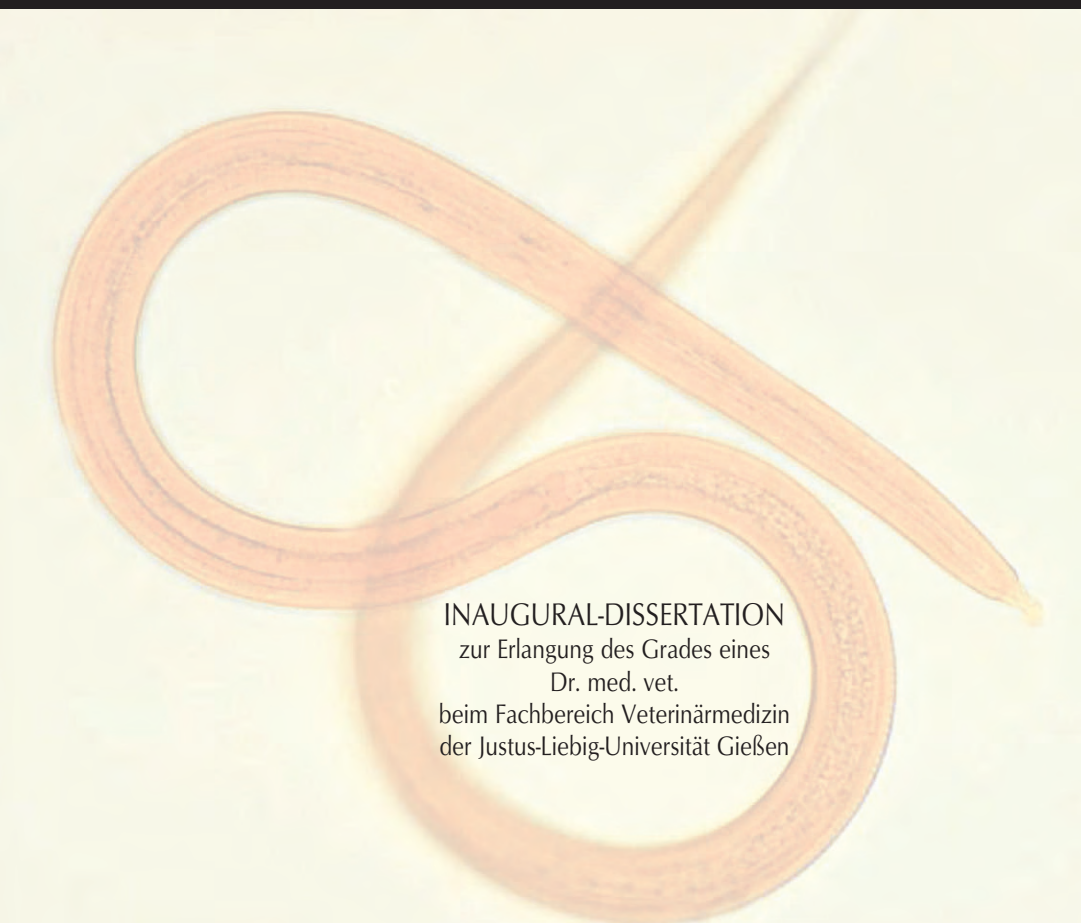
YASMINA TAZIR

STRONGYLOIDES RATTI: IDENTIFICATION, ISOLATION AND CHARACTERISATION OF HEAT SHOCK PROTEIN 10 AND HEAT SHOCK PROTEIN 60



INAUGURAL-DISSERTATION
zur Erlangung des Grades eines
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beim Fachbereich Veterinärmedizin
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eingereicht von

Yasmina Tazir
Tierärztin aus Wiesbaden

Giessen 2009

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1 Introduction

1.1 Intestinal parasites

Nematodes are extremely successful widespread organisms, many of which have evolved a parasitic life style, being relevant pathogens of humans and animals. More than two billion humans, mostly among the world's poorest (Awasthi *et al* 2003; WHO, 2003), are infected by nematodes and 3.5 billion are exposed to them (Chan 1994; Hotez 2008). The vast majority of the nematode burden of the human population is due to gastro-intestinal nematodes. The total number of people with high intensity infection in the world is 152 million for hookworms, 60 million for *Ascaris lumbricoides* and 45 million for *Trichuris trichiura* (Stephenson *et al* 2000). These infection burdens can be measured in terms of disability-adjusted life-years (DALYs) (Stephenson *et al* 2000; WHO World Health Report 2002). For theses three species of intestinal parasitic nematodes, it has been shown that 39 million DALYs are caused per year (*hookworm* 22.1 million; *A. lumbricoides* 10.5 million; *T. trichiura* 6.4 million). This is comparable to other significant infectious diseases: tuberculosis 46.5 million; malaria 35.7 million; measles 34.1 million (Stephenson *et al* 2000).

On the other hand, substantial evidence from animal models of human disease supports the hypothesis that infection with helminths can suppress the development of other maladies. In the past, there has been an exponential increase in the incidence of autoimmune and idiopathic inflammatory disorders such as inflammatory bowel disease (IBD), diabetes and atopic diseases in westernised societies, and a similar pattern is emerging in urbanised areas of developing countries (Smits & Yazdanbakhsh 2007).

This thesis focuses on the identification, isolation and characterisation of potential immune modulating molecules to better understand the parasite-host interaction.

Nematodes of the genus *Strongyloides* infect a wide variety of terrestrial vertebrate hosts (Speare 1987). *Strongyloides*, containing some 50 species of obligate gastrointestinal parasites, is a relatively common infection of wild and domestic animals, but also infects birds, reptiles and amphibians. *Strongyloides stercoralis* (*S. stercoralis*, threadworm), a species naturally infecting humans, is considered the fourth most important intestinal nematode infection, after hookworm, *A. lumbricoides*, and *T. trichiura*, with an estimate of 100-200 million infected people (Viney 2006; Viney *et al* 2007). It is endemic in tropical and subtropical countries, but also in the United States of America, Europe and Asia. Due to its low incidence in industrialised countries, misdiagnoses and medical errors may occur (Boulware *et al* 2007). Human infection occurs when

infective (filariform) larvae penetrate intact skin. This most commonly happens when the host's bare feet come in direct contact with soil contaminated with infective *Strongyloides* larvae (Figure 1.1). Once infected, the pathology of *Strongyloides* infection is often not overt (Genta & Caymmi Gomes 1989). However, because of the unique ability of *S. stercoralis* to complete its life cycle within the human host, the burden of worms can dramatically increase through a cycle of autoinfection. During autoinfection, the rhabditiform larvae become infective filariform larvae, which can penetrate either the intestinal mucosa (internal autoinfection) or the skin of the perianal area (external autoinfection). Cycles of auto-infection lead to chronic disease that can persist for several decades, more than 50 years. In either case, the filariform larvae may follow the route described in Figure 1.1, and migrate successively to the lungs, the bronchial tree, the pharynx and the small intestine, where they mature into adults, or they may disseminate widely in the body. To date, the occurrence of autoinfection in humans with helminthic infections is recognised only in *S. stercoralis* and *Capillaria philippinensis* infections. In the case of *S. stercoralis*, autoinfection may explain persistent infection in people who have not been in a disease-endemic area for many years and hyperinfection in immunodepressed patients. No other human parasitic nematode has been associated with such a broad spectrum of manifestations and different clinical syndromes as has *S. stercoralis*. Chronic infection with *S. stercoralis* often is clinically inapparent or can lead to cutaneous, gastrointestinal, or pulmonary symptoms. Gastrointestinal manifestations include vomiting, diarrhoea, abdominal discomfort, nausea, and anorexia. In contrast to other soil-transmitted helminthes (STH), the unique life cycle of *S. stercoralis* encompasses both obligate parasitic (direct, asexual, homogonic) and facultative free-living (indirect, sexual, heterogonic) generations (Viney 2006). These multiple peculiarities and singularities of *Strongyloides* infection stress the determining influence of the immune system and its partial protection of the host.

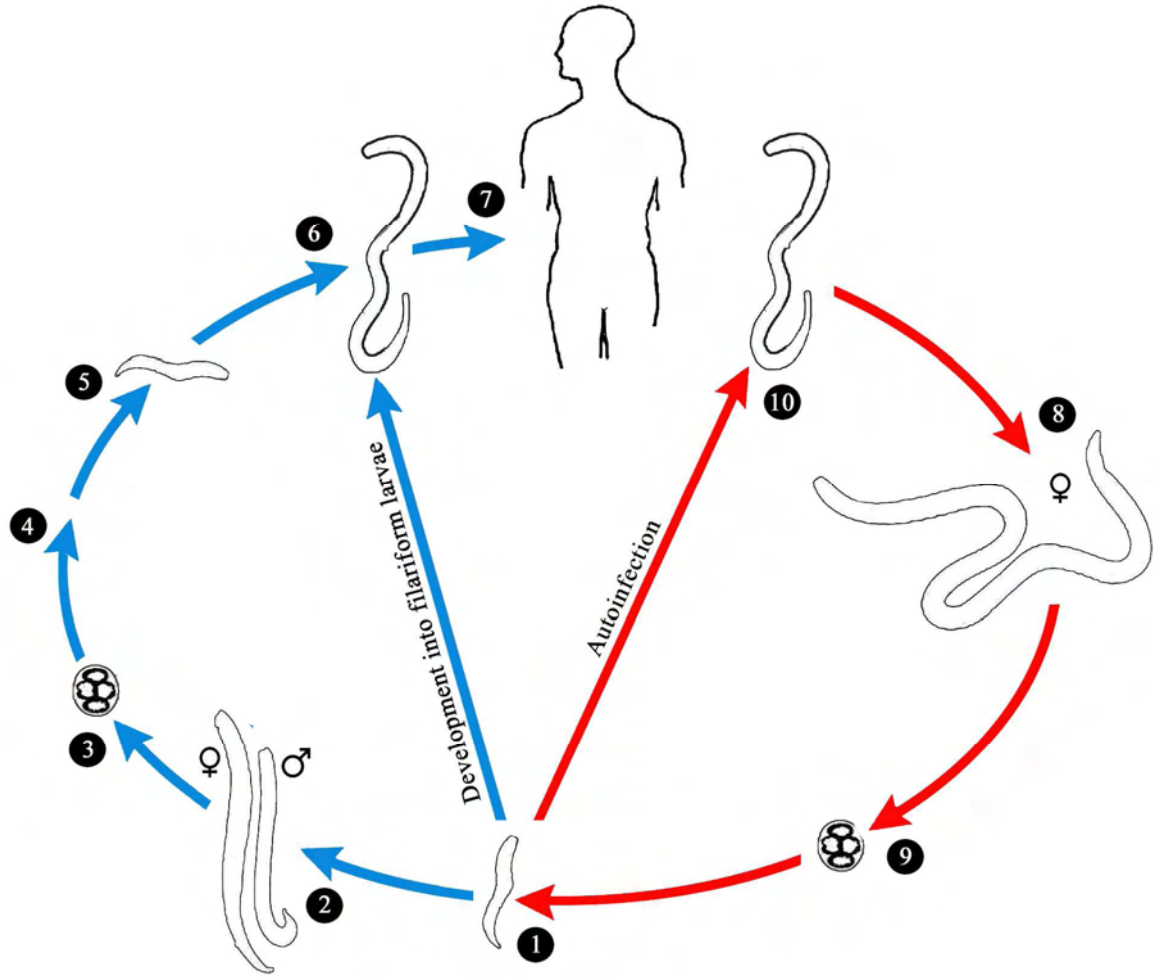

Figure 1.1: The life cycle of *Strongyloides stercoralis*

In the free-living cycle, the rhabditiform larvae passed in the stool (1) can either moult twice and become infective filariform larvae (6) or moult four times and become free-living adult males or females (2) that mate and produce eggs (3), from which rhabditiform larvae hatch (4). The rhabditiform larvae (5) in turn can develop into the infective filariform larvae (6). The filariform larvae penetrate the human host skin to initiate the parasitic cycle (7). In the parasitic cycle, filariform larvae are transported to the lungs, where they penetrate the alveolar spaces. They migrate through the bronchial tree to the pharynx, are swallowed and then reach the small intestine. Here, they moult twice and become adult female worms (8). The females live in the epithelium of the small intestine and, parthenogenetically, produce eggs (9), which yield rhabditiform larvae (1). The rhabditiform larvae (1) can either be passed in the stool or cause autoinfection (10).

Immunocompetent persons, when chronically infected with *S. stercoralis*, can control the worm burden at a very low level over decades, sustained by autoinfection. A hyperinfection and dissemination, often with fatal outcome, can occur only when the immune control collapses. Following immunocomprising conditions, e.g. immunosuppressive drug therapy (glucocorticoids, cyclosporine, azathioprine), hematologic malignancies, transplantations, HTLV-1 infection, hypogammaglobulinemia, tuberculosis or protein-caloric malnutrition syndrome, hyperinfection and dissemination, complete disruption of the mucosal structures or ulcerations have been

observed (Genta *et al* 1989; Fardet *et al* 2006). Dissemination may involve gut, stomach, lung and/or cerebrospinal fluid. Furthermore, larval penetration of the intestinal wall during dissemination may result in bacteraemia, due to the introduction of bowel flora. Recent reports indicate the underestimation of strongyloidiasis and its hyperinfection syndrome, which was evaluated as an emerging infectious disease that has migrated from developing regions to industrialised areas (Marcos *et al* 2008).

In the developed countries, helminth infections including strongyloidiasis in humans can be widely controlled by primary health care programmes and effective public sanitation. In developing counties, helminth diseases are still widespread and drug treatment does not protect against rapid re-infection (Anthony *et al* 2007). In general, the options for the treatment of helminth infections offer a range of different substances for example benzimidazoles, macrocyclic lactones, tetrahydrompyrimidines and emodepsides. The drug of choice for strongyloidiasis is the macrocyclic lactone ivermectin (www.dpd.cdc.gov.2008), which is derived from the bacterium *Streptomyces avermitilis* (Li *et al* 2008). It binds to and activates glutamate-gated chloride channels which can be predominantly found in neurons and myocytes of non-vertebrates. This leads to an influx of calcium ions causing hyperpolarisation of the cell membrane and ultimately death. Albendazole, belonging to the chemical class of benzimidazoles, is the recommended alternative (www.dpd.cdc.gov.2008) to ivermectin for treatment. Its proposed way of action is the inhibition of tubulin polymerisation in intestinal parasites which leads to metabolic interception including the loss of energy metabolism. These pathophysiological alterations lead to parasite death. Both drugs can be administered orally in non severe strongyloidiasis, whereas in hyperinfection syndrome a combination therapy has been proposed (Lim *et al* 2004). Most of the active substances were introduced into the market many years ago and were widely used in humans and animals. The resulting reduced efficacy of common anthelmintic drugs in veterinary medicine show the need for the development of new therapeutic agents for the treatment of parasitic nematode infections.

The diagnosis of strongyloidiasis can only be made in a laboratory because the only pathognomic clinical sign of a *Strongyloides* infection is the larva currens which does not necessarily occur. Baermann technique, Harada-Mori filter paper technique, Koga agar plate method and direct staining of faeces can be performed for the examination of stool samples. A single stool examination detects larvae in only 30% of infections, which makes it necessary to examine stool probes over several days. Other diagnostic tools include the detection of anti-strongyloides antibody, using the enzyme-linked immunosorbent assay (ELISA). However, the ELISA test cannot distinguish antibodies produced by past or current infections. A real-time

polymerase chain reaction (RT-PCR) targeting the small subunit of the rRNA gene was recently developed for the detection of *S. stercoralis* DNA in faecal sample (Verweij *et al* 2009). The use of this assay could facilitate monitoring the prevalence and intensity of *S. stercoralis* infections during helminth intervention programmes. Moreover, the use of this assay in diagnostic laboratories could make the introduction of molecular diagnostics feasible in the routine diagnosis of *S. stercoralis* infections, with a two-fold increase in the detection rate as compared with the commonly used Baermann sedimentation method. As an invasive method, endoscopy can reveal mucosal erythema and edema in the duodenum, and a probe of duodenal fluid can contain both eggs and larvae.

1.2 *Strongyloides ratti*

In comparison to *S. stercoralis*, the rat-invading parasite *Strongyloides ratti* (*S. ratti*) is an ideal organism to work with in the laboratory. The human parasite *S. stercoralis* can be maintained in dogs (Lok 2007) and gerbils (Nolan *et al* 1999). However, the use of these animals is costly, and of ethical concern. *S. ratti* has a parasitic and a non-parasitic phase, thus, in contrast to most other nematodes, there is no insect vector needed for the perpetuation of the cycle. The absence of an insect vector allows comparing parasitic and non-parasitic stages at the molecular level in order to study genes and gene products that might be important in the process of infection or in parasite survival within its host. Furthermore, due to the absence of an insect vector, the handling is less time consuming. Also, *S. ratti* has a remarkable life cycle, including both a parasitic and a free-living phase (Figure 1.2). The parasitic phase consists solely of adult parasitic females, which live embedded in the mucosa of the small intestine of rats. The eggs produced by these females are passed with the faeces into the external environment. Two types of development can then occur, termed homogonic and heterogonic. In homogonic (or direct) development, larvae moult via two larval stages into infective third-stage larvae (iL3s), which infect new hosts by skin penetration. The iL3 stage is developmentally arrested and will not develop further until it encounters a host. In heterogonic (or indirect development), larvae moult via four larval stages, finally maturing as free-living adult males and females. These mate, and the progeny develops, as in the homogonic development. Thus, despite being an obligate parasite, a significant part of the Strongyloides life cycle occurs outside the host. This has the practical advantage that the free-living stages can be readily grown and manipulated in faeces from infected animals. This life cycle is shared by all *Strongyloides spp*. However, there are species differences in the number of free-living generations that occur. For example, *Strongyloides planiceps* (a parasite of cats) was

found to undergo up to nine, decreasingly fecund, free-living generations (Yamada *et al* 1991). For *S. ratti* no more than one free-living generation has been observed.

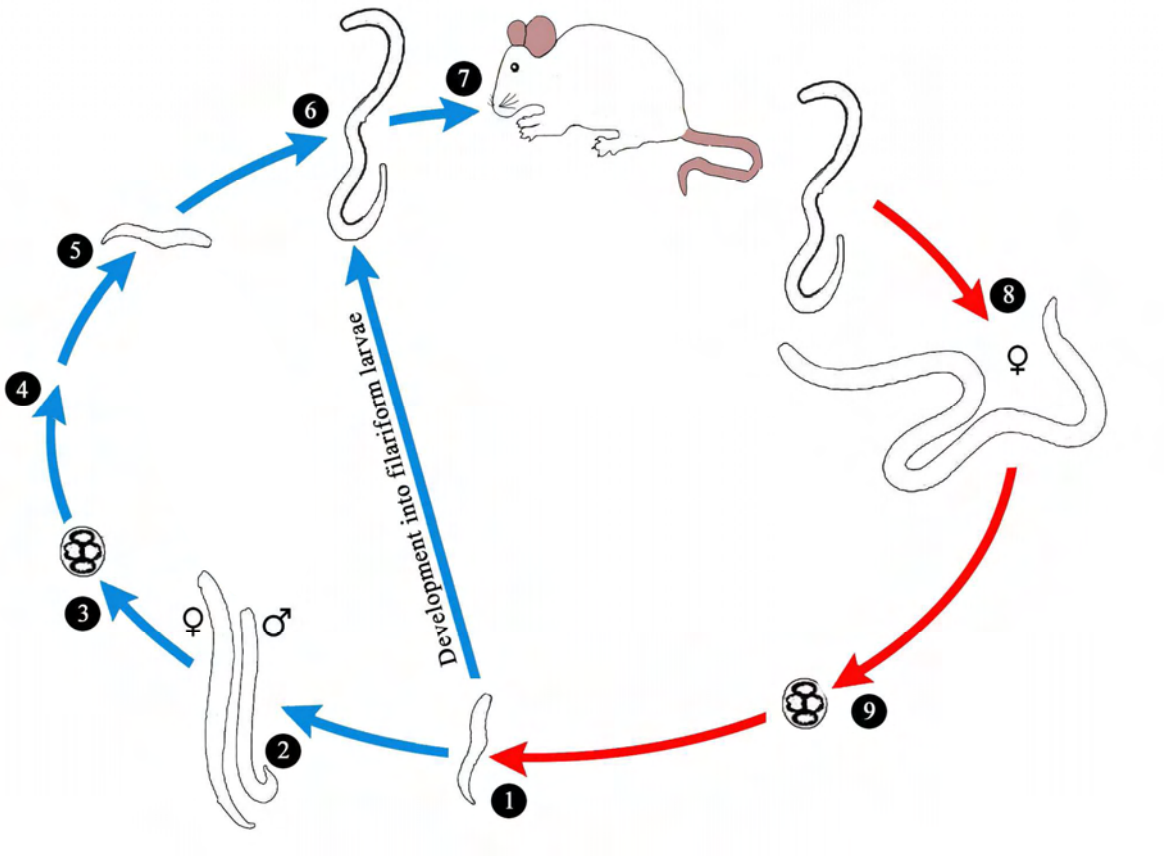

Figure 1.2: The life cycle of *Strongyloides ratti*

In the free-living cycle, the rhabditiform larvae passed in the stool (1) can either moult twice and become infective filariform larvae (6) or moult four times and become free-living adult males or females (2) that mate and produce eggs (3), from which rhabditiform larvae hatch (4). The rhabditiform larvae (5) in turn can develop into the infective filariform larvae (6). The filariform larvae penetrate the rat host skin to initiate the parasitic cycle (7). In the parasitic cycle are transported to the lungs, where they penetrate the alveolar spaces. They migrate through the bronchial tree to the pharynx, are swallowed and then reach the small intestine. Here, they moult twice and become adult female worms (8). The females live embedded in the epithelium of the small intestine and, parthenogenetically, produce eggs (9), which yield rhabditiform larvae (1). The rhabditiform larvae are passed in the stool.

1.3 Heat shock proteins

Heat shock proteins (HSPs) comprise ubiquitous, highly conserved molecular chaperones and proteases that are essential for cell viability. Furthermore, HSPs are required by many proteins to reach their native conformation (Hartl & Hayer-Hartl 2002). Selected HSPs, also known as chaperones, play crucial roles in folding/unfolding of proteins, assembly of multiprotein complexes, transport/sorting of proteins into correct subcellular compartments, cell-cycle control and signalling, and protection of cells against stress or apoptosis. HSPs have been implicated in infectious diseases, being primary targets for the host immune system, and several HSPs have been found to be immunogenic (Zugel & Kaufmann 1999b). In particular HSP60 attracted attention as a highly immunogenic molecule that activates T cells (Kaufmann 1992) and cross-reacts with eukaryotic HSP has been implicated in autoimmune and inflammatory diseases (Kiessling *et al* 1991). Chaperonins belong to the group of chaperones and are divided in two groups. Group I chaperonins, such as chaperonin 60 (Hemmingsen *et al* 1988) also known as HSP60 and called groEL in bacteria, require a co-chaperone to carry out the refolding reaction, HSP10 also known as GroES in bacteria. The complex structure of HSP60 and HSP10 was elucidated at high resolution (Xu *et al* 1997). The functional unit of the HSP60 species is a homotetradecameric complex consisting of two stacked rings with seven subunits each. HSP10 heptamers form the lid. Group II chaperonins are found in archaea and the eukaryotic cytosol, where a single polypeptide, called thermosome or TRiC, respectively, exerts the function of both HSP60 and HSP10. Detailed descriptions of the chaperonin-mediated folding cycle have been proposed (Bukau & Horwich 1998, Walter & Buchner 2002). It is thought that unfolded polypeptides are captured through hydrophobic contacts within the chaperonin barrel and then forced to refold in the central cavity. Once the native conformation is reached, the protein is released into the cytoplasm. The HSP60/10 complex is believed to be responsible for accelerating the folding of polypeptides imported into mitochondria. Furthermore, the complex reactivates denaturated proteins, and diminishes aggregation of non-native polypeptides and partially unfolded kinetically trapped intermediates. Its potential to smooth the energy landscape necessary for the folding and to prevent intermolecular interactions between non-native polypeptides has been widely investigated (Ziemienowicz *et al* 1993, Weissman *et al* 1995, Bukau & Horwich 1998, Brinker *et al* 2001, Walter & Buchner 2002).

1.3.1 Heat shock protein 60 (HSP60)

HSP60 is a highly conserved and ubiquitously expressed protein. It belongs to the chaperonin family, which is subject to inducible expression following heat shock (Lund 1995). HSP60 is one of the major molecular chaperones in both stressed and unstressed cells. In mammalian cells it is primarily found in mitochondria (Soltys & Gupta 1996; Gupta 1990). The HSP60 found in mitochondria is the mature form of the protein that lacks the mitochondrial targeting sequence (Ikawa & Weinberg 1992; Jones *et al* 1994; Khan et *al* 1998). Besides being part of the protein folding machinery, HSP60 has a number of significant functions in the immune response of an organism. Immune responses to HSP60 from bacteria, protozoa, fungi and helminths have frequently been detected after infection with these organisms. Moreover, during bacterial infections, the bacterial members of the HSP60 family (GroEL) are common targets of humoral and cell-mediated immune responses in mammals (Kaufmann 1992; van Noort *et al* 1995). Analysis of T cell responses in mice immunised with *Mycobacterium tuberculosis* showed that ~10-20% of the *M. tuberculosis*–specific T cells respond to *M. tuberculosis* HSP60 (Kaufmann *et al* 1987). They were identified as the main goals for antibodies and T-cell immune responses in patients infected by pathogenic bacteria, fungi or parasites (Weigl *et al* 1999). Furthermore, various studies have shown that HSP-reactive T cells have an immunoregulatory phenotype, indicating that in particular HSP60 is an autoantigen with the potential to trigger immunoregulatory pathways, which can suppress immune responses that occur in human inflammatory diseases, such as rheumatoid arthritis, type 1 diabetes, and possibly atherosclerosis and allergy (Graeff-Meeder *et al* 1991; Jones *et al* 1993; Xu *et al* 1993; Elias *et al* 1990; Barton *et al* 1998). This has led to an interest in studying HSP molecules with respect to the biology of parasitism, from the dual perspectives of up-regulated expression by parasites coming from a poikilothermic environment entering a homothermic environment, and over-expression by host cells responding to the stress of infection (Maresca & Carratu 1992; Young 1990). Auto-immune responses to HSP60, potentially triggered by infection, have been associated with the pathogenesis of a range of illnesses– including rheumatoid arthritis, atherosclerosis, diabetes mellitus and gastro-duodenal disease.

1.3.2 Heat shock protein 10 (HSP10)

Among other presumptive functions such as immunosuppressive activity (Akyol *et al* 2006), HSP10 serves as a co-chaperone for HSP60 during protein folding. HSP10 is a heptameric complex and functions as “lid” for the HSP60 cavity. The opening and closing of the cage depends on the ATPase activity of HSP60 (Ellis 2003). Besides being localised in the matrix compartment of mammalian mitochondria, it is localised in the cellular localisations, such as zymogene granules, hormone granules, secretory granules, and mature red blood cells (Sadacharan *et al* 2001). Many clinical studies have shown the discrepancy between HSP60 and HSP10 expression in normal (Cappello *et al* 2004) and tumoral (Cappello *et al* 2003b) cells. High levels of HSP10 in the cytoplasm of cancer cells have been reported in the past (Sadacharan *et al* 2001, Cappello *et al* 2003a, 2003c; Hansen *et al* 2003). HSP10 was additionally related to immunosuppressive activity (Akyol *et al* 2006). A protein corresponding to HSP10 was originally independently identified as early pregnancy factor (EPF) (Morton *et al* 1992). 70% of the human platelet-derived EPF amino acid sequence has been purified (Cavanagh & Morton 1994). Except for a single residue, the sequence is identical to that of rat HSP10. Not only the identical amino acid sequence is surprising, but also the differences in cellular localisation and function. EPF is now known to be essential for the initiation and maintenance of pregnancy (Athanasas-Platis *et al* 1989, 1991, 2000), but is not confined to gestation. EPF is secreted by normal, transformed, and neoplastic cells during growth and division, but not during quiescent phases and is required for continued cellular growth, both in vitro and *in vivo* (Quinn *et al* 1990, 1994; Quinn & Morton 1992). In addition to growth-regulatory effects, EPF exhibits immunomodulatory properties (Morton 1998). It suppresses the delayed type hypersensitivity (DHT) response (Noonan *et al* 1979; Zhang *et al* 2003), prolongs skin graft survival time (Morton *et al* 2000) and suppresses clinical signs of experimental autoimmune encephalomyelitis (EAE) (Zhang *et al* 2000, 2003), the animal model of multiple sclerosis (MS).

Nevertheless, little is known to date about either the physical interactions of HSP10 with other proteins within the cell, or its involvement in signal transduction pathways. Most literature describes a well-known interaction between HSP10 and HSP60 (Ryan *et al* 1997). According to my knowledge, nothing is known about the role of HSP10 and HSP60 in the nematode *S. stercoralis* and *S. ratti*.

1.4 Objective of this thesis

Strongyloidiasis is a neglected disease, mostly confined to tropical regions. According to World Health Organisation (WHO) estimates, 100 to 200 million individuals are infected by *Strongyloides* (Awasthi *et al* 2003; WHO, 2003). Individuals, who are immunosuppressed either by drug treatment or by other disease, e.g. HTLV, are susceptible to a disseminated, and usually fatal, *Strongyloides* infection. The hyperinfection syndrome has a high mortality rate (15% to 87%). Once diagnosed, the disease may be managed effectively with anthelmintic drugs, including ivermectin. But the hyperinfection syndrome causes diverse symptoms and signs, with unusual manifestations leading to misdiagnosis and medical errors. Hence, the hyperinfection syndrome is an emerging tropical infection migrating to developed countries and requires attention (Marcos *et al* 2008).

To better understand the parasite-host relationship, the study of host defence-modulating molecules in parasitic systems may likely reveal new principles of their function. HSP60 and HSP10 are of fundamental significance for protein biosynthesis, have a potential significance for the host immune response, and can be utilised as immunodominant antigens for vaccination. Moreover, these two proteins may, on the other hand, play a role as model for other helminth parasite proteins, revealing novel ways to manipulate the human immune system to treat autoimmune and idiopathic inflammatory diseases. Therefore, the objective of this study was to identify, isolate and molecularly characterise the *S. ratti* HSP10 (*Sr*HSP10) and *S. ratti* HSP60 (*Sr*HSP60) genes and proteins.

