IDENTIFICATION AND CHARACTERIZATION OF PROTECTIVE ANTIGENS FOR CONTROL OF *Ixodes scapularis* INFESTATIONS

by

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…This work is dedicated to the memories of my sister and my father

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ABBREVIATIONS

AMV, avian myeloblastosis virus

BLAST, basic local algorithm search tool

ELI, expression library immunization

EST, expression sequence tags

FIA, Freud's incomplete adjuvant

IDE8, *Ixodes scapularis* embryonic cell line

LB, Luria Bertani

ME, minimum evolution

MP, minimum parsimony

NCBI, National Center for Biotechnology Information

NJ, neighbor joining

OD, optical density

ORF, open reading frame

PBST, phosphate buffer saline-tween

RT-PCR, reverse transcriptase-polimerase chain reaction

SDS-PAGE, sodium duodecyl sulphate-polyacrylamide gel electrophoresis

TBR, tree bisection and reconection

TBS, tris-buffer saline

UTR, untranslated region

Chapter 1

INTRODUCTION

Economic Importance of Ticks

Ticks have been serious pests of humans and domestic animals since ancient times, and they surpass all other arthropods in the number and variety of pathogens that they transmit to domestic animals. Ticks rank second only to mosquitoes as vectors of human diseases including fungi, viruses, rickettsia, bacteria, protozoa and filarial nematodes (Bowman et al. 1996).

Ticks affect the production of over 1000 million cattle and sheep worldwide. The effect of tick feeding on cattle includes growth reduction and milk production, paralysis, transmission of tick-borne pathogens and secondary infections with other parasites (Estrada-Peña and Jongejan, 2001). Eighty percent of the 1288 million cattle worldwide were estimated to be at risk of tick-borne diseases, with a global cost estimated between US $13.9 and 18.9 billions (Castro, 1997).

Classification of ticks

Ticks are obligated hematophagous ectoparasites of terrestrial vertebrates. They belong to the class Arachnida and are closely related to scorpions and spiders (USDA, 1976). Ticks are classified in the subclass Acari, order Parasitiformes, suborder Ixodida (Sonenshine, 1991). Three families are included into the suborder Ixodida. The family Ixodidae or the hard ticks has a scutum and it is the largest and economically the most important, containing 13 genera and approximately 650 species (Sonenshine, 1993). The family Argasidae includes the soft ticks which lack a scutum and have a leathery cuticle (USDA, 1976; Sonenshine, 1991). The

Argasidae comprises 5 genera and approximately 170 species (Sonenshine, 1991). The third familiy, Nutalliellidae, consists of only one species (Sonenshine, 1991). In total, approximately 820 species of ticks have been identified which are geographically widespread (USDA, 1976; Wang and Nuttall, 1999). Ticks have adapted to climatic extremes and a diverse range of hosts, demonstrating that they are a biologically well established group (Wang and Nuttall, 1999).

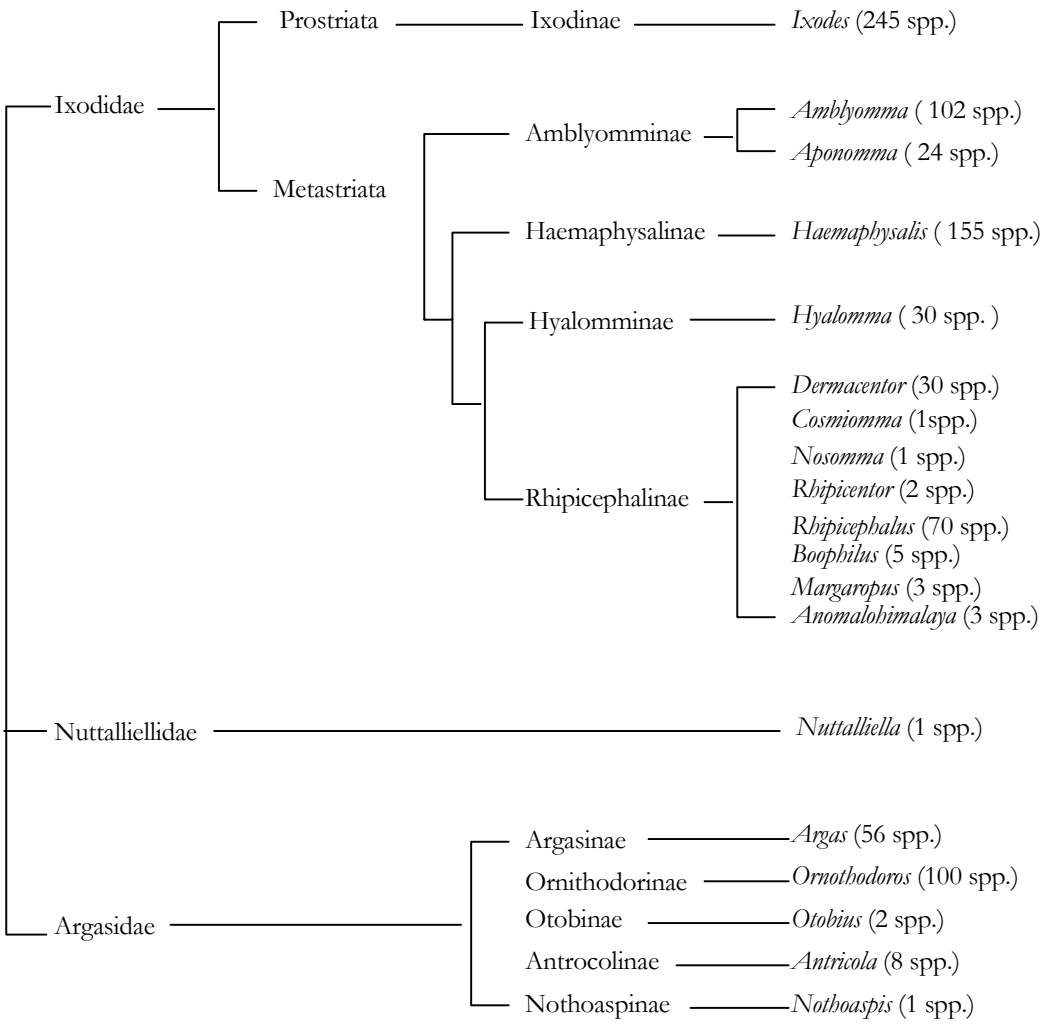

Figure 1. Classification of ticks (Adapted from Sonenshine, 1991).

Ecology and life cycle of ticks

Ticks undergo a life cycle that includes four stages: egg, larvae, nymph, and adult (male and female). Adults and nymphs have 4 pairs of legs while the larvae have 3 pairs of legs. Transition form one stage to the next occurs by molting (USDA, 1976). In most species, each active stage seeks a host, takes a bloodmeal, and drops off to develop in the natural environment, (Sonenshine, 1991). In the Argasidae, development is gradual involving multiple nymphal stages before becoming adults (multi-host life cycle), while in Ixodidae, development is accelerated and involves a single nymphal stage followed directly by adult stage (Sonenshine, 1991).

The number of tick generations may vary from 3 or 4 per year in the one host species such as *Boophilus microplus*, every 2 or 3 years in the Agasidae, or one every 2 or 3 years in some three host species, such as *Dermacentor andersoni* and *Ixodes scapularis* (USDA, 1976). In one host ticks such as *Boophilus* spp. all stages remain on the host after the larval attachment. Larvae and nymphs feed and remain *in situ* following molting to the adult stage, the males and females remain to feed and mate, and only the fed, mated females drop to oviposit in the natural environment (Sonenshine, 1991). In the two host life cycle, fed larvae remain on the host, molt *in situ* and the unfed nymphs reattach. Following their engorgement the nymphs detach, they molt off the host to the adult stage, after which they seek a new host (Sonenshine, 1991). In three host ticks, the fed larvae drop from their hosts and undergo a molt after which the unfed nymphs seek and feed on a new host again. Engorged nymphs drop from their hosts and molt, after which the adults emerge to seek a host, feed, and mate. The engorged females drop off of the host at the completion of feeding and oviposit, thus completing the life cycle

(Sonenshine, 1991). The three host life cycle is the most common developmental pattern for ticks (Sonenshine, 1991).

Ticks as vectors of diseases

Ticks, perhaps more than any other group of arthropods, are well suited for transmission of disease agents and they serve as vectors of protozoa, rickettsiae, viruses and bacteria to a variety of vertebrates throughout the world (reviewed by Kocan, 1995). In humans, ticks can cause tick paralysis and toxicosis, irritation and allergic reactions (Estrada Peña and Jongejan, 1999). In North America, ticks are considered to be the most important arthropod vector of pathogens (Parola and Raoult, 2001). Many important tick-borne diseases such as Rocky Mountain spotted fever, crimean hemorrhagic fever and tick borne encephalitis have been known for some time, while recently emergent diseases include lyme disease, human monocytic ehrlychiosis and human granulocytic ehrlichiosis (Bowman *et al.* 1996).

Ixodes spp. serve as vectors of human diseases caused by *Borrelia burgdorferi* (lyme disease), *Anaplasma phagocytophilum* (human granulocytic ehrlichiosis), *Coxiella burnetti* (Q fever), *Francisella tularensis* (tularemia), *Rickettsia helvetica, R. japonica* and *R. australis, Babesia divergens, B. microti,* tick-borne encephalitis (TBE), and omsk hemorrhagic fever viruses (Table 1; Estrada-Peña and Jongejan, 1999; Parola and Raoult, 2001). *I. scapularis* is the main vector of *B. burgdorferi*, the causative agent of human lyme disease in the eastern USA and Canada. The white-footed mouse (*Peromuscus leucopus*) serves as the primary reservoir of *B. burdorferi* and the major host for immature *I. scapularis* ticks. (McQuiston *et al.* 1999; Estrada-Peña and Jongejan, 1999). Before and during the larval tick feeding, infected nymphs transmit *B. burgdorferi* to reservoir hosts. The newly hatched spirochete-free larvae acquire the bacteria from the reservoir host and retain the infection through molting process. In spring, nymphs derived

from infected larvae transmit infection to susceptible animals, which will serve as hosts for larvae later in the summer (Fish, 1993). Lyme disease is currently the most important tick-borne disease of humans worldwide and the reported incidence of the disease in the USA has increased over recent years (Bowman et al. 1996). Most cases of Lyme disease have been reported along the northeastern coast, although the distribution of the vector appears to be spreading (Estrada Peña, 2001).

I. scapularis also transmits *Babesia microti*, the protozoan parasite responsible for human babesiosis in the Neartic. The major reservoir host for *B. microti* is the white-footed mouse, *Peromyscus leucopus*, although mead wolves (*Microtus pennsylvanicus*) may also act as reservoirs (Reviewed by Estrada-Peña and Jongejan, 1999). Nymphs are the primary vectors, although adults may transmit the infection. Human babesiosis caused by *B. microti* has remained a minor public health concern in the USA. Until 1993, about 200 cases had been recognized (Sonenshine, 1993).

Human granulocytic ehrlichiosis, caused by *Ehrlichia phagocytophila* is a disease distributed in the midwestern and northeastern USA and also in California. Larval *I. scapularis* ticks acquire the infection by feeding on infected mice, and they also efficiently transmit *Ehrlichiae* after molting to nymphs. Granulocytic ehrlichiosis is a zoonotic disease and dogs may contribute to the enzootic cycle and human infection (Estrada-Peña and Jongejan, 1999).

Table 1. *Ixodes* species, pathogens transmitted and distribution (adapted from Estrada-Peña and Jongejan, 1999)

Species	Pathogen	Distribution
I. holocyclus	*Rickettsia australis*	Australia
I. ovatus	*Borrelia japonica*	Japan
I. pacificus	*B. burgdorferi*	USA, Canada
I. persulcatus	Omsk hemorrhagic fever virus	Japan, former USSR
	B. afzelii	
	B. garinii	
	B. burgdorferi	
	TBE virus	
I. ricinus	*B. afzelii*	Europe, Western former USSR, Northern Africa
	B. garinii	
	B. lusitaniae	
	B. valasiana	
	B. burgdorferi	
	Ehrlichia phagocytophilum	
	TBE virus	
	Babesia divergens	
	Rickettsia Helvetica	
I. scapularis	*B. burgdorferi*	USA (Atlantic coast), Southeastern Canada
	Babesia microti	
	Ehrlichia phagocitophilum	

Distribution of *Ixodes* spp.

I. scapularis is found along the eastern and central United States (Denis *et al.* 1998) and the hosts range extends from Canada to Mexico (USDA, 1976). The upper boundary is located in Maine westward to Minnesota and Iowa (Wilson *et al.* 1988). The *I. scapularis* distribution correlates with the distribution of its principal host, the white-tailed deer (*Odocoileus virginianus*) (Wilson *et al.* 1988). Only deer or other large animals appear capable of supporting high populations of these ticks (Duffy *et al.* 1994). *I. scapularis* may be locally common and abundant

in periods of good climate conditions, while almost absent or with greatly reduced densities in periods of adverse climatic conditions (Estrada-Peña and Jongejan, 1999).

The western black legged tick, *I. pacificus*, is distributed primarily throughout the Pacific cost of the United States, where the western fence lizards (*Sceloporus occidentalis*) and Columbian black-tailed deer (*Odocoileus hemionus comlumbianus*) serve as the major hosts (Dennis *et al.* 1998). In Canada *I. pacificus* is endemic in localized areas of southern British Columbia and on the Gulf Islands and Vancouver Island (CCDR, 1998). *I. dentatus, I. spinipalpis*, and *I. noeotomae* are present in the United States; however, these ticks rarely feed on humans, and therefore this tick is of lesser importance compared with *I. scapularis* or *I. pacificus.*

I. ricinus is distributed in Europe from Ireland, Britain, and France where populations are associated with sheep pastures, to southern Scandinavia eastwards across Europe to northern Iran and southward to the Mediterranean littoral (Sonenshine, 1993; Vasallo, 2000). This species is also found in small numbers in forested areas in North Africa and in the Levant countries of the eastern Mediterranean (Sonenshine, 1993). The Taiga tick, *I. persulcatus,* is found in a large portion of southern Siberia, Far East, and middle Asia (Durden and Keirans, 1996).

I. scapularis **life cycle**

I. scapularis is a three-host tick and each stage feeds on a different host. Adult female ticks feed for five to seven days, while the male ticks feed only intermittently. Adult ticks feed on large animals, preferentially white-tailed deer (*Odocoileus virginianus*) (Wilson *et al.* 1990), but they also feed on cattle, horses, dogs, sheep, hogs, and human. Nymphs feed primarily on birds, small mammals, and occasionally lizards (USDA, 1976). Replete females lay between 1000-3000 eggs (Wilson *et al.* 1990), and the eggs are deposited in the spring and hatch in mid-summer (Sonenshine, 1993).

The *I. scapularis* larvae fed during the warm summer period, prior to late September, molt to nymphs that survive the winter as unfed nymphs (Wilson et al. 1990; Sonenshine, 1993). Larvae fed later in the fall are believed to overwinter as engorged larvae and molting to the nymphal stage occurs the following spring or summer. In contrast, larvae fed in spring molt immediately, and the subsequently-molted nymphs seek hosts throughout the late spring and early summer months, with the peak populations occurring in June. Nymphs that feed and incubate during the long photoperiod of the northern summer molt to adults and commence questing soon after their emergence. These patterns of feeding activity and development result in a 2-year life cycle (Sonenshine, 1993).

As reviewed by Estrada-Peña and Jongejan (1999), *I. scapularis* ticks require moist microclimates for survival, including habitats with leaf-litter and one in which a high canopy of mixed deciduous forests provides protection from extreme temperatures. *I. scapularis* accounts for 76.2% of the ticks collected on humans in southern New York, but comprise only 3.9% of the ticks collected in Georgia and South Carolina.

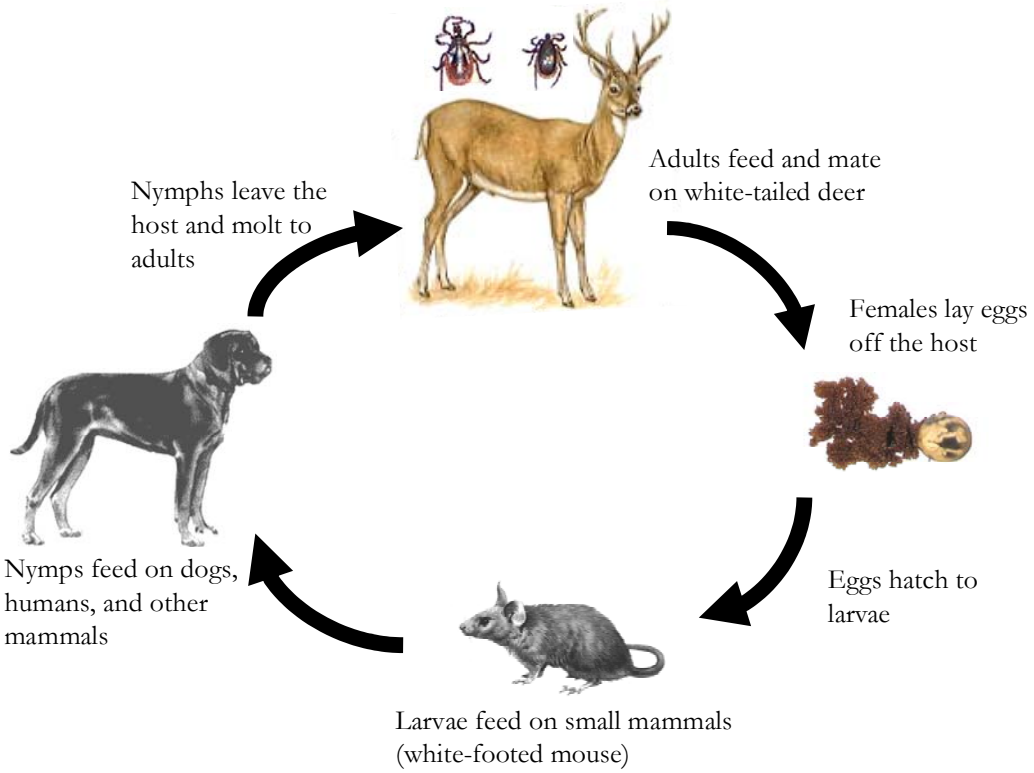

Figure 1. Life cycle of *I. scapularis*.

Host immune response to tick infestations

Compared with other ectoparasites, ticks have a unique and long-term association with the host (Bowman *et al.* 1996). After attachment to a host, ixodid ticks ingest a copious meal of blood during a prolonged attachment. Females imbibe more than 100 times their unfed body weight (Bowman *et al.* 1997) and may feed for several days or even weeks during which their mouth parts become embedded into the dermis of their vertebrate hosts (Valenzuela *et al.* 2000). Ixodid ticks feed for extended periods of two weeks or more. As the tick feeds, it alternates between imbibing blood components infiltrating into the feeding lesion and

returning excess of fluid and ions back to the host via the saliva, thus concentrating the blood meal nutrients in the gut for future egg production (Bowman *et al.* 1996).

Ticks must overcome their host's innate immune mechanisms in order to complete engorgement (Lawrie *et al.*1999). Protracted attachment and feeding is possible largely because of properties of the tick's saliva, which contains bioactive substances that counter host immune, inflammatory and hemostatic responses at the feeding site (Bowman *et al.* 1997). Pathogens are most often transmitted from infected ticks to the host via the salivary secretions (USDA, 1976). Salivary secretions also play an important role in the transmission of these pathogens by serving as a medium of transport for pathogens and may contribute to their establishment.

Tick attachment and feeding often causes an immune response that result in tick rejection or anti-tick immunity (Valenzuela, 2002). The primary effect of immune rejection is infiltration of the wound site with watery fluids instead of hemoglobin-rich blood which is the target of tick feeding and digestion. Therefore, ticks feeding performance, attachment success and/or engorged weight are greatly reduced when ticks are allowed to feed on a previously-infested host (Sonenshine, 1993). In addition to being unable to feed on blood, a secondary effect of the host immune response is the direct damage to tick tissues and physiological processes (Sonenshine, 1993).

Although hosts are usually unable to develop resistance during the initial tick challenge, they mount an effective immune response when challenged by subsequent feedings resulting in an acquired immunity (Sonenshine, 1993). Tick exposure typically leads to immune responses by the hosts that are directed against antigenic moieties at the attachment site and in tick tissues (Willadsen, 2001).

I. scapularis is able to feed repeatedly on its natural host, the white footed mouse, *Peromyscus leucopus,* primarily because of anticomplement activity in the saliva (Valenzuela *et al.* 2000). Rabbits or guinea pigs infested with *I. scapularis* acquire resistance to tick bites, which is partially mediated by antibodies (Das *et al.* 2001). Repeated exposure of rabbits, cattle, dogs, and guinea pigs to ticks has been shown to interfere with tick feeding, molting and fecundity (Das *et al.* 2001).

Infestations with *I. scapularis* nymphs were shown to modulate the host T-lymphocyte cytokine production (Schoeler *et al.* 1999). Tick-induced suppression of cytokines not only enhances the ability of the tick to feed but may also contribute to pathogen transmission (Schoeler *et al.* 1999). Host reaction to tick antigens, including humoral or cellular responses, will then affect subsequent tick infestations (Das *et al.* 2001).

Tick resistance has been demonstrated by observing a reduction in the number of feeding ticks, weights, feeding rates, a reduction in the ability of fed larvae or nymphs to molt to the next stage, and/or reduced progeny (Allen, 1989). Increased feeding periods, decreased egg production, inhibited molting and egg and tick mortality have been all well documented after ticks feed on resistant hosts (Schoeler, 1999). Acquired resistance is most evident in recent or unstable host-parasite interactions, abnormal hosts on which the tick do not usually feed or in non-specific host-parasite associations (Sonenshine, 1993). For example, infestation of raccoons with *I. scapularis* resulted in a significant decrease in the proportion of engorged larvae after repeated applications of both nymphs and larvae (Craig *et al.* 1996). In contrast, ticks in long established host parasite relationships have evolved mechanisms to suppress or evade the immune response (Sonenshine, 1993).

Control of tick infestations

Currently, the most effective method of tick control is by using chemical acaricides (USDA, 1976) which are commonly applied to the host by dipping animals in tanks or vats containing a solution of acaricide. Dipping is more effective than spraying for achieving satisfactory coverage of cattle with the acaricide. Other means of applying acaricides that are less commonly used include spot or pour-on application, slow release acaricide boluses, and acaricide-impregnated ear tags (Norval *et al.*1992). Systemic treatments like ivermectin have also been used for tick control (Wilson, 1993), but are often impractical because of the expense of the chemicals. Since the usual method of tick dispersion is by movement of the host, chemical control can be most effectively used when animals are in quarantine or when animal movements are regulated (USDA, 1976). Besides the expense, acaricides require stringent application regimens and management strategies, and may contribute to environmental pollution. In addition, ticks may develop resistance to acaricides, and cattle, especially calves, may be susceptible to toxic effects of acaricides (Kocan, 1995). In addition, acaricide applications may not reduce tick populations in a given area. Ticks are often not attracted to treated cattle; therefore tick populations may remain essentially unaffected (Kocan, 1995).

Control of tick infestations is difficult and often impractical for multi-host ticks such as *Ixodes* spp. Currently, tick control is effected most often by integrated pest management in which different control methods are adapted to a given geographic location or against one tick species with due consideration to their environmental effects (de la Fuente *et al.* 1998).

Tick vaccines

In the past years efforts had focused on development of vaccines against tick-borne pathogens. However, the tick may vector two or more diseases. Therefore, vaccination may

serve as an alternative for prevention of tick-borne diseases that would be directed against a tick antigen with the aim of interrupting pathogen transmission (Kay and Kemp, 1994). Two strategies have been adopted for anti-tick vaccine development: (1) mimicking acquired resistance and (2) targeting internal tick organs (Wang and Nuttall, 1999). Development of vaccines against ticks is feasible because ticks feed slowly, remaining in contact with the host's immune system for days or weeks. This long-term association provides the opportunity for the tick gut epithelium to be exposed to host antibodies ingested with the blood meal. Because tick digestion is largely intracellular the gut environment has a neutral pH and is relatively free of proteases (Willadsen, 2001).

Development of vaccines against the one-host tick *Boophilus* spp. has demonstrated the feasibility of using protective antigens for immunization against tick infestations (Willadsen and Kemp, 1998; de la Fuente *et al.* 1999; de la Fuente 2000; de Vos *et al.* 2001). Control of ticks by vaccination avoids environmental contamination and selection of drug resistant ticks that results from repeated acaricide applications (de la Fuente *et al.* 1998).

Anti-tick vaccines would also allow for inclusion of multiple antigens in order to target a broad range of tick species and for incorporation of pathogen-blocking antigens. Willadsen and Kemp (1998) isolated and characterized genes expressed in tick cells that are essential for tick survival. These antigens encode protective antigens for vaccination against the cattle tick *B. microplus*. This strategy relies on identification of tick antigens that are not naturally exposed to the host and are capable of eliciting a protective response upon immunization. A protective antigen, Bm86, was identified from the gut of semi-engorged adult female *B. microplus* ticks and produced in large quantities by recombinant DNA technology for use in a vaccine (de la Fuente *et al.* 1999).

In addition to Bm86, other gut antigens have been isolated from *B. microplus* and evaluated in vaccination experiments. The Bm91 gut and salivary antigen elicits partial protection against *B. microplus* with an added effect when used in combination with Bm86 (Riding *et al.* 1994). The Bm95 gut antigen was isolated from an Argentinean strain of *B. microplus* and was found to protect against a wider range of *B. microplus* strains when compared to Bm86 (Garcia-Garcia *et al.* 2000). Bm86 vaccine induces production of antibodies that bind to the intestinal cells causing them to lyse, and thereby interfering with the blood-feeding activity of the tick (Dalton and Mulcahy, 2001). However, immunization with Bm86 failed to protect against *Amblyomma* spp. (de Vos *et al.* 2001), and the vaccine did not have an immediate effect on reducing the numbers of ticks (Dalton and Mulcahy, 2001). In addition, efforts to develop a vaccine against 3-host ticks, particularly *Rhipicephalus appendiculatus*, have been unsuccessful (Castro, 1997). Therefore the screening and identification of novel protective antigens is necessary for the identification of vaccine candidates against tick infestations of these species of medical and veterinary importance.

