Variations in the Human hsp60 Gene between Cases of Sudden Infant Death Syndrome and non–affected Children

Von dem Fachbereich Biologie
der Universität Hannover
zur Erlangung des Grades einer
DOKTORIN DER NATURWISSENSCHAFTEN
–Dr. rer. nat.–
genehmigte Dissertation

von
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2005

Referent: Prof. Dr. H.–J. Jacobsen
Korreferent: Prof. Dr. H.–J. Hedrich
Tag der Promotion: 21.02.2005

Menschen treten in unser Leben und begleiten uns eine Weile.
Einige bleiben für immer,
denn sie hinterlassen Spuren in unseren Herzen.

For the SIDS babies and their families.

For my own family, Helga and Sven.

For the Children, who taught me about nature, nurture and improvisational talent:
Annika Jasmin Krönke
Maik Jason Bahrenfuss
Elisa Yvonne Krönke
Christian Klaus Böllet

And for my husband Andreas, with Love.

Abstract

The objective of this thesis is the examination of variations in the human heat shock protein gene 60 (chaperonin60, hsp60) between SIDS cases and controls. The SIDS (Sudden Infant Death) cases showed typical characteristics of the Sudden Infant Death Syndrome (SIDS). The controls were age– and sex–matched children from paternity testing and children who died of causes other than SIDS. The Sudden Infant Death Syndrome is a multifactorial process. A genetic defect is thus assumed to be *necessary, but not sufficient* for the genesis of SIDS.
No difference between SIDS cases and controls in the DNA sequence of the exon 5 of the hsp60 gene could be detected. However, the SIDS cases showed a statistically significant higher number of deletions than the controls in the intron 5 adjacent to exon 5. The deletions in the intron 5 disrupt no known regulatory sequences (donor, acceptor, branch site). Yet, intron 5 belongs to the rare GC–AG intron class, the latter are assumed to be highly regulated. Thus, the deletions may have an impact on other regulatory sites. The increased number of mutations confirms the findings in previous publications on DNA variations in SIDS cases. Overall, more mutations are found in the DNA of SIDS cases than in the DNA of controls.
During the research on the human hsp60 gene, another gene of the Chaperonin (HSP60) family was found. It shows a high similarity in length and sequence (97.5 %) to the mRNA of the hsp60 gene. Its genomic sequence does not contain introns, compared to the regular hsp60 gene it is thus much shorter. It was named hsp60s (s for short). Due to both the similarity of the hsp60s gene and the hsp60 mRNA, and a mitochondrial leader peptide sequence at the N–terminal end of the putative Hsp60s protein, it could be concluded that it can be an inducible variant of the hsp60 gene. After fully sequencing it, two open reading frames instead of the single one of hsp60 were found. A readthrough to the stop codon of the first open reading frame (ORF) would lead to a continuous ORF, ending at the stop codon of the second ORF. Such stop codon readthroughs occur in the human genome, but only on rare occasions. An expression analysis (reverse transcriptase PCR on RNA from blood) produced no positive results. Based on these findings, it can be concluded that the hsp60s gene is a pseudogene of the human chaperonin60 gene. However, it may still be possible that hsp60s is only expressed under stress (our expression analysis was carried out under non–stress conditions) and/or in tissues other than blood.
The techniques developed for this work can be practically applied to forensics: The amplification and direct sequencing of genomic DNA from corpses is becoming more and more important. It was demonstrated in the present thesis that these techniques work reliable for lengths up to 400 bases in 15 year old blood samples and might also be applicable to even older samples.
Keywords: Chaperonin60, Hsp60s, Heat Shock Proteins, Stress Proteins, Heat Stress, Hyperthermia, Sudden Infant Death Syndrome.

Kurzfassung

In der vorliegenden Arbeit sollten Teile des Gens des humanen Hitzeschockprotein 60 (chaperonin60, hsp60) auf Variationen bei Fällen des plötzlichen Säuglingstodes und einer Kontrollgruppe untersucht werden. Die Fälle wiesen typische Merkmale des plötzlichen Säuglingstodes (engl. SIDS = Sudden Infant Death Syndrome) auf. Die Kontrollen waren Kinder gleichen Alters und Geschlechts aus Vaterschaftstests und Kinder, die nicht am plötzlichen Säuglingstod verstorben sind.
Es wurden keine Unterschiede in der DNA–Sequenz des Exon 5 im humanen hsp60 Gen bei SIDS–Fällen und Kontrollen gefunden. Die SIDS (Sudden Infant Death Syndrome)–Fälle wiesen aber statistisch signifikant eine höhere Anzahl von Deletionen im Intron 5 des hsp60 Gens auf, als die Kontrollen. Dies deckt sich mit anderen molekulargenetischen Arbeiten, in denen die SIDS–Fälle ebenfalls stets eine erhöhte Anzahl an Mutationen aufweisen. Da der plötzliche Säuglingstod ein multifaktorielles Geschehen ist, liegt die Vermutung nahe, dass eine genetische Komponente existiert, die *notwendig, aber nicht ausreichend* für die Genese des plötzlichen Säuglingstodes ist. Intron 5 gehört zur seltenen Klasse der GC–AG Introns, für die umfangreiche Steuerfunktionen vermutet werden. Möglicherweise sind eine oder mehrere solcher Funktionen durch die Deletionen gestört.
Im Zuge der Forschung am humanen hsp60 Gen wurde ein weiteres Gen der Chaperoninfamilie (HSP60 Familie) entdeckt. Es ist in Länge und Sequenz der Boten–RNA des hsp60 Gens zu 97,5 % ähnlich. Die genomische Sequenz zeigt aber keine Introns. Im Vergleich mit dem regulären hsp60 Gen (16986 bp) ist es sehr kurz (2176 bp), daher wird es hsp60s (s = short, eng. kurz) benannt. Zunächst wurde aufgrund der Ähnlichkeit mit hsp60 und der mitochondrialen Signalsequenz am N–terminus des putativen Proteins eine induzierbare Variante des regulären hsp60 Gens vermutet. Eine vollständige Sequenzierung zeigte jedoch anstelle eines durchgehenden, zwei kürzere offene Leseraster. Ein Überlesen des ersten Stopcodons, im menschlichen Genom möglich, hätte jedoch ein durchgehendes Leseraster zur Folge. Eine Expressionsanalyse (reverse Transkriptase PCR an RNA aus Blut) zeigte kein positives Ergebnis. Nach den vorliegenden Informationen ist es wahrscheinlich, dass es sich bei hsp60s um ein Pseudogen handelt. Es ist aber nicht auszuschließen, dass es entweder nur unter Stress (die Expressionsanalyse fand unter non–Stress Bedingungen statt) und/oder in anderen Geweben als Blut exprimiert wird.
Die Techniken der vorliegenden Arbeit können in der forensischen Forschung angewandt werden. Die Amplifikation und direkte Sequenzierung von genomischer DNA aus Leichenblut gewinnt immer mehr an Bedeutung. Es wird gezeigt, dass in Bereichen bis 400 Basen ein solches Verfahren bei bis zu 15 Jahre alten Blutproben möglich ist (eventuell auch bei noch älteren Proben).
Schlagwörter: Chaperonin60, Hsp60s, Hitzeschockproteine, Stressproteine, Hitzestress, Hyperthermie, Plötzlicher Säuglingstod.

Acknowledgments

I would like to thank Prof. Dr. Jacobsen and Prof. Dr. Hedrich for refereeing my thesis on such a short notice. I am also grateful to PD Dr. Elisabeth Esch for joining them to form the disputation committee and to Prof. Dr. Schmidt for being the committees chairman.

For support during the long nights spent working in the lab, my thanks goto my colleague and good friend, Ingo Reimann.

In answering all questions pediatric, ranging from the utter basics to the most advanced topics, my parents-in-law Drs. Hartmut and Elke Koch provided crucial advice.

My mother Helga Teske and brother Sven Teske kept my spirits up when experiments didn't proceed as planned. In this, they were aided by my friends in the Wülmpoint-Team.

I am indebted to Andrea Bahrenfuss for suffering through the first round of proof reading, I sincerely hope that this was the last dissertation she had to bother with.

The wonderful music of the motion picture soundtrack "The Lord of the Rings" accompanied me during the whole writing of this thesis. My thanks go to its composer Howard Shore for his "light in dark places, when all other lights go out".

The broad support by my husband Andreas, from the moral to the practical, was invaluable. You are certainly the "good" part in the good and bad times of marriage. Thank you for everything.

Finally, I would like to thank all the people at the Institute of Legal Medicine, Medical Hannover, who actively aided me in my endeavor.

Contents

List of Tables

List of Figures

Abbreviations

5–HT 5–Hydroxytryptamine = Serotonin

5–HTT 5–Hydroxytryptamine Transporter

Agp–1 Protein Kinase Essential for Autophagy, mouse

BiP Immunoglobulin Heavy Chain Binding Protein

bp base pair

CCT Chaperonin Containing TCP–1

DBD DNA Binding Domain

DNA Deoxyribonucleic Acid

ddNTP Dideoxy Nucleotide Triphosphate

dNTP Deoxy Nucleotide Triphosphate

EDTA Ethylenediaminetetraacetic Acid

EP Eppendorf 5415 D bench–top centrifuge

ER Endoplasmic Reticulum

ERK Extracellular Signal–Related Kinase

GAPDH Glyceraldehyde-3-Phosphate Dehydrogenase

GAN GenBank Accession Number

GrP Glucose Regulated Protein

hnRNA heterogeneous nuclear RNA

HPLC High Pressure Liquid Chromatography

HS Haereus Sepatech Megafuge 1.0 R

HSF Heat Shock Transcription Factor

hsp Heat Shock Protein Gene

Hsp Heat Shock Protein

HSP Heat Shock Protein Family

ICD International Classification of Diseases

IFN Interferon

kB kilo Base

kD kilo Dalton, also kDa

kV kilo Volt

MAPK Mitogen–Activated Protein Kinase

MDP Muramyl Dipeptide

MHH Medizinische Hochschule Hannover (Medical School Hanover)

mRNA Messenger RNA

mt–DNA mitochondrial DNA

ORF Open Reading Frame

PRP Platelet–Rich Plasma

PrP^c Prion Protein, wild type

PrP^{sc} Prion Protein, scrapie

Pu Purine

Py Pyrimidine

RFLP Restriction Fragment Length Polymorphism

RM Institut für Rechtsmedizin (Institute for Legal Medicine)

RNA Ribonucleic Acid

SIDS Sudden Infant Death Syndrome

SNP Single Nucleotide Polymorphism

snRNA small nuclear RNA

SPAN Swiss–Prot Accession Number

SPSS Statistical Package for the Social Sciences

TBE Tris–Borate–EDTA

TCP–1 Tailless Complex Polypeptide

TriC TCP–1 Ring Complex

tRNA Transfer RNA

VNTR Variable Number of Tandem Repeats

Databases and Accession Numbers

hsp60s gene GenBank AF380943

Human Chromosomes

human chromosome 3 clone RP11–344C13 GenBank AC097360

human chromosome 4 clone RP13–539F13 GenBank AC109811

human chromosome 5 clone CTD–2577N22 GenBank AC091873

human chromosome 5 clone RP11–704G19 GenBank AC138940

human chromosome 8 clone RP11–416I21 GenBank AC131269

human chromosome 12 clone RP11–153M3 GenBank AC097104

Human heat shock related genes and proteins

hsp60/hsp10 gene GenBank AJ250915

hsp60 mRNA GenBank M34664

Hsp60 protein Swiss–Prot P10809

hsp60 mRNA GenBank BC002676

hsp60 mRNA GenBank NM_002156

hsp60 mRNA GenBank M22382

hsp60 mRNA GenBank NM_199440

hsp60 mRNA GenBank BC067082

hsp60 mRNA GenBank BC003030

hsp60 mRNA GenBank BC 047350

hsp70 gene GenBank BC002453

Hsp70 protein Swiss–Prot P08107

hsc70 GenBank Y00371

Hsc70 protein Swiss–Prot P11142

Pseudogenes of the human hsp60 gene

Pseudogene 1 GenBank NG_001145, M34660

Pseudogene 2 GenBank NG_001146, M34662

Pseudogene 3 GenBank NG_001147, M34661

Pseudogene 4 GenBank NG_001148, M34663

Pseudogene 7 GenBank NG_000920

Transcription factors

HSF1 protein human heat shock transcription factor 1, Swiss–Prot Q00613

HSF1 protein heat shock transcription factor 1, Saccharomyces cerevisiae Swiss–Prot P10961

HNF3A Hepatocyte Nuclear Factor 3 Alpha, Swiss–Prot P55317

MEF–2A Myocyte–specific enhancer factor 2A, Swiss–Prot Q02078

MEF–2D Myocyte–specific enhancer factor 2D, Swiss–Prot Q14814

RNA Quality

GAPDH Human glyceraldehyde–3–phosphate dehydrogenase (GAPDH) mRNA, GenBank M33197

Animal sequences

P1 protein gene Chinese hamster equivalent of hsp60, GenBank M22383.1

hsp60 mRNA Rattus norvegicus, GenBank NM_022229

hsp60 mRNA Mus musculus, GenBank BC016400

chromosome 22 clone Pan troglodytes, GenBank BS000169

1 Introduction

In this chapter, the fundamentals of the research area of the project are established. It starts with an overview of heat shock and stress proteins, their function as molecular chaperones and protein folding. Furthermore, the stress response and the phenomenon of stress tolerance are being described. Because a mutation analysis was performed on one of its members, the HSP60 family is described in detail. Finally, the current knowledge on Sudden Infant Death Syndrome is documented, and the main question of the thesis is presented.

1.1 Terminology and Notations

Since no uniform naming system for stress and heat shock protein genes and stress and heat shock proteins exists, the conventions according to the *Cell Stress Society International* will be used in this work (Cell Stress & Chaperones 2000). Gene names are written entirely in lower case letters, e.g., hsp60. Protein names start with a capital letter, e.g., Hsp60. Protein family names are written entirely in capital letters, e.g., HSP60. The inducible and constitutively expressed members should be distinguished, if possible. For example the inducible member of the HSP70 family is named Hsp70, the constitutively expressed (sometimes called cognate) member is named Hsc70.

1.2 Heat Shock Proteins/Stress Proteins

The ability to cope with heat by expressing a certain type of proteins is a key function of cells. This ability was first described by Ferrucio Ritossa in 1962 (Ritossa 1962) in a short paper titled "A New Puffing Pattern Induced by Temperature Shock and DNP in Drosophila." Unintentionally, he described a process in Drosophila that is essential also for human survival: Heat Shock Response. In 1974, the proteins were named heat shock proteins by Tissiéres et al. (Tissiéres et al 1974; Pockley 2003). Later on, the name was changed to the more general description *stress proteins*, because heat shock and other proteins protect the cells against various kinds of stress.

Other environmental stress factors include cold, heavy metals, oxygen free radicals, ethanol, caloric restriction and the absence of O_2. Some "clinical" stress factors (medical conditions) are for example acute hypertension, anoxia, circulatory shock, endotoxins, hemorrhagic shock, ischemia/reperfusion and hyperthermia. Living stress factors include a broad range of bacteria, viruses and parasites (Pockley 2003; Verbeke et al 2001; De Maio 1999; Fink 1999; Hartl 1996; De Maio 1995; Minowada and Welch 1995; Becker and Craig 1994; Morimoto et al 1994a; Sztankay et al 1993; Welch 1993a; Welch 1993b).

Stress proteins are ubiquitous. They exist in every compartment of a cell and in every organism. This ubiquity is well documented. The following examples give an overview of the stress proteins' involvement in physiological processes and diseases.

- Stress proteins are involved in aging and thermotolerance (Verbeke et al 2001).
- Many neurodegenerative diseases show inclusion bodies associated with ubiquitin and the proteasome: Alzheimer's, Parkinson's, Huntingdon's (Sherman and Goldberg 2001).
- Stress proteins also play a role in cardiovascular biology (Benjamin and McMillan 1998).
- Members of the HSP70 family are presumed to be involved in the apoptosis pathway (Beere and Green 2001).
- Gp96, also a member of the HSP70 family, derived from tumors was used in an experimental clinical trial as a cancer vaccine (Srivastava and Amato 2001). The purified Gp96 from a certain cancer was able to elicit a protective immunity specific to that cancer.
- Stress proteins play a distinct role in autoimmune diseases (Van Noort and Amor 1998).

Stress proteins in general are highly conserved in the evolution. They are named for their approximate molecular weight, e.g., Hsp60 (60 kD). Six large stress protein families are currently known: Small HSPs, HSP40, HSP60, HSP70, HSP90 and HSP110. An overview is given in Table 1.1 that summarizes results from the following publications: Pockley 2003; Verbeke et al 2001; De Maio 1999; Fink 1999. For some of the stress proteins, location, function and expression profiles are known. The HSP60 family and the HSP10 family will be discussed separately in Section 1.6. An overview can be found in Table 1.2.

Family, Members	Intracellular location	Function	Expression	
			Normal	Stress
Ubiquitin	Cytosol	Tag protein for degradation	+	++
HSP10, see Table 1.2				
Small HSP				
αB–crystallin	Cytoplasm	Cytoskeletal stabilisation		
Hsp27	Cytoplasm, Nucleus	Actin Dynamics	+	++
Hsp32 (Haem Oxygenase)	Cytoplasm	Haem catabolism; antioxidant of properties	+	++
Hsp35 (G3PDH)	Cytosol		+	++
HSP40				
Hsp40	Cytoplasm, Nucleus	Regulates the activity of Hsp70; Binds non–native proteins	+	++
Hsp47	ER	Processing of pro–collagen; Processing and/or secretion of collagen		
DnaJ	E. Coli	partner of DnaK	+	++
HSP60, see Table 1.2				
HSP70				
Hsp70 Inducible	Cytoplasm, Nucleus	Bind to extended polypeptides; prevent aggregation of unfolded peptides; dissociate some oligomers; ATP binding; ATPase activity; downregulates HSF1 activity	–	+++
Hsp70hom Inducible	Cytoplasm, Nucleus	as Hsp70; testis–specific	–	+++
Hsc70 constitutive, cognate	Cytoplasm Peroxisome	Bind to extended polypeptides; prevent aggregation of unfolded peptides; dissociate some oligomers; ATP binding; ATPase activity	++	
Grp78/BiP	ER	as Hsc70	++	+++*
mtHsp70/Grp75	Mitochondria	as Hsc70	+	++
DnaK	E. Coli	partner of DnaJ	–	+++
HSP90				
Hsp90 (α and β)	Cytoplasm, Nucleus	Bind to other proteins; regulate protein activity; prevent aggregation of refolded peptide; correct assembly and folding of newly synthesised protein; assists in the maintenance of the HSF1 monomeric state in non–stressful conditions	++	+++
Grp94/gp96/Hsp100	ER	Bind to other proteins; regulate protein activity; prevent aggregation of refolded peptide; correct assembly and folding of newly synthesised protein	+	++*
HSP110				
Hsp110 (human)	Nucleolus, Nucleus Cytoplasm	Thermal tolerance	+	++
Apg–1 (mouse)	Cytoplasm	Protein refolding		
Hsp105	Cytoplasm			
Hsp104	Cytosol		+	+++
Grp100	ER, Golgi		+	++*

Normal = normal expression level, **Stress** = upregulated during stress, * = non–heat induced, **empty box** = no information available, **–** = no expression, **+, ++, +++** = weak, moderate, strong expression

Table 1.1: Classification of Heat Shock Proteins; Small HSPs, HSP40, HSP70, HSP90, HSP110

1.3 Molecular Chaperones and Protein Folding

In 1972, Christian Anfinsen received the nobel prize in chemistry for his work on protein folding. Anfinsen denatured and refolded ribonuclease in vitro from an inactive state back to a 100 % active enzyme (Anfinsen 1973). He postulated that *"all information necessary to achieve the native conformation of a protein is contained in its amino acid sequence"*. Contrary to Anfinsen stands the so–called *Levinthal Paradox*, named by Levinthal in 1968 (Levinthal 1968; Yon 2002). Levinthal proposed, that for a polypeptide chain of hundred amino acids, assuming only two possible conformations for each residue, there are 10^{30} possible conformations. Estimating the required time for converting the conformations into another as 10^{-11} seconds, it would take 10^{11} years generating all conformations! In the actual cell, far more protein folding is performed in minutes or seconds, indicating the existence of additional mechanisms of protein folding.

Many stress proteins are molecular chaperones. The name was originally used by Laskey et al. in 1978 (Laskey et al 1978) as a description of nucleoplasmin, a protein involved in the building of nucleosomes in Xenopus eggs. Later on, in 1987, Ellis used the term *molecular chaperone* for **all** proteins involved in folding and assembly of other proteins and protein complexes (Ellis 1987). The definition of Ellis is still valid. Molecular chaperones are highly conserved in evolution. Similar to enzymes, they are a part of the reaction, but not a part of the later protein target. The targets of molecular chaperones are newly synthesized proteins, mutated proteins (mutations in a gene may lead to misfolding of the appendant protein) and damaged proteins (due to endogenous or exogenous stress factors like heat). Molecular chaperones are a very important part of the house–keeping machinery of the cell.

Many newly synthesized proteins need their help in developing the correct three–dimensional structure. According to Yon (Yon 2002) the Anfinsen postulate is not violated during this process: Molecular chaperones associate with nascent, stress–destabilized and translocated proteins, preventing improper folding and aggregation. The ability of the newly synthesised proteins to achieve native conformation is not impeded. It must not be forgotten, that a significant proportion of the volume in a cell is physically occupied, limiting the space for protein folding. Compared to in vitro experiments, the cell in vivo is a crowded environment, with an effective protein concentration of up to 300 mg/ml, in addition to macromolecules, organelles and a cytosceleton (Ellis 2001, Frydman 2001). Chaperones minimize the space required for successful protein folding.

Molecular chaperones, combined with proteases, work as a posttranslational quality control system in the cell, minimizing the amount of misfolded and degraded polypeptides during protein synthesis (Wickner et al 1999).

The best known chaperones belong to the HSP40, HSP60, HSP70 and HSP90 families. Hsp40 and Hsc70 bind to nascent polypeptides emerging from the ribosome. Hsp60 provides a protected environment for folding. Hsp90 is a component of the machinery assembling multiproteins (Frydman 2001). The HSP60 family will be discussed in detail in Section 1.6.

1.4 The Stress Response

The stress response is an ancient and highly conserved mechanism. It guarantees the survival of cells during various stress situations, e.g., heat, cold and exposure to chemicals (Section 1.2). In this thesis, the focus will be on heat as a stress factor, due to its importance in the genesis of the Sudden Infant Death Syndrome. Stress generates molecular damage in compartments and cells. One characteristic trait following all stress reactions is the accumulation of abnormally folded proteins. This is a universal trigger for the stress response.

As an example of stress response, the heat shock response in mammals (humans) will be discussed in this Section with a simplified overview being shown in Figure 1.1 (based on Latchman 2001 and Morimoto 1998). The heat stressed cell reacts to the abnormally folded proteins with the production of heat shock proteins. This process requires the transcription (synthesis of mRNA on a DNA template) of heat shock protein genes, followed by their translation (synthesis of protein on the mRNA template). The heat stress response starts with the trimerization of the heat shock transcription factor 1 (HSF1). This factor is essential for the RNA polymerases (enzyme for mRNA synthesis) binding to the heat shock gene. HSF1 exists as a monomer in the cell. Upon heat shock it assembles into a homotrimer, that binds to the heat shock response element (HSE). The latter is a characteristic short sequence in the promoter (the region of DNA involved in binding of RNA polymerase to initiate transcription) of a heat shock gene. After synthesis of the heat shock gene mRNA, the latter is translated into the heat shock protein. The protein now displays its protective properties in the cell, e.g., refolding of proteins.

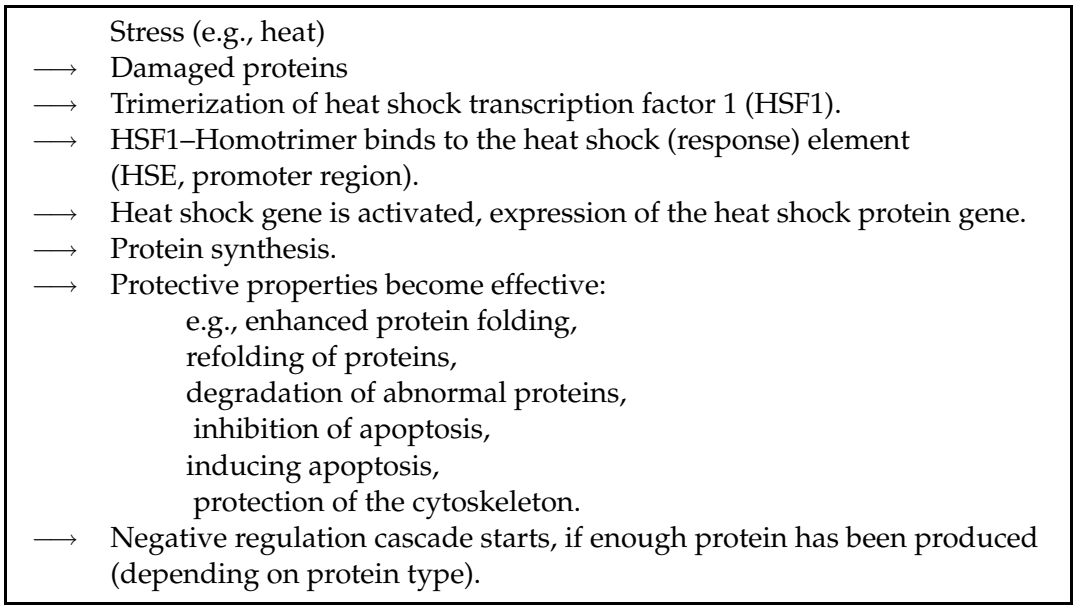


Figure 1.1: The Stress (Heat Shock) Response (based on Latchman 2001 and Morimoto 1998)

In mammals three heat shock transcription factors (HSFs) are known: HSF 1, 2 and 4. In avians an additional factor HSF3 exists. All HSFs are highly conserved in the evolution. They are modular constructions and show distinct domains: an amino–terminal helix–turn–winged–helix DNA binding domain (DBD), regulatory domains, one or more coiled–coil trimerization domains, nuclear localization domains and a carboxy–terminal transcriptional trans–activation domain (Ahn and Thiele 2003; Pirkkala et al 2001; Morimoto1998; Wu 1995; Wu et al 1994; Morimoto et al 1994b).

HSF1 is a stress–inducible factor. Heat stress and H_2O_2 activate HSF1, both in vivo and in vitro. The cystein residues C35 and C105 in HSF1 build redox–regulated disulfide bonds during this process (Ahn and Thiele 2003). Under non–stress (basal) conditions, it exists as a monomer, a form incapable of DNA binding. HSF1 is integrated in a complex control system in the cell; including the Hsp90 multichaperone system, mitogen–activated (MAP) kinases and extracellular signal–related kinase (ERK 1; Hasday and Singh 2000).

HSF2 and HSF4 exist in at least two isoforms A and B, arising from alternative splicing of the genes. HSF2 and HSF4 work in combination with HSF1 (He et al 2003; Zhang et al 2001). Also, HSF2 is negatively regulated by the stress factor heat. Under the influence of heat stress, it is deactivated (Mathew et al 2001). The isoform HSF4a is a repressor of heat shock transcription for hsp27 and hsp90 (Zhang et al 2001; Frejtag et al 2001; Tanabe et al 1999).

The HSE is the heat shock (response) element, the promoter element for binding of the heat shock transcription factors. The consensus sequence is 5'nGAAn'3. Typical heat shock elements consist of 3 to 5 repeats, e.g., 5'nGAAn nTTCn nGAAn'3. The location of the element in the promoter is variable and ranges from forty to hundreds of bases upstream the starting point of the gene (Fernandes et al 1994; Morimoto et al 1994b).

1.5 Stress Tolerance/Thermotolerance

Heat and other stress tremendously affect the living organism, down to the cellular level. For example, the cytoskeleton is disrupted due to collapsing microfilaments. The number of mitochondria decreases, the respiratory chain inside the mitochondria is disturbed. Polysomes disappear rapidly and the splicing machinery is disturbed or stopped. However, the heat shock proteins continue to be transcribed, translated and processed.

A special phenomenon known as *thermotolerance* occurs when light heat stress is induced prior to severe heat stress. A cell pretreated with a light heat shock is coping much better with a following severe heat treatment. The mild heat treatment induces the production of heat shock proteins, equipping the cell with a faster repair mechanism for the damaged proteins. In some cases (yeast, bacteria), even lethal stress can be countered following a mild heat shock. The general term applying to different kinds of stress is *stress tolerance* (Parsell and Lindquist 1994).

1.6 HSP60, Chaperonin

The HSP60 heat shock protein family is also known as the *Chaperonin* family. This family is of special interest to this thesis, because of the possible involvement of a family member (Hsp60) in the genesis of Sudden Infant Death Syndrome. The term Chaperonin was introduced by R. John Ellis in 1990 referring to non–heat–induced Heat Shock Protein 60 (Hsp60; Ellis 1990; Fink1999). As with all stress proteins and molecular chaperones, members of the Chaperonin family are highly conserved in the evolution. They exist in every organism and every cell compartment. The only exception are some parasites, microsporidia (Katinka et al 2001) and mycoplasma (Glass et al 2000; Ranford and Henderson 2002). Chaperonins usually work in combination with a smaller protein, the co–Chaperonin.

In Homo sapiens and other mammals the terms Hsp60, Chaperonin and Chaperonin60 are used synonymously. Some publications also use the abbreviation Cpn60 instead of Hsp60 for certain members of the type I subfamily of Chaperonins. Preferentially, Cpn60 names the members of the HSP60 family active in chloroplasts.

1.6.1 The HSP60 family: Subfamilies and Members

The first sequencing of the mRNA of the human hsp60 was done in 1989 by Jindal et al. (Jindal et al 1989). Later, the sequence was confirmed by Venner et al. in 1990 (Venner et al 1990). The current sequence in the GenBank Database is based on Venner et al. (Venner et al 1993; GenBank Accession Number M34664). The term GenBank Accession Number will be abbreviated as GAN in this treatise. The first expression of the human protein in E. Coli and the development of monoclonal antibodies was performed by Singh and Gupta in 1992 (Singh and Gupta 1992). In 1996, Pochon and Mach gave the first description of the human hsp60 gene regarding its exon/intron structure (Pochon and Mach 1996). The final sequencing of the human hsp60 gene, revealing the complete exon/intron structure, was done by Hansen et al. in 2003 (Hansen et al 2003b; publication, the sequence was submitted to GenBank in 1999 with a GAN AJ250915 (Hansen et al 2003a)). Hansen et al. also demonstrated, that human hsp60 is localised with its partner hsp10 (co–chaperonin) on chromosome 2 (2q33.1); in a "head to head" (starting point to starting point) direction, sharing the bidirectional promoter. The hsp60 gene contains 12 exons, of whom the first one is non–coding. This configuration is the same in the hsp60/hsp10 genes in rat (Rattus norvegicus; Ryan et al 1997a); pufferfish (Fugu rubripes), zebrafish (Danio rerio), the nematode Caenorhabditis elegans (Martin et al 2002); and the mouse (Mus musculus), indicated in a "Homologene" search in the GenBank (GENBANK 2004), no direct publication available.

The equivalent gene in Escherichia Coli is groEL (protein GroEL), its partner is groES (protein GroES). The genes are located similarly to to the abovementioned eukaryotic genes in the E. Coli groE operon (Zeilstraryalls 1991). In plant chloroplasts, the genes are named cpn60 (protein Cpn60) and cpn10 (protein Cpn60).

The Chaperonins can be divided into two subfamilies: type I Chaperonins, also known as the GroEL/Hsp60–subfamily and type II Chaperonins, also known as the TCP–1 (t–

Family, Members	Intracellular location	Function	Expression	
			Normal	Stress
HSP10 (Co–Chaperonins)				
Hsp10	Mitochondria	assists Hsp60	+	++
GroES	E. Coli	assists GroEL	+	++
Cpn10	Chloroplasts	assists Cpn60	+	++
HSP60 (Chaperonins)				
Hsp60	Mitochondria	Bind to partly folded polypeptides and assist correct folding; assembly of multimeric complexes		
GroEL	E. Coli	as Hsp60	+	+
Cpn60	Chloroplasts	as Hsp60	+	+
Thermophilic Factor 55	Archaea	as Hsp60		
TriC/CCT	Cytoplasm	as Hsp60		

Normal = normal expression level, **Stress** = upregulated during stress, * = non–heat induced, **empty box** = no information available, – = no expression, **+, ++, +++** = weak, moderate, strong expression

Table 1.2: The HSP60 family, Chaperonins and Co–Chaperonins

complex polypeptide 1) ring complex subfamily. Type I Chaperonins are present in prokaryotes (eubacteria) and organelles (mitochondria and chloroplasts). Type II Chaperonins are present in archaebacteria and in the eukaryotic cytosol. They are known under two different names: TriC = TCP–1 ring complex and CCT = Chaperonin Containing TCP–1 (Levy–Rimler et al 2002; Fink 1999). An overview of the Chaperonins and their co–Chaperonins is shown in Table 1.2.

1.6.2 The HSP60 family: Proteins, Structure and Function

All members of the HSP60 family (type I and II) have a similar structure: They are ring–shaped oligomeric protein complexes with a large central cavity, working either as a single or double ring. Nonnative protein structures bind in the central cavity, which is sometimes referred to as the *Anfinsen Cage* or folding cage. This cage represents a compartment with a defined environment and certain steric conditions and restrictions. The information on the molecular weight (60 kD) represents the weight of the monomeric protein. The members of the HSP60 family work as multimers. Graphical descriptions for the type I family are shown in Figures 1.2 (Slavotinek and Biesecker 2001) and 1.3 (Bukau and Horwich 1998). The latter shows a side and top view of a type I Chaperonin with a protein inside. The gap shown between the two heptamers indicates the double ring structure, but does not represent an unpassable vertical separation.

The type II subfamily (TCP–1) multimers are heteromeric and consist in the single ring form as an octamer or nonamer (8 or 9 60kD subunits). In contrast to the single gene encoding of the homomeric subunits, the heteromeric subunits are encoded by different unique genes. The CCT in the eukaryotic cytosol plays a fundamental role in the folding of cytoskeletal proteins (Carrascosa et al 2001; Slavotinek and Biesecker 2001; Fink 1999; Kubota et al 1994; Willison and Kubota 1994).

In thermophilic and hyperthermophilic archaea, the TCP–1 complexes are called thermosomes. They play a crucial role in constantly heat–stressed environments with temperatures above $100^{\circ}C$. This indicates that a great amount of metabolic energy is needed to maintain protein stability with constant folding and refolding occurring. Unlike the members of the type I subfamily, the thermosome of the Thermosoma species seems to have a built–in lid for covering the folding cavity (Sterner and Liebl 2001).

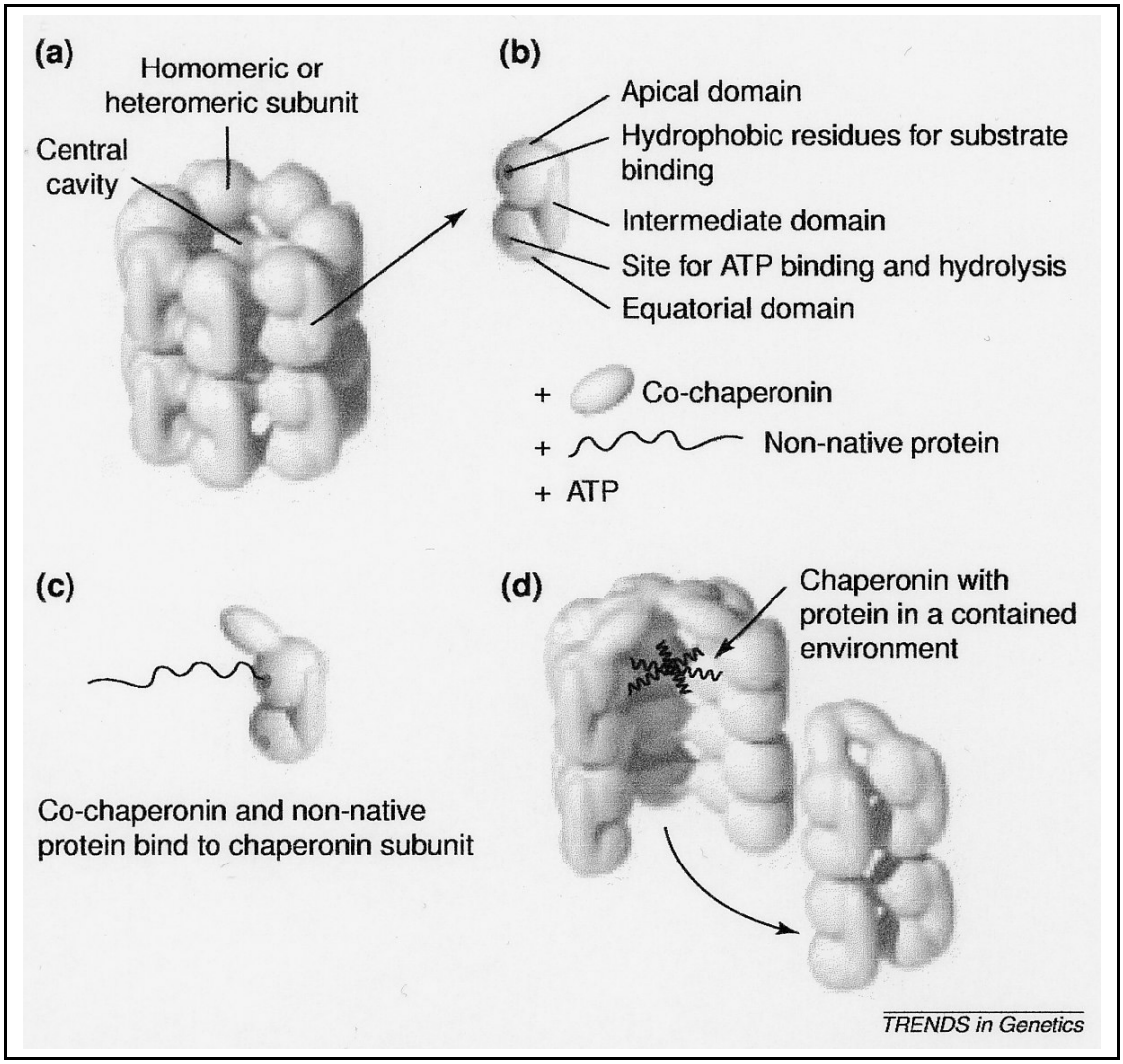


Figure 1.2: Chaperonin60: Structure and Function (Slavotinek and Biesecker 2001)

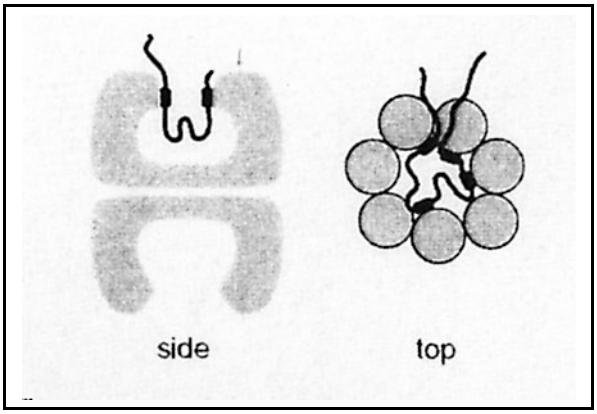


Figure 1.3: Chaperonin60: Side and Top View (Bukau and Horwich 1998)

The multimers of the type I subfamily (GroEL/Hsp60) are homomeric and exist in the single ring form in a heptamer (7 60kD subunits). The typical structure in the cell is the double ring form of 14 identical 60kD subunits in two heptameric rings. The rings are stacked on top of each other in an inverted orientation. This is a barrel–shaped structure, sometimes referred to as the "double donut" (named by Bukau and Horwich in 1998 (Bukau and Horwich 1998)), see Figure 1.3. The co–Chaperonins (Hsp10, GroES) consist of 7 subunits forming a single ring. The small partners build the caps sitting on either side of the double ring structure of the Hsp60/GroES. The activity of type I Chaperonins is modulated by the binding and hydrolysis of ATP. X–ray cristallography and electron microscopy of the GroEL/GroES complex are complete (Xu et al 1997).