2 Materials and Methods

2.1 Reagents, disposals, instruments

2.1.1 Solutions and buffers

Solutions & buffers	Composition	Application
APS:	10% *ammonium* peroxodisulphate (Amersham) in dH_2O	Protein gel
Acetylation buffer:	100 mM triethanolamine (Sigma), 5 M 0,9% NaCl, 250ml/l acetic anhydride (Sigma)	Kryo histology
Cracking buffer (stock solution):	8 M urea; 5% (w/v) SDS; 40 mM Tris (pH 6.8); 0.1 mM EDTA; 0.4 mg/ml bromphenol blue; in dH_2O	Yeast protein extraction
Buffer 1:	100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween	*In situ* hybridisation
Buffer 2:	100 mM Tris-HCl, pH 9.5; 100 mM NaCl	*In situ* hybridisation
Buffer a	2xSSC; 0.5% SDS	Southern blot
Buffer b	0.2x SSC; 0.1XSDS	Southern blot
Buffer B:	8 M urea, pH 8.1; 10 mM Tris-HCl; 100 mM NaH_2 PoH_4	Protein purification
Buffer C:	8 M urea, pH 6.3; 10 mM Tris-HCl; 100 mM NaH_2 PoH_4	Protein purification
Buffer D:	8 M urea, pH 5.9; 10 mM Tris-HCl; 100 mM NaH_2 PoH_4	Protein purification
Buffer E:	8 M urea, pH 4.5; 10 mM Tris-HCl; 100 mM NaH_2 PoH_4	Protein purification
Blotting buffer (Bjerrum-Schöfer-Nielsen Buffer):	48 mM Tris, 39 mM glycine, 20% methanol; pH 9.2	Western blot
Coomassie blue staining solution:	0.05% (w/v) Coomassie brilliant-blue R-250; 40% ethanol; 10% ice acetic acid; 50% dH_2O	Protein gel staining

Coomassie blue destaining solution:	40% ethanol; 10% ice acetic acid 50% dH_2O	Protein gel staining
CTAB:	5% CTAB (trimethyl cetylammonium bromide) 0.5 M NaCl	Mini scale plasmid preparation
DEPC- dH_2O:	0.1% diethylpyrocarbonate in dH_2O; autoclaved	RNA isolation
Developer solution:	2.5% Na_2CO_3, 0.01% formaldehyde (v/v); pH 11.3-11.8 at 25°C	Silver staining
Ethidium bromide (EtBr):	1 g/100 ml dH_2O; stored shaded	Staining of nucleic acids
Fixation buffer I:	30% ethanol (v/v); 10% acetic acid (v/v)	Silver staining
Fixation buffer II:	30% ethanol (v/v); sodium acetate 0.5 M; 25% glutaraldehyde (v/v); 0.2% $Na_2S_2O_3$ (v/v)	Silver staining
Herring sperm DNA:	10 mg/ml in H_2O; boil in microwave 3x; cool on ice; 15 min ultra sound; freeze	Southern blot analysis
Loading buffer:	0.1% bromphenol blue; 0.1% xylencyanol; 50% glycerol; in 1 x TAE-buffer	DNA gel electrophoresis
PBS (10x):	1.37 M NaCl; 27 mM KCl; 74 mM Na_2HPO_4; 15 mM KH_2PO_4; dH_2O ad 1 l (pH 7.0 – 7.2); autoclaved	Washing buffer
Phenol:	phenol, saturated with equivalent volume 0.5 mM Tris pH 7.8	Nucleic acid purification
PEG solution:	50% PEG 1500 (polyethylene glycol)	Yeast transformation
PEG/LiAc solution:	40% PEG 4000; 1 x TE-solution; 1 x LiAc-solution	Yeast transformation
Polyacrylamide stock solution:	0.8% N'N'-methylbisacrylamide; 30% acrylamide; in dH_2O (Rotiphorese® Gel 30; Carl Roth)	Protein gel preparation
Ponceau-red S:	2% ponceau-red S; 30% sulfoacyl acid; 30% sodium deoxycholate aqueous; in dH_2O	Western blot
Proteinase K buffer:	Qiagen	*In situ* hybridisation
RNA Loading buffer:	900 µl formamide (deionised); 100 µl MOPS-buffer (1 x); 160 µl formaldehyde; 20 µl bromphenol blue (1% in DEPC-dH_2O); 20 µl xylene cyanol (1% in DEPC-dH_2O); 10 µl ethidium-bromide (10 µg/µl)	RNA gel electrophoresis
SDS-PAGE stacking gel buffer (4 x):	0.5 M Tris (pH 6.8); 0.4% SDS	Protein gel preparation
SDS-PAGE running buffer (10 x):	1.92 M glycine; 250 mM Tris; 10% (w/v) SDS; dH_2O ad 1 l (pH 8.3; to be adjusted before SDS addition)	Protein gel preparation

SDS-PAGE running buffer (1 x):	100 ml 10 x stock solution; dH_2O ad 1 l	Protein gel preparation
SDS-PAGE sample buffer:	8 ml glycerin; 4 ml ß - mercaptoethanol; 12 ml 20% SDS; 16 ml 4 x staking gel buffer; (if necessary 4% bromphenol-blue)	Protein gel preparation
SDS-PAGE separation buffer (4 x):	1.5 M Tris (pH 8.8); 0.4% SDS	Protein gel preparation
SSC (20 x):	3 M NaCl; 0.3 M trisodium citrate $2H_2O$; dH_2O ad 1 l; pH 7.0, autoclaved	*In situ* hybridisation, Southern blot analysis
SSPE(20 x):	3 M NaCl; 0,2 M $NaH_2PO_4xH_20$; 0.02 M EDTA; pH 7.4 with NaOH	Southern blot analysis
Staining solution:	0.1% silver nitrate (v/v); 0.01% formaldehyde (w/v)	Silver staining
STET-buffer:	8% sucrose; 50 mM Tris (pH 8.0); 50 mM EDTA; 5% Triton X-100	Mini-scale plasmid preparation
Stop solution:	0.005 M EDTA sodium salt	Silver staining
TAE-buffer (50 x):	2 M Tris base, 50 mM EDTA, 5.71% glacial acetic acid pH 8.0	DNA gel electrophoresis
TE-solution (10 x):	0.2 M Tris; 10 mM EDTA; pH 7.5; autoclaved	Yeast transformation
1T 1/10E (1x):	10 mM Tris, pH 8.0; 1 mM EDTA, pH 8.0, autoclaved	gDNA precipitation
TEMED:	tetramethylethylenediamine, Amersham Biosciences	Protein gel preparation
TRIzol®-Reagenz:	phenyl, guanidine-isothiocyanate (GIBCO)	RNA isolation
Washing buffer A:	2 x SSC; 0.5% SDS	Southern blot analysis
Washing buffer B:	0.2 x SSC; 0.1 x SDS	Southern blot analysis
X-Gal-stock solution:	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-Gal) in N, N -dimethylformamide (DMF); 20 mg/ml	ß-galactosidase assay
Z-buffer:	60 mM $Na_2HPO_4 \cdot 7H_2O$; 40 mM $NaH_2PO_4 \cdot H_2O$; 10 mM KCl; 1 mM MgSO4·$7H_2O$; pH 7.0; autoclaved	ß-galactosidase assay
Z-buffer/X-Gal-solution:	100 ml Z-buffer; 0.27 ml β-mercaptoethanol; 1.67 ml X-Gal stock solution	ß-galactosidase assay

2.1.2 Media and additives

Media & additives	Composition	Application
DYT-medium:	1.6% bacto tryptone; 1% bacto yeast extract; 100 mM NaCl	*E. coli* - medium (fluid)
LB-agar:	see LB-medium; additional 15 g/l agar; autoclaved	*E. coli* - medium (solid)
SOC-medium:	2% bacto-tryptone, 0.5% yeast-extract; 10 mM NaCl; 2.5 mM KCl; 5 N NaOH pH 7.0; autoclaved; subsequent addition of 10 mM $MgCl_2$; 10 mM glucose solution (sterile)	*E. coli* - medium [following heat shock] (fluid)
Ampicillin:	Ampicillin trihydrate [D-(-)-α-aminobenzyl penicillin]; stock conc. 100 m/ml dH_2O; end conc. 100 µg/ml	LB-medium and LB-plate additives (selection)
X-Gal:	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside; stock conc. 2% in dimethylformamide; end conc. 0.004%	Blue-white selection
IPTG:	isopropylthiogalactoside; stock conc. 1 M in dH_2O; end conc. 0.2 mM	Blue-white selection
YPD-medium:	20 g/l peptone; 10 g/l yeast extract; 950 ml dH_2O; autoclaved; subsequent addition of 50 ml 40% glucose solution (sterile)	Yeast fluid culture (full medium)
YPD-plates:	see YPD-medium; additional 20 g/l agar; autoclaved; subsequent addition of 50 ml 40% glucose solution (sterile)	Yeast culture plates (full medium)
YPDA-medium:	see YPD-medium; additional 0.2% adenine hemisulfate solution (sterile; end conc. 0.003%)	Yeast fluid culture (full medium)
SD-medium:	6.7 g/l yeast nitrogen base (YNB, without amino acids); addition of the corresponding 100 x amino acid stock solution; dH_2O ad 950 ml; autoclaved; subsequent addition of 50 ml 40% glucose solution (sterile)	Yeast fluid culture (minimal medium)
SD-Agar:	see SD-medium; additional 20 g/l agar; autoclaved; subsequent addition of 50 ml 40% glucose solution (sterile)	Yeast culture plates (minimal medium)
SG-medium:	6.7 g/l yeast nitrogen base (YNB, without amino acids); 20 g galactose; dH_2O ad 1 l; autoclaved	Yeast fluid culture (minimal medium)
Amino acids (Merck; Sigma):	adenine (20 mg/l), L-histidine (20 mg/l), L-leucine (100 mg/l), L-lysine (30 mg/l), L-methionine (20 mg/l), L-tryptophan (20 mg/l), uracil (20 mg/l); prepared in 100 x stocks in dH_2O	SD-medium and SD plate additives

2.1.3 Enzymes

Enzymes	Company/Origin	Description
alkaline phosphatase	New England Biolabs	Calf Intestinal Mucosa Phosphatase (CIP)
DNase	Roche Diagnostics, Mannheim, Germany	RNase-free DNase I
Proteinase K	Qiagen	Cystein-protease
Restriction enzymes	New England Biolabs, Fermentas, Roche	Type II restriction endonuclease
Reverse transcriptase	Qiagen Invitrogen	SensiScript SuperScriptII, SuperScriptIII
RNaseA	Roche	DNase-free RNase
RNasin	Promega	RNase-inhibitor
Taq-polymerase	Invitrogen	DNA-polymerase
T4-DNA-ligase	New England Biolabs, Fermentas	DNA ligation
T7- , T3- RNA-polymerase	Roche	*In situ* hybridisation

2.1.4 Molecular weight standards

- DNA Standard: 1 KB ladder (Invitrogen)
  75; 135; 154; 201; 220; 298; 344; 396; 506; 1,010; 1,636; 2,036; 3,054; 4,072; 5,090; 6,108; 7,126; 8,144; 9,162; 10,180; 11,198; 12,216 [bp]
- RNA Standard: RNA Ladder High Range (Fermentas)
  6,000; 4,000; 3,000; 2,000; 1,500; 1,000; 500; 200 [bp]
- Protein Standard: Roti-Mark 10-150 (Carl Roth)
  150; 100; 80; 60; 40; 30; 20; 10 [kDa]

2.1.5 Primer

The primers sequences and temperature T_m values used in this study are as given below. The T_m values were calculated using the formula $T_m = 2x (A + T) + 4x (G + C)$. Artificially added restriction sites are printed in grey, start- or stop-codons are bold.

Primer name	Tm (°C)	Primer sequence (5' → 3')
SR_HSp60_RACE_GSP_F	68°C	gcc att gct aca gga gct aaa gtc ttt g
SR_HSp60_RACEGSP_R	67°C	ctt tag ctc ctg tag caa tgg caa tat c
Spliced Leader (SL1)	56°C	ctc aaa ctt ggg taa tta aac c
HSP60_PCR1_FW	50°C	ggt tca aga t g ttg cag
HSP60_PCR1_RV	55°C	ctt cag caa tgg taa taa gtg
HSP60_PCR2_FW	56°C	gtt cca gct ctt gaa tta gc
HSP60_PCR2_RV	55°C	caa gaa ctt tga cag cac g
HSP60Sal-For (*Sal*I)	72°C	a gtc gac atg ctt ctt tcc gct gtt cg tag
HSP60Stop_rev	67.64°C	tta gaa cat tcc acc acc cat tcc gcc
HSP60-Xba-rev (*Xba*I)	72°C	atc tag att aac cac cca ttc cgc cca tac ct

HSP60Xba-rev-D50 (*Xba*I)	68°C	atc tag att aaa taa tac cag ttt gga tca tat caa ca
HSP60_Sonde1_for	59.86°C	gtcaaagatcttaaatttggagctga
HSP60_Sonde1_rev	59.86°C	acggctaatactttcaaatccttctt
pJC45HSP60_HindIII (*Hind*III)	64.63°C	aagcttatgcttcgtcttgctgctcataat
pJC45HSP60_52HindIII (*Hind*III)	64.64°C	ggaagcttgctaaagatcttaaatttggagc
pjC45HSP60_BamHIR (*Bam*HI)	64.63°C	ggatccttagaacattccaccacccattcc
GSP_HSP60rev3'UTR	61.3°C	ggaaagattggaagcagaaggtaaaa
SSHSP10FAN	65°C	ctt act gtt acc cgt aga tgt tgt tc
SSHSP10Rend2	62°C	gtt ggt gag ttt agc aat gag atc
OdT-T7 primer	78°C	gag aga gga tcc aag tac taa tac gac tca cta tag gga gat ttt ttt ttt ttt ttt ttt ttt ttt
gene specific 1	69°C	agt acg aag tcc tgg acc agc agc
ODT-T7 II	74°C	gag aga gga tcc aag tac taa tac gac tca cta tag g
SL	56°C	ggt tta att acc caa gtt tga g
gene specific 2	69°C	gct gct ggt cca gga ctt cgt act
HSP10salfor (*Sal*I)	72°C	a gtc gac atg ctt ctt tcc gct gtt cgt ag
HSP10xbarev (*Xba*I)	67°C	a tct aga tta gtt agt gag ttt ggc gat ga
HSP10Sondefor14	62.86°C	ct gtt cgt aga tgt tca tca gct c
HSP10Sonderev330	61.15°C	tta gtt agt gag ttt ggc gat gag
HSP10rev174xba (*Xba*I)	66°C	tct aga **tta** acc atc ctc agt acg aag tcc
HSP10for168sal (*Sal*I)	67.98°C	gtc gac ggt cca gga ctt cgt act

HSP10BDpAct3sense (*Sal*I, *Xba*I)	74.78°C	tcg acg ctg ctg aag taa aat cta aag gag gta ttt aca ttc cag aga aag ccc aag gaa ag gtt ctt gaa ggt **taa** t
HSP10BDpAct3Antisense (*Sal*I, *Xba*I)	74.78°C	ct aga **tta** acc ttc aag aac ctt tcc ttg ggc ttt ctc tgg aat gta aat acc tcc ttt aga ttt tac ttc agc agc g
pBridgeHSP10EcoR1for (*Eco*RI)	66°C	agaa ttc atc ttc ttt ccg ctg ttc gta g
pBridgeHSP10Sal1rev (*Sal*I)	67.29°C	a gtc gac tta gtt gtg agt ttg gcg atg ag
pAct2HSP10Nco1for (*Nco*I)	69.78°C	acc atg gag atg ctt ctt tcc gct gtt cgt ag
pAct2HSP10EcoR1rev (*Eco*RI)	64.64°C	agaa ttc tta gtt agt gag ttt ggc gat gag
HSP10for168NCOI (*Nco*I)	69.16°C	acc atg gag ggt cca gga ctt cgt act
HSP10rev174EcoRI (*Eco*RI)	66°C	gaa ttc **tta** acc atc ctc agt acg aag tcc
HSP10BDpAct2Sense (*Eco*RI, *Nco*I)	75.56°C	c atg gag gct gct gaa gta aaa tct aaa gga ggt att tac att cca gag aaa gcc caa gga aag gtt ctt gaa ggt taa g
HSP10BDpAct2Antisense (*Eco*RI, *Nco*I)	74.54°C	aa ttc **tta** acc ttc aag aac ctt tcc ttg ggc ttt ctc tgg aat gta aat acc tcc ttt aga ttt tac ttc agc agc ctc
HSP10pJC45for (*Nde*I)	67.75°C	ggg cat atg ctt ctt tcc gct gtt cg
HSP10pJC45rev (*Eco*RI)	59.44°C	gaa ttc tta gtt agt gag ttt ggc
pAcSG2_HSP10EcoRIfor (*Eco*RI)	64.6°C	gaa ttcacc atg ctt ctt tcc gct gtt
pAcSG2_HSP10NotIrev (*Not*I)	78.07°C	gc ggc cgc cta gtg atg gtg gtg atg atg ttt atc atc gtc gtc ttt gta gtc gtt agt gag ttt ggc gat
GSP_HSP10_330-304for	61.44°C	aat caa tca ctc aaa ccg cta ctc ta
GWHSP10revnested	64.4°C	cat cta cga aca gcg gaa aga agc ata

2.1.6 Plasmids

Plasmid	Description	Company/Origin
pGemTeasy	Cloning vector	Promega
TOPT TA	Cloning vector	Invitrogen
pBluescript II SK +/–	Cloning vector, Southern blot, *in situ* hybridisation	Fermentas
pBINDdelta renilla	Mammalian two-hybrid GAL4-BD vector	Promega, modified by Dr. Borgmeyer, ZMNH
pAct	Mammalian two-hybrid GAL4-AD vector	Promega
pBridge	Yeast two-hybrid GAL4-BD vector	Clontech
pAct2	Yeast two-hybrid GAL4-AD vector	Clontech
pJC45	Expression vector	Kindly provided by Dr. J. Closs BNI, Hamburg
pAcSG2	Baculo virus transfer vector	BD Bio Sciences

The following recombinant plasmids have been constructed on the basis of the above-mentioned plasmids (* NucleoBond Xtra Midi (Plus) (Macherey and Nagel 2007) plasmid prep preparation). All mini and midi plasmid preps were sequenced.

Plasmid names	Description	Origin
TOPOTA_HSP10	Insert: *S. ratti* HSP10 sequence obtained from *S. stercoralis* primers, lacking 3'and 5'end Backbone: TOPOTA	Self made
TOPOTA_HSP10_3'RACE	Insert: HSP10 3' RACE product Backbone: TOPOTA	Self made
TOPOTA_HSP10_5'SL	Insert: HSP10 5' Spliced leader product Backbone: TOPOTA	Self made
pGemTeasy_HSP10_FL	Insert: HSP10 full-length cDNA sequence (5'*Sal*I, 3'*Xba*I) Backbone: pGemTeasy	Self made

pGemTeasy_HSP10_FL	Insert: HSP10 full-length cDNA sequence for pBridge cloning (*5'Eco*RI, *3'Sal*I) Backbone: pGemTeasy	Self made
pGemTeasy_HSP10_FL	Insert: HSP10 full-length cDNA sequence for pAct2 cloning (*5'Nco*I, *3'Eco*RI) Backbone: pGemTeasy	Self made
pGemTeasy_HSP10_FL	Insert: HSP10 full-length cDNA sequence for *E. coli* expression (5'*Nde*, 3'*Eco*RI) Backbone: pGemTeasy	Self made
pGemTeasy_HSP10_FL	Insert: HSP10 full-length cDNA sequence for pACSG2 cloning (*5'Nco*I, *3'Eco*RI) Backbone: pGemTeasy	Self made
pGemTeasy_HSP10_BD	Insert: HSP10 binding domain sequence (*5'Nco*I, *3'Eco*RI) Backbone: pGemTeasy	Self made
pGemTeasy_HSP10_5'	Insert: HSP10 5' end cDNA sequence gained from SL PCR Backbone: pGemTeasy	Self made
pGemTeasy_HSP10_3'	Insert: HSP10 3' end cDNA sequence gained from RACE PCR Backbone: pGemTeasy	Self made
PCR.2.1HSP10GEN	Insert: HSP10 subfragment (gen1; gen2, gen3) for binding domain localisation Backbone: PCR.2.1	MWG
pGemTeasy_HSP10_1gen	Insert: HSP10 subfragment for binding domain localisation (*5'Nco*I, *3'Xba*I) Backbone: pGemTeasy	Self made
pGemTeasy_HSP10_2gen	Insert: HSP10 subfragment for binding domain localisation (*5'Bam*HI, *3'xho*I) Backbone: pGemTeasy	Self made

pGemTeasy_HSP10_3gen	Insert: HSP10 subfragment for binding domain localisation (*5'sma*I, *3'sac*I) Backbone: pGemTeasy	Self made
pGemTeasy_HSP10_Sonde	Insert: HSP10 subfragment for pBluescript cloning (*5'Bam*HI, *3'Not*I) Backbone: pGemTeasy	Self made
pGemTeasy_pACSG$_2$_HSP10	Insert: HSP10 subfragment for baculo virus expression (*5'Not*I, *3'Stu*I) Backbone: pGemTeasy	Self made
pGemTeasy_HSP60	Insert: HSP60 cDNA sequence lacking 3'end (5'*Sal*I, 3'*Xba*I) Backbone: pGemTeasy	Self made
pGemTeasy_HSP60_3'RACE	Insert: HSP60 3' end cDNA sequence gained from RACE PCR Backbone: pGemTeasy	Self made
pGemTeasy_HSP60_5'SL	Insert: HSP60 5' end cDNA sequence gained from SL PCR Backbone: pGemTeasy	Self made
pGemTeasy_HSP60_FL	Insert: HPS60 full-length cDNA sequence Backbone: pGemTeasy	Self made
pGemTeasy_HSP60_FL	Insert: HPS60 full-length cDNA sequence (5'*Sal*I, 3'*Xba*I) Backbone: pGemTeasy	Self made
pGemTeasy_HSP60_FL	Insert: HPS60 full-length cDNA sequence for pBridge cloning (*5'Bam*HI, *3'Eco*RI) Backbone: pGemTeasy	Self made
pGemTeasy_HSP60_FL	Insert: HPS60 full-length cDNA sequence for pAct2 cloning (*5'Sma*I, *3'Eco*RI) Backbone: pGemTeasy	Self made
pGemTeasy_HSP60_FL	Insert: HPS60 full-length cDNA sequence (5'*Hind*III, 3'*Bam*HI) Backbone: pGemTeasy	Self made

pGemTeasy_HSP60-52	Insert: HSP60 exclusive mitochondrial leading sequence subfragment for pJC45 cloning (5'*Hind*III, 3'*Bam*HI) Backbone: pGemTeasy	Self made
pGemTeasy_HSP60_1	Insert: HSP60 subfragment for pAct2, pAct3 and pBind cloning (5'*Sal*I, 3'*Xba*I) Backbone: pGemTeasy	Self made
pGemTeasy_HSP60_2	Insert: HSP60 subfragment for pAct2, pAct3 and pBind cloning (5'*Sal*I, 3'*Xba*I) Backbone: pGemTeasy	Self made
pGemTeasy_HSP60_3	Insert: HSP60 subfragment for pAct2, pAct3 and pBind cloning (5'*Sal*I, 3'*Xba*I) Backbone: pGemTeasy	Self made
pGemTeasy_HSP60_4	Insert: HSP60 subfragment for pAct2, pAct3 and pBind cloning (5'*Sal*I, 3'X*ba*I) Backbone: pGemTeasy	Self made
pGemTeasy_HSP60_Sonde1	Insert: HSP60 subfragment for pBluscript cloning (5'*Bam*HI, 3'*Not*I) Backbone: pGemTeasy	Self made
pGemTeasy_HSP60_Sonde2	Insert: HSP60 subfragment for pBluscript cloning (5'*Bam*HI, 3'*Not*I) Backbone: pGemTeasy	Self made
pGemTeasy_HSP60_Sonde3	Insert: HSP60 subfragment for pBluscript cloning (5'*Bam*HI, 3'*Not*I) Backbone: pGemTeasy	Self made
pGemTeasy_HSP60_Sonde4	Insert: HSP60 subfragment for pBluscript cloning (*5'Not*I, *3'Bam*HI) Backbone: pGemTeasy	Self made
pBindSRHSP10fl	Insert: HSP10 full-length cDNA sequence (5'*Sal*I, 3'X*ba*I) Backbone: pBind	Self made*

pAct3SRHSP10fl	Insert: HSP10 full-length cDNA sequence (5'*Sal*I, 3'X*ba*I) Backbone: pAct3	Self made*
pAct3SRHSP10a	Insert: HSP10 subfragment for binding domain localisation (5'*Sal*I, 3'X*ba*I) Backbone: pAct3	Self made*
pAct3SRHSP10b	Insert: HSP10 subfragment for binding domain localisation (5'*Sal*I, 3'X*ba*I) Backbone: pAct3	Self made*
pAct3SRHSP10c	Insert: HSP10 subfragment for binding domain localisation (5'*Sal*I, 3'X*ba*I) Backbone: pAct3	Self made*
pBind_HSP60_FL	Insert: HSP60 full-length cDNA sequence (5'*Sal*I, 3'X*ba*I) Backbone: pBind	Self made*
pBind_HSP60_1	Insert: HSP60 subfragment for binding domain localisation (5'*Sal*I, 3'*Xba*I) Backbone: pBind	Self made*
pBind_HSP60_2	Insert: HSP60 subfragment for binding domain localisation (5'*Sal*I, 3'*Xba*I) Backbone: pBind	Self made*
pBind_HSP60_3	Insert: HSP60 subfragment for binding domain localisation (5'*Sal*I, 3'*Xba*I) Backbone: pBind	Self made*
pBind_HSP60_4	Insert: HSP60 subfragment for binding domain localisation (5'*Sal*I, 3'*Xba*I) Backbone: pBind	Self made*

pAct_HSP60_FL	Insert: HSP60 subfragment for binding domain localisation (5'*Sal*I, 3'*Xba*I) Backbone: pAct3	Self made*
pAct_HSP60_1	Insert: HSP60 subfragment for binding domain localisation (5'*Sal*I, 3'*Xba*I) Backbone: pAct3	Self made*
pAct_HSP60_2	Insert: HSP60 subfragment for binding domain localisation (5'*Sal*I, 3'*Xba*I) Backbone: pAct3	Self made*
pAct_HSP60_3	Insert: HSP60 subfragment for binding domain localisation (5'*Sal*I, 3'*Xba*I) Backbone: pAct3	Self made*
pAct_HSP60_4	Insert: HSP60 subfragment for binding domain localisation (5'*Sal*I, 3'*Xba*I) Backbone: pAct3	Self made*
pBridgeSRHSP10	Insert: HSP10 full-length cDNA sequence (*5'Eco*RI, *3'Sal*I) Backbone: pBridge	Self made*
pAct2SRHSP10fl	Insert: HSP10 full-length cDNA sequence (*5'Nco*I, *3'Eco*RI) Backbone: pAct2	Self made*
pAct2SRHSP10a	Insert: HSP10 subfragment for binding domain localisation (*5'Nco*I, *3'Eco*RI) Backbone: pAct2	Self made*
pAct2SRHSP10b	Insert: HSP10 subfragment for binding domain localisation (*5'Nco*I, *3'Eco*RI) Backbone: pAct2	Self made*

pAct2SRHSP10c	Insert: HSP10 subfragment for binding domain localisation (*5'Nco*I, *3'Eco*RI) Backbone: pAct2	Self made*
pAct2SRHSP10gen	Insert: HSP10 subfragment for binding domain localisation (*5'Bam*HI, *3'Nco*I) Backbone: pAct2	Self made*
pAct2SRHSP10gen2	Insert: HSP10 subfragment for binding domain localisation (*5'Bam*HI, *3'Xho*I) Backbone: pAct2	Self made*
pAct2SRHSP10gen3	Insert: HSP10 subfragment for binding domain localisation (*5'Sma*I, *3'Sac*I) Backbone: pAct2	Self made*
pAct2_HSP60_FL	Insert: HSP60 full-length cDNA sequence (*5'Sma*I, *3'*EcoRI) Backbone: pAct2	Self made*
pBluescript_HSP10_Sonde	Insert: HSP10 subfragment for Southern blotting and *in situ* hybridisation (*5'Bam*HI, *3'Not*I) Backbone: pBluescript	Self made*
pBluescript_HSP60_Sonde1	Insert: HSP60 subfragment for Southern blotting and *in situ* hybridisation (*5'Bam*HI, *3'Not*I) Backbone: pBluescript	Self made*
pBluescript_HSP60_Sonde2	Insert: HSP60 subfragment for Southern blotting and *in situ* hybridisation (*5'Bam*HI, *3'Not*I) Backbone: pBluescript	Self made*
pBluescript_HSP60_Sonde3	Insert: HSP60 subfragment for Southern blotting (*5'Bam*HI, *3'Not*I) Backbone: pBluescript	Self made*

pBluescript_HSP60_Sonde4	Insert: HSP60 subfragment for Southern blotting (*5'Not*I, *3'Bam*HI) Backbone: pBluescript	Self made*
pJC45_HSP10	Insert: HSP10 full-length cDNA sequence for *E. coli* expression (5'*Nde*, 3'*Eco*RI) Backbone: pJC45	Self made*
pJC45_HSP60	Insert: HSP60 full-length cDNA sequence for *E. coli* expression (*5'Bam*HI, *3'Hind*III) Backbone: pJC45	Self made*
pJC45_HSP60-52	Insert: HSP60 exclusive mitochondrial leading sequence subfragment for *E. coli* expression (*5'Bam*HI, *3'Hind*III) Backbone: pJC45	Self made*
pACSG$_2$_HSP10	Insert: HSP10 full-length cDNA sequence for *baculo virus* expression (*5'Eco*RI, *3'Nco*I) Backbone: pACSG2	Self made*

2.1.7 Bacteria and yeast strains

Bacterial strains	
XL1-Blue	Heat-shock-competent *E. coli*–cells (Bullock *et al* 1987) [endA1, hsdR17 (r_k^-,m_k^+), supE44, thi-1, λ^-, recA1, gyrA96, relA1,Δ(lac), F', Pro A^+B^+, $lacI^q$ Z ΔM15, Tn10(tet^1)]
BL21(DE3)-pLysS	Heat-shock-competent *E. coli*–cells for proteinexpression (Novagen) [F-, omT, hsdS(r_{B-}, m_{B-}), gal dcm (DE3) pLysS (Cam^R)]

Yeast strains	
AH109	Mat a, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::$Gal1_{UAS}$ - $Gal1_{TATA}$-HIS3, $GAL2_{UAS}$ -GAL2 $_{TATA}$ -ADE2, URA3::$MEL1_{UAS}$ -MEL1 $_{TATA}$ -lacZ (James *et al* 1996)

2.1.8 Antibodies

Goat Anti-Mouse IgG, Goat Anti-Rabbit IgG (Dianova; peroxidase-conjugated)

Anti-FLAG, polyclonal, rabbit (Sigma, F7425)

Anti-His, monoclonal, mouse (Qiagen; Anti-His Antibody Selector Kit, 34698)

Anti-Digoxygenin (Fab-Fragment) (Roche)

Anti-GAL monoclonal

2.1.9 Computer-based sequence analysis

Computer-based sequence analysis and alignments of DNA or protein sequences were performed using the Lasergene- program (DNASTAR, Inc., www.dnastar.com). The following public databases were used:

- NCBI (http://www.ncbi.nlm.nih.gov)
- Pubmed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi)
- BLASTN and BLASP server of NCBI (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov);
- ExPASy Proteomis server (http://expasy.org/);
- Ensemble (http://www.ensembl.org/index.html);
- Nematode.net (http://www.nematode.net)
- Promotoranalyse (MatInspector): (http://www.genomatix.de/cgi-bin/eldorado/main.pl)
- GENSCAN: http://genes.mit.edu/GENSCAN.html
- Protein Pilot (Applied Biosystems)
- iPSORT: http://hc.ims.u-tokyo.ac.jp/iPSORT/

2.2 *Strongyloides ratti* life cycle and culturing

2.2.1 Animals

S. ratti infectious larva (iL_3) and parasitic females (Bernhard-Nocht-Institute) were obtained from infected male Wistar rats (*Rattus norvegicus*), 3-4 weeks of age. There was no systematic bias in terms of weight. The Wistar rats were housed singly in stainless steel cages with food and water available ad libitum.

2.2.2 Host infection

Each rat was infected with 2,000 iL_3, diluted in 0.3 ml tapwater and directly injected with a 1 ml injection subcutaneously between the scapulae.

2.2.3 Host immunisation

Six male Wistar rats were immunised with *E. coli*-expressed protein by subcutaneous injection of r*Sr*-HSP10 protein in the neck. The animals were primed at 10 weeks of age with 20 µg r*Sr*-HSP10 protein in alum/PBS and boosted in the same way 14 days later. Sera were obtained 14 days post prime immunisation and 14 days post boost immunisation, respectively. For each sample, the respective pre-immune serum served as the negative control.

2.2.4 Baermann technique

After subcutaneous infection of the Wistar rats with 2000 iL3 (Keiser *et al* 2008; Lok 2007) millions of iL3 were harvested from charcoal coprocultures during days 6-21 applying the Baermann technique (WormBook 2007; Figure 2.1). The charcoal coprocultures were set up with stool pellets from the infected rats and stored at 26°C for 5 days in H_2O. The *S. ratti* infectious larvae are harvested after 3 hours. After washing in a ceramic sieve (Filternutsche Por 3., Roth) the larvae are stored at 4°C in deionised water until further use. For the recovery of parasitic females, male Wistar rats were sacrificed on day 6 post infection with 2000 iL3. The small intestine was removed, cleaned and opened longitudinally. After collection using the Baermann method the parasitic females were extensively washed in HBSS.

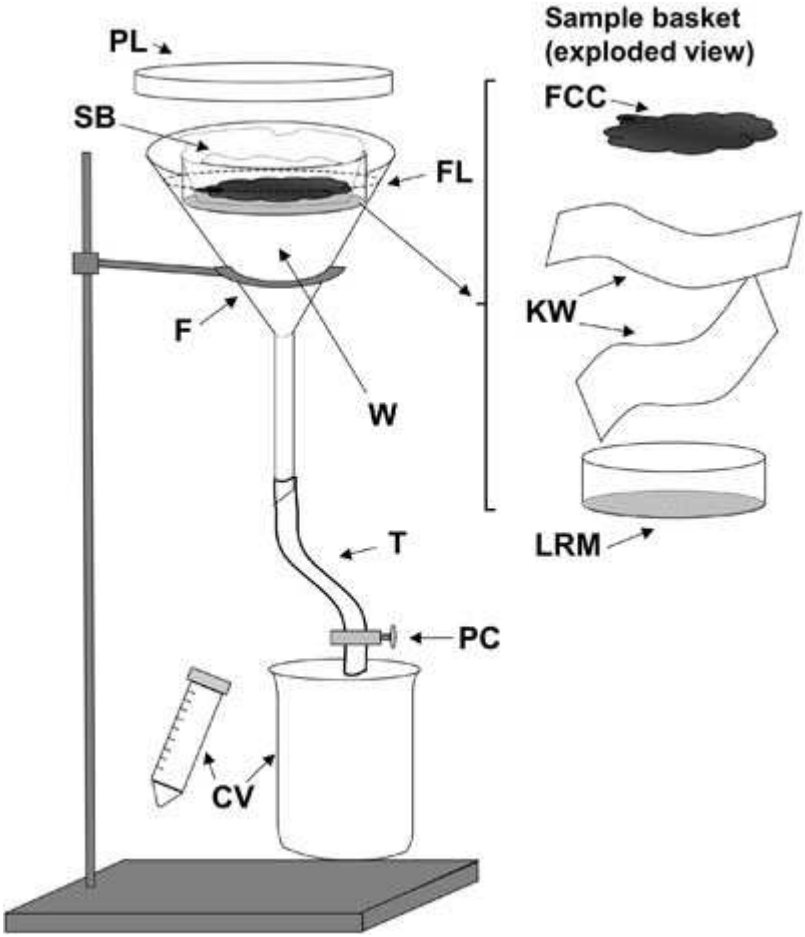

Figure 2.1: Baermann apparatus

A Baermann apparatus to isolate *Strongyloides ratti* stages from coprocultures. **F**, 150mm glass or plastic funnel; **W**, tap water at 43°C; **FL** (dashed line) fluid level in funnel; **SB**, Sample basket shown on the right in exploded view (**FCC**, fecal charcoal culture contents; **KW**, two layers of Kimwipes or other laboratory tissue; **LRM** Lucite ring with nylon mesh affixed with cement); **T**, rubber or plastic tubing; **PC**, pinch clamp; **CV**, catch vessels, conical centrifuge tube, or beaker. Source: www.wormbook.org

2.3 Molecular biological methods

2.3.1 Production of competent bacteria (Nishimura *et al* 1990)

Bacteria (*E .coli* XL1 blue) were grown on LB-agar dishes overnight at 37°C. 2 ml of Y-broth were inoculated with several colonies picked with a pipette tip and grown for 2 hours at 37°C under constant shaking (>200). Subsequently, 500 ml of Y-broth was inoculated with the preparatory culture until the culture had reached a density (OD_{600}) of 0.5. The culture was split into two polypropylene tubes and the growth of bacteria was stopped by a 5-min incubation step

on ice. Cells were pelleted at 2,000 g for 15 min (4°C) and – after removal of the supernatant - resuspended in 15 ml prechilled TFB 1 solution. Following 60-90 min incubation on ice, the centrifugation was repeated. The cell pellet was resuspended in 2 ml TFB and immediately frozen as 200 µl aliquots in liquid nitrogen and stored at -80°C. Transformation efficacy of cells was tested by transformation with a defined amount of purified supercoiled plasmid DNA.

