of partially unfolding proteins trapped in misfolded structures to help them refold into their innate conformation (Walter and Buchner, 2002). Besides the general presence of Hsp10 and Hsp60 proteins in organelles with endosymbiotic origin, Hsp10 is found in the serum of pregnant mammals as “early pregnancy factor” (Morton, 1998). It is also overexpressed during carcinogenesis of large bowel and uterine exocervix (Cappello et al., 2003). Hsp60 is also present at discrete extramitochondrial sites, including foci on ER, on the cell surface, and in unidentified vesicles, cytoplasmic granules in pituitary growth hormone secretory, and granules and pancreatic zymogen granules. These extramitochondrial localizations are responsible for Hsp60 immunoreactivity (Cechetto et al., 2000)

Chaperonins were originally discovered in eubacteria as GroEL and GroES (Chen et al., 1994; Roseman et al., 1996) which are analogs of Hsp60 and Hsp10, respectively. They are also known as Cpn60 and Cpn10 (Rothman, 1989). Bacterial chaperonin GroEL and its co-chaperonin GroES are the best known among those of all different species. For a comprehensive review on GroEL please see (Fenton and Horwich, 1997).

Table 1-1. HSPs: locations and function

HSP	Members	Locations	Functions
HSP110	HSP110/104	Cytosol/nucleus	Cytoprotection
HSP90	GRP94	Endoplasmic reticulum	Chaperone
	HSP90 α	Cytosol/nucleus	Endogenous steroid receptor antagonist
	HSP90 β	Cytosol/nucleus	Cytoprotection
HSP70	GRP78 (Bip)	Endoplasmic reticulum	Chaperone
	HSP75 (GRP75)	Mitochondria	Chaperone
	HSP73	Cytosol/nucleus	Chaperone
	HSP72	Cytosol/nucleus	Cytoprotection
HSP60	HSP60	Mitochondria	Cohort to HSP-75
	HSP56	Cytosol	Binds to steroid receptors and FK506
HSP40	HSP47	Endoplasmic reticulum	Collagen chaperone
HSP20	HSP27	Cytosol/nucleus	Chaperone; cytoprotection; Lysine- and glutamine-donor
	HSP24	Cytosol/nucleus	Chaperone
	HSP20	Cytosol/nucleus	Chaperone; Lysine- and glutamine-donor
	HSPB8	Cytosol	Lysine- and glutamine-donor
	HSPB2	Cytosol	Glutamine-donor
	HSPB3	Cytosol	---
	α B-crysatlline	Cytosol	Lysine-donor
HSP10	HSP10	Mitochondria	Cohort to HSP-60

Some members of the eukaryotic Hsp family are only induced by stressful conditions although some others are synthesized constitutively at a basic level (Gragerov et al., 1992). In heat shock conditions when proteins denature, the constitutively produced proteins are not sufficient and an excess of these proteins are required (Pelham, 1986). Hsp10 and Hsp60 are both constitutive and stress induced heat-shock chaperones.

Chaperones adopt various shapes and stoichiometries. Some such as Hsp70 are present as single monomers for each substrate molecule. This ratio is 14 to 1 and 7 to 1 for Hsp60 and Hsp10, correspondingly. Chaperonin (Hsp60) and co-chaperonin (Hsp10) are present in a collective multimeric ring forming a functional chaperone. Hsp60 forms a double heptameric back-to-back ring (Hendrix, 1979) and Hsp10 is attached to it as a single heptameric ring (Chandrasekhar et al., 1986). The functional part of a chaperonin is its central cavity formed by the rings (Ellis, 1996; Fenton and Horwich, 1997) where they facilitate protein folding away from the cytoplasmic hydrophilic environment (Braig et al., 1993). Nonetheless, chaperonin is much more than a passive box and is actively involved in facilitating protein folding (Horwich et al., 2007)

Interactions between chaperonin, co-chaperonin and ATP not only cause global change of the shape but also modify the character of the central cavity (from a hydrophilic environment to a hydrophobic one). Binding of ATP to the chaperonin ring causes rapid, forced unfolding of the substrate protein (Lin et al., 2008). This enlarges the central cavity by two-fold and closes the open side by a dome-shaped co-chaperonin. Therefore, the nonnative protein will be encapsulated inside the closed cavity (Bukau and Horwich, 1998). Folding will happen in the isolated environment of the central cavity known as “Anfinsen cage” as this process is comparable to the folding of a single protein in a test tube (Ellis, 1994a and b; Ellis, 1996; Walter and Buchner 2002) (Figure 1)

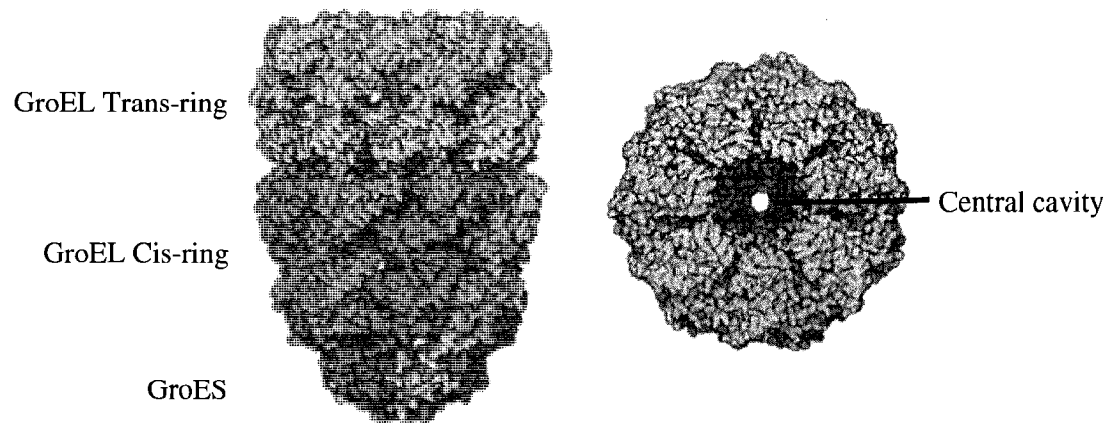


Figure 1-1. GroEL/ GroES chaperonin complexes: a) cis-ring proximal to GroES, trans-ring Distal from GroES. b) From top showing central cavity (Walter and Buchner, 2002)

The mechanism of chaperonin activity is as follows: (figure 2) (Mayhew et al., 1996, Weissman et al., 1996, Bukau and Horwich, 1998, Hartl and Hayer-Hartl, 2002)

- 1) Exposed hydrophobic residues of the nonnative substrate interact with multiple subunits of the hydrophobic cavity of GroEL. This is how it discriminates between the substrate and native protein (Hunt et al., 1996).
- 2) A GroES heptameric ring binds to the GroEL *cis*-ring following 7ATP molecules
- 3) The size of the cavity doubles and the hydrophobic residues of GroEL subunits hide, making the cavity hydrophilic (change in shape and nature)
- 4) Consequently, the substrate displaces into the central cavity
- 5) ATP hydrolysis and folding takes place (within 10-15 msec)
- 6) 7ATP molecules bind to the *trans*-ring of GroEL
- 7) An allosteric signal is sent to the *cis*-ring
- 8) GroES is released
- 9) The opening is unblocked and the folded or partially-folded protein leaves the compartment
- 10) Multiple cycles might be needed to complete this process as the protein reaches its final unique three-dimensional structure.

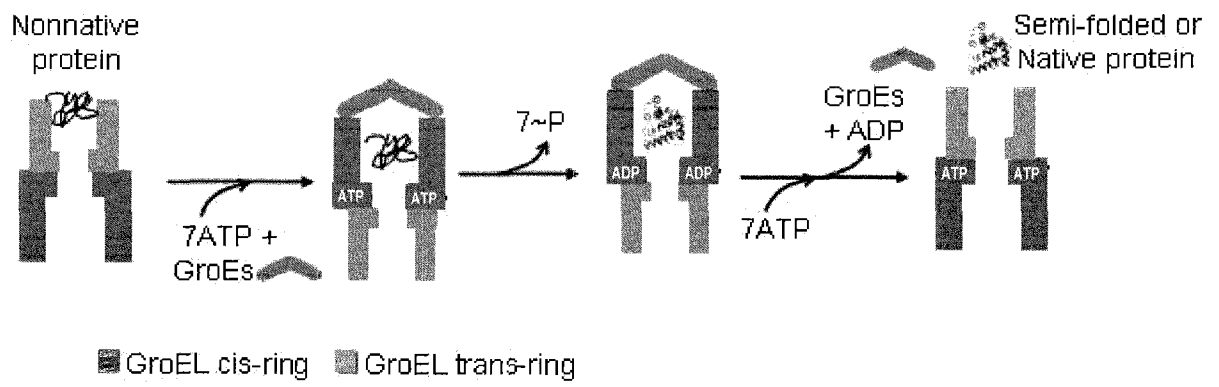


Figure 1-2. Mechanism of protein folding in GroEL: For description, please see the text.

Each subunit of Hsp60 contains three domains: 1, an equatorial domain, which is an ATP binding domain, located in the junction point of the two rings, 2, an intermediate domain that transfers the signals (Ellis 2001; Richardson et al., 1998; Roseman et al., 1996), and 3, an apical domain with hydrophobic amino acid residues where the nonnative proteins bind. The latter could accommodate secondary structures like α -helices and β -hairpins (Chatellier et al., 1999; Chen and Sigler, 1999).

Molecular chaperones induce conformational changes in their target proteins. Some chaperones are highly specific with exclusive targets or very limited number of target proteins like PapD, which only interacts with procollagen in the ER as substrate (Kuehn et al., 1993) while others are more general, folding diverse proteins. Hsp10 and Hsp60 assist in the folding of a large variety of proteins (Bukau and Horwich, 1998, Hartl and Hayer-Hartl, 2002, Ellis and Hartl, 1996).

The chaperonin family includes two sub-groups: group I are GroE-like proteins present in eubacteria and in the organelles with endosymbiotic origin (mitochondria, chloroplast) (Fink, 1999). Group II is the TriC (CTT or TCP) family located in the cytosol of eukaryotic cells. It is known as thermosome in archaeobacteria (Kubota et al., 1994). GroEL type chaperonins need co-chaperonins and are not functional individually. Group II chaperonins consist of 8-9 hetero-oligomeric subunits and are co-chaperone-independent (Kubota et al., 1994), with a built-in lid instead (Klumpp, 1997, Ditzel, 1998, Llorca, 1999).

1.4 Heat Shock Response

The heat shock response is a conserved reaction of cells and organisms to elevated temperatures and other sorts of stress. It was first discovered in 1962 when Ritossa observed the special pattern of the puffs in *Drosophila* salivary gland chromosomes, in response to heat shock. Almost all heat shock response inducers share common pathways, triggered by imbalance in protein homeostasis

(Morimoto, 1998). Accumulation of the improperly folded proteins causes the molecular responses and dramatic changes in gene expression in order to activate the expression of heat-shock genes, while suppressing the transcription and translation of most of the other genes. This prevents the introduction of new nascent proteins into the system misfolding in the suboptimal conditions. Therefore, the accumulation of nonnative proteins is prevented (Morimoto 1998, Wu, 1995). As a result, cells and organisms will be protected from severe damage and it allows maintenance of normal cellular and physiological activities, which leads to a higher level of thermotolerance.

1.5 Regulation of heat shock response

Heat shock proteins are a highly regulated family of proteins (Satyal et al., 1998). The response to stress is mediated by heat shock factor (HSF). HSF is a transcriptional activator ubiquitously expressed in all organisms (Lis and Wu, 1993; Morimoto, 1993; Morimoto et al., 1994; Wu et al., 1994; Wu, 1995). It works as a *trans*-acting factor binding to heat shock element (HSE) which is a highly conserved *cis*-acting element(s) located within the upstream promoter region of *hsp* genes (Pelham, 1982, Amin, 1988, Wu, 1990).

The regulation of heat-shock genes happens at the transcriptional level when heat-shock factors (HSF) activate the transcription of *hsp* genes by binding to its heat shock element (HSE). In normal conditions, HSF is in its inert monomeric state, which is the result of a limitation in forming active homotrimers due to intra-molecular interactions between two different domains of the protein. Accumulation of denatured proteins causes conformational changes in HSF and releases the interactions between different domains of each HSF protein. Consequently, the domain responsible for trimerization is free to interact with two other HSFs and form the homotrimer with DNA-binding activity (Lis and Wu, 1993; Morimoto, 1993). Subsequently, the trimerized HSF, which now can act

as a transcriptional factor, binds to HSE located within the heat-shock promoter and activates their transcription, resulting in the heat shock response. The process is shown in Figure 1-3 (Morimoto, 1998).

1.5.1 Heat Shock Element

HSE is the stress responsive element within the promoter, which is essential for heat shock inducibility. HSE is composed of adjacent tandem arrays (Amin et al., 1988; Fernandes et al., 1994). Each array contains three repeats of the 5'-nGAAn-3' motif. All *hsp* gene promoters contain at least one array of heat shock element (reviewed by Wu, 1995). HSE forms an inverted highly conserved palindromic motif present within the promoter region of all of the *hsp* genes. Arrays of HSE provide binding sites for HSF trimers (Wu, 1995).

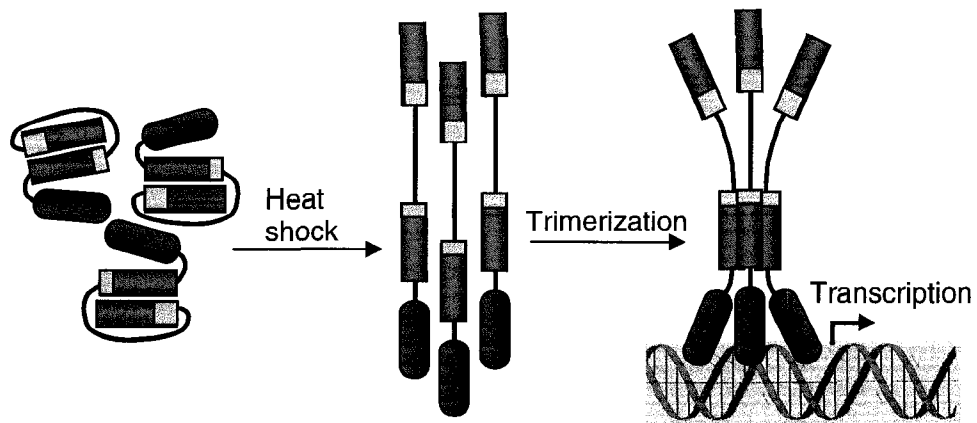


Figure 1-3. Regulation of *hsp* genes at the transcriptional level leading to adaptive response; Following heat shock, inactive heat shock factors (HSF) trimerize to form active transcription factors that could bind on heat shock element (HSE) within the promoter of *hsp* genes.

1.5.2 Regulation of Heat Shock factor 1

Since the heat shock response is a rapid, inducible response, it requires immediate upregulation of the *hsp* gene. In order to accommodate this need, HSF is constitutively expressed in the cells and is present in the inert form ready to be activated upon stress. Damaged, denatured, aggregated, or misfolded proteins are the inducers of HSF1 activity (Kampinga et al., 1995, Kampinga, 1995, Ananthan et al., 1986).

Inactivation of HSF is also necessary when it is no longer required (Tonkiss and Calderwood, 2005). HSF includes both transactivating and negative regulatory domains. These domains also have roles in repressing HSF itself (Shi et al., 1995), in an unclear manner (Larson et al., 1988; Rabindran et al., 1991; Sarge et al., 1991, 93; Baler et al., 1993; Westwood and Wu, 1993, Zuo et al., 1944). Chaperons such as Hsp70, Hdj-1/Hsp40, and Hsp90 have been shown to have a role in maintaining HSF in its inert state to repress it in normal conditions through an unknown mechanism (Mosser et al., 1993, Rabindran et al., 1994, Shi et al., 1998, Ali et al., 1998, Zuo et al., 1998). Sequestration of Hsp90 in response to heat shock, trimerizes and activates the HSFs, leading to heat shock response (Zuo et al., 1998). Hsp90 molecules sequester inactive HSF monomers. As a result, activation of HSF is prevented under normal conditions. This would be consistent with the hypothesis that HSP90 may actually be a “molecular buffer” that maintains the general balance and fidelity of cellular signaling networks (Shamovsky and Nudler, 2008). On the contrary, however, it appears that some HSF molecules exist in a complex with HSP70/HSP40. Such complexes may only act as intermediates during the formation of HSP90-HSF monomer complex or during trimerization following heat shock. The interaction of HSF with HSP70/HSP40 per se is unlikely to be the major regulatory mechanism (Shamovsky and Nudler, 2008). Furthermore, HSF is subjected to regulation by phosphorylation (Brunet et al., 2002).

Various numbers of heat shock factors have been found in different organisms (Sorger and Pelham, 1988; Wiederrecht et al., 1988; Clos et al., 1990; Rabindran et al., 1991; Schuetz et al., 1991; Nakai et al., 1997; Nakai and Morimoto, 1993; Scharf et al., 1990; Sarge et al., 1991). The stress response has been extensively studied in mammals, especially in human cell lines. Human has a few different HSFs including HSF1, HSF2, and HSF3. HSF1 is the best known and appears to play the key role. HSF1 is mainly regulated at the post transcriptional level through oligomerization, nuclear translocation and hyperphosphorylation play regulatory roles as well (Baler et al., 1993; Cotto et al., 1996).

There are some studies investigating heat shock proteins in fish (reviewed by Iwama et al., 1998). HSF1 has three different isoforms in zebrafish, zHSF1a, zHSF1b, and zHSF1c (Rabergh et al., 2000). Their role is not clearly understood. The zHSF1 isoform ratio markedly changes in response to heat shock (Airaksinen et al., 2003).

HSF1 is one of the best characterized factors stimulating transcription initiation, but the process by which it acts is more complex and might require the assembly of many different factors at the promoter (Trinklein et al., 2004). While, the activation of HSF is necessary for the heat shock response (Morimoto, 1998), it seems that more factors are required (Shamovsky and Gershon 2004). Only after binding of HSF1 to HSE, downstream regulatory factors are recruited. Secondary factors bind to regions of the *hsp* promoter that are located downstream of the HSE and result in higher mRNA transcription (Boellmann et al., 2004; Ni et al., 2004). The activation of RNA polymerase also requires a complex elongation factor that binds to it (Hahn, 2004). It has been observed that HSF also binds to the promoter of genes that are not induced by stress. HSF might have a repressing role for at least some of these genes (Trienkein et al., 2004).

1.6 Genomic structure of *hsp10* and *hsp60* genes

Most studies on chaperonins have been performed at the protein level. Relatively little is known about the genomic structure and organization of the genes coding for Hsp10 and Hsp60 proteins. Hsp10 and Hsp60 are encoded by nuclear DNA (in zebrafish they are located on chromosome 9). They are synthesized as larger precursor forms containing an N-terminal targeting sequence which is cleaved during maturation to the mature form in the mitochondrial matrix. One of the initial functional studies of human *hsp60* gene regulation and expression as well as its molecular structure was carried out in 1996 by Pochon and Mach. Their research led to the first successful cloning of the human *hsp60* gene. They reported the extensive genomic complexity of *hsp60* gene including multiple introns and exons, even though the gene was thought to be intronless (Venner et al., 1990) until then. This is probably one of the reasons causing failures in the earlier attempt to identify the *hsp60* gene. Another source of difficulty was the presence of *hsp60* pseudogenes in the genome of different organisms. The existence of multiple pseudogenes has been reported for several other *hsp* genes as well (Hickey et al., 1986 a and b, Walter et al., 1989, Sorger and Pelham, 1987).

The genomic organization of *hsp10* and *hsp60* has been studied in few organisms including prokaryotes, flies, human, rat, flounder, zebrafish, and yeasts. The most remarkable feature of the *hsp10* and *hsp60* genes is that they contain a bidirectional promoter. There are many examples of bidirectional structures in different animals, which will be discussed later. In such a genomic organization, genes are located on opposite strands and coding regions are transcribed in reverse directions; also, the two genes share identical *cis*-acting elements. The promoter of *hsp10/60* genes contains one array of heat shock element while other promoters of heat shock proteins have multiple heat shock elements. One 3-tandem HSE with almost perfect match is enough for heat shock inducibility (Fischer-Kierzkowska et al., 2003). Human and rat have a 4-tandem (5'-nTTCngGAAn

gTTCnGAA_n-3') HSE, whereas it is a 3-tandem (5'-nGAA_nTTC_nGAA_n-3') array in flounder and zebrafish. In different organisms, all of the studied bidirectional *hsp10/60* promoters are TATA-less, contrasting with other *hsp* promoters (Bienz and Pelham, 1986). The absence of a TATA box has been shown to facilitate bidirectional transcription (Grichnik et al., 1998). TATA-less promoters are also often found in house-keeping genes (Liao et al., 1994). Additional putative *cis*-acting factors found in different bidirectional *hsp10/60* promoters include CCAAT box and GC box located downstream of the HSE (Hansen et al., 2003, Ryan et al., 1997, Nam et al., 2006). The GC box is the core recognition site for transcription factor SP1, which has been shown to play a role in activating TATA-less promoters (Pugh and Tjian 1990). Putative SP1 sites have been found in the human, rat, and flounder *hsp10/60* promoters (two, three, and one copy respectively) and a putative CCAAT box has been found in the flounder and rat promoters (Hansen et al., 2003, Ryan et al., 1997, Nam et al., 2006). Mutations in the putative SP1 site of flounder had no significant effect on the function of the promoter.

Studies performed with a fragment of the rat *hsp10/60* genes, containing the intergenic region covering the 5'UTRs of *hsp10* and *hsp60* genes, showed that transcription on the *hsp60* orientation is slightly higher than on the *hsp10* orientation. However, the *hsp10* orientation was more inducible. Heat shock for 2 hours at 43°C resulted in 5.2 and 3.4 fold induction in *hsp10* and *hsp60* transcription, respectively (Ryan et al., 1997). The intergenic region and UTRs in human cover a 656-bp GC-rich sequence. Expression in the *hsp60* direction is twice as much as in the *hsp10* direction under normal conditions. Under heat shock, in human a 12-fold increase was observed with 2-fold difference preserved (Hansen et al., 2003). The potential promoter region of flounder *hsp10/60* is 544-bp long and the same levels of expression have been measured for both orientations (Nam et al., 2006).

In the human *hsp60* gene, three different alternative forms have been detected in the splicing of exon-1 to exon-2. About 98% of splicing shows the same pattern, and the other two represent only 2-3% of cases (Hansen et al., 2003). Human *hsp10* (*HspD1*) and *hsp60* (*HspE1*) cDNA exon-intron configurations are the same as zebrafish. Mouse *hsp60* shows different spliced transcripts. *Drosophila* has a non-coding first exon (Perezgasga et al., 1999) and spliced products show two forms of exon-1 (Hansen et al., 2003).

1.7 The *hsp10/60* promoter in zebrafish

Bioinformatics analyses have shown (Martin et al., 2002) that the zebrafish *hsp10* and *hsp60* genes are arranged in a head-to-head manner. The proteins coded in zebrafish are highly similar to those of mouse, rat, human, and other eukaryotes. According to sequencing results performed by Martin et al., the two genes are 860-bp apart and are transcribed on opposite strands in two divergent orientations. A single 3-tandem HSE is located within this region. The first exon of *hsp10* contains a 44-bp 5'UTR. Translation does not start in *hsp60* until the beginning of the second exon and the whole 63-bp first exon forms the 5'UTR, which is separated from the second exon by a 2818-bp intron (figure 1-4). Analysis of *hsp10* cDNA clones suggested the existence of only one transcriptional start site for *hsp10* (Martin et al., 2002). Similar to results reported in rat (Ryan et al., 1995), evidence obtained through northern blot analysis suggested the existence of alternative transcriptional start sites in *hsp10* that result in the two different *hsp10* transcripts (Martin et al., 2001). Presence of two different *hsp10* transcripts could also be due to alternative splicing.

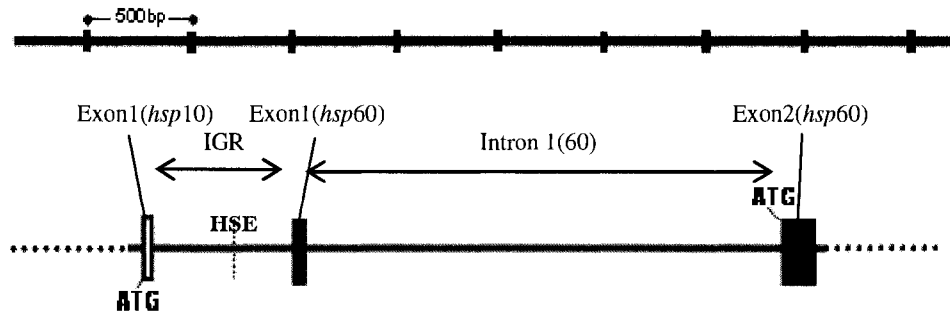


Figure 1-4. Schematic maps of a region of the *hsp10/60* locus in zebrafish: The maps contain a DNA segment that illustrates the intergenic region (IGR), the first exon (the open boxes) of *hsp10* and the first and second exons of *hsp60* (filled boxes) in zebrafish. The thick line represents introns and intergenic region. The intergenic region (IGR) is located between the first exon of *hsp10* and the first exon of *hsp60*. The position of the heat shock element (dashed vertical line) and the relative orientation and position of the ATG start codons for both transcripts (on the opposite strands) are shown. For simplicity, the two strands of DNA are shown as one line. The scale is shown at the top.

