Analysis of RNA interference in the parasitic nematode *Haemonchus contortus*

Buddhini Samarasinghe
MBiol (hons) Molecular and Cellular Biology

Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Division of Infection and Immunity
Faculty of Veterinary Medicine
University of Glasgow

Abstract

Parasitic nematode infections worldwide cause a significant impact on human health, as well as economic and welfare losses to the animal and agriculture industries. The principal method of control for parasitic nematode infections is currently limited to repeated treatments with anthelmintic drugs, but widespread resistance to all major classes of these drugs is a growing problem. As a result, there is an urgent need for alternative methods of controlling these infections, and the development of molecular vaccines and novel drugs represent two possible approaches. However, both these approaches require a deeper understanding of gene function in order to identify suitable control targets. This project examines RNA interference (RNAi) in the parasitic nematode *Haemonchus contortus* to determine if this could be developed as a functional tool and advance the discovery of novel control targets for parasitic nematodes.

RNAi has proven less effective in parasitic nematodes than in the free-living model nematode *Caenorhabditis elegans* and it is unclear why this is so. This project examined the reliability of RNAi in *H. contortus,* and several genes were successfully silenced using RNAi. Further analysis of RNAi susceptible genes revealed that RNAi silencing appears to be related to the site of expression of the target gene; genes expressed in tissues which are accessible to the environment such as intestine, excretory cell and amphids were silenced by RNAi. Upstream promoter regions of RNAi susceptible genes were examined for the presence of motifs which may regulate spatial gene expression, an approach that could be used to predict gene susceptibility to RNAi. RNAi treated larvae were subsequently used to infect sheep in the first *in vivo* RNAi study, resulting in a significant impact on worm survival *in vivo*. In addition, several components of the RNAi pathway in *H. contortus* were characterised in this project, demonstrating the presence of a functional RNAi pathway that is capable of reliably silencing genes. In conclusion, the findings presented in this project suggest that RNAi may be used in the future to evaluate the function of a novel vaccine or drug target for controlling *H. contortus* infections in sheep.

Table of Contents

List of Tables

List of Figures

Acknowledgements

First and foremost I would like to thank my supervisor Collette Britton for her guidance, discussions, endless positivity and support throughout this project and when proof-reading this thesis. Thanks also to my supervisor Dave Knox at Moredun Research Institute for the constant supply of project feedback, advice and worms! Thanks to my assessor Eileen Devaney for invaluable discussions throughout this project. Many thanks also to Linda, Brett, Alan, Willie and everyone else in the Parasitology Group for showing me the ropes and always being so patient with my questions in the lab. I would also like to thank the funding bodies involved in this work; the ORS scholarship, the Sutherland scholarship and the University of Glasgow Vet Faculty.

A special thanks to Ananda for helping me with the statistical analysis and patiently explaining it all to me. Many thanks also for the immense support from my friends and family. Thank you Thomas for all the encouragement and support and for putting up with Nancy these past few months! Finally I would like to thank my parents; my mother who has always been an inspiration to me and my father who would have been so proud.

Declaration

The work reported in this thesis was carried out under the supervision of Dr Collette Britton at the Faculty of Veterinary Medicine, University of Glasgow. All results presented, unless otherwise stated, are the sole work of this author, as is the composition of this thesis.

Buddhini Samarasinghe

March 2010

Abbreviations

ANOVA	Analysis of variance
BAC	Bacterial artificial chromosome
Bp	Base pair
cDNA	Complementary DNA
CO_2	carbon dioxide
Contigs	Contiguous sequence
DALY	Disability adjusted life year
d.f.	Degrees of freedom (statistical analysis)
DIC	Differential Interference Contrast
DMSO	dimethyl sulfoxide
dNTPs	Deoxynucleoside 5'-triphosphates
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dsRNA	Double-stranded ribonucleic acid
EBI	European Bioinformatics Institute
EBSS	Earle's Balanced Salt Solution
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetra-acetic acid

EST	Expressed sequence tag
FEC	Faecal egg count
Hc	*Haemonchus contortus*
hrs	hours
Ce	*Caenorhabditis elegans*
g	gram
GFP	Green fluorescent protein
HRP	Horse radish peroxidise
IDV	Integrated Density Value
IPTG	Isopropyl-ß-D-thiogalactoside pyranoside
kDa	Kilo Dalton
λ	wavelength
l	Litre
LB	Luria-Bertani
M	Molar
MCS	Multiple cloning site
mM	millimolar
mm	millimeter
mg	milligram

mRNA	Messenger ribonucleic acid
miRNA	micro RNA
µg	microgram
µl	microlitre
µM	micromolar
ng	nanogram
nm	nanometer
p	*p*-value of a hypothesis (statistical analysis)
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
psi	pound per square inch
PVDF	polyvinylidene fluoride
RACE	Rapid Amplification of cDNA Ends
RNA	Ribonucleic acid
RNase	Ribonuclease
RNAi	RNA interference
rpm	revolutions per minute
RSAT	Regulatory Sequence Analysis Tool

RT-PCR	Reverse transcription polymerase chain reaction
SAGE	Serial analysis of gene expression
SDS	Sodium dodecyl sulphate
siRNA	small interfering ribonucleic acids
ssRNA	single stranded ribonucleic acid
SSU	Small subunit
t	Value of the test statistic in a Student's *t-test* for the difference between two means (statistical analysis)
UV	Ultra violet
V	Volts
VSR	Viral suppressors of RNAi
v/v	Volume/volume
w/v	Weight/volume

Chapter 1

Introduction

1. Introduction

1.1 Parasitic nematode infections

Parasitic nematodes cause a wide range of diseases in humans, plants and animals. The global burden of disease in humans is assessed using the disability-adjusted life year (DALY), a time-based measure that combines years of life lost due to premature mortality and years of life lost due to time lived in states of less than full health (http://www.who.int). The global burden of disease in humans caused by the three major intestinal nematodes (hookworm, *Ascaris lumbricoides*, and *Trichuris trichiura*) combined is an estimated 39 million DALYs (World Bank, 1993). River blindness and lymphatic filariasis caused by infections with filarial nematodes also result in considerable morbidity, estimated at a combined 6 million DALYs (http://www.who.int/tdr).

Plant parasitic nematodes infect most cultivated plant species and cause crop losses that significantly affect the food and textile industries. The total annual yield losses caused by plant parasitic nematodes are estimated to be >US$ 125 billion worldwide (Chitwood, 2003). In animal hosts, disease caused by parasitic nematodes is generally considered in terms of economic and welfare burden. Parasitic nematodes represent a serious health problem for grazing livestock (sheep, cattle and goats) in the UK and throughout the world, causing significant disease, animal welfare problems and economic loss. For example, the total cost of gastro-intestinal (GI) parasites across the entire sheep industry of the UK has been estimated at £84 million per annum (Nieuwhof, 2005). GI parasite infections are currently controlled with repeated anthelmintic treatments, thus reduction in performance is part of the cost of the disease along with the costs associated with the antiparasitic treatment itself. Of the various GI parasites that infect livestock, *Haemonchus contortus* is considered to be one of the most pathogenic and economically important.

1.1.1 Pathology of *Haemonchus contortus* infections

The pathogenicity of *H. contortus* is primarily due to the blood feeding lifestyle of the adult parasite which results in anaemia. The hyper acute form of the

infection occurs in animals exposed over a short period of time to thousands of parasites; it has been estimated that a sheep infected with 5000 *H. contortus* may lose 250 ml of blood daily (Urquhart, 1987). In the acute form of the disease, animals of all ages show dark faeces and anaemia, and the anaemia develops in three phases. The first phase is characterised by a pronounced anaemia and a dramatic fall in packed cell volume (the proportion of blood volume that is occupied by red blood cells) during the first two weeks post infection. Over the subsequent weeks the packed cell volume does not decrease further due to the compensatory mobilisation of the haemopoietic system which occurs during the second phase. The third phase occurs because this expansion of the red blood cells cannot be kept up indefinitely during the continual blood loss, resulting in a further drop in packed cell volume (Urquhart, 1987). This may be followed by death.

Chronic infection is less clinically obvious compared to acute infection, resulting in symptoms such as malnourishment leading to weight loss, wool-peeling in adult animals and stunted growth of lambs (Urquhart, 1987). Thus all forms of infection are economically important.

1.1.2 Overview and prevalence of *H. contortus*

Adult *H. contortus* worms are 2-3 cm in length and can be identified by the white ovaries winding spirally around the blood-filled intestine, producing a characteristic 'barber's pole' appearance. The male worms can be distinguished from females as the males have an asymmetrical dorsal lobe and barbed spicules, whereas the females have a vulval flap.

H. contortus has a worldwide distribution, but infection is most important in tropical and sub-tropical regions of the globe. Annual treatment costs due to *H. contortus* infections alone have been estimated to be US$ 26 million, $46 million and $103 million for Kenya, South Africa and India, respectively (Peter & Chandrawathani, 2005). Although *Teladorsagia circumcincta* has been identified as the most prevalent parasitic nematode in UK sheep farms, *H. contortus* infections currently show a 60% prevalence and as these infections are more pathogenic, represent a serious concern for sheep farms across the UK (Elizabeth Redman, personal communication).

1.1.3 Life cycle of *H. contortus*

The life cycle of *H. contortus* is direct, without a secondary host. Each female lays between 5000-10000 eggs per day which are passed out in the faeces. These eggs hatch in soil or water to L1 stage larvae and subsequently L2 larvae over a period of several days. The cuticle of the L2 larvae is retained during the moult to the L3 stage infective larvae. The optimal conditions for development from egg to L3 is a temperature of 28C° and humidity greater than 70% (Rossanigo & Gruner, 1995). The L3 larvae are ingested by grazing ruminants, and subsequently moult to form the L4 stage which penetrate the lining of the abomasum and begin to feed on the host blood. Adult male and female worms mate and the cycle repeats itself as illustrated in Figure 1.1. *H. contortus* larvae have the ability to undergo hypobiosis, which tends to occur at the start of a prolonged dry season (Gatongi *et al.*, 1998). It allows the worm to survive in the host as arrested L4, instead of maturing and producing eggs which would fail to develop on a dry pasture. Normal development resumes prior to the onset of seasonal rains. During the period of maturation of these hypobiotic larvae, clinical signs of acute infection may occur and in ewes this often coincides with lambing. In addition, it has been suggested that these infections of hypobiotic larvae occur due to a temporary depression of immunological capacity of ewes brought about by endocrine changes associated with lactation (Blitz & Gibbs, 1972). Therefore it appears that *H. contortus* is capable of adjusting it's life cycle and development based on environmental conditions and host life cycle.

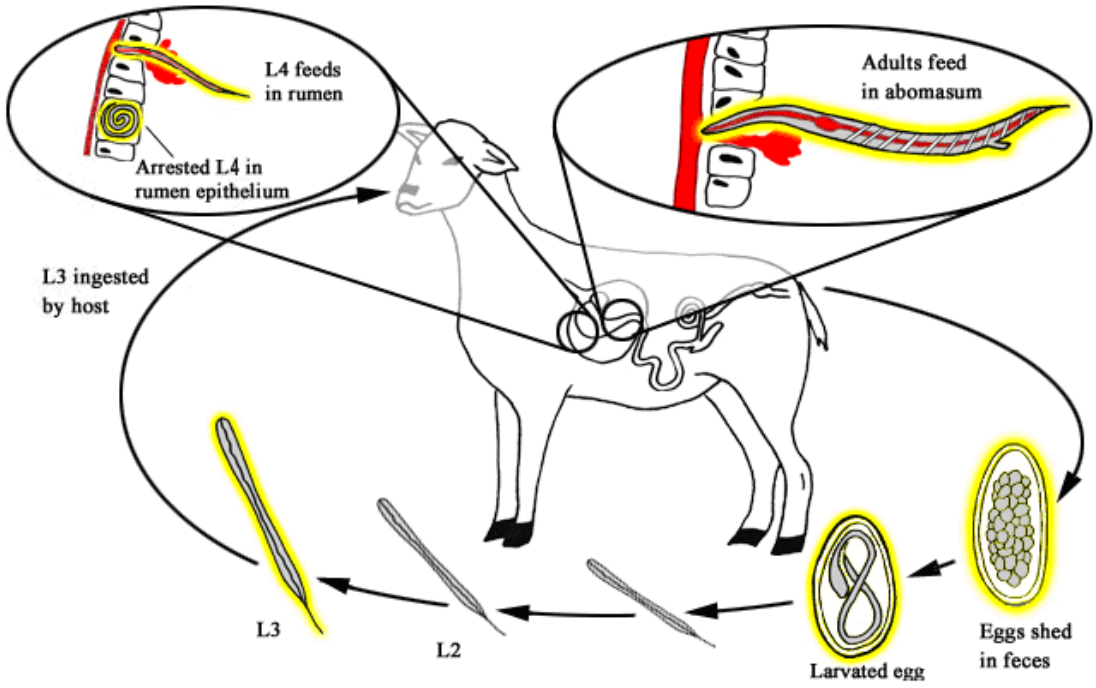


Figure 1.1. Life cycle of *Haemonchus contortus.*

Eggs are passed out in faeces from infected sheep which hatch and moult to the L3 stage larvae. These larvae are infective, and are ingested by grazing sheep. Once in the abomasum, the L3 larvae undergo two moults and mature into adults. (Image courtesy of University of Pennsylvania School of Veterinary Medicine http://cal.vet.upenn.edu/parasitc/haemonc/index.htm)

The time of development from ingested L3 to mature adult worm is approximately three weeks. Eggs can be extracted from faecal samples and isolated by floatation in a concentrated salt solution (Christie & Jackson, 1982). These eggs can be incubated at 25°C and hatch into L1 and develop through to L2 stage larvae, but viability and development beyond the L3 stage is difficult to achieve *in vitro*. Adult worms have severely limited survival outside of the sheep host. Therefore, it is difficult to culture *H. contortus in vitro*. Different culture techniques have been attempted, ranging from using simple culture media to enriched media but these methods are impractical, unreliable and not cost-effective. For example, successful culture of *H. contortus* adult males and egg-laying females was reported *in vitro* using the gastric contents of calves and sheep as well as stomach mucosa from both hosts along with other culture media with the pH carefully monitored (Stringfellow, 1986), but this method is difficult, time consuming and not cost effective for most purposes.

1.1.4 Control of parasitic nematode infections by anthelmintics

Most parasitic nematode infections, including *H. contortus* are currently controlled using antihelmintic drugs. There are several different classes of these drugs, the three major ones being benzimidazoles, tetrahydropyrimidines/imidazothiazoles and macrocyclic lactones. Much of the work on understanding the mechanisms of action of anthelmintics has come from studies in the free-living nematode *Caenorhabditis elegans* (reviewed by Brown *et al.*, 2006; Gilleard, 2006).

Benzimidazoles are broad spectrum anthelmintics which cause ultrastructural alterations in intestinal cells of nematodes by affecting the structure of the cytoskeleton (Borgers & De Nollin, 1975). The cytoskeleton is composed of microtubules, which are polymers of the tubulin proteins, alpha and beta tubulin. Microtubules exist in a dynamic steady state, with a balance between the assembly and disassembly of the soluble tubulin subunits. Benzimidazoles bind irreversibly to beta tubulin, which causes the steady state to shift, resulting in a net loss of microtubules (Lacey, 1988). This destruction of microtubules eventually leads to the death of the helminth. At a cellular level, benzimidazoles inhibit intestinal secretory vesicle transport by the depolymerisation of microtubules (Jasmer *et al.*, 2000). Benzimidazoles display a highly selective toxicity towards helminths, despite targeting beta tubulin which is a protein that can also be found in the mammalian host; it is thought that this selective toxicity is due to the much stronger and irreversible binding between the drug and helminth beta tubulin, as compared with mammalian beta tubulin (Lacey, 1988).

The tetrahydropyrimidines/imidazothiazoles, which include levamisole, act on the nervous system of the parasite. The muscle cell surfaces of nematodes possess nicotinic acetylcholine receptors, and these can be opened by nicotinic anthelmintics such as tetrahydropyrimidines/imidazothiazoles. The binding of the drugs to the receptors produces depolarisation, causing the spastic paralysis of nematode muscle, resulting in parasite expulsion (Martin, 1996). The selective toxicity is thought to be based upon the unique properties of the nematode

nicotinic acetylcholine receptor, which appears to be distinct from the homologous receptors in the mammalian hosts (Kohler, 2001).

The macrocyclic lactones include the avermectins and the milbemycins. Ivermectin is perhaps the most well known of this class of drug, as it is used to treat onchocerciasis in humans. Ivermectin can bind to and open invertebrate-specific glutamate-gated chloride channels (Vassilatis *et al.*, 1997). The presence of a low-affinity binding site, likely to be a γ-aminobutyric acid (GABA) receptor has also been suggested (Blackhall *et al.*, 2003). The increased open time of the channels results in an irreversible current of chloride ions, leading to hyperpolarisation of the cell membrane and muscle paralysis. The paralysis also causes the pharyngeal pumping action of the nematode to cease, leading to the inhibition of feeding, and it is thought that starvation is the ultimate cause of death (Paiement *et al.*, 1999). The selective effect of these drugs is again explained by their action on the distinct glutamate-gated chloride channels that are present in the invertebrate nematode but absent in the vertebrate hosts (Jagannathan *et al.*, 1999).

In the 1990s the cyclooctadepsipeptides, a new class of anthelmintic drugs were introduced for the treatment of gastrointestinal parasitic nematodes (Harder & von Samson-Himmelstjerna, 2002). Of these, the drug emodepside is currently licensed for use in cats. Emodepside inhibits muscle function and pharyngeal pumping in *C. elegans* and is thought to function via a latrophillin-like receptor which regulates these activities in the cell (Harder *et al.*, 2003).

Another novel class of anthelmintics, the AADs (amino-acetonitrile derivates), has been recently characterised by Kaminsky and colleagues (Kaminsky *et al.*, 2008). Of the AADs, monepantel was the first compound to be developed for use in sheep (Kaminsky *et al.*, 2009). These compounds were tested against adult *H. contortus*, resulting in many phenotypic effects such as muscle paralysis, spasmodic contractions of the pharynx and ultimately death. These phenotypes were distinct from the effects of any single known anthelmintic. The target of the AADs in *H. contortus* has been described as the DEG-3 group of nicotinic acetylcholine receptors, a nematode-specific subfamily that is not found in mammals (Rufener *et al.*, 2009). Of this subfamily of receptors, the DES-2 and ACR-23 receptors are important in conferring susceptibility to the drug. It was

also shown that resistance to the AADs was not conferred by loss of the levamisole receptor (Kaminsky *et al.*, 2008). This illustrates that the AADs target a unique set of nicotinic acetylcholine receptors in the helminth that are not already targeted by levamisole.

1.1.5 Anthelmintic resistance in parasitic nematodes

Anthelmintic resistance is extremely serious in the parasitic nematodes of sheep and goats, where resistance to all three major classes of anthelmintics has been reported. Parasitic nematode populations tend to be large and genetically heterogeneous, and this diversity is an essential factor for the parasite to avoid susceptibility to drugs. In fact, resistance to anthelmintics is generally not very far behind the discovery of a novel anthelmintic compound, as illustrated in Figure 1.2.

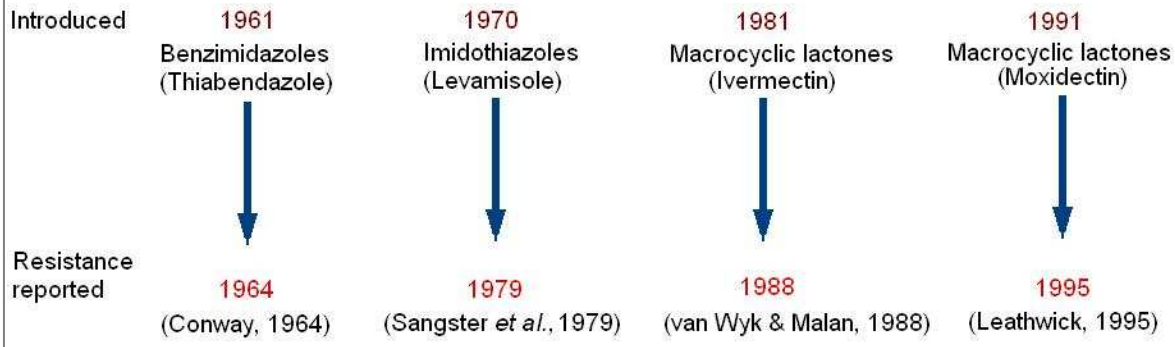


Figure 1.2. The development of resistance to anthelmintics in sheep.

Year for resistance reported indicates the publication of the first documented resistance. In many cases there are earlier published reports of suspected resistance.

Resistance in parasites to anthelmintics is defined as the genetically transmitted loss of sensitivity in worm populations that were previously sensitive to the same drug. In this process, the anthelmintic drug selectively removes individual susceptible worms from a genetically heterogeneous population leading to an increase in individuals carrying genes conferring drug resistance that are passed to the offspring generation (Kohler, 2001). There are many possible molecular mechanisms of drug resistance; (i) a change in a gene encoding a drug receptor that results in weaker drug binding, (ii) a change in gene expression that leads to an increased production of the drug target and (iii), a change resulting in enhanced detoxification that inactivates or removes the drug (Wolstenholme *et al.*, 2004). Drug metabolising enzymes such as the glutathione-thioredoxin

systems or the cytochrome P450 enzymes can detoxify the effects of the drug. Changes in these enzymes can potentially give rise to multidrug resistance (Urquhart *et al.*, 2007). Most of the known examples of anthelmintic resistance fall into one of the categories listed above.

The molecular mechanisms of resistance to benzimidazole have been investigated extensively, especially in *H. contortus*. Several different polymorphisms of the beta tubulin genes have been linked with benzimidazole resistance. Of these, a phenylalanine to tyrosine substitution at amino acid 200 of the protein encoded by the beta tubulin isotype-1 gene is considered to be an important mutation in conferring resistance in *H. contortus* (Kwa *et al.*, 1994). The resistance to benzimidazole conferred by this mutation was confirmed in an experiment by Kwa and colleagues using *C. elegans* (Kwa *et al.*, 1995). When expressed in a *C. elegans* beta tubulin *ben-1* mutant strain, the wildtype *H. contortus* beta tubulin isotype-1 gene changed a *C. elegans* population from benzimidazole resistant to benzimidazole susceptible. In contrast, expression of the *H. contortus* beta tubulin gene constructs carrying the phenyalanine to tyrosine codon substitution did not alter the resistant phenotype. This substitution has also been found in other benzimidazole resistant parasites, including *Teladorsagia circumcincta*, *Trichostrongylus colubriformis* and *Cooperia oncophora* (Grant & Mascord, 1996; Silvestre & Humbert, 2002; Winterrowd *et al.*, 2003), but not in benzimidazole resistant hookworms (Albonico *et al.*, 2004), indicating that there are exceptions to the rule. Another phenyalanine to tyrosine substitution, at position 167 of beta tubulin isotype-1 has also been detected in benzimidazole resistant populations of *H. contortus* (Silvestre & Cabaret, 2002). These same two polymorphisms also appear in the beta tubulin isotype-2 gene, and can also confer resistance to benzimidazole (Prichard, 2001). Quantitative PCR assays have now been developed to detect these mutations in parasite populations, and this provides a molecular diagnostic tool for the detection of benzimidazole resistance (von Samson-Himmelstjerna, 2006; von Samson-Himmelstjerna *et al.*, 2009). Thus far it appears that benzimidazole resistance is conferred by point mutations in the beta tubulin drug target, but it is possible that additional mechanisms could also contribute towards resistance.

The mechanism of resistance to macrocyclic lactones, such as ivermectin, is less clear-cut than benzimidazole resistance. Different selection pressures can produce different mechanisms of resistance; gradual selection using serial experimental infections with drug concentrations below therapeutic levels are more likely to select for a multi-gene basis for resistance. Alternatively, rapid selection using drug concentrations at therapeutic levels are more likely to select for a single gene effect (Gilleard, 2006). Polymorphisms in the glutamate-gated chloride channel targets have been implicated in resistance (Blackhall *et al.*, 1998; Njue *et al.*, 2004), but other genes and mechanisms for resistance have also been implied. For example P-glycoprotein (Pgp), a membrane transporter involved in removing toxic compounds from the cell, has been increasingly linked to ivermectin resistance (Kerboeuf *et al.*, 2003). Compellingly, Pgp inhibitors have been shown to increase the bioavailability of ivermectin *in vivo* in animals (Lespine *et al.*, 2008). In addition, the presence of Pgp inhibitors was shown to increase the susceptibility to ivermectin of both ivermectin sensitive and resistant isolates of *H. contortus* and *T. circumcincta* (Bartley *et al.*, 2009). This result is important, because it demonstrates that it is possible to restore ivermectin sensitivity in resistant nematodes by inhibiting the action of Pgp. Polymorphisms in cellular enzymes involved in detoxification processes, such as the cytochrome P450 enzymes, have also been linked with anthelmintic resistance, as anthelmintics have been reported to be metabolised by these enzymes (Cvilink *et al.*, 2009).

Resistance to levamisole and other tetrahydropyrimidines/imidazothiazoles is thought to be associated with a change in the target site of the drug. Studies in the intestinal roundworm *Oesophagostomum dentatum* have shown that resistant worms have a lower number of active receptors compared with levamisole sensitive worms, suggesting that resistant worms have levamisole receptors that are less sensitive to levamisole (Robertson *et al.*, 1999). This study also showed that the open time and the probability of open state of the receptors was also lower in levamisole resistant worms, compared with the susceptible populations. In *C. elegans,* resistance to levamisole can arise from an absence of functional levamisole receptors. There are two types of nicotinic acetylcholine receptors in the neuromuscular junction in *C. elegans,* one of which is the levamisole receptor; the other is preferentially activated by

nicotine and thus the levamisole resistant worms could survive without functional levamisole receptors (Richmond & Jorgensen, 1999). Differences in levamisole susceptibility in heterogeneous populations of nematodes can thus be explained by a shift in the relative proportion of nicotinic acetylcholine receptor subtype in favour of receptors that are less sensitive to the drug (Kohler, 2001).

The problem of anthelmintic resistance is complicated by reports of parasitic nematodes that display a multidrug resistant phenotype (Kaplan, 2004). As discussed above, this can arise from a change resulting in enhanced detoxification that inactivates or removes the drug from the nematode. The ability to follow the emergence and spread of resistant alleles within a nematode population would be extremely useful for monitoring the development of resistance. For example, it is possible to use microsatellite markers to differentiate between various isolates of *H. contortus* commonly used in different laboratories (Redman *et al.*, 2008b). This allows a method to monitor the relationship between laboratory isolates and field populations. A recent study in analysing the selection pressures of anthelmintic treatment upon a worm population showed that some species can be more prone to developing resistance than others due to changes in allele frequency (Churcher & Basanez, 2008). For example, resistant allele frequency can change depending on the population size of the parasite, and also the stage in the life cycle at which the anthelmintic is introduced. Efforts need to be undertaken to develop non-chemical methods of nematode control, in addition to monitoring the development of resistance so that the current anthelmintics can be used in a sustainable manner in the future.

1.1.6 Vaccine development for parasitic nematodes

The development of anthelmintic resistance over the past few decades has led to the search for immunological means of controlling parasitic nematode infections; however there is currently only one commercially available vaccine for helminth infections. The vaccine Dictol™, developed using live irradiated L3 larvae of the lungworm *Dictyocaulus viviparous* in cattle has been commercially available for the past 40 years (Jarrett *et al.*, 1957), and a similar vaccine for the sheep lungworm *D. filaria* has also been developed (Sharma *et al.*, 1988).

These partially inactivated larvae migrate to the lungs where they are eliminated before further development, and in this process an immune response to further challenge is developed. This is currently the only vaccine available for use against a parasitic nematode. Early attempts at developing a vaccine for *H. contortus* infections in a similar manner using irradiated larvae were unsuccessful; a high level of protection was obtained for older sheep but younger lambs, which are most vulnerable to nematode infection, were unprotected (Smith & Angus, 1980). Currently, the use of live attenuated larvae in a commercial vaccine requires animal hosts to be chronically infected and therefore is not an option that is ethically or economically favourable. Hence, attention has shifted towards the discovery of antigens that are of immunological importance upon which a molecular vaccine could be designed.

Antigens from parasitic nematodes can be broadly grouped into two categories; (1) natural antigens – capable of inducing a protective response in the host during the course of an infection, or (2) hidden antigens – incapable of inducing a protective response during an infection since they are hidden from the host immune system. Natural antigens, such as those excreted/secreted from parasites, are responsible for the limited natural immunity that is observed in older animals against GI nematodes. However the degree of natural immunity can vary greatly within sheep populations, and has been shown to have a genetic basis (Windon, 1996). Work is currently underway to map the quantitative trait loci responsible for resistance to *H. contortus* in sheep (Marshall *et al.*, 2009) which may eventually make it possible to selectively breed sheep which are resistant to *H. contortus* infections. Unfortunately natural immunity to GI nematodes is poor in young lambs which are most susceptible to infection (Manton *et al.*, 1962) and therefore antigens that are capable of inducing protection in young lambs are necessary.

A number of antigens expressed on the intestinal surface of *H. contortus* have been identified as protective when used to immunise sheep against challenge infection with the parasite (Knox *et al.*, 2003). Since *H. contortus* is a blood-feeder, these intestinal antigens are exposed to the host antibodies present in the ingested blood. Because some of these antigens are not presented to the host immune system during infection and cannot elicit a natural immune response, they are designated as hidden antigens. Hidden antigens have the

additional advantage of not exerting selection pressure which could lead to the evolution of worms capable of evading the immune response by mechanisms such as antigenic variation (Newton & Meeusen, 2003). The first hidden antigen described for *H. contortus* was an extracellular microvillar surface-associated polymer named contortin (Munn, 1977). Lambs vaccinated using a contortin enriched preparation showed a 78% reduction in worm burden after challenge infection, and it was the first demonstration that an antigen from the surface of the adult parasite intestine can induce a protective response (Munn *et al.*, 1987). Subsequent work has shown that contortin is composed of two prolyl-carboxypeptidases *Hc*-PCP-1 and *Hc*-PCP-2 (Geldhof & Knox, 2008). *Hc-pcp-1* and *Hc-pcp-2* transcripts are expressed from the blood feeding L4 stage onwards, and the two proteins encoded by these genes were able to inhibit blood coagulation in a dose-dependent manner. Therefore these proteins are thought to be intestinal anticoagulants used by *H. contortus* to interfere with blood coagulation.

Another important hidden antigen isolated from the *H. contortus* intestine is the microsomal aminopeptidase H11 (Smith *et al.*, 1997). Numerous studies have been carried out in sheep using H11 protein enriched from intestine extracts, most of them showing a reduction of greater than 90% in worm burden and faecal egg counts after challenge infection (Andrews *et al.*, 1995; Munn *et al.*, 1993). Importantly, H11 was successful at protecting lambs from challenge infection, and this protection persisted for up to 23 weeks and did not interfere with the development of natural immunity (Andrews *et al.*, 1997). H11 vaccinations were also successful at protecting sheep from benzimidazole resistant worms (Smith & Smith, 1993). Microsomal aminopeptidases are thought to have a function in the digestive process, and antibodies from animals vaccinated with H11 appear to inhibit the aminopeptidase activity of H11 *in vitro* by up to 80% (Smith *et al.*, 1997). The amino acid sequence of H11 predicts a single transmembrane region; it is possible that H11 could have a role as a transmembrane protein which could be blocked by the binding of antibodies to H11. Subsequently there is some controversy regarding the precise mechanism of protection provided by H11; the actual inhibition of the aminopeptidase activity, or the blocking of a possible transmembrane function by the binding of

antibodies to H11 (or even a combination of both) could be responsible for the detrimental effect on the worms.

High levels of protection can also be obtained by another hidden antigen H-gal-GP (*Haemonchus* galactose-containing glycoprotein). A study in sheep showed a reduction of worm burdens by 72% and faecal egg counts by 93% after vaccination with H-gal-GP fraction (Smith *et al.*, 1994). The H-gal-GP complex is thought be made of four major protein components and aspartyl, metallo and cysteine protease activities have been attributed to the complex (Longbottom *et al.*, 1997; Newlands *et al.*, 2006; Redmond *et al.*, 1997; Smith *et al.*, 1999). It is thought that H-gal-GP is involved in the digestive process and functions in a similar manner to H11 (Knox *et al.*, 2003). However most of these studies were conducted in worm-free sheep kept in pens, given single artificial challenge doses of larvae. A recent study examined the effectiveness of intestine extracts enriched for H11 and H-gal-GP at controlling *H. contortus* infections in lambs grazed in paddocks contaminated with *H. contortus* larvae, conditions that more closely resemble those encountered by commercial sheep farmers (LeJambre *et al.*, 2008). The results were encouraging, with significantly reduced numbers of death, degrees of anaemia and salvage anthelmintic treatments. Several other promising vaccine candidates have been described for *H. contortus*. Three intestinal surface proteins from *H. contortus* collectively termed gut antigen 1 (GA1) were used to immunise goats prior to challenge infection and have shown reductions of 60% and 50% in worm burden and faecal egg counts, respectively (Jasmer *et al.*, 1993). Cysteine proteases are also of great interest as vaccine candidates and there are several studies into the protection provided by vaccination with intestine extracts enriched for these proteins prior to challenge infection (Knox *et al.*, 2005; Ruiz *et al.*, 2004).

Unfortunately despite the numerous successes reported with the use of native antigens described above, efforts to develop effective recombinant versions of these antigens are still ongoing. An effective recombinant form of the promising native antigen must be developed in order to make the large-scale production of the antigen possible. This is necessary for the vaccine candidate to be considered for commercial development. Vaccination of sheep with recombinant H11, expressed by baculovirus in insect cells gave an extremely low level of protection (30% reduction in worm burden) when compared with the native

antigen (greater than 90%) (Reszka *et al.*, 2007). Recombinant versions of H-gal-GP have proven equally ineffective at protecting sheep from challenge infection (Newton & Meeusen, 2003). Denaturing H11 and H-gap-GP sequentially results in the progressive loss of their ability to protect sheep from challenge infection (Munn *et al.*, 1997; Smith & Smith, 1996). These results indicate that a specific conformational epitope or post-translational modification such as glycosylation could be required for the recombinant versions of these antigens to remain protective. Indeed H11 has three predicted sites for glycosylation (Smith *et al.*, 1997) and detailed glycan analysis has identified a unique core fucosylation (Haslam *et al.*, 1996). Bacterial and insect cell systems used to produce these recombinant proteins could be ineffective at replicating the exact nematode-specific conformational and post-transcriptional modifications present in the native parasite antigens, and thus an alternative system for producing these antigens would be extremely useful. The free-living nematode *C. elegans* is closely related to *H. contortus* and both nematodes share a similar pattern in carbohydrate modifications (Haslam *et al.*, 2002; Redmond *et al.*, 2004). *C. elegans* can be cultivated in large-scale cultures and this could provide a method for the large-scale production of nematode antigens. Parasite proteins can thus be expressed in a similar form to the native proteins by using *C. elegans* as an alternative expression system. This was demonstrated by the expression of active and glycosylated *H. contortus* cathepsin-L cysteine protease in *C. elegans* (Murray *et al.*, 2007).

The various methods for controlling parasitic nematode infections discussed above also highlight the challenges associated with each method. A deeper understanding of the fundamental biology of the organism can improve and strengthen the drug and vaccine development process in parasitic nematodes. Newer information such as genome, transcriptome and proteome data can thus accelerate the progress towards developing more efficient and sustainable control programmes.

1.1.7 Parasitic nematode genomes

In the last few years there has been a significant increase in the amount of genome and expressed sequence tag (EST) data for parasitic nematodes. The

availability of genome sequence data for important parasites will be useful for drug and vaccine development, and also for a broader biological understanding of the parasite. Genome information provides the ability to identify which genes, and therefore which biological pathways are present in the organism. Comparative analysis with *C. elegans* and other model organism genomes can also allow the discovery of parasite specific and nematode specific genes, which may be potential targets for vaccine or drug development.

The filarial nematode *Brugia malayi* was the first parasitic nematode to have its genome sequenced (Ghedin *et al.*, 2007). To date, 11,500 protein coding genes have been identified in the *B. malayi* genome data. This is significantly less than the free living *C. elegans* and the closely related *C. briggsae* genomes, both of which have approximately 20,000 protein coding genes (WormBase data release WS205), but a number of gaps exist in the *B. malayi* genome data and additional genes may yet be identified. It appears that this difference in gene number can be attributed to the extent to which gene families in *B. malayi* and *C. elegans* have undergone lineage specific expansion; for example, more than 8% of the 5780 *B. malayi - C. elegans* ortholog clusters were expanded in *C. elegans*. Comparing the proteomes of the nematodes *B. malayi*, *C. elegans* and *C. briggsae* with the fruit fly *Drosophila melanogaster* has also made it possible to identify genes present in the nematodes but absent in the fly; it is thought that these lineage restricted proteins define a molecular 'bodyplan' of the Nematoda (Ghedin *et al.*, 2007). The genome sequences from two plant parasitic nematodes *Meloidogyne incognita* and *M. hapla* show a gene number of 19,212 and 14,420, respectively (Abad *et al.*, 2008; Opperman *et al.*, 2008). This suggests that some parasitic nematode genomes may contain fewer genes than their free living counterparts. Interestingly *Pristionchus pacificus*, a free-living nematode often used as a satellite model organism to *C. elegans,* has 23,500 protein coding genes in its genome (Dieterich *et al.*, 2008). *P. pacificus* leads a necromenic lifestyle, living on the outside of a beetle as arrested larvae until the beetle's death and then feeding on the bacteria, fungi and nematodes that grow on the beetle's carcass and subsequently developing onto adulthood (Hong & Sommer, 2006). Because of this necromenic lifestyle, it has been suggested that *P. pacificus* has an intermediate position between the bacteria-consuming

C. elegans and true parasitic nematodes (Srinivasan & Sternberg, 2008), and this necromenic lifestyle may be a pre-adaptation to parasitism.

Another interesting insight from the genomes of the parasitic nematodes involves the detoxification processes. The *M. incognita* genome has fewer genes coding for enzymes involved in xenobiotic metabolism and protection against peroxidative damage compared to *C. elegans* (Abad *et al.*, 2008). In contrast, proteins involved in detoxification in the *P. pacificus* genome have expanded compared to *C. elegans* (Dieterich *et al.*, 2008). This suggests that *P. pacificus* has evolved towards an ability to ward off xenobiotics from the environment. In contrast, by living inside plant root tissues, *M. incognita* is protected from a variety of environmental stresses and can afford to reduce the enzymes required for detoxification. The genomes of the plant parasitic nematodes also highlight the role of horizontal gene transfer in nematode evolution. Both *M. incognita* and *M. hapla* genomes have a high number of plant-parasitism genes, coding for enzymes that are involved in plant cell-wall degradation (Abad *et al.*, 2008; Opperman *et al.*, 2008). Most of these genes were not found in any other metazoan, and the most similar proteins outside of the plant parasitic nematodes were homologues from bacteria and plant-pathogenic fungi. The genomes of *B. malayi, M. incognita* and *M. hapla* can also be used to identify genes unique to the respective organism which can then be investigated further as potential targets for control strategies. These examples serve to illustrate the invaluable insights into parasitic nematode biology that can be obtained by genome sequencing projects.

Currently there are several genome projects that are in progress for parasitic nematode and trematode species. The *Schistosoma mansoni* genome has been published (Berriman *et al.*, 2009) while *H. contortus, Strongyloides ratti, Nippostrongylus brasiliensis, Onchocerca volvulus, Teladorsagia circumcincta, Trichuris muris, Globodera pallida* and *Ascaris suum* genome sequencing projects are in progress (http://www.sanger.ac.uk/Projects/Helminths). The *H. contortus* genome sequencing project was initiated in 2004 by the Sanger Institute, UK (http://www.sanger.ac.uk/Projects/H_contortus/) with the overall goal of producing reference quality genome sequence. The project used the MHco3 (ISE) isolate of *H. contortus* as this was the most inbred strain available when the project first commenced. Two main approaches to the genome

sequencing project were a BAC clone-by-clone sequencing approach and more recently, the Sanger capillary sequencing method. As of September 2009, there is approximately 800 Mb of genome sequence available for *H. contortus* (Wellcome Trust Sanger Institute). This has been assembled into 129088 contigs which are further assembled into 70309 supercontigs. However the assembly and annotation of the *H. contortus* genome is complicated by the large number of sequence polymorphisms observed (Gary Saunders, PhD thesis 2009, University of Glasgow). This high degree of genetic variation arises from the large population sizes of the worms (Anderson *et al.*, 1998), but polyandry is another mechanism for creating variation. Polyandry was demonstrated in *H. contortus* by a recent study analysing the inheritance of autosomal genetic markers which showed that a minimum of at least four different male worms have contributed to broods of a single female (Redman *et al.*, 2008a). The natural genetic variation between *H. contortus* sequence reads from the same genetic locus is suggested to be the main reason for the poor assembly of sequence information. For example, if the two alleles of each locus present in a single *H. contortus* worm are suitably dissimilar that they cannot assemble together to form a contiguous sequence, it can be hypothesised that they would assemble independently (Gary Saunders, personal communication). Thus it appears that although there is a large amount of sequence data for *H. contortus*, the assembly of this sequence data is more complicated than was initially anticipated. As an aside, the *B. malayi* and *M. hapla* genome sequencing projects used highly inbred strains for sequencing in order to enhance assembly fidelity and to avoid complications arising due to polymorphisms. Recent studies have also indicated that the MHco3 (ISE) isolate of *H. contortus* is not as inbred as was first thought (Redman *et al.*, 2008b). In the future it should be possible to use a more inbred strain of *H. contortus* using next generation sequencing technologies in order to complete the genome sequencing project.

The parasitic nematode EST sequencing projects from *Ascaris suum, H. contortus, Necator americanus, Teladorsagia circumcincta* and *Trichuris muris* is also in progress (Wellcome Trust Sanger Institute). In addition, the NEMBASE database (http://www.nematodes.org/nematodeESTs/nembase.html) provides access to EST sequences from 37 different parasitic nematodes, giving a snapshot of gene expression in the different parasites. In addition, it is possible

to associate changes in gene expression level with the biology of the organism by creating libraries based on sex, tissue and different developmental stages.

While *C. elegans* continues to be an invaluable model system for understanding nematode biology, insights obtained from the genome projects of the parasitic nematodes described above highlight the differences between the species. It is essential to obtain genome data from representative species from across the diverse nematode phylum in order to identify genes which maybe conserved or unique to different species and parasitic lifestyles. These genes can then be studied further as possible targets for the next generation of nematode control strategies.

1.1.8 Phylogenetic relationship between nematodes

The phylum Nematoda is the largest phylum of the Animal kingdom, with up to one million species estimated (Sommer, 2000). The vast majority are free-living but many have adopted a parasitic lifestyle. Most plants and animals including humans have at least one parasitic nematode species uniquely adapted to exploit the ecological niche that host species represents. Given this vast diversity, the classification of nematodes into subsequent taxonomic ranks has been difficult in the past; much of the data has been from morphological characteristics observed by light microscopy and ecological habitats (Anderson, 1992). However since the advent of molecular phylogenetics and bioinformatics, the tools have been improved, dramatically changing the landscape of nematode classification. The now widely accepted phylogenetic classification of nematodes, as illustrated in Figure 1.3, is based on the small subunit (SSU) ribosomal DNA sequences from 53 different nematode species (Blaxter *et al.*, 1998). The SSU analyses confirms that many important features have arisen repeatedly during evolution; animal parasitism arose independently at least four times and plant parasitism three times. Inconsistencies between the SSU analyses and the earlier morphological analysis due to the process of convergent evolution have also been highlighted. For example, the *Steinernema* and *Heterorhabditis* are both entomopathogenic nematodes that infect insects and then release toxic bacterial symbionts which kill the insect host, but they do not share an exclusive common ancestry.

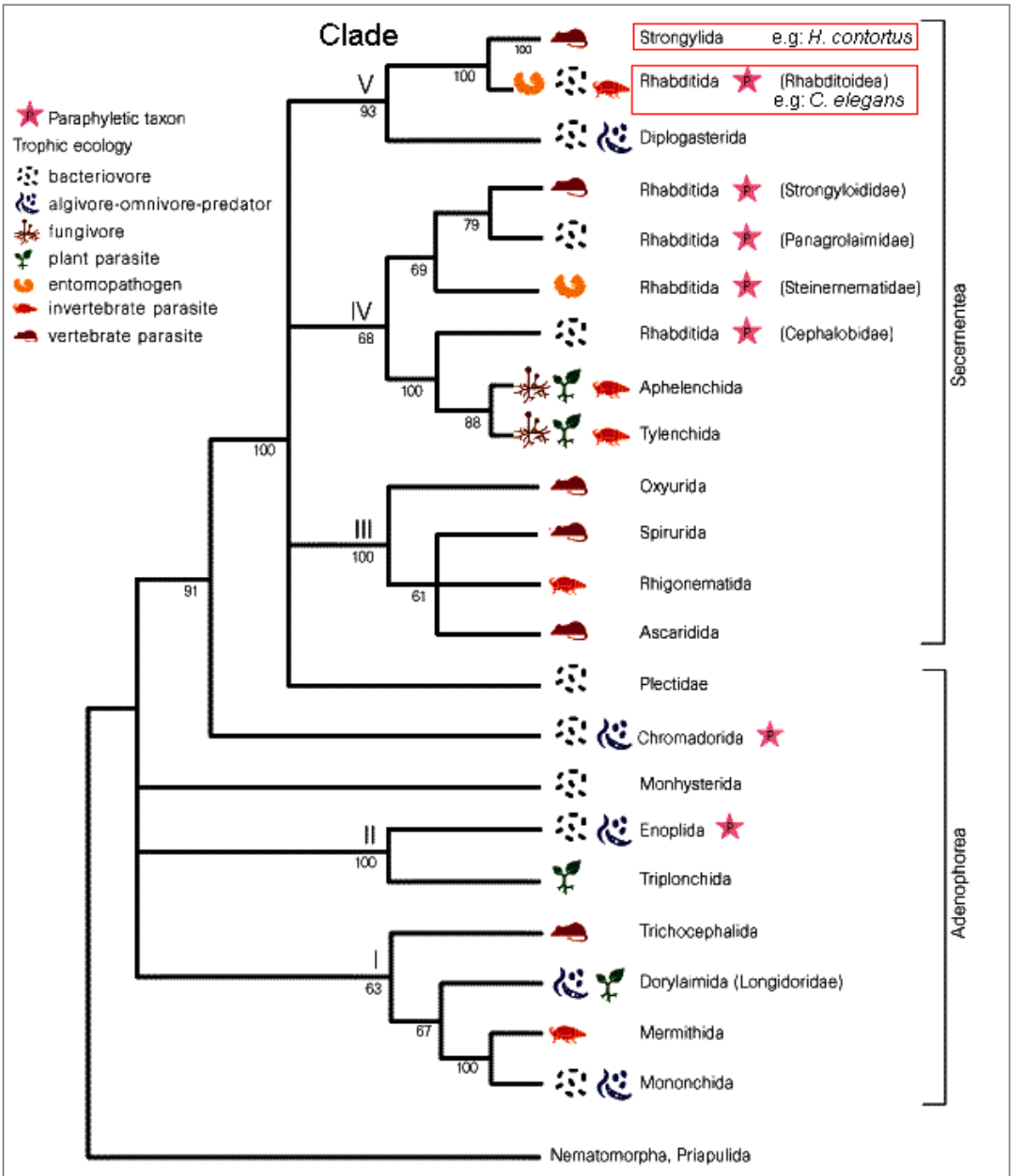


Figure 1.3. Phylogenetic relationship between nematodes

Dendrogram summarising the phylogenetic relationship between nematodes with the five major clades in the Nematoda illustrated. The close relationship shared between *C. elegans* and *H. contortus,* both Clade V nematodes, is highlighted (adapted from Blaxter *et al.*, 1998).

Although sequence data is becoming available for representative species of different nematode Clades, identifying the function of parasitic nematode genes remains difficult. Currently, there is a lack of reverse genetic techniques for most parasitic nematodes including *H. contortus,* making it difficult to elucidate gene function directly in the parasite. Genes involved in essential functions

such as development, reproduction and moulting are likely to be conserved across nematode species to some degree. Both *H. contortus* and *C. elegans* are Clade V nematodes and share a close phylogenetic relationship, as illustrated in Figure 1.3. The wealth of data already available on *C. elegans* gene function and expression makes *C. elegans* an extremely useful model system to investigate gene function in *H. contortus* and other related Clade V parasitic nematodes.

1.1.9 Overview of *C. elegans*

C. elegans is a free living soil nematode about 1 mm in length. Research into the molecular biology of *C. elegans* began in 1965 by Sydney Brenner, and was considered ideal for research purposes due to several factors; the rapid 3-day life cycle at 25°C, small size and ease of laboratory cultivation. *C. elegans* is typically cultured in agar plates seeded with a lawn of *Escherichia coli* strain OP50. In addition, it is a multicellular eukaryotic organism which is simple enough to be studied in detail; the developmental fate of every single somatic cell has now been mapped. *C. elegans* has five pairs of autosomes and one sex chromosome, therefore sex is determined on a XX system. Eggs are laid by hermaphrodite (XX) adults and pass through four larval stages (L1-L4) after hatching. The male (XO) population is rare (0.1% of total population). The hermaphrodites produce both oocytes and sperm and can thus reproduce by self fertilisation. The resulting populations are clonal and thus it is possible to culture and maintain strains in the laboratory. Strains can also be frozen and remain viable when subsequently thawed, allowing for long-term storage. Genetic crosses can be performed by mating hermaphrodites with males. If crowded or starved, *C. elegans* enters a third larval stage known as the 'dauer' stage and these are stress-resistant and do not age. When food is available the dauer stage larvae can moult to a normal L4 and then develop into an adult. *C. elegans* senses its environment using amphid neurons which are located in the anterior head region. These neurons are the primary chemoreceptive, olfactory and thermoreceptive organs and are either directly or indirectly exposed to the environment through openings in the amphid sheath cell. The amphid sheath cell is large and contains distinctive secretory vesicles that are released into the amphid channel, a narrow space formed by the amphid sheath cell and the accessory cells of the amphid neurons.

Much of the information obtained from *C. elegans* research is available online at the electronic bioinformatics database Wormbase (http://wormbase.org/). It is used by the *C. elegans* and wider research community both as an information source and as a mode to publish and distribute findings. The fully annotated genome sequence of *C. elegans* is available on Wormbase, as is the genome sequences of related nematodes *C. briggsae* and *C. remanei*. For each characterised gene, expression profiles based on stage, tissue and cell-type are available, as well as genetic mutant and RNA interference (RNAi) phenotypes. *C. elegans* has provided invaluable contributions in many areas of biological research, but perhaps the most significant contribution in recent years towards understanding gene function, and one of the main focuses of this project, is the process of RNA interference.

1.2 RNA interference

1.2.1 Overview of RNA interference

RNA interference, or RNAi, is a mechanism for RNA guided silencing of gene expression that is common in eukaryotic cells. It was first observed by plant scientists attempting to alter flower colour in petunias by the introduction of additional copies of a gene involved in flower pigmentation, chalcone synthase. Instead of darker flowers resulting from the over expressed gene, less pigmented or fully or partially white flowers were seen (Napoli *et al.*, 1990). This phenomenon was subsequently characterised in *C. elegans* where a potent gene silencing effect after injecting double stranded RNA was reported and was formally named ‘RNA interference’ (Fire *et al.*, 1998).

Gene silencing is induced by double stranded RNA (dsRNA), and initiated when dsRNA is processed into small interfering RNAs (siRNAs) 21-26 nucleotides in length (Matzke & Birchler, 2005). This processing is carried out by the RNase III enzyme Dicer. The degradation of the specific mRNA is carried out by a protein complex containing ribonuclease, known as the RNA-induced silencing complex (RISC). Base pairing interactions between the mRNAs and the siRNAs guide RISC to its mRNA targets, which it then destroys. An overview of this generalised RNAi pathway is illustrated in Figure 1.4.

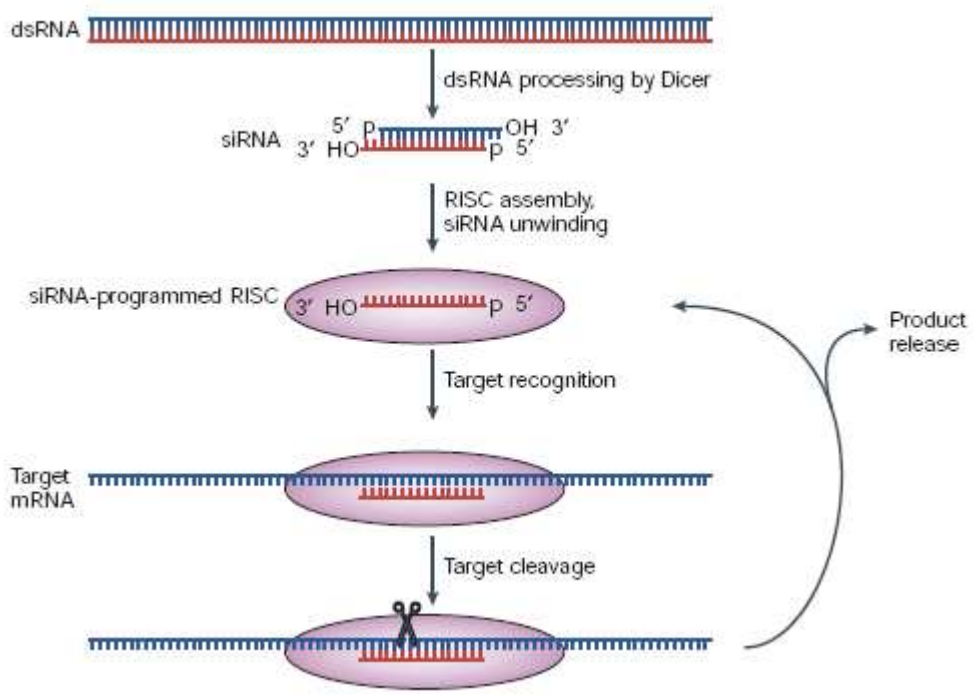


Figure 1.4. Generalised RNAi pathway

The RNase III enzyme Dicer cleaves dsRNA into siRNA molecules which then assemble into the RNA-induced silencing complex (RISC). Specific mRNA recognition occurs by siRNA-mRNA base pairing and the mRNA strand is subsequently cleaved by endoribonuclease activity within the RISC (Sontheimer, 2005).

RNAi is thought to function as an adaptive antiviral immune mechanism in eukaryotic organisms. *C. elegans* worms that are defective in RNAi are more susceptible to viral infection, and in contrast, viral infection is attenuated in worms with an enhanced RNAi response (Wilkins *et al.*, 2005). Generally, eukaryotes can use dsRNA to recognise self from non-self; viruses typically produce long stretches of dsRNA during replication, while eukaryotes do not. The RNAi pathway is therefore thought to have evolved as a protective mechanism for recognising and destroying foreign dsRNA molecules (Obbard *et al.*, 2009). RNAi has been identified as an important antiviral defence in animals and plants (reviewed by Li & Ding, 2005; Ruiz-Ferrer & Voinnet, 2009 respectively), and more recently, in fungi (Hammond *et al.*, 2008). Considering how widespread viral suppression by RNAi is, it is unsurprising that many viruses can inhibit the RNAi pathway in turn by expressing viral suppressers of RNAi (VSRs) (Li & Ding, 2005). There is also evidence that some viruses might have evolved to subvert the host RNAi pathway for their own benefit (Ding & Voinnet, 2007), resulting in an arms race where the host RNAi pathway continually evolves new ways to

escape suppression by the VSRs, which then leads to counter adaptations by the virus that restore suppression (reviewed by Obbard *et al.*, 2009). For example, three key proteins in the RNAi pathway of *Drosophila* (Dcr-2, R2D2 and Ago-1) are among the top 3% of the most rapidly evolving in the entire genome (Obbard *et al.*, 2006).

Over the last decade, a large amount of research has gone towards understanding the molecular mechanisms of RNAi in detail. Much insight has been gained by dissecting the classical RNAi pathway in *C. elegans*.

1.2.2 Classical RNAi pathway in *Caenorhabditis elegans*

RNAi in *C. elegans* has several unique features. It is heritable, in that gene silencing effects can persist for several generations after induction (Grishok, 2005). The heritability of RNAi in *C. elegans* is thought to be associated with chromatin remodelling, suggesting that the inheritance of gene silencing occurs at the transcriptional level (Vastenhouw *et al.*, 2006). RNAi in *C. elegans* is also systemic; locally initiated gene silencing can spread to distant tissues of the organism. Subsequently, key genes involved in the process of systemic RNAi have been identified as *sid-1* and *sid-2* (**s**ystemic RNA **i**nterference **d**efective, reviewed by van Roessel & Brand, 2004). Systemic RNAi has been documented in several other organisms such as planaria, honey bees and plants (Newmark *et al.*, 2003; Patel *et al.*, 2007; Voinnet & Baulcombe, 1997), but is absent in *Drosophila* and mammals (Roignant *et al.*, 2003). It appears that systemic RNAi is broadly conserved within the *Caenorhabditis* species, but the ability to take up dsRNA from the environment is rare and limited to *C. elegans* and another distantly related unnamed species of *Caenorhabditis* C. sp. SB341 (Winston *et al.*, 2007).

An impermeable cuticle covers nearly the entire surface of *C. elegans*, forming a highly impervious barrier between the worm and its environment. *C. elegans* is thought to take up environmentally available dsRNA through the intestinal lumen while feeding. The dsRNA is then taken up into the intestinal epithelial cells by the single-pass transmembrane protein **SID-2;** SID-2 is expressed in intestinal cell apical membranes and it is thought that it enables the import of ingested dsRNA from the intestinal lumen (Winston *et al.*, 2007). SID-2 activity may also require

SID-1, a protein with 11 transmembrane domains that is thought to function as a dsRNA channel, passively transporting dsRNA between cells (Winston *et al.*, 2002). Systemic RNAi spreading through the worm also involves SID-1 and proteins from vesicle trafficking pathways and endocytosis (Jose *et al.*, 2009; Saleh *et al.*, 2006).

The dsRNA binding protein **RDE-4** binds to the internalised dsRNA to form a complex with the RNase III enzyme **Dicer** and the argonaute protein **RDE-1.** The dsRNA is thus processed into primary siRNAs, 21-25 nucleotides in length. RDE-1 binds to the processed siRNAs, bringing them to the next step in the RNAi pathway, forming the RISC loading complex. The **RISC complex** consists of a single stranded siRNA and the **argonaute proteins.** The argonaute proteins are key components of the RISC complex and contain two distinct RNA binding domains, the PAZ and PIWI domains. These domains interact with the 3' and 5' ends of the single stranded siRNA leaving the internal nucleotides available for base pairing with the target mRNA (reviewed in Song & Joshua-Tor, 2006). Once the active RISC complex is formed, the argonaute proteins mediate target mRNA sensing, subsequent recognition and complementary base pairing between the RISC complex and target mRNA. The base pairing places the target mRNA in proximity to the argonaute protein PIWI domain, and the RNase activity of this domain is responsible for the cleavage of the target mRNA (Yigit *et al.*, 2006). There are no less than 27 argonaute proteins in *C. elegans*; it is likely that these proteins provide a degree of functional redundancy in the RNAi pathways, most likely as components of the RISC (Grishok, 2005).

RNAi in some organisms, including *C. elegans,* is made more efficient by a siRNA amplification step in the pathway, with the involvement of RNA dependent RNA polymerases (RdRPs) **EGO-1** and **RRF-1**. EGO-1 is required for RNAi of germline genes (Smardon *et al.*, 2000) whereas RRF-1 is necessary for somatic gene targeting by RNAi (Sijen *et al.*, 2001). The RdRPs are required for the accumulation of siRNAs *in vivo*; it is thought that the primary siRNAs produced by Dicer act as primers for the RdRP, using the target mRNA as a template. This produces dsRNA which is then again processed by Dicer and thus the RNAi response is amplified. **RDE-3** functions in a complex with the RdRPs and is necessary for the amplification step in RNAi (Chen *et al.*, 2005a).