Expression library immunization (ELI)

Expression library immunization (ELI) is an alternative approach for identification of protective antigens (Moore *et al.* 2002). Unlike methods used previously, ELI does not require prior knowledge of possible antigenic targets and has the potential to screen the entire genome. ELI involves the construction of genomic libraries, vaccination of hosts with naked DNA from the libraries, and screening through a disease model to demonstrate whether clones from the library confer protection against ticks or disease agents (Moore *et al.* 2002). Studies in mice suggest that DNA immunization is one of the most simple and yet versatile methods of inducing both humoral and cellular host immune responses (van Drunen Littel-van den Hurk et *al.* 2001).

To obtain a cDNA vaccine, RNA is extracted from a given parasite. Then, by reverse transcription, the cDNA coding for a potentially protective antigen is amplified, and subsequently cloned into a plasmid vector under the control of a strong eukaryotic promoter. The vector is then administered to a host organism where it undergoes expression and the expressed protein elicits an immune response that results in resistance to any particular disease **(**Kofta and Wedrychowiez 2001). DNA vaccines are strongly immunogenic, inducing immune mechanisms different from the active response, which would be a crucial factor in their efficiency. In addition, DNA itself can act as an adjuvant (Kofta and Wedrychowiez, 2001).

ELI was first used to identify protective antigens against *Mycoplasma pulmonis* after immunization of mice with cloned genomic DNA in pools of 3000-27000 plasmids (Barry *et al.* 1995). Subsequently, ELI has been used for antigen identification of other parasites, primarily protozoa such as *Tripanosoma cruzi.* An expression genomic library was constructed using pcDNA3 plasmid for immunization of mice and expression of *T. cruzi* antigens was detected 7 days after intramuscular immunization of mice (Alberti *et al.* 1998).

A genomic *Plasmodium chabaudi* expression library was constructed comprising ten separate pools containing 3000 plasmids. In three vaccine trials using pools composed of 616 to 30,000 clones, 63% protection was found in mice challenged with *P. chabaudi adami* (Smooker *et al.* 2000).

Melby *et al.* (2000) immunized BALB/c mice with plasmid DNA isolated and pooled from 15 cDNA sub-libraries, following systemic challenge with *L. donova*ni. Mice immunized with 6 of these 15 sub-libraries shown a significantly reduction of hepatic parasite burden. Several groups of cDNAs that afforded protection were identified, including a set of nine novel cDNAs and a group of cDNAs that encoded *L. donovani* histone proteins.

This methodology was applied then to helminths for development of a DNA vaccine against rats experimentally infected with the liver fluke *Fasciola hepatica*. A reduction of 74% in the fluke burden per animal was found in vaccinated animals and flukes the livers appeared histologically normal in contrast to livers from unvaccinated rats that were markedly damaged (Kofta *et al.* 2000).

Vaccination with DNA and cDNA molecules has been used to induce a protective immune response against *B. microplus* (De Rose *et al.* 1999). However, identification of individual protective clones was not reported. The difficulty in identifying protective antigens will most likely increase with the complexity of the organism genome.

Analysis of expressed sequence tags (EST)

The analysis of expressed sequence tags (ESTs) has proven to be a valuable approach in gene discovery and has generated a large pool of coding sequences (Boguski *et al.* 1993). This approach has resulted in valuable information for the study of biological systems and for the identification of potential vaccine candidates (Lizotte-Waniewski *et al.* 2000; Kressler *et al.* 2002). Hill and Gutierrez (2000) reported the EST approach to study the genome of *Amblyomma americanum*. Analysis of ESTs has been used to characterize gene expression in salivary glands of *I. scapularis* and *I. ricinus* (Valenzuela *et al.* 2000; Valenzuela *et al.* 2002). Other tick species such as *A. variegatum* (Nene *et al.* 2002), and *Dermacentor variabilis* (Mulenga *et al.* 2003) have been studied by ESTs. The characterization of *I. scapularis* EST sequences will provide a basis for future research in anti-tick vaccines, with an important role in the reduction of transmission of tick-borne pathogens.

Expression profile and functional analysis

Evaluation of the extent of antigens in tick's tissues is important in order to detect protective antigens. Penichet *et al.* (1994) detected gut cell recognition to antiserum from cattle

vaccinated with the recombinant Bm86 antigen. Expression of Troponin I-like protein from the hard tick *Haemaphysalis longicornis* was detected by immunohistochemistry (You *et al.* 2001). In addition, the expression profile can also be tested in different phases from the tick development by reverse transcription-polymerase chain reaction (RT-PCR).

RNA interference (RNAi), or the induction of sequence specific gene silencing by double stranded RNA (dsRNA), is accomplished when expression of double-stranded RNA leads to specific decreases in the abundance of cognate mRNAs (Sorensen, 2003). dsRNAs can be delivered in a variety of ways, including introduction of large or small dsRNA directly, and through expression from appropriate expression vectors following transfection (Hannon, 2002). This approach was first described in the nematode *Caenorhabditis elegans* (Fire *et al.* 1998). Messenger RNA-specific anti-sense oligonucleotides were used to inhibit the *in vitro* gene expression of the protozoa *Trypanosoma congolense* (Inoue *et al.* 2002). In arthropods, RNAi allowed for the targeting of specific genes in *Drosophila melanogaster* adult fly (Kalidas and Smith, 2002). Recently, the first application of RNAi in ticks was reported (Aljamali *et al.* 2003). A dsRNA from an *A. americanum* histamine binding protein was cloned and incubated with tick salivary glands. Results showed a lower histamine binding ability, suggesting that RNAi might be an important tool for target encoded gene proteins (Aljamali *et al.* 2003). Experiments *in vivo* can be performed (Kalidas and Smith, 2002) in which molecules of interest may be injected into the hemolymph in order to interrupt vital functions of the tick.

Tick genomics and proteomics are likely to evolve into projects addressing the sequencing, annotation and functional analysis of entire tick genomes, providing invaluable information for the development of tick vaccines. EST databases provide an inclusive catalog of potential vaccine candidate antigens. The use of this information in conjunction with bioinformatics, RNAi, mutagenesis, immunomapping, transcriptomics, proteomics, ELI and

other emerging technologies should allow for a systematic and comprehensive approach to vaccine discovery. In addition, the screening of protective clones by ELI of cDNA libraries constructed from different tick tissues, developmental stages and from genes expressed in response to various stimuli, including tick feeding or infection with pathogens, will provide exciting possibilities for the identification of new antigens protective against tick infestations and may also allow for identification of antigens that interfere with pathogen development and transmission. Vaccination trials can be also designed to evaluate the effect of selected tick antigens, in combination with pathogen-specific antigens, in reducing transmission of tick-borne pathogens.

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RESEARCH PROBLEM

Because of the importance of ticks to livestock and public health and the difficulty of controlling them, vaccines would be an improved and effective control method for ticks of medical and veterinary importance. The use of acaricides for tick control is less desirable because of the risk of environmental contamination and selection of resistant ticks. Recently, a successful vaccine against the cattle tick *B. microplus* was produced by recombinant technology (de la Fuente *et al.* 1999). However, this vaccine did not protect against *Amblyomma* spp. (de Vos *et al.* 2001). The screening and identification of novel protective antigens is therefore needed in order to identify vaccine candidates that would protect against multiple tick species. Expression library immunization (ELI) is a new method for discovery of potential vaccine antigens and, unlike more traditional methods, ELI does not require prior knowledge of possible antigenic targets and also provides the opportunity to screen the entire organism genome. ELI in combination with analysis of expressed sequence tags (ESTs) provides an alternative approach for identification of protective antigens through the rapid screening of the expressed genes in immunized hosts. Several reports have demonstrated that antigens identified by ELI have provided a degree of protection when used in vaccine formulations (Barry *et al.* 1995; Alberti *et al.* 1998; Smooker *et al.* 2000; Melby *et al.* 2000). However, this methodology has not been used in arthropods and, specifically in ticks. In this research we hypothesized that cDNAs encoding protective antigens against *I. scapularis* infestations can be identified by ELI of a cDNA library constructed from a tick cell line (IDE8) that was derived from embryonic *I. scapularis*. Herein, we proposed to screen and characterize genes expressed in *I. scapularis* that are essential for tick larval development and encode protective antigens by cDNA-ELI. The

putative function were determined by analysis of ESTs. Candidate vaccine antigens for control of tick infestations were then tested in mice and rabbits model systems and in a tick capillary feeding system.

The specific objectives of the research proposed herein are:

1. To construct a cDNA expression library from cultured IDE8 tick cells.
2. To identify genes encoding protective antigens against tick larvae by expression library immunization using a mouse model of *I. scapularis* infestation.
3. To clone and sequence selected genes and to predict putative protein function according to sequence databases.
4. To express protective antigens in *E. coli* to produce recombinant antigens for protein characterization and vaccine formulations.
5. To characterize the biological function, expression profile and sequence conservation of genes encoding protective antigens across ixodid tick species.
6. To test the efficacy of recombinant vaccine formulations in mice and rabbits against the different developmental stages of *I. scapularis*.

C h a p t e r 2

IDENTIFICATION OF PROTECTIVE ANTIGENS FOR THE CONTROL OF *Ixodes scapularis* INFESTATIONS USING cDNA EXPRESSION LIBRARY IMMUNIZATION

Consuelo Almazán, Katherine M. Kocan, Douglas K. Bergman, Jose C. Garcia-Garcia, Edmour F. Blouin and José de la Fuente. *Vaccine 2003, 21: 1492-1501.*

Abstract

Identification of antigens that induce an immune response against tick infestations is required for the development of vaccines against these economically important ectoparasites. In order to identify protective antigens, we constructed a cDNA expression library from a continuous *Ixodes scapularis* cell line (IDE8) that was initially derived from tick embryos. cDNA clones were subjected to several rounds of screening in which mice were immunized with individual pools and then challenge-exposed by allowing *I. scapularis* larvae to feed on the immunized and control mice. Immunity against tick infestation was determined by the reduction in the ability of the larvae to attach, feed to repletion and molt to the nymphal stage. Individual clones in pools that induced immunity to larval infestations were partially sequenced and grouped according to their putative protein function by comparison with sequence databases. The screening identified several individual antigens that induced a protective immune response against *I. scapularis* infestations. Our studies demonstrated for the first time that cDNA expression library immunization (ELI) combined with sequence analysis is a powerful and efficient tool for identification of candidate antigens for use in vaccines against ticks.

Keywords: tick, vaccine, tick cell culture, cDNA library immunization

Introduction

Ticks are ectoparasites of wild and domestic animals and humans, and they transmit pathogens including fungi, bacteria, viruses and protozoan. Currently, ticks are considered to be second in the world to mosquitoes as vectors of human diseases, but they are considered to be the most important vector of pathogens in North America [1]. *Ixodes* spp. are distributed worldwide and are vectors of human pathogens, including *Borrelia burgdorferi* (Lyme disease), *Anaplasma phagocytophila* (human granulocytic ehrlichiosis), *Coxiella burnetti* (Q fever), *Francisella tularensis* (tularemia), *B. afzelii*, *B. lusitaniae*, *B. valaisiana* and *B. garinii*, *Rickettsia helvetica*, *R. japonica* and *R. australis*, *Babesia divergens*, as well as tick-borne encephalitis (TBE) and Omsk Hemorrhagic fever viruses [1, 2]. Throughout eastern and southeastern United States and Canada, *I. scapularis* (the black legged tick) is the main vector of *B. burgdorferi* sensu stricto and *A. phagocytophila* [1, 2].

Control of tick infestations is difficult and often impractical for multi-host ticks such as *Ixodes* spp. Presently, tick control is effected by integrated pest management in which different control methods are adapted in a geographic area against one tick species with due consideration to their environmental effects. Recently, development of vaccines against one-host *Boophilus* spp. has provided new possibilities for the identification of protective antigens for use in vaccines for control of tick infestations [3-7]. Control of ticks by vaccination would avoid environmental contamination and selection of drug resistant ticks that result from repeated acaricide application [8, 9]. Anti-tick vaccines also allow for inclusion of multiple antigens in order to target a broad range of tick species as well as pathogen-blocking antigens.

A new technique, expression library immunization (ELI), in combination with sequence analysis, provides an alternative approach for identification of potential vaccine antigens that is based on rapid screening of the expressed genes without prior knowledge of

the antigens encoded by the cDNAs. ELI was first reported for *Mycoplasma pulmonis* [10] and since then has been used for unicellular and multicellular pathogens and viruses [11-17]. However, the identification of individual protective clones has not been reported and it was predicted that the identification of protective antigens would be more difficult as the complexity of the genome increases.

Herein we describe the first application of ELI to arthropods, specifically ticks. A combination of cDNA ELI and sequence analysis resulted in the identification of individual protective antigens against *I. scapularis* infestations.

2. Materials and Methods

2.1. Tick cells

Monolayers of IDE8 (ATCC CRL 1973) cells, originally derived from embryonic *I. scapularis*, were maintained in 25 cm^2 flasks at 31°C in L-15B medium supplemented with 5% fetal bovine serum, tryptose phosphate broth and bovine lipoprotein concentrate (ICN, Irvine, CA) after Munderloh et al. [18]. Cells were subcultured at a density of 2 x 10^5 cells/cm^2. Medium was replaced weekly.

2.2. Library construction

A cDNA expression library was constructed in the vector pEXP1 containing the strong human cytomegalovirus major immediate early promoter/enhancer (CMV_{IE}) (Clontech, Palo Alto, CA). We chose to construct our library from cultured embryonic *I. scapularis* IDE8 cells-derived poly(A)+ RNA in order to target the early larval stages of *I. scapularis*. The cDNA library contained 4.4 x 10^6 independent clones and a titer of approximately 10^{10} cfu/ml with more than 93% of the clones with cDNA inserts. The average cDNA size was 1.7 kb (0.5-4.0 kb).

2.3. Library screening by ELI

2.3.1. Primary screen

The overall schema for identification of protective antigens through ELI, sequential fractionation and sequence analysis is shown in Fig. 1.

Ninety six LBA (master) plates containing an average of 41 (30-61) cDNA clones per plate were prepared. Replicas were made and clones from each plate were pooled, inoculated in Luria-Bertani with 50 μg/ml ampicillin, grown for 2 hr in a 96-well plate and plasmid DNA purified from each pool (Wizard SV 96 plasmid DNA purification system, Promega, Madison, WI). BALB/c female mice, 5-6 weeks of age at the time of first vaccination, were used. Mice were cared for in accordance with standards specified in the Guide for Care and Use of Laboratory Animals.

Mice were injected using a 1 ml tuberculin syringe and a 27½G needle at days 0 and 14. Three mice per group were each immunized IM in the thigh with 1 μg total DNA/dose in 50 μl PBS. Two groups of 3 mice each were included as controls. One group was injected with 1 μg vector DNA alone and the second with saline only. Two weeks after the last immunization, mice were infested with 100 *I. scapularis* larvae per mouse. Ticks were reared at the Oklahoma State University Tick Rearing Facility by feeding larvae on mice, nymphs on rabbits and adults on sheep. For these experiments, larvae were obtained from the eggs oviposited by sister females. Twelve hours after tick infestation, larvae that did not attach were counted in order to calculate the number of attached larvae per mouse. Mice were then transferred to individual cages with an elevated ¼" mesh wire platform with water (½" deep) in the bottom of the cage. Replete larvae dropping from each mouse were collected daily from the water and counted during 7 days. Time for larval development was evaluated from the day of tick infestation to the day in which the maximum number of replete larvae was collected.

The inhibition of tick infestation (I) for each test group was calculated with respect to vector-immunized controls as [1-(RLn/RLc x RLic/RLin)] x 100, where RLn is the average number of replete larvae recovered per mouse for each test group, RLc is the average number of replete larvae recovered per mouse for control group, RLic is the average number of larvae attached per mouse for control group, and RLin is the average number of larvae attached per mouse for each test group.

2.3.2. Secondary screen

After the primary screen of 66 cDNA pools (2705 clones), 9 pools (351 clones) with I≥60% were selected for the secondary screen (re-screening) employing 5 mice per group as described above. Control mice were immunized with the negative (I=0%) F2 cDNA pool or saline only. A group was included that was immunized SC with two doses of 100 μg of total IDE8 tick cell proteins per dose in Freund's incomplete adjuvant. Engorged larvae were held in a 95% humidity chamber and allowed to molt. Molting of engorged larvae was evaluated 34 days after the last larval collection by visual examination of ticks under a dissecting light microscope. The inhibition of molting (M) for each test group was calculated with respect to controls as [1-(MLn/MLc x RLc/RLn)] x 100, where MLn is the number of nymphs for each test group, MLc is the number of nymphs for the control group, RLc is the number of larvae recovered for the control group, and RLi is the number of larvae recovered for each test group.

2.3.3. Sequence analysis

Immunization of mice with all 9 pools (351 cDNA clones) tested in the secondary screen effected immunity to larval tick infestation. Therefore, DNA from individual clones in these pools was purified (Wizard SV 96 plasmid DNA purification system, Promega, Madison, WI) from the master plate and partially sequenced with a 5' vector-specific primer (5'-

CGACTCACTATAGGGAG-3') at the Core Sequencing Facility, Department of Biochemistry and Molecular Biology, Noble Research Center, Oklahoma State University, using ABI Prism dye terminator cycle sequencing protocols developed by Applied Biosystems (Perkin-Elmer Corp., Foster City, CA). In most cases a sequence larger than 700 nucleotides was obtained. Nucleotide sequences were analyzed using the program AlignX (Vector NTI Suite V 5.5, InforMax, North Bethesda, MD). BLAST [19] was used to search the NCBI databases to identify previously reported sequences with homology to those that we sequenced. Sequence analysis allowed grouping the clones according to sequence identity to DNA databases and predicted protein function.

2.3.4. Tertiary screen

For the tertiary screen, 64 clones were grouped in 16 sub-pools each containing 1 to 17 plasmids according to the predicted function of encoded proteins (e.g. all the plasmids that encoded histone proteins were grouped together) and used, along with 4 sub-pools containing 182 clones of unknown function or with sequences without homology to sequence databases, to immunize 4 mice per group. Mice were immunized with 0.3 μg/plasmid/dose in 50 μl PBS and evaluated as described above. Control mice were immunized with a pool of 20 plasmids containing mitochondrial cDNAs. The inhibition of molting (M) for each test group was calculated with respect to controls as described above. The protection efficacy was calculated for cDNA sub-pools resulting in I>1 and M>1 as E (%) = 100 x [1-(RI x RM)] where RI (reduction in tick infestation) = 1-I/100 and RM (reduction in molting) = 1-M/100.

2.4. Microscopy

Selected ticks were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2),

dehydrated in a graded series of ethanol washes and embedded in epoxy resin after Kocan et al. [20]. Thick sections (1.0 μm) were cut and stained with Mallory's stain [21] for light microscopic observation.

2.5. Characterization of the immune response in vaccinated mice by Western blot

Mice were euthanatized by cervical dislocation, blood was collected and the serum removed. One hundred micrograms of IDE8 tick cell proteins were loaded in an 8% polyacrylamide gel [22] using a preparative comb. SDS-PAGE gels were transferred to a nitrocellulose membrane. Strips were made and membranes were blocked with 5% skim milk for 1 hr at room temperature. Sera from immunized and control mice were diluted 1:10 in TBS and incubated with the membrane for 1 hr at room temperature. The membrane was washed 3 times with TBST and incubated for 1 hr at room temperature with goat anti-mouse IgG alkaline phosphatase conjugate (KPL, Gaithersburg, MD) diluted 1:10,000. The membrane was washed again and the color developed using Sigma Fast BCIP/NBT alkaline phosphatase substrate tablets.

2.6. Statistical analysis

In each round of screening, the number of larvae attached per mouse and the number of engorged larvae recovered per mouse 7 days after infestation were compared by Student's t-test between cDNA-immunized and control vector DNA-immunized mice (primary screen), between mice immunized with positive cDNA pools and the control negative F2 cDNA pool (secondary screen) or between test cDNA sub-pools-immunized and control mice immunized with mitochondrial cDNAs (tertiary screen).

3. Results

3.1. Primary screen

Pools of approximately 41 (30-61) *I. scapularis* cDNA clones were screened by ELI. Only 33 cDNA pools and 2 control groups were analyzed per experiment. The average tick infestation level in two experiments was 50±13 and 56±15, and 56±15 and 54±18 larvae/mouse for cDNA immunized and control mice, respectively ($P>0.05$) (Table 1). The average number of engorged larvae recovered per mouse was 9±3 and 13±4 in the cDNA-immunized mice and 16±4 and 17±3 in the control vector-immunized group ($P<0.05$) (Table 1). Reduction in the number of larvae collected from mice that received the vector DNA was not observed compared with the saline-immunized controls (data not shown). The average inhibition of tick infestation (I) was 49±28% and 30±22% (Table 1). The maximum number of engorged larvae was collected 3 to 4 days after infestation. However, when larvae were fed on mice immunized with cDNA pools B5, A8 and A10 (Fig. 2), a retardation of larval development in 1 to 2 days was observed (data not shown). After two experiments covering the analysis of 66 pools (2705 clones), 9 protective pools (351 clones) were selected producing inhibition of tick infestations I≥60% (Fig. 2A and 2B and Table 1). Because of the complexity of the screening procedure in mice vaccinated and challenged with tick larvae, it was difficult to work with more than 9 protective cDNA pools. Therefore, we did not continue screening new cDNA pools and focused our attention on the 9 pools selected after the primary screen.

3.2. Secondary screen and sequence analysis

The secondary screen was done to verify the protective capacity of the cDNA pools selected after the primary screen (Fig. 2A and 2B). Positive results were observed in all 9 protective cDNA pools in the secondary screen. Tick infestation levels were higher in this

experiment (average 85±6 and 84±3 larvae/mouse for cDNA-immunized and control mice, respectively; P>0.05). Nevertheless, the average number of engorged larvae recovered per mouse was 39±7 and 26±6 for control and cDNA-immunized mice, respectively (P<0.05). The group immunized with total IDE8 tick cell proteins was protected with I=33%. Again, no reduction was observed in the number of larvae collected from mice that were injected with the control cDNA (F2 negative pool after the primary screen; Fig. 2A) compared to saline-immunized controls (data not shown).

In the secondary screen, molting of engorged larvae was evaluated after 34 days. Molting was affected in all but one test cDNA-immunized group. Inhibition of molting in test cDNA-immunized mice compared to the control cDNA-immunized group varied from 0% to 12% (6±4%). Differences were not observed between control cDNA and saline-immunized mice. Among the larvae that did not molt to the nymphal stage, some were visibly different from the controls and had a bright red color. The percent of red larvae in cDNA-immunized mice varied between 3% to 18 % (7±5%) while in the saline and control cDNA-immunized groups red larvae represented the 6% and 4%, respectively.

Plasmid DNA from the 351 individual clones in the 9 protective pools was isolated and partially sequenced. Comparison to sequence databases permitted identification of sequence identity with previously reported genes with known function in 152 (43%) of the clones (Table 2). Fifty seven percent of the sequences were homologous to genes with unknown function or had no significant identity to previously reported sequences (Table 2). Of the clones with sequence identity to genes with known function, 85% were homologous to arthropod sequences. Ninety three clones (61%) contained sequences homologous to *Drosophila melanogaster*, 5 (3%) to other insects and 32 (21%) to Ixodid tick species. Thirty

percent of the clones were eliminated from further analysis based on their sequence identity, including those containing similar sequences (Table 2).

3.3. Tertiary screen

For the tertiary screen, mice were immunized with 64 clones grouped in 16 sub-pools according to the predicted function of encoded proteins and with 4 sub-pools containing 182 clones with unknown function or with sequences without homology to sequence databases (Table 3). Control mice were immunized with a pool of mitochondrial cDNAs. Tick infestation levels were similar in all test groups (72±2 larvae/mouse) and in control mice (69±2 larvae/mouse) ($P>0.05$). The number of engorged larvae recovered per mouse was also similar between test (16±7) and control (14±6) mice ($P>0.05$). However, the groups immunized with cDNA sub-pools containing clones with putative endopeptidase, nucleotidase, ribosomal proteins, heat shock proteins, glutamine-alanine-rich proteins and 3 of the sub-pools with clones of unknown function or with sequences without homology to sequence databases had $I \geq 15\%$ (Fig. 3). Furthermore, among them, the groups immunized with sub-pools containing clones with a putative endopeptidase, nucleotidase and two of the cDNA sub-pools with unknown function or with sequences without homology to sequence databases resulted in lower infestation levels compared to control mice ($P<0.05$) and $I \geq 40\%$ (Fig. 3). Retardation of larval development was not observed. Molting was affected only in the groups immunized with sub-pools containing clones with a putative endopeptidase, nucleotidase and three of the cDNA sub-pools with unknown function or with sequences without homology to sequence databases (Table 4). Three of these sub-pools were the same giving the highest I value (Fig. 3 and Table 4). The protection efficacy of cDNA sub-pools was calculated considering the effect of vaccination on tick infestation and molting and varied from

44% to 73% (Table 4). Only in mice immunized with a putative beta-adaptin-encoding cDNA sub-pool, the proportion of red larvae (6%) was higher than in the control group (2%). The red coloration observed in this group of ticks appeared to be associated with an extended gut (Fig. 4). Sera from each group of mice immunized with cDNAS sub-pools containing clones with a putative endopeptidase, nucleotidase, ribosomal proteins, heat shock proteins, glutamine-alanine-rich proteins, beta-adaptin and from control mice immunized with vector DNA were pooled and analyzed by Western blot against IDE8 tick cell proteins. Distinctive bands were identified in some of the sera from mice immunized with cDNA sub-pools compared to control serum (Fig. 5).