1.8 Coordinated regulation

Hsp10 and Hsp60 are essential proteins in different organisms; in addition, they play roles during development. Presence of *hsp10* and *hsp60* mRNA in the embryos prior to mid-blastula transition stage when gene expression initiates, confirms their importance for zebrafish embryos (Martin et al., 2002). It has also been reported that the complete absence of GroEL and GroES is lethal in *E. coli* (Fayet et al., 1989). The presence of highly conserved homologues of GroEL/ES in the mitochondria of eukaryotic cells suggests the same degree of importance of Hsp10 and Hsp60 proteins in these endosymbiotic essential organelles. Not only the existence of these proteins but also their cooperation is vital for the cells. The protein level shows the same pattern in different organisms which is 2 molecules of Hsp60 per each molecule of Hsp10. According to western blot data (Martin et al., 2002) the zebrafish Hsp10 and Hsp60 monomers are available within the cells in a 2:1 ratio. This stoichiometric ratio is necessary for cooperation of chaperonin machinery, as the functional barrel shaped compartment is composed of 14 subunits of Hsp60 and 7 subunits of Hsp10. The precise stoichiometry of this arrangement suggests that their synthesis is regulated by the cell. In *E. coli*, the molar equivalents of GroEL and GroES are maintained through a polycistronic operon under the control of a common promoter (Tilly et al., 1981). Bacterial operons often consist of genes that are functionally related, for instance being part of the same metabolic pathway. However some polycistronic transcripts are reported in some eukaryotes (Agabian 1990; Nanbru et al., 2002; Blumenthal, 2004), operons are mainly found in prokaryotes. A more abundant similar concept in eukaryotes is bidirectional promoters. Therefore, co-expression of adjacent genes with closely linked loci can be attributed to a bidirectional promoter that exists between the two genes.

Organization of genes in head-to-head configuration, sharing the same regulatory elements constitutes an efficient mechanism for coordinated regulation. As a substitute to the genomic

organization found in *E.coli*, bidirectional promoters have been described for the *hsp10* and *hsp60* genes in rat (Ryan et al., 1997) human (Hansen et al., 2003), and flounder (Nam et al., 2006). This organization has been found in all investigated vertebrates (Martin et al., 2002). However, this is not a requirement, as yeast and *Drosophila hsp10* and *hsp60* genes are not organized in a head-to-head manner (Perezgasga et al., 1999; Garrels, 1995). These separate genes still maintain a degree of coordinate regulation by containing same *cis*-acting elements in their separate promoters upon which only identical transcription factors can bind and activate transcription. Therefore, stoichiometric ratios can be supported (Britten and Davidson, 1969). The latter is not exclusive to *hsp10* and *hsp60* genes, it has been seen in many physically unlinked eukaryotic genes that require coordinate regulation (Ryan et al., 1997).

1.9 Bidirectional promoters

It seems that bidirectional promoters in eukaryotes take place in bacterial operons to assure effective gene co-regulation. In prokaryotes, operons also help to minimize the length of the genes. It helps them to cope with their small dimensions and the limited size of their genome (Adachi and Lieber, 2002) which does not seem to be necessary in vertebrates. There are two possible explanations for the presence of bidirectional transcription. 1) It is an ancestral characteristic that survived evolution, 2) it occurs in genes that are functionally related (Zhang et al., 2003). Functional relationships include: maintaining stoichiometric ratios such as histone genes (Ahn and Gruen, 1999), co-regulating genes that participate in the same biological pathways (Momota et al., 1998), controlling genes required at specific time points such as genes involved in the cell cycle (Guarguaglini et al., 1997), or genes with coordinated response to inducing signals such as heat (Ryan et al., 1997). In fact, the bidirectional *hsp10/60* promoter provides both maintenance of stoichiometric ratio and coordinated response (Hansen et al., 2003). Not all of the genes in close head-to-head arrangements have a known

functional relationship but in at least some cases, their expressions are co-regulated (Platzer et al., 1997; Shimada et al., 1989).

Although vertebrate genes are more dispersed within their genome compared to those of lower organisms, genes are sometimes arranged into clusters (Lander et al., 2001). In 2002, Adachi and Lieber identified a major class of gene pairs arranged in a head-to-head manner located on opposite strands in which a sequence less than 1000 base pairs separates their transcription start sites. This arrangement of gene pairs was previously named "bidirectional". However, such extremely close loci were assumed exceptions; recent studies revealed that bidirectional promoters are not rare. A number of closely linked loci, which are arranged in a bidirectionally divergent fashion, have been reported within the human genome (Adachi and Lieber, 2002). A genome wide analysis has shown that this arrangement would represent around 10% of the human genome (Trinklein et al., 2004). Bidirectional promoters in vertebrates are part of a growing list and some of those reported in human and other animals are as follows: *TLX1/HOX11*, *SIRT3/PSMD13*, *BALI/BBAP*, *ACACA/TADA2L*, *FEN1/C11orf10*, *BRCA1/NBR2*, *DNA-PKcs/MCM4*, *ATM/NPAT*, *DHFR/MSH3*, *G6PD/NEMO*, and *Ku86(KARP-1)/TERP*, (Greene et al., 2007, Bellizzi et al., 2007, Juszczynski et al., 2006, Travers et al., 2004, Adachi and Lieber, 2002; Braastad et al., 2002; Connelly et al., 1998, Galgoczy et al., 2001; Platzer et al., 1997; Shimada et al., 1989, Xu et al., 1997).

Bidirectional promoters have variable properties. Nonetheless, the flanking genes are located on opposite strands and are all less than 1-kb apart. In human 77 percent of bidirectional promoters, are located within a CpG island; this number is only 38 percent for unidirectional promoters. CpG islands are often found in housekeeping genes (Gardiner-Garden and Frommer, 1987). Since CpG islands consist of cytosine and guanine arrays they could act in a bidirectional manner. Sharing same CpG islands in both promoter orientations could assist coordinated expression. Bidirectional promoters are

often GC rich as the median GC content of human bidirectional promoters is 66 percent versus 53 percent in unidirectional promoters. Another prevalent feature in bidirectional promoter is that many of them seem to lack TATA boxes in both orientations. This might facilitate bidirectionality, since there are no elements to specify a unique transcriptional orientation (Burbelo et al., 1988). However, in some cases, both directions of the promoter include TATA boxes (Hentschel and Birnstiel, 1981).

Some studies have described the presence of an enhancer within the noncoding first intron of one of the two genes flanking a bidirectional promoter (Killen et al., 1988; Oshima et al., 1990). These promoters require the enhancer elements located within the first intron for the optimal activity of one of the two genes. For instance, bidirectional transcription of human collagen gene requires sequences present in the first intron (Pollner et al., 1990).

Although 90 percent of bidirectional promoters show activity in both directions at all times, 10 percent of them are active in just one direction under some circumstances. Trinklein et al., (2003) reported that a bidirectional promoter could show unidirectional activity in the forward direction in one cell line, and unidirectional activity in the opposite direction in a different cell line. Most bidirectional promoters share at least some regulatory elements but in a competitive manner. Deleting some promoter sequences, especially from the centre of the promoter, truncates transcriptional activity in both directions indicating the importance of these elements in the transcriptional activity of both directions since both flanking genes use them in common (Trinklein et al., 2004).

1.10 Zebrafish as a model organism

Zebrafish (*Danio rerio*) is a small, freshwater, tropical fish native to India and Southeast Asia (Eisen, 1996). It represents teleosts or the class of bony fish. Teleosts form half of the existing vertebrates. This makes zebrafish a good model to represent vertebrates. The advances obtained in applicable

methods in zebrafish biology can help in setting up experimental approaches that could be used in other members of the large fish species. In recent years, zebrafish became a popular model system not only for development and genetics studies (Driever et al., 1994) but also for a number of other areas such as toxicological studies and biotechnology research beside its classical usage as model system in fisheries (Lele et al., 1996).

Embryos and larvae of zebrafish offer a number of distinctive characteristics that make them well suited for biological studies. They are small and easy to grow. Their assets include simple husbandry requirements, large number of spawned eggs, which are available daily in all seasons, complete external fertilization and development, and short generation time (comparable to mouse). Zebrafish is reproductive within three months of fertilization. The body axis patterning and segmentation of the embryos complete in the first day of development and primary organogenesis happens in the second day. The entire gastrulation stage completes ten hours after fertilization and the heart is functional in the first day. Organogenesis is complete within the first five days of their life and most of the organs become functional within this period.

Zebrafish live 50% longer than commonly used mouse strains (Gerhard and Cheng, 2002). They have the majority of organs that are found in mammals. The only exceptions are a four-chambered heart, lungs, prostate, and mammary glands (Rubinstein, 2003). The small size and external development of embryos and fry (1-5 mm) makes assays in 96-well plates possible (Goldsmith, 2004). Adequate chromosomal integration frequency for transgenic production and good germ line transmission make zebrafish an ideal model organism especially for producing transgenic animals and even gives them some advantages over more established models such as mouse. Transparent eggs make microinjection by hand-held glass needles possible. Furthermore, optically clear embryos in combination with the use of fluorescent reporter transgenes can be used to study living tissues,

visualizing changes in the gene expression and developmental events, as well as detailed morphological movement studies (Udvardia and Linney, 2003). This allows monitoring the expression of reporter genes in whole mount preparations and *in vivo*. It also enables non-invasive experimental manipulations combined with live imaging. Studies such as transient expression and misexpression of genes are also possible in zebrafish (Udvardia and Linney, 2003).

Available molecular tools and databases permit detailed molecular studies on zebrafish (Westerfield, 1995; Driever et al., 1994). The gene complexity in zebrafish is comparable to that of vertebrate genes in general. As a result, it is used to establish basic development process in vertebrates (Teh et al., 2005). Large-scale genetic screening has been performed in zebrafish and thousands of mutations have been generated (Rasooly et al., 2003.).

Zebrafish are poikilothermic (ectothermic) organisms and have been used in many heat shock studies; their body temperature is the same as that of their surrounding environment. Poikilothermal organisms are subjected to daily and seasonal temperature fluctuations. Their metabolic rate is closely related to the water temperature, as a result the higher the temperature the greater the metabolism rate will be. However, this does not imply that all temperatures are suitable for fish normal physiological and developmental needs (Schirone and Gross, 1968). Nonetheless, they can grow in a wide range of temperatures, from 27°C to 34°C (Engeszer et al, 2007). One of the key factors that influence animal behavior, physiology, population size, and geographic range is temperature (Brett, 1971). Acclimation temperature has a positive correlation with the temperature in which molecular chaperones become activated (Dietz and Somero, 1992; Buckley and Hofmann, 2002). Usually young fish are more sensitive to temperature than adults are. Nevertheless, in tropical fish like zebrafish the larvae and juveniles are as tolerant to temperature as adults (Rombough, 1997). The latter also makes zebrafish more desirable in heat shock studies.

1.11 *hsp10/60* promoter and transgenic technology

Transgenic animal technology and the ability to introduce functional genes into animals is a powerful, dynamic tool for analyzing complex biological processes. A better understanding of the events involved in the development of a unicellular zygote into a complex organism can be achieved using transgenic animals (Teh et al., 2005). The questions to be addressed by this technique span a wide spectrum from biomedical and biological applications to agricultural utilizations. Transgenic organisms are recognized as good tools in understanding gene expression, regulation, and function. Understanding the developmental and tissue-specific regulation of gene expression can ultimately only be achieved through *in vivo* whole-animal studies and zebrafish have been widely used for this purpose. The first stable line of transgenic zebrafish was generated about two decades ago (Stuart et al., 1988; 1990). It was only recently that this technology was extended to developmental stage- and tissue-specific gene regulation research, as well as studies in cell migration and targeted misexpression (Udvardia and Linney, 2003). This includes transgenic lines with permanent homogenous gene expression and primary fish with transient, mosaic expression of the transgene. When misexpression of a gene has an impact on the vitality or productivity of the fish, producing a stable line is impossible and transient misexpression is a good alternative. Since only a limited number of cells express the transgene, the endogenous genes still function normally in the non-transgenic-type cells. The effect of the gene of interest can then be studied in the transgenic cells.

In order to produce a transgenic fish, the construct should be introduced into the fish zygote. Constructing the transgenic cassette and inserting it into the cells and genome of young embryos are fundamental procedures in producing transgenic lines as well as transient transgenic mosaics.

1. Transgenic cassette: The most important building blocks of a reporter transgene cassette are a set of regulatory elements and a reporter gene. Expression of the reporter gene should be easily detected, demonstrating the activity of the regulatory element(s). Reporter genes are genes with readily measurable or visible phenotype that can be easily distinguished from the background or endogenous proteins. The most commonly used reporter genes are: bacterial sequences such as chloramphenicol acetyltransferase (CAT), β -galactosidase (LacZ), neomycin-resistance (NEO) genes, as well as animal sequences like green fluorescent proteins (GFP), mouse tyrosinase, and chum salmon melanin concentrating hormone (Inoue et al., 1992; Maclean, 1998). Each of these reporters has its own advantages. For instance, fluorescent proteins (FP) especially green fluorescent proteins (GFP) are widely used reporter genes. GFP was isolated from jellyfish, *Aequorea victoria*, and it is visualized under specific wavelengths (excitation= 480/40, emission= 535/50) of light. The most important feature of this reporter gene is the opportunity to visualize the reporter in the live animal without harming it (Maclean, 1998).

Regulatory elements, which are employed to drive the transgenic constructs, might be either from the same species or from a different origin. Regulatory regions of non-teleost species such as the CMV promoter (Maclean, et al., 1996), the SV40 early promoter (Houdebine and Chourrout, 1991), and the human *hsp70* promoter (Seok et al., 2006) have been successfully used and are effective. However, it might be preferable to use sequences with fish origin due to their higher compatibility with the fish transcription machinery (Volckaert et al., 1991). The following, fish-derived, regulatory elements have been used successfully: β -actin (Liu et al., 1990), H3 histone promoter (Hanley et al., 1998), metallothionein upstream regulatory region (Zafarullah et al., 1989), and heat-inducible heat shock protein 70 (Adam et al., 2000).

The unidirectional promoter of the zebrafish *hsp70* gene has been widely used in producing transgenic zebrafish with good results (Halloran et al., 2000; Adam et al., 2000). Using this inducible promoter, misexpression in the fish can be kept under control. Inducibility is a desirable feature in transgenic systems to control the production of the transgene product during the experiments (Teh et al., 2005). The presence of an HSE within the *hsp70* promoter makes it an excellent promoter for zebrafish transgenesis. The *hsp70* promoters from human, mouse, *Xenopus*, tilapia, and zebrafish have been cloned and shown to work efficiently in zebrafish embryos (Adam et al., 2000; Molina et al., 2000; Halloran et al., 2000; Teh et al., 2005).

The *hsp10/60* promoter, whose characterization is the object of this thesis, is predicted to have the same properties. Moreover, it has another valuable potential of being used to express two different transgenes simultaneously due to its bidirectional property. Therefore, the *hsp10/60* promoter might be a good candidate to be utilized in making transgenic fish expressing two independent genes under the control of identical promoter and regulatory elements.

Methods have been previously described that make simultaneous misexpression possible. These methods however useful have some downsides. Here are a few examples:

Using two different cassettes on separate vectors: the expression of the reporter gene might not be a very accurate representative of the gene of interest since the diffusion pattern of the two independent constructs might not be completely the same. A more accurate way is using a single vector that includes both cassettes.

Tagging the gene of interest with the desired reporter: This method allows for the simultaneous expression of the gene of interest and of the reporter. Nevertheless, attaching one protein or

polypeptide to another one might interfere with their folding and their proper tridimensional functional structure, making them non-functional.

Using "internal ribosomal entry site" (IRES): IRES drives translation-initiation of bicistronic mRNA. Coordinate expression of two different genes could be achieved resulting in two separate proteins. However, such artificial eukaryotic operons may exceed the cloning capacity of the vector and bring up problems for the downstream molecular procedures. Furthermore, it has been shown that the function of IRES sequence in fish is too weak to be used reliably (Bai et al., 2007).

2. Introducing DNA: This is the second fundamental step in producing transgenic animals. Conventionally, transgenic lines are often produced by direct transgenesis in which DNA is microinjected into the cells of 1-4 cell-stage embryos. There are also reports of successful trials with electroporation, liposome, and sperm-mediated gene transfer (Inoue et al., 1990; Szelei et al., 1994; Muller et al., 1992). Direct transgenesis depends on the random genomic integration of the transgene for producing lines. It is simple, but results in low frequency of germline transmission. Besides, insertion of concatemers of the transgene into the genome happens commonly. This may cause intrachromosomal recombination that could alter the gene expression profile (Udvardia and Linney, 2003).

The use of transposons (Kawakami et al., 2000; Davidson et al., 2003) and bacteriophage recombination (Branda and Dymecki, 2004) systems are alternative methods that were shown to yield better results in term of germline transmission. Such methods can overcome the problem of position and transgenic copy number (Izsvak and Ivics, 2004; Branda and Dymecki, 2004). One of the approaches that have been used in zebrafish transgenesis is the use of transposable elements. Transposons are repetitive elements capable of moving around to different positions within the genome. They can cause heritable changes in the genome. High transgenesis efficiency could be

achieved by using transposons as vectors. Tol2 (Kawakami et al., 2000 and 2004; Parinov et al 2004) and sleeping beauty (SB) (Davidson et al., 2003; Balciunas et al., 2004) are transposable elements that have been successfully used in generating stable transgenic zebrafish lines. Moreover, they provide reliable transgene expression and reduce concatamerization. Transposons enable precise transposition by assisting the cut of the desired sequence and re-entering it into a new location. These elements could be used as generalized DNA vectors due to the extremely broad range of organisms they could be used in. Moreover, this technique is simple and could be used for large transgenic constructs.

Contrary to the majority of transposon systems that cannot act in a site-specific manner, Cre-lox P system can mediate site-specific recombination. It is usually utilized for producing conditional knockouts or stage- tissue-specific gene targeting to delete, insert, or invert a gene in a particular order. This method requires two different transgenic lines. One line includes the desired promoter controlling the *cre* gene, which encodes Cre recombinase, a 38-KDa protein, isolated from bacteriophage P1. The second transgenic line contains the gene of interest flanked by two Lox P sequences. Lox P is the specific recognition site for the recombinase (Abremski et al., 1983, 1984). The two Lox P sites might be in the same direction or in opposite orientation, leading to deletion or addition of a flanked gene, respectively. When the aim is conditional deletion, the gene should be placed between the two Lox P sites in the same orientation. After crossing with a Cre-expressing animal, the gene will be selectively eliminated wherever the promoter driving the Cre is active. When the aim is the conditional addition of a gene, a stop gene (i.e. a coding sequence containing a stop codon) is flanked by the Lox P sites and the gene of interest is located after the second Lox P site. Conditional presence of Cre results in the deletion of the stop gene and activation of the gene of interest. This scheme has successfully been used in double reporter systems. In such systems, two reporter genes are utilized. One is flanked by the two Lox P sites and the second one is located after

the second site. Expression of the first reporter gene will be detected until the Cre recombinase is expressed under the control of a specific promoter. Recombinase expression will result in the deletion of the first reporter and the second reporter will become detectable in those tissues or cells where Cre is expressed.

The use of activator/effector systems in combination with tissue- or stage- specific promoters constitutes another approach for the production of transgenic zebrafish. It helps decreasing mosaicism and improves the transgenic expression in zebrafish. Gal4-VP16 is one of such systems, which has improved transgenic expression in zebrafish embryos by decreasing mosaicism in comparison to promoter-driven misexpression in conventional expression vectors (Koster et al., 2001). The Gal4-VP16 system is a chimeric transcriptional activator composed of the Gal4-DNA binding domain of yeast and the VP16 transcriptional activation domain of herpes simplex virus (Sadowski et al., 1988). The activator, Gal4-VP16, acts as a *trans*-acting element binding to a promoter that includes an upstream activating sequence (UAS) (Koster et al., 2001). In this system, two cassettes are involved, one cassette includes the promoter of interest (for example a tissue-specific one) regulating *gal4-vp16* gene. The second cassette includes a UAS-including promoter which drives the reporter or structural gene of interest. It would be more efficient if both cassettes were located within the same construct (Teh et al., 2005). Using two effectors makes it possible to conduct simultaneous coexpression. For this purpose, the two genes that need to be coexpressed are cloned downstream of two identical UAS-including promoters. When a single *gal4/vp16* activator is expressed, the produced transcriptional activators turn on both UAS-driven genes simultaneously, resulting in their coexpression. Therefore, Gal4-VP16 efficiently drives the expression of two effector cassettes from the same construct (Koster et al., 2001). This system requires empirical optimization of the concentration of Gal4-VP16 activator constructs adding some complexities.

Another approach that improves the rate of germline transmission is *I-SceI* meganuclease-mediated transgenesis. *I-SceI* is a homing endonuclease with an 18-bp recognition site which is expected to be found only once in 7×10^{10} bp of a random DNA sequence. In this approach, the vector should be designed in such a way to include two meganuclease recognition sites flanking the transgenic sequence that needs to be integrated into the germline. The enzyme digests the injected DNA only at the recognition sites and is predicted to cut the fish genome on average once. As a result, co-injection of *I-SceI*-including plasmids with the meganuclease enzyme facilitates its integration into the genome (Thermes et al., 2002).

1.12 Project outline and hypotheses

This study presents the functional analysis of the regulatory region of the zebrafish chaperonin genes. It characterizes the transcriptional control region of *hsp10* and *hsp60* genes using sequence analysis, reporter assays, and mutagenesis. Reporter genes used in this study include *gfp*, *firefly luciferase*, and *Renilla luciferase* (for dual luciferase assay). This study will not only provide a better understanding of transcriptional regulation of these genes, but also will add more information to the growing database of the identified bidirectional promoters in the vertebrate genomes. Moreover, promoters of heat shock proteins are ideal promoters for transgenic studies in which heat shock (elevated temperature) or stress causes transcriptional activation of the genes cloned downstream of the promoter. This promoter could be particularly useful in simultaneous co-expressions.

With respect to these aims, the questions to be addressed in this project are as follow:

- What are the similarities between the *hsp10/60* promoter of zebrafish and of some other organisms?
- How does the promoter control downstream genes?
- Is *hsp10/60* promoter bidirectional?
- Are there any enhancer elements located nearby?
- What are the differences in the promoter activity in the *hsp10* orientation compared to the *hsp60* orientation?
- How different is the expression profile of the promoter in different cell lines in comparison to the whole zebrafish embryos?

The aims of this project were to:

- Describe the structure of *hsp10/60* promoter

- Characterize the activity and inducibility of the promoter

In silico analyses were performed on the sequence of the genomic regions surrounding the *hsp10* and *hsp60* gene in zebrafish. The transcription start sites of both *hsp10* and *hsp60* had been previously determined. Yet, the functionality of this region was not shown experimentally. Former analyses also showed that the initiation codons are located far from the transcription start site and the genes include relatively large 5'UTRs especially in the case of *hsp60*. Based on this information, the following were hypothesized and predicted:

1) There are some similarities in the genomic organizations and sequences of the zebrafish and other organisms for which the *hsp10/60* sequences are known. In addition, it is expected to find some *cis*-acting elements in this region.

2) The short intergenic region between *hsp10* and *hsp60* genes and the fact that the two genes are located on opposite strands suggests the presence of a bidirectional promoter between these two genes. However, this was not demonstrated experimentally. It was hypothesized that the two genes are under the control of common regulatory elements. When tested with a reporter gene such as *gfp*, the *hsp10/60* promoter should function in both *hsp10* and *hsp60* orientations.

3) The *hsp10/60* promoter, like any other promoter, is likely to be composed of core sequences, as well as interval sequences, which technically do not have a critical major contribution in the promoter activity. If the promoter is bidirectional, at least some *cis*-acting elements should be shared between the two orientations. The alternative is that transcription in the *hsp10* and *hsp60* orientations are controlled by two different sets of regulatory elements. Therefore, deleting some sequences especially from the region located close to the middle of the promoter will inactivate the promoter in both directions and subsequently no reporter gene expression will be detected in either orientation.

The 5' untranslated region (5'UTR) should not have a significant role in transcription and gene expression unless they include some transcriptional regulatory elements, which are not still core promoter elements. Therefore, excluding the UTRs from the sequence should not have profound impact on transcriptional activity.

4) If the 5'UTR contains any enhancer element, however they are not necessary for basic promoter activity deleting them may affect the transcription to some extent. In case of removing this element from the sequence, different pattern might be detected in the expression of reporter genes in the cells, or whole embryos or at least in some parts of embryos. Other possibility was alteration in tissue specific expression due to elimination of tissue-specific regulatory elements.

5) There are reports that some bidirectional promoters do not show the same level of activity in both directions under all conditions. Hence, to be able to detect the differences in the level of *hsp10/60* gene expression, quantitative approaches are necessary. If the promoter shows different strengths it might be noticeable in the intensity of the GFP expression, however using more accurate techniques might be necessary. For example, different promoter activities could be measured qRT-PCR analysis of the *gfp* mRNA or by using luciferase assays.

6) Since Hsp10 and Hsp60 are members of heat shock family, their genes should include an inducible promoter. Therefore, stressful conditions such as heat shock should result in an increase in their transcription

7) It is anticipated that *hsp* promoter properties could be observed in both zebrafish embryos *in vivo* and in cultured cells. These include promoter activity, bidirectionality and, inducibility. We do not expect to see many differences between an ectothermal zebrafish cell line and a homeothermal mammalian cell line.

The aim of this study was to have a better understanding of a bidirectional regulatory region in a vertebrate organism. A thorough understanding of this promoter makes it possible to use the promoter in practical applications. These include:

As a bidirectional inducible promoter: Inducibility is a great asset in transgenic animals. It makes it possible to control the expression of the transgene. It is especially important when the transgene is lethal at early stages of life. Moreover, a bidirectional promoter provides more conveniences. It can be used to study two different genes simultaneously. Many studies take advantage of reporter genes. In such cases, one direction of the promoter could control a gene of interest, while the other direction controls expression of the reporter gene. Therefore, a reporter gene could be coexpressed with any transgene of interest. Due to simultaneous coexpression, the reporter gene could be an accurate representation of transgene-expressing cells. When the reporter gene is a fluorescent protein, this allows real-time, *in vivo* imaging of the behavior of the cells that have received the transgene in mosaic organisms. It allows visual genotyping of transgenic fish by fluorescence microscopy.