Most studies on structure, function and the mechanism of protein folding in type I Chaperonins examine the GroEL/GroES system of E. coli. Each ring consists of three domains: the equatorial, the intermediate and the apical. The apical, forming the mouth of the central cavity undergoes major conformational changes on binding of ATP and the co–Chaperonin GroES, which lead to substantial changes in the hydrophobic nature of the cavity. In addition to their folding tasks, the type I Chaperonins assemble oligomeric proteins. For GroEL/GroES evidence for the mechanism is present (Wynn et al 2000). The folding mechanism in the type I Chaperonin can be described as follows:

1. Capture

   The protein enters the proximal Chaperonin ring. It is a in a special conformation and energy state called a folding intermediate and thus prone to aggregation. Typical molecular distinguishing marks for this state are exposed hydrophobic surfaces. The Chaperonins bind these hydrophobic surfaces to prevent them from incorrect interaction and aggregation.

2. Encapsulation

   The binding of the co–Chaperonin enlarges the central cavity by inducing a conformational change in the proximal Chaperonin ring. The environment in the central cavity is changed from a hydrophobic to a hydrophilic one. This environment forces a change in the conformation of the substrate. The folding then occurs in the central cavity under ATP hydrolysis.

3. ATP hydrolysis and ring conditioning

   This is a timing device, controlling the lifetime of the encapsulated state.

4. Ligand release and domain relaxation

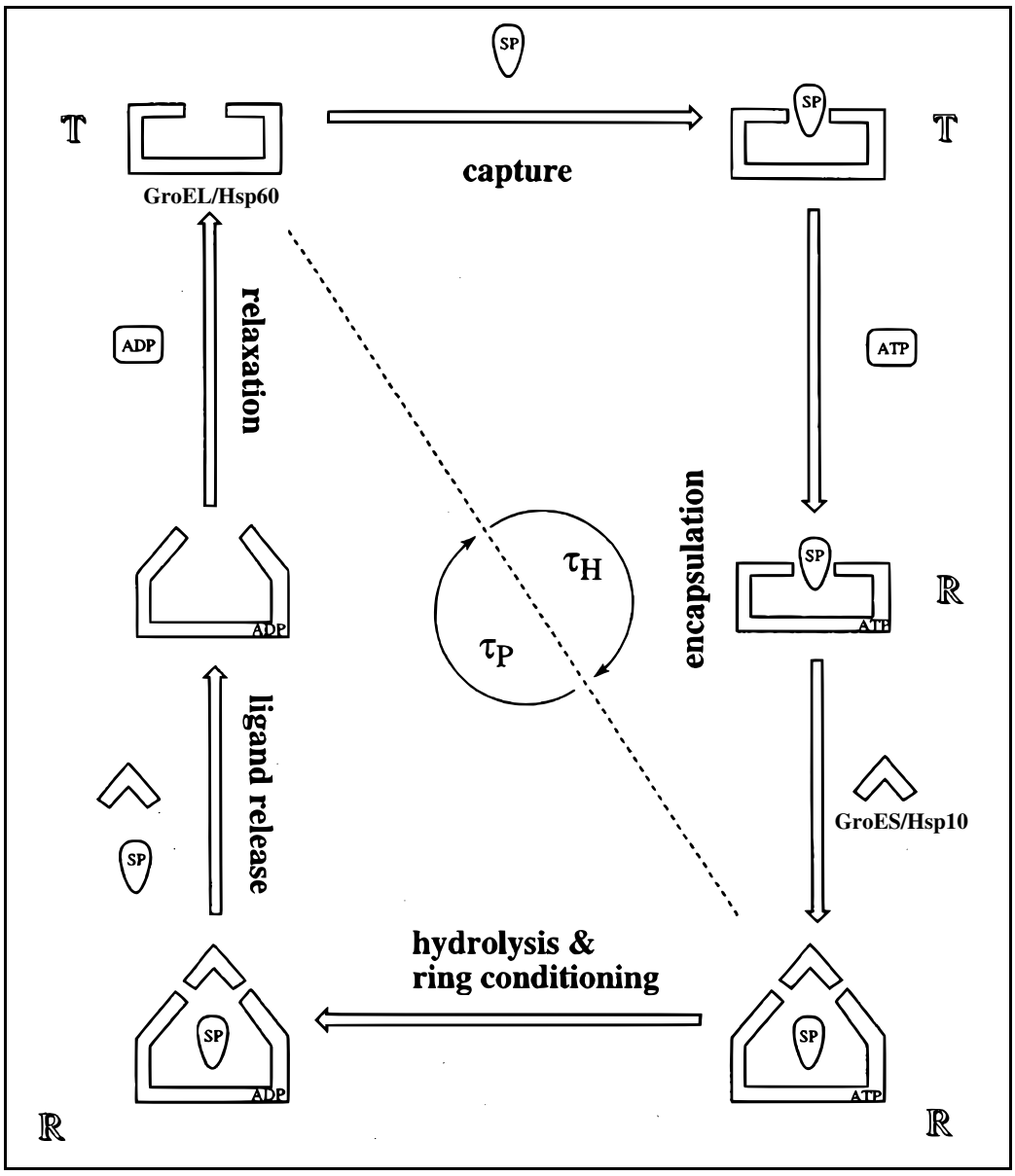
   After folding, the ATP hydrolysis of the distal Chaperonin ring drives the release of the ligands, protein, co–Chaperonin and ADP.

   If a protein begins to misfold after synthesis, Hsp60 is even able to *unfold* it partially. From this newly corrected energy state, the protein can reach its native conformation more easily.

It is still unclear if a certain protein undergoes a single or multiple cycles of folding in the type I Chaperonins. Both alternatives appear to be possible. Also, the energy states

of a protein and their influence on folding are under discussion (Walter 2002; Thirumalai and Lorimer 2001; Grantcharova et al 2001; Lewin 2000; Fink 1999; Bukau and Horwich 1998; Frydman et al 1994).

A graphical description is shown in Figure 1.4. For simplicity, only the proximal or cis ring of the GroEL tetradecamer is shown. The original figure appeared in Thirumalai and Lorimer 2001.



SP = substrate protein; **T** = tightly bound, the state in which the substrate protein is most tightly bound; **R** = relaxed bound, the state in which the substrate protein is less tightly bound; τ_H = time with hydrophobic interaction between SP and Chaperonin; τ_P = time with hydrophilic interaction between SP and Chaperonin

Figure 1.4: Chaperonin60: Mechanism of Protein Folding (based on Thirumalai and Lorimer 2001)

1.6.3 The Human Chaperonin60

The human Chaperonin Hsp60 is located in the mitochondria, where it exists most likely in a dynamic equilibrium between monomers, single ring heptamers and double ring tetradecamers. A high concentration of Hsp60 and the presence of ATP and Hsp10 (the most likely condition in a cell) favors the formation of the tetradecameric structure.

Many proteins located in the mitochondrial matrix are encoded in the nucleus, not in the mitochondrial DNA. They are thus translated in the ribosomes of the cytoplasm. It is not possible for a folded protein to pass the mitochondrial membrane, only a linear unfolded structure can pass. For recognition, the proteins carry a leader peptide, that is later cleaved at their amino terminal end. This leader or signal peptide marks a protein for transport into mitochondria. E.g., in Hsp60, the leader consists of the first 26 amino acids. In a typical eukaryotic cell, about 10 % of all proteins are targeted at the mitochondria. A complex system exists that directs the protein into the appropriate inner section of the mitochondria (outer membrane, intermembrane space, inner membrane and matrix). Hsp60 is located in the innermost compartment, the mitochondrial matrix. In combination with the mitochondrial Hsp70 (mt–Hsp70), the Hsp60 machinery is the most important chaperone machinery in the mitochondria. In the human mitochondria, the chaperoning of new proteins is a more vital function of Chaperonin60 than its stress response (Levy–Rimler et al 2001; Fink 1999; Ryan et al 1997b). Additionally, evidence exists that Hsp60 is exported from the mitochondria into extramitochondrial settings such as the cell surface, peroxisomes and the ER (Soltys and Gupta 1999).

1.6.4 Involvement of Hsp60 in physiological processes

Chaperonins are essential for cellular functions and processes:

- The deletion of the genes in yeast (Saccharomyces cerevisiae: hsp60/hsp10) and bacteria (E. Coli: groEL/groES) are lethal for the organisms (Levy–Rimler et al 2002).
- In humans, many diseases with Hsp60/Hsp10 involvement are known. Hereditary spastic paraplegia spg13 is associated with a mutation in the hsp60 gene: The amino acid 72 valine is changed to isoleucine (mutation V72I; Hansen et al 2002).
- A patient with congenital lactic acidaemia caused by insuffiency of mitochondrial enzymes, suffered from a Hsp60 deficiency and died shortly after (Ranford and Henderson 2002).
- Recombinant Human Hsp60 interacts with the prion protein PrP^c (Edenhofer et al 1996). Chaperonins may be involved in the conversion process from the wild type prion PrP^c to the disease type prion PrP^{sc}. The latter is the pathogenic isoform, associated with scrapie (sc), where the disease type prion was first noticed (Gauczynski et al 2001).

The human immune system preferentially targets the members of Type I subfamily (GroEL/Hsp60) in pathogens such as bacteria, viruses, protozoa and worms. They are

members of the so–called common antigen group. In humans, immune responses to the Hsp60 of several microorganisms are known:

- Tuberculosis (Mycobacterium tuberculosis)
- Leprosy (Mycobacterium leprae)
- Trachoma (Chlamydia trachomatis)
- Lyme Disease (Borrelia burgdorferi)
- Q–fever (Coxiella burnetti)
- Legionnaires' Disease (Legionella pneumophila)
- Brucellosis (Brucella abortus)
- Whooping Cough (Bordetella pertussis)
- Gastric Ulcer, Gastritis (Helicobacter pylori)
- Yersiniosis (Yersinia enterocolitica)
- Syphilis (Treponema pallidum)
- Listeriosis (Listeria monocytogenes)

Some of the infections can even be diagnosed based on the presence of antibodies against Hsp60. The downside of a potent immune reaction to microbial Chaperonins are autoimmune diseases and immunodeficiency diseases. GroEL for example, the best studied Chaperonin from E. coli, affects both the innate and the acquired immune system of humans and other mammals.

An infectional invasion stresses both, the pathogen and the infected host. Both react with an upregulated expression of stress proteins, including Chaperonins (Zügel and Kaufmann 1999; Fracella and Rensing 1995).

Heat shock proteins are involved in the allograft rejection process after transplantation. However, the precise influence of these proteins is unknown. A fine balance may exist between protective and damaging effects (Pockley 2001).

The Chaperonins are highly conserved in the evolution and thus very similar. It is hence possible, that an immune system attacks both its own and the foreign Chaperonins. This process is called molecular mimicry: the immune system, e.g., T cells and antibodies, is not able to discriminate sharply between different sources of antigens, e.g., amino acid sequences of Chaperonins (Van Noort and Amor 1998). Often, the antibodies bind to the target tissues and cause an autoaggressive response. Examples for autoimmune diseases and other diseases in humans in which Hsp60 plays a role are summarized in Table 1.3 (Falkowska–Podstawka and Wernicki 2003; Ranford and Henderson 2002; Neuer et al 2000; Ranford et al 2000; Zügel and Kaufmann 1999; Edenhofer et al 1996; Fracella and Rensing 1995).

In connection with the immune system, Chaperonins are also involved in cellular communication. Different Chaperonins can bind to different cell–surface receptors. They may be intercellular stress signals, secreted into the extracellular fluid in times of stress (Maguire et al 2002; Ranford et al 2000; Soltys and Gupta 1999).

Autoimmune Diseases
Insulin–dependent Diabetes mellitus (IDDM)
Adjuvant Arthritis (AA)
Pristan–induced Arthritis
Reactive Arthritis Response of $\alpha\beta$ T cells from synovial fluid to human Hsp60, to stressed host cells, and to mononuclear cells isolated from inflamed joints.
Juvenile Arthritis Anti–Hsp60 antibodies found.
Rheumatoid Arthritis (RA) Increased levels of Hsp60–specific antibodies in serum; response of crossreactive $\alpha\beta$ T cells from inflamed sites to mycobacterial Hsp60 and human Hsp60
Morbus Bechterew
Multiple Sclerosis (MS) Detection of Hsp70–reactive and Hsp60–reactive $\alpha\beta$ T cells in spinal fluid of MS patients.
Systemic Lupus Erythematosus (SLE)
Chronic Gastritis Colocalization of $\gamma\beta$ T cells with Hsp60 expression of imflammatory gastritis epithelium.
Behcets Disease Hsp60–specific antibodies in serum from patients; T cell response to self Hsp60.
Arteriosclerosis
Systemic Sclerosis Increased levels of Hsp60–specific antibodies (mycobacterial) in serum.
Atherosclerosis Increased levels of Hsp60–specific antibodies in serum; presence of Chaperonin60 in atherosclerotic lesions; lysis of macrophages expressing Hsp60 from patients by antibodies against self hsp60.
Psoriasis Increased levels of Hsp60–specific antibodies (mycobacterial) in serum.
Kawasaki Disease Increased levels of Hsp60–specific antibodies in serum; both against mycobacterial and human.
Inflammatory Bowel Diseases
Crohn's Disease Increased levels of Hsp60–specific antibodies in serum
Ulcerative Colitis Increased levels of Hsp60–specific antibodies in serum
Borderline Hypertension Increased levels of Hsp60–specific antibodies in serum
Prion Diseases Hsp60 interacts with the prion protein
Immune mediated pregnancy failure Suggested mechanism: strong immune response to self Hsp60 after persistent infection with Chlamydia trachomatis disturbing the embryo development

Table 1.3: Hsp60, Involvement in Diseases

1.7 Sudden Infant Death Syndrome (SIDS)

The **S**udden **I**nfant **D**eath **S**yndrome (SIDS) is still an enigma. Even in our current times only theories exist on its genesis. One of the earliest mentions of this phenomenon is described at about 4000 B. C. in the cultures of the orient. The branch of legal medicine is engaged in this topic since 1895 (Rißе 2002).

SIDS (the code in the International Classification of Diseases (ICD) is 798.0 (Sudden Infant Death Syndrome), ICD 9th Edition, and R 95, ICD 10th edition) is one of the most frequent causes of death in Germany and other Western countries (Switzerland, Netherlands, France, Great Britain, Ireland, Denmark, Norway, Finland, USA, Australia, New Zealand) in children up to 1 year. The annual rate of SIDS infant mortality in these countries over the last twenty years lies between 0.4 and 1.7 per 1000 live births (Hunt and Hauck 2004; Jorch 2000).

SIDS remains a diagnosis of exclusion. Only after ruling out other possible causes of death, is the diagnosis "Sudden Infant Death" given. The definition of Beckwith (Beckwith 2003) is the most substantial: *"The sudden and unexpected death of an infant younger than 1 year and usually beyond the immediate perinatal period, which remains unexplained after a thorough case investigation, including performance of a complete autopsy and review of the circumstances of death and of the clinical history. Onset of the lethal episode was presumably during sleep (ie, the infant was not known to be awake). Minor inflammatory infiltrates or other abnormalities insufficient to explain the death are acceptable."*

Hunt and Hauck (Hunt and Hauck 2004), Rißе (Rißе 2002), Hunt (Hunt 2001) and Roll (Roll 2000) also emphasise a thorough postmortem examination to eliminate other causes of death such as congenital anomalies, deadly infections and traumatic child abuse (filicide). An older definition described by Beckwith is still in use (Beckwith1970; Beckwith 2003): "The sudden death of any infant or young child, which is unexpected by history, and in which a thorough postmortem examination fails to demonstrate an adequate cause of death." Also commonly used, especially in the United States of America, is the definition by Willinger (Willinger 1991), Valdes–Dapena (Valdes–Dapena 1992) and Beckwith (Beckwith 1993; Kurz 2000a): "The sudden unexpected death of an infant under one year of age, which remains unexplained after a thorough case investigation, including performance of a complete autopsy, examination of death scene, and review of clinical history."

Despite the complexity and problems of diagnosis, certain characteristics are known. This includes risk factors, autopsy findings (pathological features), findings at the death scene and epidemiological features. These are typical characteristics found solely or more likely in SIDS victims compared to controls. A complete overview of these characteristics is shown in Section 1.7.1. A subset of the characteristics will be designated *SIDS criteria* and is of greater interest in this doctoral thesis (see Section 1.7.1 and Section 1.7.3).

Based on their pathological findings, SIDS cases are categorised. In this thesis, the pathological categories (1–4) of the SIDS cases were classified according to the German Study on Sudden Infant Death (GeSID; Findeisen et al. 2003), see Figure 1.5.

Category 1 includes all children without pathological findings from autopsy and additional investigations.

Category 2 (minor pathological findings):

- minor infections of the respiratory tract
  - rhinitis, otitis media, pharyngitis tracheitis
  - bronchitis, mild/intermediate forms of peribronchitis
  - mild/intermediate forms of bronchiolitis and of interstitial pneumonia;
- tonsilitis
- mild abnormalities and congenital disorders
  - mild forms of hepatitis/hepatosis
  - enteritis without exsiccosis
  - mild forms of nephritis/nephrosis
  - local infections with cytomegalovirus
  - mild forms of metabolic disorders without symptoms

Category 3 (major pathological findings):

- metabolic disorders
- interstitial pneumonia with bacterial superinfection
- severe bronchiolitis
- enteritis with exsiccosis
- peri–/myocarditis, especially of the borderline type
- mild forms of meningo–encephalitis

Category 4 (includes all non SIDS cases, children with a clear cause of death): e.g.,

- bronchopneumonia
- interstitial pneumonia with carditis
- severe meningo–encephalitis
- major congenital disorders
- generalised infection
- myocarditis with sarcolysis

Figure 1.5: Pathological Categories of Sudden Infant Death Cases

1.7.1 SIDS Characteristics

SIDS characteristics can be divided in two sets. The first contains autopsy results (pathological features) including histological analyses. The second includes all other charac-

teristics, with most of them being determined epidemiologically (this also applies to the findings at the death scene). By now, many of the characteristics that are associated with an increased risk of SIDS are known. In many countries, SIDS prevention programs were introduced to give parents advice on how to minimize the risks (Hunt and Hauck 2004), Deutsche Akademie für Kinderheilkunde und Jugendmedizin (Poets and Jorch 2000), American Academy of Pediatrics, Task Force on Infant Sleep Position and Sudden Infant Death Syndrome (1999) .

Characteristic autopsy results are :

- Inthrathoracic petechiae (mostly subepicardial, subpleural and beneath the thymus)
- Pulmonary edema
- White or bloody foamy secrete/fluid around the nose, also in mouth, pharynx, larynx, trachea and bronchus, indicating a pulmonary edema
- Certain brainstem abnormalities
- Liquid blood in the right heart
- Cyanosis of lips and nailbeds
- Anemic abdominal organs
- Empty bladder
- Infections of the upper respiratory tract
- Lymphatic system with big lymph node in thymus, neck and intestines

(Hunt and Hauck 2004; Byard 2003; Kleemann and Rognum 2000; Kleemann 2000; Kleemann 1996; Berry 1992).

General characteristics associated with a higher risk of Sudden Infant Death are an age between 2 and 4 months and male sex. Also a "winter peak" exists (highest SIDS incidence during the winter months). The high rate of infections in victims of Sudden Infant Death shortly before they died is often associated with the cold season (Byard 2003; Kurz 2000c; Jorch 2000; Vege and Rognum 1999; Blackwell 1999; Blackwell and Weir 1999; Helweg–Larsen et al 1999; Forsyth 1999; Douglas et al 1998; Mage and Donner 1997; Kleemann 1996; Kleemann 1991).

Social characteristics include:

- Ethnicity (for example a higher risk in African–Americans than in Caucasian Americans or Asians)
- Socioeconomic status (a lower status and income indicates a higher risk)

- Age of the parents (younger parents undergo a higher risk than older parents)
- Educational level of the parents, especially the mother (mothers with a lower educational level undergo a higher risk)

(Pollack and Frohna 2002; Einspieler 2000; Blackwell 1999; Kleemann 1996; Willinger 1995).

Pregnancy, birth and neonatal care are also important in determining a possible risk of Sudden Infant Death:

- Twin births and multiple births undergo a higher risk
- Children born prematurely or those who have a low birth weight undergo a higher risk
- Short interpregnancy intervals and high birth order undergo a higher risk

Poor prenatal care is associated with a higher risk.

In the postnatal care the greatest risk factors are:

- Absence of breast feeding
- Co–sleeping (bedsharing)
- Maternal drug use
- Sleeping outside parents bedroom (lesser factor)

Smoking is one of the highest known risk factors. Parental/maternal smoking and exposure to cigarette smoke (during and after pregnancy) leads to greater vulnerability against infections, breathing problems during sleep, and disturbs the general development of a child. It also increases the carbon dioxide threshold necessary for arousal (Byard 2003; Cohen et al 2002; Köstl and Kerbl 2000; Einspieler 2000; Kurz 2000c; Blackwell 1999; Raza et al 1999; Kleemann 1996; Schlaud et al 1996; Willinger 1995; Poets et al 1994).

Prone sleeping is often considered along with smoking as the highest risk factor at all. Children sleeping prone undergo a significantly higher risk of Sudden Infant Death than children sleeping supine. The back–to–sleep campaign (advice for parents to put their children to sleep supine, if not medically contraindicated) in some countries, e.g., USA, Great Britain, in the early Nineties (1991–1995) reduced the SIDS incidence tremendously. No uniform theory exists, why prone sleeping increases the risk of SIDS. A possibility is a greater vulnerability to infections or heat stress. Others indicate a reduction in physiological control related to respiratory, cardiovascular and autonomic control mechanisms.

The latter is important for arousal during sleep. A delayed arousal or no arousal at all may have deadly consequences. A delayed arousal is especially dangerous, if the child becomes hypoxic, in consequence of having an infection of the upper airways (Byard 2003; Galland et al 2002; Pollack and Frohna 2002; Einspieler 2000; Gibson 2000; Blackwell 1999; Schlaud et al 1999; Douglas et al 1998; Kleemann et al 1998; Willinger 1995; Hardy Havens and Zink 1994; Kleemann 1991).

1.7.2 SIDS and Heat Stress

Due to a possible connection between Sudden Infant Death and heat stress (heat stress and/or a deficiency in the heat stress response because of mutated heat shock proteins), this risk factor is of special interest in this thesis.

Human beings have a very complex thermoregulation. A human is homeotherm, the internal temperature is kept constant. Thus, the heat storage in the body must be zero, heat gain and heat loss are balanced. Body temperature is a function of three variables: internal heat production, external environmental temperature and thermal insulation determining the exchange of thermal energy between the body and its environment. In an infant, heat production depends, for example, on muscular activity and metabolism. Heat loss is regulated, for example, via sweating and an outstretched body position.

Following Guntheroth and Spiers (Guntheroth and Spiers 2001) a differentiation is made between thermal stress and hyperthermia. Thermal stress describes a threat to the organism's thermal regulation, that is mild enough to permit the core temperature to remain within normal limits. Hyperthermia describes a condition with an increased core temperature, such as a fever.

During sleep, the heat exchange with the environment can be accomplished in four different ways: Conduction (exchange between two surfaces, e.g., skin and mattress), radiation (exchange between body and walls due to infrared electromagnetic energy), convection (exchange between skin and air) and evaporation (exchange due to the conversion of liquid into a gas phase – sweating). Heat exchange in neonates is more rapid and overall greater than in adults. Thus an increased risk exists for a neonate to suffer from hyperthermia or hypothermia. The most important aspect of this characteristic is, that neonates cannot maintain a thermoregulatory response during long exposures (Bach et al 2002).

As mentioned before, many SIDS victims die between 2 and 4 months of age. At this age, the thermal balance of a child is shifted in favour of heat conservation (Bach et al 2002). This fact also points to heat stress as an important environment and/or internal factor in Sudden Infant Death. Typical environmental factors are overheated rooms and a larger thermal insulation due to winter clothes and bedding (remember the "winter peak" – seasonality of SIDS); a typical internal factor is an infection, see Section 1.7.1.

Bach et al. (Bach et al 2002) theorize that homeostatic challenges (coping with heat stress) could require a protective arousal of the child. If this mechanism is faulty due to a delayed maturation of the arcuate nucleus, a brain–stem area implicated in the hypercapnic ventilatory response, chemosensitivity and blood pressure responses, the arousal may be not possible. Empiric research documented certain risk factors that are consistent

with thermal (heat) stress:

- Prone sleeping
- Child's head covered
- Soft mattress and pillows in child's bed
- Sheepskin in child's bed
- Swaddling the baby in combination with prone sleeping
- Excessive sweating of the children
- Overheated rooms
- Children having infections with fever
- Seasonal distribution of deaths
- Excess bedclothes – children wearing to much clothing in bed
- Passive cigarette smoking

(Guntheroth and Spiers 2001; Einspieler 2000; Kurz 2000c; Kleemann et al 1998; Sawczenko and Fleming 1996; Kleemann et al 1996; Kleemann1996; Ponsonby et al 1992; Fleming et al 1992; Stanton 1984).

These factors document that, despite any other potential trigger for Sudden Infant Death Syndrome, heat stress plays an important role. Nelson et al. (Nelson et al 2002) worked with a neonatal rat model and performed an interesting experiment, that combined the two risk factors heat stress and infection. They induced hyperthermia in neonatal rats and mimicked an infection with the chemical Muramyl Dipeptide (MDP). These risk factors lead to an increased production of specific interleukins (IL–1β, IL–6) that were also documented in SIDS victims (Rognum 2000). Furthermore, the mortality rate of the neonatal rats confronted with the infection–equivalent MDP and heat stress was increased.

Elabbassi et al. (Elabbassi et al 2001) argue, that body position and the risk of thermal stress induced by increased body heat storage are two independent risk factors for Sudden Infant Death. Their assumptions are based on an experiment with a thermal mannequin for testing the effects of clothing and body position (prone vs. supine). However, the model is without adaptive thermoregulatory processes, which are essential to human heat regulation. This reduces the adaptability in comparison with human beings.

Hahn et al. (Hahn et al 1991) examined the combined impact of heat and nicotine in cell culture (chinese hamster cells of the ovary). Nicotine alone does not induce the stress response, but nicotine in combination with heat induces the heat shock proteins Hsp28 and Hsp70 via binding of heat shock transcription factors to the heat shock element.

1.7.3 SIDS Criteria

The following characteristics are termed SIDS criteria. In context of this thesis, they are the most common characteristics and/or risk factors for Sudden Infant Death Syndrome. These criteria are:

- Preterm birth (risk factor)
- Body position and body temperature in death scene (risk factors prone sleeping and heat stress)
- Nicotine abuse by mother (risk factor smoking)
- Petechiae in pleura, epicard and thymus (characteristic autopsy results)
- Oedemata, foam and hemorrhage in the respiratory tract (characteristic autopsy results)

1.7.4 SIDS and Gene Variations

Until recently, research on Sudden Infant Death Syndrome was conducted mainly based on autopsy results (including histological findings) and epidemiological factors/risk factors. In the last ten years more and more molecular biology findings have made a valuable contribution to the known facts. It is very important to realize though, that even now all known genetic variations are insufficient to explain the genesis of Sudden Infant Death. No genetic trait or mutation has been identified as the sole trigger or actual reason for Sudden Infant Death.

The possibility of an x–chromosomal factor that would be a necessary but not sufficient factor for causing Sudden Infant Death Syndrome was theorized by Mage and Donner (Mage and Donner 1997). This theory was based on the significantly higher male SIDS fraction compared to the male birth fraction. Mage and Donner investigated 67378 SIDS cases from 36 global data sets and applied the Hardy–Weinberg genetic principle. Even today, no such factor has been identified.

Weese–Mayer et al. (Weese–Mayer et al 2003) theorize an influence of serotonin on Sudden Infant Death Syndrome because of its functions: Serotonin is involved in the regulation of breathing, the cardiovascular system, temperature control and the sleep–wake cycle. Narita et al. (Narita et al 2001) and Weese–Mayer et al. researched a polymorphism in the promoter region of the serotonin transporter gene (5–HTT; serotonin = 5–HT, 5–hydroxytryptamine). The polymorphism is a VNTR (variable number of tandem repeats) and has been shown to differentially modulate gene expression. Three human alleles are known: S, short, 14 repeats of the 20–23 base pair repeat unit; L, long, 16 repeats; XL, extra long, 18–20 repeats. SIDS victims have a statistically significant higher frequency of the long alleles (genotype L/L) compared to gender/ethnicity matched controls. Another VNTR is located in intron 2 of the 5–HTT gene, which so far has not been noticeable in Sudden Infant Death Syndrome.

In a pilot study, Gordon et al. (Gordon et al 2002) found a polymorphism in the interferon–γ gene (IFN). This polymorphism, called genotype 3/3, was significantly higher in SIDS families (40 %) than in control families (15.4 %).

In a recent study, Puffenberger et al. discovered a disorder named SIDDT (Sudden Infant Death with dysgenesis of the testes syndrome, OMIM Accession Number 608800; Puffenberger et al 2004). This disorder was found in 21 individuals of the Belleville Amish Community. 4 of the 21 SIDS cases showed a specific gene variant in both copies of a gene: A frameshift mutation 457–458insG at codon 153 in the TSPYL1 gene (testis–specific Y–like gene), which was identified by sequence analysis. The mutation leads to a truncation of translation at codon 169.

Defects of the mitochondrial β–oxidation (fatty acid metabolism), for example MCAD deficiency (medium chain acyl–CoA dehydrogenation), and glucose metabolism have long been mistaken as a genetic component in Sudden Infant Death Syndrome. Actually, these defects are diseases of the metabolism and do not belong into the genesis of Sudden Infant Death. For a review of these diseases, see Vockley and Whiteman 2002.

Some theories link the Sudden Infant Death Syndrome to a prolongation of the QT interval and to mutations in sodium channel and potassium channel genes, e.g., sodium channel alpha–subunit gene SCN5A (Wedekind et al 2001; Schwartz et al 1998). Wedekind et al. (Wedekind et al 2001) published a case report linking a missense mutation (A1330P) in the sodium channel alpha–subunit gene (SCN5A) to a Sudden Infant Death. The QT interval is a part of the electrocardiogram: It represents the time between the start of ventricular depolarization and the end of ventricular repolarization. It is useful as a measure of the duration of repolarization. Fatal arrhythmias from occult long QT syndrome may be responsible for some SIDS cases. Patients who suffer from long QT syndrome with sodium channel gene (SCN5A) defects show an increased frequency of cardiac events during sleep. It is suggested that mutations in cardiac ion channels may provide a lethal arrhythmogenic substrate in some infants at risk for SIDS.

Mutations in the mitochondrial genome were found by Opdal et al. (Opdal et al 1998) and Hofmann et al. (Hofmann et al 1997). Both documented differences between the mitochondrial DNA of SIDS cases and controls. Opdal et al. described a tendency towards a higher substitution rate in the displacement–loop (D–loop, the non–coding part of the mitochondrial genome containing the promoter regions) of the SIDS cases compared to controls.

Rahim et al. (Rahim et al 1996) described fragment length polymorphisms in the human heat shock protein genes hsp60, hsp70 and hsp90. For the genes hsp70 and hsp90, no difference was found between SIDS cases and controls. The gene hsp60 showed a MspI–polymorphism that exists highly significant less often in SIDS cases than in controls.

Bross et al. (Bross et al 2001) described DNA variations in the human heat shock protein gene hsp60. Two of them are in the coding region and alter the amino acid code. The change G537A (glycine to alanine) is present in similar frequency in SIDS cases and controls. The change N158S (asparagine to serine) was observed in one SIDS case and no controls and Bross et al. assumed a possible rare disease allel of the hsp60 gene.

1.8 Research focus

Due to the large number of differences in characteristics which occur in SIDS cases, a high probability exists, that the Sudden Infant Death Syndrome is multifactorial:

1. Factor One is an infant in a vulnerable state of development. The highest SIDS incidence is between 2 and 4 months, up to 6 months. During that time, for example, the immune system of the child changes from the fetal immune system provided by the mother to its own. The autonomous regulation in sleep (respiration, thermoregulation, circulation) is not entirely stable (Forsyth 1999; Vege and Rognum 1999; Blackwell and Weir 1999).

2. Factor Two is an exogenous stress factor. Almost all of the SIDS victims had viral or bacterial infections shortly before their deaths. These infections often cause high fever and considerable heat stress. Other exogenous factors are prone sleeping, problems with respiration due to nicotine influence and overheating.

3. Factor Three is an endogenous factor. One possibility is a physiological defect such as magnesium deficiency (Durlach et al 2002). Another possibility is a genetic variant or defect.

In context of this thesis, the multifactorial genesis was examined with the following hypothesis:

1. Factor One is a vulnerable infant, because of its developmental stage.

2. Factor Two is an exogenous factor: heat stress.

3. Factor Three is an endogenous factor: A possible genetic variation in a heat shock protein/gene, hsp60.

Thus, the fundamental question is: Is there any difference at the DNA level between children, who died of Sudden Infant Death Syndrome and children, who are either healthy or died of other causes? In context of this work, a more specific form of that question is asked as:

Is there a difference in the DNA of the human chaperonin gene hsp60 between SIDS victims and other children?

2 Materials and Methods

The discussion in Chapter 1 now leads to the experimental body of this thesis. DNA was obtained both from SIDS cases and control children to perform a mutation analysis of the partial human chaperonin60 gene, employing the techniques PCR and direct sequencing.

2.1 Materials

2.1.1 SIDS cases

91 SIDS cases from the years 1987–2001, 56 male and 35 female infants, were examined. Their ages ranged from 13 to 341 days (1–12 months). The cause of death in each of the cases was diagnosed as Sudden Infant Death, if, after a thorough postmortem examination, no other cause of death could be detected. All autopsies were performed in the Institute of Legal Medicine, Medical School Hanover, Germany. Hence, the Institute for Legal Medicine, Medical School Hanover, Germany, will be abbreviated as RM/MHH. The analysis of the autopsy protocols was performed in cooperation with the physicians Ingo Reimann and Yvonne Schulz, RM/MHH. For the analysis of the variations in the hsp60 gene, the DNA of the SIDS cases was isolated from blood samples obtained during autopsies at the RM/MHH.

All 91 cases discussed here belong to the pathological categories of SIDS cases 1 to 3 (see Section 1.7). Typical SIDS criteria (characteristic findings in autopsy and at death scene, well known risk factors) were documented, if mentioned in the autopsy protocols (see Section 1.7.3).

2.1.2 Controls

It is very difficult to obtain control children for studies on Sudden Infant Death. As SIDS is the leading cause of death of children under one year in Western countries (Hunt and Hauck 2004; Jorch 2000), only a comparatively small number of children died of causes other than SIDS. As an alternative, the head of the DNA laboratory of the RM/MHH specified a control group matched in age and sex to the SIDS cases as the basis for this research project.

In this thesis 108 controls from the years 1994–1999, 59 male and 49 female children, were examined. Their ages ranged from 103 to 361 days (3–12 months). Most of the controls (N=104) are children who were subject to paternity testing at the RM/MHH. Very few (N=4) died of other causes and underwent autopsy at the RM/MHH. They belong to category 4 according to the German Study on Sudden Infant Death (GeSID; Findeisen et

al 2003; see Section 1.7). For the analysis of the variations in the hsp60 gene, the DNA of the controls was isolated from blood samples of the children.

2.1.3 Databases

Various databases were accessed for this research:

Gene sequences were retrieved from the GenBank/EMBL/DDBJ Database (GENBANK 2004). GenBank Accession Numbers are abbreviated GAN.

Genomes As resources for information on entire genomes the GenBank/EMBL/DDBJ Database (GENBANK 2004) and the Genome Database (GDB 2004) were used. For information on the human mitochondrial genome the Mitomap Database was used (MITOMAP 2004).

Genetic disorders As a resource for information on human genes and genetic disorders the OMIM Database was used (OMIM 2000).

Protein sequences were retrieved from the Swiss–Prot Database (SWISSPROT 2004) and the TrEMBL Database (TrEMBL 2004). Swiss–Prot Accession Numbers are abbreviated SPAN.

Transcription factors As a resource for transcription factors and other factors regarding the gene regulation on the level of transcription, the Transfac Professional 7.4.1 Database (Matys et al 2003) was used, courtesy of Biobase Biological Databases GmbH Wolfenbüttel, Germany. TRANSFAC is a database on eukaryotic transcription factors, their genomic binding sites and DNA–binding profiles.

2.1.4 DNA for PCR and sequencing of hsp60s

For the PCR and the sequencing of the newly discovered member of the HSP60 family hsp60s, DNA was isolated from blood samples of three adult test persons (two female and one male).

2.1.5 RNA for RT–PCR and sequencing of hsp60s

For the RT–PCR and the sequencing of the newly discovered member of the HSP60 family hsp60s, RNA was isolated from blood samples of one test person (one of the female human adults, whose DNA was used for PCR and sequencing of the hsp60s gene), see last Section.

2.1.6 hsp60 – Human Chaperonin60 gene

The sequence of hsp60 was retrieved from the GenBank Database, GAN AJ250915 (Hansen et al 2003a). The corresponding paper is from Hansen et al. (Hansen et al 2003b).

The original sequence of the hsp60 mRNA was described by Venner et al. (Venner et al 1990), GAN M34664 (Venner et al. 1993). There is a difference between the mRNA from Hansen et al. and the mRNA from Venner et al.: In position 67 of the protein, Hansen et al. described the amino acid serine, Venner et al. described a glycine. In the RNA, it reads an *A* in position 259 from Hansen et al., corresponding to a *G* in position 223 from Venner et al. This difference may be a SNP (single nucleotide polymorphism). For this thesis, its occurrence is not relevant.

2.2 Methods

All of the original protocols and programs were customized to adjust them for the experiments of the research project.

2.2.1 Laboratory Equipment and Reagents

Equipment:

- **Centrifuges.** Centrifugation steps with 15–ml sterile test tubes were carried out at a Haereus Sepatech Megafuge 1.0 R (abbreviated HS). An Eppendorf 5415 D bench–top centrifuge (abbreviated EP) was used for micro test tubes, 1.5 and 2.0 ml, its maximum speed is 13.200 rpm.
- **Rotator.** For extracting DNA fragments, from agarose gel, a rotator was used (REAX2 Rotator, REAG 2050, Heidolph Company, Germany).
- **Interval Shaker.** For resolving a DNA pellet, an interval shaker was used (LKB Wallace 1292 Rack Shaker).
- **Plastic materials for DNA experiments** were disposables: 1.5–ml and 2.0–ml micro test tubes were supplied by Eppendorf AG, Germany. 15–ml test tubes were supplied by Sarstedt AG, Germany.
- **Plastic materials for RNA experiments.** For RNA experiments, the plastic materials used were disposables and RNAsefree. The pipet tips (epTIPS Filter) and the micro test tubes 1.5 and 2.0 ml (Biopur Safe–Lock) were supplied by Eppendorf AG, Germany. The 15–ml sterile test tubes were supplied by Sarstedt AG, Germany.
- **PCR Engine and plastic material.** PCR and RT–PCR reactions were performed in 0.2 ml PCR tubes (supplied by Biozym Scientific GmbH, Germany) on a PTC–200 Peltier Thermal Cycler DNA Engine (MJ Research).
- **PCR Purification.** The purification reactions for either PCR or RT–PCR were performed with the QIAquick PCR Purification Kit (supplied by Qiagen GmbH, Germany). The only exception is the PCR of hsp60s. These fragments were extracted by preparation of agarose gels with the Easypure DNA Purification Kit (supplied

by Biozym Scientific GmbH, Germany). PCR products of different sizes in addition to hsp60s were generated in the PCR reaction. These would have interfered with the following sequencing reaction, thus the need to isolate hsp60s from these contaminants.