4. Discussion

The feasibility of controlling tick infestations through immunization of hosts with tick antigens has been demonstrated for *Boophilus* spp. (reviewed by [3-6]). The recombinant *B. microplus* BM86 gut antigen included in commercial vaccine formulations, TickGARD (Hoechst Animal Health, Australia) and Gavac (Heber Biotec S. A., Havana, Cuba), also confers partial protection against phylogenetically related *Hyalomma* and *Rhipicephalus* species [6, 7]. However, immunization with BM86 failed to protect against the more phylogenetically distant *Amblyomma* spp. [7]. These results suggested that use of BM86 or a closely related antigen for the production of vaccines against *Ixodes* spp. or other tick genera phylogenetically distant from *Boophilus* spp. [23] may be impractical. Therefore, the screening for novel protective antigens would be necessary for identification of candidate vaccine antigens for control of these tick species of medical and veterinary importance.

Vaccination with DNA and cDNA molecules has been used to induce a protective immune response against *B. microplus,* as well as several pathogens in laboratory animals and livestock [24-27]. Here, cDNA ELI, combined with sequence analysis, resulted in a rapid

method for the identification of protective antigens against *I. scapularis* infestations. Although several reports in the literature have demonstrated by ELI that libraries can offer a degree of protection [10-17], this is the first report in which sub-fractionation identified individual protective clones.

When we started these experiments, we planned to screen over 4000 cDNA clones considering the complexity of the tick genome. However, 9 protective cDNA pools were identified after screening only 66 pools containing 2705 cDNA clones. This result probably reflects the possibility of interfering with tick infestations at many different levels that involve a plethora of gene products. Results from vaccination experiments against ticks employing recombinant tick antigens support this view (reviewed by [28]).

The effect of cDNA vaccination on *I. scapularis* experimental infestations of mice was evidenced by the reduction of the number of engorged larvae, the retardation of larval development, the inhibition of molting to nymphal stages and the appearance of visibly affected larvae. These effects were also recorded in vaccination experiments with recombinant BM86 and BM95 antigens against infestations with *B. microplus* [29] and the protective effect of these antigens correlated with the concentration of specific antibodies [8]. Immunoglobulins trigger a variety of effector mechanisms and are specifically transported into the tick hemolymph during feeding [30, 31]. Some of the sera collected from mice after the tertiary screen showed discrete bands against IDE8 tick cell proteins, thus suggesting an immune response against cDNA vaccination. The failure to detect an immune response in all the immunized mice may be due to poor immunogenicity or underrepresentation of the encoded proteins in the IDE8 protein extract.

After the tertiary screen, tick infestation levels were lower in mice immunized with several cDNA sub-pools and inhibition of molting to nymphal stage was apparent when

compared to controls. Two of these cDNA sub-pools contained single clones homologous to an endopeptidase from *D. melanogaster* and to a human 5'-nucleotidase. Proteinases have been identified in many tick species and are involved in different physiological and developmental processes [32-37]. Furthermore, tick proteins with proteinase activity have been recently targeted for development of anti-tick vaccines [28, 38-42]. A 5'-nucleotidase was identified and characterized in *B. microplus* by Liyou et al. [43, 44] but they did not assay its protection capacity.

Although a preliminary observation, the highest number of red larvae was recorded in the group immunized with a cDNA homologous to a beta-adaptin from *D. melanogaster*. Beta adaptins are adaptor components required in the assembly of clathrin-coated plasma membrane pits, which function during endocytosis [45]. The process of endocytosis is actively involved in blood digestion by ticks and other hematophagous arthropods [33]. Therefore, an inhibition of endocytosis may impair the acquisition and digestion of the blood meal in ticks and results in extension of the midgut as observed in larvae fed on immunized mice. Development of vaccines against tick infestations by targeting tick proteins essential for acquisition and digestion of blood meal has been reported by various groups [46, 47]. Furthermore, the protective BM86 antigen from *B. microplus* has been suggested to be involved in endocytosis and ticks fed on vaccinated cattle also show a red coloration associated with tick damage [3, 29].

The sub-pools identified in the tertiary screen with higher capacity to inhibit tick infestations were composed of 50 and 49 clones with unknown function or with sequences without homology to sequence databases. Furthermore, the protection elicited by cDNA vaccination was higher in the primary and secondary screens of library pools than in the tertiary screen of sub-pools. Results of previous ELI studies have demonstrated that

immunization with pools confers a better protection than sub-pools after fractionation [14]. Therefore, protection induced by immunization with the larger pools appears to be mediated by the combined and/or synergistic effect of different antigens or, alternatively, by the nonspecific adjuvant activity of some DNA sequences on antigenic cDNAs [14].

The other 3 cDNA sub-pools identified in our experiments to induce partial protection contained sequences homologous to glutamine-alanine-rich, ribosomal and heat shock proteins. Although initially surprising, the protection capacity of ribosomal and heat shock protein preparations has been previously documented in other organisms [14, 48-50].

Several vaccines have been developed to protect humans against *Ixodes*-transmitted pathogens including TBE virus and *B. burgdorferi*. However, it is not clear whether these vaccines will protect against all pathogen strains and genotypes. The inclusion of tick immunogens in pathogen-specific vaccines could enhance their protective effect and increase efficacy [51]. This transmission-blocking approach is supported by evidence that host resistance to ticks provides some protection against tick-borne transmission of viruses and *B. burgdorferi* [52]. Furthermore, vaccination against *B. microplus* has been demonstrated to contribute to the control of tick-borne diseases [5, 8].

In summary, we have used ELI and sequence analysis to identify cDNAs that induce protection to experimental infestations of mice with *I. scapularis*. Two of these cDNAs conferred protection when used individually to immunize mice. We anticipate that use of recombinant proteins and/or modified cDNAs in combination with adjuvant will enhance the protective efficacy of vaccine preparations. Ultimately, the combination of anti-tick antigens with pathogen antigens may provide a means to control tick-borne infections through immunization of the human population at risk or by immunization of the mammalian reservoir to reduce pathogen transmission to humans.

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Table 1. Primary screen of the *I. scapularis* cDNA library by ELI in mice.

Experimental group[a]	Number of pools screened (clones)	Average number of larvae attached per mouse[b]	Average number of engorged larvae recovered per mouse[c]	Average inhibition of tick infestation (I)[d]	Number of pools selected for the secondary screen
Experiment 1	33 (1383)	50±13 (33-80)	9±3 (2-42)	49±28% (0-87%)	6 (I>75%)
Vector DNA-immunized controls for experiment 1	---	56±13 (45-67)	16±4 (5-27)	---	---
Experiment 2	33 (1322)	56±15 (29-79)	13±4 (1-27)	30±22% (0-89%)	3 (I>60%)
Vector DNA-immunized controls for experiment 2	---	54±18 (36-73)	17±3 (6-28)	---	---

[a]Ninety six LBA plates containing an average of 41 cDNA clones per plate were prepared. Replicas were made and clones from each plate were pooled, inoculated, grown for 2 hr in a 96 wells plate and plasmid DNA purified from each pool for ELI. Three mice per group were each immunized IM twice with 1 μg DNA/dose in 50 μl PBS two weeks apart. Two groups of 3 mice each were included as controls. One group was injected with vector DNA and the second with saline only.

[b]Fifteen days after the last immunization, mice were infested with 100 *I. scapularis* larvae per mouse. Twelve hrs later, larvae that did not attach were counted to calculate the number of attached larvae per mouse and mice were transferred to new cages.

[c]Engorged larvae dropping from each mouse were collected daily and counted after 7 days.

[d]The inhibition of tick infestation (I) for each test group was calculated with respect to vector-immunized controls as [1-(<RL>n/<RL>c x <RL>ic/<RL>in)] x 100, where <RL>n is the average number of replete larvae recovered per mouse for each test group, <RL>c is the average number of replete larvae recovered per mouse for control group, <RL>ic is the average number of larvae attached per mouse for control group, and <RL>in is the average number of larvae attached per mouse for each test group.

Table 2. Classification of the *I. scapularis* cDNA clones in protective pools by putative protein function according to identity to sequence databases.

Putative Protein Function	Number of clones
Biosynthetic[a]	2
Catabolism	4
Cell adhesion	2
Cell cycle[a]	2
Cytoskeletal[a]	8
Defense	2
DNA structure or replication[a]	3
Extracellular matrix	3
Endocytosis	2
Energy metabolism	10
Homeostasis	2
Morphogenetic	9
Mitochondrial[a]	34
Protein synthesis or processing[a,b]	34
RNA synthesis or processing[a]	7
Heat-shock proteins	4
Signal transduction	16
Transport	8
Unknown	199
Total	351

[a]Eliminated from further screening of protective antigens, [b]except for ribosomal proteins. Other clones were eliminated from further screening for containing similar sequences.

Table 3. Grouping of the *I. scapularis* cDNA clones according to the predicted function of encoded proteins in sub-pools for the tertiary screen.

cDNA sub-pool (No. of clones)	Clone	Pool[a]
Unknown I (50)	NR	NR
Unknown II (50)	NR	NR
Unknown III (49)	NR	NR
Unknown IV (33)	NR	NR
Ribosomal (17)	1A2, 1A10, 1C11	A5
	1F6	D1
	2B8	A10
	2F8, 2F10	E8
	3A10, 2C3, 3D2, 3D10	B4
	3G9, 3G10	E3
	4D11, 4D12, 4E7, 4F7	F1
	1D8, 1D11, 1E10	D1
Membrane protein (7)	2B12	A10
	2H5	E8
	3C9	B4
	3G11	E3
ATPase (6)	1A9, 1B2, 1C9	A5
	2C9	A10
	4A4	C3
	4G12	F1
Cell channel/Transporter (5)	1F4	D1
	2H11	E8
	4A12	C3
	4G10, 4G11	F1
Early development-specific (4)	1C8	A5
	3F4	E3
	4C7	C3
	4G9	F1
G protein-coupled receptor (4)	2B7, 2C12	A10
	2F12	E8
	4C9	C3
Growth factor receptor (3)	2E8	B5
	3B8, 3C8	B4
Lectin (3)	3E10	E3
	4B8, 4C8	C3
Vitellogenin (3)	1F12	D1
	4A6	C3
	4G2	F1
Heat shock (3)	1C10	A5
	1F10	D1

EGF-like (2)	3F6	E3
	2H4	E8
	4C10	C3
Secreted protein (2)	2F9	E8
	3C12	B4
Glutamine-Alanine rich (2)	4D6, 4E6	F1
Adaptin (1)	3E1	E3
Endopeptidase (1)	4D8	F1
Nucleotidase (1)	4F8	F1

[a]cDNA pools refer to positive pools after primary and secondary screens (Fig. 2A and 2B). NR, not relevant.

Table 4. Inhibition of tick infestation, inhibition of molting and protection efficacy of cDNA sub-pools after the tertiary screen by ELI.

cDNA sub-pool	I (%)	M (%)	E (%)
Endopeptidase	40	7	44
Nucleotidase	50	17	58
Unknown II	57	37	73
Unknown III	44	18	54
Unknown IV	0	14	---

Sub-pools refer to those in table 3. Only cDNA sub-pools that produced an inhibition of molting to nymphal stages (M) are shown. The protection efficacy (E) was calculated considering the effect of vaccination on the reduction of tick infestation (I) and molting as E (%) = 100 x [1-(RI x RM)] = 100 x [I x M – (I-M/100)].

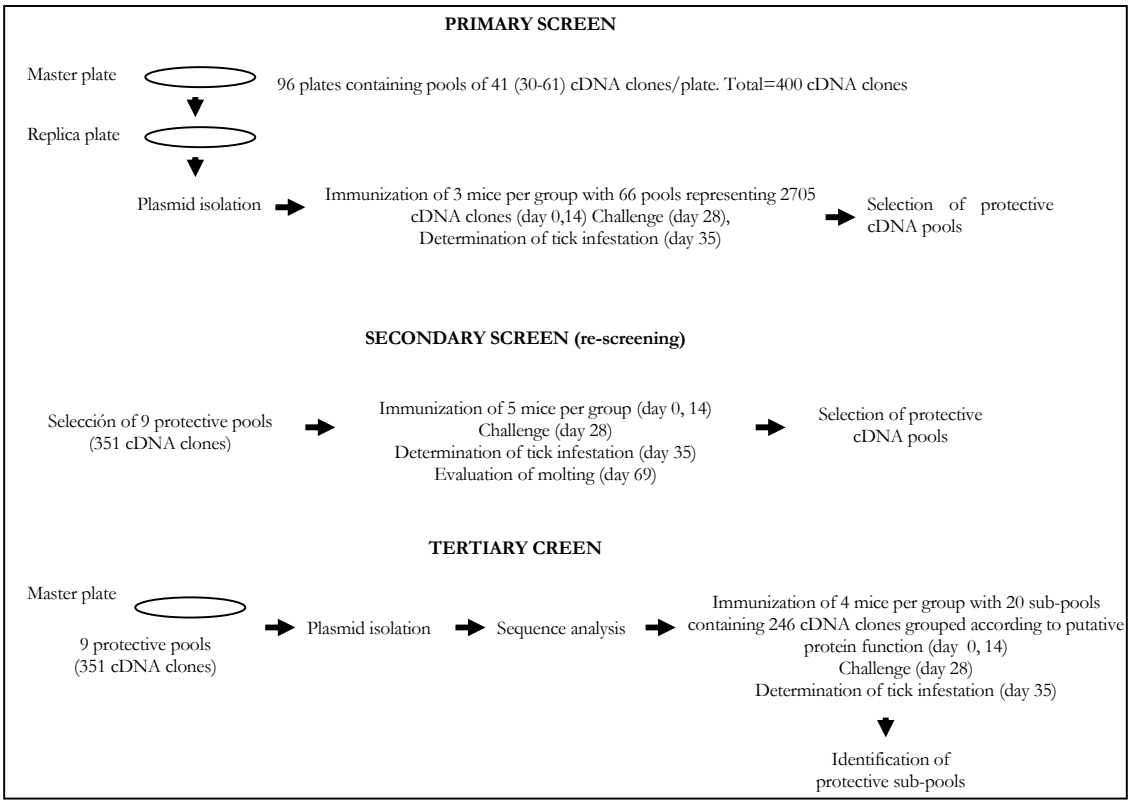
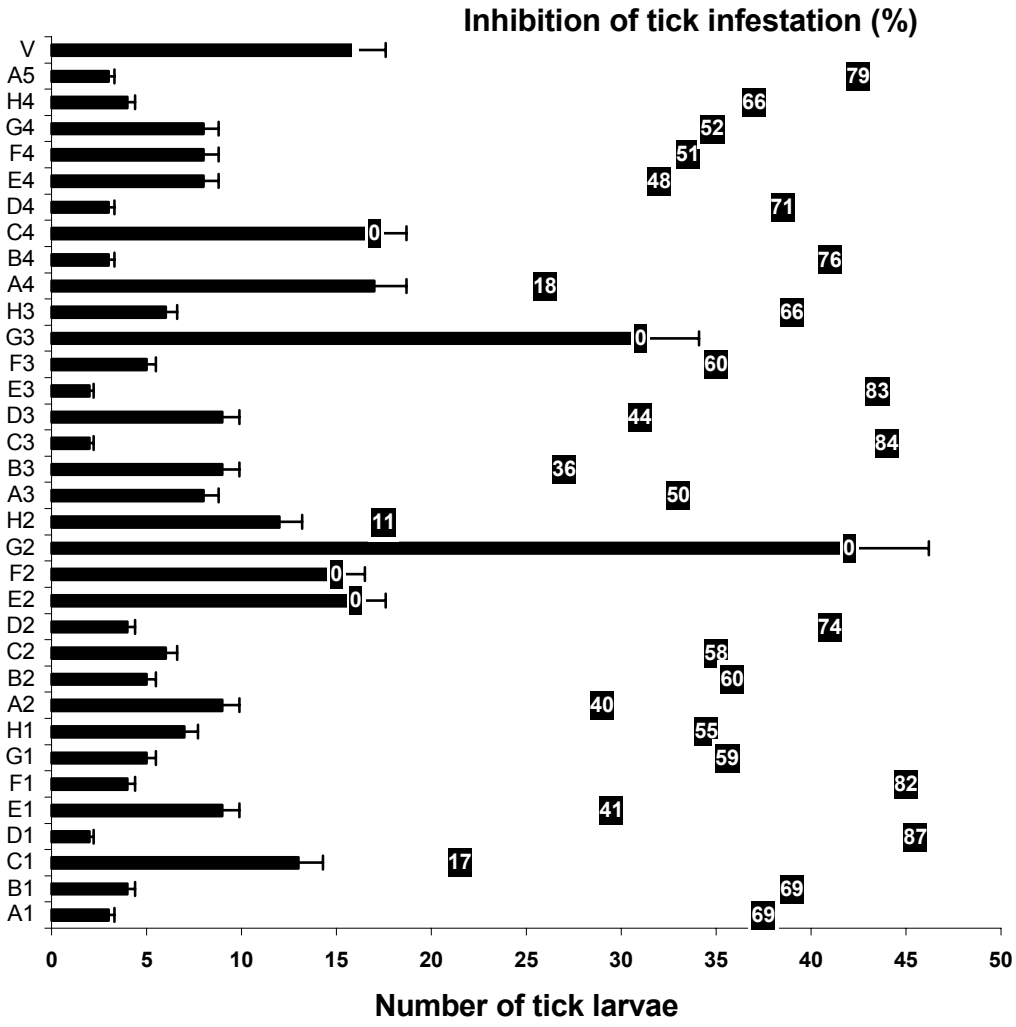
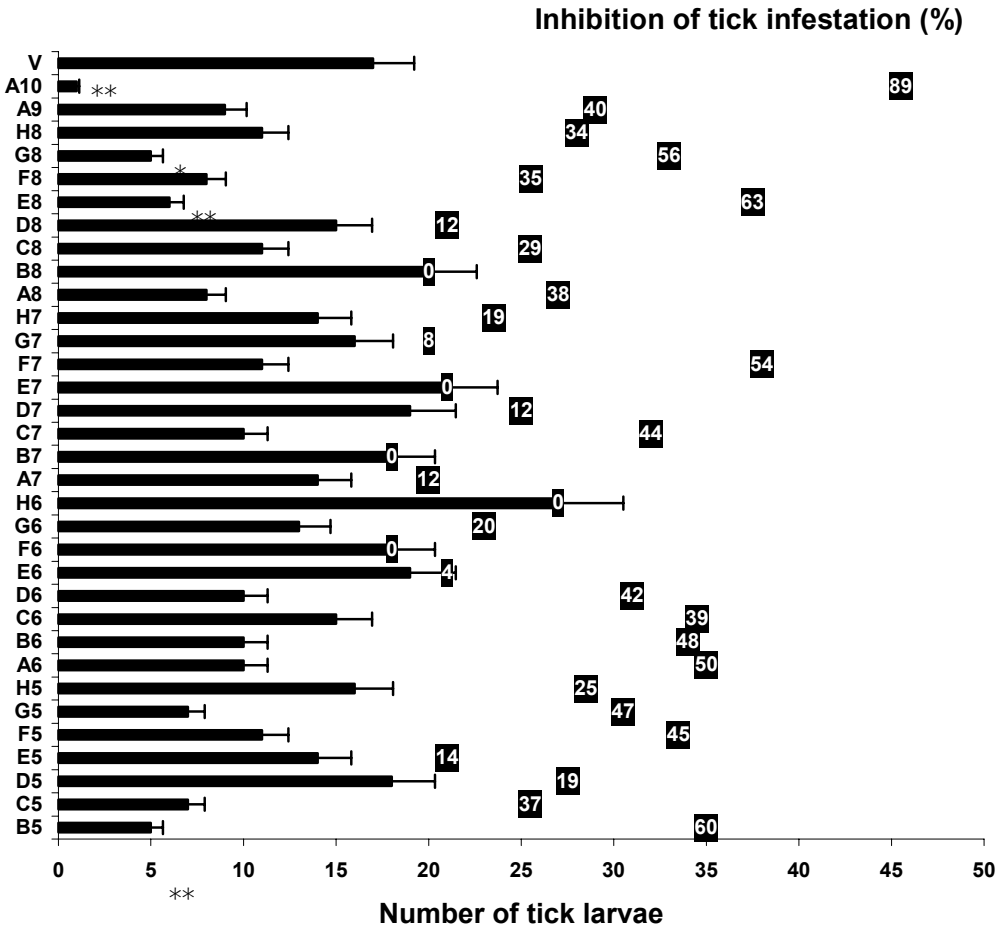


Figure 1. Summary of the cDNA ELI approach used to identify protective antigens against *I. scapularis* infestations.


Inhibition of tick infestation (%)
V
A5
H4
G4
F4
E4
D4
C4
B4
A4
H3
G3
F3
E3
D3
C3
B3
A3
H2
G2
F2
E2
D2
C2
B2
A2
H1
G1
F1
E1
D1
C1
B1
A1
79
66
52
51
48
71
0
76
18
66
0
60
83
44
84
36
50
11
0
0
0
74
58
60
40
55
59
82
41
87
17
69
69
0
5
10
15
20
25
30
35
40
45
50
Number of tick larvae

(A)



(B)

Figure 2. Primary screen of *I. scapularis* cDNAs pools by ELI. (A) Experiment 1 (cDNA pools A-H 1-4, A5). *P<0.05, **P<0.03 (Student's t-test). (B) Experiment 2 (cDNA pools A6-A10, B-H 5-8). *P<0.05, **P<0.03 (Student's t-test). The inhibition of tick infestation (I) and the number of engorged larvae per mouse (mean±SD) is presented. V, control mice injected with 1 μg vector DNA alone.

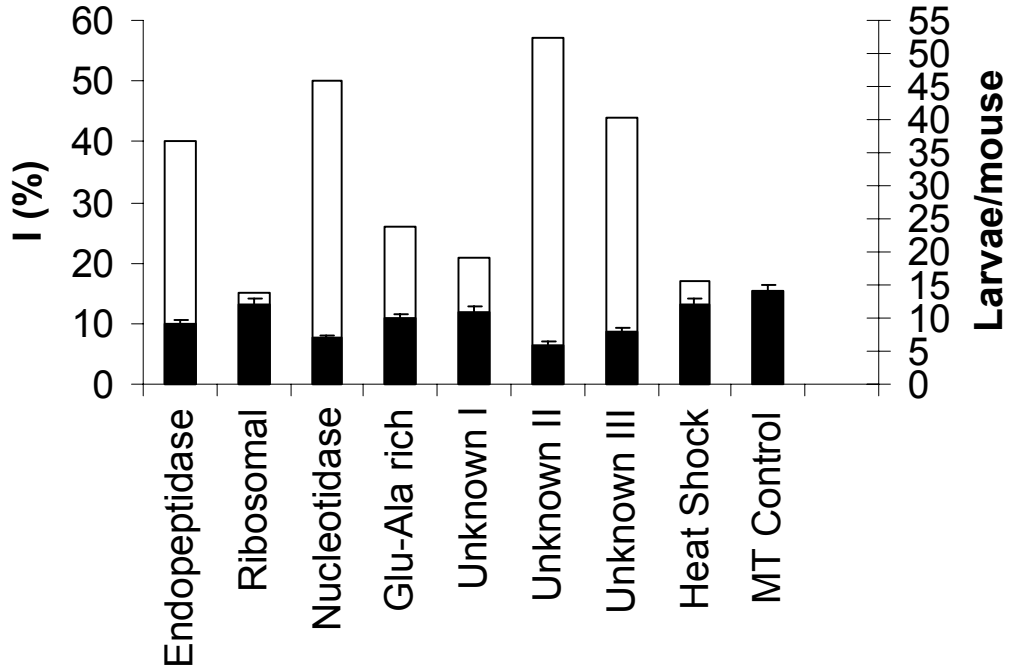


Figure 3. Tertiary screen by ELI of *I. scapularis* cDNA sub-pools formed according to the predicted function of encoded proteins. Only groups with I≥15% are shown (white bars). The number of engorged larvae per mouse is expressed as mean±SD (black bars). Control mice were injected with mitochondrial (MT) cDNAs. *P≤0.05 (Student's t-test).

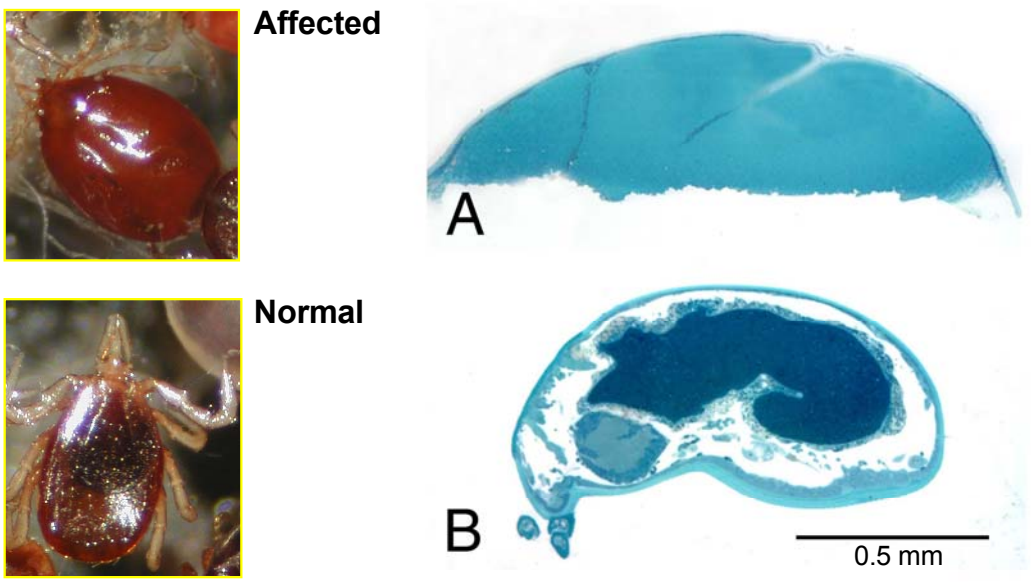


Figure 4. Normal and affected ticks. Replete larvae were kept for molting in a 95% humidity atmosphere. Larvae with normal appearance molt to nymphal stage while affected red larvae did not. Microscopic analysis of larval sections denoted extended guts in affected larvae.