The RNAi pathways in *C. elegans* have been studied in great detail, which indicate that in addition to the classical RNAi pathway activated in response to environmentally introduced dsRNA, there are many separate but overlapping pathways responsible for other processes such as the silencing of repetitive elements (Slotkin & Martienssen, 2007), post transcriptional gene silencing in epigenetic regulation (Almeida & Allshire, 2005) and endogenous regulation by micro RNAs (miRNAs, Ambros, 2001). These RNAi pathways can result in the degradation of target mRNA, the recruitment of additional factors to alter gene expression or even long-term gene expression changes via heterochromatin formation and epigenetic modification (reviewed by Chapman & Carrington, 2007). There is evidence for substantial cross-regulation and interactions among these different silencing processes in *C. elegans* (Lee *et al.*, 2006). Many of these pathways share certain components; for example Dicer is required for both the exogenous RNAi pathway and the endogenous miRNA pathway which is involved in developmental regulation in *C. elegans* (Ketting *et al.*, 2001). Hence if the components of an RNAi pathway are limiting, then a load on one RNAi pathway can impact the efficiency of another RNAi pathway. For example, studies in mice indicate that the saturation of the endogenous RNAi pathway (miRNA pathway) results in mouse fatality (Grimm *et al.*, 2006). Negative regulators of the classical RNAi pathway therefore play an important role in ensuring that the efficiency of the various endogenous RNAi pathways remains unaffected. For example, the exonuclease **ERI-1** targets siRNAs for degradation, thus suppressing the classical RNAi pathway, but allowing other pathways to proceed (Kennedy *et al.*, 2004). Similarly, **RRF-3** acts as an inhibitor of RdRP-directed siRNA amplification, reducing the effectiveness of RNAi (Simmer *et al.*, 2002). RRF-3 is thought to compete with the siRNA amplification step involving RRF-1/EGO-1 RdRPs, negatively regulating the RNAi process. Details of the different proteins involved in the RNAi pathway in *C. elegans* are shown in Figure 1.5.

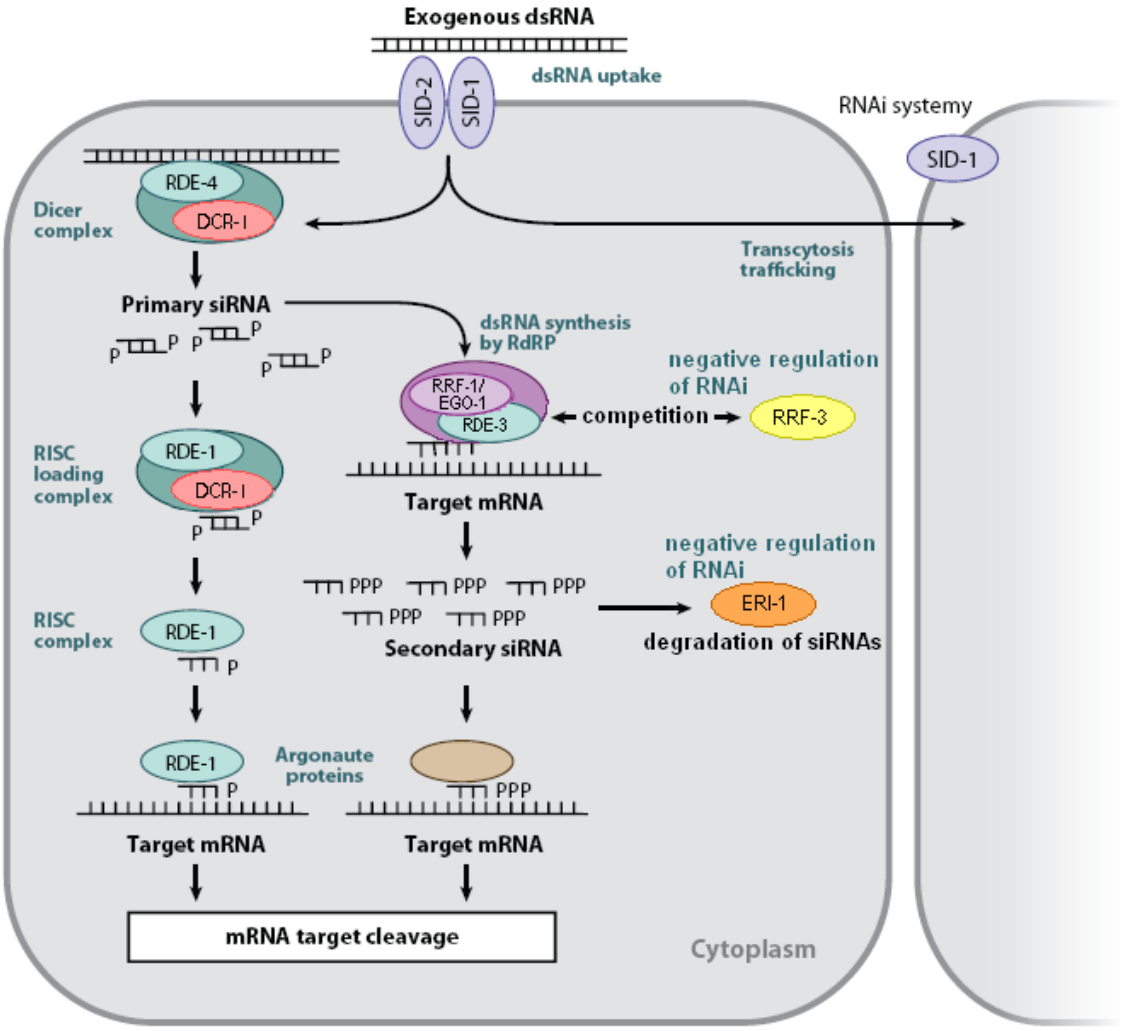


Figure 1.5. Schematic representation of the classical exogenous RNAi pathway in *C. elegans*

(adapted from Rosso et al., 2009).

1.3 RNAi in parasitic nematodes

The ease with which RNAi can be carried out in *C. elegans* has led to large-scale genome-wide screens to elucidate the function of different genes, discussed further in Chapter 3. This data can to some extent be extrapolated to related parasitic nematodes to identify essential genes which may be novel vaccine and drug targets. However as discussed in Section 1.1.7, many parasite genes have no identifiable homologues in *C. elegans* and could be involved in the parasitic lifestyle and likely be important targets for parasite control. RNAi, when applied directly on the parasite, would be an important tool for understanding the function of these unique parasite genes. Therefore, attempts to establish RNAi in several parasitic nematode species have been carried out to date, summarised in Table 1.1 and discussed further in Chapter 3.

Table 1.1. RNA interference studies in parasitic nematodes

Organism	Stage	Gene target(s)	Method	Phenotype and Evaluation	Reference
Nippostrongylus brasiliensis	Adult	Acetylcholinesterase A isoform	Soaked in 1 mg/ml dsRNA for 16hrs, washed and cultured *in vitro* for 6 days	↓ 80-90% of the secretion of acetylcholinesterase isoforms A, B and C, measured by enzyme activity assay. **Transcript levels not assessed.**	(Hussein *et al.*, 2002)
Brugia malayi	Adult	Beta-tubulin (*Bm-tub-1*), RNA polymerase II large subunit (*Bm-ama-1*), microfilarial sheath protein (*Bm-shp-1*).	Soaked in ~3.5 mg/ml dsRNA for 24 hrs	↓ *ama-1* and *tub-1* transcript after 14-17hrs, death after 24hrs. ↓ *shp-1* transcript after 10-14hrs, ↓ microfilariae release, 50% of the released microfilariae did not have fully elongated sheaths.	(Aboobaker & Blaxter, 2003)
Onchocerca volvulus	L3	Cathepsin L (*Ov-cpl*), cathepsin Z-like (*Ov-cpz*) cysteine protease	Soaked in 0.5 mg/ml dsRNA for 18 hrs	92% (*cpl* treated) and 86% (*cpz* treated) ↓ in moulting rate compared to untreated controls. **Non-specific effects** in larvae soaked in control dsRNAs. Immunolocalisation used to show reduced staining with antibodies. **Transcript levels not assessed.**	(Lustigman *et al.*, 2004)
	L3	Serine protease inhibitors (*Ov-spi-1* and *Ov-spi-2*)	Soaked in 0.5 mg/ml dsRNA for 20 hrs	↓ ecdysis (84.2), ↓ viability (39.4% death) 7 days post-treatment. 200-fold ↓ in *spi-1* and *spi-2* transcript levels, loss of SPI-1/SPI-2 native proteins.	(Ford *et al.*, 2005)
Ascaris suum	L3	Inorganic pyrophosphatase	Soaked in 2 mg/ml dsRNA for 24hrs, washed and cultured *in vitro* for 9 days	31% ↓ moulting, 56% ↓ enzyme activity. Complete ↓ of transcript, suppression of native protein. **Control larvae not cultured in presence of non-target dsRNA**	(Islam *et al.*, 2005)
Litomosoides sigmodontis	Adult	Actin (*Ls-act*)	Soaked in 0.35 mg/ml dsRNA for 24 hrs	↓ motility 48hrs post treatment, ↓ microfilariae release, ↓ transcript levels to less than 10%.	(Pfarr *et al.*, 2006)

Trichostrongylus colubriformis	L1	Ubiquitin (*Tc-ubq-1*), tropomyosin (*Tc-tmy-1*)	Feeding Soaked in 2 mg/ml dsRNA or 2 µM siRNA for 6 hrs at 20°C Electroporation with 1-2 mg/ml dsRNA or 2 µM siRNA	No effect with feeding or soaking with dsRNA. Soaking with siRNA showed 69% ↓ in number of L3 larvae 6 days later. Death or developmental delays seen in 90% of larvae after electroporation. **Transcript level not assessed. Protein levels not assessed.**	(Issa *et al.*, 2005)
Haemonchus contortus	L3, L4, Adult L1, L3	Beta tubulin [(*tub 8-9* (isotype 1) and *tub 12-16* (isotype 2)] Beta tubulin (*Hc-ben-1*), COPII component (*Hc-sec-23*), Ca2+ binding protein, heat shock protein hsp70 (*Hc-hsp-1*), vacuolar ATPase (*Hc-vha-10*), cathepsin-L (*Hc-cpl-1*), paramyosin *(Hc-unc-15*), superoxide dismutase (*Hc-sod-1*), intermediate filament (*Hc-mua-6*), Type IV collagen (*Hc-let-2*), GATA transcription factor (*Hc-elt-2*).	Soaked in 1 mg/ml dsRNA for 24 hrs Feeding Soaked in 1 mg/ml dsRNA and 50-0.5 µg/ml siRNA Electroporation with 1 mg/ml dsRNA	↓ transcript levels in all three life stages. Fewer L3s developed into L4s and showed decreased motility 6 days post treatment. No change in transcript level or phenotype with feeding. Complete ↓ of *Hc-ben-1* transcript. Significant ↓ of *Hc-sec-23* transcript. No decrease in transcript levels seen for any of the other 9 targets. No phenotypes. ***Hc-sec-23* knockdown not always reproducible.** ↓ *Hc-ben-1* transcript, but ↑ larval death. No decrease in transcript levels seen for any of the other 10 targets. No phenotypes.	(Kotze & Bagnall, 2006) (Geldhof *et al.*, 2006)
Ostertagia ostertagi	L3	Tropomyosin, beta-tubulin, ATP-synthetase, superoxide dismutase, polyprotein allergen, ubiquitin, transthyretin-like protein, 17 kDa ES protein	Soaked in 1 mg/ml dsRNA for 24, 48 or 72 hrs at 37°C Electroporation	↓ beta tubulin, tropomyosin, ATP-synthetase, superoxide dismutase, polyprotein allergen. No decrease in transcript levels for any of the other 3 targets. ↓ **not always reproducible.** ↓ beta tubulin and tropomyosin, but **not reproducible**.	(Visser *et al.*, 2006)

Heligmosomoides polygyrus	L1, Adult	Tropomyosin (*Hp-tm-1*)	Feeding Soaked in 0.05 – 2 mg/ml dsRNA for 18, 24 hrs and 6 days at 37°C. Electroporation	No change in transcript level, No phenotype. No change in transcript level. Higher proportion of worms showed symptoms of ageing compared to controls after 6 days soaking. No change in transcript level. 70-90% larval death.	(Lendner *et al.*, 2008)
Heterorhabditis bacteriophora	L1	T-complex chaperonin (*Hba-cct-2*), Hsp-90 (*Hba-daf-21*), BTF3 transcription factor (*Hba-icd-1*), Ribosome biogenesis (*Hba-nol-5*), RNA polymerase subunit (*Hba-WO1G7.3*), G-protein beta subunit (*Hba-rack-1*), ADP-ribosylating factor (*Hba-arf-1*), Beta tubulin (*Hba-ben1*), multidrug resistance (*Hba-mrp-4*), nuclear hormone receptor (*Hba-nhr-47)*	Soaked in 1-1.5 mg/ml dsRNA for >24 hrs at 28°C (eggs hatch in the dsRNA)	↓ transcript of all targeted genes. *cct-2, daf-21, icd-2, nol-5, WO1G7.3* RNAi gave high penetrance of RNAi phenotypes *rack-1* and *arf-1* gave moderate penetrance of RNAi phenotypes. *ben-1, mrp-4, nhr-47* did not show any phenotypes.	(Ciche & Sternberg, 2007)
Globodera pallida	J2	Cysteine proteinase *(Gp-cp-1)*	Soaked in 2-5 mg/ml dsRNA + octopamine for 4 hrs	↓ number of established nematodes, ↑ male: female ratio, ↓ transcript.	(Urwin *et al.*, 2002)
	J2	FMRFamide-like peptides (*Gp-flp-6, flp-12, flp-14, flp-1, flp-18*)	Soaked in 0.1 mg/ml dsRNA for 24 hrs, 2 days, 7 days.	↓ migration inhibition (motility impairment), 'straight' (paralysed) phenotype, ↓ transcript	(Kimber *et al.*, 2007)
	J2	FMRFamide-like peptide (*Gp-flp-12*)	Soaked in 0.1 mg/ml 21bp siRNAs for 24 hrs.	↓ migration inhibition, ↓ of transcript. **Aberrant phenotype from one non-target control siRNA.**	(Dalzell *et al.*, 2009a)
Globodera rostochiensis	J2	ß-1,4-endoglucanases (*Gr-eng-1*), amphid secreted protein (*Gr-ams-1*)	Soaked in 2-5 mg/ml dsRNA + octopamine for 24 hrs.	↓ of all targeted transcripts. *eng-1* RNAi ↓ number of established nematodes. Impaired host location and invasion for *ams-1* RNAi. **Non-target dsRNA control not used.**	(Chen *et al.*, 2005b)

Meloidogyne incognita	J2	Calreticulin (*Mi-crt*), polygalacturonase (*Mi-pg-1*)	Soaked in 4 mg/ml dsRNA + resorcinol for 4 hrs	↓ transcript shown by RT-PCR and *in situ* hybridisation.	(Rosso *et al.*, 2005)
	J2	Dual oxidase	Soaked in 2 mg/ml dsRNA + octopamine for 4 hrs	↓ number and size of established females, ↓ rate of development, 70% ↓ egg output. ↓ transcript level.	(Bakhetia *et al.*, 2005)
	J2	Splicing factor, Integrase	Host (plant) generated hair-pin dsRNA (*in planta* RNAi)	↓ size and number of root knots formed. ↓ number of females, abnormally developed within root knots. Complete ↓ of transcript.	(Yadav *et al.*, 2006)
	J2	Parasitism gene *16D10*	Soaked in 1 mg/ml dsRNA + resorcinol for 4 hrs	93-97% ↓ transcript, 65-69% ↓ peptide, ↓ infectivity. **Non-target dsRNA control not used.**	(Huang *et al.*, 2006)
			Host (plant) generated hair-pin dsRNA (*in planta* RNAi)	↓ size and number of root knots formed when tested with *M. incognita, M. javanica, M. arenaria* and *M. hapla*. **Non-target dsRNA control not used.**	(Huang *et al.*, 2006)
	J2	cathepsin L cysteine proteinase (*Mi-cpl-1*)	Soaked in 2-5 mg/ml dsRNA + octopamine for 4 hrs	↓ nematode growth and infection in plants. ↓ cysteine proteinase activity, ↓ transcript level.	(Shingles *et al.*, 2007)
	J2	Glutathione-S transferase (*Mi-gsts-1*)	Soaked in 4 mg/ml dsRNA + resorcinol + serotonin for 4hrs	↓ fecundity, ↓ transcript	(Dubreuil *et al.*, 2007)
	J2	FMRFamide-like peptide (*Mi-flp-18*)	Soaked in 0.1 mg/ml 21 bp siRNAs for 24 hrs.	↓ migration inhibition, ↓ of transcript.	(Dalzell *et al.*, 2009a)
	J2	Troponin C (*Mi-tnc*), Calreticulin (*Mi-crt*)	Host (plant) infected with Tobacco rattle virus engineered to produce dsRNA	**variable ↓ of transcript** in eggs. No change in fecundity. ↓ ability of juveniles to extrude from the egg shell. ↓ of transcript observed in progeny.	(Dubreuil *et al.*, 2009)
Meloidogyne javanica	J2	Putative transcription factor (*Mj-Tis11*)	Host (plant) generated hair-pin dsRNA (*in planta* RNAi)	↓ transcript level, no phenotype.	(Fairbairn *et al.*, 2007)
	J2	Avirulence gene *Cg-1*	Soaked in 0.5 mg/ml dsRNA + octopamine for 48 hrs.	Virulent on *Mi-1* plants	(Gleason *et al.*, 2008)
Meloidogyne artiellia	eggs	Chitin synthase	Soaked in 1 mg/ml dsRNA at 20°C for 24-72 hrs.	59-70% ↓ chitin in eggs. Hatching defect. ↓ transcript. **Non-target dsRNA control not used.**	(Fanelli *et al.*, 2005)

Heterodera glycines	J2	Cysteine proteinase (*Hg-cp-1*), C-type lectin (*Hg-ctl*), major sperm protein.	Soaked in 2-5 mg/ml dsRNA + octopamine for 4 hrs	↓ of all targeted transcripts. ↓ number of established nematodes, ↑ male: female ratio. No phenotype seen with major sperm protein	(Urwin *et al.*, 2002)
	J2	Aminopeptidase (*Hg-amp-1*)	Soaked in 2-5 mg/ml dsRNA + octopamine for 4 hrs.	↓ number of established nematodes. ↑ male: female ratio. ↓ transcript. **Non-target dsRNA control not used.**	(Lilley *et al.*, 2005)
	J2	major sperm protein	Host (plant) generated hairpin dsRNA (*in planta* RNAi)	↓ number of eggs, ↓ fecundity. **Transcript levels not assessed.**	(Steeves *et al.*, 2006)
	J2	ribosomal protein (*Hg-rps-23*)	Soaked in 10 mg/ml dsRNA + octopamine for 4 hrs.	↓ in viability, ↓ transcript.	(Alkharouf *et al.*, 2007)
	J2	Pectate lyase (*Hg-pel-1*), Parasitism gene Hg-4EO2.	Soaked in 2.5-5mg/ml dsRNA + octopamine at 28°C for 24hrs.	↓ of all targeted transcripts. No phenotype reported.	(Sukno *et al.*, 2007).
	J2	B-1, 4-endoglucanase (*Hg-eng-1*), pectate lyase (*Hg-pel*), chorismate mutase (*Hg-cm*), gland protein (*Hg-gp*), unknown protein *Hg-syv-46*.	Soaked in 2 mg/ml dsRNA + octopamine for 16 hrs.	↓ number of established nematodes, ↑ male: female ratio. ↓ transcript levels.	(Bakhetia *et al.*, 2007)
	J2	Dorsal pharyngeal gland cell genes (*dg21, dg22, dg13, dg14*)	Soaked in 2-5 mg/ml dsRNA + octopamine for 4-16 hrs.	↑ male: female ratio, ↑ number of established nematodes. **↓ transcript when dsRNA was used singly, ↑ transcript with combinatorial RNAi.**	(Bakhetia *et al.*, 2008)
Heterodera schachtii	J2	GAPDH	Host (plant) infected with Tobacco rattle virus engineered to produce dsRNA	10-15% ↓ in female size, ↓ transcript.	(Valentine *et al.*, 2007)
	J2	Cellulose binding protein (3B05), ubiquitin-like (4G06), polyubiquitination complex components (8H07, SKP1), zinc finger protein (10A06).	Host (plant) generated hairpin dsRNA (*in planta* RNAi)	↓ of all targeted transcripts except 10A06 which was not measured. ↓ number of developing females.	(Sindhu *et al.*, 2008)

Bursaphelenchus xylophilus	L2-L3	Myosin heavy chain (*Bx-myo-3*), tropomyosin (*Bx-tmy-1*), heat shock protein 70 (*Bx-hsp-1*), cytochrome C (*Bx-cyt-2.1*)	Microinjection of 10 nl of dsRNA at 1 mg/ml. Adult females injected and mated with untreated males. Soaked in 1 mg/ml dsRNA for 1 day at 25°C. Electroporation with 1 mg/ml dsRNA.	46% ↓ hatching rate in the F1 generation. **Transcript level not assessed.** 25% ↓ of survival to adulthood. 33% ↓ viability at high temperature with *hsp-1* RNAi. Abnormal locomotion with *myo-3, tmy-1* RNAi. 35% ↓ of transcript levels for all targeted transcripts. 32% ↓ of survival to adulthood. No significant difference between soaking and electroporation. **Transcript level not assessed.**	(Park *et al.*, 2008)

RNAi experiments performed on parasitic nematodes of animals are indicated in text on white background. Experiments on plant parasitic nematodes are indicated on grey background. Genes efficiently silenced by plant-mediated RNAi in plant parasitic nematodes are indicated in blue text. ↓ indicates decrease, ↑ indicates increase.

Nippostrongylus brasiliensis, a GI nematode of rats, was the first parasitic nematode in which RNAi was attempted (Hussein *et al.*, 2002). This was followed by many reports of RNAi in various parasitic nematode species (see Table 1.1), although recently there has been paucity in the number of reports describing successful RNAi in parasitic nematodes of animals. In addition, the methods used to evaluate the success of RNAi vary widely between different studies, making direct comparisons of results difficult. Not all studies evaluated the success of RNAi by directly investigating a decrease in transcript level; instead, indirect methods such as phenotypic effects and protein immunolocalisation assays were used in several studies (Issa *et al.*, 2005; Lustigman *et al.*, 2004). Problems with susceptibility, reliability and reproducibility in addition to off-target effects of dsRNA treatment have also been reported, and these problems with RNAi in parasitic nematodes are discussed further in Chapter 3.

It is worth mentioning that significant advances have been made towards developing RNAi in the plant parasitic nematodes. Initial studies focused on *in vitro* RNAi experiments in which nematodes were routinely soaked in dsRNA, with a resulting knockdown of transcript levels and associated phenotypes being reported (Table 1.1). Subsequent work has since been carried out *in planta*; transgenic plants were engineered to produce hairpin dsRNA to be processed by the RNAi machinery of the plant. This generated siRNAs which can then be ingested by the nematode to produce an RNAi silencing of the desired nematode genes (Yadav *et al.*, 2006). *In planta* RNAi experiments thus raise the attractive possibility of parasitic nematode control by developing transgenic crops with potential host resistance to parasitic nematodes. In addition it could be possible to analyse the genomes of the plant parasitic nematodes functionally by carrying out large-scale RNAi screens as with *C. elegans*. Unfortunately it seems that results from plant parasitic nematode RNAi experiments need to be interpreted with caution, as non-nematode derived dsRNAs designed for use as controls in RNAi screens appear to induce aberrant phenotypes, implying off-target effects (Dalzell *et al.*, 2009b).

Developing RNAi to analyse gene function directly in the parasite would be immensely useful in identifying genes that may be utilised for future control methods. Better knowledge of where and when genes of interest are expressed can also be important to understanding their function; at present, little is known

of the spatial and temporal regulation of parasitic nematode genes. The wealth of genome sequence data available could be used to identify promoter regions and regulatory motifs, and promoter reporter constructs can be helpful in examining expression patterns of specific genes in transgenic *C. elegans*. Thus understanding the function and regulation of *H. contortus* genes is an important step towards ultimately discovering possible drug and vaccine targets for future use.

1.4 Aims and objectives of the project

As highlighted above, *H. contortus* infections cause a significant loss of productivity in the sheep industry, and current methods of control appear to be inadequate at controlling infection when faced with the problem of anthelmintic resistance. Understanding the biology of *H. contortus* at a fundamental level, by analysing the function and expression of genes, could lead to novel targets for control which may alleviate this problem, allowing for the sustainable use of control strategies in the future.

The overall objectives of this project are to analyse gene function and expression in *H. contortus*. More specifically, the aims are;

i. To examine the reproducibility and reliability of RNAi in *H. contortus* L3 stage larvae.

ii. To attempt to answer the question of why RNAi is successful for some genes and not others.

iii. To analyse important components of the RNAi pathway.

iv. To examine the expression pattern and regulation of *H. contortus* genes targeted by RNAi.

Chapter 2

Materials and methods

2.1 *Haemonchus contortus* methods

The MHco3 (ISE) isolate of *H. contortus*, chosen for full genome sequencing as part of the *H. contortus* genomic project (http://www.sanger.ac.uk/Projects/H_contortus), was used throughout this study. This worm isolate is susceptible to the three major classes of anthelmintic drugs currently in use.

2.1.1 Infective larvae

H. contortus L3 larvae, developed from infected sheep faecal cultures, were kindly provided by Professor Dave Knox at the Moredun Research Institute, Pentlands Science Park, Penicuik, Midlothian EH26 0PZ, UK. Larvae were collected from faecal cultures and stored at 4°C where they remain viable for several months.

2.1.2 Exsheathing L3 larvae

The required number of L3 larvae was aliquoted into eppendorf tubes and centrifuged (bench top Biofuge Pico centrifuge) at 6000 rpm for 2 minutes. The supernatant was removed and 200 µl of Phosphate Buffered Saline (PBS, Appendix 1) with 5 µl of sodium hypochlorite solution (10-13% concentration, Sigma-Aldrich) or 5 µl of Milton solution (Milton sterilising fluid, UK) was added. The tubes were incubated horizontally at room temperature for 5 minutes and then checked under the microscope at 10X magnification for exsheathment. Where larvae had not exsheathed, they were left for longer. Once exsheathment was complete, the larvae were washed 3 times in PBS to remove the sodium hypochlorite.

2.1.3 RNA interference method in *H. contortus*

The method for RNAi in *H. contortus* was adapted from the work described by Geldhof and co-workers (Geldhof *et al.*, 2006). Briefly, exsheathed larvae were prepared for soaking in dsRNA by several washes in antibiotics. For most experiments, approximately 1000 L3 larvae were used per RNA sample. Larvae

were washed three times in filter sterilised PBS solution with added penicillin (250 units/ml), streptomycin (50 µg/ml) and fungizone (1.25 µg/ml). Larvae were then washed twice in Earle's Balanced Salt Solution (EBSS) pH 5.2 (supplied by Gibco and prepared from powder, or Sigma, diluted from 10X concentrate) containing penicillin/streptomycin/fungizone. The larvae were centrifuged briefly at 6000 rpm to obtain a final volume of 30 µl. 10 µl of dsRNA (4 µg/µl) which had been pre-incubated at room temperature for 10 minutes with 1 µl of Lipofectin reagent (Invitrogen) and RNasin (8 units, Promega) was added to each tube. Control incubation with dsRNA to a *Caenorhabditis elegans* gene (*Ce-rab-7*) was also set up. The final concentration of dsRNA was 1 µg/µl. Larvae were incubated at 37°C, 5% CO_2 for 72 hours unless stated otherwise. dsRNA and culture media was then removed as described in section 2.5.9.

2.1.4 Albendazole assay

Preliminary experiments were performed to determine the protocol most suitable for the albendazole assay. Varying concentrations and time periods were used to determine the optimum concentration of the drug and the time period required to observe effects of the drug treatment. A final concentration of 40 µg/ml for 48 hours was determined as the optimal.

Following 24 hours of soaking the larvae in *Hc-bt-iso-1* dsRNA, as described in section 2.1.3, larvae were washed 2X in PBS to remove dsRNA and set up with 40 µg/ml final concentration albendazole (Sigma, UK) dissolved in dimethyl sulfoxide (DMSO, Sigma UK). A DMSO-only control was also included in the experiment by incubating larvae in 2 µl of DMSO, resulting in a final concentration of 5% DMSO. 5 µl of larvae were removed after 48 hours and observed at 10X magnification for effects of albendazole.

2.2 *In vivo* assays in sheep

All *in vivo* experiments were carried out by animal technicians at the Moredun Research Institute, Pentlands Science Park, Penicuik, Midlothian, UK. These experiments were approved by the Ethics Committee at Moredun Research Institute. Six-month old worm-free Suffolk lambs were allocated into groups as

described for each experiment, balanced for sex and weight. Faecal egg counts (FECs) and total worm burdens were measured as previously described (Smith & Smith, 1993).

2.2.1.1 Preliminary *in vivo* RNAi experiment using L3 larvae cultured in control dsRNA or dsRNA-free medium

A preliminary experiment was set up in which sheep were infected with larvae (treated with control *Ce-rab-7* dsRNA or untreated) and the faecal egg output and total worm burdens were compared. Six sheep were used, with two sheep per experimental group. Each sheep was infected with approximately 5000 L3 larvae which were previously exsheathed as described in section 2.1.2, using Milton solution. Group A sheep were orally infected with approximately 5000 exsheathed larvae that had been soaked for 24 hours in control dsRNA (*Ce-rab-7*). Group B sheep were surgically infected with approximately 5000 exsheathed larvae soaked for 24 hours in control dsRNA (*Ce-rab-7*). Group C sheep were orally infected with approximately 5000 exsheathed larvae that were soaked in dsRNA-free culture medium for 24 hours. The FECs were measured 21 and 28 days post-infection.

2.2.1.2 *In vivo* RNAi experiment using L3 larvae cultured in control dsRNA or target dsRNA

Eight sheep were used in this experiment with four animals per experimental group. Larvae were exsheathed as described in section 2.1.2 using Milton solution. Group A sheep were each orally infected with approximately 5000 exsheathed larvae, previously soaked for 24 hours at 37^{o}C in control dsRNA (*Ce-rab-7*). Group B sheep were also orally infected with approximately 5000 exsheathed larvae, previously soaked for 24 hours 37^{o}C in target dsRNA (*Hc-H11*). FECs were measured at 15, 23, 26 and 28 days post infection. The total worm burden in each sheep was measured on day 28 by sacrificing the sheep at the conclusion of the experiment. Adult male and female worms present in a 2% abomasal wash were counted, as were the adult male and female worms present in a 2% abomasal digest. These numbers were then multiplied by 50 to estimate the total number of male and female worms present within each sheep, and

together this represented the total worm burden in each sheep. A sample of the adult worms isolated from each sheep was frozen at -80^{o}C for subsequent analysis of transcript levels as described in section 2.5.11

2.3 *Caenorhabditis elegans* methods

2.3.1 Culture and maintenance of *C. elegans*

C. elegans was grown and maintained on 5 cm Petri dishes containing NGM agar (Appendix 1) and seeded with a lawn of *E. coli* strain OP50. *C. elegans* strains were acquired from the *Caenorhabditis* Genetics Center (CGC) unless otherwise stated. Worms were cultured and maintained at 20°C unless otherwise stated.

Worm stocks were stored by freezing using the method described by Stiernagle (Stiernagle, 2006). Briefly, 800 µl of M9 solution was added to a plate of starved L1/L2 larvae. An equal amount of Freezing Solution (Appendix 1) was also added to the plates, mixed well by pipetting and then transferred to 1.5 ml cryotubes. The tubes were kept in a Styrofoam box at -80°C for a few hours and then transferred to a freezer box for storage at -80°C. Stocks were thawed as required by emptying cryotube contents onto a seeded NGM agar plate.

2.3.2 Transformation of *C. elegans* by microinjection

The injection procedure used for the transformation of DNA into *C. elegans* was described by Mello and co-workers (Mello *et al.*, 1991). DNA was injected into the gonad of adult hermaphrodites. A marker gene was also co-injected with the DNA of interest to allow the recognition of transgenic animals and to aid in the identification of individual worms carrying the transgene in the subsequent F1 and F2 progeny. The *rol-6* marker gene, present in plasmid pRF4, was used in this study which is a dominant mutant of the collagen gene *rol-6* (Kramer *et al.*, 1990). This mutation confers a roller phenotype that can be easily identified under low power magnification.

2.3.2.1 DNA preparation for microinjection

DNA for the microinjection procedure was prepared by using the Qiagen Miniprep kit as per manufacturer's instructions. DNA was diluted in distilled water to obtain a final concentration of 100 ng/µl of *rol-6* plasmid and 15 ng/µl of DNA of interest for microinjection. This injection mixture was centrifuged at 13,000 rpm for 15 minutes and only the upper portion of the supernatant was used, to prevent any insoluble contaminants from blocking the needle.

2.3.2.2 Preparation of microinjection needles

Microinjection needles were prepared from borosilicate glass capillaries of external diameter 1.2 mm and internal diameter 0.69 mm (Harvard Apparatus) using a 773 APP Micropipette Puller (Campden Instruments Ltd). The injection mixture was loaded by mouth pipette. A Zeiss Axiovert S100 inverted Differential Interference Contrast (DIC) microscope equipped with standard 10X and 40X Nomarski objectives was used to carry out the microinjections. A pressurised injection system (Tritech Research) with a needle holder was used, with pressure applied at 40 psi using a foot pedal. The loaded needle was opened to ensure that it is 'flowing' by gently rubbing the tip on the agarose pad while observing through the 10X objective and applying pressure from the foot pedal.

2.3.2.3 Mounting and injecting worms

Dried agarose pads for immobilising the worms were prepared by placing a drop of melted 2% agarose onto a 22 mm X 64 mm coverslip and flattening with another coverslip. Agarose pads were dried at room temperature for a few hours and then baked overnight in an 80°C incubator before use. Young healthy hermaphrodite *C. elegans* were picked and immobilised on the agarose pads by placing them in a drop of mineral oil. The worms were positioned so that the needle was inclined at 30-40° to the distal arm of the gonad. The needle was inserted into the gonad of the worm and pressure was applied using the foot pedal to inject the DNA into the worm, confirmed by the swelling of the gonad. The injected worm was removed from the agarose pad immediately by placing a drop of M9 buffer (Appendix 1) on the surface of the mineral oil. The worm was then transferred onto a seeded agar plate and left to recover at 20°C.

2.3.2.4 Identification of *rol-6* transformants

About 72 hours after injection, plates were examined for F1 progeny showing the roller phenotype. Roller worms were picked and transferred onto fresh plates and if any F2 progeny displayed the roller phenotype, they were picked singly onto fresh agar plates and considered to be independent transgenic lines.

2.3.2.5 Reporter gene expression in transformed worms

2% agarose pads were prepared on glass slides as described in Section 2.3.2.3. A small amount of Vaseline was placed around the agarose pad, and a drop of M9 buffer containing 0.1% sodium azide was placed on the agarose pad. Worms displaying the roller phenotype were picked onto the drop of buffer and then sealed with a coverslip and examined using the high power X40 GFP microscope.

LacZ staining was carried out on transgenic lines in the following manner; worms were washed off the plate using M9 containing 0.001% Triton (Sigma-Aldrich) into eppendorf tubes. Worms were centrifuged and washed three times using M9/triton solution. The supernatant was removed after the final wash leaving 100 µl of buffer in the eppendorf tube. 100 µl of 2.5% glutaraldehyde was added and eppendorf tubes were laid on their sides for 15 minutes of incubation at room temperature. Worms were then washed 3X in M9/trition solution to remove the glutaraldehyde and liquid was removed to 50 µl. Worms were pipetted onto a glass slide and allowed to dry at room temperature. Once dry, slides were placed in acetone at -20°C for 3-5 minutes. Slides were then dried at room temperature. X-gal stain (Appendix 1) was pipetted onto the slides and left at room temperature in a light-impenetrable humid chamber for several hours or overnight. Slides were then examined under the bright field microscope for *LacZ* expression.

2.4 General protein techniques

2.4.1 L3 larvae protein extracts

Approximately 1000 *H. contortus* L3 larvae were cultured in dsRNA (control or target) as described in section 2.1.3 for varying lengths of time (72 hours, 7 days

and 15 days). Larval total protein extracts were prepared by washing larvae in PBS to remove dsRNA and heating to 95°C for 10 minutes in 95 µl of 2X SDS-PAGE sample buffer (Appendix 1) and 5 µl of 2-mercaptoethanol (Sigma-Aldrich). Larval protein extracts were stored at -20°C until further use.

2.4.2 Protein separation by polyacrylamide gel electrophoresis

L3 larval protein extracts were separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Protein separation was carried out using 4-15% Tris-HCl precast gels (BioRad). 12 µl of larval protein extract, prepared as described in section 2.4.1, was heated at 95°C for 5 minutes prior to being loaded onto the gel. The heated samples were loaded onto a pre-cast gel with pre-stained protein ladder broad range marker (New England Biolabs). Gels were run in 1X Tris-Glycine-SDS Buffer (diluted from 10X Tris-Glycine-SDS Buffer, BioRad) using a BioRad Power Pac 200 at 200 V for 1 hour.

2.4.3 Western blotting

Proteins separated by SDS-PAGE were transferred onto a polyvinylidene fluoride (PVDF) membrane (NEN Life Sciences) in 1X Tris-Glycine blotting buffer (Appendix 1). Transfer set up consisted of pre-wetting the PDVF membrane in methanol prior to equilibration in 1X Tris-Glycine blotting buffer (Appendix 1). A sandwich was created with a sponge pre-soaked in 1X blotting buffer; one pre-soaked filter paper was laid on top, followed by the SDS-PAGE gel, the equilibrated PDVF membrane, another pre-soaked filter paper and finally another pre-soaked sponge, completing the transfer sandwich. Protein transfer was carried out at 100 V for 1 hour using a BioRad Power Pac 200. Following protein transfer, the membrane was incubated overnight in a blocking solution of 5% milk (5% w/v non-fat dried milk powder in PBST solution, Marvel, UK). PBST was prepared as described in the Appendix 1.

2.4.4 Antibody detection of transferred proteins

Test antibody was raised in rabbits following immunisation with *H. contortus* beta tubulin isotype-1 peptide (*Hc*-Bt-ISO-1). The peptide was restricted to the last 13 amino acids at the C-terminal region of *Hc*-Bt-ISO-1 which differs

between the different *H. contortus* beta tubulin isotypes (Gary Saunders, PhD Thesis University of Glasgow 2009). Control antibody was anti-human actin, raised in mice (Sigma, UK). Following overnight blocking, the primary antibody was added at the appropriate dilution in 5% milk solution. A 1:500 dilution was used for both the test and control antibodies. Primary antibody incubation was carried out overnight at 4°C. Following the primary antibody incubation, the membrane was washed 3X in 5% milk solution and incubated overnight at 4°C with the secondary antibody, horse-radish peroxidise (HRP) labelled anti-rabbit or anti-mouse antibody, diluted 1:10000. Subsequent to incubation with the secondary antibody, the membrane was washed 3X in PBST solution. Antibody was detected using Enhanced Chemiluminescence, ECL, according to manufacturer's instructions (Amersham ECL plus Western Blotting detection system, GE Healthcare). Upon exposure to HRP, a chemi-luminescence reaction occurs and the luminescence can be detected by exposure to X-ray film (Kodak Scientific Imaging Film). Exposure times were varied depending on signal strength.

2.4.5 Stripping membrane of bound antibody

Where the same membrane required re-probing with a different antibody, the membrane was stripped of the first antibody prior to being probed with another primary antibody. This step is necessary as the two primary antibodies used in this study were of approximately similar size; *Hc*-Bt-ISO-1 at 50 kDa and actin at 42 kDa. The membrane was sealed in a plastic bag containing 10 ml of stripping buffer (Appendix 1) and sealed again inside another plastic bag to ensure it was completely waterproof. The plastic bags were then immersed in a 42°C water bath for 20 minutes. The stripping buffer was removed from the plastic bag, fresh buffer added, and immersed again in a 42°C water bath for 20 minutes. The membrane was then rinsed in PBST and blocked in 5% milk solution and re-probed with a primary antibody as described in section 2.4.4.

2.4.6 Quantitative analysis of Western blot signal

The detection of the antibody signal on X-ray film, described in section 2.4.4, was subjected to quantitative analysis. The X-ray film was scanned and analysed

using FluorChem IS-5500 software. The integrated density value was measured for each sample and subsequently used to calculate the ratio of the signal relative to the control antibody signal (anti-actin antibody).

2.5 Molecular biology methods

2.5.1 Polymerase chain reaction (PCR)

2.5.1.1 Standard PCR

All Polymerase Chain Reactions (PCR) were carried out in a Techne Flexigene PCR machine, using standard techniques unless otherwise stated. Promega GoTaq Flexi DNA Polymerase was used in most experiments and reaction mixes were prepared according to manufacture's instructions: 5X Green GoTaq Flexi Buffer (or 5X Colourless GoTaq Flexi Buffer), 1 mM $MgCl_2$, 0.1 mM of each dNTP (Promega), 100 ng of Primer 1, 100 ng of Primer 2 (final concentration 4 ng each, primers synthesised by Eurofins MWG Operon), 1.25 units GoTaq Flexi DNA Polymerase (Promega), and 50-100 ng DNA template in a total volume of 25 µl. Standard PCR conditions were; 4 minutes at 94°C initial denaturation, 30-35 cycles of 45 seconds at 94°C to denature, 30 seconds at 54°C-57°C for primer annealing, 1 minute per 1 kb at 72°C for extension and a final extension of 5 minutes at 72°C. PCR using Taq polymerases result in a single deoxy-adenine base at the 3' end of the PCR product which can be exploited for TA cloning purposes as described in section 2.5.4.1.

2.5.1.2 PCR using thermostable polymerase

Where an amplified fragment was to be used in expression studies (e.g.; promoter expression) a mixture of Taq polymerase and a proofreading thermostable DNA polymerase was used for PCR in order to minimise the frequency of polymerase induced mutations. *Pfu* DNA polymerase (Stratagene) was used for this purpose. *Pfu* polymerase was used with Taq polymerase at a 1:5 ratio. For long range PCR (>3 kb in size), *Pfu* Turbo Polymerase (Stratagene) was used, which creates blunt ended PCR products; these cannot be cloned directly into TA vectors as described in Section 2.5.4.1. Therefore 3' adenosine

overhangs were added by incubating the PCR reaction mix (25 µl) with 1.25 units of GoTaq Flexi DNA Polymerase at 72°C for 8 minutes.

2.5.1.3 Fusion PCR for promoter analysis

PCR-based fusions of overlapping DNA fragments were used to generate reporter gene constructs as outlined by Hobert (Hobert, 2002). The promoter of the gene of interest is fused to the reporter gene encoding Green Fluorescent Protein (GFP) using **Primers A and B** in Figure 2.1. Briefly, the promoter of the gene of interest is amplified from genomic DNA (**PCR #1**), and in parallel, the GFP coding sequence is amplified from the pPD95.75 GFP vector (**PCR #2**, Fire vector, http://www.ciwemb.edu/pages/firelab.html) using **Primers C and D** in Figure 2.1. The 3' primer for the promoter (**Primer B**) has a 24 nucleotide overhang to the GFP vector pPD95.75. The DNA concentrations of these two primary PCRs are roughly estimated by agarose gel electrophoresis and then diluted as appropriate to a final concentration of 10-50 ng/µl. The PCRs were not cleaned or gel purified, but used directly from the reaction tubes in a second round of nested PCR using **Primers A* and D*** (Figure 2.1, **Fusion-PCR**). Following dilution, 1 µl of each diluted primary PCR is used in the fusion PCR. The concentration of DNA from this fusion PCR is also roughly estimated and diluted as appropriate to give a final concentration of 20-50 ng/µl. The diluted fusion PCR is then directly injected into the gonad of adult *C. elegans* worms with no further purification together with a marker gene such as *rol-6*. The microinjection procedure is described in detail in Section 2.3.2. This fusion PCR technique is illustrated in Figure 2.1.

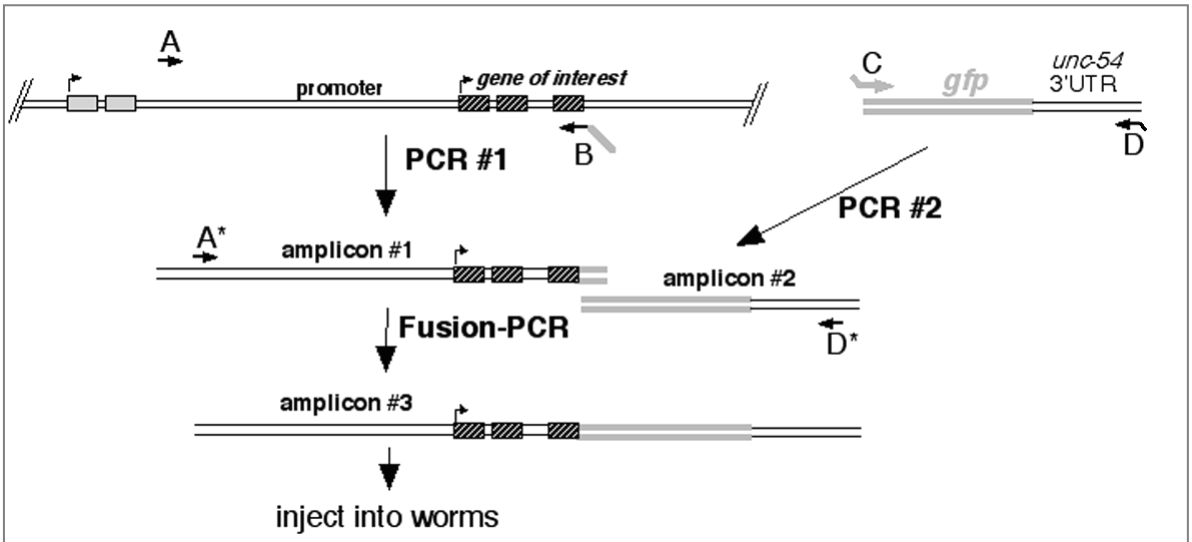


Figure 2.1. GFP fusion PCR protocol.

Primers A & B are used to amplify the promoter of the gene of interest using *H. contortus* genomic DNA as template in PCR #1. Primer B is designed with a 24 nucleotide overhang to the GFP vector pPD95.75. Primers C & D are used to amplify GFP coding region and the *unc-54* 3' UTR using GFP vector pPD95.75 as template. Fusion PCR is carried out using 10-50 ng each of PCR #1 and PCR #2 as template, using primers A* and D* in the second round of nested PCR. The resultant PCR product is injected directly into worms with an appropriate marker gene. Adapted from Hobert, 2002.

2.5.1.4 5' RACE PCR of *H. contortus dcr-1* gene

The Rapid Amplification of cDNA Ends (RACE) PCR method was used to obtain the 5' sequence of the *H. contortus dcr-1* gene (*Hc-dcr-1*). The FirstChoice RLM-RACE Kit (Ambion) was used for this procedure. This technique was used to facilitate the cloning of full-length cDNA sequence and determine the correct 5' end of the gene. cDNA was prepared from adult *H. contortus* using the Qiagen RNeasy Mini Kit (Qiagen) and used in the 1st round 5'RACE PCR: 2 µl cDNA, 150 ng RACE Outer Primer (provided in kit), 150 ng of gene specific primer (*Hc-dcr-1* exon 5), 1.25 units GoTaq Flexi DNA Polymerase, 5X Colourless GoTaq Flexi Buffer, 2.0 mM $MgCl_2$, 0.2 mM of each dNTP (Promega) in a total volume of 30 µl. The following PCR conditions were used; 5 minutes at 94°C initial denaturation; 35 cycles of 30 seconds at 94°C, 30 seconds at 56°C, 1 minute at 72°C and a final extension of 5 minutes at 72°C.

2nd round 5'RACE PCR was carried out by using the 1st round PCR as template. 0.5 µl, 1 µl and 2 µl of 1st round reaction was used with the same amounts of reagents as for the 1st round, but with 150 ng RACE Inner Primer (provided in kit), and 150 ng of gene specific primer (*Hc-dcr-1* exon 2) in a total volume of 30 µl. The same PCR conditions were used as above but with a reduction in the number of cycles from 35 to 32. Products were analysed by gel electrophoresis

and excised from the gel using a scalpel blade for subsequent cloning into the pSC-A cloning vector using the methods described in section 2.5.4.1. Primers used for 5' RACE are shown in Table 2, Appendix 2.

2.5.1.5 PCR to obtain full-length sequence of *H. contortus dcr-1* gene

The *H. contortus dcr-1* genomic DNA sequence was identified on Supercontig_0059385_cw_200808 from the Sanger Institute server (assembled *H. contortus* supercontigs release 21/08/08). PCR primers were designed to exon sequences identified to encode regions conserved between *C. elegans* and *H. contortus* DCR-1. The 5' sequence of *H. contortus dcr-1* was obtained by 5' RACE as described in Section 2.5.1.4. PCR amplification of the *H. contortus dcr-1* gene was carried out in four fragments of ~1.5 kb size as the full length sequence was too large (approximately 5.7 kb) for conventional PCR (the sequences of the primers used, and their positions on the *Hc-dcr-1* sequence is shown in Table 3, Appendix 2 and Figure 2.2). 10 ng of adult 21 day cDNA (prepared by Roz Laing, University of Glasgow) was used as template for PCR. Thermostable polymerase *pfu* Turbo was used. The PCR conditions were; 45 seconds at 94°C for one cycle, 40 cycles of 45 seconds at 94°C, 45 seconds at 56°C, 2 minutes 30 seconds at 72°C, and a final extension of 5 minutes at 72°C. 3' Adenosine overhangs were added by incubating the PCR reaction mix (25 µl) with 1.25 units of GoTaq Flexi DNA Polymerase at 72°C for 8 minutes. PCR reactions were then purified as described in Section 2.5.3 and cloned into the pSC-A vector as described in Section 2.5.4.1. Cloned inserts were sequenced using T3 and T7 primer sites present on the pSC-A vector as described in Section 2.5.6.

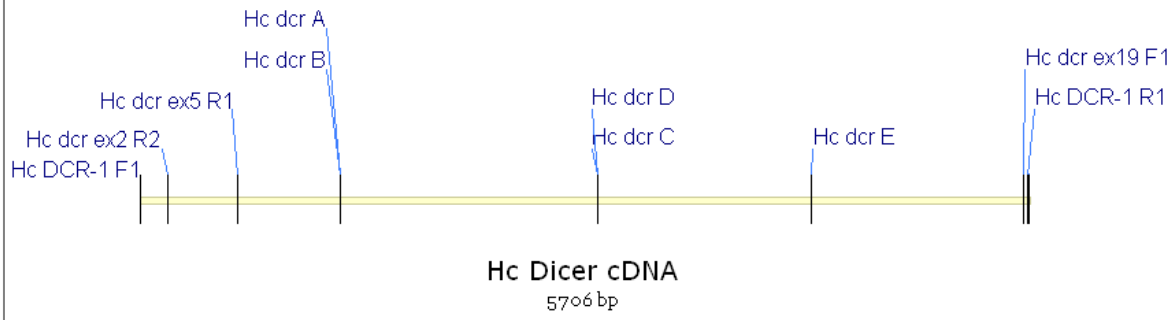


Figure 2.2. Schematic of *H. contortus dcr-1* cDNA showing primer sites used to amplify ~1.5 kb fragments which together constitute the full length cDNA sequence.

2.5.2 Agarose gel electrophoresis of nucleic acids

PCR products, RNA and DNA samples were analysed by conventional agarose gel electrophoresis. 1% (w/v) agarose gels were prepared by dissolving agarose (Invitrogen) in 1X Tris-acetate EDTA (TAE) electrophoresis buffer (Appendix 1). Nucleic acids were visualised by using ethidium bromide at a final concentration of 0.5 µg/ml or SafeView (NBS Biologicals) at a dilution of 0.05 µl/ml. Nucleic acid samples were mixed with DNA loading buffer (Appendix 1) and loaded onto the gels. Gels were run in 1X TAE using Gibco BRL Horizontal Gel Electrophoresis Apparatus at 90-120 V powered by an Amersham Pharmacia Biotech Electric Power Supply unit. Product sizes were estimated by comparing with the 1 kb DNA Ladder (Invitrogen), visualised by UV illumination using a BioRad Trans UV Illuminator (BioRad).

2.5.3 Purification of PCR products

PCR products were purified using the Qiaquick PCR Purification Kit (Qiagen) according to manufacturer's instructions. Where the PCR product was isolated directly from the agarose gel, the band of interest was excised from the gel and the Qiaquick Gel Extraction Kit (Qiagen) was used as per manufacturer's instructions. DNA samples were eluted with 4 X 50 µl distilled water heated to 50°C, ethanol precipitated by adding 8 µl 5 M NaCl and 400 µl 100% ethanol and stored at -20°C overnight. Samples were then centrifuged at 13,000 rpm for 15 minutes and washed in 70% ethanol and resuspended in 10-20 µl distilled water and stored at -20°C.

2.5.4 DNA cloning techniques

2.5.4.1 TA cloning of PCR products

Routine cloning of PCR products was performed using a TA vector system. This system utilises the 3' adenosine overhangs added to the PCR product by Taq polymerase during PCR. The vector is linearised to produce overhanging thymidine residues that will act as complementary ends for the PCR products. Two different kits were used for routine cloning during this study, both of which operate under the same general cloning strategy, 'TA cloning' (TOPO TA Cloning Kit by Invitrogen or StrataClone PCR Cloning Kit, pCS-A vector by Stratagene). The ligations and transformations were carried out as per manufacturer's instructions. Generally ~40 ng of insert DNA was used with 10 ng of cloning vector mix from kit. PCR products cloned using the StrataClone PCR Cloning Kit were ligated into the pSC-A vector and transformed into StrataClone Solo Pack competent cells (50 µl) also provided with the kit. PCR products cloned using the TOPO TA Cloning Kit were ligated into the TOPO vector and transformed into 40 µl XL10 Gold Ultracompetent cells (Stratagene).

2.5.4.2 Selection of positive transformants

Both pSC-A and TOPO vectors utilise blue/white colony selection to identify colonies that contain the plasmid with an insert. Blue-white colony selection of positive transformants was carried out by incubation overnight at 37°C on agar plates containing 100 µl of 0.1 M IPTG (Appendix 1) and 100 µl of 2% X-Gal (Appendix 1). Agar plates were made with LB agar (Appendix 1) and ampicillin (100 µg/ml). Positive colonies were screened for the presence of the correct insert by colony PCR. Briefly, positive colonies were lysed in 100 µl of distilled water in an eppendorf tube, boiled to 100°C for 5 minutes and then centrifuged for 15 minutes to pellet bacterial cell debris. 2 µl of the lysed colony preparation was used as template in a standard PCR reaction described in Section 2.5.1.1. Appropriate insert specific gene primers were used in the PCR reaction. Colonies containing the correct insert were grown overnight at 37°C in LB media (Appendix 1) containing ampicillin (100 µg/ml).

2.5.4.3 Cloning procedure for dsRNA preparation

For dsRNA preparation, DNA was sub-cloned from pSC-A or TOPO into the L4440 vector (originally designed by A. Fire and kindly provided by Julie Ahringer, University of Cambridge). The L4440 vector has a double T7 promoter which transcribes RNA from either end to produce dsRNA, and can be used to generate feeding libraries or to produce dsRNA for soaking. Both recipient vector and insert fragment were digested with appropriate restriction enzymes and subsequently gel purified (using Qiaquick Gel Extraction Kit as per manufacturer's instructions). Generally approximately 10 ng of linearised vector with varying ratios of insert DNA (1:1, 1:3 and excess insert) was used in a final volume of 10 µl. Ligations were set up using T4 DNA Ligase/10x ligase Buffer (New England Biolabs) following the manufacturer's instructions. Ligation reactions were incubated overnight at 15°C. 1 µl of this ligation mixture was used to transform 40 µl of XL-10 Gold Ultracompetent Cells (Stratagene) using standard procedures (Sambrook, 1989).

The genes selected for investigating RNAi susceptibility along with primers for producing dsRNA and subsequent RT-PCR are listed in Table 1, Appendix 2.

2.5.4.4 Cloning procedure for expression in *C. elegans*

For expression in *C. elegans*, DNA was sub-cloned from pSC-A or TOPO into the GFP/*LacZ* pPD96.04 expression vector (Fire vector, http://www.ciwemb.edu/pages/firelab.html). Both recipient vector and insert fragment were digested with appropriate restriction enzymes and subsequently gel purified (using Qiaquick Gel Extraction Kit as per manufacturer's instructions). Generally approximately 10 ng of linearised vector with varying ratios of insert DNA (1:1, 1:3 and excess insert) was used in a final volume of 10 µl. Ligations were set up using T4 DNA Ligase and 10x ligase Buffer (New England Biolabs) following the manufacturer's instructions. Ligation reactions were incubated overnight at 15°C. 1 µl of this ligation mixture was used to transform 40 µl of XL-10 Gold Ultracompetent Cells (Stratagene) using standard procedures (Sambrook, 1989).

Primers used for promoter amplification for expression studies in *C. elegans* are shown in Table 2, Appendix 2.

2.5.5 Purification of plasmid DNA

Bacterial cells containing the correct insert from cloning procedures (Section 2.5.4) were grown overnight in 10 ml of LB broth with 100 µg/ml ampicillin. 3 ml of this culture was centrifuged at 13,000 rpm for 15 minutes to obtain a bacterial pellet. The supernatant was removed and plasmid DNA was isolated using the Qiaquick Spin Miniprep Kit (Qiagen) following manufacturer's instructions. Plasmid DNA was eluted in 2 X 10 µl of distilled water heated to 50°C.

2.5.6 Sequencing of plasmid inserts

All sequencing reactions were carried out by MWG Eurofins Operon using the cycle sequencing technology (dideoxy chain termination/cycle sequencing) on ABI 3730XL sequencing machines. When inserts were cloned into pSC-A, the T3 and T7 primer sites flanking the Multiple Cloning Site (MCS) were used for sequencing reactions. When inserts were cloned into L4440 vector, L4440 sense and M13 universal (-21) were used for sequencing. When inserts were cloned into pPD96.04 vector, M13 reverse (-29) and 96.04 reverse were used for sequencing. The sequences of these primers are indicated in Table 5, Appendix 2.

2.5.7 Restriction enzyme digests of DNA

Restriction enzyme digests were carried out according to manufacturer's instructions (New England Biolabs). Incubations were generally performed overnight using 1-10 units of restriction enzyme for each µg of DNA digested with the appropriate buffer.

2.5.8 Double stranded RNA (dsRNA) production

A region of the gene of interest (generally 200-400 bp) was PCR amplified from *H. contortus* L3 stage cDNA or adult *H. contortus* day 11 cDNA library, kindly provided by Professor David Knox. PCR products were cloned into pSC-A or

TOPO, then sub-cloned into the L4440 vector using methods described in Section 2.5.4. Primers used to amplify the gene of interest are shown in Table 1, Appendix 2 as 'gene-RNAi F1/R1'. dsRNA was prepared using the T7 Ribomax RNAi kit (Promega). Briefly, plasmids were linearised by restriction enzyme digest (generally *Xba*I and *Xho*I unless otherwise stated) on opposite sides of the DNA insert. Separate reactions were set up using 5 µg of linearised DNA template according to the manufacturer's instructions to allow the transcription of single stranded RNA (ssRNA). The concentrations of the two complementary ssRNA strands were then measured by spectrophotometer at λ 260 nm and 280 nm. Equal amounts of the two complementary ssRNAs were mixed with 10X injection buffer (Appendix 1) and annealed at 37°C for 30 minutes to form dsRNA. The resulting dsRNA was analysed on a 1% agarose gel along with a sample of each ssRNA to observe a shift in band size due to the annealing of the two ssRNAs.

In all except one instance of soaking *H. contortus* larvae in dsRNA, the dsRNA was prepared using the T7 Ribomax RNAi kit (Promega). However, dsRNA was also prepared using the MegaScript T7 kit (Ambion) according to the manufacturer's instructions. The yield of dsRNA obtained from using the Ambion kit was significantly lower compared to the Promega kit, therefore the Promega kit was used to prepare dsRNA for all subsequent reactions.

2.5.9 Total RNA extraction from cultured *H. contortus* L3 larvae

H. contortus exsheathed L3 larvae were cultured as described in Section 2.1.3 and washed 2X in PBS to remove culture media and dsRNA. 180 µl of RT PCR buffer (Appendix 1) was added to the larvae, followed by 10 µl of 10 mg/ml Proteinase K (Sigma-Aldrich). The mixture was vortexed for 2 minutes and then 10 µl of 2-mercaptoethanol (Sigma-Aldrich) was added and vortexed again. The samples were then frozen at -80°C for a minimum of 30 minutes, preferably overnight.

Samples were removed from the freezer and incubated at 55°C for 1 hour and left on ice for 10-15 minutes. 500 µl of Total RNA Isolating Reagent (Advanced Biotechnologies Ltd) was added. Samples were mixed well by inverting and incubated on ice for 10 minutes. 100 µl of chloroform (pH 8.0, Sigma-Aldrich) was then added to the samples and mixed by inverting the tubes and incubated

on ice for a further 15 minutes. Phases were separated by centrifugation at 13,000 rpm for 15 minutes and the upper aqueous phase containing the RNA was removed. RNA was precipitated by adding 500 µl of isopropanol and incubating on ice for 30-60 minutes, centrifuging at 13,000 rpm for 30 minutes and removing the supernatant. The RNA pellet was washed with 70% (v/v) ethanol, briefly air dried and resuspended in 20 µl of DEPC-treated water (Appendix 1). RNA was stored at -80°C until required.