- **Primers** were manufactured by Applied Biosystems/Applera Deutschland GmbH, Germany and MWG–Biotech AG, Germany.

If not specified otherwise, the following reagents were used in the experiments:

- **96% Ethanol**.This ethanol absolute was used in all DNA experiments (supplied by J. T. Baker).
- **96% Ethanol**, ethanol absolute, molecular biology grade. This RNAse–free ethanol was used in all RNA experiments (supplied by Applichem GmbH, Germany).
- **HPLC–analyzed H_2O.** This HPLC–grade water was used, in all but RNA experiments (supplied by J. T. Baker).
- **H_2O**, molecular biology grade. This RNAse–free water was used in the RNA experiments (supplied by Applichem GmbH, Germany).
- **β–Mercapthoethanol**, molecular biology grade. This reagent was used for RNA isolation (supplied by SIGMA–ALDRICH Chemie GmbH, Germany).
- **dNTPs** (deoxy nucleotide triphosphate). Since the kits do not contain a sufficient number of dNTPs, additional dNTPs (GeneAmp 10 mM dNTP Mix with dTTP) were supplied by Applied Biosystems/Applera Deutschland GmbH, Germany.

2.2.2 Isolation of DNA – SIDS samples and Controls

The DNA of the controls was isolated from leukocytes in buffy coat samples for Factor XIIIA protein isolation (Section 2.2.2.1). These samples were originally prepared for paternity testing at the RM/MHH. For DNA isolation, either a Qiagen QiAmp Mini Kit (blood protocol) or a Qiagen Blood Mini Kit was used (same protocol as for QiAmp Mini Kit, see Section 2.2.2.2). The RNAse A used in the isolation procedure was also provided by Qiagen. The Qiagen protocols were customized, using longer incubation time with application of buffer AL. The amount of DNA isolated was determined photometrically (Section 2.2.4).

The DNA of the SIDS cases was isolated from whole blood (either heart or femoral vein blood), which was taken during the autopsies. The same protocol as for the controls was used (see Section 2.2.2.2). Instead of the buffy coats, whole blood was used in the first step.

The DNA for the PCR and the sequencing of the hsp60s gene was also isolated from whole blood (basilic vein blood), which was taken from the test persons at the RM/MHH. The same protocol as for the SIDS cases and controls was used, starting with whole blood, see Section 2.2.2.2.

2.2.2.1 Preparation of Buffy Coats, Factor XIIIA isolation

1. Centrifuge whole blood samples (EDTA–blood) of at least 4 ml volume for 1 minute at 500 x g (1750 rpm) without brake on the HS centrifuge.
2. Centrifuge recovered platelet–rich plasma (PRP, upper phase) once again for 10 minutes at 2000 x g (3500 rpm) on the HS centrifuge.
3. Discard supernatant retaining only approximately 2 droplets.
4. Swirl pellet up homogenously.
5. Freeze at -18° C to initiate "rhexis" of thrombocyte membrane for setting free intracellular Factor XIIIA protein.

2.2.2.2 Isolation of DNA

1. Pipet 200 μl buffy coat or whole blood into a 1.5–ml microcentrifuge tube.
2. For RNA–free genomic DNA add 4 μl RNase A stock solution (100) mg/ml to sample, before adding Buffer AL. Vortex and centrifuge briefly. Incubate at room temperature for 5 min.
3. Add 20 μl Qiagen Proteinase K to sample, ensure proper mixing by vortexing.
4. Warm Buffer AL at 56° C for 5 minutes.
5. Add 200 μl Buffer AL to the sample. For efficient lysis, proper mixing is needed. Mix by pulse–vortexing for 15 sec. Centrifuge briefly. Do not add Qiagen Protease or Proteinase directly to Buffer AL.
6. Incubate at 56° C for at least 12–15 hours.
7. Centrifuge briefly to remove drops from the lid.
8. Add 200 μl ethanol (96–100%), mix by pulse–vortexing for 15 sec, centrifuge briefly to remove drops from the lid.
9. Carefully apply the mixture from step 8 to the QIAmp spin column (in a 2–ml collection tube) without wetting the rim, close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAmp spin column in a clean 2–ml collection tube and discard the tube containing the filtrate.
10. Carefully open the QIAmp spin column and add 500 μl Buffer AW 1 without wetting the rim. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAmp spin column in a clean 2–ml collection tube and discard the tube containing the filtrate.
11. Carefully open the QIAmp spin column and add 500 μl Buffer AW 2 without wetting the rim. Close the cap and centrifuge at 13.200 rpm for 3 min.
12. Place the QIAmp spin column in a new 2–ml collection tube (or use 1.5–ml tube) and discard the collection tube with the filtrate. Centrifuge at 13.200 rpm for 1 min.

13. Place the QIamp spin column in a new sterile 1.5–ml tube (not included in kit) and discard the collection tube containing the filtrate. Dry off the open spin column at 56° C for max. 3 min to evaporate remaining EtOH. Add 100 μl Buffer AE and then 100 μl sterile HPLC water or mix buffer AE and water before. Incubate at room temperature for 5 min, and then centrifuge at 6000 x g (8000 rpm) for 1 min.

14. The purified DNA can be used immediately or stored at -20°C for later use.

2.2.3 Isolation of RNA – hsp60s

The RNA for the expression analysis of hsp60s was isolated using the Qiagen QIAmp RNA Blood Mini Kit from fresh whole blood (basilic vein blood), see following Section. EDTA was used as an anticoagulant. The blood was processed immediately after taking the blood sample. The Qiagen protocols were customized: The amount of blood used in the isolation was increased to 2.0 ml instead of 1.5 ml. The centrifugation speed for the steps with the HS centrifuge was increased to 800 x g compared to 400 x g in the original Qiagen protocol. Step 1 to step 5 must be performed on ice. Beginning with step 6, the isolation is performed at room temperature.

1. Mix 1 volume of whole human blood with 5 volumes of buffer EL in a 15–ml sterile test tube: 2.0 ml blood + 10 ml Buffer EL. Pipet the blood into the tube filled with buffer EL. The volume of the mixture should not exceed three quarters of the tube to allow efficient mixing.

2. Incubate for 10–15 minutes on ice. Mix by vortexing briefly 2 times during incubation. The cloudy suspension becomes translucent during incubation, indicating lysis of erythrocytes. If necessary, incubation time can be extended to 20 min.

3. Centrifuge at 800 x g (HS) for 10 min at 4°C. Leukocytes will form a pellet after centrifugation. Ensure supernatant is completely removed. Trace amounts of erythrocytes, which give the pellet a red tint, will be eliminated in the following washing step. If erythrocyte lysis is incomplete, the white pellet may not be visible and large amounts of erythrocytes will form a red pellet. If this happens, incubate for an additional 5–10 min on ice after addition of Buffer EL at step 4.

4. Add Buffer EL to the cell pellet (use 2 volumes of Buffer EL per volume of whole blood employed in step 1): 4 ml Buffer EL. Resuspend cells by vortexing briefly.

5. Centrifuge at 800 x g (HS) for 10 min at 4°C, and completely remove and discard supernatant. Incomplete removal of the supernatant will interfere with lysis and subsequent binding of RNA to the QIAmp spin column, resulting in lower yield.

6. Add 600 μl buffer RLT to pelleted leukocytes. The buffer must be freshly made: 10 μl β–Mercaptoethanol (β–ME) + 1 ml RLT. Then, the buffer RLT is stable for 1 month at room temperature (15–25 °C) after addition of β–ME.

7. Pipet lysate directly into a QIAshredder spin column sitting in a 2–ml collection tube and centrifuge for 2 min at 13.200 rpm (EP) to homogenize. Discard QIAshredder spin column and save homogenized lysate. To avoid aerosol formation, adjust pipet to $\geq$ 750 μl to ensure that the lysate can be added to the QIAshredder spin column in a single step. If too many

cells have been used, the lysate will be too viscous to pipet after homogenization. In this case divide the sample into two aliquots and adjust the volumes of each aliquot to 600 μl with Buffer RLT. Continue with the procedure in step 7. The β–ME waste is hazardous material and must be disposed of separately.

8. Add 1 volume (600 μl) of 70 % ethanol to the homogenized lysate and mix by pipetting. Do not centrifuge. A precipitate may form after the addition of ethanol. This will not affect the QIAmp procedure.

9. Carefully pipet sample, including any precipitate which may have formed, into a new QIAmp spin column sitting in a 2–ml collection tube (provided) without moistening the rim. Centrifuge for 15 sec at $\geq$ 8000 x g ($\geq$ 10000 rpm). Maximum loading volume is 700 μl. If the volume of the sample exceeds 700 μl, successively load aliquots onto the QIAamp spin column and centrifuge as above. Discard flow–through and collection tube.

10. Transfer the QIAmp spin column into a new 2–ml collection tube. Apply 350 μl Buffer RW1 to the QIAmp spin column and centrifuge for 15 sec at $\geq$ 8000 x g ($\geq$ 10000 rpm). Discard flow–through and collection tube.

11. Add 10 μl DNAse I stock solution (DNAse I is from the RNasefree DNase Set, supplied by Qiagen) to 70 μl Buffer RDD. Mix by gently inverting the tube. DNAse I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube. Do not vortex.

    a) Prepare DNAse I stock solution before using the RNAse–Free DNAse Set for the first time. Dissolve the solid DNAse I (1500 Kunitz units) in 550 μl of the RNAse–free water provided. Take care that no DNAse I is lost when opening the vial. Mix gently by inverting the tube. Do not vortex.

    b) Do not vortex the reconstituted enzyme. DNAse I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube.

    c) For long term storage, remove the stock solution from the glass vial, divide it into single–use aliquots, and store at -20$^{\circ}C$ for up to 9 months. Thawed aliquots can be stored at 2–8$^{\circ}C$ for up to 6 weeks. Do not refreeze after thawing.

12. Pipet the DNAse I incubation mix (80 μl) directly onto the spin column membrane, and incubate for 15 min. at 20–30$^{\circ}C$. Note: Make sure to pipet the DNase I incubation mix directly onto the spin–column membrane. DNAse digestion will be incomplete if part of the mix sticks to the walls or the O–ring of the spin column.

13. Pipet 350 μl Buffer RW1 into the spin column and centrifuge for 15 sec at $\geq$ 8000 x g ($\geq$ 10000 rpm). Discard flow–through and collection tube.

14. Place the spin column in a new 2–ml collection tube. Pipet 500 μl of Buffer RPE into the spin column, and centrifuge for 15 sec at $\geq$ 8000 x g ($\geq$ 10000 rpm). Discard flow–through and collection tube. Note: Ensure ethanol is added to Buffer RPE before use.

15. Pipet 500 μl of Buffer RPE into the spin column, and centrifuge for 2 min at 13.200 rpm to dry the silica gel membrane. It is important to dry the silica–gel membrane, since residual alcohol may interfere with subsequent reactions. This 2 min spin ensures that no ethanol is carried over during elution. Following the spin, remove the spin column from the collection

tube carefully so the column does not contact the flow–through as this will result in a carry–over of ethanol.

16. Transfer spin column to a new 1.5–ml collection tube, and pipet 30–50 μl of RNAse–free water directly onto the silica–gel membrane. Centrifuge for 1 min at $\geq$ 8000 x g ($\geq$ 10000 rpm) to elute.

17. Repeat step 16 if the expected RNA yield is >30 μg. If a second elution step is performed, elute into the same collection tube using another 30–50 μl of RNAse–free water. With the QIAmp RNA Blood Mini Kit, a second elution is recommended if more than 0.5 ml of whole blood (or >2x10^6 leukocytes) have been processed.

The amount of RNA was determined photometrically. The procedure is described in the next Section.

2.2.4 Quantification and purity of nucleic acids

The quantification of both DNA and RNA was carried out on a Pharmacia Biotech Ultrospec 2000 UV/Visible Spectrophotometer. For DNA, a quartz glass cuvette was used (Hellma Type No. 105.202–QS, layer thickness 10mm). A 1:10 dilution was used for measuring the DNA concentration (10 μl DNA–solution and 90 μl HPLC–H_2O).

For RNA, sterile, disposable plastic cuvettes were used (Eppendorf Uvette with Universal Adapter). A 1:5 dilution was used for measuring the RNA concentration (20 μl RNA–solution and 80 μl RNAse–free H_2O). All measurements were carried out against HPLC–H_2O as reference.

Calf thymus DNA (Gibco BRL Life Technologies, stock solution 10mg/ml) of a known concentration (20 ng/μl) was used for calibration of the photometer. The concentration of the DNA– and RNA–solutions was measured using absorbance at 260 nm. A solution of DNA with an optical density of 1.0 has a concentration of 50 μg/ml, for RNA it is 40 μg/ml.

The purity of DNA– and RNA–solutions is determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm. Pure DNA has an absorbance ratio A_{260}/A_{280} of 1.8, pure RNA has one of 2.0.

$$\boxed{\text{Concentration of DNA [ng/}\mu\text{l]} = \text{absorption at } \lambda 260 \text{ *50 ng/}\mu\text{l *10}}$$

$$\boxed{\text{Concentration of RNA [ng/}\mu\text{l]} = \text{absorption at } \lambda 260 \text{ *40 ng/}\mu\text{l *5}}$$

$$\boxed{\text{Absorbance ratio (Purity)} = \frac{A_{260}}{A_{280}}}$$

2.2.5 Polymerase Chain Reaction (PCR) and Reverse Transcriptase Polymerase Chain Reaction (RT–PCR)

The Polymerase Chain Reaction (PCR) is used for the amplification of DNA fragments. This method was originally developed by Mullis et al. in 1986 (Mullis et al 1986). The Reverse Transcriptase Polymerase Chain Reaction (RT–PCR) is used for the amplification of RNA fragments. It is a two step reaction. First, the reverse transcriptase transcribes the RNA into a cDNA, then the normal PCR starts. Reverse transcriptases were at first isolated from retroviruses. Their genome consists of single–stranded RNA, which needs to be converted into DNA for integrating the virus genome into the genome of a cell (Lewin 2000).

All primers used in the PCR and RT–PCR experiments were self constructed, except the GAPDH primers for testing the RNA quality. The software used for this procedure was Primer3 (Rozen and Skaletsky 2000). Furthermore, all PCR and RT–PCR protocols and programs for this thesis, following the appropriate guidelines in the manuals, were customized. For PCR, the ProofStart DNA Polymerase (supplied by Qiagen GmbH, Germany; Qiagen Proofstart DNA Polymerase 2002) and the GeneAmp XLPCR Kit (supplied by Applied Biosystems/Applera Deutschland GmbH, Germany; XLPCR Kit 1997) were used. For RT–PCR the Qiagen OneStep RT–PCR Kit (Qiagen GmbH, Germany; Qiagen OneStep RT–PCR Kit 2002) was used. The customization affected the reagent concentrations in the reactions, the time and temperature of the different reaction steps, and the number of cycles.

2.2.5.1 Purification of PCR products and RT–PCR products

This purification method is designed to purify single– or doublestranded PCR products ranging from 100 bp to 10 kb from primers, nucleotides, polymerases and salts, using QIAquick spin columns in a microcentrifuge. All centrifugation steps were performed at 13.200 rpm. The method was customized: The elution buffer was diluted 1:8 with water.

1. Add 5 volumes of Buffer PB to the PCR reaction and mix. It is not necessary to remove mineral oil or kerosene. For 25 (50, 100) μl reaction use 125 (250, 500) μl PB.
2. Place the QIAquick spin column in a 2.0–ml micro test tube.
3. To bind DNA, apply the sample to the QIAquick column and centrifuge for 1 min.
4. Discard the flow–through. Place the QIAquick column carefully into a new 2.0–ml tube.
5. To wash, add 600 μl Buffer PE to the column and centrifuge for 30–60 sec.
6. Discard the flow–through. Place the QIAquick column into a new tube and centrifuge the column for an additional minute.
7. Place the QIAquick column into a sterile 1.5 ml micro test tube.
8. Open the column lid and let the remaining EtOH evaporate for 3 min at 56°C.

9. To elute the DNA, add 50 μl Buffer EB (10mM Tris–Cl, pH 8.5, diluted 1:8 with water, for 1 ml: 125 μl EB + 875 μl H_2O) to the center of the QIAquick column, incubate for 5 min at room temperature, and centrifuge for 1 min. Alternatively, for increased DNA concentration: Add 30 μl Buffer EB to the center of the QIAquick column, incubate for 1–5 min at room temperature, and then centrifuge. Ensure that the elution buffer (EB) is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume for 50 (30) μl Elution Buffer is 48 (28) μl.

For analyzing DNA yield and purity in the eluate, use 10 μl DNA–solution and 90 μl H_2O.

2.2.5.2 Purification of PCR products – Agarose Gels

The protocol was customized by adding an extra step for extracting more DNA.

1. Cut the DNA band out of the gel and apply it to a 15–ml test tube. Weigh the gel piece to measure the volume (100 mg = 100 μl). The volumes of the solutions MELT and SALT are dependent on the volume of the gel band.
2. Dissolving of the agarose (TBE gels).
   a) First add 0.5 volume MELT solution (pink) and then 4.5 volume SALT solution (yellow) to the gel fragments.
   b) Incubate for 10 min at 55°C. Mix by gentle vortexing. Repeat 3 to 4 times if necessary. The agarose must be fully dissolved. Vortexing is dependent on fragment size.
3. Binding of the DNA:
   a) Vortex the BIND solution (red) thoroughly.
   b) Take 5 μl plus 1 μl per μg of expected DNA amount Matrix (BIND). Use a yellow pipet tip.
   c) Mix for 15 min at room temperature in the rotator, speed level 3.
   d) Centrifuge briefly (maximum 5 sec). Take away the supernatant and keep it.
4. Washing:
   a) Resolve the pellet in 1 ml fresh washing solution (240 μl Concentrate, 4.76 ml Water, 5.00 ml EtOH). Shake gently. The pellet must be fully resolved. Transfer the solution into a micro test tube (1.5 ml). Incubate for 15 to 30 min at room temperature without mixing.
   b) Centrifuge briefly (for maximal 5 sec) and discard supernatant. Use a blue pipet tip.
   c) Centrifuge briefly (for maximal 5 sec) and discard supernatant. Use a yellow pipet tip.
   d) Dry the pellet at 56°C for 10 min; or until all solution has evaporated.

5. Elution:

   a) Resolve the pellet in 100 μl HPLC–H_2O. Incubate for 10 min at room temperature on an interval shaker.

   b) Centrifuge for 1 min in the EP at 13.200 rpm. Take away the supernatant carefully into a sterile new 1.5–ml micro test tube, take no more than 60 μl.

   c) Additional step for extracting more DNA: resolve the pellet again in 40 μl HPLC–H_2O, incubate for 10 min on the interval shaker, and centrifuge for 10 min in the EP at 13.200 rpm. Then take 50 μl from the supernatant.

For analyzing DNA yield and purity, use 10 μl DNA–solution and 90 μl HPLC–H_2O.

2.2.6 PCR of partial human hsp60 gene

For the amplification of the partial hsp60 gene, exon 5 with 5′ and 3′ boundaries, the Qiagen ProofStart DNA Polymerase (with ProofStart PCR Buffer, Q–Solution, $MgSO_4$; Qiagen GmbH Germany) was used. The parameters for the ProofStart DNA Polymerase reactions were obtained from the manual of Qiagen (Qiagen ProofStart DNA Polymerase 2002). The quantity of the starting template was calculated as follows: The size of the human genomic DNA is $3.3 \cdot 10^9$ base pairs; this number corresponds to $4.7 \cdot 10^{-7}$ pmol/μg and $2.8 \cdot 10^5$ Molecules/μg ($2.8 \cdot 10^4$ Molecules/ng) for single copy genes. Thus, the number of copies of the starting template is $1 \cdot 10^3$ to $5 \cdot 10^4$. This refers to 3.6–179 ng of human genomic DNA. The individual primer concentration used was 1 μM (50 pmol per 50 μl reaction). The dNTP concentration used was 1.5 μl for a 50 μl reaction (final concentration 300 μM).

2.2.6.1 Primers for the PCR of partial human hsp60 gene

The primers for the amplification of the partial hsp60 gene were constructed based on the sequence of the human hsp60 genomic DNA and are shown in table 2.1. The letter L stands for left (forward primer), the letter R stands for right (reverse primer). The original annealing temperature (T_A) is 5 °C lower than the melting temperature (T_M), but may be adjusted in the PCR optimization process.

Primer	**Sequence,** $5' \longrightarrow 3'$	T_M	**% GC**	**length**
XXLHSEXON5IL	GGT AAG GGA ACT GAC TGA GG	56.25°C	55.00	20
XXLHSEXON5IR	GAT GGA TTC ATT TCA TGC AG	56.47°C	40.00	20
XXLHSEXON5IIR	GAT CAT CAG ATA ACT CAA AC	46.57°C	35.00	20
XXLHSEXON5IIIR	CTA TAC ATG TGC TGA GAC TAC	47.30°C	42.86	21

T_M = melting temperature; **%GC** = amount of GC nucleotides; **length** = number of nucleotides

Table 2.1: hsp60, Exon 5 with Boundaries: PCR Primers

The primer pair XXLHSEXON5IL and XXLHSEXON5IR was used for the partial amplification of hsp60 from human genomic DNA. The primer XXLHSEXON5IIR was used

as an additional sequencing primer. The primer XXLHSEXON5IIIR was used only for testing purposes during PCR optimization, but not for the actual experiments.

The partial sequence of the human chaperonin gene 60 exon 5 with 5′ and 3′ boundaries is shown in Table 2.2. The exon 5 is marked in boldface. Primer sequences are underlined, their direction is marked. The expected fragment has a length of 380 base pairs (GAN AJ250915: base position 8701–9040). The exon 5 ranges from base position 8748–8843 with a size of 96 bp; the upstream adjacent intron 4 ranges from base position 8207–8747 with a size of 541 bp and the downstream adjacent intron 5 ranges from base position 8844–9251 with a size of 408 bp.

8641	CCCCTGGATG	GAAGTATCAA	TTTGGAAGTT	ACTAGGAAAA	*GGTAAGGGAA* *>8681–8700>*	*CTGACTGAGG* *>XXLHSEXON5IL>*
8701	ACACTAGGCT	TACCTTTTAA	TTTCGAAGAG	TAACTGGATT	ATTTCAG**GTT**	**GCTACGATTT**
8761	**CTGCAAACGG**	**AGACAAAGAA**	**ATTGGCAATA**	**TCATCTCTGA**	**TGCAATGAAA**	**AAAGTTGGAA**
8821	**GAAAGGGTGT**	**CATCACAGTA** *8748–8843*	**AAG**GCAAGTG *exon 5*	TGTTTGTATT	TTTAAAGATA	ATTTTGAGTT
8881	ATCTTATGAT	CAAAA *GTTTG* *<8896–8915<*	*AGTTATCTGA* *<XXLHSEXON5IIR<*	*TGATC*AAAAC	TGAATTTTTC	AAACAGAAAA
8941	ATTCTGTTTT	AAAAACAGTG	CTTTTTTTAT	TCCTTCATAT	*GTAGTCTCAG* *<8981–9001<*	*CACATGTATA* *<XXLHSEXON5IIIR<*
9001	*G*AATAAAGTA	CTTACTGTGT	TTTATGTGAA	TTAGCCTTTG	*CTGCATGAAA* *<9041–9060<*	*TGAATCCATC* *<XXLHSEXON5IR<*

boldface = exon 5; **underlined** = primer

Table 2.2: hsp60: Exon 5 with boundaries (AJ250915, base position 8641–9060); Position of PCR Primers

2.2.6.2 Protocols and Programs for the PCR of partial human hsp60 gene

The protocol and program are shown in Tables 2.3 and 2.4. The reagents were mixed at room temperature.

Template DNA	100–200 ng
Primer forward XXLHSEXON5IL	50 pmol
Primer reverse XXLHSEXON5IR	50 pmol
10x ProofStart Buffer	5 μl
10mM dNTP Mix	1.5 μl
Q–Solution 5x	10 μl
Proofstart Polymerase	2.5 Units
Water	ad 50 μl

Table 2.3: hsp60, Exon5 with Boundaries: PCR Protocol with Q–Solution, 50 μl

1.	5 min 95°C	Activation Proofstart DNA Polymerase
2.	1 min 94°C	Denaturation
3.	1 min 57°C	Annealing
4.	1 min 72°C	Extension
5.	Goto 2 41 times	Cycles
6.	10 min 72°C	Final Extension
7.	Forever 4°C	Hold

Table 2.4: hsp60, Exon5 with Boundaries: PCR Program XXLHS5V2

2.2.7 PCR of hsp60s

Initially, the PCR of hsp60s occurred unintentionally. The primer pair XLLEFT/XLRIGHT was originally constructed to amplify the coding part of the genomic hsp60 gene (exons 2–12 with introns, size 13248 base pairs). Thus, all PCR parameters were chosen for a single copy gene in the human genome, the size parameters were adjusted to 13248 base pairs. However, instead of the desired hsp60 fragment, a previously unknown gene was discovered. It was named hsp60s (GAN AF380943; Teske et al 2001). The complete analysis of hsp60s is described in Section 3.3.

For the amplification of the hsp60s gene, the GeneAmp XLPCR Kit (Applied Biosystems/Applera GmbH Deutschland) was used. Thus, all primer names start with XL. The parameters for the XLPCR reactions were adapted according to the manual of Applied Biosystems/Applera GmbH Deutschland (XLPCR Kit 1997).

2.2.7.1 Primers for the PCR of hsp60s

The primers for the amplification of the hsp60s gene were constructed based on the sequence of the human hsp60 mRNA and the sequence of the human hsp60 genomic DNA (Table 2.5). The suffix LEFT stands for the forward primer, the suffix RIGHT stands for the reverse primer. The original annealing temperature (T_A) is 5 °C lower than the melting temperature (T_M), but may be adjusted in the PCR optimization process.

Primer	**Sequence,** $5' \longrightarrow 3'$	T_M	**% GC**	**length**
XLLEFT	GAC GAC CTG TCT CGC CG	61.65°C	70.59	17
XLLEFTII	CTC GCC GAG CCA CGC CT	68.55°C	76.47	17
XLLEFTIII	TCT CGC CGA GCC ACG CCT	70.19°C	72.22	18
XLRIGHT	ACA CAA AGG TTG TCA CAT AAT TGG	60.08°C	37.50	24
XLRIGHTII	GCC TTG GGC TTC CTG TC	59.28°C	64.71	17
XLRIGHTIII	TAA CTT CTC ATC TGG TGG TGG CAA	65.04°C	45.83	24
XLRIGHTIV	ACT GCT TAA CTT CTC ATC TGG TGG TGG CAA	71.67°C	46.67	30

T_M = melting temperature; **%GC** = amount of GC nucleotides; **length** = number of nucleotides

Table 2.5: hsp60s: PCR Primer

The primer pair XLLEFT/XLRIGHT was used for the amplification of hsp60s from human genomic DNA. The other primers were used only for testing purposes during PCR optimization. The partial sequence of the human chaperonin60 gene with the primer XLLEFT is shown in Table 2.6; the illustration for XLRIGHT is shown in Table 2.7. The exons are marked with boldface. Primer sequences are underlined, their direction is marked. The expected fragment has a length of 13248 base pairs (GAN AJ250915, base position 3669–16917).

3361	CCCGGAAATG	ACGCGATTTG	ACCCTTGAGC	CGTAGGGAGC	GCGGCAT*TTT* *3408–3428*	*CTGGAAAGTT* *putative heat*
3421	*CTGGAACC*GA *shock element*	GCGAGGCCCG	GGAACTAGAC	TAAGCCGGCC	GGAGAGGGCT	GAGCGCGCTA
3481	GCACACCCTG	CGCGGGTAGG *3504–3510*	GAG*GGGCGGG* *SP1 element*	GCTCGCGCGC	AGGGTGTGCA	GATTGCAGGG
3541	CCCGGGCTGA	CGGGAAGTGG	GTGGGAGCTG	CCTGCACACG	CGGTGCCGCG *3591–3597*	*GGGCGGG*AGT *SP1 element*
3601	AGAGGCGGAG	GGAGGGGACA	CGGGCTCATT	GCGGTGTGCG	CCCTGCACTC *C3654: start p10gene*	TGTC**CCTCAC** *C3655: start p60gene/mRNA*
3661	**TCGCCGCC GA** *>3669–3685>*	**CGACCTGTCT** *>XLLEFT>*	**CGCCGAGCGC**	**ACGCCTTGCC**	**GCCGCCCCGC** *3655–3712*	**AG**GTACGCGG *exon 1*

boldface = exons; **underlined** = primer

Table 2.6: hsp60: AJ250915, base position 3361–3720; Position of Primer XLLEFT

16261	CATATTTTAC	TAAGGGAAAA	CTAATTAATT	AAATTTTTTT	CTAG**GTTGTG** *16873–16917*	**AGAACTGCTT** *exon 12*
16321	**TATTGGATGC**	**TGCTGGTGTG**	**GCCTCTCTGT**	**TAACTACAGC**	**AGAAGTTGTA**	**GTCACAGAAA**
16381	**TTCCTAAAGA**	**AGAGAAGGAC**	**CCTGGAATGG**	**GTGCAATGGG**	**TGGAATGGGA**	**GGTGGTATGG**
16441	**GAGGTGGCAT**	**GTTCTA***A***CTC** *A16457*	**CTAGACTAGT** *end p60cds*	**GCTTTACCTT**	**TATTAATGAA**	**CTGTGACAGG**
16501	**AAGCCCAAGG**	**CAGTGTTCCT**	**CACCAATAAC**	**TTCAGAGAAG**	**TCAGTTGGAG**	**AAAATGAAGA**
16561	**AAAAGGCTGG**	**CTGAAAATCA**	**CTATAACCAT**	**CAGTTACTGG**	**TTTCAGTTGA**	**CAAAATATAT**
16621	**AATGGTTTAC**	**TGCTGTCATT**	**GTCCATGCCT**	**ACAGATAATT**	**TATTTTGTAT**	**TTTTGAATAA**
16681	**AAAACATTTG**	**TACATTCCTG**	**ATACTGGGTA**	**CAAGAGCCAT**	**GTACCAGTGT**	**ACTGCTTTCA**
16741	**ACTTAAATCA**	**CTGAGGCATT**	**TTTACTACTA**	**TTCTGTTAAA**	**ATCAGGATTT**	**TAGTGCTTGC**
16861	**GTAGAGAAGT**	**AT CCAATTAT** *<16873–16896<*	**GTGACAACCT** *<XLRIGHT<*	**TTGTGT**AATA	**AAAATTTGTT** *T16917*	**TAAAGTT**AAT *end p60gene/mRNA*

boldface = exons; **underlined** = primer

Table 2.7: hsp60: AJ250915, base position 16261–16720; Position of Primer XLRIGHT

2.2.7.2 Protocols and Programs for the PCR of hsp60s

The protocol and program are shown in Tables 2.8 and 2.9. The reagents were assembled on ice. The PCR fragments were extracted from a 0.4% agarose gel (Seakem Gold Agarose, 1X TBE–Buffer, Section 2.2.5.2). The fragments obtained were used as a template for a second amplification ("template from template" method) with the same protocol and program as the first time. The purification after the second amplification was performed with the QIAquick PCR Purification Kit (Section 2.2.5.1). The purified fragments from the second processing were used for direct sequencing.

Template DNA	25–50 ng
Primer forward XLLEFT	15 pmol
Primer reverse XLRIGHT	15 pmol
3.3x XL Buffer	15.2 μl
10 mM dNTP Mix	6.0 μl
25 mM $Mg(Oac)_2$	3 μl
Proofstart Polymerase	1.5 Units
Water	ad 50 μl

Table 2.8: hsp60s: PCR Protocol, 50 μl

1.	10 min 4°C	Cooling down the reaction, "Manual Hot Start"
2.	45 sec 94°C	Denaturation
3.	15 sec 94°C	Denaturation
4.	30 sec 55°C	Annealing
5.	8.5 min 67.5°C	Extension
6.	Goto 2 29 times	Cycles
7.	10 min 72°C	Final Extension
8.	Forever 4°C	Hold

Table 2.9: hsp60s: PCR Program AT1

2.2.8 PCR of different fragment sizes for direct sequencing with DNA from SIDS victims

The DNA isolated from blood or tissues of corpses is often degraded due to cell degradation after death. This causes problems: Only comparatively short fragments (a few hundred bases) that can be amplified and sequenced are left intact. Thus, a PCR/direct sequencing approach on this DNA is costly and time–consuming. To improve the research regarding the Sudden Infant Death Syndrome, the applicability of this methods was extended to longer fragments (up to 2200 bases). As a test subject for short fragments, the partial human hsp60 gene (about 400 bases, see Section 2.2.6), was used. For longer fragments, the human hsp60s gene (about 2200 bases, see Section 2.2.7) was used.

2.2.9 RT–PCR (PCR with Reverse Transcriptase) of hsp60s

For the amplification of the hsp60s gene from RNA the Qiagen OneStep RT–PCR Kit (Qiagen GmbH, Germany) was used. This Kit provides an enzyme mix of two reverse transcriptases (Omniscript and Sensiscript) and a HotStarTaq DNA Polymerase (Qiagen OneStep RT–PCR Kit 2002). The reverse transcriptases perform at a reaction temperature of 50°C. Then the temperature is increased to 95°C to activate the HotStarTaq Polymerase. This step deactivates the reverse transcriptases. The parameters for the RT–PCR reactions were obtained from the Qiagen OneStep RT–PCR Kit Handbook (Qiagen OneStep RT–PCR Kit 2002): The mRNA is classified based on abundance. For GAPDH, $7 \cdot 10^6 - 7 \cdot 10^7$ (7–70 Million) copies are estimated in 100 ng RNA. This leads to 25–30 cycles in the RT–PCR reaction. For hsp60s, $7 \cdot 10^4 - 7 \cdot 10^6$ (70.000–7 Millioned) copies are estimated in 100 ng RNA, leading to 25–35 cycles. The water used in all RT–PCR reactions was RNAse-free water included in the Qiagen OneStep RT–PCR Kit. As a RNAse inhibitor during the RT–PCR reactions, rRNAsin(R) RNase Inhibitor (Recombinant RNAsin Ribonuclease Inhibitor) supplied by Promega GmbH, Germany was used.

2.2.9.1 Primer for RT–PCR of hsp60s

For testing the quality of the RNA, a human GAPDH primer pair (R&D Systems GmbH, No. RDP–30–025) was used. This primer pair amplifies a 576 base pair cDNA observed from pseudogenes of the human genome, GAN M33197 (Tokunaga et al 1994). The results were verified on an agarose gel (1%, NuSieveGTG Agarose/ TBE–Buffer).

The primer for the amplification of the hsp60s cDNA are shown in Table 2.12. They were constructed based on the sequence of the human hsp60s genomic DNA, the sequence of a human chromosome 5 clone (GAN AC091873; DOE Joint Genome Institute and Stanford Human Genome Center 2002), the sequence of the human hsp60 mRNA and the sequence of the human hsp60 genomic DNA. The name Start stands for the forward primer, the name Stop stands for the reverse primer. The original annealing temperature (T_A) is 5°C lower than the melting temperature (T_M), but may be adjusted in the PCR optimization process. The prefixes ”53”, ”5”, and ”3” indicate the differences in the primer sequence at the 5′ and 3′ end between the putative mRNA of the hsp60s gene and the mRNA of the hsp60 gene. The partial sequence of the human chromosome 5 clone with the hsp60s gene included is shown in Tables 2.10 and 2.11. The putative coding sequences are marked in boldface. Primer sequences are underlined, their direction is marked.