Potential biomarker for monitoring environmental pollution: Since heat shock proteins, respond to a variety of stressful conditions they may be used in toxicological studies. For instance, overexpression of the *hsp10/60* genes has been observed in zebrafish exposed to cadmium (Ivanina et al., 2008), high concentrations of lead (Köhler et al., 2005), and pesticides like endosulphan (organochlorine) and monocrotophos (organophosphate) (Sharma et al 2007). This has also been observed in some physiological conditions such as oxidative stress resulting from type two diabetes mellitus (Calabrese et al., 2007). The *hsp10/60* promoter could be used to monitor the effect of a toxin on a particular gene. For this purpose, *hsp10/60* can simultaneously co-express the reporter gene along with the gene that is suspected to be affected by the toxin.

Materials and Methods

2.1 Zebrafish as an experimental organism

Adult zebrafish (*Danio rerio*) were obtained from Mirdo Importations (Montreal, QC). Fish were maintained in aquaria at 28°C on a cycle of 14 and 10 hours of light and dark, respectively. Fish tanks (Aquatic Habitats) were connected to fish water reservoirs keeping the water temperature at 28°C. Fish were fed once or twice daily with #1 Crumble (Aquatic Habitats). Embryo-collection traps were placed inside each tank of 20-30 fish in the evening and the embryos were collected on the next morning. Fish spawn at 9 AM when the lights came on, and embryos were collected from the traps shortly thereafter. Embryos were then incubated at 28°C and staged according to hours post fertilization (hpf) (Kimmel et al., 1995). General zebrafish care was according to Westerfield (1995).

2.2 DNA constructs

Plasmids were extracted either using standard boil prep protocols (Sambrook and Russel, 2001) or miniprep kits (Sigma Canada Ltd, Oakville, Ontario) for general use or being introduced to the embryos or cells, respectively.

a) GFP Constructs: For the first *cpn10/60* fragment which is the longest one (fragment A), the region of interest was amplified using standard PCR protocols. The designed primers matched the regions beyond and in frame with the ATG start codons of *hsp10* and *hsp60*. After purification of the amplified region, it was cloned into pCRII-TOPO vector of TOPO-TA cloning kit (Invitrogen Canada, Burlington, ON). At the next step, this region was excised and inserted into a promoterless EGFP-N1 vector, in both orientations, using suitable restriction enzyme sites. The promoter of

EGFP-N1 vector (Clontech, Palo Alto, CA) had been removed in the Dr. Ekker lab previously. Construct C was also constructed the same way, but different primers were employed (table 2-1). For constructs B, D, and E, the construct A was digested with *SceI*, *PstI* and *XbaI*, respectively. These enzymes had two or more recognition sites in the suitable locations. The linearized constructs were re-ligated to obtain the desired circular plasmids next.

b) Luciferase Constructs: All of the *hsp10/60* fragments were excised from the GFP constructs and were inserted into PGL3-basic vector (Promega Canada, Nepean, ON), in both directions, using suitable restriction sites.

c) Internally deleted constructs: For constructing these plasmids, fragment "A" was used as the template. The method was adopted from Makarova et al. (2000) with some modifications. A primer was designed in a way flanking the sequence that needed to be deleted. Primers were usually around 50 bases and the designated mutations were located in the center of the primers. Those primers have been used in a conventional PCR one at a time where the template was a circular plasmid. Following a 30 cycle PCR using PfuUltra Hotstart DNA polymerase (Stratagene, La Jolla, CA), the product was digested by *DpnI* restriction enzyme to eliminate the template and leave behind the potential mutated PCR copies. *DpnI* only digests the methylated, nonmutated parental supercoiled dsDNA. The mutant plasmid containing nicked circular strands was transformed into DH5 competent cells and grown on a selective plate. Most of the resulting bacteria contained the desired mutant plasmids with large deletion.

Table 2-1 includes the primers used for generating the transgenic constructs. All the generated plasmids were sequenced to confirm the accuracy of the constructs.

Table 2-1. Primers used for generating transgenic constructs.

Construct	Primers	
Frag A	Forward primer	5' AGGATCCCGCGTCTTACCATTTT 3'
	Reverse primer	5' GGATCCTGTCTCATGACACTGG 3'
Frag C	Forward primer	5' AGGATCCCGCGTCTTACCATTTT 3'
	Reverse primer	5' GGATGAGGATAATGCTGAGTG 3'
Del I	5'CTGTGTCGTGACGCGGAGTGATTTT ¹ CGGGGCCACGTCATTGATTAAATCA3'	
Del II	5'CTGTGTCGTGACGCGGAGTGATTTT ¹ TCCCTTGCTGGTTAGGGATTGTTCA3'	
Del III	5'TCCCTTGCTGGTTAGGGATTGTTCA ¹ CGGGGCCACGTCATTGATTAAATC3'	
Del IV	5'GCCTAACCGAACACCGAAATTGT ¹ CGGGGCCACGTCATTGATTAAATC3'	

2.3 Microinjection

Microinjections were performed on one- or two-cell-stage embryos (0-1.5 hpf) using a Narishige IM-300 pneumatic microinjector (Narishige, Japan) system connected to nitrogen gas and hand-held joystick micromanipulator model MN-151 (Narishige). The procedure was performed under a stereomicroscope. The embryos were held in wedged-shaped grooves made with agarose. They were injected into the cells usually penetrating through the yolk unless when the side or ventral side of the embryos was not readily accessible. In such cases the needle penetrated into the cell from the top. Needles were made from glass filaments with an outer diameter of 1.0 mm and an inner diameter of 0.5 mm. Needles were pulled using a Kopf needle/pipette puller model 730 (David Kopf Instr., Tujunga, Calif) and manually beveled using a sharp razor blade. Around 100 μ l of plasmids were injected at the concentration of 100 ng/ μ l in 1X injection buffer. The volume of the injected drop was about one-third of the size of the cell. Injection solution was dyed with 0.02% (vol/vol) phenol red (Sigma Canada Ltd, Oakville, Ontario) to help visualizing the injection volume.

2.4 Fluorescence microscopy

Following injecting the newly spawned fish with GFP constructs, they were raised to 24-hpf. The chorion of these embryos was removed using sharp forceps (Dumont No. 5). Then they were anesthetized using Tricaine solution. The reporter was observed using a Nikon stereomicroscope equipped with a fluorescent filter (excitation= 480/40, emission= 535/50).

2.5 Cell culture

2.5.1 Zebrafish ZF-4 cells

ZF-4 cells were seeded and split at high confluency. They were grown in a medium containing a combination of DMEM/F12, 10% (vol/vol) fetal bovine serum (FBS), and 1% (vol/vol) antibiotic-antimycotic at 28°C and 5% CO₂.

2.5.2 Mammalian 293 cells

Mammalian 293 cells were cultured in DMEM medium supplemented with 10% (vol/vol) horse serum (HS) and 3% (vol/vol) antibiotic-antimycotic at 37°C and 5% CO₂. All cell culture materials well supplied by Gibco (Burlington, ON; through Invitrogen)

2.6 Transfection

Liposome based techniques have been used to transfect the cells. All cells received both the experimental plasmid including firefly luciferase and *Renilla* luciferase for normalization in the approximate ratio of 3:1.

2.6.1 Transfecting ZF-4 cells

Effectene Transfection reagent (Qiagen Canada, Mississauga, ON) was used to transfect zebrafish cells. This was a less toxic reagent usually used for cells that are more sensitive. Moreover, this reagent requires using less DNA which makes it more cell-friendly. Transfection was carried out according to the manufacturers suggested protocol with some modifications to optimize it for these cells. Cells were seeded at the concentration of 3×10^5 per well of a six-well plate 24 hrs prior to transfection. The transfection mixture for each well contained 0.8µg plasmid DNA (0.53µg experimental plasmid and 0.27µg Rluc), 6.4µl enhancer (a solution provided in the kit), and 8µl

Effectene in the final volume of 100µl in the provided buffer (EC). The solution was added to 75% confluent cells in 1600 µl of fresh medium. This medium had to be removed in 6 hours and replaced by fresh medium. PBS wash was not necessary at this step. 24 hours later cells were heat shocked and harvested.

2.6.2 Transfecting 293 cells

Lipofectamine 2000 (Invitrogen Canada, Burlington, ON) was used according to the manufacturers guidelines for six-well plates. Cells were seeded 24 hrs prior to transfection and kept in Opti-MemII Reduced Serum Medium without serum (Invitrogen) during transfection. Twenty-four hours later cells were heat shocked and harvested in passive lysis buffer from the luciferase assay kit.

2.7 Heat shock

Embryos were heat shocked by being placed in a 37°C incubator for the desired duration. Embryos were placed in a six-well plate containing 37°C fish-water. Similar approach was used for the cells. The heated medium was added to the cells and they were incubated in an incubator with the desired temperature for the chosen duration. This temperature was 37°C for ZF-4 cells and 42°C for 293 cells.

2.8 Luciferase assay

2.8.1 Procedure

Dual-Luciferase Reporter Assay System (Promega, Madison, WI) was used for luciferase assay. The procedure was carried out according to the manufacturer's protocol with the exception that 300 µl of 1X passive lysis buffer was dispensed per each 35-mm well or each embryo. Chemiluminescence was measured for the cell lysate from each well to quantify luciferase production using an Lmax II384 luminometer with Softmax Pro software (Molecular Devices, Sunnyvale, Calif). All the wells were

cotransfected with a vector containing *Renilla* luciferase, pRL-TK (Promega Canada, Nepean, Ont), simultaneously. 100µl of prepared Luciferase Assay ReagentII (LARII) and Stop & Glo reagents were used per each 20µl of cell lysate through the P and M injector of the instrument, respectively. LARII measured the amount of firefly luminescence while Stop & Glo indicated the activity of *Renilla*.

2.8.2 Analysis

Normalization for each sample was carried out by dividing relative luminescence of firefly luciferase by the relative luminescence of *Renilla* luciferase of the same sample. The ratio presented differences in promoter activity. The mean and standard deviation from all replicates for each treatment group were calculated using Microsoft Excel software (Redmond, WA).

2.8.3 Statistics

ANOVA test was performed to determine significant differences in the relative mRNA expression. Significance was accepted at $P < 0.05$.

2.9 RT-PCR

Two different semiquantitative RT-PCR reactions have been carried out. One has been performed on the *hsp10* and *hsp60* mRNA of wild type zebrafish embryos that have been subjected to heat shock. The other one was executed on *egfp* mRNA from the transient transgenic zebrafish embryos. All these groups included both normal condition and heat shocked samples no-template and no reverse transcriptase controls have been run in parallel.

2.9.1 Sample collection and treatment

For a set of experiments, newly spawned embryos were first microinjected with one of the constructs. The injected embryos, wild-type ones, or control samples were grown in normal growth condition in a 28°C incubator for 24 hours. Then the experimental group was heat shocked at 37°C for the durations of 1, 2 or 4 hours. Heat shocked embryos were immediately ground in TRIzol (Invitrogen Canada, Burlington, ON), frozen in liquid nitrogen, and stored at -80°C for future follow up.

2.9.2 RNA extraction

RNA was extracted using TRIzol (Invitrogen Canada, Burlington, ON) and chloroform, followed by standard isopropanol precipitation protocols. Quantity of extracted mRNA was assessed through UV spectrophotometry.

2.9.3 cDNA synthesis

The total RNA was used as a template in cDNA synthesis. Approximately 5 µg of RNA was mixed with 250 ng random primer, 0.5 mM dNTP mix, 1X buffer, 10 mM DTT, 40 units RNase inhibitor and 200 units M-MLV reverse transcriptase. The reaction was incubated at 37°C for one hour and then stopped by heating at 70°C for 15 minutes. The product was treated with DNase and purified using DNase-free kit (Ambion, Inc., Austin, TX). The quality of cDNA was assessed by PCR with β-actin primers. β-actin primer sequences were as follows:

Forward primer 5' ACTGGTATTGTGATGGACTCTGGTG 3' (T_m 55.5°C)

Reverse primer 5' ACGCTCGGTCAGGATCTTCAT 3' (T_m 54.8°C)

2.9.4 Primers

RT-PCR was performed on *hsp10*, *hsp60* and *egfp* mRNA molecules. Primers were designed to amplify a 150-500 bp region of the selected gene, and to have an annealing temperature of

approximately 58°C (table 2-1). A sequence from *efl-α* gene was used as the control. *hsp10* and *hsp60* primers spanned the intron-exon boundaries.

Table 2-2. Primers used in RT-PCRs

Gene	Primer sequence (F, R)	T_m (°C)
β-actin	5' ACTGGTATTGTGATGGACTCTGGTG 3'	58
	5' ACGCTCGGTCAGGATCTTCAT 3'	58
Ef1-α	5' TACGCCTGGGTGTTGGACAAA 3'	59
	5' TCTTCTTGATGTATCCGCTGA 3'	55.7
Hsp10	5' GCAGCACTTTGGCTTGAGACTT 3'	59
	5' TGTCGGACGCGGTTTTCTTA 3'	58
Hsp60	5' TGATGGTGTCGAAGCCCTCTTT 3'	60
	5' CTGCTGTTACCCAGTGTCAT 3'	56
Hsp70	5' CCAGCCTATTTCAACGACTCCC 3'	58
	5' TCAGGATGGACACGTCGAAGGT 3'	60
GFP	5' TGCTGCTGCCCGACAACAC 3'	63
	5' GTCCATGCCGAGAGTGATCC 3'	58

2.9.5 Standard curves

Standard curves were produced for each primer set to assess the efficiency of the reactions. A pool of obtained cDNAs from different embryos was used as a template to generate standard curve. The template was serially diluted over eight concentrations ranging from 1X to 10^{-7} X .

2.9.6 Reactions

RT-PCR reactions of 20 μ l were set up using a Stratagene Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA) according to the instruction manual. Master mixture contains DNA polymerase, PCR buffer, SYBR Green dye, and $MgCl_2$. Reactions consisted of 12.5 μ l (1X) master mix, 55 ng cDNA and 25 μ M of each primer in the total volume of 25. Reactions were run in a Stratagene MX3000 machine for 40 cycles with an annealing temperature of 58°C.

2.9.7 Analysis

Threshold-crossing cycle numbers (CT values) were analyzed using Microsoft Excel software. Values for ΔCT were calculated as $CT_{(gene)} - CT_{(reference)}$. $\Delta\Delta CT$ values were calculated for each time-point by comparing the experimental samples to the corresponding control samples. $\Delta\Delta CT$ was calculated as $\Delta CT_{(treated)} - \Delta CT_{(control)}$. Efficiencies were calculated by the formula " $E = 10^{(-1/slope)}$," where the slope is that of the standard curve (Pfaffl, 2001). The efficiency of each primer set was taken into account by applying a mathematical equation suggested by Pfaffl (2001). The mean and standard deviation from all replicates for each treatment group were calculated using Excel software (Redmond, WA).

2.9.8 Statistics

ANOVA test was performed to determine significant differences in the relative mRNA expression. Significance was accepted at $P < 0.05$.

Results

3.1 *hsp10/60* Sequence Analysis

Comparisons of the complete *hsp10/60* gene sequences from a few different eukaryotic organisms available in Genbank (accession numbers: human AJ250915; rat U68562; flounder DQ250132 and zebrafish AY112664) showed that the coding regions of all these genes are highly similar (Martin et al., 2002). Less similarity was found in the intergenic regions and the 5'UTRs. However, the organization of intergenic region, transcription initiation sites (TIS), untranslated regions (UTR), location of the ATG start codons, and intron-exons location and boundaries, at least for the first few exons of both genes, are the same, but the length of each of the noncoding regions (figure 3-1) and their nucleotide contents are different (figure 3-2).

The transcription initiation sites (TIS), the first two exons of *hsp10*, and the first three exons of *hsp60* had been previously identified by Martin et al. in 2002. Partial sequence of the cDNA had been determined from mRNA extracted from zebrafish using a reverse transcriptase-PCR approach with degenerate primers. The intron-exon boundaries of the first few exons had been found by comparing this cDNA sequence with the genomic DNA sequence of *hsp10* and *hsp60* genes obtained from a zebrafish genomic library macroarray filter (filter set #706). According to the prediction from Ensembl database (www.ensembl.org), the total numbers of the exons in the *hsp10* and *hsp60* genes are 4 and 11, respectively. In order to find the transcription start site, the 5' rapid amplification of cDNA ends (5'RACE) technique was used (Martin et al., 2002). According to this analysis, the TISs of *hsp10* and *hsp60* are 860-bp apart. Comparisons between the sequences of Hsp10 and Hsp60 proteins from other organisms and predicted amino acid sequences based on the cDNAs suggested that the first ATG codon of *hsp10* is located at the end of its first exon and the ATG start codon of

hsp60 is located at the beginning of its second exon. The same organization has been found in rat, human, and flounder except the differences in the sizes of each region.

The intergenic region of the *hsp10/60* genes, which is the region located between the two TISs, was analyzed to find *cis*-acting elements. The intergenic region contains three tandem inverted repeats of the nGAAn sequence, forming a single heat shock element (HSE) (nGAAn nCTTn nGAAn) (shown as dashed boxes in figures 3-2). The HSE found in flounder also contains three tandems but the reported HSE in mammalian species contains four tandems. Comparison of the nucleotide content and alignment have been performed using "AliBee-multiple alignment, release 2.0" online tool (http://www.genebee.msu.su/services/malign_reduced.html). "Multiple sequence alignment" is the arrangement of several DNA sequences with postulated gaps in a way that similar residues are juxtaposed.

We also observed a few short GC-rich sequences present in the *hsp10/hsp60* intergenic region of all the compared organisms. They are shown as solid boxes in figures 3-2.

We did not find any Sp1 binding site, CCAAT boxes, or TATA boxes in the zebrafish *hsp10/60* intergenic region using TFSEARCH online software tool (<http://www.cbrc.jp/research/db/TFSEARCH.html>).

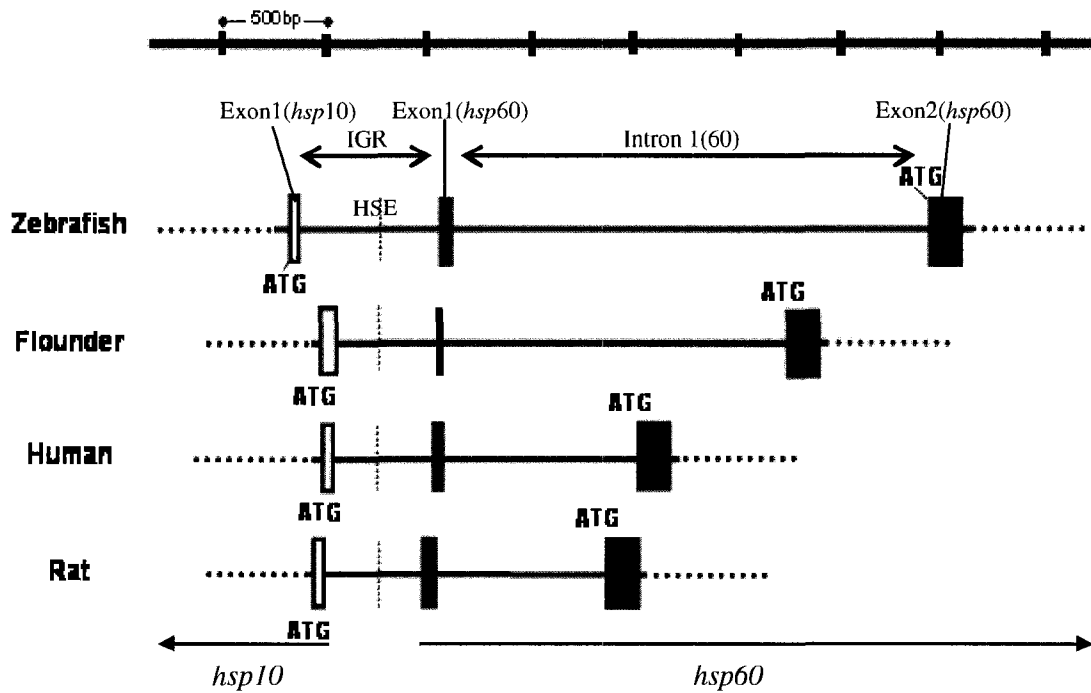


Figure 3-1. Comparative schematic maps of a part of *hsp10/60* genes in different organisms:

The maps contain a DNA segment that illustrates the intergenic region (IGR), the first exon (the open boxes) of *hsp10* and the first and second exons of *hsp60* (filled boxes) in zebrafish, flounder, human, and rat (eukaryotic genes available in Genebank). The thick lines represent introns and intergenic region. The intergenic region (IGR) is located between the first exon of *hsp10* and the first exon of *hsp60*. The position of the heat shock element (dashed vertical line) and the relative orientation and position of the ATG start codons for both transcripts (on the opposite strands) are shown. For simplicity, the two strands of DNA are shown as one line. The scale is shown at the top.

3.2 Bidirectionality of the promoter

Since the two chaperonins are adjacent genes arranged in a head-to-head manner, it was expected to find their promoter in the intergenic region located between these two genes. Lack of a TATA box made finding the promoter region difficult. To find the promoter of these two genes, we searched for a region capable of activating the expression of downstream genes. Therefore, a fragment spanning from the ATG start codon of *hsp10* to the first ATG codon of *hsp60* was chosen (figure 3-3). This 3.3-kb fragment (fragment A, figure 3-3) included the intergenic region (containing the heat shock element), the first exon of *hsp10*, the first exon plus three nucleotides of the second exon of *hsp60*, as well as the first intron of *hsp60*. It was also hypothesized that *hsp10* and *hsp60* genes might share a common bidirectional promoter due to their head-to-head organization and short intergenic region. Based on this and the following reports about bidirectional promoters, it was speculated that enhancers might occur in the first intron of *hsp60* gene, which is entirely noncoding (forms the 5'UTR). Some studies have described the presence of an enhancer within the noncoding first intron of one of the two genes flanking a bidirectional promoter. Examples include the bidirectional promoter of alpha1 (IV)/alpha2 (IV) collagen genes (Killen et al., 1988) and the bidirectional promoter of K18/ thymidine kinase genes (Oshima et al., 1990). These promoters require the enhancer elements located within the first intron of one of the two genes for optimal activity (Pollner et al., 1990). Such examples encouraged us to use "fragment A" first. Moreover, we preferred to use the ATG start codon of *hsp10/60* genes instead of the ATG codon of the reporter genes for the first attempt. Fragment "A" was designed in a way to include possible *cis*-acting elements, which might be present in the region spanning from the start codon of *hsp10* to the start codon of *hsp60*. Moreover, the 5'UTRs (i.e. *hsp10* exon one except the last three nucleotides and the entire first exon of *hsp60*) may contain sequences that regulate translation efficiency or affect mRNA stability.

To characterize the promoter activity of fragment "A" experimentally, it was cloned in both orientations into a *gfp* reporter vector as follows. "Fragment A" that included the non-coding intron and 5'UTRs was first cloned upstream of a *gfp* reporter gene in the same orientation as in *hsp60* gene: "fragment A60". Then the same fragment was cloned in the opposite orientation, to drive GFP in the same orientation as in *hsp10*: "fragment A10". The "A10-GFP" and "A60-GFP" constructs were injected into one cell-stage zebrafish embryos and, 24 hours later, the expression was observed for both reporter constructs (figure 3-4).

Embryos injected with both "A10-GFP" and "A60-GFP" showed GFP expression. Therefore, the "A" genomic fragment was able to drive expression in both orientations. Embryos showed sporadic expression in different tissues covering all major cell types when all observations were combined. Combined observations of more than three hundred injected embryos have shown that the "A fragments" targets expression in all major cell types and tissues. Transgenic embryos showed the typical mosaic expression generally observed in this species (figure 3-4). A larger number of GFP-expressing cells were observed when the "A60-GFP" fragment was investigated in comparison to "A10-GFP". While in the *hsp10* orientation about 80-90 GFP-expressing cells were detected, this number was about 5 times higher with the *hsp60* orientation. Thus, the expression of "A60-GFP" was seemingly stronger than that of "A10-GFP".

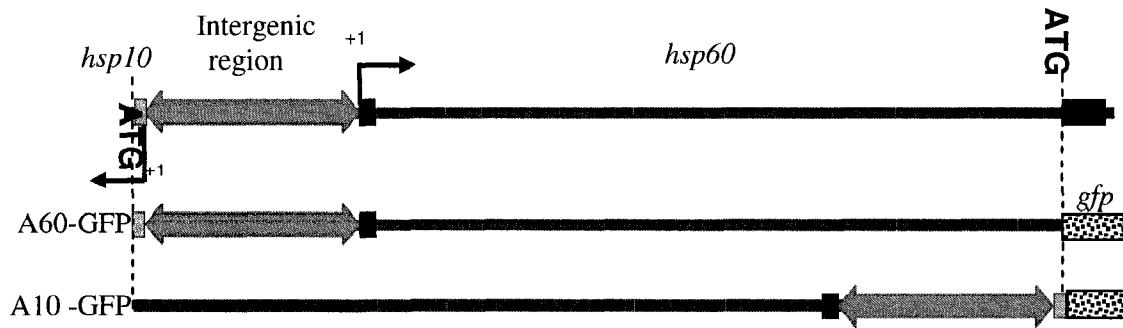


Figure 3-3. Schematic map of part of *hsp10/60* genes spanning from the ATG start codon of *hsp10* to the ATG codon of *hsp60*: the top part shows the start codon of each gene as well as introns (the thick lines), exons (the boxes), transcription start sites (bent arrows), and intergenic region (bidirectional arrow). Grey boxes represent *hsp10* exons and black boxes represent *hsp60* exons. Reporter constructs are depicted to show the relative orientation of the "A fragment" with respect to *gfp* coding sequences. Throughout the entire results section, *hsp60* exons are shown in black and *hsp10* exons in grey.