2.3.2 Transformation of DNA into bacteria

Plasmid DNA (0.5 ng), or 5 µl of a ligation mixture were added to 50 µl of ice-thawed competent XL 1 blue cells and incubated for 30 min on ice. After a heat shock (3 min, 37°C) and successive incubation on ice (3 min), 600 µl of SOC medium were added to the bacteria and incubated at 37°C for 30 min with constant agitation. 50 µl and 250 µl of transformed cells were plated on LB plates containing the appropriate antibiotics and incubated at 37°C for 12-16 h to allow single colonies to grow.

2.3.3 Purification of plasmid DNA

2.3.3.1 Plasmid purification from bacterial cultures

2.3.3.1.1 Mini-scale plasmid isolation

Three ml LB/Amp-Medium (200 µg/ ml ampicillin) were inoculated with a single colony and incubated overnight at 37°C with constant agitation. Cultures were transferred into 2 ml Eppendorf tubes, and the cells pelleted by centrifugation (13,000 g; 1 min; RT). Plasmids were isolated from the bacteria using the Macherey-Nagel NucleoSpin Plasmid prep system, according to the manufacturer's protocol. The DNA was eluted in 50 µl elution buffer with subsequent centrifugation (13,000 g; 2 min; RT). Plasmid DNA was stored at -20°C.

2.3.3.1.2 Alternative mini-scale plasmid isolation

All steps were performed at RT. The overnight cultures were transferred into 1.5 ml Eppendorf tubes, the cells pelleted by centrifugation (2,400 g, 1 min), and the supernatant discarded. Subsequently, 200 µl STET/lysozyme (1 µg/ml STET) solution was added and resuspended under constant agitation. Prior to 15 min centrifugation at 16,000 g, the solution was heated at 95°C for 2 min. The cell detritus was removed. To bind the DNA, 10 µl 5% CTAB was added, vortexed and 10 min centrifuged at 16,000 g. The supernatant was removed, 300 µl 7.5 M Na-acetate were added to the pellet and shaken for 5 min. Subsequently, 750 µl 100% ethanol

were added, vigorously vortexed and centrifuged for 10 min at 16,000 g. The supernatant was discarded, and the DNA washed with 1 ml 75% ethanol followed by a 5 min centrifugation step at 16,000 g. The supernatant was discarded, and the DNA pellet air-dried and resuspended in 40 µl dH_20.

2.3.3.1.3 Plasmid-DNA isolation from 100 ml cultures (Midi preps)

For the preparation of large quantities of DNA, the Macherey-Nagel Nucleobond AX Xtra Midi kit was used. A single colony was inoculated in 5 ml LB/amp (100 µg/ml ampcillin) medium and grown at 37°C for 8 h with constant agitation. Afterwards, this culture was added to 100 ml LB/amp (100 µg/ml ampcillin), and the culture was incubated at 37°C with constant agitation overnight. Cells were pelleted (6,000 g; 15 min; 4°C), and DNA was isolated as described in the manufacturer's protocol. Finally, the DNA pellet was resuspended in 200 µl of TE (10 mM, pH 8.0), and the DNA concentration was determined (2.2.13).

2.3.3.1.4 DNA-fragment purification

For the purification of DNA fragments, the Macharey-Nagel NucleoSpin Extract II kit was used according to the manufacturer's protocol. The DNA was eluted from the column by the addition of 25-50 µl of elution buffer, depending on the DNA gel concentration.

2.3.3.1.5 DNA-fragment extraction from agarose gels

For the isolation and purification of DNA fragments from agarose gels, ethidium-bromide-stained gels were illuminated with UV-light, and the appropriate DNA band was excised from the gel with a clean scalpel and transferred into an Eppendorf tube. The fragment was isolated using the Macherey-Nagel NucleoSpin Extract II kit according to the manufacturer's protocol. The fragment was eluted from the column by the addition of 25-50 µl of elution buffer, depending on the DNA gel concentration (optical gel evaluation by comparison with the 1600 bp band of the 1 kb ladder).

2.3.4 Total RNA isolation from *S. ratti* infectious larvae or parasitic females

2.3.4.1 Total RNA isolation

The Trizol LS reagent (Invitrogen) was used to isolate the RNA from *S. ratti* (larval or adult stages). The reagent is formulated for use with tissues in buffer solution, therefore, the procedure was based on the volume of worms not on the weight of tissue.

Worms for RNA isolation were washed in 1xPBS buffer in a 2 ml Eppendorf tube, quickly frozen in liquid nitrogen, and stored at -80°C degrees. For RNA isolation, the worms were defrosted on ice, and for every 250 µl of worm solution (worms in buffer) 750 µl Trizol LS (1:3) was added. Subsequently, one 3 mm stainless steel ball (peqGold RNAPure™, peqLAB; Erlangen) was added and vortexed at highest speed, using a special vortex adaptor (Mo Bio Laboratories, Inc. vortex adapter), for 30 minutes. 200 µl chloroform (per 750 µl of Trizol LS) were added and vortexed for 15 seconds. After incubation at RT, the entire sample including smashed worms, Trizol LS, and chloroform, but without the bead, was transferred to a clean Eppendorf tube and centrifuged at 4°C for 15 min at 13,000 g. The aqueous phase (top phase) was transferred to a fresh tube and kept on ice. To precipitate the RNA sample, 500 µl ice-cold isopropanol (Merck) per 750 µl of Trizol LS were added, followed by vortexing. After 10 min incubation at RT, the sample was centrifuged at 4°C for 10-30 min at 13,000 g. The supernatant was removed with an RNase-free pipette. To ensure optimal purity, the pellet was washed with 1 ml 75% ethanol (Merck) made with DEPC H_2O (Roth) and centrifuged at 4°C for 5 min at 5,300 g. The supernatant was carefully removed, inverted, and air-dried for 5-10 min. Depending on the yield, the pellet was resuspended with 50-100 µl of DEPC H_2O (Roth) and measured (see below). The RNA was precipitated as described below and stored at -80°C.

2.3.4.2 Phenol/chloroform extraction

200 µl of TE (pH 8.0) was added to a 100 µl DNA-containing sample to obtain a volume of 300 µl (1V). An equal volume of phenol/chloroform/isoamylalcohol (25:24:1) was added to the DNA-TE-containing sample and vortexed for 1-2 min to create an emulsion. To avoid shearing, samples containing genomic DNA were mixed gently. After centrifugation at 16,000 g (RT) for 5 min, the aqueous layer was carefully transferred to a new tube, avoiding any flocculent material at the interface. The phenol/chloroform/isoamylalcohol (25:24:1) step was repeated. In order to remove residual phenol, 1 V of chloroform/isoamylalcohol (24:1) was added to the aqueous DNA-containing sample, vortexed and centrifuged for 5 min at 16,000 g (RT). The aqueous layer was transferred to a new Eppendorf tube, designated for further DNA precipitation (2.2.7.3).

2.3.4.3 Precipitation of RNA

The salt concentration of aqueous RNA solutions was adjusted by adding 1/10 volume of 3 M sodium acetate, pH 5.2. Cold 96% ethanol (-20°C, 2.5 volumes) was then added, and the samples were mixed well. The samples were centrifuged for 30 min (16,000 g, RT). For optimal

purity, the pellet was washed in 75% ethanol. After removal of the supernatant, a quick (1-2 seconds) centrifugation step was performed, and residual ethanol was aspirated. The supernatant was removed, and the DNA pellets dried on air (approximately 5 min at RT). The RNA was resuspended in 25 µl or 50 µl RNase- free water, depending on the size of the pellet.

2.3.5 gDNA isolation from *S. ratti* infectious larvae and precipitation

A pellet of approximately 250,000 *S. ratti* iL_3 was washed three times with 1xPBS and digested overnight at 56°C with 40 µl proteinase K (Qiagen) and 200 µl ATL buffer (Qiagen) under constant agitation. The sample was then precipitated as described below and stored at 4°C. For precipitation, the salt concentration of the aqueous DNA solution was adjusted by adding 1/10 volume of 3 M sodium acetate (pH 5.2). Cold 96% ethanol (-20°C, 2.5 volumes) was then added, and the samples were mixed (not vortexed to avoid shearing). For optimal purity, the pellet was fished from the tube, washed with 80% ethanol (-20°C), and air dried in a new Eppendorf tube (approximately 15 min at RT). The DNA was resuspended in 25µl or 50µl 1xT 1/10xE buffer depending on the pellet size.

2.3.6 Polymerase chain reaction (PCR)

To amplify DNA, qTaq (Invitrogen) was used. The PCR reactions were performed in 0.2 ml Eppendorf tubes according to the standard protocols. The template (~100 ng DNA) used was plasmid DNA, or first strand cDNA. Primer sequences were designed manually. The number of cycles required for optimal amplification varied between 25 and 35 depending on the amount of starting material and the efficiency of each amplification step. A final incubation step at the extension temperature ensured complete formation of double-stranded molecules from all nascent products. Following cycling (T-Gradientencycler, Biometra), aliquots (10-15 µl) were analysed by agarose gel-electrophoresis to visualise the amplified products, cloned into the pGemTeasy vector, and sequenced.

2.3.7 5'and 3' cDNAs amplification

To obtain the 3'cDNA ends, 3'RACE (rapid amplification of cDNA ends) experiments were performed. To this end, full-length cDNAs were generated by using 3'oligo-dT-containing primers complementary to the poly(A) tail of mRNAs. 5 µg total RNA was reverse-transcribed using SuperscriptIII (Invitrogen) and GeneRacer (Invitrogen) oligo(dT) primers according to the manufacturer's instructions. RACE fragments were then amplified by RT-PCR (Sambrook *et al*

1989) using Taq polymerase, gene-specific forward primers and the GeneRacer 3'oligo as reverse primers (primer sequences given in chapter 2.1.5). The amplified 3'cDNA fragments were analysed by agarose gel-electrophoresis to visualise the amplified products, cloned into the pGemTeasy vector and sequenced. 5' cDNA fragments were obtained performing Spliced Leader (SL) RT-PCR. For this, 5 µg total RNA was reverse-transcribed using SuperscriptIII (Invitrogen) and a gene-specific primer according to the manufacturer's instructions. RT-PCR was performed using Taq polymerase (Invitrogen), SL forward primers and the gene-specific reverse primers (primer sequences given in chapter 2.1.5). The amplified 5'cDNA fragments were analysed by agarose gel-electrophoresis to visualise the amplified products, cloned into the pGemTeasy vector, and sequenced.

2.3.8 DNA agarose gel electrophoresis

To analyse restriction digestions or the quality of nucleic acid preparations, horizontal agarose gel-electrophoresis was performed. Gels were prepared by heating 0.8-2.5% (w/v) agarose (SeaKem LE agarose) in Tris–acetate buffer (TAE), depending on the size of fragments to be separated. DNA samples were diluted in 1 x DNA sample buffer and were adjusted to electrophoresis at 10 V/cm in Kodak BioMAX MP1015 gel chambers in 1xTAE running buffer. Afterwards, gels were stained in 0.5 µg/ ml ethidium bromide containing 1xTAE solution for approximately 20 min RT. Thermographs of transilluminated gels were taken, or bands were made visible on a UV-screen (λ = 360nm). If required, fragments were cut out of the gel with a clean scalpel. The extraction of fragments from agarose gel is described in chapter 2.3.3.1.4.

2.3.9 Sequencing of DNA

DNA sequence analysis was carried out using the Sanger method (Sanger *et al* 1977) by the service laboratory of the Center for Molecular Neurobiology Hamburg (ZMNH) using ABI Prism 377 DNA sequencer (*Perkin Elmer*, www.perkin-elmer.de) and the Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (*Perkin Elmer*).

2.3.10 Enzymatic manipulation of DNA

2.3.10.1 Restriction analysis

Restriction digests were performed by incubating dsDNA molecules with an appropriate amount of restriction enzyme(s), the respective buffer as recommended by the supplier(s), and at

the optimal temperature for the specific enzyme(s). In general, 20 µl digests were done. For preparative restriction digests, the reaction volume was scaled up to 50 µl. Digestion reactions were set up with DNA, 1 x restriction buffer, the appropriate number of units of the respective enzyme(s) (due to the glycerol content, the volume of the enzyme(s) added should not exceed 1/10 of the digested volume), and nuclease-free H_2O. After incubation at the optimal temperature (25°C to 37°C) and time (2-3 h or overnight), digests were stopped by incubation for 20 min at 65°C. If reaction conditions of enzyme mixes were incompatible, DNA was digested successively with the individual enzymes. Between individual reactions, DNA was purified (see DNA fragment purification).

2.3.10.2 Plasmid DNA fragment

For cloning of distinct regions of plasmid DNA, donor molecules were digested with appropriate restriction enzyme(s). Even though direct ligation using DNA from inactivated restriction digest was possible, mostly complete digests were applied to agarose gel electrophoresis. Appropriate bands were cut out, and the DNA was eluted from agarose plugs (2.3.3.1.4), thus avoiding unwanted by-products during subsequent ligation reactions. Non-complementary ends were converted to blunt ends prior to ligation using the Klenow enzyme according to the manufacturer's protocol.

2.3.10.3 Enzymatic manipulation of vector DNA prior to ligation

When used as vectors, plasmids were digested by two restriction enzymes to achieve insertion of target DNA in a defined orientation. Digestion reactions were carried out using 5-10 µg of plasmid DNA as starting material. Digestions were checked by agarose gel-electrophoresis, and subjected to preparative agarose gel-electrophoresis. Appropriate bands representing digested vectors were cut out, and vector DNA was extracted from agarose plugs. The plasmid DNA was used for ligation without further purification.

2.3.10.4 Ligation of plasmid vector and insert DNA

Ligation of DNA fragments was performed by mixing 50 ng vector with the threefold molar excess of insert DNA. One µl of T4-Ligase (Fermantas) and 1 µl of 10x ligation buffer (Fermantas) were added, and the reaction mix was brought to a final volume of 10 µl. The reaction was incubated overnight at room temperature. The reaction mixture was used directly for transformation of bacteria (2.3.2) without any further purification.

2.3.11 Photometric quantification of nucleic acids

DNA, RNA and oligonucleotide concentrations were estimated in TE solutions (1:200) by measuring absorptions at λ = 260 nm against blank probes (Spectrophotometer Shimadzu UV 160A). The determined optical density (OD) values were multiplied by 50, 40 and 30 for double stranded dsDNA, RNA or oligonucleotides, respectively, to calculate concentrations in g/ ml (1OD at 260 nm = 50 µg DNA/ml, 1OD at 260 nm = 40 µg RNA/ml, 1 OD at 260 nm = 30 µg oligonucleotides/ml). Interference by contaminants was recognised by calculation of ratios. The ratio A260/280 was used to estimate the purity of nucleic acid, since proteins absorb at 280 nm. Pure DNA should have an A260/A280 ratio of 1.8, whereas pure RNA should give a value of approximately 2.0. Absorption at λ = 230 nm reflects contamination of the sample by substances such as carbohydrates, peptides, phenols or aromatic compounds. In the case of pure samples, the ratio A 260/230 should be approximately 2.2.

2.3.12 Southern blot analysis

S. ratti genomic DNA (10 µg) was digested with *Bam*HI, *Eco*RI, *Eco*RV and *Hind*III (Roche) restriction enzymes (50 u at 37°C for 16 hours) and electrophoresed in a 1% (w/v) agarose gel. The DNA was depurinated by incubation of the gel in 0.25 M HCL for 10 min at RT - until the bromphenol blue turned yellow – and equilibrated for 15 min in a solution containing 0.4 M NaOH. The capillary blot procedure was used to transfer the DNA onto Hybond XL1-membrane (GE Health Care) by 0.4 M NaOH solution. After transfer overnight, the membrane was floated for not more than 10 min in 2x SSC and baked for 3 h at 80°C. The membranes were tightly rolled and placed into hybridisation bottles with pre-warmed hybridisation buffer and denatured herring sperm DNA. The pre-hybridisation was performed at 65°C in a hybridisation oven for >60 min. The pre-hybridisation mixture was discarded.

The *S. ratti* HSP10 and HSP60 probes were derived by *Bam*HI and *Not*I cleavage of the pBluescript +/+ vector resulting in 300-350 bp DNA fragments. The DNA fragments were eluted from the gel and their concentration adjusted to 5 ng/ µl. (For random prime labelling, see 2.3.12.1)

The random prime labelled probes ($2.5x10^5$-$5x10^5$ cpm/ ml hybridisation mixture) were denaturated at 95°C for 5 min. The genomic DNA was hybridised with each of the radioactively labelled probes in prewarmed (65°C) fresh hybridisation solution containing denatured herring sperm DNA overnight at 65°C in a hybridisation oven. Unspecifically bound probe was washed from the membrane by washing steps with prewarmed buffer a. The buffer was directly discarded,

and new buffer a was added for 10-15 min at 65°C. The washing steps were repeated until <30 radioactive decays per s were detected on the membrane. Subsequently, the membrane was washed with buffer b for 10 min. The procedure was repeated until no radioactivity was detected in the wash buffer b. Prior to detection of the signals (Fuji FLA 3000 Imaging reader) the blot was saran wrapped and exposed to the Fuji (Raytest) Imaging Plate for 1 hour and 18 hours respectively.

2.3.12.1 Random prime RNA labelling

The cDNA probes used for Southern blot analysis were [$_{32}$P]-dCTP-labelled with the Ready-to-go DNA labelling beads (Amersham Biosciences). The DNA was first denatured by heating for 3 min at 95°C and placed immediately on ice for 2 min. 25 ng of the designated cDNA probes were filled up with DEPC-H_2O to 45 µl and together with 5 µl [$_{32}$P]-dCTP added to the reaction mix bead. For labelling, mixtures were incubated at 37°C for 15 min. Prior to the application of the probes to the prehybridised blots, the molecules were denatured by boiling for 5 min.

2.3.13 Whole mount *in situ* hybridisation

2.3.13.1 Tissue preparation

2.3.13.1.1 Primary fixation of iL$_3$

Approximately 250,000 iL$_3$ were washed with M9 by centrifugation (375 g; 1 min; 4°C). The worms were transferred into 2 ml Eppendorf tubes at 200 µl (packed volume) worms per tube. To let the worms settle down, the tubes were centrifuged at 1,100 g for 10 sec at 4°C and left standing for 30 sec. The supernatant was removed, and 10mM DTT, 0.1% Tween-20 in 1x BO_3, (pH9) was added and equilibrated at 22°C. The tubes were rotated for 20 min at 22°C. The buffer was changed to PBS (4°C) and rotated for 2 min at RT, this was repeated twice. For Proteinase K (Qiagen) digestion, PBT (22°C) to a total of 1 ml and 5 µl of Proteinase K (20 mg/ ml) was added. The tubes rotated for 12 min at 22°C. The buffer was changed to glycine in PBT (at 4°C) and the tubes rotated for 2 min at RT. The buffer was changed to PBS and the tubes rotated again for 2 min at RT; this was repeated twice. To fix the worms the buffer was changed to Dent (MeOH:DMSO=8:2), pre-cooled at -20°C and the worms rotated for 5 min in a cold room. For redydration, the buffers were changed and the tubes rotated as follows: MeOH 4°C 5 min, MeOH:0.2N HCl = 1:1 4°C 10 min, PBS 4°C 2 min, PBS 22°C 5 min, 10mM DTT in 1x BO3

(pH9) 22°C 10 min, 1x BO3(pH9) 22°C 3 min, 2 min, 2 min (3 times), 0.6% H_2O_2 in 1x BO3 22°C 10 min, PBS 22°C 2 min (3 times), 3.7% formaldehyde in hepes-PBS 22°C 2 hrs (freshly prepared and stored in a refrigerator until use.) For dehydration, the buffers were changed and the tubes rotated at RT as follows: EtOH:PBS = 3:7 5 min, EtOH:PBS = 1:1 5 min, EtOH:PBS = 7:3 5 min, EtOH 5 min (twice). The fixed worms were stored at -20°C in EtOH.

2.3.13.1.2 Fixation of iL3 onto slides

The fixed iL3 (stored in ethanol at -20°C) were resuspended and 900 µl/ tube of iL3 quickly transferred into siliconised 2 ml Eppendorf tubes. For rehydration, the buffers were changed, and the tubes rotated at RT as follows: EtOH:PBS = 7:3 5 min, EtOH:PBS = 1:1 5 min, EtOH:PBS = 3:7 5 min. The larvae were washed with PBT 3 times for 5 min each and resuspended in about 700 µl of PBT. The density of the iL3 was checked by counting in an aliquot of the suspension under a dissecting microscope. The iL3 were stuck to the slides as follows: poly-L-lysine coated test slides were placed on the top of an aluminium block pre-cooled on ice. Ice-cold PBS was dispended to the slides at 240 µl/slide. The rehydrated iL3 were dispensed to the slides at 40 µl/slide. After 5 min to settle iL3 to the bottom, they were fixed by soaking the slides in MeOH pre-cooled at 4°C arranging the slides in a stainless steel holder (15 slides/holder) placed in MeOH for 5 min. The holder with the slides was then soaked in the following series of solutions at 4°C in a cold room: MeOH:formaldehyde in hepes-PBS = 7:3 2 min, MeOH:formaldehyde in hepes-PBS = 1:1 2 min, MeOH:formaldehyde in hepes-PBS = 3:7 2 min, 3.7% formaldehyde in hepes-PBS 60 min, PBT 5 min x 5 times at RT. A second Proteinase K digestion was performed by adding 60 µl of Proteniase K (20 mg/ml, Qiagen) in 180 ml of PBT pre-warmed at 37°C (final conc. µg/ml). The solution was well mixed by stirring; the holder containing the slides was soaked in this Proteinkinase K solution and subsequently incubated at 37°C for 30 min. The holder was transferred to glycine in PBT (pre-cooled at 4°C) and incubated for 2 min to stop digestion. The holder was then soaked in 0.1% triethanol for 2 min at RT and in 0.05% acetic anhydride in triethanol for 10 min. Subsequently, it was dehydrated by soaking in the following series of solutions at RT: PBT 2 min, PBT 2 min, formaldehyde in hepes-PBS 20 min, EtOH:PBS = 3:7 5 min, EtOH:PBS = 1:1 5 min, EtOH:PBS = 7:3 5 min, EtOH 5 min twice. Finally, the slides were stored in EtOH at -80°C.

2.3.13.1.3 Kryo-block preparation

Approximately 1000 iL3 were washed 3 times with tapwater and carefully transferred to micro-boxes made of plastic 1×1×1 cm in size. The box was filled with tissue freezing medium

(Tissue Freezing Medium, Jung Leica Microsystems, Nussloch) and stored overnight on dry ice. Up to 10 tissue sections, cut at -20°C (Leica Kryostat CM 3000), with 16 µm thickness are mounted on a positively charged glass slide (SuperFrost Plus; Menzel, Braunschweig, Germany), fixed in 4% paraformaldehyd for 20 min at 4°C and washed twice for 5 min in 1xPBS. For consistent results the slides were soaked in acetylation buffer for 10 min. Subsequently, they were dehydrated in following series of solution at RT: 60%, 80%, 90%, 95%, 100% chloroform for 5 min each and air dried.

2.3.13.2 Dig-labelling of RNA probes

Non-radioactively labelled RNA probes were prepared by *in vitro* transcription using linearised, phenol-extracted plasmids (pBluescript) containing *S. ratti* HSP10 and HSP60 sequences, 300 – 350 bp respectively. The RNA probe-labelling was performed according to the manufacturer's protocol (Dig RNA labelling mix, Roche), using 1 µg DNA/20 µl assay. Sense (negative control) and anti-sense RNA probes were synthesised using T7 or T3 RNA polymerase (T7 and T3 RNA Polymerase, Roche). After 1 hour incubation at 37°C, labelled probes were resuspended in 50 µl STET buffer. The probes were cleaned on a probe Quant column according to the manufacturer's advice (GE Healthcare illustra Microspin G 50 Column) and stored at -20°C until used. The final concentration of nucleotides was as follows: ATP, GTP, CTP: each 1000 µM (= 20 µM in 20 µl), UTP: 650 µM; Dig-UTP: 300 µM.

2.3.13.3 Prehybridisation, hybridisation and posthybridisation procedures

Prehybridisation was performed to prevent background staining. The prehybridisation mixture contained all components of the hybridisation mixture except for the probe and dextran sulfate. The slides were placed horizontally in a humidified chamber. 0.5 - 1 ml prehybridisation buffer was applied to each slide and incubated at 50°C for 2 - 3 hours.

The prehybridisation buffer was removed, and 100 µl hybridisation buffer was added to each slide containing 20 ng DIG-labelled RNA probes. The slides were covered with coverslips and incubated in a humidified chamber overnight at 50°C. After incubation the coverslips were removed by rinsing in 2 x SSC (4 x 5 min in 2 x SSC at RT). Subsequently, a series of washing steps was performed: 20 min at 50°C in 2 x SSC/50% formamide/0.05% Tween 20, 30 min at 50°C in 1 x SSC/50% formamide/0.05% Tween 20 and 30 min at 50°C in washing solution in 0.1 x SSC/50% formamide/0.05 % Tween 20.

2.3.13.4 Detection procedure

The detection procedure for the performed *in situ* hybridisation is based on the alkaline phosphatase enzyme reaction. The slides were washed for 5 min in buffer 1. Unspecific background was blocked by incubating the sections for 30 min in buffer 1 (buffer 1 with 1% Blocking Reagent, Boehringer). Afterwards, the slides were incubated with 100 µl anti-DIG-antibody conjugated with alkaline phosphatase (FAB-fragments) (diluted 1:500 in buffer 1 with 1% blocking reagent) for 2 hours at RT. After rinsing the slides in buffer 1 at RT and washing them twice for 15 min, the slides were incubated for 5 min in buffer 2 at RT. 500 µl staining solution (containing 5-Bromo-4-chloro-3-indolylphosphate/Nitro-blue tetrazolium (BCIP/NBT)) was applied, and the slides were incubated in a humidified chamber for several hours, up to 24 hours depending on the staining, to obtain optimal staining results. The staining was stopped by rinsing the slides in dH_2O for 10 min at RT and the slides were mounted with glycerin gelatine.

2.4 Cell cultures

Spodoptera frugiperda cells (SF21) were grown in serum-free BD BaculoGold Max-XP medium supplemented with 1% penicillin and streptomycin at 27°C.

Spodoptera frugiperda cells (SF9) were grown in serum-free Novagen BacVector® Insect Cell Medium supplemented with 1% penicillin and streptomycin at 27°C.

Murine neuroblastoma cells (N2a) were cultured in DMEM (Dulbecco's Modified Eagle Medium Invitrogen) supplemented with 5% FCS and 1% penicillin and streptomycin at 37%, 5% CO_2.

Human embryonic kidney cells (HEK) were cultured in DMEM supplemented with 5% FCS and 1% penicillin and streptomycin at 37%, 5% CO_2.

2.4.1 Yeast cell culture

The glycerol stocks were prepared according to the Yeast Protocols Handbook, 2001.

The liquid overnight cultures were prepared according to the Yeast Protocols Handbook, 2001.

2.5 Protein biochemical methods

2.5.1 Mammalian two-hybrid

The two-hybrid system is used to detect protein:protein interactions *in vivo*. Originally developed in yeast (Fields & Song 1989; Chien *et al* 1991), the two-hybrid system has been adapted for use in mammalian cells (Dang *et al* 1991; Fearon *et al* 1992). The CheckMate Mammalian Two-Hybrid System (Promega) used here is based on the pBIND, pACT and pG5*luc* vector. The pBind vector contains the yeast GAL4 DNA-binding domain upstream of a multiple cloning region. The pBIND3 vector used is a derivate of pBIND (Promega). The oligonucleotide GATC GTC GAC CCG CGG GGA TCC A was cloned into the *Bam*HI/ Mlu digested vector. *Bam*HI and *Sal*I site have been replaced by *Sal*I and *Bam*HI sites, and the reading frame was shifted. Furthermore, the Renilla luciferase site was eliminated (Hentschke *et al* 2009). All modifications were kindly performed by PD Dr. Uwe Borgmeyer, Insitut for Developmental Neurobiology, (ZMNH, Hamburg). The pACT Vector contains the herpes simplex virus VP16 activation domain upstream of a multiple cloning region. The pACT3 Vector used is a derivate of pACT. The oligonucleotide GATC GTC GAC CCG CGG GGA TCC A was cloned into the *Bam*HI/*Mlu*I digested vector. The *Bam*HI and *Sal*I sites were replaced by *Sal*I and *Bam*HI sites, and the reading frame was shifted. The pG5*luc* Vector contains five GAL4 binding sites upstream of a minimal TATA box, which in turn is upstream of the firefly luciferase gene (*luc*+). The pGAL4 and pVP16 fusion constructs were transfected along with the pG5*luc* Vector into mammalian HEK and N2A cells. The cells were lysed for 24 h and 48 hours respectively after transfection, and the amount of firefly luciferase was quantified using the Bright-Glo Luciferase Assay System. The empty pBIND3 and pACT3 vectors were used as negative control. The following vectors were used for mammalian two-hybrid analyses:

Table 2.1: Overview of the bait (pBind) and prey (pAct3) vectors used in the mammalian two-hybrid assay

Mammalian two-hybrid vector name	Vector description	Primer
pBindSRHSP10fl	Includes the full-length *S. ratti* HSP10 sequence. Subcloned to bait vector pBind3delta renilla *Xba*I and *Sal*I site.	HSP10flfor HSP10re330xba
pAct3SRHSP10fl	Includes full-length *S. ratti* HSP10 sequence. Subcloned to prey vector pAct3 X*ba*I and S*al*I site.	HSP10flfor HSP10re330xba
pAct3SRHSP10a	Includes *S. ratti* cDNA HSP10 fragment ranging from bp 1 to bp 174. Subcloned to prey vector pAct3 *Xba*I and *Sal*I site.	HSP10flfor HSP10rev174xba
pAct3SRHSP10b	Includes *S. ratti* cDNA HSP10 fragment ranging from bp 85 to bp 138. Richardson *et al* 2001 described this HSP10 binding site. Subcloned to prey vector pAct3 *Xba*I and *Sal*I site.	HSP10BDpAct3sense HSP10BDpAct3Antiense
pAct3SRHSP10c	Includes *S. ratti* cDNA HSP10 fragment ranging from bp 168 to bp 330. Subcloned to prey vector pAct3 *Xba*I and *Sal*I site.	HSP10for168 HSP10rev330
pBindHSP60fl	Includes full-length *S. ratti* HSP60 sequence. Subcloned to bait vector pBind3delta renilla X*ba*I and *Sal*I site.	Hsp60Sal-For
pBindHSP60_1-6	Includes *S. ratti* cDNA HSP60 fragment ranging from bp 1 to bp 873. Subcloned to bait vector pBind3delta renilla X*ba*I and *Sal*I site.	Hsp60Sal-For HSP60-Xba291rev
pBindHSP60_4-6	Includes *S. ratti* cDNA HSP60 fragment ranging from bp 624 to bp 873. Subcloned to bait vector pBind3delta renilla X*ba*I and *Sal*I site.	HSP60-Sal208F HSP60-Xba291rev
pBindHSP60_5-6	Includes *S. ratti* cDNA HSP60 fragment ranging from bp 739 to bp 873. Subcloned to bait vector pBind3delta renilla X*ba*I and *Sal*I site.	HSP60-Sal247F HSP60-Xba291rev
pBindHSP60_4-2	Includes *S. ratti* cDNA HSP60 fragment ranging from bp 624 to bp 1683. Subcloned to bait vector pBind3delta renilla X*ba*I and *Sal*I site.	HSP60-Sal208F HSP60Xba-rev

pAct3HSP60fl	Includes full-length *S. ratti* HSP60 sequence. Subcloned to prey vector pAct3 X*ba*I and S*al*I site.	Hsp60Sal-For HSP60Stop_rev
pAct3HSP60_1-2	Includes *S. ratti* cDNA HSP60 fragment ranging from bp 1to bp 1683. Subcloned to prey vector pAct3 *Sba*I and *Sal*I site.	Hsp60Sal-For HSP60Xba-rev
pAct3HSP60_4-6	Includes *S. ratti* cDNA HSP60 fragment ranging from bp 624 to bp 873. Subcloned to prey vector pAct3 *Xba*I and *Sal*I site.	HSP60-Sal208F HSP60-Xba291rev
pAct3HSP60_4-2	Includes *S. ratti* cDNA HSP60 fragment ranging from bp 624 to bp 1683. Subcloned to prey vector pAct3 *Xba*I and *Sal*I site.	HSP60-Sal208F HSP60Xba-rev

2.5.1.1 Transfection

For transfection of human embryonic kidney cells, the cells were separated. One day before transfection, the HEK cells were stripped with 10x Trypsin EDTA for 1 min at 37°C in a 5% CO_2 incubator. Depending on the density, the cells were diluted with Opti-MEM (5% FCS Gibco/Invitrogen, 1% Pen/Strep PAA Laboratories) and plated in a 96-well plate (Greiner) at a concentration of 1 x 10^4/well. The HEK cells were transfected using 3:1 ratios of FuGENE6 (Roche) Transfection Reagent (µl) to DNA (µg), respectively (2 µg DNA/well : 6 µl FuGENE6/well). (2 µg DNA/well containing 0.3 µg pBind Vector DNA, 0.7 µg pAct Vector DNA, 0.7 µg EGFP, 0.3 µg UAS.) 1010 µl Opti-MEM (Invitrogen) was then mixed with 80 µl FuGENE in a polysterol tube. After 5 min incubation, 85 µl Opti-MEM/FuGENE were added to each well (96-well plate), containing the DNA mixture. After 30 min of incubation, 12 µl of the FuGENE- Opti-MEM-DNA mixture was plated onto HEK cells in a 96-well plate, leaving the last row blank.