C h a p t e r 3

CHARACTERIZATION OF GENES TRANSCRIBED IN AN *Ixodes scapularis* CELL LINE THAT WERE IDENTIFIED BY EXPRESSION LIBRARY IMMUNIZATION AND ANALYSIS OF EXPRESSED SEQUENCE TAGS

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Summary

Expression library immunization (ELI) combined with the analysis of expressed sequence tags (ESTs) was used to analyze genes transcribed in the *Ixodes scapularis* embryonic IDE8 cell line. A cDNA expression library was constructed from IDE8 cells. cDNA clones were screened by ELI in mice injected with cDNAs and then infested with *I. scapularis* larvae. cDNA clones affecting larval development were subjected to single pass 5' sequence analysis. Non-redundant sequences were putatively identified based on sequence identity using the protein Basic Local Alignment Search Tool (BLAST) algorithm and grouped according to the predicted function of encoded proteins. The screening identified 351 cDNAs affecting larval development in the mouse model of tick infestation. Of them, 316 cDNA clones contained non-redundant sequences and 102 produced a significant identity to previously reported sequences. Gene ontologies could be assigned to 87 clones. Vaccination of mice with plasmid DNA and tick infestation resulted in some cDNA clones producing inhibition of tick infestation and other promoting tick feeding. The cDNAs inhibiting tick infestation were identical to nucleotidase, heat shock proteins, beta-adaptin, chloride channel, ribosomal proteins and proteins with unknown function. Tick pro-feeding activity was produced by cDNA clones identical to beta-amyloid precursor, block of proliferation, mannose-binding

lectin, RNA polymerase III, ATPases and a protein of unknown function. In this paper we describe the sequence analysis of *I. scapularis* ESTs selected by ELI for affecting tick larval development. These proteins might be used in vaccine preparations to interrupt the life cycle of *I. scapularis* and help to control this ectoparasite and/or to reduce its ability to transmit pathogens.

Keywords: tick, vaccine, tick cell culture, cDNA library immunization, EST

I. Introduction

Ticks are ectoparasites of wild and domestic animals and humans, and are considered to be the most important vector of pathogens in North America (Parola and Raoult, 2001). *Ixodes* spp. (Acari: Ixodidae) are distributed worldwide and are vectors of human pathogens, including *Borrelia burgdorferi* (Lyme disease), *Anaplasma phagocytophilum* (human granulocytic ehrlichiosis), *Coxiella burnetti* (Q fever), *Francisella tularensis* (tularemia), *B. afzelii*, *B. lusitaniae*, *B. valaisiana* and *B. garinii*, *Rickettsia helvetica*, *R. japonica* and *R. australis*, *Babesia divergens*, as well as tick-borne encephalitis (TBE) and Omsk Hemorrhagic fever viruses (Estrada-Peña and Jongejan, 1999; Parola and Raoult, 2001). Throughout eastern and southeastern United States and Canada, *I. scapularis* (the black legged tick) is the main vector of *B. burgdorferi* sensu stricto and *A. phagocytophilum* (Estrada-Peña and Jongejan, 1999; Parola and Raoult, 2001).

Control of tick infestations is difficult, particularly for multi-host ticks such as *Ixodes* spp. Presently, tick control is effected by integrated pest management in which different control methods are adapted in a geographic area against one tick species with due consideration to their environmental effects. Recently, development of vaccines against one-host *Boophilus* spp. has provided new possibilities for the identification of protective antigens for use in vaccines for control of tick infestations (Willadsen, 1997; Willadsen and Jongejan, 1999; de la Fuente et al., 1999, 2000a; de Vos et al., 2001). Control of ticks by vaccination

would avoid environmental contamination and selection of drug resistant ticks that result from repeated acaricide application (de la Fuente et al., 1998; Garcia-Garcia et al., 1999). Anti-tick vaccines also allow for inclusion of multiple antigens in order to target a broad range of tick species as well as pathogen-blocking antigens.

Development of high throughput DNA sequencing technologies and bioinformatic tools facilitate assignment of provisional function to expressed sequence tags (ESTs; Boguski et al., 1993). This approach has resulted in valuable information for the study of biological systems and for the identification of potential vaccine candidates (Lizotte-Waniewski et al., 2000; Knox et al., 2001; Tarleton and Kissinger, 2001; Touloukian et al., 2001; Kessler et al., 2002). In ticks, the construction of EST databases has been reported for *B. microplus* (Crampton et al., 1998), *Amblyomma americanum* (Hill and Gutierrez, 2000) and *A. variegatum* (Nene et al., 2002). The application of EST approach has been used for the characterization of gene expression in salivary glands of *I. scapularis* (Valenzuela et al., 2002), *I. ricinus* (Valenzuela, 2002), *A. americanum* and *Dermacentor andersoni* (Bior et al., 2002) and for the identification of genes differentially expressed in *D. variabilis* ovaries in response to rickettsial infection (Mulenga et al., 2003) and in *I. ricinus* salivary glands in response to blood feeding (Leboulle et al., 2002).

A new technique, expression library immunization (ELI), in combination with sequence analysis of ESTs, provides an alternative approach for identification of potential vaccine antigens that is based on rapid screening of the expressed genes without prior knowledge of the antigens encoded by the cDNAs. ELI was first reported for *Mycoplasma pulmonis* (Barry et al., 1995) and since then has been used for unicellular and multicellular pathogens and viruses (Manoutcharian et al., 1998; Alberti et al., 1998; Brayton et al., 1998; Melby et al., 2000; Smooker et al., 2000; Moore et al., 2001; Singh et al., 2002; Leclercq et al.,

2003). Recently, we described the first application of ELI to arthropods, specifically to *I. scapularis* (Almazán et al., 2003). A combination of cDNA ELI and EST analysis resulted in the selection of 351 cDNA clones affecting tick larval development (Almazán et al., 2003). After grouping the clones according to the putative function of predicted proteins, some cDNA pools resulted in the inhibition of tick infestation and other promoted tick feeding after ELI (Almazán et al., 2003).

Herein we describe the sequence analysis and characterization *I. scapularis* ESTs that were identified by Almazan et al. (2003) using cDNA ELI and a mouse model for tick infestation.

II. Materials and Methods

A. Construction of the *I. scapularis* expression cDNA library.

The cDNA library was constructed from *I. scapularis* cultured embryonic IDE8 cells (Munderloh et al., 1994) as previously reported (Almazán et al., 2003). The expression library was constructed in the vector pEXP1 containing the strong human cytomegalovirus major immediate early promoter/enhancer (CMV_{IE}) (Clontech, Palo Alto, CA). The cDNA library contained 4.4 x 10^6 independent clones and a titer of approximately 10^{10} cfu/ml with more than 93% of the clones with cDNA inserts. The average cDNA size was 1.7 kb (0.5-4.0 kb).

B. DNA vaccination and tick infestation.

Vaccinations with plasmid DNA and tick infestations were done as previously reported for the screening of the expression cDNA library by ELI using the mouse model of *I. scapualris* infestations (Almazán et al., 2003). Briefly, plasmid DNA was purified (Wizard SV 96 plasmid DNA purification system, Promega, Madison, WI) and used to inject CD-1 female mice, 5-6 weeks of age at the time of first vaccination. Mice were cared for in accordance with standards specified in the Guide for Care and Use of Laboratory Animals. Mice were injected

using a 1 ml tuberculin syringe and a 27½G needle at days 0 and 14. Three to 6 mice per group were each immunized IM in the thigh with 1 μg total DNA/dose in 50 μl PBS. Control mice were injected with 1 μg vector DNA alone. Two weeks after the last immunization, mice were infested with 100 *I. scapularis* larvae per mouse. For tick infestations, mice were retrained in a small wire cage in a cardboard carton. On hundred larvae were counted and applied to the mice with a brush. Ticks were reared at the Oklahoma State University Tick Rearing Facility by feeding larvae on mice, nymphs on rabbits and adults on sheep. For these experiments, larvae were obtained from the eggs oviposited by sister females. Twelve hours after tick infestation, larvae in the bottom of the cage that did not attach were counted in order to calculate the number of attached larvae per mouse. Mice were then transferred to individual cages with an elevated ¼" mesh wire platform with water (½" deep) in the bottom of the cage. Replete larvae dropping from each mouse were collected daily from the water and counted during 7 days. Time for larval development was evaluated from the day of tick infestation to the day in which the maximum number of replete larvae was collected. The inhibition of tick infestation (I) for each test group was calculated with respect to vector-immunized controls as [1-(RLn/RLc x RLic/RLin)] x 100, where RLn is the average number of replete larvae recovered per mouse for each test group, RLc is the average number of replete larvae recovered per mouse for control group, RLic is the average number of larvae attached per mouse for control group, and RLin is the average number of larvae attached per mouse for each test group. Engorged larvae were held in a 95% humidity chamber and allowed to molt. Molting of engorged larvae was evaluated 34 days after the last larval collection by visual examination of ticks under a dissecting light microscope. The inhibition of molting (M) for each test group was calculated with respect to controls as [1-(MLn/MLc x RLc/RLn)] x 100, where MLn is the average number of nymphs for each test group, MLc is the average number of nymphs for

the control group, RLc is the average number of larvae recovered for the control group, and RLi is the average number of larvae recovered for each test group.

C. Plasmid DNA preparation and sequencing.

Bacterial colonies were inoculated in Luria-Bertani with 50 μg/ml ampicillin, grown for 16 hr in a 96-well plate and plasmid DNA purified (Wizard SV 96 plasmid DNA purification system, Promega, Madison, WI) and partially sequenced with a 5' vector-specific primer (5'-CGACTCACTATAGGGAG-3') at the Core Sequencing Facility, Department of Biochemistry and Molecular Biology, Noble Research Center, Oklahoma State University, using ABI Prism dye terminator cycle sequencing protocols developed by Applied Biosystems (Perkin-Elmer Corp., Foster City, CA). In most cases a sequence larger than 700 nucleotides was obtained.

D. Data analysis.

Nucleotide sequences were analyzed using the program AlignX (Vector NTI Suite V 5.5, InforMax, North Bethesda, MD). Multiple sequence alignment was performed using an engine based on the Clustal W algorithm (Thompson et al., 1994). Nucleotides were coded as unordered, discrete characters with five possible character-states; A, C, G, T, or N (missing) and gaps were coded as missing data. Phylogenetic trees were constructed based on a sequence distance method utilizing the Neighbor Joining algorithm of Saitou and Nei (1987). BLAST (Altschul et al., 1990) was used to search the NCBI databases to identify previously reported sequences with identity to those that we sequenced. Sequence analysis allowed grouping the clones according to predicted protein function. Gene ontology assignments were made according to Ashburner et al. (2000) for non-redundant EST sequence data with the help of GoFish v.1.0 (Berriz et al., 2003).

III. Results

The screening of the *I. scapularis* expression cDNA library by ELI and EST analysis resulted in 351 cDNAs affecting larval development in the mouse model of tick infestation (Alamazán et al., 2003). Of them, 316 cDNA clones contained non-redundant sequences and 101 (32%) produced a significant identity to previously reported sequences by BLAST analysis of NCBI nucleotide and protein databases (Table 1). Gene ontologies could be assigned to 87 clones (27.5% of non-redundant sequences and 86.1% of clones with identity to sequences reported previously) (Table 2).

The majority of clones with gene ontology assigned corresponded to non-nuclear gene products involved in cell growth and maintenance, including genes with ligand binding, carrier or enzymatic activities (Table 2). Seventeen clones contained sequences corresponding to tick mitochondrion and were not submitted to the EST database. Other clones such as 2A9 and 1D6, although probably coding for mitochondrial proteins, were analyzed and submitted to the EST database. Interestingly, 11 clones encoded gene products localized in the cell nucleus (Table 2).

The average G + C content of the EST dataset (47,503 bases excluding the poly-A tails with 171 (0.4%) undetermined nucleotide positions) was 54%, but some sequences, such as clone 2A9 which probably codes for a mitochondrial protein, had only a 25% G + C content. Some short ESTs in clones 1D1 and 2D5 contained a long stretch of T.

Vaccination of mice with plasmid DNA followed by tick infestation resulted in some cDNA clones that had an inhibitory effect on tick infestations, while others appeared to promote tick feeding (Table 3). The cDNAs inhibiting tick infestation were identical to nucleotidase, heat shock proteins, beta-adaptin, chloride channel, ribosomal proteins and proteins with unknown function. cDNA clones identical to beta-amyloid precursor, block of

proliferation, mannose-binding lectin, RNA polymerase III, ATPases and a protein of unknown function enhanced tick feeding.

Further characterization of cDNAs that affected larval development (Table 3) was conducted for all clones except for 4D8, 4F8, 4D6 and 4E6, which produced high inhibition of tick infestation and are currently being studied separately as recombinant proteins expressed in *Escherichia coli*.

The pool of hsp70 and hsp60 cDNAs conferred partial protection against tick infestations and did not affect molting (Table 3). The cDNA sequences for hsp70 and hsp60 in clones 1C10 and 3F6, respectively, were partial and contained the region coding for the C-terminal of the protein, and were highly identical to other hsp70 sequences (data not shown).

The sequence of hsp70 contained a 3' untranslated region (UTR) of 299 bp before the poly-A tail. The clone 3E1 contained a cDNA identical to the beta-adaptin that produced a 27% inhibition of tick infestation and a 5% inhibition of molting to the nymphal stage after vaccination and tick challenge (Table 3). The complete sequence was determined for the clone 3E1 (Fig. 1A), and contained an insert of 1,942 bp encoding for a predicted protein of 191 amino acids. The sequence of this protein was shorter than that for other beta-adaptins (Fig. 1B), suggesting that it could encode for a beta-adaptin appendage or it may be a partial cDNA sequence because of a long 3' UTR of 1,334 bp located before the poly-A tail.

The cDNA in clone 4G11 was identical to a chloride channel but it contained only a partial sequence (Fig. 2A). This sequence protected against tick infestations and inhibited larval molting (Table 3). Chloride channels have been found in living organisms from bacteria to mammals, with some amino acid positions being conserved in all sequences (Fig. 2A). As expected, phylogenetic analysis of chloride channel sequences demonstrated that the *I.*

scapularis sequence comprised a sister group to other insect sequences that have been reported (Fig. 2B).

Vaccination with ribosomal sequences had some inhibitory effect on tick infestations but did not affect molting (Table 3). The pool of ribosomal cDNAs included EST sequences coding for cellular and mitochondrial ribosomal proteins and translation factors (Table 4), and these genes are highly conserved across species. However, proteins encoded by *I. scapularis* ESTs were 43% to 95% identical to arachnida or insect sequences and 36% to 85% identical to mouse sequences (Table 4). The cDNA in clone 2C12 that was found to be identical to the beta-amyloid precursor protein (APP) contained a fragment encoding for the C-terminal of the protein (Fig. 3), suggesting that it contains a partial cDNA with a long (1,400 bp) 3' UTR. Nonetheless, the C-terminal sequence of the *I. scapularis* APP contained regions of amino acids identical to fly and mosquito sequences (Fig. 3). Vaccination with this cDNA resulted in 8% enhancement of larval feeding (Table 3). The complete sequence of clone 4F1 cDNA was determined and contained an isnert of 2475 bp woth 30 bp and 66 bp of 5' and 3' UTR, respectively and a poly-A tail of 114 bases.

An open reading frame of 2,265 bp encoded for a protein of 754 amino acids that was identical to mouse block of proliferation (Bop 1) (Fig. 4).

Similar proteins have been identified in other organisms including *Drosophila melanogaster, Anopheles gambiae* and humans (Fig. 4), suggesting that this protein has been highly conserved during evolution. The clone 3E10 had a pronounced stimulatory effect on larval feeding (Table 3). This clone was completely sequenced and contained an insert of 1,848 bp with 50 bp and 279 bp of 5' and 3' UTR, respectively and a short poly-A tail of 24 bases. An open reading frame of 1,494 bp encoded for a protein of 497 amino acids that was identical to

mannose-binding lectins found in many eukaryotes (Fig. 5). A similar sequence was described in *A. variegatum* ESTs, which clustered together with the *I. scapularis* sequence (Fig. 5).

The clone 3C12, together with clone 2F9, produced the greatest enhancement of tick feeding after vaccination and tick challenge (Table 3). The clone 3C12 was completely sequenced and contained an insert of 447 bp with 5 bp and 86 bp of 5' and 3' UTR, respectively and a short poly-A tail of 29 bases. An open reading frame of 327 bp encoded for a protein of 108 amino acids that was identical to RNA polymerase III, and had a high degree of identity with human and insect sequences (Fig. 6A). The EST in clone 2F9 was identical to human and *A. variegatum* sequences coding for proteins of unknown function (Fig. 6B).

Vaccination with the pool of ESTs identical to ATPases resulted in a 57% increase in larval feeding (Table 3). This pool originally contained 6 sequences (Almazán et al., 2003) but only 3 were non-redundant (clones 1A9, 1B2 and 4A4). All sequences were identical to vacuolar proton pump ATPases (EC 3.6.1.34). The sequence of 1A9 was identical to *D. melanogaster* (TC112371) V-ATPase subunit D, 1B2 was identical to *A. americanum* (AAU03374) V-ATPase subunit C and 4A4 was identical to *D. melanogaster* (TC112172) V-ATPase subunit E.

Six clones of the *I. scapularis* ESTs contained short tandem repeat (STR) microsatellite sequences. STRs were found in 5 clones (1F4, 2C7, 3B6, 4G12 and 4H2) containing sequences of unknown function and in one clone (1A9) that was identical to the *D. melanogaster* V-ATPase subunit D (Table 1). Microsatellite sequences contained perfect and imperfect STRs (Table 5). Clones 1A9, 4G12 and 3B6 contained 9, 6 and 12 TA repeats, respectively. Clone 1F4 contained an imperfect repeat of 15 GC/T and the clone 2C7 contained 9 GT repeats. The clone 4G12 contained a second STR of 10 CA/GA/CT repeats.

IV. Discusion

The feasibility of controlling tick infestations through immunization of hosts with tick antigens has been demonstrated previously for *Boophilus* spp. (reviewed by Willadsen, 1997; Willadsen and Jongejan, 1999; de la Fuente et al., 1999, 2000a). However, a limiting step for development of effective anti-tick vaccines is the identification of tick protective antigens. In the past, tick protective antigens were identified by (a) evaluating proteins after host immunization and tick challenge that were derived from progressive fractionation of crude tick extracts, (b) immunomapping of tick antigens which elicit an antibody response in the infested host, and (c) testing tick proteins in vaccination experiments that were considered to be important for the parasite function and/or survival.

However, construction of cDNA libraries and EST databases from different tick tissues, developmental stages and from genes expressed in response to various stimuli (i.e., tick feeding or infection with pathogens) of cDNAs encoding for tick immunosuppressants, anticoagulants and other proteins with low antigenicity that may enhance tick feeding provides new exciting possibilities for screening and identifying antigens protective against tick infestations. This approach may also allow for identification of antigens that interfere with pathogen development and transmission. Alternatively, they may encode for proteins homologous to host proteins associated with anti-tick or growth suppression activity which neutralization results in a tick pro-feeding effect. The former could be the case for ATPases. These proteins are highly conserved across species and, therefore, could elicit a poor immune response. However, ATPases are expressed in tick embryos and salivary glands of unfed adults and adult females at all stages of feeding and some evidences suggest that these proteins may participate in salivary fluid secretion in *A. americanum* (McSwain et al., 1997).

Therefore, although the mechanism is not known, DNA vaccination with ATPase-coding cDNAs could produce enhanced larval feeding. Although we presently do not have evidence to support the latter hypothesis, proteins of unknown function, such as the one encoded by clone 2F9 that is identical to host proteins of unidentified function, and Bop 1, a nonribosomal protein that is highly conserved from yeast to human with a growth suppressor function that plays a key role in the formation of mature 28S and 5.8S rRNAs and in the biogenesis of the 60S ribosomal subunit (Pestov et al., 1998; Strezoska et al., 2000), are examples that may enhance tick feeding.

Nonetheless, cDNAs associated with enhanced tick feeding could be made as recombinant proteins to modify their immunogenicity and then be evaluated as candidate protective antigens. Additionally, these antigens may also be good candidates for blocking the transmission of tick-borne pathogens (Wikel et al., 1997; Labuda et al., 2002).

The enhanced feeding effect of cDNA clones with identity to App (2C12), mannose-binding lectin (3E10) and RNA polymerase III (3C12) is difficult to explain. The beta-amyloid protein precursor is involved in different physiological processes, including development of the embryonic nervous system in *D. melanogaster* (Rosen et al., 1989) and pharyngeal pumping in *Caenorhabditis elegans* (Zambreano et al., 2002). The sequence contained in clone 2C12 corresponded to the beta-amyloid peptide (ß-AP), a ≈40 amino acids peptide derived from the APP protein found as the major component of dense plaques in brains of Alzheimer disease patients (reviewed by Cummings, 2003). Vaccination with ß-AP prevented the formation of ß-AP plaques in transgenic mice, opening a new possible approach for treatment of Alzheimer disease (McGeer and McGeer, 2003). However, we do not understand the apparent enhanced

feeding effect of the tick ß-AP in cDNA-vaccinated mice. The lectin in clone 3E10 was identical to mannose-binding endoplasmic reticulum-Golgi intermediate compartment protein (Arar et al., 1995; Lahtinen et al., 1996). However, the carbohydrate-binding domain is shared by other lectins found in different cell compartments. The clone 3C12 encoded for an RNA polymerase III. Enhanced tick feeding was produced in mice vaccinated with a DNA pool containing this clone and clone 2F9 of unknown function. It is therefore possible that the enhanced feeding effect on tick larvae was due to clone 2F9 with little or no contribution of clone 3C12.

Microsatellites are a class of genetic markers that are composed of STR sequences flanked by unique DNA sequences (Hearne et al., 1992). STRs are highly polymorphic and widely distributed through the genome. The analysis of tick STRs has been used for identification of strains of *B. microplus* (de la Fuente et al., 2000b) and for the development of a preliminary genetic linkage map of *I. scapularis* (Ullman et al., 2003). The STR sequences described in this study could be used for completion of the genetic map of *I. scapularis* as the first step toward the sequencing of this tick genome.

Most sequences in the *I. scapularis* EST data set were relatively G + C rich, with an average G + C content of 54%, similar to the 52% reported by Nene et al. (2002) for *A. variegatum*. The few sequences with a high A + T content probably corresponded to mitochondrial genes, corroborating the hypothesis that there is a marked difference in codon usage between mitochondrial and nuclear protein coding genes in the Ixodidae (Nene et al., 2002).

Recently, Almazán et al. (2003) used cDNA ELI combined with EST analysis as a rapid method for the identification of protective antigens against *I. scapularis* infestations, demonstrating the role of sequence information in conjunction with new technologies such as bioinformatics and ELI for a systematic and comprehensive approach to vaccine discovery.

One of the advantages of ELI for identification of protective antigens is that *a priori* criteria are not introduced to direct the selection of candidate genes. This approach, as shown in this study, resulted in potential vaccine antigens otherwise not predicted, such as clone 4F8 that was found to be identical to a nucleotidase. However, nucleotidases are essential for cell growth and the inhibition of its enzymatic activity would be cytotoxic (Spiegelberg et al., 1999), providing a possible explanation for their protective properties against tick infestations. The *I. scapularis* sequence in clone 4F8 was different from the 5'-nucleotidase that was identified and characterized previously by Liyou et al. (1999, 2000) in *B. microplus*. However, the protective capacity of this protein has not been evaluated.

As discussed previously by Almazán et al. (2003), a possible explanation for the inhibitory effect on larval tick development of other vaccine candidates that were identified in this study is based on the role that they play in cell growth and maintenance, which is evident for clones identical to beta-adaptin (3E1) and chloride channel (4G11). Beta adaptins are adaptor components required in the assembly of clathrin-coated plasma membrane pits that function in cell vesicular transport mechanisms including endocytosis (Camidge and Pearse, 1994; Boehm and Bonifacino, 2002), a process actively involved in blood digestion by ticks and other hematophagous arthropods (Akov, 1982). Chloride channels are also involved in vital cell functions including the catalysis of counter ion currents that accompany primary

proton fluxes in endosomal and lysosomal acidification (Koprowski and Kubalski, 2001; Iyer et al., 2002). Therefore, interference with the process of endocytosis may impair acquisition and digestion of the tick bloodmeal and result in inhibition of tick infestations. Another *I. scapularis* EST (clone 3E12) encoded for a protein identical to *D. melanogaster* clathrin heavy chain, a protein involved in synaptic vesicle endocytosis (Chang et al., 2002). This cDNA is also a candidate protective antigen because it interfers with endocytosis in feeding larvae.

The protection capacity of ribosomal and heat shock protein preparations has been documented previously in other organisms (Elad and Segal, 1995; Silva, 1999; Melby et al., 2000; Cassataro et al., 2002). Recently, Hsp70 was demonstrated to be induced in *I. ricinus* salivary glands during blood feeding (Leboulle et al., 2002), documenting the role of heat shock proteins in physiological responses in ticks. Even in the case where substantial homology exists between tick proteins and host (mouse) proteins, analysis of ribosomal proteins suggests that differences in the amino acid sequence could direct the host immune response against distinctive, non-self epitopes, which could be sufficient to induce a protective response.