2.5.10 Reverse transcription PCR (RT-PCR)

Reverse transcription PCR (RT-PCR) was carried out using the SuperScript One-Step RT-PCR System (Invitrogen). Total RNA isolated in section 2.5.9 was split into two tubes, into which either control (non target gene primers, generally *H. contortus* superoxide dismutase *Hc-sod-1* unless otherwise stated) or RT-PCR primers for the target gene were added. RT-PCR primers were positioned external to the primers used to amplify DNA for dsRNA production and also incorporated an intronic region to ensure that if genomic DNA contamination is present in the extracted RNA, a size difference could be seen between cDNA and genomic DNA on the agarose gel. The primer design process is explained in Section 2.7.5, Figure 2.3 and Figure 2.4. Amplification of non-target gene *Hc-sod-1* was used as an internal control for the quality of RNA extracted. Primers used for RT-PCR to check for transcript knockdown are shown in Table 1, Appendix 2 as 'gene RTPCR F1/R1'.

Standard RT-PCR conditions were 30 minutes at 50°C followed by 2 minutes at 94°C for one cycle, 30-35 cycles of 30 seconds at 94°C, 30 seconds at 56°C, 1 minute at 72°C, and a final extension of 5 minutes at 72°C.

2.5.11 Total RNA extraction from adult *H. contortus*

Adult *H. contortus* were isolated following the *in vivo* RNAi assay described in section 2.2.1.2 and frozen at -80°C. Samples of frozen worms, weighing approximately 0.20 g, were used to isolate total RNA using the Qiagen RNeasy Mini kit (Qiagen), according to the manufacturer's instructions. The RNA was resuspended in 50 µl of RNAse-free water. The quality of the RNA was assessed by gel electrophoresis analysis; 1 µl of the RNA was loaded onto a 1% agarose gel

as described in section 2.5.2. Two distinct bands depicting the 28S ribosomal subunit and the 18S ribosomal subunit were indicative of good quality RNA. The concentration of the RNA was measured using the spectrophotometer at λ 260 nm and 280 nm. The total RNA sample was stored at -80°C until further use.

2.5.12 1st strand cDNA synthesis using total RNA from adult *H. contortus*

2 µg of total RNA from adult worms, extracted as described in section 2.5.11, was used to synthesise 1st strand cDNA. The AffinityScript Multiple Temperature cDNA synthesis kit (Stratagene) was used according to the manufacturer's instructions. The oligo (dT) primer supplied with the kit was utilised, and the reaction mixture was incubated at 65°C for 5 minutes, followed by 10 minutes at room temperature to allow primer annealing. Reverse transcription was carried out at 42°C for 1 hour followed by heat inactivation of the reaction at 70°C for 15 minutes. The adult *H. contortus* cDNA was stored at -20°C until further use in a standard PCR reaction as described in section 2.5.1.1.

2.6 Statistical analysis

All statistical analysis was performed using Microsoft Office Excel 2003.

2.7 Bioinformatics methods

2.7.1 Software and databases used

All bioinformatics analysis was carried out on Vector NTI Advance software (Invitrogen, version 10 and 11). All alignments of protein and nucleotide sequences were carried out on the Align X programme, a part of the Vector NTI Advance software package. *C. elegans* data was accessed on the Wormbase website (http://www.wormbase.org). *H. contortus* genome data was accessed on the Sanger Institute BLAST server (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/h_contortus).

Nematode Expressed Sequence Tag (EST) data, including *H. contortus* EST data, was accessed on the European Bioinformatics Institute (EBI) server (http://www.ebi.ac.uk/Tools/blast2/parasites.html) and the Nembase database (http://www.nematodes.org/nembase3/index.shtml).

2.7.2 Phylogenetic analysis

Phylogenetic analysis was carried out using the amino acid sequences of proteins of interest. Protein sequences were aligned using the multiple sequence alignment programme CLUSTAL X (Thompson *et al.*, 1997). Phylogenetic tree was constructed by the Neighbour-Joining (NJ) method in MEGA Version 4 (Tamura *et al.*, 2007). Bootstrap sampling analysis from 500 replicates was adopted to evaluate internal branches.

2.7.3 Search for *H. contortus* genes with a high expression level in L3 stage larvae

Search for genes highly expressed in L3 stage larvae was carried out using the Nembase database (www.nematodes.org/nembase3/stageSpec.shtml). Genes were identified using the 'Lifecyle Stage' search, using 'Haemonchus contortus' and '>6' for larval L3 growth stage EST expression levels. Three genes were selected as candidates for RNAi experiments in *H. contortus* in this manner. A fourth gene, GTP cyclohydrolase, was selected due to a published account of 10 ESTs at the L3 stage (Hoekstra *et al.*, 2000). The genes are listed in Table 3.1.

2.7.4 Expression pattern search on *C. elegans* and identification of homologues in *H. contortus*

Searches for genes with a specific expression pattern in *C. elegans* were carried out using the Expression Pattern Search on Wormbase (http://wormbase.org/db/searches/expr_search). The search terms used were 'Intestine' (WBbt:0005772), 'Amphid neuron' (WBbt:0005394), 'Amphid Sheath Cell' (WBbt:0006754) and 'Excretory Cell' (WBbt:0005812). The protein sequences of the *C. elegans* genes from this search were then used to carry out a search amongst the *H. contortus* genomic contigs (tBLASTn against the *H.*

contortus combined worms supercontigs 20/08/08). The resultant *H. contortus* genomic sequence was then used to carry out a reciprocal BLAST search against the *C. elegans* database, to confirm that the originally identified *C. elegans* gene is the putative homologue (BLASTn against the *C. elegans* WormPep database). The *H. contortus* gene sequences were then used to search the ESTs located at the Washington University Parasite Genomes Database (http://www.ebi.ac.uk/Tools/blast2/parasites.html) to check if they were expressed genes in *H. contortus* or other parasites. Only genes that fulfilled all these criteria were considered as candidates for RNAi experiments in *H. contortus*. Lack of full gene sequence data limited the region of genes which could be amplified for dsRNA production. A list of selected genes is shown in Table 3.2.

2.7.5 Primer design for RNAi and RT-PCR

Where possible, *H. contortus* amino acid sequence for a target gene was aligned with the *C. elegans* amino acid sequence to reliably identify exon sequences to which PCR primers could be designed. Primers were designed to span an intron, to ensure that any amplification of contaminating genomic DNA during RT-PCR could be detected. A schematic of this is shown in Figure 2.3 and Figure 2.4. A list of primers designed in this manner for dsRNA production and subsequent RT-PCR is shown in Table 2.

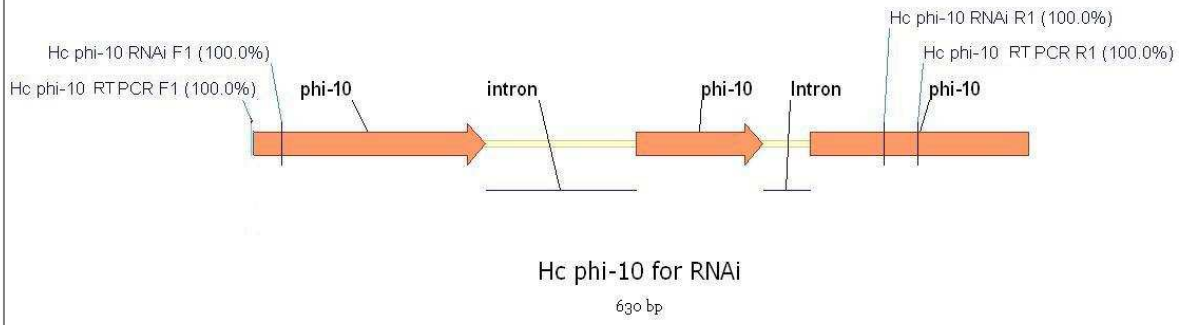


Figure 2.3. Primer design for RNAi of the *Hc-phi-10* gene.

Exons of *Hc-phi-10* are shown in orange and were identified from translation of *Hc-phi-10* genomic DNA sequence and aligning with the putative homologue protein in *C. elegans*. RT-PCR F1/R1 refers to the primers used for RT-PCR to check for transcript knockdown. RNAi F1/R1 refers to the primers used to amplify DNA for dsRNA production.

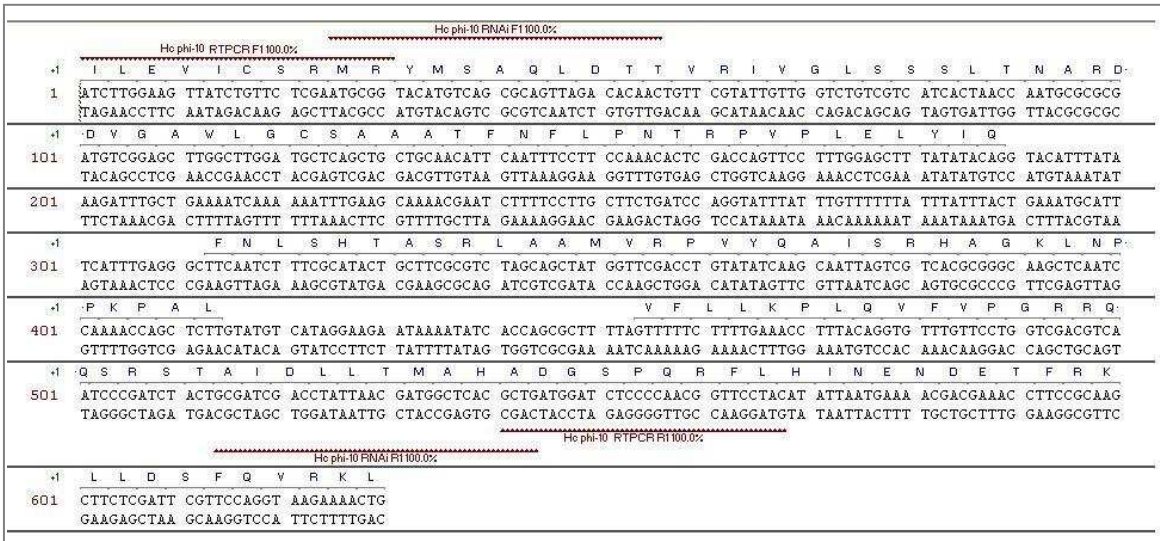


Figure 2.4. Primer design for RNAi of *Hc-phi-10* at the nucleotide level

Coding sequence is shown translated. RT-PCR primers lie external to the RNAi primers. The expected products using primers Hc-phi-10 RTPCR F1/Hc phi-10 RTPCR R1; 406 bp (on cDNA), 568 bp (on gDNA). Expected products using primers Hc-phi-10 RNAi F1/Hc-phi-10 RNAi R1; 358 bp (on cDNA), 520 bp (on gDNA).

2.7.6 Analysis of *H. contortus* promoters for regulatory motifs

Sequences of *H. contortus* promoter regions were PCR amplified and cloned as described previously in Sections 2.5.1, 2.5.1.3 and 2.5.4. Primers used for amplifying promoter regions by PCR are listed in Table 4, Appendix 2. Promoter regions were analysed by using Align X to align nucleotide regions to search for any obvious regions of similarity visually. Promoters were also analysed using the MotifSampler Programme located at http://homes.esat.kuleuven.be/~thijs/Work/MotifSampler.html (Thijs *et al.*, 2002). This programme finds over-represented motifs in the promoter sequences, to be expected if the identified motifs were involved in gene regulation.

The promoter sequences were then searched for these motifs in order to ascertain if the position was conserved within different promoters. The Regulatory Sequence Analysis Tools (RSAT) database was used, located at http://rsat.ulb.ac.be/rsat/ (van Helden, 2003).

Chapter 3

RNA interference in *Haemonchus contortus*

3.1 Introduction

RNA interference, or RNAi, is a mechanism for RNA guided silencing of gene expression that is common in eukaryotic cells. It was first characterised in *C. elegans,* where RNAi is induced by exposure to double-stranded RNA (dsRNA) which leads to the degradation of mRNA sequences homologous to the introduced dsRNA (Fire *et al.*, 1998). dsRNA was injected into the gonads of young adult hermaphrodites and this technique was successful in completely silencing the mRNA transcript of tested genes. Subsequent to the development of the dsRNA injection protocol, *C. elegans* was also soaked in dsRNA with successful knockdown of gene expression, although slightly less efficiently than with direct microinjection (Tabara *et al.*, 1998). An RNAi response was also achieved by feeding *C. elegans* with bacteria engineered to produce dsRNA, but again this technique was slightly less efficient compared with direct microinjection (Timmons & Fire, 1998).

Nevertheless, these dsRNA delivery methods along with the complete availability of *C. elegans* genome sequence information (*C. elegans* Genome Sequencing Consortium, 1998) allowed the global study of gene function by large scale RNAi screens. The first such screen used RNAi by bacterial feeding to target almost 90% of predicted genes on *C. elegans* chromosome I (Fraser *et al.*, 2000). Subsequently, the first genome-wide RNAi screen for *C. elegans* was performed with the construction of an RNAi feeding library, in which 16,757 of the predicted 19,757 genes in *C. elegans* were cloned and dsRNA expressed in bacteria under the control of double T7 promoters in plasmid L4440 (Kamath *et al.*, 2003). Both these screens were performed in a wild-type genetic background (*C. elegans* Bristol N2 strain). Following the discovery that the *C. elegans* gene *rrf-3* encodes an RNA directed RNA polymerase, mutation of which leads to enhanced RNAi gene silencing (see pathway described in Section 1.2 and Simmer *et al.*, 2002) another genome-wide screen was performed in RNAi sensitive *rrf-3* mutant worms, allowing the detection of phenotypes that were previously not observed in a wild-type background (Simmer *et al.*, 2003).

While RNAi is a robust tool for analysing gene function in *C. elegans*, recent evidence has indicated that applying RNAi to parasitic nematodes is not as

straightforward as may have been expected. Several difficulties are encountered when attempting RNAi in parasitic nematodes, including, but not limited to, reproducibility, specificity and susceptibility (reviewed by Geldhof *et al.*, 2007). Not all studies have investigated a decrease in target transcript level, and instead only reported indirect evidence for RNAi success, such as phenotype or protein levels (see Table 1.1). It is therefore difficult to determine whether effects are specific to the knockdown of the target gene or non-specific effects of the procedure used. For example, in *Onchocerca volvulus*, RNAi against cathepsin-L and Z like cysteine proteases used immunolocalisation studies to demonstrate a reduction in both protein levels in moulting larvae, in addition to a reduction of 92% and 86% in moulting rate compared to untreated controls (Lustigman *et al.*, 2004). However immunolocalisation studies do not quantitatively show how many worms display this effect, and a decrease in transcript level of the target genes was not investigated.

It has also been difficult to reproduce some of the published studies. RNAi of beta-tubulin, RNA polymerase II large subunit and microfilarial sheath protein in *Brugia malayi* adults showed that the transcript levels of these three genes start to decrease after 14-17hrs of soaking (Aboobaker & Blaxter, 2003). However, attempts to repeat RNAi in the related nematode *Brugia pahangi* were unsuccessful as adult worms did not survive in culture (Eileen Devaney, personal communication). An extensive study of RNAi in *H. contortus* tested eleven different genes for RNAi efficacy (Geldhof *et al.*, 2006). In this study, only two genes showed a decrease in transcript level; beta tubulin showed consistently repeatable knockdown while *sec-23*, a secretory pathway gene, showed a decrease in transcript levels only three times in five different attempts. Another study in *Ostertagia ostertagi* for RNAi silencing showed a lack of consistently reproducible results, and was the first study to report these inconsistencies (Visser *et al.*, 2006). For example, when the tropomyosin gene was targeted by soaking *O. ostertagi* larvae in dsRNA, a decrease in transcript level was observed five times and no decrease observed twice. Transcript level of the superoxide dismutase gene showed a decrease once and no decrease twice. Thus it is clear that at least for some parasitic nematodes, consistently reproducible RNAi induced gene silencing is difficult.

The specificity of RNAi in parasitic nematodes has also been called into question in some studies. Non-specific effects of soaking in dsRNA have been reported for *O. volvulus*; soaking larvae in control dsRNAs, designed to an unrelated *C. elegans* gene and an intronic region of an *O. volvulus* gene, resulted in a reduction in moulting rate relative to the untreated controls (Lustigman *et al.*, 2004). This indicates that soaking in exogenous dsRNA could have a non-specific toxic effect upon larvae. In *B. malayi*, a reduction in motility was also reported in control dsRNA treated worms compared to controls incubated in medium alone, but this reduction was not deemed significant (Aboobaker & Blaxter, 2003). Off-target effects have also been reported in plant parasitic nematode RNAi experiments; non-nematode derived dsRNAs designed for use as controls in RNAi screens appear to induce aberrant phenotypes such as decreased motility (Dalzell *et al.*, 2009b). It has since been shown that discrete 21 bp siRNAs might be more suitable for plant parasitic nematode RNAi as off target effects are limited (Dalzell *et al.*, 2009a).

However one of the biggest difficulties encountered with RNAi in parasitic nematodes is susceptibility. It appears that not all genes are equally susceptible to RNAi silencing. Of eleven genes tested for RNAi in *H. contortus,* only beta tubulin was consistently silenced in two independent studies (Geldhof *et al.*, 2006; Kotze & Bagnall, 2006). In *O. ostertagi*, genes encoding ubiquitin, transthyretin-like protein and a 17 kDa ES protein showed a complete lack of susceptibility to RNAi. More recent work with *Heligmosomoides polygyrus* attempted RNAi of the tropomyosin gene by feeding, soaking and electroporation with dsRNA. This study showed that no decrease in tropomyosin transcript level could be observed following any delivery method of dsRNA (Lendner *et al.*, 2008).

These mixed results cast doubt upon the application of RNAi as a robust and successful technique to study gene function in parasitic nematodes. Without reliability and reproducibility, RNAi cannot be used as a functional genomics tool in *H. contortus* as it is with *C. elegans.* The main aims of this chapter are:

- To examine the reproducibility and the reliability of RNAi in *H. contortus*

- Attempt to answer the question of why RNAi is successful for some genes but not others

- To search for phenotypic effects of RNAi in *H. contortus*, both *in vitro* and *in vivo*.

3.2 Results

3.2.1 RNAi silencing of beta tubulin isotype-1

Previous studies have demonstrated the specific knockdown of beta tubulin by RNAi in *H. contortus* (Geldhof *et al.*, 2006; Kotze & Bagnall, 2006). The first aim of this study was to confirm the knockdown and determine how reliable beta tubulin silencing is and whether this is subject to the same variations as other genes previously targeted by RNAi. A 260 bp region of *H. contortus* beta tubulin isotype-1 gene (*tub 8-9*, Accession number M76493) (Geary *et al.*, 1992), hereafter referred to as *Hc-bt-iso-1*, was amplified as described in section 2.5.8. The PCR product was cloned using standard techniques and dsRNA was synthesised as described in the materials and methods.

H. contortus L3 larvae were exsheathed and soaked in target (*H. contortus bt-iso-1*) or control (*C. elegans rab-7*) dsRNA for 72 hours at 37°C, 5% CO_2. RT-PCR was carried out on RNA extracted from treated larvae (section 2.5.9), and transcript levels were compared between target and control dsRNA treated larvae, relative to the non-target control gene (*Hc-sod-1*), as described in section 2.5.10. *Hc-sod-1* has been previously shown to be constitutively expressed and therefore selected as a control gene for RT-PCR studies (Liddell & Knox, 1998). Primers used for RT-PCR were designed external to the RNAi targeted region, to prevent the amplification of the added dsRNA as described in section 2.7.5. The sequence of primers used and the expected size of the products are shown in Table 1, Appendix 2.

As shown in Figure 3.1, control *Ce-rab-7* dsRNA treated larvae express both target *Hc-bt-iso-1* and non-target *Hc-sod-1*. Larvae treated with *Hc-bt-iso-1* dsRNA express only *Hc-sod-1*, showing the complete and specific knockdown of the *Hc-bt-iso-1* transcript. This silencing was consistently reproducible in over 15 separate experiments during the course of this project, using different batches of dsRNA and different batches of larvae.

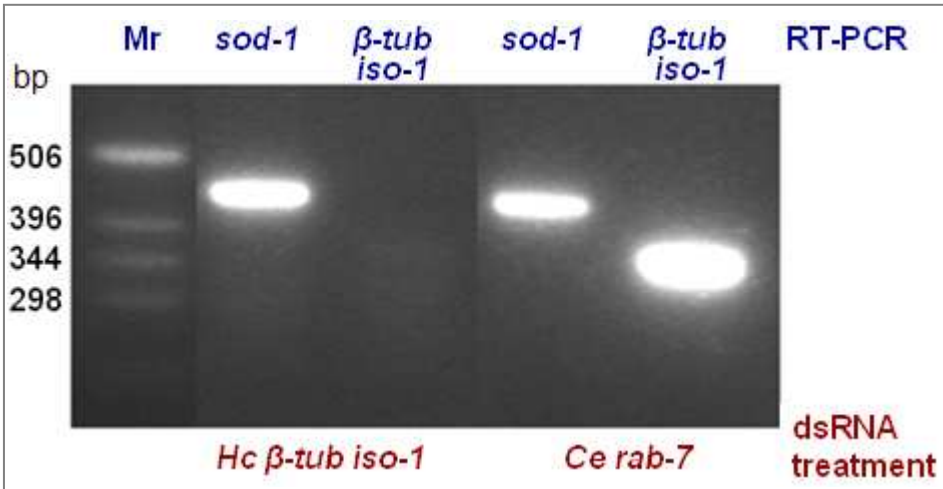


Figure 3.1. *H. contortus beta tubulin isotype-1* is reliably silenced by dsRNA soaking

RT-PCR detection of *Hc-bt-iso-1* mRNA levels, following 72 hours of soaking in *Hc-bt-iso-1* dsRNA or control *Ce-rab-7* dsRNA. Expression of a non-target gene (*Hc-sod-1*) in the same RNA sample is shown for comparison. One kilobase DNA marker is shown in bp (Mr).

3.2.2 Time course RNAi silencing of beta tubulin isotype-1

A time course treatment of dsRNA was carried out in order to establish when the decrease in transcript levels could be observed. Larvae were soaked in dsRNA for 24 or 48 hours, RNA extracted and RT-PCR carried out.

As shown in Figure 3.2A, after 24 hours of soaking in *Hc-bt-iso-1* dsRNA, the *Hc-bt-iso-1* transcript level remains unchanged. After 48 hours of soaking in *Hc-bt-iso-1* dsRNA (Figure 3.2B), a significant decrease in transcript level can be seen, although a faint band can be observed upon close inspection of the RT-PCR gel analysis. After 72 hours of soaking, complete knockdown of transcript levels can be observed as shown in Figure 3.1.

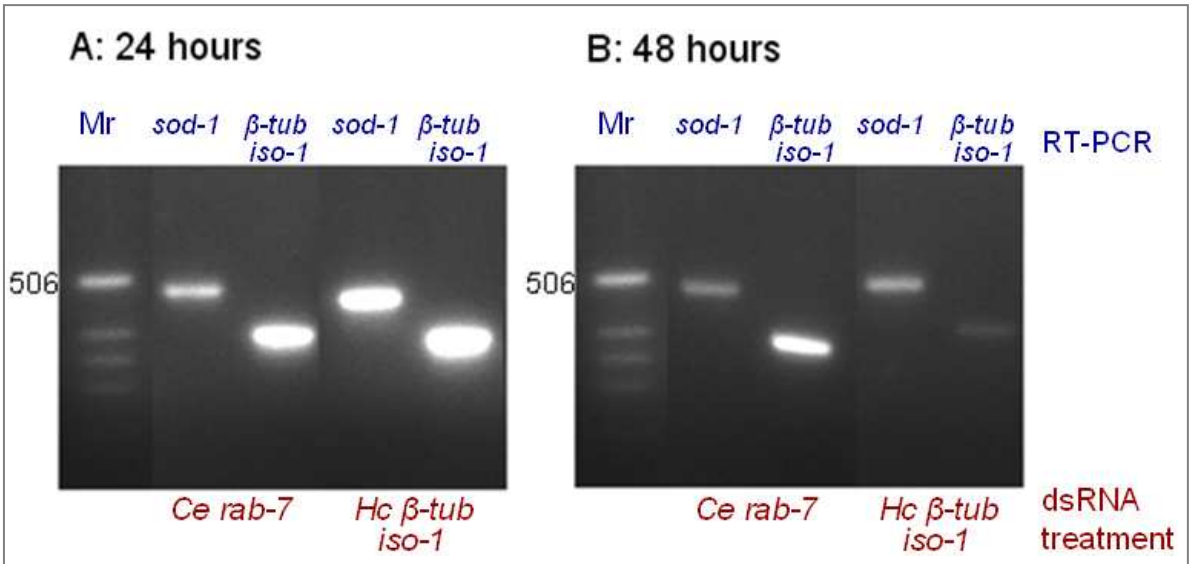


Figure 3.2. Transcript levels of RNAi targeted *beta tubulin iso-1* following soaking of *H. contortus* L3 larvae in dsRNA for 24 hours and 48 hours

(A) *Hc-bt-iso-1* and *Hc-sod-1* transcript levels following 24 hours of soaking in control *Ce-rab-7* dsRNA or *Hc-bt-iso-1* dsRNA. Expression of a non-target gene (*Hc-sod-1*) in the same sample is shown for comparison. **(B)** *Hc-bt-iso-1* and *Hc-sod-1* transcript levels following 48 hours of soaking in control *Ce-rab-7* dsRNA or *Hc-bt-iso-1* dsRNA. Expression of a non-target gene (*Hc-sod-1*) in the same sample is shown for comparison.

3.2.3 RNAi silencing of highly expressed genes

Beta tubulin is involved in microtubule polymerisation and is therefore anticipated to be expressed in all cell types. RT-PCR suggests that beta tubulin is expressed at high levels in *H. contortus* L3 larvae. RNAi was successful in knocking down *Hc-bt-iso-1* transcript levels as shown in Figure 3.1. In order to investigate the possibility that successful knockdown might be related to the abundance of the transcript, the analysis was extended to *H. contortus* genes identified as being highly expressed, based on EST data. Four genes highly expressed at the L3 stage were chosen for RNAi analysis as described in section 2.7.3. The genes chosen showed the highest number of ESTs from a *H. contortus* L3 SL1 library (http://www.nematodes.org/nembase3/stageSpec.shtml and Hoekstra *et al.*, 2000) and are listed in Table 3.1.

Table 3.1. Genes highly expressed in the L3 larval stage in *H. contortus*

Name **(Cluster ID)**	HCC00700 (‘*Hc*-700’)	HCC00623 (‘*Hc*-623’)	HCC00645 (‘*Hc*-645’)	*Hc*-GTPch
No. of ESTs at L3 stage	7	12	16	10
Description (similar to)	Microsomal signal peptidase subunit in *C. elegans*	Large ribosomal protein subunit in *C. elegans*	Ribosomal protein S27 mRNA sequence in *H. contortus*	GTP cyclohydrolase precursor (Hoekstra *et al.*, 2000)
Putative *C. elegans* homologue	C34B2.10	Y48B6A.2	F56E10.4	F32G8.6

The four chosen genes, referred to as *Hc-700, Hc-623, Hc-645* and *Hc-GTPch*, were amplified from *H. contortus* L3 larvae cDNA and cloned using standard procedures (section 2.5.4.3). The sizes of the dsRNAs were 310, 189, 253 and 369 bp for *Hc-700, Hc-623, Hc-645* and *Hc-GTPch*, respectively. Larvae were soaked for 72 hours in dsRNA specific to each gene. RT-PCR was carried out on RNA isolated from the treated larvae and the levels of target and non-target (*Hc-sod-1*) transcripts were compared to assess any decrease in target transcript.

As indicated in Figure 3.3A, complete and specific knockdown of the *Hc-700* transcript following dsRNA treatment is demonstrated. A decrease, but not complete loss, in transcript level of *Hc-GTPch* was also observed (Figure 3.3B). A faint band above the *Hc-GTPch* transcript representing the amplification of genomic DNA could also be detected. During this work, amplification from contaminating genomic DNA was occasionally found when transcript knockdown occurred. No decrease in transcript level was seen for either *Hc-623* or *Hc-645* (Figure 3.3C and 3D) and this was consistent in three subsequent experiments. However, the successful knockdown of *Hc-700* and *Hc-GTPch* was not reproducible following this initial experiment, despite three additional attempts at repeating the experiment. Therefore, from the finding that two of the target genes could not be silenced and the other two target genes showed variable results, it was concluded that genes that are highly expressed in L3 larvae are not necessarily susceptible to RNAi.

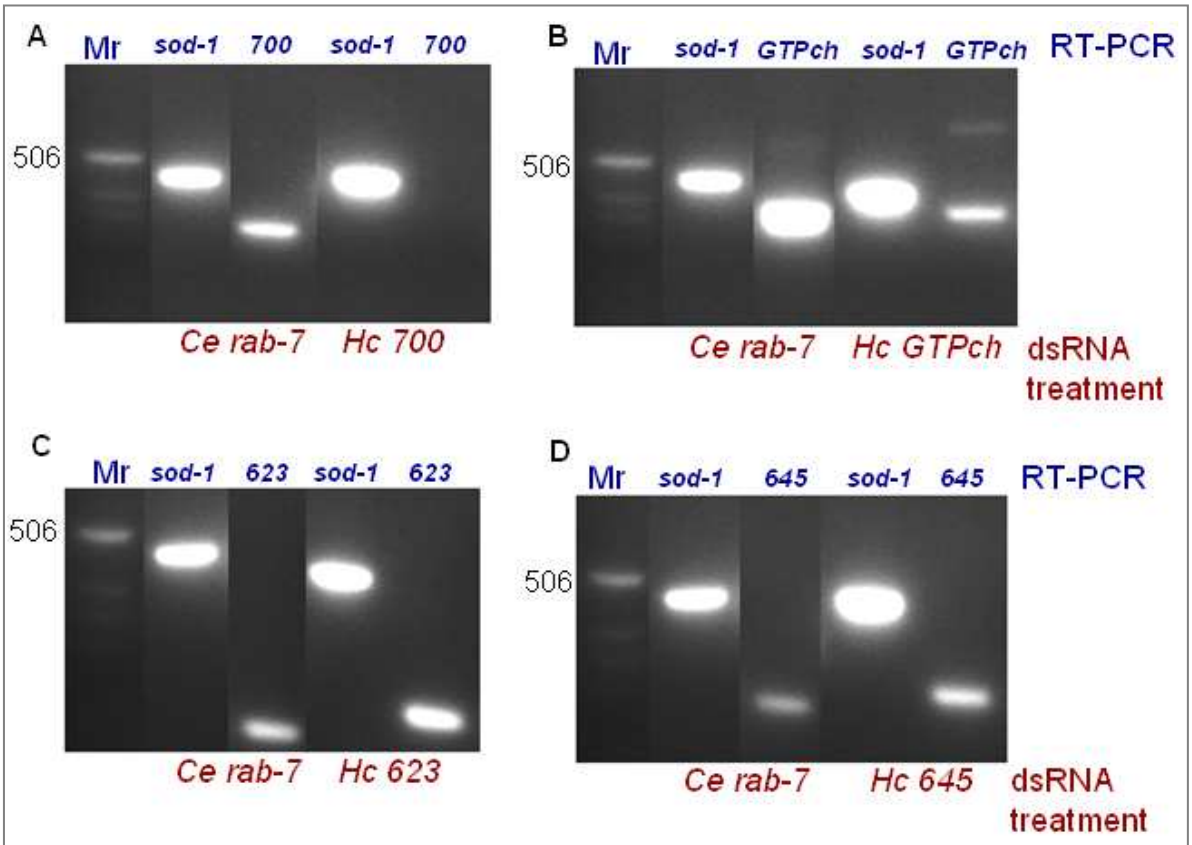


Figure 3.3. Transcript levels of RNAi targeted genes which are highly expressed at the L3 larval stage in *H. contortus.*

(A) *Hc-700* and *Hc-sod-1* transcript levels following 72 hours of soaking in control *Ce-rab-7* dsRNA or *Hc-700* dsRNA. Expression of a non-target gene (*Hc-sod-1*) in the same sample is shown for comparison. **(B)** *Hc-GTPch* and *Hc-sod-1* transcript levels following 72 hours of soaking in control *Ce-rab-7* dsRNA or *Hc-GTPch* dsRNA. **(C)** *Hc-623* and *Hc-sod-1* transcript levels following 72 hours of soaking in control *Ce-rab-7* dsRNA or *Hc-623* dsRNA. **(D)** *Hc-645* and *Hc-sod-1* transcript levels following 72 hours of soaking in control *Ce-rab-7* dsRNA or *Hc 645* dsRNA. The decrease in transcript levels of *Hc-700* and *Hc-GTPch* in A and B respectively was observed in only one in four experiments.

3.2.4 RNAi silencing of genes expressed at accessible locations within the *H. contortus* L3 larvae

Although beta tubulins are thought to be ubiquitously expressed, benzimidazole effects on nematode beta tubulins are most significant in the intestine (Jasmer *et al.*, 2000). In addition, recent work has shown that the *Hc-bt-iso-1* promoter region is strongly expressed in the posterior intestine and amphid neurons in transgenic *C. elegans* (Gary Saunders, PhD Thesis University of Glasgow 2009). If this expression in intestine and amphids is representative of expression in *H. contortus*, it is possible that these sites are accessible to dsRNA from the environment. Therefore the site of gene expression, rather than amount of gene expressed, may be an important factor in susceptibility to RNAi.

This hypothesis was examined by carrying out RNAi on *H. contortus* genes which are thought to be expressed in sites which are accessible to the environment. Genes were selected based on previous experiments in *H. contortus*. This data is currently limited due to the lack of detailed information on spatial expression, particularly for the L3 stage of *H. contortus*. The aminopeptidase encoding gene *H11* (accession number X94187) was selected for RNAi as H11 was identified in intestine extracts from adult *H. contortus* and antibody raised against native H11 localises to the worm intestine surface (Newton & Meeusen, 2003; Smith *et al.*, 1997). In addition, analysis of the expression pattern directed by the *Hc-H11* promoter, at least in transgenic *C. elegans,* supported the selection of this gene as an RNAi target (Chapter 5).

A 400 bp region of *Hc-H11* was amplified from *H. contortus* L3 cDNA. dsRNA was synthesised and larvae were incubated in this for 72 hours, followed by total RNA extraction and RT-PCR. Figure 3.4 shows the complete and specific knockdown of *H. contortus H11* following dsRNA treatment. This knockdown was consistently repeatable in ten separate experiments.

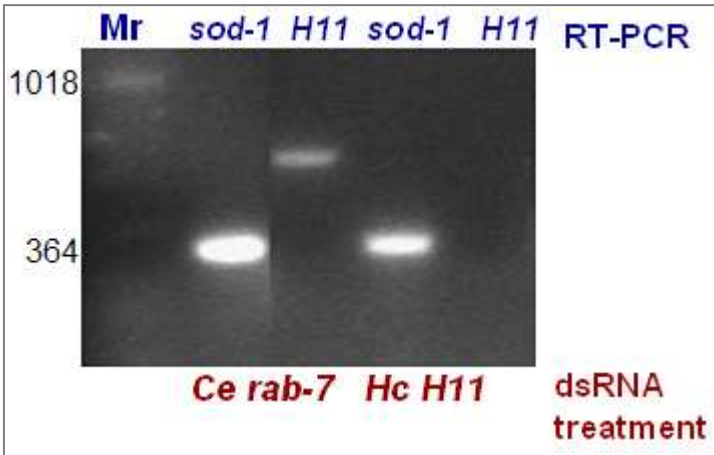


Figure 3.4. Transcript levels of RNAi targeted *Hc-H11* following soaking of *H. contortus* L3 larvae in *Hc-H11* dsRNA

RT-PCR detection of *Hc-H11* mRNA levels following 72 hours soaking in *Hc-H11* dsRNA or control *Ce-rab-7* dsRNA. Expression of a non-target gene (*Hc-sod-1*) in the same RNA sample is shown for comparison.

Another candidate gene for RNAi was the *Hc-asp-1* gene (Accession Number A30245). *Hc-asp-1* (activation associated secretory protein) shows similarity to *asp* genes from other nematodes, and also referred to as venom allergen-like proteins (VAP). *C. elegans* also expresses *asp*-like genes, and the *C. elegans vap-1* promoter has been shown to direct expression in the amphid cells (Aline Visser, PhD Thesis, University of Ghent 2008). However, their precise function is currently unknown. In *H. contortus,* L3 stage larvae express the *Hc-asp-1* gene and also secrete the ASP-1 protein when maintained under *in vitro* culture conditions (Douglas Clark, PhD Thesis University of Glasgow 2006). This was consistent with previous work showing that hookworm *Ancyclostoma caninum* L3 larvae secrete ASP-1 protein into culture medium (Hawdon *et al.*, 1996). In addition, *Onchocerca volvulus* ASP-1 protein localises to the secretory granules of L3 larvae (MacDonald *et al.*, 2004). Therefore, as nematode secreted proteins are often released from excretory/secretory cells, which have access to the environment, *Hc-asp-1* was selected as a candidate gene for RNAi.

A 620 bp region of *Hc-asp-1* gene was amplified from *H. contortus* L3 larvae cDNA and cloned using standard procedures (section 2.5.4.3). Larvae were soaked for 72 hours in *Hc-asp-1* dsRNA, following which the total RNA was isolated for RT-PCR analysis. As found for *Hc-H11*, complete and specific knockdown of *Hc-asp-1* transcript following *Hc-asp-1* dsRNA treatment was

observed (Figure 3.5). This knockdown was consistently repeatable in five separate experiments.

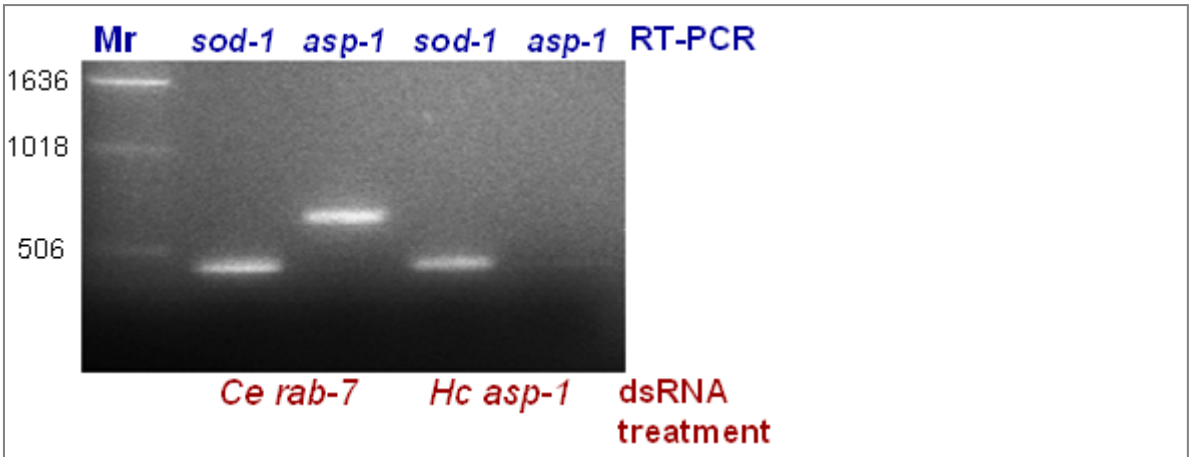


Figure 3.5. Transcript levels of RNAi targeted *Hc-asp-1* following soaking of *H. contortus* L3 larvae in dsRNA

RT-PCR detection of *Hc-asp-1* mRNA levels following 72 hours of soaking in *Hc-asp-1* dsRNA or control *Ce-rab-7* dsRNA. Expression of a non-target gene (*Hc-sod-1*) in the same sample is shown for comparison.

The successful knockdown of both *Hc-H11* and *Hc-asp-1* genes using RNAi may be related to the possibility that these genes are expressed in sites that are accessible to the environment; the intestine for *Hc-H11* and secretory cells for *Hc-asp-1*. Hence, more candidate genes for RNAi studies were selected, based on their putative sites of expression. Currently, direct information on gene expression in the L3 stage of *H. contortus* is limited. However, there is a wealth of data on spatial expression patterns of *C. elegans* genes from promoter-reporter studies (Hope, 1991). Given the close phylogenetic relationship of *C. elegans* and *H. contortus,* expression data from *C. elegans* was used to identify putative homologues in *H. contortus* which may have conserved expression patterns. *C. elegans* genes expressed in sites of interest (intestine, amphid cells and excretory cell) were identified using the expression pattern search function in Wormbase (http://wormbase.org/db/searches/expr_search). The sequences of these *C. elegans* genes were then used to search the *H. contortus* genome information for putative homologues using tBLASTn, as described in detail in Section 2.7.4. The genes selected in this manner were *H. contortus* putative homologues of *C. elegans aqp-2, ceh-6, exc-4, ins-1* and *phi-10,* as shown in Table 3.2.

Table 3.2. *C. elegans* genes expressed in sites of interest (excretory cell, intestine, amphid cells).

Genes were selected based on expression pattern data in *C. elegans* with expression in but not restricted to the cells indicated. Putative homologues of these genes were used in RNAi in *H. contortus*.

Gene	*aqp-2*	*phi-10*	*exc-4*	*ins-1*	*ceh-6*
Site of expression in *C. elegans*	excretory cell	excretory cell and intestine	excretory cell	amphid sensory cells and intestine	excretory cell and neurons
Function in *C. elegans*	Encodes an aquaglyceroporin protein, involved in regulating osmosis	Encodes a RNA helicase DEAD-box superfamily protein	Encodes a member of the chloride intracellular channel (CLIC) family of anion channels	Encodes an insulin-like peptide, orthologous to human insulin	Encodes a POU-family homeodomain protein, transcription factor

Regions of putative *H. contortus aqp-2, ceh-6, exc-4, ins-1* and *phi-10* genes were amplified from *H. contortus* L3 cDNA (section 2.5.4.3) and dsRNA was prepared (section 2.5.8). PCR amplified regions ranged from 203 bp – 410 bp. Larvae were incubated in dsRNA for 72 hours and subsequently the total RNA was extracted and used in RT-PCR to assess transcript levels (section 2.5.9 and 2.5.10, respectively).

As shown in Figure 3.6A, a significant decrease in *Hc-aqp-2* transcript level is seen following dsRNA treatment. This decrease in transcript level was repeated in two subsequent experiments. Successful silencing of *Hc-phi-10* gene expression was also observed in this and in two subsequent experiments (Figure 3.6B). A faint band representing the amplification of *Hc-phi-10* in genomic DNA was also observed, due to lower levels of the target cDNA following RNAi. RNAi was unsuccessful at silencing *Hc-exc-4* expression, since *Hc-exc-4* transcript levels are unchanged between target and control dsRNA treatments in three experiments (Figure 3.6C). RNAi silencing of *ins-1* appears to be similarly unsuccessful although detection of *Hc-ins-1* in control and treated larvae is difficult due to low expression of the gene (Figure 3.6D). *Hc-ceh-6* RNAi seems show increased expression of the target gene; in all three experiments, treating larvae with target specific *Hc-ceh-6* dsRNA seems to show an upregulation of *Hc-ceh-6* transcript level (Figure 3.6E). A similar result in which the upregulation of

the target gene was observed when attempting RNAi of *Hc-vha-10* using *Hc-vha-10* dsRNA has been reported (Geldhof *et al.*, 2006).

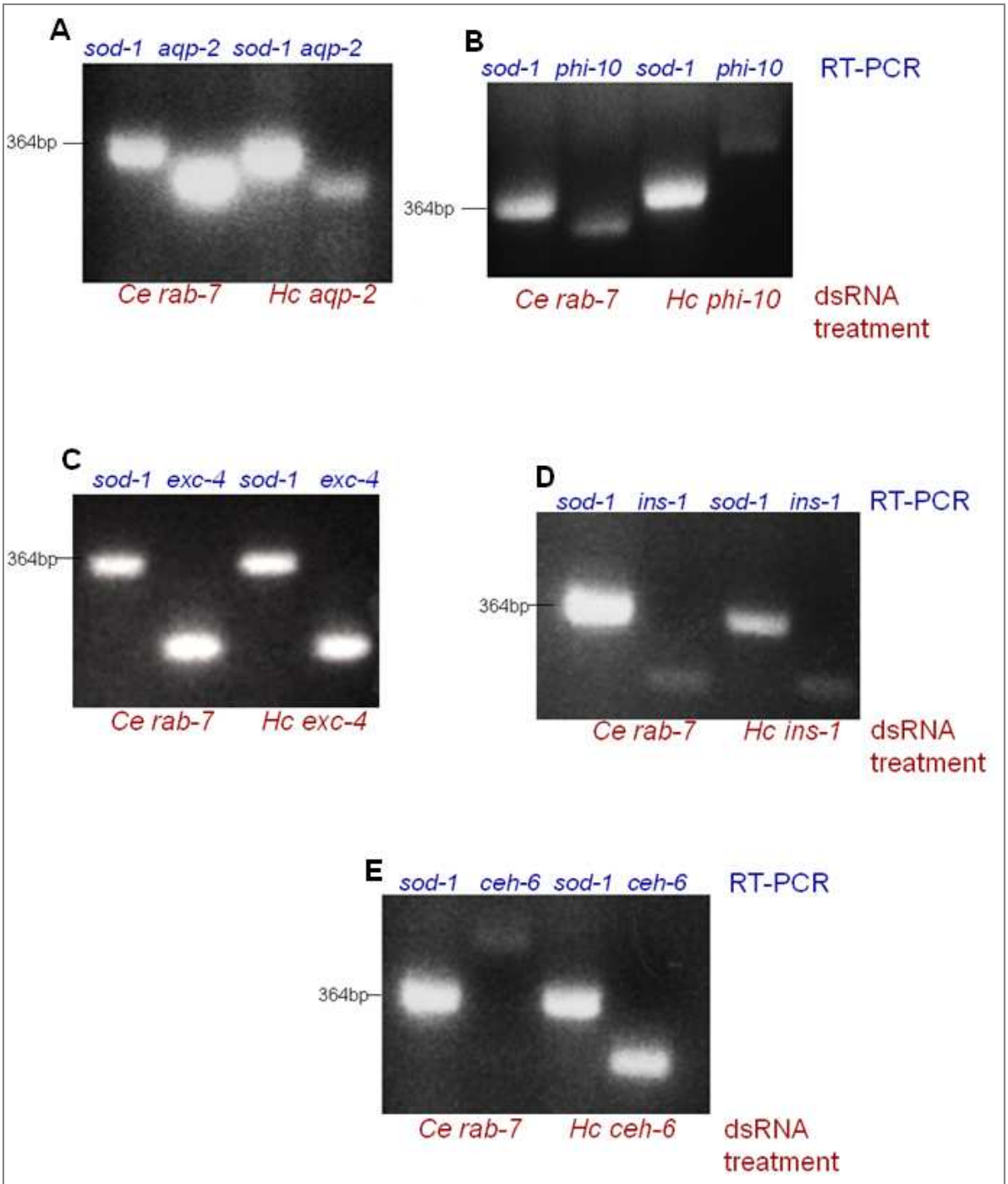


Figure 3.6. Transcript levels of RNAi targeted genes thought to be expressed in accessible sites in the L3 larval stage in *H. contortus,* based on *C. elegans* expression pattern data.

(A) *Hc-aqp-2* and *Hc-sod-1* transcript levels following 72 hours of soaking in control *Ce-rab-7* dsRNA or *Hc-aqp-2* dsRNA. Expression of a non-target gene (*Hc-sod-1*) in the same sample is shown for comparison. **(B)** *Hc-phi-10* and *Hc-sod-1* transcript levels following 72 hours of soaking in control *Ce-rab-7* dsRNA or *Hc-phi-10* dsRNA. **(C)** *Hc-exc-4* and *Hc-sod-1* transcript levels following 72 hours of soaking in control *Ce-rab-7* dsRNA or *Hc-exc-4* dsRNA. **(D)** *Hc-ins-1* and *Hc-sod-1* transcript levels following 72 hours of soaking in control *Ce-rab-7* dsRNA or *Hc-ins-1* dsRNA. **(E)** *Hc-ceh-6* and *Hc-sod-1* transcript levels following 72 hours of soaking in control *Ce-rab-7* dsRNA or *Hc-ceh-6* dsRNA. Knockdown of *Hc-aqp-2* and *Hc-phi-10* was found in three separate experiments.

These results indicate that several genes that are thought to be expressed in accessible locations within *H. contortus* can be susceptible to RNAi silencing; successful silencing of *Hc-bt-iso-1, Hc-H11, Hc-asp-1, Hc-aqp-2* and *Hc-phi-10* transcripts were obtained following target dsRNA treatment. Importantly, the silencing of these genes was repeatable on all occasions tested. Silencing was also specific to the target dsRNA used to induce the silencing; for example, transcript levels of *Hc-H11* were unaffected by *Hc-asp-1* or *Hc-bt-iso-1* dsRNA, and only *Hc-H11* dsRNA was able to silence the *Hc-H11* transcript. Similarly, *Hc-asp-1* and *Hc-bt-iso-1* were only silenced with homologous and not heterologous dsRNA (data not shown). However, three of the tested genes (*Hc-exc-4, Hc-ins-1* and *Hc-ceh-6*) were not susceptible to silencing, suggesting that other factors may also be important, or that expression of these genes in *C. elegans* and *H. contortus* is not conserved.

3.2.5 *In vitro* assays for phenotypes for RNAi silenced genes

Despite the successful silencing of some genes with RNAi, no obvious phenotypic effects on larval survival or motility were observed for any of the dsRNA treated larvae. Larvae were therefore subjected to specific assays that might highlight a phenotypic difference between the control and treated larvae, summarised below.

3.2.5.1 Albendazole resistance assay

Albendazole belongs to the benzimidazole class of drugs which act by targeting beta tubulin in nematodes, as discussed in section 1.1.4. The MHco3 (ISE) isolate of *H. contortus* is susceptible to this class of drug, which acts by targeting beta tubulin and has an observable impact on motility by causing paralysis (O'Grady & Kotze, 2004). Resistance to benzimidazole has been linked with several different polymorphisms of the beta tubulin genes, most notably a phenylalanine to tyrosine substitution at amino acid 200 of the *Hc*-Bt-ISO-1 protein (Kwa *et al.*, 1994). It is possible that larvae that have been treated with dsRNA targeting *Hc-bt-iso-1* would be resistant to effects of the drug.

H. contortus larvae were soaked for 24 hours in *Hc-bt-iso-1* dsRNA and then transferred to culture medium containing albendazole dissolved in DMSO or DMSO only, as described in section 2.1.4. Larvae were then observed for any phenotypes following drug treatment. Paralysis of both control *Ce-rab-7* and *Hc-bt-iso-1* dsRNA treated larvae was observed after 72 hours exposure to the drug. There was no significant difference in drug susceptibility between the control larvae and those in which *Hc-bt-iso-1* transcript had been silenced.

3.2.5.2 Beta tubulin isotype-1 protein levels in dsRNA treated larvae

Although successful knockdown of *Hc-bt-iso-1* transcript can be achieved by RNAi, no phenotype was observed as a result of this knockdown. The dsRNA treated larvae were not detectably resistant to the effects of albendazole. It is possible that although the transcript has been effectively silenced by RNAi, the protein could still be present in the larvae; the *Hc*-Bt-ISO-1 protein could have a low turnover rate. Alternatively, other *H. contortus* beta tubulin isotypes may compensate for loss of *Hc*-Bt-ISO-1 and maintain drug sensitivity. Therefore it was necessary to assess the *Hc*-Bt-ISO-1 protein levels in dsRNA treated larvae to examine this further.

Western blot analysis was carried out on *H. contortus* L3 larvae that had been soaked for 72 hours in *Hc-bt-iso-1* dsRNA or control *Ce-rab-7* dsRNA using an *Hc*-Bt-ISO-1 specific antibody (kindly provided by Gary Saunders), as described in section 2.4.3. The blot was then stripped of the *Hc*-Bt-ISO-1 antibody and screened again with an actin antibody as a loading control for protein levels. As indicated in Figure 3.7A, the *Hc*-Bt-ISO-1 protein remains present in the larvae following soaking in *Hc-bt-iso-1* dsRNA for 72 hours. RT-PCR analysis on a sample of worms from the same experiment indicated that the transcript level of *Hc-bt-iso-1* was successfully knocked down at this time point, similar to that shown in Figure 3.1. Therefore it appears that although the *Hc-bt-iso-1* transcript can be successfully silenced after 72 hours of soaking in dsRNA, the *Hc*-BT-ISO-1 protein level remains unaffected.

It appears likely that the *Hc*-Bt-ISO-1 protein possesses a low turnover rate, and incubating the larvae in dsRNA for longer than 72 hours might lead to a decrease in the target protein level compared to the control. Therefore the experiment

was repeated by incubating the larvae for 7 and 15 days in *Hc-bt-iso-1* dsRNA. A sample of the worms were analysed by RT-PCR, and the *Hc-bt-iso-1* transcript was successfully silenced in the 7 and 15 day samples (data not shown). As shown in Figure 3.7B and C, the resultant Western blots show a slight decrease in *Hc*-Bt-ISO-1 protein levels after the larvae have been treated with dsRNA for 7 days, and a greater decrease after 15 days of target dsRNA treatment. A repeat of the experiment produced results which were consistent with these observations.

The Western blots were scanned and the amount of *Hc*-Bt-ISO-1 protein was quantified using the integrated density value (IDV) reading for each sample. This was achieved by calculating the IDVs for *Hc*-Bt-ISO-1 relative to the actin control for *Hc-bt-iso-1* dsRNA treated larvae compared to control *Ce-rab-7* dsRNA treated larvae, as described in section 2.4.6. The percentage of *Hc*-Bt-ISO-1 protein remaining in the larvae following target dsRNA treatment was calculated in this manner, the results of which are shown graphically in Figure 3.8. This analysis indicated the *Hc*-Bt-ISO-1 level remained at 94% following 72 hours incubation in target dsRNA and subsequently decreased to 86% and 70% following 7 and 15 days of incubation in target dsRNA, respectively.

These results indicate that most of the *Hc*-BT-ISO-1 protein remains present in the larvae despite 15 days of RNAi treatment, even though the transcript can be successfully knocked down after just 72 hours of RNAi treatment. The lack of observable phenotypes in the larvae following *Hc-bt-iso-1* RNAi, or any resistance to the paralytic effects of albendazole treatment could thus be explained, as the *Hc*-BT-ISO-1 protein remains present within the larvae for at least 15 days or longer, even in the presence of *Hc-bt-iso-1* dsRNA.

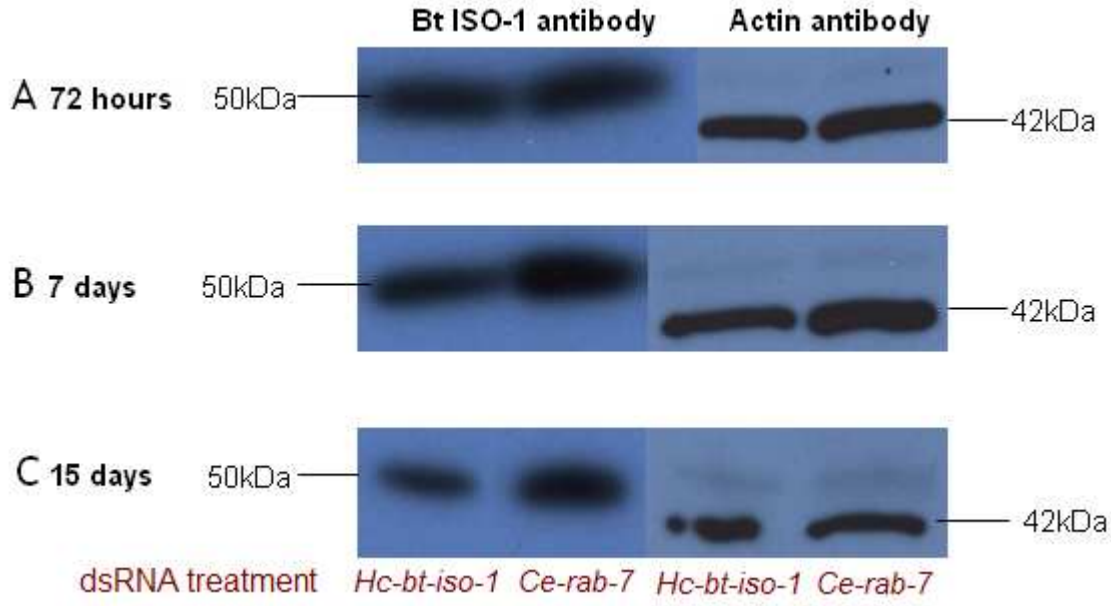


Figure 3.7. Western blot detection of *Hc*-Bt-ISO-1 protein following exposure of L3 larvae to *Hc-bt-iso-1* dsRNA.

(A) *Hc*-Bt-ISO-1 protein detection following 72 hours of soaking in *Hc-bt-iso-1* dsRNA or control *Ce-rab-7* dsRNA. Reactivity of the same blot with actin antibody is shown and was used to measure the relative amounts of protein present in the different samples (**B**) *Hc*-BT-ISO-1 protein following 7 days of soaking in *Hc-bt-iso-1* dsRNA or control *Ce-rab-7* dsRNA. (**C)** *Hc*-BT-ISO-1 protein following 15 days of soaking in *Hc-bt-iso-1* dsRNA or control *Ce-rab-7* dsRNA. The relative molecular masses (Mr) of *H. contortus* Bt-ISO-1 and actin are indicated.

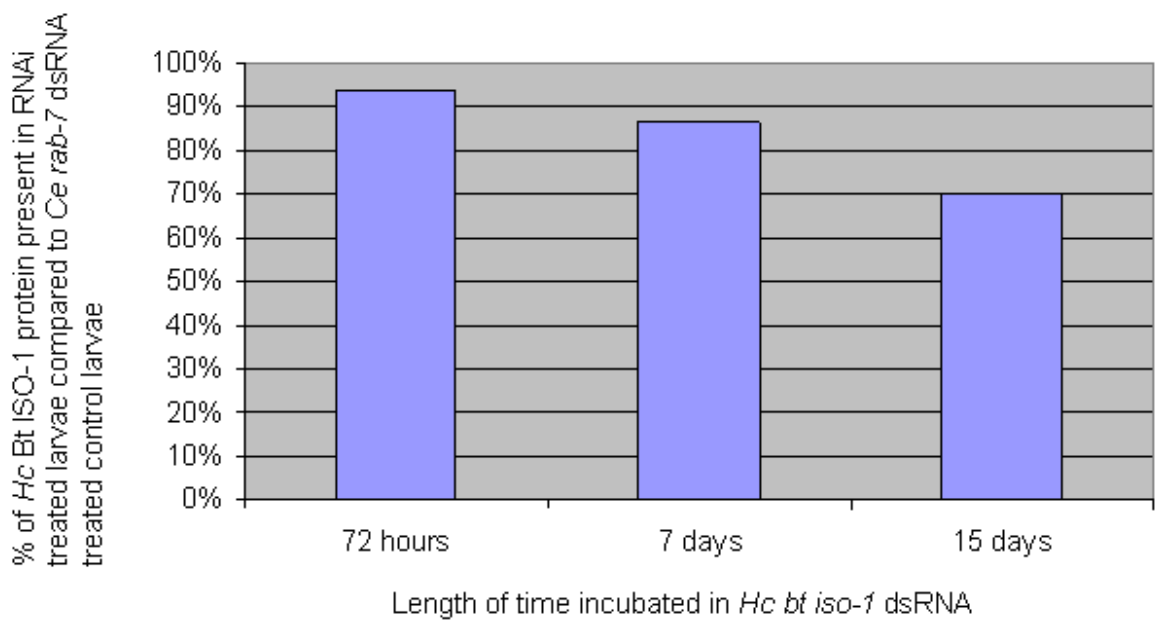


Figure 3.8. *Hc*-Bt-ISO-1 protein levels following *Hc-bt-iso-1* RNAi treatment compared to control dsRNA treated larvae

Reactivity with *Hc*-Bt-ISO-1 antibody and actin antibody on the Western blots shown in Figure 3.7 was quantified using the Integrated Density Value (IDV) for each sample. The value for *Hc*-Bt-ISO-1 protein relative to actin was calculated. This was used to determine the level of *Hc*-Bt-ISO-1 protein remaining in *Hc-bt-iso-1* RNAi treated larvae compared to *Ce-rab-7* control treated larvae, which is shown as a percentage.

3.2.6 RNAi silencing of genes after 24 hours exposure to dsRNA

The lack of any *in vitro* effects of RNAi silencing of *H. contortus* genes led to considering the feasibility of *in vivo* RNAi studies. This would require infective larvae to be pre-soaked in dsRNA prior to infection. L3 larvae do not survive for long in culture, and viability starts to decrease after approximately a week of *in vitro* culture, soon after the moult from L3 to L4 stage larvae. Previous experiments have shown that the RNAi silencing effects of gene expression take approximately 48 hours to be detected by RT-PCR (Figure 3.2B). It was of interest to determine the minimum time of exposure to dsRNA in culture required to obtain a silencing effect. This would be important for determining any *in vivo* effects of RNAi silencing as the less time in culture, the healthier the larvae will be for infection.

In order to investigate the time required for complete transcript knockdown to occur, an experiment was set up in which the larvae were only exposed to dsRNA for 24 hours. The larvae were then washed three times in culture medium and then incubated for a further 48 hours in culture medium without dsRNA (total 72 hours). *Hc-bt-iso-1, Hc-H11* and *Hc-asp-1* dsRNA was tested in this experiment.