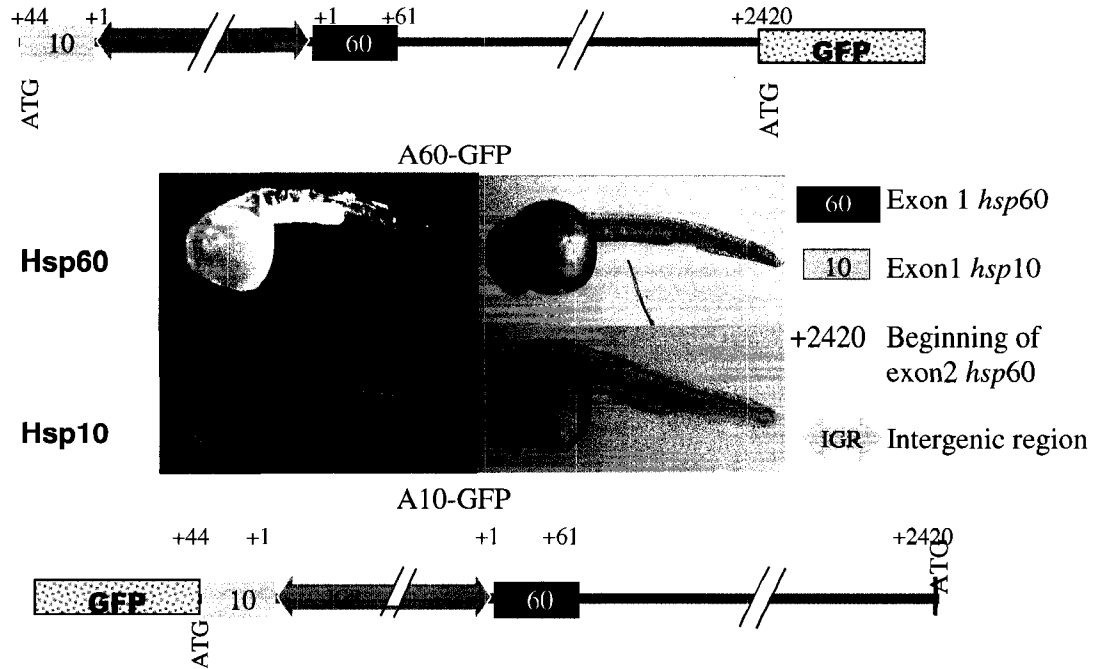


Figure 3-4. 24-hpf zebrafish embryos injected with "A10-GFP" and "A60-GFP" cassettes: Lateral views of 24-hpf embryos which were injected with the A60 construct is shown at the top and those injected with A10 at the bottom. Fluorescent image at left and bright field at right. Reporter expression is detectable in both orientations. The schematic maps of the corresponding construct are shown above and below the photographs of transgenic embryos.

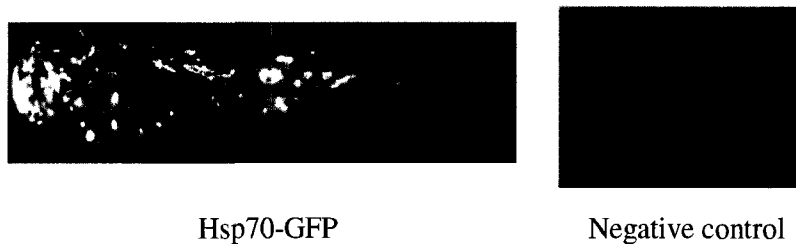


Figure 3-5. Positive and negative controls for promoter activity: Hsp70 is expressed ubiquitously in a transient transgenic embryo on the left panel and a promoterless GFP vector, shown on the right panel, does not produce detectable expression. Lateral views of 24-hpf embryos are shown in the photographs.

The positive control for this experiment consisted of an "hsp70-GFP" construct in which the promoter region of the *hsp70* gene was cloned upstream of the same *gfp* reporter gene. This construct was injected into embryos of the same batch as for the other constructs. The *hsp70* gene contains a well-characterized promoter that is expressed ubiquitously upon heat shock. The "hsp70-GFP" construct was introduced into embryos at one cell-stage and the embryos were subjected to a 2-hour heat shock 24 hours later. A promoter-less GFP vector was used as a negative control. A large number of GFP-expressing cells including all major cell types was observed in the positive control group as expected. The negative control group did not produce any detectable GFP expression except for a few occasional cells (1-2 cells in approximately 10% of the embryos) which would be attributable to leakiness in the promoter-less construct or possible positional effect following integration of this plasmid (figure 3-5).

Significant amounts of GFP expression were detected in the yolk of almost all the embryos injected with the "A60-GFP" construct. Since the strong expression in the yolk was an unexpected observation, the next step was determining the origin of the strong GFP expression in the yolk. The first visible GFP expression around the yolk started approximately at 5-6 hours post fertilization (hpf), that is, at the beginning of gastrula stage. It started from the yolk syncytial layer (YSL), increased over time, and was followed by the gradual diffusion of the fluorescence into the yolk (figure 3-6) until it became a glowing ball. Yolk expression remained as such afterwards until the end of the period during which embryos were examined (15-hpf). The YSL is a layer of cells separating the embryo from the yolk. During the initial steps of zebrafish development, the embryo lies against the yolk and remains connected to it throughout cleavage. During the mid-blastula period, the marginal blastomeres, which are adjacent to the yolk, form a syncytial layer by losing their lower borders where they join the yolk cell producing the yolk syncytial layer (YSL), which is the region in the yolk where we first observed GFP expression. The marginal area of the YSL is termed external

(eYSL) located on the peripheral parts and the central parts internal (iYSL) forming a plate. GFP was expressed in eYSL as shown by arrows in figure 3-6.

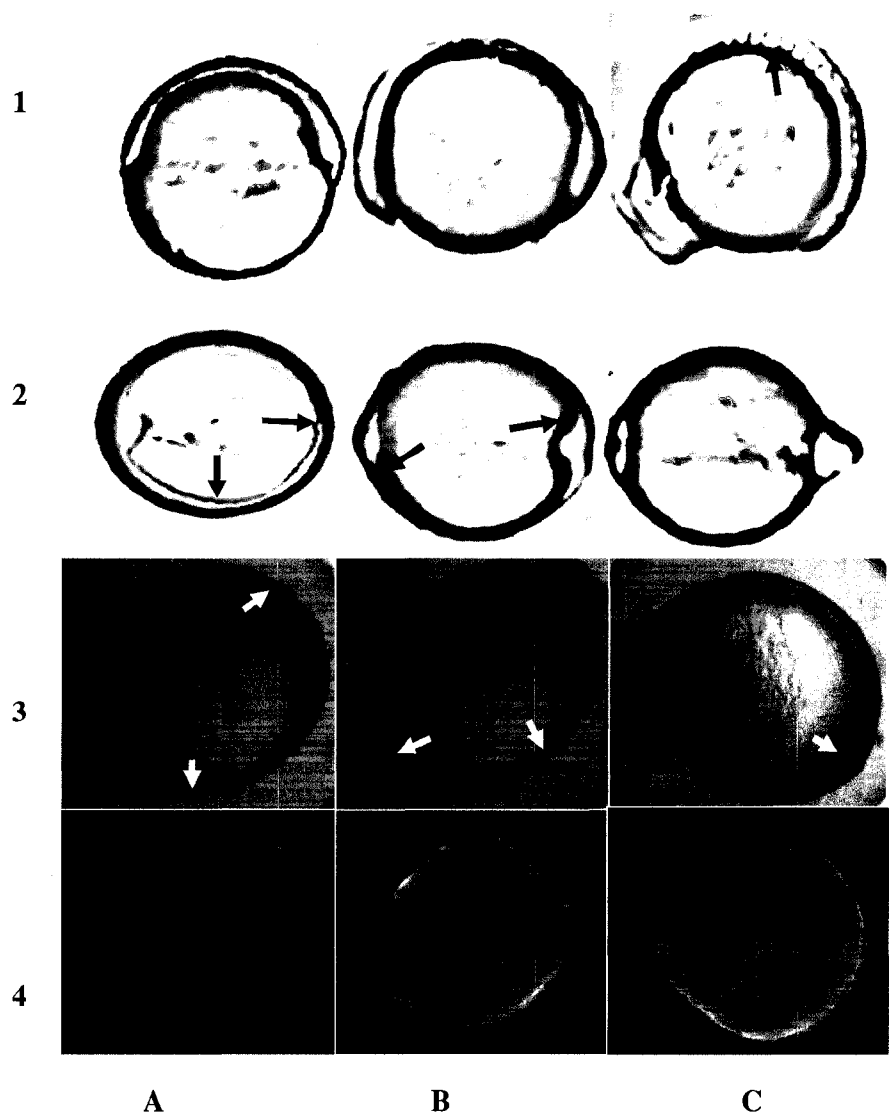


Figure 3-6. Expression in the yolk: Panel 1, lateral representation of the embryos; panel 2, dorsal representation (Animal pole); arrows show yolk syncytial layer; panel 3, bright field pictures showing the position of the gastrula stage embryos; panel 4, fluorescence microscopy pictures showing the dorsal view in A and B and lateral view in C. In 4A, a line of fluorescence is seen only in the external YSL; in 4B, the fluorescence area is wider. Finally, in 4C, it is diffused all over the yolk.

3.3 End deletions

Using "fragment A", the bidirectional functionality of a relatively large fragment of the *hsp10/60* locus was detected. Finding a smaller region that was still functional as a promoter was the next aim. To determine a region within the "A fragment" where the core promoter elements of the *hsp60* and *hsp10* genes are located (Figure 3-7), a series of deletions were generated starting from the ends of this fragment. Most of the end deletions were carried out on the *hsp60* side, because this end was preceded by the intron, which is eliminated during splicing; while the *hsp10* end was only 44-bp away from the transcription initiation site. First, sequences of different lengths were deleted from the 3'-end of the fragment on the *hsp60* side, step by step, as shown in (figure 3-7 a). The resulting sequences were all cloned upstream of the *gfp* reporter gene in both *hsp10* and *hsp60* orientations*. These constructs were introduced into one cell-stage embryos by microinjection and the embryos were inspected for GFP-expression 24 hours later, using fluorescence microscopy. For each construct, more than three hundred embryos were examined. "Fragment B" whose size is about 1-kb, lacked a 2.2-kb region from "fragment A". In "fragment B", most of the *hsp60* first intron was deleted. It contained the intergenic region, exon one of both genes and a small piece of intron one of *hsp60* (figure 3-7, B). The next fragments contained no intronic sequence. "Fragment C", included only the intergenic region and the 5'UTRs of both genes and was about 100-bp shorter than "fragment B". "Fragment D" which was 500- bp shorter than "C" contained only the intergenic

* Throughout this thesis, we will mention that a given genomic fragment is placed in the *hsp60* orientation when the GFP coding sequence is placed downstream of the desired fragment in the same orientation as in *hsp60* gene fragment. Similarly, a fragment will be in the *hsp10* orientation when GFP is placed downstream of it relative to *hsp10* in the same orientation as in *hsp10* gene.

region and the first exon of *hsp10*. Finally, fragment "E" whose size was 710-bp was 190-bp shorter than "fragment D" and covered only a part of the intergenic region, more specifically, from the location -108 from *hsp10* gene to -24 from *hsp60*. Both transcription start sites (location +1s) were missing (figure 3-7).

When GFP was cloned downstream of "fragment B" in the *hsp60* orientation (B60-GFP), it resulted in fewer GFP-expressing cells (about 80-90 cells) at 24-hpf, compared to the "A60-GFP" construct. Equal numbers of GFP-expressing cells were seen when "B60-GFP" and "B10-GFP" (GFP in the *hsp10* orientation) were examined. In other words, the differential expression of the two orientations was lost when "fragment B" (figure 3-7 b) was used; however, the expression pattern and the distribution of GFP-expressing cells did not change. When the "C10-GFP" and "C60-GFP" constructs (GFP driven by *hsp10* orientation and *hsp60* orientation of "fragment C", respectively) were injected into the embryos, similar numbers of GFP-expressing cells were observed in both orientations. "Fragment C" seemed to be less active than "fragment B" (figure 3-7 c). "Fragment D" which lacked the transcription start site of *hsp60* produced no expression when injected into the embryos in the form of "D60-GFP". However, GFP-expressing cells were observed following the injection of "D10-GFP". This showed that the *hsp10* orientation was still active while the *hsp60* orientation was not able to act as a promoter anymore (figure 3-7 d). Finally, "fragment E" that lacks both transcription start sites, was inactive in both orientations (not shown)

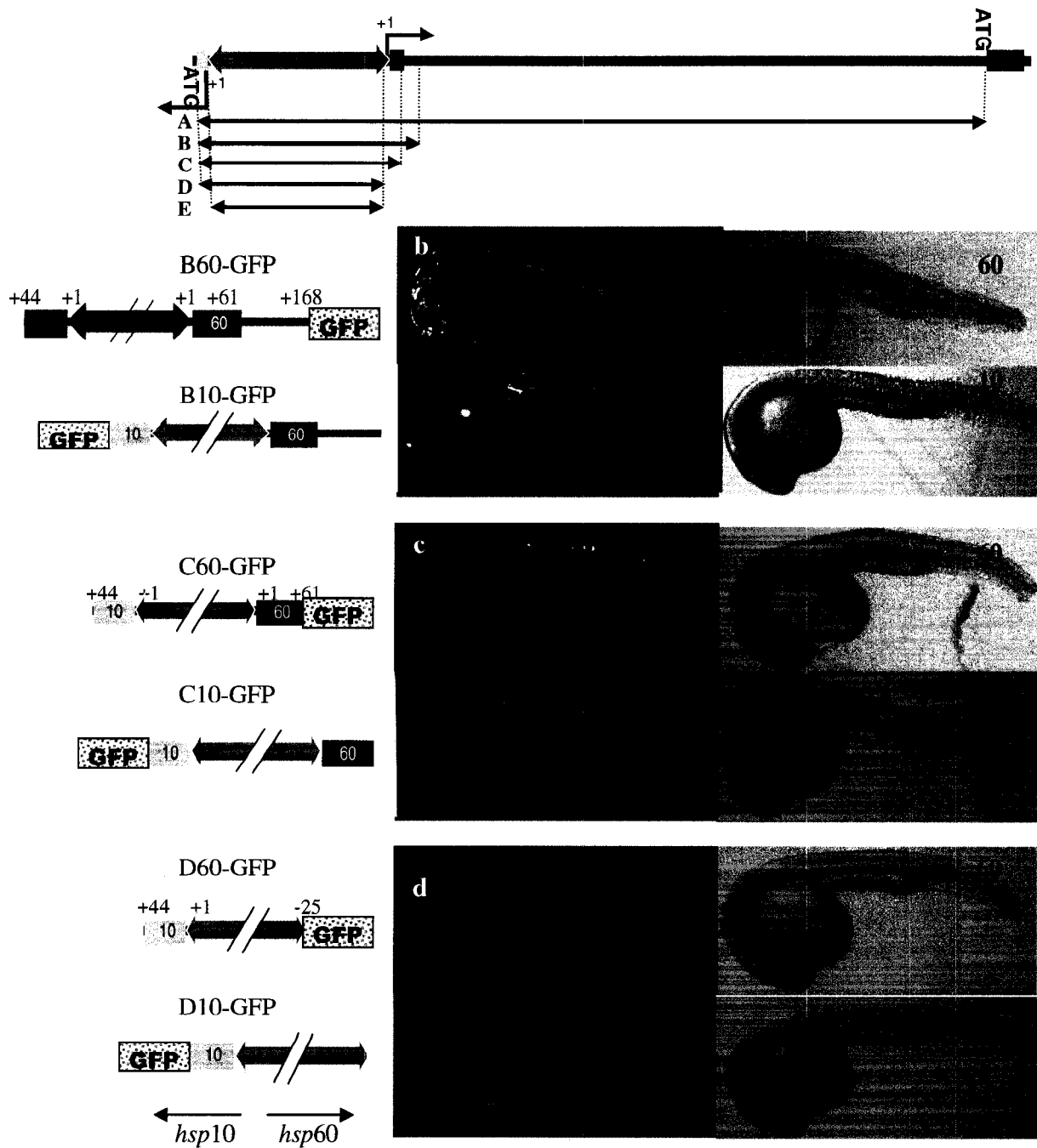


Figure 3-7. Schematic map and qualitative analysis of the activity of different promoter-including fragments: a) comparative schematic map of the deletion fragments; b, c, and d, fluorescent microscopy images (lateral views) of 24-hpf embryos injected with the deletion fragments (middle panel) along with the bright field images (right panel). The related constructs are shown on the left. For legends please refer to figure 3-4

3.4 Quantifying promoter activity

Using GFP reporter and fluorescence microscopy enabled us to estimate the strength of promoter activity to some extent. Nevertheless, quantitative measurements of the promoter strength appeared necessary to better assess differences between the various genomic fragments. For this purpose, we used both quantification of GFP transcripts by RT-PCR and luciferase constructs.

3.4.1 RT-PCR using GFP specific primers in embryos

We injected three constructs: "A60-GFP", "C60-GFP", and "C10-GFP" into single-cell stage embryos. For each construct, one hundred transient transgenic 24-hpf embryos that showed at least one GFP-expressing cell were collected. The one hundred embryos were divided into three groups of about equal size. The mRNA from pooled embryos was extracted and was used in RT-PCR reactions using PCR primers specific to *gfp* mRNA. The RT-PCR assay was semi-quantitative. This provided information about the levels of transcripts without the interference of factors involved in translation of the reporter protein. Among the three different constructs used in this experiment, the "A60-GFP" construct displayed the highest level of expression, which was twice as much as the other two constructs. The "C60-GFP" and "C10-GFP" constructs showed almost the same level of activity as illustrated in figure 3-8.

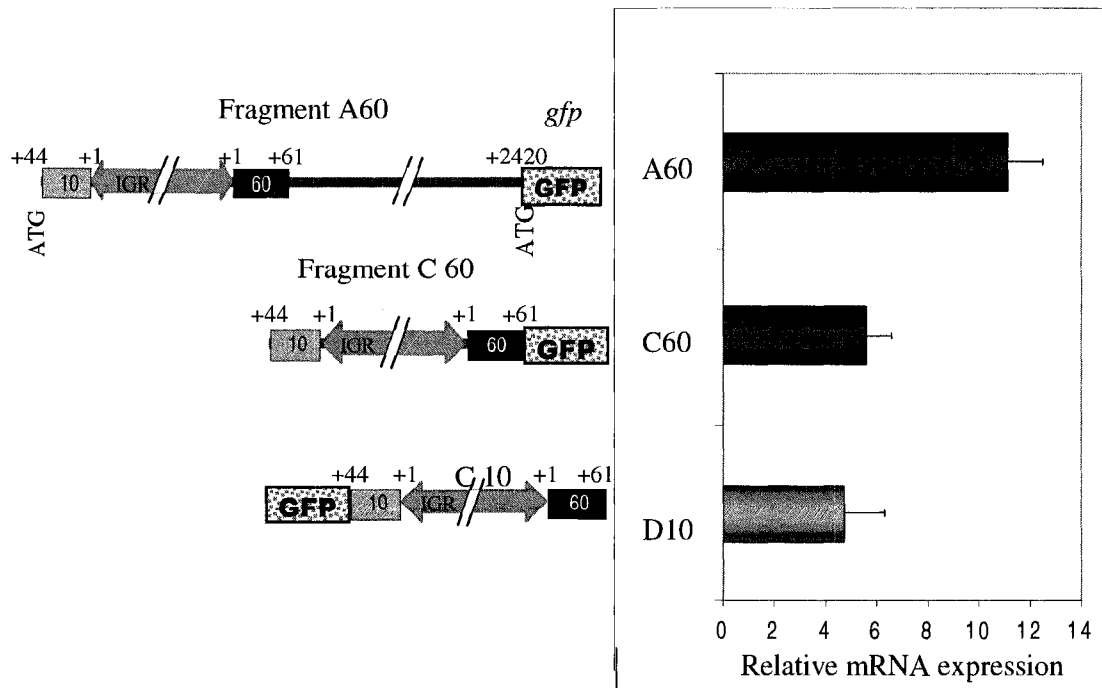


Figure 3-8. Analysis of the promoter activity of different *hsp10/60* promoter-including fragments in different orientations: showing the amount of GFP expression in 24hr transient transgenic zebrafish embryos. Embryos were injected with "A60-GFP", "C60-GFP" and "C10-GFP". Constructs corresponding to each sample are shown on the left panel. For all the graph results, error bars show standard error on the mean; n=3.

3.4.2 Luciferase assay

We also used luciferase reporter constructs and luciferase assays to quantify gene expression. Using luciferase enabled us to measure the light intensity precisely and the short half-life of the protein, compared to GFP, allowed us to avoid reporter protein saturation. This ensured a more accurate estimate because when the levels of GFP expression exceed a certain threshold, it becomes impossible to detect higher expression levels. Thus, all fragments were cloned upstream of a firefly luciferase reporter gene (Fluc) in the pGL3-basic vector. The constructs were either introduced into zebrafish embryos, or transfected into two different types of cells in culture. Subsequently, luciferase assays were performed on the lysates obtained from the 24-hpf transient transgenic embryos or on cell lysates prepared 24-hour post-transfection.

3.4.2.1 Activity in zebrafish embryos

To quantify the promoter activity of "fragment A" in zebrafish embryos, constructs "A10-Fluc" and "A60-Fluc" were injected into zebrafish embryos at the one-cell stage. After 24 hours, embryos were homogenized and the luciferase activity was measured in the lysate. For each construct, lysates of 10 different embryos were prepared, individually. Luciferase catalyzes the oxidation of luciferin and the intensity of the light produced by the reaction reflects the strength of the promoter driving the production of Fluc. The "A60-Fluc" construct resulted in twice as much luciferase expression in comparison to "A10-Fluc". In agreement with the results from GFP in the previous sections, the promoter was active in both orientations and the *hsp60* orientation was more active than the *hsp10* orientation (figure 3-9).

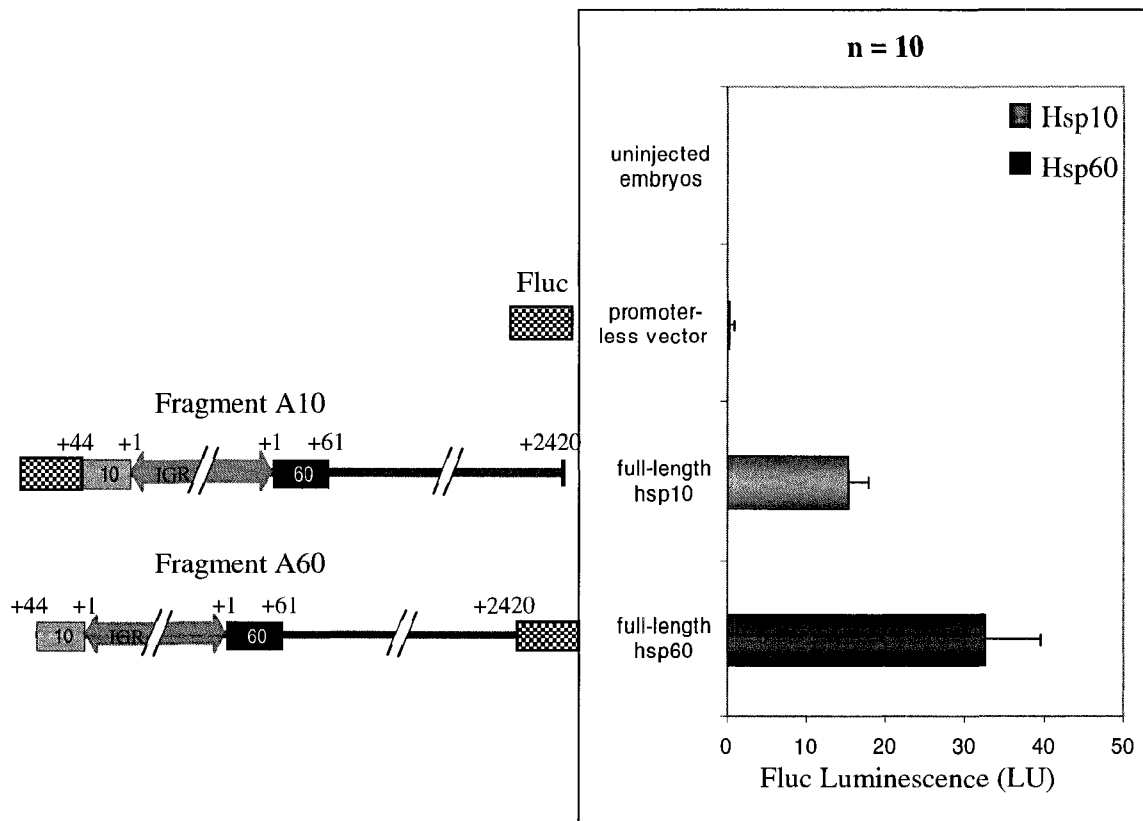


Figure 3-9. Analysis of the activity of fragment A by luciferase reporter assay in 24 hour transient transgenic zebrafish embryos: the right panel shows the average of light intensity in light units (LU) obtained from luciferase assays performed on 24 hr embryos injected with "A60-Fluc" and "A10-Fluc" plasmids (n=10). Negative control in this case includes luciferase assay performed on wild type embryos. The corresponding constructs are shown on the left panel.

3.4.2.2 Activity in cultured cells

Constructs containing firefly luciferase (Fluc) driven by the fragments from figure 3-7 were used to quantify the transcriptional activity of each fragment. To eliminate the problem of mosaicism, encountered in primary transgenic zebrafish embryos, cell lines were used. Cells were co-transfected with a construct containing *Renilla* luciferase (Rluc) driven by the HSV-TK (herpes simplex virus thymidine kinase) promoter to normalize for transfection efficiency in the different samples.

The constructs were tested in two different cell lines: ZF-4 zebrafish embryonic cells, and 293 human embryonic kidney cells. ZF-4 is a fibroblast-like cell line, derived from one-day-old zebrafish embryos. Driever and Rangini (1993), who developed the ZF-4 cell-line, have confirmed the functionality of the HSV-TK promoter in ZF-4 cells. Mammalian 293 cells were used as well to compare the bidirectional activity of the promoter in a different cell type, as transcription from bidirectional promoters could be cell-specific as mentioned in section 1-9 (Trinklein et al., 2004).