137941	AACCAGACAT	AGATTGGCTG	TATTGCTCTC	ATAATTTGGC	AGATGTCTGT	ATGTTCCATT
138001	TATAGTACAA	CTGCCACATT	TATTTGCTTT	TTAAAAGTGA	TATATTAGCA	CTCTGTCCCT
138061	CACTCGCCGC	CGACAACCTG	TCTCGCCGCG *>138095–112>*	*138098 first base hsp60s* CGCATGCCCT *>53start1v1>*	GCAGCCGCCC *138118–20 Startcodon*	CACAGAA***ATG*** *hsp60s ORF 1*
138121	**CTTCGGTTAC** *>138138–56>*	**CCACAGT CTT** *>3start1v3>*	**TCGCCAGATG** *>138156–74>*	**AGACCAGTGT** *>5start1v4>*	**CCAGGGTACT**	**GGCTCCTCAT**
138181	**CTCACTCGAG**	**CTTATGCCAA**	**AGATGTAAAA**	**TTTGGTGCAG**	**ATGCCCGAGC**	**CTTAATGCTT**
138241	**CAAGGTGTAG**	**ACCTTTTAGC**	**CGATGCTGTG**	**GCCGTTACAA**	**TGGAGCCAAA**	**GGGAAGAACA**
138301	**GTGATTATTG**	**AGCAGAGCTG** *>138318–33>*	**GGGAAGTCCC** *>53start1v5>*	**AACGTAACAA**	**AAGATGGTGT**	**GACTGTTGCA**
138361	**AAGTCAATTG**	**ACTTGAAGGA**	**TAAATATAAA**	**AACATTGGAG**	**CTAAACTTGT**	**TCAAGATGTT**
138421	**GCCAATAACA**	**CAAATGAAGA**	**ATCTGGGGAT**	**GGCACTACCA**	**CTGCTACTGT**	**ACTGGCAGGC**
138481	**TCTATAGCCA**	**AGGAAGGCTT**	**CCAGAAGATT**	**AGCAAAGGTG**	**CTAATCCAGT**	**GGAAATCAGG**
138541	**AGAGGTGTGA**	**TGTTAGCTGT**	**TGATGCTGTA**	**ATTGCTGAAC**	**TTAAAAAGCA**	**GTCTAAACCT**
138601	**GTGACCACCC**	**CTGAAGAAAT**	**TGCACAGGTT**	**GCTATGATTT**	**CTGCAAATGG**	**AGACAAAGAA**
138661	**ATTGGCAATA**	**TC ATCTCTGA** *<138673–93<*	**TGCAATGAAA** *<5stop1v1<*	**AAGGTTGGAA** *<138693–712<*	**GAAAGGGTGT** *<3stop1v2<*	**CATCACAGTA**
138721	**AAGGATGGAA**	**AAACACTGAA**	**TGATGAATTA** *<138761–78<*	**GAAATTATTG** *<5stop1v3<*	**AAGGCATGAA** *138778–80 Stopcodon*	**GTTTGAT*TGA*** *hsp60s ORF 1*
138781	GGGTATATTT	CTCCATACTT	TATTAATACA	TCAAAAGGTC	AGAAATGTGA	ATTCCAGGAT
138841	GCCTATGTTC	TGTTGAGTGA	AAAGAAAATT	TCTAGTGTCC	AGTCCATTGT	ACCTGCTCTT
138901	GAAATTGCCA	ATGCTCACCA	TAAGCCTTTG	GTGATAATCG	CTGAAGATGT	TGATGGAGAA
138961	GCTCTAAGTA	CACTCATCTT	GAATAGGCTA	AAGGTTGGTC	TTCAGGTTGT	GGCAGTCAAG
139021	GCTCCAGGGT	TTGGTGACAA	TAGAAAGAAC	CAACTTAAAG *139063–65 Startcodon*	AT***ATG*GCTAT** *hsp60s ORF 2*	**TGCTACTGGT**
139081	**GGTGCAGTGT** *>139085–104>*	**TTGGAGAAGA** *>3start2v1>*	**GGGGTTGACC** *>139104–25>*	**CTGAATCTTG** *>53start2v2>*	**AAGATGTTCA**	**GCCTCATGAC**

boldface = putative coding sequence; **underlined** = primer

Table 2.10: hsp60s: Chr. 5 Clone (AC091873, base position 137941–139140, Reverse Complementary Sequence); Position of RT–PCR Primer

139141	**TTAGGAAAAG**	**TTGGAGAGGT**	**CATTGTGACC**	**AAAGACGATG**	**CCATGCTCTT**	**AAAAG GAAAA** *>139196–216>*
139201	**GGTGACAAGG** *>3start2v3>*	**CTCAACTTGA** *>139216–36>*	**AAAACGTATT** *>5start2v4>*	**CAAGAAATCA**	**TTGGGCAGTT**	**AGATGTCACA**
139261	**ACTAGTGAAT**	**ATGAAAAGGA**	**AAAACTGAAT**	**GAATGGCTGG**	**CAAAACTTTC**	**AGATGGAGTA**
139321	**GTTGTGCTGA**	**AGTTTGGTGG**	**GACAAGTGAT**	**GTTGAAGTGA**	**ATGAAAAGAA**	**AGACAGAGTT**
139381	**ACAGATGCCC**	**TTAATGCTAC**	**AAGAGCTGCT**	**GTTGAAGGAG**	**GCATTGTTTT**	**GGGAGGGGGT**
139441	**TTTGCCCTCC**	**TTCGATGCAT**	**TCCAGCCTTG**	**GACTCATTGA**	**CTCCAGCTAA**	**TGAAGATCAA**
139501	**AAAATTGGTA**	**TGGAAATTGT**	**TAAAAGAACA**	**CTCAAAATTC**	**CAGCAATGAC**	**CACTGCTACG**
139561	**AATGCAGGTG**	**TTGAAGGATC**	**TTTGATAGTT**	**GAGAAAATTA**	**TGCAAAATTC**	**CTCAGAAGTT**
139621	**GGTTATGATG**	**CTATGG TTGG** *<139637–59<*	**AGATTTTATG** *<53stop2v1<*	**AATATGGTAG**	**AAAAAGGAAT**	**TATTGACCCA** *<139671–87<*
139681	**ACAAAGCTTG** *<53stop2v2<*	**TGAGAACTGC** *<139687–708<*	**TTTATTGGAT** *<3stop2v3<*	**GCTGCTGGTG**	**TGGCCTCTCT**	**GTTAACTACA**
139741	**GCAGAAGTTG**	**TAGTCACAGA**	**AATTCCTAAA**	**GAAGAGAAGG**	**ACCCTGGAAT**	**GGGTGCAATG**
139801	**GGTGGAATGG**	**GAGGTGGTAT**	**GGGAGGTGGC** *139837–39 Stopcodon*	**ATGTTC*TAA*C** *hsp60s ORF 2*	TCCTAGACTA	GTGCTTTACC
139861	TTTATTAATG	AACTGTGACA	GGAAGCCCAA	GGCAGTGTTC	CTCACCAATA	ACTTCAGAGA
139921	AGTCAGTTGG	AGAAAATGAA	AAAAAGGCTG	GCTGAAAATC	ACTATAACCA	TCAGTTACTG
139981	GTTTCAGTTG	ACAAAATATA	TAATGGTTTA	CTGCTGTCAT	TGTCCATGCC	TACAGATAAT
140041	TTATTTTGTA	TTTTTGAATA	AAAAACATTT	GTACATTCCT	GATACTGGGT	ACAAGAGCCA
140101	TGTACCAATG	TACTGCTTTC	AACCTAAATC	ACTGAGGCAT	TTTTACTACT	ATTCTGTTAA
140161	AATCAGGATT	TTAGTACTTG	CCACCACCAG	ATGAGAAGTT	AAGCAGTCTT	TCTATGGAGA
140221	GTGAGAATAG	TTGTGTACAA	AGTAGAGAAA	TATCCAATTA	TGTGACAACC *140273 last base*	TT*T*GTGTAAT *hsp60s*
140281	AAAAATTTGT	TTAGTTAAAA	AAAAGTGATG	TATTAGACAA	CATTCTGTGT	TTTCCCTTTT
140341	TAAACATATT	CACTTAATTT	TTGAAACTAC	TGTATGGGGT	AATAACTTAT	TATAAAAGGC

boldface = putative coding sequence; **underlined** = primer

Table 2.11: hsp60s: Chr. 5 Clone (AC091873, base position 139141–140400, Reverse Complementary Sequence); Position of RT–PCR Primer

Primer	**Sequence,** $5' \longrightarrow 3'$	T_M	**% GC**	**length**
53Start1v1	TGCCCTGCAGCCGCCCCA	76.14	77.78	18
3Start1v3	CTTTCGCCAGATGAGACCA	59.93	52.63	19
5Start1v4	AGTGTCCAGGGTACTGGCT	57.13	57.89	19
53Start1v5	CTGGGGAAGTCCCAAC	54.31	62.50	16
5Stop1v1	CTTTTTCATTGCATCAGAGAT	54.62	33.33	21
3Stop1v2	TGACACCCTTTCTTCCAACC	59.94	50.00	20
5Stop1v3	AATCAAACTTCATGCCTT	50.34	33.33	18
3Start2v1	CAGTGTTTGGAGAAGAGGGG	59.69	55.00	20
53Start2v2	GTTGACCCTGAATCTTGAAGAT	57.25	40.91	22
3Start2v3	GAAAAGGTGACAAGGCTCAAC	58.83	47.62	21
5Start2v4	CTTGAAAAACGTATTCAAGAA	52.54	28.57	21
53Stop2v1	TACCATATTCATAAAATCTCCAA	53.36	26.09	23
53Stop2v2	GCTTTGTTGGGTCAATA	49.76	41.18	17
3Stop2v3	CCAATAAAGCAGTTCTCACAAG	56.76	40.91	22

T_M = melting temperature; **% GC** = amount of GC nucleotides; **length** = number of nucleotides

Table 2.12: hsp60s: RT–PCR Primer

2.2.9.2 Protocols and Programs for the RT–PCR of hsp60s

The protocol is shown in Table 2.13. For all reactions, the thermocycler was preheated to $50°C$ before use. All base pair positions in the program tables for RT–PCR of hsp60s refer to the human chromosome 5 clone CTD–2577N22, reverse complementary sequence. Due to their complexity, the programs are shown in detail in Appendix A.3, Tables A.5, A.6, A.7, A.8 and A.9.

Template RNA	100–200 ng
Primer forward	0.6 μM = 30 pmol in 50 μl
Primer reverse	0.6 μM = 30 pmol in 50 μl
5x OneStep RT–PCR Buffer	10.0 μl
5x Q–Solution	10.0 μl
dNTP Mix (10 mM = 400 μM in 50 μl)	2.0 μl
RNase Inhibitor (10 Units)	0.25 μl
One Step Enzyme Mix	2.0 μl
RNAse free water	ad 50 μl

Table 2.13: hsp60s: RT–PCR Protocol

2.2.10 Agarose gel electrophoresis

Agarose gel electrophoresis was used for separation, identification and purification of the PCR and RT–PCR products (DNA). DNA is a negatively charged molecule. Under

electrical current, it moves from the cathode to the anode. The agarose matrix separates the molecules according to their size; small linear molecules are moving faster in the gel matrix than larger ones.

For identification and PCR optimization of the partial hsp60 gene (exon 5 with 5' and 3' boundaries) and all gel electrophoresis of RT–PCR products, NuSieve GTG Agarose (BMA by Biozym Scientific GmbH, Germany) was used with gel concentrations between 1 % and 2 %. For the separation und purification of the hsp60s gene, Seakem Gold Agarose (BMA/FMC by Biozym Scientific GmbH, Germany) was used with a gel concentration of 0.4%.

Running buffer for all gels was 1X TBE (Tris–Borate–EDTA), stock solution 10X TBE. The chemicals Boric Acid, EDTA and Tris Base were molecular biology grade (Merck Eurolab GmbH, Calbiochem–Novabiochem Corporation, Germany). All gels were stained with Gel Star Nucleic Acid Gel Stain (BMA/FMC by Biozym Scientific GmbH), final concentration of 1X. For loading DNA on all gels, Triple Dye Loading Buffer 6X (BMA by Biozym Scientific GmbH, Germany) was used.

The gel results were documented on a LKB Bromma MacroVue Transilluminator (LKB Bromma, Sweden) at a wave length of 302 nm. Pictures were taken with a Polaroid GelCam with the appropriate filter kit for the Gel Star staining, using a Polaroid Film Type 667 Electrophoresis Film (Polaroid GmbH, Germany). For determining the size of DNA fragments, the marker Gene ruler 100 bp DNA ladder plus, as well as the Lambda DNA/*Eco*130I(StyI)/*MluI* Marker, 17 and Lambda Mix Marker, 19 (MBI Fermentas GmbH, Germany) were used. The fragment sizes of the individual marker ladders are shown in Table 2.14.

Gene Ruler 100 bp		**Lambda Marker 17**		**Lambda Marker 19**	
3000 bp	700 bp	26282 bp	2419 bp	48502 bp	17053 bp
2000 bp	600 bp	19329 bp	2205 bp	38416 bp	15004 bp
1500 bp	500 bp	9824 bp	1882 bp	33498 bp	12220 bp
1200 bp	400 bp	7743 bp	1489 bp	29946 bp	10086 bp
1031 bp	300 bp	6223 bp	1268 bp	24508 bp	8614 bp
900 bp	200 bp	5090 bp	956 bp	23994 bp	8271 bp
800 bp	100 bp	4254 bp	925 bp	19397 bp	1503 bp
		3472 bp	458 bp		
		2690 bp	421 bp		
			74 bp		

Table 2.14: DNA Marker Ladders

2.2.11 DNA Sequencing

The sequencing of the DNA was performed using *Cycle Sequencing* with fluorescent marked ddNTPs (one for each nucleotide in the DNA), described in the Training Course Manual for Sequencing of Applied Biosystems/Applera Deutschland GmbH, Germany (ABI PRISM Training Course 1999). The Genetic Analyzer and all reagents were supplied

by the same company. The sequencing reaction is similar to a PCR reaction. Statistically, for each normal nucleotide a fluorescent marked ddNTP (dideoxy nucleotide triphosphate) is inserted into the DNA chain at any given position. The ddNTP lacks the 3'–OH group, thus it can not accept an incoming nucleotide in a polymerase reaction and the DNA chain is interrupted. The products marked in this manner differ by exactly one base and are separated by capillary electrophoresis. The Genetic Analyzer 200/240V 310 (GA310) was used for capillary electrophoresis and analysis. The fluorescence was detected by an argon laser and captured by a CCD camera. Later on, the collected data was analysed by the GA310 collection software.

All sequencing reactions were performed with Big Dye Terminator Cycle Sequencing Ready Reaction Kits, versions 1.0, 1.1, and 2.0. All reactions were performed at room temperature. Sequencing reactions with kits version 1.1 and above needed a preheating step in the program. The purification of the sequencing reactions was performed using Centri Sep Spin columns. The polymer used in the capillary electrophoresis was POP–6, performance optimized polymer 6. For short DNA fragments (up to 400 base pairs), the green capillary was used (47cm x 50 μmid) with the Seq POP6 Rapid (1ml) Filterset E module: injection time 10–30 seconds, injection voltage 2.5 kV, run voltage 15.0 kV, run temperature 50°C, run time 20–36 min. For longer fragments, the red capillary (61 cm x 50 μmid) was used with the Seq POP6 (1ml) Filterset E module: injection time 30 seconds, injection voltage 2.5 kV, run voltage 12.2 kV, run temperature 50°C, run time 120 min.

All sequencing primers were self constructed. The software used in this process was Primer3 (Rozen and Skaletsky 2000). Furthermore, all sequencing protocols and programs to the research project were adapted according to the ABI PRISM Training Course 1999.

2.2.11.1 Sequencing of hsp60 exon 5 with boundaries

The sequencing of the partial hsp60 gene was performed with the PCR primers (XXLHSEXON5IL, XXLHSEXON5IR) and the additional sequencing primer XXLHSEXON5IIR (Section 2.2.6). The protocol and programs are shown in Tables 2.15 and 2.16. The programs SXXLHSV1 and SXXLHSV2 were used with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit version 1.0.; SXXLHSV3 and SXXLHSV4 were used with the Kit versions 1.1 and 2.0.

Template DNA	80–200 ng
Big Dye Reaction Mix	4 μl
Sequencing Primer	5 pmol
HPLC–water	ad 20 μl

Table 2.15: hsp60s, Exon 5 with Boundaries: Sequencing Protocol

Program Steps	Program, Primer	Program, Primer
	SXXLHSV1	**SXXLHSV2**
	XXLHSEXON5IL/IR	**XXLHSEXON5IIR**
1. Denaturation	10 sec 96°C	10 sec 96°C
2. Annealing	05 sec 55°C	05 sec 45°C
3. Extension	4 min 60°C	4 min 60°C
4. Cycles	Goto 1 24 times	Goto 1 24 times
5. Hold	Forever 8°C	Forever 8°C

Program Steps	Program, Primer	Program, Primer
	SXXLHSV3	**SXXLHSV4**
	XXLHSEXON5IL/IR	**XXLHSEXON5IIR**
1. Preheating	1 min 96°C	1 min 96°C
2. Denaturation	10 sec 96°C	10 sec 96°C
3. Annealing	05 sec 55°C	05 sec 45°C
4. Extension	4 min 60°C	4 min 60°C
5. Cycles	Goto 1 24 times	Goto 1 24 times
6. Hold	Forever 8°C	Forever 8°C

Table 2.16: hsp60, Exon 5 with Boundaries: Sequencing Programs SXXLHSV1, SXXLHSV2, SXXLHSV3 and SXXLHSV4

2.2.11.2 Sequencing of hsp60s

The sequencing of the hsp60s gene was performed with the PCR primer (Section 2.2.7) and additional sequencing primers. The primers for the sequencing of the hsp60s gene were constructed based on the sequence of the human hsp60 mRNA and the sequence of the human hsp60 genomic DNA (Table 2.17). The letter L stands for the forward primer "left", the letter R stands for the reverse primer "right". The mRNA sequence of the human chaperonin gene 60 with the primers is shown in Tables 2.18, and 2.19. The coding sequence is located from base 25 to 1746. Primer sequences are underlined, their direction is marked. The protocol and program are shown in Tables 2.20 and 2.21.

Primer	**Sequence,** $5' \longrightarrow 3'$	T_M	**% GC**	**length**
XLS1L (XLLEFT)	GAC GAC CTG TCT CGC CG	61.65°C	70.59	17
XLS2R	TTC TTT GTC TCC GTT TGC AG	60.50°C	50.00	20
XLS3L	CCT GTG ACC ACC CCT GAA G	61.55°C	63.16	19
XLS4R	AGA GCA TGG CAT CGT CTT TG	59.96°C	47.62	21
XLS5L	GAG AAG AGG GAT TGA CCC TG	58.66°C	55.00	20
XLS6R	TCA ATG ATT CCT TTT TCC ACC	58.87°C	38.10	21
XLS7L	GTT CCT CAG AAG TTG GTT ATG ATG	59.07°C	41.67	24
XLS8R(XLRIGHT)	ACA CAA AGG TTG TCA CAT AAT TGG	60.08°C	37.50	24

T_M = melting temperature; **% GC** = amount of GC nucleotides; **length** = number of nucleotides

Table 2.17: hsp60s: Sequencing Primers

5′			*genomic hsp60*	*GACGACCTGT* *>3669–3685>*	*CTCGCCG*AGC *>XLS1L>*	…
1	CACGCTTGCC	GCCGCCCCGC *25–27*	AGAA*ATG*CTT *startcodon*	CGGTTACCCA	CAGTCTTTCG	CCAGATGAGA
61	CCGGTGTCCA	GGGTACTGGC	TCCTCATCTC	ACTCGGGCTT	ATGCCAAAGA	TGTAAAATTT
121	GGTGCAGATG	CCCGAGCCTT	AATGCTTCAA	GGTGTAGACC	TTTTAGCCGA	TGCTGTGGCC
181	GTTACAATGG	GGCCAAAGGG	AAGAACAGTG	ATTATTGAGC	AGGGTTGGGG	AAGTCCCAAA
241	GTAACAAAAG	ATGGTGTGAC	TGTTGCAAAG	TCAATTGACT	TAAAAGATAA	ATACAAGAAC
301	ATTGGAGCTA	AACTTGTTCA	AGATGTTGCC	AATAACACAA	ATGAAGAAGC	TGGGGATGGC
361	ACTACCACTG	CTACTGTACT	GGCACGCTCT	ATAGCCAAGG	AAGGCTTCGA	GAAGATTAGC
421	AAAGGTGCTA	ATCCAGTGGA	AATCAGGAGA	GGTGTGATGT	TAGCTGTTGA	TGCTGTAATT
481	GCTGAACTTA	AAAAGCAGTC	TAAA*CCTGTG* *>505–523>*	*ACCACCCCTG* *>XLS3L>*	*AAG*AAATTGC	ACAGGTTGCT
541	ACGATTT*CTG* *<548–567<*	*CAAACGGAGA* *<XLS2R<*	*CAAAGAA*ATT	GGCAATATCA	TCTCTGATGC	AATGAAAAAA
601	GTTGGAAGAA	AGGGTGTCAT	CACAGTAAAG	GATGGAAAAA	CACTGAATGA	TGAATTAGAA
661	ATTATTGAAG	GCATGAAGTT	TGATCGAGGC	TATATTTCTC	CATACTTTAT	TAATACATCA
721	AAAGGTCAGA	AATGTGAATT	CCAGGATGCC	TATGTTCTGT	TGAGTGAAAA	GAAAATTTCT
781	AGTATCCAGT	CCATTGTACC	TGCTCTTGAA	ATTGCCAATG	CTCACCGTAA	GCCTTTGGTC
841	ATAATCGCTG	AAGATGTTGA	TGGAGAAGCT	CTAAGTACAC	TCGTCTTGAA	TAGGCTAAAG
901	GTTGGTCTTC	AGGTTGTGGC	AGTCAAGGCT	CCAGGGTTTG	GTGACAATAG	AAAGAACCAG
961	CTTAAAGATA	TGGCTATTGC	TACTGGTGGT	GCAGTGTTTG	*GAGAAGAGGG* *>1001–1020>*	*ATTGACCCTG* *>XLS5L>*
1021	AATCTTGAAG	ACGTTCAGCC	TCATGACTTA	GGAAAAGTTG	GAGAGGTCAT	TGTGACCAAA
1081	*GACGATGCCA* *<1081–1096<*	*TGCTCT*TAAA *<XLS4R<*	AGGAAAAGGT	GACAAGGCTC	AAATTGAAAA	ACGTATTCAA
1141	GAAATCATTG	AGCAGTTAGA	TGTCACAACT	AGTGAATATG	AAAAGGAAAA	ACTGAATGAA
1201	CGGCTTGCAA	AACTTTCAGA	TGGAGTGGCT	GTGCTGAAGG	TTGGTGGGAC	AAGTGATGTT
1261	GAAGTGAATG	AAAAGAAAGA	CAGAGTTACA	GATGCCCTTA	ATGCTACAAG	AGCTGCTGTT
1321	GAAGAAGGCA	TTGTTTTGGG	AGGGGGTTGT	GCCCTCCTTC	GATGCATTCC	AGCCTTGGAC
1381	TCATTGACTC	CAGCTAATGA	AGATCAAAAA	ATTGGTATAG	AAATTATTAA	AAGAACACTC
1441	AAAATTCCAG	CAATGACCAT	TGCTAAGAAT	GCAGGTGTTG	AAGGATCTTT	GATAGTTGAG
1501	AAAATTATGC	AAA*GTTCCTC* *>1514–1537>*	*AGAAGTTGGT* *>XLS7R>*	*TATGATG*CTA	TGGCTGGAGA	TTTTGTGAAT
1561	AT*GGTGGAAA* *<1563–1583<*	*AAGGAATCAT* *<XLS6R<*	*TGA*CCCAACA	AAGGTTGTGA	GAACTGCTTT	ATTGGATGCT

underlined = primer

Table 2.18: hsp60 mRNA: Sequencing Primer on M34664 with additional 5′ bases (1–1620)

1621	GCTGGTGTGG	CCTCTCTGTT	AACTACAGCA	GAAGTTGTAG	TCACAGAAAT	TCCTAAAGAA
1681	GAGAAGGACC	CTGGAATGGG	TGCAATGGGT	GGAATGGGAG	GTGGTATGGG	AGGTGGCATG
1741	TTC*TAA*CTCC *1744–1746*	TAGACTAGTG *Stopcodon*	CTTTACCTTT	ATTAATGAAC	TGTGACAGGA	AGCCCAAGGC
1801	AGTGTTCCTC	ACCAATAACT	TCAGAGAAGT	CAGTTGGAGA	AAATGAAGAA	AAAGGCTGGC
1861	TGAAAATCAC	TATAACCATC	AGTTACTGGT	TTCAGTTGAC	AAAATATATA	ATGGTTTACT
1921	GCTGTCATTG	TCCATGCCTA	CAGATAATTT	ATTTTGTATT	TTTGAATAAA	AAACATTTGT
1981	ACATTCCTGA	TACTGGGTAC	AAGAGCCATG	TACCAGTGTA	CTGCTTTCAA	CTTAAATCAC
2041	TGAGGCATTT	TTACTACTAT	TCTGTTAAAA	TCAGGATTTT	AGTGCTTGCC	ACCACCAGAT
2101	GAGAAGTTAA	GCAGCCTTTC	TGTGGAGAGT	GAGAATAATT	GTGTACAAAG	TAGAGAAGTA
2161	T*CCAATTATG* *<2162–2185<*	*TGACAACCTT* *<XLS8R<*	*TGTGT*AATAA	AAATTTGTTT	AA	

underlined = primer

Table 2.19: hsp60 mRNA: Sequencing Primer on M34664 (bases 1621–2202)

Template DNA	100 ng
Big Dye Reaction Mix	4 μl
Sequencing Primer	4 pmol
HPLC–water	ad 20 μl

Table 2.20: hsp60s: Sequencing Protocol

1.	05 sec 96°C	Denaturation
2.	1.5 min 60°C	Annealing
3.	1.5 min 50°C	Extension
4.	Goto 1, 24 times	Cycles
5.	Forever 4°C	Hold

Table 2.21: hsp60s: Sequencing Program AT3S, Primer XLS1L–XLS8R

2.2.11.3 Direct sequencing of PCR fragments with different size from SIDS DNA

The sequencing parameters are the same as for the partial hsp60 gene (Section 2.2.11.1), and the hsp60s gene (Section 2.2.11.2).

2.2.12 Software tools used

2.2.12.1 Primer construction

For primer construction, the Primer3 software was used (Rozen and Skaletsky 2000). This software calculates certain characteristics of a primer. Some of these can be constrained by the user to constant values beforehand. The most important feature is the melting temperature T_M, which directly influences the annealing temperature T_A. At this temperature, the binding between primer and DNA occurs in the PCR. Additionally, Primer3 determines the GC–concentration and the length of the primer, that also influence the melting temperature.

2.2.12.2 Expression analysis

The most important feature of a gene is its expression (production of a protein). Before actual experiments are performed, newly discovered genes can be tested with software. Certain characteristics, such as transcription factor binding sites in the promoter region of a gene, show consensus sequences. Based on the latter, software can detect these sites in a DNA sequence and assumptions on a putative expression can be made.

The MATCH software was used for testing of putative transcription factor binding sites of hsp60s. Match is a weight matrix–based tool for searching putative transcription binding sites in DNA sequences. It uses the matrix library of the TRANSFAC Database, version 7.4.1 (Section 2.1.3). Also the TRANSPLORER promoter and gene finder software (TRANSPLORER 2004), version 1.2, was used for analysis of putative promoter sequences of hsp60s. The matrices used in Transplorer 1.2 are from the TRANSFAC Database. MATCH, TRANSPLORER and TRANSFAC were made available for this research, courtesy of Biobase Biological Databases GmbH.

2.2.12.3 Protein parameters and alignment

If an open reading frame (ORF) is found in a DNA sequence, it can be translated into a protein sequence. For humans, the standard genetic code is used. The translation of the hsp60s gene from DNA to protein was performed with the EditSeq module of the DNASTAR Lasergene 99 Software (EditSeq 1999; GATC GmbH, Germany). Based on the protein sequence, various physical and chemical parameters can be calculated to classify the putative protein. For determining the protein parameters of Hsp60s1 and Hsp60s2, the ProtParam tool (`http://au.expasy.org/tools/protparam.html`) of the Swiss–Prot Database (SWISSPROT 2004) was used. This tool allows, among others, the computation of the molecular weight, of the amino acid composition and the estimated half–life.

The protein sequence can also be used for an alignment. An alignment is the basic principle of a sequence analysis (DNA and protein). Two or more sequences are compared in a nucleotide by nucleotide (amino acid by amino acid) fashion. The same nucleotides or amino acids in the same position are called a match; if they are different, they are called a mismatch. Insertions or deletions between positions are called gaps. The Lipman–Pearson algorithm was used for the protein alignment of Hsp60 and Hsp60s1/Hsp60s2.

It constructs tables of *k*–tuples to find regions of similarity. The alignment is then optimized using a trimmed–down version of the Needleman–Wunsch method. The original research was done by Lipman and Pearson 1985 (Lipman and Pearson 1985). This algorithm was applied using the MegAlign software (MegAlign 1999).

2.2.12.4 DNA sequence analysis

One major part of this thesis consists of a mutation analysis. SIDS cases and controls were examined for genetic differences. Another major part of this thesis consists of the analysis of a newly discovered gene, including an analysis of its possible phylogenetic tree. Both parts are essentially performed with a sophisticated analysis of the DNA sequence.

The collection software of the Genetic Analyzer GA310 presents the results of a sequencing analysis in two forms: The DNA sequence, in FASTA format, e.g., ACTTACGT.... This is derived by interpreting the so–called electropherogram, a waveform representation of the analogue signal captured by the CCD camera. If the incoming signal is distorted, the software may not be able to to interpret it correctly. In these cases, manual verification and/or translation of the electropherogram is required. For electropherogram viewing and editing, the CHROMAS software, version 1.45, was used (McCarthy 1998). For editing the FASTA–sequences, the EditSeq module of the DNASTAR Lasergene 99 Software (EditSeq 1999; GATC GmbH, Germany) was used.

The DNA sequences obtained from the SIDS cases and controls were then aligned with a reference sequence (pairwise alignment), to detect any mutation. For the construction of a phylogenetic tree, a multiple alignment is the prerequisite. Sequence alignment was performed locally with the MegAlign module of the DNASTAR Lasergene 99 Software (MegAlign 1999; GATC GmbH, Germany), except the Jotun–Hein algorithm, which was performed with the more recent DNASTAR Lasergene Software, version 5.08, courtesy of GATC GmbH. Pairwise alignment of DNA sequences was performed with the Wilbur–Lipman and the Martinez/Needleman–Wunsch algorithms. Multiple alignment was performed using the Jotun–Hein algorithm.

The Wilbur–Lipman method is used for highly divergent DNA sequences, e.g., sequences of unknown ancestry. Another application is the comparison of large nucleotide sequences. Both applications are performed with a small *k*–tuple. For example if *k* has the value of 5, the algorithm searches for exact matches of the length 5 in the matrix. The Wilbur–Lipman method can be interpreted as a word method in a DotPlot setting (Hansen 2001). The original research on this method was done by Wilbur and Lipman 1983 (Wilbur and Lipman 1983). The Martinez/Needleman–Wunsch method is used for the pairwise alignment of closely related DNA sequences. The original research on this method was done by Needleman and Wunsch (Needleman and Wunsch 1970) and Martinez (Martinez 1983). The Jotun–Hein Method constructs a phylogenetic tree by examining sequence pairs and creating the best possible arrangement of ancestral branches. This method is used for multiple sequences that are related or possibly related. The original research on this method was done by Hein (Hein 1990).

Multiple alignment can be performed on a grand scale. A DNA sequence can be aligned to an entire database. For genomewide alignments with GenBank the Blast Tool was used. The Blast algorithm (**B**asic **L**ocal **A**lignment **S**earch **T**ool) is a heuristic search algorithm

for fast database alignments. It is used for many different inputs (e.g., DNA, proteins; small and large sequences) at GenBank (GENBANK 2004). The original research on this method was done by Atschul et al. (Atschul et al 1990). The current version of this method has been programmed by Atschul et al. (Atschul et al 1997).

2.2.12.5 Statistics

Statistical methods were applied to determine, if the results of the mutation analysis were correlated with the Sudden Infant Death Syndrome or could be explained by chance. The purpose of a statistical test is to determine if the obtained results provide a reason to reject the null hypothesis (also called statistical hypothesis). This defines the probability that a given result could also be a product of chance factors. An example of the null hypothesis would be, that the sex has no influence on the risk of dying from Sudden Infant Death. A significant difference between a SIDS group and a control group rejects the null hypothesis, indicating that the *sex* does have an influence on the risk of dying on Sudden Infant Death. A finding (for example, the influence of the sex on Sudden Infant Death) is described as statistically significant, if it can be demonstrated, that the probability of obtaining such a difference by chance only is relatively low. The significance level, or p–rating, describes the probability that a given result could have been originated by chance. A p–rating of 0.05 (5 %) is considered significant, a p–rating of 0.01 (1 %) highly significant. A p–rating of 0.10 (10 %) is considered a trend.

In this thesis, two types of data were analysed statistically: nominal and metric data. Nominal characteristics are qualitative characteristics, e.g., sex. For these characteristics the Chi–Square–Test was used. In case of an insufficient sample size, the exact Fisher–Test was used. The tests were used in a two–tailed manner, testing for differences between groups, e.g., male and female, SIDS cases and controls. Additionally, the binary logistic regression was used. It allows an analysis of more than two nominal characteristics as well as the integration of metric data in the results. Metric characteristics are quantitative characteristics, e.g., the age. For testing if metric data was Gauss–distributed, the Kolmogoroff–Smirnov–Test was used in a two–tailed manner. It is also possible to take the skew as an indication for a Gauss–distribution, if it is used as a prerequisite for the t–test (mean test). A skew between -1.5 and +1.5 can be assumed to be Gauss–distributed, and enables the application of the t–test (Geerlings 2004). Statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS), version 11.5.1 (SPSS 2002).

3 Results

The following text presents the major findings discovered by applying the experimental procedures described in Chapter 2.

3.1 SIDS cases and controls

The SIDS cases are classified according to their SIDS criteria (defined in Section 1.7.3). To this end, these risk factors and autopsy results were analysed based on the detail in the autopsy protocols, supported by information from the German Study on Sudden Infant Death (Findeisen et al 2003). Statistical methods were then applied to verify these results, so that a random formation of the SIDS criteria could be ruled out.

The controls were chosen by the RM/MHH matched in age and sex to the SIDS cases, thus only these characteristics of the controls could be evaluated. The age and sex data of the individual SIDS cases and controls, as well as the information of the SIDS criteria is listed in the Appendix (Tables A.1, A.2, A.3, A.4).

3.1.1 Sex Distribution

The sex distribution is shown in Table 3.1 and in Figure 3.1. 56 male and 35 female SIDS cases were examined in this thesis. Statistically, there is a significant difference between the number of male and female samples (Chi–Square–Test: p=0.028). More SIDS cases are male than female. For the control group, 59 male and 49 female children were examined. Statistically, there is no significant difference between the number of male and female samples (Chi–Square–Test: p=0.336). No significant statistical difference was found comparing the sex of SIDS cases (N=91) and controls (N=108), Chi–Square–Test p=0.326.

	SIDS		**Control**	
Sex	**Frequency**	**%**	**Frequency**	**%**
1 (♂)	56	61.54	59	54.63
2 (♀)	35	38.46	49	45.37
$\sum$	91	100	108	100

Table 3.1: SIDS Cases and Controls: Sex Distribution and Frequencies

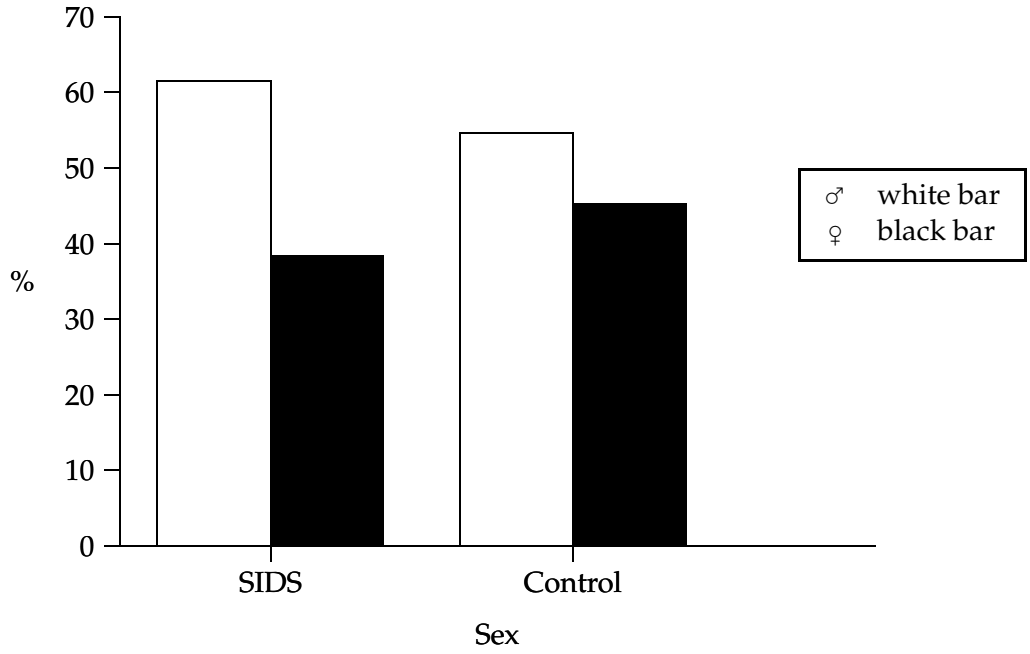


Figure 3.1: SIDS cases and Controls: Sex – Distribution and Frequency

3.1.2 Age Distribution

91 SIDS cases were examined, ranging in age from 13 to 341 days (1–12 months), and 108 controls, ranging from 103 to 361 days (3–12 months) of age. The statistical parameters for the age in days are shown in Table 3.2.

Statistics	**SIDS**	**Control**
N	91	108
Arithmetic mean	116.5165	274.3704
Standard error arithmetic mean	8.05029	4.50798
Median	95.000	266.000
Deviation	76.79487	46.84825
skew	1.216	-0.709
Standard error skew	0.253	0.233
Minimum	13.00	103.00
Maximum	341.00	361.00

Table 3.2: SIDS Cases and Controls: Age in Days – Statistics

The frequencies and percentages of the SIDS cases and controls according to age and sex are shown in Tables 3.3 and 3.4, the corresponding Figures are 3.2 and 3.3. The frequencies and percentages comparing the ages of SIDS cases and controls are shown in Table 3.5 and Figure 3.4. The SIDS cases are Gauss–distributed. The Kolmogoroff–Smirnov–Test calculated a p–rating of 0.053. For the controls, the same test calculated a p–rating

of 0.001, indicating that the control are not Gauss–distributed. The reason for this oddity lies within the nature of the controls: Most of the controls (N=104) are older than 8 months due the nature of paternity testing with blood phenotypes. Certain tests are only possible after the child is about 8 months old (the phenotypes of the blood factors are not developed until then). All younger controls are children who did not die of SIDS (N=4). For comparison with SIDS cases, the chosen controls are suitable: By definition the age of SIDS cases ranges between 8 and 365 days (Beckwith 2003, Findeisen et al 2003), all controls are within these bounds.