The results of vaccination and tick infestation demonstrated that some cDNAs enhance tick feeding. This effect could be due to the expression corroborating the hypothesis that there is a marked difference in codon usage between mitochondrial and nuclear protein coding genes in the Ixodidae (Nene et al., 2002)

Most of the ESTs in our database, although initially identified by ELI of cDNA pools that produced inhibition of tick infestation, were not characterized further and remain potential candidate antigens for vaccine development against *I. scapularis* infestations. Particularly interesting were cDNAs that may be involved in developmental processes. Clone

4B2, identical to *D. melanogaster* sequence NP_523710, encoded for calmodulin, a Ca^{++}-binding protein of 149 amino acids that is involved in fly development. This protein was found to be expressed in several larval and adult tissues, including the larval midgut (Takamatsu et al., 2002). Clone 1C8 had a low degree of identity to *D. melanogaster* virilizer, a gene involved in Sex-lethal (Sxl) splicing and essential for fly male and female viability and embryonic development (Niessen et al., 2001). Clone 2A11 also had a low degree of identity to *D. melanogaster* developmental regulator, Notchless, a key player in the signaling by Notch family receptors that are involved in many cell-fate decisions during development (Royet et al., 1998). Similarly, clone 4A10 had partial identity to the putative homeodomain transcriptional factor, phtf, a member of a gene family that plays an important role during development and is conserved between fly, mouse and human (Manuel et al., 2000). Other clones with special interest as vaccine candidates may include those identical to membrane proteins (1D8, 1D11, 3G11) and those putatively involved in G-protein-coupled signaling (2B7, 2F12, 4C9). In fact, the clone 3G11 was identical to *D. melanogaster* BM-40, a protein of the group of extracellular basement membrane proteins which includes the protective antigen p29 from *Harmaphysalis longicornis* (Mulenga et al., 1999).

In summary, we have characterized *I. scapularis* EST sequences that were selected by cDNA ELI in the mouse/tick challenge model because they affected tick development. Characterization of these ESTs provides a basis for future research on ticks and is a source of candidate antigens for use in vaccine development designed to control tick infestations and/or reduce transmission of pathogens. The combination of ELI with EST appears to be a productive systematic and comprehensive approach to vaccine discovery.

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Table 1. cDNA clones with identity to previously reported sequences.

cDNA clone	Predicted protein	GenBank accession number
1C11	Translation initiation factor 5A (Eif5A)	CD052489
1E6	Translation initiation factor 5C (eIF-5C)	CD052490
2D2	Initiate factor 5 (if5)	CD052491
1A10	Elongation factor 2	CD052492
4F7	Elongation factor 1alpha	CD052493
1F6	Ribosomal protein S4 (RpS4)	CD052494
2B8	Ribosomal protein S11 (RpS11)	NR
2F8	Laminin receptor 1 (ribosomal protein SA)	CD052496
2F10	Ribosomal protein L3 (RpL3)	NR
3A10	Ribosomal protein L7A (RpL7A)	CD052497
3G9	Ribosomal protein S8 (RpS8)	CD052495
3G10	Ribosomal protein L27A (RpL27A)	CD052498
3C3	QM homolog (DQM) ribosomal protein	CD052499
4D12	Proteasome/Signalosome subunit	CD052500
4E7	Proteasome subunit	CD052501
4D11	Proteasome subunit	CD052502
3D10	Ribophorin I	CD052503
1B12	Ubiquitin-conjugating enzyme	CD052504
1D10	Ubiquitin	CD052505
1A9	V-ATPase Conjugated enzyme Contains mcirosatellite sequence	CD052506
1B2	V-ATPase C subunit	CD052507
4A4	V-ATPase E subunit	CD052508
1C5	Na+/K+ ATPase, alpha subunit	CD052509
2A9	NADH dehydrogenase	CD052510
1D6	NADH dehydrogenase subunit 5 (nad5)	CD052511
1A4	Aldehyde dehydrogenase	CD052512
1C8	Virilizer (vir)	CD052513
1C10	Hsp70	CD052514
3F6	Hsp60	CD052515
1D1	Nucleotide binding protein 1 (Nubp1)	CD052516
1D8	Identity to *D. melanogaster* GH03607 full length cDNA coding for a putative membrane protein	CD052517
1D11	Putative membrane protein	CD052518
1E7	Sterol carrier protein	CD052519
1F3	Cyclin C (CycC)	CD052520
3D9	Alpha tubulin	CD052521
2A7	Beta tubulin	CD052522
2A11	Notchless (Nle)	CD052523
2B2	Export factor binding protein 2 (Refbp2)	CD052524
2B7	G protein-coupled receptor	CD052525
2B9	Succinate dehydrogenase B (SdhB)	CD052526

2C12	Beta-amyloid precursor protein (APP	CD052527
2D1	Fructose-1,6-bisphosphatase (fbp gene)	CD052528
2D5	DNA repair protein Rad1 (Rad1)	CD052529
2D6	Identity to *S. pombe* dim1+, helicase protein 1	CD052530
2E8	Esterase	CD052531
2F9	Identity to AvGI TC255 (*A. variegatum*) & hypothetical protein FLJ12475 (*H. sapiens*)	CD052532
2F12	Transmembrane G-protein-responsive adenylyl cyclase	CD052533
2G8	Lysyl-tRNA synthetase	CD052534
2H11	Sodium- and chloride-dependent taurine transporter	CD052535
3C12	RNA polymerase III	CD052536
3E1	Beta-adaptin	CD052537
3E2	Microtubule-associated protein, RP/EB family	CD052538
3E4	Myosin II regulatory light chain	CD052539
3E6	Unknown Zinc finger like protein	CD052540
3E10	Mannose binding lectin (rhea)	CD052541
3E12	Clathrin heavy chain (Chc)	CD052542
3F4	Identity to *M. musculus* adult male testis cDNA	CD052543
3F10	Identity to *D. melanogaster* P-element somatic inhibitor (Psi)	CD052544
3G11	Identity to *D. melanogaster* BM-40 extracellular basement membrane protein	CD052545
4A8	Identity to *D. melanogaster* regulator of gene transcription (Chi)	CD052546
4A10	Identity to D. melanogaster homeoprotein phtf	CD052547
4A12	Amino acid transporter system A (ATA2)	CD052548
4B2	Calmodulin	CD052549
4B7	Alpha-tubulin	CD052550
4C9	Identity to *D. melanogaster* transducin (G protein)-like enhancer of split 3, homolog of E(spl)	CD052551
4C11	Intracellular receptor of activated protein kinase C1 (Rack1)	CD052552
4D6	Identity to *D. melanogaster* CG10395 cDNA	CD052553
4D7	Identity to *D. melanogaster* LD23959 cDNA	CD052554
4E6	Identity to *D. melanogaster* CG13597 cDNA	CD052555
4D8	Identity to *H. sapiens* hypothetical protein FLJ10342	CD052556
4E1	Pre-mRNA splicing factor	CD052557
4E3	Receptor signaling protein serine/threonine kinase	CD052558
4F8	Nucleotidase	CD052559
4F1	Block of proliferation 1 (Bop1)	CD052560
4G1	Identity to *H. sapiens* hypothetical protein MGC2404	CD052561
4G2	LRP/alpha-2-macroglobulin receptor	CD052562
4G5	Disulfide isomerase	CD052563
4G8	Fumarate hydratase	CD052564
4G10	Rab3D (member of the Ras superfamily of small GTPases)	CD052565

4G11	Chloride channel	CD052566
4H4	Solute carrier protein	CD052567
1B7	Mitochondrion	NR
1B8	Mitochondrion	NR
2E9	Mitochondrion	NR
2G11	Mitochondrion	NR
3C6	Mitochondrion	NR
3G4	Mitochondrion	NR
4A2	Mitochondrion	NR
4E9	Mitochondrion	NR
2A6	Mitochondrion	NR
4G7	NAD-dependent malate dehydrogenase	NR
3D4	Cytochrome c oxidase I (COI)	NR
1C2	Cytochrome c oxidase II (COII)	NR
4D2	Cytochrome c oxidase III (COIII)	NR
1G4	Cytochrome b (cytb)	NR
2G9	16S ribosomal RNA	NR
	Unknown	CD052568
1F4	Identity to *I. scapularis* clone AC22 microsatellite sequence (AF331735)	
2C7	Unknown Contains microsatellite sequence	CD052569
3B6	Unknown Contains a microsatellite sequence	CD052570
4G12	Unknown Contains microsatellite sequence	CD052571
4H2	Unlnown Contains microsatellite sequence	CD052572

NR, Not reported to the EST database for being identical to mitochondrial sequences.

Table 2. *I. scapularis* gene ontology assignments.

Category	Number of clones	% of 87 clones with gene ontology assignments	% of 102 clones with identity to reported sequences
Cellular component			
Cell	32	36.78	31.88
Mitochondria	17	15.54	16.83
Cell membrane	14	16.09	13.86
Nucleus	11	12.64	10.89
Extracellular	2	2.30	1.98
Unlocalized	2	2.30	1.98
Unknown	9	10.34	8.91
Biological process			
Cell growth or maintenance	61	70.11	60.40
Physiological process	8	9.20	7.92
Developmental process	5	5.75	4.95
Cell communication	2	2.30	1.98
Unknown	11	12.64	10.89
Molecular function			
Ligand binding or carrier	30	34.48	29.70
Enzyme	29	33.33	28.71
Transporter	9	10.34	8.91
Chaperone	2	2.30	1.98
Structural molecule	7	8.05	6.98
Unknown	10	11.49	9.90

Gene ontology assignments were made according to Ashburner et al. (2000) for non-redundant EST sequence data with the help of GoFish v.1.0 (Berriz et al., 2003). The number of clone sequences falling into each category are listed and then calculated as a percent of clones for which gene ontology was assigned and the total number of clones for which identity to previously published sequences was found.

Table 3. Summary of results of DNA vaccination and challenge with *I. scapularis* larvae in the mouse model of tick infestations.

cDNA clone	Predicted protein	Inhibition of tick infestation I (%)	Inhibition of molting M (%)
4D8	Identity to *H. sapiens* hypothetical protein FLJ10342 with unknown function	40 [a]	7 [a]
4F8	Nucleotidase	50 [a]	17 [a]
1C10 [b]	Hsp70	17 [a]	0 [a]
3F6 [b]	Hsp60		
4D6	Identity to *D. melanogaster* CG10395 cDNA with unknown function	61	11
4E6	Identity to *D. melanogaster* CG13597 cDNA with unknown function	20	ND
3E1	Beta-adaptin	27	5
4G11	Chloride channel	38	30
17 clones [b]	Ribosomal proteins	15 [a]	0 [a]
2C12	Beta-amyloid precursor protein (APP)	-8 [c]	ND
4F1	Block of proliferation Bop1	-39 [c]	ND
3E10	Mannose binding lectin	-48 [a, c]	ND
3C12 [b]	RNA polymerase III		
2F9 [b]	Identity to *A. variegatum* AvGI TC255 & *Homo sapiens* hypothetical protein FLJ12475 with unknown functions	-104 [a, c]	ND
1A9, 1B2, 4A4 [b]	ATPase	-57 [a, c]	ND

[a] Data reported by Almazán et al. (2003). For all other experiments, mice were immunized with cDNA-containing expression plasmid DNA as described above. I and M were calculated as described in Materials and Methods section. ND, not determined.

[b] Pooled together for vaccination experiments by ELI (Almazán et al., 2003) (1C10 and 3F6, cDNA pool "Heat shock"; 3C12 and 2F9, cDNA pool "Secreted protein"; ribosomal clones, cDNA pool "Ribosomal"; 1A9, 1B2 and 4A4, cDNA pool "ATPase").

[c] Resulted in a tick pro-feeding activity after mouse vaccination and tick challenge.

Table 4. Characterization of *I. scapularis* ESTs encoding for ribosomal proteins.

Clone	Encoded protein	Identical amino acids	Species	GenBank accession number
4F7	Elongation factor 1-alpha	95%	*Neacarus texanus*	AAK12660
1A2		85%	*Mus musculus*	NP_031932
1A10	Elongation factor-2	88%	*Mastigoproctus giganteus*	AAK12348
		80%	*Mus musculus*	BAC26203
1C11	eIF-5A	65%	*Drosophila melanogaster*	AAM68297
		59%	*Mus musculus*	XP_203336
1F6	RpS4	79%	*Spodoptera frugiperda*	AAL26580
2C3		75%	*Mus musculus*	AAH09100
2B8	RpS11	92%	*Dermacentor variabilis*	AAO92287
		80%	*Mus musculus*	XP_133477
2F8	Laminin receptor 1 (RpSA)	66%	*Anopheles gambiae*	EAA00413
		73%	*Mus musculus*	NP_035159
2F10	RpL3	70%	*Spodoptera frugiperda*	AAL62468
		68%	*Mus musculus*	AAH09655
3A10	RpL7A	55%	*Drosophila melanogaster*	NP_511063
		60%	*Mus musculus*	A30241
3D10	Ribophorin I	57%	*Drosophila melanogaster*	AAN71150
		50%	*Mus musculus*	BAC26679
3G9	RpS8	70%	*Spodoptera frugiperda*	AAL62472
		71%	*Mus musculus*	XP_134904
3G10	RpL27A	42%	*Spodoptera frugiperda*	AAK92158
		36%	*Mus musculus*	XP_137118
4D11	Proteasome subunit	60%	*Drosophila melanogaster*	NP_524115
		55%	*Mus musculus*	NP_035315
4D12	Proteasome/Signalosome subunit	43%	*Anopheles gambiae*	EAA11895
		56%	*Mus musculus*	AAC33900
4E7	Proteasome subunit	84%	*Anopheles gambiae*	EAA10351
		85%	*Mus musculus*	NP_036096

The sequences of *I. scapularis* ESTs identical to ribosomal proteins, which were pooled for DNA vaccination in Almazán et al. (2003), were compared to all non-redundant sequences in GenBank DNA and protein databases (1,419,727 sequences total; Apr-09-2003) using BLASTX 2.2.6 (Altschul et al., 1997). The percent of identical amino acids to arachnida or insect and mouse sequences are shown together with their corresponding GenBank accession number. The GenBank accession numbers for *I. scapualris* sequences are shown on Table 1.

Table 5. Microsatellite STR sequences in *I. scapularis* ESTs.

cDNA clone	Microsatellite sequence
1A9	TATATATATATATATATA
4G12	CACACACAGACACACTCACA ATATATATATATA
1F4	GCGCGCGCGTGTGCGTGTGTGTGTGTGTGT
2C7	GTGTGTGTGTGTGTGTGT
3B6	TATATATATATATATATATATATATA
4H2	TGAAATGAAATGAAATGAAA

A

cg**ATG**CAGGCGATGACGGGCTTTGCGGTGCAGTTCAACAAAAACAGTTTCGGGCTGACTC
CAGCTCAGCCGCTGCAGTTGCAGATTCCCCTGCAGCCCAACTTCCCAGCTGATGCGAGCT
TGCAGCTGGGAACCAACGGTCCCGTGCAGAAGATGGACCCCCTCACCAACCTTCAGGTGG
CCATCAAGAACAATGTGGACGTGTTCTACTTCAGCTGCCTGGTGCCCATGCACGTGCTGA
GCACGGAGGACGGCCTGATGGACAAGCGGGTGTTCCTGGCCACCTGGAAAGACATCCCCG
CCCAAAACGAGGTCCAGTACACCCTCGACAACGTCAACCTCACTGCAGACCAAGTTTCCC
AGAAGCTGCAGAACAACAACATTTTCACGATAGCCAAGAGGAACGTGGACGGCCAGGACA
TGCTGTACCAGTCCCTGAAGCTCACCAACGGCATTTGGGTGTTGGCGGAGCTCAAGATAC
AGCCCGGCAATCCAAGGATCACGTTGTCTTTGAAGACAAGAGCACCTGAAGTGGCAGCAG
GTGTACAACAAACTTACGAACTCATTCTACACAGC**TGA**ggctgctgtgaatgaaactctt
ctcccacccccttcttttgatggcagtcaatgtctcgtttcattttcttgttttctttttg
cggcgtgctacggaacaaggtcctacattcccaagttatatggtgttgtcgcgtaggggg
cagagtgccgctgagcccgcgacagccttgtttctgaggagagccgaacgcaccacttcg
aaaaagaaaaagtgaaaacggaaaaatgaaaaattttccagttgcttcaaattaacattc
ctcgtagtcagtctgtggccgttgagtttggtgtaaagaagaaaaaggtgtctcttttag
tgaaaatggttgctttttattggtatcccctatcacaccgagcacgaacataagaaatcc
tgacaaggattctcctttagttgtattatggtggctggagcacacgaggcacctgttgcc
aattcgacccagcaaatgcccaattctcaagatttgagttcattgaggttgttttgctcc
tccccccccacccccccaactttgtcgttggattgtctaacagtgtaaatgggcgacgact
cgttattctttttttcttcattctttcttttgttgtcacgcgccccgggggacgcgaca
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tgaaactctgctccgccgctgctagtgtcatcacgggacgaccatcgcgttttctctgac
tatttaaacaaaactgcatagcttaggggggcagtctgtgcaaagtggaacaaccaaactg
agccctgccctttcggtgtgtgtacaagcatctctgtgtaacatgaactactttacatga
actacattgcatgaacgggagaagtttagttgtttttttgtttttttttttcaggtgacta
tgtcaacagattagaaccatttttggaacggctggaaagataaccgctcattttgtttc
tactaaaagactacgaaaagtgttgacttttgcatcggtttggcaacgtttgtttggca
tgcatgtagttgagcgtaatggtatcacccctcgtaaacaataacagtgcaatggagcag
tactgtagtgtccattaaagagcgagagtttggttaaaggttgttaattgaggtccgtgt
tatcctttgagtaggagagcggcacttttgcaaatagcgctgctgggggcgtcatatct
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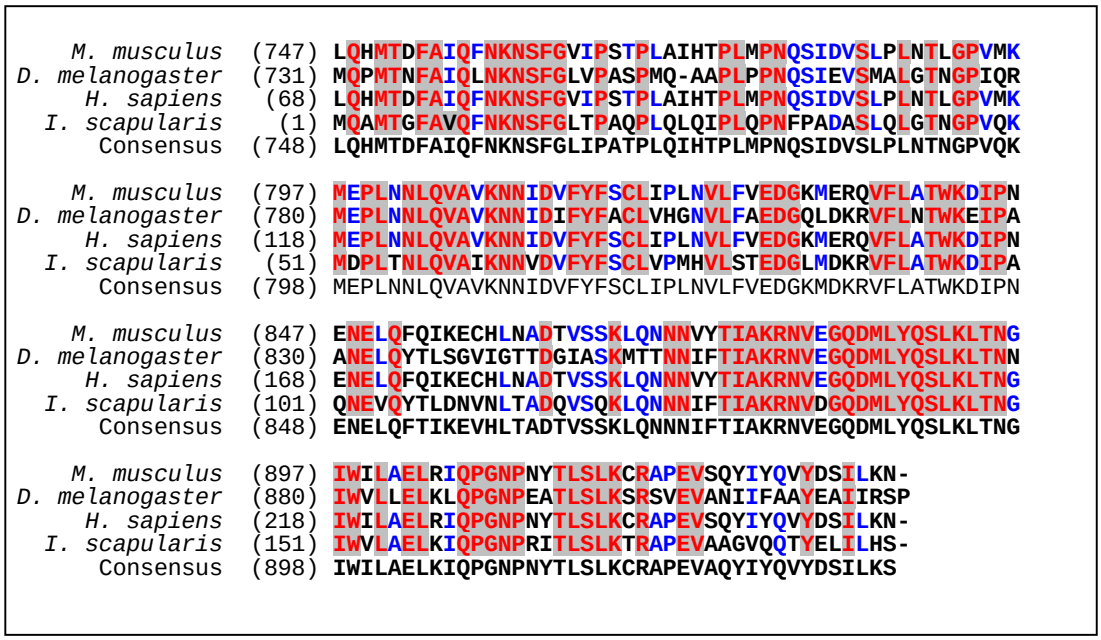
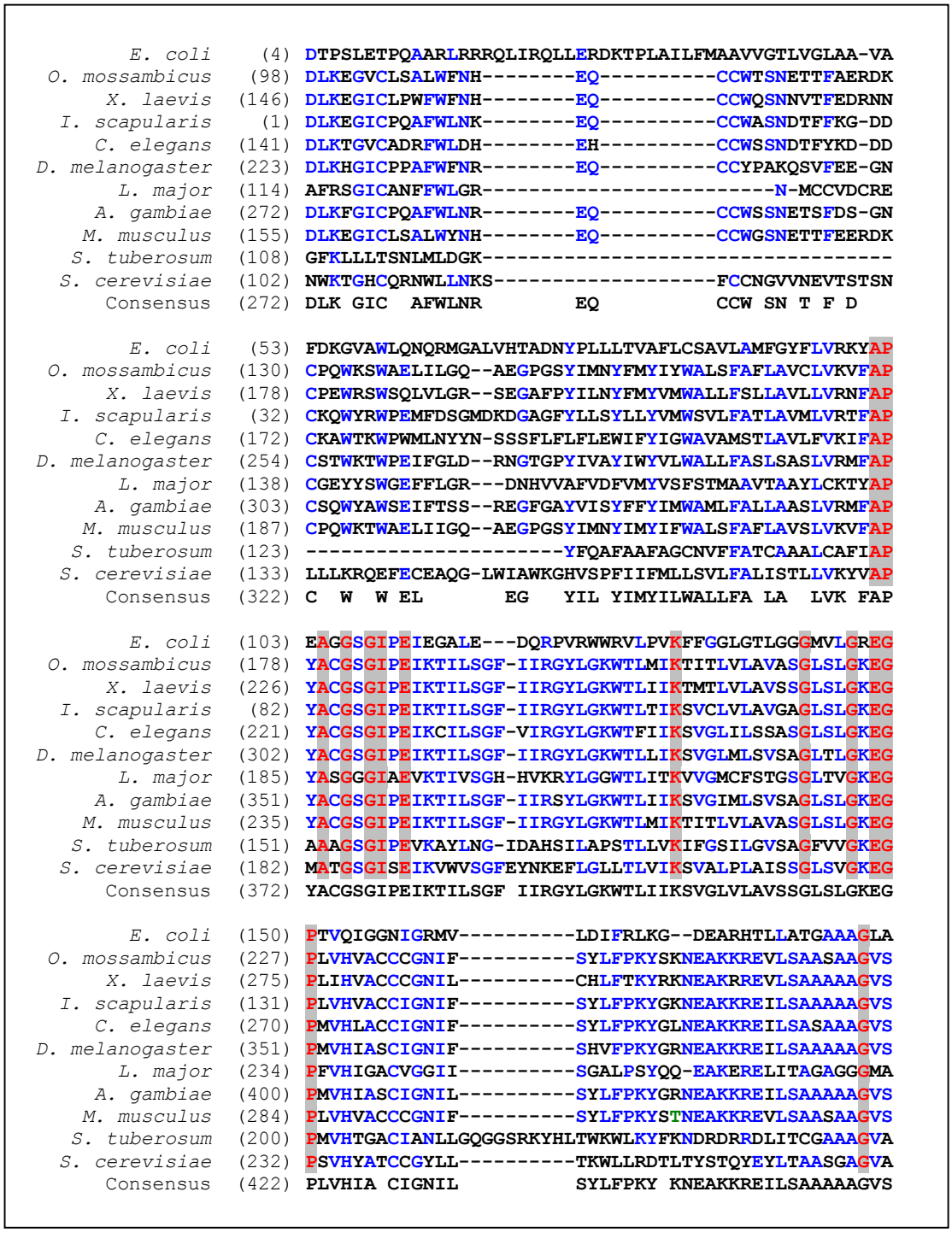
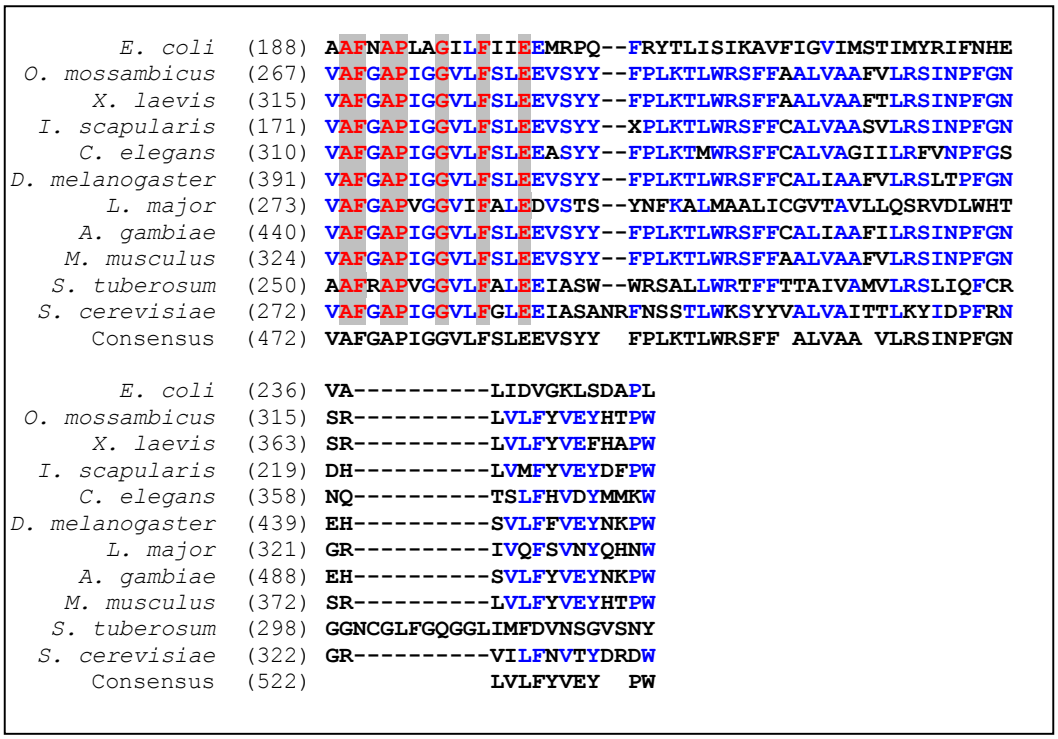


Figure 1. Analysis of clone 3E1 identical to beta-adaptin. (A) Nucleotide sequence of full cDNA. Non-coding sequence is shown in lower case letters and coding sequence is shown in capital letters with translation initiation and termination codos in bold letters. (B) Alignment of *M. musculus* (GenBank accession number XP_109938), *D. melanogaster* (CAA53509) and *Homo sapiens* (AAA35583) protein sequences and the translation product of clone 3E1 identified as *I. scapularis* beta-adaptin appendage (AY296113). Protein sequences are shown in the single letter amino acid code. Identical amino acids are shown in red and amino acids conserved in 3 of 4 sequences are shown in blue.