The results obtained from luciferase reporter assays from ZF-4 (figure 3-10) and 293 (figure 3-11) cultured cells were similar to those observed with GFP constructs in zebrafish embryos. "Fragment A60" showed twice as much expression as "fragment A10". This suggested that the promoter is more active in the *hsp60* orientation (similar to the observation from luciferase assay in zebrafish embryos). After deleting a significant part of the first intron of *hsp60* the 2:1 ratio was missing and the expression in both directions was about the same. Fragments "B10", "B60", "C10", and "C60" as well as "D10" displayed a level of activity which was similar to "A10-Fluc". The *hsp60* orientation of "fragment D" (D60), fragment "E10", and fragment "E60" did not produce any detectable expression (figure 3-10). The lack of promoter activity in the latter fragments suggested the lack of some necessary elements. A construct containing the well-characterized *hsp70* promoter was used as a positive control. A promoterless vector, which contained Fluc, was used as a negative control to

reveal a possible residual transcriptional activity originating from plasmid sequences or from genomic integration. Results obtained from 293 cells (figure 3-11) were in good agreement with those obtained from ZF-4 cells.

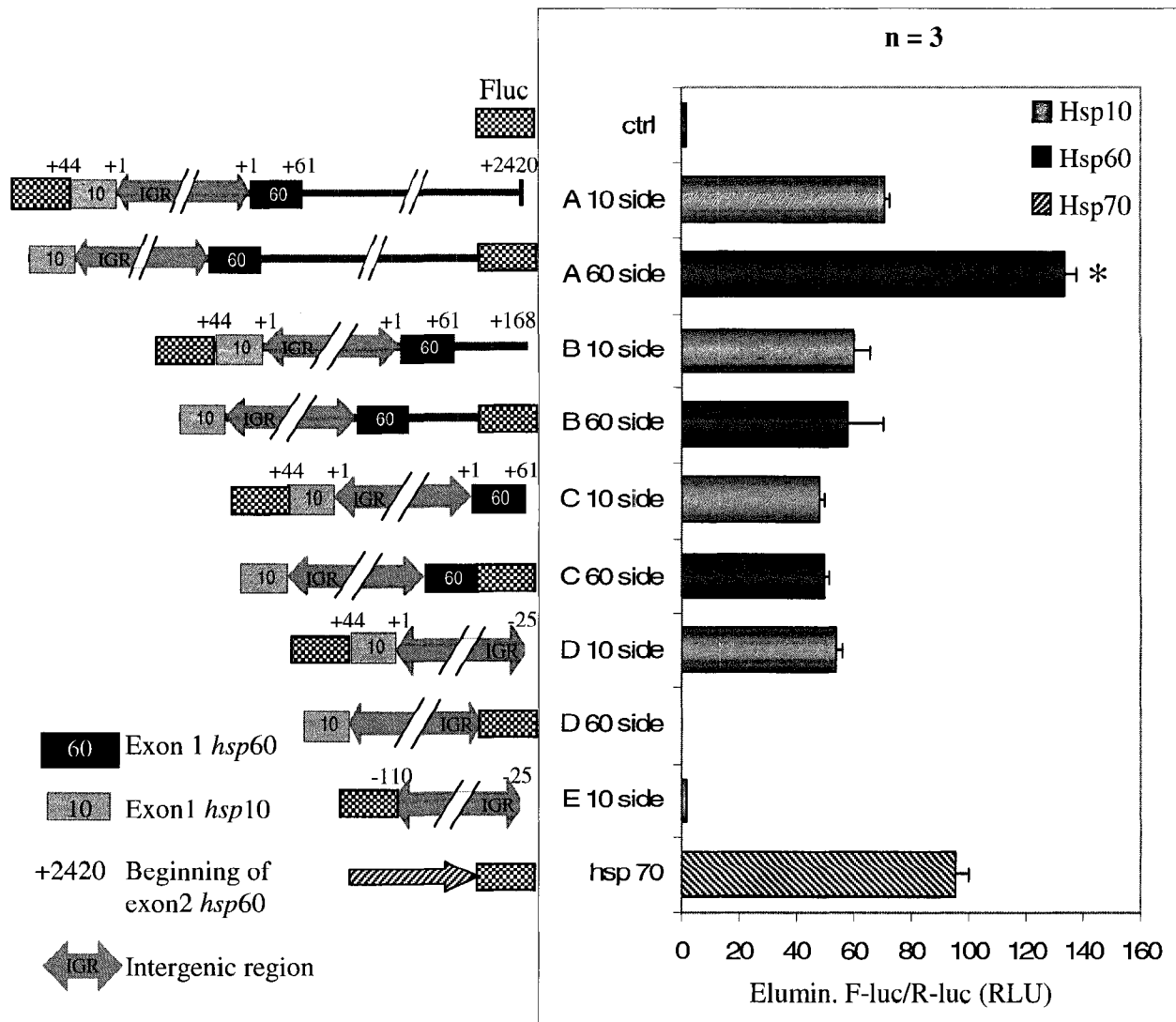


Figure 3-10. Promoter activity of constructs containing different fragments from the zebrafish *hsp10/60* locus in ZF-4 cells: on the left, a schematic map of the constructs is displayed; normalized luciferase activity, in relative light units (RLU), of the transiently transfected cells is shown on the right. (n = 3). A promoter-less vector was used as a negative control. The "*" indicates a significant difference compared to the other constructs; (P<0.05).

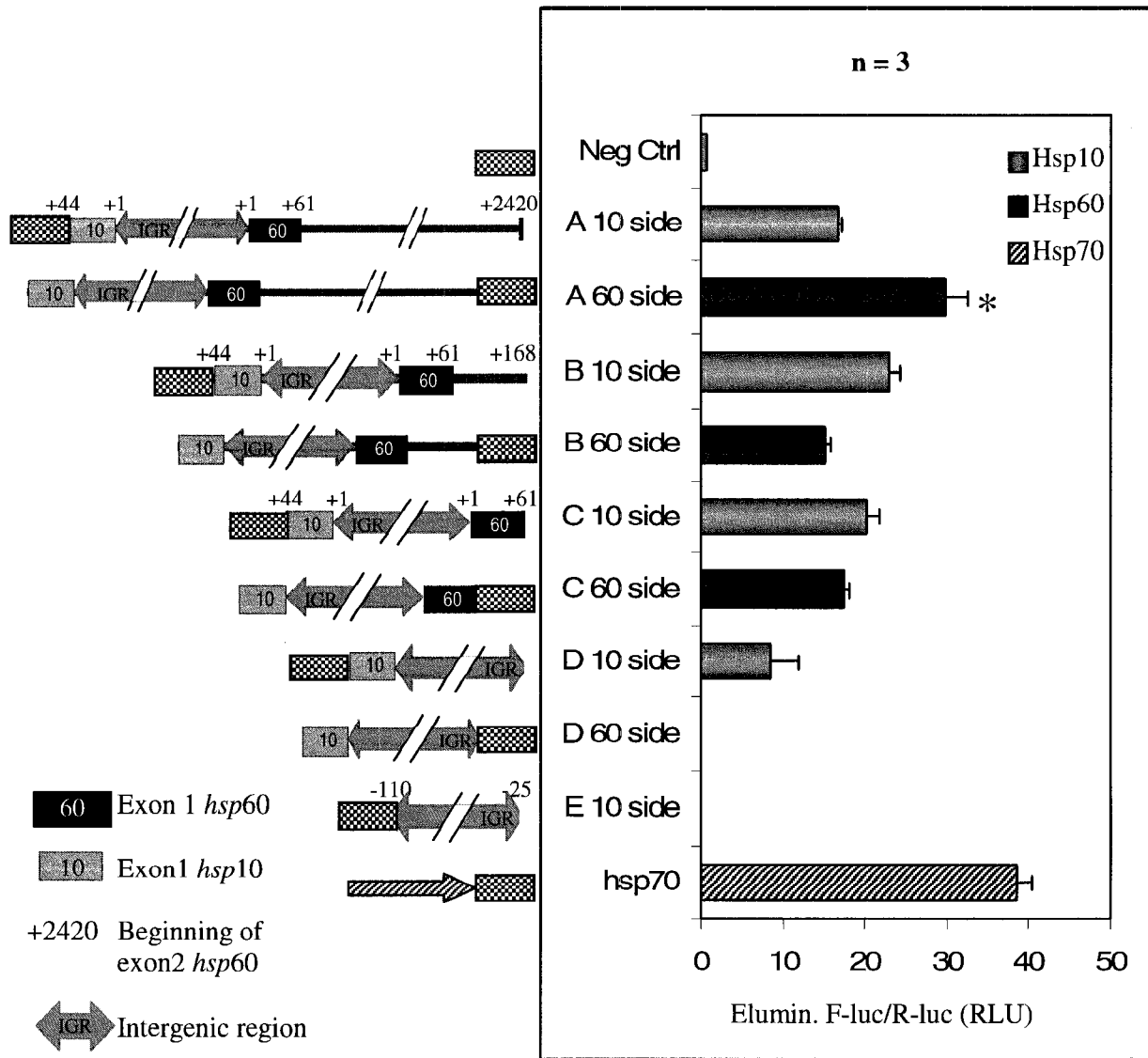


Figure 3-11. Promoter activity of the constructs containing different fragments from the zebrafish *hsp10/60* locus in 293 cells: on the left, a schematic map of the constructs is displayed; average of normalized luciferase activity, in relative light units (RLU), of the transiently transfected cells is shown on the right (n = 3). A promoter-less vector was used as a negative control. The "*" indicates a significant difference from the other constructs (P<0.05)

3.5 Inducibility

The intergenic region of the *hsp10* and *hsp60* genes in all studied organisms contains a heat shock element (HSE) which is responsible for promoter inducibility. Heat shock was applied as the typical stress condition used in most of the studies involving characterization of the promoter of a heat shock gene. Zebrafish is a tropical fish whose normal thermal condition is slightly higher than that of some other teleosts. The normal temperature for the zebrafish is around 28°C. Fish maintained at temperatures above 31°C will often develop abnormally and breed poorly. With a normal temperature in the wild for this species rarely exceeding 34°C, the standard heat shock condition for zebrafish is 37°C. This temperature would mimic the condition when the fish are subjected to multiple simultaneous stressors in their natural habitat. The durations of heat shock used in published studies for different heat shock gene promoters usually vary between 1 to 6 hours.

3.5.1 Inducibility of the endogenous promoter

To examine heat shock response in zebrafish embryos, wild type 24-hpf embryos were heat-shocked at 37°C for 1, 2, or 4 hours. Three hundred embryos were used for each heat shock treatment. Subsequently, mRNA was extracted shortly after heat shock, and RT-PCR was performed on the extracted mRNA. PCR primers used in this experiment were *hsp10* and *hsp60* gene-specific primers. Following heat shock, higher levels of *hsp10* and *hsp60* mRNA were detected with a maximum induction occurring after two hours of heat shock when expression levels almost doubled. A four-hour induction caused too much stress on the embryos resulting in high mortality (in about 10% of embryos while this number was less than two percent at other time points). The results are summarized in figure 3-12. A "no heat shock" group (28-hpf embryos) was added as controls and no

differences in expression were observed in this group compared to measurement at the onset of heat shock (24-hpf) (figure 3-12).

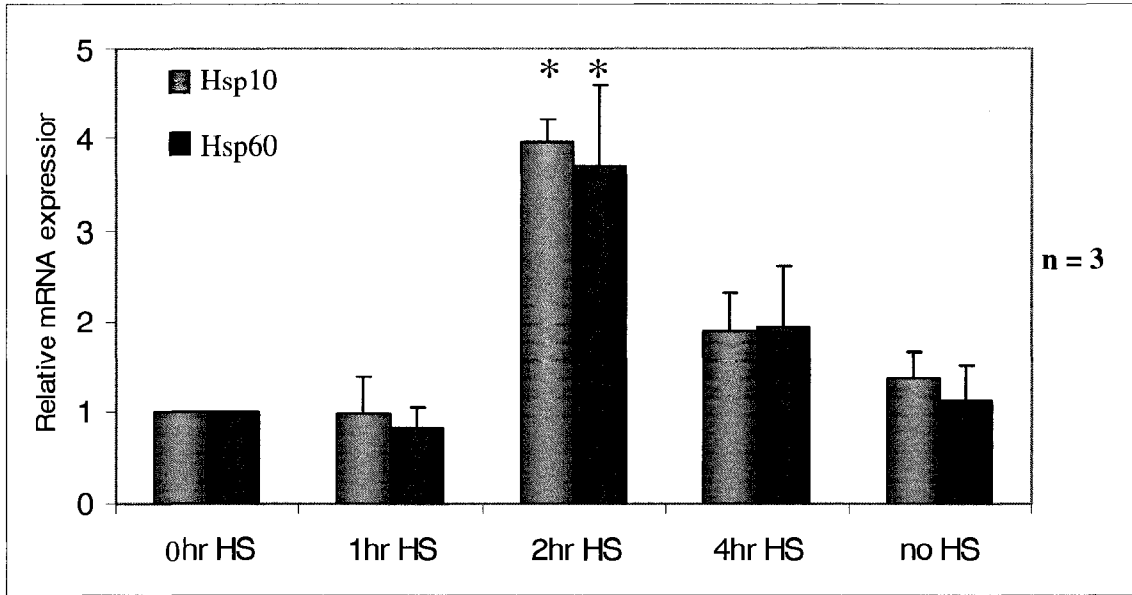


Figure 3-12. Inducibility of the endogenous *hsp10/60* promoter in 24hpf zebrafish embryos subjected to heat shock: Using real-time semi-quantitative PCR, the amount of endogenous *hsp10* and *hsp60* mRNA in 24hr wild type zebrafish embryos were measured using *hsp10* and *hsp60* gene-specific primers. Embryos were heat shocked (HS) for 1, 2 , or 4 hours; 24 hr non-heat shocked embryos were used as control (0hr HS). No heat shock group are wild type 28-hpf embryos kept at normal temperature; n = 3. The "*" indicates a significant difference from the 0hr HS group (P<0.05)

3.5.2 Promoter inducibility

To dissect the inducibility of the intergenic region of *hsp10/60*, we used the same reporter constructs as for promoter analysis (sections 3-2 to 3-4). In order to avoid the added complications resulting from the mosaicism of primary transgenic embryos, we used cultured ZF-4 or 293 cells for these experiments. To characterize promoter inducibility in both directions, the fragments containing the shortest active promoter were cloned into a firefly luciferase (Fluc) vector. Transfection efficiency was normalized by cotransfecting *Renilla* luciferase (Rluc).

3.5.2.1 Inducibility in ZF-4 cell line

Considering possible differences between ectothermal and homeothermal organisms, it was important to carry out these experiments in a cell line of zebrafish origin. Constructs were, therefore, transfected into ZF-4 cells. Following transfection, cells were maintained at 28°C (same as the maintenance temperature for zebrafish) for 24 hours. Subsequently, the transfected cells were heat shocked at 37°C for 1, 2, or 4 hours. An *hsp70* promoter construct was used as a positive control. Induction became detectable following 2 and 4 hours of heat shock. Approximately 2-3 fold induction was detected in response to heat shock for both orientations using "D10" and "C60" fragments (figure 3-13).

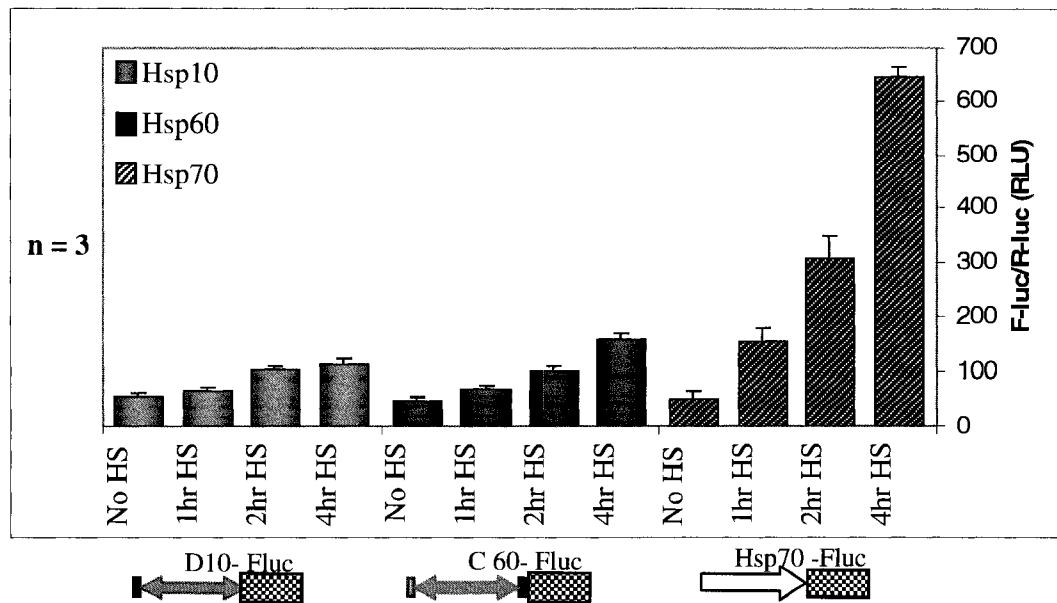


Figure 3-13. Analysis of inducibility of *hsp10/60* promoter in ZF-4 cells subjected to heat shock: Cells were co-transfected with *Renilla* luciferase (Rluc) and the indicated construct and the cells were heat shocked for 1, 2, or 4 hours. Luciferase assay was performed measuring the amount of expressed firefly luciferase (Fluc) upon heat shock (HS). The values were normalized against Rluc. Hsp70-Fluc was used as the positive control. A two-fold higher expression following two hrs of heat shock was significant comparing to the non-heat shocked group. The corresponding constructs are shown below the graph (n = 3).

3.5.2.2 Inducibility in mammalian cell line

We performed a similar inducibility study in mammalian 293 cells except that the cells were maintained at 37°C and heat shocked at 42°C. Heat shock response is a well-conserved reaction present in all organisms and induction of the *hsp10/60* is, therefore, also expected to take place in a mammalian cell context. Similar to what was observed in ZF-4 cells, following a one-hour heat shock, the expression levels did not change much, and maximum up-regulation happened after two hours of heat shock. A 2-fold induction was detected in both orientations in the "D10" and "C60" constructs. Expression levels observed after the 4-hour heat shock were low due to a high mortality of the cells and harsh experimental condition (figure 3-14).

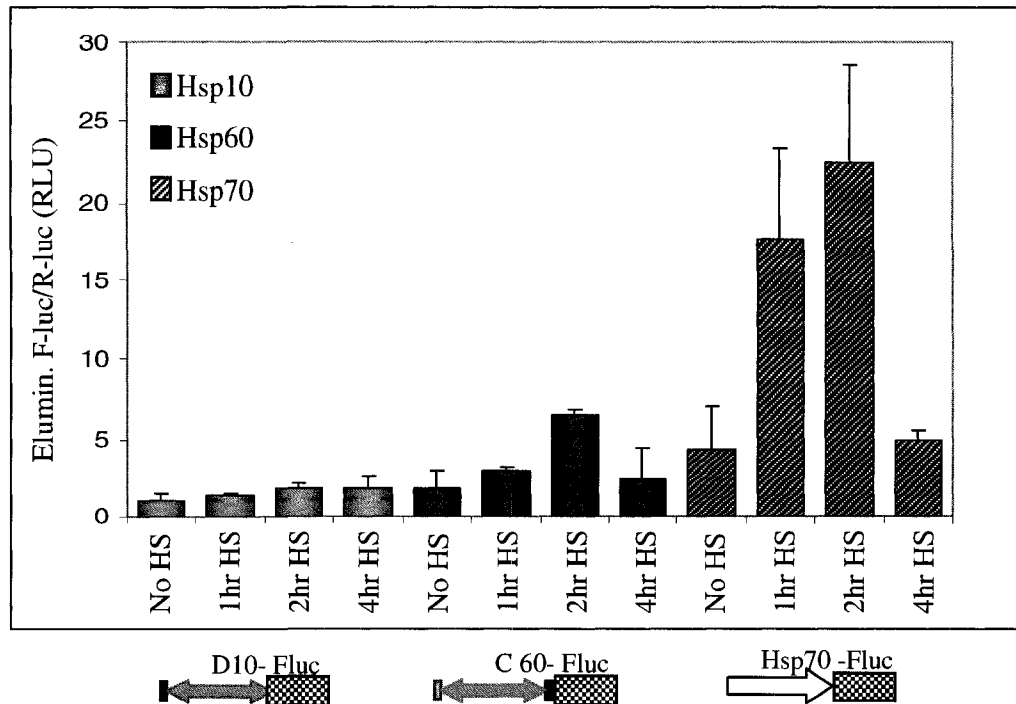


Figure 3-14. Inducibility analysis of *hsp10/60* promoter in mammalian 293 cells subjected to heat shock: Cells were co-transfected with *Renilla* luciferase (Rluc) plus the indicated construct and the cells were heat shocked for 1, 2, or 4 hours. Luciferase assays were performed measuring the amount of expressed firefly luciferase (Fluc) upon heat shock (HS). The values were normalized against Rluc. Hsp70-Fluc was used as the positive control. The induced expression following two hrs of heat shock was significant compared to non-heat shocked group. The corresponding constructs are shown below the graph (n = 3).

3.6 Internal deletions

When we compared the activities of the different promoter-containing fragments under basal (non heat-shock) conditions, only one displayed a significantly different activity. The promoter activity of "fragment A60" was twice as much as that of all the other constructs mentioned in section 3-3. Following deletion of the major part of the first intron of *hsp60* (Figure 3-15a), the promoter activity in the *hsp60* orientation showed a dramatic reduction although the 5'UTRs of both genes were still present in some of the constructs. The first exons of *hsp10* and *hsp60* contain the 5'UTRs. The 5'UTR might contain sequences that regulate translation efficiency or mRNA stability. Thus, there is probably a region within the intron that makes major contribution to transcriptional activity, rather than an mRNA stabilizing factor or a translation efficiency regulator within the 5'UTR. Otherwise, the asymmetrical expression should have been observed in all the fragments except fragment "D". Furthermore, following the deletion of the intron, strong *gfp* reporter expression is no longer observed in the yolk (compare figures 3-4 with 3-7b). This suggests the presence of an element within the intron enhancing the promoter activity in the *hsp60* direction. Deleting the intron might have eliminated the putative element from the construct causing the loss of the 2:1 expression ratio between the two orientations. We performed an *in silico* analysis using *Cis*-element Cluster Finder (Cister) software (<http://zlab.bu.edu/~mfrith/cister.shtml>) to find out if the intron sequence contains regions predicted as *cis*-acting elements. According to this analysis, two regions with high probability of occurrence of *cis*-acting elements were found (Fig. 3-15b). These are indicated as boxes in figure 3-15a. The region with larger coverage and higher probability, corresponding to the hatched box (figure 3-15a), was selected for further examination. To observe the impact of absence of this particular site, it was internally deleted from the "A60-GFP" construct. We also deleted subsets of the hatched region from "A60-GFP" (figure 3-16). All these constructs were injected into three hundred one cell-stage embryos and the expression pattern was investigated at 24-hpf.

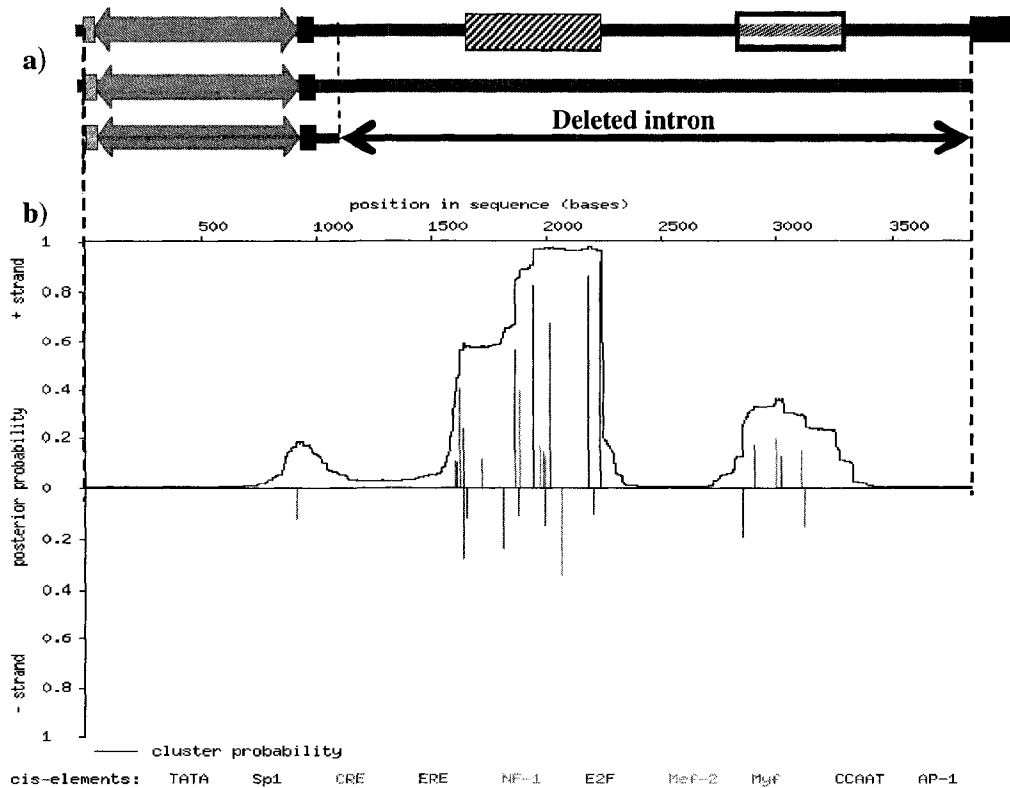
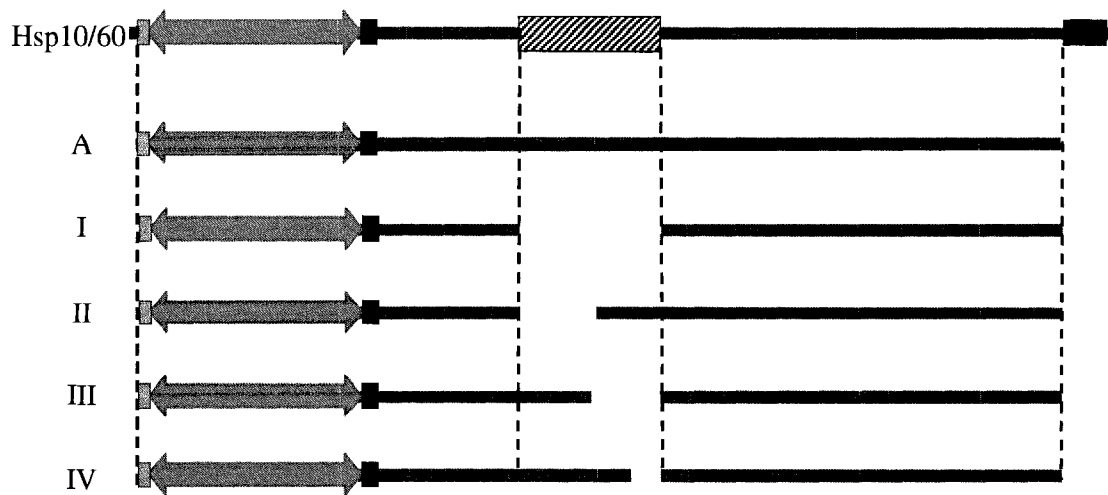


Figure 3-15. Prediction of *cis*-acting elements: a, Structure of the bidirectional transcriptional region of the zebrafish *hsp10/60* genes and of related fragments. The solid filled boxes show the exons (the grey box *hsp10* exon1 and the black ones *hsp60* exon1 and 2); the hatched and open boxed illustrate the *cis*-acting element prone regions; the hatched box is the dissected region. b, Cister analysis graph predicting the putative *cis*-acting elements within the sequence. Vertical lines within the graph indicate the probabilities that regulatory factors bind to the listed *cis*-elements at the given positions; the curve indicates the overall probability of being within a cluster of *cis*-elements bound by their factors; lines in the upper half of the plot indicate *cis*-elements on the direct strand, and lines in the lower half refer to the complementary strand; "Position in sequence" (the horizontal vector) values shows the nucleotide position starting from the end of the first exon of *hsp10* on the left end to the beginning of exon 2 of *hsp60* around 3800-bp downstream of the transcriptional start site (Martin et al., 2002) at the far right end.