Age	SIDS		♂		♀	
Month	**Frequency**	**%**	**Frequency**	**%**	**Frequency**	**%**
1	5	5.5	2	3.6	3	8.6
2	18	19.8	14	25.0	4	11.4
3	20	22.0	14	25.0	6	17.1
4	13	14.3	7	12.5	6	17.1
5	11	12.1	6	10.7	5	14.3
6	9	9.9	5	8.9	4	11.4
7	5	5.5	4	7.1	1	2.9
8	1	1.1	1	1.8	0	0.0
9	4	4.4	2	3.6	2	5.7
10	0	0.0	0	0.0	0	0.0
11	3	3.3	1	1.8	2	5.7
12	2	2.2	0	0.0	2	5.7
$\sum$	91	100.00	56	100.00	35	100.00

Table 3.3: SIDS cases: Age and Sex – Distribution and Frequency

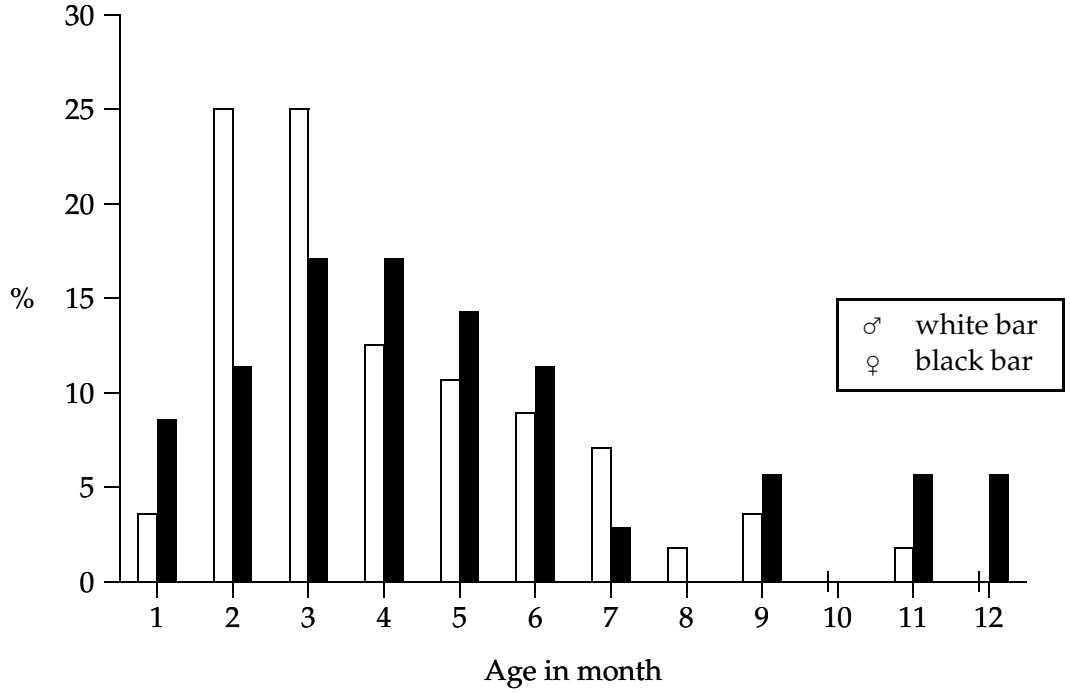


Figure 3.2: SIDS cases: Age and Sex – Distribution and Frequency

Age	SIDS		♂		♀	
Month	**Frequency**	**%**	**Frequency**	**%**	**Frequency**	**%**
1	0	0.0	0	0.0	0	0.0
2	0	0.0	0	0.0	0	0.0
3	0	0.0	0	0.0	0	0.0
4	2	1.9	1	1.7	1	2.0
5	1	0.9	1	1.7	0	0.0
6	1	0.9	1	1.7	0	0.0
7	1	0.9	1	1.7	0	0.0
8	0	0.0	0	0.0	0	0.0
9	55	50.9	29	49.2	26	53.1
10	19	17.6	11	18.6	8	16.3
11	15	13.9	7	11.9	8	16.3
12	14	13.0	8	13.6	6	12.2
$\sum$	108	100.00	59	100.00	49	100.00

Table 3.4: Controls: Age and Sex – Distribution and Frequency

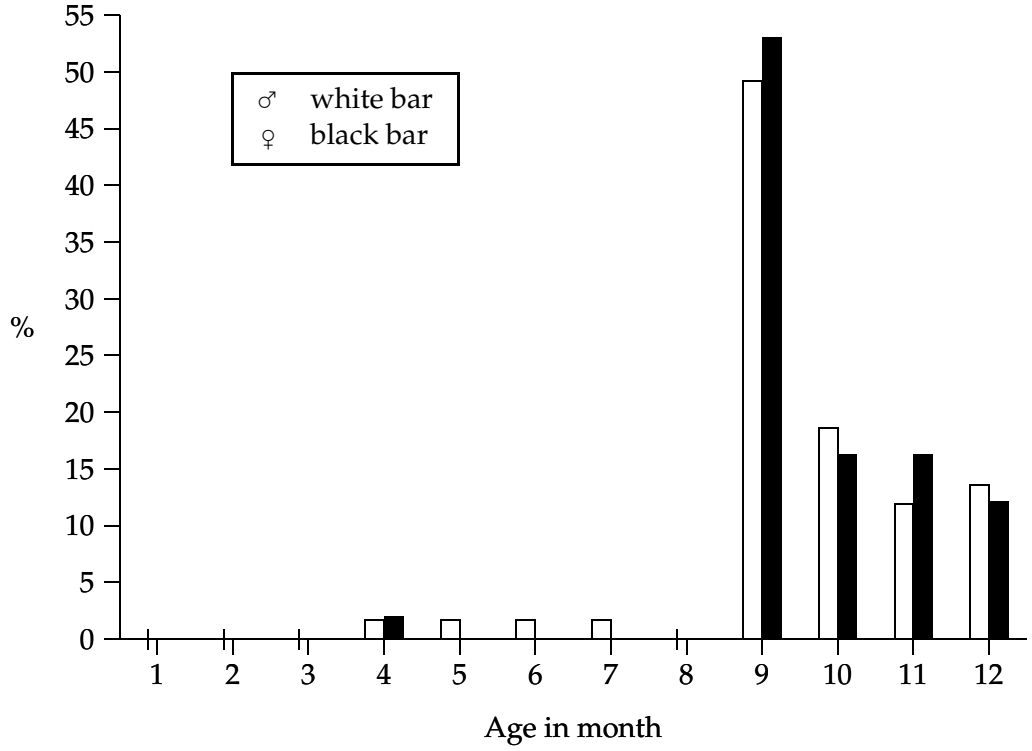


Figure 3.3: Controls: Age and Sex – Distribution and Frequency

Age	SIDS		Control	
(Month)	Frequency	%	Frequency	%
1	5	5.5	0	0.0
2	18	19.8	0	0.0
3	20	22.0	0	0.0
4	13	14.3	2	1.9
5	11	12.1	1	0.9
6	9	9.9	1	0.9
7	5	5.5	1	0.9
8	1	1.1	0	0.0
9	4	4.4	55	50.9
10	0	0.0	19	17.6
11	3	3.3	15	13.9
12	2	2.2	14	13.0
$\sum$	91	100	108	100

Table 3.5: SIDS Cases and Controls: Age in Month – Distribution and Frequency

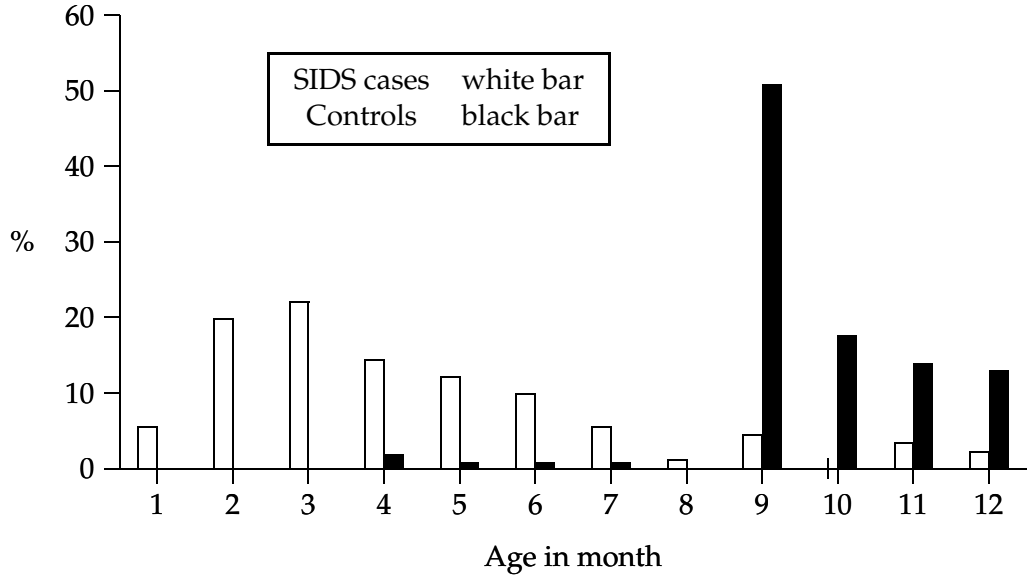


Figure 3.4: Sid cases and Controls: Age in Month – Distribution and Frequency

3.1.3 Analysis of SIDS criteria

The SIDS criteria are shown in Table 3.6. The individual criteria are classified as being ”present” (asserted by the autopsy protocol) or ”not present” (controverted by the autopsy protocol or unknown). The complete information on the SIDS criteria for the individual SIDS cases are shown in the Appendix (Section A.2, Tables A.3 A.4). The results of the evaluation of epidemiological/general characteristic and autopsy data follow:

Male sex The SIDS cases contain more male than female children. The Chi–Square–Test indicates a significant difference between the sexes, p=0.028 (Section 3.1.1).

Age between 2 to 4 month More than half of the SIDS cases died at this age (51 the 91, see also Section 3.1.2).

Preterm Birth Only 13 of the 91 SIDS cases were documented preterm births. Most of the autopsy protocols contained no information regarding a preterm birth. The ratio between male and female children concurs with the entire sample.

Prone sleeping Over 70 % of the cases with a documented position in the death scene (N=61) were found prone.

Heat stress The binary logistic regression shows, that age is statistically significant (p=0.017). The higher the age, the higher is the probability of SIDS cases showing heat stress in the death scene. This result should be taken cautiously due to the relatively small number of samples showing heat stress (N=12).

Smoking Nicotine abuse by the mother was documented in 10 of 91 SIDS cases. Most of the autopsy protocols contained no information regarding parents smoking.

Petechiae in Pleura About two third of the SIDS cases showed petechiae in the pleura. The Chi–Square–Test indicates a significant difference between the sexes, p=0.004 (binary logistic regression: p=0.002). Male children dying on Sudden Infant Death Syndrome have a much higher probability showing petechiae in the pleura than female children.

Petechiae in Epicardium About two third of the SIDS cases showed petechiae in the epicardium. The binary logistic regression shows that age is statistically significant (p=0.047). The higher the age, the higher the probability of petechiae in the epicardium of SIDS cases.

Petechiae in Thymus About two third of the SIDS cases showed petechiae in the thymus. The binary logistic regression shows that age is statistically significant (p=0.026). The higher the age, the higher the probability of petechiae in the thymus of SIDS cases.

Oedemata in the respiratory tract About one third of the SIDS cases showed oedemata in the respiratory tract.

Criteria	∑ SIDS				present in ♂		present in ♀	
	present		not present					
	Frequency	%	Frequency	%	Frequency	%	Frequency	%
Gen. Characteristics								
Sex	91	100.0	–	–	56	100.0	35	100.0
Age: 2–4 months	51	56.04	40	43.96	35	68.63	16	31.37
Preterm Birth	13	14.30	78	85.70	8	61.54	5	38.46
Death Scene Position	61	67.03	30	32.97	39	63.93	22	36.07
Prone	43	70.50	–	–	25	58.14	18	41.86
Supine	14	22.95	–	–	11	78.57	3	21.43
Side	4	4.40	–	–	3	75.00	1	25.00
Death Scene Temperature	12	13.20	79	86.80	6	50.00	6	50.00
Nicotine Abuse By Mother	10	11.00	81	89.00	5	50.00	5	50.00
Autopsy Results								
Petechiae in Pleura	63	69.20	28	30.80	45	71.43	18	28.57
Petechiae in Epicardium	57	62.60	34	37.40	37	64.91	20	35.09
Petechiae in Thymus	63	69.20	28	30.80	38	60.32	25	39.68
Oedemata in Resp.Tract	36	39.60	55	60.40	25	69.44	11	30.66
Foam in Resp. Tract	30	33.00	61	67.00	27	90.00	3	10.00
Hemorrhage in Resp.Tract	57	62.60	34	37.40	31	54.39	26	45.61

Table 3.6: SIDS cases – Frequencies of SIDS criteria

Foam in the respiratory tract About one third of the SIDS cases showed foam in the respiratory tract. The Chi–Square–Test indicates a highly significant difference between the sexes, p=0.000 (binary logistic regression p=0.001). 27 male (90.00 % of the 30 documented cases), but only 3 female children (10.00 %) showed foam in the respiratory tract. Male children dying on Sudden Infant Death Syndrome have a much higher probability of showing foam in the respiratory tract than female children.

Hemorrhage in the respiratory tract About two thirds of the SIDS cases showed hemorrhage in the respiratory tract. The Chi–Square–Test indicates a trend in difference between the sexes, p=0.069. The binary logistic regression confirms the trend: p=0.039. Female children dying on Sudden Infant Death Syndrome have a higher probability of showing hemorrhage in the respiratory tract than male children. The binary logistic regression also shows a trend (p=0.094), indicating a connection between the age and hemorrhage in the respiratory tract. The higher the age, the lower the probability of hemorrhage occurring in the respiratory tract of SIDS cases.

Based on the SIDS criteria it can be concluded, that the SIDS cases examined in this thesis are typical SIDS cases.

3.2 Mutations in the human chaperonin gene hsp60

The research on gene variations in SIDS cases leads to the following conclusion: SIDS cases show more variations than controls (see also Section 1.7.4). It was examined, whether this effect occurs in the partial human hsp60 gene, namely in its exon 5 with boundaries. To detect any mutation, both strands of the exon 5 and parts of the adjacent introns 4 and 5 were amplified and sequenced in all SIDS cases and all controls. The mutation analysis was verified using statistical methods, in order to disprove a possible random formation of the mutations.

3.2.1 The mutation N158S in exon 5 of hsp60

The size of the hsp60 PCR fragment of exon 5 with boundaries (380 bp) was verified on an agarose gel (Figure 3.5). The outer lanes, 1 and 6, show the Gene Ruler 100bp marker ladder, the inner lanes, 2. . . 5, show the hsp60 PCR fragment. The DNA and protein sequence of exon 5 are shown in Table 3.7.

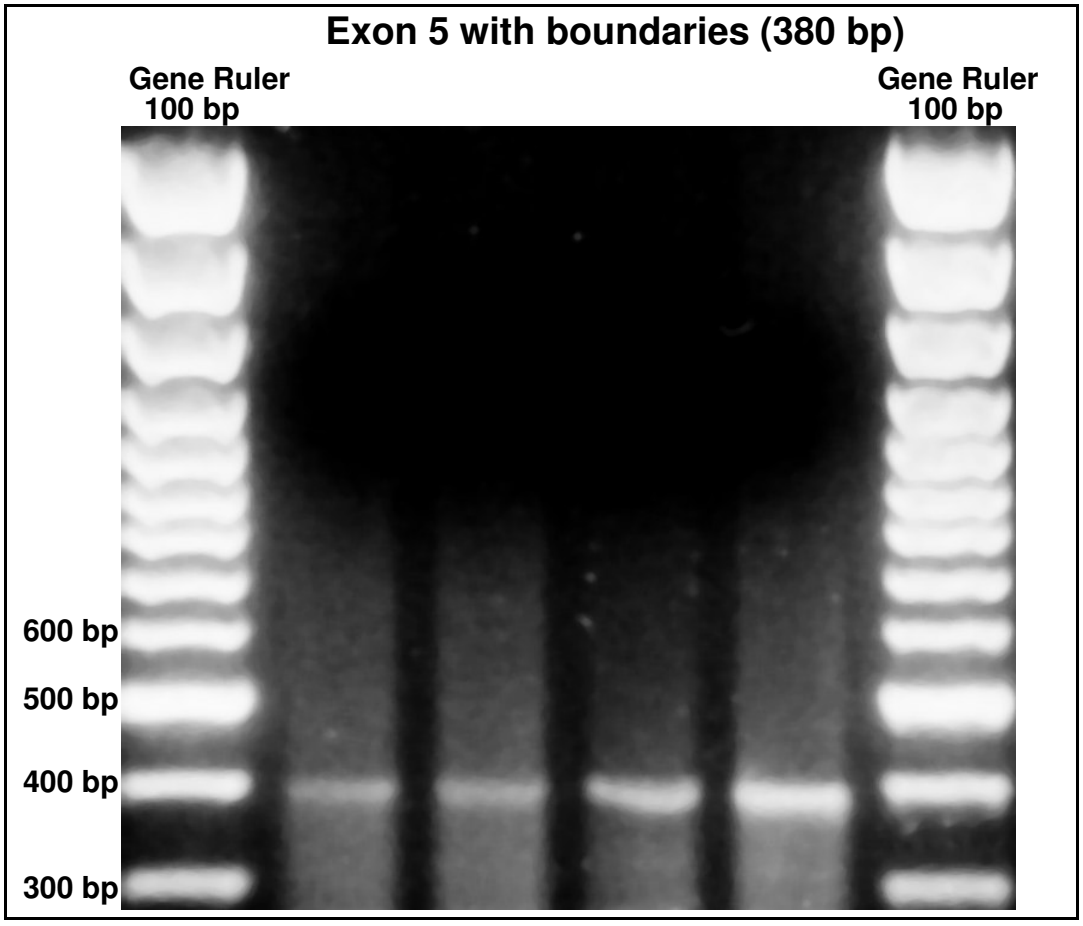


Figure 3.5: 1% Agarose Gel; PCR product of the partial human hsp60 gene, including exon 5 with 5′ and 3′ boundaries just below 400 bp

gtt	gct	acg	att	tct	gca	aac	gga	gac	aaa	gaa	att
V	A	T	I	S	A	N	G	D	K	E	I
145	146	147	148	149	150	151	152	153	154	155	156
ggc	**aat**	atc	atc	tct	gat	gca	atg	aaa	aaa	gtt	gga
G	**N**	I	I	S	D	A	M	K	K	V	G
157	**158**	159	160	161	162	163	164	165	166	167	168
aga	aag	ggt	gtc	atc	aca	gta	aag				
R	K	G	V	I	T	V	K				
169	170	171	172	173	174	175	176				

Table 3.7: Exon 5 of the human hsp60 gene: DNA and protein sequence

The sequences obtained in this manner were aligned against the human hsp60 gene, using the Martinez/Needleman–Wunsch algorithm. No amino acid variation N158S (mutation in the AAT codon) was found in either of the SIDS cases or controls. Furthermore, no other mutation in the complete exon 5 of human hsp60 could be discovered (Table 3.8). A subset of these results (sequencing of exon 5 in 60 SIDS cases and 60 controls) were published in 2002 (Teske et al 2002).

DNA Sequence	SIDS cases	Controls
Variation N158S	0/91 (0 %)	0/108 (0 %)
Other mutations in exon 5	0/91 (0 %)	0/108 (0 %)

Table 3.8: Number of mutations in the exon 5 of the human hsp60 gene

3.2.2 The deletions in intron 5 of hsp60

In the search for the mutation N158S in the exon 5, parts of the adjacent upstream and downstream introns (number 4 and 5) to exon 5 were also amplified and sequenced. In the case of intron 4, no variations in the DNA of the hsp60 gene were found. However, two kinds of deletion in the intron 5 of the human hsp60 gene were discovered, in both SIDS cases and controls. The two deletions did not occur simultaneously in either SIDS cases or controls. The distribution of the deletions is shown in Table 3.9.

The first deletion affects the bases *AG* at positions 8957–8958 in the human hsp60 gene, hence named *deletion AG*; the second is a deletion of 24 bases (*TATGAT-CAAAAGTTTGAGTTATCT*) at positions 8885–8908, hence named *deletion 24* (see Table 3.10). The deletions are marked in boxes.

DNA Sequence	SIDS cases	Controls
Deletions in intron 5	16/91 (17.60 %); 11 ♂, 5 ♀	11/108 (10.20 %); 3 ♂, 8 ♀
Deletion AG	12/91 (13.20 %); 10 ♂, 2 ♀	8/108 (7.40 %); 3 ♂, 5 ♀
Deletion 24	4/91 (4.40 %); 1 ♂, 3 ♀	3/108 (2.80 %); 0 ♂, 3 ♀

Table 3.9: Number of mutations in the intron 5 of the human hsp60 gene

Deletion 24 The binary logistic regression with a p–rating of 0.043 indicates a statistically significant difference between the SIDS cases and controls regarding the sex. All 3 controls with the *deletion 24* are female. Of the 4 SIDS cases 3 were female. This result should be considered cautiously due to the small number of deletions in each group.

Deletion AG The binary logistic regression detects a statistically significant difference between the SIDS cases and controls (p=0.036): More SIDS cases than controls show the deletion AG (Figure 3.6). Among the SIDS cases, the male children show more *deletions AG* than the female.

Accumulated deletions in the entire intron 5 The binary logistic regression detects a statistically significant difference between the SIDS cases and controls (p=0.036) in the overall intron 5. The SIDS cases seem to be affected more with deletions in the hsp60 gene than the controls (Figure 3.7).

8641	CCCCTGGATG	GAAGTATCAA	TTTGGAAGTT
	ACTAGGAAAA	*GGTAAGGGAA* *>8681–8700>*	*CTGACTGAGG* *>XXLHSEXON5IL>*
8701	ACACTAGGCT	TACCTTTTAA	TTTCGAAGAG
	TAACTGGATT	ATTTCAG**GTT**	**GCTACGATTT**
8761	**CTGCAAACGG**	**AGACAAAGAA**	**ATTGGCAATA**
	TCATCTCTGA	**TGCAATGAAA**	**AAAGTTGGAA**
8821	**GAAAGGGTGT**	**CATCACAGTA** *8748–8843*	**AAG**GCAAGTG *exon 5*
	TGTTTGTATT	TTTAAAGATA	ATTTTGAGTT
8881	ATCT TATGAT	CAAAA *GTTTG* *<8896–8915<*	*AGTTATCT GA* *<XXLHSEXON5IIR<*
	*TGATC*AAAAC	TGAATTTTTC	AAACAGAAAA
8941	ATTCTGTTTT	AAAAAC AG TG	CTTTTTTTAT
	TCCTTCATAT	*GTAGTCTCAG* *<8981–9001<*	*CACATGTATA* *<XXLHSEXON5IIIR<*
9001	*G*AATAAAGTA	CTTACTGTGT	TTTATGTGAA
	TTAGCCTTTG	*CTGCATGAAA* *<9041–9060<*	*TGAATCCATC* *<XXLHSEXON5IR<*

boxes = deletions in intron 5

Table 3.10: Deletions in the intron 5 of the human hsp60 gene

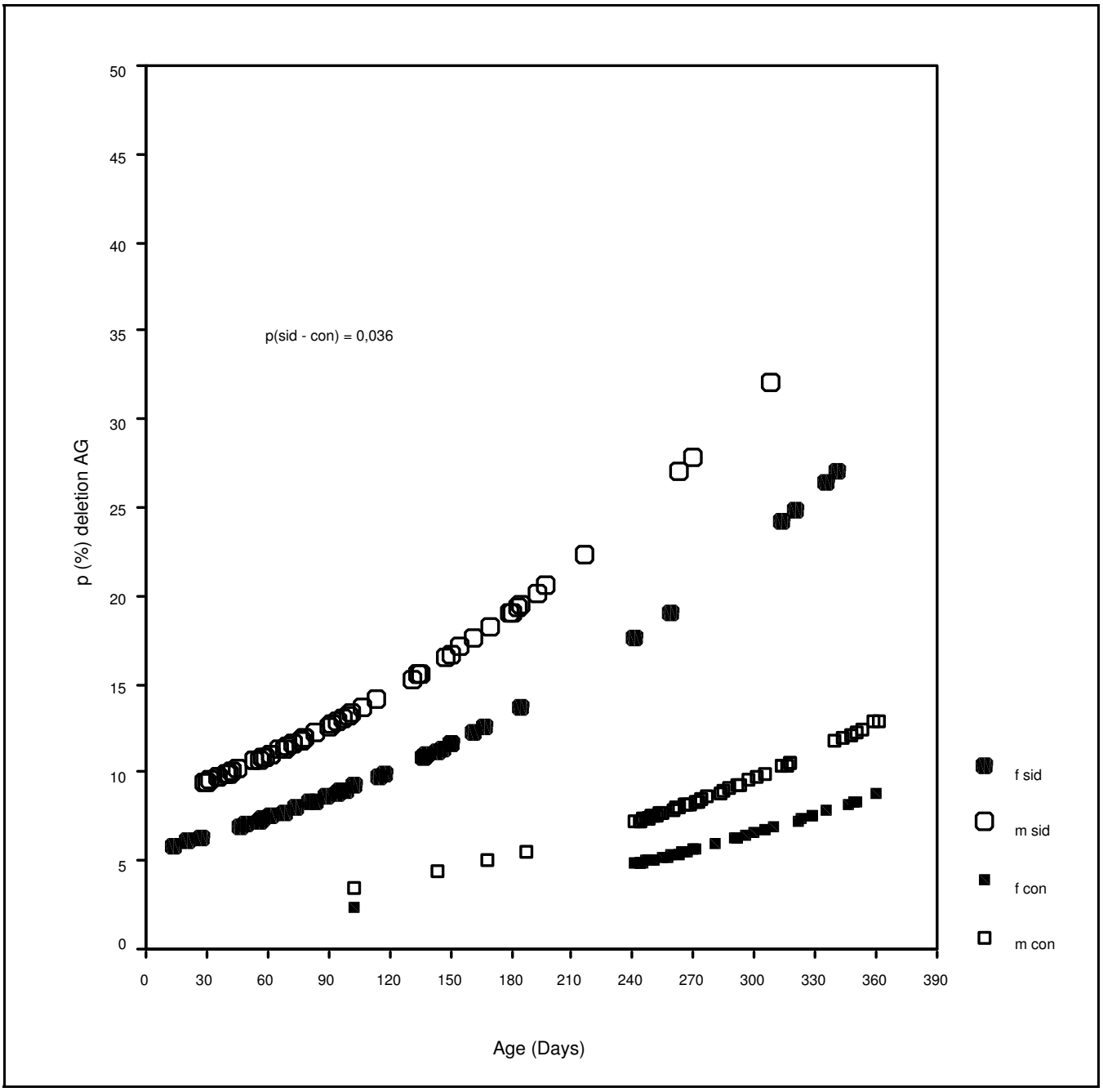


Figure 3.6: Binary logistic regression of the *deletion AG* in intron 5 in the human hsp60 gene in SIDS cases and Controls

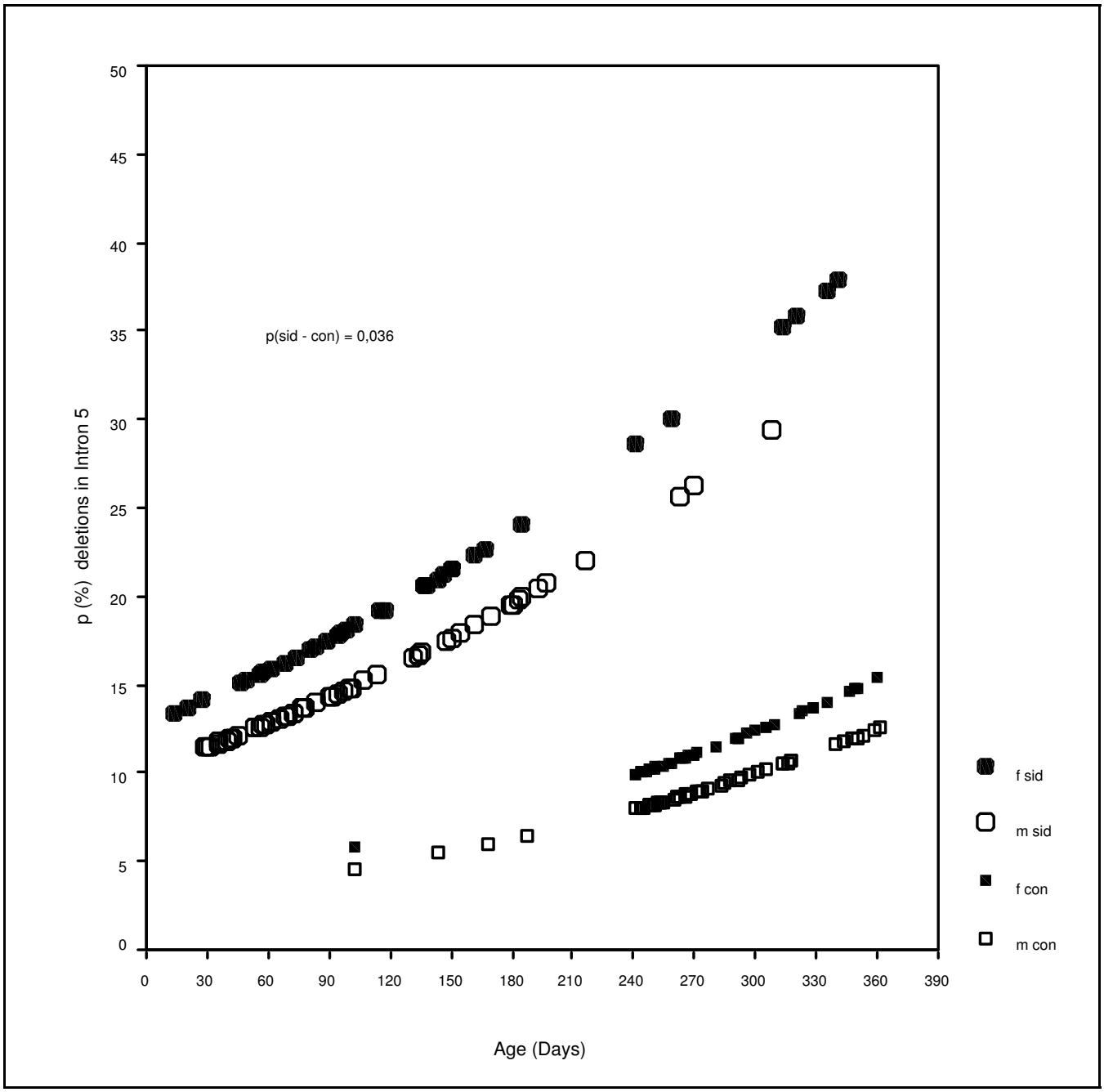


Figure 3.7: Binary logistic regression of the *accumulated deletions in intron 5* in the human hsp60 gene in SIDS cases and Controls

A comparison between SIDS cases and controls with regard to correlations of SIDS criteria and deletions in the intron 5 of the human hsp60 gene, discovered only two significant results:

- Male children seem to be affected more with a deletion AG than female, confirming the risk factor "male sex", already explained above.
- Statistically significant more SIDS cases with a deletion AG showed no petechiae in the thymus (binary logistic regression: p=0.017).

These results of the mutation analysis of exon 5 with boundaries are similar to other genetic variations in Sudden Infant Death. All show the same phenomenon: SIDS cases seem to have more genetic variations (deletions, polymorphisms, mutations) than controls (for a detailed description, see Section 4.2).

3.3 hsp60s, a new member/pseudogene of the HSP60 family

3.3.1 Gene Sequence of hsp60s

The original intention was to amplify the coding part of the human genomic hsp60 gene (exons 2–12 with introns, size 13248 base pairs) for the direct sequencing approach. The latter was planned to be used in mutation analysis of SIDS cases and controls. When the size of the expected hsp60 PCR fragment (13248 bp) was verified on an agarose gel (Figure 3.8), no such fragment was found on the gel. Instead, a very prominent fragment showed up at 2205 bp of the Lambda 17 marker ladder.

The fragment was extracted from the gel and the primers XLLEFT and XLRIGHT were used for sequencing. The sequences obtained appeared to be very similar to the mRNA of the human chaperonin gene (hsp60 gene). The fragment size (about 2200 bp) was also similar to the size of the hsp60 mRNA (2202 bp). It was suspected to be either a pseudogene or an inducible alternative to the hsp60 gene.

Inducible alternatives are known for some heat shock proteins, e.g., the human heat shock protein gene hsp70. It exists in two forms: The first is the cognate or constitutive form hsc70 (GAN Y00371), protein Hsc70 (SPAN P11142). It contains introns and is a regular molecular chaperone and house–keeping gene (Tavaria et al 1996; Tavaria et al 1995). The second is the stress (especially heat) inducible intronless form hsp70 (GAN BC002453), protein Hsp70 (SPAN P08107), which can be expressed very quickly in emergency situations (Tavaria et al 1996; Wu et al 1985).

Based on the assumption that there may be a similar occurrence for the hsp60 gene, it was decided to use the sequence of the hsp60 mRNA as a template for constructing additional sequencing primers (Section 2.2.11.2). With these primers, it was possible to sequence the entire fragment (Table 3.11) between the PCR primer pair XLLEFT and XLRIGHT. The putative start and stop codons are marked in boxes, the putative coding

sequences are marked in boldface. The fragment was named hsp60s (s for short) analogously to the regular hsp60 gene, which is much longer. The GAN for hsp60s is AF380943 (Teske et al 2001).

The grade of similarity between the hsp60s gene and the human hsp60 gene mRNA was determined, using the Martinez/Needleman–Wunsch algorithm. The result showed a high similarity of 97.5 %, indicating that they are closely related. The hsp60s gene may therefore be a member of the Chaperonin (HSP60) gene family. The alignment is shown in Figures 3.9, 3.10, 3.11 and 3.12. The differences (substitutions) between the two genes are marked in boxes.

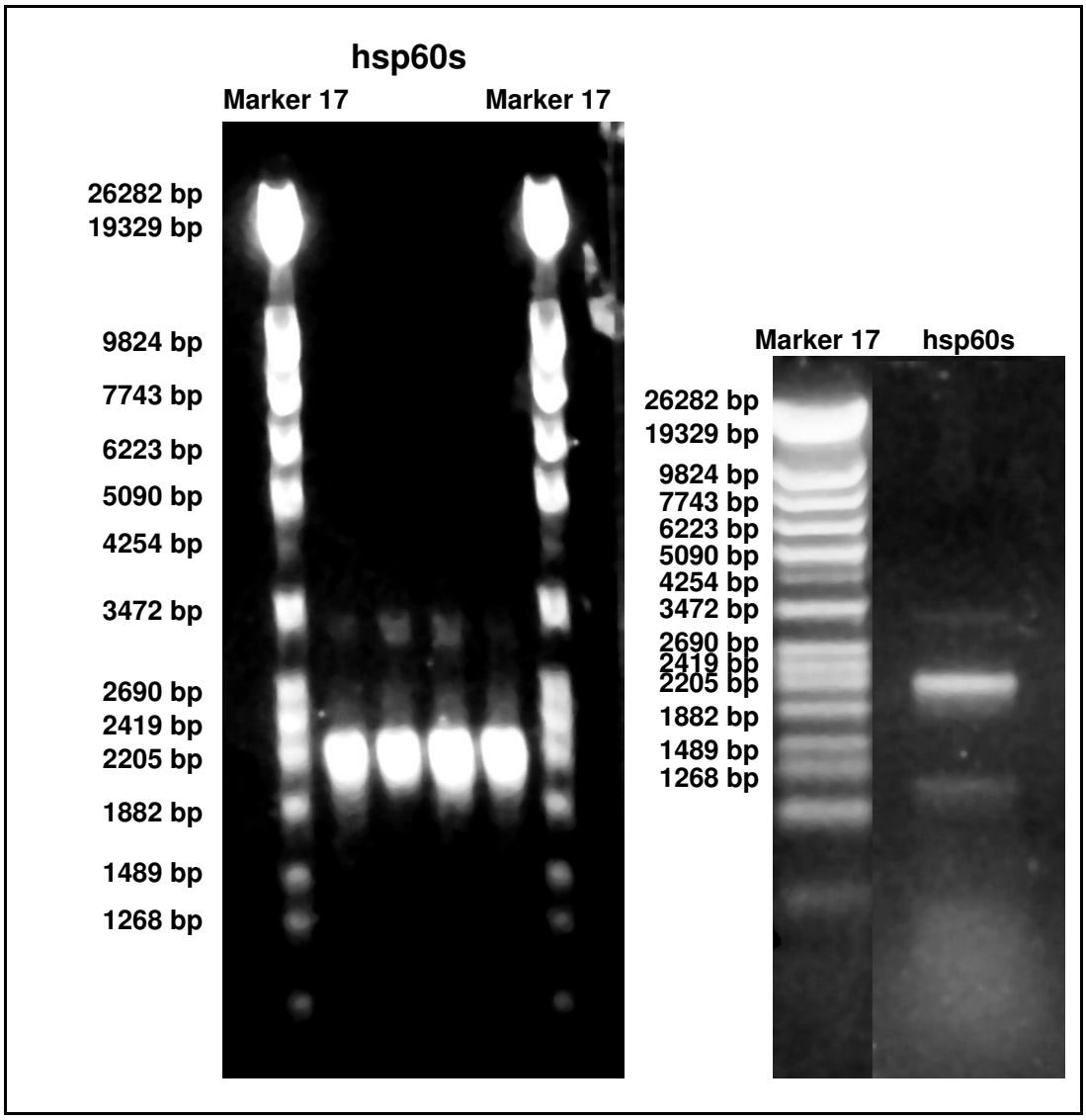


Figure 3.8: 0.4% Agarose gel: PCR product of hsp60s at 2205 bp of the Lambda 17 marker ladder

1	CCTGCAGCCG	CCCCACAGAA	*ATG* **CTTCGGT**	**TACCCACAGT**	**CTTTCGCCAG**	**ATGAGACCAG**
			putative Start Codon hsp60s1			
61	**TGTCCAGGGT**	**ACTGGCTCCT**	**CATCTCACTC**	**GAGCTTATGC**	**CAAAGATGTA**	**AAATTTGGTG**
121	**CAGATGCCCG**	**AGCCTTAATG**	**CTTCAAGGTG**	**TAGACCTTTT**	**AGCCGATGCT**	**GTGGCCGTTA**
181	**CAATGGAGCC**	**AAAGGGAAGA**	**ACAGTGATTA**	**TTGAGCAGAG**	**CTGGGGAAGT**	**CCCAACGTAA**
241	**CAAAAGATGG**	**TGTGACTGTT**	**GCAAAGTCAA**	**TTGACTTGAA**	**GGATAAATAT**	**AAAAACATTG**
301	**GAGCTAAACT**	**TGTTCAAGAT**	**GTTGCCAATA**	**ACACAAATGA**	**AGAATCTGGG**	**GATGGCACTA**
361	**CCACTGCTAC**	**TGTACTGGCA**	**GGCTCTATAG**	**CCAAGGAAGG**	**CTTCCAGAAG**	**ATTAGCAAAG**
421	**GTGCTAATCC**	**AGTGGAAATC**	**AGGAGAGGTG**	**TGATGTTAGC**	**TGTTGATGCT**	**GTAATTGCTG**
481	**AACTTAAAAA**	**GCAGTCTAAA**	**CCTGTGACCA**	**CCCCTGAAGA**	**AATTGCACAG**	**GTTGCTATGA**
541	**TTTCTGCAAA**	**TGGAGACAAA**	**GAAATTGGCA**	**ATATCATCTC**	**TGATGCAATG**	**AAAAAGGTTG**
601	**GAAGAAAGGG**	**TGTCATCACA**	**GTAAAGGATG**	**GAAAAACACT**	**GAATGATGAA**	**TTAGAAATTA**
661	**TTGAAGGCAT**	**GAAGTTTGAT**	*TGA* GGGTATA	TTTCTCCATA	CTTTATTAAT	ACATCAAAAG
			putative Stop Codon hsp60s1			
721	GTCAGAAATG	TGAATTCCAG	GATGCCTATG	TTCTGTTGAG	TGAAAAGAAA	ATTTCTAGTG
781	TCCAGTCCAT	TGTACCTGCT	CTTGAAATTG	CCAATGCTCA	CCATAAGCCT	TTGGTGATAA
841	TCGCTGAAGA	TGTTGATGGA	GAAGCTCTAA	GTACACTCAT	CTTGAATAGG	CTAAAGGTTG
901	GTCTTCAGGT	TGTGGCAGTC	AAGGCTCCAG	GGTTTGGTGA	CAATAGAAAG	AACCAACTTA
961	AAGAT *ATG* **GC**	**TATTGCTACT**	**GGTGGTGCAG**	**TGTTTGGAGA**	**AGAGGGGTTG**	**ACCCTGAATC**
		putative Start Codon hsp60s2				
1021	**TTGAAGATGT**	**TCAGCCTCAT**	**GACTTAGGAA**	**AAGTTGGAGA**	**GGTCATTGTG**	**ACCAAAGACG**
1081	**ATGCCATGCT**	**CTTAAAAGGA**	**AAAGGTGACA**	**AGGCTCAACT**	**TGAAAAACGT**	**ATTCAAGAAA**
1141	**TCATTGGGCA**	**GTTAGATGTC**	**ACAACTAGTG**	**AATATGAAAA**	**GGAAAAACTG**	**AATGAATGGC**
1201	**TGGCAAAACT**	**TTCAGATGGA**	**GTAGTTGTGC**	**TGAAGTTTGG**	**TGGGACAAGT**	**GAtGTTGAAG**
1261	**TGAATGAAAA**	**GAAAGACAGA**	**GTTACAGATG**	**CCCTTAATGC**	**TACAAGAGCT**	**GCTGTTGAAG**
1321	**GAGGCATTGT**	**TTTGGGAGGG**	**GGTTTTGCCC**	**TCCTTCGATG**	**CATTCCAGCC**	**TTGGACTCAT**
1381	**TGACTCCAGC**	**TAATGAAGAT**	**CAAAAAATTG**	**GTATGGAAAT**	**TGTTAAAAGA**	**ACACTCAAAA**
1441	**TTCCAGCAAT**	**GACCACTGCT**	**ACGAATGCAG**	**GTGTTGAAGG**	**ATCTTTGATA**	**GTTGAGAAAA**
1501	**TTATGCAAAA**	**TTCCTCAGAA**	**GTTGGTTATG**	**ATGCTATGGT**	**TGGAGATTTT**	**ATGAATATGG**
1561	**TAGAAAAAGG**	**AATTATTGAC**	**CCAACAAAGC**	**TTGTGAGAAC**	**TGCTTTATTG**	**GATGCTGCTG**
1621	**GTGTGGCCTC**	**TCTGTTAACT**	**ACAGCAGAAG**	**TTGTAGTCAC**	**AGAAATTCCT**	**AAAGAAGAGA**
1681	**AGGACCCTGG**	**AATGGGTGCA**	**ATGGGTGGAA**	**TGGGAGGTGG**	**TATGGGAGGT**	**GGCATGTTC** *T*
1741	*AA* CTCCTAGA	CTAGTGCTTT	ACCTTTATTA	ATGAACTGTG	ACAGGAAGCC	CAAGGCAGTG
		putative Stop Codon hsp60s2				
1801	TTCCTCACCA	ATAACTTCAG	AGAAGTCAGT	TGGAGAAAAT	GAAAAAAAGG	CTGGCTGAAA
1861	ATCACTATAA	CCATCAGTTA	CTGGTTTCAG	TTGACAAAAT	ATATAATGGT	TTACTGCTGT
1921	CATTGTCCAT	GCCTACAGAT	AATTTATTTT	GTATTTTTGA	ATAAAAAACA	TTTGTACATT
1981	CCTGATACTG	GGTACAAGAG	CCATGTACCA	ATGTACTGCT	TTCAACCTAA	ATCACTGAGG
2041	CATTTTTACT	ACTATTCTGT	TAAAATCAGG	ATTTTAGTAC	TTGCCACCAC	CAGATGAGAA
2101	GTTAAGCAGT	CTTTCTGTGG	AGAGTGAGAA	TAGTTGTGTA	CAAAGTAGAG	AAATATCCAA
2161	TTATGTGACA	ACCTTT				

boldface = putative coding sequences

Table 3.11: DNA Sequence of hsp60s; GAN AF380943 (Teske et al 2001)