As shown in Figure 3.9, successful knockdown of all three target genes occurs following a 24 hour exposure to the target dsRNA. It appears that the larvae do not need to be exposed to the target dsRNA for the full duration of the culture period required for the RNAi silencing to be seen (72 hours). These results suggested that it should be possible to induce specific gene silencing by culturing the larvae in dsRNA for 24 hours. The treated larvae could then be used to infect sheep to assay for any *in vivo* effects of RNAi silencing.

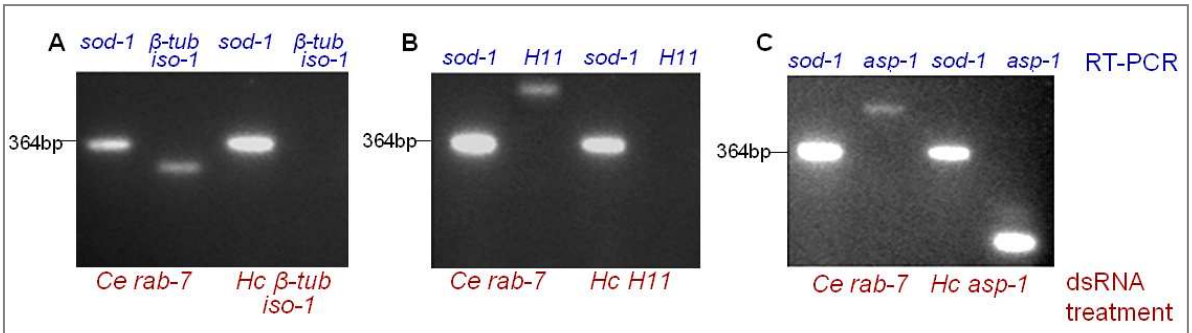


Figure 3.9. RNAi silencing of *beta tubulin isotype-1, Hc-H11* and *asp-1* after 24 hours of exposure to dsRNA

(A) *Hc-bt-iso-1* and *Hc-sod-1* transcript levels following 24 hours of exposure to dsRNA by soaking in control *Ce-rab-7* dsRNA or *Hc-bt-iso-1* dsRNA, followed by 48 hours of culture in dsRNA free medium. Expression of a non-target gene (*Hc-sod-1*) in the same sample is shown for comparison. **(B)** *Hc-H11* and *Hc-sod-1* transcript levels following 24 hours of exposure to dsRNA by soaking in control *Ce-rab-7* dsRNA or *Hc-H11* dsRNA, followed by 48 hours of culture in dsRNA free medium. **(C)** *Hc-asp-1* and *Hc-sod-1* transcript levels following 24 hours of exposure to dsRNA by soaking in control *Ce-rab-7* dsRNA or *Hc-asp-1* dsRNA, followed by 48 hours of culture in dsRNA free medium. Bright band representing unbound *Hc-asp-1* RT-PCR primers was also observed, where transcript silencing occurred.

3.2.7 *In vivo* assay following RNAi silencing of target genes

Prior to testing for any effects of target gene silencing on the course of *H. contortus* infection, it was important to assess if dsRNA treatment itself can affect the ability of the larvae to infect sheep. Therefore, a preliminary experiment was set up in which sheep were infected with larvae (treated with dsRNA or untreated) and the faecal egg output and total worm burdens were compared, as described in section 2.2.1.1. Six sheep were used in this experiment, with two sheep per experimental group. Exsheathed larvae were used in this experiment as previous observations have shown better silencing with exsheathed larvae, compared to sheathed larvae (data not shown). Each sheep was infected with approximately 5000 larvae. Group A sheep were orally infected with 5000 exsheathed larvae that had been soaked for 24 hours in control *Ce-rab-7* dsRNA. Group B sheep were surgically infected with 5000 exsheathed larvae soaked for 24 hours in control *Ce-rab-7* dsRNA. Group C sheep were orally infected with 5000 exsheathed larvae that were soaked in dsRNA-free culture medium for 24 hours. Oral and surgical infections were compared to determine whether direct implantation of the larvae into the abomasum would lead to a better take of the infection. The faecal egg counts (FECs) were measured 21 days post-infection and 28 days post-infection. The results from this preliminary *in vivo* RNAi experiment are summarised in Table 3.3.

Table 3.3. Faecal egg counts (FEC) from preliminary *in vivo* RNAi experiment with *Ce-rab-7* dsRNA

	FEC 21 days post infection			FEC 28 days post infection		
Group/ treatment	**Sheep # 1**	**Sheep # 2**	**Mean**	**Sheep # 1**	**Sheep # 2**	**Mean**
Group A - *Ce-rab-7* dsRNA, oral infection	1863	2322	2092.5	2340	6642	4491
Group B - *Ce-rab-7* dsRNA, surgical implant	432	3843	2137.5	2115	8712	5413.5
Group C – no dsRNA, oral infection	4698	2331	3514.5	20466	189	10327.5

As the data in Table 3.3 indicate, incubating the larvae in control *Ce-rab-7* dsRNA does not affect their ability to infect sheep with high numbers of eggs being produced. No significant difference between the mean FECs of groups A, B and C was observed on day 21 (Single Factor ANOVA; F (2,3)=0.45; p=0.67) nor on day 28 (Single Factor ANOVA; F(2,3)=0.25; p=0.79). It was concluded that control *Ce-rab-7* dsRNA treatment has no significant effect on larval survival and development *in vivo*. The FECs obtained from infecting sheep by surgical implantation are not significantly different to the FECs from oral infection. As oral infection is easier to carry out and is better for animal welfare, this method was chosen for the subsequent *in vivo* RNAi study. A wide variation in FECs is observed, and this is typical of assessing infectivity using FECs as a measure, where the number of eggs produced can vary between individual sheep and vary on different days post infection (Professor David Knox, personal communication). However, the dsRNA treated larvae are still able to infect sheep successfully and mature into adults and produce eggs.

Following this initial study, an experiment was set up to assess the *in vivo* effects of RNAi knockdown of *Hc-H11*, as described in section 2.2.1.2. *Hc-H11* was chosen for this experiment because it has been previously characterised as an important antigen in vaccine trials, capable of inducing protective immunity in lambs, resulting in a reduction of 90% in worm burden and 92% faecal egg counts after challenge infection(Andrews *et al.*, 1995; Munn *et al.*, 1993). The successful silencing of *Hc-H11* transcript has also been possible, as described in section 3.2.4 and 3.2.6. Therefore, effects of *Hc-H11* silencing on the ability of

H. contortus L3 larvae to infect sheep, mature to adult worms and reproduce was examined, and assessed by measuring FECs at regular intervals and the total worm burden four weeks post infection.

Eight sheep were used in this experiment with four animals per experimental group. Group A sheep were each orally infected with approximately 5000 exsheathed larvae, previously soaked for 24 hours at 37^{o}C in control *Ce-rab-7* dsRNA. Group B sheep were also orally infected with approximately 5000 exsheathed larvae, previously soaked for 24 hours at 37^{o}C in *Hc-H11* dsRNA. Faecal egg counts were measured and sheep were sacrificed four weeks post infection, and the total worm burden measured. The faecal egg counts (FECs) at various days during this experiment are indicated in Table 3.4.

Table 3.4. Faecal egg counts (FEC) from *Hc-H11 in vivo* RNAi experiment

		FEC post infection			
Group/treatment	**Sheep ID #**	**15 days**	**23 days**	**26 days**	**28 days**
Group A – control *Ce-rab-7* dsRNA, oral infection	1C	1	1656	1107	7812
	2C	0	1233	2556	2502
	3C	0	1431	5670	5454
	4C	2	3114	2655	2808
	MEAN (Control *rab-7* treated)	**1**	**1859**	**2997**	**4644**
Group B – *Hc-H11* dsRNA, oral infection	1i	2	1899	1107	1098
	2i	0	1683	2466	2142
	3i	1	639	918	864
	4i	0	2160	4158	3816
	MEAN (*Hc-H11* RNAi treated)	**1**	**1595**	**2162**	**1980**

The mean FECs were plotted on a graph against the number of days post-infection, as shown in Figure 3.10. Infection with control *Ce-rab-7* dsRNA treated larvae resulted in a rise in FECs from day 15 onwards, with a maximum FEC of 4644 on day 28. In contrast, infection with *Hc-H11* dsRNA treated larvae led to a comparatively smaller rise in FECs from day 15, with a maximum FEC of 2162 on day 26, following which the FEC decreased to 1980 by day 28. Therefore a 57.4% reduction in the faecal egg output can be seen between the *Hc-H11* RNAi treated group and the control *Ce-rab-7* treated group by the conclusion of the experiment, on day 28. A two sample *t-test* assuming equal variances was used to compare mean FECs on day 28. The mean FEC on day 28 of Group A was significantly higher than that of Group B (t=1.88; $d.f.$=6; p<0.1).

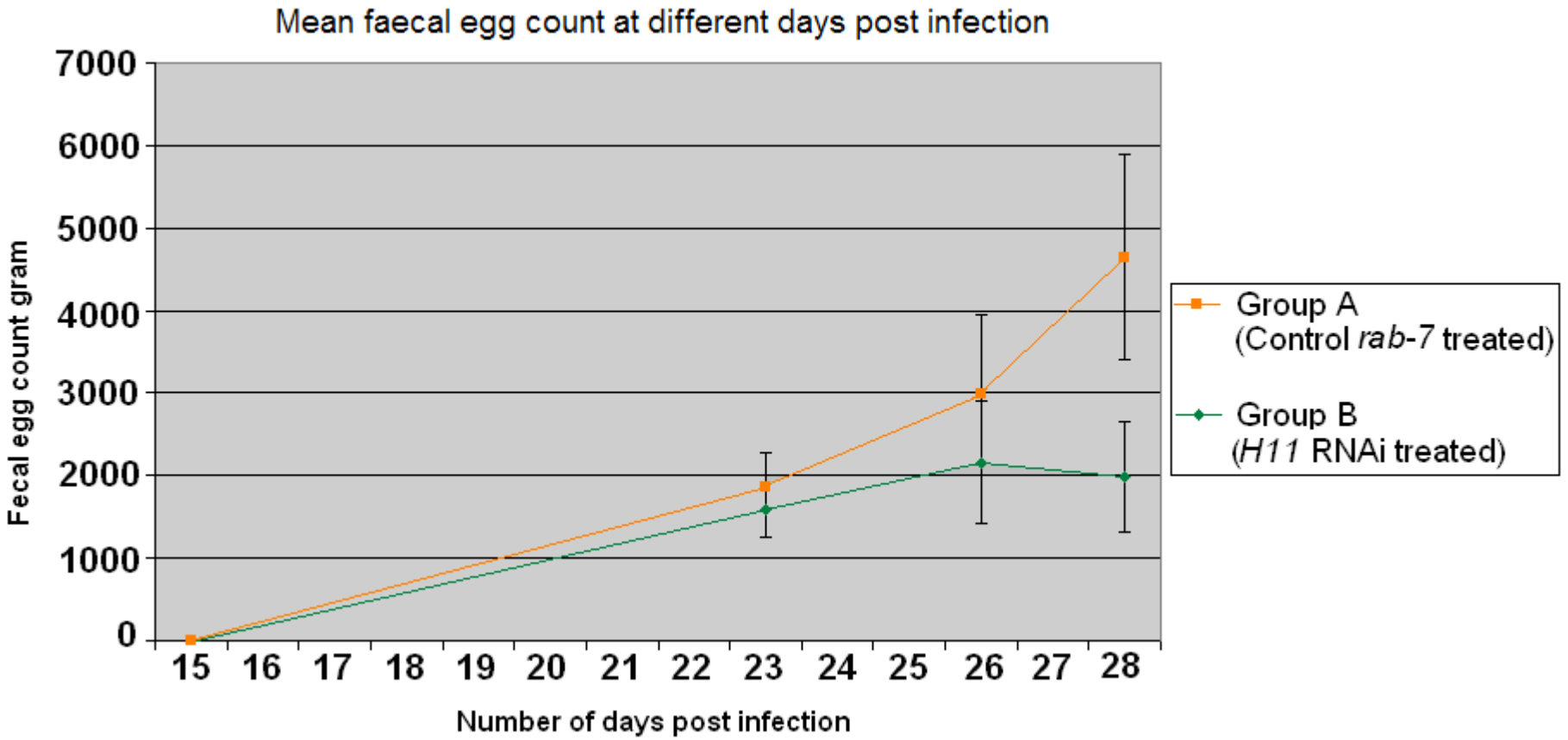


Figure 3.10. Graph of mean faecal egg count, measured at various days post infection.

FECs were measured 15, 23, 26 and 28 days post infection, as indicated by the data points on the graph. Group A sheep, infected with control *Ce-rab-7* dsRNA treated larvae shown in orange line. Group B sheep, infected with *Hc-H11* dsRNA treated larvae shown in green line. Error bars show the standard deviation.

The total worm burden in each sheep was also measured on day 28 by sacrificing the sheep at the conclusion of the experiment, as described in section 2.2.1.2. Adult male and female worms present in a 2% abomasal wash were counted, as were the adult male and female worms present in a 2% abomasal digest. These numbers were then multiplied by 50 to estimate the total number of male and female worms present within each sheep. This data is shown in Table 3.5.

Table 3.5. Total worm burdens from *Hc-H11 in vivo* RNAi experiment

		Abomasal wash (2%)		Abomasal digest (2%)					
Group/ treatment	**Sheep ID #**	**Male**	**Female**	**Male**	**Female**	**Total male (100%)**	**Total female (100%)**	**Total worm burden**	**Mean total worm burden**
Group A – control *Ce-rab-7* dsRNA, oral infection	1C	13	14	6	11	950	1250	2200	**2950**
	2C	20	11	10	13	1500	1200	2700	
	3C	22	26	26	25	2400	2550	4950	
	4C	18	17	2	2	1000	950	1950	
Group B – *Hc-H11* dsRNA, oral infection	1i	5	8	6	4	550	600	1150	**1750**
	2i	5	6	13	7	900	650	1550	
	3i	7	10	1	3	400	650	1050	
	4i	10	9	25	21	1750	1500	3250	

The mean total worm burdens recovered from each sheep in both groups was calculated and plotted on a graph, as shown in Figure 3.11. A 40.7% reduction was seen in the total number of adult worms recovered from the *Hc-H11* RNAi treated group, compared with the control *Ce-rab-7* treated group.

A two sample *t-test* assuming equal variances compared the worm burdens using the abomasal wash data; Group A sheep had a significantly higher mean worm burden than Group B (two sample equal variances *t-test*; t=4.13; *d.f.*=6; p<0.005). However the total worm burden, calculated by aggregating the worms isolated using both the abomasal wash and abomasal digest data, showed that the mean total worm burden of Group A was not significantly higher than that of Group B (t=1.40; *d.f.*=6; p=0.105). As evidenced by the data shown Table 3.5, the number of worms in the abomasal digest from each sheep showed more variability than the abomasal wash data, which influences the statistical analysis. This variability is further demonstrated by comparing the two groups'

mean worm burdens using the abomasal digest data, which showed no significant difference between the groups (two sample equal variances *t-test*; t=0.28; $d.f.$=6; p=0.396). In the future, using a larger sample size would reduce variability and may further demonstrate the significance of *in vivo* RNAi.

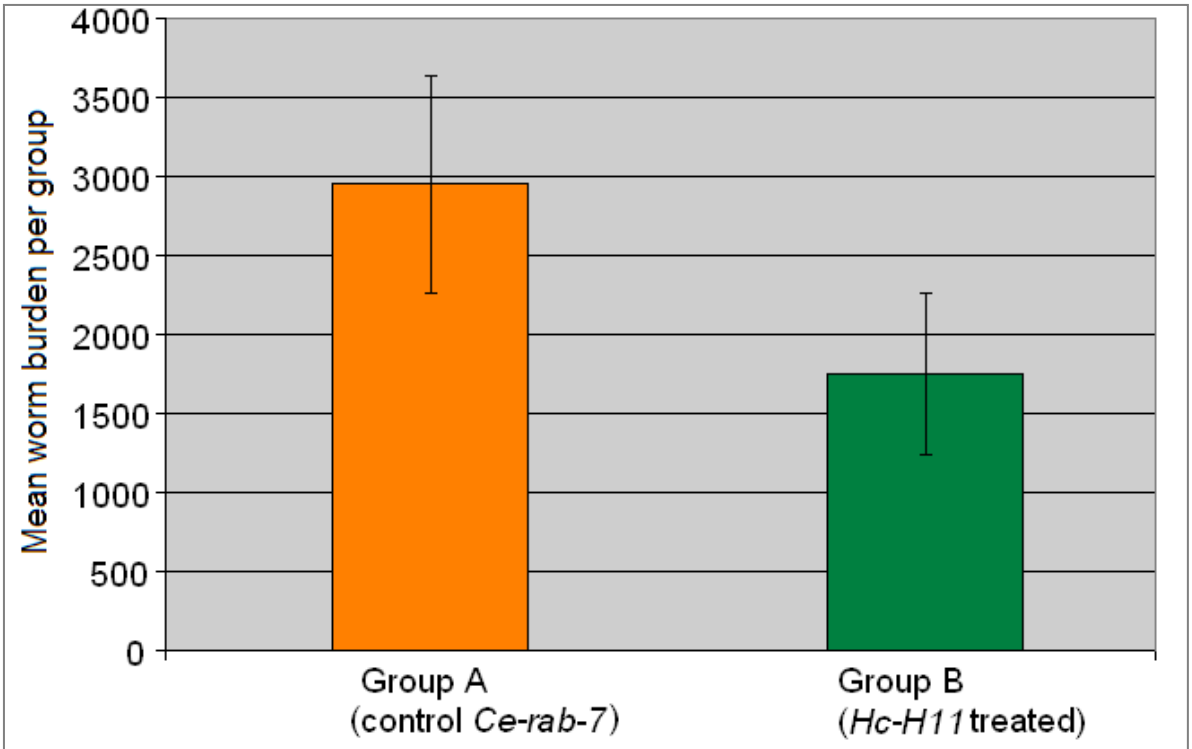


Figure 3.11. Mean worm burden from *Hc-H11 in vivo* RNAi experiment

Group A sheep were orally infected with larvae that were soaked in control *Ce-rab-7* dsRNA for 24 hours (orange). Group B sheep were orally infected with larvae that were soaked in *Hc-H11* dsRNA for 24 hours (green). Total worm burden was estimated by counting the adult male and female worms from a 2% abomasal wash and a 2% abomasal digest, 28 days post infection. Error bars indicate the standard deviation.

3.2.8 Assessment of *Hc-H11* transcript levels in adult *H. contortus* following *in vivo* RNAi experiment

The *Hc-H11* transcript can be successfully silenced in L3 larvae as demonstrated in section 3.2.4, and this silencing can be achieved by culturing the larvae in *Hc-H11* dsRNA for 24 hours as shown in section 3.2.6. It was of interest to determine whether the *Hc-H11* transcript remains silenced in the adult worms recovered from sheep in Group B.

At termination of the *Hc-H11 in vivo* experiment on day 28, adult worms were collected from each of the eight sheep in Group A and B. Samples of adult worms were used to isolate total RNA, as described in section 2.5.11, in order to assess the transcript levels of *Hc-H11* in worms from individual sheep. First-strand cDNA was prepared (section 2.5.12) and *Hc-H11* transcript levels were assessed by comparing with the relative levels of *Hc-sod-1* following PCR amplification. 20 cycles of amplification were used in the PCR reaction in order to obtain a more quantitative indication of any possible changes to *Hc-H11* transcript levels. The results of this analysis are shown in Figure 3.12. There was no detectable difference in the *Hc-H11* transcript level in the RNAi treated worms, isolated from Group B sheep, compared to the *Hc-H11* transcript level in the control *rab-7* dsRNA treated worms isolated from Group A sheep. The PCR analysis was repeated, with the same results showing that *Hc-H11* transcript level remained unchanged between worms isolated from Group A and Group B sheep.

In summary, the results from the *in vivo* RNAi experiment indicate that a significant phenotypic impact on worm survival can be observed, as demonstrated by a reduced faecal egg output and a reduced worm burden in sheep infected with *Hc-H11* RNAi treated worms. However, the RNAi treated adult worms collected from sacrificed sheep do not show a decrease in *Hc-H11* transcript levels compared to the control RNAi treated worms, suggesting that not all worms are affected by the Hc-H11 dsRNA treatment or that worms can recover from gene silencing.

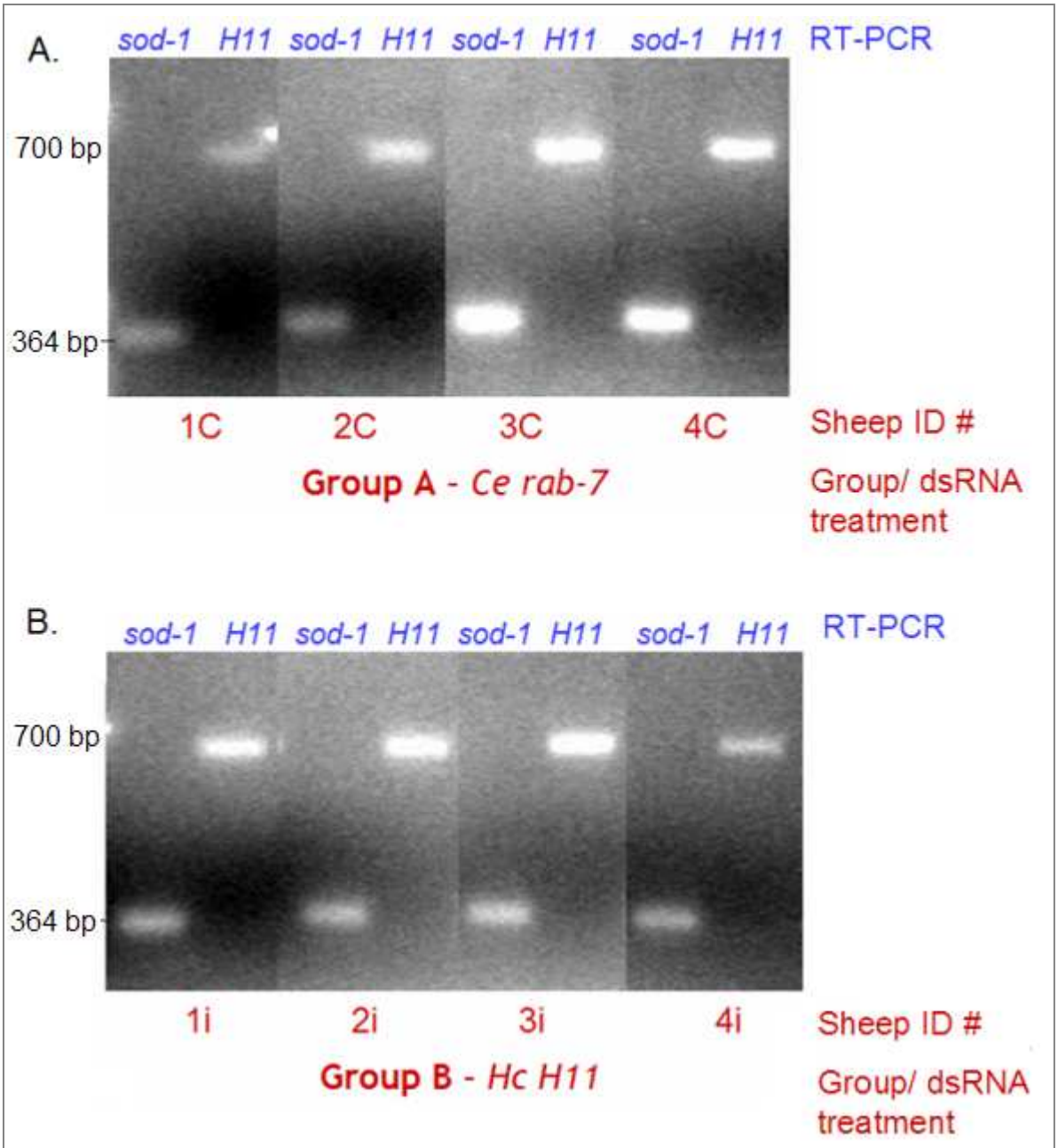


Figure 3.12. *Hc-H11* transcript levels from adult worms isolated from *in vivo* RNAi experiment

PCR amplification on cDNA template, isolated from adult *H. contortus*. **(A)** *Hc-H11* transcript levels in adult *H. contortus,* isolated from Group A sheep which were orally infected with larvae soaked in control *Ce-rab-7* dsRNA for 24 hours. Expression of non-target control gene *Hc-sod-1* is shown for comparison. **(B)** *Hc-H11* transcript levels in adult *H. contortus*, isolated from Group B sheep, orally infected with larvae soaked in *Hc-H11* dsRNA for 24 hours. 20 cycles of PCR amplification was carried out.

3.3 Discussion

The primary aim of this chapter was to reliably silence genes in *H. contortus* using RNAi, examine the possible reasons why RNAi can silence some genes but not others and observe any phenotypic effects of gene silencing *in vitro* and *in vivo*. The reliable silencing of the *Hc-bt-iso-1* transcript using dsRNA indicates that *H. contortus* does possess the necessary core RNAi pathway machinery to silence genes. However, the abundance of transcript does not appear to be a factor in RNAi susceptibility, as genes selected based on abundance of ESTs in the L3 larval stage of *H. contortus* were either unsusceptible to RNAi or gave variable results.

Importantly, the site of gene expression appears to play a role in RNAi susceptibility. *Hc-bt-iso-1, Hc-H11, Hc-asp-1, Hc-aqp-2* and *Hc-phi-10* genes were all successfully silenced in several separate experiments. This study has significantly expanded the number of genes targetable by RNAi in *H. contortus*. In addition to being repeatable, the silencing obtained for these genes was extremely specific; the introduced dsRNA silenced only the target gene. All of these genes are thought to be expressed in sites that are accessible to the environment such as the intestine, excretory/secretory cells or amphid cells. There is currently limited information about the site of gene expression in *H. contortus*, particularly the L3 stage. As a result, indirect methods such as promoter-reporter constructs active in transgenic *C. elegans* or expression pattern data from putative homologues in *C. elegans* were used to gain information on possible expression of *H. contortus* genes for which no prior expression data was known. Although *Hc-exc-4, Hc-ins-1* and *Hc-ceh-6* were not susceptible to RNAi, it is possible that the expression of these genes is not spatially conserved between *C. elegans* and *H. contortus*. It may be hypothesised that although these three genes are expressed in the amphid cells, intestine or excretory cell in *C. elegans*, the putative homologues in *H. contortus* have different sites of expression. The site of expression of these genes in *H. contortus* is unknown; limited genome sequence information currently prevents the construction of promoter-reporter constructs for these genes, to examine the spatial expression they direct in transgenic *C. elegans*. In addition,

antibodies are available for only a few *H. contortus* proteins which would allow localisation directly in the parasite.

Although several genes could be silenced with RNAi in *H. contortus*, no observable phenotypes affecting motility or development *in vitro* were seen in any of the worms. This was surprising for a gene such as *Hc-bt-iso-1*, expected to be expressed in all cell types and important for microtubule formation and cell structure; the loss of a functionally significant gene would be expected to have phenotypic consequences on the larvae. However, mutations in the homologous *ben-1* gene in *C. elegans* result in a wild-type phenotype, with the exception of conferring resistance to benzimidazole drugs such as albendazole (Driscoll *et al.*, 1989). Therefore, *H. contortus* larvae were also exposed to albendazole, in order to assess if *Hc-bt-iso-1* dsRNA treated larvae might be resistant to the paralytic effects of the drug; no difference in drug susceptibility was seen between the control and RNAi larvae. It is possible that although the *Hc-bt-iso-1* transcript has been effectively silenced by RNAi, the *Hc*-Bt-ISO-1 protein still remains in the larvae. This hypothesis was tested by Western blot detection using an *Hc*-Bt-ISO-1 specific antibody, which showed that the *Hc*-Bt-ISO-1 protein indeed remained present in larvae even when the transcript has been effectively silenced. Soaking the larvae for 7 days in *Hc-bt-iso-1* dsRNA resulted in a slight decrease in target protein levels, compared to the control *Ce-rab-7* dsRNA treated larvae. A more obvious decrease in target protein levels was obtained by soaking the larvae for 15 days in *Hc-bt-iso-1* dsRNA. However, as the viability of the larvae is significantly reduced following this time in culture, it is difficult to accurately assess reduced drug susceptibility. Therefore in summary, these results indicate that the *Hc*-BT-ISO-1 protein has a low turnover rate, which could explain the lack of any observable phenotypes following silencing of the *Hc-bt-iso-1* transcript. It would be interesting to examine this turnover of other *H. contortus* proteins. This is currently limited by a lack of appropriate antibodies.

Three different isoforms of beta tubulin in *H. contortus* have been identified and characterised (Gary Saunders, PhD Thesis, University of Glasgow 2009). Of these, RNAi was successful for isoform-1, but not isoform-2 or 3 (data not shown). Interestingly, promoter regions of the three beta tubulin isoforms seem to direct different spatial expression, as elucidated using promoter-reporter constructs in

transgenic *C. elegans* (Gary Saunders, PhD Thesis, University of Glasgow 2009). A *Hc-bt-iso-1*:*GFP* promoter-reporter gene fusion showed strong expression in the posterior intestine and amphid neurons in transgenic *C. elegans*. In contrast, the *Hc-bt-iso-2:GFP* promoter-reporter gene fusion showed faint expression in the intestine, although this was found using a short promoter region of 802 bp. Expression in the touch receptors and head neurons was seen for the *Hc-bt-iso-3:GFP* promoter reporter gene fusion. These expression patterns may explain the susceptibility of *Hc-bt-iso-1* to RNAi silencing, since it appears to be expressed in the posterior intestine and amphid neurons; sites that may be accessible to the environmental uptake of dsRNA. Neuronal expression for *Hc*-Bt-ISO-1 was also confirmed in *H. contortus* using antibody; however no intestine reactivity was observed which could be due to loss of intestine structure during fixation (Gary Saunders, PhD Thesis, University of Glasgow 2009). The lack of susceptibility of *Hc-bt-iso-2* and *Hc-bt-iso-3* to RNAi may also be explained by their more restricted and perhaps less accessible spatial expression.

The lack of any detectible phenotypes following RNAi knockdown of several genes in *H. contortus* is perhaps not unexpected when compared to *C. elegans*; a genome-wide RNAi screen on *C. elegans* showed that 86% of genes targeted were susceptible to RNAi, and only 10.3% of these genes display phenotypes such as sterility, embryonic lethality and other such defects (Kamath *et al.*, 2003). Therefore it appears that only a tenth of RNAi targeted genes display an observable phenotype in *C. elegans*. If this is also representative of RNAi phenotypes in *H. contortus*, then it could explain the lack of discernible phenotypes following the RNAi silencing of *H. contortus* genes described in this chapter.

Limitations of *in vitro* culture may also explain the lack of discernible phenotypes following the RNAi silencing of *H. contortus* genes. It is worth noting that *C. elegans* can be cultured in the laboratory, and all life stages can be examined for phenotypes following RNAi with relative ease. In contrast, *H. contortus* cannot be cultured in the laboratory, and only the L3/L4 larval stages can be observed for phenotypes following RNAi. It is difficult to determine if the genes which can be reliably silenced as described in this chapter would necessarily display a phenotype in the L3/L4 stages; it is possible that the

silencing of these genes could have an effect in a different life stage that cannot be observed in the laboratory.

In order to examine this possibility, an *in vivo* RNAi experiment was designed to examine the effects of RNAi on the infectivity of larvae and development to adults in sheep. A preliminary experiment determined that dsRNA treatment itself does not affect the ability of the larvae to infect sheep. Subsequently the *in vivo* RNAi experiment examined any detrimental effects on infectivity following silencing of *Hc-H11*. A marked decrease in both FEC and total worm burden was observed in sheep that were infected with RNAi treated larvae, compared to control larvae. Therefore it appears that the RNAi silencing of *Hc-H11* has phenotypic effects, impacting worm survival in sheep. The *Hc-H11* protein is an important antigen in inducing protection in sheep as discussed previously, and this experiment was the first example of RNAi being used as a tool *in vivo* to have an impact on worm survival.

Surprisingly, analysis of the adult worms recovered from sheep showed that *Hc-H11* transcript levels remain unchanged in the adult worms between the RNAi treated and control dsRNA treated worms. It is possible that the RNAi treatment affected worm survival *in vivo,* resulting in a reduction in faecal egg output and worm burden. Surviving adult worms which were isolated from the sheep could therefore be larvae that had recovered from or were unaffected by *Hc-H11* dsRNA treatment. This hypothesis could be examined by setting up a similar *in vivo* experiment, in which sheep would be sacrificed at various time points following infection, to isolate the developing worms. This would make it possible to continuously follow the level of the *Hc-H11* transcript after exposure to dsRNA, from the silencing of the transcript to the possible recovery back to normal levels at a later time point.

Previous studies using lambs immunised with *Hc*-H11 in vaccine trials showed reductions of 90% in worm burden and 92% faecal egg counts after challenge infection with *H. contortus* (Andrews *et al.*, 1995; Munn *et al.*, 1993). The precise mechanism of protection provided by immunising lambs with *Hc*-H11 could be the actual inhibition of aminopeptidase activity or the blocking of a possible transmembrane function by the binding of antibodies to *Hc*-H11, or even a combination of both. In contrast to protection provided by vaccination, pre-

treatment of *H. contortus* larvae with *Hc-H11* dsRNA resulted in a reduction of 40.7% in worm burden and 57.4% faecal egg counts. These differences may be explained by different mechanisms of *Hc-H11* RNAi silencing compared to vaccination with *Hc*-H11, affecting worm survival; silencing the *Hc-H11* transcript using RNAi and inhibiting the function of *Hc*-H11 protein using antibodies are two different mechanisms. It would be important to confirm *Hc*-H11 protein levels *in vivo* following RNAi; this was difficult to do in the present study as the available *Hc*-H11 antibody showed high reactivity with many other proteins.

In conclusion, several genes in *H. contortus* can be successfully silenced by RNAi, consistent with previous work suggesting that a functional RNAi pathway exists in *H. contortus* and possibly other parasitic nematodes. This technique could potentially be used to elucidate the function of different parasitic nematode genes. However, it appears that not all genes can be silenced by RNAi, and the findings presented here suggest that susceptibility to RNAi may be linked to the site of expression of target genes. It may be therefore possible to predict which genes might be susceptible to RNAi silencing and allow RNAi to be developed as a functional genomics tool. The half-life of the protein encoded by the RNAi target gene also appears to be a factor in producing an observable phenotype; silencing the target gene may not necessarily ablate the protein to produce a phenotype, which in turn would be necessary to elucidate the function of the target gene. The limitations of *in vitro* culture of *H. contortus* prevent the parasitic life stages being observed for phenotypic effects of RNAi, and *in vivo* experiments using sheep for the continuous monitoring of parasite development and transcript levels are difficult and expensive to achieve. Improved *in vitro* culture techniques which allow development from larval stages to adulthood could potentially result in a more straightforward approach to functionally annotate parasitic nematode genes using RNAi.

Chapter 4

Analysis of *Haemonchus contortus* Dicer and other components of the RNAi pathway

4.1 Introduction

The RNA interference pathway is an ancient mechanism for gene silencing that is conserved across most eukaryotic organisms, and is explained in detail in Section 1.2. The pathway has multiple roles within the organism, including but not limited to functioning as a defence mechanism against viruses (Obbard *et al.*, 2009) and genome modification through the creation of heterochromatin (Hawkins & Morris, 2008). Elements of the RNAi pathway are also used to regulate development through the endogenous miRNA pathway (reviewed by Breving & Esquela-Kerscher, 2009). Hence there is substantial cross regulation and interactions amongst these different processes, and many of them share certain components of the pathway (reviewed by Lee *et al.*, 2006).

A key component of the RNAi pathway is the RNase III enzyme Dicer (Bernstein *et al.*, 2001), responsible for processing dsRNA molecules into small interfering RNAs (siRNAs) 21-26 nucleotides in length. Dicer is also responsible for processing precursor-miRNA (pre-miRNA) into miRNA in the endogenous miRNA pathway in the cytoplasm (reviewed by Nelson & Weiss, 2008). Dicer belongs to a conserved family of proteins found in plants, fungi and the Metazoa. A typical Dicer contains several different protein domains (reviewed by Collins & Cheng, 2005); an N-terminal DEAD box RNA helicase domain, the RNA binding domain PAZ, a divergent dsRNA binding domain, two ribonuclease (RNase III) domains and an additional dsRNA binding motif (DSRM). Numerous biochemical and structural studies of the Dicer proteins have resulted in a deeper understanding of how Dicer functions at the molecular level (Wang *et al.*, 2009). In addition to unwinding dsRNA substrate, the N-terminal helicase domain is thought to regulate Dicer activity, becoming active only upon interaction with specific partner proteins of the Dicer complex such as RDE-4 (discussed in Chapter 1 and Ma *et al.*, 2008; Parker *et al.*, 2008). The PAZ domain of Dicer is thought to bind to the dsRNA, positioning it for cleavage by the two RNase III domains at a distance of ~25 nucleotides from the end (Macrae *et al.*, 2006). The specificity of Dicer for dsRNA is thought to be provided by the dsRNA binding domains such as the PAZ domain, the divergent dsRNA binding domain and the dsRNA binding motif (Dlakic, 2006). Intriguingly, an additional level of specificity may be provided by the 3' overhang of the dsRNA molecule itself; the overhang length

and sequence composition have been shown to play a role in determining the position of Dicer cleavage (Vermeulen *et al.*, 2005).

Plants are thought to possess several Dicers; for example, *Arabidopsis thaliana* contains four Dicers (Margis *et al.*, 2006). In contrast, mammals and nematodes each possess only one Dicer protein whilst insects and fungi each possess two (Catalanotto *et al.*, 2004; Kadotani *et al.*, 2004). In *Drosophila melanogaster*, the two Dicers have different roles; Dicer-1 functions in the miRNA pathway whilst Dicer-2 is required for RNAi (Lee *et al.*, 2004). Similarly, in *A. thaliana* four Dicer-like proteins (*At*DCL) each have specific roles; *At*DCL1 generates miRNA from pre-miRNA, *At*DCL2 produces siRNAs necessary for viral defence, *At*DCL3 generates siRNAs required for chromatin modification, and *At*DCL4 generates trans-acting siRNAs that regulate vegetative phase change (Borsani *et al.*, 2005; Gasciolli *et al.*, 2005; Park *et al.*, 2002; Xie *et al.*, 2004; Xie *et al.*, 2005). Rice and poplar plants have five and six Dicers respectively, and it appears that the number of Dicer-like proteins have continued to increase in plants over evolutionary time, whereas in the evolution of mammals the number has decreased (Margis *et al.*, 2006). It has been suggested that the higher numbers of Dicers in plants is related to antiviral immunity (Blevins *et al.*, 2006). For example, in *A. thaliana*, *At*DCL2 responds to the turnip crinkle virus but not the cucumber or turnip mosaic viruses, which are specifically targeted by *At*DCL4 (Xie *et al.*, 2004).

Given that Dicer plays such a fundamental role in the RNAi pathway, an obvious point to consider would be the correlation of a functional Dicer protein with a functional RNAi pathway in different organisms, leading to successful RNAi in those organisms. RNAi studies and analysis of the genome sequences of protozoan parasites have yielded some interesting results. For example, *Trypanosoma brucei* has the RNAi machinery to effect gene silencing and this is now the method of choice to down regulate gene expression (Bastin *et al.*, 2000; LaCount *et al.*, 2000; Li *et al.*, 2009; Shi *et al.*, 2000; Singha *et al.*, 2009). The *T. brucei* RNAi pathway appears to rely on a single Argonaute protein and two Dicer-like enzymes; *Tb*DCL1 is thought to function in the cytoplasm while *Tb*DCL2 is thought to function in the nuclear RNAi pathway (Patrick *et al.*, 2009). Genes of *T. congolense* are also similarly susceptible to RNAi (Inoue *et al.*, 2002), but surprisingly neither *T. cruzi* nor the related trypanosomatids

Leishmania major or *L. donovani* appear to be susceptible to RNAi (DaRocha *et al.*, 2004; Robinson & Beverley, 2003). In addition, the genome databases for these three organisms also lack any identifiable homologues of genes involved in the RNAi pathway, including Dicer (Ullu *et al.*, 2004). Interestingly, although both *L. major* and *L. donovani* appear to lack a functional RNAi pathway, the genome of *L. braziliensis* appears to possess several RNAi pathway genes (Peacock *et al.*, 2007). Despite the publication of a few reports of RNAi in the malaria parasite *Plasmodium* (Malhotra *et al.*, 2002; McRobert & McConkey, 2002), the success of these studies appear to be a matter of debate given that exhaustive database searches and molecular studies in *Plasmodium* has thus far yielded no homologues of RNAi pathway genes, including Dicer (Baum *et al.*, 2009). Analyses of the RNAi pathway genes in budding yeasts have yielded similarly interesting results. Although homologues of RNAi pathway genes such as the Argonaute proteins can be found in the genomes of *Saccharomyces castellii, Kluyveromyces polysporus* and *Candida albicans*, a gene with the domain architecture of known Dicers has not been found (Drinnenberg *et al.*, 2009). However it appears that these three fungal genomes contain a gene possessing two dsRNA binding domains but only a single RNase III domain and no helicase or PAZ domains. This gene has since been identified as encoding the Dicer of budding yeasts (Drinnenberg *et al.*, 2009).

It appears that the presence of a functional RNAi pathway, with the necessary key components, is fundamental to a robust RNAi response. The main aims of this chapter are:

- To confirm that a *dcr-1* gene is present and expressed in *H. contortus*,
- To PCR amplify and sequence the full length cDNA of the *Hc-dcr-1* gene,
- Compare the *Hc*-DCR-1 sequence with other known DCR-1 sequences, particularly other helminth DCR-1 sequences,
- To investigate other RNAi pathway components in the *H. contortus* genome

4.2 Results

4.2.1 Identification of *H. contortus dcr-1* genomic sequence

The presence of a functional RNAi pathway is a requisite for RNAi knockdown of target genes. The successful knockdown of several genes by RNAi in *H. contortus* was obtained as detailed in Chapter 3, implying an active RNAi pathway in *H. contortus*. A preliminary search of the *H. contortus* genome information has shown that several known RNAi pathway genes are present in *H. contortus* as summarised in Table 4.2, including *dcr-1*, a key component of the RNAi pathway (Geldhof *et al.*, 2007).

In order to analyse the *H. contortus dcr-1* gene in greater detail, the *H. contortus* genome database (assembled worm contigs release 12/11/07) was searched using the *C. elegans* DCR-1 protein sequence by tBLASTn analysis. *dcr-1* sequence was present on contigs 0001464, 0007048 and 0007949, and a subsequent search on newer *H. contortus* genome sequence (assembled supercontigs release 21/08/08) identified *H. contortus dcr-1* (*Hc-dcr-1*) as present on a single supercontig 0059385. This covered a genomic region of approximately 12 kb. No other similar sequences were identified in the *H. contortus* genomic database, and it therefore appears that *H. contortus* possesses a single *dcr-1* gene.

Due to the large size of the *Hc-dcr-1* gene, annotation to identify each exon proved difficult. Identification of exons and deduced amino acid sequence would be useful for comparison with known DCR-1 sequences from other organisms. Consequently, attempts were made to obtain the full coding sequence of *Hc-dcr-1*. The start codon (ATG) of *Hc-dcr-1* was not easily identifiable by amino acid alignment with *C. elegans* DCR-1. Therefore, 5' RACE was carried out on adult cDNA as described in section 2.5.1.4, and the start codon was identified. The gene specific primer used for 5' RACE (*Hc-dcr-1* exon 5 R1) and its relative position on *Hc-dcr-1* cDNA is indicated in Figure 4.1. The sequence of the 3' terminus of *Hc-dcr-1* was also identified by PCR amplification on an adult *H. contortus* cDNA using an oligo dT primer and an internal gene specific primer (*Hc*-dcr 19F1, also shown in Figure 4.1). The two gene specific primers used for

5’ RACE and 3’ terminus identification were designed to an exon sequence identified to encode a region conserved between *C. elegans* and *H. contortus* DCR-1. A full list of primer sequences used is shown in Table 2, Appendix 2. The stop codon (TAA) and the 3’ untranslated region were identified in this manner.

4.2.2 PCR amplification of full length *H. contortus dcr-1*

The identification of the 5’ and 3’ end sequences of the *Hc-dcr-1* gene from adult cDNA firstly confirmed that *Hc-dcr-1* is expressed, and secondly allowed primers to be designed to try to PCR amplify the full length coding sequence of the gene. These primers were named *Hc*-DCR-F1 and *Hc*-DCR-R1, as shown in Figure 4.1. The length of *Hc-dcr-1* coding region was estimated at 5.5 kb, based on tBLASTn results which identified conserved exons between *H. contortus* and *C. elegans* DCR-1. Initial attempts at PCR amplification of the full length gene using H. *contortus* adult or larval (L3) cDNA proved unsuccessful. This was most likely due to the large size of the expected PCR product, and the low level of expression of the *dcr-1* gene (see section 4.2.3). PCR amplification attempts were also made using several different thermostable polymerases intended for use in long range PCR, but no resultant gene product was obtained.

Since it was not possible to PCR amplify the full length of the *Hc-dcr-1* coding sequence in a single round of amplification, internal primers were synthesised to amplify the gene in four smaller fragments of approximately 1.5 kb each, as described in section 2.5.1.5. These eight primers were synthesised based on exon sequences, identified using tBLASTn analysis, to encode regions conserved between *C. elegans* and *H. contortus* DCR-1. The relative position of these primers on *Hc-dcr-1* cDNA is indicated in Figure 4.1.

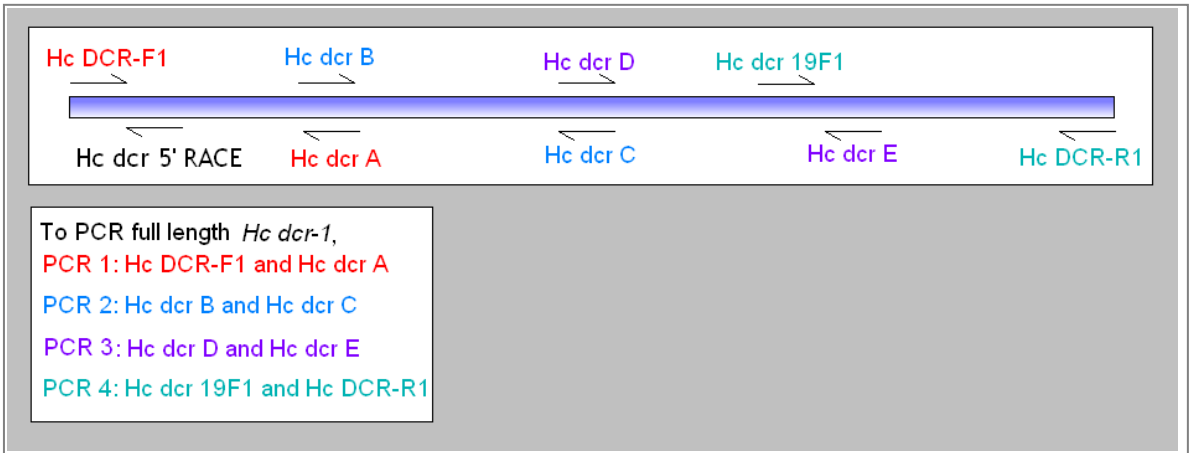


Figure 4.1. Schematic showing positioning of PCR primer pairs used to amplify full length *Hc-dcr-1* cDNA in four separate PCR reactions.

Four separate PCRs were carried out using the primers indicated; PCR 1 used Hc DCR-F1 and Hc dcr A primers, indicated in red text. PCR 2 used Hc dcr B and Hc dcr C primers, as indicated in blue text. PCR 3 used Hc dcr D and Hc dcr E primers as indicated in purple text, and finally PCR 4 used Hc dcr 19F1 and Hc DCR-R1 primers as indicated in green text. 5' RACE was carried out using 'Hc dcr 5' RACE' while 3' RACE used an oligo dT primer in conjunction with 'Hc dcr 19F1'. A full list of primer sequences is shown in Appendix 1. PCRs were carried out using adult *H. contortus* cDNA as template.

Four PCR products were obtained from the four separate PCR reactions outlined in the schematic in Figure 4.1. Together, these four PCR products encompass the full length coding region of *Hc-dcr-1*. Each PCR product was approximately 1.5 kb in length, as shown in Figure 4.2.

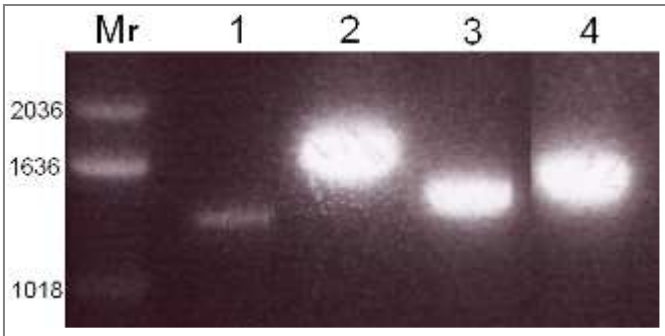


Figure 4.2. PCR of *Hc-dcr-1* using adult *H. contortus* cDNA, in four separate PCR reactions.

(Lane 1) PCR 1, using Hc DCR-F1 and Hc dcr A primers on adult *H. contortus* cDNA. **(Lane 2)** PCR 2, using Hc dcr B and Hc dcr C primers, **(Lane 3)** PCR 3, using Hc dcr D and Hc dcr E primers and **(Lane 4)** PCR 4, using Hc dcr 19F1 and Hc DCR-R1 primers. The position of these primers relative to the cDNA sequence is outlined in Figure 4.1. All four PCR products approximately 1.5 Kb in length.

It is unclear why PCR 1 appears to be less efficient than PCR 2, 3 and 4. Only one PCR product was obtained from 5' RACE, used to elucidate the 5' sequence of the *Hc-dcr-1* gene, upon which the Hc DCR-F1 primer was based, suggesting that alternatively spliced variants of *Hc-dcr-1* are not transcribed. Nevertheless,

these four PCR products were cloned into pSC-A vector using standard techniques described in section 2.5.4.1 and then sequenced as described in section 2.5.6. In most cases, complete insert sequence was obtained using T3 and T7 vector primers. Where overlapping sequence was not obtained, internal primers were synthesised and the insert was re-sequenced. The resultant sequences were assembled by aligning overlapping regions to construct the full length coding sequence of *Hc-dcr-1* using Vector NTI software. The full cDNA sequence, and the translated amino acid sequence is shown in Appendix 4.

4.2.3 Expression of *Hc-dcr-1* in adult and L3 larval stages

It was of interest to examine the expression of *Hc-dcr-1* in adult and L3 larval stages, by comparing the relative amount of *Hc-dcr-1* expression in each stage relative to the amount of *Hc-sod-1* expression. *Hc-sod-1* has been previously shown to be constitutively expressed and therefore selected as a control gene for RT-PCR studies (Liddell & Knox, 1998). Total RNA from cultured *H. contortus* L3 larvae (section 2.5.9) were used to carry out RT-PCR analysis using *Hc-dcr-1* primers (*Hc* dcr 19F1 and *Hc* dcr 20R1, expected product size of 310 bp) and *Hc-sod-1* primers (expected PCR product size of 364 bp), as described in section 2.5.10 . 1st strand cDNA from adult *H. contortus* and total RNA from exsheathed L3 larvae cultured for 72 hours was used for PCR analysis using the same primers as above. The results of this analysis are indicated below in Figure 4.3. *Hc-dcr-1* shows strong expression in the adult stage of *H. contortus*. A slightly lesser amount of *Hc-dcr-1* is expressed in the L3 larval stage. A faint band representing the amplification of genomic DNA was also observed for *Hc-dcr-1*, possibly due to lower levels of the target cDNA.

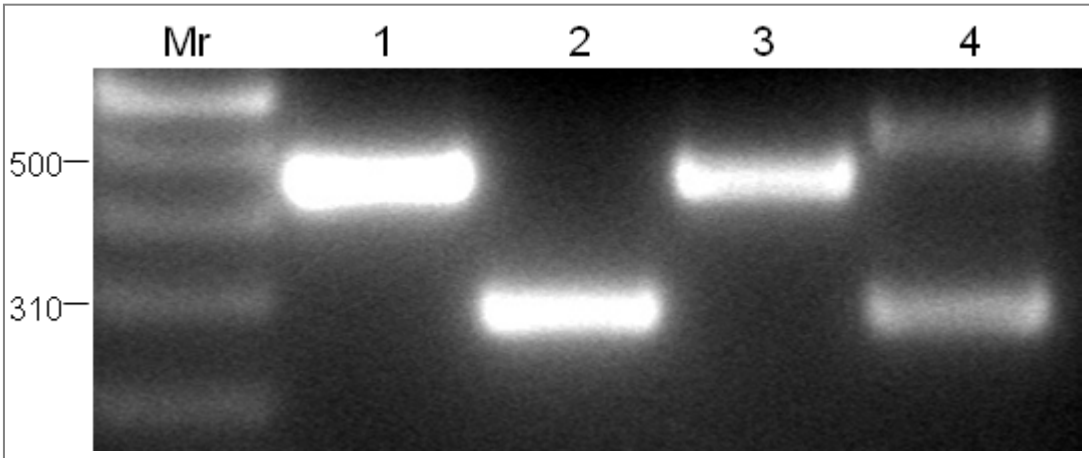


Figure 4.3. *Hc-dcr-1* expression levels relative to *Hc-sod-1* control.

(Lane 1) *Hc-sod-1* gene expression in adult *H. contortus*, (Lane 2*) Hc-dcr-1* gene expression in adult *H. contortus*. 1st strand cDNA synthesised from adult *H. contortus* was used as a template for the PCRs shown in Lane 1 and 2. (Lane 3) *Hc-sod-1* gene expression in *H. contortus* L3 larvae, (Lane 4) *Hc-dcr-1* gene expression in *H. contortus* L3 larvae. Cultured L3 larvae were used to extract total RNA for use as a template for the RT-PCRs shown in Lane 3 and 4. Mr indicates 100 bp DNA ladder.

4.2.4 Analysis of *H. contortus dcr-1* sequence

Comparison of the *Hc-dcr-1* cDNA sequence with the *Hc-dcr-1* genomic region allowed the intron/exon boundaries to be identified. A schematic representation of the *Hc-dcr-1* gene, with the *C. elegans dcr-1* gene for comparison, is shown in Figure 4.4. The size of *Hc-dcr-1* is at least 12,177 bp; the precise size cannot be determined at this point because the region between exons 13 and 15 is present on a region for which incomplete genomic sequence is currently available. *Hc-dcr-1* has 41 exons, ranging in size from the smallest at 63 bp (exon 33) to the largest at 431 bp (exon 3). The mean exon size was determined as 139 bp. All exons combine to form a 5706 bp open reading frame, encoding a 1902 amino acid protein with a predicted molecular weight of 215 kDa.

As shown in Figure 4.4, *Hc-dcr-1* has 40 introns with variable sizes, ranging from the smallest at 51 bp (intron 4) to the largest at 542 bp (intron 3), with a mean intron size of 162 bp. All of the introns in *Hc-dcr-1* possess canonical GT : AG splice donor : acceptor sites except for intron 14. However the complete cDNA sequence indicates that the predicted exons 14 and 15 are spliced as expected. As indicated in Figure 4.4, a comparison with the *Ce-dcr-1* open reading frame shows that the *C. elegans* predicted cDNA is comparatively smaller with 5538 bp, while the *Hc-dcr-1* is 5706 bp in length. The *Ce-dcr-1* gene also has fewer exons than the *Hc-dcr-1*; 26 exons in *Ce-dcr-1* compared with 41 exons in *Hc-dcr-1* and

the *Ce-dcr-1* genomic sequence is 8359 bp in length. This is consistent with previous observations in which the putative *C. elegans* homologue of a *H. contortus* gene tends of be smaller and have fewer exons (for example, Figure 5.6 describes a similar comparison between the *H. contortus H11* gene with the putative *C. elegans* homologue T07F10.1). This is also found for the *H. contortus* and *C. elegans* beta tubulin genes (Gary Saunders, PhD Thesis University of Glasgow 2009) and the cathepsin L protease gene (Britton & Murray, 2002).

4.2.5 Domain architecture of *H. contortus* DCR-1

The *Hc-dcr-1* gene encodes all the domains that are characteristic of typical metazoan dicers, as shown in Figure 4.5. An amino terminal helicase domain, a domain of unknown function (DUF 283) which has since been identified as a divergent dsRNA binding domain (Dlakic, 2006), a PAZ domain, two RNAse III domains and a carboxyl terminal double stranded RNA binding motif (dsRBD) are all present in *Hc*-DCR-1. These domain regions are illustrated in Figure 4.5 and discussed further in section 4.3.

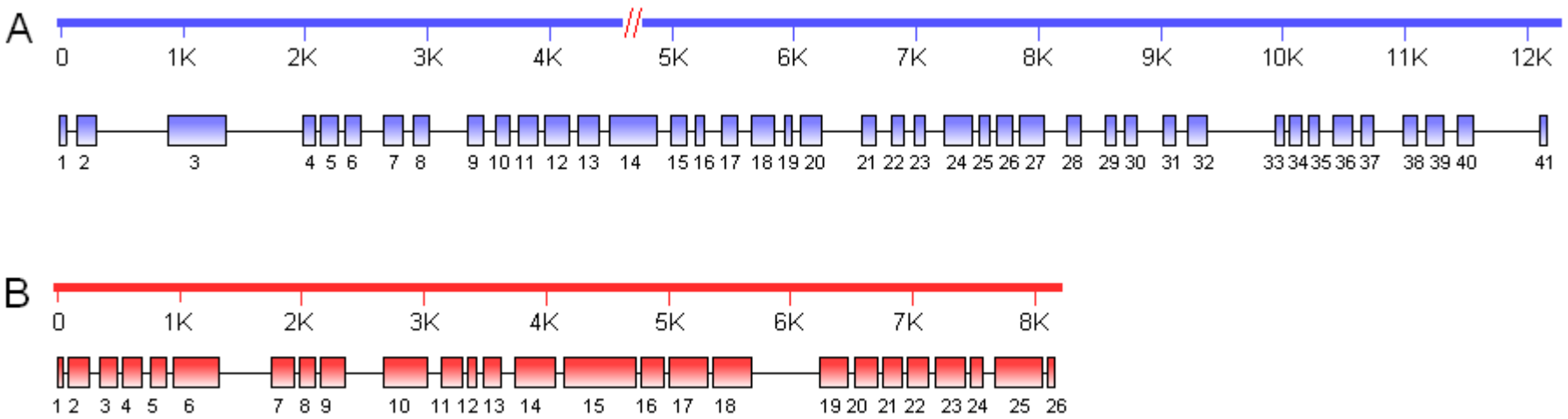


Figure 4.4. Schematic representation of the *H. contortus dcr-1* gene on supercontig 0059385, with the *C. elegans dcr-1* gene for comparison.

(A) The genomic region of *H. contortus dcr-1* indicated in a solid blue line. 'K' indicates kilobase pairs. Exons 1-41 of *Hc-dcr-1* are indicated by blue boxes. Numbers underneath the exons refer to the exon number. -//- on the solid blue line indicates the gap in the genomic region which has not been fully sequenced, but cDNA sequence shows Exon 14 is contained within the gap. The size of *Hc-dcr-1* (genomic copy) is at least 12,177 bp in length.

(B) The genomic region of *C. elegans dcr-1*, indicated in a solid red line. 'K' indicates kilobase pairs. Exons 1-26 of *Ce-dcr-1* are indicated by red boxes. Numbers underneath the exons refer to the exon number. The size of *Ce-dcr-1* (genomic copy) is 8,359 bp in length.

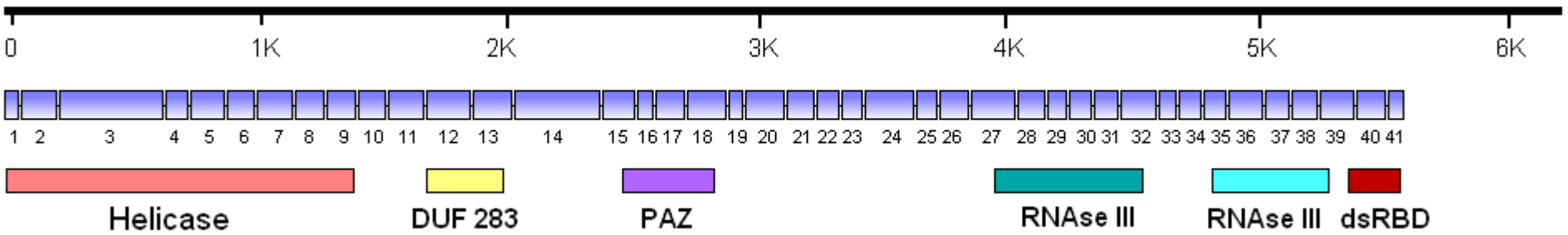


Figure 4.5. *H. contortus dcr-1* coding DNA, with domains characteristic of Dicer proteins highlighted.