For transfection with murine neuroblastoma cells (N2a), the cells were separated. One day before the transfection, the N2a cells were lysed with 10x Trypsin EDTA for 1 min at 37°C in a 5% CO_2 incubator. Depending on the cell density, the cells were diluted in Opti-MEM (5% FCS Gibco/Invitrogen, 1% Pen/Strep PAA Laboratories) and plated in a 96-well plate (Greiner) at a concentration of 1 x 10^4/well. The N2a cells were transfected using a 2.5 : 1 ratio of Lipofectamine 2000 (Invitrogen) : DNA (µl/µg), (5 µl Lipofectamine 2000/well : 2 µg DNA/well). (2 µg DNA/well containing 0.3 µg pBind Vector DNA, 0.7 µg pAct Vector DNA, 0.7 µg EGFP, 0.3 µg UAS.)

1235 µl Opti-MEM (Invitrogen) were mixed with 65 µl Lipofectamine 2000 in a polysterol tube. After a 5 min incubation, 100 µl Opti-MEM/Lipofectamine 2000 was added to each well (96 - well plate) containing the DNA mixture. After 30 min of incubation, 25 µl Lipofectamine 2000-Opti-MEM-DNA mixture was plated onto the N2a cells of a 96-well plate, leaving the last row blank.

2.5.1.2 Renilla luciferase assay

24 hours (HEK cells), or 48 hours (N2a) respectively after transfection, the cells were lysed, and the amount of firefly luciferase was quantitated using the Bright-Glo Luciferase Assay System (Promega).

The supernatant was discarded from the 96-well plate. 25 µl Opti-MEM/well and 25 µl Bright-Glo/well were added, the 96-well plate wrapped in aluminium foil and shaken for 5 min at 200 rpm. The 50 µl Opti-MEM-Bright-Glo mixture was immediately transferred to an opaque 96-well plate (Microplate 96-well PS white, Greiner) and the luminescence was measured. The EGFP and Luciferase luminescence was measured in the Wallac Victor^3, 1420 Multilabel Counter, luminometer (PerkinElmer). The data were evaluated with the Wallac 1420 Manager Program.

2.5.2 Yeast two-hybrid

The yeast two-hybrid system (YTH-system) identifies the interaction of two proteins *in vivo* (Chien *et al* 1991). In this study the Matchmaker GAL4 Two-Hybrid System was used (Clontech). Matchmaker systems use the transcription-activating (AD) and DNA-binding domains (DNA-BD) of GAL4, a well-characterised yeast transcription factor (Zhu & Hanes 2000). In a two-hybrid assay, a cDNA is fused to the GAL4 DNA-binding domain and expressed, while another gene or cDNA is expressed as a fusion to the GAL4 activation domain, prey protein, (Fields & Song 1989; Chien *et al* 1991). When bait and prey proteins interact in a yeast reporter strain such as AH109, the DNA-BD and AD are brought into a physical proximity as a prerequisite for the activation of reporter gene transcription: *ADE2, HIS3, lacZ,* and *MEL1*.

Gal4 DNA-BD and AD fusions were created by cloning cDNAs into the yeast expression vectors pBridge or pACT. pBridge expresses proteins as fusions with the GAL4 DNA-BD, while pACT expresses proteins as fusions with the GAL4 AD. In yeast, both fusions are expressed from the constitutive *ADH1* promoter (P_{ADH1}). The following vectors were used for yeast two-hybrid analyses:

Table 2.2: Overview of the bait (pBridge) and prey (pAct2) vectors used in the yeast two-hybrid assay

Yeast two-hybrid vector name	Vector description	Primer
pBridgeSRHSP10	Includes full-length *S. ratti* HSP10 sequence subcloned to bait vector pBridge *Eco*RI and *Sal*I site.	pBridgeHSP10EcoR1for pBridgeHSP10Sal1rev
pAct2SRHSP10fl	Includes full-length *S. ratti* HSP10 sequence subcloned to prey vector pAct2 *Nco*I and *Eco*RI site.	pAct2HSP10Nco1for pAct2HSP10EcoR1rev
pAct2SRHSP10a	Includes *S. ratti* cDNA fragment ranging from bp 1 to bp 174. Subcloned to prey vector pAct2 pAct2 *Nco*I and *Eco*RI site.	pAct2HSP10Nco1for HSP10rev174EcoRI
pAct2SRHSP10b	Includes *S. ratti* cDNA fragment ranging from bp 85 to bp 138. Richardson *et al* 2001 described this HSP10 binding site. Subcloned to prey vector pAct2 multiple cloning site.	HSP10BDpAct2Sense HSP10BDpAct2Antisense
pAct2SRHSP10c	Includes *S. ratti* cDNA fragment ranging from bp 168 to bp 330. Subcloned to prey vector pAct2 *Nco*I and *Eco*RI site.	HSP10for168NCOI HSP10revEcoRI
pAct2SRHSP10gen1	Includes *nco*I and *xba*I digested out of pCR2.1-pAct2_HSP10 plasmid. Ranging from bp 1 to bp 84. *Subcloned* to prey vector pAct2 *Nco*I and *xba*I site.	
pAct2SRHSP10gen2	Includes *bam*HI and *Xho*I digested out of pCR2.1-pAct2_HSP10 plasmid. Ranging from bp 46 to bp 138. Subcloned to prey vector pAct2 *Bam*HI and *Xho*I site.	
pAct2SRHSP10gen3	Includes *sma*I and *sac*I digested out of pCR2.1-pAct2_HSP10 plasmid. Ranging from bp 85 to bp 168. Subcloned to prey vector pAct2 *sma*I and *Eco*RI site.	
pBridgeSRHSP60	Includes full-length *S. ratti* HSP60 sequence. Subcloned to bait vector pBridge *Bam*HI and *Eco*RI site.	
pAct2SRHSP60	Includes full-length *S. ratti* HSP60 sequence. Subcloned to prey vector pAct2 *sma*I and *Eco*RI site.	

2.5.2.1 Filter and fluid ß-Galactosidase assays

Filter ß-Galactosidase assays were carried out according to the Yeast Protocols Handbook, 2001. The fluid ß-Galactosidase assays were carried out according to the Yeast Protocols Handbook, 2001.

2.5.3 Western blot analyses

Western blot analyses were performed to detect *Sr*HPS10 and *Sr*HSP60 proteins in *S. ratti* extracts, to confirm the expression of HSP10 and HSP60 recombinant proteins, and to detect specific antibodies against HSP10 and HSP60 in *S. ratti* infected rats. Protein extracts of *S. ratti* iL_3 or recombinantly expressed proteins were separated according to their protein molecular weight by SDS-PAGE (2.5.3.1) and transferred (2.5.3.2) to a nitrocellulose membrane by electroblotting. Subsequently, the proteins were probed with specific antibodies.

2.5.3.1 Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed by mixing an aliquot of a protein extract with sample buffer, which was incubated at 95°C for 10 min before the samples were loaded onto 6-13% slab gels, depending on the protein size to be detected.

Table 2.3: Separating and stacking gel composition

	Separating gel							**Stacking gel**
Percentage	**6**	**7**	**8**	**9**	**10**	**12**	**13**	**3.90**
Polyacrylamide-Solution [ml]	3.00	3.5	4.00	4.50	5.00	6.00	6.50	0.65
Stacking gel-/Separating gel buffer [ml]	3.75	3.75	3.75	3.75	3.75	3.75	3.75	1.25
dH_2O [ml]	8.25	7.75	7.25	6.75	6.25	5.25	4.75	3.05
+ 50 µl 10% APS, 10 µl TEMED								

2.5.3.2 Electropheric transfer (semi dry blot) and immunological detection of proteins

After electrophoresis, the proteins were transferred onto nitrocellulose membranes by using a mini-Trans Blot Cell in Bjerrum-Schafer-Nielsen transfer buffer for 50 min at 50 V. The membrane was blocked with 5% skim milk in PBS (w/v) overnight under constant agitation at 4°C. Subsequent to a wash step with TBS/ 0.05 % Tween 20 for 10 min at RT, the nitrocellulose membrane was probed with 1:20 dilutions of sera from *S. ratti* infected Wistar rats. Unbound antibodies were removed by 2 wash steps with TBS/0.05 % Tween 20 and PBS for 10 min respectively. The appropriate diluted (5% skim milk in PBS (w/v)) horseradish peroxidase-conjugated goat anti-rat IgG (Roche) secondary antibody was used. Immunoreactive bands were visualised using 4-chloro-1-naphtol (Roche) colour development reagent according to the manufacturer's protocol.

2.5.3.3 Coomassie staining of polyacrylamide gels

After SDS-PAGE, the gel was washed three times in 2.5% Triton X-100 mM NaCl and stained with 0.1% (v/v) Coomassie brilliant blue, Roti®-Blue (Carl Roth). Under acidic conditions, the dye is most stable as a doubly-protonated red form. Upon binding to protein, however, it is most stable as an unprotonated, blue form. After destaining (Coomassie destaining solution), proteolytic degradation of the gelatine substrate was visible as unstained bands on the blue gel.

2.5.3.4 Silver staining of polyacrylamide gels

After SDS-PAGE, gels were fixed with fixation solution I and II for 10 min at RT respectively, washed in distilled water during at least 10 min, and visualised by incubating in freshly prepared silver nitrate solution. Afterwards, the gels were silvered, washed twice in distilled water and developed, depending on the protein density, for 3-30 min with developing solution. When a sufficient degree of staining was obtained, the reaction was stopped by washing the gels in stopping solution. The gel was stored in distilled water until drying.

2.5.3.5 Ponceau-S staining

Ponceau-S staining was performed using 1.0% Ponceau-S in 5% acetic acid for 5 min followed by a wash step in distilled water. To completely remove the stain from the protein bands, the membrane was washed several times in distilled water.

2.5.3.6 Drying of polyacrylamide gels

Polyacrlyamide gels were incubated in 10% glycerol for 30 min at RT and dried using Gel Drying Frame (Roth). The gels were placed between two cellophane sheets, fixed using a metal frame and plastic clips, and dried for 1 day at RT.

2.5.4 Protein expression

2.5.4.1 Expression of recombinant protein in *E. coli*

For protein expression, appropriate DNA inserts were cloned in-frame into the pJC45 Vector (kindly provided by Dr. Joachim Closs, BNI) containing a N-terminal His tag. Competent bacteria, *E. coli* strain DH5-α, were transformed with plasmid DNA and plated onto LB plates containing appropriate antibiotics. After overnight culture, positive colonies were transferred to 25 ml LB medium with 100 µg/ ml ampicillin and incubated overnight at 37°C under constant agitation. The non-induced overnight culture was transferred to a 1 l LB expression-medium and cultivated at 37°C under constant agitation until the culture reached an optical density (OD_{600}) of 0.6. The protein synthesis was induced by adding 1 M IPTG (isopropyl-D-thiogalactopyranoside). The protein expression was controlled by collecting small aliquots of the culture after IPTG induction every hour. After 4 h of growth at 37°C, the bacteria were harvested by centrifugation (4,000 g; 30 min; 4°C).

The bacterial pellet was resuspended in 50 ml Buffer B for 2 hours under constant rotation. Subsequently, the bacteria were centrifuged at 15,000 g for 30 min. For purification, the proteins were loaded onto a NINTA Agarose Column according to the manufacturer's protocol (Qiagen). The purified protein was then washed twice in 16 ml Buffer C (pH 6.3) and eluted 4 x in 2 ml Buffer D (pH 5.9), 4 x in 2 ml Buffer E (pH 4.5). The eluted protein fractions were loaded onto SDS PA gels, and the positive fractions pooled. The pooled proteins were dialysed (VISKING Dialysis Tubing), according to the manufacturer's protocol, overnight in 4 M Urea Buffer (pH 8.0). Subsequently, PEG 1500 (Merck) was used to a final concentration of 114 µg/ ml. The concentrations were measured using the Bradford assays.

2.5.4.2 Transfection and expression of recombinant protein in the Baculovirus system

The baculovirus expression system (FlashBAC^{TM} System, Oxford Expression Technologies LTD) uses a transfer vector, which contains sequences that flank the polyhedron gene in the virus. The transfer-vectors, containing the foreign gene, and the virus genome are introduced into the

host insect cell. The homologous recombination between the flanking sequences, common to both DNA molecules, effects insertion of the foreign gene into the virus genome. This results in a recombinant virus genome.

The 6-well plate was seeded 1 hour prior to transfection with a cell density of $1.5x10^6$ SF21 or $1x10^6$ SF9 cells/well in a 2 ml volume of appropriate medium (BD BaculoGold Max-XP Insect Cell Medium from BD Biosciences Pharmingen; BacVector Insect Cell Medium from Novagen respectively). To ensure the use of healthy cells before passaging, cell samples were taken and observed under a phase-contrast microscope. 20 µl of cell medium were counted using the Neubauer chamber. The co-transfection mix of DNA and liposome reagent (GeneJuice® Transfection Reagent, Novagen) was prepared as follows:

Table 2.4: Overview of the baculovirus assay reagents

Reagent 1	pAcSG2 HSP10 (500ng)	+	Baculo DNA (100ng)
Reagent 2	pAcSG2 HSP10 (500ng)	+	Baculo DNA (100ng)
Reagent 3	pAcSG2 HSP10 (500ng)	+	Baculo DNA (100ng)
Reagent 4	negative control mock-transfection (media with omitted DNA)		
Reagent 5	positive control K+ (500ng)	+	Baculo DNA (100ng)
Reagent 6	pIEX-GFP (2500ng) transfection control		

Appropriate media, depending on the cell type (SF21: BD BaculoGold Max-XP Insect Cell Medium from BD Biosciences Pharmingen; SF9: BacVector Insect Cell Medium from Novagen respectively) was added to a total volume of 60 µl/reagent. 18 µl GeneJuice/600 ng DNA and 12.5 µl GeneJuice/2500ng DNA were added and incubated for 15 minutes. Just before the end of the incubation period, the culture medium from the 6 well plate was removed, 940 µl/reagent of appropriate media was added and 1 ml reagent/well was transferred to the insect cells. After 5 hours incubation, the reagent medium was discarded and 2 ml fresh appropriate media was added. Following a 5-day incubation period, the media containing the recombinant virus (seed

stock) was harvested into sterile 1.5 ml Eppendorf tubes and stored at -70°C until use. The cells were also harvested into sterile 1.5 ml Eppendorf tubes and stored at -20°C until use. To amplify the recombinant virus, 200 µl seed stock/1000 µl medium was transferred to a 6-well plate with freshly split cells (cell density: $1.5x10^6$ SF21 or $1x10^6$ SF9 cells/well in a 2 ml volume of appropriate medium). Prior to transfer, the cell media was discarded. Following a 1 hour-incubation period, 1 ml of appropriate media was added and incubated for 5 days. After incubation, the media containing the recombinant virus (1st passage) was harvested into sterile 1.5 ml Eppendorf tubes and stored at -70°C until use. The cells were also harvested into sterile 1.5 ml Eppendorf tubes and stored at -20°C until use. To verify the expression success, the harvested cells were analysed by Western blot.

2.5.5 Enzyme-linked immunosorbent assay (ELISA)

Rat sera was analysed by ELISA for IgG antibodies to *S. ratti* HSP10 antigens. 96-well microtiter plates (polystyrene microtiter plate, Maxi-Sorb, Nunc) were coated with 100 µl/well *S. ratti* extract and *S. ratti* HSP10 recombinant protein respectively at a concentration of 200 ng/well in carbonate buffer (pH 9.6), sealed with Saran wrap, and incubated over night at 4°C. After removal of unbound *S. ratti* extract and *S. ratti* HSP10 recombinant protein respectively by washing three times with 300 µl/well washing-buffer PBS/0.05% Tween 20, the plate was blocked with 200 µl/well 5% BSA in PBS for 1 hour at 37°C. Serum was diluted 1:300, 1:600, 1:1200 in PBS/0.5%BSA prior to incubation at 37°C for 1 hour. Unspecifically bound antibodies were removed by 3 washing steps. 100 µl/well 1:5000 diluted Anti-Rat IgG peroxidase–conjugate antibody was added and incubated at RT for 1 hour followed by 3 washing steps. 100 µl/well peroxidase substrate solution was added for 5 min prior to 100 µl/well 1 N H_2SO_4 stop solution. For detection of antibody binding in all ELISA experiments, the optical density (OD) was measured at the absorbance at 450 nm (ref. 630 nm) using an ELISA reader (Dynatech).

2.5.6 Mass spectrometry (Liquid Chromatography Electrospray Ionisation Tandem MS-LC ESI MS/MS)

2.5.6.1 Supernatant extraction

The iL3 and parasitic females were extensively washed with Hanks balanced salt solution (Invitrogen) supplemented with 200 IU penicillin/200 mg streptomycin/ml. The iL3 and parasitic

females were either (i) homogenised using one steel bead (PeqLab Biotech., Erlangen, Germany) and subsequently the proteins extracted in the presence of proteinase inhibitors, or (ii) incubated for the generation of E/S products. Infective larvae ($4x10^4$/ml) and parasitic females (100/ml) were cultured in RPMI 1640 medium, at 37°C using previously described protocols (Maruyama *et al* 2003). Secreted adhesion molecules of *Strongyloides venezuelensis* are produced by oesophageal glands and are components of the wall of tunnels constructed by adult worms in the host intestinal mucosa. The incubation times were 24 h for the iL_3 and 72 h for parasitic females with changes of media every 24 h. E/S products were concentrated 250x (Amicon Ultra 10.000 MWCO filters; Millipore GmbH, Schwalbach, Germany) after supplementing protease inhibitors (Roche). In experiments inhibiting the protein synthesis, cycloheximide (Sigma-Aldrich) was added to the culture medium with a final concentration of 70 µM.

The larvae were pelleted by centrifugation, the excretory and secretory protein-containing supernatants (ESP-SN) harvested and analysed for sterility by blood agar growing test. The ESP-SN was concentrated 100-200 fold by 10 kDa ultrafilter centrifugation and analysed for protein composition by SDS-PAGE as well as for proteolytic activity by gelatine gel electrophoresis according to earlier reports (Borchert *et al* 2007). The fractions were subjected to 12% SDS-PAGE followed by staining with colloidal Coomassie (Invitrogen). The entire lane was cut into 36 gel blocks of equal size. All gel blocks were in-gel digested with trypsin using published procedures (Shevchenko *et al* 1996).

The mass spectrometric analyses were kindly performed by Hanns Soblik (Bernhard-Nocht Institute) supervised by Hanno Steen, Director of the Proteomics Center, Department of Pathology, Children's Hospital, Harvard Medical School in Boston. All MS datasets were searched against combined protein sequence database containing EST sequences from *S. ratti* and *S. stercoralis* as well as the RefSeq protein sequences for *C. elegans* and *C. briggsae*. Searches were performed using the ProteinPilot search engine (v2.0). The following search parameters were selected: Sample Type: Identification; Cys. Alkylation: Iodoacetamide; Digestion: Trypsin; Instrument: LTQ; Special Factors: Gel-based ID; ID Focus: Amino acid substitutions; Search Effort: Thorough. Proteins were identified based on a minimum of 4.00 'unused score' equivalent to two or more unique peptides of confidence 99.

3 Results

3.1 Identification and cloning of the putative *S. ratti* HSP10 sequence

To identify a homologue of HSP10 in *S. ratti*, a nucleotide search for potential *S. stercoralis* HSP10 ESTs (expressed sequence tags) was performed using the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov). The search resulted in a designated *S. stercoralis* chaperonin 10-like EST (GenBank accession number BE580000) (Figure 3.1).

cttactgttacccgtagatgttgttcagctcttaagaatgtccaaccacttttgatcgtgtaatggttaa
aaaagctgctgcagaagtacaatctaagggtggcatttatattccagaaaaagcccaaggaaaagttcttg
aaggaactgttgttgctgctggtccaggacttcgtactgaagatggtaaagtaattcctttatctgttggt
gttggggatcgtgtaatgttaccagagtatggtggaaataaagttgttatggatgataaagaatacttcat
ctaccgtgagtcggatctcattgctaaactcaccaactaagaaatacatgaagtgtaattttatataaata
t

Figure 3.1: HSP10 homologue of *S. stercoralis*

HSP10 homologue of *S. stercoralis* (GenBank accession number BE580000). Degenerate primers used for the performed *S. ratti* RT-PCR are underlined.

Using the degenerate primer pair SSHSP10FAN and SSHSP10Rend2, an RT-PCR was performed with cDNA of *S. ratti* iL3 as template. Agarose gel electrophoresis of the RT-PCR product showed a band of the expected size of about 300bp. The RT-PCR product was cloned into the TOPO TA vector and sequenced. The sequence analysis confirmed a product of 295 bp. The BLAST search (BLAST resources available at HTTP://www.ncbi.nlm.nih.gov.blast) revealed a high degree of sequence similarity to *S. stercoralis* HSP10. To obtain the full-length sequence, 3'RACE and spliced leader (SL)-RT-PCRs were performed. For the 3'RACE, *S. ratti* mRNA was reverse transcribed with an oligodt-T7 primer and the PCR was performed using the gene-specific 1 and ODT-T7 II primers.

SLs have been identified in a variety of eukaryotes including nematodes (Krause & Hirsh 1987). SL1 (a non-coding 22 bp-nucleotide sequence) was the first SL sequence identified in nematodes (Krause & Hirsh 1987; Bektesh *et al* 1998). Since it was speculated that the mRNA of *S. ratti* could also contain a SL, a SL RT-PCR was performed to obtain the 5'end. *S. ratti* cDNA was used as template, SL as sense primer and the gene-specific 2 as antisense primer.

Agarose gel electrophoresis of the 3'RACE product revealed a band of approximately 250 bp, the SL RT-PCR product had a size of approximately 300 bp. The RT-PCR products were cloned separately into TOPO TA vectors. The subsequent sequence analysis showed a 229 bp insert for the 3'RACE RT-PCR product and a 302 bp insert for the SL RT-PCR product. The

overlapping sequence fragments of the already obtained putative *S. ratti* HSP10 sequence and the 3'end and 5'RACE products showed 100% identity.

Finally, to amplify the full-length *S. ratti* HSP10 mRNA/cDNA, an RT-PCR was done with *S. ratti* iL3 cDNA as template using the primer combination HSP10flfor and HSP10flrev. The RT-PCR product was cloned into pGemTeasy vector. Subsequent sequence analyses confirmed the correct putative full-length sequence of HSP10. The pGemTeasy_HSP10_FL clone served then as basis for further subclonings to generate expression-clones coding for *S. ratti* HSP10 full-length or *S. ratti* HSP10 fragments.

3.2 Characterisation of the *S. ratti* HSP10 transcript

The full-length cDNA of the *S. ratti* HSP10 is 507 bp long. The translation initiation codon (ATG) is 43 bp downstream of the 5' untranslated region (UTR) starting with the SL sequence. There are 2 stop codons (TAA), one at position bp 327-330, followed by one in-frame stop at position bp 339-342 (Figure 3.2). The 5' UTR does not exhibit any Kozak consensus sequences, or Kozak consensus-like sequences upstream of the ATG codon (Kozak 1987). The sequence contains a majority of A-T bases (61%) within the open reading frame (ORF), of which 69% are in the 5'UTR and 82% in the 3' UTR. Alignment results indicated that the *S. ratti* HSP10 full-length cDNA has significant homology (93%) to the HSP10 homologue of *S. stercoralis* (Fig. 3.3). The ORF comprises 330 bp and codes for 110 amino acid residues and has a predicted molecular mass of 11.8 kDa. Moreover, the *Sr*HSP10 ORF has a mitochondrial targeting peptide (AA 1-30, iPSORT prediction). Furthermore, its N-terminal sequence has the ability to form an amphipathic alpha helix, which enables it to cross the mitochondrial membrane. The *S. ratti* HSP10 protein has a high homology (93%) to *S. stercoralis*, with the exception of 8 amino acid exchanges, of which 4 are conservative (Figure 3.4). An additional multi alignment analysis indicated that the amino acid sequence of *S. ratti* HSP10 is 63% identical with the HSP10 homologue of *C. elegans* and 57% identical with the HSP10 homologue of *homo sapiens*. Furthermore, the *S. ratti* HSP10 sequence shows similarity of 57% to the *Brugia malayi* (*B. malayi*) HSP10 (Figure 3.5).

```
-43  ggtttaattacccaagtttgaggttcttgtaattttggtaaatatgcttctttccgctgtt  18
     V  -  L  P  K  F  E  V  L  V  I  L  V  N  M  L  L  S  A  V
 19  cgtagatgttcatcagctcttaaaaatgttcaaccacttttgatcgtgttatgatcaaa   78
     R  R  C  S  S  A  L  K  N  V  Q  P  L  F  D  R  V  M  I  K
 79  aaagctgctgctgaagtaaaatctaaaggaggtatttacattccagagaaagcccaagga  138
     K  A  A  A  E  V  K  S  K  G  G  I  Y  I  P  E  K  A  Q  G
139  aaggttcttgaaggtactgttgttgctgctggtccaggacttcgtactgaggatggtaaa  198
     K  V  L  E  G  T  V  V  A  A  G  P  G  L  R  T  E  D  G  K
199  cttatacctttatcagttagtgttggagatcgtgttatgttgccagagtatggtggaaat  258
     L  I  P  L  S  V  S  V  G  D  R  V  M  L  P  E  Y  G  G  N
259  aaagttgttatggatgatacagaatatttcatctatcgtgagtcagatctcatcgccaaa  318
     K  V  V  M  D  D  T  E  Y  F  I  Y  R  E  S  D  L  I  A  K
319  ctcactaactaaggaatacattaaagtgtaatttttatatattatttgttgttttttgc  378
     L  T  N  -  G  I  H  -  S  V  I  F  I  Y  Y  F  V  V  F  C
379  atagttgatattgtagtttgtattcttgttggttggttaaagactataaaaaatttttaca  438
     I  V  D  I  V  V  C  I  L  V  G  W  L  K  T  I  K  N  F  T
439  caaaaaaaaaaaaaaaaaaaaaaa                                      464
     Q  K  K  K  K  K  K  K
```

Figure 3.2: Complete coding sequence and deduced amino acid sequence of the *S. ratti* HSP10 gene

The translation initiation and termination codons are printed in bold. The predicted amino acid sequence is shown below the nucleotide sequence. The spliced leader sequence is pinted in grey. The putative mitochondrial targeting sequence is shaded grey. Numbering starts at the first nucleotide of the initiation codon, with upstream sequences indicated as negative numbers.

```
SrHSP10         ggtttaattacccaagtttgaggttcttgtaattttggtaaatatgcttctttccgctgt 60
SsHSP10         --------------------------------------------------cttactgttac 11
                                                                   *** * * *

SrHSP10         tcgtagatgttcatcagctcttaaaaatgttcaaccacttttgatcgtgttatgatcaa 120
SsHSP10         ccgtagatgttgttcagctcttaagaatgtccaaccacttttgatcgtgtaatggttaa 71
                 **********  ********** ***** ******************** *** * **

SrHSP10         aaaagctgctgctgaagtaaaatctaaaggaggtatttacattccagagaaagcccaagg 180
SsHSP10         aaaagctgctgcagaagtacaatctaagggtggcatttatattccagaaaaagcccaagg 131
                ************ ****** ******* ** ** ***** ******** ***********

SrHSP10         aaaggttcttgaaggtactgttgttgctgctggtccaggacttcgtactgaggatggtaa 240
SsHSP10         aaaagttcttgaaggaactgttgttgctgctggtccaggacttcgtactgaagatggtaa 191
                *** *********** ************************************ ********

SrHSP10         acttatacctttatcagttagtgttggagatcgtgttatgttgccagagtatggtggaaa 300
SsHSP10         agtaattcctttatctgttggtgttggggatcgtgtaatgttaccagagtatggtggaaa 251
                * * ** ******** *** ******* ******** ***** *****************

SrHSP10         taaagttgttatggatgatacagaatatttcatctatcgtgagtcagatctcatcgccaa 360
SsHSP10         taaagttgttatggatgataaagaatacttcatctaccgtgagtcggatctcattgctaa 311
                ******************** ****** ******** ******** ******** ** **

SrHSP10         actcactaactaaggaatacattaaagtgtaatttttatatattatttgttgtttttg 420
SsHSP10         actcaccaactaagaaatacat-gaagtgtaatttt---atataaatat----------- 356
                ****** ******* *******  ************   **** * * *

SrHSP10         catagttgatattgtagtttgtattcttgttggttggttaaagactataaaaaatttac 480
SsHSP10         ------------------------------------------------------------

SrHSP10         acaaaaaaaaaaaaaaaaaaaaaaa 507
SsHSP10         -------------------------
```

Figure 3.3: Nucleotide sequence alignment of the *S. ratti* HSP10 and the HSP10 homologue of *S. stercoralis*

Nucleotide sequence alignment (CLUSTALW) of the *S. ratti* HSP10 (*Sr*HSP10) sequence, including the 3' and 5'ends, with the *S. stercoralis* EST (*Ss*HSP10) (GenBank accession number BE580000). Symbol (*) indicates identical nucleotides. Numbering starts at the first nucleotide of the initiation codon.

```
SrHSP10         MLLSAVRRCSSALKNVQPLFDRVMIKKAAAEVKSKGGIYIPEKAQGKVLEGTVVAAGPGL 60
SsHSP10         --LTVTRRCCSALKNVQPLFDRVMVKKAAAEVQSKGGIYIPEKAQGKVLEGTVVAAGPGL 58
                  *:..***.**************:*******:**************************

SrHSP10         RTEDGKLIPLSVSVGDRVMLPEYGGNKVVMDDTEYFIYRESDLIAKLTN---------- 109
SsHSP10         RTEDGKVIPLSVGVGDRVMLPEYGGNKVVMDDKEYFIYRESDLIAKLTN-EIHEV-FYIN 116
                ******:*****.******************.***************
```

Figure 3.4: Amino acid sequence alignment of the *S. ratti* HSP10 and the HSP10 homologue of *S. stercoralis*

Amino acid sequence alignment (CLUSTALW) of the *S. ratti* HSP10 (*Sr*HSP10)-like sequence with the HSP10 homologue of *S. stercoralis* (*Ss*HSP10) (GenBank accession number BE580000). Symbol (*) indicates identical amino acids, (:) indicates strongly similar amino acids and (.) indicates weakly similar amino acids. Numbering starts at the first amino acid.

```
SrHSP10         MLLSAVRRCS--SALKNVQPLFDRVMIKKAAAEVKSKGGIYIPEKAQGKVLEGTVVAAGP 58
SsHSP10         --LTVTRRCC--SALKNVQPLFDRVMVKKAAAEVQSKGGIYIPEKAQGKVLEGTVVAAGP 56
BmHSP10         MASMSVARKALGDLIKAFKPLSDRVLVERFAAETKTKGGIMIPDKAQGKVLEATVISTGP 60
CeHSP10         MFLTAVRRSS--NVLKTFKPLYDRVLVERVAAETKTKGGIMLPEKSQGKVLEATVVSAGA 58
                     . * .  . :* .:** ***:::: ***.::**** :*:*:******.**:::*.

SrHSP10         GLRTEDGKLIPLSVSVGDRVMLPEYGGNKVVMDDTEYFIYRESDLIAKLTN 109
SsHSP10         GLRTEDGKVIPLSVGVGDRVMLPEYGGNKVVMDDKEYFIYRESDLIAKLTN 107
BmHSP10         GGRDSKGNLVPMTVQAGDHVLLPEYGGTKVVVDEKEYHIFREADILGKFDQ 111
CeHSP10         GLRNEKGELVALTVKPGDRVLLPEYGGTKVVVEDKEYSIFRESDLLGVFH- 108
                * * ..*::::::*  **:*:******.***:::.** *:**:*::. :
```

Figure 3.5: Amino acid alignment of the *S. ratti* HSP10 and the HSP10 homologues of *S. stercoralis*, *C. elegans* and *Brugia malayi*

Amino acid alignment (CLUSTALW) of the *S. ratti* HSP10 (*Sr*HSP10)-like sequence with *S. stercoralis* (*Ss*HSP10, GenBank accession number BE580000), *C. elegans* (*Ce*HSP10, GenBank accession number NP497428) and *Brugia malayi* (*Bm*HSP10, GenBank accession number XM001902716). Symbol (*) indicates identical amino acids, (:) indicates conserved substitutions, (.) indicates semi-conserved substitutions. Numbering starts at the first amino acid.

3.3 Identification and cloning of the putative *S. ratti* HSP60 transcript

To identify the putative HSP60 sequence of *S. ratti*, a BLAST search was performed using the *Onchocerca volvulus* HSP60 sequence (AF121264) as a matrix. The data entry in the library Nematode.net for *S. ratti* showed a 1078 bp sequence comprising 5 ESTs:

Table 3.1: Overview of the S. ratti HSP60 EST Nematode.net data entry

EST	EST description	Derived primers
EST 1	ku55f08.y1 bp 1 - 412 equivalent to *S. ratti* ORF bp 268 - 679. Database entry CB098615.	PCR1 FW
EST 2	kt27c10.y3 bp 1 – 470 equivalent to *S. ratti* ORF bp 613 - 1083. Database entry BI703957.	PCR1 RV PCR2 FW
EST 3	kt26f04.y3 bp 389 - 523 equivalent to *S. ratti* ORF bp 1083 - 1216. Database entry BI703954.	
EST 4	kt26f04.y3 bp 237 - 395 equivalent to *S. ratti* ORF bp 1217 – 1375. Database entry CB097948.	PCR2 RV PCR3 FW
EST 5	kt72a12.y1 bp 41 - 372 equivalent to *S. ratti* ORF bp 1814 – 2141. Database entry BI323502.	PCR3 RV

To obtain the putative *S. ratti* HSP60 full-length cDNA sequence, three separate RT–PCR analyses were performed. First strand *S. ratti* iL_3 cDNA served as template for each RT–PCR. PCR1 FW / PCR1 RV, PCR2 FW / PCR2 RV and PCR3 FW / PCR3 RV, primer pairs derived from the 5 ESTs were used as forward (FW) and reverse (RV) primers. Two RT-PCR products were obtained from the PCR1 and PCR2 primer pairs. They consisted each of a cDNA fragment of about 500 bp. The amplicons were separately cloned into pGemTeasy. Sequencing and alignment analyses confirmed their identity as putative *S. ratti* HSP60 cDNAs. No amplicon was obtained using the primer pair PCR3 FW/RV. To obtain the full-length sequence of the putative *S. ratti* HSP60 transcript, again, 3'RACE fragments were generated and a spliced leader (SL) RT-PCR was performed.