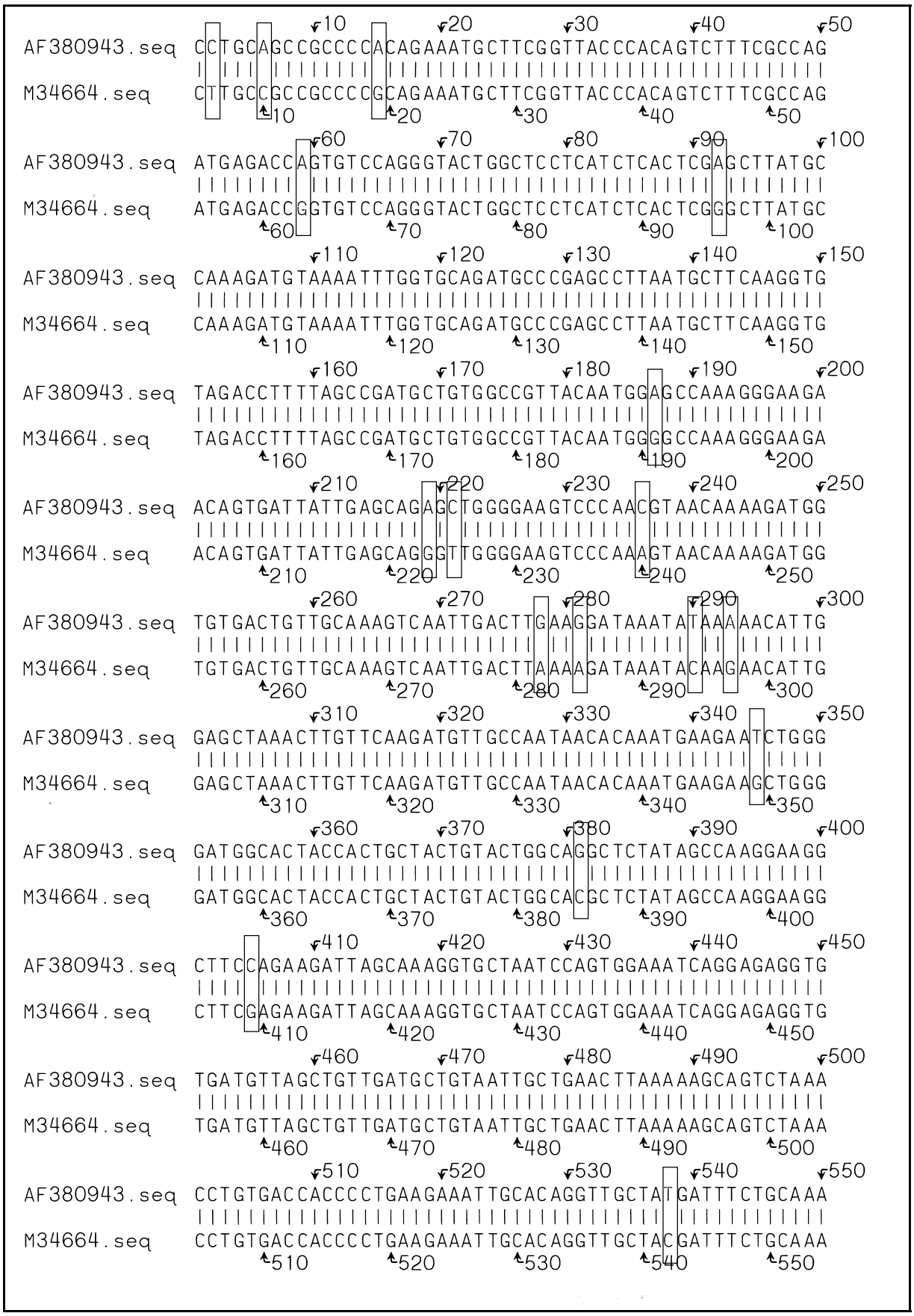


Figure 3.9: Alignment between human hsp60s DNA (AF380943) and human hsp60 mRNA (M34664), Bases AF380943 1–550

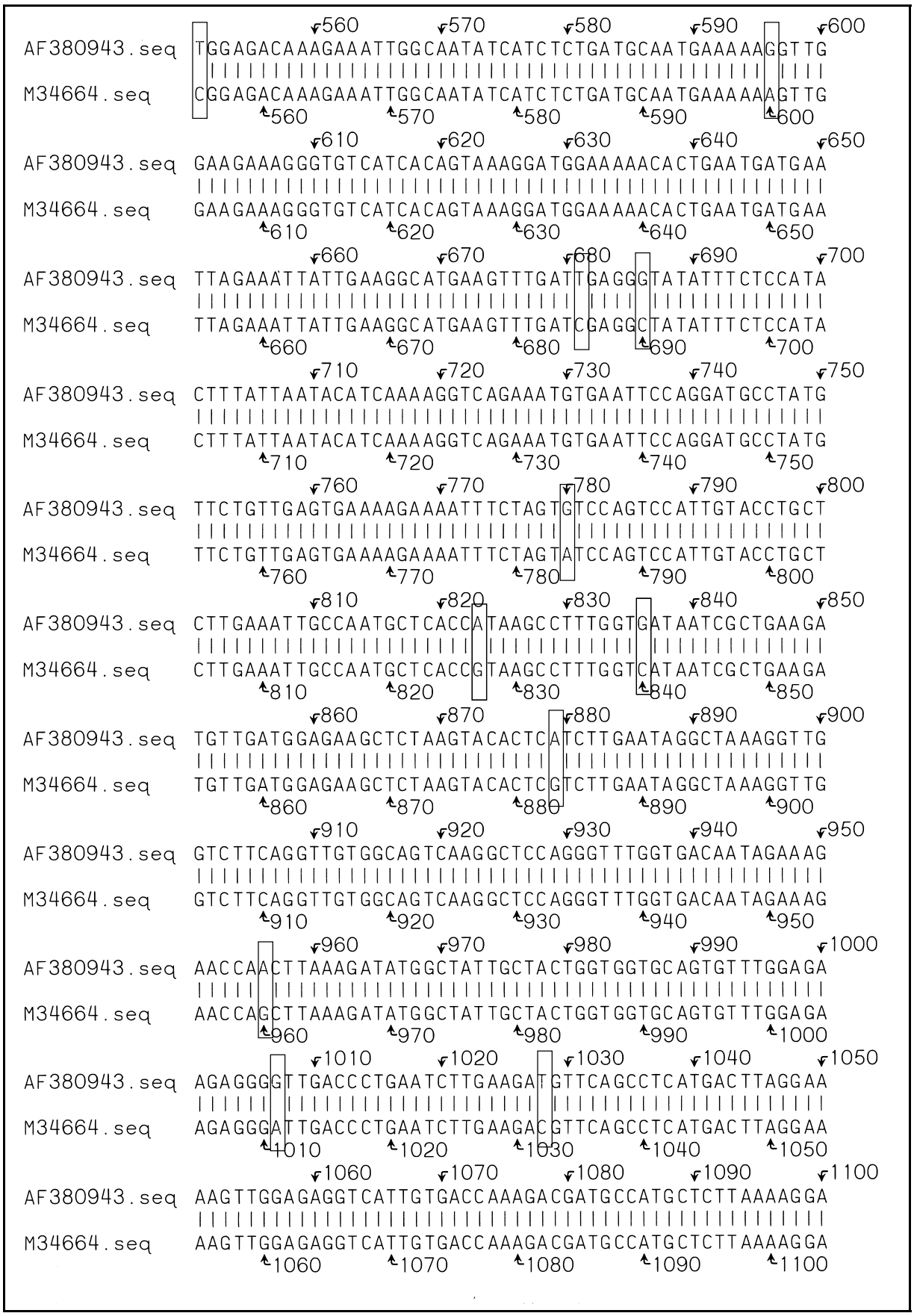


Figure 3.10: Alignment between human hsp60s DNA (AF380943) and human hsp60 mRNA (M34664), Bases AF380943 551–1100

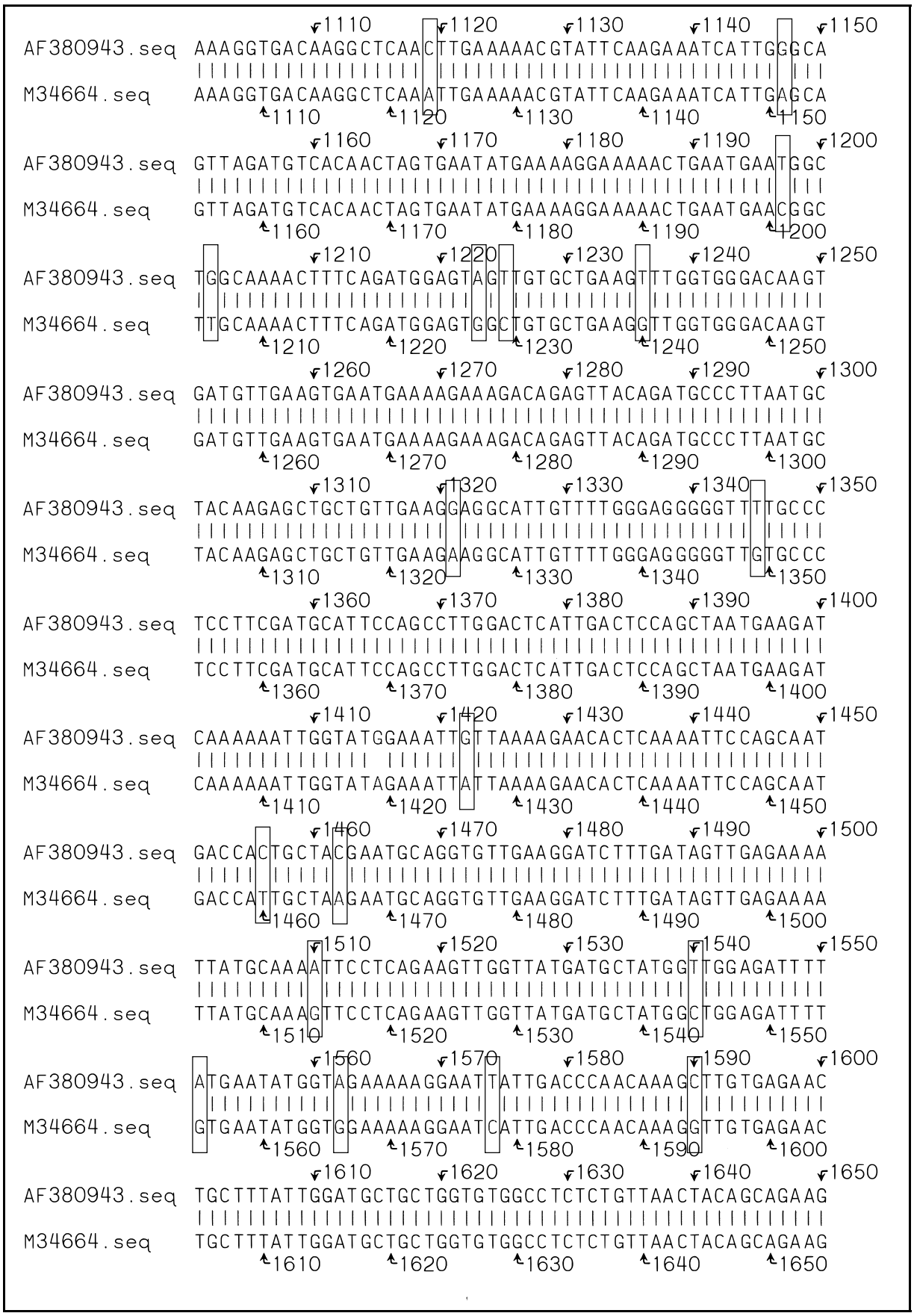


Figure 3.11: Alignment between human hsp60s DNA (AF380943) and human hsp60 mRNA (M34664), Bases AF380943 1101–1650

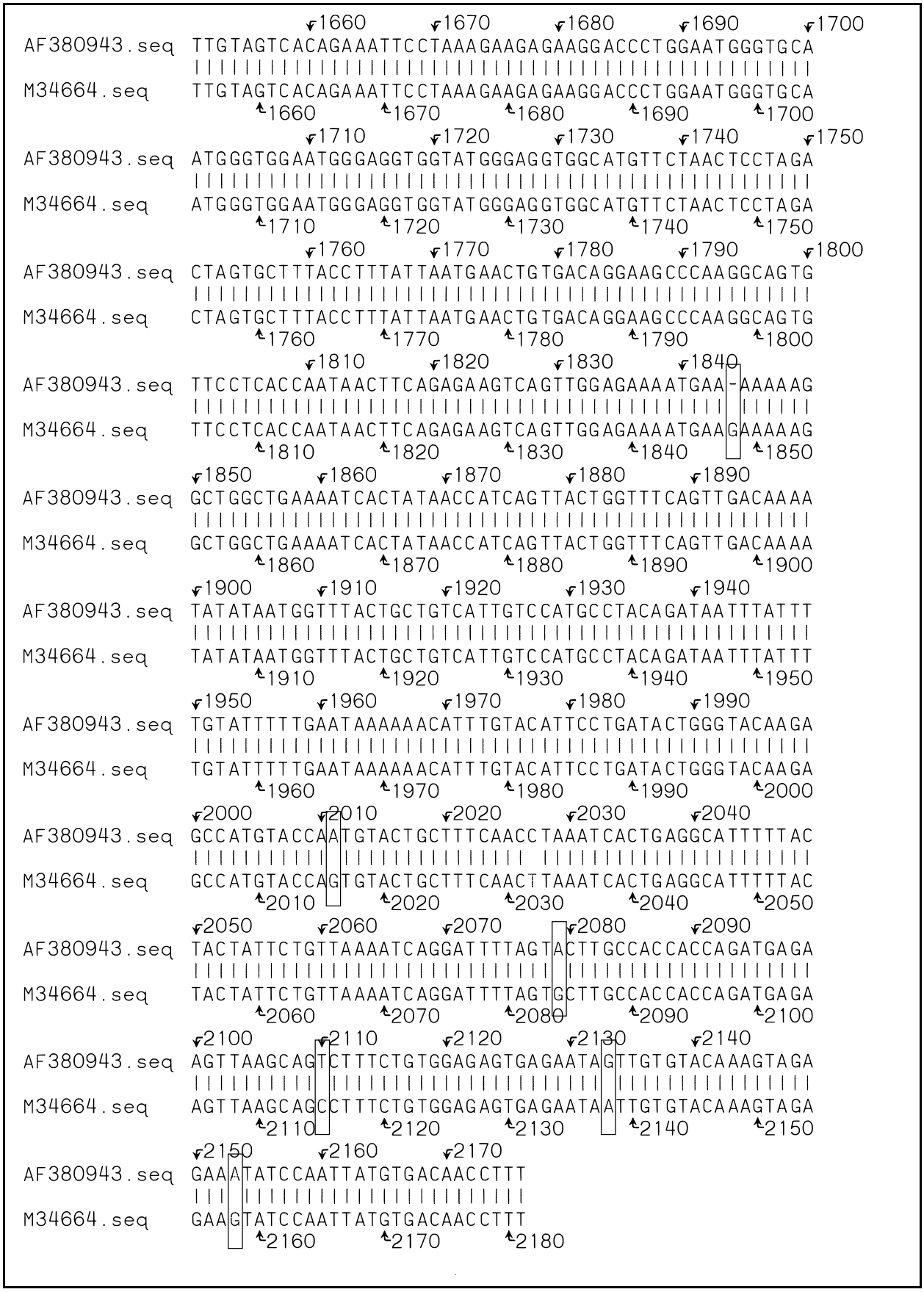


Figure 3.12: Alignment between human hsp60s DNA (AF380943) and human hsp60 mRNA (M34664), Bases AF380943 1651–2176

3.3.2 Protein Sequence of hsp60s

The first question to ask about any newly discovered DNA sequence concerns an open reading frame. If one exists of a sufficient size, the gene may actually encode for a protein. Using the EditSeq software with the standard genetic code, the hsp60s gene was translated into a protein sequence. Two open reading frames were found which are sufficiently long to encode for a protein. They were named Hsp60s1 (GAN AAK60260, TrEMBL Accession Number Q96RI4, Table 3.12) and Hsp60s2 (GAN AAK60261, TrEMBL Accession Number Q96RI3, Table3.13). Hsp60s1 contains 220 amino acids, base 21–683 in hsp60s, and Hsp60s2 contains 258 amino acids, base 966–1742 in hsp60s.

1	*MLRLPTVFRQ*	*MRPVSRVLAP*	*HLTRAY*AKDV	KFGADARALM	LQGVDLLADA	VAVTMEPKGR
	putative mitochondrial leader peptide					
61	TVIIEQSWGS	PNVTKDGVTV	AKSIDLKDKY	KNIGAKLVQD	VANNTNEESG	DGTTTATVLA
121	GSIAKEGFQK	ISKGANPVEI	RRGVMLAVDA	VIAELKKQSK	PVTTPEEIAQ	VAMISANGDK
181	EIGNIISDAM	KKVGRKGVIT	VKDGKTLNDE	LEIIEGMKFD		

boxes = putative mitochondrial leader peptide

Table 3.12: Putative protein sequence of Hsp60s1 (AAK60260), amino acids 1–220

1	MAIATGGAVF	GEEGLTLNLE	DVQPHDLGKV	GEVIVTKDDA	MLLKGKGDKA	QLEKRIQEII
61	GQLDVTTSEY	EKEKLNEWLA	KLSDGVVVLK	FGGTSDVEVN	EKKDRVTDAL	NATRAAVEGG
121	IVLGGGFALL	RCIPALDSLT	PANEDQKIGM	EIVKRTLKIP	AMTTATNAGV	EGSLIVEKIM
181	QNSSEVGYDA	MVGDFMNMVE	KGIIDPTKLV	RTALLDAAGV	ASLLTTAEVV	VTEIPKEEKD
241	PGMGAMGGMG	GGMGGGMF				

Table 3.13: Putative protein sequence of Hsp60s2 (AAK60261), amino acids 1–258

An analysis with the ProtParam tool obtained theoretical data on the chemical and physical parameters of Hsp60s1 and Hsp60s2. The molecular weight of Hsp60s1 is about 23 kD, Hsp60s2 weighs about 27 kD. The TrEMBL entries also contain information on theoretical molecular functions. Both proteins, Hsp60s1 and Hsp60s2, are categorized into the Chaperonin (HSP60) protein family, because of their similarity with other members. Theoretical functions of both proteins include ATP binding, heat shock protein activity and protein folding. Based on these findings, it was concluded that the hsp60s gene belongs to the Chaperonin (HSP60) family.

The grade of similarity between the Hsp60s proteins and the human Hsp60 protein was then determined, using the Lipman–Pearson algorithm. The protein translation of the EditSeq software was used, covering the entire hsp60s gene, to obtain an equivalent of the 573 amino acids of the Hsp60 protein (Figures 3.13 and 3.14). The similarity index between the Hsp60 protein and the two putative proteins of 95.5 % is high, confirming the suspected membership of hsp60s in the Chaperonin (HSP60) family. However, the stop codon at the end of hsp60s1 could also indicate a pseudogene nature of the hsp60s gene.

3.3.3 The mitochondrial leader peptide

The next step in protein analysis of unknown proteins is a search for characteristic sequences, indicating, for example, a cell compartment as destination or a putative structure. Both Hsp60s1 and Hsp60s2 were examined for such characteristics and one was found in Hsp60s1. The first part of the Hsp60s1 putative protein resembles the leader peptide for transport through the mitochondrial membrane.

Nearly all mitochondrial proteins are encoded in the nucleus and are synthesized in the cytoplasm. They are carrying a special signaling peptide sequence, the so–called leader peptide or transit peptide, at their amino terminal end. This short sequence marks the proteins for import into the mitochondria (Lewin 2000). For reference, the first 26 amino acids of Hsp60s1 are shown below their Hsp60 equivalent. Obviously, both leader peptides are identical (Table 3.14). Under the assumption, that hsp60s is an expressed gene, its protein product would thus be targeted to the mitochondrial matrix.

Hsp60	M(Met)	L(Leu)	R(Arg)	L(Leu)	P(Pro)	T(Thr)	V(Val)	F(Phe)	R(Arg)	Q(Gln)
Hsp60s1	M(Met)	L(Leu)	R(Arg)	L(Leu)	P(Pro)	T(Thr)	V(Val)	F(Phe)	R Arg)	Q(Gln)
Hsp60	M(Met)	R(Arg)	P(Pro)	V(Val)	S(Ser)	R(Arg)	V(Val)	L(Leu)	A(Ala)	P(Pro)
Hsp60s1	M(Met)	R(Arg)	P(Pro)	V(Val)	S(Ser)	R(Arg)	V(Val)	L(Leu)	A(Ala)	P(Pro)
Hsp60	H(His)	L(Leu)	T(Thr)	R(Arg)	A(Ala)	Y(Tyr)				
Hsp60s1	H(His)	L(Leu)	T(Thr)	R(Arg)	A(Ala)	Y(Tyr)				

Table 3.14: Mitochondrial leader peptide of Hsp60 and Hsp60s1

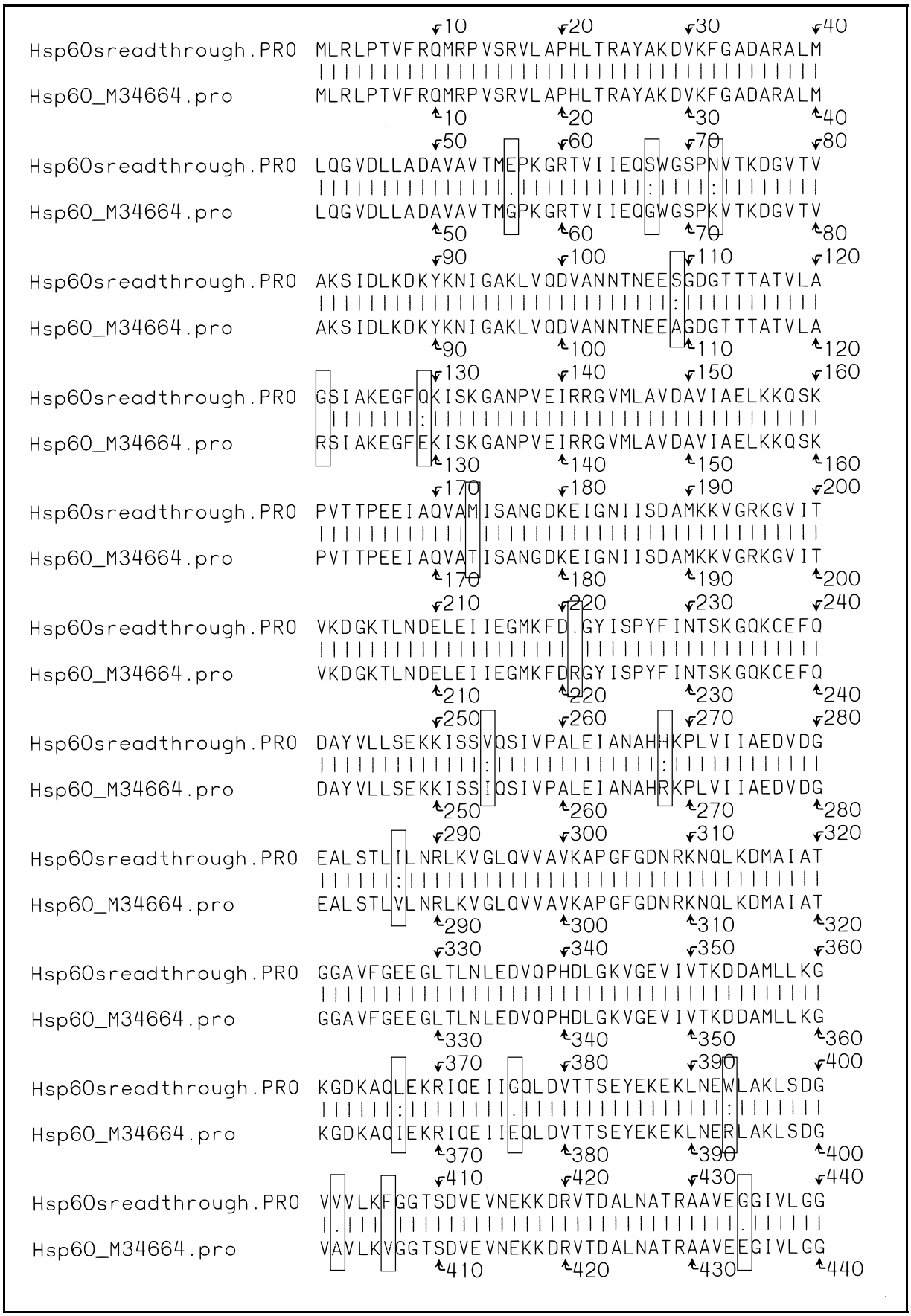


Figure 3.13: Alignment between human Hsp60s1/Hsp60s2 putative proteins and human Hsp60 protein; amino acids 1–440 translated from AF380943 with standard genetic code

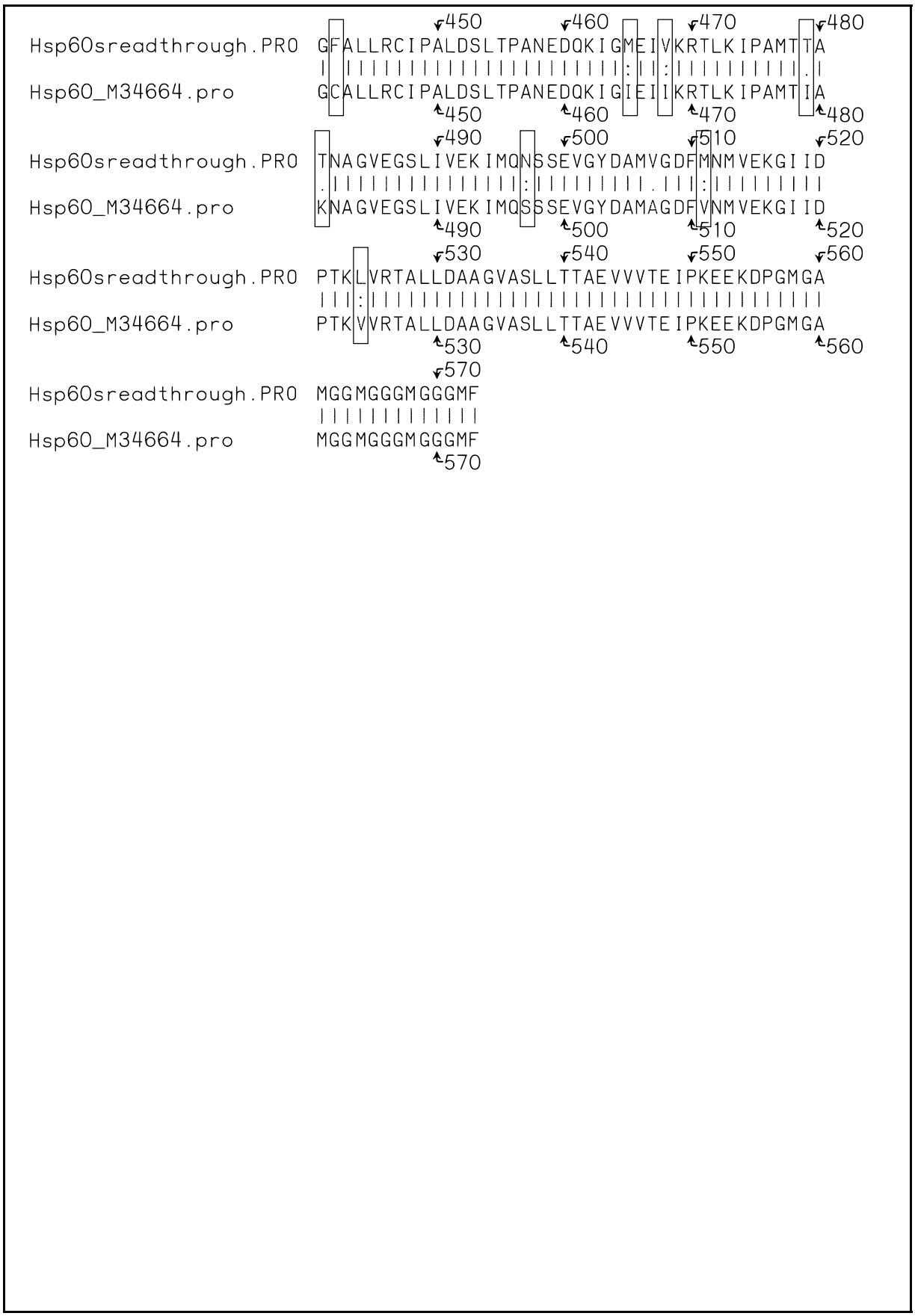


Figure 3.14: Alignment between human Hsp60s1/Hsp60s2 putative proteins and human Hsp60 protein; amino acids 441–573 translated from AF380943 with standard genetic code

3.3.4 Blast Search against the entire GenBank Database with AF380943 (hsp60s genomic sequence)

Due to these contradictory results regarding the nature of the hsp60s gene, it was not possible to decide, whether the hsp60s gene is an expressed member, or a pseudogene member (pseudogene of hsp60) of the HSP60 family. To obtain further information, a Blast search with the hsp60s sequence against the entire GenBank Database was conducted. Especially the genomic upstream region of the hsp60s gene was of great interest, to determine possible promoter sequences such as a heat shock element.

In Table 3.15 a selection of sequences with the highest similarities (scores) to the hsp60s gene is shown. The score is an assigned value of the GenBank alignment, referring to a match between two sequences: The higher the score, the better the alignment. Most of the hits in the Blast search are different clones of the hsp60 gene mRNA.

The highest similarity between a chromosomal location and the hsp60s gene existed for chromosome 5. Thus, it was assumed that the hsp60s gene is located on chromosome 5. For an analysis of the genomic surroundings of the hsp60s gene, the chromosome 5 clone CTD–2577N22 (GAN AC091873) was used. The base position of hsp60s on this clone is 28788–266613. Here, the reverse complementary sequence of the clone is employed, placing the hsp60s gene from bases 138098–140723. The open reading frame hsp60s1 is positioned from base 138118–138780, and the open reading frame 2 (hsp60s2) is positioned from base 139063–139839. The only difference between hsp60s and its equivalent sequence on chromosome 5 is a substitution of a *G* in position 2117 of hsp60s to an *A* on chromosome 5.

To gain further understanding of a putative pseudogene nature of the hsp60s gene, the sequences of the other hsp60–pseudogenes were examined. Five pseudogenes of the hsp60 gene are known, but more may exist in the human genome. The sequences on the chromosomes 12 (GAN AC097104), 4 (GAN AC109811), 3 (GAN AC097360) and 8 (GAN AC131269) may be pseudogenes.

GAN and Description	Similarity with AF380943 (%) and Score
Homo sapiens	
AC091873 Homo sapiens chromosome 5 clone CTD–2577N22	99 % (4264)
AC138940 Homo sapiens chromosome 5 clone RP11–704G19	99 % (4230)
BC002676 Homo sapiens heat shock 60kDa protein 1 (Chaperonin), mRNA, cDNA clone MGC:4084, complete cds	97% (3858)
NM_002156 Homo sapiens heat shock 60kDa protein 1 (Chaperonin) (HSPD1), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA	97 % (3858)
M22382 HUMPMMPP1 Human mitochondrial matrix protein P1 (nuclear encoded) mRNA, complete cds	97 % (3850)
NM_199440 Homo sapiens heat shock 60kDa protein 1 (Chaperonin) (HSPD1), nuclear gene encoding mitochondrial protein, transcript variant 2, mRNA	97 % (3846)
BC067082 Homo sapiens heat shock 60kDa protein 1 (Chaperonin), transcript variant 1, mRNA (cDNA clone MGC:71259), complete cds	97 % (3842)
M34664 HUMHSP60A Human chaperonin (HSP60) mRNA, complete cds	97 % (3842)
BC003030 Homo sapiens heat shock 60kDa protein 1 (Chaperonin), mRNA (cDNA clone MGC:4335), complete cds	97 % (3834)
BC047350 Homo sapiens heat shock 60kDa protein 1 (Chaperonin), mRNA (cDNA clone IMAGE:4214709)	97 % (3715)
AC097104 Homo sapiens (chr.) 12 BAC RP11–153M3 (Roswell Park Cancer Institute Human BAC Library) complete sequence	95 % (3491)
AC109811 Homo sapiens BAC clone RP13–539F13 (chr.) 4, complete sequence	93 % (3118)
AC097360 Homo sapiens chromosome 3 clone RP11–344C13, complete sequence	92 % (2991)
AC131269 Homo sapiens chromosome 8, clone RP11–416I21, complete sequence	91 % (2179)
Homo sapiens, known pseudogenes	
NG_001148 Homo sapiens heat shock 60kDa protein 1 (chaperonin) pseudogene 4(HSPDP4), also: M34663 HUMHP604A	89 % (2016)
NG_001146 Homo sapiens heat shock 60kDa protein 1 (chaperonin) pseudogene 2 (HSPDP2); also: M34662 HUMHP602A	88 % (1532)
NG_001147 Homo sapiens heat shock 60kDa protein 1 (chaperonin) pseudogene 3 (HSPDP3) on chromosome 8; also: M34661 HUMHP603A	90 % (1445)
NG_000920 Homo sapiens heat shock 60kDa protein 1 (chaperonin) pseudogene 7 (HSPDP7) on chromosome 21	90 % (1257)
NG_001145 Homo sapiens heat shock 60kDa protein 1 (chaperonin) pseudogene 1 (HSPDP1); also: M34660 HUMHP601A	90 % (1216)
Animals	
M22383.1 CRUP1P Chinese hamster P1 protein mRNA, complete cds	89 % (2399)
NM_022229 Rattus norvegicus heat shock protein 60 (liver) (Hsp60), mRNA	89 % (2137)
BC016400 Mus musculus heat shock protein 1 (Chaperonin), mRNA (cDNA clone MGC:6709), complete cds	89 % (2135)
BS000169 Pan troglodytes chromosome 22 clone: RP43–042E01, map 22, complete sequences	90 % (1279)

Table 3.15: Results of the Blast search; searching the GenBank database with hsp60s (AF380943)

Based on the results of the Blast search, a genealogical tree of the hsp60s gene was constructed (Figure 3.15). It traces the different relationships between the hsp60s gene, the hsp60 gene mRNA and the pseudogenes of hsp60. The similarity indices between the genes were obtained with the Martinez/Needleman–Wunsch algorithm (Table 3.16). The phylogenetic tree was created using the Jotun–Hein Method (see Section 2.2.12.4). The closest relative to the hsp60s gene is the hsp60 mRNA, while the pseudogenes of hsp60 show fewer similarities.

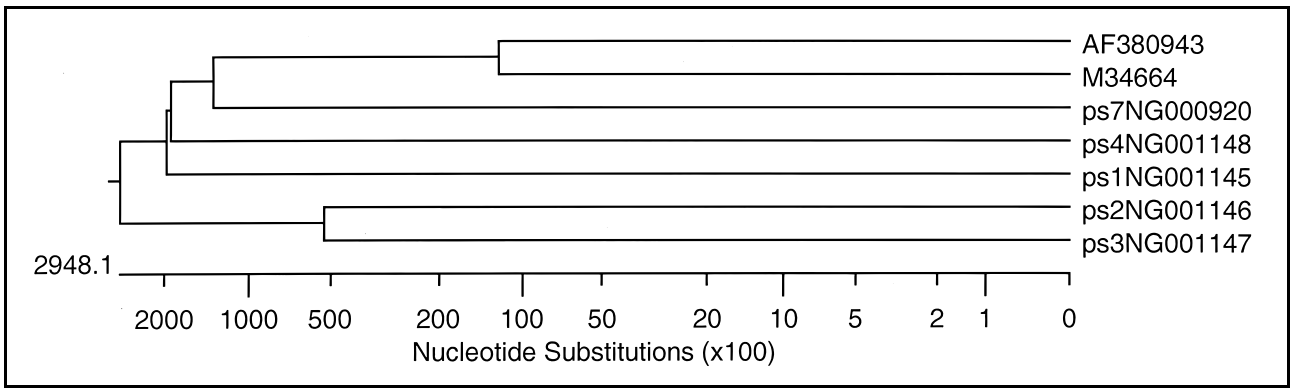


Figure 3.15: Phylogenetic Tree of the human hsp60s, hsp60 and hsp60–pseudogenes using the Jotun–Hein–Method

Sequence	Length	Similarity to hsp60s	Consensus Length
pseudogene 1 (NG001145)	1868 bp	87.4 %; AF380943: 1–1831, NG001145: 67–1868	1842 bp
pseudogene 2 (NG001146)	1875 bp	85.0 %; AF380943: 1–1841, NG001146: 99–1875	1853 bp
pseudogene 3 (NG001147)	1879 bp	88.8 %; AF380943: 1–1817, NG001147: 91–1879	1822 bp
pseudogene 4 (NG001148)	1950 bp	87.3 %; AF380943: 1–1852, NG001148: 91–1950	1887 bp
pseudogene 7 (NG000920)	2549 bp	72.4 %; AF380943: 1–2176, NG000920: 165–2549	2511 bp
hsp60 mRNA (M34664)	2202 bp	97.5 %; AF380943: 1–2176, M34664: 5–2181	2177 bp

Table 3.16: Length and Similarity of the human hsp60s, hsp60 and pseudogenes of hsp60

The hsp60s gene is related more closely to the hsp60 gene mRNA than to any hsp60 pseudogene. However, even this additional data cannot ascertain the true nature of the hsp60s gene.

3.3.5 Analysis of the hsp60s gene and flanking sequences

With the Blast Search, see last Section, it was possible to identify the human chromosome of the hsp60s gene: Chromosome 5. The clone CTD–2577N22 was used for analysing the upstream region of the hsp60s gene, looking for putative promoter elements. Assuming the possibility of the hsp60s gene being the ”emergency” (stress inducible) variant of hsp60, it was tried to identify promoter regions in the hsp60s gene similar to those in the hsp60 gene. Especially it was searched for the characteristic promoter element of heat shock genes (heat shock element, see also Section 1.4).

The promoter sequence of the hsp60 gene is shown in Table 3.17. Its heat shock element is located in the bases 3408–3428.

5′T–TTC–T3′	5′G–GAA–A3′	5′G–TTC–T 3′	5′G–GAA–CC3′

Two protein binding sequences, otherwise known as GC–boxes or SP1–elements, are located in bases

3504–3510 GGGCGGG and 3591–3597 GGGCGGG.

The heat shock element is located 1238 bases upstream of the ATG and 991 bases upstream of the beginning of the hsp60 mRNA. Using the EditSeq, Transplorer and Match programs, a search for putative promoter sequences was conducted on the hsp60s gene. Eukaryotic promoter sequences can be composed of several modules spanning more than one thousand bases. Thus, 1500 bases upstream of the hsp60s gene's ORF1 start codon were examined (see Table 3.18). A putative heat shock element was found, located in the bases 137140–137159.

5′G–GAA–T3′	5′T–GAA–C3′	5′T–TTC–T3′	5′A–GAA–A3′

The putative heat shock element is located 978 bases upstream of the ATG from Hsp60s1. No GC–boxes similar to the ones in the hsp60 gene were found. But just upstream the ORF 1 start codon, a highly GC–rich region (bases 138065–72, 138084–93, 138104–110) exists, which may include possible GC–boxes.

The Match Software was used to test the promoter regions for the binding of transcription factors. The Transfac database contains a great number of transcription factors, but is not complete. It may be possible, that more than the following transcription factors bind. Again, the hsp60 gene was used as a template for identifying similar regions in the hsp60s gene. The only transcription factor in the Transfac Database binding the heat shock element of the hsp60 gene is the heat shock transcription factor 1 (HSF1) of Saccharomyces cerevisiae, SPAN P10961. For hsp60s, the program produced the same hit as for hsp60, heat shock transcription factor 1 (HSF1) of Saccharomyces cerevisiae. Additionally, the human heat shock transcription factor 1 (HSF1) was found, SPAN Q00613. The latter is the typical eukaryotic DNA–binding protein that specifically binds heat shock promoter elements (HSE) and activates transcription. The putative heat shock element and binding site for the typical eukaryotic transcription factor point to an expression of the hsp60s gene.