Exons 1-41 are indicated in blue boxes. Numbers underneath the exons refer to the exon number. 'K' indicates kilobase pairs. Domains conserved among members of the Dicer protein family (Helicase, DUF 283, PAZ, two RNAse III domains and the double stranded RNA binding domain dsRBD) are indicated below in the exonic regions encoding each domain.

4.2.6 Alignment of *H. contortus* DCR-1 with other DCR-1 proteins

An alignment of the protein sequence of *Hc*-DCR-1 with other DCR-1 proteins from different organisms was carried out using the Vector NTI Align-X programme. Domains characteristic of DCR-1 proteins are highlighted as shown in Figure 4.6. For this analysis, the predicted DCR-1 proteins from *C. elegans, C. briggsae, Homo sapiens, Drosophila melanogaster, Schistosoma mansoni* and *Brugia malayi* were used, along with the *H. contortus* DCR-1 amino acid sequence.

The amino terminus of *Hc*-DCR-1 contains a large helicase domain, homologous to DEAD-box helicases and thought to possess ATP-dependent RNA unwinding activity (Ma *et al.*, 2008). Figure 4.6A shows an alignment of Dicer helicase domains. The conserved DEAD/DExH motif characteristic of these helicases is also present in *Hc*-DCR-1 (^{146}DECH^{149}, motif II in Figure 4.6A) as well as several other motifs conserved within the DEAD-box helicase family (motifs I-V, Figure 4.6A).

The *Hc*-DCR-1 protein also contains a conserved domain of unknown function (DUF 283) which has since been characterised as a divergent dsRNA binding domain (Dlakic, 2006). This domain is largest in the *Sm* DCR-1 protein, while the *Hc*-DCR-1 protein appears to have a more typically sized 86 amino acid DUF 283, as indicated in Figure 4.6B. The PAZ domain, so called because the sequence is also found in proteins belonging to the Piwi, Argonaute and Zwille families, is involved in binding to and positioning RNA for cleavage by the two RNAse III domains (Yan *et al.*, 2003). The *Hc*-DCR-1 PAZ domain is well conserved with the other Dicers analysed, as shown in Figure 4.6C. The two carboxy-terminal RNAse III domains are thought to position the dsRNA target for cleavage approximately 21-25 nucleotides apart, to create siRNAs. These two RNAse III domains are indicated in Figure 4.6D and 4.6E. Finally, the double stranded RNA binding domain (dsRBD), required for binding RNA is well conserved between all the Dicers analysed in this alignment, shown in Figure 4.6F.

The *S. mansoni* DCR-1 protein, at 2,641 amino acids, is significantly larger than the other DCR-1 proteins analysed in this chapter. As a result there appear to be several regions unique to the *Sm* DCR-1 protein which are not found in any of the

other DCR-1 proteins. It is surprising that these unique regions appear within areas conserved between the other DCR-1s, encompassing various functional domains; for example, *Sm* DCR-1 shows unique regions within the helicase domain, DUF 283, PAZ and the RNAse III domain 2, as shown in the alignment in Figure 4.6. The human *Hs* DCR-1 also shows a unique amino acid region within the RNAse III domain 1.

Of particular interest is a region found between the helicase domain and the DUF 283, indicated with a dotted black line in Figure 4.6. This region appears to be fairly well conserved between all the DCR-1 proteins analysed here except for the *C. elegans* and *C. briggsae* DCR-1s, which are both entirely lacking in this area of amino acids. This is surprising considering the phylogenetic relationship of these organisms to each other; areas conserved across mammals, flies and parasitic nematodes would be anticipated to be found in the two free-living nematodes as well, but this is not the case. In order to assess if this region was truly absent from the *C. elegans* DCR-1 protein, the genomic region of the *C. elegans dcr-1* gene was examined. Translating this region into amino acids resulted in a sequence that was somewhat conserved upon alignment with the rest of the DCR-1 proteins (data not shown). Hence it appears that the *C. elegans dcr-1* coding region (Accession number NP_498761.1) might be missing this region of the gene due to incorrect annotation of the exon/intron boundaries. This possibility could be tested experimentally by designing primers to PCR amplify across this region on *C. elegans* cDNA and sequencing the resultant PCR product.

Figure 4.6. Alignment of DCR-1 protein sequences of different organisms.

Accession numbers are given in parentheses. *Hc, Haemonchus contortus*; *Ce, Caenorhabditis elegans* (NP_498761.1); *Cb, Caenorhabditis briggsae* (XP_002643058.1)*; Hs, Homo sapiens* (NP_085124); *Dm, Drosophila melanogaster* (NP_524453); *Bm, Brugia malayi* (translated from mRNA sequence AY352639); *Sm, Schistosoma mansoni* (ABQ02405). Shading denotes amino acid conservation. Regions boxed in colours indicate the amino acid regions encompassed by each domain in the *H. contortus* DCR-1; (A) helicase, in pink (numbers I-V refer to conserved motifs); (B) domain of unknown function 283, in yellow; (C) PAZ domain, in purple; (D) RNAse domain 1, in green; (E) RNAse domain 2, in green; (F) double stranded RNA binding domain, in red (dsRBD). Area boxed in black dotted line indicates amino acid region present in all five DCR-1 proteins analysed except for *C. elegans* and *C. briggsae* DCR-1s. Protein domains were identified using the *S. mansoni* DCR-1 protein as a guide (Krautz-Peterson & Skelly, 2008).

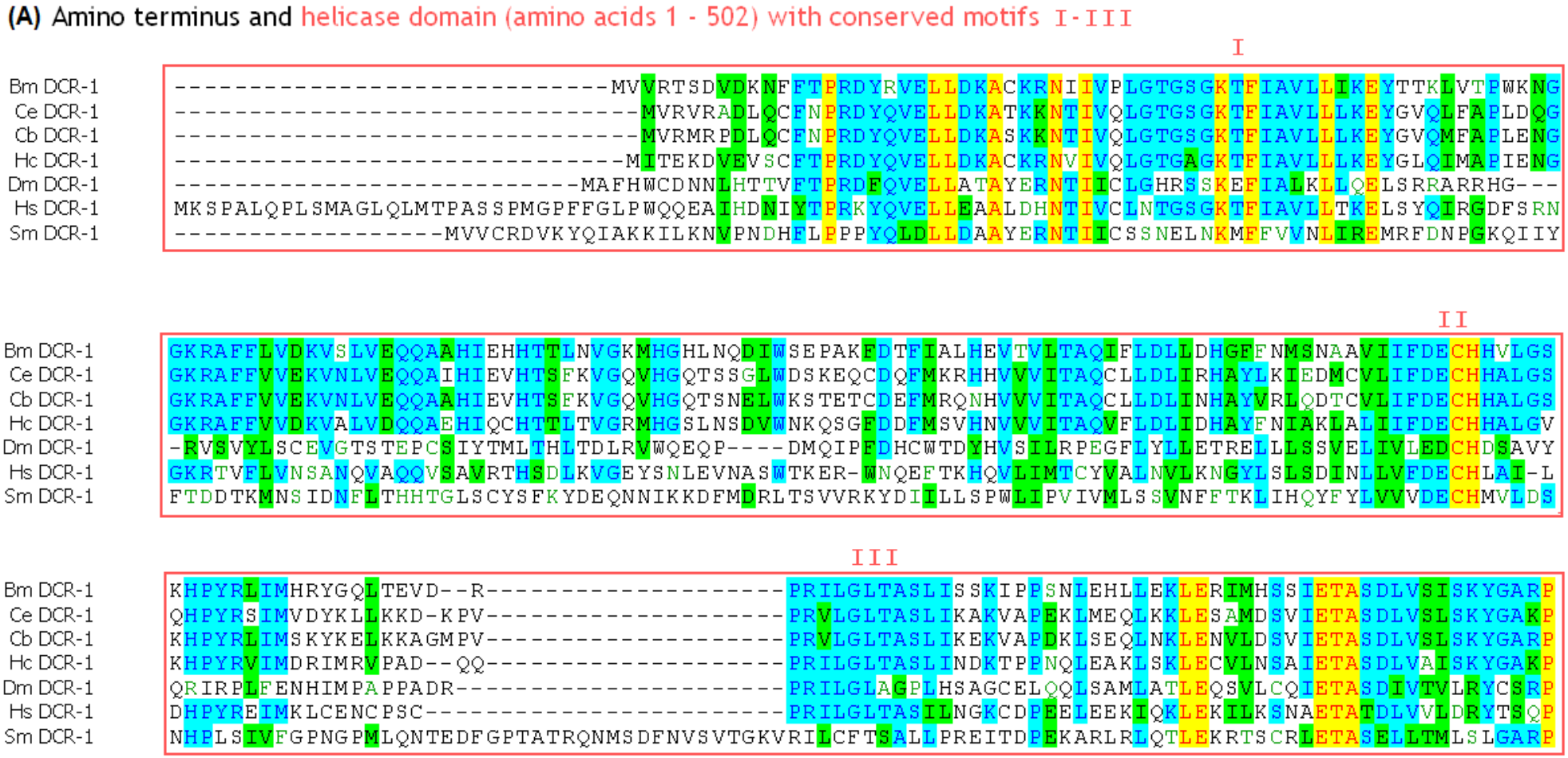


Figure 4.6 ***(continued)***

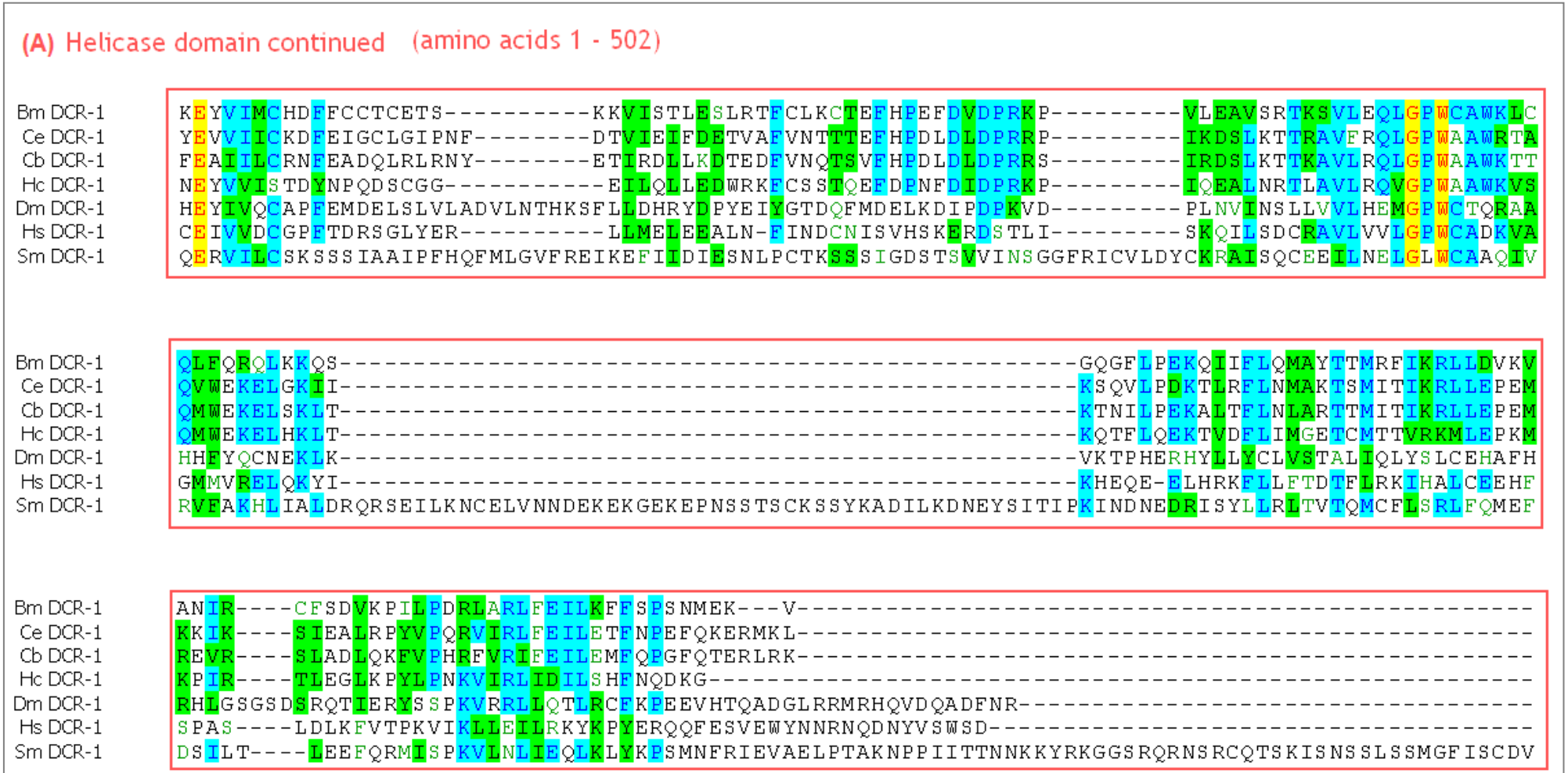


Figure 4.6 ***(continued)***

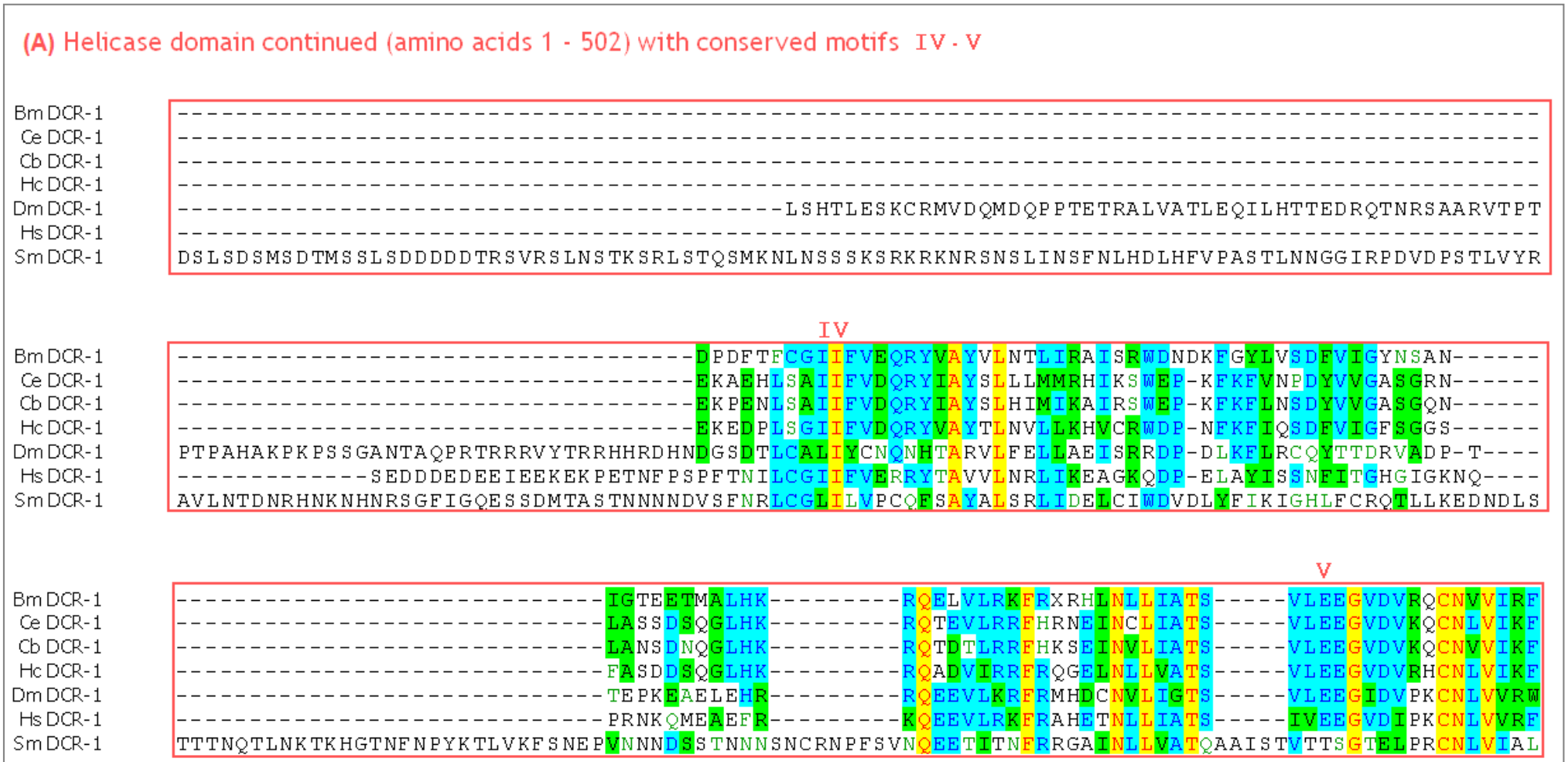


Figure 4.6 ***(continued)***

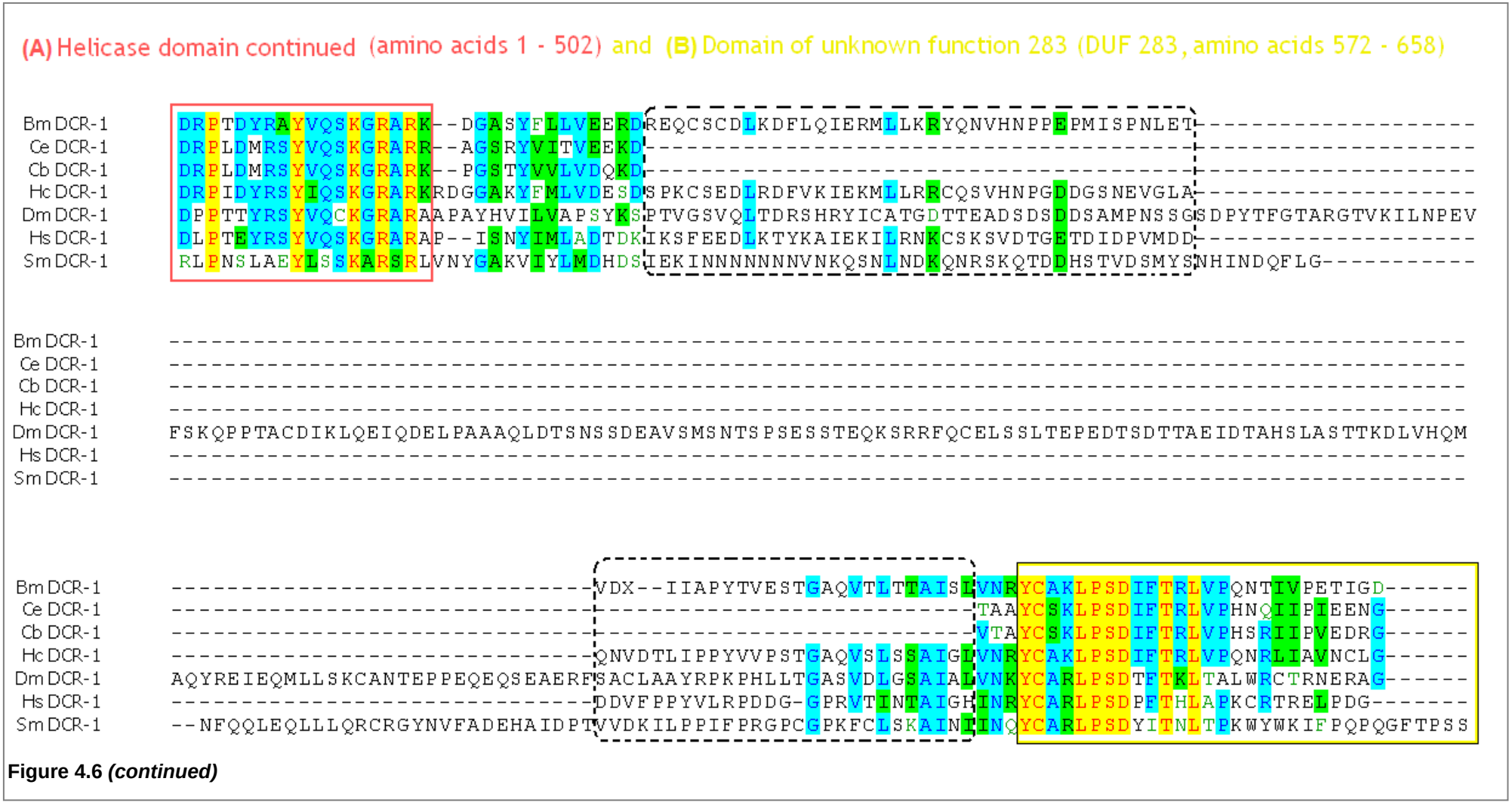


Figure 4.6 ***(continued)***

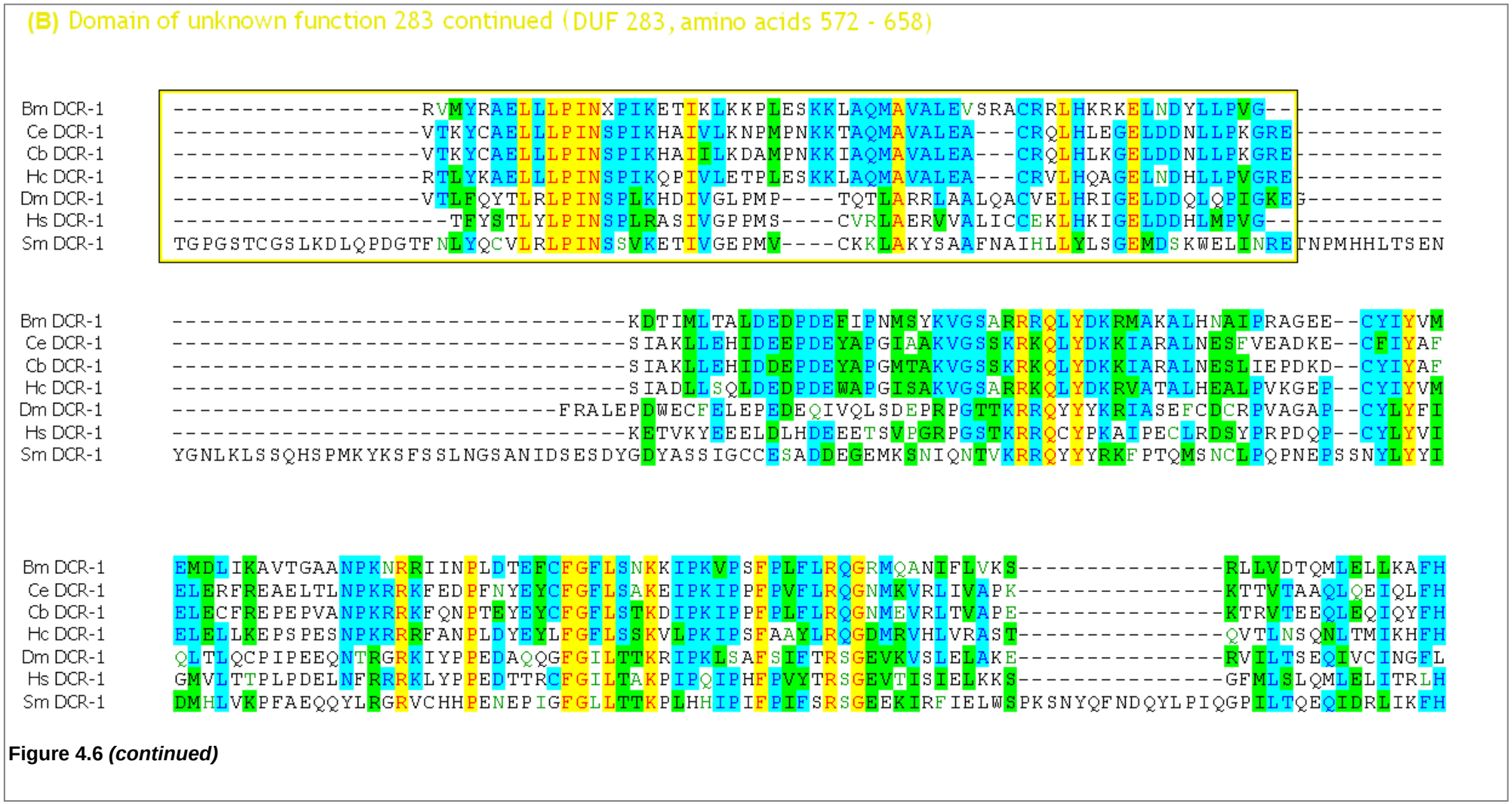


Figure 4.6 ***(continued)***

(C) PAZ domain (amino acid 850 - 1014)

Figure 4.6 ***(continued)***

(C) PAZ domain continued (amino acid 850 - 1014)

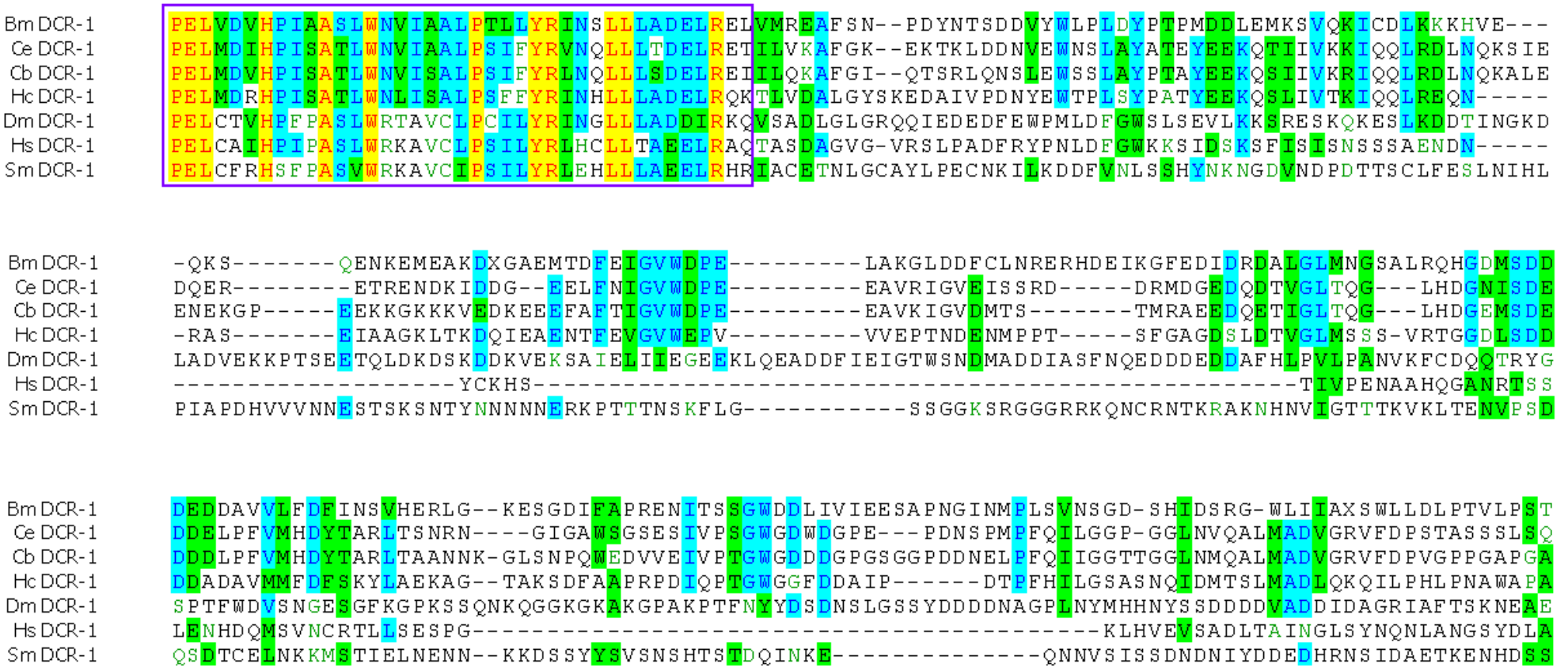


Figure 4.6 ***(continued)***

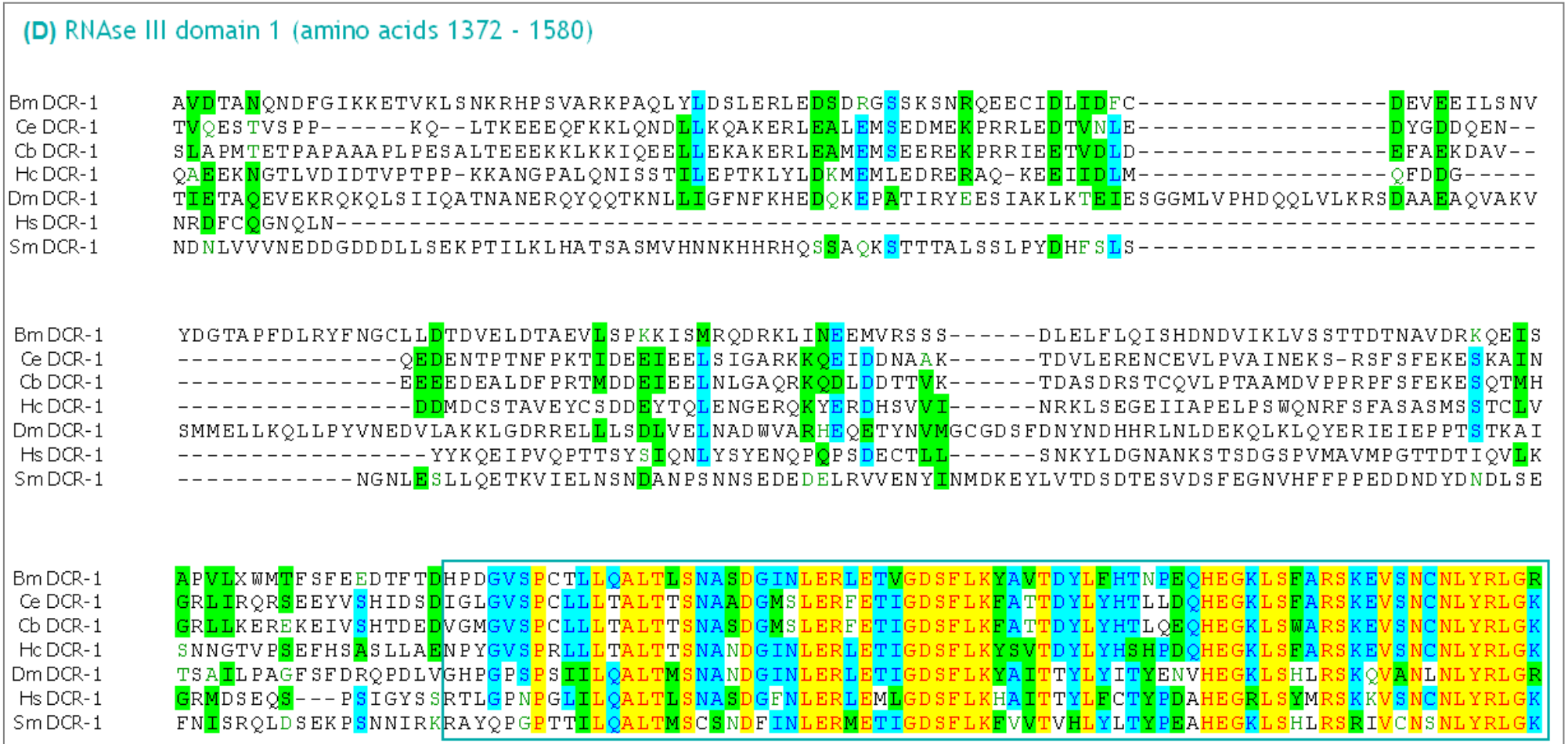


Figure 4.6 ***(continued)***

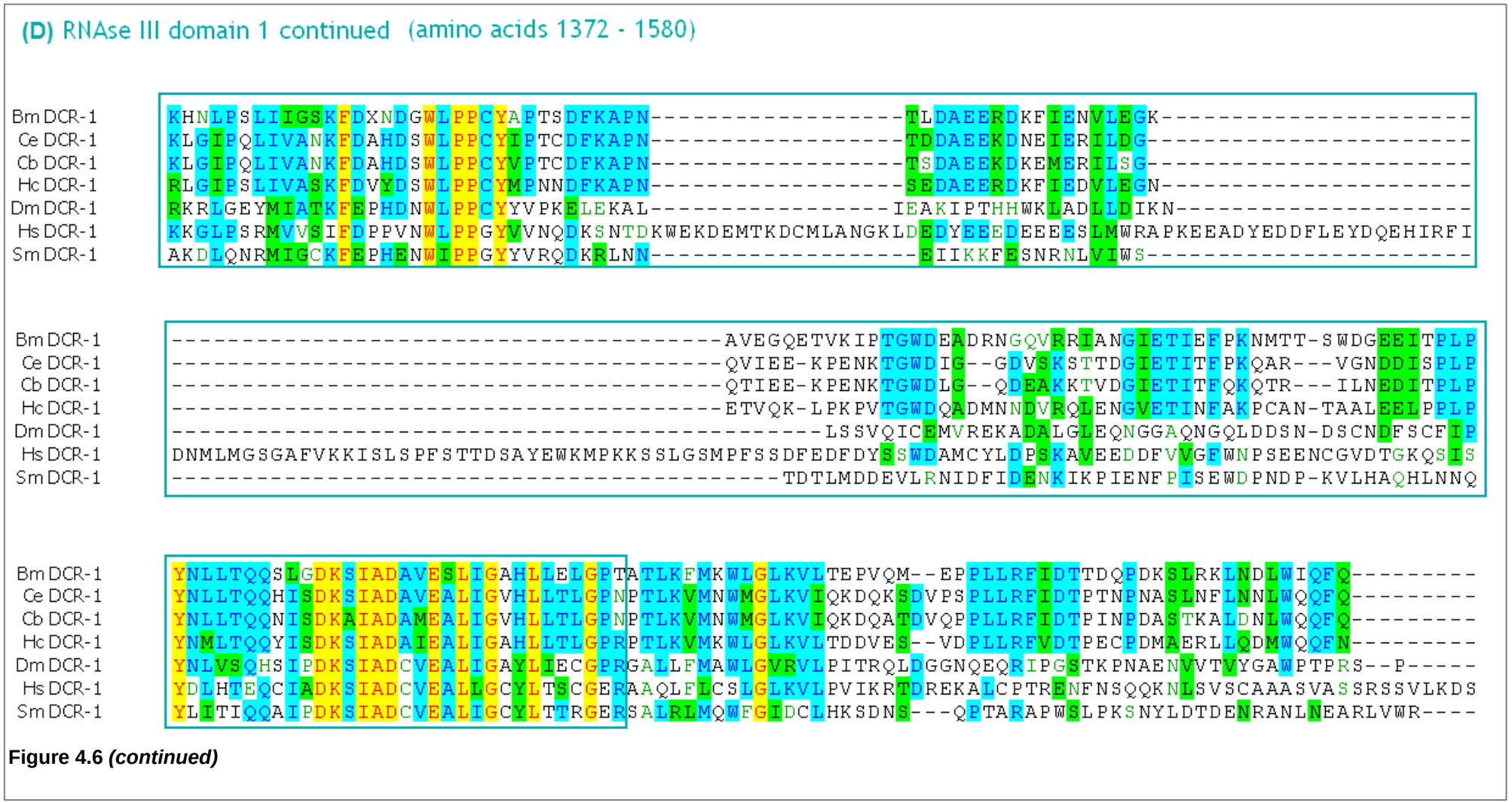


Figure 4.6 ***(continued)***

(E) RNAse III domain 2 (amino acids 1664 - 1791)

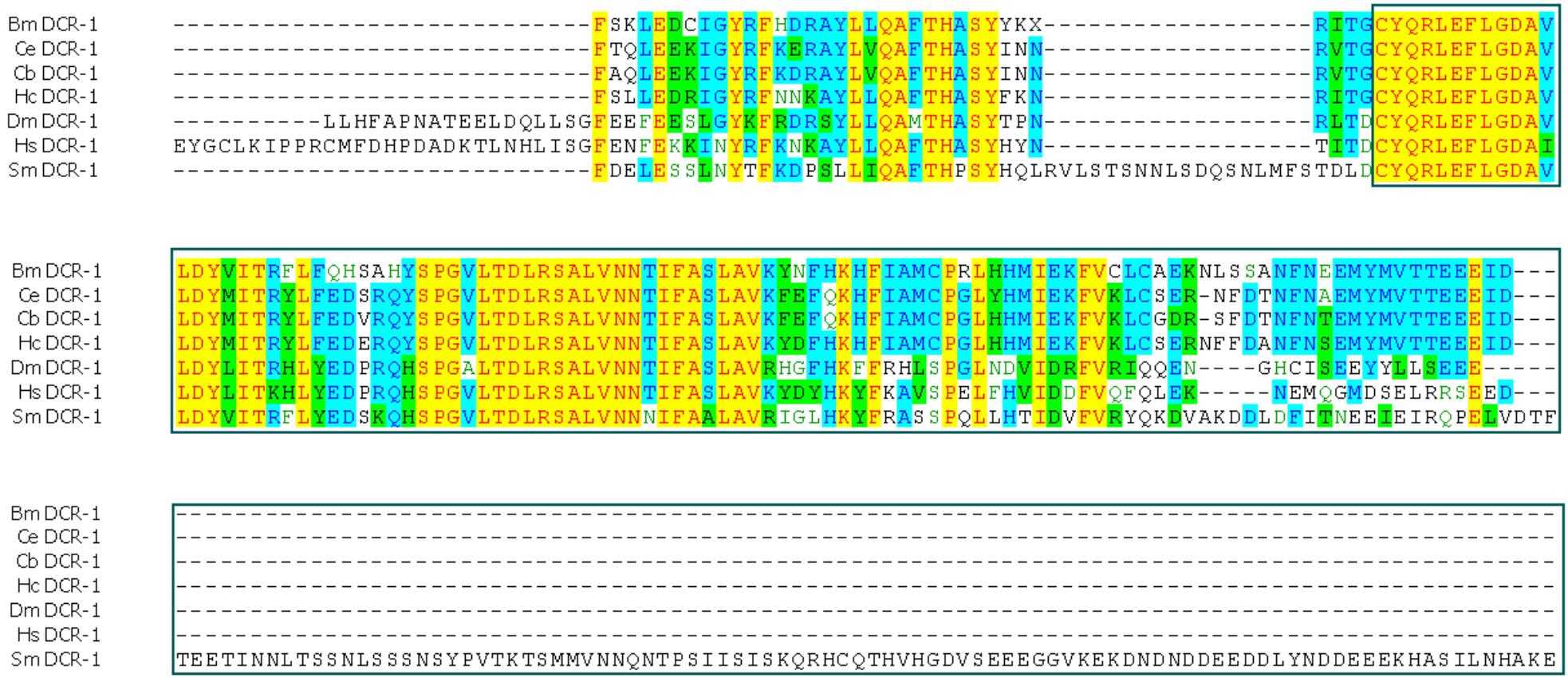


Figure 4.6 ***(continued)***

(E) RNAse III domain 2 continued (amino acids 1664 - 1791) and **(F)** Double stranded RNA binding domain (dsRBD, amino acids 1818 - 1883)

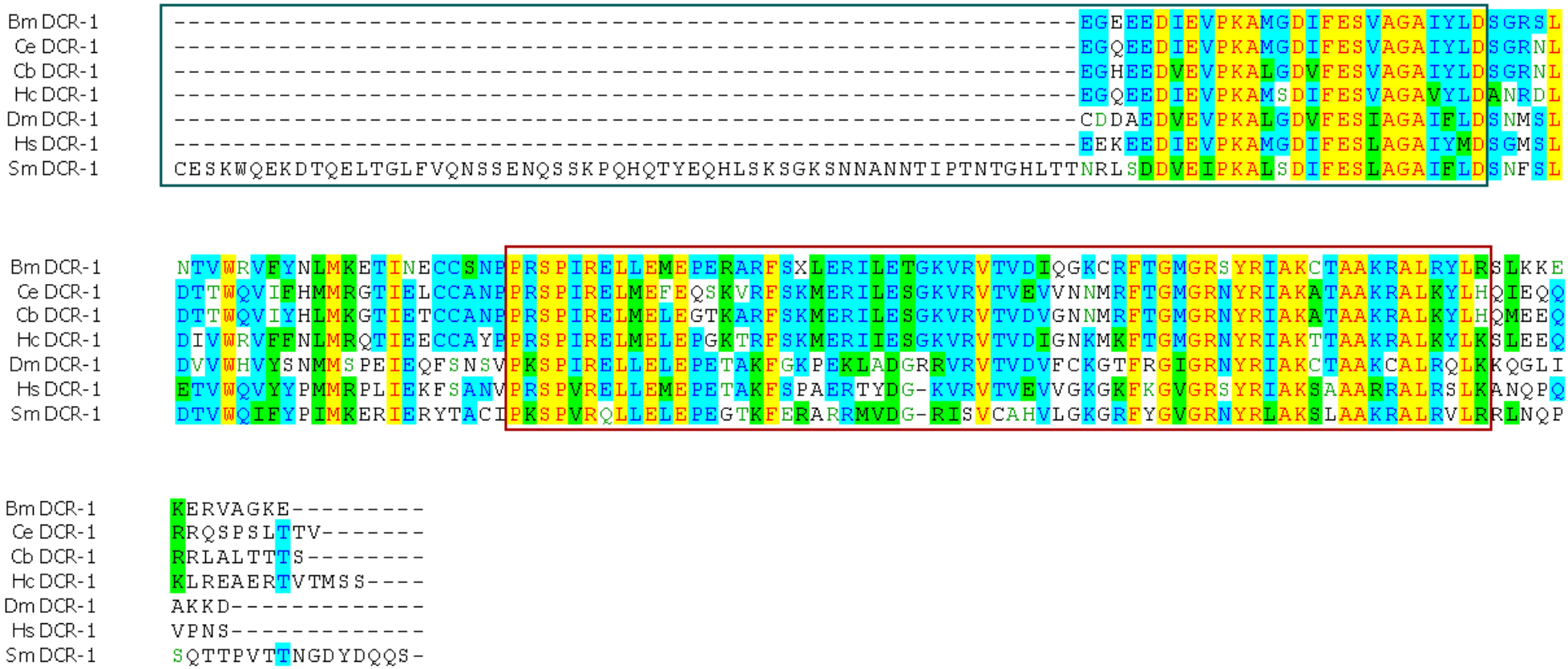


Figure 4.6 ***(continued)***

The *Hc*-DCR-1 protein sequence was also individually aligned with the *C. elegans, C. briggsae, H. sapiens, D. melanogaster, S. mansoni* and *B. malayi* DCR-1 sequences. The percentage identity and similarity of amino acids were calculated, as shown in Table 4.1 for each of these alignments. From this result, it appears that the *S. mansoni* DCR-1 sequence is indeed the most divergent of the Dicer proteins; it has the lowest percentage identity and similarity compared to all six other Dicer proteins analysed here.

Table 4.1. Percentage identity/similarity of DCR-1 amino acid sequences from different organisms

Hc, Haemonchus contortus DCR-1*; Ce, Caenorhabditis elegans* DCR-1 (NP_498761.1*); Cb, Caenorhabditis briggsae* DCR-1 (XP_002643058.1); *Hs, Homo sapiens* DCR-1 (NP_085124); *Dm, Drosophila melanogaster* DCR-1 (NP_524453); *Bm, Brugia malayi* DCR-1 (translated from mRNA sequence AY352639); *Sm, Schistosoma mansoni* DCR-1 (ABQ02405). The identity/similarity of each pair of protein sequences are shown as a percentage.

	Hc	***Ce***	***Cb***	***Hs***	***Dm***	***Bm***	***Sm***
Hc	100 / 100	52 / 64	51 / 63	31 / 42	27 / 39	52 / 64	20 / 30
Ce	52 / 64	100 / 100	74 / 84	30 / 40	26 / 36	46 / 58	19 / 28
Cb	51 / 63	74 / 84	100 / 100	29 / 40	26 / 36	45 / 58	19 / 28
Hs	31 / 42	30 / 40	29 / 40	100 / 100	29 / 38	30 / 41	22 / 30
Dm	27 / 39	26 / 36	26 / 36	29 / 38	100 / 100	27 / 39	22 / 31
Bm	52 / 64	46 / 58	45 / 58	30 / 41	27 / 39	100 / 100	19 / 28
Sm	20 / 30	19 / 28	19 / 28	22 / 30	22 / 31	19 / 28	100 / 100

4.2.7 Phylogenetic analysis of Dicer proteins

The amino acid sequence of *Hc*-DCR-1 was aligned using the multiple sequence alignment programme CLUSTAL X (Thompson *et al.*, 1997) with the corresponding amino acid sequences of DCR-1 from *C. elegans, C. briggsae, H. sapiens, D. melanogaster, S. mansoni* and *B. malayi*, as described in section 2.7.2. A phylogenetic tree was constructed by the neighbour-joining method in MEGA Version 4 (Tamura *et al.*, 2007). Bootstrap sampling analysis from 500 replicates was adopted to evaluate internal branches. The resultant phylogenetic tree is shown in Figure 4.7.

As expected, both *C. elegans* and *C. briggsae* DCR-1 proteins group together, as shown in Figure 4.7. Of the two parasitic nematode DCR-1s analysed, the *H.*

contortus DCR-1 is more closely related to the *C. elegans* and *C. briggsae* DCR-1s than *B. malayi* DCR-1. This was unsurprising as *B. malayi* is classified as a Clade III nematode while *H. contortus*, *C. elegans* and *C. briggsae* are all classified as Clade V nematodes (Blaxter *et al.*, 1998). The *S. mansoni* DCR-1 appears to be the most divergent of the DCR-1 proteins analysed. The large gaps seen in the alignment (Figure 4.6) may result in the *S. mansoni* DCR-1 sequence being described as the most divergent of the DCR-1 proteins in this phylogenetic analysis. It is possible that these gaps represent regions unique to the *S. mansoni* DCR-1 sequence, and may be involved in a novel function for the DCR-1 protein not necessarily related to the RNAi and miRNA pathways.

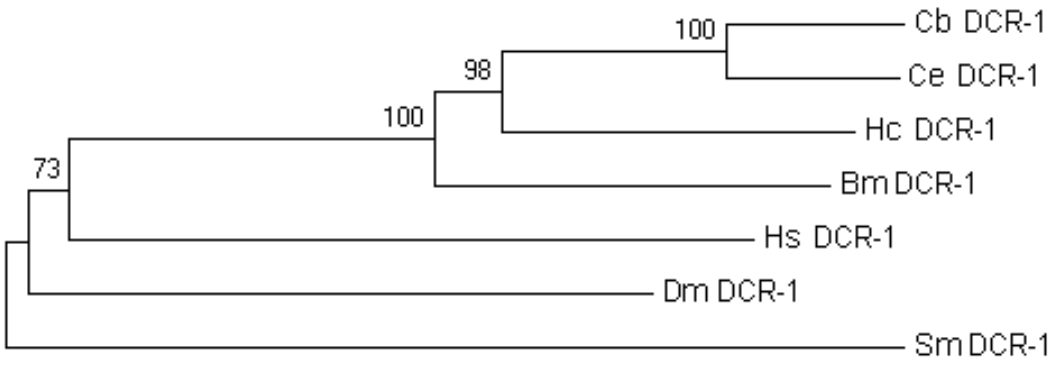


Figure 4.7. Phylogenetic tree based on DCR-1 amino acid sequences.

Protein sequences were aligned by the multiple alignment programme CLUSTAL X (Thompson *et al.*, 1997) and the phylogenetic tree was constructed by the neighbour-joining method using phylogenetic software MEGA Version 4 (Tamura *et al.*, 2007). The bootstrap values from 500-replicates analysis are given at the nodes in percentages. Accession numbers are given in parentheses. *Cb, Caenorhabditis briggsae* (XP_002643058.1); *Ce, Caenorhabditis elegans* (NP_498761.1); *Hc, Haemonchus contortus*; *Bm, Brugia malayi* (translated from mRNA sequence AY352639); *Hs, Homo sapiens* (NP_085124); *Dm, Drosophila melanogaster* (NP_524453); *Sm, Schistosoma mansoni* (ABQ02405).

4.2.8 Bioinformatic search for other RNAi pathway genes in *H. contortus*

Previous work has shown that the *H. contortus* genome has several putative homologues of *C. elegans* genes which are essential for the RNAi pathway (Geldhof *et al.*, 2007). These genes are summarised in Table 4.2. However there were four *C. elegans* RNAi pathway genes for which no homologous sequence could be found in *H. contortus*; *sid-2, rde-2, rde-4* and *rsd-2*. At the time of the initial study, the *H. contortus* genome sequence was at 6-fold coverage and it is

possible that the putative homologues of these genes might be present in more recent *H. contortus* sequence information. Therefore the *H. contortus* genome sequence (assembled supercontigs release 21/08/08) was examined for putative homologues of these genes. SID-2 is involved in dsRNA transportation into the cell and was therefore of particular interest as the uptake of exogenous dsRNA might be a factor in the efficacy of RNAi in *H. contortus* as described in Chapter 3.

Table 4.2. Analysis of *H. contortus* genomic database for putative homologues of *C. elegans* RNAi pathway genes

C. elegans **gene name**	**Size (amino acids)**	**Protein function**	**Putative homologue in *H. contortus* (*H. contortus* assembled supercontigs 21/08/08 all reads)**
dicer	1845	dsRNA specific ribonuclease	Yes, present on Supercontig_0059385
sid-1	776	dsRNA transporter required for systemic RNAi	Yes, present on Supercontig_0024404
sid-2	311	dsRNA transporter required for systemic RNAi	No direct hit, but may be present on Supercontig_ 0059226 as identified in this study.
rde-4	385	dsRNA binding protein (in dicer complex)	No hit
rde-1	1020	Transports siRNAs from initiator complex to rest of pathway	Yes, present on Supercontig_0036504
ego-1	1632	RNA dependent RNA polymerase (required for germline RNAi)	Yes, present on Supercontig_0014903
rrf-1	1601	RNA dependent RNA polymerase (required for somatic RNAi)	Yes, present on Supercontig_0028876
rde-3	441	Functions in complex with RNA dependent RNA polymerases	Yes, present on Supercontig_0035025
eri-1	448	RNAi antagonist (exonuclease)	Yes, present on Supercontig_0047877
rrf-3	1780	RNAi antagonist (RNA dependent RNA polymerase)	Yes, present on Supercontig_0034153
rde-2	578	siRNA accumulation	No hit
rsd-2	1319	Systemic RNAi	No hit

An alignment of the amino acid sequences of *C. elegans*, *C. briggsae* and *C. remanei* SID-2 proteins is shown in Figure 4.8. Several regions which appear to be well conserved between the three sequences are seen, particularly towards the C terminal region which is predicted to be the transmembrane region (Winston *et al.*, 2007). However, several regions of low conservation are also seen, making it apparent that even within the *Caenorhabditis* species, the SID-2 proteins show a high degree of variability.

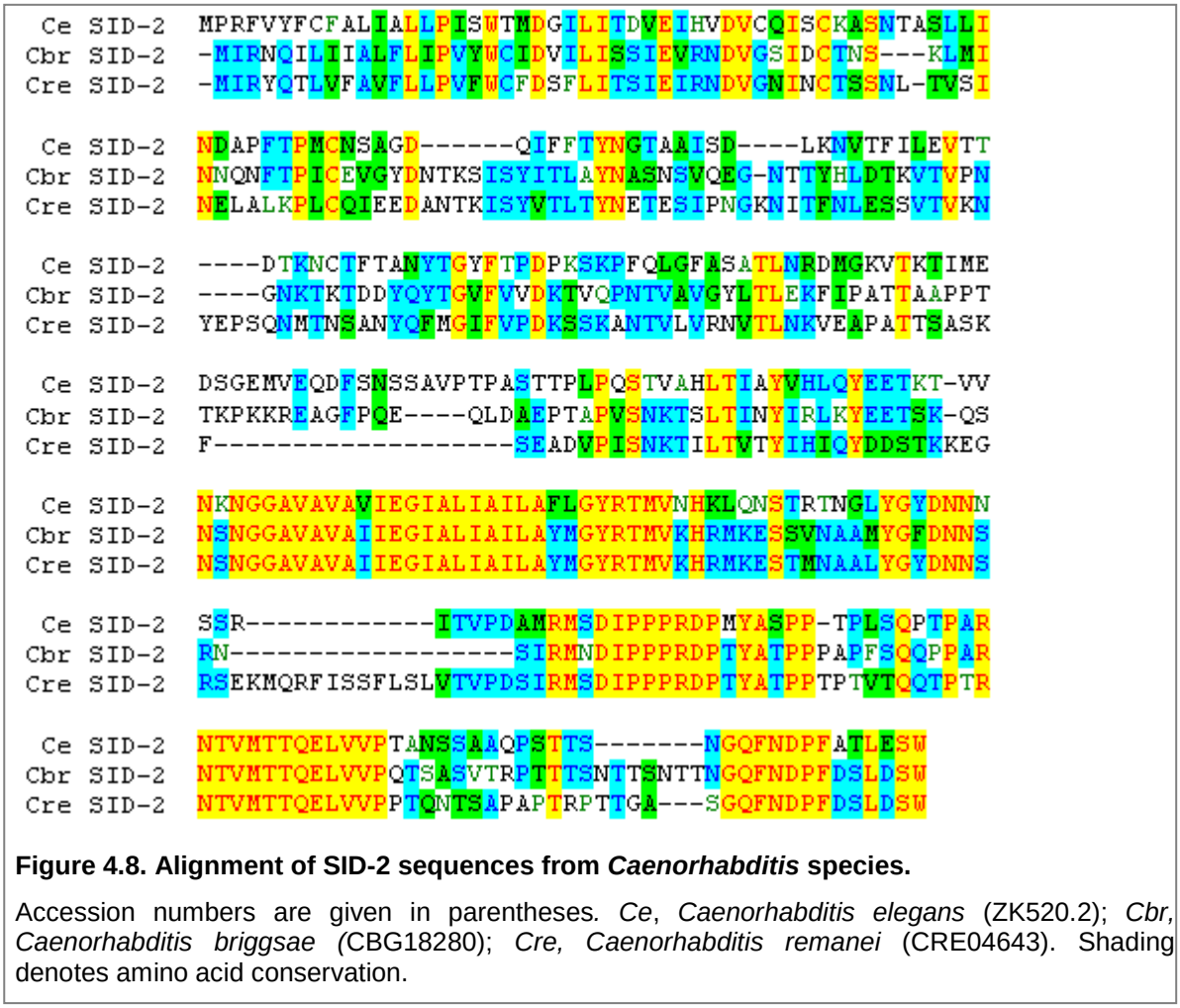


Figure 4.8. Alignment of SID-2 sequences from *Caenorhabditis* species.

Accession numbers are given in parentheses. *Ce*, *Caenorhabditis elegans* (ZK520.2); *Cbr, Caenorhabditis briggsae (*CBG18280); *Cre, Caenorhabditis remanei* (CRE04643). Shading denotes amino acid conservation.

A tBLASTn search using the *C. elegans* SID-2 amino acid sequence against the *H. contortus* genome sequence (assembled supercontigs release 21/08/08 and supercontigs release 26/08/2009) failed to identify any *H. contortus* sequence that might be considered a putative *sid-2* sequence. It is possible that *sid-2* in parasitic nematodes, if present, is extremely divergent from the *C. elegans* sequence and would therefore not be recognised by a tBLASTn analysis. In order to investigate this possibility, the *C. elegans* SID-2 sequence was used to carry out tBLASTn search on the ESTs located at the Washington University Parasite Genomes Database (http://www.ebi.ac.uk/Tools/blast2/parasites.html). This

search resulted in an alignment with an *Ancylostoma ceylanicum* cDNA sequence from L3 larvae (accession number CB175864), which has been annotated as being similar to *C. elegans* SID-2 (ZK520.2). Several regions of conservation were seen between the two sequences, although the overall identity is low at only 35%.

A translation of the *A. ceylanicum* cDNA sequence (accession number CB175864) was then used to search the *H. contortus* genome sequence (assembled supercontigs release 21/08/08) using tBLASTn analysis. A region of the *H. contortus* supercontig 0059226 appears to align with this sequence, with several regions of conserved sequence (71% identity on average at the amino acid level, but over short regions. The *H. contortus* sequences were assembled into a putative protein covering approximately 150 amino acids, hereafter referred to as putative *H. contortus* SID-2. An alignment of the translated *A. ceylanicum* cDNA sequence (accession number CB175864), *C. elegans* SID-2, *C. briggsae* SID-2, *C. remanei* SID-2 and the putative *H. contortus* SID-2 sequence is shown in Figure 4.9. From the alignment, it appears that the *A. ceylanicum* and *H. contortus* sequences are incomplete, as the N terminal regions of these proteins appear to be missing. However, several regions of conservation can be seen between all five proteins, particularly towards the C terminal region predicted to be the transmembrane region. It would be necessary to confirm by RT-PCR analysis that this gene is expressed in *H. contortus*. It appears that both *A. ceylanicum* and *H. contortus* possess a sequence which might be an extremely divergent version of the *C. elegans sid-2* gene. A search of the *B. malayi* genome did not reveal a gene characterised as *sid-2*.

A similar search was carried out for the dsRNA binding protein RDE-4, described in section 1.2.2. The *H. contortus* genome appears to lack any sequencing coding for a putative homologue of *rde-4*. The *B. malayi* genome sequence also appears to lack any sequence encoding a homologue of RDE-4.

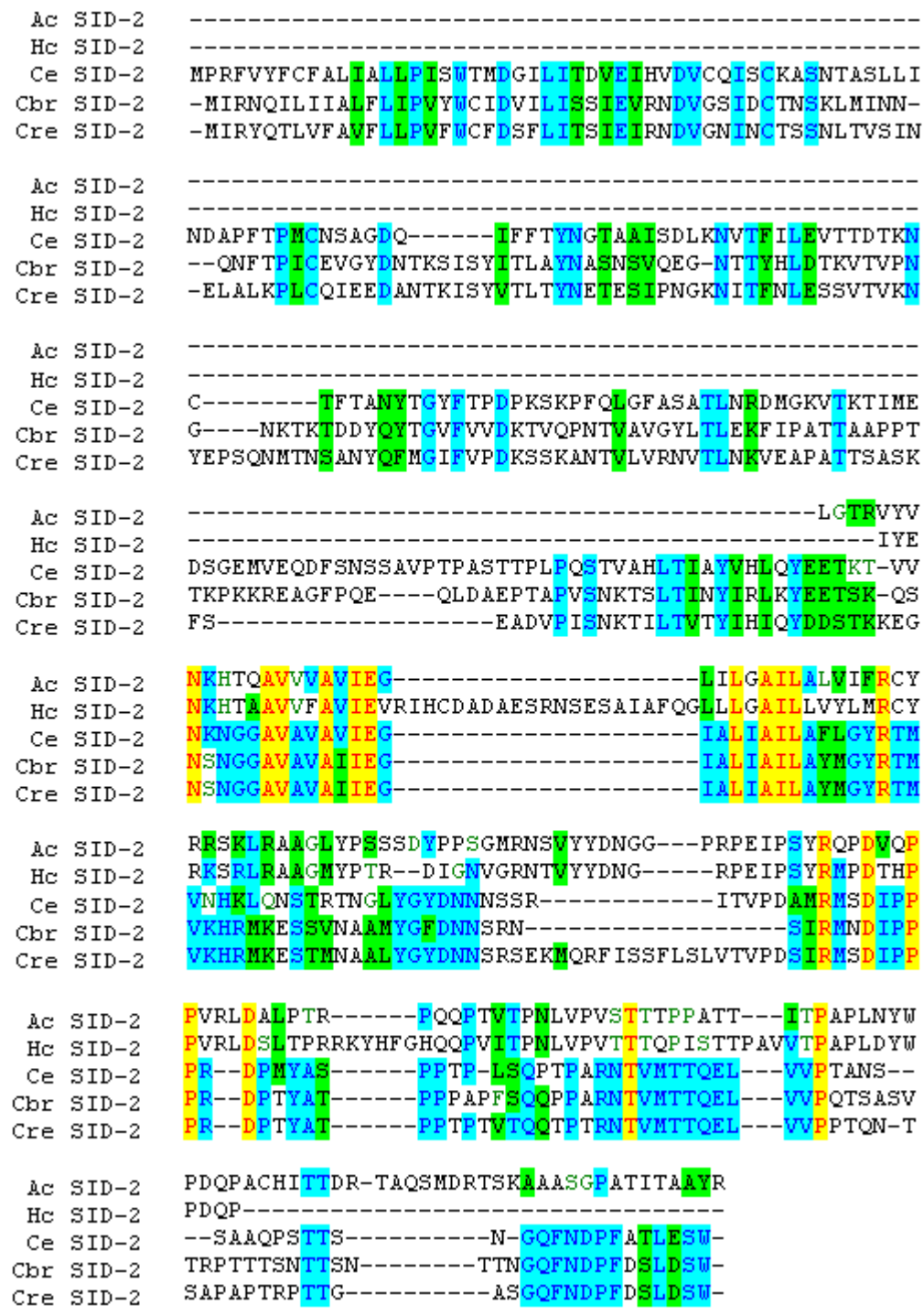


Figure 4.9. Alignment of SID-2 sequences from Caenorhabditis and parasitic species.