For 3'RACE, *S. ratti* mRNA was reverse transcribed with the oligo(dT)-T7 primer, subsequently an RT-PCR was performed using the primers SR_HSp60_RACE_GSP_F and ODT-

T7 II. For SL-RT-PCR, *S. ratti* iL3 cDNA was used as template, SL as sense primer and the SR_HSp60_RACEGSP_R as antisense primer. The obtained 3'RACE and SL-RT-PCR fragments were cloned each into pGemTeasy (pGemTeasy_HSP60_3'RACE, pGemTeasy_HSP60_5'SL). Agarose gel electrophoresis showed a band of approximately 1 kb for the 3'RACE, and a band of approximately 0.8 kb for the SL-RT-PCR. Sequencing confirmed their identity as putative *S. ratti* HSP60 cDNAs.

To confirm the full-length cDNA sequence of HSP60 of *S. ratti*, a RT-PCR analysis was performed using *S. ratti* iL3 cDNA as template, and HSP60Stop_rev and HSP60Sal-For as primers. The PCR product was checked on agarose gel and cloned into pGemTeasy. Subsequent sequencing and alignment with the already obtained putative *S. ratti* HSP60 transcript fragments showed 100% identity.

3.4 Characterisation of *S. ratti* HSP60 iL3 transcript

The full-length HSP60 cDNA of *S. ratti* is 1914 bp in size. The translation initiation codon starts 52 bp downstream of the 5' UTR. The stop codon (TAA) is located at position 1742, followed by two in-frame stop codons within the 174 bp long 3' UTR. The open reading frame (ORF) is 1695 bp in size and codes for 565 amino acid residues (Figure 3.6). The sequence contains a majority of A-T bases (67%) within the ORF, of which 75% are in the 5'UTR and 80% in the 3'UTR. A putative mitochondrial targeting sequence with 25 amino acid residues was identified in the N-terminal region (Figure 3.6, grey shaded sequence) (Claros and Vincens 1996*)*. The deduced amino acid sequence has a predicted molecular mass of 64 kDa, which is expected for the products of HSP60 genes (Hemmingsen *et al* 1988; Jindla *et al* 1989; Lund 1995). The sequence contains the canonical chaperonin 60 signature (AAAVEEGIVPGGG, Figure 3.6 (ProSite document PS00296 1997)) between amino acid residues 420 and 432. BLAST P analysis revealed high sequence similarity with HSP60 genes from *Strongyloides sterocaris* (Figure 3.7a) and other organisms, including the human, *Onchocerca volvulus (O. volvulus*) and *B. malayi* HSP60 (Figure 3.7b). The HSP60 gene promoter is characterised by the absence of a classical TATA box and by the presence of a single heat shock response element (HSE), nGAAn, located 25 bp upstream of the start codon (ATG). Promoters of other heat shock protein genes frequently contain several such elements (Xiao & Lis 1988; Amin *et al* 1988; Xiao *et al* 1991).

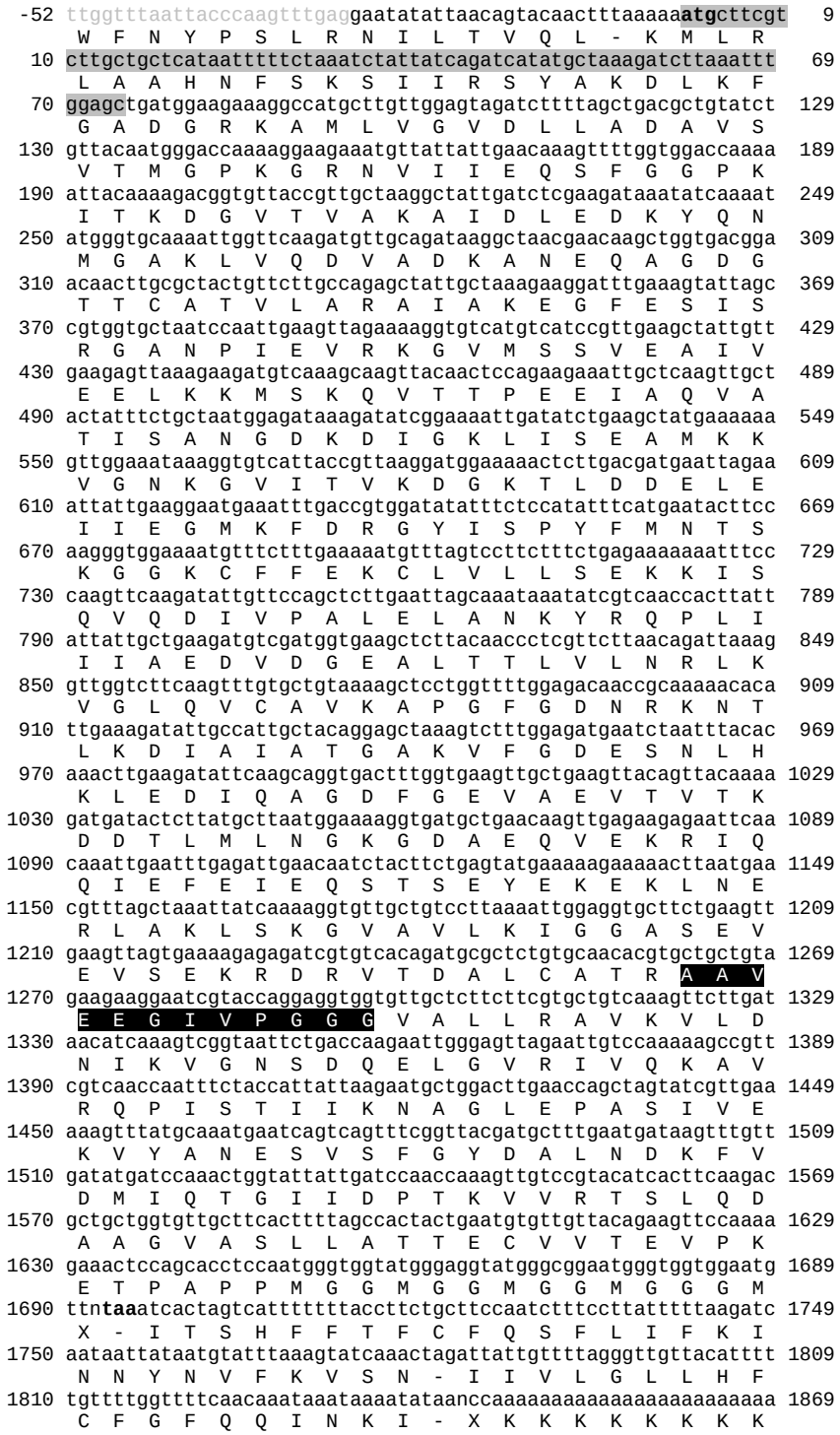

Figure 3.6: Complete coding sequence and deduced amino acid sequence of the *S. ratti* HSP60 gene

The translation initiation and termination codon is printed in bold. The predicted amino acid sequence is shown below the nucleotide sequence. The spliced leader sequence is printed in grey. The putative mitochondrial targeting sequence is shaded grey. The canonical HSP60 signature is shaded black. Numbering starts at the first nucleotide of the initiation codon, with upstream sequences indicated as negative numbers.

```
SrHSP60         MLRLAAHNFSKSIIRSYAKDLKFGADGRKAMLVGVDLLADAVSVTMGPKGRNVIIEQSFG 60
SsHSP60         ---LATRNFSKSFLRSYAKDLKFGADGRKAMLVGVDLLADAVSVTMGPKGRNVIIEQSFG 57
                   **::*****::*********************************************

SrHSP60         GPKITKDGVTVAKAIDLEDKYQNMGAKLVQDVADKANEQAGDGTTCATVLARAIAKEGFE 120
SsHSP60         GPKITKDGVTVAKAIDLEDKYQNMGAKLVQDVADKANEQAGDGTTCATVLARAIAKEGFE 117
                ************************************************************

SrHSP60         NISRGANPIEVRKGVMSSVEAIVEELKKMSKQVTTPEEIAQVATISANGDKDIGKLISEA 180
SsHSP60         NISRGANPIEVRKGVMSSVEAIVGEVNEMSKQVATPEEIASVATISANGEKEMGKCISTO 177
                ********************** *:::*****:******.********:*::** **
```

Figure 3.7a: Amino acid sequence alignment of the *S. ratti* HSP60 and the HSP60 homologue of *S. stercoralis*

Amino acid alignment (CLUSTALW) of the *S. ratti* HSP60 (*Sr*HSP60)-like sequence with HSP60 homologue of *S. stercoralis* (*Ss*HSP60) (Nematode.net accession number kq35f01.y1). Symbol (*) indicates identical amino acids, (:) indicates strongly similar amino acids and (.) indicates weakly similar amino acids. Numbering starts at the first amino acid.

```
SrHSP60         ---------MLRLAAHNFSKSIIRSYAKDLKFGADGRKAMLVGVDLLADAVSVTMGPKGR 51
HhHSP60         MLRLPTVFRQMRPVSRVLAPHLTRAYAKDVKFGADARALMLQGVDLLADAVAVTMGPKGR 60
OvHSP60         ---------MARLVSR-TVRSGLRHYAKDVKFGADGRASMLYGVDTLADAVAVTMGPKGR 50
BmHSP60         -MFRIGGQSVARLVSR-TVQSGLRHYAKDVKFGADGRASMLYGVDTLADAVAVTMGPKGR 58
                          * .::       * ****:*****.*  ** *** *****:********

SrHSP60         NVIIEQSFGGPKITKDGVTVAKAIDLEDKYQNMGAKLVQDVADKANEQAGDGTTCATVLA 111
HhHSP60         TVIIEQSWGSPKVTKDGVTVAKSIDLKDKYKNIGAKLVQDVANNTNEEAGDGTTTATVLA 120
OvHSP60         NVVIEQSWGSPKITKDGVTVAKAIDFKDKYKNLGAKLVQDVANKTNEEAGDGTTCATVLA 110
BmHSP60         NVVIEQSWGSPKITKDGVTVAKAIDFKDKYKNLGAKLVQDVANKTNEEAGDGTTCATVLA 118
                .*:****:*.**:*********:**::***:*:*********:::**:****** *****

SrHSP60         RAIAKEGFENISRGANPIEVRKGVMSSVEAIVEELKKMSKQVTTPEEIAQVATISANGDK 171
HhHSP60         RSIAKEGFEKISKGANPVEIRRGVMLAVDAVIAELKKQSKPVTTPEEIAQVATISANGDK 180
OvHSP60         RAIAKEGFENISKGANPVEVRRGVMKAVELLVAELKKMSKDVTTPEEIAQVATISANGDS 170
BmHSP60         RAIAKEGFENISKGANPVEVRRGVMKAVELLVAELKKMSKDVTTPEEIAQVATISANGDS 178
                *:*******:**:****:*:*:*** :*: :: **** ** ******************.

SrHSP60         DIGKLISEAMKKVGNKGVITVKDGKTLDDELEIIEGMKFDRGYISPYFMNTSKGGKCFFE 231
HhHSP60         EIGNIISDAMKKVGRKGVITVKDGKTLNDELEIIEGMKFDRGYISPYFINTSKGQKCEFQ 240
OvHSP60         SVGKLISEAMKTVGNKGVITVKDGKTLHDELETIEGMKFDRGYISPYFINTSKGAKVEFE 230
BmHSP60         TVGKLISEAMKKVGNKGVITVKDGKTLHDELETIEGMKFDRGYISPYFINTTKGAKVEFE 238
                 :*::**:***.**.************.**** ***************:**:** *  *:

SrHSP60         KCLVLLSEKKISQVQDIVPALELANKYRQPLIIIAEDVDGEALTTLVLNRLKVGLQVCAV 291
HhHSP60         DAYVLLSEKKISSIQSIVPALEIANAHRKPLVIIAEDVDGEALSTLVLNRLKVGLQVVAV 300
OvHSP60         KCLLLFSEKKISQVQDIVPALELANKYRKPIVIIAEDVDGEALTTLVLNRLKVGLQVAAV 290
BmHSP60         KCLLLFSEKKISQVQDIVPALELANKYRKPIVIVAEDVDGEALTTLVLNRLKVGLQVAAV 298
                .. :*:******.:*.******:** :*:*::*:*********:************* **

SrHSP60         KAPGFGDNRKNTLKDIAIATGAKVFGDESNLHKLEDIQAGDFGEVAEVTVTKDDTLMLNG 351
HhHSP60         KAPGFGDNRKNQLKDMAIATGGAVFGEEGLTLNLEDVQPHDLGKVGEVIVTKDDAMLLKG 360
OvHSP60         KAPGFGDNRKNTLKDMAIATGGTVFGDDANLLKIEDVQISDLGEAEEVSITKDDTLILRG 350
BmHSP60         KAPGFGDNRKNTLKDMAIATGGTVFGDDANLLKIEDVQISDLGEA--------------- 343
                *********** ***:*****. ***::.   ::**:*  *:*:.
```

Figure 3.7b: Amino acid sequence alignment of *S. ratti* HSP60 and the HSP60 homologues Homo sapiens, *Onchocerca volvulus and Brugia malayi*

Amino acid sequence alignment (CLUSTALW) of *S. ratti* HSP60 (*Sr*HSP60)-like sequences with *Homo sapiens* (*Hh*HSP60, GenBank accession number NM002156); *Onchocerca volvulus* (*Ov*HSP60, GenBank accession number AF121264); *Brugia malayi* (*Bm*HSP60, GenBank accession number XM001902738). Symbol (*) indicates identical amino acids, (:) indicates conserved substitutions, (.) indicates semi-conserved substitutions. Numbering starts at the first amino acid.

3.5 Characterisation of putative *S. ratti* HSP10 and HSP60 transcripts of parasitic females

To detect the presence and putative isoforms of HSP10 and HSP60 transcripts in parasitic females of *S. ratti*, further RT-PCR experiments were performed as follows. Primers were derived based on the putative full-length cDNA sequence of *S. ratti* iL3 HSP10 and HSP60, respectively. RNA of *S. ratti* parasitic females was isolated and reverse transcribed. HSP10 and HSP60 amplicons were generated separately using appropriate primers (HSP10flfor and HSP10flrev or HSP60for and HSP60rev, respectively). Agarose gel electrophoresis revealed an *S. ratti* HSP10 amplicon of 330 bp, and for HSP60 an amplicon of about 1700 bp (Figure 3.8). These products were cloned into pGemTeasy (pGemTeasy_HSP10_FLPF, pGemTeasy_HSP60_FL). Subsequent sequence analysis showed a 330 bp cDNA insert for HSP10 and a 1695 bp cDNA insert for HSP60. Sequence alignment analysis of the obtained HSP10 cDNAs showed 100% identity. It contains a 330 bp-long open reading frame, including start (ATG) and stop (TAA) codons. The deduced amino acid sequence has a predicted molecular mass of 10 kDA and is identical with the putative full-length HSP10 cDNA sequence of *S. ratti* iL3. The identity of the putative *S. ratti* HSP60 parasitic female cDNA sequence was confirmed by BLAST analysis revealing a 100% similarity with HSP60 from iL3 and comprising a 1695 bp open reading frame including start (ATG) and stop (TAA) codons. The deduced amino acid sequence has a predicted molecular mass of 60 kDA and showed 100% similarity to the *S. ratti* HSP60 translation product of iL3.

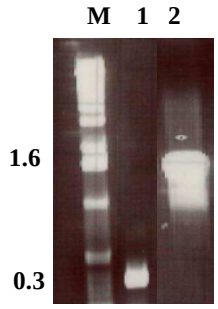

Figure 3.8: Analyses of the putative parasitic female *S. ratti* HSP10 and *S. ratti* HSP60 transcripts

RT-PCR analyses of the putative *S. ratti* parasitic female HSP10 (1) and HSP60 (2) transcripts. *S. ratti* total RNA was extracted from the small intestine of *S. ratti* infected Wistar rats at day 7 *post infectionem*. PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide. Numbers on the left panels are in kb. M = molecular weight marker (Invitrogen)

3.6 Localisation of putative HSP10 and HSP60 transcripts in *S. ratti* iL3 by *in situ* hybridisation

In situ hybridisation was done to localise the HSP10 transcripts in *S. ratti* iL3. To this end, cryo- and whole-mount techniques were used. The *S. ratti* iL3 are thin filamenteous structures and approximately 300–500 µm thick. The whole-mount slides contained approximately 100 *S. ratti* iL3. For cryo-sectioning, all blocks contained multiple cross- and longitudinal sections. Prior to hybridisation, RNA-probes of HSP10 and HSP60 were generated. To amplify the HSP10 probe with the appropriate primers (HSP10_sonde_for and HSP10_sonde_rev), the pGemTeasy_HSP10_FL clone served as template. For the amplification of the *S. ratti* HSP60 probe, the pGEmteasy_HSP60FL clone served as template, HSP60_sonde1_for and HSP60_sonde1_rev served as primers. The resulting PCR products, were cloned into pGemTeasy (pGemTeasy_HSP10_Sonde, pGemTeasy_HSP60_Sonde1). Sequence analyses confirmed the identity of both probes (HPS10: 307 bp, HSP60: 321 bp). Both clones were digested, each with *Not*I and *Bam*HI, the resulting HSP10 and HSP60 fragments each ligated directionally into pBluescript (pBluescript_HSP10_Sonde and pBluescript_HSP60_Sonde1). To generate antisense probes, the pBluescript_HSP10_Sonde and pBluescript_HSP60_Sonde1 clones were each linearised with the restriction enzyme *Not*I, and DIG-labelled transcripts were generated by T7 RNA polymerase. The sense probes were generated by linearising the clones pBluescript_HSP10_Sonde and pBluescript_HSP60_Sonde1 with the restriction enzyme *Bam*HI, respectively, and Dig-labelled transcripts were generated by T3 RNA polymerase. The sense probes served as a negative control.

For HSP10 and HSP60 *in situ* hybridisations, 3 *S. ratti* iL3 whole-mounts and 3 *S. ratti* iL3 cryo-slides were each hybridised with 20ng DIG-labelled RNA sense and antisense HSP10. For HSP60 hybridisation, 3 *S. ratti* iL3 whole-mounts and 3 *S. ratti* iL3 cryo-slides were each hybridised with 20ng DIG-labeled RNA sense and antisense HSP60 probes as described (see 2.3.13.2). The detection procedure used is based on the alkaline phosphatase enzyme reaction. The slides were incubated with anti-DIG-antibody conjugated with alkaline phosphatase and subsequently stained in 500µl staining solution (see 2.3.13.4). The anti-DIG antibody is then visualised with colometric NTB and BCIP alkaline phosphatase substrates. After 3 and 18 hours, respectively, the staining was stopped.

Both the HSP60 sense probe (Figure 3.9 a) and the HSP10 sense probes (Figure 3.9 c+d) showed no hybridisation after 3 hours and weak signals after 18 hours staining.

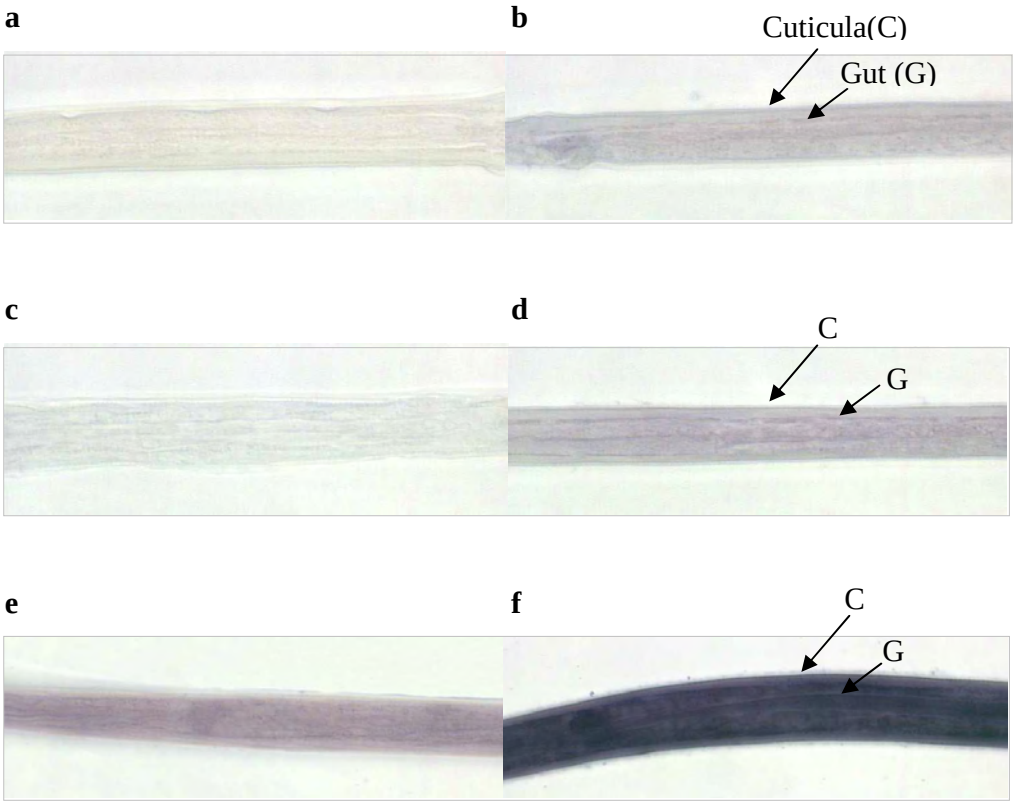

Figure 3.9: *In situ* hybridisation in *S. ratti* iL3

(a) *S. ratti* iL3 cryo-slides stained for 18 hours with HSP60 sense probes (b) *S. ratti* iL3 cryo-slides hybridised for 18 hours with HSP60 antisense probes (c) *S. ratti* iL3 cryo-slides hybridised for 18 hours with HSP10 sense probes (d) *S. ratti* iL3 cryo-slides hybridised for 18 hours with HSP10 antisense probes (e) *S. ratti* iL3 wholemount-slides hybridised for 18 hours with HSP10 sense probes (f) *S. ratti* iL3 wholemount-slides hybridised for 18 hours with HSP10 antisense probes

The HSP60 antisense probe hybridised with the cryo cut *S. ratti* iL3. A pattern of transcription was indicated within the digestive tract and not in the cuticula (Figure 3.9 b)

The HSP10 antisense probe also hybridised with the cryo (Figure 3.9 d) cut *S. ratti* iL3 and additionally with the whole mount *S. ratti* iL3 (Figure 3.9 f). A pattern of transcription was observed in the digestive tract and not in the cuticula.

3.7 Identification of HSP10 and HSP60 proteins in supernatants of *S. ratti* iL3 and parasitic females

The secreted products of *in vitro*-cultured *S. ratti* iL3 and parasitic females were collected under serum-free conditions using previously optimised protocols (Kwan-Lim *et al* 1989). SDS-PAGE analysis of all stages revealed a general consistency in protein concentration and composition of 10 kDa bands and 60 kDa bands. To address the question whether *S. ratti* iL3 and parasitic female worms selectively secrete a specific subset of proteins, the excretory/secretory *S. ratti* products were compared to a soluble worm homogenate. SDS gel electrophoresis revealed that both excretory/secretory *S. ratti* products and the soluble worm homogenate had distinct banding patterns at 10 kDa and 60 kDA (Figure 3.10). To identify the proteins present in excretory/secretory *S. ratti*, the excretory/secretory band and the soluble worm homogenate band at 10 kDa and 60 kDA were excised from the SDS gel and subjected to LC ESI MS/MS analysis. By using LC-MS/MS and the Protein Pilot database (Protein Pilot™ Software), *S. ratti* HSP10 and HSP60 were identified in excretory/secretory *S. ratti* products and soluble worm homogenate. Prior to the database search, the Protein Pilot database was complemented with the *S. ratti* HSP10 and HSP60 amino acid sequences previously obtained.

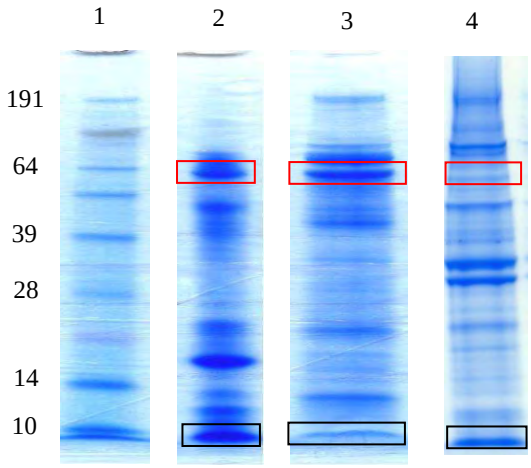

Figure 3.10: One-dimensional SDS-PAGE of iL3, parasitic females and *S. ratti* worm homogenate proteins

10 independent batches of *S. ratti* iL3 excretory/ secretory products (2) and *S. ratti* parasitic female (3) were compared to *S. ratti* soluble worm homogenate (4) by one-dimensional SDS-PAGE; each lane was loaded with 2 µg of protein. Molecluar weight markers (1) are indicated on the left in kDA. 60 kDa bands are red framed, 10 kDa bands are black framed

3.8 Characterisation of the putative *S. ratti* HSP10 and HSP60 genes

3.8.1 *S. ratti* HSP10 and HPS60 Southern blot analyses

To determine the genomic structure and the approximate site of the putative *S. ratti* HSP10 and HSP60 genes, a Southern blot analysis was carried out with *S. ratti* genomic DNA. The gDNA was isolated from 250 000 *S. ratti* iL3. The *S. ratti* genomic DNA was digested with the restriction enzymes *Bam*HI, *Eco*RI, *Eco*RV and *Hind*III, split into 2 equivalent portions, separately electrophoresed in a 1% (w/v) agarose gel, and transferred onto a Hybond XL1-membrane.

To generate the Southern blot probe, both, the pGemTeasy_HSP10_Sonde clone and the pGemTeasy_HSP60_Sonde1 clone were digested separately with the restriction enzymes *Not* I and *Spe* I and each ligated directionally to the pBluescript II SK +/– vector multiple cloning site (pBluescript_HSP10_Sonde, pBluescript_HSP60_Sonde1). Subsequent sequence analyses of the pBluescript_HSP10_Sonde clone (307 bp) and pBluescript_HSP60_Sonde1 (313 bp) confirmed the correctness of DNA insert and direction.

Both clones (pBluescript_HSP10_Sonde clone and pBluescript_HSP60_Sonde1) were digested separately with the restriction enzymes *Not* I and *Spe* I and the inserts eluted from the gel and purified. 25 ng HSP10 probe and 25 ng HSP60 probe were each random prime-labelled.

The filter-bound genomic DNA of *S. ratti*, was separately hybridised with the *S. ratti* HSP10 or the *S. ratti* HSP60 radioactively labelled probes. Hybridisations with both probes showed a 7 kb band with *Eco*RV-digested DNA, and a 5 kb band with *Hind*III-digested DNA (Figure 3.11). The detection of one common *Eco*RV and *Hind*III fragment suggests a cistronic arrangement as single copy genes.

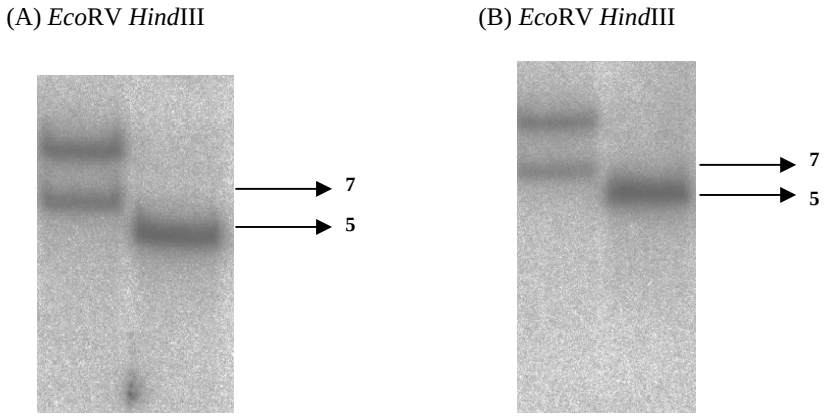

Figure 3.11: Southern blot analyses of *S. ratti* genomic DNA

DNA (10µg) was digested with *Eco*RV and *Hind*III, fractionated on 1% agarose gels, blotted to nylon membranes and separately hybridised to (A) a HSP10 probe or to (B) a HSP60 probe. Numbers on the right indicate fragment sizes in kb.

3.8.2 *S. ratti* HSP10 and HSP60 gene analyses

The Southern blot data suggested that the putative *S. ratti* HSP10 and HSP60 genes are located in a cistronic manner. To confirm this, PCR analyses were done. For amplification 1 µg *S. ratti* genomic DNA was used as template and HSP10_fl_for as well as HSP10_fl_rev as primers. A band of approximately 400 bp was detected on agarose gels and cloned into pGemTeasy. Sequence analysis showed a 372 bp DNA insert. Alignment with the putative *S. ratti* full-length HSP10 cDNA sequence revealed that the coding region shows 100% identity with the putative *S. ratti* full-length HSP10 cDNA sequence. Furthermore, alignment showed that the coding region is interrupted by one intron at position bp 27 to bp 69 with a length of 42 bp (Figure 3.12).

atgcttctttccgctgttcgtagatgtgtaggttattttatttctatataaaattatatttctttttagtc
atcagctcttaaaaatgttcaaccacttttgatcgtgttatgatcaaaaaagctgctgctgaagtaaaat
ctaaaggaggtatttacattccagagaaagcccaaggaaaggttcttgaaggtactgttgttgctgctggt
ccaggacttcgtactgaggatggtaaacttatacctttatcagttagtgttggagatcgtgttatgttgcc
agagtatggtggaaataaagttgttatggatgatacagaatatttcatctatcgtgagtcagatctcatcg
ccaaactcactaac**taa**

Figure 3.12: *S. ratti* HSP10 genomic DNA sequence

The intron (position 27 to position 69) is shaded grey. The translation initiation and termination codons are printed in bold.

To further investigate the gene location of HSP60 to HSP10, again, PCR was performed using 1 µg *S. ratti* genomic DNA as template and HSP60 and HSP60Stop_rev as primers. A product of approximately 1600 bp was obtained and cloned into pGemTeasy. Sequence analysis showed a 1600 bp DNA insert containing the HSP60 sequence. Alignment with the *S. ratti* full-length HSP60 cDNA revealed a 100% identity and showed that the coding region is without intron (Figure 3.13).

atgcttcgtcttgctgctcataatttttctaaatctattatcagatcatatgctaaagatcttaaatttgg
agctgatggaagaaaggccatgcttgttggagtagatcttttagctgacgctgtatctgttacaatgggac
caaaaggaagaaatgttattattgaacaaagttttggtggaccaaaaattacaaaagacggtgttaccgtt
gctaaggctattgatctcgaagataaatatcaaaatatgggtgcaaaattggttcaagatgttgcagataa
ggctaacgaacaagctggtgacggaacaacttgcgctactgttcttgccagagctattgctaaagaaggat
ttgaaagtattagccgtggtgctaatccaattgaagttagaaaaggtgtcatgtcatccgttgaagctatt
gttgaagagttaaagaagatgtcaaagcaagttacaactccagaagaaattgctcaagttgctactatttc
tgctaatggagataaagatatcggaaaattgatatctgaagctatgaaaaaagttggaaataaaggtgtca
ttaccgttaaggatggaaaaactcttgacgatgaattagaaattattgaaggaatgaaatttgaccgtgga
tatatttctccatatttcatgaatacttccaagggtggaaaatgtttctttgaaaaatgtttagtccttct
ttctgagaaaaaaatttcccaagttcaagatattgttccagctcttgaattagcaaataaatatcgtcaac
cacttattattattgctgaagatgtcgatggtgaagctcttacaaccctcgttcttaacagattaaaggtt
ggtcttcaagtttgtgctgtaaaagctcctggttttggagacaaccgcaaaaacacattgaaagatattgc
cattgctacaggagctaaagtctttggagatgaatctaatttacacaaacttgaagatattcaagcaggtg
actttggtgaagttgctgaagttacagttacaaaagatgatactcttatgcttaatggaaaaggtgatgct
gaacaagttgagaagagaattcaacaaattgaatttgagattgaacaatctacttctgagtatgaaaaaga
aaaacttaatgaacgtttagctaaattatcaaaaggtgttgctgtccttaaaattggaggtgcttctgaag
ttgaagttagtgaaaagagagatcgtgtcacagatgcgctctgtgcaacacgtgctgctgtagaagaagga
atcgtaccaggaggtggtgttgctcttcttcgtgctgtcaaagttcttgataacatcaaagtcggtaattc
tgaccaagaattgggagttagaattgtccaaaaagccgttcgtcaaccaatttctaccattattaagaatg
ctggacttgaaccagctagtatcgttgaaaaagtttatgcaaatgaatcagtcagtttcggttacgatgct
ttgaatgataagtttgttgatatgatccaaactggtattattgatccaaccaaagttgtccgtacatcact
tcaagacgctgctggtgttgcttcacttttagccactactgaatgtgttgttacagaagttccaaaagaaa
ctccagcacctccaatgggtggtatgggaggtatgggcggaatgggtggtggaatgttc**taa**

Figure 3.13: Putative *S. ratti* HSP60 genomic DNA sequence

Putative *S. ratti* HSP60 genomic DNA sequence. The translation initiation and termination codons are printed in bold

3.8.3 Analyses of the intergenic region of *S. ratti*

To analyse the entire genomic structure of the two genes, PCR analysis was performed using 1 µg *S. ratti* genomic DNA as template and GSP_HSP60rev3'UTR and GSP_HSP10_330-304for as primers. A PCR product of approximately 2500 bp was obtained and cloned into pGemTeasy. By sequencing, the entire genomic structure of the two genes covering approximately 2.5 kb was determined (GenBank accession no. FJ694974). This analysis demonstrated that the HSP10 and HSP60 genes are organised in a head-to-head orientation with a 413 bp sequence separating the two ATG start codons (Figure 3.14). To confirm this sequence structure, 3 further PCRs with *S. ratti* genomic DNA were performed using a primer pair in the 5'UTR HSP10 region (GWHSP10revnested) and in the HSP60 3'UT region (HSP60_3'UTR).