3001	CGTACGCCTC	AGACAGGCCC	GGGCCTGCAG	GCCTCGGGGC	CACGCGGGAC	TCAC***CATTAC*** *T3057: start p10cds*
3061	**TCCCTCCGCC**	**TCAGACTCGT**	**ACTCTGCTCT**	**AGTGTAGC***GC* *C3098: start p10mRNA*	CGCAAAGAGA *G3099: start pro p60*	CTCGCAGTCC
3121	GGCCCTGGCG	ACACGTGAAA	AAATCACCCA	GCGCGCAGGC	GCGCTCGCCC	CAGCGGTCGC
3181	CGCTAGCCCC	CTCGCCCCTC	CCAGGGAGGG	AGGGCGGTCG	CCCCGACGCG	CACCGCGATT
3241	CGCCCCAAGG	GCCCTGCGCA	GGACGCTGAC	GCGAAGACTC	GGAGGCGGAA	GAAAAAAGGA
3301	GCTGTTTCTA	GGCTTTTCTA	GGCGCCCAGC	CGAGGTGAAA	GAACTACCCC	TTCGTCCCCT
3361	CCCGGAAATG	ACGCGATTTG	ACCCTTGAGC	CGTAGGGAGC	GCGGCAT *TTT*	*CTGGAAAGTT* *3408–3428 heat shock element*
3421	*CTGGAACC* GA	GCGAGGCCCG	GGAACTAGAC	TAAGCCGGCC	GGAGAGGGCT	GAGCGCGCTA
3481	GCACACCCTG	CGCGGGTAGG *3504–3510*	GAG *GGGCGGG* *proteinbind seq*	GCTCGCGCGC	AGGGTGTGCA	GATTGCAGGG
3541	CCCGGGCTGA	CGGGAAGTGG	GTGGGAGCTG	CCTGCACACG	CGGTGCCGCG *3591–3597*	*GGGCGGG* AGT *proteinbind seq*
3601	AGAGGCGGAG	GGAGGGGACA	CGGGCTCATT	GCGGTGTGCG	CCCTGCACTC *C3654 start p10gene; C3655 p60gene/mRNA*	TGTC***CCTCAC***
3661	**TCGCCGCCGA**	**CGACCTGTCT**	**CGCCGAGCGC**	**ACGCCTTGCC**	**GCCGCCCCGC**	**AG**GTACGCGG
3721	CCTTCCCGCA	CCACCTCCCC	CCGGCTCCTG	CGACCCCCGA	GTGGGCCTTCA	CTCCTCTGCG
3781	GTCCAGCTGG	AGGGTGCGGG	CGGCGACAAC	CTGGAGTTCC	GCACCGCGCC	GCACCGCAGG
3841	CCCAGGCCTG	CGGGTCCAGG	CCTCGCGGGT	CCATGCGGGT	TCCATCACGT	GCGACGCGGA
3901	TCCTGGACCT	CGGCTGGGCG	CGGGAGGGAC	AACTCGTGGG	GTTTGAATTT	GGGGGATTGT
3961	AACCACTTAG	GCCCGCTGGT	GCAACCTGCG	CCTGGCCGAC	TTCAGGGAAC	CGGGGAGGGT
4021	GGTGTACGTG	AGGGAACCGC	TTTTTTCCCC	CGCAGGCAGC	TGGGATACCG	CGCCTCCAGT
4081	CTTGACCTTG	CTGGGGGCGT T	CGGAAAGAA	GGACACGTGG	GCTCGCGCCG	AGTGGGCCGG
4141	GCCGCCGCCC	CCAGCATCGC	GGCCTTGCTG	GTTGCTCTGA	TACAGTAGCC	TTGGGTGGAT
4201	CCATGGAGAC	ACTTTGCTGA	GCGGCTATTT	GCACCGTCAG	GAATACTTTT	AACTTCCCTG
4261	GTCACTTGGA	TTTGTAACTT	GCCTCTCTCC	GGCTGACTTG	GTTTCTCAGG	GGCCACAGTT
4321	GGAGTGGGTC	TGCCCACAAG	GCCGTTTTCG	CGGTGAAAGA	TTAACTCGTT	TTTGGCGGCA
4381	GGGATAGGTG	GCCGTGGGGA	AGGAGCCGCA	TGTGCTTTGT	GCTGGTGCTA	TAGGTCAGGC
4441	GCCGGCCGGG	GCGGCCTCTT	TAGCTTGCGC	TAGTGGCTGA	TGTTCCGAAG	CTGCGGCGCC
4501	TTCCGTCATT	CAGGCGTAGT	GGCTGGCGGA	GGTCAGCCAG	GCTCAGCATC	TCACGTTGCT
4561	TGCTCTGTTG	CACAGGTGGG	CATGTTTGTT	CTGCAGCGCA	CCACGGGTGG	TAGTTCTGAT
4621	GATCTTTTTG	CTTCCATCCC *4646–4648*	CAG***AA ATG*CT** *Start (Begin CDS)*	**TCGGTTACCC**	**ACAGTCTTTC**	**GCCAGATGAG**
4681	**ACCGGTGTCC**	**AGGGTACTGG**	**CTCCTCATCT**	**CACTCGGGCT**	**TATGCCAAAG**	**ATGTAAAATT**
4741	**TGGTGCAGAT**	**GCCCGAGCCT**	**TAATGCTTCA**	**AGGTGTAGAC**	**CTTTTAGCCG**	**ATGCTGTGGC**
4801	**CGTTACAATG**	**GGGCCAAAG**G	TACCAGTATT	TTTATTTTAT	TGTAATTACG	GTATTATTAA

boxes = promoter elements; boldface = exons; Startcodon = ATG in exon 2 bases 4646–4648

Table 3.17: Promoter region of the human hsp60 gene; Bases 3099–3654 (AF250915)

136321	CAGTGCACCC	CAGCTCTCAG	TATTTTTGAT	CGTGAGAGAG	AAAGTTTCAT	GGAGTCAGGG
136381	TTTATCAATT	TCTTGTTCTG	AAGAGTGGGA	TTTTGCACTC	AAGTGCAGAG	TTGAATCATC
136441	CATCATTTCC	AGAGTACAAA	AACATAGGAA	ACTTCACCTG	TGTTTCTAGT	GACTGTCTCT
136501	AAAGTTATAT	ATTTAATGCC	AGGGCACTTC	TTTAGCTACC	CCTCGGGGAG	AATAATAAAA
136561	TCTTAGTTTT	TTATTTATAA	GCCATCTATT	AAGTGACCAC	TATTTTAGCA	GCTCACACTG
136621	CACTATGCAC	AGAGAATATA	AGGTATAAAA	ATGAATGAAA	AAGAAAAATG	AAAAAAAACT
136681	GCTGAAATGT	AGTATTCTAA	AAACTTTATT	TTATATGATT	TCTTTTGGGA	AACTCAGAAA
136741	ATACTTCTCA	TTTGCAACTC	TCTCTTCTTT	CCTAAAACAG	GAAAGGAACT	TAACAATATT
136801	TATGGAGCTA	TTACAATGTG	TCAAACATTG	TGCAAAACAT	TTTATATGCA	TTCTTATATT
136861	TAATGCTTAA	ACCCATCCTA	TTTTCTATTA	TTTGCACCAT	TTCTATAAAT	GCAAAACTAA
136921	CATCTAGATG	ATGACACGGG	TTGTCTGGAG	TCTTGACTTT	TAGTTAAGTG	GTGGAGCTGG
136981	GGCAAACTCA	AACAGTTGAC	ACCAAAGCCA	TTGCTCACAG	CCACTACCCT	AGTCCACCTC
137041	CAGGGAACAG	AGAAGTTCTT	CATAAAATAA	CTAAGCTGTT	TTATTTTGTT	TGTTTGGACA
137101	TTCTTGGCAC	AACATATTTT	TTCCATTTTT	TTTAAATTT *G*	*GAATTGAACT*	*TTCTAGAAA* T
				137140–137159 putative heat shock element		
137161	ACACTACTCA	TTTTTGTTTA	CTTAAGTAAA	CAAATAGTCT	ATTTGGTTAA	ACTTCGTATC
137221	ATTTTTATAT	ATTAGTTTAT	CTGATACAAA	ATGGTTACTA	ATGGATCTGC	ATATATAATT
137281	TCAAATTACC	TGAATACTTA	AAAAGTATGC	AAGGAATTTT	CCACAGTAGC	TACACTTGCC
137341	TAAGATCAGA	TATGCATAAT	ATGTTTTGAA	TCATGGATCT	TTTTTCTTTT	GTTCTTATTA
137401	ATATCATTTA	AAAATCTAGC	ACCATTGTAA	TTCACATGTT	GATACTCTGC	AAAAATGAGA
137461	AGAAATCAGT	CTAATATCCA	GGCTGTGAAT	GTAGATGCAT	TTTGATAGTT	TATGATGTAT
137521	TGATTGGAAA	TTCAAGATAA	ATAATAAGCG	TTTTACTAAA	ATGAAAACTA	GATTACAAAG
137581	CAATATTTGT	AAAGATATAT	CAAACAATAT	TGCCAGTATT	TAATTAGAGC	CAATGTTCAG
137641	GTTTCAGTAA	TACCATTTTA	GCTTCAGACG	GTTATTGTCA	CTGGATTTCT	TTGTTATGCC
137701	ATTATAAATT	AAGCAATGAT	GCATCATGAT	GATTCTTCCA	TTACTGGAAG	TCACATTTTA
137761	TCTGAATTTA	TTCTCATGTC	CAAATGTTTG	TGACGTATAT	TTAATTTGCT	TATTGCCACA
137821	TGCACAGCCA	CACACAGAGG	ATGACGTAAA	GCAATCATAG	AAATTAACAA	AAAGACGTAT
137881	TTTTATTACT	TTATCAGTAT	TTCAAATTGA	TTTTTTAATC	AGAAAAAAAC	AGGTTATTTG
137941	AACCAGACAT	AGATTGGCTG	TATTGCTCTC	ATAATTTGGC	AGATGTCTGT	ATGTTCCATT
138001	TATAGTACAA	CTGCCACATT	TATTTGCTTT	TTAAAAGTGA	TATATTAGCA	CTCTGTCCCT
			138098	*Begin hsp60s*	*138118–20 Startcodon*	*hsp60s ORF 1*
138061	CACT *CGCCGC*	*CG* ACAACCTG	TCT *CGCCGCG*	*CGC* ATGCCCT	GCA *GCCGCCC*	CACAGAA***ATG***
	138065–72, 138084–93, 138104–110: GC–rich region, possible GC–boxes					
138121	**CTTCGGTTAC**	**CCACAGT CTT**	**TCGCCAGATG**	**AGACCAGTGT**	**CCAGGGTACT**	**GGCTCCTCAT**
138181	**CTCACTCGAG**	**CTTATGCCAA**	**AGATGTAAAA**	**TTTGGTGCAG**	**ATGCCCGAGC**	**CTTAATGCTT**

boxes = putative promoter elements; boldface = putative coding sequence; Startcodon = ATG bases 138118–138120

Table 3.18: Putative Promoter sequences of the human hsp60s gene on chromosome 5 (AC091873)

3.3.6 Test of expression

Based on the results of the alignments and the computational promoter analysis, the possibility of the hsp60s gene being expressed exists. RT–PCR primer were constructed for various alternatives deemed likely (shown below) of a putative hsp60s mRNA. To construct primer specific to hsp60s, the hsp60s and hsp60 genes were aligned. Due to the high sequence similarity, in some cases the suitable primer regions differed only by one nucleotide. The primer and other reaction parameters are shown in Section 2.2.9.1.

Because of the ubiquitous nature of stress and heat shock proteins, they are expressed in almost every tissue (see Section 1.2). Blood was used for the isolation of the messenger RNA (mRNA) for the RT–PCR, because it is easily obtained and can be processed quickly after withdrawal. Messenger RNA is generally short lived compared to DNA. Its half life ranges from seconds up to several minutes (depending on the nature of the gene, e.g., the mRNAs of house–keeping genes show a longer stability). The blood was obtained from one of the test persons whose hsp60s genomic DNA was previously sequenced. The isolation protocol is shown in Section 2.2.3. A number of possible alternatives for the nature of a potential mRNA of hsp60s were considered:

Alternative 1: One mRNA exists, including both ORFs, ORF1 and ORF2. The mRNA starts between the putative HSE (137140–137159) and the start codon of hsp60s1 (138118–20) and ends downstream of the second stopcodon (between 139839–140723). This would necessitate a readthrough of the TGA stopcodon of ORF1.

Alternative 2: One mRNA exists, including both ORFs, ORF1 and ORF2. The mRNA starts at the start codon of hsp60s1 (138118) and ends downstream of the second stopcodon (between 139839–140723). This would necessitate a readthrough of the TGA stopcodon of ORF1.

Alternative 3a/3b: Two mRNAs exist, one for ORF1 and one for ORF2. The mRNA for ORF1 (3a) starts between the putative HSE (137140–137159) and the start codon of hsp60s1 (138118) and ends downstream of the ORF1 stopcodon (between 138780–139063). The mRNA for ORF2 (3b) starts at the start codon of hsp60s2 (139063) and ends downstream of the ORF2 stopcodon (between 139839–140723).

Alternative 4a/4b: Two mRNAs exist, one for ORF1 and one for ORF2. The mRNA for ORF1 (4a) starts at the start codon of hsp60s1(138118) and ends downstream of the ORF1 stopcodon (between 138780–139063). 4b is the same as 3b.

Table 3.19 shows a graphical view of the different mRNA alternatives that were considered. The human chromosome 5 clone CTD–25777N22 is shown as the reverse complementary sequence, because the original clone found in the Blast search was the minus strand. All positions are referring to the reverse complementary sequence. The specific primers for each of the alternatives are shown in Table 3.20. The detailed description is shown in Section 2.2.9.1. The primers are chosen to be hsp60s–specific and are set inside the putative mRNAs. Each forward primer was paired with each reverse primer. The primer combinations and programs are described in detail in Appendix A.3. For all forward and reverse primer combinations associated with an alternative, a RT–PCR was

performed. The results were verified on agarose gels (1% gel NuSieve GTG agarose/ TBE–Buffer).

5′	Chromosome 5 clone CTD–2577N22 (AC091873), rev. complementary sequence: Bases 137041–140400								3′
	Chr. 5		hsp60s (AF380943, 1–2176): Bases 138098–140723					Chr. 5	
	putative HSE 137140–137159	Chr. 5	Begin hsp60s 138098	ATG TGA ORF1 138118–138780	hsp60s	ATG TAA ORF2 139063–139839	End hsp60s 140723		
		mRNA alternative 1							
			mRNA alternative 2						
		mRNA alternative 3a			mRNA alternative 3b				
			mRNA alternative 4a		mRNA alternative 4b (3b)				

Table 3.19: Putative RNA alternatives of the human hsp60s gene

None of the alternatives tested lead to a positive match: Thus, It can be stated with certainty, that the hsp60s gene is not expressed in unstressed blood. However, the possibility remains that the gene is expressed in other tissues. Furthermore, it may be possible, that the hsp60s gene is only expressed under stress conditions. For example, the inducible form of the human hsp70 gene can only be detected under stress. The blood used in the experiment was obtained under non–stressed conditions. An attempt to procure blood from heat–stressed patients (children with high fever) for an appropriate analysis was initiated, but ultimately failed due to lack of support by MHH pediatric clinic.

5′	Chromosome 5 clone CTD–2577N22 (AC091873), rev. complementary sequence: Bases 137041–140400								3′
	Chr. 5		hsp60s (AF380943, 1–2176): Bases 138098–140723					Chr. 5	
	putative HSE 137140–137159	Chr. 5	Begin hsp60s 138098	ATG TGA ORF 1 138118–138780	hsp60s	ATG TAA ORF2 139063–139839	End hsp60s 140723		
			mRNA alternative 1 53Start1v1 ⟹			⟸ 53Stop2v1 ⟸ 53Stop2v2 ⟸ 3Stop2v3			
				mRNA alternative 2 3Start1v3 ⟹ 5Start1v4 ⟹ 53Start1v5 ⟹		⟸ 53Stop2v1 ⟸ 53Stop2v2 ⟸ 3Stop2v3			
		mRNA alternative 3a 53Start1v1 ⟹ ⟸ 5Stop1v1 ⟸ 3Stop1v2 ⟸ 5Stop1v3			mRNA alternative 3b 3Start2v1 ⟹ 53Start2v2 ⟹ 3Start2v3 ⟹ 5Start2v4 ⟹ ⟸ 53Stop2v1 ⟸ 53Stop2v2 ⟸ 3Stop2v3				
			mRNA alternative 4a 3Start1v3 ⟹ 5Start1v4 ⟹ 53Start1v5 ⟹ ⟸ 5Stop1v1 ⟸ 3Stop1v2 ⟸ 5Stop1v3		mRNA alternative 4b (3b)				

Table 3.20: RT–PCR Primer for the different putative RNA alternatives of the human hsp60s gene

3.4 Direct sequencing of PCR products obtained from DNA of SIDS victims

For forensic and research applications, it is often necessary to amplify and sequence DNA obtained from corpses. Here, the degradation of the DNA is an everpresent problem, limiting the length of PCR fragments and the quality of a sequencing reaction. In this thesis, PCR was performed on genomic DNA isolated from victims of Sudden Infant Death. The blood samples themselves were obtained during the autopsies. In times before the

widespread use of DNA analysis, such blood samples were taken and simply frozen for successive toxicological analyses. Some of these samples were only cooled in a refrigerator for a few months before actually being frozen. In some of the SIDS cases, the genomic and mitochondrial DNA had to be isolated from 15 year old frozen blood samples. The newest samples were less than one day old (the DNA was isolated immediately after taking the sample during the autopsy). These DNAs were then used for the PCR reaction (see Section 2.2.8) with a direct sequencing approach. The success of the PCR reactions was verified on agarose gels before the sequencing reaction was carried out.

The PCRs were performed with the GeneAmp XLPCR Kit and the Qiagen ProofStart DNA Polymerase (Section 2.2.8). The PCR products were either purified from agarose gels or were directly purified (Section 2.2.5.1). The sequencing reactions were performed using the "Cycle Sequencing" method with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit; the purification of the sequencing reactions was performed using Centri Sep Spin columns (Section 2.2.11). Up to a fragment length of 400 bases, a PCR/direct sequencing approach was successful for all blood samples from SIDS cases regardless of the samples' age. A gel picture is shown in Figure 3.5. Above 400 bp, the success of this approach depended on the date of DNA isolation after taking the blood sample during the autopsy. In Figure 3.16, a fragment of 2.2 kB is shown.

The difference between the successes of PCR is clearly visible. The PCR was most successful when genomic DNA isolated at the day of the autopsy, was amplified. The fragments from samples with an age of 1 year, 5 years and 11 years were less distinct, but showed almost no differences on the gel. The fragments from samples 14 and 15 years old, appeared only as a barely visible band on the gel. Even with an apparent success in the PCR step, a following sequencing reaction succeeded only for the most recent sample. In summary, for a successful PCR/direct sequencing approach on DNA from corpse blood: If the fragments to be amplified are longer than 400 bases, it is most important to isolate the DNA quickly after taking the sample.

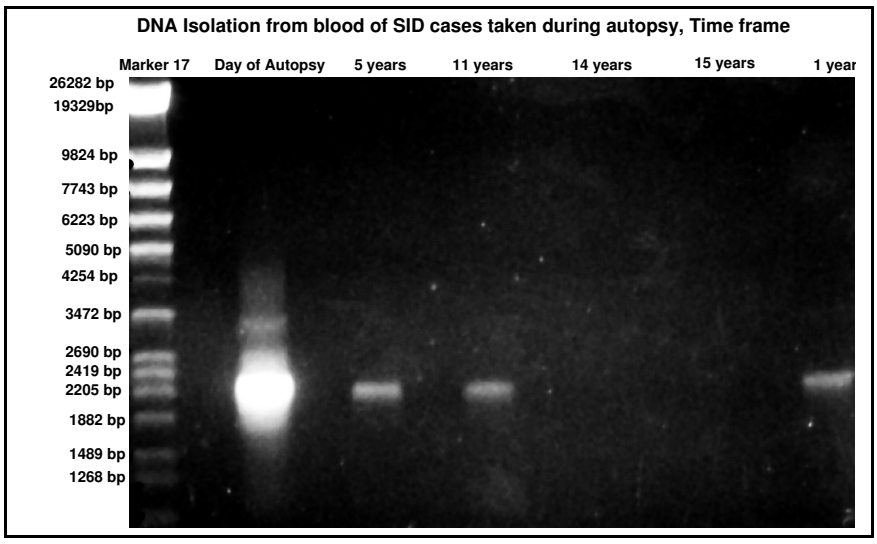


Figure 3.16: 0.4% Agarose gel: PCR of the hsp60s DNA from SIDS cases (fragment at 2205 bp of the Lambda marker 17)

4 Discussion

The interpretation of the experimental results of this thesis (Chapter 3), confirm the increased occurrence of genetic variations in Sudden Infant Death Syndrome.

4.1 SIDS cases

4.1.1 Epidemiological data

In this thesis, 91 SIDS cases from the years 1987 to 2001 and 108 controls from the years 1994 to 1999 were investigated. Both sample sets originated from Lower Saxony, Germany. Epidemiological data on birth rates and the incidence of Sudden Infant Death in Lower Saxony (Table 4.1) were obtained from the "Niedersächsisches Landesamt für Statistik, Hannover" (department of statistics, state of Lower Saxony, Germany). As usual, the SIDS incidence is expressed as the annual rate of SIDS infant mortality, the number of cases per 1000 live births.

Year	SIDS incidence	Year	SIDS incidence	Year	SIDS incidence
1987	1.1	1992	1.2	1997	0.6
1988	1.3	1993	1.0	1998	0.8
1989	1.6	1994	0.8	1999	0.6
1990	1.6	1995	0.8	2000	0.6
1991	1.5	1996	0.8	2001	0.7

Table 4.1: SIDS incidence in Lower Saxony in the years 1987–2001

The incidence in Lower Saxony corresponds to the rates in other Western countries in these years, including all of Germany (Hunt and Hauck 2004; Jorch 2000). It is noteworthy, however, that the incidence significantly decreases after 1992. In some countries (e.g., United States of America, Great Britain) the "back–to–sleep" campaigns were launched at this time, after the prone sleeping position was discovered as a risk factor. This campaign encouraged parents to put the children on their backs for sleeping (if not medically contraindicated). Germany did not launch a direct campaign, but the supine sleeping position was recommended by pediatricians. It is often assumed that the reduced incidence of Sudden Infant Death was due to the propagation of supine sleeping.

4.1.2 SIDS criteria

The sample of SIDS cases examined in this thesis exhibited the typical autopsy results: About two thirds of the SIDS cases showed petechiae in the pleura, epicardium and thymus; often in all three tissues. Also, two thirds of the SIDS cases featured hemorrhage in the respiratory tract. About one third had foam or oedemata in the respiratory tract.

The typical epidemiological factors were also present. The sample contained more male than female cases. More than half of the SIDS cases died within the age of 2 to 4 months. For 61 SIDS cases, the death scene position was documented in the autopsy protocols. 70.5 % of these 61 cases were discovered prone. Evidence of the additional risk factors preterm birth, heat stress and smoking could not be verified due to insufficient detail in the case reports. In summary, the cases studied in this thesis showed the typical characteristics associated with Sudden Infant Death.

4.2 Mutations in the human chaperonin gene 60

In recent years, research into Sudden Infant Death was extended to the molecular level, leading to knowledge of many genetic variations. The latter include metabolic disorders (Vockley and Whiteman 2002) as well as mutations in sodium and potassium channel genes (Wedekind et al 2001) and polymorphisms in the serotonin transporter gene 5–HTT (Weese–Mayer et al 2003; Narita et al 2001). Furthermore, a polymorphism was described for the interferon–γ gene (Gordon et al 2002) and an insertion mutation for the TSPYL gene (Puffenberger et al 2004). Additionally, many mutations of the mitochondrial genome are now known, being associated with SIDS (Reimann et al 2004; Opdal et al 1998; Hofmann et al 1997). All research yielded the same result: SIDS cases exhibit more mutations/genetic variations than controls. A selection of studies on genetic variants of genomic DNA in Sudden Infant Death Syndrome is shown in Table 4.2.

Heat stress has been associated with the Sudden Infant Death Syndrome for more than 20 years by now (Guntheroth and Spiers 2001; Einspieler 2000; Kurz 2000c; Kleemann et al 1998; Sawczenko and Fleming 1996; Kleemann et al 1996; Kleemann 1996; Ponsonby et al 1992; Fleming et al 1992; Stanton1984). Despite this fact and despite the advances in molecular biology over the last 20 years, very few studies have examined a possible connection between SIDS and heat shock protein genes. Rahim et al (Rahim et al 1996) described restriction fragment length polymorphisms (RFLPs) for certain restriction enzymes in human heat shock protein genes (hsp60, 70, 90). Bross et al. (Bross et al 2001) reported two mutations in the coding region of the hsp60 gene.

The only heat shock protein gene that showed distinctive features in Sudden Infant Death was the hsp60 gene. Thus, the efforts were concentrated on this gene. At first, it was tried to confirm what Bross et al. called a rare disease allele: the mutation N158S, an amino acid exchange in position 158 from asparagine to serine. This codon is located in the exon 5 of the human chaperonin60 gene. Neither the N158S mutation, nor any other mutation in the exon5 was found.

With the PCR and sequencing protocols, parts of the adjacent upstream and downstream introns to exon 5, introns 4 and 5, were also amplified and sequenced. Intron 4

Study, Author	Gene	Variation	SIDS cases, tissue for DNA isolation	Controls, tissue for DNA isolation	SIDS associated
Bross et al. 2001	hsp60	N158S	65	10	yes
Bross et al. 2001	hsp60	G573A	65	10	no
Rahim et al. 1996	hsp60	MspI–RFLP	12, liver	26, placenta	yes
Rahim et al. 1996	hsp60	ScaI–RFLP	12, liver	21, placenta	no
Rahim et al. 1996	hsp70	BglI–RFLP	12, liver	19, placenta	no
Rahim et al. 1996	hsp70	PstI–RFLP	12, liver	19, placenta	no
Rahim et al. 1996	hsp70	PvuII–RFLP	12, liver	28, placenta	no
Rahim et al. 1996	hsp90	Sau961–RFLP	12, liver	26, placenta	no
Weese–Mayer et al. 2003	5–HTT	LP	87	87	yes
Narita et al. 2001	5–HTT	LP	27, brain	115, blood	yes
Gordon et al. 2002	IFN–γ	LP	10, blood	10, blood	yes
Puffenberger et al. 2004	TSPYL1	457–458insG	4, blood	no controls	yes
Teske 2004	hsp60	N158S	91, blood	108, blood	no
Teske 2004	hsp60	deletions intron 5	91, blood	108, blood	yes
Teske 2004	hsp60	deletion AG	91, blood	108, blood	yes
Teske 2004	hsp60	deletion 24	91, blood	108, blood	no

Table 4.2: Known genetic variants in Sudden Infant Death Syndrome

showed no mutations, but in intron 5, two different kinds of deletion were found. The first is a deletion of the bases *AG* in positions 8957–8958. The second is a deletion of 24 bases (*TATGATCAAAAGTTTGAGTTATCT*) in positions 8885–8908.

In summary, the SIDS cases show significantly more mutations of *deletion AG* (p=0.036 in the binary logistic regression) than the controls and more mutations (deletions) in the entire intron 5 (p=0.036 in the binary logistic regression) than the controls. These findings correspond to other studies: SIDS cases show more mutations than the controls. However, no mutation so far can be interpreted as sole reason for Sudden Infant Death Syndrome.

4.2.1 Deletions in intron 5 as putative sites for splicing and transcription regulation

In higher eukaryotes most genes are interrupted, having a distinct exon–intron sequence. The coding exons are often very small compared to the large non–coding introns. At the primary transcript on the hnRNA level the introns are spliced and the exon sequences remain as the protein template. According to Lewin (Lewin 2000) the splice junctions show a characteristic consensus sequence, also known as the GT–AG rule: An intron starts 5′ (also called *left or donor site*) with a GT and ends 3′ (also called *right or acceptor site*)with

an AG. 18 to 40 bases upstream of the 3′ splice site is the location of the branch site (animal consensus sequence $Py_{80}\mathrm{N}Py_{80}Py_{87}Pu_{75}\mathrm{A}Py_{95}$), which is crucial for the splicing process. It cannot be verified, whether the deletions in intron 5 do disrupt the splicing process. Since the deletions are located near the 5′ end, they most likely neither affect the donor and acceptor sites, nor the branch site.

However, it interesting to notice that intron 5 belongs to class of introns with an alternative donor site *GC–AG*. Not all introns do have the GT–AG donor–acceptor sites. Different splice sites are known: GC–AG, GG–AG, GT–TG, GT–CG, CT–AG (Burset et al 2000). According to Clark and Thanaraj (Clark and Thanaraj 2002; Thanaraj and Clark 2001), GC splice sites account only for 1.1 % of the donor sites. GT–AG introns and GC–AG introns are spliced by the U2–type spliceosome, which is the standard spliceosome in metazoans. For the GC–AG introns, a mismatch exists between the donor site and the U1 snRNA in the intron position 2 (C). It has been proposed, that there may be a compensation in the sequence around the donor site to maximize base pair formation with the other positions in the U1 snRNA. GC–AG introns have a high average length and a prevalence toward alternative forms. It is suggested, that they are highly regulated. It could not be demonstrated, that the *deletion AG* and the *deletion 24* are located within an important regulatory region for the splicing of the GC–AG intron 5 of the human hsp60 gene.

The Match software was used in conjunction with matrices from the Transfac Database to check the possibility, whether the deletions affect the gene regulation on the level of transcription of the hsp60 gene. In the region of *deletion 24*, Match found two hits:

1. The first is the transcription factor HNF–3α in two species, human (Homo sapiens) and rat (Rattus norvegicus). HNF–3α is Hepatocyte Nuclear Factor 3 Alpha (HNF3A). According to the Swiss–Prot entry, HNF3A is transcription activator for a number of liver genes such as AFP, albumin, tyrosine aminotransferase and PEPCK. It interacts with the cis–acting regulatory regions of these genes.

2. The second is the Cdc5 transcription factor of human Homo sapiens (cell division control protein 5, no Swiss–Prot entry). According to the Transfac Database, Cdc5 is a transcriptional activator and a transcriptional regulator of cell cycle G2 phase progression and mitotic entry.

In the region of *deletion AG* the Match software found one hit: MEF–2 (Myocyte–specific enhancer factor 2) in three species, human (Homo sapiens), mouse (Mus musculus) and rat (Rattus norvegicus). In humans, four MEF–2 factors exist, MEF2 A, B, C, D. The hits in the Transfac Database refer to the factors MEF2A (SPAN Q02078) and MEF2D (SPAN Q14814). MEF2A is a transcription factor which binds specifically to the MEF2 element present in the regulatory regions of many muscle–specific genes. It activates transcription via this element and may be involved in muscle–specific and/or growth factor–related transcription. MEF2D has the same functions as MEF2A. The nature of the deletions (positions inside of the hsp60 gene) suggests that they are most likely not affected by transcription factors. However, it may be possible, that the deleted positions are part of an enhancer binding site. The Transfac Database did not contain any information on this possibility.

4.2.2 A putative mechanism for the influence of a hsp60 gene mutation on Sudden Infant Death

It is known, that mutations in the human hsp60 gene can have fatal effects: The amino acid exchange V721I (valine to isoleucine) is a disease–causing mutation for hereditary spastic paraplegia SPG13 (Hansen et al 2002). Assuming a mutation either affecting the coding sequence or an important binding site in the non–coding part, the following scenario for a connection between Sudden Infant Death and the human Chaperonin60 could be constructed: In Figure 4.1, possible general mechanisms following Chaperonin mutations, are shown (Slavotinek and Biesecker 2001).

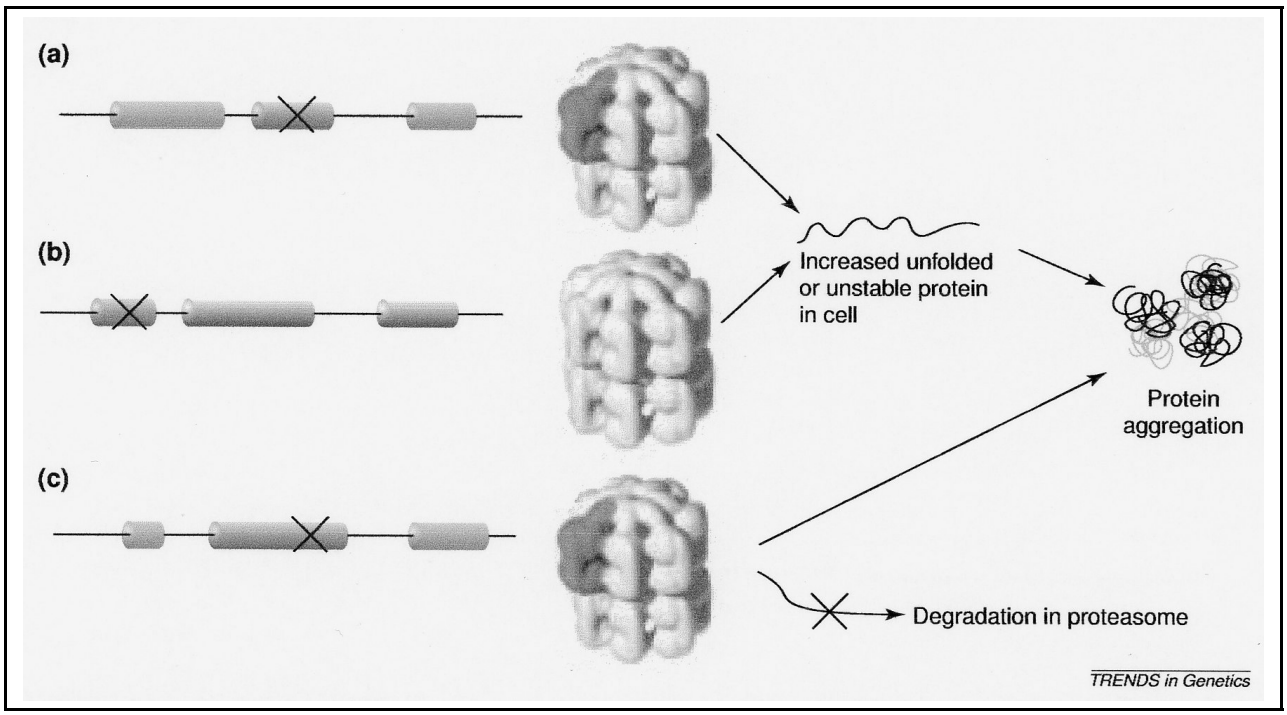


Figure 4.1: Possible disease mechanisms following Chaperonin mutations (based on Slavotinek and Biesecker 2001)

- Mechanism a) represents a mutation, that creates a defective subunit either unable to bind a substrate or with a reduced binding ability.
- Mechanism b) represents a mutation that produces an unstable protein or an expanded polyQ–tract (Glutamin) prone to increased aggregation.
- Mechanism c) represents a mutation that impairs the ability of a Chaperonin to target a misfolded protein for degradation in the proteasome.

These mutation possibilities in the gene can lead, for example, to amino acid exchanges and splicing defects, ultimately altering the protein structure. The Chaperonin is unable to carry out its normal function in protein folding. The crucial factor for this kind of

mutation is, that the protein still exists, but may not be able to function properly. Such a damaged Chaperonin, emerging from a mutation in the hsp60 gene, could be the endogenous factor in the multifactorial genesis of the Sudden Infant Death Syndrome (Kurz 2000b). The other two factors are a vulnerable infant, because of its developmental stage and an exogenous factor, in this case, heat stress.

In summary, the child has a weakened immune system or a weakened thermoregulation because of its developmental state. It suffers from considerable heat stress due to an infection with high fever and/or a much too warm environment. Prone sleeping may increase heat stress. Because of the damaged Chaperonin, it can not cope fully with the heat stress. Each factor alone is not severe enough. All factors in conjunction provoke deadly consequences. Thus, a mutation in the hsp60 gene may be a *necessary, but not sufficient* factor in the genesis of SIDS.

4.3 hsp60s, a new member/pseudogene of the HSP60 family

The original intention to amplify the coding part of the human hsp60 gene, resulted in an unexpected outcome: Instead of the anticipated 13.2 kB fragment, a very prominent 2.2 kB fragment showed up on the agarose gel. The fragment was extracted from the gel and the PCR primer were used for sequencing. The sequences obtained were very similar to the mRNA of the human hsp60 gene. The putative 5' part of the gene contained a start-codon, ATG, followed by an open reading frame. The first part of this translated ORF (26 amino acids) is identical to that of the Hsp60 protein. It resembles the so–called mitochondrial leader peptide, a small protein sequence serving as a targeting signal for the mitochondrial import. The GAN for the newly discovered fragment, which was named hsp60s (s for short, comparing it to the much longer regular hsp60 gene), is AF380943 (Teske et al 2001).

The high similarity between the fragment and the mRNA of hsp60 and the existence of a putative ORF with a mitochondrial leader peptide could have been evidence of an expressed gene. It was suspected to be an inducible variant of the hsp60 gene, which is already known for other heat shock genes, such as hsp70. The gene exists in two forms: A constitutive or cognate form for house keeping purposes and an intronless inducible form for the stress response. Based on the assumption that the fragment could be the inducible variant of the hsp60 gene, the hsp60 mRNA was used as a template to construct primer for sequencing the entire fragment (2176 bp). It showed a 97.5 % similarity to the human hsp60 mRNA in an alignment with the Martinez/Needleman–Wunsch algorithm.

Instead of a single open reading frame as in the regular hsp60 gene, two putative ORFs were found. They were translated from the DNA with the EditSeq software using the standard genetic code and named Hsp60s1 and Hsp60s2 (short Heat Shock protein 60 1 and 2). Both are sufficiently long to encode proteins: Hsp60s1 contains 220 amino acids, Hsp60s2 contains 258 amino acids. Generally, a peptide longer than 100 amino acids is considered a protein. An analysis with the ProtParam tool confirmed the similarity with the Hsp60 protein. Theoretically, both putative proteins show Chaperonin functions.

Gupta 1995 (Gupta 1995) documented certain characteristics based on the sequences of the members of the HSP60 family and their evolution (see below). These characteristics indicate clearly that the hsp60s gene is a member of the chaperonin gene family:

1. "Most Hsp60 sequences contain one or more repeats of the amino acid sequence *GGM* or *GGGM* at their C–terminal end." The end of the ORF Hsp60s2 reads as follows: putative amino acid sequence *GGMGGGMGGGM*.

2. "The mitochondrial homologues in all of the eukaryotic species examined are encoded by nuclear genes and contain a characteristic N–terminal leader sequence (rich in basic and hydroxylated amino acids such as Arg, Lys, Ser and Thr) that is required for the mitochondrial import of the protein." The N–terminal sequence (26 amino acids, mitochondrial leader peptide) of the ORF Hsp60s1 is exactly the same as in the Hsp60 protein.

3. "The global alignment of Hsp60 sequences has identified a number of signature sequences that are uniquely shaped among (a) members of the Gram–negative α–purple subdivision of bacteria and mitochondrial homologues, and (b) between cyanobacteria and chloroplast homologues." The signature sequences between Hsp60 and Hsp60s1/Hsp60s2 are identical.

However, the stop codon at the end of Hsp60s1 could be evidence of a pseudogene nature of hsp60s. Many pseudogenes originate from failed copies of mRNA that are reintegrated into the DNA, and have an altered sequence. Often they incorporate stop codons.