Accession numbers are given in parentheses. Ac, Ancylostoma ceylanicum (CB175864); Hc, Haemonchus contortus (supercontig 0059226); *Ce*, *Caenorhabditis elegans* (ZK520.2); *Cbr, Caenorhabditis briggsae (*CBG18280); *Cre, Caenorhabditis remanei* (CRE04643). Shading denotes amino acid conservation.

4.3 Discussion

The main focus of this chapter was to identify and characterise key components of the RNAi pathway, with particular attention to the *H. contortus dcr-1* gene. The complete coding sequence of the *Hc-dcr-1* gene was obtained by PCR amplification and subsequently sequenced, resulting in a gene that has an open reading frame of 5706 bp. Comparative analysis of the *Hc*-DCR-1 protein sequence with DCR-1 sequences from other organisms allowed identification of important domains characteristic of DCR-1 proteins, conserved across species.

C. elegans and *H. contortus* are both Clade V nematodes, and the *C. elegans* DCR-1 sequence was useful for identifying the complete sequence of *Hc*-DCR-1. A comparison of the DCR-1 proteins between the two nematodes shows that the *Hc-dcr-1* gene is larger in size and has more exons and introns than the *Ce-dcr-1* gene. This observation is consistent with other comparisons between *C. elegans* genes and their putative homologues in *H. contortus*; for example, Figure 5.6 shows a juxtaposition of the *H. contortus H11* family with the putative *C. elegans* homologue T07F10.1. The *Hc*-DCR-1 protein is 1,902 amino acids in length, while both *C. elegans* and *C. briggsae* DCR-1 proteins are smaller than the *Hc*-DCR-1 protein, being 1,845 and 1,863 amino acids in length respectively. In contrast, the *D. melanogaster* and *S. mansoni* DCR-1 proteins are larger than the *Hc*-DCR-1 protein, with lengths of 2,249 and 2,261 amino acids respectively. It is surprising that the *S. mansoni* DCR-1 protein shows such a high degree of divergence from the other DCR-1 proteins analysed in this chapter; regions of the proteins conserved across diverse phyla such as Arthropods, Chordates and Nematoda are variant in the *S. mansoni* DCR-1 protein, with a significant number of insertions compared to the other Dicer proteins. Phylogenetic analysis using *Hc*-DCR-1 with the other DCR-1 proteins positioned the *H. contortus* protein not surprisingly between the *Caenorhabditis* DCR-1 proteins and the *B. malayi* DCR-1 protein. This is expected because *C. elegans*, *C. briggsae* and *H. contortus* are all Clade V nematodes while *B. malayi* is classified as a Clade III nematode (Blaxter *et al.*, 1998). A few regions of divergent sequence in which the *Hc*-DCR-1 protein does not seem to align well with the *Ce*-DCR-1 can be observed, but these regions are variant in the other DCR-1 proteins too and possibly not involved in a critical function of DCR-1. It is probable that there is low selection

pressure for these regions to remain conserved over evolutionary time. The theory of exon shuffling refers to the idea that each exon can encode a single protein domain which then allows novel genes to be created by duplication and rearrangement (Patthy, 1999). However, this cannot be applied to *Hc*-DCR-1 because each of the protein domains spans more than one exon.

During the PCR amplification of *Hc*-DCR-1, a region across intron 12 was amplified on *H. contortus* genomic DNA in order to define the intron-exon boundaries. The sequence of this PCR fragment was different to the *H. contortus* genomic supercontig 0059385 (data not shown). The region is most likely an indel region as the variation is present in the intronic region only. This was surprising as the genomic DNA template used for the PCR amplification was from the same MHco3 (ISE) isolate of *H. contortus* as used for the *H. contortus* genome sequencing project. Previous work has also identified indel regions during PCR amplification of the promoter region of *H. contortus* beta tubulin isotype-3 gene (Gary Saunders, PhD Thesis University of Glasgow 2009). This observation highlights the extensive sequence polymorphisms that complicate the annotation and assembly of the *H. contortus* genome data.

DCR-1 has an overlapping role in both RNAi and miRNA pathways; DCR-1 is involved in processing long dsRNA molecules into siRNAs in the RNAi pathway and in processing dsRNA hairpins into miRNAs in the miRNA pathway. Both processes require several functions, which in turn require several specialised protein domains capable of carrying out these functions. The dsRNA needs to be unwound, held in place and then cleaved at either end to produce siRNA or miRNAs molecules. The domain architecture of the *Hc*-DCR-1 protein is ideally suited to carry out these functions; the amino terminal helicase domain is involved in unwinding the dsRNA while the divergent dsRNA binding domain (DUF-283), PAZ domain and carboxy terminal dsRNA binding domain positions the dsRNA for cleavage at two separate locations by the two RNAse III domains.

The results shown in Chapter 3 describe the RNAi silencing of genes in the L3 larval stage of *H. contortus*. Currently it is difficult to culture the larvae beyond the L3/L4 stage into adult worms, and it is therefore not known if better RNAi silencing would occur in a later stage of the life cycle. The *Hc-dcr-1* gene is expressed in larval L3 and adult stages, and it appears that the level of *Hc-dcr-1*

is slightly less in the L3 larval stage compared to the adult, relative to the *Hc-sod-1* control. However it is worth noting that the *Hc-dcr-1* mRNA levels in L3 larvae do not change when larvae are exposed to dsRNA; inducing the RNAi pathway does not seem to upregulate *Hc-dcr-1* expression (data not shown). Similarly, there was no significant upregulation of *Ce-dcr-1* upon soaking *C. elegans* in dsRNA to induce RNAi silencing (Collette Britton, unpublished data). The finding that *Hc-dcr-1* is expressed in both L3 and adult stages is important; it has been suggested that lack of RNAi silencing in *S. mansoni* schistosomulae is due to low levels of expression of RNAi pathway genes (Krautz-Peterson & Skelly, 2008; Krautz-Peterson *et al.*, 2009).

A considerable number of known RNAi pathway genes which were initially characterised in *C. elegans* have putative homologues in the *H. contortus* genome (Geldhof *et al.*, 2007). Several *H. contortus* genes have been consistently silenced using RNAi, as detailed in Chapter 3. Therefore it appears that *H. contortus* possesses a functional RNAi pathway, capable of silencing some genes. A database search identified a putative homologue of *C. elegans* DRSH-1, a protein involved upstream of DCR-1 in the miRNA pathway, is present on *H. contortus* supercontig 0032681 (assembled supercontigs release 21/08/08, data not shown). Several miRNAs have also been identified in *H. contortus* both bioinformatically (Collette Britton, personal communication) and experimentally (Alan Winter, personal communication). Based on this information, it appears that *H. contortus* also has a functional miRNA pathway, in addition to the functional RNAi pathway. Given the overlapping role played by DCR-1 in both RNAi and miRNA pathways, mutations in *dcr-1* have lethal consequences in several different species. The inactivation of *dcr-1* in mice results in early embryonic lethality (Bernstein *et al.*, 2003) and the RNAi mediated silencing of human *dcr-1* leads to defects in both miRNA production and RNAi (Hutvagner *et al.*, 2001). Mutations in the *C. elegans dcr-1* gene show defects in RNAi and developmental abnormalities (Grishok *et al.*, 2001; Ketting *et al.*, 2001; Lee & Ambros, 2001). These developmental abnormalities observed with *dcr-1* mutants have since been attributed to the malfunctioning of the miRNA pathway (Grishok *et al.*, 2001; Lee *et al.*, 1993; Reinhart *et al.*, 2000). Most of the RNAi and developmental defects were rescued by introducing a wild type *Ce-dcr-1* gene to the *C. elegans dcr-1* mutants. It would be interesting to test the *Hc-dcr-1* gene

functionally in a similar manner. Although there are currently no protocols developed for direct transgenesis in *H. contortus*, it would be possible to test any rescue effects by introducing the *Hc-dcr-1* gene into *C. elegans dcr-1* mutants. This could be done by using a construct consisting of the *C. elegans dcr-1* promoter, *Hc-dcr-1* coding region and *C. elegans dcr-1* 3' UTR, based on the rescue protocol used to test *Hc-cpl-1* function in *C. elegans* (Britton & Murray, 2002). If rescue of the *C. elegans dcr-1* mutant phenotype is indeed possible by *Hc-dcr-1*, it would provide more evidence for a functional role for *Hc-dcr-1* in the RNAi and miRNA pathway in *H. contortus*. The full length coding sequence of *Hc-dcr-1* described in this chapter would enable the above described experiment to be carried out in the future.

The dsRNA binding protein RDE-4, encoded by the *rde-4* gene, appears to be absent from the *H. contortus* genome. *rde-4* also appears to be absent from the *B. malayi* genome (data not shown). It is possible that the *rde-4* gene in both these organisms has not yet been characterised as the genome information may still be incomplete. Alternatively, it is possible that another dsRNA binding protein has taken over the function of RDE-4, or the RDE-4 protein in parasitic nematodes is so divergent from the *C. elegans* RDE-4 sequence that it cannot be identified using tBLASTn alignment programmes. Furthermore, although RDE-4 is regarded as essential for RNAi in *C. elegans* (Parrish & Fire, 2001), recent work has shown that *rde-4* deficient worms are capable of RNAi silencing in the presence of high concentrations of dsRNA (Habig *et al.*, 2008).

It is unclear if the dsRNA channel protein SID-2 is absent from the *H. contortus* genome. In *C. elegans*, SID-2 is required for the uptake of dsRNA from the environment as elaborated in section 1.2.2. Previous database searches using *C. elegans* SID-2 to search for a putative homologue in *H. contortus* have thus far failed to result in any matching sequence, but it appears that an extremely diverged version of SID-2 might be present in *A. ceylanicum* and *H. contortus*. Although the related nematode *C. briggsae* also possesses a homologous SID-2 protein, its function in transporting environmentally introduced dsRNA is not conserved in *C. briggsae* (Winston *et al.*, 2007). An alignment of *C. elegans* and *C. briggsae* SID-2 proteins show that they are not very well conserved between the two species (Winston *et al.*, 2007), particularly in the luminal domain (region of the protein predicted to be in the intestinal lumen of the worm),

despite the relatively recent divergence between *C. elegans* and *C. briggsae* estimated at 25-50 million years ago (Kent & Zahler, 2000). Therefore it is perhaps unsurprising that *H. contortus* appears to lack a SID-2 protein which is similar to the *C. elegans* SID-2 protein. It is possible that if SID-2 is present in *H. contortus*, it might have diverged in function and therefore the sequence might not be well conserved with *C. elegans* SID-2. Alternatively, it is possible that an entirely different protein is involved in the uptake of dsRNA from the environment. As a result, it may be hypothesised that this uptake of dsRNA is not an efficient as it is in *C. elegans*, possibly explaining why RNAi works for some genes but not others.

Chapter 5

Haemonchus contortus gene expression and regulation

5.1 Introduction

Our knowledge of gene expression and regulatory mechanisms in free-living and parasitic nematodes is increasing through studies carried out in the free-living model nematode *C. elegans*. Methods developed for analysing the regulation of single genes have now been expanded to include large-scale analysis of gene regulation at a global level. For instance, experimental analysis of the promoters of a number of intestine-specific genes in *C. elegans* show that they are all controlled by GATA-related sequences, recognised by specific GATA transcription factors (Britton *et al.*, 1998; Egan *et al.*, 1995; Fukushige *et al.*, 2005; MacMorris *et al.*, 1992; MacMorris *et al.*, 1994; Pauli *et al.*, 2006). On a wider scale, techniques such as Serial Analysis of Gene Expression (SAGE) have allowed the study of the global regulation of transcription, for example in intestinally expressed genes in *C. elegans* (McGhee *et al.*, 2007).

Specific motifs thought to regulate expression in specific tissues can be verified by transgene expression in *C. elegans*. Transgenic animals can be generated carrying promoter regions fused *in vitro* to *lacZ* (Fire *et al.*, 1990) or *gfp* reporter genes (Chalfie *et al.*, 1994), allowing the expression pattern of the gene of interest to be elucidated. A large range of plasmid vectors, the Fire Vectors, containing modular features such as *lacZ* and *gfp* reporter genes are available for studying gene expression in various eukaryotic systems (Fire *et al.*, 1990). However, this approach to generating reporter gene fusions involves numerous steps of DNA purification and sub-cloning which can be time consuming. As a result, a newer method for generating reporter gene fusions has been developed which involves PCR-based fusions of overlapping DNA fragments, as outlined in Figure 2.1 (Hobert, 2002). *gfp* fusion constructs can thus be ready for injection into *C. elegans* within one day, and this approach has been utilised in high-throughput studies of gene expression involving large numbers of reporter gene constructs. For example, the spatial and temporal tissue expression profiles of 1,886 genes in *C. elegans* was analysed in this manner, identifying many tissue specific 5' regulatory regions (Hunt-Newbury *et al.*, 2007).

Another approach used to characterise the *in vivo* expression state of the genome is the construction of the *C. elegans* promoterome (Dupuy *et al.*, 2004). The promoterome contains a third of the predicted *C. elegans* promoters (~6000) in a cloning format that allows their transfer into various destination vectors that can then be utilised in a high-throughput setting to probe the dynamic aspects of gene regulation. The promoterome database, located at http://vidal.dfci.harvard.edu/promoteromedb, can be searched by gene name and displays the upstream promoter region of the gene, the primer sequences used, cloning and sequence information. The individual clones can also be purchased from the *C. elegans* promoterome library, located at http://www.geneservice.co.uk/products/clones/Celegans_Prom.jsp. These can subsequently be used in various downstream applications such as tissue-specific RNAi, in which a tissue specific promoter is used to control the expression of double stranded hairpin RNA encoded by an inverted repeat of the target gene, or yeast-one-hybrid analysis to characterise DNA-protein interactions (Briese *et al.*, 2006; Deplancke *et al.*, 2004). The promoterome could potentially be applied to any other organism, subject to availability of an annotated genome sequence and transgenesis methods. Although transgenesis methods are currently lacking for *H. contortus*, successful reports of heritable DNA transformation and transgene expression have been reported in *Strongyloides stercoralis* (Li *et al.*, 2006).

It is worth noting that regulatory information that affects the expression pattern of a gene is not always found exclusively within the 5' upstream promoter region of a gene, and can often be found within introns or downstream of a gene. For example, an increasing number of micro RNAs (miRNAs) have been identified that can bind to and regulate gene expression via the 3' untranslated region. The *C. elegans* miRNAs *lin-4* and *let-7* miRNAs were the first miRNAs identified in any organism; they play essential roles in controlling the timing of development by negatively regulating expression of their target genes (Lee *et al.*, 1993; Reinhart *et al.*, 2000). Reporter genes covering large genomic regions are more likely to capture all the *cis*-regulatory information and may be expected to represent most aspects of endogenous gene expression. Therefore a recent approach to reporter gene constructs involves engineering fluorescent proteins

into large genomic clones within BACs or fosmid vectors, using homologous recombination in bacterial cells (Tursun *et al.*, 2009).

In contrast to the studies in *C. elegans,* the mechanisms of gene expression and regulation in *H. contortus* and other parasitic nematodes are poorly understood because there is a lack of direct information about the site of gene expression within the parasite, particularly in the larval stages. However, some information is available through tissue-specific EST libraries; for example, intestinal sequences from *H. contortus* and *Ascaris suum* have been used to construct an intestine-specific EST library (Yin *et al.*, 2008). An alternative approach, analysing where the encoded protein is localised using antibody staining, is an expensive and lengthy process as it is necessary to raise and manufacture the appropriate antibody for each protein of interest.

Nevertheless, due to the close phylogenetic relationship shared between *H. contortus* and *C. elegans,* and the numerous techniques available for use in *C. elegans,* these problems can be somewhat overcome. For instance, it is possible to express some *H. contortus* genes in transgenic *C. elegans*, as demonstrated by the functional analysis of a *H. contortus* beta tubulin gene in *C. elegans* (Kwa *et al.*, 1995). Similarly, *C. elegans* can be used as a heterologous transformation system to examine the expression of parasitic nematode gene promoters. For example, the expression pattern of a *H. contortus* cysteine protease gene AC-2 was analysed by fusing a 2 kb upstream promoter region of the gene to a reporter (*lacZ*) construct which was then injected into *C. elegans* (Britton *et al.*, 1999). This technique has been used in a number of other studies examining the expression of parasite genes in transgenic *C. elegans* (Gomez-Escobar *et al.*, 2002; Grant *et al.*, 2006). However, it is worth noting that while spatial expression may be conserved, the temporal expression pattern of genes appears to be less well conserved between *C. elegans* and the parasite. For instance, the *Ostertagia circumcincta* cuticular collagen gene *colost-1* showed a spatially conserved expression pattern when compared to *C. elegans*, but the temporal expression pattern was not conserved (Britton *et al.*, 1999).

From the findings presented in Chapter 3, it appears that the susceptibility of *H. contortus* genes to RNAi may depend on the site of expression of the targeted genes. Therefore it is important to obtain as much information as possible about

the expression pattern of parasite genes. To date this has been difficult due to the lack of gene expression information on parasitic species; however, with the wealth of genomic sequence available for species such as *H. contortus* and *B. malayi*, it is becoming more straightforward to manually identify upstream gene sequences.

The main aims of this chapter are;

- To examine the expression pattern of the *H. contortus H11* gene which was successfully knocked down using RNAi as described in Chapter 3

- Compare this expression pattern to that of the putative *C. elegans* homologue of *Hc-H11*, T07F10.1

- Analyse the gene locus of *Hc-H11* in *H. contortus*

- Analysis of the upstream regions of excretory cell expressed genes, to identify motifs that may allow the prediction of additional RNAi susceptible target genes.

5.2 Results

5.2.1 Expression pattern of *H. contortus* H11 promoter in transgenic *C. elegans*

The findings described in Chapter 3 suggest that the site of gene expression may be a factor in susceptibility to RNAi knockdown. *H. contortus H11* was reliably silenced using RNAi as detailed in Chapter 3. Therefore it was of interest to examine in detail the expression of the *Hc-H11* gene and identify motifs which may regulate expression. *Hc*-H11 (Accession number X94187) is a microsomal aminopeptidase, isolated from intestine extracts from adult *H. contortus* and localized to the microvillar surface of the intestine (Redmond *et al.*, 1997; Smith *et al.*, 1997). Expression of a 1.5 kb upstream region of the *Hc-H11* gene fused to the *gfp* reporter gene (section 2.5.1.3) was examined in transgenic *C. elegans*. The worms were analysed using UV microscopy for sites of gene expression, as shown in Figure 5.1. From the GFP expression, the *Hc-H11* promoter region is expressed strongly in the posterior intestine in transgenic *C. elegans*, consistent with localisation data in the parasite. In addition, strong expression was observed in the amphid cells on either side of the pharynx.

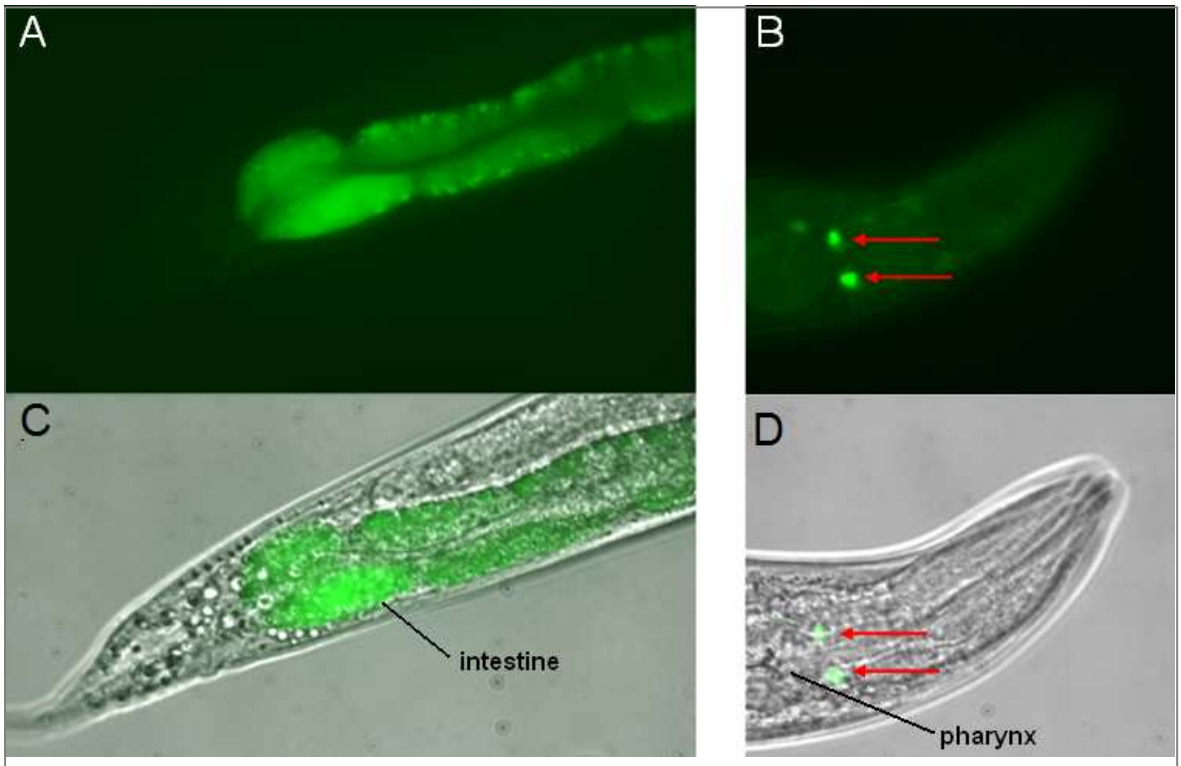


Figure 5.1. *H. contortus Hc-H11* promoter GFP fusion in transgenic *C. elegans.*

Gene expression in posterior intestine region (A) and amphid cells is indicated with red arrows (B). An overlay of both GFP and bright-field images is shown (C and D).

5.2.2 Putative *C. elegans* homologue of *H. contortus* H11

Analysis of the *C. elegans* genome sequence showed that the gene T07F10.1 is a putative homologue of the *H. contortus* H11 gene, although the encoded proteins are only 61% identical at the amino acid level, which is lower than other identified *H. contortus* and *C. elegans* homologous pairs. An alignment of *Hc*-H11 with *Ce*-T07F10.1 is shown in Figure 5.2, with characteristic motifs highlighted.

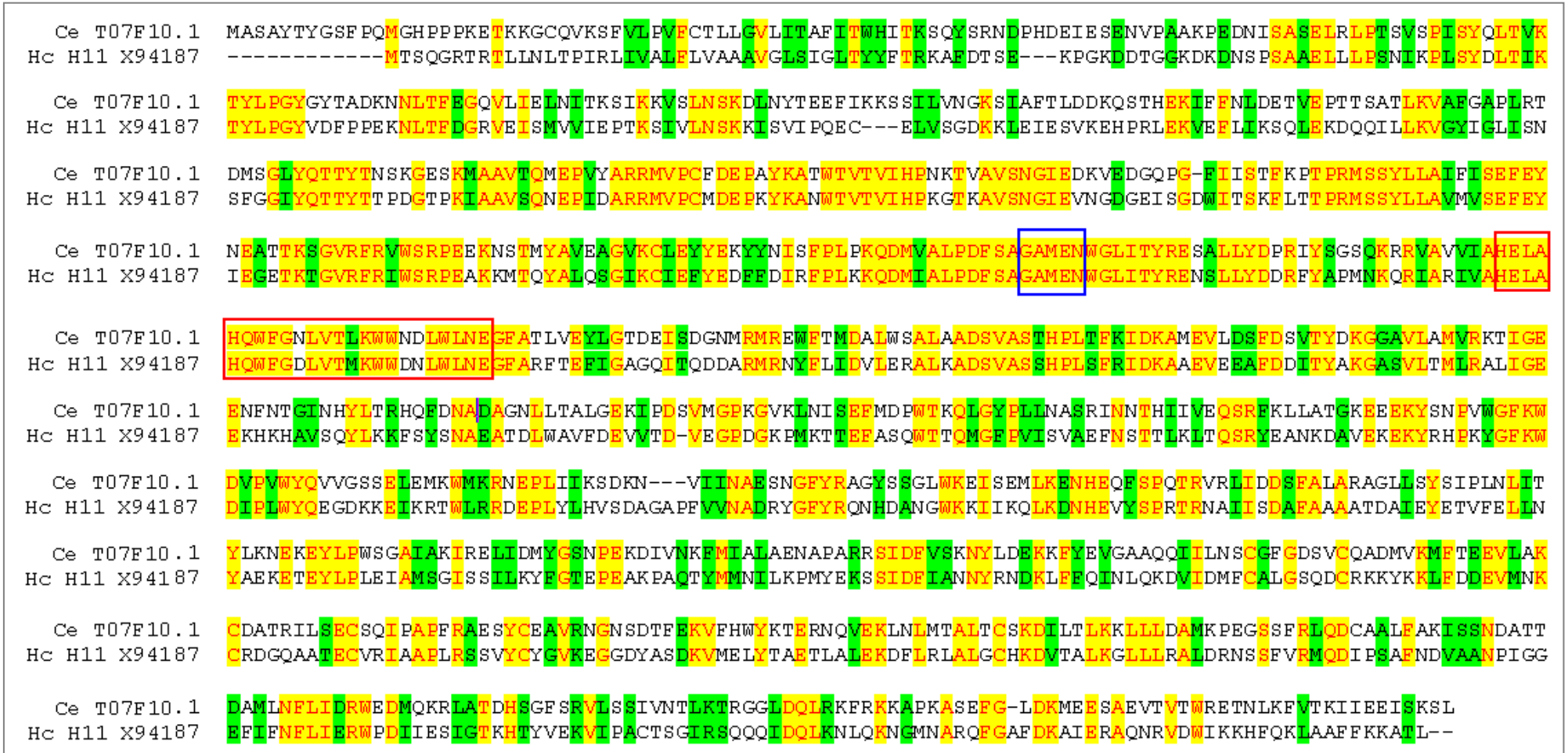


Figure 5.2 Alignment of *H. contortus* H11 with *C. elegans* T07F10.1

Area boxed in blue indicates GAMEN motif, characteristic of aminopeptidase activity. Area boxed in red indicates the HEXXH(X)18E metal ion coordination site (Zn^{2+} binding site). The two proteins show a 61% identity at the amino acid level.

In order to examine the expression pattern of the T07F10.1 gene in *C. elegans* and compare with *Hc-H11* expression, a 1.2 kb region upstream of the T07F10.1 gene was cloned into the *C. elegans lacZ/gfp* expression vector pPD 96.04 as described in section 2.5.4.4. This would enable the visualisation of the expression pattern of this gene, the results of which are shown in Figure 5.3. In contrast to *H. contortus H11* expression in *C. elegans*, the T07F10.1 promoter region is strongly expressed exclusively in the excretory cell.

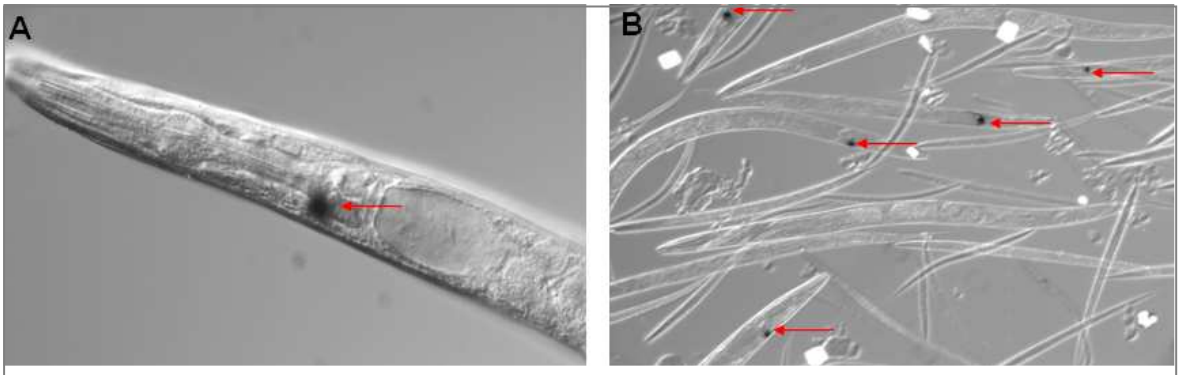


Figure 5.3. *C. elegans* T07F10.1 promoter expression pattern in *C. elegans* using a *lacZ* reporter gene construct

(A) *LacZ* expression is restricted to the excretory cell, indicated by red arrow (X40 magnification). (B) Staining can be observed from L1 larval stage to adult *C. elegans*. Expression in the excretory cell is indicated by red arrows (X10 magnification).

In order to determine if the restricted nature of the expression pattern obtained using the 1.2 kb promoter region of T07F10.1 was dependent on the length of promoter used, a longer 3.7 kb promoter region was used. An expression pattern identical to that with the 1.2 kb promoter, specific to the excretory cell was obtained (data not shown). In conclusion, while the *H. contortus H11* promoter region in *C. elegans* directs expression in the intestine and in amphid cells, expression of the promoter region of the putative *C. elegans* homologue T07F10.1 shows a very restricted pattern in the excretory cell.

5.2.3 Analysis of *H. contortus H11* promoters for potential regulatory motifs

Previous studies have shown that there are four different isoforms of *H. contortus H11* which all have been molecularly cloned and sequenced (Newton, 1993). The different *Hc-H11* isoforms are thought to be intestine expressed and as described in section 5.2.1, the promoter of the original *H. contortus H11*

sequence is expressed in the intestine and amphid cells of transgenic *C. elegans*. In order to identify any regulatory motifs that may be common to different *Hc-H11* promoters, the 5' upstream regions of *Hc-H11* and *Hc-H11-4* genes (accession numbers X94187 and AJ311316 respectively) were identified from *H. contortus* genomic sequence (section 5.2.6) and compared. 1.5 kb of upstream regions of *Hc-H11* and *Hc-H11-4* were first aligned using the Vector NTI – Align X programme, and then using the NCBI Blast 2 Sequences (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi); no regions of identity were found between the two promoters. Alignment attempts were repeated with shorter lengths of promoter regions (1 kb, 500 bp, 300 bp) upstream of the start codon, but again no significant identities were found between any regions of the sequences.

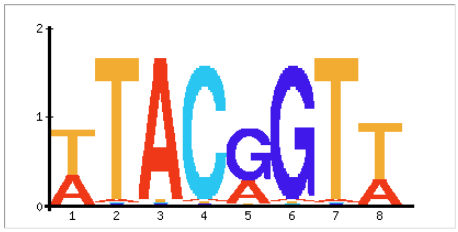
5.2.4 Analysis of the promoter regions of *C. elegans* and *C. briggsae* excretory cell expressed genes for potential regulatory motifs

As described above, the *C. elegans* T07F10.1 promoter drives reporter gene expression specifically in the excretory cell. As excretory cell expressed genes may be suitable targets for RNAi silencing, it was of interest to determine if specific promoter regions or motifs determined this expression, at least in *Caenorhabditis* species. In order to identify any potential motifs that may be involved in regulating this specific expression pattern, the MotifSampler programme (Thijs *et al.*, 2002) located at http://homes.esat.kuleuven.be/~thijs/Work/MotifSampler.html was used. This programme finds motifs that are over-represented in the upstream region of a gene, which may be expected if a particular motif was involved in gene regulation. Four promoter regions of genes thought to be involved in excretory cell specific expression were included in this analysis; (i) the *C. elegans* T07F10.1 gene, (ii) the putative *C. briggsae* homologue of *C. elegans* T07F10.1, CBG09515, (iii) the *C. elegans* fumarate reductase gene F48E8.3 expressed in the excretory cell (Zhao *et al.*, 2005) and finally (iv) the putative *C. briggsae* homologue of F48E8.3, CBG05192. The resultant motif obtained from the analysis of these four promoter regions is shown in Table 5.1. From this analysis,

it appears that the motif WTACRGTW can be found in the upstream region of all four genes.

Table 5.1. MotifSampler programme analysis on the promoter regions of genes thought to be expressed in the excretory cell.

A=Adenine, C=Cytosine, G=Guanine, T=Thymine, R=Adenine or Guanine, W=Adenine or Thymine (IUPAC Nucleotide Codes).

Promoter region (name)	Reason used /homologous to	Over-represented motifs (Result)
C.e. T07F10.1	Putative homologue of *H.c. H11* X94187.	
C.b. CBG09515	Putative homologue of *C.e.* T07F10	
C.e. F48E8.3	Fumarate reductase gene expressed in the *C.e.* excretory cell (Zhao *et al.*, 2005)	*i.e.* **WTACRGTW**
C.b CBG05192	Putative homologue of *C.e.* F48E8.3	

5.2.5 Analysis of the promoter regions of *C. elegans*, *C. briggsae* and *H. contortus* genes for the positions of regulatory motifs

Both the sequence and the position of the regulatory motif(s) within the upstream region of a gene can control gene expression. Therefore, it was necessary to ascertain if the position of the WTACRGTW motif identified in the previous section is conserved within the excretory cell expressed gene promoters. In addition, the *Caenorhabditis* excretory cell expressed gene promoters were also searched for the presence and position of any Ex-1 motifs, described as critical for excretory cell specific expression (Zhao *et al.*, 2005). Therefore the Ex-1 motif (CCATACATTA) was included in the search, as well as Ex-1 variants (identified by Zhao *et al.*, 2005) as described in Table 5.2. These different variations of the Ex-1 motif were entered into the search in order to maximise potential hits. Simultaneously, the *H. contortus H11* and *H11-4* gene promoters were both analysed for any motifs common to these two promoters. Since the *Hc-H11* genes are expressed in the intestine, this expression may be regulated by GATA motifs (McGhee *et al.*, 2007). For this analysis, the Regulatory Sequence Analysis Tools (RSAT) database was used (http://rsat.ulb.ac.be/rsat/) (van Helden, 2003). The RSAT database allows *cis*-

regulatory elements to be identified on both strands of promoter sequences; their positions are displayed schematically on the upstream region of the genes as shown in Figure 5.4.

Table 5.2. Different motif sequences used in the Regulatory Sequence Analysis Tools programme.

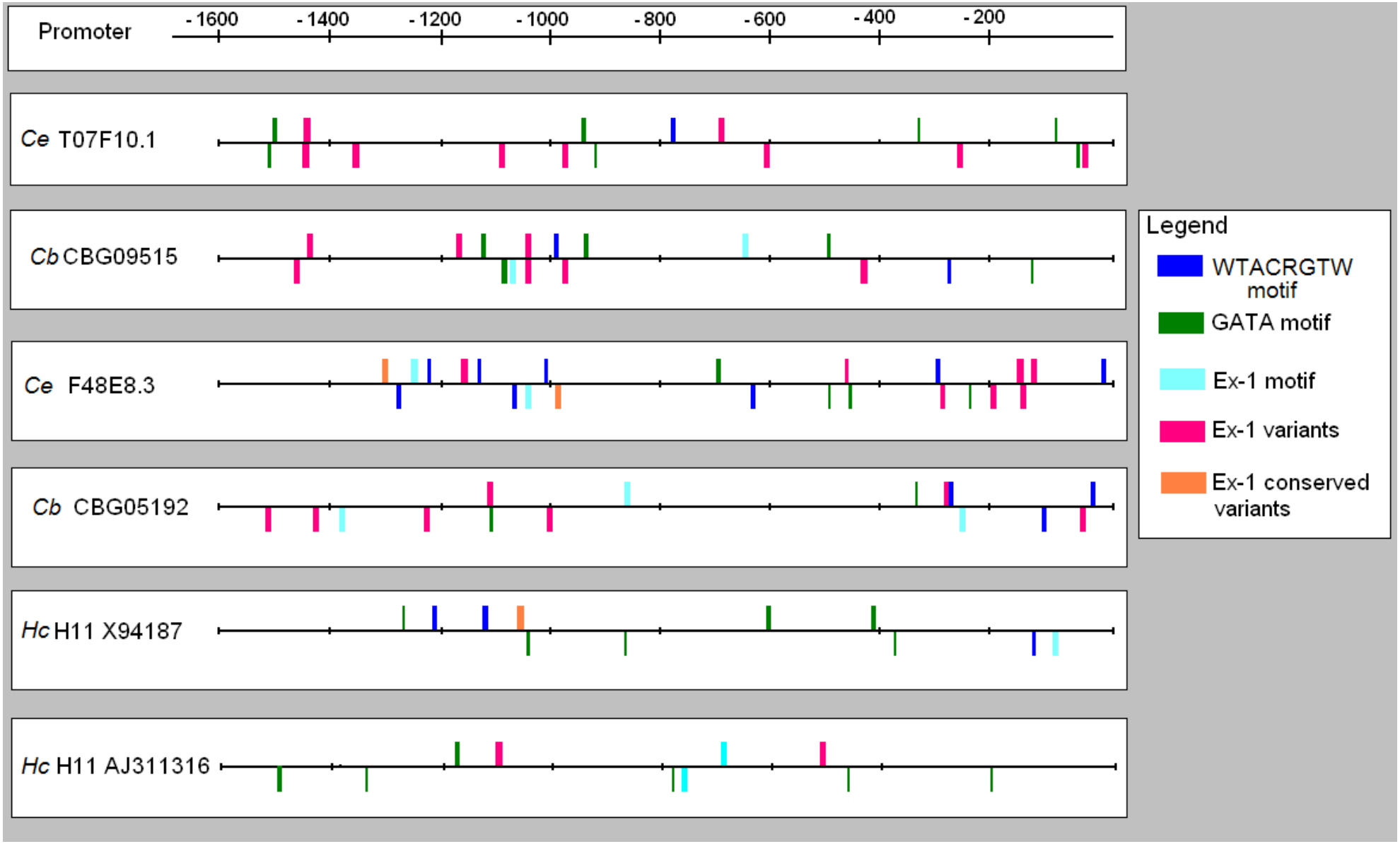
A=Adenine, C=Cytosine, G=Guanine, T=Thymine, R=A or G, Y=C or T, S= G or C, W=A or T, K=G or T, M=A or C, B=C or G or T, D= A or G or T, H=A or C or T, V=A or C or G, N= any base (IUPAC Nucleotide Codes).

Motif	Sequence	Relevance
Excretory cell specific motif	WTACRGTW	Obtained from MotifSampler analysis.
GATA motif	GATAA	Intestine-specific promoter region (*H.c H11* is intestine-expressed).
Ex-1 motif	CCATACATTA	(Zhao *et al.*, 2005)
Ex-1 variant	MMAYWVWTHW	Integrates every single base change seen within promoters containing Ex-1 sequences (Zhao *et al.*, 2005)
Ex-1 conserved variant	CCATWSATWW	Integrates base change variation only if it occurs more than once within promoters containing Ex-1 sequences (Zhao *et al.*, 2005)

Figure 5.4 shows the positions of the regulatory motifs, described in Table 5.2, on the upstream regions of the *C. elegans, C. briggsae* and *H. contortus* genes analysed. There are a high number of Ex-1 and Ex-1 variant motifs, involved in excretory cell expression in the promoters of the *C. elegans* and *C. briggsae* genes. These genes have shown/been predicted to show excretory cell expression and the presence of the large number of Ex-1 and Ex-1 variant motifs were therefore an expected result. Although these are abundant, the positioning of these motifs in the promoters of the *C. elegans* and *C. briggsae* putative homologues is not highly conserved. As might be expected, there are fewer and less conserved identifiable Ex-1 motifs in *Hc-H11* and *Hc-H11-4* promoters. The *Hc-H11* promoter showed activity in the posterior intestine and amphid cells, and indeed both *Hc-H11* and *Hc-H11-4* promoters show the presence of a number of GATA factors which have been shown to regulate intestine expression. As with the Ex-1 motifs, the positioning of the GATA motifs in the different *Hc-H11* genes does not appear to be conserved. Other genes expressed in the amphid cells in *C. elegans* appear to have roles in chemosensation, but thus far no specific motifs involved in regulating gene expression in the amphid cells have been characterised.

Figure 5.4. Positions of regulatory motifs on 1.5 kb promoter sequences of *C. elegans*, *C. briggsae* and *H. contortus* genes.

Numbering is relative to the ATG start codon. Promoters are represented by black horizontal lines. Coloured vertical bars on either side of the promoters represent the positions of the respective regulatory motifs; bars above the promoter signify regulatory motifs present 5' to 3' orientation, and bars below the promoter signify regulatory motifs present 3' to 5' orientation. The WTACRGTW motif, found using the Motif Sampler programme as described in section 5.2.4, is shown in blue vertical bars. The GATA motif is shown in green vertical bars. The Ex-1 motif and the different variations of the Ex-1 motif are shown in light blue, pink and orange vertical bars respectively. *Hc, H. contortus*; *Ce, C. elegans*; *Cb, C. briggsae.* The analysis was carried out using the Regulatory Sequence Analysis Tools (RSAT) database.


Promoter
-1600
-1400
-1200
-1000
-800
-600
-400
-200
Ce T07F10.1
Cb CBG09515
Ce F48E8.3
Cb CBG05192
Hc H11 X94187
Hc H11 AJ311316
Legend
WTACRGTW motif
GATA motif
Ex-1 motif
Ex-1 variants
Ex-1 conserved variants

5.2.6 *Hc-H11* family gene organisation

Analysis of the *Hc-H11* promoter expression pattern in *C. elegans* required the manual annotation of the *Hc-H11* gene. This was followed by PCR amplification of the promoter region from *H. contortus* genomic DNA to identify the upstream gene region. Primers for this were designed using sequence reads from the *H. contortus* genome sequence database. The PCR product obtained was ~3 kb in length, and was cloned into the pSC-A TA vector, and the insert DNA was sequenced. The resulting sequence was aligned with the *H. contortus* genome sequence database (assembled supercontig release 21/08/08 all reads) using BLASTN analysis to verify that the insert DNA was indeed the *Hc-H11* promoter region. This identified that the *Hc-H11* sequence was present on supercontig 0057272.

BLAST analysis showed that the sequence of the PCR product aligns with both the 5' coding region of the *Hc-H11* gene (accession number X94187) as expected, and also with the 3' end of another *Hc-H11* gene, previously identified as *Hc-H11* isoform 4 (Newton, 1993, accession number AJ311316). This is shown schematically in Figure 5.5. Further BLASTN analysis of the supercontig using the cDNA sequence of a third *Hc-H11* family member, previously identified as *Hc-H11* isoform 2 (accession number AJ249942) showed that a 78 bp coding region of *H11-2* aligns with the extreme 5' end of the supercontig. Therefore supercontig 0057272 contains the sequence of three different *Hc-H11* isoforms next to each other in the *H. contortus* genome. The organisation on the supercontig was already suggested experimentally by the PCR amplification of the *Hc-H11* gene promoter region. Identification of the full genomic region of *Hc-H11-2* and *Hc-H11-4* is not possible at this time due to gaps in the sequence of the supercontig (indicated by Ns in Figure 5.5), but will be possible once sequence for this gap becomes available.

Figure 5.5 shows a schematic diagram of supercontig 0057272. The positions of the three *Hc-H11* genes (H11-2, H11 and H11-4) are also shown. The size of the region between *Hc-H11* and *Hc-H11-4* on genomic DNA is 1902 bp. The size of the region between *Hc-H11-2* and *Hc-H11* is unknown due to the gap in the genomic DNA sequence. Since the 3 kb upstream region of *Hc-H11* includes the 3' end of

Hc-H11-4 (AJ311316), the *Hc-H11* promoter used for gene expression analysis in transgenic *C. elegans* was limited to a 1.5 kb region, as described in Section 5.2.1.

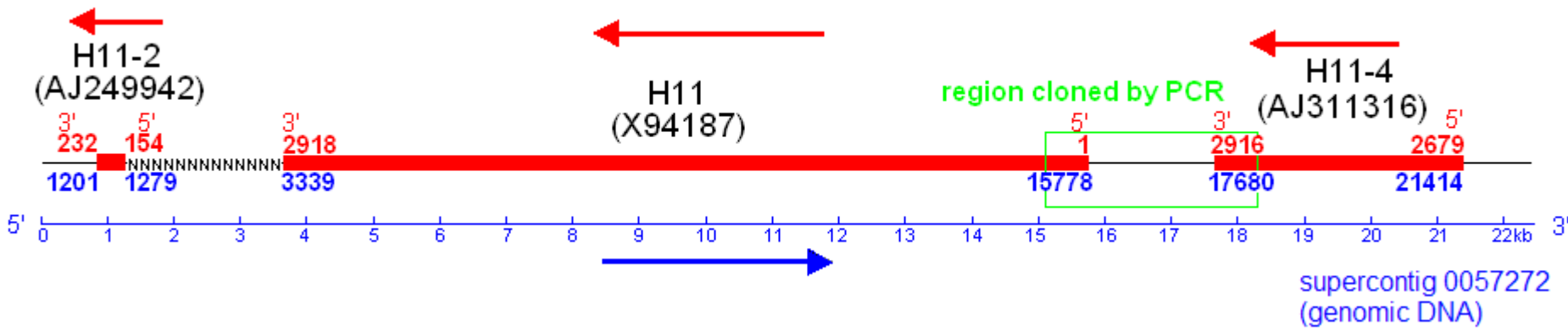


Figure 5.5. Genomic organisation of *H. contortus* H11 genes *Hc*-H11-2, *Hc*-H11 and *Hc*-H11-4.

Three *Hc-H11* isoforms, *Hc-H11-2*, *Hc-H11* and *Hc-H11-4* (accession numbers AJ249942, X94187 and AJ311316, respectively) are shown in red boxes. The red arrows above the three genes show the orientation of the genes, 5' to 3'. The red numbering refers to cDNA sequence of the *H11* genes. The three *H11* isoforms are shown next to each other on the genomic sequence of supercontig 0057272, approximately 22 kb in size and indicated in a blue line. The numbers indicated in blue text refer to supercontig 005727 genomic DNA sequence. The blue arrow below supercontig 0057272 indicates orientation, 5' to 3'. Area boxed in green indicates H11 (X94187) upstream region amplified by PCR. This region includes the 5' region of *H11* and the 3' region of *H11-4*. The gap between the two genes *H11* and *H11-4* was calculated at 1902 bp on genomic DNA. Ns indicate gap in genomic sequence.

The supercontig sequence also allowed the intron-exon boundaries of *Hc-H11* and *Hc-H11-4* genes to be annotated by comparing it with the cDNA sequences. The findings of this analysis are presented in Figure 5.6. Both *H. contortus* H11 and H11-4 genes have a larger number of introns compared to the putative *C. elegans* homologue T07F10.1 gene. These introns are also much larger in size; some in excess of 1 kb, making the genomic copy of the gene in excess of 11 kb in size compared to 4.39 kb for T07F10.1. Exons with similar sequences are colour coded, demonstrating that the genomic structure of the exons appear to be well conserved between the two *Hc-H11* genes and the putative *C. elegans* homologue T07F10.1 gene. In addition, the exon/intron boundaries appear to be conserved between all three genes. This suggests firstly that the *H. contortus* and *C. elegans* genes are descended from a common ancestral gene and secondly that the *Hc-H11* gene family has arisen through gene duplication events. The observation that *H. contortus* introns are larger in size and number compared to *C. elegans* genes is consistent with other instances where *C. elegans* and *H. contortus* gene structure have been compared; the beta tubulin genes (Gary Saunders, PhD thesis, University of Glasgow 2009), the cysteine protease genes (Stephanie Johnston, unpublished data) and the *dcr-1* gene (described in Chapter 4).

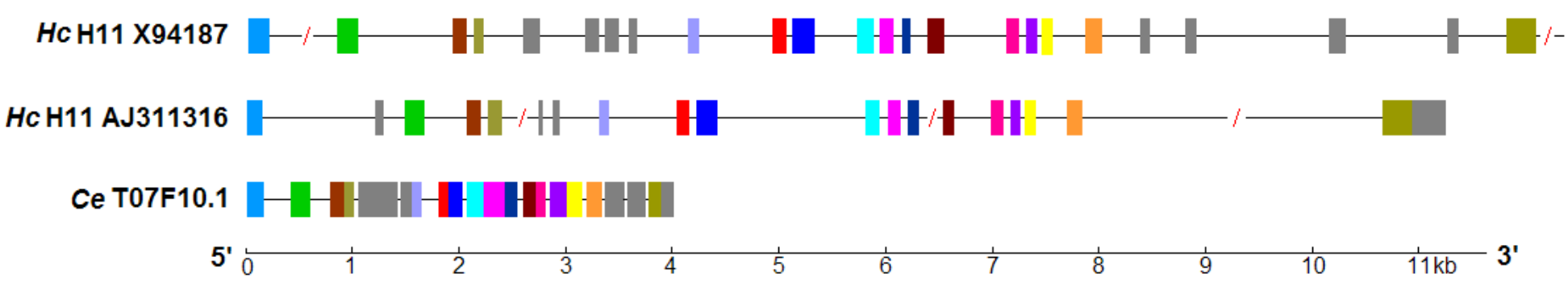


Figure 5.6. Gene structures of *H. contortus* H11, H11-4 and *C. elegans* T07F10.1 genes

The structure of *H. contortus* H11 genes (X94187 and AJ311316) compared with putative *C. elegans* homologue T07F10.1. Boxes of similar colours indicate exons with similar sequences between the three genes. Boxes in grey indicate exons with no similar sequences between the genes. Introns are shown between the exons as lines. Gaps in *H. contortus* genomic sequence of supercontig 0057272 are indicated by /.

5.3 Discussion

This chapter analysed the expression pattern of the *H. contortus H11* promoter and compared this with the expression pattern for the putative *C. elegans* homologue T07F10.1 promoter. In addition, analysis was carried out to identify motifs that might be important in the regulation of gene expression. Since the site of gene expression appears be a possible factor in whether a gene is susceptible to RNAi knockdown or not, establishing expression data and identifying motifs involved in this expression may allow the prediction of additional RNAi susceptible targets.

As described in Chapter 3, previous work using antibody localisation studies and promoter reporter constructs in transgenic *C. elegans* have shown that the *H. contortus* beta tubulin isotype-1 gene is localised and expressed in the amphid neurons and posterior intestine (Gary Saunders, PhD thesis, University of Glasgow 2009). The successful knockdown of this gene using RNAi in *H. contortus* is described in Chapter 3, as well as other genes expressed in the intestine, excretory cell and amphid cells. RNAi was also attempted on the *H. contortus* aminopeptidase H11 (Accession number X94187), and similarly successful transcript knockdown using RNAi was obtained (Chapter 3). H11 is expressed on the intestinal microvilli and shows microsomal aminopeptidase A and M activities (Smith *et al.*, 1997). Enzyme activity is localised to the intestinal brush border and is reported to be inhibited by H11 antisera *in vitro* (Smith *et al.*, 1997). The work carried out in this chapter describes the localisation of H11 promoter activity using transgenic *C. elegans*. A *gfp* reporter gene fusion shows that the *H. contortus H11* promoter is expressed in the amphid cells and posterior intestine region. In contrast, the putative *C. elegans* homologue of *H11*, T07F10.1 is expressed exclusively in the excretory cell of the worm. This result was surprising, as often the spatial expression pattern tends to be conserved between parasite genes and *C. elegans* homologues. For example, the *B. malayi phy-1* gene is involved in cuticle collagen modification and a hypodermal localisation is therefore predicted; analysis of the *B. malayi phy-1* promoter in transgenic *C. elegans* also showed a hypodermal localisation, as did the homologous *C. elegans phy-1* (Winter & Page, 2000; Winter *et al.*, 2003). Comparative studies of acetylcholinesterase (*ace*) genes show that the

promoters of both *C. elegans ace-2* and *G. pallida ace-2* direct expression in inner labial (IL) neurons (Combes *et al.*, 2003; Costa *et al.*, 2009). Therefore it might have been anticipated that the intestine expressed *H. contortus H11* would show a similar conservation in spatial expression when compared with the putative *C. elegans* homologue T07F10.1. It is possible that *C. elegans* T07F10.1 is not the true homologue of *H. contortus* H11 (despite being the closest match by sequence comparison), or the two genes have evolved separate functions in the free-living and parasitic nematode since diverging approximately 400 million years ago (Vanfleteren *et al.*, 1994) and therefore have different spatial expression patterns. It is worth noting that two different methods were used to analyse the expression pattern of the promoter regions of *H. contortus* H11 and *C. elegans* T07F10.1; the former involved using the PCR fusion method to generate a *gfp* reporter construct (Hobert, 2002) and the latter involved the more traditional sub-cloning into the *C. elegans lacZ/gfp* expression vector pPD 96.04. While both methods have advantages and disadvantages, for *C. elegans* genes the expression patterns obtained using the PCR fusion method agree with previously annotated expression patterns in Wormbase (Hunt-Newbury *et al.*, 2007).

Further analysis of the promoters of four *C. elegans* and *C. briggsae* genes, some of which show excretory cell expression, was carried out in order to identify any motifs that might regulate excretory cell expression. This analysis was carried out using the Motif Sampler programme and identified the WTACRGTW motif that was over-represented in all four *C. elegans* and *C. briggsae* promoters. The RSAT database was then used to ascertain the positions of the WTACRGTW motifs on the promoters of the four *C. elegans* and *C. briggsae* genes, as well as other motifs thought to be involved in excretory cell expression such as the Ex-1 and Ex-1 variant motifs identified previously (Zhao *et al.*, 2005). As expected, several of these motifs were present on the promoters of the four *C. elegans* and *C. briggsae* genes. It was surprising that the promoter regions of the two *H. contortus H11* genes showed the presence of a small number of these excretory cell motifs, despite being expected to be expressed in the intestine, and not the excretory cell. However, the four *C. elegans* and *C. briggsae* promoters appear to have more excretory cell motifs than the two *H. contortus H11* gene promoters. Therefore it is possible that the small number of

excretory cell motifs present on the two *H. contortus H11* gene promoters may not have a functional impact on directing gene expression to the excretory cell.

In summary, this analysis was significant as some genes, which may be expressed in the excretory cell, have been successfully silenced using RNAi in *H. contortus* as described in Chapter 3. Identifying motifs involved in this expression could allow the prediction of additional RNAi susceptible targets. It is worth noting that although the analysis was carried out on the promoters of *C. elegans* and *C. briggsae* genes, a similar motif could regulate gene expression in *H. contortus* and this could be experimentally tested when further genome sequence for *H. contortus* is obtained. This would allow the promoter regions of putative *H. contortus* excretory cell expressed genes to be analysed in a similar manner in the future.

A comparison of the promoter regions of the two *H. contortus H11* genes was carried out in order to identify any motifs that may be involved in regulating gene expression in the intestine. Despite the high (74% at the nucleotide level) sequence similarity between the coding regions of *H11* and *H11-4*, no significant similarity was found between the promoter regions. Given that the coding sequences are subject to a higher level of selection pressure than non-coding promoter regions, it was expected that the promoter regions would display more variation in the sequence. However, as the genomic organisation suggests these are recently duplicated genes, some conservation of regulatory sequence might have been expected. The promoter regions were also examined for GATA motifs which have been shown to regulate gene expression in the intestine of *C. elegans* (McGhee *et al.*, 2007). Several GATA motifs were identified on the two *H. contortus H11* gene promoter regions, although the positioning of these motifs was not highly conserved. Interestingly, the *H. contortus H11* promoter drove reporter gene expression in the amphid cells in addition to the posterior intestine, in transgenic *C. elegans*. Thus far, no motifs which may be involved in regulating gene expression in the amphid cells in *C. elegans* have been identified. Genes expressed in the amphid cells in *C. elegans* appear to play a role in chemosensation, being involved in the recognition of environmental chemicals (Bargmann, 2006). Identifying more *H. contortus* genes with similar expression patterns in the amphid cells could enable the identification of possible motifs involved in regulating gene expression in the amphid cells.

During the isolation of the *Hc-H11* promoter region for this analysis, it was found that at least three *Hc-H11* family members lie adjacent to each other on the genomic sequence, suggesting possible gene duplication events. It has been known previously that four different isoforms of *Hc-H11* exist (Newton, 1993). However it was not known if these isoforms were variant alleles of each other or different gene loci. This is the first identification of *Hc-H11* genes as distinct but adjacent genes which suggests that they have arisen through gene duplication. This is true of some other genes in *C. elegans* and in *H. contortus*; for example, a cysteine protease gene family of *H. contortus* comprises eight distinct tandemly arrayed genes in *H. contortus*, and all but one are expressed (Stephanie Johnston, University of Glasgow, unpublished data). It is possible that the complicated parasitic lifestyle of *H. contortus*, with development into adulthood in the sheep host, requires a greater functional diversity of genes than the relatively simpler free-living lifestyle of *C. elegans*. In turn, this functional diversity could be provided by gene duplication events which could explain the observation of multiple isoforms of *H. contortus* genes. Alternatively, it is possible that these genes are required in higher levels in the parasite, and thus multiple copies are required. A further examination of the *Hc-H11* genes will be possible once complete assembled genomic sequence for this region is obtained. The gene organisation of the *Hc-H11* genes was analysed, and compared with the putative *C. elegans* homologue, showing that the genomic copy of the *H. contortus* genes are much larger than the *C. elegans* gene. However, intron/exon boundaries were conserved, suggesting they are descended from a common ancestral gene.

In conclusion, this chapter shows that the promoter of the *H11* gene, susceptible to RNAi, drives reporter gene expression in the posterior intestine and amphid cells in transgenic *C. elegans*. The promoter of the putative *C. elegans* homologue of *Hc-H11*, T07F10.1 localises to the excretory cell. The successful knockdown of other genes expressed in sites that are similarly accessible to the environment, as detailed in Chapter 3, was also obtained. The promoter regions of other genes that are susceptible to RNAi (Chapter 3) can also be analysed in this manner once more complete genomic sequence data is obtained. For example, the *H. contortus asp-1* gene is susceptible to RNAi but promoter activity or motifs could not be identified as assembled genomic information for

this region is currently limited. In the future it may be possible to identify motifs involved in particular expression patterns which may then be used to predict RNAi susceptible genes.

Chapter 6

General Discussion

Parasitic nematodes exact a significantly large toll on the animal industry. These infections represent a considerable health problem to grazing livestock, causing a substantial amount of disease, animal welfare problems and economic loss. Of the various parasitic nematodes that infect livestock, *H. contortus* is considered to be one of the most pathogenic and economically important.

The methods of control for *H. contortus* and other parasitic nematodes are currently limited to repeated treatments using anthelmintic drugs. As elaborated in Chapter 1, there is widespread resistance to all three major classes of anthelmintic drugs currently in use. Consequently, there is an urgent need to find novel and alternative methods for treating and controlling these infections in livestock. The development of molecular vaccines and novel drugs are two approaches which could potentially be used in the future. However, both of these approaches require a deeper understanding of the function of different parasite genes to identify potential drug/vaccine targets. RNA interference would be an invaluable functional tool and advance the discovery of new control targets for parasitic nematodes.

The silencing of specific genes by RNA interference was first described in *C. elegans* and has since helped to functionally annotate the complete genome of the free-living nematode (Kamath & Ahringer, 2003). Given the ease with which RNAi can be utilised in *C. elegans*, together with the discovery that the RNAi pathway is widely conserved across most eukaryotic organisms, there was considerable anticipation that RNAi might be used as a tool for analysing gene function in parasitic nematodes. However, as discussed in Chapter 1 and Chapter 3, the application of RNAi to parasitic nematodes has not been as straightforward as may have been expected. Numerous difficulties have been encountered in attempts to develop RNAi in various parasitic nematodes, encompassing problems with reproducibility, specificity and susceptibility leading to doubts on the reliability of RNAi in parasitic nematodes.

Is the classical RNAi pathway functional in *H. contortus*?

The primary aim of this project was to examine in detail the application of RNAi to the parasitic nematode *H. contortus*. The successful silencing of several *H. contortus* genes using RNAi was described in Chapter 3. Importantly, the

silencing obtained for these genes was specific for the target gene and, reproducible. In addition, the *H. contortus dcr-1* gene, an essential component of the RNAi pathway, was fully sequenced and shown to be expressed in *H. contortus* L3 and adult stages (Chapter 4). This is important as it has been suggested that higher levels of *Schistosoma mansoni dcr-1* in older parasites may explain why older schistosomula are more susceptible to RNAi than younger forms (Krautz-Peterson & Skelly, 2008). Several known RNAi pathway genes which were initially characterised in *C. elegans* also appear to have putative homologues in *H. contortus* (Geldhof *et al.*, 2007). Together, these findings indicate the presence of a functional RNAi pathway in *H. contortus* that is capable of reliably silencing the expression of some specific genes.

Why is RNAi in *H. contortus* successful for some genes but not others?

Although some *H. contortus* genes have been reliably silenced as described in Chapter 3, several other genes have not been similarly susceptible to RNAi. Interestingly, the RNAi susceptible genes appear to be expressed in sites that are accessible to the environment. These sites include the intestine, amphid cells and excretory cell. Although direct information on the sites of gene expression is difficult to obtain in *H. contortus* L3 stage larvae, indirect methods were used to elucidate the site of expression of these genes. Hence it appears that *H. contortus* genes expressed in sites that are accessible to the environment such as the intestine and excretory cell may be susceptible to silencing by RNAi.