A


E. coli (4) DTPSLETPQAARLRRRQLIRQLLERDKTPLAILFMAAVVGTLVGLAA-VA
O. mossambicus (98) DLKEGVCLSALWFNH--------EQ----------CCWTSNETTFAERDK
X. laevis (146) DLKEGICLPWFWFNH--------EQ----------CCWQSNNVTFEDRNN
I. scapularis (1) DLKEGICPQAFWLNK--------EQ----------CCWASNDTFFKG-DD
C. elegans (141) DLKTGVCADRFWLDH--------EH----------CCWSSNDTFYKD-DD
D. melanogaster (223) DLKHGICPPAFWFNR--------EQ----------CCYPAKQSVFEE-GN
L. major (114) AFRSGICANFFWLGR----------N-MCCVDCRE
A. gambiae (272) DLKFGICPQAFWLNR--------EQ----------CCWSSNETSFDS-GN
M. musculus (155) DLKEGICLSALWYNH--------EQ----------CCWGSNETTFEERDK
S. tuberosum (108) GFKLLLTSNLMLDGK----------
S. cerevisiae (102) NWKTGHCQRNWLLNKS----------FCCNGVVNEVTSTSN
Consensus (272) DLK GIC AFWLNR EQ CCW SN T F D
E. coli (53) FDKGVAWLQNQRMGALVHTADNYPLLLTVAFLCSAVLAMFGYFLVRKYAP
O. mossambicus (130) CPQWKSWAELILGQ--AEGPGSYIMNYFMYIYWALSFAFLAVCLVKVFAP
X. laevis (178) CPEWRSWSQLVLGR--SEGAFPYILNYFMYVMWALLFSLLAVLLVRNFAP
I. scapularis (32) CKQWYRWPEMFDSGMDKDGAGFYLLSYLLYVMWSVLFATLAVMLVRTFAP
C. elegans (172) CKAWTKWPWMLNYYN-SSSFLFLFLEWIFYIGWAVAMSTLAVLFVKIFAP
D. melanogaster (254) CSTWKTWPEIFGLD--RNGTGPYIVAYIWYVLWALLFASLSASLVRMFAP
L. major (138) CGEYYSWGEFFLGR---DNHVVAFVDFVMYVSFSTMAAVTAAYLCKTYAP
A. gambiae (303) CSQWYAWSEIFTSS--REGFGAYVISYFFYIMWAMLFALLAASLVRMFAP
M. musculus (187) CPQWKTWAELIIGQ--AEGPGSYIMNYIMYIFWALSFAFLAVSLVKVFAP
S. tuberosum (123) ----------YFQAFAAFAGCNVFFATCAAALCAFIAP
S. cerevisiae (133) LLLKRQEFECEAQG-LWIAWKGHVSPFIIFMLLSVLFALISTLLVKYVAP
Consensus (322) C W W EL EG YIL YIMYILWALLFA LA LVK FAP
E. coli (103) EAGGSGIPEIEGALE---DQRPVRWWRVLPVKFFGGLGTLGGGMVLGREG
O. mossambicus (178) YACGSGIPEIKTILSGF-IIRGYLGKWTLMIKTITLVLAVASGLSLGKEG
X. laevis (226) YACGSGIPEIKTILSGF-IIRGYLGKWTLIIKTMTLVLAVSSGLSLGKEG
I. scapularis (82) YACGSGIPEIKTILSGF-IIRGYLGKWTLTIKSVCLVLAVGAGLSLGKEG
C. elegans (221) YACGSGIPEIKCILSGF-VIRGYLGKWTFIIKSVGLILSSASGLSLGKEG
D. melanogaster (302) YACGSGIPEIKTILSGF-IIRGYLGKWTLLIKSVGLMLSVSAGLTLGKEG
L. major (185) YASGGGIAEVKTIVSGH-HVKRYLGGWTLITKVVGMCFSTGSGLTVGKEG
A. gambiae (351) YACGSGIPEIKTILSGF-IIRSYLGKWTLIIKSVGIMLSVSAGLSLGKEG
M. musculus (235) YACGSGIPEIKTILSGF-IIRGYLGKWTLMIKTITLVLAVASGLSLGKEG
S. tuberosum (151) AAAGSGIPEVKAYLNG-IDAHSILAPSTLLVKIFGSILGVSAGFVVGKEG
S. cerevisiae (182) MATGSGISEIKVWVSGFEYNKEFLGLLTLVIKSVALPLAISSGLSVGKEG
Consensus (372) YACGSGIPEIKTILSGF IIRGYLGKWTLIIKSVGLVLAVSSGLSLGKEG
E. coli (150) PTVQIGGNIGRMV----------LDIFRLKG--DEARHTLLATGAAAGLA
O. mossambicus (227) PLVHVACCCGNIF----------SYLFPKYSKNEAKKREVLSAASAAGVS
X. laevis (275) PLIHVACCCGNIL----------CHLFTKYRKNEAKRREVLSAAAAAGVS
I. scapularis (131) PLVHVACCIGNIF----------SYLFPKYGKNEAKKREILSAAAAAGVS
C. elegans (270) PMVHLACCIGNIF----------SYLFPKYGLNEAKKREILSASAAAGVS
D. melanogaster (351) PMVHIASCIGNIF----------SHVFPKYGRNEAKKREILSAAAAAGVS
L. major (234) PFVHIGACVGGII----------SGALPSYQQ-EAKERELITAGAGGGMA
A. gambiae (400) PMVHIASCIGNIL----------SYLFPKYGRNEAKKREILSAAAAAGVS
M. musculus (284) PLVHVACCCGNIF----------SYLFPKYSTNEAKKREVLSAASAAGVS
S. tuberosum (200) PMVHTGACIANLLGQGGSRKYHLTWKWLKYFKNDRDRRDLITCGAAAGVA
S. cerevisiae (232) PSVHYATCCGYLL----------TKWLLRDTLTYSTQYEYLTAASGAGVA
Consensus (422) PLVHIA CIGNIL SYLFPKY KNEAKKREILSAAAAAGVS


E. coli (188) AAFNAPLAGILFIIEEMRPQ--FRYTLISIKAVFIGVIMSTIMYRIFNHE
O. mossambicus (267) VAFGAPIGGVLFSLEEVSYY--FPLKTLWRSFFAALVAAFVLRSINPFGN
X. laevis (315) VAFGAPIGGVLFSLEEVSYY--FPLKTLWRSFFAALVAAFTLRSINPFGN
I. scapularis (171) VAFGAPIGGVLFSLEEVSYY--XPLKTLWRSFFCALVAASVLRSINPFGN
C. elegans (310) VAFGAPIGGVLFSLEEASYY--FPLKTMWRSFFCALVAGIILRFVNPFGS
D. melanogaster (391) VAFGAPIGGVLFSLEEVSYY--FPLKTLWRSFFCALIAAFVLRSLTPFGN
L. major (273) VAFGAPVGGVIFALEDVSTS--YNFKALMAALICGVTAVLLQSRVDLWHT
A. gambiae (440) VAFGAPIGGVLFSLEEVSYY--FPLKTLWRSFFCALIAAFILRSINPFGN
M. musculus (324) VAFGAPIGGVLFSLEEVSYY--FPLKTLWRSFFAALVAAFVLRSINPFGN
S. tuberosum (250) AAFRAPVGGVLFALEEIASW--WRSALLWRTFFTTAIVAMVLRSLIQFCR
S. cerevisiae (272) VAFGAPIGGVLFGLEEIASANRFNSSTLWKSYYVALVAITTLKYIDPFRN
Consensus (472) VAFGAPIGGVLFSLEEVSYY FPLKTLWRSFF ALVAA VLRSINPFGN
E. coli (236) VA----------LIDVGKLSDAPL
O. mossambicus (315) SR----------LVLFYVEYHTPW
X. laevis (363) SR----------LVLFYVEFHAPW
I. scapularis (219) DH----------LVMFYVEYDFPW
C. elegans (358) NQ----------TSLFHVDYMMKW
D. melanogaster (439) EH----------SVLFFVEYNKPW
L. major (321) GR----------IVQFSVNYQHNW
A. gambiae (488) EH----------SVLFYVEYNKPW
M. musculus (372) SR----------LVLFYVEYHTPW
S. tuberosum (298) GGNCGLFGQGGLIMFDVNSGVSNY
S. cerevisiae (322) GR----------VILFNVTYDRDW
Consensus (522) LVLFYVEY PW

B

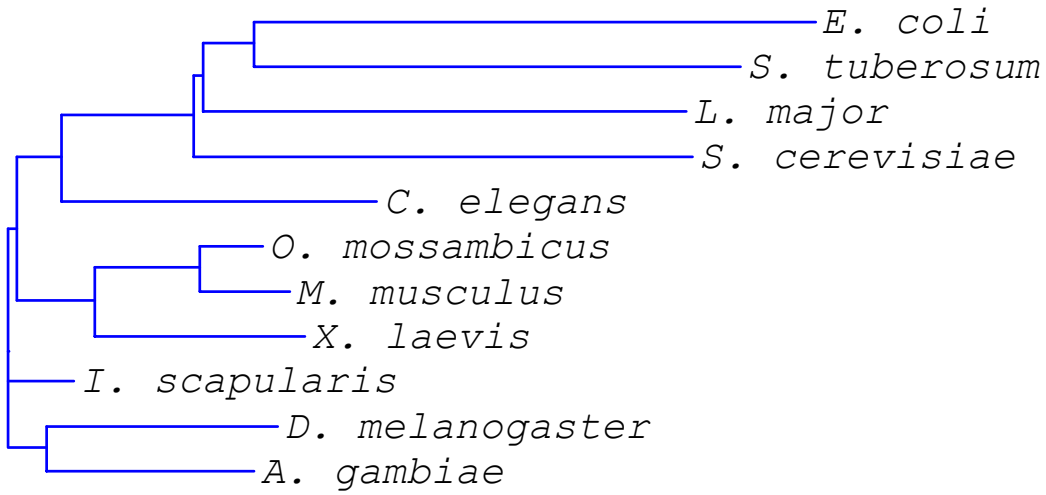


Figure 2. Analysis of clone 4G11 identical to chloride channel. (A) Alignment of *M. musculus* (XP_134186), *D. melanogaster* (AAM76180), *Solanum tuberosum* (T07608), *Oreochromis mossambicus* (AAD56388), *A. gambiae* (EAA11899), *C. elegans* (NP_495940), *Leishmania major* (strain Friedlin) (T02805), *Saccharomyces cerevisiae* (P37020), *Escherichia coli* K12 (AAC73266), and *Xenopus laevis* (CAA71071) protein sequences and the translation product of clone 4G11 identified as a fragment of *I. scapularis* chloride channel (AY296114). Protein sequences are shown in the single letter amino acid code. Identical amino acids are shown in red and amino acids conserved in 6-10 of 11 sequences are shown in blue. (B) Phylogenetic tree constructed from analysis of chloride channel protein sequences based on a sequence distance method utilizing the Neighbor Joining algorithm of Saitou and Nei (1987).

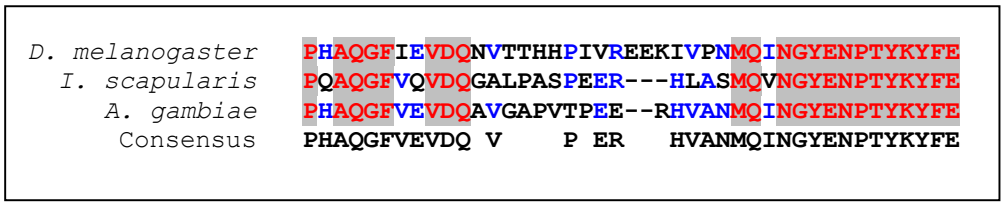
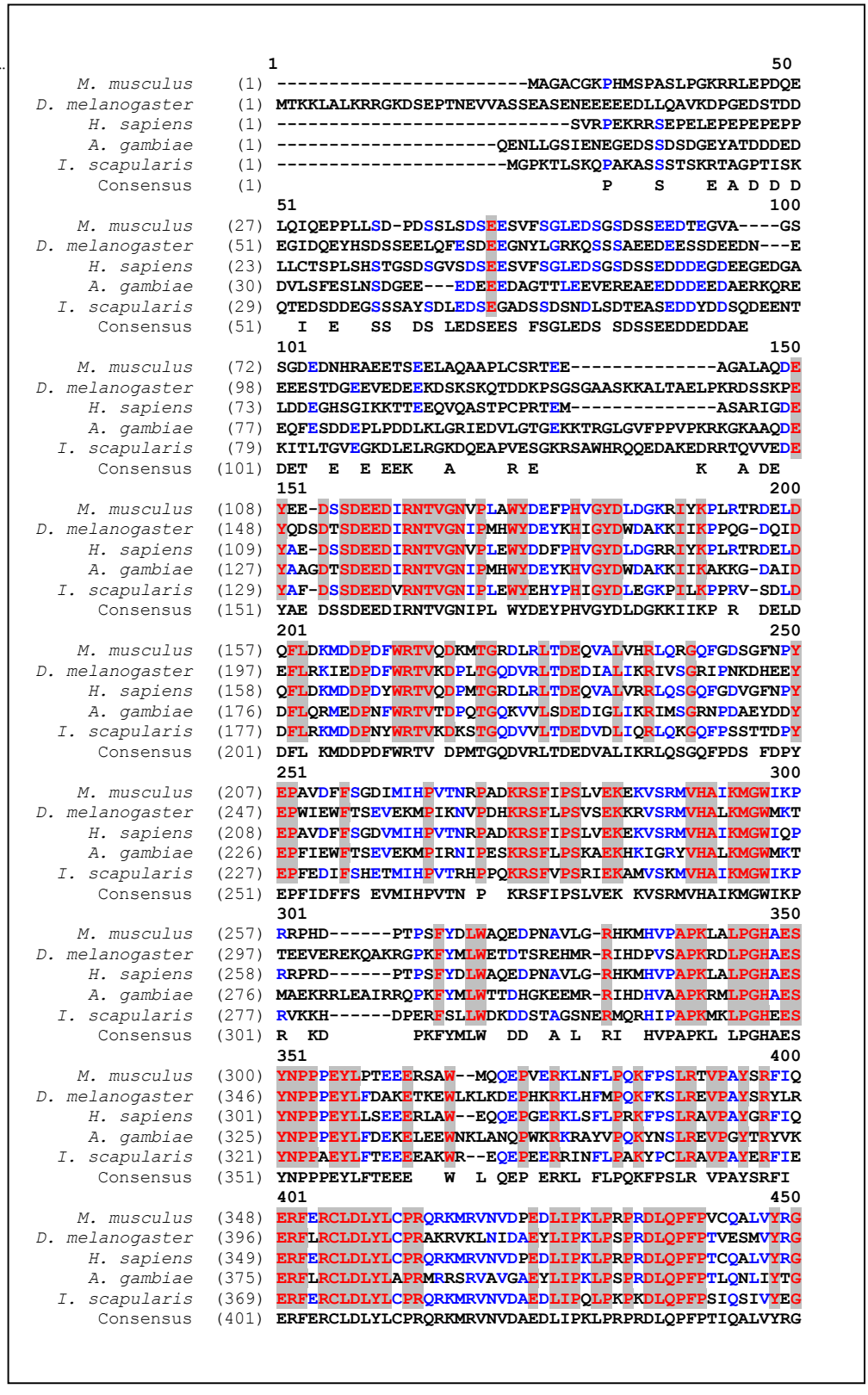


Figure 3. Analysis of clone 2C12 identical to beta-amyloid precursor protein. Alignment of *D. melanogaster* (AF181628) and *A. gambiae* (EAA07868) protein sequences and the translation product of clone 2C12 identified as *I. scapularis* beta-amyloid peptide (ß-AP) (AY296115). Protein sequences are shown in the single letter amino acid code. Identical amino acids are shown in red and amino acids conserved in 2 of 3 sequences are shown in blue.


1 50
M. musculus (1) ----------MAGACGKPHMSPASLPGKRRLEPDQE
D. melanogaster (1) MTKKLALKRRGKDSEPTNEVVASSEASENEEEEEDLLQAVKDPGEDSTDD
H. sapiens (1) ----------SVRPEKRRSEPELEPEPEPEPP
A. gambiae (1) ----------QENLLGSIENEGEDSSDSDGEYATDDDED
I. scapularis (1) ----------MGPKTLSKQPAKASSSTSKRTAGPTISK
Consensus (1) P S E A D D D
51 100
M. musculus (27) LQIQEPPLLSD-PDSSLSDSEESVFSGLEDSGSDSSEEDTEGVA----GS
D. melanogaster (51) EGIDQEYHSDSSEELQFESDEEGNYLGRKQSSSAEEDEESSDEEDN---E
H. sapiens (23) LLCTSPLSHSTGSDSGVSDSEESVFSGLEDSGSDSSEDDDEGDEEGEDGA
A. gambiae (30) DVLSFESLNSDGEE---EDEEEDAGTTLEEVEREAEEDDDEEDAERKQRE
I. scapularis (29) QTEDSDDEGSSSAYSDLEDSEGADSSDSNDLSDTEASEDDYDDSQDEENT
Consensus (51) I E SS DS LEDSEES FSGLEDS SDSSEEDDEDDAE
101 150
M. musculus (72) SGDEDNHRAEETSEELAQAAPLCSRTEE--------------AGALAQDE
D. melanogaster (98) EEESTDGEEVEDEEKDSKSKQTDDKPSGSGAASKKALTAELPKRDSSKPE
H. sapiens (73) LDDEGHSGIKKTTEEQVQASTPCPRTEM--------------ASARIGDE
A. gambiae (77) EQFESDDEPLPDDLKLGRIEDVLGTGEKKTRGLGVFPPVPKRKGKAAQDE
I. scapularis (79) KITLTGVEGKDLELRGKDQEAPVESGKRSAWHRQQEDAKEDRRTQVVEDE
Consensus (101) DET E E EEK A R E K A DE
151 200
M. musculus (108) YEE-DSSDEEDIRNTVGNVPLAWYDEFPHVGYDLDGKRIYKPLRTRDELD
D. melanogaster (148) YQDSDTSDEEDIRNTVGNIPMHWYDEYKHIGYDWDAKKIIKPPQG-DQID
H. sapiens (109) YAE-DSSDEEDIRNTVGNVPLEWYDDFPHVGYDLDGRRIYKPLRTRDELD
A. gambiae (127) YAAGDTSDEEDIRNTVGNIPMHWYDEYKHVGYDWDAKKIIKAKKG-DAID
I. scapularis (129) YAF-DSSDEEDVRNTVGNIPLEWYEHYPHIGYDLEGKPILKPPRV-SDLD
Consensus (151) YAE DSSDEEDIRNTVGNIPL WYDEYPHVGYDLDGKKIIKP R DELD
201 250
M. musculus (157) QFLDKMDDPDFWRTVQDKMTGRDLRLTDEQVALVHRLQRGQFGDSGFNPY
D. melanogaster (197) EFLRKIEDPDFWRTVKDPLTGQDVRLTDEDIALIKRIVSGRIPNKDHEEY
H. sapiens (158) QFLDKMDDPDYWRTVQDPMTGRDLRLTDEQVALVRRLQSGQFGDVGFNPY
A. gambiae (176) DFLQRMEDPNFWRTVTDPQTGQKVVLSDEDIGLIKRIMSGRNPDAEYDDY
I. scapularis (177) DFLRKMDDPNYWRTVKDKSTGQDVVLTDEDVDLIQRLQKGQFPSSTTDPY
Consensus (201) DFL KMDDPDFWRTV DPMTGQDVRLTDEDVALIKRLQSGQFPDS FDPY
251 300
M. musculus (207) EPAVDFFSGDIMIHPVTNRPADKRSFIPSLVEKEKVSRMVHAIKMGWIKP
D. melanogaster (247) EPWIEWFTSEVEKMPIKNVPDHKRSFLPSVSEKKRVSRMVHALKMGWMKT
H. sapiens (208) EPAVDFFSGDVMIHPVTNRPADKRSFIPSLVEKEKVSRMVHAIKMGWIQP
A. gambiae (226) EPFIEWFTSEVEKMPIRNIPESKRSFLPSKAEKHKIGRYVHALKMGWMKT
I. scapularis (227) EPFEDIFSHETMIHPVTRHPPQKRSFVPSRIEKAMVSKMVHAIKMGWIKP
Consensus (251) EPFIDFFS EVMIHPVTN P KRSFIPSLVEK KVSRMVHAIKMGWIKP
301 350
M. musculus (257) RRPHD------PTPSFYDLWAQEDPNAVLG-RHKMHVPAPKLALPGHAES
D. melanogaster (297) TEEVEREKQAKRGPKFYMLWETDTSREHMR-RIHDPVSAPKRDLPGHAES
H. sapiens (258) RRPRD------PTPSFYDLWAQEDPNAVLG-RHKMHVPAPKLALPGHAES
A. gambiae (276) MAEKRRLEAIRRQPKFYMLWTTDHGKEEMR-RIHDHVAAPKRMLPGHAES
I. scapularis (277) RVKKH------DPERFSLLWDKDDSTAGSNERMQRHIPAPKMKLPGHEES
Consensus (301) R KD PKFYMLW DD A L RI HVPAPKL LPGHAES
351 400
M. musculus (300) YNPPPEYLPTEEERSAW--MQQEPVERKLNFLPQKFPSLRTVPAYSRFIQ
D. melanogaster (346) YNPPPEYLFDAKETKEWLKLKDEPHKRKLHFMPQKFKSLREVPAYSRYLR
H. sapiens (301) YNPPPEYLLSEEERLAW--EQQEPGERKLSFLPRKFPSLRAVPAYGRFIQ
A. gambiae (325) YNPPPEYLFDEKELEEWNKLANQPWKRKRAYVPQKYNSLREVPGYTRYVK
I. scapularis (321) YNPPAEYLFTEEEEAKWR--EQEPEERRINFLPAKYPCLRAVPAYERFIE
Consensus (351) YNPPPEYLFTEEE W L QEP ERKL FLPQKFPSLR VPAYSRFI
401 450
M. musculus (348) ERFERCLDLYLCPRQRKMRVNVDPEDLIPKLPRPRDLQPFPVCQALVYRG
D. melanogaster (396) ERFLRCLDLYLCPRAKRVKLNIDAEYLIPKLPSPRDLQPFPTVESMVYRG
H. sapiens (349) ERFERCLDLYLCPRQRKMRVNVDPEDLIPKLPRPRDLQPFPTCQALVYRG
A. gambiae (375) ERFLRCLDLYLAPRMRRSRVAVGAEYLIPKLPSPRDLQPFPTLQNLIYTG
I. scapularis (369) ERFERCLDLYLCPRQRKMRVNVDAEDLIPQLPKPKDLQPFPSIQSIVYEG
Consensus (401) ERFERCLDLYLCPRQRKMRVNVDAEDLIPKLPRPRDLQPFPTIQALVYRG

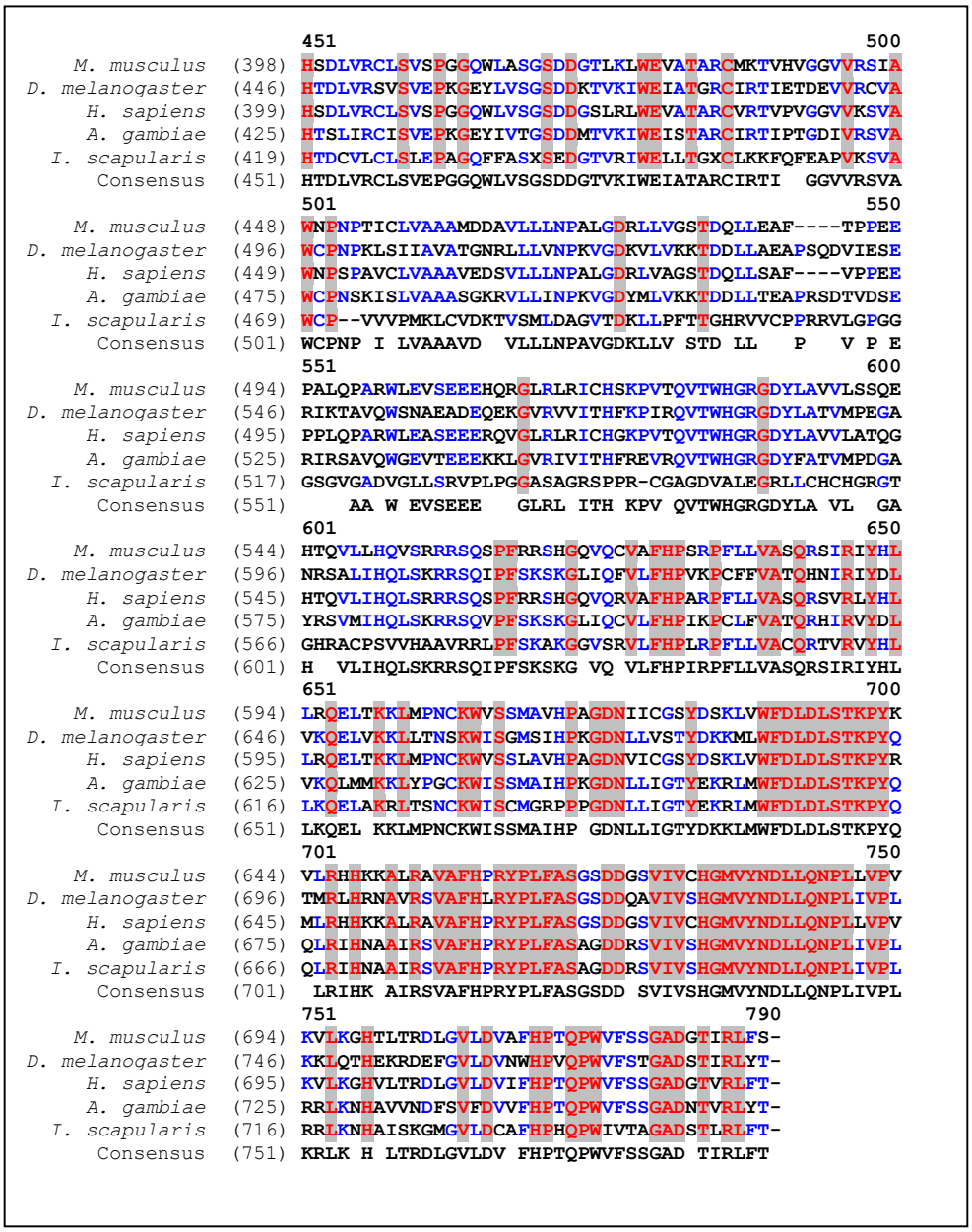


Figure 4. Analysis of clone 4F1 identical to block of proliferation (Bop1). (A) Alignment of *M. musculus* (AAH12693), *D. melanogaster* (NP_611270), *A. gambiae* (EAA04116), and *H. sapiens* (AAH07274) protein sequences and the translation product of clone 4F1 identified as *I. scapularis* Bop (_AY296116). Protein sequences are shown in the single letter amino acid code. Identical amino acids are shown in red and amino acids conserved in 3-4 of 5 sequences are shown in blue.