To obtain further information on the nature of the hsp60s gene, a Blast search against GenBank was conducted and chromosome 5 was determined as the location of the hsp60s gene. With this data, the greatest interest lay in the upstream region of the gene. If the promoter contains a heat shock element, a characteristic promoter element in heat shock genes, an expression could yet be possible. A putative heat shock element was discovered, located 978 bases upstream of the ATG from Hsp60s1. A test with the Match software against the transcription factor database Transfac found the putative heat shock element suitable for binding the human heat shock transcription factor 1. HSF1 is the typical eukaryotic DNA–binding protein that specifically binds heat shock promoter elements (HSE) and activates transcription.

With these findings it was hypothesized, that the hsp60s gene could be expressed. For verification, several hypothetical mRNA sequences were formulated for hsp60s, including those requiring a readthrough to the TGA stop codon of the hsp60s1 ORF. RT–PCRs were performed with diverse primers to test these hypotheses.

However, none of the sequences considered could be confirmed: Based on these results, it can be concluded, that the hsp60s gene is most likely a pseudogene of the hsp60 gene. It may still be possible, that the hsp60s gene is expressed in tissue other than blood. Additionally, it may only be expressed under stress conditions (our expression analysis was carried out under non–stress conditions). While the required experiments are outside the scope of this work, they could be considered in future research.

4.3.1 Stop codon readthrough in eukaryotes, application to hsp60s

If the stop codon of hsp60s1 is overread, a continuous open reading frame emerges with a slightly altered protein sequence, but the same length (573 amino acids) as the regular Hsp60 protein. The similarity in an alignment with the Lipman–Pearson algorithm between the putative protein of a continous ORF of hsp60s and the Hsp60 protein was 95.5 %.

Considering the possibility of a stop codon readthrough, known to exist in the human genome, it was decided to take a closer look on this phenomenon. The stop codon of the hsp60s1 open reading frame is *UGA*, called *opal*. Termination codons are not represented by a tRNA. Release factors catalyze the termination of the protein synthesis. A so–called nonsense suppressor tRNA is able to read the stopcodon and incorporates an amino acid; the protein translation carries on to the next stop codon. The description nonsense suppressor was named after the nonsense mutation: a point mutation creating a stop codon and thus a premature termination of the protein synthesis. Nonsense suppressor tRNAs, making a readthrough of a stop codon possible (translational readthrough), exist in prokaryotes and eukaryotes. They are known in bacteria (E. Coli), yeast, mammals (e.g., rabbits), avians (e.g., chickens) and amphibia (O'Neill et al 1985; Pratt et al 1985). The bacterial UGA suppressor tRNA, derived from the tryptophan tRNA, has an altered conformation of the anticodon loop. It allows a pairing between the anticodon $\overleftarrow{CCA}$ and the regular codon for tryptophan (UGG) as well as a pairing with the termination codon UGA. In eukaryotes, the bovine liver contains a $tRNA^{Ser}$ with the anticodon $\overleftarrow{CCA}$, that should respond to tryptophan, but in fact it responds to UGA. "*The codon–anticodon recognition of either wild type or mutant tRNA cannot be predicted entirely from the relevant triplet sequences, but is influenced by other features of the molecule.*" (Lewin 2000).

O'Neill et al. (O'Neill et al 1985) discovered a gene for an opal suppressor tRNA in humans with a $\overleftarrow{TCA}$ anticodon, demonstrating that the mature tRNA reads the termination codon UGA. Lee et al. (Lee et al 1989; Lee et al 1990) proved the existence of a unique opal suppressor seryl–tRNA in higher vertebrates. They found a single gene for this tRNA in humans and chicken. More important this tRNA serves as a selenocysteyl–tRNA ($tRNA^{Sec}$), being the donor for the unique amino acid selenocysteine in response to specific UGA codons. A readthrough of the UGA stop codon to incorporate selenocysteine needs a *secis sequence (selenocysteine insertion sequence)* downstream of the UGA, the $tRNA^{Sec}$ and a specific, GTP–dependant translation factor (SelB in bacteria). The secis element forms a distinct secondary structure of the mRNA, a stem loop with a non–Watson–Crick motif downstream of the UGA in the 3' untranslated region (Grundner–Culemann et al 1999).

The incorporation of selenocysteine is a characteristic of selenoproteins like glutathione peroxidase enzymes, iodothyronine deiodinase enzymes and thioredoxin reductase (TR). The human heat shock protein 60 does not belong to this protein class. Based on the close relationship between the hsp60 gene and the hsp60s gene it can be assumed, that the hsp60s gene does not encode for a selenoprotein either. Despite the low likelihood, the prior findings do not completely exclude the possibility for a readthrough of the opal stop codon at the end of the hsp60s1.

4.4 Direct sequencing of PCR products obtained from DNA of SIDS victims

Most of the current forensic test kits work with sequence lengths between 100 and 350 bases for fragment length analysis of genomic DNA (for example the AmpFLSTR Profiler Plus, Cofiler, and Profiler Plus ID PCR Amplification Kits of Applied Biosystems). These test kits contain specified primers to identify individual humans. A similar approach (with appropriate primers) would be possible for any fragment with a maximum length of 350 bases. However, these methods do not include an analysis of the *DNA sequence*. Thus, they are not usable for a mutation analysis of all bases on an entire fragment.

A successful PCR/direct sequencing approach was demonstrated for fragments up to 400 bases for DNA from blood samples of SIDS victims. Even DNA from 15 year old blood samples could be analysed with these methods. If the DNA is isolated from corpse blood shortly after blood withdrawal during an autopsy, the genomic DNA can be used for PCR and direct sequencing up to lengths of 2.2 kB. However, this requires a relatively fresh corpse, the autopsy must be performed no more than a few days after death. In SIDS cases, the autopsy often takes place shortly after death. Blood samples should be taken during the autopsy and the DNA should be isolated as quickly as possible, enabling the application of molecular biology methods for mutation analysis and further research on Sudden Infant Death Syndrome.

5 Summary and Future Work

Sudden Infant Death Syndrome (SIDS) is still one of the leading causes of infant death in Germany and other Western countries. It is certainly the most enigmatic one. Today, many risk factors and other characteristics are known, but the genesis is still not completely understood. A genetic predisposition or a genetic defect have long been suspected to be a part of Sudden Infant Death Syndrome. As one candidate for such a defect, variations in the human heat shock protein gene 60 (chaperonin60, hsp60) were examined between SIDS cases and controls.

The cases showed typical characteristics of Sudden Infant Death Syndrome (SIDS). For example, a characteristic epidemiological trait and by now well known risk factor is prone sleeping. Over 70 % of 61 SIDS cases, where the body position in death was documented, were found prone. Sex is another risk factor: male children are more likely to suffer from Sudden Infant Death than female children. The sample includes about two thirds of boys. Overall, the SIDS cases show the typical age distribution, with a peak between 2 and 4 months. Typical autopsy results include petechiae in the pleura, epicardium and thymus, as well as hemorrhage in the respiratory tract. Over two thirds of the examined cases showed either one or all of the characteristics. The controls were age and sex matched children encountered in paternity testing.

A connection between heat stress and Sudden Infant Death has been known for over twenty years. Many SIDS victims were found soaked in sweat or had infectious diseases, that induce high fever, shortly before death. Hyperthermia and ultimately heat shock are strong risk factors for SIDS. The most important molecular factors for heat stress response in the cell are heat shock (stress) proteins. These proteins have two different tasks in the cell: As molecular chaperones, they assist in protein folding following synthesis. As part of the stress response, they maintain protein stability under stress circumstances. Damaged proteins are refolded; if the damage is too severe, the proteins are degraded. The human Hsp60, whose exon 5 and boundaries were analysed in SIDS cases and controls, is a protein of the mitochondrial matrix. It binds to partly folded proteins and assists in correct folding. Furthermore, it is involved in the assembly of multimeric complexes.

No difference between SIDS cases and controls in the DNA sequence of the exon 5 of the hsp60 gene could be detected. But the SIDS cases showed a statistically significant higher amount of deletions in the intron 5 than the controls. This confirms other research results on DNA variations in SIDS cases. Overall, the SIDS cases showed more mutations than the controls. The Sudden Infant Death Syndrome is generally viewed as a multifactorial process. A genetic defect is thus assumed to be *necessary, but not sufficient* for the genesis of SIDS. The deletions in intron 5 do not disrupt any known regulatory sequences (donor, acceptor, branch site). But since intron 5 belongs to the rare GC–AG intron class, which is assumed to be highly regulated, the deletions may have an impact on other regulatory sites.

During the research in the human hsp60 gene, a previously undocumented gene of the Chaperonin (HSP60) family was discovered. It shows a high similarity in length and sequence (97.5 %) to the mRNA of the hsp60 gene. The genomic sequence does not contain introns and is much shorter compared to the regular hsp60 gene. Thus, it was named hsp60s (s for short). Because of the similarity and a mitochondrial leader peptide sequence at the N–terminal end of the putative protein, it was assumed to be an inducible variant of the hsp60 gene. After fully sequencing it, two open reading frames instead of the single one in hsp60 were found. A readthrough to the stop codon of the first ORF would lead to a continous ORF. Stop codon readthrough exists in the human genome, but only on rare occasions. An expression analysis (reverse transcriptase PCR on RNA from blood) of the hsp60s gene showed no results. It can be concluded, that the hsp60s gene is a pseudogene of the human chaperonin60 gene. However, it may still be possible, that hsp60s is only expressed under stress (the expression analysis was carried out under non–stress conditions) and/or in tissues other than blood.

The results from the present thesis can be applied to practical forensics. DNA fragments of different sizes from up to 15 year old blood samples of SIDS cases were amplified and sequenced. The amplification and direct sequencing of genomic DNA from corpses is becoming more and more important in forensics as older unsolved criminal cases will be examined again. It was demonstrated, that the approach of PCR and direct sequencing of DNA isolated from corpses, is possible for lengths up to 400 bases, even in older samples.

Research on Sudden Infant Death is like a puzzle. Due to the multifactorial genesis every result adds another piece to the whole picture. Until recently, most of the research was conducted in either epidemiological or anatomical fashion. This lead to the recognition of risk factors, such as prone sleeping, heat stress and smoking. The following "back–to–sleep" campaigns (putting babies in a supine sleep position, instead of a prone position, if not medically contraindicated) reduced the SIDS incidence in many countries. An autopsy is absolutely necessary in any case of Sudden Infant Death. It enables the diagnosis "Sudden Infant Death" and distinguishes it from filicide and other causes of death.

This thesis clearly shows, that a molecular biological approach should be included in the research of the Sudden Infant Death Syndrome. As mentioned before, Sudden Infant Death probably has a genetic component. These genetic defects are most likely to differ out in different SIDS cases. It was suspected, that the heat shock response plays a vital role in Sudden Infant Death. Therefore, more genes of heat shock proteins should be analysed in SIDS cases compared to appropriate controls. Applying the micro array technique could be very promising. Before specific genes or parts of these genes are analysed, a general human micro array analysis and/or a micro array analysis emphasizing on heat shock genes should be carried out to determine differences and abnormalities in the total gene expression between SIDS cases and controls.

A very important part in the ongoing research on Sudden Infant Death should be strong partnership between epidemiology, forensic medicine, and molecular biology. The knowledge and abilities of the researchers in epidemiology and forensic medicine established the foundation for further work. Now it is time to broaden that knowledge and let molecular biologists apply the methods of the 21st century to the research on Sudden Infant Death Syndrome.

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A Appendix

A.1 Individual SIDS cases and controls

Sample	Sex	Age	Sample.	Sex	Age	Sample	Sex	Age
1	m	135	45	f	74	82	f	117
4	m	43	46	m	30	83	f	28
5	m	78	47	m	90	84	m	95
6	f	58	48	m	56	85	f	98
7	m	66	49	f	49	86	m	97
8	m	179	50	m	54	87	f	115
9	m	32	51	f	47	88	f	89
10	m	39	52	m	72	89	m	41
11	m	57	53	f	13	90	m	180
12	f	68	54	m	70	91	m	101
13	f	151	55	m	62	92	m	43
14	m	134	56	m	84	93	m	193
15	m	183	57	f	185	94	m	72
16	m	131	58	m	148	95	m	35
17	m	263	59	m	36	98	f	96
18	m	78	60	m	45	102	f	81
19	m	169	61	f	56	103	f	143
20	f	137	62	f	61			
21	m	100	63	f	146			
22	m	92	64	m	162			
23	m	77	65	m	29			
24	m	270	66	f	95			
26	f	335	67	f	258			
27	f	241	68	m	155			
28	m	185	69	m	54			
31	f	151	70	m	216			
32	f	320	71	f	162			
33	f	84	72	m	78			
34	f	103	73	m	62			
35	m	77	74	f	21			
38	m	197	75	m	134			
39	m	150	76	f	138			
40	m	107	77	f	314			
41	f	137	78	m	39			
42	m	59	79	m	114			
43	f	167	80	f	341			
44	m	308	81	m	68			

Sample = Sample Number, Sex: m = male, f = female, Age = age in days

Table A.1: SIDS cases – Age and Sex

Sample	Sex	Age	Sample	Sex	Age	Sample	Sex	Age
1	f	350	43	f	247	93	m	276
2	m	301	44	m	241	94	f	271
3	f	291	45	m	293	95	f	360
5	f	346	46	f	243	96	m	248
6	m	243	49	f	263	97	f	267
7	f	245	50	m	262	98	f	309
8	f	266	51	f	323	99	m	247
9	m	359	52	m	248	108	f	247
10	m	288	53	m	314	110	m	267
11	f	305	54	m	252	111	m	247
12	m	297	55	f	349	112	f	321
13	f	251	56	m	347	114	m	188
14	f	270	57	m	263	116	f	349
15	m	250	59	f	292	118	f	246
16	m	318	61	f	300	120	f	296
19	f	241	62	m	260	122	m	316
20	m	247	63	m	305	123	f	290
21	m	353	64	f	254	126	f	264
22	m	361	65	m	273	127	m	249
23	f	263	66	m	283	128	m	254
24	f	250	67	m	253	130	m	253
25	m	347	68	f	329	131	f	264
f6	m	317	69	m	243	138	m	350
27	m	340	70	f	280	139	f	247
28	m	274	71	f	291	140	m	243
29	m	344	72	f	241	141	m	265
31	m	247	73	m	272	145	f	247
32	m	292	75	m	285	147	f	328
33	m	244	76	f	244	149	f	328
34	f	257	77	m	250	156	m	262
36	m	268	80	m	314	200	m	103
37	m	247	85	f	244	201	m	144
38	f	243	87	m	248	204	f	103
39	f	258	89	f	244	205	m	168
40	f	335	90	m	271			
41	f	270	91	m	245			
42	f	305	92	m	266			

Sample = Sample Number, Sex: m = male, f = female, Age = age in days

Table A.2: Controls – Age and Sex

A.2 Individual SIDS Criteria

Sample Sample number

Sex Sex: 1 = male, 2 = female

Age Age in days

PrBi Preterm birth: 1 = yes, 0 = no, or no information available

DSIP Death Scene Investigation Position: 1 = prone, 2 = supine, 3 = side, 0 = no information available

DSIT Death Scene Investigation Temperature: 1 = indication of possible hyperthermia (high body temperature, fever or sweating), 0 = none, or no information available

NiMo Nicotine Abuse by mother: 1 = yes, 0 = no, or no information available

PetP Petechiae in Pleura: 1 = yes, 0 = no, or no information available

PetE Petechiae in Epicardium: 1 = yes, 0 = no, or no information available

PetT Petechiae in Thymus: 1 = yes, 0 = no, or no information available

RToe Respiratory tract oedemata: 1 = yes, 0 = no, or no information available

RTfo Respiratory tract foam: 1 = yes, 0 = no, or no information available

RThe Respiratory tract hemorrhage: 1 = yes, 0 = no, or no information available

Sample	Sex	Age	PrBi	DSIP	DSIT	NiMo	PetP	PetE	PetT	RToe	RTfo	RThe
1	1	135	0	1	0	0	1	1	1	0	0	0
4	1	43	0	0	0	0	0	1	0	1	1	1
5	1	78	0	3	0	0	1	0	0	1	1	0
6	2	58	0	2	0	1	0	1	1	1	0	1
7	1	66	1	2	0	1	1	0	0	0	0	0
8	1	179	0	2	0	1	1	1	1	1	0	0
9	1	32	0	1	0	0	1	0	0	1	0	0
10	1	39	1	0	0	0	0	1	1	1	0	1
11	1	57	0	1	0	0	1	1	1	1	0	0
12	2	68	0	0	0	0	0	1	1	0	0	1
13	2	151	0	1	0	0	0	0	0	0	0	1
14	1	134	0	1	0	0	1	1	1	0	0	0
15	1	183	0	1	0	0	1	0	1	0	1	0
16	1	131	1	1	0	0	1	0	1	1	1	0
17	1	263	0	1	0	0	1	1	1	1	1	0
18	1	78	0	0	0	0	1	0	1	0	0	0
19	1	169	0	2	0	0	1	1	1	1	0	0
20	2	137	0	1	0	0	1	0	1	1	0	1
21	1	100	0	1	0	0	1	0	0	1	0	0
22	1	92	0	2	0	0	1	1	0	1	0	1
23	1	77	0	1	0	0	0	0	0	1	1	0
24	1	270	0	1	1	0	0	0	1	1	0	0
26	2	335	0	2	1	0	1	1	1	1	0	1
27	2	241	1	1	1	0	1	1	1	0	0	1
28	1	185	0	0	0	0	1	1	1	0	1	0
31	2	151	0	1	1	1	1	1	1	0	0	0
32	2	320	0	0	0	0	1	1	1	0	0	0
33	2	84	0	1	0	0	1	0	0	1	0	1
34	2	103	0	1	0	0	0	0	0	1	0	1
35	1	77	1	1	0	1	1	1	0	1	1	0
38	1	197	0	3	0	0	1	1	1	1	1	0
39	1	150	0	1	1	0	1	1	1	0	1	0
40	1	107	0	1	0	0	1	1	0	1	1	1
41	2	137	0	0	0	0	0	0	0	1	0	0
42	1	59	0	0	0	0	0	0	1	0	0	1
43	2	167	1	1	1	1	0	1	1	0	0	1
44	1	308	0	1	0	0	0	1	0	0	1	1
45	2	74	0	1	1	0	1	1	1	0	0	1
46	1	30	0	3	0	0	1	1	0	1	1	1
47	1	90	0	2	0	0	1	1	1	1	1	0
48	1	56	0	0	0	0	1	1	0	0	0	1
49	2	49	0	0	0	0	0	0	1	0	0	1
50	1	54	1	1	1	0	0	0	1	1	0	1
51	2	47	0	0	0	0	0	0	0	0	0	0
52	1	72	0	0	0	0	1	0	0	0	0	1

Table A.3: SIDS cases – SIDS criteria, samples 1–52

Sample	Sex	Age	PrBi	DSIP	DSIT	NiMo	PetP	PetE	PetT	RToe	RTfo	RThe
53	2	13	0	3	0	1	0	0	0	1	0	1
54	1	70	0	1	0	0	1	1	1	1	1	1
55	1	62	0	0	0	0	1	1	1	0	0	1
56	1	84	0	1	0	1	1	1	1	1	1	1
57	2	185	0	1	0	0	1	1	1	0	0	1
58	1	148	0	1	1	0	0	1	1	0	0	0
59	1	36	0	2	0	0	1	1	1	0	0	1
60	1	45	0	0	0	0	0	0	0	0	0	1
61	2	56	0	0	0	0	0	1	1	0	1	0
62	2	61	0	0	0	0	0	0	1	0	1	1
63	2	146	0	1	0	0	1	1	1	0	1	1
64	1	162	1	2	0	0	1	0	1	0	0	1
65	1	29	0	0	0	0	1	1	0	0	1	1
66	2	95	0	1	0	0	1	1	1	0	0	1
67	2	258	0	1	0	0	1	1	1	0	0	1
68	1	155	0	0	0	0	1	1	1	0	1	1
69	1	54	0	0	0	0	1	1	1	0	0	1
70	1	216	0	0	1	0	1	0	0	1	0	1
71	2	162	0	0	0	0	0	0	1	0	0	0
72	1	78	1	1	0	0	1	1	1	1	1	1
73	1	62	0	2	0	0	1	1	1	0	0	1
74	2	21	0	0	0	0	0	0	1	0	0	1
75	1	134	0	0	0	0	0	1	1	0	0	1
76	2	138	1	0	0	0	1	1	1	1	0	1
77	2	314	0	0	0	0	0	1	1	0	0	1
78	1	39	0	1	0	1	0	1	1	0	1	1
79	1	114	0	2	0	0	1	0	1	0	1	1
80	2	341	0	1	0	0	1	1	1	0	0	1
81	1	68	0	0	0	0	1	0	1	0	0	1
82	2	117	0	2	0	0	0	0	0	0	0	1
83	2	28	0	1	0	0	1	0	0	1	0	0
84	1	95	0	2	0	0	1	1	1	0	0	1
85	2	98	0	0	0	0	1	0	1	0	0	1
86	1	97	0	0	0	0	1	0	1	0	0	1
87	2	115	1	1	0	0	1	1	1	1	0	1
88	2	89	1	0	0	0	0	0	0	0	0	1
89	1	41	0	1	0	0	1	1	1	0	1	1
90	1	180	1	1	1	0	1	1	1	1	1	0
91	1	101	0	2	0	0	1	0	0	0	1	1
92	1	43	0	1	0	0	1	1	1	0	1	1
93	1	193	0	1	0	0	1	1	1	0	0	0
94	1	72	0	1	0	0	1	1	1	1	1	0
95	1	35	0	0	0	0	1	1	0	0	1	0
98	2	96	0	1	0	0	1	1	1	0	0	0
102	2	81	0	1	1	1	0	1	1	1	0	1
103	2	143	0	1	0	0	1	1	0	0	0	0

Table A.4: SIDS cases – SIDS criteria, samples 53–103

A.3 RT–PCR programs for verifying hsp60s mRNA Possibilities

RT–PCR step	Program, Primer	Program, Primer	
	RT1V1A	**RT1V2A**	
	53Start1v1	**53Start1v1**	
	53Stop2v1	**53Stop2v2**	
1.Reverse Transcription	30 min 50°C	30 min 50°C	
2.Initial PCR Activation	15 min 95°C	15 min 95°C	
3.Denaturation	1 min 94°C	1 min 94°C	
4.Annealing	1 min 48°C	1 min 45°C	
5.Extension	1.45 min 72°C	1.45 min 72°C	
6.Cycles	Goto 3 30 times	Goto 3 30 times	
7.Final Extension	10 min 72°C	10 min 72°C	
8.Hold	Forever 4°C	Forever 4°C	
Expected Fragment Size	1565 bp (138095–139659)	1593 bp(138095–139687)	

RT–PCR step	Program, Primer	Program, Primer	Program, Primer
	RT1V3A	**RT1V3B**	**RT1V3D**
	53Start1v1	**53Start1v1**	**53Start1v1**
	3Stop2v3	**3Stop2v3**	**3Stop2v3**
1.Reverse Transcription	30 min 50°C	30 min 56°C	30 min 50°C
2.Initial PCR Activation	15 min 95°C	15 min 95°C	15 min 95°C
3.Denaturation	1 min 94°C	1 min 94°C	1 min 94°C
4.Annealing	1 min 52°C	1 min 52°C	1 min 56°C
5.Extension	1.45 min 72°C	1.45 min 72°C	1.45 min 72°C
6.Cycles	Goto 3 30 times	Goto 3 30 times	Goto 3 30 times
7.Final Extension	10 min 72°C	10 min 72°C	10 min 72°C
8.Hold	Forever 4°C	Forever 4°C	Forever 4°C
Expected Fragment Size	1614 bp(138095–139708)	1614 bp(138095–139708)	1614 bp(138095–139708)

Table A.5: RT–PCR Programs for verifying hsp60s mRNA Alternative 1

RT–PCR step	**Program, Primer**	**Program, Primer**	**Program, Primer**
	RT2V1A	**RT2V2A**	**RT2V3A**
	3Start1v3	**3Start1v3**	**3Start1v3**
	53Stop2v1	**53Stop2v2**	**3Stop2v3**
1.Reverse Transcription	30 min 50°C	30 min 50°C	30 min 50°C
2.Initial PCR Activation	15 min 95°C	15 min 95°C	15 min 95°C
3.Denaturation	1 min 94°C	1 min 94°C	1 min 94°C
4.Annealing	1 min 48°C	1 min 45°C	1 min 52°C
5.Extension	1.45 min 72°C	1.45 min 72°C	1.45 min 72°C
6.Cycles	Goto 3 30 times	Goto 3 30 times	Goto 3 30 times
7.Final Extension	10 min 72°C	10 min 72°C	10 min 72°C
8.Hold	Forever 4°C	Forever 4°C	Forever 4°C
Expected Fragment Size	1522 bp (138138–139659)	1550 bp (138138–139687)	1571 bp (138138–139708)
RT–PCR step	**Program, Primer**	**Program, Primer**	**Program, Primer**
	RT2V4A	**RT2V5A**	**RT2V6A**
	5Start1v4	**5Start1v4**	**5Start1v4**
	53Stop2v1	**53Stop2v2**	**3Stop2v3**
1.Reverse Transcription	30 min 50°C	30 min 50°C	30 min 50°C
2.Initial PCR Activation	15 min 95°C	15 min 95°C	15 min 95°C
3.Denaturation	1 min 94°C	1 min 94°C	1 min 94°C
4.Annealing	1 min 48°C	1 min 45°C	1 min 52°C
5.Extension	1.45 min 72°C	1.45 min 72°C	1.45 min 72°C
6.Cycles	Goto 3 31 times	Goto 3 30 times	Goto 3 30 times
7.Final Extension	10 min 72°C	10 min 72°C	10 min 72°C
8.Hold	Forever 4°C	Forever 4°C	Forever 4°C
Expected Fragment Size	1504 bp (138156–139659)	1532 bp (138156–139687)	1553 bp (138156–139708)
RT–PCR step	**Program, Primer**	**Program, Primer**	**Program, Primer**
	RT2V7A	**RT2V8A**	**RT2V9A**
	53Start1v5	**53Start1v5**	**53Start1v5**
	53Stop2v1	**53Stop2v2**	**3Stop2v3**
1.Reverse Transcription	30 min 50°C	30 min 50°C	30 min 50°C
2.Initial PCR Activation	15 min 95°C	15 min 95°C	15 min 95°C
3.Denaturation	1 min 94°C	1 min 94°C	1 min 94°C
4.Annealing	1 min 48.5°C	1 min 45°C	1 min 50°C
5.Extension	1.30 min 72°C	1.30 min 72°C	1.30 min 72°C
6.Cycles	Goto 3 30 times	Goto 3 30 times	Goto 3 30 times
7.Final Extension	10 min 72°C	10 min 72°C	10 min 72°C
8.Hold	Forever 4°C	Forever 4°C	Forever 4°C
Expected Fragment Size	1370 bp (138318–139659)	1549 bp(138318–139687)	1391 bp (138318–139708)

Table A.6: RT–PCR Programs for verifying hsp60s mRNA Alternative 2

RT–PCR step	**Program, Primer**	**Program, Primer**	**Program, Primer**
	RT3AV1A	**RT3AV2A**	**RT3AV2B**
	53Start1v1	**53Start1v1**	**53Start1v1**
	5Stop1v1	**3Stop1v2**	**3Stop1v2**
1.Reverse Transcription	30 min 50°C	30 min 50°C	30 min 56°C
2.Initial PCR Activation	15 min 95°C	15 min 95°C	15 min 95°C
3.Denaturation	1 min 94°C	1 min 94°C	1 min 94°C
4.Annealing	1 min 50°C	1 min 55°C	1 min 55°C
5.Extension	1 min 72°C	1 min 72°C	1 min 72°C
6.Cycles	Goto 3 30 times	Goto 3 30 times	Goto 3 30 times
7.Final Extension	10 min 72°C	10 min 72°C	10 min 72°C
8.Hold	Forever 4°C	Forever 4°C	Forever 4°C
Expected Fragment Size	599 bp (138095–138693)	618 bp (138095–138712)	618 bp (138095–138712)

RT–PCR step	**Program, Primer**	**Program, Primer**	
	RT3AV2D	**RT3AV3A**	
	53Start1v1	**53Start1v1**	
	3Stop1v2	**5Stop1v3**	
1.Reverse Transcription	30 min 50°C	30 min 50°C	
2.Initial PCR Activation	15 min 95°C	15 min 95°C	
3.Denaturation	1 min 94°C	1 min 94°C	
4.Annealing	1 min 60°C	1 min 46°C	
5.Extension	1 min 72°C	1 min 72°C	
6.Cycles	Goto 3 30 times	Goto 3 30 times	
7.Final Extension	10 min 72°C	10 min 72°C	
8.Hold	Forever 4°C	Forever 4°C	
Expected Fragment Size	618 bp (138095–138712)	684 bp (138095–138678)	

RT–PCR step	**Program, Primer**	**Program, Primer**	**Program, Primer**
	RT3BV1A	**RT3BV2A**	**RT3BV3A**
	3Start2v1	**3Start2v1**	**3Start2v1**
	53Stop2v1	**53Stop2v2**	**3Stop2v3**
1.Reverse Transcription	30 min 50°C	30 min 50°C	30 min 50°C
2.Initial PCR Activation	15 min 95°C	15 min 95°C	15 min 95°C
3.Denaturation	1 min 94°C	1 min 94°C	1 min 94°C
4.Annealing	1 min 48°C	1 min 45°C	1 min 52°C
5.Extension	1 min 72°C	1 min 72°C	1 min 72°C
6.Cycles	Goto 3 30 times	Goto 3 30 times	Goto 3 30 times
7.Final Extension	10 min 72°C	10 min 72°C	10 min 72°C
8.Hold	Forever 4°C	Forever 4°C	Forever 4°C
Expected Fragment Size	575 bp (139085–139659)	603 bp (139085–139687)	624 bp (139085–139708)

Table A.7: RT–PCR Programs for verifying hsp60s mRNA Alternatives 3a and 3b

RT–PCR step	**Program, Primer**	**Program, Primer**	**Program, Primer**
	RT3BV4A	**RT3BV5A**	**RT3BV6A**
	53Start2v2	**53Start2v2**	**53Start2v2**
	53Stop2v1	**53Stop2v2**	**3Stop2v3**
1.Reverse Transcription	30 min 50°*C*	30 min 50°*C*	30 min 50°*C*
2.Initial PCR Activation	15 min 95°*C*	15 min 95°*C*	15 min 95°*C*
3.Denaturation	1 min 94°*C*	1 min 94°*C*	1 min 94°*C*
4.Annealing	1 min 48°*C*	1 min 45°*C*	1 min 52°*C*
5.Extension	1 min 72°*C*	1 min 72°*C*	1 min 72°*C*
6.Cycles	Goto 3 30 times	Goto 3 30 times	Goto 3 30 times
7.Final Extension	10 min 72°*C*	10 min 72°*C*	10 min 72°*C*
8.Hold	Forever 4°*C*	Forever 4°*C*	Forever 4°*C*
Expected Fragment Size	556 bp (139104–139659)	584 bp (139104–139687)	605 bp (139104–139708)

RT–PCR step	**Program, Primer**	**Program, Primer**	**Program, Primer**
	RT3BV7A	**RT3BV8A**	**RT3BV9A**
	3Start2v3	**3Start2v3**	**3Start2v3**
	53Stop2v1	**53Stop2v2**	**3Stop2v3**
1.Reverse Transcription	30 min 50°*C*	30 min 50°*C*	30 min 50°*C*
2.Initial PCR Activation	15 min 95°*C*	15 min 95°*C*	15 min 95°*C*
3.Denaturation	1 min 94°*C*	1 min 94°*C*	1 min 94°*C*
hline 4.Annealing	1 min 48°*C*	1 min 45°*C*	1 min 52°*C*
hline 5.Extension	1 min 72°*C*	1 min 72°*C*	1 min 72°*C*
6.Cycles	Goto 3 30 times	Goto 3 30 times	Goto 3 30 times
7.Final Extension	10 min 72°*C*	10 min 72°*C*	10 min 72°*C*
8.Hold	Forever 4°*C*	Forever 4°*C*	Forever 4°*C*
Expected Fragment Size	464 bp (139196–139659)	492 bp (139196–139687)	513 bp (139196–139708)

RT–PCR step	**Program, Primer**	**Program, Primer**	**Program, Primer**
	RT3BV10A	**RT3BV11A**	**RT3BV12A**
	5Start2v4	**5Start2v4**	**5Start2v4**
	53Stop2v1	**53Stop2v2**	**3Stop2v3**
1.Reverse Transcription	30 min 50°*C*	30 min 50°*C*	30 min 50°*C*
2.Initial PCR Activation	15 min 95°*C*	15 min 95°*C*	15 min 95°*C*
3.Denaturation	1 min 94°*C*	1 min 94°*C*	1 min 94°*C*
4.Annealing	1 min 48°*C*	1 min 45°*C*	1 min 48°*C*
5.Extension	1 min 72°*C*	1 min 72°*C*	1 min 72°*C*
6.Cycles	Goto 3 30 times	Goto 3 30 times	Goto 3 30 times
7.Final Extension	10 min 72°*C*	10 min 72°*C*	10 min 72°*C*
8.Hold	Forever 4°*C*	Forever 4°*C*	Forever 4°*C*
Expected Fragment Size	444 bp (139216–139659)	472 bp (139216–139687)	493 bp (139216–139708)

Table A.8: RT–PCR Programs for verifying hsp60s mRNA Alternative 3b

RT–PCR step	**Program, Primer**	**Program, Primer**	**Program, Primer**
	RT4AV1A	**RT4AV2A**	**RT4AV3A**
	3Start1v3	**3Start1v3**	**3Start1v3**
	5Stop1v1	**3Stop1v2**	**5Stop1v3**
1.Reverse Transcription	30 min 50°*C*	30 min 50°*C*	30 min 50°*C*
2.Initial PCR Activation	15 min 95°*C*	15 min 95°*C*	15 min 95°*C*
3.Denaturation	1 min 94°*C*	1 min 94°*C*	1 min 94°*C*
hline 4.Annealing	1 min 50°*C*	1 min 55°*C*	1 min 46°*C*
hline 5.Extension	1 min 72°*C*	1 min 72°*C*	1 min 72°*C*
6.Cycles	Goto 3 30 times	Goto 3 30 times	Goto 3 31 times
7.Final Extension	10 min 72°*C*	10 min 72°*C*	10 min 72°*C*
8.Hold	Forever 4°*C*	Forever 4°*C*	Forever 4°*C*
Expected Fragment Size	556 bp (138138–138693)	575 bp (138138–138712)	641 bp (138138–138778)

RT–PCR step	**Program, Primer**	**Program, Primer**	**Program, Primer**
	RT4AV4A	**RT4AV5A**	**RT4AV6A**
	5Start1v4	**5Start1v4**	**5Start1v4**
	5Stop1v1	**3Stop1v2**	**5Stop1v3**
1.Reverse Transcription	30 min 50°*C*	30 min 50°*C*	30 min 50°*C*
2.Initial PCR Activation	15 min 95°*C*	15 min 95°*C*	15 min 95°*C*
3.Denaturation	1 min 94°*C*	1 min 94°*C*	1 min 94°*C*
4.Annealing	1 min 50°*C*	1 min 53°*C*	1 min 46°*C*
5.Extension	1 min 72°*C*	1 min 72°*C*	1 min 72°*C*
6.Cycles	Goto 3 30 times	Goto 3 31 times	Goto 3 31 times
7.Final Extension	10 min 72°*C*	10 min 72°*C*	10 min 72°*C*
8.Hold	Forever 4°*C*	Forever 4°*C*	Forever 4°*C*
Expected Fragment Size	538 bp (138156–138693)	557 bp (138156–138712)	623 bp (138156–138678)

RT–PCR step	**Program, Primer**	**Program, Primer**	**Program, Primer**
	RT4AV7A	**RT4AV8**	**RT4AV9A**
	53Start1v5	**53Start1v5**	**53Start1v5**
	5Stop1v1	**3Stop1v2**	**5Stop1v3**
1.Reverse Transcription	30 min 50°*C*	30 min 50°*C*	30 min 50°*C*
2.Initial PCR Activation	15 min 95°*C*	15 min 95°*C*	15 min 95°*C*
3.Denaturation	1 min 94°*C*	1 min 94°*C*	1 min 94°*C*
4.Annealing	1 min 49.5°*C*	1 min 50°*C*	1 min 46°*C*
5.Extension	1 min 72°*C*	1 min 72°*C*	1 min 72°*C*
6.Cycles	Goto 3 31 times	Goto 3 31 times	Goto 3 31 times
7.Final Extension	10 min 72°*C*	10 min 72°*C*	10 min 72°*C*
8.Hold	Forever 4°*C*	Forever 4°*C*	Forever 4°*C*
Expected Fragment Size	376 bp (138318–138693)	395 bp (138318–138712)	623 bp (138318–138678)

Table A.9: RT–PCR Programs for verifying hsp60s mRNA Alternative 4a

Persönliche Daten

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Lebenslauf

2005	Gebietsrepräsentantin Baden–Württemberg für den Bereich Molekularbiologie/Verkauf Großgeräte bei der Firma Applied Biosystems/Applera Deutschland GmbH.
2003–2004	Annotation der TRANSFAC Database (Transkriptionsfaktoren–Datenbank) bei der Firma Biobase, Biological Databases GmbH Wolfenbüttel.
1999–2004	Wissenschaftliche Mitarbeiterin am Institut für Rechtsmedizin der Medizinischen Hochschule Hannover; Dissertation: "Variations in the Human hsp60 Gene between Cases of Sudden Infant Death Syndrome and non–affected Children".
1999	Analyse von Tierknochenfunden des Grundstücks Weberstr. 10 für die Bezirksregierung Braunschweig, Dezernat 406.
1998–1999	Auslandssemester an der University of California at Berkeley, USA.
1998	Analyse von Knochenfunden der arch"aologischen Ausgrabung „Burg Wolfstein“ in Neumarkt/Oberpfalz.
1997–1998	Diplomarbeit im Lehrgebiet Anthropologie, Institut für Zoologie. „Anthropologische Bearbeitung des slawischen, mittelalterlichen Gräberfeldes Tulln, Ländgasse“.
1989–1998	Studium der Biologie an der Gesamthochschule Kassel und Technischen Universität Braunschweig mit Abschluß Sehr Gut.
1989	Zeugnis der allgemeinen Hochschulreife.
1986–1989	Besuch der Albert–Schweitzer–Schule (Gymnasium) in Kassel.
1980–1986	Besuch des Wolfgang–Ernst–Gymnasiums in Büdingen/Wetterau.
1976–1980	Besuch der Grundschule in Bergheim/Erft.

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Curriculum Vitae

2005	Sales Representative Molecular Biology/Instruments in Baden–Württemberg at Applied Biosystems/Applera Deutschland GmbH.
2003–2004	Annotation of the TRANSFAC Database (Transcription factor database) at the Biobase Biological Databases Company Wolfenbüttel.
1999–2004	Graduate student at the Institute of Legal Medicine at the Medical School Hannover; doctoral thesis: "Variations in the Human hsp60 Gene between Cases of Sudden Infant Death Syndrome and non–affected Children".
1999	Freelance Work: Analysis of animal skeletal remains of the archaeological excavation Weberstr. 10 for the Department of City Archaeology, Braunschweig.
1998–1999	Semester abroad at the University of California at Berkeley, USA.
1997–1998	Field Work: Analysis of skeletal remains at the archaeological excavation "Burg Wolfstein" in Neumarkt/Oberpfalz.
1997–1998	Diploma thesis at the Department of Anthropology, Institute for Zoology, Technical University (TU) Braunschweig: "Anthropological Analysis of the Medieval, Slavonian Burial Ground of Tulln, Ländgasse (Austria)".
1989–1998	Undergraduate studies of Biology at the University of Kassel and the Technical University of Braunschweig.
1989	Final secondary-school examinations (University-entrance degree).
1986–1989	Secondary School Albert–Schweitzer–Schule (Gymnasium) in Kassel.
1980–1986	Secondary School Wolfgang–Ernst–Gymnasiums in Büdingen/Wetterau.
1976–1980	Elementary School in Bergheim/Erft.