The RNAi silencing of genes induced by environmentally delivered dsRNA first requires the uptake of the exogenous dsRNA into cells. In *C. elegans*, this is thought to be achieved by the dsRNA transporter protein *Ce*-SID-2, which is expressed in the intestinal cell apical membranes. *Ce*-SID-2 is thought to enable the import of the ingested dsRNA from the intestinal lumen into the intestinal epithelial cells (Winston *et al.*, 2007). The systematic RNAi spreading through the worm is thought to involve SID-1, shown to be expressed in nearly all non-neuronal cell types in *C. elegans* (Winston *et al.*, 2002). The failure to detect *Ce*-SID-1 in the neuronal cells is consistent with the observation that neuronal cells are generally resistant to systemic, but not autonomous RNAi in *C. elegans* (Tavernarakis *et al.*, 2000).

The *H. contortus* genome does not appear to contain a putative homologue of the *sid-2* gene, even though putative homologues of many other RNAi pathway genes including *sid-1* are present in the genome sequence (Geldhof *et al.*, 2007 and Chapter 4). This suggests that although *H. contortus* seems to posses the RNAi pathway genes to elicit a systematic spreading RNAi response, the uptake of dsRNA from the environment might not be as efficient as in *C. elegans* due to the absence of a putative *sid-2* homologue. An extremely divergent sequence in *H. contortus,* which may be distantly related to the *C. elegans sid-2* gene, was described in Chapter 4, but it is unclear if this sequence is a true homologue of the *C. elegans sid-2* gene. If SID-2 is present in *H. contortus*, its function and sequence may have diverged from *Ce-sid-2* and it is therefore difficult to identify from sequence data. Alternatively it is also possible that an entirely different protein is involved in the uptake of dsRNA from the environment in *H. contortus,* and possibly other parasitic nematodes. A third possibility is that the uptake of dsRNA from the environment in *H. contortus* is not facilitated by a transporter protein, SID-2 or otherwise, and occurs by another mechanism; for example, the endocytic pathway has been suggested as a dsRNA entry mechanism into cells and this dsRNA uptake mechanism seems to be evolutionarily conserved (Saleh *et al.*, 2006). As a result of any one of these possibilities, the uptake of dsRNA might not be as efficient as it is in *C. elegans*, possibly explaining why not all *H. contortus* genes seem to be susceptible to RNAi. The fact that some genes, which may be expressed in sites that are accessible to the environment, are susceptible to RNAi supports this hypothesis.

This is further supported by the fact that putative homologues of *sid-1* and *sid-2* appear to be absent in the genome or ESTs of the plant parasitic nematodes such as *M. incognita*, *M. hapla*, *G. rostochiensis*, *G. pallida,* and *H. glycines* despite several reports of successful gene silencing using RNAi (see Table 1.1). Interestingly, many of the plant parasitic nematode genes successfully silenced by RNAi appear to be expressed in tissues such as oesophageal glands, amphids, reproductive system and intestine (Bakhetia *et al.*, 2005; Bakhetia *et al.*, 2007; Chen *et al.*, 2005b; Lilley *et al.*, 2005; Sukno *et al.*, 2007; Urwin *et al.*, 2002). These tissues may be accessible sites for dsRNA to enter the worms. Therefore as the findings of Chapter 3 suggest, the site of gene expression may be an

important factor in susceptibility to RNAi as the uptake of exogenous dsRNA may be more efficient in tissues which are accessible to the environment.

Is the uptake of environmental dsRNA inefficient in *H. contortus*?

Previous work in *H. contortus* examining the uptake of fluorescently labelled dsRNA from the environment showed that although L1/L2 larvae take up sufficient amounts of dsRNA to observe green fluorescence in the intestine, very little or no dsRNA was taken up by L3 larvae (Geldhof *et al.*, 2006). Interestingly, fluorescence was observed in the pharynx and intestine of some exsheathed L3 larvae which had moulted to L4 larvae *in vitro* (Geldhof et al., 2006). The exsheathed L3 larvae used in this project were also observed to moult onto L4 larvae, recognised by the loss of the L3 cuticle and the development of the intestine. This could explain why RNAi knockdown of some genes can occur even though it appears that L3 larvae are not very effective at taking up dsRNA.

In order to examine whether inefficient uptake of dsRNA explains the variable gene susceptibility to RNAi in *H. contortus*, it would be useful to elucidate the precise role of SID-2 in dsRNA uptake. A similar experiment was performed to examine the functional role of the SID-2 protein in *C. briggsae* (Winston *et al.*, 2007). The *C. briggsae* genome appears to possess a putative homologue of the *Ce-sid-2* gene, but *C. briggsae* is not susceptible to RNAi by soaking in dsRNA, even though successful RNAi is possible by injecting dsRNA directly into *C. briggsae*. An alignment of *Cb*-SID-2 with *Ce*-SID-2 shows that these two proteins are not very well conserved between the two species, particularly in the region of the protein predicted to be in the intestinal lumen (Winston *et al.*, 2007). Expression of *Ce*-SID-2 in *C. briggsae* allowed the transgenic *C. briggsae* worms to be susceptible to RNAi induced by soaking in dsRNA. It therefore appears that the *C. elegans* SID-2 protein confers sensitivity to RNAi by soaking in *C. briggsae* worms which were previously unsusceptible.

Although successful trangenesis has been described in *Strongyloides stercoralis* (Li *et al.*, 2006), currently there are no stable DNA transformation techniques for other parasitic nematodes. If developed, it would be interesting to express *Ce-sid-2* in *H. contortus* to determine if RNAi susceptibility to any tested gene

would be possible. It may be possible to carry out the reverse experiment and test if the putative *Hc-sid-2* identified in the genome data can rescue RNAi defects of *Ce-sid-2* mutants. This would confirm if this gene can function as a dsRNA transporter for RNAi. Although dsRNA can be delivered to *C. elegans* by microinjection of adult worms, this is not feasible or practical for *H. contortus*. Larval stages do not survive microinjection well, a lot of larvae would need to be injected, and it is very difficult to maintain and develop the worms into adults *in vitro*.

Is it possible to predict gene susceptibility to RNAi?

Since not all genes in *H. contortus* appear to be susceptible to RNAi, it would be immensely useful to predict if a gene may be susceptible to RNAi. Since the site of gene expression appears to be an important factor in determining susceptibility to RNAi, if expression site could be predicted it may be possible to specifically target susceptible genes. As shown in Chapter 5, specific motifs may be involved in controlling gene expression in tissues such as the excretory cell. The site of expression of a gene could, in theory, be predicted based on motifs present in the 5' upstream region of that gene. This is currently possible for some motifs present on the 5' upstream regions of *C. elegans* genes, such as the presence of GATA motifs on intestinally expressed genes. However, little is currently known of motifs controlling other spatial gene expression patterns in *C. elegans*. In the future, with more parasite genome sequence available, it may be possible to identify motifs involved in particular expression patterns in parasitic nematodes which may then be used to predict RNAi susceptible genes.

What is the ideal candidate gene for RNAi in *H. contortus*?

In addition to being expressed in a location that is accessible to environmentally delivered dsRNA, transcripts for the ideal candidate gene should be expressed in the target stage and ideally in a stage easily maintained in culture (for example, L3/L4 *H. contortus*). It would also be helpful to assess gene silencing with a phenotypic effect, in addition to analysing transcript levels. This would firstly require that the target gene be involved in a process that results in an observable phenotype upon being silenced; for example in moulting or movement which can be readily monitored. Secondly, that both the transcript

and protein coded by the candidate gene has a short half-life such that phenotypic effects can be detected within a few days of soaking in dsRNA.

What are the practical applications of RNAi in *H. contortus*?

Given that reliable RNAi silencing for some *H. contortus* genes has been demonstrated in this project, the next question would be to investigate the practical applications of RNAi to further knowledge of *H. contortus* gene functions and to identify essential genes. The *in vivo Hc-H11* RNAi experiment carried out in this project (Chapter 3) is the first demonstration that *in vivo* RNAi is possible for *H. contortus*. The findings of the preliminary study demonstrated that pre-soaking in dsRNA per se has no detrimental effects on *H. contortus* larval survival *in vivo*. It is also an example of RNAi being used as a tool to investigate the effects of silencing a gene on worm survival *in vivo*. Pre-treatment of infective larvae with *Hc-H11* dsRNA resulted in a significant reduction in egg output and adult worm survival in sheep, indicating an important role for *Hc*-H11. In this study, the *Hc-H11* dsRNA was specific to one H11 isoform. However, it should be possible in future experiments to target additional Hc-H11 family members.

In vivo studies such as this are limited by cost and welfare considerations, but could be used very effectively to test essential function of genes identified in previous studies or *in vitro* RNAi. In the future it would be of interest to target genes which may be required earlier in infection, particularly in view of potential of worms to recover and re-express targeted genes on removal from dsRNA. For example, the *Hc-asp-1* gene was effectively silenced *in vitro* using RNAi as described in Chapter 3. *Hc*-ASP-1 is secreted from infective larvae *in vitro* (Douglas Clark, PhD Thesis University of Glasgow 2006) and may have an important function early in infection, which could be examined using a similar *in vivo* RNAi study. Such experiments would be extremely useful for identifying the *in vivo* function of parasite genes and evaluating essential genes as novel control targets.

Appendices

Appendix 1: Common buffers and reagents

2X SDS-PAGE sample buffer	0.09M Tris-HCl (pH 6.8), 20% Glycerol, 2% SDS, 0.02% bromophenol blue. Stored at room temperature.
Diethylpyrocarbonate (DEPC) treated water	0.1% (v/v) diethylpyrocarbonate (Sigma) in sterile distilled H_2O mixed overnight and autoclaved. Stored at room temperature.
DNA loading buffer	0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll (Type 400; Pharmacia) in ddH_2O. Stored at room temperature.
Freezing solution	10% 1 M NaCl, 5% 1 M KH_2PO_4. pH to 6.0. 30% glycerol diluted in sterile distilled H_2O. Autoclave and add 0.3% sterile 0.1 M $MgSO_4$. Stored at room temperature.
Injection buffer	20 mM KPO_4, 3 mM potassium citrate, 2% PEG 600, pH 7.5. Stored at 4°C.
IPTG	Isopropyl-β-D-thiogalactoside (Promega) in sterile distilled H_2O. Stock concentration of 1 M filter sterilised and stored at – 20°C.
LB agar	LB broth + 15g/L bacto-agar (Oxoid). Autoclaved and stored at room temperature.
LB broth	1% bacto tryptone (Oxoid), 0.5% yeast extract (Oxoid), 0.5% NaCl in sterile distilled H_2O. Autoclaved and stored at room temperature.
M9 buffer	3% KH_2PO_4, 6% Na_2HPO_4, 5% NaCl, 10 mM $MgSO_4$. 10X stock autoclaved and stored at room temperature.
NGM agar	0.3% NaCl, 1.7% agar (Oxoid), 0.25% peptone (Oxoid), 0.0003% cholesterol (1 ml/L of 5 mg/ml stock in ethanol) in sterile H_2O. Autoclaved and 1 ml/L 1 M $CaCl_2$, 1 ml/L 1 M $MgSO_4$ and 25 ml/L KPO_4 (pH 6.0) added.
Phosphate Buffered Saline (PBS)	137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.47 mM KH_2PO_4 in sterile distilled H_2O. pH 7.2. Sterilised by autoclaving and stored at room temperature.
Phosphate Buffered Saline Tween-20 (PBST)	Prepare PBS as described above and add 0.002% Tween-20. Sterilised by autoclaving and stored at room temperature.
RT-PCR Lysis buffer	0.5% SDS, 10 mM EDTA, 10 mM Tris-HCl, pH 7.5. Filter sterilised and stored at room temperature.
Stripping buffer	1.5% (w/v) glycine, 0.1% SDS, 1% Tween-20. pH 2.2 in sterile distilled H_2O.
Tris-acetate-EDTA (TAE) buffer (50X)	2 M Tris-HCl, 5.17% glacial acetic acid, 0.05 M EDTA pH 8.2 in sterile distilled H_2O. Dilute to 1X for working concentration. Stored at room temperature.
Tris-glycine blotting buffer	20% methanol, 0.01% SDS in 1X Tris-glycine solution (BioRad). Made up fresh as required and stored at 4°C.
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactosidase (Promega) dissolved in N,N'-dimethly-formamide and stored at -20°C out of light. Stock concentration of 2% (w/v) X-gal. Stored at -20°C.
X-gal stain	40 µl of 1 M NaH_2PO_4, 120 µl of 1 M Na_2HPO_4, 10 µl of 1 M $MgCl_2$, 5 µl of 1M K_4FeCN_6, 5 µl of 1M K_3FeCN_6, 10 µl of 20 µl of 20% SDS, 18 µl of 2% (w/v) X-gal and distilled H_20 up to 1ml. Made up fresh as required.

Appendix 2: Primer sequences

Table 1: Primers used for RNAi, for dsRNA synthesis and subsequent RT-PCR analysis

Primer Pair	Sequence (5' to 3')	Tm °C	Product size on cDNA (bp)	Purpose of primers
Ce-rab-7-RNAi F1	ATCGTCGACCCGATTG CTGTGTGCTGGC	71	395	Amplify cDNA region of *C. elegans rab-7* for dsRNA synthesis (*C. elegans* ***W03C9.3*** gene, used as a control dsRNA for all RNAi experiments)
Ce-rab-7 RNAi R1	GTGGGATCCCAATTGC ATCCCGAATTCTGC	70		
Hc-sod-1 RT-PCR F1	CAAAGGCGAAATCAAG GGTTTG	58	364	RT-PCR primers to check for transcript level of non-target *H. contortus sod-1*, used as an internal control for the quality of RNA extracted (based on NCBI sequence **Accession No: Z69621**)
Hc-sod-1 RT-PCR R1	AATAACTCCGCAAGCG ACAC	57		
Hc-bt-iso1 RNAi F1 XhoI restriction site	ACCCTCGAGTGCTACC CTTTCCGTCCATCAACT G	73	260	Amplify cDNA region of *H. contortus beta tubulin iso-1* for dsRNA synthesis (based on NCBI sequence **Accession No: X67489**)
Hc-bt-iso1 RNAi R1 XbaI restriction site	CAGTCTAGAGAGCAAA ACCGGGCATGAAGAAG	70		
Hc-bt-iso1 RT-PCR F1	CGTTGTTCCATCACCCA AGGTATC	63	370	RT-PCR primers to check for transcript knockdown of *H. contortus beta tubulin iso-1*.(based on NCBI sequence **Accession No: X67489**)
Hc-bt-iso-1 RT-PCR R1	CTGTGTAAGCTCAGCA ACTGTCGAA	63		
Hc-bt-iso2 RNAi F1 (XhoI)	CCCCTCGAGTCATCCT TCTCAGTGGTACCTTCA CCA	73	393	Amplify cDNA region of *H. contortus beta tubulin iso-2* for dsRNA synthesis (based on **Supercontig_0059653_cw_200808**)
Hc-bt-iso2 RNAi R1 (XbaI)	CCCTCTAGAGAGCTCA GAAACAGTTAGTGCTC GGTAG	71		
Hc-bt-iso2 RT-PCR F1	AGTTCGCTCCGGACCT TTTGGTGCT	66	1112	RT-PCR primers to check for transcript knockdown of *H. contortus beta tubulin iso-2* (based on **Supercontig_0059653_cw_200808**)
Hc-bt-iso2 RT-PCR R1	CATACGTGTCGTTTTCA ACGGCACCTTCCATT	70		
Hc-bt-iso3 RNAi F1	TAGCCCGAAAGTCTCT GATACGGTAGTCG	68	348	Amplify cDNA region of *H. contortus beta tubulin iso-3* for dsRNA synthesis (based on **Supercontig_0013827_cw_200808**)
Hc-bt-iso3 RNAi R1	GATACCGATACGGCGC GATACTGCTGATTGC	71		

Hc-bt-iso3 RT-PCR F1 Hc-bt-iso3 RT-PCR R1	GAACACGTTTTCCGTG GTTCCTAGCCCG GGCATCGAAACACTGT TGTGTCAATTCGG	70 67	397	RT-PCR primers to check for transcript knockdown of *H. contortus beta tubulin iso-3* (based on **Supercontig_0013827_cw_200808**)
Hc-623 RNAi F1 (XbaI) Hc-623 RNAi R1 (XhoI)	CCCTCTAGAGGTCAAG AAGATGGAGGTGACAC AGCAC CCCCTCGAGGATCGCA CAGTTGCGGCAGTGAT AGTTCC	74 77	189	Amplify cDNA region of *H. contortus 623* for dsRNA synthesis (based on **Nembase Cluster HCC00623**)
Hc-623 RT-PCR F1 Hc-623 RT-PCR R1	CGGTACCCGATATGGA GCGTCACTGCG CTTTAAGATCGCGCAG ACGACGGATTG	71 67	233	RT-PCR primers to check for transcript knockdown of *H. contortus 623*. (based on **Nembase Cluster HCC00623**)
Hc-645 RNAi F1 (XbaI) Hc-645 RNAi R1 (XhoI)	CCCTCTAGATACACCCT GACCCAGAAATTGAAC G CCCCTCGAGCTTTAGT GTTGCTTCTTACGGAAC GAG	71 73	253	Amplify cDNA region of *H. contortus 645* for dsRNA synthesis (based on **Nembase Cluster HCC00645**)
Hc-645 RT-PCR F1 Hc-645 RT-PCR R1	AATCATGCCTTTGGCTA TTGATTTGC CAAAAACAATTTATGTA ACACAACAACGCAC	60 62	291	RT-PCR primers to check for transcript knockdown of *H. contortus 645*. (based on **Nembase Cluster HCC00645**)
Hc-700 RNAi F1 (XbaI) Hc-700 RNAi R1 (XhoI)	CCCTCTAGAACTGGTC AAAAACAGCTGAGAGA AC CCCCTCGAGGACAACC ATAAGAACCAAATAACA G	70 70	310	Amplify cDNA region of *H. contortus 700* for dsRNA synthesis. (based on **Nembase Cluster HCC00700**)
Hc-700 RT-PCR F1 Hc-700 RT-PCR R1	ATGTTCTCGACTCACAT CGATTTCAC CAGAATTTATACCCCGC AGTAAATAAG	62 61	343	RT-PCR primers to check for transcript knockdown of *H. contortus 700*. (based on **Nembase Cluster HCC00700**)
Hc-GTPch RNAi F1 (XhoI) Hc-GTPch RNAi R1 (XbaI)	GCACTCGAGGATGCCG AGCATAAGAC CCCTCTAGAGCCAATTT TGAGAGACCCAGAAC	68 70	369	Amplify cDNA region of *H. contortus GTP-cyclohydrolase precursor* for dsRNA synthesis (based on **Accession No AW670739**)
Hc-GTPch RT-PCR F1 Hc-GTPch RT-PCR R1	AAGCGGTTTTATCTCGT CCGACAG CGTCGACTAAACATCTC GACAATTC	63 61	410	RT-PCR primer to check for transcript knockdown of *H. contortus GTP-cyclohydrolase precursor* for dsRNA synthesis (based on NCBI sequence **Accession No AW670739**)

Hc-H11 RNAi F1 Hc-H11 RNAi R1	ACTTCCTGATTGATGTACTTGAACG CTCCACAGCGTCTTTATTCGCCTC	60 64	400	Amplify cDNA region of *H. contortus H11* for dsRNA synthesis (based on NCBI sequence **Accession No: X94187**)
Hc-H11 RT-PCR F1 Hc-H11 RT-PCR R1	CATAAGCATGCAGTATCGCAGTACC TTCATCATGTATGTTTGAGCTGGC	63 60	700	RT-PCR primers to check for transcript knockdown of *H. contortus H11* (based on NCBI sequence **Accession No: X94187**)
Hc-H11-4 RNAi F1 (XbaI) Hc-H11-4 RNAi R1 (XhoI)	CCCTCTAGAGGAGACCATTACTTCAACATGCCGG CCCCTCGAGCAGAAGTGGCATTGAACGCTTCCACCG	72 76	409	Amplify cDNA region of *H. contortus H11-4* for dsRNA synthesis (based on NCBI sequence **Accession No: AJ311316**)
Hc-H11-4 RT-PCR F1 Hc-H11-4 RT-PCR F1	GCCACTTCTGTAAAAATATCGC GAGCTGTAACATCCCTGTGG	57 59	1000	RT-PCR primers to check for transcript knockdown of *H. contortus H11-4* (based on NCBI sequence **Accession No: AJ311316**)
Hc-asp RNAi F1 Hc-asp RNAi R1	GGCAACGAAAAATTGCGAGACTTCAGAACCT TTAATATAAGTTTGTCTTTTTCGGGCAGAG	67 61	626	Amplify cDNA region of *H. contortus asp-1* for dsRNA synthesis (based on NCBI sequence **Accession No: A30245**)
Hc-asp RT-PCR F1 RT-PCR carried out using Hc-Asp RT-PCR F1 and Hc-Asp RNAi R1.	GATTCTGGTGGCTTCGGCATCTTC	64	648	RT-PCR primer to check for transcript knockdown of *H. contortus asp-1*. Used in combination with Hc Asp RNAi R1. (based on NCBI sequence **Accession No: A30245**)
Hc-aqp-2 RNAi F1 Hc-aqp-2 RNAiR1	ATCGGAATAAACGTCGGTTTCGGTTTGGCC GGCTGGGTAGGAAGCGAAAATGCCCGCGG	68 74	315	Amplify cDNA region of *H. contortus aqp-2* for dsRNA synthesis. (based on *H. contortus* **Supercontig_0059432_cw_200808**)
Hc-aqp-2 RT-PCR F1 Hc-aqp-2 RT-PCR R1	GTTGTTGCCCAAAGTGTGCTACCACGTCC CTGGTCAATGAGTCCTCCGAAAAGACCC	70 68	396	RT-PCR primers to check for transcript knockdown of *H. contortus aqp-2* (based on *H. contortus* **Supercontig_0059432_cw_200808**)
Hc-ceh-6 RNAi F1 Hc-ceh-6 RNAiR1	CCACCCTGCCCAGTCATCTTTTCGAAGGCC GACCTGAGGTAATCCAGCGACTTCCTCATC	72 70	373	Amplify cDNA region of *H. contortus ceh-6* for dsRNA synthesis (based on *H. contortus* **Supercontig_0055281_cw_200808**)

Hc-ceh-6 RT-PCR F1 Hc-ceh-6 RT-PCR R1	ACTCACCTCTATACTGG TTCTCTTCTTTCG ACGCTACTTAAAAGTGT GCAACTTCAGG	65 64	450	RT-PCR primers to check for transcript knockdown of *H. contortus ceh-6* (based on *H. contortus* **Supercontig_0055281_c w_200808**)
Hc-exc-4 RNAi F1 Hc-exc-4 RNAi R1	GGATTGGAGCATGTCT GTTTTGCCAAG GATTTCACGATTGTCCG TGTAGGTGGCC	65 68	203	Amplify cDNA region of *H. contortus exc-4* for dsRNA synthesis (based on *H. contortus* **Supercontig_0026239_c w_200808**)
Hc-exc-4 RT-PCR F1 Hc-exc-4 RT-PCR R1	CAGGCGTCCGGTATTG ATAGCAGAAGGATTG CAAGGTGAAAGATTCG TCCTTCGATTTC	70 64	250	RT-PCR primers to check for transcript knockdown of *H. contortus exc-4*. (based on *H. contortus* **Supercontig_0026239_c w_200808**)
Hc-ins-1 RNAi F1 Hc-ins-1 RNAiR1	CCATCGTTGCTGCTGC TTCTGCTGTTCATC CTGAATGAACATCGATT TTCACAGC	70 60	206	Amplify cDNA region of *H. contortus ins-1* for dsRNA synthesis (based on *H. contortus* **Supercontig_0054445_c w_200808**)
Hc-ins-1 RT-PCR F1 Hc-ins-1 RT-PCR R1	TACAAACCGCAGTGTT CCAGTCCATCG ATTACAGCAGTAAGTCA GGAGATATCTGAATG	67 64	252	RT-PCR primers to check for transcript knockdown of *H. contortus ins-1*. (based on *H. contortus* **Supercontig_0054445_c w_200808**)
Hc-phi-10 RNAi F1 Hc-phi-10 RNAiR1	ATGCGGTACATGTCAG CGCAGTTAGACACAAC CAGCGTGAGCCATCGT TAATAGGTCGATCGC	70 71	358	Amplify cDNA region of *H. contortus phi-10* for dsRNA synthesis (based on *H. contortus* **Supercontig_0057368_c w_200808**)
Hc-phi-10 RT-PCR F1 Hc-phi-10 RT-PCR R1	ATCTTGGAAGTTATCTG TTCTCGAATGCGG GTAGGAACCGTTGGGG AGATCCATCAGC	65 70	406	RT-PCR primers to check for transcript knockdown of *H. contortus phi-10* (based on *H. contortus* **Supercontig_0057368_c w_200808**)
Hc-nas-37 RNAi F1 Hc-nas-37 RNAi R1	GAAACAAGTTGACGTC CACCCGTTCGTCC GGTGAAGGAACTACTC GCGCAGTACTACCG	70 71	421	Amplify cDNA region of *H. contortus nas37* for dsRNA synthesis (based on *H. contortus* **Supercontig_0047489_c w_200808**)
Hc-nas-37 RT-PCR F1 Hc-nas-37 RT-PCR R1	GTGACTTACCGAATCA CATCCGTATCC CATCGCAAATGACGTG GTCAGTGACTACGC	65 70	486	RT-PCR primers to check for transcript knockdown of *H. contortus nas37* (based on *H. contortus* **Supercontig_0047489_c w_200808)**

Table 2: List of primers used to obtain *H. contortus dcr-1 5'* end

Primers	Sequence (5' to 3')	Tm °C	Product size on cDNA (bp)	Purpose of primers
5' RACE Outer Primer Hc *dcr-1* exon 5 R1	GCTGATGGCGATGAATGAACACTG CACGAGATCAGAGGCTGTTTCGATG	63 65	664	1st round of 5' RACE to amplify *H. contortus dcr-1* 5' end. 5' RACE Outer Primer provided with FirstChoice RLM-RACE Kit (Ambion).
5' RACE Inner Primer Hc *dcr-1* exon 2 R2	CGCGGATCCGAACACTGCGTTTGCTGG CTTTGATG CGCCATTTTCAATCGGAGCCATG	74 62	186	2nd round of 5' RACE to amplify *H. contortus dcr-1* 5' end. 5' RACE Inner Primer provided with FirstChoice RLM-RACE Kit (Ambion).

Table 3: Primers used to amplify *H. contortus dcr-1* cDNA

Primer Pair	Sequence (5' to 3')	Tm °C	Product size on cDNA (bp)
Hc DCR F1 Hc dcr A	CAGAGCTATCATGATCACCGAGAAAGATG CTGCTTGTCGTTTGTGCAATCCTTGGCTGTC	65 70	1303
Hc dcr B Hc dcr C	GACAGCCAAGGATTGCACAAACGACAAGCAG CGCCGATATCGGGTGTCGATCCATAAGTTC	70 70	1686
Hc dcr D Hc dcr E	GAACTTATGGATCGACACCCGATATCGGCG GCCAAGTCGATAAAGGTTGCAATTTGAGAC	70 65	1404
Hc dcr 19 F1 Hc DCR R1	GGAAACGATAGGAGACTCTTTCCTAA CCAATGGCGACTAACTGCTCATAGTCACGG	62 70	1515

Table 4: Primers used to amplify upstream promoter regions

Primers	Sequence (5' to 3')	Tm °C	Expected product size (bp)	Purpose of primers
Hc-H11 AJ311 Prom F1 (Pst1)	CCCCTGCAGATCTCTT ATCAGAGGCTTCCACG TAGGAAAG	75	Hc-H11 AJ311 Prom F1/ Hc-H11 AJ311 Prom R1 = 1510	Amplify upstream promoter region of *H. contortus* H11-4 (**Accession No: AJ311316**).
Hc-H11 AJ311 Prom F2 (Sph1)	CCCGCATGCGTAGTCC TCAACATTAATCGAATT CTTTATTGC	71	Hc-H11 AJ311 Prom F2/Hc-H11 AJ311 Prom R2 = 1448	
Hc-H11 AJ311 Prom R1 (BamH1)	CCCGGATCCCTGAACA ATATTTTTATGGAAAGG TTAATACATAGAG	70		
Hc-H11 AJ311 Prom R2 (BamH1)	CCCGGATCCGAAAGGT TAATACATAGAGCAGA AATCCGAACAATC	73		
Hc-H11 X94 Prom F1 (Pst1)	CCCCTGCAGCAGGACA GATTGTGTTCAGGCGG CATAAGC	77	1500	Amplify upstream promoter region of *H. contortus* H11-1 (**Accession No:X94187**).
Hc-H11 X94Prom Fus R1	AGTCGACCTGCAGGCA TGCAAGCTCTAGATGG AGACCCTGGAATGAGC TGAAG	80		
Ce-TO7 Prom EF1 (Sph1)	CCCGCATGCTGTATCA TCTACCTACAACTG	68	Ce-TO7 Prom EF1/ Ce-TO7 Prom ER1 = 1200	Amplify upstream promoter region of *C. elegans* **T07F10.1** (Sequence obtained from Wormbase).
Ce-TO7 Prom ER1 (BamH1)	GTGGGATCCGAACACA GTGAGAAAACGAAATA A	67		
Ce-TO7 Prom3.8F1 (Sph1)	CCCGCATGCGGACCGA TGCTTACACTAAGAC	72	Ce-TO7 Prom3.8F1/ Ce-TO7 Prom ER1 = 3800	
Hc-asp-1 Prom F1	CCCGCATGCATGAGAA ACAATCGTATACGCAG AGAACTC	75	957	Amplify upstream promoter region of *H. contortus* asp-1 gene (based on NCBI sequence **Accession No: A30245**)
Hc-asp-1 Prom R1	CCCGGATCCCATTTTG GTAGTCCCTATGATCA CGATAC	73		

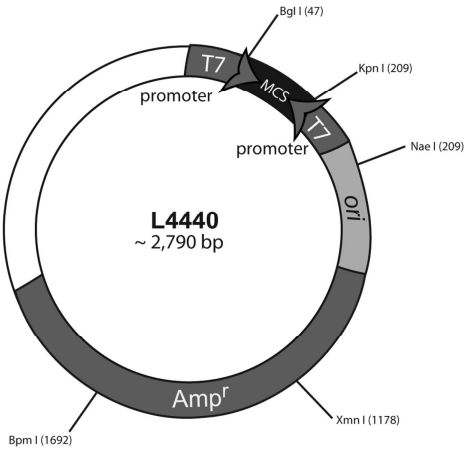
Table 5: Sequencing primers

Primer	**Sequence (5' to 3')**	**Tm ° C**	**Purpose of primers**
T3	ATTAACCCTCACTAAAG	45	Sequencing primers for **pSC-A or TOPO** vector insert DNA
T7	AATACGACTCACTATAG	45	
L4440 Sense	CGACTCACTATAGGGAGACC	59	Sequencing primers for on **L4440** vector
M13 universal (-21)	TGTAAAACGACGGCCAGT	54	
M13 reverse (-29)	CAGGAAACAGCTATGACC	54	Sequencing primers for **pPD96.04** vector
96.04 reverse	TCTGAGCTCGGTACCCTCCAAGGG	68	

Appendix 3: Vector maps

1. L4440 vector with double T7 promoters.

RNA is transcribed from T7 promoters at either end of the multiple cloning site (MCS) to produce dsRNA, and can be used to generate feeding libraries or to produce dsRNA for soaking. (Originally designed by A. Fire and kindly provided by Julie Ahringer, University of Cambridge).



T7 promoter

Bgl II Sac I Sac II Eag I Not I
CCGGCAGATCTGATATCATCGATGAATTCGAGCTCCACCGCGGTGGCGGCCGCTC

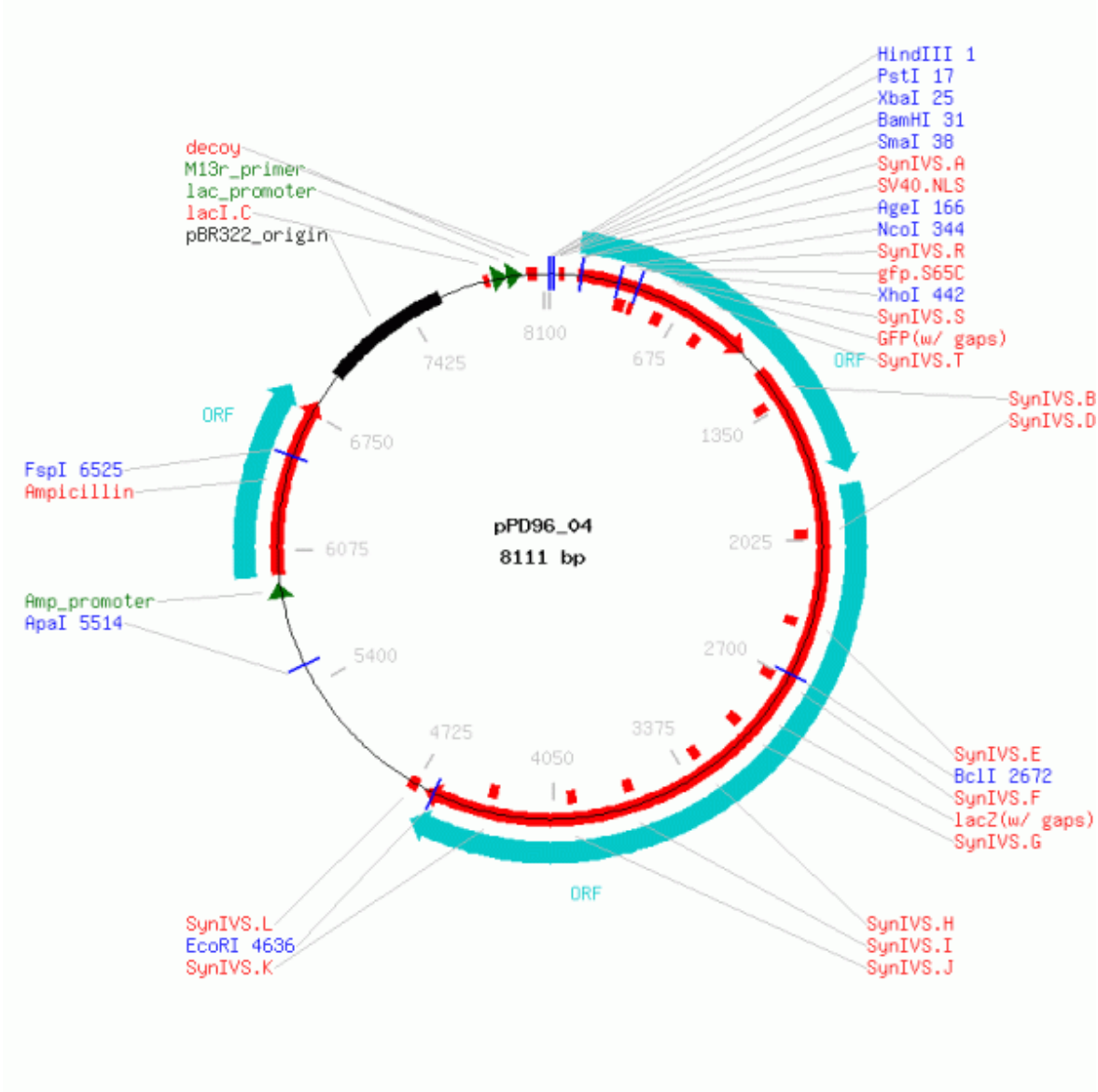
Xba I Spe I Age I Nco I Nhe I Mlu I Xma I Sma I Pst I
TAGATCTAGAACTAGTGGATCCACCGGTTCCATGGCTAGCCACGTGACGCGTGGATCCCCCGGGCTGCA

Sal I
Hind III Hinc II Xho I Apa I Kpn I
GGAATTCGATATCAAGCTTATCGATACCGTCGACCTCGAGGGGGGGCCCGGTACCCAATT

T7 promoter

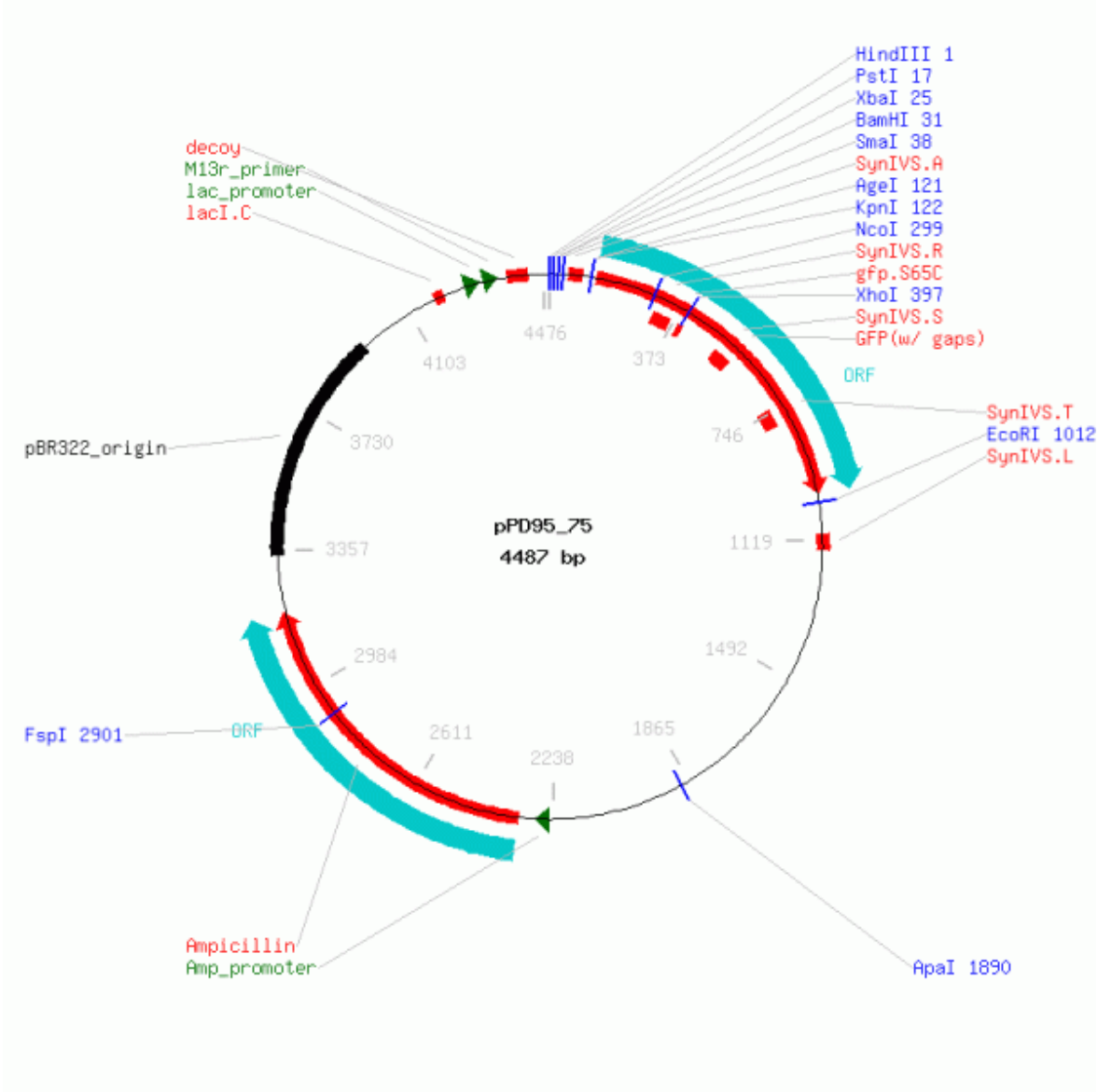
2. pPD 96.04 *lacZ*/GFP expression vector

Fire vector, http://www.ciwemb.edu/pages/firelab.html



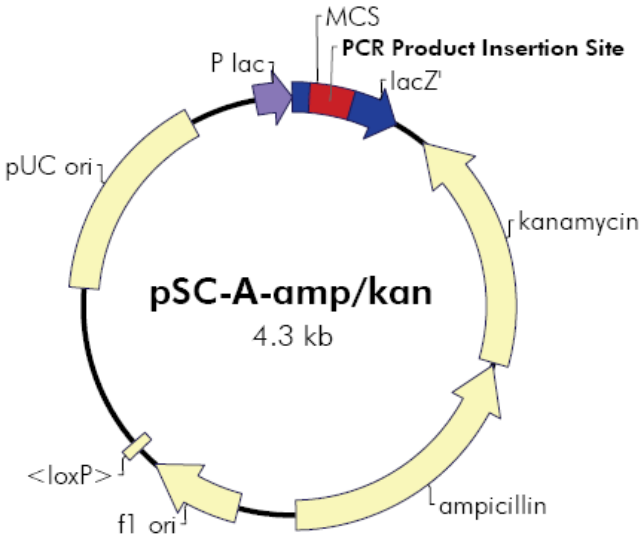
3. pPD 95.75 GFP expression vector

Fire vector, http://www.ciwemb.edu/pages/firelab.html

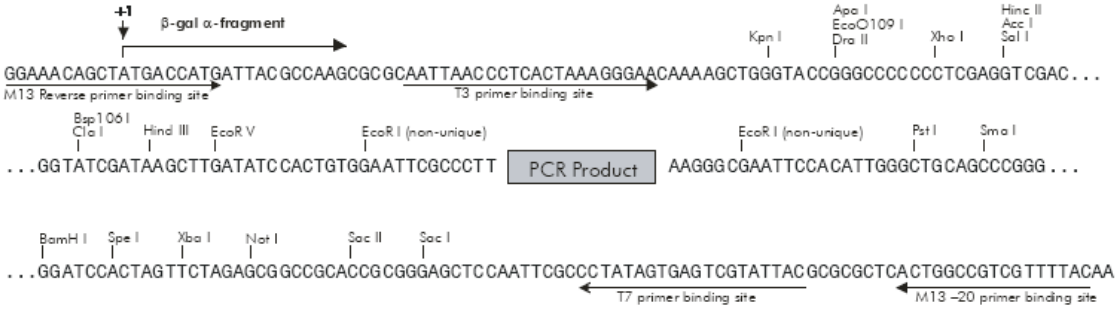


4. pSC-A PCR cloning vector

Supplied by Stratagene.

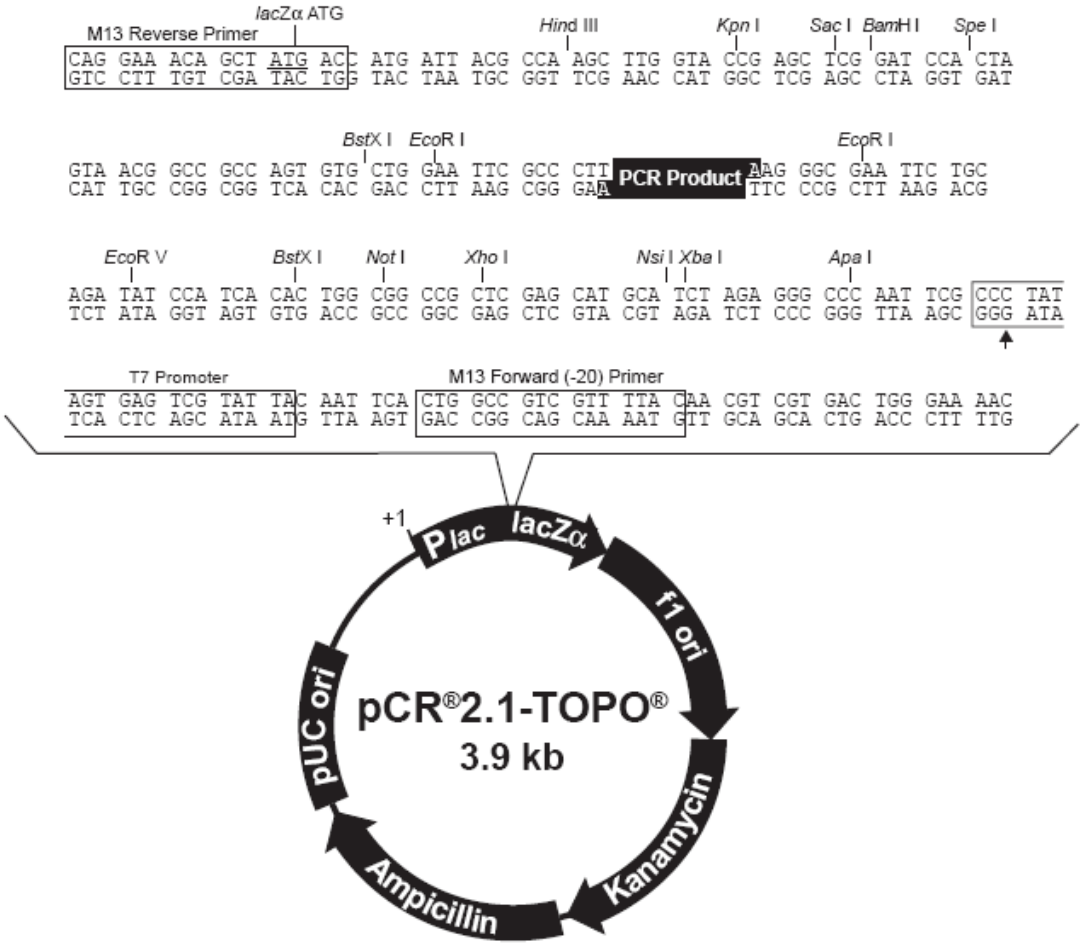


pSC-A-amp/kan PCR Cloning Vector PCR Product Insertion Site Region (sequence shown 4261–4270, 1–250)



5. pCR 2.1 TOPO PCR cloning vector

Supplied by Invitrogen.



Comments for pCR®2.1-TOPO®
3931 nucleotides

Appendix 4: Sequence data

Hc-dcr-1 cDNA sequence

atgatcaccgagaaagatgttgaagtcagctgcttcacaccgagagattatcaggttgaacttcttgacaaggcct
gtaaacgcaatgttatcgttcaactcggtactggtgctggaaaaacattcatagccgttctactgctgaaggagta
tggacttcaaatcatggctccgattgaaaatggcggaaaacgagcgtttttcgtagttgataaagtagctctggtg
gatcagcaagccgagcatattcagtgtcatactacgcttactgttggcagaatgcatggcagtttgaacagtgatg
tctggaataagcagagtggattcgatgatttcatgtctgtacataatgtagtggtcattaccgctcaagtgtttctc
gatttgatagatcatgcgtacttcaatatcgcgaaactcgctcttattatttttgatgagtgtcaccatgcccttggc
gtgaaacatccatatcgtgtgattatggatcgtattatgcgagttcccgcagatcagcaacctcggatattgggcc
tgaccgcttctcttattaacgataaaacaccaccaaatcaattggaagcaaaactttccaagttggagtgcgtatt
gaacagtgccatcgaaacagcctctgatctcgtggcgatatccaaatacggtgccaagcccaacgagtatgtcgt
catttcaacagattacaaccctcaggatagctgtggcggagaaattctgcagctattggaagattggcgaaaattc
tgctcgtcgacgcaagagttcgatccaaacttcgacatcgatcctcgaaagcccattcaggaagcgctcaacaga
actctggcggtacttcgtcaagttggaccatgggctgcttggaaagtttctcaaatgtgggagaaagaactgcaca
aactgacaaagcagactttccttcaagagaagaccgtggacttttgattatgggagagacctgtatgacaactgt
tcggaaaatgctcgagcctaagatgaagccgatcagaactcttgaaggattgaagccttatctgccaaacaaggt
tatacgattgatagacattctctcacacttcaatcaagacaagggtgagaaagaggatccgttaagtggcatcat
cttcgtcgatcaacgttatgtggcctatactctcaacgttttgctaaagcacgtttgtcgatgggatccaaacttca
agtttattcagtctgattttgtaattggattcagtggtgggagtttcgcatctgatgacagccaaggattgcacaaa
cgacaagcagatgtaattcgacgttttcgacaaggtgaactgaacttgttagtggcaacgagtgttttggaagagg
gcgtggatgtaaggcattgcaacttggtgattaaattcgatcggccgatcgactatcgatcgtacatccagtcgaa
agggcgagctcgcaaacgtgatgggggagcgaagtatttcatgttggttgatgagtcagatagtccgaaatgttct
gaagaccttcgagattttgttaaaatcgaaaagatgcttcttcgtcgatgtcaaagtgttcacaatccaggtgatg
atggaagtaacgaagttggtctagctcaaaatgtggataccttaattcctccatacgttgtgccgtctactggtgcg
caagtttccttgtcttcagcgatagggctagtgaataggtattgcgcaaaactaccgtctgatattttcacacgact
tgttcctcaaaatcgtttgatagctgtgaattgtctgggaagaacactttacaaggctgagcttttactaccgataa
actcacctataaagcaaccgatagtattggagacgcctctggaaagtaagaagctagcgcaaatggctgtggctc
ttgaggcctgtcgcgttcttcaccaagcaggtgaactgaatgaccacttgcttccggtaggacgagaatctattgc
cgatttgctaagccagctggacgaagacccggacgagtgggctcccggtatctccgctaaagtcgggtcggcgag
aaggaaacaactttatgataaaagagttgcgacagctttacatgaggcgctccctgtaaagggagaaccgtgtta
tatttatgtaatggagttagaattgctcaaagaaccgtcaccggagtcgaatccgaaacgacgacgttttgcaaat
cctctagattacgagtacttatttggatttttaagctcaaaagtgttaccgaagattccgtccttcgcggcctacct
acgtcaaggagatatgcgggtccatctcgttcgtgcttctactcaggttacactcaacagtcagaatctcactatg

atcaaacattttcatcattatattttcaaaaatgtgttacaattgtgcaaagccaatttggatttccacctggatgc
atcaacacctataaatactctcattgtacctctccacagaactgcctcatcgaccagtgataaatgggaatacag
tataaacatgaagtacgtggaagaagttgttcaaatgatgggtgacaccccaagaatacccagcgaagaggagc
gaaggaactttgtattcaagcccgaagactacagagatgccgttgtcatgccatggtacaggaatattgaacagc
cggtattttactatgttgccgagatacttgaaaatcttacgccgtcatcaccattccctgatgaagaatactcatca
ttcaacgagtatttcatcaagaaatacaatttggagatttatgatcagaagcagaatttactcgacgtcgacttta
cttcaagtcggctgaacttactgctaccgcgtgctggtggagggcgacgaaagacggctgcagtgaaatccgaag
acaactctgctttgtcacggcaacgtcagatctacgttcctgaacttatggatcgacacccgatatcggcgacatt
atggaatttgatatctgcattaccaagcttcttctacaggataaatcacctattgctggcggatgagctgcgtcaga
agactctggtggacgctttaggatattccaaagaggacgccattgtgcccgacaattatgagtggacaccgctttc
ttatcctgccacatatgaggagaaacaatcgctgattgttacgaagattcaacaactcagagaacaaaatcgcgc
ctctgaaatagccgcagggaaactcacaaaggaccagatcgaagcagagaatactttcgaagttggagtatggg
agccagtggtcgtcgagccgactaacgacgagaatatgcccccaacatcatttggtgctggtgattcacttgatac
cgttggattaatgtcctcctccgtacgaactggtggggatctctcggacgacgatgatgcggatgcggtgatgatg
tttgacttctccaaatatcttgctgaaaaggctggtactgcgaaatcagattttgctgccccacgtccggatatcca
gccgactggatggggtggtttcgatgatgctattcctgatacaccatttcatatccttggaagtgcatcgaatcaaa
tagatatgacgagtctcatggctgatctccaaaagcaaattctacctcatcttcccaatgcatgggctcccgccca
agcagaagaaaagaacggaacccttgttgatatcgacactgtgcctacacctccgaaaaaagcgaacggtcctg
ctctacaaaacatcagctccactattcttgagcctacaaaactgtatttggataagatggagatgttagaagatcg
ggaaagagcacaaaaggaggaaatcattgatttgatgcagtttgatgatggagatgatatggattgtagtactgcg
gttgagtactgttcggacgatgagtacacccagttagaaaatggtgaacggcaaaagtatgaacgtgatcattca
gtagtcatcaatcgaaaactttctgagggagaaatcatcgctccagaattacccagctggcagaatcgtttctctt
ttgcttctgcatctatgtcctcaacatgtctggtgtcgaataatgggacagttccatcagaatttcattctgcatctc
ttcttgccgaaaatccttacggtgtatctcctcgtttgctgttgacggctctcactacctcaaatgccaatgacggt
atcaatttggagcgattggaaacgataggagactctttcctaaaatactcggtcaccgactacctctatcattctc
atccagaccaacatgagggcaaattatctttcgctagaagtaaagaggtctcaaactgcaacctttatcgacttg
gcaaacggctaggcataccatcgctaattgttgcctccaagtttgacgtgtacgactcttggcttccccttgttac
atgccgaacaacgacttcaaagctcctaattctgaagatgcagaggagcgtgataagtttatcgaggatgttcttg
aagggaacgaaactgttcagaaattgccgaaaccggtaactgggtgggatcaggcggacatgaataacgatgtg
cgacaactagagaatggtgtggagactatcaactttgccaagccatgtgccaatactgcggcgctcgaagaattg
ccacctttaccatacaatatgttaacgcaacaatacatcagtgacaagtcgatcgccgatgcaattgaagctctc
atcggtgcccatctgttaaccttaggacctcgacctacgctgaaagttatgaaatggcttggtctaaaagtactga
cggatgatgtcgaaagtgtggatccgttgttgagatttgtagacactcccgaatgtcctgatatggccgagcggct
gttacaggatatgtggcaacaattcaacttcagtctgctggaagatcggatagggtatcgattcaacaacaaagc
ctacttactgcaagcattcactcatgctagctatttcaaaaatagaattacgggttgttatcagcgtttggaattcc

ttggtgatgctgtactcgattacatgatcacacgatacctattcgaagatgaacgacagtatagtcctggggtgct
gacagatttgcgctcagctctcgtgaataacacgatttttgcaagtcttgctgtaaagtacgacttccacaagcatt
ttattgcgatgtgtcccggtctccaccatatgatcgagaagtttgtgaaactttgcagtgagcggaacttcttcgat
gccaacttcaattctgagatgtacatggttacaaccgaggaagaaattgatgaaggtcaggaagaggacattgaa
gtgccgaaagcgatgagtgatattttcgagagcgttgcgggtgccgtctacctcgatgcaaatcgtgatttggata
ttgtgtggagggtgtttttcaacctaatgagacagacgatagaggaatgctgcgcgtatccgcctagatcgccgat
tcgtgagctcatggaacttgaaccgggaaagactcgtttcagtaagatggagcgaataattgagagcggaaaggt
gcgagtaacagtggatattggaaataagatgaaattcacagggatgggacgaaattatcgtatagcaaagacga
cagcagccaagagggcgctcaagtatctcaagagcttggaagagcagaaactacgcgaagctgaacgaaccgt
gactatgagcagttag

Translation oh *Hc-dcr-1* cDNA

MITEKDVEVSCFTPRDYQVELLDKACKRNVIVQLGTGAGKTFIAVLLLKEYGLQIMAPIENGGK
RAFFVVDKVALVDQQAEHIQCHTTLTVGRMHGSLNSDVWNKQSGFDDFMSVHNVVVITAQVF
LDLIDHAYFNIAKLALIIFDECHHALGVKHPYRVIMDRIMRVPADQQPRILGLTASLINDKTPPNQ
LEAKLSKLECVLNSAIETASDLVAISKYGAKPNEYVVISTDYNPQDSCGGEILQLLEDWRKFCSST
QEFDPNFDIDPRKPIQEALNRTLAVLRQVGPWAAWKVSQMWEKELHKLTKQTFLQEKTVDFLI
MGETCMTTVRKMLEPKMKPIRTLEGLKPYLPNKVIRLIDILSHFNQDKGEKEDPLSGIIFVDQRY
VAYTLNVLLKHVCRWDPNFKFIQSDFVIGFSGGSFASDDSQGLHKRQADVIRRFRQGELNLLVA
TSVLEEGVDVRHCNLVIKFDRPIDYRSYIQSKGRARKRDGGAKYFMLVDESDSPKCSEDLRDFVK
IEKMLLRRCQSVHNPGDDGSNEVGLAQNVDTLIPPYVVPSTGAQVSLSSAIGLVNRYCAKLPSDI
FTRLVPQNRLIAVNCLGRTLYKAELLLPINSPIKQPIVLETPLESKKLAQMAVALEACRVLHQAG
ELNDHLLPVGRESIADLLSQLDEDPDEWAPGISAKVGSARRKQLYDKRVATALHEALPVKGEPC
YIYVMELELLKEPSPESNPKRRRFANPLDYEYLFGFLSSKVLPKIPSFAAYLRQGDMRVHLVRAS
TQVTLNSQNLTMIKHFHHYIFKNVLQLCKANLDFHLDASTPINTLIVPLHRTASSTSDKWEYSIN
MKYVEEVVQMMGDTPRIPSEEERRNFVFKPEDYRDAVVMPWYRNIEQPVFYYVAEILENLTPSS
PFPDEEYSSFNEYFIKKYNLEIYDQKQNLLDVDFTSSRLNLLLPRAGGGRRKTAAVKSEDNSALS
RQRQIYVPELMDRHPISATLWNLISALPSFFYRINHLLLADELRQKTLVDALGYSKEDAIVPDNYE
WTPLSYPATYEEKQSLIVTKIQQLREQNRASEIAAGKLTKDQIEAENTFEVGVWEPVVVEPTND
ENMPPTSFGAGDSLDTVGLMSSSVRTGGDLSDDDDADAVMMFDFSKYLAEKAGTAKSDFAAP
RPDIQPTGWGGFDDAIPDTPFHILGSASNQIDMTSLMADLQKQILPHLPNAWAPAQAEEKNGT
LVDIDTVPTPPKKANGPALQNISSTILEPTKLYLDKMEMLEDRERAQKEEIIDLMQFDDGDDMDC
STAVEYCSDDEYTQLENGERQKYERDHSVVINRKLSEGEIIAPELPSWQNRFSFASASMSSTCLV
SNNGTVPSEFHSASLLAENPYGVSPRLLLTALTTSNANDGINLERLETIGDSFLKYSVTDYLYHS
HPDQHEGKLSFARSKEVSNCNLYRLGKRLGIPSLIVASKFDVYDSWLPPCYMPNNDFKAPNSED

AEERDKFIEDVLEGNETVQKLPKPVTGWDQADMNNDVRQLENGVETINFAKPCANTAALEELP
PLPYNMLTQQYISDKSIADAIEALIGAHLLTLGPRPTLKVMKWLGLKVLTDDVESVDPLLRFVDT
PECPDMAERLLQDMWQQFNFSLLEDRIGYRFNNKAYLLQAFTHASYFKNRITGCYQRLEFLGD
AVLDYMITRYLFEDERQYSPGVLTDLRSALVNNTIFASLAVKYDFHKHFIAMCPGLHHMIEKFVK
LCSERNFFDANFNSEMYMVTTEEEIDEGQEEDIEVPKAMSDIFESVAGAVYLDANRDLDIVWRV
FFNLMRQTIEECCAYPPRSPIRELMELEPGKTRFSKMERIIESGKVRVTVDIGNKMKFTGMGRNY
RIAKTTAAKRALKYLKSLEEQKLREAERTVTMSS*

List of References

Abad, P., Gouzy, J., Aury, J. M. *et al*. (2008). Genome sequence of the metazoan plant-parasitic nematode *Meloidogyne incognita*. *Nature Biotechnology* **26**, 909-915.

Aboobaker, A. A. & Blaxter, M. L. (2003). Use of RNA interference to investigate gene function in the human filarial nematode parasite *Brugia malayi*. *Molecular and Biochemical Parasitology* **129**, 41-51.

Albonico, M., Engels, D. & Savioli, L. (2004). Monitoring drug efficacy and early detection of drug resistance in human soil-transmitted nematodes: a pressing public health agenda for helminth control. *International Journal for Parasitology* **34**, 1205-1210.

Alkharouf, N. W., Klink, V. P. & Matthews, B. F. (2007). Identification of *Heterodera glycines* (soybean cyst nematode [SCN]) cDNA sequences with high identity to those of *Caenorhabditis elegans* having lethal mutant or RNAi phenotypes. *Experimental Parasitology* **115**, 247-258.

Almeida, R. & Allshire, R. C. (2005). RNA silencing and genome regulation. *Trends in Cell Biology* **15**, 251-258.

Ambros, V. (2001). microRNAs: tiny regulators with great potential. *Cell* **107**, 823-826.

Anderson, R. C. (1992). Nematode parasites of vertebrates. Their development and transmission. *CAB International* **Wallingford, England**.

Anderson, T. J., Blouin, M. S. & Beech, R. N. (1998). Population biology of parasitic nematodes: applications of genetic markers. *Advances in Parasitology* **41**, 219-283.