Sequence analyses confirmed the predicted structure. The intergenic sequence was analysed by Genomatix MatInspector (http://www.genomatix.de) on the basis of the transcription factor databank TRANSFEC (Quandt *et al* 1995; Heinemeyer *et al* 1999). Core and matrix similarities are the parameters used. The "core sequence" of a matrix is defined as the highest conserved positions of the matrix. The maximum core similarity of 1.0 is only reached when the highest conserved bases of a matrix matches exactly in the sequence. The matrix similarity is calculated as described in Cartharius *et al* 2005. Given that each sequence position corresponds to the highest conserved nucleotide at that position in the matrix, a perfect match to the matrix exists. This is valuated with a score of 1.0. Analysis revealed several putative promoter elements including heat shock element binding sites (HSEs), TATA-box binding sites and GATA element binding sites. All these binding sites indicate individual transcriptional functions. The putative *S. ratti* HSP60 promoter region shows two family matches with putative HSE at position bp 213-237 and at position bp 285-309 with 1.00 core and 0.97 matrix similarity, respectively. Family matches with putative TATA-box binding sites are located at bp 33-48 with 0.75 core and 0.81 matrix similarity. The putative *S. ratti* HSP60 promoter region also shows a putative GATA element binding site at bp 318-330 with 1.00 core- and matrix similarity. The *S. ratti* HSP10 promoter region contains two putative HSEs at bp 213-237 and at bp 276-300 with 1.00 core and 0.97 matrix similarity. A putative TATA-box binding site is located at bp 222-238 with 1.00 core and 0.91 matrix similarity. Furthermore, the entire intergenic region is AT-rich (88%), which is typical for regions of strong promoters (Maseda & Hoshino 1995).

```
catttttaaagttgtactgttaatatattcctaaaaatttaaatattaaacaaacttttaattgaaaaaa
ttttcaaaaaaattataatttttaaaaactgaaaactaatttttataacgaaagtattaaagatatacttt
aaattaaaataaattggaatagaataaacatttcttgctgcactattgcgaaatattaatataggaatttt
cgcgaaatttctttaaaaaaaatctacaaattaataaattgctcctacaacgatagtagagacgatcttgt
tgcagaatgttctagaatgacttttaagttatgatggtaatctgcggctcgtccatataacttagagatta
ttttaattttaaattatttttaactaattgcaggttcttgtaattttggtaaatatg
```

Figure 3.14: Intergenic region of HSP10 and HSP60 of *S. ratti*

The *S. ratti* HSP10 start codon is shaded black and printed grey, the *S. ratti* HSP60 start codon is shaded grey. The 5'upstream region of each gene is printed in bold. The remaining sequence comprises the intergenic region. Within this region, the black-shaded base pairs (bp) show family matches with the HSP10 TATA-box. The grey-marked bp show family matches with the HSP60 TATA-box.

Table 3.2: Overview of the *Sr*HSP10 and *Sr*HSP60 intergenic sequence analysis

Heat shock elements binding site	HSP60 upstream sequence bp 276-300	aaagaaatttcgCGAAaattcctat
Heat shock elements binding site	HSP10 upstream sequence bp 213-237; bp 276-300	taggaattttcgCGAAatttcttta
TATA-box binding site	HSP60 upstream sequence bp 33-48	taatattTAAAttttta
TATA-box binding site	HSP10 upstream sequence bp 105-121	ttcttTAAAaaaaatct
AT-rich sequence	HSP60 upstream sequence bp 37-51	tttAATAtttaaatt
Special AT-rich sequence	HSP10 upstream sequence bp 197-211	attAATAtaggaatt
GATA element binding site	HSP60 upstream sequence bp 318-330	cgcaGATTaccat

3.9 Characterisation of the *S. ratti* HSP10 and HSP60 as putative binding partners using two-hybrid approaches

To investigate the putative binding of HSP10 to HSP60 and test whether *S. ratti* HSP10 occurs as homo-dimers, mammalian and yeast two-hybrid assays were performed

3.9.1 Mammalian two-hybrid analysis

Co-chaperonin interaction with chaperonin is mediated by the co-chaperonin mobile loop that folds into a ß-hairpin conformation upon binding to the chaperonin. The ability of HSP10 to interact specifically with HSP60 lies within the mobile loop sequence (Richardson *et al* 2001). Since the mobile loop of the co-chaperonin mediates interaction with the chaperonin, it is probable that this specific mobile loop sequence is also important for HSP10-HSP10 or HSP10-HSP60 binding in *S. ratti*. Alignment analyses between the human and *E. coli* HSP10 mobile loop sequences were performed, to identify the putative *Sr*HSP10 mobile loop. To perform the mammalian two-hybrid analysis, the *S. ratti* HSP10 full-length and *S. ratti* HSP60 full-length cDNAs were cloned separately into the prey (pAct3) and bait (pBind) vectors. Furthermore, HSP10 fragments (HSP10a, HSP10b and HSP10c) surrounding the putative mobile loop sequence were chosen. To investigate the HSP60 binding capacity of *S. ratti*, different HSP60 cDNA fragments were chosen (HSP60-1_2, HSP60-4_6, HSP60-5_6, HSP60-1_6, HSP60-4-2). *S. ratti* HSP10 and HSP60 fragments were amplified separately by RT-PCR. For HSP10 full-length and HSP10 fragment amplifications, the linearised pGemTeasy_HSP10_FL clone served as template. The linearised pGemTeasy_HSP60_FL clone also served as template for the amplification of HSP60 full-length cDNA and HSP60 fragments. The resulting putative *S. ratti* HSP10 and HSP60 PCR products were cloned into pGemTeasy. Sequence analyses confirmed their identity. Subsequently, the insert-containing plasmids were digested with the appropriate restriction enzymes and subcloned into the bait and prey vectors, respectively.

To optimise transfection and expression, the *S. ratti* HSP10 and *S. ratti* HSP60 cDNAs were cloned separately into both bait and prey vectors, confirmed by restriction mapping and sequencing. To verify the protein expression, the translated Gal4 binding site of the bait vector served to perform a Western blot analysis. The analysis showed molecular weight bands consistent with the size deduced from the cDNA sequence of the *S. ratti* HSP10 and HSP60 proteins and *S. ratti* HSP10 and HSP60 protein fragments.

Table 3.3: Overview of the mammalian two-hybrid bait (pBind) and prey (pAct3) vectors

Vector name	Vector Description
pBindSRHSP10fl	Includes the full-length *S. ratti* HSP10 sequence.
pAct3SRHSP10fl	Includes full-length *S. ratti* HSP10 sequence.
pAct3SRHSP10a	Includes *S. ratti* cDNA HSP10 fragment ranging from bp 1 to bp 174.
pAct3SRHSP10b	Includes *S. ratti* cDNA HSP10 fragment ranging from bp 85 to bp 138.
pAct3SRHSP10c	Includes *S. ratti* cDNA HSP10 fragment ranging from bp 169 to bp 330.
pBindHSP60fl	Includes full-length *S. ratti* HSP60 sequence.
pBindHSP60_1-6	Includes *S. ratti* cDNA HSP60 fragment ranging from bp 1 to bp 873.
pBindHSP60_4-6	Includes *S. ratti* cDNA HSP60 fragment ranging from bp 625 to bp 873.
pBindHSP60_5-6	Includes *S. ratti* cDNA HSP60 fragment ranging from bp 739 to bp 873.
pBindHSP60_4-2	Includes *S. ratti* cDNA HSP60 fragment ranging from bp 625 to bp 1683.
pAct3HSP60fl	Includes full-length *S. ratti* HSP60 sequence.
pAct3HSP60_1-2	Includes *S. ratti* cDNA HSP60 fragment ranging from bp 1to bp 1683.
pAct3HSP60_4-6	Includes *S. ratti* cDNA HSP60 fragment ranging from bp 625 to bp 873.
pAct3HSP60_4-2	Includes *S. ratti* cDNA HSP60 fragment ranging from bp 625 to bp 1683.

For control, the individual vectors, including the prey and bait cloning vector, were each transformed into N2a cells. Briefly, 24 h after transfection, luciferase activities were measured, and the values were compared with the basal binding activity of the bait and prey vectors, which was set to 1. pBind basic-vector/pAct3SRHSP10, pBind basic-vetor/pAct3SRHSP10a, pBind basic-vetor/pAct3SRHSP10b, and pBind basic-vetor/pAct3SRHSP10c displayed very low activity (1.02-fold, 0.87-fold, 0.94-fold and 0.80-fold respectively). pBind basic vectot/pAct3SRHSP60 showed very low activity, too.

In a second step, the direct binding activities of pBindSRHSP10fl full-length with the pAct3SRHSP60 full length, with the pAct3SRHSP10 full-length and with the pAct3SRHSP10 fragments a, b and c respectively were tested. Furthermore, the direct binding activities between pBindSRHSP10fl and pAct3SRHSP60 fragments (1-2; 4-6; 5-6;4-2 respectively), and between the pBindHSP60fl and pActSRHSP60 (1-2; 4-6; 5-6;4-2 respectively) fragments were tested. Again, 24 h after transfection of N2a cells, luciferase activities were measured. A strong activity was detected after transfection of the pBindSRHSP10fl full-length and pAct3SRHPS10fl full-length vectors (2.41-fold). No significant luciferase activities were observed after transfection of the pBindHSP10 full-length with the pAct3SRHSP60, or with the pAct3SRHSP10a, b and c constructs respectively (Figure 3.15).

Furthermore, no significant luciferase binding activities were observed between the pBindSRHSP10 and pAct3SRHSP60 fragments (1-2; 4-6; 5-6;4-2 respectively), or between pBindSRHSP60fl and pAct3SRHSP60 fragments (1-2; 4-6; 5-6;4-2 respectively).

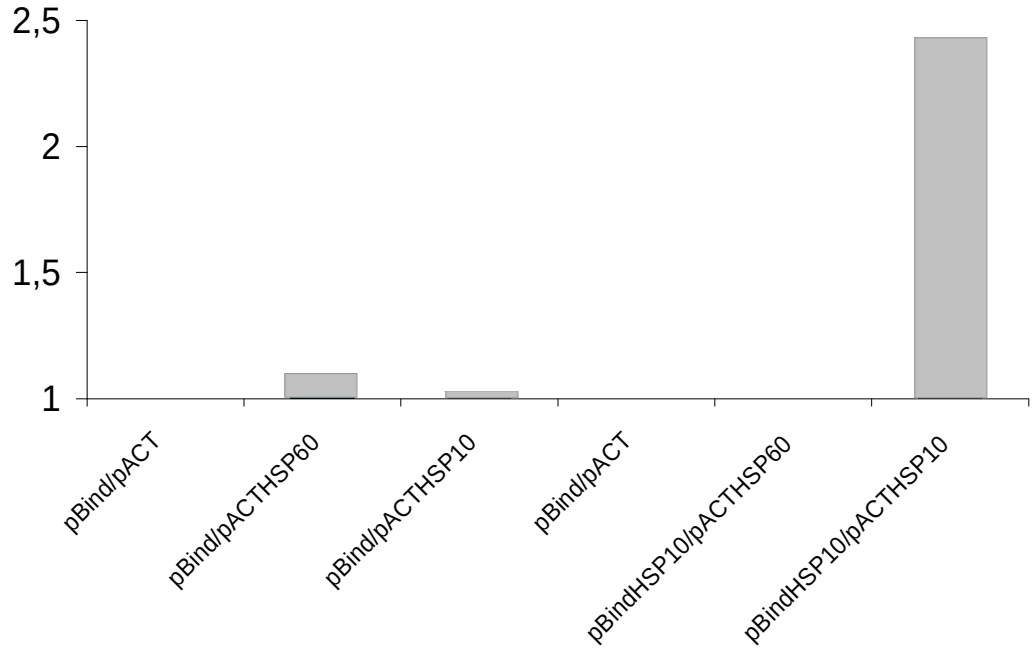

Figure 3.15: Luciferase binding assay

Binding activities of the indicated constructs in N2a cells were determined by luciferase-based reporter gene assays. Luciferase activity is shown as fold activation beyond basal activation due to pBind and pACT binding control. The experiment was technically performed sevenfold and biologically repeated three times.

3.9.2 Yeast two-hybrid analysis

To confirm the binding results in an independent system, yeast two-hybrid analyses were performed. To this end *S. ratti* HSP10 full-length cDNA and the pertinent *S. ratti* HSP10 fragments a, b and c were amplified separately with the appropriate primers and cloned into the pGemTeasy vector. Subsequently, the encoding sequences were subcloned to the appropriate yeast two-hybrid bait (pBridge) and prey (pAct2) vectors respectively. Sequence analyses confirmed the identity of the DNA inserts and the in-frame ligations.

The pBridge *S. ratti* HSP60 full-length plasmids were obtained by *Bam*HI and *Eco*RI digestion of the linearised pBindHSP60FL clone. The HSP60 DNA insert was subcloned into the pBridge vector. The pAct2 *S. ratti* HSP60 full-length plasmid was obtained by *Sma*I and *Eco*RI digestion of the linearised pBindHSP60FL clone. The resulting HSP60 DNA insert was subcloned into the pAct2 vector.

Table 3.4: Overview of the yeast two-hybrid bait (pBridge) and prey (pAct2) vectors

Vector name	Vector Description
pBridgeSRHSP10	Includes full-length *S. ratti* HSP10 sequence.
pAct2SRHSP10fl	Includes full-length *S. ratti* HSP10 sequence.
pAct2SRHSP10a	Includes *S. ratti* cDNA fragment ranging from bp 1 to bp 174.
pAct2SRHSP10b	Includes *S. ratti* cDNA fragment ranging from bp 85 to bp 138.
pAct2SRHSP10c	Includes *S. ratti* cDNA fragment ranging from bp 169 to bp 330.
pAct2SRHSP10gen1	Includes *nco*I and *xba*I digested out of pCR2.1-pAct2_HSP10 plasmid.
pAct2SRHSP10gen2	Includes *bam*HI and *Xho*I digested out of pCR2.1-pAct2_HSP10 plasmid.
pAct2SRHSP10gen3	Includes *sma*I and *sac*I digested out of pCR2.1-pAct2_HSP10 plasmid.
pBridgeSRHSP60	Includes full-length *S. ratti* HSP60 sequence.
pAct2SRHSP60	Includes full-length *S. ratti* HSP60 sequence.

The pBridgeSRHSP10fl plasmid was co-transformed into yeast-AH109 cells with the pAct2HSP60, the pAct2SRHSP10fl plasmids, or the pAct2SRHSP10a, b and c plasmids respectively. The pBridgeSRHSP60 plasmid was co-transformed into yeast-AH109 cells with the pAct2SRHSP60 plasmid. These cells were selected on SD-Trp/-Leu/-His/-Ade-plates, a dropout media for growth and interaction selection, and incubated for 5 days at 30°C. Furthermore, the pBridgeSRHSP60 plasmid was co-transfected with the pAct2SRHSP60 plasmid into yeast-AH109 cells. Then, the cells were selected on SD-Trp/-Leu/-His/-Ade-plates and incubated for 5 days at 30°C.

The β-galactosidase filter assay (Breeden & Nasmyth 1985) is a colorimetric control of clones that survived the *HIS3-/ADE2*-Selection. During interaction, the lacZ gene encodes the enzyme ß-galactosidase, and clones expressing this enzyme were identified by a blue-staining with the chromogenic substrate X-gal. For the quantification of the HSP10 two–hybrid interaction, a sensitive colorimetric method, the ß-galactodisase fluid assay (Yeast Protocols Handbook, 2001) was used. The ß-galactosidase fluid assay relies on the ability of the enzyme to catalyse the hydrolysis of ONPG (o-nitrophenyl-ß-D-galactopyranoside) to free o-nitrophenol, which absorbs light at 420 nm. Interactions were detected by performing the β-galactodisase filter and fluid assays, between the pBridgeSRHSP10fl protein and the pAct2SRHSP10fl protein

(Figure 3.16 A, B). Furthermore, the β-galactodisase filter assay showed an interaction between the pBridgeSRHSP10fl protein with the N-terminal pAct2SRHSP10a protein fragment including binding domain (Figure 3.16 C). The analysis was extended to investigate a possible interaction between the pBridgeSRHSP10fl protein and fragments more exactly defining the HSP10 binding domain. No further interactions were detected. The yeast two-hybrid assay showed no interaction between the pBridge2SRHSP60 protein and the pAct2SRHSP60 protein, and no interaction between the pBridgeSRHSP10 protein and the pAct2SRHSP60 protein. The yeast two-hybrid ß-galactosidase filter and fluid assay confirmed the mammalian two-hybrid analyses results (Figure 3.16 A, B).

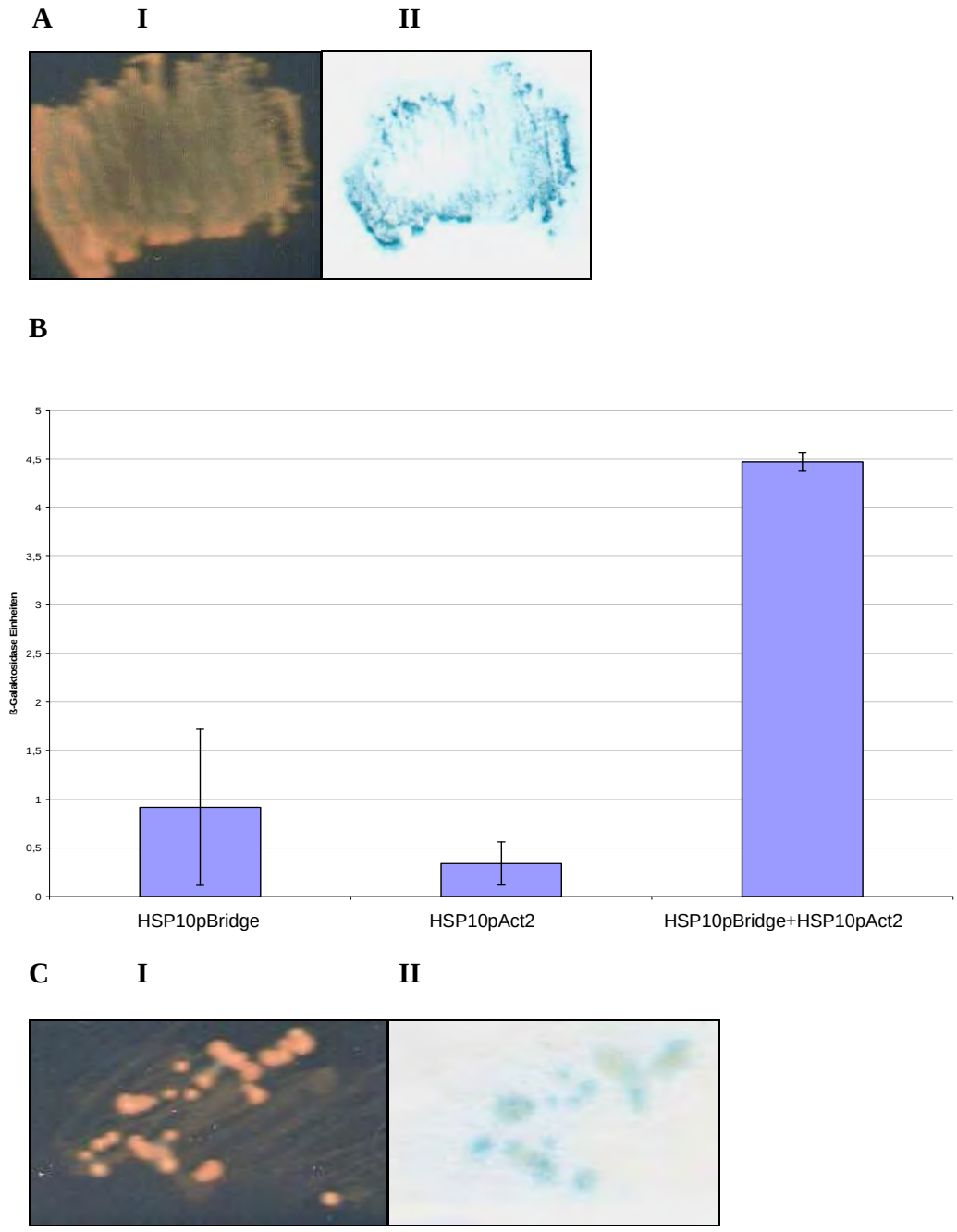

Figure 3.16: Yeast two-hybrid protein-protein interaction analysis

Yeast two-hybrid analyses of protein-protein interaction between *S. ratti* HSP10 full-length with *S. ratti* HSP10 full-length and *S. ratti* HSP10 N- terminal. Representative interaction profiles between **A**: pBridgeSRHSP10fl and pAct2SRHSP10fl co-transfected yeast AH-109 cells. SD-Trp/-Leu/-His/-Ade-plates after 5 days incubation at 30°C (I) and the ß-galactosidase filter assay (II). **B**: Quantification of the binding strength by ß galactosidase fluid assays (n=6); **C**: pBridgeSRHSP10fl and pAct2SRHSP10a co-transfected yeast AH-109 cells. SD-Trp/-Leu/-His/-Ade-plates after 5 days incubation at 30°C (I) and the ß-galactosidase filter assay (II).

3.10 DNA immunisation with recombinantly expressed *S. ratti* HSP10

To investigate the presumptive immunological role of *S. ratti* HSP10, sera of immunised rats were obtained and analysed by ELISA and by Western blot. For immunisation the *S. ratti* HSP10 protein was used which was recombinantly expressed in *E. coli*.

To generate recombinant *S. ratti* HSP10, PCR was performed using the linearised pGemTeasy_HSP10_FL clone as template, HSP10pJC45 and HSP10pJC45rev as primers. The amplicon was cloned into pGemTeasy (pGemTeasy_HSP10_FL_Nde/EcoRI). Sequence analyses revealed the identity of the pGemTeasy_HSP10_FL_Nde/EcoRI clone. 3 µg plasmid DNA was digested with the appropriate amount of the restriction enzymes *Nde*I and *Eco*RI and subsequently subcloned into the expression vector pJC45 (Clos and Brandau 1994). Further sequence analyses confirmed the in-frame cloning of the DNA insert and affirmed the identity of the sequence.

The histidine tag of the expression vector pJC45 allows the easy purification of recombinant proteins from *E. coli*. Expression was induced by IPTG. Bacteria were pelleted, the protein extracted and analysed by SDS-PAGE and immunoblot analysis. Histidine tag antibodies recognised bands at the expected molecular weight of 10 kDa (Figure 3.17).

6 Wistar rats were immunised with 40 µg *S. ratti* HSP10 protein at 10 weeks of age, and boosted with another 40 µg of protein 16 days after prime immunisation. Sera were obtained 16 days post prime immunisation and 19 days post boost immunisation, respectively. Sera of 6 non-immunised Wistar rats (16 days post prime immunisation and 19 days post boost) served as negative control. In Western blots, the serum of the immunised rats not only recognised r*Sr*-HSP1*0*, but also the native *Sr*-HSP1*0* in the extracts of iL3 (Figure 3.17).

For the ELISA analysis, 2 µg *S. ratti* HSP10 recombinantly expressed protein was applied as antigen. The 50µl sera from the 6 pre immunised, post primed and post boosted rats, respectively, were tested in different dilutions starting from 1:100 up to 1:20,480 in comparison to 6 sera from non-immunised rats.

Post-prime and post-boost sera showed production of high levels of specific IgG antibody detectable at high serum dilutions by ELISA (Figure 3.18) as well as by Western blot analyses (Figure 3.17). Significantly higher IgG reactivities were found for the post boost sera compared to the post prime sera. The pre-immunised sera and the negative control group sera showed no IgG reactivity.

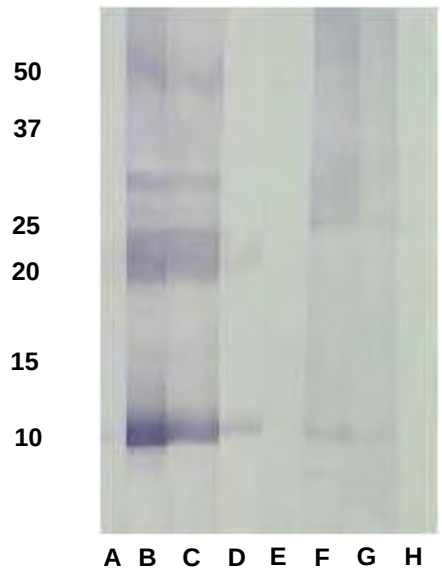

Figure 3.17: Western blots of recombinant *Sr*Hsp10

In the Western blot, high titre *Sr*Hsp10-specific rat immune serum recognised r*Sr*Hsp10 and detected *Sr*Hsp10 in whole worm extract of *S. ratti* iL3. Lanes A-D show r*Sr*Hsp10 protein, lanes E-H show detection of *Sr*Hsp10 in *S. ratti* iL3 extract. Lanes A and E: incubation with serum of the non immunised rats (1:50), all other lanes: incubation with immune serum taken day 14 post boost. Dilutions: lanes B and F = 1:50, lanes C and G = 1:1000, lanes D and H = $1:1x10^4$.

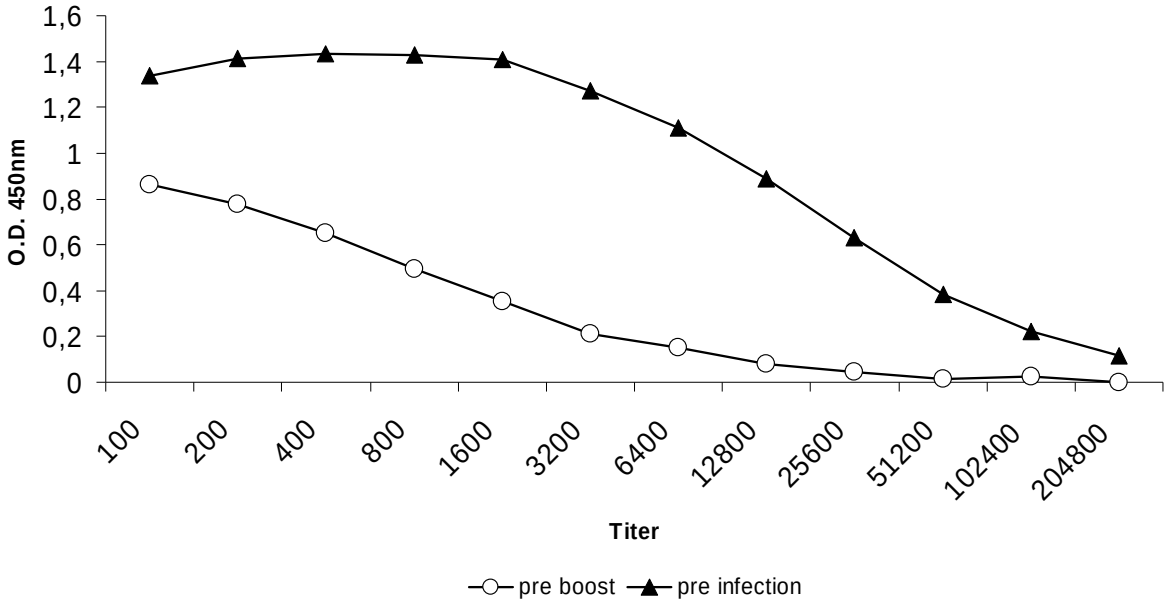

Figure 3.18: Humoral response to recombinant *Sr*HSP10

Humoral response to recombinant *Sr*HSP10 after rat immunisation with recombinantly expressed *Sr*HSP10 protein. The mean serum concentration of 6 serum samples of parasite-specific IgG in boosted (arrowed line) and primed (dotted line) rats, respectively. The extinction is measured in different serum dilutions (1 : 100; 1 : 200; 1 : 400; 1 : 800, 1 :1,2800: 1 : 3,200, 1 : 6,400, 1 : 12,800, 1 : 25,600, 1 . 51,200, 1 : 102,400, 1 : 204,800).

3.11 Putative *S. ratti* HSP60 expression in *E. coli*

In addition to its essential role as chaperonin, HSP60 has received much attention as a dominant cross-reactive antigen, and it has been implicated in the pathogenesis of autoimmune diseases (Young 1990, Kaufmann 1992). HSP60 proteins from several microorganisms, including human pathogens such as *Mycobacterium tuberculosis*, elicit a strong and dominant immune response in their mammalian hosts (Zugel & Kaufmann 1999a; Shinnick *et al* 1988). To investigate the immunological importance of *S. ratti* HSP60 protein by ELISA, an attempt to express *S. ratti* HSP60 full-length protein in *E. coli* was carried out. The open reading frame of the HSP60 gene was amplified using the linearised pGemTeasy_HSP60_FL clone as template and pJC45HSP60_HindIII and pJC45HSP60_BamHI primers. The PCR product was cloned into pGemTeasy. Sequence analysis confirmed the identity of the DNA insert. Subsequently, the pGemTeasy_HSP60 clone was *Hind*III and *Bam*HI digested, and subcloned into the bacterial expression vector pJC45 (pJC45_HSP60), a derivative of the pJC40 (Clos and Brandau 1994). The vector allows expression of proteins and carries a 10-residue histidine tag for recognition by histidine tag antibodies. *E. coli* was grown and expression was induced by IPTG. Bacteria were pelleted, the protein extracted and analysed by SDS-PAGE and immunoblot analysis. Histidine tag antibodies did not recognise bacterial protein.

To further try to induce bacterial expression, an HSP60 version without the mitochondrial leading sequence (atgcttcgtcttgctgctcataatttttctaaatctattatcagatcatat) was cloned into pGemTeasy (pGemTeasy_HSP60-52). After sequence confirmation, the HSP60 DNA insert was subcloned into the bacterial expression vector pJC45 (pJC45_HSP60-52). To verify the expression, SDS-PAGE and immunoblot analyses were carried out. Again, histidine tag antibodies did not recognise a recombinant protein.

Attempts to express *Sr*HSP60 recombinantly failed, which was probably due to *S. ratti* HSP60 endotoxicity. Further approaches that may provide a successful expression of *Sr*HSP60 could be the use of the baculovirus expression system, or to explore the feasibility of expressing recombinant *Sr*HSP60 peptides on both the *E. coli*- and the baculovirus expression systems.

3.12 Putative *S. ratti* HSP10 expression in the baculovirus system

While high levels of expression in bacterial systems are common, problems of proper folding and lack of post-translational processing may produce functionally inactive molecules. As with other eukaryotic expression systems, baculovirus expression of heterologous genes permits folding, post-translational modification and oligomerisation in manners that are often identical to those that occur in mammalian cells. The insect cytoplasmic environment allows proper folding and S-S bond formation, unlike the reducing environment of the *E. coli* cytoplasm. Post-translational processing identical to that of mammalian cells has been reported for many proteins (O'Reilly *et al* 1992). Here, the *flash*BAC baculovirus expression system was tested to express the *S. ratti* HSP10. To generate a HSP10 expression clone PCR was performed using the linearised pGemTeasy_HSP10_FL clone as template, and pAcSG2_HSP10EcoRIfor as well as pAcSG2_HSP10NotIrev as primers. The reverse primer contained a 42 bp flag-tag residue. The amplicon was cloned into pGemTeasy (pGemTeasy_pACSG$_2$_HSP10). Sequence analyses revealed the identity of the pGemTeasy_pACSG$_2$_HSP10 clone. The plasmid was digested with the appropriate amount of the restriction enzymes *Nde*I and *Eco*RI, and the insert subsequently subcloned into the transfer vector pAcSG2 (Oxford expression technologies, flashback^{TM} System). Further sequence analyses confirmed the in-frame cloning of the DNA insert and affirmed the correctness of the sequence. The *S. ratti* HSP10 gene was cloned into the transfer vector pAcSG2. The pAcSG2 vector contains sequences that flank the polyhedron gene in the virus genome. The virus genome and the pAcSG2HSP10 vector were introduced into the host insect cells, which were incubated for 5 days at 28°C before harvesting. To upscale the amount of recombinant virus, the suspension cultures of cells were passaged. The passaging was performed twice. The suspension cultures were stored cell free at -70°C. The cells were harvested and stored at -20°C until use. To verify the expression success, the harvested cells were analysed by Western blot. Flag tag antibodies recognised bands at the expected molecular weight of the expressed *S. ratti* HSP10 protein. The basis has been provided to express *Sr*HSP10 recombinantly using the baculovirus expression. Further studies are needed to verify if the baculovirus expression system is more convenient in comparison to the *E. coli* expression.

4 Discussion

4.1 Identification of *Sr*HSP10 and *Sr*HSP60

Drug resistance in parasites of livestock is reaching alarming levels and is consequently becoming an issue of growing importance in animal health and well-being. This phenomenon also affects humans, particularly with respect to mass drug administration programmes, which exert selective pressure on parasitic populations.

An estimated 100-200 million people are infected with *Strongyloides stercoralis* (*S. stercoralis*, threadworm) (Viney 2006; Viney *et al* 2007). Recent reports indicate an increasing prevalence of strongyloidiasis and its hyperinfection syndrome, which was evaluated as an emerging global infectious disease that has migrated from developing regions to industrialised areas (Marcos *et al* 2008). *S. stercoralis* is unique in its ability to replicate and multiplicate within the human host. Cycles of auto-infection lead to chronic disease that can persist for several decades in dangerous disseminated infections, in most cases with a fatal outcome (≥87%) (Keiser & Nutman 2004). Furthermore, in comparison to soil-living nematodes, parasitic nematodes such as *S. stercoralis* possess additional genes (Viney 2006).