3.6.1 Activity of internally deleted constructs in zebrafish embryos

By deleting the ~600-bp region (shown as a hatched box in figure 3-15) from "A60-GFP", the Deletion I construct (Figure 3-16) was produced. This internally deleted construct was introduced into one-cell stage zebrafish embryos. Embryos were inspected 24 hours later. The fluorescence produced in the embryos that received "Deletion I-GFP" construct was compared to the expression observed in embryos injected with the "A60-GFP" and "C60-GFP" (Figure 3-7 c). Deletion I-GFP showed expression in about 30-40 cells of 24-hpf primary embryos (Figure 3-17 D), a lower number than for "A60-GFP" but comparable to "C60-GFP".

Next, subsets of the ~600-bp region were deleted from the "A60-GFP" construct (Figure 3-16 Deletions II, III, and IV). Following injection into zebrafish embryos, "Deletion II-GFP", lacking a ~300-bp sequence, showed more GFP-expression cells comparing to "Deletion I-GFP", and was comparable to that of the full-length fragment (fragment A). Injection of "Deletion III-GFP" into zebrafish embryos resulted in reporter expression in fewer cells in comparison to the original construct (fragment A60) and similar to that of "C60-GFP". The high expression in the yolk was also missing (Figure 3-17). Finally, injecting "Deletion IV-GFP" into fish embryos resulted in an expression similar to that of "Deletion II-GFP" (figure 3-17), with GFP expression similar to that of the full-length construct. The latter was missing a ~200-bp sequence that overlaps with the deleted part in "Deletion III". The region missing from "Deletion III" seemed to be responsible for the higher expression and the YSL expression while the region missing from "Deletion IV" appeared to contribute little to the overall transcription. Therefore, fragment missing in "Deletion III" but present in "Deletion IV" contained the enhancer element shown as a bidirectional arrow in figure 3-17 "Deletion IV". For all of the internally deleted constructs, only the *hsp60* orientation was studied.



3-16 Internal deletions from the "A60-GFP" construct: Schematic map of the *hsp10/60* locus in zebrafish. The bidirectional arrow shows the intergenic region; the solid black boxes from left to right show exon 1 and exon 2 of *hsp60*, respectively. The grey box shows the first exon of *hsp10*. The hatched box shows the 600bp cis-acting element-prone region within the first intron of *hsp60*. "A" represents "fragment A". I to IV represent internal deletion of A60-GFP. The gaps are sequences that were deleted from fragment A.

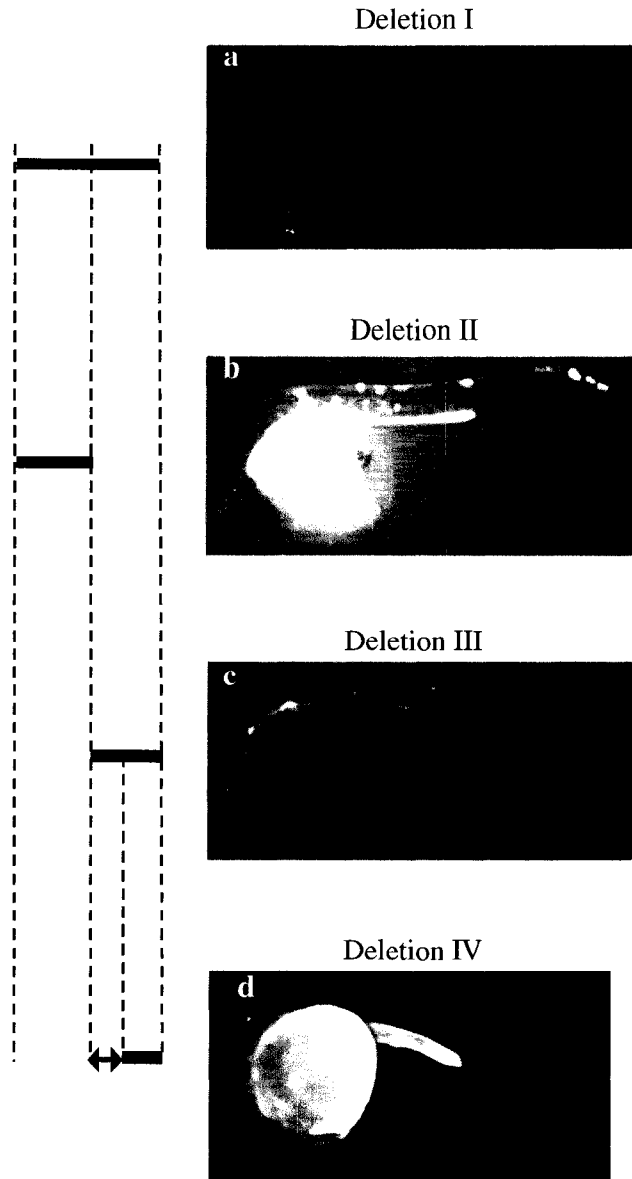


Figure 3-17. Analysis of A60-GFP deletion construction in primary embryos: Fluorescence microscopy images (lateral view) of 24-hpf primary transgenic embryos injected with the constructs carrying internal deletion of "A60-GFP". The number of cells expressing the transgene in embryos injected with "Deletion I-GFP" is much lower than that obtained with "A60-GFP"; "Deletion II-GFP" is expressed with levels comparable to those observed with "A60-GFP"; "Deletion III-GFP" is expressed with levels comparable to those observed with "C60-GFP"; "Deletion IV-GFP" is expressed with levels comparable to A60-GFP. The region that might include the putative YSL element is shown by the bidirectional arrow.

3.6.2 Quantitative analysis of transgene expression for the internal deletion constructs

We quantified the expression of deletion constructs in primary embryos using real-time PCR. RT-PCR was performed on the mRNA extracted from 24-hpf embryos that received different constructs containing the *hsp10/60* promoter with internal deletions and *gfp* as the reporter gene. For each construct, one hundred transient transgenic 24-hpf embryos that showed at least one GFP-expressing cell were collected. The 100 embryos were divided into three groups of about equal size. The mRNA from pooled embryos was extracted and was used in RT-PCR reactions. The comparative levels of GFP expression were measured using PCR primers specific to *gfp* mRNA. The semiquantitative results obtained from these embryos roughly matched the fluorescence microscopy observations. However, despite what was observed in figure 3-17, differences were observed between the intact "A60-GFP" and all the internal deletion constructs. The largest deletion, "Deletion I" showed the lowest expression and was used as the reference to generate relative expression ratios. "Deletion III-GFP" showed an expression comparable to that of "C60-GFP". This result might be due to the absence of expression in the YSL. "Deletion II" and "Deletion IV" fragments, expression levels were lower than the expression observed with "A60-GFP" but higher than those obtained with "Deletion III-GFP". According to these results, deleting subsets of the fragment removed from "Deletion I", led to different levels of expression, which are all less than the intact "A60-GFP" as shown in Figure 3-18.

We also subjected one hundred embryos to a 2-hour heat shock. The mRNA was extracted immediately and reporter gene expression was determined by RT-PCR. The expression levels obtained from the heat shock group were compared to the results obtained from embryos that were kept under normal condition. All constructs displayed an approximate 2-fold induction, as shown in Figure 3-18.

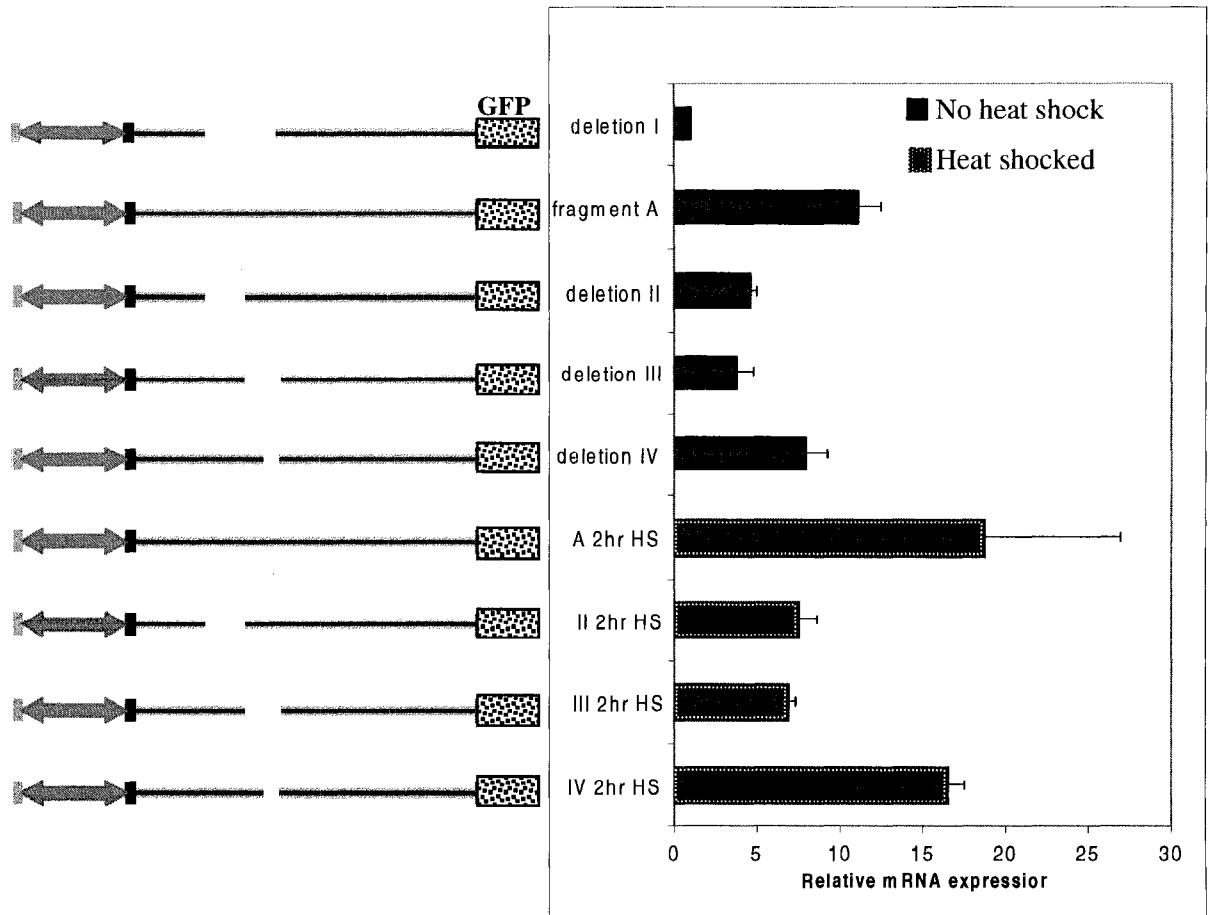


Figure 3-18. Semiquantitative activity and inducibility analysis of internal deletion constructs in zebrafish embryos: The graph shows the levels of *gfp* expression under the control of internally deleted fragments in 24-hpf embryos subjected to normal or heat shock (HS) conditions. Embryos were heat shocked (HS) for two hrs; embryos that received constructs containing "Deletion I" are used as reference. For normal conditions, sample size $n = 6$ and for heat-shocked, samples size $n = 3$. Constructs are schematized on the left. The black solid boxes are exon 1 of *hsp60* followed by the thick line representing the first intron of *hsp60*. The grey boxes show the first exon of *hsp10*. GFP coding region is shown as the patterned boxes. The gaps within the intron demonstrates region of internal deletions. These results did not show a significant statistical difference but they corollate with other observation.

Discussion

Heat shock proteins are required for protein folding and adaptive response against stressful conditions in all cells. Their expression is upregulated when cells are exposed to elevated temperatures or other sorts of stress. The dramatic upregulation of the heat shock protein genes requires an inducible promoter responding efficiently to stressful conditions. The induction of *hsp10* and *hsp60* genes is transcriptionally regulated. The bidirectionality of the promoter of the *hsp10/60* gene, which enables it to control the expression of two different genes simultaneously, makes it particularly interesting.

Using GFP and luciferase reporter genes, we were able to demonstrate the bidirectionality of the promoter of *hsp10/60* genes. We were also able to assess the functionality of a promoter-including region in the two orientations and, after deleting some sequences, in both normal and heat shock conditions. Although, the use of reporter genes is an indirect measurement of the transcriptional properties of the test DNA, the reporter gene activity is, generally, proportional to transcriptional activity of the studied promoter and associated regulatory elements (Alam and Cook, 1990).

4.1 The *hsp10/60* promoter

The genomic organization and the predicted amino acid sequences of the *hsp10* and *hsp60* genes in zebrafish are similar to those of flounder, rat, and human indicating the importance of these well-conserved genes. The size of the intergenic region, in other words, the DNA sequence that separates the two transcription start sites, is about 860-bp. This is consistent with the size of the intergenic region of other bidirectional transcription units, which is generally less than 1 kb.

HSE is the consensus sequence found in the promoter region of all of the heat shock protein genes. It is the element providing inducibility in response to heat shock and other forms of stress. Unlike other

hsp promoters, the *hsp10/60* genes of many organisms contain only one HSE element. The zebrafish *hsp10/60* promoter contains a single three-tandem HSE, a situation similar to what has been found in other organisms. The sequence of the HSE is "cGAAa tTTCt cGAAC" with a very good match to the consensus sequence. The perfect HSE sequence is "aGAAn" or "gGAAn" (Maniak and Nellen, 1990), but a sequence with an almost perfect match (nGAAn) is capable of responding to heat shock (Fischer-Kierzkowska et al., 2003). HSE sequences in human, rat, and flounder show the perfect match for all of the three tandems. Their HSE sequences are: "aGAAa aTTCt gGAAG" in flounder, "gGAAG gTTCt aGAAC" in rat, and "gGAAa gTTCt gGAAC" in human. Since HSE is a palindromic sequence, it could work on either DNA strand and could act bidirectionally.

Bidirectional promoters in mammals are usually TATA-less and GC-rich. No TATA box was found in the zebrafish *hsp10/60* promoter region. TATA-less promoters usually control housekeeping genes (Liao et al., 1994). The TATA box is one of the elements that regulate directionality of transcription. However, some TATA-less promoters show strong directionality and some bidirectional promoters include a TATA box.

A couple of well-aligned 10-17 bp GC-rich (70%- 91%) regions were found in all the four *hsp10/60* gene sequences. No GC box (SPI binding site) was found within the intergenic region of the zebrafish *hsp10/60* gene. The perfect sequence of a GC box consensus site is "(G/T)GGGCGGGC" however "GGGCGG" works as well. Unlike zebrafish, putative GC box *cis*-acting elements have been found in the *hsp10/60* promoters of human, rat, and flounder. The GC rich regions found in the zebrafish promoter might act as non-perfect GC boxes. Bidirectional promoters usually have a high median G+C content. The G+C content of bidirectional promoters in mammalian species is 66%, which is 53% for mammalian unidirectional promoters (Trinklein et al., 2004). The G+C content of the zebrafish *hsp10/60* bidirectional promoter is 41.2%. This value is 47.5% in flounder (Nam et al.,

2006). Considering the general lower G+C content in lower vertebrates as an evolutionary phenomenon, these numbers are quite high for fish species. It is suggested that the higher G+C content in higher vertebrates is an adaptation for the homeothermal organisms considering their thermal profile.

No major putative CAAT box was detected in the zebrafish sequence as opposed to the rat and flounder orthologs. No CAAT box was found in the human promoter. The CAAT box is a eukaryotic *cis*-acting element whose consensus sequence is "GGNCAATCT". General transcription factors bind to this site. CAAT box is required for expression of sufficient amount of the transcription in some genes. The position of the CAAT box usually occurs at about 75-80 nucleotides upstream of the transcription initiation site (TIS). The putative CAAT box in rat *hsp10/60* is located at around -360 from *hsp60* TIS and within the first intron of *hsp10*. In flounder, it is located at around -340 from *hsp60* TIS and -200 from the *hsp10* TIS. Experimental alterations of the CAAT box in flounder decreased the expression level of the promoter, especially at the *hsp60* orientation, but the promoter with degenerated or deleted nucleotides at the CAAT site was still able to drive the bidirectional expression of a luciferase reporter gene (Nam et al., 2006).

Outside the coding region, not much sequence similarities is found between *hsp10/60* and characterized orthologs. Thus, limited similarity is found in non-coding sequences such as the UTRs and intergenic regions. The zebrafish locus has both the largest intergenic region and *hsp60* 5'UTR among these four organisms. It is almost twice as large as those of rat and human. The zebrafish intergenic region shows higher similarities to the orthologous region in flounder than to its mammalian counterparts. The size of the 5'UTR region in the fish species is larger than the 5'UTR in rat and human. Table 4.1 compares the size of these regions in zebrafish, flounder, rat, and human.

Table 4-1. Differences in the size of intergenic region, UTR and studied regions in different organisms

	Intergenic region	Hsp60 1st intron	Size of the studied regions
Zebrafish	860bp	2365	860-3300bp
Flounder	550	1666	604 bp
Human	538	923	602 bp
Rat	482	817	582 bp

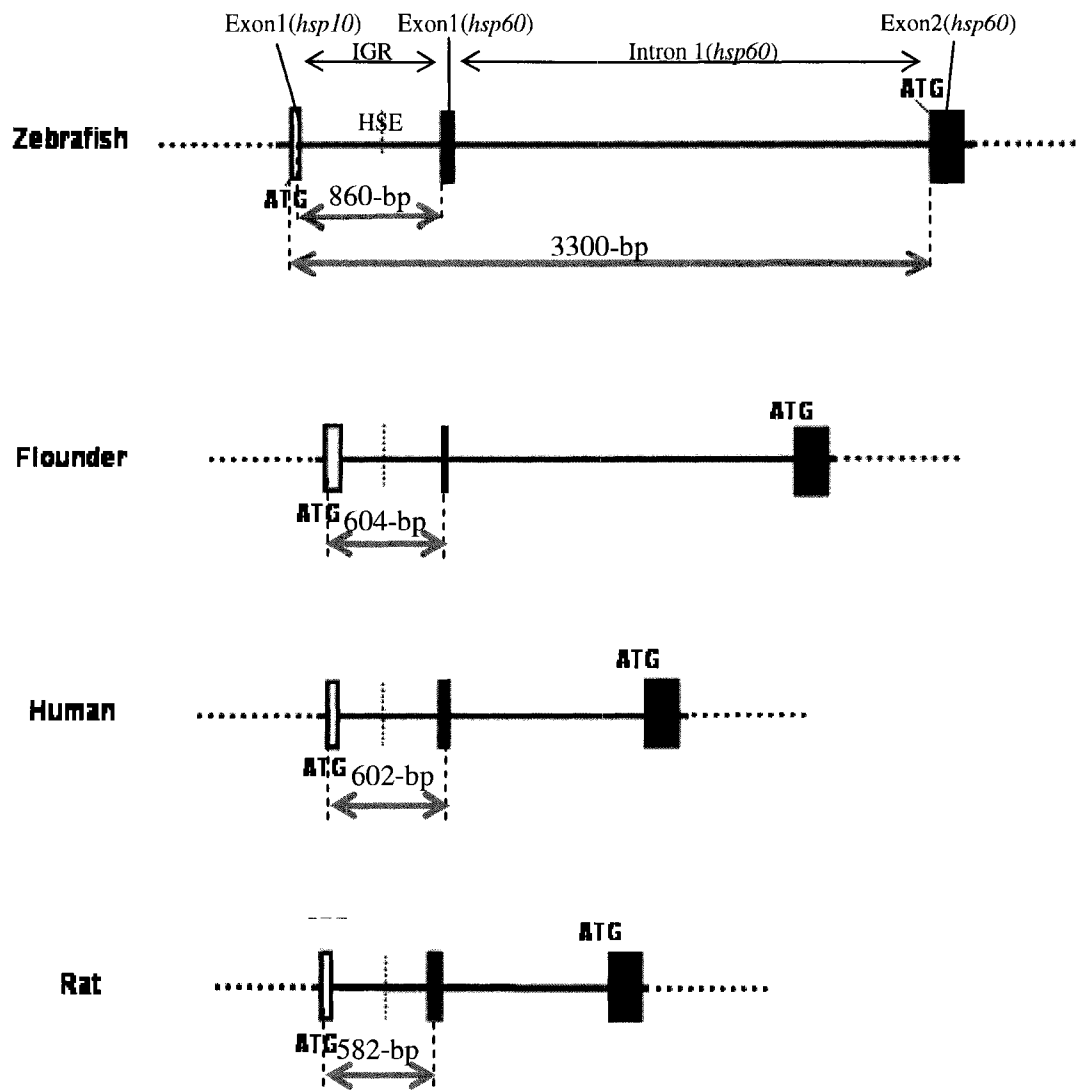


Figure 4-1. Fragments studied in different organisms: the schematic map is a duplicate of figure 3-1. The grey arrows indicate the fragment used in studies conducted in different organisms to study the *hsp10/60* promoter.

4.2 Bidirectional promoter

Bidirectional promoters are not common; however, they are not very rare. The genome-wide studies conducted recently have rejected the idea of complete random genetic order in vertebrates. Instead, they have revealed the existence of gene clusters when comparable and/or coordinated expression of genes are needed (Trinklein et al., 2004; Li et al., 2006). Transcription of many genes is coupled by sharing a bidirectional promoter in an antisense orientation (Bellizzi et al., 2007; Chen et al., 2007). Bidirectional promoters have been shown to facilitate the control of functionally linked related genes (Gavalas and Zalkin 1995; Segal et al., 2001).

Almost all of the studies on bidirectional promoters have been conducted on mammalian species. At this time, *hsp10/60* is the only known bidirectional promoter in zebrafish. The present study has showed that the 980-bp intergenic region of these genes is able to act as a promoter regardless of its orientation. As a result, cloning any structural gene in either the *hsp10* or *hsp60* orientations is able to drive the expression of a downstream gene. Since we have used reporter genes for this study, we were able to observe or measure promoter activity with both orientations of the *hsp10/60* regulatory region in zebrafish embryos, as well as in two different types of cell lines. Moreover, the bidirectional expression was assessed at the transcription level using RT-PCR on the *gfp* mRNA. Trinklein et al. (2004) have shown that a bidirectional promoter might not act bidirectionally in all situations and only one direction might be active in a cell-specific manner. To address this phenomenon to some extent, two different cell lines with various origins have been used in this study. In both mammalian 293 cells and zebrafish ZF-4 cells, both orientations were active and resulted in expression of downstream genes.

4.3 Expression profile of the bidirectional promoter

In bidirectional promoter studies, the expression of the promoter in each direction and comparison of the activity on the different directions are commonly investigated. In some cases, the expression of both orientations is the same; however, some other promoters reveal a different level of expression in each direction. In transient experiments, the *hsp10/60* fragment "A" displayed an asymmetric bidirectional promoter activity with a high promoter activity for the *hsp60* direction. This has been confirmed through different experimental approaches. These approaches include direct viewing of GFP utilizing fluorescence microscopy, luciferase assays on zebrafish embryos and transfection experiments in ZF-4 and 293 cells. RT-PCR was used as a sensitive method to measure mRNA levels. Contrarily to reporter gene expression, RT-PCR also provided an estimate of promoter activity without the interference of post-translational steps.

Direct inspection of the GFP expression in embryos provided the advantage of *in-situ* observation of gene expression and a rough estimate of the relative level of expression in different cells and tissues. However, the stability of the long-lasting GFP version used in this experiment might have impacted the accuracy of quantitative measurements. Luciferase assays, which take advantage of the short turnover time of luciferase protein, provided accuracy.