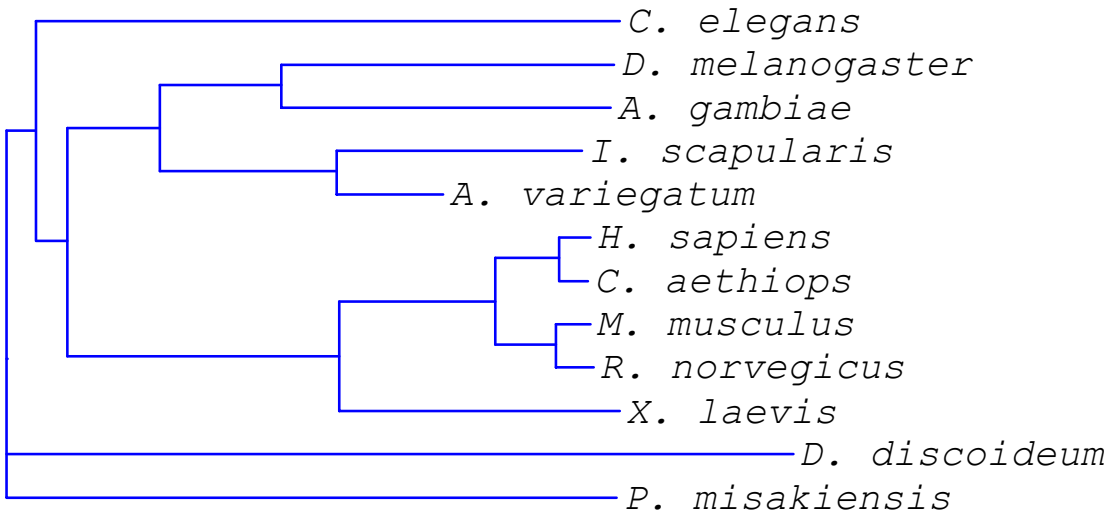
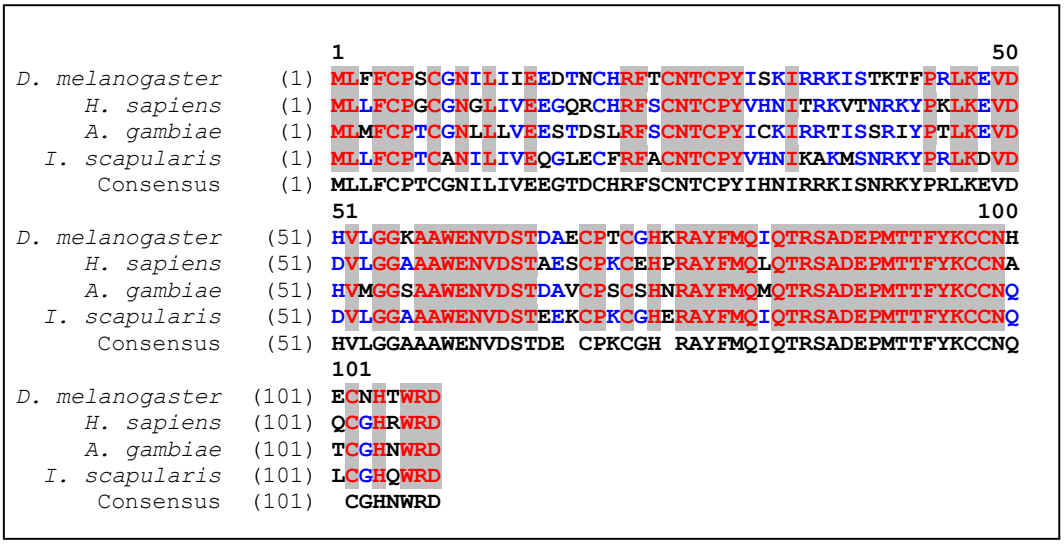


Figure 5. Analysis of clone 3E10 identical to mannose-binding lectin. Phylogenetic tree constructed from analysis of *C. elegans* (NP_492548), *A. gambiae* (EAA11908), *D. melanogaster* (NP_524776), *M. musculus* (XP_128952), *R. norvegicus* (NP_446338), *Cercopithecus aethiops* (Q9TU32), *H. sapiens* (NP_005561), *Polyandrocarpa misakiensis* (BAB20045), *X. laevis* (AAC59755), *Dictyostelium discoideum* (AAL92589), *A. variegatum* (BM290898) and *I. scapularis* (AY296117) protein sequences based on a sequence distance method utilizing the Neighbor Joining algorithm of Saitou and Nei (1987).

A



B

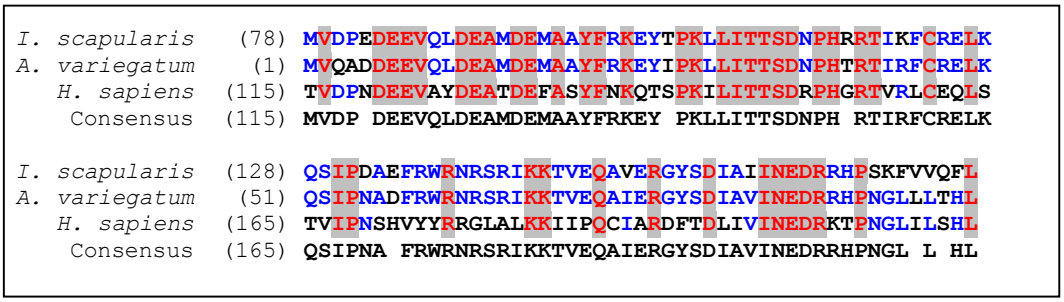


Figure 6. Analysis of clones 3C12 and 2F9 identical to RNA polymerase III and a hypothetical protein of unknown function, respectively. (A) Alignment of *D. melanogaster* (AAF57437), *A. gambiae* (TC6088), and *H. sapiens* (AAK61210) RNA polymerase III protein sequences and the translation product of clone 3C12 identified as *I. scapularis* RNA polymerase III (AY296118). (B) Alignment of *A. variegatum* (TC255), *H. sapiens* (FLJ12475) and *I. scapularis* clone 2F9 (AY296119) partial protein sequences. Protein sequences are shown in the single letter amino acid code. Identical amino acids are shown in red and amino acids conserved in 2-3 of 4 (A) and 2 of 3 (B) sequences are shown in blue.

C h a p t e r 4

CHARACTERIZATION OF GENES TRANSCRIBED IN AN *IXODES SCAPULARIS* CELL LINE THAT WERE IDENTIFIED BY EXPRESSION LIBRARY IMMUNIZATION AND ANALYSIS OF EXPRESSED SEQUENCE TAGS

Consuelo Almazán, Uriel Blas-Machado, Katherine M. Kocan, Joy H. Yoshioka, Edmour F. Blouin, Atilio J. Mangold and José de la Fuente. *(Vaccine, in press).*

Abstract

cDNA expression library immunization (ELI) and analysis of expressed sequenced tags (EST) in a mouse model of tick infestations was used to identified cDNA clones that affected *I. scapularis*. Three protective antigens against larval tick infestations, 4F8, with homology to a nucleotidase, and 4D8 and 4E6 of unknown function, were selected for further characterization. All three antigens were expressed in all *I. scapularis* stages and localized in adult tick tissues. 4D8 was shown to be conserved in 6 other tick species. Based on immunization trials with synthetic polypeptides against larvae and nymphs and on artificial feeding experiments of adults, these antigens, especially 4D8, appear to be good candidates for continued development of a vaccine for control of tick infestations and may be useful in a formulation to target multiple species of ticks.

Keywords: tick, vaccine, expression library immunization, EST, recombinant protein

1. Introduction

Ticks are ectoparasites of wild and domestic animals and humans, and are considered to be the most important vector of pathogens in North America [1]. *Ixodes* spp. (Acari: Ixodidae) are distributed worldwide and are vectors of pathogens affecting humans and wild and domestic animals [1, 2]. Members of the *I. ricinus* complex are the primary tick vectors of *Borrelia burgdorferi* and *Anaplasma phagocytophilum*, the causative agents of Lyme disease and

human granulocytic anaplasmosis, respectively [1, 2]. Throughout eastern and north-central United States, southern Canada and northern Mexico, *I. scapularis* (the black-legged tick) is the main vector of these pathogens [1, 2], while the closely related species, *I. pacificus* and *I. ricinus*, vector these pathogens in western United States and Europe, respectively [2].

Some tick species such as *Boophilus microplus* complete the life cycle while feeding on a single host. In other tick species such as *I. scapularis*, larvae, nymphs and adults feed on different hosts. Therefore, the control of tick infestations is especially difficult for multi-host ticks because several host species may need to be considered when implementing tick control strategies. Presently, tick control is effected by integrated pest management in which different control methods are adapted to a geographic area against one tick species with consideration to their environmental effects (reviewed by [3]). Recently, development of vaccines against one-host *Boophilus* spp. has provided new possibilities for identification of protective antigens for use in vaccines for control of tick infestations (reviewed by [4, 5]). Control of ticks by vaccination would avoid environmental contamination and the selection of drug resistant ticks that result from repeated acaricide applications. Tick vaccines could also be designed to include multiple tick and pathogen antigens that may target a broad range of both tick species and associated pathogens (reviewed by [4]).

Development of high throughput screening and sequencing technologies and bioinformatic tools facilitate the study of biological systems and provide information for the identification of potential vaccine candidates [6-10]. Recently, we reported the use of cDNA expression library immunization (ELI) and analysis of expressed sequenced tags (EST) in a mouse model of tick infestations for identification of cDNAs protective against *I. scapularis* [11, 12]. The combination of cDNA ELI and EST analysis resulted in the selection of 351 cDNA clones that affected tick larval development [11, 12]. These clones were grouped

according to their putative function, and some cDNAs resulted in inhibition of tick infestation while others promoted tick feeding [11, 12].

Herein we describe the characterization of three *I. scapularis* cDNAs, 4F8, 4D8 and 4E6, which reduced tick infestations in cDNA-vaccinated mice [11, 12].

2. Materials and Methods

2.1. Sequence analysis of protective tick cDNAs and deduced proteins

A strategy using primer walking was developed at the Core Sequencing Facility, Department of Biochemistry and Molecular Biology, Noble Research Center, Oklahoma State University, for sequencing both strands of cDNA inserts contained in protective clones 4F8, 4D8 and 4E6, using ABI Prism dye terminator cycle sequencing protocols developed by Applied Biosystems (Perkin-Elmer Corp., Foster City, CA, USA). Nucleotide sequences were analyzed using the program AlignX (Vector NTI Suite V 5.5, InforMax, North Bethesda, MD, USA). Open reading frames (ORFs) were found and the deduced amino acid sequence of encoded proteins determined. BLAST [13] was used to search the NCBI databases and the TIGR *Amblyomma variegatum* Gene Index (AvGI; [14]) to identify previously reported sequences with identity to those obtained by ELI. Conserved domains in proteins were analyzed by searching CDD, a curated Entrez database of conserved domain alignments [15] at NCBI web site. Protein topology was analyzed using TMpred and TMHMM v.2.0 algorithms for the prediction of transmembrane helices in proteins [16, 17], and the TargetP v1.01 algorithm was used to predict the localization of proteins in cells [18].

Phylogenetic analysis were implemented with 4D8 protein sequences using MEGA version 2.1 [19]. Protein sequences similar to *I. scapularis* 4D8 were included in the analysis from *Drosophila melanogaster* (Genbank accession number AAF50569), *A. variegatum* (T10865), *Danio rerio* (AAQ94594), mouse (XP_131324), human (NP_060534), *Xenopus laevis*

(AAH43949), *Anopheles gambiae* (EAA04195) and *Caenorhabditis elegans* (NP_491304). Maximum parsimony (MP) tree searches were heuristic, using tree-bisection-and-reconnection (TBR) branch swapping for 10 random addition sequence replicates. Minimum evolution (ME) and Neighbor Joining (NJ) trees were constructed based on p-distances and pairwise deletion of gaps. Stability or accuracy of inferred topology(ies) were assessed via bootstrap analysis [20] of 1000 replications.

2.2. Production and characterization of tick antigens

2.2.1. Cloning and expression in Escherichia coli of 4F8 and 4D8 recombinant proteins

For expression of 4F8 and 4D8 cDNAs in *E. coli*, coding regions were amplified from plasmid DNA by PCR using specific oligonucleotide primers introducing Eco RI and Sal I restriction sites in the 5' and 3' primers, respectively, to insert amplified fragments into the cloning site of pFLAG-CTC expression vector (Sigma, St. Louis, MO, USA). Recombinant plasmids were named pFNUC1 and pFEND2 for 4F8 and 4D8, respectively. In these constructs, the inserted genes were under the control of the inducible *tac* promoter and yielded full-length polypeptides with a C-terminal fusion of a FLAG marker octapeptide. The fidelity and orientation of the constructs was verified by sequencing. For expression of recombinant polypeptides, pFNUC1 and pFEND2 expression plasmids were transformed into *E. coli* K-12 (strain JM109). Transformed *E. coli* cells were inoculated in LB containing 50 μg/ml ampicillin and 0.4% glucose. Cultures were grown at 37°C to OD_{600nm}=0.4. IPTG was then added to 0.5 mM final concentration, and incubation continued during 4 h for induction of recombinant protein expression. Cells were collected by centrifugation and later analyzed by SDS-PAGE and Western blot, or then used for the purification of recombinant proteins.

2.2.2. Synthesis of 4E6 peptide

A peptide corresponding to the sequence of 4E6 ORF (NH_2-MEISVKPRPTKRKRKAIIIMARMRTAFPTRSGNSFSRT-COOH) was synthesized, analyzed by HPLC and mass spectrometry by Sigma-Genosys (The Woodlands, TX, USA), and shown to be 99% pure.

2.2.3. Protein purification

E. coli cells expressing recombinant 4F8 and 4D8 proteins were disrupted by sonication. Recombinant proteins were extracted with 0.1% Triton X-100 in Tris-buffered saline (TBS) and purified by FLAG-affinity chromatography (Sigma) following the manufacturer's instructions. The purity of recombinant proteins was estimated to be ≥90% as assayed by densitometry scanning of protein gels.

2.2.4. Protein gel electrophoresis and Western blot analysis

Expression and purification of the recombinant proteins was confirmed by SDS-PAGE [21] and immunoblotting. Protein samples were loaded on 12.5% polyacrylamide gels that were stained with Coomassie Brilliant Blue or transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk for 60 min at room temperature. Western blot analysis was performed using anti-FLAG M2 monoclonal antibodies (Sigma) for detection of recombinant fusion 4F8 and 4D8 proteins or 4F8-, 4D8-, 4E6-monospecific rabbit sera prepared in New Zealand White rabbits that were immunized subcutaneously with 3 doses (weeks 0, 4 and 7) each containing 50 μg purified 4F8 and 4D8 proteins or 4E6 synthetic peptide per dose in Freud's incomplete adjuvant (FIA) (Sigma). After washing with TBS, the membranes were incubated with 1:10,000 goat anti-mouse IgG or goat anti-rabbit IgG alkaline phosphatase conjugate (KPL, Inc., Gaithersburg, MD, USA). The membranes were washed again, and the color was developed using BCIP/NBT alkaline phosphatase substrate (Sigma).

2.3. Ticks

I. scapularis females, nymphs, larvae and eggs and *A. americanum*, *Dermacentor variabilis* and *Rhipicephalus sanguineus* nymphs and adults were obtained from the Oklahoma State University Tick Rearing Facility. *I. pacificus* females were field collected and kindly provided by Dr. Robert B. Kimsey (University of California, Davis, CA, USA). *I. ricinus* and *B. microplus* were kindly provided by Drs. Milan Labuda (Institute of Zoology, Slovak Academy of Sciences, Bratislava, Slovakia) and Robert J. Miller (Cattle Fever Tick Research Laboratory, USDA, Edinburg, TX, USA), respectively.

2.4. RNA extraction and reverse transcriptase (RT)-PCR

Total RNA was extracted from guts and salivary glands dissected from unfed adult ticks or from homogenates of eggs and whole unfed larvae and nymphs. Thirty *I. scapularis* females, approximately 100 and 1000 *I. scapularis* nymphs and larvae, respectively, the egg mass oviposited by one *I. scapularis* female, 10 *D. variabilis* males, 20 *A. americanum* adults, 20 *R. sanguineus* adults, 10 *B. microplus* adults, 10 *I. pacificus* females and 100-150 *I. ricinus* larvae were used. Total RNA was extracted from homogenized tick samples using TRI Reagent (Sigma), except for *I. ricinus* and *B. microplus* RNA which were extracted using the RNA Instapur kit (Eurogentec, Seraing, Belgium) and the RNeasy mini kit (Qiagen, Valencia, CA, USA), respectively, according to the manufacturer's instructions. The final RNA pellet was resuspended in 50-100 µl diethyl pyrocarbonate-treated distilled deionized sterile water. RT-PCR reactions were performed using the Access RT-PCR system (Promega, Madison, WI, USA). One µl RNA was reverse transcribed in a 50 µl reaction mixture (1.5 mM $MgSO_4$, 1 X avian myeloblastosis virus (AMV) RT/*Thermus flavus* (*Tfl*) reaction buffer, 10 mM random hexamers, 0.2 mM each deoxynucleoside triphosphate (dNTP), 5 U AMV RT, 5u *Tfl* DNA polymerase (Promega), (10 pmol of each primer) at 48°C for 45 min. After 2 min incubation at

94°C, PCR was performed in the same reaction mixture with specific primers (4F8, 4F8R5: 5´-GCGTCGTGTGGAGCATCAGCGAC-3´ and 4F8-R: 5´-TCGCAACGGACAACGGCAGGTTG-3´; 4D8, 4D8R5: 5´-GCTTGCGCAACATTAAAGCGAAC-3´and 4D8-R: 5´-TGCTTGTTTGCAGATGCCCATCA-3´; 4E6, 4E6R5: 5´-GAAATATCTGTGAAACCAAGGCC-3´ and 4E6U-R: 5´-ATTGCACAACACATCATTAACTG-3´) and amplification conditions (4F8, 30 sec at 61°C and 2 min at 68°C; 4D8, 30 sec at 56°C and 1 min at 68°C; 4E6, 30 sec at 52°C and 1 min at 68°C for annealing and extension steps, respectively). Control reactions were performed using the same procedures but without RT to control for DNA contamination in the RNA preparations and without RNA added to control contamination of the PCR reaction. Positive control reactions for the PCR were performed with plasmid DNA containing the cloned tick cDNAs and with genomic DNA extracted from *I. scapularis* IDE8 cells. PCR products were electrophoresed on 1% agarose gels to check the size of amplified fragments (972 bp, 577 bp and 138 bp for 4F8, 4D8 and 4E6 amplicons, respectively) by comparison to a DNA molecular weight marker (1 Kb Plus DNA Ladder, Promega).

2.5. Immunohistochemistry of tick tissue sections

Adult *I. scapularis* and IDE8 tick cells derived originally from *I. scapularis* embryos [22] were fixed in formaldehyde and embedded in paraffin. Sections (4 μm) were prepared and mounted on microscope slides that were stored at 4°C. For immunohistochemistry studies, tissue sections were deparaffinized and dehydrated twice for 5 min in xylene, 100% ethanol, 95% ethanol, followed by a 5 min wash in 80% ethanol. For antigen retrieval, slides were incubated with 0.05% pronase (DakoCytomation, Glostrup, Denmark) diluted in TBS, pH 7.2 during 15 min. The slides were incubated for 1 hr with rabbit 4F8, 4D8 or 4E6 antisera prepared as

described previously and diluted 1:400 in PBS, pH 7.2. A preimmune rabbit serum and a monospecific rabbit serum prepared with total IDE8 proteins as described previously for recombinant proteins were used as negative and positive controls, respectively. The slides were blocked in PBS/0.5% Tween 20, pH 7.2 (PBST) with 10% goat serum and 5% skim milk for 1 hr and then incubated for 1 hr with peroxidase–labeled goat anti-mouse IgG (KPL) diluted 1:3000 in PBST. To inactivate the endogenous peroxidase activity, slides were incubated with 3% H_2O_2 in PBS, pH 7 and 10% ethanol for 1 hr prior to a 1 min incubation with the substrate 3',3'-diaminobenzidine tetrahydrochloride Fast DAB set (Sigma) followed by staining with hematoxylin for 2 min. After each treatment, the slides were rinsed twice for 5 min in PBST, unless otherwise indicated. All incubations were done at room temperature. For microscopic examination, the slides were rinsed with distilled water and dehydrated 2 times for 2 min each in 95% ethanol, 100% ethanol, and finally xylene, and mounted in permount.

2.6. Biological function of recombinant 4F8 putative tick nucleotidase

In order to characterize the biological activity of the of recombinant 4F8 putative tick nucleotidase, an affinity purification experiment was conducted based on the nucleotidase's activity on 3'-phosphoadenosine 5'-phosphate (PAP) [23]. PAP-agarose resin (Sigma) was swelled in PAP-agarose buffer (50 mM HEPES, pH 7.5, 10 mM $CaCl_2$, and 50 mM KCl) and 3 ml were poured into a 4-ml glass chromatography column (Sigma). Calcium was added to satisfy the metal requirement for substrate binding while preventing the hydrolysis of immobilized PAP by homologous phosphomonoesterases. Purified recombinant 4F8 (0.4 mg) was resuspended in 1 ml PAP-agarose buffer and applied to the column by gravity flow. The column was washed with 10 column volumes of PAP-agarose buffer plus 0.5 M NaCl and then reequilibrated with 3 column volumes of PAP-agarose buffer containing no additional salt. The enzyme was eluted with 3 ml PAP-agarose buffer containing 300 μM 2'/3',5'-PAP (Sigma).

Protein fractions in the column pass flow (unbound protein) and after elution with 2'/3',5'-PAP were collected and concentrated in Amicon Ultra-15 centrifugal filter devices, 10,000 nominal molecular weight limit (Millipore; Bedford, MA, USA). Both protein samples were then analyzed by Western blot using anti-FLAG M2 monoclonal antibodies (Sigma) as described above.

2.7. Protective properties of tick antigens

Three experiments were conducted to evaluate the effect of 4D8, 4F8 and 4E6 antigens on larval, nymphal and adult tick infestations. The first experiment was designed to compare the effect elicited by protein antigens with that previously obtained with tick cDNAS following the same protocol of mouse immunization and infestation with *I. scapularis* larvae [11]. The second experiment was conducted to obtain preliminary data on the inhibitory effect of the tick protective antigens on nymphal infestations by *I. scapularis*, *D. variabilis* and *A. americanum*. For this experiment, rabbits were chosen because they are a better host to support the infestation with nymphs of several tick species and were immunized following the protocol described above to elicit a strong antibody response with 4D8, 4F8 and 4E6 antigens. The third experiment was conducted in order to obtain preliminary data on the effect of the immune response to recombinant protective antigens against adult *I. scapularis* stages using an artificial feeding method followed by completion of the feeding period on a sheep, which supports adult tick infestations and is the host routinely used to feed *I. scapularis* adults at the Oklahoma State University Tick Rearing Facility.