To better understand the parasite-host relationship, the study of host defence-modulating molecules in parasitic systems may likely reveal new principles of their function. Intensive study of immune functions of HSPs within the last years clearly demonstrated that they possess immune stimulatory potential. It is well established that HSPs participate in the initiation of adaptive immune response by chaperoning peptide antigens for cross-presentation. Moreover, it is now emerging that HSPs bind bacterial endotoxins and modulate PAMP-induced stimulation, while, on the other hand, there are several hints arguing for an intrinsic immune stimulatory function of HSPs that cannot be ascribed to bacterial contaminants (Osterloh & Breloer 2008). HSP10 may not only be a component of the folding machinery but also an active player of the cell signalling network, influencing cell cycle, nucleocytoplasmic transport, and may be involved in cancer ethiology as well as in the inhibition of apoptosis (Czarnecka *et al* 2006). HSP60, was shown to induce the activation of NFκB (Vabulas *et al* 2001), and MyD88 and TRAF6 were found to be involved in HSP-mediated APC stimulation (Vabulas *et al* 2001). Furthermore, TLR2 and TLR4 were identified to be responsible for HSP60 activation. Expression of TLR2 or TLR4 together with MD2, which is also part of the TLR4 receptor complex (Takeda & Akira 2004), in otherwise non-responsive human cell lines conferred responsiveness to HSP60 as indicated by activation of NFκB-controlled reporter genes (Vabulas *et al* 2001). Moreover, APC derived from C3H/HeJ

mice expressing a nonfunctional mutant TLR4 receptor failed to respond to HSP60 (Ohashi *et al* 2000; Flohe *et al* 2003).

The objective of this study was to identify, isolate and characterise *S. ratti* HSP10 (*Sr*HSP10) and HSP60 (*Sr*HSP60). *S. ratti* was used as a model for the human pathogen *S. stercoralis*, because *S. ratti* is not infectious for humans and, in addition, both nematodes share almost identical life cycles.

Initially, we detected *Sr*HSP10 and *Sr*HSP60 in supernatants of all stages of *S. ratti* by mass spectrometry. Supernatants were devoid of proteins when the *S. ratti* stages were exposed to the protein-synthesis inhibitor cycloheximide or to azide, confirming the secretion of *Strongyloides* proteins including HSPs. Based on the knowledge that HSP10 and HSP60 play not only intracellular roles as chaperones to prevent the aggregation of proteins or to activate the renaturation of unfolded proteins, it is tempting to speculate that these HSPs also exhibit extracellular roles in parasite-host interaction such as influencing immune responses of the host. A similar role for a HSP was suggested for the small heat shock protein 12.6 from *B. malayi*, which functions as a human IL-10 receptor-binding protein (Gnanasekar *et al* 2008). On the other hand, HSP60 is able to bind to LPS and enhance the LPS-stimulated TNFα production in macrophages such as human HSP60 (Habich *et al* 2005).

4.2 Characterisation of *Sr*HSP10 and *Sr*HSP60

The genomes of *S. ratti* and *S. stercoralis* have been subjected to expressed sequence tag (EST) analyses. Some 14,000 and 11,000 ESTs from both parasites have been discovered, representing more than 4,000 genes and about 20% of the nematodes' genomes (Thompson *et al* 2005; Viney 2006; Lok 2007). In the absence of sequence information for *Sr*HSP10 and *Sr*HSP60, nucleotide searches for allied *S. stercoralis* HSP10 ESTs were performed and resulted in the designated *S. stercoralis* HSP10-like EST (GenBank accession number BE580000). For HSP60, the data entry in the library Nematode.net for *S. ratti* showed a 1078 bp sequence comprising 5 overlapping EST sequences. These EST sequences were used to construct one contig. By using specific primers putative cDNA fragments of *S. ratti* (*Sr*HSP10, *Sr*HSP60) were amplified. To gain the full-length sequences, 3'RACE and spliced leader (SL)-RT-PCRs were performed. SLs have been identified in a variety of eukaryotes including nematodes (Krause and Hirsh 1987). SL1 (a non-coding 22-nucleotide sequence used in this study) was the first SL sequence identified in nematodes (Krause and Hirsh 1987; Bektesh *et al.* 1988). One-fifth of the genes of, for example, *C. elegans* are organised as sets from 2-8 genes expressed from the same promoter, similar to bacterial "operons". The pre-mRNAs made from these operons are processed

by an intermolecular ligation process involving SL-sequences which is called SL trans-splicing (Guiliano and Blaxter 2006). Therefore, it was speculated that the HSP genes of *S. ratti* may posses SL-sequences in their 5'UTRs. Indeed, sequence analyses of the obtained and cloned RT-PCR products confirmed the presence of SL-sequences as well as the identity of the full-length *Sr*HSP10 and *Sr*HSP60 sequence. The full-length *Sr*HSP10 cDNA comprised 507 bp. Alignment results showed that the cDNA of *Sr*HSP10 has significant homology (87%) to the cDNA of the *S. stercoralis* HSP10 EST. An additional nucleotide alignment analysis showed that *Sr*HSP10 is 60% identical with the HSP10 homologue of *C. elegans* and 57% with the HSP10 homologue of *Homo sapiens*. The open reading frame (ORF) comprised 330 bp and codes for 110 amino acid residues. Having a predicted molecular mass of 11.8 kDa, *Sr*HSP10 belongs to the group of small heat shock proteins (sHSP) which constitutes a diverse family of proteins ranging from 12-43 kDa proteins found in several organisms (Ingolia & Craig 1982; Yeh *et al* 1994). *Sr*HSP10 mainly consists of hydrophilic amino acids with short hydrophobic N- and C- terminal peptides. However, the *Sr*HSP10 ORF is predicted to have a mitochondrial targeting peptide (AA 1-30, iPSORT prediction), and its N-terminal sequence has the ability to form an amphipathic alpha helix, which may enable it to cross the mitochondrial membrane. Amino-acid alignment analyses showed that *Sr*HSP10 has a very high homology (93%) to HSP10 of the human pathogen *S. stercoralis*, with the exception of eight amino acid exchanges, of which four are conserved. In addition, *Sr*HSP10 shows similarity to HSP10 of other nematodes including *C. elegans* (63% identity) and *Brugia malayi* (57% identity). *Sr*HSP10 has a low homology to *B. malayi* 12.6 (19%), however a more pronounced homology to the purified amino acid sequence of the human early pregnancy factor (EPF) (<40%). The first nonfolding HSP10 activity to be identified was similar to that of EPF (Summers *et al* 1998), which was finally shown to be an extracellular homologue of HSP10 (Summer *et al* 1996, Morton 1998). This suggests the existence of a further immunoregulative HSP within the group of sHSPs.

The full-length *Sr*HSP60 cDNA is 1914 bp in size. The HSP60 gene promoter is characterised by the absence of a classical TATAA box, and by the presence of a single heat shock response element (HSE), nGAAn. Here, the HSE is located 25 bp upstream of the start codon (ATG). Promoters of other heat shock protein genes frequently contain several HSEs (Xiao & Lis 1988; Amin *et al* 1988). Furthermore, the cDNA sequence contains the canonical chaperonin 60 signature (AAAVEEGIVPGGG; ProSite document PS00296 1997). The ORF, comprising 1695 bp, codes for 565 amino acid residues. There is a majority of A-T bases (67%) within the ORF, of which 75% are in the 5'UTR and 80% in the 3'UTR. The resulting *Sr*HSP60 protein has a predicted molecular mass of 64 kDa, a typical size for HSP60 orthologues or

homologues (Hemmingsen *et al* 1988; Jindla *et al* 1989; Lund 1995). A putative mitochondrial targeting sequence with 25 amino acid residues was identified in the N-terminal region. BLAST P analysis revealed high sequence similarity with HSP60 proteins from *O. volvulus* (72.5%), *B. malayi* (72%) and the human pathogen *S. stercoralis* (94%).

Using *in situ* hybridisation (ISH) analyses, *Sr*HSP10 and *Sr*SHP60 transcripts were localised in *S. ratti* iL3. A pattern of *Sr*HSP10 and *Sr*HSP60 transcription was detected in the subcuticular and pharyngeal tract tissues. This ubiquitous staining under non-stressed conditions indicates additional biological roles to the originally known co-chaperone functions under stress conditions. Although there is evidence for secretion, it remains unclear how *Sr*HSP10 and *Sr*HSP60 proteins are transported. In filarid nematodes, such as *O. volvulus*, the existence of a system capable of efficiently transporting proteins to the cuticle of the nematode body has been hypothesised (McKerrow *et al* 1999). Based upon immunolocalisation, a more definite localisation of the molecules within tissues could be performed to obtain additional information for excretory/secretory pathways. This could be the subject of further studies.

4.3 Characterisation of *Sr*HSP10 and *Sr*HSP60 genes

Although HSP proteins are well studied, the regulatory features of their coding genes have not yet been characterised in detail. To determine the genomic arrangement of the putative *Sr*HSP10 and *Sr*HSP60 genes, a Southern blot analysis was carried out with *S. ratti* genomic DNA. Both the *Sr*HSP10 probe and the *Sr*HSP60 probe hybridisations showed two similar bands with *Eco*RV-digested DNA, and hybridisation with the *Hind*III-digested *S. ratti* genomic DNA showed one 5 kb band in each case. The detection of one fragment suggested a single-copy tandem arrangement of both genes. Head-to-head gene arrangements have previously been defined as "bidirectional", and the divergent gene pairs have been termed as "bidirectional genes". A "bidirectional gene pair" is defined as two adjacent genes, which are located on opposite strands of DNA with transcription start sites (TSSs) not more than 1000 base pairs apart. The intergenic region between two TSSs is designated as a putative "bidirectional promoter" (Adachi & Lieber 2002; Trinklein *et al* 2004). However, it is not clear whether this region has regulatory activity, or is enriched of regulatory elements, which are essential for initiation of transcription.

The postulated "bidirectional" structure of the *Sr*HSP10 and *Sr*HSP60 genes, covering approximately 2.5 kb, was confirmed by PCR, amplification, cloning and sequencing. Alignment analyses revealed that the *Sr*HSP10 gene is interrupted by one intron (position bp 27 to bp 69) with a length of 42 bp. In contrast, the *Sr*HSP60 gene is without an intron. In addition, the *Sr*HSP10 and *Sr*HSP60 genes are found to be linked in a head-to-head configuration. The

intergenic region comprises 413 bp separating the two ATG start codons. This sequence was mapped and analysed *in silico*. Heat shock elements (HSEs)- and TATA-box factor binding-sites have been identified in the intergenic region. The binding to the TATA box is crucial for transcription initiation. HSEs are functionally conserved from yeasts to humans, consisting of tandem inverted repeats of the short consensus sequence 5_-nGAAn-3 (Xiao *et al* 1991; Wu 1995; Liu *et al* 1997). HSE sequences confer strong heat-inducibility on heterologous promoters, and can act both as TATA-proximal elements and as enhancer (Pelham 1982; Pelham & Bienz 1982; Bienz & Pelham 1986). In addition, HSEs are required for promoter activation (Jakobsen and Pelham 1988). In eukaryotes, the transcription of heat-shock genes is mediated by HSEs found in multiple copies in the promoter regions of heat-shock genes (reviewed by Bienz & Pelham 1987). Heat shock transcription factors (HSFs) bind to HSEs, and it has been demonstrated in target-deletion studies of the stress-responsive mouse HSF1 gene that it is essential for both basal and stress activation of, for example, HSP, gene expression and inflammatory response (Christians *et al* 2002; Xiao *et al* 1999). The regulatory activity of the *Sr*HSP10 and *Sr*HSP60 promoter region and, especially the putative HSEs activation under control or stressed conditions, remain to be clarified.

Individual examples of bidirectional gene pairs have been reported for years, and a few genome-wide analyses have been studied in mammalian and human genomes (Lennard & Fried 1991; Brayton *et al* 1994; Adachi *et al* 2002; Trinklein *et al* 2004). "Head-to-head" gene organisations also occur in plants. Furthermore, the bidirectional gene pairs are more correlated in expression and are associated functionally, which confirms that this structure is prevalent in the plant genome (Wang *et al* 2009). However, neither individual examples of bidirectional gene pairs, nor genome-wide analyses for bidirectional genes of nematodes have been performed (Figure 4.1). Thus, the *S. ratti* HSP10 and HSP60 gene pair shows a probability of being coordinately regulated and functionally associated, which would be consistent with results in mammalians (Trinklein *et al* 2004).

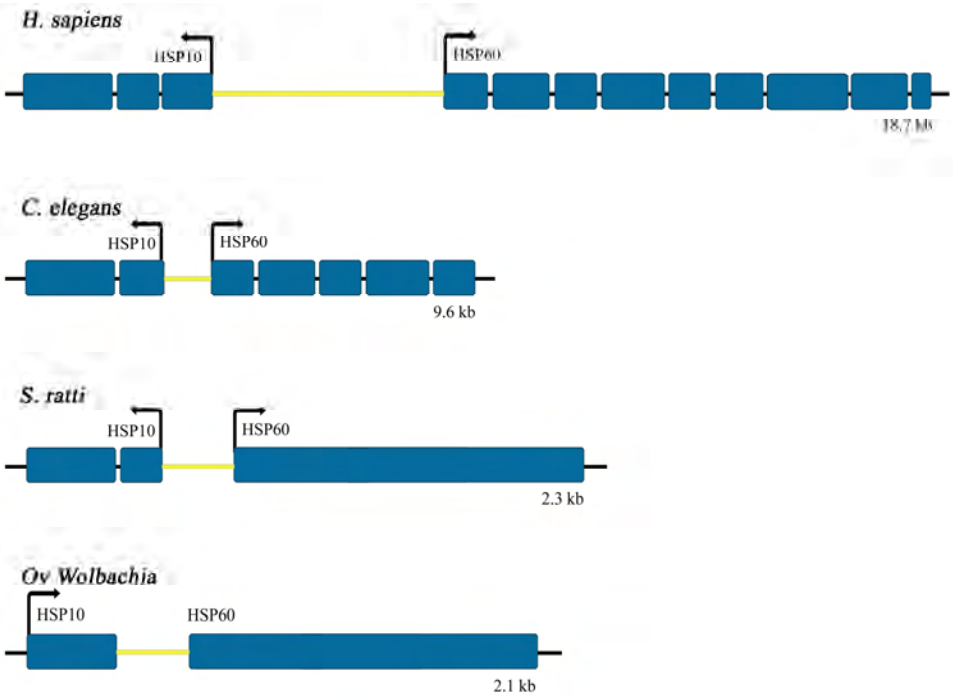

Figure 4.1: Diagram of the genomic organisation of HSP10 and HSP60 genes of different species

H. sapiens, C. elegans, S. ratti or O. volvulus Wolbachia HSP10 and HSP60 exons are printed in blue; the intergenic region is printed in yellow. The HSP10 and HSP60 transcription start sites are marked by arrows. With the exception of bacteria, all genes are arranged in a tandem head-to-head configuration, of which the H. sapiens bidirectional nature has been analysed and verified (Hansen et al 2003).

4.4 Characterisation of *Sr*HSP10 and *Sr*HSP60 as binding partners

In mammalian systems, HSP10 and HSP60 are known to be vital in the protein folding machinery (Fink 1999). Both HSP60 and HSP10 have essential functions in promoting the ATP-dependent folding of proteins, under normal growth conditions or under stress. Furthermore, the binding between HSP10 and HSP60 is essential for providing a folding compartment (Hartl 1995). In bacteria, HSP60 also requires a co-chaperonin HSP10 for full function. The binding of HSP10 to HSP60 leads to substantial changes in the hydrophobic nature of the cavity. An unfolded substrate protein binds to the relatively hydrophobic cavity lining before HSP10, which is then required for productive polypeptide release (Fink 1999). To date, strong HSP10-HSP10 and HSP10-HSP60 interactions were detected between the *E. coli* homologues GroES/GroES, and between the human HSP10 and HSP60, respectively, by yeast two-hybrid assays (Lee *et al* 2002). As approaches to investigate the interaction of *Sr*HSP10-*Sr*HSP60, mammalian and yeast two-hybrid assays were performed in this study. However, no binding was detected between *Sr*HSP10 and *Sr*HSP60 or between *Sr*HSP60 and *Sr*HSP60. Interestingly, HSP10 showed a dimerisation potential in the mammalian two-hybrid system, which was confirmed in the yeast

two-hybrid assays. A detailed analysis of HSP10 dimerisation revealed a potential HSP10 binding domain (AAEVKSKGGIYIPEKAQGPEKAQG). Many organisms share similar motifs in HSP10 as conserved protein-protein interactions sites (Richardson *et al* 2001). Several *Sr*HSP10 fragments surrounding the potential *Sr*HSP10 binding domain were examined. Here, the yeast two-hybrid analysis revealed that the N-terminus including the binding domain hybridised with the full length *Sr*HSP10, whereas the C-terminus did not show any binding activity. This dimerisation of *Sr*HSP10 may be a further indicator of additional, *Sr*HSP60-independent functions.

One explanation of the failure to detect *Sr*HSP10-*Sr*HSP60 interaction may be the lack of target proteins to be folded. To date, HSP10-HSP60 interactions in a yeast two-hybrid assay were only demonstrated during protein folding activities (Lee *et al* 2002).

4.5 The immunological role of *Sr*HSP10

Since an immunological role of HSP10 protein has been demonstrated in other systems (Akyol *et al* 2006), and since HSP10 was detected in supernatants of worms and larvae, indicating its secretion as a factor putatively involved in parasite-host interaction, the immunological potential was investigated. To this end, HSP10 was recombinantly expressed in *E. coli* and used for immunisation studies. Rats were immunised with recombinant *Sr*HSP10, and the serum analysed by ELISA and Western blot. A 12 kDa protein in *S. ratti* infective larvae extracts was recognised, representing the predicted molecular mass of the amino acid sequence. The high immunogenicity of *Sr*HSP10 was demonstrated by a very strong reactivity of serum IgG with the recombinantly expressed *Sr*HSP10 showing a titer >1:10,000 as compared to a very weak reactivity of the pre-immune serum.

These results manifest the expression of HSP10 during an infection process when host and parasite interact. This suggests that *S. ratti* may also secrete *Sr*HSP10 in the host, and confirms that *Sr*HSP10 found in the *S. ratti* supernatant is no *in vitro* artefact. Furthermore, these findings suggest a role of *Sr*HSP10 during parasite-host interaction.

B. malayi infects humans and lives in the lymphatics, successfully evading host immune responses (Blaxter *et al* 1992; Behnke *et al* 1992). Many hypotheses have been put forward for the parasite's successful establishment and longevity in the mammalian host (Maizels *et al* 1993); among these are the production of parasite-encoded products that mimic host regulatory factors (Maizels *et al* 2001a; Maizels *et al* 2001b). In *B. malayi*, recombinant *Bm*HSP12.6 bound to human IL10R in a dose-dependent fashion and inhibited the binding of human IL10 to human IL10R *in vitro*. *Sr*HSP10 may also have the capacity to bind to human IL10R, blocking the

binding of human IL10R. To corroborate these assumptions, further immunological studies are needed to clarify a possible binding of the *Sr*HSP10 to host immune cells and to elucidate subsequently how *Sr*HSP10 might mediate its effect. In addition, immunofluorescence and electron microscopic localisation studies should be performed to obtain more detailed information of the *Sr*HSP10 expression.

4.6 Outlook

The data presented in this study provide a molecular basis for further studies on *Sr*HSP10 and *Sr*HSP60, including immunological investigations. Furthermore, the high homology of *Sr*HSP10 and *Sr*HSP60 with *S. stercoralis* HSP10 and *S. stercoralis* HSP60, respectively, suggests that there is a potential for continued studies of both proteins, also in the human host. The fact that *Sr*HSP10 and *Sr*HSP60 may function not only as chaperones but also in immune modulation promotes them as potential targets for candidate vaccination approaches or chemotherapeutic approaches for nematode control.

Moreover, there is substantial evidence from animal models of human diseases which support the hypothesis that infection with helminths can suppress the development of other maladies. The *hygiene hypothesis* suggests an inverse relationship between the incidence of parasitic infections and, for example, chronic inflammatory bowel diseases (IBD). It remains to be elucidated, whether *Sr*HSP10, being highly immunogenic, and *Sr*HSP60 may also have therapeutic potential.

5 Summary

S. stercoralis and *S. ratti* are intestinal parasitic nematodes infecting humans and rats. Both nematodes present extraordinary life cycles, comprising a free-living generation in addition to parasitic stages. To better understand the parasite-host relationship, the study of host defence-modulating molecules in parasitic systems may likely reveal new principles of their function. In search of such molecules, we performed mass spectrometry to identify excretory/secretory products of *S. ratti*. Among others, homologues of the heat shock proteins HSP10 and HSP60 (*Sr*HSP10, *Sr*HSP60) were detected. Intensive study of immune functions of HSP10 and HSP60 within the last years clearly demonstrated that they possess immune stimulatory potential and hence suggest additional roles to their protein folding function in parasite biology. To characterise *Sr*HSP10 and *Sr*HSP60, their full-length cDNA were cloned, the genomic organisation analysed, their presumptive role as interaction partners investigated, their tissue-specific transcription studied, and *Sr*HSP10 was expressed for antibody production. Based up on these results, the protective potential of *Sr*HSP10 was investigated by immunological studies. The *Sr*HSP10 cDNA contains an open reading frame of 330 bp, encoding a polypeptide of 110 amino acids with an approximate molecular weight of 10 kDa. The resulting full length *Sr*HSP60 cDNA is 1914 bp in size. The deduced amino acid sequence has a predicted molecular mass of 64 kDa. Both proteins exhibit high homology to that of the human pathogen *S. stercoralis*. The analysis of the genomic organisation of the *Sr*HSP10 and *Sr*HSP60 locus revealed that the genes are linked in a head-to-head configuration by a bidirectional promoter. Mammalian and yeast two-hybrid assays correspondingly showed dimerisation of *Sr*HSP10. *In situ* hybridisation results demonstrated *Sr*HSP10 transcription in the complete gut area. Immunisation experiments, finally, revealed a high immunogenicity of *Sr*HSP10 and provided evidence for a strong protective effect in *S. ratti*-infected rats.

6 Appendix

6.1 Mammalian and yeast two-hybrid *Sr*HSP60 bait-constructs

The cDNA sequences of the mammalian and yeast two-hybrid *Sr*HSP60 are shown below, the base pair length is bracketed. Start and stop codons are printed in bold.

6.1.1 *Sr*HSP60 cDNA sequences of the mammalian two-hybrid bait-constructs

pBindHSP60fl: *Sr*HSP60 cDNA full length cDNA sequence (1695 bp)

atgcttcgtcttgctgctcataatttttctaaatctattatcagatcatatgctaaagatcttaaatttggagctgatg
gaagaaaggccatgcttgttggagtagatcttttagctgacgctgtatctgttacaatgggaccaaaaggaagaaatgt
tattattgaacaaagttttggtggaccaaaaattacaaaagacggtgttaccgttgctaaggctattgatctcgaagat
aaatatcaaaatatgggtgcaaaattggttcaagatgttgcagataaggctaacgaacaagctggtgacggaacaactt
gcgctactgttcttgccagagctattgctaaagaaggatttgaaagtattagccgtggtgctaatccaattgaagttag
aaaaggtgtcatgtcatccgttgaagctattgttgaagagttaaagaagatgtcaaagcaagttacaactccagaagaa
attgctcaagttgctactatttctgctaatggagataaagatatcggaaaattgatatctgaagctatgaaaaaagttg
gaaataaaggtgtcattaccgttaaggatggaaaaactcttgacgatgaattagaaattattgaaggaatgaaatttga
ccgtggatatatttctccatatttcatgaatacttccaagggtggaaaatgtttctttgaaaaatgtttagtccttctt
tctgagaaaaaaatttcccaagttcaagatattgttccagctcttgaattagcaaataaatatcgtcaaccacttatta
ttattgctgaagatgtcgatggtgaagctcttacaaccctcgttcttaacagattaaaggttggtcttcaagtttgtgc
tgtaaaagctcctggttttggagacaaccgcaaaaacacattgaaagatattgccattgctacaggagctaaagtcttt
ggagatgaatctaatttacacaaacttgaagatattcaagcaggtgactttggtgaagttgctgaagttacagttacaa
aagatgatactcttatgcttaatggaaaaggtgatgctgaacaagttgagaagagaattcaacaaattgaatttgagat
tgaacaatctacttctgagtatgaaaaagaaaaacttaatgaacgtttagctaaattatcaaaaggtgttgctgtcctt
aaaattggaggtgcttctgaagttgaagttagtgaaaagagagatcgtgtcacagatgcgctctgtgcaacacgtgctg
ctgtagaagaaggaatcgtaccaggaggtggtgttgctcttcttcgtgctgtcaaagttcttgataacatcaaagtcgg
taattctgaccaagaattgggagttagaattgtccaaaaagccgttcgtcaaccaatttctaccattattaagaatgct
ggacttgaaccagctagtatcgttgaaaaagtttatgcaaatgaatcagtcagtttcggttacgatgctttgaatgata
agtttgttgatatgatccaaactggtattattgatccaaccaaagttgtccgtacatcacttcaagacgctgctggtgt
tgcttcacttttagccactactgaatgtgttgttacagaagttccaaaagaaactccagcacctccaatgggtggtatg
ggaggtatgggcggaatgggtggtggaatgttc**taa**

pBindHSP60_1-6: *Sr*HSP60 cDNA fragment (bp 1 to bp 873)

atgcttcgtcttgctgctcataatttttctaaatctattatcagatcatatgctaaagatcttaaatttggagctgatg
gaagaaaggccatgcttgttggagtagatcttttagctgacgctgtatctgttacaatgggaccaaaaggaagaaatgt
tattattgaacaaagttttggtggaccaaaaattacaaaagacggtgttaccgttgctaaggctattgatctcgaagat
aaatatcaaaatatgggtgcaaaattggttcaagatgttgcagataaggctaacgaacaagctggtgacggaacaactt
gcgctactgttcttgccagagctattgctaaagaaggatttgaaagtattagccgtggtgctaatccaattgaagttag
aaaaggtgtcatgtcatccgttgaagctattgttgaagagttaaagaagatgtcaaagcaagttacaactccagaagaa
attgctcaagttgctactatttctgctaatggagataaagatatcggaaaattgatatctgaagctatgaaaaaagttg
gaaataaaggtgtcattaccgttaaggatggaaaaactcttgacgatgaattagaaattattgaaggaatgaaatttga
ccgtggatatatttctccatatttcatgaatacttccaagggtggaaaatgtttctttgaaaaatgtttagtccttctt
tctgagaaaaaaatttcccaagttcaagatattgttccagctcttgaattagcaaataaatatcgtcaaccacttatta
ttattgctgaagatgtcgatggtgaagctcttacaaccctcgttcttaacagattaaaggttggtcttcaagtttgtgc
tgta

pBindHSP60_4-6: *Sr*HSP60 cDNA fragment (bp 625 to bp 873)

```
gaaatttgaccgtggatatatttctccatatttcatgaatacttccaagggtggaaaatgtttctttgaaaaatgttta
gtccttctttctgagaaaaaaatttcccaagttcaagatattgttccagctcttgaattagcaaataaatatcgtcaac
cacttattattattgctgaagatgtcgatggtgaagctcttacaaccctcgttcttaacagattaaaggttggtcttca
agtttgtgctgta
```

pBindHSP60_5-6: *Sr*HSP60 cDNA fragment (bp 739 to bp 873)

```
gatattgttccagctcttgaattagcaaataaatatcgtcaaccacttattattattgctgaagatgtcgatggtgaag
ctcttacaaccctcgttcttaacagattaaaggttggtcttcaagtttgtgctgta
```

pBindHSP60_4-2: *Sr*HSP60 cDNA fragment (bp 625 to bp 1683)

```
gaaatttgaccgtggatatatttctccatatttcatgaatacttccaagggtggaaaatgtttctttgaaaaatgttta
gtccttctttctgagaaaaaaatttcccaagttcaagatattgttccagctcttgaattagcaaataaatatcgtcaac
cacttattattattgctgaagatgtcgatggtgaagctcttacaaccctcgttcttaacagattaaaggttggtcttca
agtttgtgctgtaaaagctcctggttttggagacaaccgcaaaaacacattgaaagatattgccattgctacaggagct
aaagtctttggagatgaatctaatttacacaaacttgaagatattcaagcaggtgactttggtgaagttgctgaagtta
cagttacaaaagatgatactcttatgcttaatggaaaaggtgatgctgaacaagttgagaagagaattcaacaaattga
atttgagattgaacaatctacttctgagtatgaaaaagaaaaacttaatgaacgtttagctaaattatcaaaaggtgtt
gctgtccttaaaattggaggtgcttctgaagttgaagttagtgaaaagagagatcgtgtcacagatgcgctctgtgcaa
cacgtgctgctgtagaagaaggaatcgtaccaggaggtggtgttgctcttcttcgtgctgtcaaagttcttgataacat
caaagtcggtaattctgaccaagaattgggagttagaattgtccaaaaagccgttcgtcaaccaatttctaccattatt
aagaatgctggacttgaaccagctagtatcgttgaaaaagtttatgcaaatgaatcagtcagtttcggttacgatgctt
tgaatgataagtttgttgatatgatccaaactggtattattgatccaaccaaagttgtccgtacatcacttcaagacgc
tgctggtgttgcttcacttttagccactactgaatgtgttgttacagaagttccaaaagaaactccagcacctccaatg
ggtggtatgggaggtatgggcggaatgggtggt
```

6.1.2 *Sr*HSP10 cDNA sequences of the mammalian two-hybrid prey-constructs

pAct3SRHSP10fl: *Sr*HSP10 full length cDNA sequence (bp 330)

```
atgcttctttccgctgttcgtagatgttcatcagctcttaaaaatgttcaaccacttttgatcgtgttatgatcaaaa
aagctgctgctgaagtaaaatctaaaggaggtatttacattccagagaaagcccaaggaaaggttcttgaaggtactgt
tgttgctgctggtccaggacttcgtactgaggatggtaaacttatacctttatcagttagtgttggagatcgtgttatg
ttgccagagtatggtggaaataaagttgttatggatgatacagaatatttcatctatcgtgagtcagatctcatcgcca
aactcactaactaa
```

pAct3SRHSP10a: *Sr*HSP10 cDNA fragment (bp 1 to bp 174)

```
atgcttctttccgctgttcgtagatgttcatcagctcttaaaaatgttcaaccacttttgatcgtgttatgatcaaaa
aagctgctgctgaagtaaaatctaaaggaggtatttacattccagagaaagcccaaggaaaggttcttgaaggtactgt
tgttgctgctggtcca
```

pAct3SRHSP10b: *Sr*HSP10 cDNA fragment (bp 85 to bp 138)

```
gctgctgaagtaaaatctaaaggaggtatttacattccagagaaagcccaagga
```

pAct3SRHSP10c: *Sr*HSP10 cDNA fragment (bp 169 to bp 330)

```
tggtccaggacttcgtactgaggatggtaaacttatacctttatcagttagtgttggagatcgtgttatgttgccagag
tatggtggaaataaagttgttatggatgatacagaatatttcatctatcgtgagtcagatctcatcgccaaactcacta
actaa
```

6.1.3 *Sr*HSP60 cDNA sequences of the yeast two-hybrid bait-constructs

pBridgeHSP60fl: *Sr*HSP60 full-length cDNA sequence (1695 bp)

atgcttcgtcttgctgctcataatttttctaaatctattatcagatcatatgctaaagatcttaaatttggagctgatg
gaagaaaggccatgcttgttggagtagatcttttagctgacgctgtatctgttacaatgggaccaaaaggaagaaatgt
tattattgaacaaagttttggtggaccaaaaattacaaaagacggtgttaccgttgctaaggctattgatctcgaagat
aaatatcaaaatatgggtgcaaaattggttcaagatgttgcagataaggctaacgaacaagctggtgacggaacaactt
gcgctactgttcttgccagagctattgctaaagaaggatttgaaagtattagccgtggtgctaatccaattgaagttag
aaaaggtgtcatgtcatccgttgaagctattgttgaagagttaaagaagatgtcaaagcaagttacaactccagaagaa
attgctcaagttgctactatttctgctaatggagataaagatatcggaaaattgatatctgaagctatgaaaaaagttg
gaaataaaggtgtcattaccgttaaggatggaaaaactcttgacgatgaattagaaattattgaaggaatgaaatttga
ccgtggatatatttctccatatttcatgaatacttccaagggtggaaaatgtttctttgaaaaatgtttagtccttctt
tctgagaaaaaaatttcccaagttcaagatattgttccagctcttgaattagcaaataaatatcgtcaaccacttatta
ttattgctgaagatgtcgatggtgaagctcttacaaccctcgttcttaacagattaaaggttggtcttcaagtttgtgc
tgtaaaagctcctggttttggagacaaccgcaaaaacacattgaaagatattgccattgctacaggagctaaagtcttt
ggagatgaatctaatttacacaaacttgaagatattcaagcaggtgactttggtgaagttgctgaagttacagttacaa
aagatgatactcttatgcttaatggaaaaggtgatgctgaacaagttgagaagagaattcaacaaattgaatttgagat
tgaacaatctacttctgagtatgaaaaagaaaaacttaatgaacgtttagctaaattatcaaaaggtgttgctgtcctt
aaaattggaggtgcttctgaagttgaagttagtgaaaagagagatcgtgtcacagatgcgctctgtgcaacacgtgctg
ctgtagaagaaggaatcgtaccaggaggtggtgttgctcttcttcgtgctgtcaaagttcttgataacatcaaagtcgg
taattctgaccaagaattgggagttagaattgtccaaaaagccgttcgtcaaccaatttctaccattattaagaatgct
ggacttgaaccagctagtatcgttgaaaaagtttatgcaaatgaatcagtcagtttcggttacgatgctttgaatgata
agtttgttgatatgatccaaactggtattattgatccaaccaaagttgtccgtacatcacttcaagacgctgctggtgt
tgcttcacttttagccactactgaatgtgttgttacagaagttccaaaagaaactccagcacctccaatgggtggtatg
ggaggtatgggcggaatgggtggtggaatgttc**taa**