Luciferase assays carried out on both cultured cells and embryos resulted in a two-fold higher expression in the *hsp60* direction when compared to the *hsp10* orientation when fragment "A" was used. RT-PCR performed on the *gfp* mRNA extracted from transient transgenic zebrafish embryos led to the same results. In contrast, the expression on the *hsp60* orientation in zebrafish embryos based on direct fluorescent observation, seemed a lot more intense than for *hsp10* orientation. In addition, a strong GFP fluorescence was present in the yolk region. This experiment utilized a stable form of eGFP. This version of GFP allows its accumulation and easy detection in the cells but it may

causes problem in dynamic studies. The combined effect of high promoter activity and low protein turnover in the yolk were probably responsible for this observation. This problem could be resolved by using the destabilized forms of GFP.

The *hsp10* and *hsp60* genes show ubiquitous expression in zebrafish embryos. Unlike Hsp70, which is expressed in most of the tissues only after heat shock, chaperonins show background expression under normal condition. Thus, *hsp60* expression was previously observed in the yolk syncytial layer, particularly during gastrulation (Thisse et al., 2001). In our transgenic experiments, the GFP produced by the YSL reflected endogenous gene expression and diffused into the whole yolk slowly until it became totally saturated.

Despite the absence of *hsp70* expression in most cell types and tissues under normal condition, we observed some expression of an *hsp70* promoter construct prior to heat shock. Basal levels of expression of *hsp70* had been reported in some tissues such as eye lenses. Therefore, its expression in some cell types was not completely surprising.

To find a smaller region of the *hsp10/60* locus that could function as an active promoter, deletions were performed on the ends of "fragment A". A relatively large intron is located immediately before the ATG start codon of *hsp60*, at the end of "fragment A". This intron contains a number of ATG sequences in frame with codons introducing translation stop. Deletion of intron sequences involved in splicing might result in an abnormal mature mRNA that contains intronic sequences with the above start and stop codons and, therefore, prevent expression of the reporter gene. To avoid the premature start and stop of translation, we needed to either include the splicing donor, splicing acceptor, and A-branch side to enable correct splicing or exclude as many of such ATG sequences from the fragments as possible.

When the fragment contained only the intergenic region or the intergenic region plus the immediately adjacent sequences, equal levels of expression was observed for both orientations. The YSL expression was also absent. The difference between "fragment A" and "fragment B" is a major part of the first intron of *hsp60*. Thus, there is probably an element located within the first intron that is responsible for the differential expression between the two orientations. This, or another element, would also be needed for the YSL expression. Dissecting this region, *in silico*, revealed two regions qualified as *cis*-acting elements-prone sites. We carried out multiple deletions within one of these two regions but could not clearly pinpoint any important *cis*-regulatory elements. Thus, deletion of a 600bp sequence, corresponding to one of the predicted *cis*-acting-prone sites resulted in a reporter construct with little transcriptional activity. We then deleted subsets of the 600-bp sequence but such deletions did not lead to any major decrease in reporter expression. The largest deletion might have hindered downstream steps such as splicing.

Deletion of a ~300bp intronic region affected the YSL expression, specifically (figure 3-17). When a smaller subset of this 300bp region was deleted, the YSL-specific expression was reinstated, suggesting the presence of a putative element affecting this tissue-specific expression. Thus, we identified an 86-bp region within (sequence is shown in figure 4-2) the first intron of *hsp60* that is putatively responsible for the YSL expression under normal (non-heat shock) conditions. This region is located around 900bp downstream of the *hsp60* transcription initiation site. It might include the *cis*-acting element that differentiates the expression pattern from the full-length *hsp60* fragment from the rest of the constructs. The results obtained measuring GFP protein roughly matched the ones from measuring *gfp* mRNA. The fluorescence microscopy observation suggested that the expression levels of "Deletion II" and "Deletion IV" are similar to "A60-GFP". Although, the RT-PCR results did not show significant statistical differences, they showed the same trend as the mRNA results. The levels of mRNA expression obtained with "Deletion II" and "Deletion IV" embryos are lower than the

expression obtained with “A60-GFP” construct. The lasting presence of the GFP protein due to its stability results in an overestimate of expression. The finding of the region responsible for YSL expression can be supplemented with protein/DNA interaction assays such as electrophoretic mobility shift assay (EMSA), ribonuclease protection assay, and chromatin immuno precipitation (ChIP).

1 GATATTATCC TTGTTTCAGAT ATTTTTTTCT GTGTGTGACT GCCATGCATT
51 AAATGTACTC TATGCCTAAC CGAACACCGA AATTGT

Figure 4-2. Sequence of the YSL element in zebrafish.

4.4 Bidirectional inducibility of the promoter

The transcriptional activity of inducible promoters increases in the presence or absence of biotic or abiotic factors. Inducers include chemicals such as alcohols, steroids, and oxygen or physical conditions such as heat, cold, and light. In many cases, these promoters are also able to operate under normal conditions, albeit at lower levels. The typical inducer for the promoters of *hsp* genes is an elevation in temperature. When normal zebrafish embryos were subjected to heat shock (37°C), the level of *hsp10* and *hsp60* mRNA molecules increased. Maximum induction was observed following two hours of heat shock, while a one-hour heat shock did not show considerable induction, perhaps due to the time required to warm up the experimental set up. When heat shock induction was tested in transient transgenic zebrafish embryos, an approximately 2- to 3-fold induction was observed following two hours of heat shock, when we measured GFP reporter transcript levels by RT-PCR. To be able to measure the differences accurately, parallel studies were carried out with luciferase reporter constructs in transfected cells in culture. Confirming the previous results, a 2- to 3-fold increase in expression was measured in both zebrafish ZF-4 cells and mammalian 293 cells. The fold increase was almost the same, regardless of the orientation of the *hsp10/60* regulatory fragment. Slight differences were detected between the inductions in mammalian cells in comparison to the zebrafish cells. The heat shock response is a universal reaction but it might be slightly different in the cells with homeothermal origin when compared to cells with ectothermal origin. ZF-4 cells were better in handling heat shock condition for extended time and less mortality was observed in this group of cells. When the mammalian cells were subjected to long-term heat shock, visible changes in the well-being of the cells were detected. This might be due to the differences between the cells or to the fact that we had to use different heat shock conditions, as the normal temperature for the growth of cells are distinct. Quicker production of metabolites in a relatively small volume could be the

reason of high mortality after the 4-hour heat shock. Induction had been observed in the *hsp10/60* promoter of human, rat, and flounder. Studies of the human promoter were conducted using luciferase assays in 293 cells, one of the two cells lines used in my studies. Heat shock increased luciferase activity by a factor of approximately 12-fold in both directions of the human promoter (Hansen et al., 2003). Studies of the rat *hsp10/60* promoter were done in COS cells with luciferase as the reporter in the *hsp10* orientation and chloramphenicol acetyl-transferase (CAT) as a reporter gene in the *hsp60* orientation. Following heat shock, 5.2-fold induction was reported for the *hsp10* orientation while this amount was 3.4-fold in the *hsp60* orientation (Ryan et al., 1997). Finally, the flounder *hsp10/60* promoter was studied in *Epithelioma papulosum cyprinid* (EPC) cells, of carp origin. Heat shock induction caused around 2-fold induction in both orientations (Nam et al., 2006). Overall, the fold-inductions of the *hsp10/60* promoter in the three species are comparable to those we observed for the zebrafish *hsp10/60* promoter with the same order of magnitude. Induction folds were slightly lower in the fish species, which might reflect an adaptation to a wider range of temperature fluctuations fish encounter being ectothermal organisms. Being ectothermal animals, zebrafish and flounder adapt the temperature of their environment. This might also result in higher tolerance towards stress and wider fluctuations in the temperature.

Deleting the internal parts of the intron did not affect the inducibility of the promoter. As long as the intergenic region was intact, the construct was inducible as a result of the presence of the HSE in the intergenic region.

Conclusion

According to the present study, zebrafish hsp10 and hsp60 genes are linked in a head-to-head manner sharing a common promoter region. Therefore, the promoter of hsp10/60 genes is functional in two different orientations and simultaneously activates the transcription of the two genes that are located on the opposite DNA strands. The promoter contains a single three-tandem heat shock element located within the intergenic region between these genes. As a result, the promoter is inducible, beside its background activity that could be detected in any condition. Stressful conditions such as heat shock, increases the expression of these genes resulting is heat shock response. Following two hours of heat shock of wild-type embryos, primary embryos, or transient transgenic cells a two-fold induction has been observed in both orientations. When a larger fragment containing the first intron of hsp60 has been tested, an asymmetrical expression has been detected in which the activity of hsp60 orientation is double the hsp10 orientation. Moreover, this fragment causes some YSL expression seen in the external YSL at gastrula stage. When a major part of this intron was deleted, the promoter was still active and inducible, but the asymmetrical activity was turned into equal expression in both orientations and the specific expression related to YSL was absent. This observation suggests the presence of a cis-acting element within the first intron of hsp60 gene. Some manipulation on a region of this intron did not affect the 2:1 expression of the large fragment, however; the YSL expression was distracted. A 86-bp region seems to include a site that is linked to this tissue-specific activity.

A region of the promoter that includes the transcription initiation sites, which are 860-bp apart, is required for the promoter functionality and inducibility. Inducibility of this promoter and its known functionality makes it a good candidate to be used as a controllable bidirectional promoter in

producing transgenic zebrafish. It enables simultaneous study of two different genes or coexpression of a reporter gene with an experimental protein to visualize its expression.

References

- Abremski K, Hoess R (1984) Bacteriophage P1 site-specific recombination: purification and properties of the Cre recombinase protein. *Journal of Biological Chemistry* **259**, 1509-1514.
- Abremski K, Hoess R, Sternberg N (1983) Studies on the properties of P1 site-specific recombination: evidence for topologically unlinked products following recombination. *Cell* **32**, 1301-1311.
- Adachi N, Lieber MR (2002) bidirectional gene organization: A common architectural feature of the human genome. *Cell* **109**, 807-809
- Adam A, Bartfai R, Lele Z, Krone PH, Orban L (2000) Heat-inducible expression of a reporter gene detected by transient assay in zebrafish. *Experimental Cell Research* **256**, 282-290.
- Agabian N (1990) Trans splicing of nuclear pre-mRNA. *Cell* **61**, 1157-1160
- Agashe VR, Hartl FU (2000) Roles of molecular chaperones in cytoplasmic protein folding. *Cell and Developmental Biology* **11**, 15-25
- Ahn, J, Gruen, JR (1999). The genomic organization of the histone clusters on human 6p21.3. *Mammalian Genome* **10**, 768-770.
- Airaksinen S, Rabergh CMI, Lahti A, Kaatrasalo A, Sistonen L, Nikinmaa M (2003) Stressor-dependent regulation of the heat shock response in zebrafish, *Danio rerio* *Comparative Biochemistry and Physiology A* **134**, 839 -846
- Airaksinen S, Råbergh CMI, Sistonen L, Nikinmaa M. (1998). Effects of heat shock and hypoxia on protein synthesis in rainbow trout (*Oncorhynchus mykiss*) cells. *Journal of Experimental Biology* **201**, 2543-2551.
- Alam J, Cook JL (1990) Application to the study of mammalian gene transcription. *Analytical Biochemistry* **188**, 245-254.

- Ali A, Bharadwaj S, O'Carroll R, Ovsenek N (1998) HSP90 interacts with and regulates the activity of heat shock factor 1 in *Xenopus* oocytes. *Molecular and Cellular Biology* **18**, 4949-4960
- Amin J, Ananthan J, Voellmy R (1988) Key features of heat shock regulatory elements. *Molecular and Cellular Biology* **8**, 3761-3769
- Ananthan J, Goldberg AL, Voellmy R (1986) Abnormal Proteins Serve as Eukaryotic Stress Signals and Trigger the Activation of Heat Shock Genes. *Science* **232**, 522-524
- Anfinsen CB (1973). Principles that govern the folding of protein chains. *Science* **181**, 223-230
- Bai Q, Garver JA, Hukriede NA, Burton EA (2007) Generation of a transgenic zebrafish model of Tauopathy using a novel promoter element derived from the zebrafish *eno2* gene *Nucleic Acids Research* **35**, 6501-6516
- Bajoghli B, Aghaallaei N, Heimbucher T, Czerny T. (2004) An artificial promoter construct for heat-inducible misexpression during fish embryogenesis. *Developmental Biology* **271**, 416-30
- Balciunas D, Davidson AE, Sivasubbu S, Hermanson SB, Welle Z, Ekker SC (2004) Enhancer trapping in zebrafish using the Sleeping Beauty transposon. *BMC Genomics* **5**, 62-77.
- Baler R, Dahl G, Voellmy R. (1993) Activation of human heat shock genes is accompanied by oligomerization, modification, and rapid translocation of heat shock transcription factor HSF1. *Molecular and Cellular Biology* **13**, 2486-2496
- Bellizzi D, Dato S, Cavalcante P, Covello G, Di F. Passarino G, Rose G, De Benedictis G (2007) Characterization of a bidirectional promoter shared between two human genes related to aging: *SIRT3* and *PSMD13*. *Genomics* **89**, 143-150
- Bienz M, Pelham HR (1986) Heat shock regulatory elements function as an inducible enhancer in the *Xenopus hsp70* gene and when linked to a heterologous promoter. *Cell* **45**,753-760
- Blumenthal T, (2004) Operons in eukaryotes. *Brief Functional Genomic and Proteomic* **3**, 199–211

- Boellmann F, Guettouche T, Guo Y, Fenna M, Mnayer L, Voellmy R. (2004), DAXX interacts with heat shock factor 1 during stress activation and enhances its transcriptional activity, *Proceedings of the National Academy of Science U.S.A.* **101**, 4100–4105.
- Borges JC, Ramos CHI (2005) Protein folding assisted by chaperons *Protein and Peptide Letters* **12**, 257-261
- Braastad C.D., Leguia M., Hendrickson E.A. (2002) Ku86 autoantigen related protein-1 transcription initiates from a CpG island is induced by P53 through a nearby response element. *Nucleic Acids Research* **30**, 1713-1724
- Braig K, Simon M, Furuya F, Hainfeld JF, Horwich, AL (1993) A polypeptide bound by the chaperonin groEL is localized within a central cavity. *Proceedings of the National Academy of Science U.S.A.* **90**, 3978–3982
- Branda CS, Dymecki SM (2004) Talking about a Revolution: the impact of site-specific recombinase on genetic analyses in mice *Developmental Cell* **6**, 7-28
- Brett JR (1971). Energetic response of salmon to temperature. A study of some thermal relations in the physiology and freshwater ecology of sockeye salmon (*Oncorhynchus nerka*). *American Zoologist* **11**, 99–113
- Britten RJ, Davidson EH (1969) Gene regulation for higher cells: a theory. *Science* **165**, 349-357
- Brunet A, Kanai F, Stehn J, Xu J, Sarbassova D, Frangioni JV, Dalal SN, DeCaprio JA, Greenberg ME, Yaffe MB. (2002) 14-3-3 transits to the nucleus and participates in dynamic nucleocytoplasmic transport. *The Journal of Cell Biology* **156**, 817-828
- Buckley BA, Hofmann GE (2002) Thermal acclimation changes DNA-binding activity of heat shock factor 1 (HSF1) in the goby *Gillichthys mirabilis*: implications for plasticity in the heat-shock response in natural populations. *Journal of Experimental Biology* **205**, 3231–3240
- Bukau B, Horwich AL (1998) The *hsp70* and *hsp60* chaperon machines. *Cell* **92**, 351-366

- Burbelo PD, Martin GR, Yamada Y (1988) $\alpha 1(IV)$ $\alpha 2(IV)$ collagen genes are regulated by a bidirectional promoter a shared enhancer. *Proceedings of the National Academy of Science U.S.A.* **85**, 9679- 82
- Calabrese V, Mancuso C, Sapienza M, Puleo E, Calafato S, Cornelius C, Finocchiaro M, Mangiameli A, Di Mauro M, Stella AM, Castellino P (2007) Oxidative stress and cellular stress response in diabetic nephropathy. *Cell Stress Chaperones* **12**, 299-306.
- Cappello F, Bellafiore M, David S, Anzalone R, Zummo G (2003) Ten kilodalton heat shock protein (HSP10) is overexpressed during carcinogenesis of large bowel and uterine exocervix. *Cancer Letters* **196**, 35-81.
- Carvan MJ, Dalton TP, Stuart GW, Nebert DW (2000) Transgenic Zebrafish as Sentinels for Aquatic Pollution, *Annals of the New York Academy of Sciences* **919**, 133-147
- Cechetto JD, Soltys BJ, Gupta RS (2000) Localization of mitochondrial 60-kDa heat shock chaperonin protein (Hsp60) in pituitary growth hormone secretory granules and pancreatic zymogen granules. *Journal of Histochemistry & Cytochemistry* **48**, 45-56.
- Chandrasekhar GN, Tilly K, Woolford C, Hendrix R, Georgopoulos C (1986) Purification and properties of the groES morphogenetic protein of *Escherichia coli* *Journal of Biological Chemistry* **261**, 12414-12419
- Chatellier, J Buckle, AM Fersht AR (1999) GroEL recognises sequential and non-sequential linear structural motifs compatible with extended β -strands and α -helices. *Journal of Molecular Biology* **292**, 163-172
- Chen L, Sigler PB (1999) The Crystal Structure of a GroEL/Peptide Complex: Plasticity as a Basis for Substrate Diversity. *Cell* **99**, 757-768
- Chen PY, Chang WS, Chou RH, Lai, YK, Lin SC, Chi CY, Wu SW (2007) Two non-homologous brain diseases-related genes, SERPIN11 and PDCD10, are tightly linked by an asymmetric bidirectional promoter in an evolutionarily conserved manner. *BMC Molecular Biology* **8**, 2-16

- Chen S, Roseman AM, Hunter AS, Wood SP, Burston SG, Ranson NA, Clarke AR, Saibil HR (1994) Location of a folding protein and shape changes in GroEL-GroES complexes imaged by cryo-electron microscopy *Nature* **371**, 261–264.
- Clos J, Westwood JT, Becker PB, Wilson S, Lambert K, Wu C (1990) Molecular cloning and expression of a hexameric Drosophila heat shock factor subject to negative regulation. *Cell* **63**, 1085-1097
- Connelly MA, Zhang H, Kieleczawa J, Erson CW (1998) The promoter for human DNA-PKcs MCM4 divergently transcribed genes locates chromosome 8 b q11. *Genomics* **47**, 71-83
- Cotto JJ, Kline M, Morimoto RI (1996) Activation of heat shock factor 1 DNA binding precedes stress-induced serine phosphorylation. *Journal of Biological Chemistry* **271**, 3355-3358
- Davidson AE, Balciunas D, Mohn D, Shaffer J, Hermanson S, Sivasubbu S, Cliff MP, Hackett PB, et al. (2003) Efficient gene delivery and gene expression in zebrafish using the Sleeping Beauty transposon. *Developmental Biology* **263**,191-202
- Dietz TJ, Somero GN (1992). The threshold induction temperature of the 90-kDa heat shock protein is subject to acclimatization in eurythermal goby fishes (genus *Gillichthys*). *Proceedings of the National Academy of Sciences U.S.A.* **89**, 3389–3393.
- Ditzel L, Löwe J, Stock D, Stetter KO, Harber H, Huber R, Steinbacher S (1998) Crystal Structure of the Thermosome, the Archaeal Chaperonin and Homolog of CCT. *Cell* **93**, 125-138
- Dobson CM, Karplus M (1999) The fundamentals of protein folding: bringing together theory and experiment Current Opinion in Structural Biology. *Current Opinion in Structural Biology* **9**, 92-101
- Driever W, Rangini Z (1993) Characterization of a cell line derived from zebrafish (*brachydanio rerio*) embryos. *In Vitro Cellular & Developmental Biology - Animal* **29A**, 749–754
- Driever W, Stemple D, Schier A, Solnica-Krezel L (1994) Zebrafish Genetic tools for studying vertebrate development. *Trends in Genetics* **10**, 152–159
- Eisen JS (1996) Zebrafish make a big splash. *Cell* **87**, 969-977.

- Ellis RJ (1987) Proteins as molecular chaperones *Nature* **328**, 378-379
- Ellis RJ (1994a) Roles of molecular chaperones in protein folding *Current Opinions in Structural Biology* **4**, 117-122
- Ellis RJ (1994b) Opening and closing the Anfinsen cage, *Current Biology* **1**, 633-635
- Ellis RJ (1996) Editor Chaperonins: introductory perspective, *The Chaperonins*, Academic Press, San Diego, CA. pp. 2-25.
- Ellis RJ (1997) Molecular chaperones: Avoiding the crowd. *Current Biology* **7**, R531-R533
- Ellis RJ (1998) Steric chaperones *Trends in Biochemical Sciences* **23**, 43-45
- Ellis RJ (2001) Molecular chaperones: Inside and outside the Anfinsen cage. *Current Biology* **11**, R1038-R1040
- Ellis RJ, Hartl F-U (1996) Protein folding in the cell: Competing models of chaperonin function. *FASEB Journal* **10**, 20-26
- Elowitz MB, Surette MG, Wolf P-E, Stock JB, Leibler S (1999) Protein Mobility in the Cytoplasm of *Escherichia coli*. *Journal of Bacteriology* **181**, 197-203
- Fayet O, Ziegelhoffer T, Georgopoulos C (1989) The *groES* and *groEL* heat shock gene products of *Escherichia coli* are essential for bacterial growth at all temperatures. *Journal of Bacteriology* **171**, 1379-1385
- Feldman DE, Frydman J (2000) Protein folding in vivo: the importance of molecular chaperons. *Current Opinion in Structural Biology* **10**, 26-33
- Fenton WA, Horwich AL (1997) GroEL-mediated protein folding *Protein Science* **6**, 743-760
- Fernandes M, O'Brien T, Lis JT (1994) *The Biology of Heat Shock Proteins and Molecular Chaperones* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pp.375-393
- Fink AL (1999) Chaperon mediated protein folding. *Physiological Reviews* **79**, 425-449

- Fiszer-Kierzkowska A, Wysocka A, Jarzab M, Lisowska K, Krawczyk Z (2003) Structure of gene flanking regions and functional analysis of sequences upstream of the rat *hsp70.1* stress gene. *Biochimica et Biophysica Acta (BBA)* **1625**, 77-87
- Fulton AB (1982) How crowded is the cytoplasm? *Cell* **30**, 345-7
- Galgoczy P, Rosenthal A, Platzer M (2001) Human-mouse comparative sequence analysis of the NEMO gene reveals an alternative promoter within the neighboring G6PD gene. *Gene* **271**, 93-98.
- Gardiner-Garden M, Frommer MJ (1987) CpG isles in vertebrate genomes. *Journal of Molecular Biology* **196**, 261-282.
- Garrels JI, (1995) YPD-A database for the proteins of *Saccharomyces cerevisiae*. *Nucleic Acids Research* **24**, 46-49
- Gavalas A, Zalkin H (1995) Analysis of the chicken GPAT/AIRC bidirectional promoter for de novo purine nucleotide synthesis. *Journal of Biological Chemistry* **270**, 2403-2410
- Gerhard GS, Cheng KC (2002) A call to fins! Zebrafish as a gerontological model. *Aging Cell* **1**, 104-111
- Gething M-J, Sambrook J (1992) Protein folding in the cell *Nature* **355**, 33-45
- Goldsmith P (2004) Zebrafish as a pharmacological tool: the how, why and when. *Current Opinion in Pharmacology* **4**, 504-512.
- Goodsell DS (1991) Inside a living cell. *Trends in Biochemical Sciences* **16**, 203-206
- Gragerov A, Nudler E, Komissarova N, Gaitanaris GA, Gottesman ME, Nikiforov V (1992) Cooperation of GroEL/GroES and DnaK/DnaJ heat shock proteins in preventing protein misfolding in *Escherichia coli* *Proceedings of the National Academy of Science U.S.A.* **89**, 10341-10344

- Greene WK, Sontani Y, Sharp MA, Dunn DS, Kees UR and Bellgard MI (2007) A promoter with bidirectional activity is located between *TLX1/HOX11* and a divergently transcribed novel human gene. *Gene* **391**, 223-232
- Grichnik JM, French BA, Schwartz RJ (1988) The chicken skeletal α -actin gene promoter region exhibits partial dyad symmetry and a capacity to drive bidirectional transcription. *Molecular and Cellular Biology* **8**, 4587-4597
- Guarguaglini G, Battistoni A, Pittoggi C, Di Matteo G, Di Fiore B, Lavia P (1997) Expression of the murine RanBP1 and Htf9-c genes is regulated from a shared bidirectional promoter during cell cycle progression. *Biochemical Journal* **325**, 277-286.
- Hahn S (2004) Structure and mechanism of the RNA polymerase II transcription machinery, *Nature Structural & Molecular Biology* **11**, 394-403
- Halloran MC, Sato-Maeda M, Warren JT, Su F, Lele Z, Krone PH, Kuwada JY, Shoji W (2000) Laser-induced gene expression in specific cells of transgenic zebrafish. *Development* **127**, 1953-1960
- Hanley S, Muller F, Maclean N, Smith TJ (1998) Isolation functional analysis of the histone H3 promoter from Atlantic salmon (*Salmo salar* L.) *Molecular Marine Biology and Biotechnology* **7**, 165-172
- Hansen JJ, Bross P, Westergaard M, Nielsen MN, Eiberg H, Borglum AD, Mogensen J, Kristiansen K, Bolund L, Gregersen L (2003) genomic structure of the human mitochondrial chaperonin genes: Hsp60 Hsp10 are localized head to head on chromosome 2 separated by a bidirectional promoter. *Human Genetics* **112**, 71-77
- Hartl FU, Hayer-Hartl M (2002) Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* **295**, 1852-1858
- Hartl UH (1996) Molecular chaperones in cellular protein folding. *Nature* **381**, 571-580.
- Hendrick JP, Hartl FU (1993) Molecular Chaperone Functions of Heat-Shock Proteins *Annual Review of Biochemistry* **62**, 349-384

- Hendrix RW (1979) Purification and properties of *groE*, a host protein involved in bacteriophage assembly *Journal of Molecular Biology* **129**, 375-392
- Hentschel CC, Birnstiel ML (1981) The organization expression of histone gene families. *Cell* **25**, 301-313.
- Hickey E, Brandon SE, Potter R, Stein G, Stein J, Weber LA (1986a) Sequence and organization of genes encoding the human 27 kDa heat shock protein [published erratum appears in *Nucleic Acids Research* **14**, 4127-45
- Hickey E, Brandon SE, Sadis S, Smale G, Weber LA (1986b) Molecular cloning of sequences encoding the human heat-shock proteins and their expression during hyperthermia *Gene* **43**,147-154.
- Horwich AL, Fenton WA, Chapman E, Farr GW (2007) Two families of chaperonin: physiology and mechanism. *Annual Reviews in Cell and Developmental Biology* **23**, 115-145
- Houdebine LM, Chourrout D (1991) Transgenesis in fish *Cellular and Molecular Life Sciences (CMLS)* **47**,891-897
- Houry WA (2001) Chaperone-assisted protein folding in the cytoplasm *Current Protein and Peptide Science* **2**, 227-244
- Hunt JF, Weaver AJ, Landry SJ, Gierasch L, Deisenhofer J (1996) The crystal structure of the GroES co-chaperonin at 2.8 Å resolution. *Nature* **379**, 37-45
- Inoue K, Akita N, Shiba T, Satake M, Yamashita S (1992) Metal-inducible activities of metallothionein promoters in fish cells fry, *Biochemical and Biophysical Research Communications* **185**, 1108–1114.
- Inoue K, Yamashita S, Akita N, Mitsuboshi T, Nagahisa E, Shiba T, Fujita T (1990) Electroporation as a new technique for producing transgenic fish, *Cell Differentiation and Development* **29**, 123-128.