Andrews, S. J., Hole, N. J., Munn, E. A. & Rolph, T. P. (1995). Vaccination of sheep against haemonchosis with H11, a gut membrane-derived protective antigen from the adult parasite: prevention of the periparturient rise and colostral transfer of protective immunity. *International Journal for Parasitology* **25**, 839-846.

Andrews, S. J., Rolph, T. P. & Munn, E. A. (1997). Duration of protective immunity against ovine haemonchosis following vaccination with the nematode gut membrane antigen H11. *Research in Veterinary Science* **62**, 223-227.

Bakhetia, M., Charlton, W., Atkinson, H. J. & McPherson, M. J. (2005). RNA interference of dual oxidase in the plant nematode *Meloidogyne incognita*. *Molecular Plant Microbe Interactions* **18**, 1099-1106.

Bakhetia, M., Urwin, P. E. & Atkinson, H. J. (2007). QPCR analysis and RNAi define pharyngeal gland cell-expressed genes of *Heterodera glycines* required for initial interactions with the host. *Molecular Plant Microbe Interactions* **20**, 306-312.

Bakhetia, M., Urwin, P. E. & Atkinson, H. J. (2008). Characterisation by RNAi of pioneer genes expressed in the dorsal pharyngeal gland cell of *Heterodera*

glycines and the effects of combinatorial RNAi. *International Journal for Parasitology* **38**, 1589-1597.

Bank, W. (1993). *World Development Report: Investing in Health.*: Oxford, Oxford University Press.

Bargmann, C. I. (2006). Chemosensation in *C. elegans*. *WormBook*, 1-29.

Bartley, D. J., McAllister, H., Bartley, Y., Dupuy, J., Menez, C., Alvinerie, M., Jackson, F. & Lespine, A. (2009). P-glycoprotein interfering agents potentiate ivermectin susceptibility in ivermectin sensitive and resistant isolates of *Teladorsagia circumcincta* and *Haemonchus contortus*. *Parasitology* **136**, 1081-1088.

Bastin, P., Ellis, K., Kohl, L. & Gull, K. (2000). Flagellum ontogeny in trypanosomes studied via an inherited and regulated RNA interference system. *Journal of Cell Science* **113 (Pt 18)**, 3321-3328.

Baum, J., Papenfuss, A. T., Mair, G. R., Janse, C. J., Vlachou, D., Waters, A. P., Cowman, A. F., Crabb, B. S. & de Koning-Ward, T. F. (2009). Molecular genetics and comparative genomics reveal RNAi is not functional in malaria parasites. *Nucleic Acids Research* **37**, 3788–3798.

Bernstein, E., Caudy, A. A., Hammond, S. M. & Hannon, G. J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363-366.

Bernstein, E., Kim, S. Y., Carmell, M. A. *et al*. (2003). Dicer is essential for mouse development. *Nature Genetics* **35**, 215-217.

Berriman, M., Haas, B. J., LoVerde, P. T. *et al*. (2009). The genome of the blood fluke *Schistosoma mansoni*. *Nature* **460**, 352-358.

Blackhall, W. J., Pouliot, J. F., Prichard, R. K. & Beech, R. N. (1998). *Haemonchus contortus*: selection at a glutamate-gated chloride channel gene in ivermectin- and moxidectin-selected strains. *Experimental Parasitology* **90**, 42-48.

Blackhall, W. J., Prichard, R. K. & Beech, R. N. (2003). Selection at a gamma-aminobutyric acid receptor gene in *Haemonchus contortus* resistant to avermectins/milbemycins. *Molecular & Biochemical Parasitology* **131**, 137-145.

Blaxter, M. L., De Ley, P., Garey, J. R. *et al*. (1998). A molecular evolutionary framework for the phylum Nematoda. *Nature* **392**, 71-75.

Blevins, T., Rajeswaran, R., Shivaprasad, P. V. *et al*. (2006). Four plant Dicers mediate viral small RNA biogenesis and DNA virus induced silencing. *Nucleic Acids Research* **34**, 6233-6246.

Blitz, N. M. & Gibbs, H. C. (1972). Studies on the arrested development of *Haemonchus contortus* in sheep. I. The induction of arrested development and the spring rise phenomenon. *International Journal for Parasitology* **2**, 5-12.

Borgers, M. & De Nollin, S. (1975). Ultrastructural changes in *Ascaris suum* intestine after mebendazole treatment *in vivo*. *Journal of Parasitology* **61**, 110-122.

Borsani, O., Zhu, J., Verslues, P. E., Sunkar, R. & Zhu, J. K. (2005). Endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulate salt tolerance in *Arabidopsis*. *Cell* **123**, 1279-1291.

Breving, K. & Esquela-Kerscher, A. (2009). The complexities of microRNA regulation: Mirandering around the rules. *International Journal of Biochemistry & Cell Biology*.

Briese, M., Esmaeili, B., Johnson, N. M. & Sattelle, D. B. (2006). pWormgatePro enables promoter-driven knockdown by hairpin RNA interference of muscle and neuronal gene products in *Caenorhabditis elegans*. *Invertebrate Neuroscience* **6**, 5-12.

Britton, C., McKerrow, J. H. & Johnstone, I. L. (1998). Regulation of the *Caenorhabditis elegans* gut cysteine protease gene *cpr-1*: requirement for GATA motifs. *Journal of Molecular Biology* **283**, 15-27.

Britton, C., Redmond, D. L., Knox, D. P., McKerrow, J. H. & Barry, J. D. (1999). Identification of promoter elements of parasite nematode genes in transgenic *Caenorhabditis elegans*. *Molecular & Biochemical Parasitology* **103**, 171-181.

Britton, C. & Murray, L. (2002). A cathepsin L protease essential for *Caenorhabditis elegans* embryogenesis is functionally conserved in parasitic nematodes. *Molecular & Biochemical Parasitology* **122**, 21-33.

Brown, L. A., Jones, A. K., Buckingham, S. D., Mee, C. J. & Sattelle, D. B. (2006). Contributions from *Caenorhabditis elegans* functional genetics to antiparasitic drug target identification and validation: nicotinic acetylcholine receptors, a case study. *International Journal of Parasitology* **36**, 617-624.

Catalanotto, C., Pallotta, M., ReFalo, P., Sachs, M. S., Vayssie, L., Macino, G. & Cogoni, C. (2004). Redundancy of the two dicer genes in transgene-induced posttranscriptional gene silencing in *Neurospora crassa*. *Molecular & Cellular Biology* **24**, 2536-2545.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W. & Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science* **263**, 802-805.

Chapman, E. J. & Carrington, J. C. (2007). Specialization and evolution of endogenous small RNA pathways. *Nature Reviews Genetics* **8**, 884-896.

Chen, C. C., Simard, M. J., Tabara, H., Brownell, D. R., McCollough, J. A. & Mello, C. C. (2005a). A member of the polymerase beta nucleotidyltransferase superfamily is required for RNA interference in *C. elegans*. *Current Biology* **15**, 378-383.

Chen, Q., Rehman, S., Smant, G. & Jones, J. T. (2005b). Functional analysis of pathogenicity proteins of the potato cyst nematode *Globodera rostochiensis* using RNAi. *Molecular Plant Microbe Interactions* **18**, 621-625.

Chitwood, D. J. (2003). Research on plant-parasitic nematode biology conducted by the United States Department of Agriculture-Agricultural Research Service. *Pest Management Science* **59**, 748-753.

Christie, M. & Jackson, F. (1982). Specific identification of strongyle eggs in small samples of sheep faeces. *Research in Veterinary Science* **32**, 113-117.

Churcher, T. S. & Basanez, M. G. (2008). Density dependence and the spread of anthelmintic resistance. *Evolution* **62**, 528-537.

Ciche, T. A. & Sternberg, P. W. (2007). Postembryonic RNAi in *Heterorhabditis bacteriophora*: a nematode insect parasite and host for insect pathogenic symbionts. *BMC Developmental Biology* **7**, 101.

Collins, R. E. & Cheng, X. (2005). Structural domains in RNAi. *FEBS Letters* **579**, 5841-5849.

Combes, D., Fedon, Y., Toutant, J. P. & Arpagaus, M. (2003). Multiple *ace* genes encoding acetylcholinesterases of *Caenorhabditis elegans* have distinct tissue expression. *The European Journal of Neuroscience* **18**, 497-512.

Consortium, *C. e.* G. S. (1998). Genome sequence of the nematode *C. elegans*: a platform for investigating biology. *Science* **282**, 2012-2018.

Conway, D. P. (1964). Variance in the Effectiveness of Thiabendazole against *Haemonchus Contortus* in Sheep. *American Journal of Veterinary Research* **25**, 844-846.

Costa, J. C., Lilley, C. J., Atkinson, H. J. & Urwin, P. E. (2009). Functional characterisation of a cyst nematode acetylcholinesterase gene using *Caenorhabditis elegans* as a heterologous system. *International Journal of Parasitology* **39**, 849-858.

Cvilink, V., Lamka, J. & Skalova, L. (2009). Xenobiotic metabolizing enzymes and metabolism of anthelminthics in helminths. *Drug Metabolism Reviews* **41**, 8-26.

Dalzell, J. J., McMaster, S., Fleming, C. C. & Maule, A. G. (2009a). Short interfering RNA-mediated gene silencing in *Globodera pallida* and *Meloidogyne incognita* infective stage juveniles. *International Journal of Parasitology* **40**, 91-100.

Dalzell, J. J., McMaster, S., Johnston, M. J., Kerr, R., Fleming, C. C. & Maule, A. G. (2009b). Non-nematode-derived double-stranded RNAs induce profound phenotypic changes in *Meloidogyne incognita* and *Globodera pallida* infective juveniles. *International Journal of Parasitology* **39**, 1503-1516.

DaRocha, W. D., Otsu, K., Teixeira, S. M. & Donelson, J. E. (2004). Tests of cytoplasmic RNA interference (RNAi) and construction of a tetracycline-inducible

T7 promoter system in *Trypanosoma cruzi*. *Molecular & Biochemical Parasitology* **133**, 175-186.

Deplancke, B., Dupuy, D., Vidal, M. & Walhout, A. J. (2004). A gateway-compatible yeast one-hybrid system. *Genome Research* **14**, 2093-2101.

Dieterich, C., Clifton, S. W., Schuster, L. N. *et al*. (2008). The *Pristionchus pacificus* genome provides a unique perspective on nematode lifestyle and parasitism. *Nature Genetics* **40**, 1193-1198.

Ding, S. W. & Voinnet, O. (2007). Antiviral immunity directed by small RNAs. *Cell* **130**, 413-426.

Dlakic, M. (2006). DUF283 domain of Dicer proteins has a double-stranded RNA-binding fold. *Bioinformatics* **22**, 2711-2714.

Drinnenberg, I. A., Weinberg, D. E., Xie, K. T., Mower, J. P., Wolfe, K. H., Fink, G. R. & Bartel, D. P. (2009). RNAi in Budding Yeast. *Science* **326**, 544 - 550.

Driscoll, M., Dean, E., Reilly, E., Bergholz, E. & Chalfie, M. (1989). Genetic and molecular analysis of a *Caenorhabditis elegans* beta-tubulin that conveys benzimidazole sensitivity. *The Journal of Cell Biology* **109**, 2993-3003.

Dubreuil, G., Magliano, M., Deleury, E., Abad, P. & Rosso, M. N. (2007). Transcriptome analysis of root-knot nematode functions induced in the early stages of parasitism. *The New Phytologist* **176**, 426-436.

Dubreuil, G., Magliano, M., Dubrana, M. P., Lozano, J., Lecomte, P., Favery, B., Abad, P. & Rosso, M. N. (2009). Tobacco rattle virus mediates gene silencing in a plant parasitic root-knot nematode. *Journal of Experimental Botany* **60**, 4041-4050.

Dupuy, D., Li, Q. R., Deplancke, B. *et al*. (2004). A first version of the *Caenorhabditis elegans* Promoterome. *Genome Research* **14**, 2169-2175.

Egan, C. R., Chung, M. A., Allen, F. L., Heschl, M. F., Van Buskirk, C. L. & McGhee, J. D. (1995). A gut-to-pharynx/tail switch in embryonic expression of the *Caenorhabditis elegans ges-1* gene centers on two GATA sequences. *Developmental Biology* **170**, 397-419.

Fairbairn, D. J., Cavallaro, A. S., Bernard, M., Mahalinga-Iyer, J., Graham, M. W. & Botella, J. R. (2007). Host-delivered RNAi: an effective strategy to silence genes in plant parasitic nematodes. *Planta* **226**, 1525-1533.

Fanelli, E., Di Vito, M., Jones, J. T. & De Giorgi, C. (2005). Analysis of chitin synthase function in a plant parasitic nematode, *Meloidogyne artiellia*, using RNAi. *Gene* **349**, 87-95.

Fire, A., Harrison, S. W. & Dixon, D. (1990). A modular set of *lacZ* fusion vectors for studying gene expression in *Caenorhabditis elegans*. *Gene* **93**, 189-198.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. & Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806-811.

Ford, L., Guiliano, D. B., Oksov, Y., Debnath, A. K., Liu, J., Williams, S. A., Blaxter, M. L. & Lustigman, S. (2005). Characterization of a novel filarial serine protease inhibitor, Ov-SPI-1, from *Onchocerca volvulus*, with potential multifunctional roles during development of the parasite. *Journal of Biological Chemistry* **280**, 40845-40856.

Fraser, A. G., Kamath, R. S., Zipperlen, P., Martinez-Campos, M., Sohrmann, M. & Ahringer, J. (2000). Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. *Nature* **408**, 325-330.

Fukushige, T., Goszczynski, B., Yan, J. & McGhee, J. D. (2005). Transcriptional control and patterning of the *pho-1* gene, an essential acid phosphatase expressed in the *C. elegans* intestine. *Developmental Biology* **279**, 446-461.

Gasciolli, V., Mallory, A. C., Bartel, D. P. & Vaucheret, H. (2005). Partially redundant functions of *Arabidopsis* DICER-like enzymes and a role for DCL4 in producing trans-acting siRNAs. *Current Biology* **15**, 1494-1500.

Gatongi, P. M., Prichard, R. K., Ranjan, S., Gathuma, J. M., Munyua, W. K., Cheruiyot, H. & Scott, M. E. (1998). Hypobiosis of *Haemonchus contortus* in natural infections of sheep and goats in a semi-arid area of Kenya. *Veterinary Parasitology* **77**, 49-61.

Geary, T. G., Nulf, S. C., Favreau, M. A., Tang, L., Prichard, R. K., Hatzenbuhler, N. T., Shea, M. H., Alexander, S. J. & Klein, R. D. (1992). Three beta-tubulin cDNAs from the parasitic nematode *Haemonchus contortus*. *Molecular & Biochemical Parasitology* **50**, 295-306.

Geldhof, P., Murray, L., Couthier, A., Gilleard, J. S., McLauchlan, G., Knox, D. P. & Britton, C. (2006). Testing the efficacy of RNA interference in *Haemonchus contortus*. *International Journal of Parasitology* **36**, 801-810.

Geldhof, P., Visser, A., Clark, D., Saunders, G., Britton, C., Gilleard, J., Berriman, M. & Knox, D. (2007). RNA interference in parasitic helminths: current situation, potential pitfalls and future prospects. *Parasitology* **134**, 609-619.

Geldhof, P. & Knox, D. (2008). The intestinal contortin structure in *Haemonchus contortus*: An immobilised anticoagulant? *International Journal of Parasitology* **38**, 1579-1588

Ghedin, E., Wang, S., Spiro, D. *et al*. (2007). Draft genome of the filarial nematode parasite *Brugia malayi*. *Science* **317**, 1756-1760.

Gilleard, J. S. (2006). Understanding anthelmintic resistance: the need for genomics and genetics. *International Journal of Parasitology* **36**, 1227-1239.

Gleason, C. A., Liu, Q. L. & Williamson, V. M. (2008). Silencing a candidate nematode effector gene corresponding to the tomato resistance gene *Mi-1* leads to acquisition of virulence. *Molecular Plant Microbe Interactions* **21**, 576-585.

Gomez-Escobar, N., Gregory, W. F., Britton, C., Murray, L., Corton, C., Hall, N., Daub, J., Blaxter, M. L. & Maizels, R. M. (2002). *Abundant larval transcript-1* and *-2* genes from *Brugia malayi*: diversity of genomic environments but conservation of 5' promoter sequences functional in *Caenorhabditis elegans*. *Molecular & Biochemical Parasitology* **125**, 59-71.

Grant, W. N. & Mascord, L. J. (1996). Beta-tubulin gene polymorphism and benzimidazole resistance in *Trichostrongylus colubriformis*. *International Journal of Parasitology* **26**, 71-77.

Grant, W. N., Skinner, S. J., Newton-Howes, J., Grant, K., Shuttleworth, G., Heath, D. D. & Shoemaker, C. B. (2006). Heritable transgenesis of *Parastrongyloides trichosuri*: a nematode parasite of mammals. *International Journal of Parasitology* **36**, 475-483.

Grimm, D., Streetz, K. L., Jopling, C. L., Storm, T. A., Pandey, K., Davis, C. R., Marion, P., Salazar, F. & Kay, M. A. (2006). Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature* **441**, 537-541.

Grishok, A., Pasquinelli, A. E., Conte, D. *et al*. (2001). Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *C. elegans* developmental timing. *Cell* **106**, 23-34.

Grishok, A. (2005). RNAi mechanisms in *Caenorhabditis elegans*. *FEBS Letters* **579**, 5932-5939.

Habig, J. W., Aruscavage, P. J. & Bass, B. L. (2008). In *C. elegans*, high levels of dsRNA allow RNAi in the absence of RDE-4. *PLoS ONE* **3**, e4052.

Hammond, T. M., Andrewski, M. D., Roossinck, M. J. & Keller, N. P. (2008). *Aspergillus* mycoviruses are targets and suppressors of RNA silencing. *Eukaryotic Cell* **7**, 350-357.

Harder, A. & von Samson-Himmelstjerna, G. (2002). Cyclooctadepsipeptides: a new class of anthelmintically active compounds. *Parasitology Research* **88**, 481-488.

Harder, A., Schmitt-Wrede, H. P., Krucken, J., Marinovski, P., Wunderlich, F., Willson, J., Amliwala, K., Holden-Dye, L. & Walker, R. (2003). Cyclooctadepsipeptides: an anthelmintically active class of compounds exhibiting a novel mode of action. *International Journal of Antimicrobial Agents* **22**, 318-331.

Haslam, S. M., Coles, G. C., Munn, E. A., Smith, T. S., Smith, H. F., Morris, H. R. & Dell, A. (1996). *Haemonchus contortus* glycoproteins contain N-linked oligosaccharides with novel highly fucosylated core structures. *Journal of Biological Chemistry* **271**, 30561-30570.

Haslam, S. M., Gems, D., Morris, H. R. & Dell, A. (2002). The glycomes of *Caenorhabditis elegans* and other model organisms. *Biochemical Society Symposium*, 117-134.

Hawdon, J. M., Jones, B. F., Hoffman, D. R. & Hotez, P. J. (1996). Cloning and characterization of *Ancylostoma*-secreted protein. A novel protein associated with the transition to parasitism by infective hookworm larvae. *Journal of Biological Chemistry* **271**, 6672-6678.

Hawkins, P. G. & Morris, K. V. (2008). RNA and transcriptional modulation of gene expression. *Cell Cycle* **7**, 602-607.

Hobert, O. (2002). PCR fusion-based approach to create reporter gene constructs for expression analysis in transgenic *C. elegans*. *Biotechniques* **32**, 728-730.

Hoekstra, R., Visser, A., Otsen, M., Tibben, J., Lenstra, J. A. & Roos, M. H. (2000). EST sequencing of the parasitic nematode *Haemonchus contortus* suggests a shift in gene expression during transition to the parasitic stages. *Molecular & Biochemical Parasitology* **110**, 53-68.

Hong, R. L. & Sommer, R. J. (2006). *Pristionchus pacificus*: a well-rounded nematode. *Bioessays* **28**, 651-659.

Hope, I. A. (1991). 'Promoter trapping' in *Caenorhabditis elegans*. *Development* **113**, 399-408.

Huang, G., Allen, R., Davis, E. L., Baum, T. J. & Hussey, R. S. (2006). Engineering broad root-knot resistance in transgenic plants by RNAi silencing of a conserved and essential root-knot nematode parasitism gene. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 14302-14306.

Hunt-Newbury, R., Viveiros, R., Johnsen, R. *et al*. (2007). High-throughput *in vivo* analysis of gene expression in *Caenorhabditis elegans*. *PLoS Biology* **5**, e237.

Hussein, A. S., Kichenin, K. & Selkirk, M. E. (2002). Suppression of secreted acetylcholinesterase expression in *Nippostrongylus brasiliensis* by RNA interference. *Molecular and Biochemical Parasitology* **122**, 91-94.

Hutvagner, G., McLachlan, J., Pasquinelli, A. E., Balint, E., Tuschl, T. & Zamore, P. D. (2001). A cellular function for the RNA-interference enzyme Dicer in the maturation of the *let-7* small temporal RNA. *Science* **293**, 834-838.

Inoue, N., Otsu, K., Ferraro, D. M. & Donelson, J. E. (2002). Tetracycline-regulated RNA interference in *Trypanosoma congolense*. *Molecular & Biochemical Parasitology* **120**, 309-313.

Islam, M. K., Miyoshi, T., Yamada, M. & Tsuji, N. (2005). Pyrophosphatase of the roundworm *Ascaris suum* plays an essential role in the worm's molting and development. *Infection & Immunity* **73**, 1995-2004.

Issa, Z., Grant, W. N., Stasiuk, S. & Shoemaker, C. B. (2005). Development of methods for RNA interference in the sheep gastrointestinal parasite, *Trichostrongylus colubriformis*. *International Journal of Parasitology* **35**, 935-940.

Jagannathan, S., Laughton, D. L., Critten, C. L., Skinner, T. M., Horoszok, L. & Wolstenholme, A. J. (1999). Ligand-gated chloride channel subunits encoded by the *Haemonchus contortus* and *Ascaris suum* orthologues of the *Caenorhabditis elegans gbr-2* (*avr-14*) gene. *Molecular & Biochemical Parasitology* **103**, 129-140.

James, C. E., Hudson, A. L. & Davey, M. W. (2009). Drug resistance mechanisms in helminths: is it survival of the fittest? *Trends in Parasitology* **25**, 328-335.

Jarrett, W. F. H., McIntyre, W. I. M., Jennings, F. W. & Mulligan, W. (1957). The natural history of parasitic bronchitis with notes on prophylaxis and treatment. *Veterinary Record* **69**, 1329-1340.

Jasmer, D. P., Perryman, L. E., Conder, G. A., Crow, S. & McGuire, T. (1993). Protective immunity to *Haemonchus contortus* induced by immunoaffinity isolated antigens that share a phylogenetically conserved carbohydrate gut surface epitope. *Journal of Immunology* **151**, 5450-5460.

Jasmer, D. P., Yao, C., Rehman, A. & Johnson, S. (2000). Multiple lethal effects induced by a benzimidazole anthelmintic in the anterior intestine of the nematode *Haemonchus contortus*. *Molecular & Biochemical Parasitology* **105**, 81-90.

Jose, A. M., Smith, J. J. & Hunter, C. P. (2009). Export of RNA silencing from *C. elegans* tissues does not require the RNA channel SID-1. *Proceedings of the National Academy of Sciences in the United States of America* **106**, 2283-2288.

Kadotani, N., Nakayashiki, H., Tosa, Y. & Mayama, S. (2004). One of the two Dicer-like proteins in the filamentous fungi *Magnaporthe oryzae* genome is responsible for hairpin RNA-triggered RNA silencing and related small interfering RNA accumulation. *Journal of Biological Chemistry* **279**, 44467-44474.

Kamath, R. S. & Ahringer, J. (2003). Genome-wide RNAi screening in *Caenorhabditis elegans*. *Methods* **30**, 313-321.

Kamath, R. S., Fraser, A. G., Dong, Y. *et al*. (2003). Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* **421**, 231-237.

Kaminsky, R., Ducray, P., Jung, M. *et al*. (2008). A new class of anthelmintics effective against drug-resistant nematodes. *Nature* **452**, 176-180.

Kaminsky, R., Mosimann, D., Sager, H., Stein, P. & Hosking, B. (2009). Determination of the effective dose rate for monepantel (AAD 1566) against adult gastro-intestinal nematodes in sheep. *International Journal of Parasitology* **39**, 443-446.

Kaplan, R. M. (2004). Drug resistance in nematodes of veterinary importance: a status report. *Trends in Parasitology* **20**, 477-481.

Kennedy, S., Wang, D. & Ruvkun, G. (2004). A conserved siRNA-degrading RNase negatively regulates RNA interference in *C. elegans*. *Nature* **427**, 645-649.

Kent, W. J. & Zahler, A. M. (2000). Conservation, regulation, synteny, and introns in a large-scale *C. briggsae-C. elegans* genomic alignment. *Genome Research* **10**, 1115-1125.

Kerboeuf, D., Blackhall, W., Kaminsky, R. & von Samson-Himmelstjerna, G. (2003). P-glycoprotein in helminths: function and perspectives for anthelmintic treatment and reversal of resistance. *International Journal of Antimicrobial Agents* **22**, 332-346.

Ketting, R. F., Fischer, S. E., Bernstein, E., Sijen, T., Hannon, G. J. & Plasterk, R. H. (2001). Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*. *Genes & Development* **15**, 2654-2659.

Kimber, M. J., McKinney, S., McMaster, S., Day, T. A., Fleming, C. C. & Maule, A. G. (2007). *flp* gene disruption in a parasitic nematode reveals motor dysfunction and unusual neuronal sensitivity to RNA interference. *The FASEB Journal* **21**, 1233-1243.

Knox, D. P., Redmond, D. L., Newlands, G. F., Skuce, P. J., Pettit, D. & Smith, W. D. (2003). The nature and prospects for gut membrane proteins as vaccine candidates for *Haemonchus contortus* and other ruminant trichostrongyloids. *International Journal of Parasitology* **33**, 1129-1137.

Knox, D. P., Smith, S. K., Redmond, D. L. & Smith, W. D. (2005). Protection induced by vaccinating sheep with a thiol-binding extract of *Haemonchus contortus* membranes is associated with its protease components. *Parasite Immunology* **27**, 121-126.

Kohler, P. (2001). The biochemical basis of anthelmintic action and resistance. *International Journal of Parasitology* **31**, 336-345.

Kotze, A. C. & Bagnall, N. H. (2006). RNA interference in *Haemonchus contortus*: suppression of beta-tubulin gene expression in L3, L4 and adult worms *in vitro*. *Molecular & Biochemical Parasitology* **145**, 101-110.

Kramer, J. M., French, R. P., Park, E. C. & Johnson, J. J. (1990). The *Caenorhabditis elegans rol-6* gene, which interacts with the *sqt-1* collagen gene to determine organismal morphology, encodes a collagen. *Molecular & Cellular Biology* **10**, 2081-2089.

Krautz-Peterson, G. & Skelly, P. J. (2008). *Schistosoma mansoni*: The Dicer gene and its expression. *Experimental Parasitology* **118**, 122-128.

Krautz-Peterson, G., Bhardwaj, R., Faghiri, Z., Tararam, C. A. & Skelly, P. J. (2009). RNA interference in schistosomes: machinery and methodology. *Parasitology*, 1-11.

Kwa, M. S., Veenstra, J. G. & Roos, M. H. (1994). Benzimidazole resistance in *Haemonchus contortus* is correlated with a conserved mutation at amino acid 200 in beta-tubulin isotype 1. *Molecular & Biochemical Parasitology* **63**, 299-303.

Kwa, M. S., Veenstra, J. G., Van Dijk, M. & Roos, M. H. (1995). Beta-tubulin genes from the parasitic nematode *Haemonchus contortus* modulate drug resistance in *Caenorhabditis elegans*. *Journal of Molecular Biology* **246**, 500-510.

Lacey, E. (1988). The role of the cytoskeletal protein, tubulin, in the mode of action and mechanism of drug resistance to benzimidazoles. *International Journal of Parasitology* **18**, 885-936.

LaCount, D. J., Bruse, S., Hill, K. L. & Donelson, J. E. (2000). Double-stranded RNA interference in *Trypanosoma brucei* using head-to-head promoters. *Molecular & Biochemical Parasitology* **111**, 67-76.

Leathwick, D. M. (1995). A case of moxidectin failing to control ivermectin resistant *Ostertagia* species in goats. *Veterinary Record* **136**, 443-444.

Lee, R. C., Feinbaum, R. L. & Ambros, V. (1993). The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell* **75**, 843-854.

Lee, R. C. & Ambros, V. (2001). An extensive class of small RNAs in *Caenorhabditis elegans*. *Science* **294**, 862-864.

Lee, R. C., Hammell, C. M. & Ambros, V. (2006). Interacting endogenous and exogenous RNAi pathways in *Caenorhabditis elegans*. *RNA* **12**, 589-597.

Lee, Y. S., Nakahara, K., Pham, J. W., Kim, K., He, Z., Sontheimer, E. J. & Carthew, R. W. (2004). Distinct roles for *Drosophila* Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell* **117**, 69-81.

LeJambre, L. F., Windon, R. G. & Smith, W. D. (2008). Vaccination against *Haemonchus contortus*: performance of native parasite gut membrane glycoproteins in Merino lambs grazing contaminated pasture. *Veterinary Parasitology* **153**, 302-312.

Lendner, M., Doligalska, M., Lucius, R. & Hartmann, S. (2008). Attempts to establish RNA interference in the parasitic nematode *Heligmosomoides polygyrus*. *Molecular & Biochemical Parasitology* **161**, 21-31.

Lespine, A., Alvinerie, M., Vercruysse, J., Prichard, R. K. & Geldhof, P. (2008). ABC transporter modulation: a strategy to enhance the activity of macrocyclic lactone anthelmintics. *Trends in Parasitology* **24**, 293-298.

Li, H. W. & Ding, S. W. (2005). Antiviral silencing in animals. *FEBS Letters* **579**, 5965-5973.

Li, X., Massey, H. C., Jr., Nolan, T. J., Schad, G. A., Kraus, K., Sundaram, M. & Lok, J. B. (2006). Successful transgenesis of the parasitic nematode

Strongyloides stercoralis requires endogenous non-coding control elements. *International Journal of Parasitology* **36**, 671-679.

Li, Z., Umeyama, T. & Wang, C. C. (2009). The Aurora Kinase in *Trypanosoma brucei* plays distinctive roles in metaphase-anaphase transition and cytokinetic initiation. *PLoS Pathogens* **5**, e1000575.

Liddell, S. & Knox, D. P. (1998). Extracellular and cytoplasmic Cu/Zn superoxide dismutases from *Haemonchus contortus*. *Parasitology* **116 (Pt 4)**, 383-394.

Lilley, C. J., Goodchild, S. A., Atkinson, H. J. & Urwin, P. E. (2005). Cloning and characterisation of a *Heterodera glycines* aminopeptidase cDNA. *International Journal of Parasitology* **35**, 1577-1585.

Longbottom, D., Redmond, D. L., Russell, M., Liddell, S., Smith, W. D. & Knox, D. P. (1997). Molecular cloning and characterisation of a putative aspartate proteinase associated with a gut membrane protein complex from adult *Haemonchus contortus*. *Molecular & Biochemical Parasitology* **88**, 63-72.

Lustigman, S., Zhang, J., Liu, J., Oksov, Y. & Hashmi, S. (2004). RNA interference targeting cathepsin L and Z-like cysteine proteases of *Onchocerca volvulus* confirmed their essential function during L3 molting. *Molecular & Biochemical Parasitology* **138**, 165-170.

Ma, E., MacRae, I. J., Kirsch, J. F. & Doudna, J. A. (2008). Autoinhibition of human dicer by its internal helicase domain. *Journal of Molecular Biology* **380**, 237-243.

MacDonald, A. J., Tawe, W., Leon, O., Cao, L., Liu, J., Oksov, Y., Abraham, D. & Lustigman, S. (2004). Ov-ASP-1, the *Onchocerca volvulus* homologue of the activation associated secreted protein family is immunostimulatory and can induce protective anti-larval immunity. *Parasite Immunology* **26**, 53-62.

MacMorris, M., Broverman, S., Greenspoon, S., Lea, K., Madej, C., Blumenthal, T. & Spieth, J. (1992). Regulation of vitellogenin gene expression in transgenic *Caenorhabditis elegans*: short sequences required for activation of the *vit-2* promoter. *Molecular & Cellular Biology* **12**, 1652-1662.

MacMorris, M., Spieth, J., Madej, C., Lea, K. & Blumenthal, T. (1994). Analysis of the VPE sequences in the *Caenorhabditis elegans vit-2* promoter with extrachromosomal tandem array-containing transgenic strains. *Molecular & Cellular Biology* **14**, 484-491.

Macrae, I. J., Zhou, K., Li, F., Repic, A., Brooks, A. N., Cande, W. Z., Adams, P. D. & Doudna, J. A. (2006). Structural basis for double-stranded RNA processing by Dicer. *Science* **311**, 195-198.

Malhotra, P., Dasaradhi, P. V., Kumar, A., Mohmmed, A., Agrawal, N., Bhatnagar, R. K. & Chauhan, V. S. (2002). Double-stranded RNA-mediated gene silencing of cysteine proteases (falcipain-1 and -2) of *Plasmodium falciparum*. *Molecular Microbiology* **45**, 1245-1254.

Manton, V. J. A., Peacock, R., Poynter, D., Silverman, P. H. & Terry, R. J. (1962). The influence of age on naturally-acquired resistance to *Haemonchus contortus* infections in young lambs. *Research in Veterinary Science* **1**, 308-314.

Margis, R., Fusaro, A. F., Smith, N. A., Curtin, S. J., Watson, J. M., Finnegan, E. J. & Waterhouse, P. M. (2006). The evolution and diversification of Dicers in plants. *FEBS Letters* **580**, 2442-2450.

Marshall, K., Maddox, J. F., Lee, S. H., Zhang, Y., Kahn, L., Graser, H. U., Gondro, C., Walkden-Brown, S. W. & van der Werf, J. H. (2009). Genetic mapping of quantitative trait loci for resistance to *Haemonchus contortus* in sheep. *Animal Genetics* **40**, 262-272.

Martin, R. J. (1996). An electrophysiological preparation of *Ascaris suum* pharyngeal muscle reveals a glutamate-gated chloride channel sensitive to the avermectin analogue, milbemycin D. *Parasitology* **112 (Pt 2)**, 247-252.

Matzke, M. A. & Birchler, J. A. (2005). RNAi-mediated pathways in the nucleus. *Nature Reviews Genetics* **6**, 24-35.

McGhee, J. D., Sleumer, M. C., Bilenky, M. *et al*. (2007). The ELT-2 GATA-factor and the global regulation of transcription in the *C. elegans* intestine. *Development Biology* **302**, 627-645.

McRobert, L. & McConkey, G. A. (2002). RNA interference (RNAi) inhibits growth of *Plasmodium falciparum*. *Molecular & Biochemical Parasitology* **119**, 273-278.

Mello, C. C., Kramer, J. M., Stinchcomb, D. & Ambros, V. (1991). Efficient gene transfer in *C.elegans*: extrachromosomal maintenance and integration of transforming sequences. *The EMBO Journal* **10**, 3959-3970.

Munn, E. A. (1977). A helical, polymeric extracellular protein associated with the luminal surface of *Haemonchus contortus* intestinal cells. *Tissue & Cell* **9**, 23-34.

Munn, E. A., Greenwood, C. A. & Coadwell, W. J. (1987). Vaccination of young lambs by means of a protein fraction extracted from adult *Haemonchus contortus*. *Parasitology* **94 (Pt 2)**, 385-397.

Munn, E. A., Smith, T. S., Graham, M., Tavernor, A. S. & Greenwood, C. A. (1993). The potential value of integral membrane proteins in the vaccination of lambs against *Haemonchus contortus*. *International Journal of Parasitology* **23**, 261-269.

Munn, E. A., Smith, T. S., Smith, H., James, F. M., Smith, F. C. & Andrews, S. J. (1997). Vaccination against *Haemonchus contortus* with denatured forms of the protective antigen H11. *Parasite Immunology* **19**, 243-248.

Murray, L., Geldhof, P., Clark, D., Knox, D. P. & Britton, C. (2007). Expression and purification of an active cysteine protease of *Haemonchus contortus* using *Caenorhabditis elegans*. *International Journal of Parasitology* **37**, 1117-1125.

Napoli, C., Lemieux, C. & Jorgensen, R. (1990). Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. *The Plant Cell* **2**, 279-289.

Nelson, K. M. & Weiss, G. J. (2008). MicroRNAs and cancer: past, present, and potential future. *Molecular Cancer Therapeutics* **7**, 3655-3660.

Newlands, G. F., Skuce, P. J., Nisbet, A. J., Redmond, D. L., Smith, S. K., Pettit, D. & Smith, W. D. (2006). Molecular characterization of a family of metalloendopeptidases from the intestinal brush border of *Haemonchus contortus*. *Parasitology* **133**, 357-368.

Newmark, P. A., Reddien, P. W., Cebria, F. & Sanchez Alvarado, A. (2003). Ingestion of bacterially expressed double-stranded RNA inhibits gene expression in planarians. *Proceedings of the National Academy of Sciences of the United States of America* **100 Suppl 1**, 11861-11865.

Newton, S. E. & Meeusen, E. N. (2003). Progress and new technologies for developing vaccines against gastrointestinal nematode parasites of sheep. *Parasite Immunology* **25**, 283-296.

Newton, S. E., Graham, M., Knox, D.P., Munn, E.A., Oliver, J.J., Smith, T.S. (1993). Recombinant DNA molecules encoding aminopeptidase enzyme and their use in preparation of vaccine against helminth infections Patent Application WO9323542.

Nieuwhof, G. J. B., S. C. (2005). Costs of the major endemic diseases of sheep in Great Britain and the potential benefits of reduction in disease impact. *Animal Science* **81**, 23-29.

Njue, A. I., Hayashi, J., Kinne, L., Feng, X. P. & Prichard, R. K. (2004). Mutations in the extracellular domains of glutamate-gated chloride channel alpha3 and beta subunits from ivermectin-resistant *Cooperia oncophora* affect agonist sensitivity. *Journal of Neurochemistry* **89**, 1137-1147.

O'Grady, J. & Kotze, A. C. (2004). *Haemonchus contortus*: in vitro drug screening assays with the adult life stage. *Experimental Parasitology* **106**, 164-172.

Obbard, D. J., Jiggins, F. M., Halligan, D. L. & Little, T. J. (2006). Natural selection drives extremely rapid evolution in antiviral RNAi genes. *Current Biology* **16**, 580-585.

Obbard, D. J., Gordon, K. H., Buck, A. H. & Jiggins, F. M. (2009). The evolution of RNAi as a defence against viruses and transposable elements. *Philosophical Transactions of the Royal Society of London Series B Biological Sciences* **364**, 99-115.

Opperman, C. H., Bird, D. M., Williamson, V. M. *et al*. (2008). Sequence and genetic map of *Meloidogyne hapla*: A compact nematode genome for plant parasitism. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 14802-14807.

Paiement, J. P., Leger, C., Ribeiro, P. & Prichard, R. K. (1999). *Haemonchus contortus*: effects of glutamate, ivermectin, and moxidectin on inulin uptake activity in unselected and ivermectin-selected adults. *Experimental Parasitology* **92**, 193-198.

Park, J. E., Lee, K. Y., Lee, S. J., Oh, W. S., Jeong, P. Y., Woo, T., Kim, C. B., Paik, Y. K. & Koo, H. S. (2008). The efficiency of RNA interference in *Bursaphelenchus xylophilus*. *Molecules & Cells* **26**, 81-86.

Park, W., Li, J., Song, R., Messing, J. & Chen, X. (2002). CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in *Arabidopsis thaliana*. *Current Biology* **12**, 1484-1495.

Parker, G. S., Maity, T. S. & Bass, B. L. (2008). dsRNA binding properties of RDE-4 and TRBP reflect their distinct roles in RNAi. *Journal of Molecular Biology* **384**, 967-979.

Parrish, S. & Fire, A. (2001). Distinct roles for RDE-1 and RDE-4 during RNA interference in *Caenorhabditis elegans*. *RNA* **7**, 1397-1402.

Patel, A., Fondrk, M. K., Kaftanoglu, O., Emore, C., Hunt, G., Frederick, K. & Amdam, G. V. (2007). The making of a queen: TOR pathway is a key player in diphenic caste development. *PLoS One* **2**, e509.

Patrick, K. L., Shi, H., Kolev, N. G., Ersfeld, K., Tschudi, C. & Ullu, E. (2009). Distinct and overlapping roles for two Dicer-like proteins in the RNA interference pathways of the ancient eukaryote *Trypanosoma brucei*. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 17933-17938.

Patthy, L. (1999). Genome evolution and the evolution of exon-shuffling - a review. *Gene* **238**, 103-114.

Pauli, F., Liu, Y., Kim, Y. A., Chen, P. J. & Kim, S. K. (2006). Chromosomal clustering and GATA transcriptional regulation of intestine-expressed genes in *C. elegans*. *Development* **133**, 287-295.

Peacock, C. S., Seeger, K., Harris, D. *et al*. (2007). Comparative genomic analysis of three *Leishmania* species that cause diverse human disease. *Nature Genetics* **39**, 839-847.

Peter, J. W. & Chandrawathani, P. (2005). *Haemonchus contortus*: parasite problem No. 1 from tropics - Polar Circle. Problems and prospects for control based on epidemiology. *Tropical Biomedicine* **22**, 131-137.

Pfarr, K., Heider, U. & Hoerauf, A. (2006). RNAi mediated silencing of actin expression in adult *Litomosoides sigmodontis* is specific, persistent and results in a phenotype. *International Journal of Parasitology* **36**, 661-669.

Prichard, R. (2001). Genetic variability following selection of *Haemonchus contortus* with anthelmintics. *Trends in Parasitology* **17**, 445-453.

Redman, E., Grillo, V., Saunders, G., Packard, E., Jackson, F., Berriman, M. & Gilleard, J. S. (2008a). Genetics of Mating and Sex Determination in the Parasitic Nematode *Haemonchus contortus*. *Genetics* **180**, 1877-1887.

Redman, E., Packard, E., Grillo, V., Smith, J., Jackson, F. & Gilleard, J. S. (2008b). Microsatellite analysis reveals marked genetic differentiation between *Haemonchus contortus* laboratory isolates and provides a rapid system of genetic fingerprinting. *International Journal of Parasitology* **38**, 111-122.

Redmond, D. L., Knox, D. P., Newlands, G. & Smith, W. D. (1997). Molecular cloning and characterisation of a developmentally regulated putative metallopeptidase present in a host protective extract of *Haemonchus contortus*. *Molecular & Biochemical Parasitology* **85**, 77-87.

Redmond, D. L., Geldhof, P. & Knox, D. P. (2004). Evaluation of *Caenorhabditis elegans* glycoproteins as protective immunogens against *Haemonchus contortus* challenge in sheep. *International Journal of Parasitology* **34**, 1347-1353.

Reinhart, B. J., Slack, F. J., Basson, M., Pasquinelli, A. E., Bettinger, J. C., Rougvie, A. E., Horvitz, H. R. & Ruvkun, G. (2000). The 21-nucleotide *let-7* RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature* **403**, 901-906.

Reszka, N., Rijsewijk, F. A., Zelnik, V., Moskwa, B. & Bienkowska-Szewczyk, K. (2007). *Haemonchus contortus*: Characterization of the baculovirus expressed form of aminopeptidase H11. *Experimental Parasitology* **117**, 208-213

Richmond, J. E. & Jorgensen, E. M. (1999). One GABA and two acetylcholine receptors function at the *C. elegans* neuromuscular junction. *Nature Neuroscience* **2**, 791-797.

Robertson, A. P., Bjorn, H. E. & Martin, R. J. (1999). Resistance to levamisole resolved at the single-channel level. *The FASEB Journal* **13**, 749-760.

Robinson, K. A. & Beverley, S. M. (2003). Improvements in transfection efficiency and tests of RNA interference (RNAi) approaches in the protozoan parasite *Leishmania*. *Molecular & Biochemical Parasitology* **128**, 217-228.

Roignant, J. Y., Carre, C., Mugat, B., Szymczak, D., Lepesant, J. A. & Antoniewski, C. (2003). Absence of transitive and systemic pathways allows cell-specific and isoform-specific RNAi in *Drosophila*. *RNA* **9**, 299-308.

Rossanigo, C. E. & Gruner, L. (1995). Moisture and temperature requirements in faeces for the development of free-living stages of gastrointestinal nematodes of sheep, cattle and deer. *Journal of Helminthology* **69**, 357-362.

Rosso, M. N., Dubrana, M. P., Cimbolini, N., Jaubert, S. & Abad, P. (2005). Application of RNA interference to root-knot nematode genes encoding esophageal gland proteins. *Molecular Plant Microbe Interactions* **18**, 615-620.

Rosso, M. N., Jones, J. T. & Abad, P. (2009). RNAi and Functional Genomics in Plant Parasitic Nematodes. *Annual Reviews Phytopathology* **47**, 207-232.

Rufener, L., Maser, P., Roditi, I. & Kaminsky, R. (2009). *Haemonchus contortus* acetylcholine receptors of the DEG-3 subfamily and their role in sensitivity to monepantel. *PLoS Pathogens* **5**, e1000380.

Ruiz-Ferrer, V. & Voinnet, O. (2009). Roles of plant small RNAs in biotic stress responses. *Annual Reviews Plant Biology* **60**, 485-510.

Ruiz, A., Molina, J. M., Gonzalez, J. F., Conde, M. M., Martin, S. & Hernandez, Y. I. (2004). Immunoprotection in goats against *Haemonchus contortus* after immunization with cysteine protease enriched protein fractions. *Veterinary Research* **35**, 565-572.

Saleh, M. C., van Rij, R. P., Hekele, A., Gillis, A., Foley, E., O'Farrell, P. H. & Andino, R. (2006). The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing. *Nature Cell Biology* **8**, 793-802.

Sambrook, J., Fritsch, E.F., Maniatis, T, (1989). *Molecular Cloning: A Laboratory Manual* Cold Springs Harbor: Cold Springs Harbor Laboratory Press.

Sangster, N. C., Whitlock, H. V., Russ, I. G., Gunawan, M., Griffin, D. L. & Kelly, J. D. (1979). *Trichostrongylus colubriformis* and *Ostertagia circumcincta* resistant to levamisole, morantel tartrate and thiabendazole: occurrence of field strains. *Research in Veterinary Science* **27**, 106-110.

Saunders, G. (2009). Comparative Genomics of Nematodes: *Caenorhabditis elegans* as a tool to study the *Haemonchus contortus* genome.: University of Glasgow.

Sharma, R. L., Bhat, T. K. & Dhar, D. N. (1988). Control of sheep lungworm in India. *Parasitology Today* **4**, 33-36.

Shi, H., Djikeng, A., Mark, T., Wirtz, E., Tschudi, C. & Ullu, E. (2000). Genetic interference in *Trypanosoma brucei* by heritable and inducible double-stranded RNA. *RNA* **6**, 1069-1076.

Shingles, J., Lilley, C. J., Atkinson, H. J. & Urwin, P. E. (2007). *Meloidogyne incognita*: molecular and biochemical characterisation of a cathepsin L cysteine proteinase and the effect on parasitism following RNAi. *Experimental Parasitology* **115**, 114-120.

Sijen, T., Fleenor, J., Simmer, F., Thijssen, K. L., Parrish, S., Timmons, L., Plasterk, R. H. & Fire, A. (2001). On the role of RNA amplification in dsRNA-triggered gene silencing. *Cell* **107**, 465-476.

Silvestre, A. & Cabaret, J. (2002). Mutation in position 167 of isotype 1 beta-tubulin gene of *Trichostrongylid* nematodes: role in benzimidazole resistance? *Molecular & Biochemical Parasitology* **120**, 297-300.

Silvestre, A. & Humbert, J. F. (2002). Diversity of benzimidazole-resistance alleles in populations of small ruminant parasites. *International Journal of Parasitology* **32**, 921-928.

Simmer, F., Tijsterman, M., Parrish, S., Koushika, S. P., Nonet, M. L., Fire, A., Ahringer, J. & Plasterk, R. H. (2002). Loss of the putative RNA-directed RNA polymerase RRF-3 makes *C. elegans* hypersensitive to RNAi. *Current Biology* **12**, 1317-1319.

Simmer, F., Moorman, C., van der Linden, A. M., Kuijk, E., van den Berghe, P. V., Kamath, R. S., Fraser, A. G., Ahringer, J. & Plasterk, R. H. (2003). Genome-wide RNAi of *C. elegans* using the hypersensitive *rrf-3* strain reveals novel gene functions. *PLoS Biology* **1**, E12.

Sindhu, A. S., Maier, T. R., Mitchum, M. G., Hussey, R. S., Davis, E. L. & Baum, T. J. (2008). Effective and specific *in planta* RNAi in cyst nematodes: expression interference of four parasitism genes reduces parasitic success. *Journal of Experimental Botany* **60**, 315-324.

Singha, U. K., Sharma, S. & Chaudhuri, M. (2009). Downregulation of mitochondrial porin inhibits cell growth and alters respiratory phenotype in *Trypanosoma brucei*. *Eukaryotic Cell* **8**, 1418-1428.

Slotkin, R. K. & Martienssen, R. (2007). Transposable elements and the epigenetic regulation of the genome. *Nature Reviews Genetics* **8**, 272-285.

Smardon, A., Spoerke, J. M., Stacey, S. C., Klein, M. E., Mackin, N. & Maine, E. M. (2000). EGO-1 is related to RNA-directed RNA polymerase and functions in germ-line development and RNA interference in *C. elegans*. *Current Biology* **10**, 169-178.

Smith, S. K. & Smith, W. D. (1996). Immunisation of sheep with an integral membrane glycoprotein complex of *Haemonchus contortus* and with its major polypeptide components. *Research in Veterinary Science* **60**, 1-6.

Smith, S. K., Pettit, D., Newlands, G. F., Redmond, D. L., Skuce, P. J., Knox, D. P. & Smith, W. D. (1999). Further immunization and biochemical studies with a protective antigen complex from the microvillar membrane of the intestine of *Haemonchus contortus*. *Parasite Immunology* **21**, 187-199.

Smith, T. S., Graham, M., Munn, E. A. *et al*. (1997). Cloning and characterization of a microsomal aminopeptidase from the intestine of the nematode *Haemonchus contortus*. *Biochimica Biophysica Acta* **1338**, 295-306.

Smith, W. D. & Angus, K. W. (1980). *Haemonchus contortus*: attempts to immunise lambs with irradiated larvae. *Research in Veterinary Science* **29**, 45-50.

Smith, W. D. & Smith, S. K. (1993). Evaluation of aspects of the protection afforded to sheep immunised with a gut membrane protein of *Haemonchus contortus*. *Research in Veterinary Science* **55**, 1-9.

Smith, W. D., Smith, S. K. & Murray, J. M. (1994). Protection studies with integral membrane fractions of *Haemonchus contortus*. *Parasite Immunology* **16**, 231-241.

Sommer, R. J. (2000). Evolution of nematode development. *Current Opinion in Genetics & Development* **10**, 443-448.

Song, J. J. & Joshua-Tor, L. (2006). Argonaute and RNA - getting into the groove. *Current Opinion in Structural Biology* **16**, 5-11.

Sontheimer, E. J. (2005). Assembly and function of RNA silencing complexes. *Nature Reviews Molecular & Cellular Biology* **6**, 127-138.

Srinivasan, J. & Sternberg, P. W. (2008). *Pristionchus pacificus*: an appropriate fondness for beetles. *Nature Genetics* **40**, 1146-1147.

Steeves, R. M., Todd, T. C., Essig, J. S. & Trick, H. N. (2006). Transgenic soybeans expressing siRNAs specific to a major sperm protein gene suppress *Heterodera glycines* reproduction. *Functional Plant Biology* **33**, 991-999.

Stiernagle, T. (2006). Maintenance of *C. elegans*. In *WormBook*: The *C. elegans* Research Community.

Stringfellow, F. (1986). Cultivation of *Haemonchus contortus* (Nematoda: Trichostrongylidae) from infective larvae to the adult male and the egg-laying female. *Journal of Parasitology* **72**, 339-345.

Sukno, S. A., McCuiston, J., Wong, M. Y., Wang, X., Thon, M. R., Hussey, R., Baum, T. & Davis, E. (2007). Quantitative Detection of Double-Stranded RNA-Mediated Gene Silencing of Parasitism Genes in *Heterodera glycines*. *Journal of Nematology* **39**, 145-152.

Tabara, H., Grishok, A. & Mello, C. C. (1998). RNAi in *C. elegans*: soaking in the genome sequence. *Science* **282**, 430-431.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology & Evolution* **24**, 1596-1599.

Tavernarakis, N., Wang, S. L., Dorovkov, M., Ryazanov, A. & Driscoll, M. (2000). Heritable and inducible genetic interference by double-stranded RNA encoded by transgenes. *Nature Genetics* **24**, 180-183.

Thijs, G., Marchal, K., Lescot, M., Rombauts, S., De Moor, B., Rouze, P. & Moreau, Y. (2002). A Gibbs sampling method to detect overrepresented motifs in the upstream regions of coexpressed genes. *Journal of Computational Biology* **9**, 447-464.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**, 4876-4882.

Timmons, L. & Fire, A. (1998). Specific interference by ingested dsRNA. *Nature* **395**, 854.

Tursun, B., Cochella, L., Carrera, I. & Hobert, O. (2009). A toolkit and robust pipeline for the generation of fosmid-based reporter genes in *C. elegans*. *PLoS One* **4**, e4625.

Ullu, E., Tschudi, C. & Chakraborty, T. (2004). RNA interference in protozoan parasites. *Cellular Microbiology* **6**, 509-519.

Urquhart, B. L., Tirona, R. G. & Kim, R. B. (2007). Nuclear receptors and the regulation of drug-metabolizing enzymes and drug transporters: implications for interindividual variability in response to drugs. *Journal of Clinical Pharmacology* **47**, 566-578.

Urquhart, G. M., Armour, J., Duncan, J. L., Dunn, A. M. & Jennings F. W. (1987). Veterinary Parasitology. *Blackwell Science, UK* **2nd Edition**.

Urwin, P. E., Lilley, C. J. & Atkinson, H. J. (2002). Ingestion of double-stranded RNA by preparasitic juvenile cyst nematodes leads to RNA interference. *Molecular Plant Microbe Interactions* **15**, 747-752.

Valentine, T. A., Randall, E., Wypijewski, K., Chapman, S., Jones, J. & Oparka, K. J. (2007). Delivery of macromolecules to plant parasitic nematodes using a tobacco rattle virus vector. *Plant Biotechnology Journal* **5**, 827-834.

van Helden, J. (2003). Regulatory sequence analysis tools. *Nucleic Acids Research* **31**, 3593-3596.

van Roessel, P. & Brand, A. H. (2004). Spreading silence with SID. *Genome Biology* **5**, 208.

van Wyk, J. A. & Malan, F. S. (1988). Resistance of field strains of *Haemonchus contortus* to ivermectin, closantel, rafoxanide and the benzimidazoles in South Africa. *Veterinary Record* **123**, 226-228.

Vanfleteren, J. R., Van de Peer, Y., Blaxter, M. L., Tweedie, S. A., Trotman, C., Lu, L., Van Hauwaert, M. L. & Moens, L. (1994). Molecular genealogy of some nematode taxa as based on cytochrome c and globin amino acid sequences. *Molecular Phylogenetics & Evolution* **3**, 92-101.

Vassilatis, D. K., Arena, J. P., Plasterk, R. H., Wilkinson, H. A., Schaeffer, J. M., Cully, D. F. & Van der Ploeg, L. H. (1997). Genetic and biochemical evidence for a novel avermectin-sensitive chloride channel in *Caenorhabditis elegans*. Isolation and characterization. *Journal of Biological Chemistry* **272**, 33167-33174.

Vastenhouw, N. L., Brunschwig, K., Okihara, K. L., Muller, F., Tijsterman, M. & Plasterk, R. H. (2006). Gene expression: long-term gene silencing by RNAi. *Nature* **442**, 882.

Vermeulen, A., Behlen, L., Reynolds, A., Wolfson, A., Marshall, W. S., Karpilow, J. & Khvorova, A. (2005). The contributions of dsRNA structure to Dicer specificity and efficiency. *RNA* **11**, 674-682.

Visser, A., Geldhof, P., de Maere, V., Knox, D. P., Vercruysse, J. & Claerebout, E. (2006). Efficacy and specificity of RNA interference in larval life-stages of *Ostertagia ostertagi*. *Parasitology* **133**, 777-783.

Voinnet, O. & Baulcombe, D. C. (1997). Systemic signalling in gene silencing. *Nature* **389**, 553.

von Samson-Himmelstjerna, G. (2006). Molecular diagnosis of anthelmintic resistance. *Veterinary Parasitology* **136**, 99-107.

von Samson-Himmelstjerna, G., Walsh, T. K., Donnan, A. A., Carriere, S., Jackson, F., Skuce, P. J., Rohn, K. & Wolstenholme, A. J. (2009). Molecular detection of benzimidazole resistance in *Haemonchus contortus* using real-time PCR and pyrosequencing. *Parasitology* **136**, 349-358.

Wang, H. W., Noland, C., Siridechadilok, B., Taylor, D. W., Ma, E., Felderer, K., Doudna, J. A. & Nogales, E. (2009). Structural insights into RNA processing by the human RISC-loading complex. *Nature Structural & Molecular Biology* **16**, 1148-1153.

Wilkins, C., Dishongh, R., Moore, S. C., Whitt, M. A., Chow, M. & Machaca, K. (2005). RNA interference is an antiviral defence mechanism in *Caenorhabditis elegans*. *Nature* **436**, 1044-1047.

Windon, R. G. (1996). Genetic control of resistance to helminths in sheep. *Veterinary Immunology & Immunopathology* **54**, 245-254.

Winston, W. M., Molodowitch, C. & Hunter, C. P. (2002). Systemic RNAi in *C. elegans* requires the putative transmembrane protein SID-1. *Science* **295**, 2456-2459.

Winston, W. M., Sutherlin, M., Wright, A. J., Feinberg, E. H. & Hunter, C. P. (2007). *Caenorhabditis elegans* SID-2 is required for environmental RNA interference. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 10565-10570.

Winter, A. D. & Page, A. P. (2000). Prolyl 4-hydroxylase is an essential procollagen-modifying enzyme required for exoskeleton formation and the maintenance of body shape in the nematode *Caenorhabditis elegans*. *Molecular & Cellular Biology* **20**, 4084-4093.

Winter, A. D., Myllyharju, J. & Page, A. P. (2003). A hypodermally expressed prolyl 4-hydroxylase from the filarial nematode *Brugia malayi* is soluble and active in the absence of protein disulfide isomerase. *Journal of Biological Chemistry* **278**, 2554-2562.

Winterrowd, C. A., Pomroy, W. E., Sangster, N. C., Johnson, S. S. & Geary, T. G. (2003). Benzimidazole-resistant beta-tubulin alleles in a population of parasitic nematodes (*Cooperia oncophora*) of cattle. *Veterinary Parasitology* **117**, 161-172.

Wolstenholme, A. J., Fairweather, I., Prichard, R., von Samson-Himmelstjerna, G. & Sangster, N. C. (2004). Drug resistance in veterinary helminths. *Trends in Parasitology* **20**, 469-476.

Xie, Z., Johansen, L. K., Gustafson, A. M., Kasschau, K. D., Lellis, A. D., Zilberman, D., Jacobsen, S. E. & Carrington, J. C. (2004). Genetic and functional diversification of small RNA pathways in plants. *PLoS Biology* **2**, E104.

Xie, Z., Allen, E., Wilken, A. & Carrington, J. C. (2005). DICER-LIKE 4 functions in trans-acting small interfering RNA biogenesis and vegetative phase change in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 12984-12989.

Yadav, B. C., Veluthambi, K. & Subramaniam, K. (2006). Host-generated double stranded RNA induces RNAi in plant-parasitic nematodes and protects the host from infection. *Molecular & Biochemical Parasitology* **148**, 219-222.

Yan, K. S., Yan, S., Farooq, A., Han, A., Zeng, L. & Zhou, M. M. (2003). Structure and conserved RNA binding of the PAZ domain. *Nature* **426**, 468-474.

Yigit, E., Batista, P. J., Bei, Y. *et al*. (2006). Analysis of the *C. elegans* Argonaute family reveals that distinct Argonautes act sequentially during RNAi. *Cell* **127**, 747-757.

Yin, Y., Martin, J., Abubucker, S., Scott, A. L., McCarter, J. P., Wilson, R. K., Jasmer, D. P. & Mitreva, M. (2008). Intestinal Transcriptomes of Nematodes: Comparison of the Parasites *Ascaris suum* and *Haemonchus contortus* with the Free-living *Caenorhabditis elegans*. *PLoS Neglected Tropical Diseases* **2**, e269.

Zhao, Z., Fang, L., Chen, N., Johnsen, R. C., Stein, L. & Baillie, D. L. (2005). Distinct regulatory elements mediate similar expression patterns in the excretory cell of *Caenorhabditis elegans*. *Journal of Biological Chemistry* **280**, 38787-38794.