6.1.4 *Sr*HSP10 cDNA sequences of the yeast two-hybrid prey-constructs

The *Sr*HSP10 yeast two-hybrid prey-constructs are shown below. The in Richardson *et al* 2000 described mobile loop sequence is printed in grey. Start and stop codons are printed in bold. The base pair position is bracketed.

pAct2SRHSP10fl: *Sr*HSP10 full length cDNA sequence (bp 1 to bp 330)

atgcttctttccgctgttcgtagatgttcatcagctcttaaaaatgttcaaccacttttgatcgtgttatgatcaaaa
aagctgctgctgaagtaaaatctaaaggaggtatttacattccagagaaagcccaaggaaaggttcttgaaggtactgt
tgttgctgctggtccaggacttcgtactgaggatggtaaacttatacctttatcagttagtgttggagatcgtgttatg
ttgccagagtatggtggaaataaagttgttatggatgatacagaatatttcatctatcgtgagtcagatctcatcgcca
aactcactaac**taa**

pAct2SRHSP10a: *Sr*HSP10 cDNA fragment (bp 1 to bp 174)

atgcttctttccgctgttcgtagatgttcatcagctcttaaaaatgttcaaccacttttgatcgtgttatgatcaaaa
aagctgctgctgaagtaaaatctaaaggaggtatttacattccagagaaagcccaaggaaaggttcttgaaggtactgt
tgttgctgctggtcca

pAct2SRHSP10b: *Sr*HSP10 cDNA fragment (bp 85 to bp 138)

gctgctgaagtaaaatctaaaggaggtatttacattccagagaaagcccaagga

pAct2SRHSP10c: *Sr*HSP10 cDNA fragment (bp 169 to bp 330)

tggtccaggacttcgtactgaggatggtaaacttatacctttatcagttagtgttggagatcgtgttatgttgccagag
tatggtggaaataaagttgttatggatgatacagaatatttcatctatcgtgagtcagatctcatcgccaaactcacta
ac**taa**

pAct2SRHSP10gen1: *Nco*I/*xba*I digested *Sr*HSP10 subfragment (origin: pCR2.1-pAct2_HSP10 plasmid)

gcc**atg**gagatgcttctttccgctgttcgtagatgttcatcagctcttaaaaatgttcaaccacttttgatcgtgtta
tgatcaaaaaagct**taa**tctaga

pAct2SRHSP10gen2: *Bam*HI/*Xho*I digested *Sr*HSP10 subfragment (origin: pCR2.1-pAct2_HSP10 plasmid)

gggatccgagttcaaccacttttgatcgtgttatgatcaaaaaagctgctgctgaagtaaaatctaaaggaggtattt
acattccagagaaagcccaaggataactcgag

pAct2SRHSP10gen3: *Sma*I/*Sac*I digested *Sr*HSP10 subfragment (origin: pCR2.1-pAct2_HSP10 plasmid)

gccccggggctgctgaagtaaaatctaaaggaggtatttacattccagagaaagcccaaggaaaggttcttgaaggta
ctgttgttgctgcttaagagctc

6.2 *Sr*HSP60 and *Sr*HSP10 Southern blot probes

6.2.1 *Sr*HSP60 Southern blot probe cDNA sequences

The *Sr*HSP60 Southern blot probe cDNA sequences are shown below. The base pair position is bracketed.

pBluescript_HSP60_Sonde1 (bp 52-bp 372)

gctaaagatcttaaatttggagctgatggaagaaaggccatgcttgttggagtagatcttttagctgacgctgtatctg
ttacaatgggaccaaaaggaagaaatgttattattgaacaaagttttggtggaccaaaaattacaaaagacggtgttac
cgttgctaaggctattgatctcgaagataaatatcaaaatatgggtgcaaaattggttcaagatgttgcagataaggct
aacgaacaagctggtgacggaacaacttgcgctactgttcttgccagagctattgctaaagaaggatttgaaagtatta
gccgt

pBluescript_HSP60_Sonde2 (bp 378-bp 708)

taatccaattgaagttagaaaaggtgtcatgtcatccgttgaagctattgttgaagagttaaagaagatgtcaaagcaa
gttacaactccagaagaaattgctcaagttgctactatttctgctaatggagataaagatatcggaaaattgatatctg
aagctatgaaaaaagttggaaataaaggtgtcattaccgttaaggatggaaaaactcttgacgatgaattagaaattat
tgaaggaatgaaatttgaccgtggatatatttctccatatttcatgaatacttccaagggtggaaaatgtttctttgaa
aaatgtttagtcctt

pBluescript_HSP60_Sonde3 (bp 936-bp 1235)

agctaaagtctttggagatgaatctaatttacacaaacttgaagatattcaagcaggtgactttggtgaagttgctgaa
gttacagttacaaaagatgatactcttatgcttaatggaaaaggtgatgctgaacaagttgagaagagaattcaacaaa
ttgaatttgagattgaacaatctacttctgagtatgaaaaagaaaaacttaatgaacgtttagctaaattatcaaaagg
tgttgctgtccttaaaattggaggtgcttctgaagttgaagttagtgaaaagagagatcgtgt

pBluescript_HSP60_Sonde4 (bp 1334-bp 1641)

tcaaagtcggtaattctgaccaagaattgggagttagaattgtccaaaaagccgttcgtcaaccaatttctaccattat
taagaatgctggacttgaaccagctagtatcgttgaaaaagtttatgcaaatgaatcagtcagtttcggttacgatgct
ttgaatgataagtttgttgatatgatccaaactggtattattgatccaaccaaagttgtccgtacatcacttcaagacg
ctgctggtgttgcttcacttttagccactactgaatgtgttgttacagaagttccaaaagaaactccagca

6.2.2 *Sr*HSP10 Southern blot probe cDNA sequence

The *Sr*HSP10 Southern blot probe cDNA sequences are shown below. The base pair position is bracketed.

pBluescript_HSP10_Sonde (bp 14-bp 323)

```
atgcttctttccgctgttcgtagatgttcatcagctcttaaaaatgttcaaccacttttgatcgtgttatgatcaaaa
aagctgctgctgaagtaaaatctaaaggaggtatttacattccagagaaagcccaaggaaaggttcttgaaggtactgt
tgttgctgctggtccaggacttcgtactgaggatggtaaacttatacctttatcagttagtgttggagatcgtgttatg
ttgccagagtatggtggaaataaagttgttatggatgatacagaatatttcatctatcgtgagtcagatctcatcgcca
aactcactaacta
```

6.3 *Sr*HSP60 subfragment cDNA sequence for *E. coli* expression excluding the mitochondrial leading sequence

The *Sr*HSP60 subfragment cDNA sequences for *E. coli* expression excluding the mitochondrial leading sequence are shown below. The base pair position is bracketed

pJC45_HSP60-52 (bp 51-bp 1695)

```
gctaaagatcttaaatttggagctgatggaagaaaggccatgcttgttggagtagatcttttagctgacgctgtatctg
ttacaatgggaccaaaaggaagaaatgttattattgaacaaagttttggtggaccaaaaattacaaaagacggtgttac
cgttgctaaggctattgatctcgaagataaatatcaaaatatgggtgcaaaattggttcaagatgttgcagataaggct
aacgaacaagctggtgacggaacaacttgcgctactgttcttgccagagctattgctaaagaaggatttgaaagtatta
gccgtggtgctaatccaattgaagttagaaaaggtgtcatgtcatccgttgaagctattgttgaagagttaaagaagat
gtcaaagcaagttacaactccagaagaaattgctcaagttgctactatttctgctaatggagataaagatatcggaaaa
ttgatatctgaagctatgaaaaaagttggaaataaaggtgtcattaccgttaaggatggaaaaactcttgacgatgaat
tagaaattattgaaggaatgaaatttgaccgtggatatatttctccatatttcatgaatacttccaagggtggaaaatg
tttctttgaaaaatgtttagtccttctttctgagaaaaaaatttcccaagttcaagatattgttccagctcttgaatta
gcaaataaatatcgtcaaccacttattattattgctgaagatgtcgatggtgaagctcttacaaccctcgttcttaaca
gattaaaggttggtcttcaagtttgtgctgtaaaagctcctggttttggagacaaccgcaaaaacacattgaaagatat
tgccattgctacaggagctaaagtctttggagatgaatctaatttacacaaacttgaagatattcaagcaggtgacttt
ggtgaagttgctgaagttacagttacaaaagatgatactcttatgcttaatggaaaaggtgatgctgaacaagttgaga
agagaattcaacaaattgaatttgagattgaacaatctacttctgagtatgaaaaagaaaaacttaatgaacgtttagc
taaattatcaaaaggtgttgctgtccttaaaattggaggtgcttctgaagttgaagttagtgaaaagagagatcgtgtc
acagatgcgctctgtgcaacacgtgctgctgtagaagaaggaatcgtaccaggaggtggtgttgctcttcttcgtgctg
tcaaagttcttgataacatcaaagtcggtaattctgaccaagaattgggagttagaattgtccaaaaagccgttcgtca
accaatttctaccattattaagaatgctggacttgaaccagctagtatcgttgaaaaagtttatgcaaatgaatcagtc
agtttcggttacgatgctttgaatgataagtttgttgatatgatccaaactggtattattgatccaaccaaagttgtcc
gtacatcacttcaagacgctgctggtgttgcttcacttttagccactactgaatgtgttgttacagaagttccaaaaga
aactccagcacctccaatgggtggtatgggaggtatgggcggaatgggtggtggaatgttc
```
taa

6.4 *Sr*HSP60 cDNA and amino acid sequences including the 5' and 3' UTR

The *Sr*HSP60 full length cDNA sequences and the deduced amino acid sequence are shown below. Start and stop codons are printed in bold. Spliced leader sequence is shadowed grey.

```
ttggtttaattacccaagtttgaggaatatattaacagtacaactttaaaaa**atg**cttcgt
  W  F  N  Y  P  S  L  R  N  I  L  T  V  Q  L  -  K  M  L  R

cttgctgctcataatttttctaaatctattatcagatcatatgctaaagatcttaaattt
 L  A  A  H  N  F  S  K  S  I  I  R  S  Y  A  K  D  L  K  F

ggagctgatggaagaaaggccatgcttgttggagtagatcttttagctgacgctgtatct
 G  A  D  G  R  K  A  M  L  V  G  V  D  L  L  A  D  A  V  S

gttacaatgggaccaaaaggaagaaatgttattattgaacaaagttttggtggaccaaaa
 V  T  M  G  P  K  G  R  N  V  I  I  E  Q  S  F  G  G  P  K

attacaaaagacggtgttaccgttgctaaggctattgatctcgaagataaatatcaaaat
 I  T  K  D  G  V  T  V  A  K  A  I  D  L  E  D  K  Y  Q  N

atgggtgcaaaattggttcaagatgttgcagataaggctaacgaacaagctggtgacgga
 M  G  A  K  L  V  Q  D  V  A  D  K  A  N  E  Q  A  G  D  G

acaacttgcgctactgttcttgccagagctattgctaaagaaggatttgaaagtattagc
 T  T  C  A  T  V  L  A  R  A  I  A  K  E  G  F  E  S  I  S

cgtggtgctaatccaattgaagttagaaaaggtgtcatgtcatccgttgaagctattgtt
 R  G  A  N  P  I  E  V  R  K  G  V  M  S  S  V  E  A  I  V

gaagagttaaagaagatgtcaaagcaagttacaactccagaagaaattgctcaagttgct
 E  E  L  K  K  M  S  K  Q  V  T  T  P  E  E  I  A  Q  V  A

actatttctgctaatggagataaagatatcggaaaattgatatctgaagctatgaaaaaa
 T  I  S  A  N  G  D  K  D  I  G  K  L  I  S  E  A  M  K  K

gttggaaataaaggtgtcattaccgttaaggatggaaaaactcttgacgatgaattagaa
 V  G  N  K  G  V  I  T  V  K  D  G  K  T  L  D  D  E  L  E

attattgaaggaatgaaatttgaccgtggatatatttctccatatttcatgaatacttcc
 I  I  E  G  M  K  F  D  R  G  Y  I  S  P  Y  F  M  N  T  S

aagggtggaaaatgtttctttgaaaaatgtttagtccttctttctgagaaaaaaatttcc
 K  G  G  K  C  F  F  E  K  C  L  V  L  L  S  E  K  K  I  S

caagttcaagatattgttccagctcttgaattagcaaataaatatcgtcaaccacttatt
 Q  V  Q  D  I  V  P  A  L  E  L  A  N  K  Y  R  Q  P  L  I

attattgctgaagatgtcgatggtgaagctcttacaaccctcgttcttaacagattaaag
 I  I  A  E  D  V  D  G  E  A  L  T  T  L  V  L  N  R  L  K

gttggtcttcaagtttgtgctgtaaaagctcctggttttggagacaaccgcaaaaacaca
 V  G  L  Q  V  C  A  V  K  A  P  G  F  G  D  N  R  K  N  T

ttgaaagatattgccattgctacaggagctaaagtctttggagatgaatctaatttacac
 L  K  D  I  A  I  A  T  G  A  K  V  F  G  D  E  S  N  L  H

aaacttgaagatattcaagcaggtgactttggtgaagttgctgaagttacagttacaaaa
 K  L  E  D  I  Q  A  G  D  F  G  E  V  A  E  V  T  V  T  K

gatgatactcttatgcttaatggaaaaggtgatgctgaacaagttgagaagagaattcaa
 D  D  T  L  M  L  N  G  K  G  D  A  E  Q  V  E  K  R  I  Q

caaattgaatttgagattgaacaatctacttctgagtatgaaaaagaaaaacttaatgaa
 Q  I  E  F  E  I  E  Q  S  T  S  E  Y  E  K  E  K  L  N  E
```

```
cgtttagctaaattatcaaaaggtgttgctgtccttaaaattggaggtgcttctgaagtt
 R  L  A  K  L  S  K  G  V  A  V  L  K  I  G  G  A  S  E  V

gaagttagtgaaaagagagatcgtgtcacagatgcgctctgtgcaacacgtgctgctgta
 E  V  S  E  K  R  D  R  V  T  D  A  L  C  A  T  R  A  A  V

gaagaaggaatcgtaccaggaggtggtgttgctcttcttcgtgctgtcaaagttcttgat
 E  E  G  I  V  P  G  G  G  V  A  L  L  R  A  V  K  V  L  D

aacatcaaagtcggtaattctgaccaagaattgggagttagaattgtccaaaaagccgtt
 N  I  K  V  G  N  S  D  Q  E  L  G  V  R  I  V  Q  K  A  V

cgtcaaccaatttctaccattattaagaatgctggacttgaaccagctagtatcgttgaa
 R  Q  P  I  S  T  I  I  K  N  A  G  L  E  P  A  S  I  V  E

aaagtttatgcaaatgaatcagtcagtttcggttacgatgctttgaatgataagtttgtt
 K  V  Y  A  N  E  S  V  S  F  G  Y  D  A  L  N  D  K  F  V

gatatgatccaaactggtattattgatccaaccaaagttgtccgtacatcacttcaagac
 D  M  I  Q  T  G  I  I  D  P  T  K  V  V  R  T  S  L  Q  D

gctgctggtgttgcttcacttttagccactactgaatgtgttgttacagaagttccaaaa
 A  A  G  V  A  S  L  L  A  T  T  E  C  V  V  T  E  V  P  K

gaaactccagcacctccaatgggtggtatgggaggtatgggcggaatgggtggtggaatg
 E  T  P  A  P  P  M  G  G  M  G  G  M  G  G  M  G  G  G  M

ttctaaatcactagtcatttttttaccttctgcttccaatctttccttatttttaagatc
 F  -  I  T  S  H  F  F  T  F  C  F  Q  S  F  L  I  F  K  I

aataattataatgtatttaaagtatcaaactagattattgttttagggttgttacatttt
 N  N  Y  N  V  F  K  V  S  N  -  I  I  V  L  G  L  L  H  F

tgttttggttttcaacaaataaataaaatataanccaaaaaaaaaaaaaaaaaaaaaaa
 C  F  G  F  Q  Q  I  N  K  I  -  X  K  K  K  K  K  K  K  K
```

6.5 *Sr*HSP10 cDNA and amino acid sequences including the 5’ and 3’ UTR

The *Sr*HSP10 full length cDNA sequence and the deduced amino acid sequence are shown below. Start and stop codons are printed in bold. Spliced leader sequence is shadowed grey.

```
ggtttaattacccaagtttgaggttcttgtaattttggtaaatatgcttctttccgctgtt
  V  -  L  P  K  F  E  V  L  V  I  L  V  N  M  L  L  S  A  V

cgtagatgttcatcagctcttaaaaatgttcaaccacttttgatcgtgttatgatcaaa
 R  R  C  S  S  A  L  K  N  V  Q  P  L  F  D  R  V  M  I  K

aaagctgctgctgaagtaaaatctaaaggaggtatttacattccagagaaagcccaagga
 K  A  A  A  E  V  K  S  K  G  G  I  Y  I  P  E  K  A  Q  G

aaggttcttgaaggtactgttgttgctgctggtccaggacttcgtactgaggatggtaaa
 K  V  L  E  G  T  V  V  A  A  G  P  G  L  R  T  E  D  G  K

cttatacctttatcagttagtgttggagatcgtgttatgttgccagagtatggtggaaat
 L  I  P  L  S  V  S  V  G  D  R  V  M  L  P  E  Y  G  G  N

aaagttgttatggatgatacagaatatttcatctatcgtgagtcagatctcatcgccaaa
 K  V  V  M  D  D  T  E  Y  F  I  Y  R  E  S  D  L  I  A  K

ctcactaactaaggaatacattaaagtgtaattttatatattattttgttgttttttgc
 L  T  N  -  G  I  H  -  S  V  I  F  I  Y  Y  F  V  V  F  C
```

```
atagttgatattgtagtttgtattcttgttggttggttaaagactataaaaaatttaca
 I  V  D  I  V  V  C  I  L  V  G  W  L  K  T  I  K  N  F  T

caaaaaaaaaaaaaaaaaaaaaaa
 Q  K  K  K  K  K  K  K
```

6.6 *Sr*HSP60 and SrHSP10 parasitic female cDNA sequences

6.6.1 *Sr*HSP60 parasitic female cDNA sequence

The SrHSP60 full length parasitic female cDNA sequences and the alignment with the *Sr*HSP60 full length iL3 cDNA sequences are shown below. Start and stop codons are printed in bold.

```
il3     atgcttcgtcttgctgctcataatttttctaaatctattatcagatcatatgctaaagat60
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      atgcttcgtcttgctgctcataatttttctaaatctattatcagatcatatgctaaagat60

il3     cttaaatttggagctgatggaagaaaggccatgcttgttggagtagatcttttagctgac     120
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      cttaaatttggagctgatggaagaaaggccatgcttgttggagtagatcttttagctgac     120

il3     gctgtatctgttacaatgggaccaaaaggaagaaatgttattattgaacaaagttttggt     180
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      gctgtatctgttacaatgggaccaaaaggaagaaatgttattattgaacaaagttttggt     180

il3     ggaccaaaaattacaaaagacggtgttaccgttgctaaggctattgatctcgaagataaa     240
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      ggaccaaaaattacaaaagacggtgttaccgttgctaaggctattgatctcgaagataaa     240

il3     tatcaaaatatgggtgcaaaattggttcaagatgttgcagataaggctaacgaacaagct     300
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      tatcaaaatatgggtgcaaaattggttcaagatgttgcagataaggctaacgaacaagct     300

il3     ggtgacggaacaacttgcgctactgttcttgccagagctattgctaaagaaggatttgaa     360
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      ggtgacggaacaacttgcgctactgttcttgccagagctattgctaaagaaggatttgaa     360

il3     agtattagccgtggtgctaatccaattgaagttagaaaaggtgtcatgtcatccgttgaa     420
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      aatattagccgtggtgctaatccaattgaagttagaaaaggtgtcatgtcatccgttgaa     420

il3     gctattgttgaagagttaaagaagatgtcaaagcaagttacaactccagaagaaattgct     480
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      gctattgttgaagagttaaagaagatgtcaaagcaagttacaactccagaagaaattgct     480

il3     caagttgctactatttctgctaatggagataaagatatcggaaaattgatatctgaagct     540
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      caagttgctactatttctgctaatggagataaagatatcggaaaattgatatctgaagct     540
```

```
il3     atgaaaaaagttggaaataaaggtgtcattaccgttaaggatggaaaaactcttgacgat     600
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      atgaaaaaagttggaaataaaggtgtcattaccgttaaggatggaaaaactcttgacgat     600

il3     gaattagaaattattgaaggaatgaaatttgaccgtggatatatttctccatatttcatg     660
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      gaattagaaattattgaaggaatgaaatttgaccgtggatatatttctccatatttcatg     660

il3     aatacttccaagggtggaaaatgtttctttgaaaaatgtttagtccttctttctgagaaa     720
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      aatacttccaagggtggaaaatgtttctttgaaaaatgtttagtccttctttctgagaaa     720

il3     aaaatttcccaagttcaagatattgttccagctcttgaattagcaaataaatatcgtcaa     780
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      aaaatttcccaagttcaagatattgttccagctcttgaattagcaaataaatatcgtcaa     780

il3     ccacttattattattgctgaagatgtcgatggtgaagctcttacaaccctcgttcttaac     840
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      ccacttattattattgctgaagatgtcgatggtgaagctcttacaaccctcgttcttaac     840

il3     agattaaaggttggtcttcaagtttgtgctgtaaaagctcctggttttggagacaaccgc     900
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      agattaaaggttggtcttcaagtttgtgctgtaaaagctcctggttttggagacaaccgc     900

il3     aaaaacacattgaaagatattgccattgctacaggagctaaagtctttggagatgaatct     960
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      aaaaacacattgaaagatattgccattgctacaggagctaaagtctttggagatgaatct     960

il3     aatttacacaaacttgaagatattcaagcaggtgactttggtgaagttgctgaagttaca    1020
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      aatttacacaaacttgaagatattcaagcaggtgactttggtgaagttgctgaagttaca    1020

il3     gttacaaaagatgatactcttatgcttaatggaaaaggtgatgctgaacaagttgagaag    1080
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      gttacaaaagatgatactcttatgcttaatggaaaaggtgatgctgaacaagttgagaag    1080

il3     agaattcaacaaattgaatttgagattgaacaatctacttctgagtatgaaaaagaaaaa    1140
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      agaattcaacaaattgaatttgagattgaacaatctacttctgagtatgaaaaagaaaaa    1140

il3     cttaatgaacgtttagctaaattatcaaaaggtgttgctgtccttaaaattggaggtgct    1200
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      cttaatgaacgtttagctaaattatcaaaaggtgttgctgtccttaaaattggaggtgct    1200


il3     tctgaagttgaagttagtgaaaagagagatcgtgtcacagatgcgctctgtgcaacacgt    1260
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      tctgaagttgaagttagtgaaaagagagatcgtgtcacagatgcactctgtgcaacacgt    1260

il3     gctgctgtagaagaaggaatcgtaccaggaggtggtgttgctcttcttcgtgctgtcaaa    1320
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      gctgctgtagaagaaggaatcgtaccaggaggtggtgttgctcttcttcgtgctgtcaaa    1320
```

```
il3     gttcttgataacatcaaagtcggtaattctgaccaagaattgggagttagaattgtccaa    1380
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      gttcttgataacatcaaagtcggtaattctgaccaagaattgggagttagaattgtccaa    1380

il3     aaagccgttcgtcaaccaatttctaccattattaagaatgctggacttgaaccagctagt    1440
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      aaagccgttcgtcaaccaatttctaccattattaagaatgctggacttgaaccagctagt    1440

il3     atcgttgaaaaagtttatgcaaatgaatcagtcagtttcggttacgatgctttgaatgat    1500
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      atcgttgaaaaagtttatgcaaatgaatcagtcagtttcggttacgatgctttgaatgat    1500

il3     aagtttgttgatatgatccaaactggtattattgatccaaccaaagttgtccgtacatca    1560
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      aagtttgttgatatgatccaaactggtattattgatccaaccaaagttgtccgtacatca    1560

il3     cttcaagacgctgctggtgttgcttcacttttagccactactgaatgtgttgttacagaa    1620
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      cttcaagacgctgctggtgttgcttcacttttagccactactgaatgtgttgttacagaa    1620

il3     gttccaaaagaaactccagcacctccaatgggtggtatgggaggtatgggcggaatgggt    1680
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      gttccaaaagaaactccagcacctccaatgggtggtatgggaggtatgggcggaatgggt    1680

il3     ggtggaatgttctaa  1695
        ::::::::::::::
pf      ggtggaatgttctaa  1695
```

6.6.2 *Sr*HSP10 parasitic female cDNA sequence

The SrHSP10 full length parasitic female cDNA sequences and the alignment with the SrHSP10 full length iL3 cDNA sequences are shown below. Start and stop codons are printed in bold.

```
iL3     atgcttctttccgctgttcgtagatgttcatcagctcttaaaaatgttcaaccacttttt    60
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      atgcttctttccgctgttcgtagatgttcatcagctcttaaaaatgttcaaccacttttt    60

il3     gatcgtgttatgatcaaaaaagctgctgctgaagtaaaatctaaaggaggtatttacatt    120
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      gatcgtgttatgatcaaaaaagctgctgctgaagtaaaatctaaaggaggtatttacatt    120

il3     ccagagaaagcccaaggaaaggttcttgaaggtactgttgttgctgctggtccaggactt    180
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      ccagagaaagcccaaggaaaggttcttgaaggtactgttgttgctgctggtccaggactt    180

il3     cgtactgaggatggtaaacttatacctttatcagttagtgttggagatcgtgttatgttg    240
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      cgtactgaggatggtaaacttatacctttatcagttagtgttggagatcgtgttatgttg    240

il3     ccagagtatggtggaaataaagttgttatggatgatacagaatatttcatctatcgtgag    300
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
pf      ccagagtatggtggaaataaagttgttatggatgatacagaatatttcatctatcgtgag    300
```

```
il3     tcagatctcatcgccaaactcactaactaa   330
        ::::::::::::::::::::::::::::
pf      tcagatctcatcgccaaactcactaactaa   330
```

6.7 *Sr*HSP60 and *Sr*HSP10 gDNA sequences

The *Sr*HSP60 and SrHSP10 full length gDNA sequences including the intergenic sequence are shown below. Stop codons are printed in bold. The *Sr*HSP60 start codon is shadowed grey. The *Sr*HSP10 start codon is shadowed black. The intergenic region is printed in grey.

ttagaacattccaccacccattccgcccatacctcccataccacccattggaggtgctggagtttcttttggaact
tctgtaacaacacattcagtagtggctaaaagtgaagcaacaccagcagcgtcttgaagtgatgtacggacaactttgg
ttggatcaataataccagtttggatcatatcaacaaacttatcattcaaagcatcgtaaccgaaactgactgattcatt
tgcataaacttttcaacgatactagctggttcaagtccagcattcttaataatggtagaaattggttgacgaacggct
ttttggacaattctaactcccaattcttggtcagaattaccgactttgatgttatcaagaactttgacagcacgaagaa
gagcaacaccacctcctggtacgattccttcttctacagcagcacgtgttgcacagagcgcatctgtgacacgatctct
cttttcactaacttcaacttcagaagcacctccaatttaaggacagcaacacctttgataatttagctaaacgttca
ttaagtttttctttttcatactcagaagtagattgttcaatctcaaattcaatttgttgaattctcttctcaacttgtt
cagcatcacctttccattaagcataagagtatcatctttgtaactgtaacttcagcaacttcaccaaagtcacctgc
ttgaatatcttcaagtttgtgtaaattagattcatctccaaagactttagctcctgtagcaatggcaatatctttcaat
gtgtttttgcggttgtctccaaaaccaggagctttacagcacaaacttgaagaccaacctttaatctgttaagaacga
gggttgtaagagcttcaccatcgacatcttcagcaataataataagtggttgacgatatttatttgctaattcaagagc
tggaacaatatcttgaacttgggaaatttttttctcagaaagaaggactaaacatttttcaaagaaacattttccaccc
ttggaagtattcatgaaatatggagaaatatatccacggtcaaatttcattccttcaataatttctaattcatcgtcaa
gagtttttccatccttaacggtaatgacacctttatttccaacttttttcatagcttcagatatcaattttccgatatc
tttatctccattagcagaaatagtagcaacttgagcaatttcttctggagttgtaacttgctttgacatcttctttaac
tcttcaacaatagcttcaacggatgacatgacacctttttctaacttcaattggattagcaccacggctaatactttcaa
atccttctttagcaatagctctggcaagaacagtagcgcaagttgttccgtcaccagcttgttcgttagccttatctgc
aacatcttgaaccaattttgcacccatattttgatatttatcttcgagatcaatagccttagcaacggtaacaccgtct
tttgtaatttttggtccaccaaaactttgttcaataataacatttcttccttttggtcccattgtaacagatacagcgt
cagctaaaagatctactccaacaagcatggcctttcttccatcagctccaaatttaagatctttagcatatgatctgat
aatagatttagaaaaattatgagcagcaagacgaagcatttttaaagttgtactgttaatatattcctaaaaatttaa
atattaaacaaacttttaattgaaaaaattttcaaaaaaattataatttttaaaaactgaaaactaatttttataacga
aagtattaaagatatactttaaattaaaataaattggaatagaataaacatttcttgctgcactattgcgaaatattaa
tataggaattttcgcgaaatttctttaaaaaaaatctacaaattaataaattgctcctacaacgatagtagagacgatc
ttgttgcagaatgttctagaatgacttttaagttatgatggtaatctgcggctcgtccatataacttagagattatttt
aattttaaattatttttttaactaattgcaggttcttgtaattttggtaaatatgcttctttccgctgttcgtagatgtg
taggttatttttatttctatataaaattatatttctttttagtcatcagctcttaaaaatgttcaaccacttttgatcg
tgttatgatcaaaaaagctgctgctgaagtaaaatctaaaggaggtatttacattccagagaaagcccaaggaaaggtt
cttgaaggtactgttgttgctgctggtccaggacttcgtactgaggatggtaaacttatacctttatcagttagtgttg
gagatcgtgttatgttgccagagtatggtggaaataaagttgttatggatgatacagaatatttcatctatcgtgagtc
agatctcatcgccaaactcactaac**taa**

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8 Abbreviations and Units

8.1 Abbreviations

A	adenine	**HSP**	Heat shock protein
APS	ammoniumperoxodisulfate	**IPTG**	isopropyl-beta-d-thiogalactopyranoside
aa	amino acid	**iL3s**	infectious third stage larvae
BD	binding domain	**LacZ**	general structure of beta galactosidase
BSA	bovine serum albumine	**LB**	luria broth
conc.	concentrated	**MCS**	multiple cloning site
d	coat thickness	**MOPS**	3-n(morpholino) propane sulfonic acid
DEPC	diethylpyrocarbonate	**NCBI**	National Centre for Biotechnology Information
dH_2O	deionised water	**NCS**	newborn calf serum
DMSO	dimethyl sulfoxide	**ONPG**	o-nitrophenyl-ß. -d-galactopyranoside
DNA	deoxyribonucleic acid	**ORF**	open reading frame
cDNA	complementary DNA	**PAGE**	polyacrylamide gel electrophoresis
DNase	deoxyribonuclease	**PBS**	phosphate buffered saline
dNTP	deoxyribonucleotide triphosphate	**PCR**	polymerase chain reaction
DTT	dithiothreitol	**PEG**	polyethylenglycole
E	extinction	**RNA**	ribonucleic acid
EDTA	ethylenediaminetetraacetic acid	**mRNA**	messenger RNA
et al.	et alii	**rRNA**	ribosomal RNA
EtBr	ethidium bromide	**RNase**	ribonuclease
EtOH	ethanol	**RT**	room temperature
G	guanine	**RT**	reverse transcription
GFP	green fluorescent protein	**RT-PCR**	reverse transcription-PCR
HCl	hydrochloric acid	**SDS**	sodium dodecyl sulfate
HEPES	4-2-hydroxyethyl)piperazine-1-ethanesulfon i cacid	**T**	thymidine
Taq	thermophilus aquatus	**TWEEN**	poloxyethylenglykolsorbitol-monooleat

TEMED	tetramethylethylenediamine	**WHO**	World Health Organisation
TK	tyrosine kinase	**X-Gal**	5-bromo-4-chloro-3-indoyl- ß-d-galacto-pyranosid
Tris	tris(hydroxymethyl)amino- methane	**λ**	wavelength

8.2 Units

aa	amino acid	**kb**	kilo base pair	**tm**	temperature melting point
bp	base pair	**min**	minute	**u**	unit
c	concentration	**ml**	millilitre	**v**	volt
°C	Celsius	**mM**	millimolar	**(v/v)**	volume per volume
cm	centimetre	**ng**	nanogram	**w**	watt
d	day	**nm**	nanometre	**(w/v)**	weight per volume
da	dalton	**od**	optical density	**µg**	microgram
g	gram	**ph**	h+-concentration	**µl**	microlitre
g	acceleration of gravidity	**rpm**	rotations per minute	**µmol**	micromol
h	hours	**sec**	seconds	**µm**	micromolar
hz	hertz	**t**	time	**%**	percentage

8.3 Amino acids

A	Ala	alanine	**M**	Met	methionine
B	Asx	asparagine, asparagine acid	**N**	Asn	asparagine
C	Cys	cysteine	**P**	Pro	proline
D	Asp	asparagine acid	**Q**	Gln	glutamine
E	Glu	glutamine acid	**R**	Arg	arginine
F	Phe	phenylalanine	**S**	Ser	serine
G	Gly	glycine	**T**	Thr	threonine
H	His	histidine	**V**	Val	valine
I	Ile	isoleucine	**W**	Trp	tryptophane
K	Lys	lysine	**Y**	Tyr	tyrosine
L	Leu	leucine	**Z**	Glx	glutamine, glutamine acid

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Ich erkläre: Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte, fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündliche Auskünfte beruhen, sind als solche kenntlich gemacht, Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter, wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.

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