- Ivanina AV, Cherkasov AS, Sokolova IM (2008) Effects of cadmium on cellular protein and glutathione synthesis and expression of stress proteins in eastern oysters, *Crassostrea virginica* Gmelin. *Journal of Experimental Biology* **211**, 577-586.
- Iwama GK, Thomas PT, Forsyth RB, Vijayan MM (1998). Heat shock protein expression in fish *Reviews in Fish Biology and Fisheries* **8**, 35–56.
- Izsvak Z, Ivics Z (2004) Sleeping beauty transposition: biology and application for molecular therapy. *Molecular Theories* **9**, 147-156.
- Juszczynski P, Kutok JL, Li C, Mitra J, Aguiar RCT, Shipp MA (2006) *BAL1* and *BBAP* are regulated by a gamma interferon-responsive bidirectional promoter and are overexpressed in diffuse large B-Cell lymphomas with a prominent inflammatory infiltrate. *Molecular and Cellular Biology* **26**, 5348-5359
- Kampinga HH (1995) Hyperthermia, thermotolerance, and topoisomerase II inhibitors *British Journal of Cancer* **72**, 333-338
- Kampinga HH, Brunsting JF, Stege GJ, Burgman PW, Konings AW (1995) Thermal protein denaturation and protein aggregation in cells made thermotolerant by various chemicals: role of heat shock proteins. *Experimental Cell Research* **219**, 536-549
- Kawakami K, Shima A, Kawakami N (2000) Identification of a functional transposase of the Tol2 element, an Ac-like element from the Japanese medaka fish, and its transposition in the zebrafish germ lineage. *Proceedings of the National Academy of Sciences U.S.A.* **97**, 11403-11408.
- Kawakami K, Takeda H, Kawakami N, Kobayashi M, Matsuda N, Mishina M (2004) A transposon-mediated gene trap approach identifies developmentally regulated genes in zebrafish. *Developmental Cell* **7**,133-144
- Kiang JG (2004) Inducible heat shock protein 70 kD and inducible nitric oxide synthase in hemorrhage/resuscitation-induced injury. *Cell Research* **14**, 450-459.

- Killen PD, Burbelo PD, Martin GR, Yamada Y (1988) Characterization of the promoter for the alpha 1 (IV) collagen gene. DNA sequences within the first intron enhance transcription. *Biological Chemistry* **263**, 12310-12314.
- Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling T (1995) Stages of embryonic development of the zebrafish, *Developmental Dynamics* **203**, 253-310
- Kline MP, Morimoto RI (1997) Repression of the heat shock factor 1 transcriptional activation domain is modulated by constitutive phosphorylation. *Molecular and Cellular Biology* **17**, 2107-2115
- Klumpp M, Baumeister W, Essen L.O. (1997) Structure of the substrate binding domain of the thermosome, an archaeal group ii chaperonin. *Cell* **91**, 263-270
- Knauf U, Newton EM, Kyriakis J, Kingston RE (1996) Repression of human heat shock factor 1 activity at control temperature by phosphorylation, *Genes and Development* **10**, 2782-93
- Köhler HR, Alberti G, Seniczak S, Seniczak A (2005) Lead-induced *hsp70* and *hsp60* pattern transformation and leg malformation during postembryonic development in the oribatid mite, *Archeogozetes longisetosus* Aoki. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* **141**, 398-405.
- Koster RW, Fraser SE (2001) Tracing transgene expression in living zebrafish embryos. *Developmental Biology* **233**, 329-346
- Kubota H, Hynes G, Carne A, Ashworth A, Willison K (1994) Identification of six TCP-1-related genes encoding divergent subunits of the TCP-1-containing chaperonin. *Current Biology* **4**, 89-99
- Kuehn MJ, Ogg DJ, Kihlberg J, Slonim LN, Flemmer K, Bergfors T, Hultgren SJ (1993) Structural basis of pilus subunit recognition by the PapD chaperone *Science* **262**, 1234-1241
- Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W, et al. (2001) Initial sequencing and analysis of the human genome. *Nature* **409**, 860-921

- Larson JS, TJ Schuetz, R.E. Kingston (1988) Activation in vitro of sequence-specific DNA binding by a human regulatory factor. *Nature* **335**, 372-375
- Laskey RA, Honda BM, Mills, AD Finch JT (1978) Nucleosomes are assembled by an acidic protein which binds histones and transfers them to DNA. *Nature* **275**, 416-420
- Lele Z, Krone PH (1996) The zebrafish as a model system in developmental, toxicological transgenic research. *Biotechnology Advances* **14**, 57-72.
- Li YY, Yu H, Guo, ZM, Guo TQ, Tu K, Li YX (2006) Systematic analysis of head-to-head gene organization: evolutionary conservation and potential biological relevance. *PLoS Computational Biology* **2**, 688-697
- Liao WC, Ash J, Johnson LF (1994) Bidirectional promoter of the mouse thymidylate synthase gene *Nucleic Acids Research* **22**, 4044-4049
- Lin Z, Madan D, Rye HS (2008) GroEL stimulates protein folding through forced unfolding. *Nature Structural and Molecular Biology* **15**, 303-11
- Lis J, Wu C (1993) Protein traffic on the heat shock promoter: Parking, stalling, and trucking along. *Cell* **74**, 1-4
- Liu RY, Kim D, Yang SH, Li GC (1993) Dual control of heat shock response: involvement of a constitutive heat shock element-binding factor. *Proceedings of the National Academy of Science U.S.A.* **90**, 3078-3082
- Liu Z, Moav B, Faras AJ, Guise KS, Kapuscinski AR, Hackett PB (1990) Functional analysis of elements affecting expression of the b-actin gene of carp, *Molecular and Cellular Biology* **10**, 3432-3440.
- Llorca O, Smyth MG, Carrascosa JL, Willison KR, Radermacher M, Steinbacher S, Valpuesta JM (1999) 3D reconstruction of the ATP-bound form of CCT reveals the asymmetric folding conformation of a type II chaperonin. *Nature Structural and Molecular Biology* **6**, 639-642
- Maclean N (1998) Regulation exploitation of transgenes in zebrafish, *Mutation Research* **399**, 255-266

- Macleán N., Alam M.S., Iyengar A., Popplewell A. (1996) Transient expression of reporter genes in fish as a measure of promoter efficiency, in: S.J. Ennion, G. Goldspink Eds. , *Gene Expression Manipulation in Aquatic Organisms*, Soc. Exp. Biol. Seminar Series 58, Cambridge Univ. Press., pp. 165-174.
- Makarova O, Kamberov E, Margolis B (2000) Generation of deletion and point mutations with one primer in a single cloning step. *Biotechniques* **29**, 970-2
- Martin CC, Tang P, Barnardo G, Krone PH (2001) Expression of the chaperonin 10 gene during zebrafish development *Cell Stress Chaperones* **6**, 38-43.
- Martin CC, Tsang CH, Beiko RG, Krone PH (2002) Expression genomic organization of the zebrafish chaperonin gene complex. *Genome* **45**, 804-811
- Mayhew M, Da Silva ACR, Martin J, Erdjument-Bromage H, Tempst P, Hartl F (1996) Protein folding in the central cavity of the GroEL-GroES chaperonin complex. *Nature* **379**, 420-426
- Molina A, Biemar F, Muller F, Iyengar A, Prunet P, Maclean N, Martial JA, Muller M (2000) Cloning and expression analysis of an inducible HSP70 gene from tilapia fish. *FEBS Letter* **474**, 5-10.
- Momota R, Sugimoto M, Oohashi T, Kigasawa K, Yoshioka H, Ninomiya Y. (1998) Two genes, COL4A3 and COL4A4 coding for the human $\alpha 3(\text{IV})$ and $\alpha 4(\text{IV})$ collagen chains are arranged head-to-head on chromosome 2q36. *FEBS Letter* **424**, 11-16.
- Morimoto RI (1993) Cells in stress: Transcriptional activation of heat shock genes. *Science* **259**, 1409-1410
- Morimoto RI (1998) regulation of heat shock transcription response: Cross talk between a family of heat shock factors, molecular chaperones, negative regulators. *Genes and Development* **12**, 3788-3796.
- Morimoto RI, DA Jurivich, PE Kroeger, SK Mathur, SP Murphy, A Nakai, K Sarge, K Abravaya, LT Sistonen (1994) Regulation of heat shock gene transcription by a family of heat shock factors. In *The biology of heat shock proteins and molecular chaperones* (ed. R.I. Morimoto, A.

Tissieres and C. Georgopoulos), pp. 417-456 Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Morton H (1998) Early pregnancy factor: An extracellular chaperonin 10 homologue. *Immunology & Cell Biology* **76**, 483-496

Mosser DD, Duchaine J, Massie B (1993) The DNA-binding activity of the human heat shock transcription factor is regulated in vivo by *hsp70*. *Molecular and Cellular Biology* **13**, 5427-5438

Muller F, Ivics Z, Erdelyi Papp T, Varadi L, Horvath L, Maclean N, Orban L (1992) Introducing foreign genes into fish eggs with electroporated sperm as a carrier, *Molecular Marine Biology and Biotechnology* **1**, 276-281.

Nakai A, Morimoto RI (1993) Characterization of a novel chicken heat shock transcription factor, heat shock factor 3, suggests new regulatory pathway. *Molecular and Cellular Biology* **13**, 1983-1997

Nakai A, Tanabe M, Kawazoe Y, Inazawa J, Morimoto RI, Nagata K (1997) HSF4, a new member of the human heat shock factor family which lacks properties of a transcriptional activator *Molecular and Cellular Biology* **17**, 469-481

Nam B-H, Kim Y-O, Lim S-U, Cho Y-C, Kim B-S, Jee Y-J, Lee S-J, Cheong J (2006) Characterization of the Heat Shock Protein 60 and 10 Genes and their Bidirectional Promoter Region in Olive Flounder (*Paralichthys olivaceus*) *Korean Journal of Genetics* **28**, 361-368

Nanbru C, Prats A-C, Droogmans L, Defrance P, Huez G, Krays V (2001) Translation of the human c-myc P0 tricistronic mRNA involves two independent internal ribosome entry sites. *Oncogene* **20**, 4270-4280

Ni Z, Schwartz BE, Werner J, Suarez JR, Lis JT (2004) Coordination of transcription, RNA processing, and surveillance by P-TEFb kinase on heat shock genes, *Molecular Cell* **13**, 55-65

- Oshima RG, Abrams L, Kulesh D (1990) Activation of an intron enhancer within the keratin 18 gene by expression of c-fos and c-jun in undifferentiated F9 embryonal carcinoma cells. *Genes and Development* **4**, 835-848.
- Parinov S, Kondrichin I, Korzh V, Emelyanov A (2004) Tol2 transposon-mediated enhancer trap to identify developmentally regulated zebrafish genes in vivo. *Developmental Dynamics* **231**, 449-459.
- Pelham HRB (1982) A regulatory upstream promoter element in the Drosophila Hsp 70 heat-shock gene *Cell* **30**, 517-528
- Pelham HRB (1986) Speculation on the function of the major heat shock and glucose-regulated proteins *Cell* **46**, 959-961
- Perezgasga L, Segovia L, Zurita M (1999) Molecular characterization of the 5' control region of two lethal alleles affecting the *hsp60* gene in Drosophila melanogaster. *FEBS Letter* **456**, 269-273
- Pfaffl MW(2001) A new mathematical model for relative quantification in real-time RT-PCR, *Nucleic Acids Research* **29**, 2002-2007
- Platzer M, Rotman G, Bauer D, Uziel T, Savitsky K, Bar-Shira A, Gilad S, Shiloh Y, Rosenthal A (1997) ATAXIA-telangiectasia locus: sequence analysis of 148 kb of human genomic DNA containing the entire ATM gene. *Genome Research* **7**, 592-605.
- Pochon NA, Mach B (1996) Genetic complexity of the human *hsp60* gene. *International Immunology* **8**, 221-230
- Pollner R, Fischer G, Kühn K (1990) Regulation of divergent transcription of genes for basement membrane type IV collagen *Annals of the New York Academy of Sciences* **580**, 44-54
- Pugh BF, Tjian R (1990) Mechanism of transcriptional activation by Sp1: Evidence for coactivators. *Cell* **61**, 1187-1197
- Rabergh CM, Airaksinen S, Soitamo A, Bjorklund HV, Johansson T, Nikinmaa M, Sistonen L (2000) Tissue-specific expression of zebrafish (*Danio rerio*) heat shock factor 1 mRNAs in response to heat stress. *Journal of Experimental Biology* **203**, 1817-1824

- Rabindran SK, Giorgi G, Clos J, Wu C (1991) Molecular cloning and expression of a human heat shock factor, HSF1. *Proceedings of the National Academy of Science U.S.A.* **88**, 6906-6910
- Rasooly RS, Henken D, Freeman N, Tompkins L, Badman D, Briggs J, Hewitt AT (2003) Genetic and genomic tools for zebrafish research: the NIH zebrafish initiative. *Developmental Dynamics* **228**, 490-496.
- Engeszer RE, Patterson LB, Rao AA, Parichy DM (2007) *Zebrafish* **4**, 21-40.
- Richardson A, Landry SJ, Georgopoulos C (1998) The ins and outs of a molecular chaperone machine *Trends in Biochemical Sciences* **23**, 138-143
- Ritossa F (1962) A new puffing pattern induced by temperature shock and DNP in *Drosophila*. **18**, 571-573
- Rombough PJ (1997). The effects of temperature on embryonic and larval development. In *Global Warming: Implications for Freshwater and Marine Fish* (Wood CM, McDONALD DM, eds), pp. 177-224. Cambridge: Cambridge University Press
- Roseman, AM, Chen S, White H, Braig K, Saibil HR (1996) The Chaperonin ATPase Cycle: Mechanism of Allosteric Switching and Movements of Substrate-Binding Domains in GroEL *Cell* **87**, 241-251
- Rothman JE (1989) Polypeptide chain binding proteins: catalysts of protein folding and related processes in cell. *Cell* **59**, 591-601
- Rubinstein AL (2003) Zebrafish: from disease modeling to drug discovery. *Current Opinion in Drug Discovery and Development* **6**, 218-23
- Ryan MT, Herd SM, Sberna G, Samuel MM, Hoogenraad NJ, Hoj PB (1997) The genes encoding mammalian chaperonin 60 chaperonin 10 are linked head-to-head share a bidirectional promoter. *Gene* **196**, 9-17
- Ryan MT, Naylor DJ, Hoogenraad NJ, Hoj PB (1995) Affinity Purification, Overexpression, and Characterization of Chaperonin 10 Homologues Synthesized with and without N-terminal acetylation. *Journal of Biological Chemistry* **270**, 2203-2243

- Sadowski I, Ma J, Triezenberg S, Ptashne M (1988) GAL4-VP16 is an unusually potent transcriptional activator. *Nature* **335**, 563-564.
- Sambrook J, and Russell DW, (2001) Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Sarge KD, Zimarino V, Holm K, Wu C, Morimoto RI (1991) Cloning and characterization of two mouse heat shock factors with distinct inducible and constitutive DNA-binding ability *Genes and Development* **5**, 1902-1911
- Satyal SH, Chen D, Fox SG, Kramer JM, Morimoto RI (1998) Negative regulation of the heat shock transcriptional response by HSBP1 *Genes & Development* **12**, 1962-1974
- Scharf KD, Rose S, Zott W, Nover L, Schoffl F (1990). Three tomato genes code for heat stress transcription factors with a region of remarkable homology to the DNA-binding domain of the yeast HSF. *EMBO Journal* **9**, 4495-4501
- Schirone RC, Gross L (1968) Effect of temperature on early embryological development of the zebra fish, *Brachydanio rerio*, *Journal of Experimental Zoology* **169**, 43-52
- Schuetz TJ, Gallo GJ, Sheldon L, Tempst P, Kingston RE (1991) Isolation of a cDNA for HSF2: evidence for two heat shock factor genes in humans. *Proceedings of the National Academy of Science U.S.A.* **88**, 6911-6915
- Segal Y, Zhuang L, Rondeau E, Sraer JD, Zhou J (2001) Regulation of the paired type IV collagen genes COL4A5 and COL4A6. Role of the proximal promoter region *Journal of Biological Chemistry* **276**, 11791-11797
- Seok SH, Park JH, Baek MW, Lee HY, Kim DJ, Uhm HM, Honga J-J, Naa Y-R, Jinb B-H, Ryub D-Y, Parka J-H (2006) Specific activation of the human HSP70 promoter by copper sulfate in mosaic transgenic zebrafish. *Journal of Biotechnology* **126**, 406-413
- Shamovsky I and Nudler E (2008) Cellular and Molecular Life Sciences. *New insights into the mechanism of heat shock response activation* **65**, 855-861

- Shamovsky I, Gershon D (2004) Novel regulatory factors of HSF-1 activation: facts and perspectives regarding their involvement in the age-associated attenuation of the heat shock response. *Mechanisms of Ageing and Development* **125**, 767-775
- Sharma S, Rohilla MS, Reddy PV, Tiwari PK (2007) In Vitro Induction of 60-kDa and 70-kDa Heat Shock Proteins by Endosulphan and Monocrotophos in Sheep Blowfly *Lucilia cuprina*. *Archives of Environmental Contamination and Toxicology* Advanced online publish.
- Shi Y, Kroeger PE, Morimoto RI (1995) the carboxyl terminal Transactivation domain of heat shock factor 1 is negatively regulated and stress responsive. *Molecular and Cellular Biology* **15**, 4309-4318
- Shi Y, Mosser DD, Morimoto RI (1998) Molecular chaperones as HSF1-specific transcriptional repressors *Genes & Development* **12**, 654-666
- Shimada T, Fujii H, Linn HJ (1989) A 165-base pair sequence between the dihydrofolate reductase gene and the divergently transcribed upstream gene is sufficient for bidirectional transcriptional activity. *Journal of Biological Chemistry* **264**, 20171-20174
- Sorger PK, Pelham HR (1987) Cloning and expression of a gene encoding hsc73, the major hsp70-like protein in unstressed rat cells *EMBO Journal* **6**, 993-998
- Sorger PK, Pelham RB (1988) Yeast heat shock factor is an essential DNA-binding protein that exhibits temperature-dependent phosphorylation. *Cell* **54**, 855-864
- Stuart GW, McMurray JV, Westerfield M (1988) Replication, integration stable germ-line transmission of foreign sequences injected into early zebrafish embryos. *Development* **103**, 403-412.
- Stuart GW, Vielkind JR, McMurray JV, Westerfield M (1990) Stable lines of transgenic zebrafish exhibit reproducible patterns of transgene expression, *Development* **109**, 577-584
- Szelei J, Varadi L, Muller F, Erdelyi F, Orban L, Horvath L, Duda E (1994) Liposome-mediated gene transfer in fish embryos, *Transgenic Research* **3**, 116-119

- Teh C, Vladimir Korzh SP (2005) New ways to admire zebrafish: progress in functional genomics research methodology. *BioTechniques* **38**, 897-906
- Thermes V, Grabher C, Ristoratore F, Bourrat F, Choulika A, Wittbrodt J, Joly JS (2002) I-SceI meganuclease mediates highly efficient transgenesis in fish. *Mechanism of Development* **118**, 91-98.
- Thisse B, Pflumio S, Fürthauer M, Loppin B, Heyer V, Degraeve A, Woehl R, Lux, A, Steffan T, Charbonnier XQ, Thisse C (2001) Expression of the zebrafish genome during embryogenesis (NIH R01 RR15402) ZFIN Direct Data Submission (<http://zfin.org>)
- Tilly K, Murialdo H, Georgopoulos C (1981) Identification of a second *Escherichia coli groE* gene whose product is necessary for bacteriophage morphogenesis. *Proceedings of the National Academy of Science U.S.A.* **78**, 1629-1633
- Tonkiss, J Calderwood SK (2005) Regulation of heat shock gene transcription in neuronal cells. *International Journal of Hyperthermia* **21**, 433-444
- Travers MT, Cambot M, Kennedy HT, Lenoir GM, Barber MC, Joulin V (2004) Asymmetric expression of transcripts derived from the shared promoter between the divergently oriented ACACA and TADA2L genes. *Genomics* **85**, 71-84
- Trinklein ND, Aldred SJ, Saldanha AJ, Myers RM (2003) Identification and functional analysis of human transcriptional promoters. *Genome Research* **13**, 308-312
- Trinklein ND, Murray JI, Hartman SJ, Botstein D, Myers RM (2004) The role of heat shock transcription factor 1 in the genome-wide regulation of the mammalian heat shock response. *Molecular Biology of the Cell* **15**, 1254-1261
- Udvardia AJ, Linney E (2003) Windows into development: historic, current, future perspectives on transgenic zebrafish. *Developmental Biology* **256**, 1-17
- Venner TJ, Singh B, Gupta RS (1990) Nucleotide sequences and novel structural features of human and Chinese hamster *hsp60* (chaperonin) gene families. *DNA and Cell Biology* **9**, 545-552

- Volckaert F, Hellemans B, Daemen D, Ollevier F (1991) Gene transfer expression of the CMV b gal fusion gene in embryos of the African catfish the zebra fish, in, Second Int. Mar. Biotechnol. Conf., Baltimore, MD, p. 91.
- Walter S, Buchner J (2002) Molecular chaperons-cellular machines for protein folding. *Angewandte Chemie International Edition* **41**, 1098-1113
- Walter T, Drabent B, Krebs H, Tomalak M, Heiss S Benecke BJ (1989) Cloning and analysis of a human 86-kDa heat-shock-protein-encoding gene *Gene* **83**, 105-115
- Weissman JS, Rye HS, Fenton WA, Beechem JM, Horwich AL (1996) Characterization of the active intermediate of a GroEL-GroES-mediated protein folding reaction. *Cell* **84**, 481-490
- Westerfield M (1995). *The Zebrafish Book*, Eugene: University of Oregon
- Westwood JT, Wu C (1993) Activation of Drosophila heat shock factor: conformational change associated with a monomer-to-trimer transition. *Molecular and Cellular Biology* **13**, 3481-3486
- Wiederrecht G, Seto D, Parker CS (1988). Isolation of the gene encoding the *S. cerevisiae* heat shock transcription factor *Cell* **54**, 841-853
- Wu C (1995) Heat shock response factors: structure and regulation. *Annual Reviews in Cell and Developmental Biology* **11**, 441-469
- Wu C, Clos J, Giorgi G, Haroun RI, Kim S-J, Rabindran SK, Westwood JT, Wisniewski J, Yim G (1994) Structure and regulation of heat shock transcription factor. In *The biology of heat shock proteins and molecular chaperones* (ed. R.I. Morimoto, A. Tissieres and C. Georgopoulos), pp. 395-416. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Wu C, Zimarino V, Tsai C, Walker B, Wilson S (1990) in *stress proteins in biology and medicine* (ed. R.I. Morimoto, A. Tissieres and C. Georgopoulos), pp. 429-442. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Xu CF, Chambers JA, Soloon EJ (1997) Complex regulation of BRCA1 gene. *Journal of Biological Chemistry* **272**, 20994-20997

- Young JC, Agashe VR, Siegers K, Hartl FU (2004) Pathway of chaperone-mediated protein folding in the cytosol. *Molecular Cell Biology* **5**, 781-791
- Zafarullah M, Olsson PE, Gedamu L (1989) Rainbow trout metallothionein gene structure regulation, in: N. Maclean. Ed., Oxford Surveys on Eukaryotic Genes 6, Oxford Univ. Press, pp. 111–143.
- Zhang L-F, Ding J-H, Yang B-Z, He G-C, Roe C (2003) Characterization of the bidirectional promoter region between the human genes encoding VLCAD and PSD-95 *Genomics* **82**, 660-668
- Zuo J, Baler R, Dahl G, Voellmy R (1994) Activation of the DNA-binding ability of human heat shock transcription factor 1 may involve the transition from an intramolecular to an intermolecular triple-stranded coiled-coil structure. *Molecular and Cellular Biology* **14**, 7557-7568
- Zuo J, Guo Y, Guettouche T, Smith DF, Voellmy R (1998) Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. *Cell* **94**, 471-480
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