2.7.1. Immunization of mice and infestation with I. scapularis larvae

Five groups of 6 CD-1 female mice, 5-6 weeks of age at the time of first immunization, were used in this experiment. Experimental groups included immunization with 10 µg/dose of 4F8 or 4D8 recombinant proteins, 4E6 synthetic peptide, and recombinant *Anaplasma marginale*

MSP1a [24], which did not affect tick feeding [25], or vehicle (TBS)/adjuvant alone to serve as controls. The mice were cared for in accordance with standards specified in the Guide for Care and Use of Laboratory Animals. Mice were injected subcutaneously with each antigen or TBS at weeks 0 and 2 with 100 µl/dose in FIA (Sigma) using a 1 ml tuberculin syringe and a 27½G needle as described previously [11]. In separate experiments we have shown that immunization with FIA did not have an effect on tick feeding (unpublished results). Two weeks after the last immunization, mice were infested with 100 *I. scapularis* larvae per mouse as described previously [11]. The unattached larvae were counted and removed 12 hrs after infestation, and engorged larvae were collected daily for 7 days from each mouse and counted. After tick feeding, the mice were euthanized by cervical dislocation, the blood was collected and the serum removed and stored. The engorged larvae were held in a humidity chamber for 34 days, after which molting was evaluated using a dissecting light microscope. The inhibition of tick infestation (I) and inhibition of molting (M) for each test group with respect to the MSP1a-immunized controls and the overall efficacy of the vaccine (E) were calculated as described previously [11]. The protection efficacy of the vaccine was calculated as E = 100 x [1-(RI x RM)] where RI (average reduction in tick infestation) = 1-I/100 and RM (average reduction in molting) = 1-M/100.

2.7.2. Characterization of the immune response in the immunized mice by Western blot

Ten micrograms of 4F8 and 4D8 recombinant proteins and 4E6 synthetic peptide were loaded on a 12.5% polyacrylamide gel using a preparative comb for Western blot analysis of mouse immune response as described previously [11].

2.7.3. Characterization of the immune response in the immunized mice by ELISA

The antibody response against tick antigens in immunized and control mice was evaluated by ELISA. Antibody levels to *I. scapularis* recombinant 4F8, 4D8 and 4E6 were

detected by indirect ELISA. Purified recombinant proteins were used to coat ELISA plates over night at 4°C. Sera were serially diluted to 1:10 and 1:100 in PBST and 10% fetal bovine serum (Sigma). The plates were incubated with the diluted sera for 1 hour at 37°C and then incubated with 1:10,000 sheep anti-mouse IgG-HRP conjugate (Sigma) for 1 hour at 37°C. The color reaction was developed with 3,3',5,5'-tetramethylbenzidine (Sigma) and the OD_{450nm} was determined. After incubations the plates were washed with PBST. Antibody titers were considered positive when yielded an OD value at least twice as high as the negative control serum and were expressed as the geometric mean (mean ± S.D.) at the 1:100 serum dilution of the six immunized mice OD minus the OD of the control group.

2.7.4. Immunization of rabbits and infestation with I. scapularis, D. variabilis and A. americanum nymphs

Preliminary data on the inhibitory effect of the three tick protective antigens on nymphal infestations of *I. scapularis*, *D. variabilis* and *A. americanum* was obtained using immunized and control rabbits. One New Zealand White rabbit per group was each immunized with 3 doses (weeks 0, 4 and 7) containing 50 µg/dose purified 4F8 and 4D8 proteins or 4E6 synthetic peptide, a combination of 4F8+4D8+4E6 or bovine serum albumin (Sigma) as control in FIA (Sigma). Rabbits were cared for in accordance with standards specified in the Guide for Care and Use of Laboratory Animals. Rabbits were injected subcutaneously with 500 µl/dose using a 1 ml tuberculin syringe and a 27½G needle. Two weeks after the last immunization, each rabbit was infested in ear bags with 100 *I. scapularis* nymphs per rabbit in one ear and 110 nymphs of each *D. variabilis* and *A. americanum* on the other ear. The unattached nymphs were counted and removed 24 hrs after infestation. The ear bag was then removed from the *D. variabilis* and *A. americanum* infested ear and replete nymphs from each rabbit were collected on each of the next 7 days from either the ear bag left

(for *I. scapularis*) or from water in the bottom of the cage (for *D. variabilis* and *A. americanum*), and were weighed and counted. *D. variabilis* and *A. americanum* engorged nymphs were separated under a dissecting light microscope. The inhibition of tick nymphal infestation was calculated in comparison with the control group as described above for larval tick infestations. Reduction in the weights of engorged nymphs was determined in experimental groups with respect to the nymphs collected from the control groups.

2.7.5. Capillary feeding of adult I. scapularis with rabbit immune sera against tick protective antigens

Twenty unfed female *I. scapularis* per group were used for capillary feeding (CF) experiments as described previously [26]. Five groups were included in the CF experiment that were fed tick meals with rabbit immune sera against 4F8, 4D8, 4E6, 4F8+4D8+4E6, or rabbit preimmune serum as control. The 4F8+4D8+4E6 serum contained 4F8, 4D8 and 4E6 sera combined in equal amounts prior to preparing the tick meal for CF. The quantity of serum consumed per tick was determined by measuring the difference in level of serum in the micropipets when first filled and prior to changing of the meal. The height of the ingested serum meal column (mm) was determined daily and used to calculate the total volume of ingested serum per tick. After two days of CF, the ticks were removed from the tape and placed with 10 unfed males per group in separate orthopedic stockinettes glued to the side of a sheep and allowed to attach and feed for 11 days. Replete ticks were collected daily and the number recorded, and the ticks were weighed prior to incubation in a humidity chamber and held until oviposition was completed (40 days). The egg mass from each tick was weighed and recorded. The number of dead ticks during feeding and oviposition was also recorded.

2.7.6. Statistical analysis

The ratio of the number of engorged larvae recovered per mouse 7 days after infestation (RL) and the number of larvae attached per mouse (RLi) and the ratio of the

number of nymphs (ML) and the number of engorged larvae per mouse (RL) were compared by Student's t-test between tick antigen-immunized and control MSP1a-immunized mice in the mouse vaccination experiment. In the CF experiment, ingested serum volume, tick weights and weight of egg mass of ticks capillary fed on immune sera were compared using a Student's t-test with the control ticks fed on preimmune serum. The ratios of dead to fed ticks were compared by $\chi 2$–test. The number of ticks on the sheep was recorded daily and compared using a Wilcoxon signed rank test. Wilcoxon signed rank and $\chi 2$ tests were implemented using Mstat 4.01.

3. Results

3.1. Characterization of protective tick cDNAs

The *I. scapularis* cDNA clones 4F8, 4D8 and 4E6 that caused inhibition of tick infestation in ELI experiments were sequenced and the ORFs identified. The cDNA clone 4F8 contained a cDNA insert of 1791 bp plus a poly-A tail of 30 bases. An ORF of 951 bp was identified encoding for a protein of 316 amino acids with a predicted molecular weight of 34.7 kDal. The protein was predicted to be a soluble polypeptide localized in the cytoplasm of the cell. Protein domain analysis resulted in identity to highly conserved protein families KOG3099, bisphosphate 3'-nucleotidase BPNT1/Inositol polyphosphate 1-phosphatase involved in nucleotide transport and metabolism (E=4e-84) and KOG3853, inositol monophosphatase involved in signal transduction mechanisms (E=8e-38). The 4F8-encoded protein contained the consensus for the sequence involved in metal binding and catalysis (D-X_n-EE-X_n-DP(i/l)D(s/g/a)T-X_n-WD-X_{11}-GG; ref. [23]) and was similar to *A. variegatum* (TIGR gene index TC259; 64%), mouse nucleotidase (Genbank accession number AAH11036; 61%), human nucleotidase (AAD17329; 60%), *A. gambiae* (XP_316740; 61%), *D. melanogaster* (AAF56941; 58%), *C. elegans* (NP_494780; 46%) and *X. laevis* (AAH59974; 16%)

sequences. Sequence analysis suggested that 4F8 encoded for a tick nucleotidase. The nucleotidase activity of 4F8 was then confirmed in a biological assay showing that the recombinant protein bound to the immobilized substrate PAP and specifically eluted with 2'/3',5'-PAP (data not shown).

The cDNA clone 4D8 contained a cDNA insert of 2,664 bp plus a poly-A tail of 79 nucleotides. This cDNA contained a large 3' untranslated region (UTR) of 2,030 bp excluding the poly-A tail and a 5' UTR of 79 bp. An ORF of 555 bp was identified encoding for a putative soluble cytoplasmic protein of 184 amino acids with a predicted molecular weight of 20.7 kDal. A conserved protein domain (KOG4330) was localized in the 4D8-encoded protein that was present in uncharacterized conserved proteins of unknown function. The protein encoded by 4D8 was similar to *A. variegatum* (T10865; 32%; 100% similarity in the 40 amino acids reported for *A. variegatum*), *D. rerio* (AAQ94594; 56%), mouse (XP_131324; 51%), human (NP_060534; 49%), *X. laevis* (AAH43949; 48%), *A. gambiae* (EAA04195; 47%), *D. melanogaster* (AAF50569; 46%) and *C. elegans* (NP_491304; 24%) sequences. Phylogenetic analysis of 4D8 protein sequences revealed similar tree structures for MP, ME and NJ algorithms (Fig. 1 and data not shown). Tick sequences clustered together, separated from insect and vertebrate sequences (Fig. 1).

The cDNA clone 4E6 contained a small insert of 320 bp excluding a 29 bases poly-A tail. This insert contained an ORF of 117 bp encoding for a soluble peptide of 38 amino acids with a predicted molecular weight of 4.4 kDa or corresponding to a truncated protein from an incomplete cDNA. The only sequence found in the databases with homology to 4E6 corresponded to a *D. melanogaster* protein of unknown function (AAL90160; 39% similarity in the region comprising the 38 amino acids of 4E6).

3.2. Expression of recombinant proteins

Recombinant 4F8 and 4D8 proteins were expressed in *E. coli* fused on the C-terminal to a FLAG marker octapeptide for purification by FLAG-affinity chromatography. Expression levels of recombinant proteins after induction reached approximately the 5-10% of total cellular proteins (Fig. 2, lanes 2, 4). Rabbit immune sera were prepared with recombinant proteins 4F8 and 4D8 and with the synthetic peptide 4E6. Rabbit sera specifically recognized 4F8 and 4D8 proteins in induced *E. coli* protein extracts (Fig. 3A, lanes 2, 4) and in purified protein preparations (Fig. 3A, lanes 3, 5). The rabbit serum against the 4E6 peptide recognized the peptide in Western blots (Fig. 3A, lane 6). The anti-FLAG M2 monoclonal antibody specifically recognized the FLAG peptide fused to the C-terminal of 4F8 and 4D8 proteins. Combined analysis of Western blots of *E. coli*-induced and purified proteins with the anti-FLAG M2 monoclonal antibody and the rabbit immune sera evidenced the presence of higher molecular weight proteins in 4F8 (Fig. 3B, diamond in lane 2) and 4D8 (Fig. 3A and 3B, diamond in lanes 4, 5), also present in the PAGE (Fig. 2, lines 3, 5), and lower molecular weight products in 4D8 (Fig. 3A and 3B, circle in lines 4, 5). Higher molecular weight products corresponded in size to protein dimers and lower molecular weight polypeptides could represent degradation products. After purification, the purity of recombinant proteins was estimated to be ≥90% as assayed by densitometry scanning of protein gels (Fig. 2, lanes 3, 5) considering all products specifically recognized in Western blot analyses (Fig. 3A and 3B).

3.3. Expression of protective cDNAs

Expression of genes encoding for tick protective antigens was analyzed at mRNA and protein levels by RT-PCR and immunohistochemistry, respectively. Expression of 4F8, 4D8 and 4E6 mRNA was detected in *I. scapularis* eggs, larvae and nymphs and in guts and salivary glands from adult ticks (Fig. 4). Control reactions ruled out contamination with genomic DNA or during the PCR (Fig. 4). Furthermore, PCR of tick genomic DNA showed that the size of

the amplified DNA fragments was higher than the size corresponding to cDNA fragments, probably due to the presence of intron sequences in the analyzed genes (data not shown).

Protective polypeptides were detected by immunohistochemistry in *I. scapularis* IDE8 cells (Fig. 5). Furthermore, proteins 4D8 and 4E6 were also detected in *I. scapularis* gut sections (Fig. 5). The positive control anti-IDE8 proteins serum labeled IDE8 cells and tick gut sections (Fig. 5). Labeling was not seen in sections reacted with the negative control rabbit preimmune serum (Figs. 5).

Sequence conservation and expression of 4F8, 4D8 and 4E6 were analyzed in *I. scapularis* related species, *I. pacificus* and *I. ricinus*, and in *D. variabilis*, *R. sanguineus*, *B. microplus* and *A. americanum* by RT-PCR using the primers derived from *I. scapularis* sequences. Expression was detected in *I. ricinus* for all three genes and in *I. pacificus* and *A. americanum* for 4D8 and 4E6 (Fig. 4). Expression of 4D8 was also detected in *D. variabilis*, *B. microplus* and *R. sanguineus* (Fig. 4).

3.4. Protective properties of tick antigens against I. scapularis larval infestations

The first vaccination experiment was designed to evaluate the effect of recombinant or synthetic proteins on larval *I. scapularis* infestations as evaluated previously for cDNA immunizations [10]. Mice were immunized with 10 μg/dose of purified recombinant 4F8 and 4D8 proteins and with the synthetic 4E6 peptide. Control mice received 10 μg/dose of the unrelated recombinant MSP1a protein or saline/adjuvant alone. Antibody response against protective antigens was confirmed in mice after immunization by ELISA (mean OD ± SD of 1.0 ± 0.5, 0.9 ± 0.6 and 0.4 ± 0.04 for 4D8, 4F8 and 4E6 immunized mice, respectively) and Western blot (data not shown). Sera from mice immunized with adjuvant/vehicle alone did not recognize 4F8, 4D8, 4E6 or MSP1a (data not shown). Vaccination with recombinant proteins 4F8 and 4D8 and with the synthetic peptide 4E6 resulted in protection of mice

against tick larval infestations. Mice vaccinated with tick protective antigens showed a decrease in tick infestations (RL/RLi) when compared to controls, although it was statistically significant for 4F8 and 4D8 only (Table 1). The inhibition of tick infestations (I) was ≥46% for all three tick antigens (Table 1). A decrease in the molting of tick larvae to nymphs was found in ticks that fed on mice immunized with 4D8 and 4E6 proteins, while molting of larvae that fed on 4F8-immunized mice was similar to controls (Table 1). Differences in tick infestations were not observed between MSP1a-immunized mice and mice that received saline/adjuvant alone (data not shown). The overall efficacy of immunization was calculated for each tick antigen considering the effect on larval infestations and on molting to nymphs and ranged from 62% to 71% (Table 1).

3.5. Protective properties of tick antigens against I. scapularis, D. variabilis and A. americanum nymphal infestations

The detection of 4D8 and 4E6 expression in non-Ixodes tick species and the evaluation of the effect of tick protective antigens on tick nymphal infestations were the basis for an experiment in which rabbits were immunized and challenged with *I. scapularis*, *D. variabilis* and *A. americanum* nymphs. Although viewed as preliminary data because of the use of one rabbit per group only, the results demonstrated an effect of 4D8 immunization on the inhibition of nymphal infestations in all three tick species. In addition, a notable effect of immunization with 4D8 was observed on *D. variabilis* nymphs by the number of visibly damaged ticks (Fig. 6) and a reduction in the weight of engorged nymphs (Table 2). Immunization with 4F8 affected *I. scapularis* nymphal infestations and immunization of rabbits with 4E6 affected *A. americanum* infestations and the weights of *D. variabilis* engorged nymphs (Table 2).

3.6. Effect of rabbit immune sera against recombinant proteins on adult I. scapularis

Capillary feeding experiments were conducted in order to obtain preliminary data on the effect of the immune response to recombinant protective antigens against adult *I. scapularis* stages. Female ticks that were capillary fed for two days with rabbit immune sera prepared against recombinant proteins and were then allowed to feed on a sheep for 11 days had reduced feeding periods as compared to controls ($P<0.05$; Fig. 7). In addition, the weight of these ticks at repletion was reduced in comparison with the control group, with significant differences in the weights of ticks capillary fed on 4D8 and 4E6 immune sera ($P<0.05$; Table 3). All ticks survived CF, but tick mortality during 11 days of feeding on the sheep was higher in ticks fed on 4D8, 4E6 and the combined immune sera ($P<0.05$; Table 3). For ticks that survived feeding and completed engorgement, a significant reduction in weight was observed in ticks that fed on 4D8 immune serum ($P<0.05$; Table 3). When egg mass weight and tick mortality were determined at 40 days post-oviposition, the reduction in oviposition observed for ticks fed on 4D8 immune serum was statistically significant ($P<0.05$; Table 3). Furthermore, the overall tick mortality was significantly higher for ticks that fed on 4D8 immune serum ($P<0.01$; Table 3). Although ticks capillary fed on 4F8, 4D8 and 4E6 immune sera ingested more serum than the controls fed on preimmune serum or those fed on the combined immune sera ($P<0.05$; Table 3), a correlation was not observed between the volume of ingested serum by CF and the effect on ticks. Notably, the effect of the 4D8 immune serum had the most pronounced effect on tick feeding, oviposition and survival (Table 3).

4. Discussion

The feasibility of controlling tick infestations through immunization of hosts with tick antigens has been demonstrated for *Boophilus* spp. (reviewed by [4, 5]). Although proteins with

the capacity to control tick infestations have been described in various tick species, identification of tick protective antigens has continued to be the limiting step in the process of developing new effective tick vaccines (reviewed by [4, 5]). Recently, we used a high throughput screening method for the identification of tick protective antigens in *I. scapularis* by using cDNA ELI and EST analysis [11, 12]. Immunization of mice with the *I. scapularis* 4F8 and 4D8 cDNAs resulted in 50% and 40% inhibition of tick infestations, respectively [10, 11], and these cDNAs were therefore selected for further characterization. 4E6 cDNA, although inhibited tick infestations at a lower level (20%) compared to 4D8 and 4F8 [12], was selected for further evaluation because it encodes a small protein of 38 amino acids which could be interesting to use in chimeric polypeptides or in combination with other antigens for vaccination against ticks.

Recombinant polypeptides were produced for 4F8 and 4D8 in *E. coli* and a synthetic peptide was prepared for the short 4E6 protein. Vaccination of mice with recombinant 4F8 and 4D8 proteins and with the synthetic peptide 4E6 resulted in an overall vaccine protection efficacy of >60% against *I. scapularis* larval infestations. Previous pen vaccine trials using Bm86 and Bm95 recombinant antigens with the one-host cattle tick *B. microplus* showed that a vaccine efficacy higher than 50% resulted in control of tick populations in the field (reviewed by [4]). However, in the three-host tick, *I. scapularis,* the efficacy of immunization with 4F8, 4D8 and 4E6 on all tick developmental stages is unknown. Nonetheless, preliminary immunization trials in rabbits and CF studies suggested that immune sera against 4F8, 4D8 and 4E6 have an inhibitory effect on *I. scapularis* nymphs and adults. Although statistical analyses could not be done in the rabbit immunization trial because of the use of one rabbit per group, the results suggested an effect of 4D8 and 4F8 immunizations on *I. scapularis* nymphs. The lack of

statistical significance in some analyses of the CF experiment may reflect differences in the antibody concentration between the different immune sera, differences in the activity of immune sera against adult ticks or the result of the small number of ticks used in the CF experiment. Nevertheless, these results are particularly encouraging because adult ticks were exposed to specific antibodies for two days only, which represents 15% of the total feeding time. Ticks feeding on artificial membrane systems or on ascitic mice producing tick-specific IgGs and inoculation of female ticks with immune sera have been used before to test the inhibitory effect of antibodies against tick antigens in *B. microplus* [27, 28], *Ornithodoros moubata* [29] and *Haemaphysalis longicornis* [30].

The degree of protection obtained with 4F8, 4D8 and 4E6 protein vaccine formulations was higher than that obtained with cDNA vaccination [11, 12]. The inhibition of tick infestation for protein vs. cDNA vaccination was 64% vs. 50% for 4F8, 61% vs. 40% for 4D8 and 46% vs. 20% for 4E6. The inhibition of molting was assayed with cDNA immunization for 4F8 (M=17%) and 4D8 (M=7%) in previous experiments [11]. Although inhibition of molting was not observed in the experiments described herein for the group immunized with recombinant 4F8, the vaccination efficacy observed was higher for 4D8 protein formulation when compared to cDNA vaccination (71% vs. 44%). Additionally, antibodies against protective antigens were detected in all protein-immunized mice, a finding that contrasted with the poor antibody response observed in cDNA-vaccinated mice [11]. The higher protection efficacy obtained with protein vaccine formulations probably reflects a higher stimulation of the host antibody response by protein immunization (reviewed by [31]) and suggests a role for antibodies in the mechanism of protection with tick vaccine formulations [32]. Immunoglobulins trigger a variety of effector mechanisms and are

specifically transported into the tick hemolymph during feeding [33, 34]. Therefore, immunization with tick antigens may target a variety of tick cell proteins.

The expression of 4F8, 4D8 and 4E6 in all *I. scapularis* developmental stages suggested that these genes are constitutively expressed throughout the life cycle of the tick. In addition, expression was detected for all three genes in *I. ricinus* and for 4D8 and 4E6 in *I. pacificus*, also members of the *I. ricinus* complex. Recent analyses indicate that members of the *I. ricinus* species complex are closely related despite the fact that they are distributed in different regions of the world [35]. These results strongly suggest the possibility for *I. scapularis* protective antigens to be cross-protective against other tick species of the *I. ricinus* complex.

Expression of 4F8, 4D8 and 4E6 mRNA was detected in tick guts and the expression of 4D8 and 4E6 was confirmed at the protein level in tick gut sections. Although it is possible to control tick infestations using antigens expressed in different tick tissues (reviewed by [4]), many of the tick protective antigens evaluated as recombinant proteins, including the *B. microplus* Bm86 and Bm95 antigens used in commercial tick vaccine formulations, are expressed in tick guts (reviewed by [4]). Targeting gut antigens may result in impairment of tick development by interfering with uptake of the blood meal and digestion, which may cause the visible damage observed in *D. variabilis* nymphs fed on the 4D8-immunized rabbit as previously noted on *I. scapularis* [11] and *B. microplus* [36, 37] fed on mice and cattle immunized with tick cDNAs and Bm86 and Bm95 recombinant proteins, respectively.

The protein encoded by 4D8 was found to be highly conserved and tracked ancestors to the bilateria (Taxblast threshold 10^{-3}). With the exception of the position of fish and amphibian sequences, 4D8 trees were in accordance with the phylogenetic relationships proposed for eukaryotes [38]. Expression of 4D8 mRNAs were detected in all *I. scapularis* developmental stages (eggs, larvae, nymphs and adults). Furthermore, the detection of 4D8

mRNA expression using primers identical to the *I. scapularis* sequence in related tick species, *I. ricinus* and *I. pacificus*, and in the more phylogenetically distant species, *A. americanum*, *B. microplus*, *R. sanguineus* and *D. variabilis* [39], suggests that the function of 4D8 is widely conserved among tick species. Expression of the human 4D8 homologue has been detected in a variety of adult and fetal tissues (expression information for NCBI UniGene Cluster Hs.201864 *Homo sapiens*) and in *C. elegans* the 4D8 homologue was expressed in all developmental stages [40]. Interestingly, the biological function of 4D8 is unknown, but may be involved in the control of developmental processes as deduced from the possible involvement of the *D. melanogaster* homologue in the dorsal-thorax formation of the embryo [41] and the embryonic lethal phenotype obtained by RNA interference with the *C. elegans* homologue [40]. Importantly, conservation of 4D8 in different tick species suggests that this antigen may be useful in vaccine formulations designed for the control of multiple tick species, as evidenced by our preliminary experiments in which immunized rabbits were challenged with both *D. variabilis* and *A. americanum* nymphs.

The cDNA clone 4F8 encodes for a metal-dependent tick nucleotidase, a member of the phosphomonoesterase protein family that is involved in nucleotide transport and metabolism and signal transduction [23]. In general, 5'-nucleotidase has been considered as a marker enzyme for the plasma membrane, and is considered to be a key enzyme in the generation of adenosine, a potential vasodilator [42]. However, from its wide range of localization in tissues it is also considered to be related to the membrane movement of cells in the transitional epithelium, cellular motile response, transport process, cellular growth, synthesis of fibrous protein and calcification, lymphocyte activation, neurotransmission, and oxygen sensing mechanisms [42]. The inhibition of nucleotidases results in the accumulation of PAP and 3'-phosphoadenosine 5'-phosphosulfate (PA) leading to cell toxicity [23].

Although a 5'-nucleotidase (AAB38963) with 6% similarity to 4F8 was identified and characterized in *B. microplus* by Liyou et al. [43, 44], the protection capacity of this antigen has not been reported.

Presently, little is known about 4E6, but immunization with this short 38 amino acid peptide resulted in the inhibition of tick infestations. Immunizations with Bm86-derived peptides have resulted in control of *B. microplus* infestations ([45]; de la Fuente J and Garcia-Garcia JC, unpublished results). These results suggest that chimeric vaccine antigens containing protective epitopes derived from multiple tick proteins may enhance development of new tick vaccines.

In summary, we identified and characterized three *I. scapularis* tick protective antigens, 4D8, 4F8 and 4E6. These antigens, and especially 4D8, appear to be good candidates for continued development of a commercial product for control of tick infestations. Although these antigens are expressed in all tick developmental stages and preliminary immunization trials and capillary feeding experiments have provided encouraging results, further studies are needed to test the efficacy of these proteins for the control of all *I. scapularis* stages and of other tick species.

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Table 1. Results of vaccination with recombinant tick protective antigens on *I. scapularis* larvae.

Experimental Group	RL/RLi	I (%)	ML/RL	M (%)	E (%)
4F8	0.14±0.07 [a]	64	0.84±0.05	-5	62
4D8	0.15±0.10 [a]	61	0.59±0.30 [a]	26	71
4E6	0.21±0.17	46	0.54±0.29 [a]	32	63
Control (MSP1a)	0.39±0.33	--	0.80±0.17	--	--

The inhibition of tick larval infestation (I) for each test group was calculated with respect to controls as [1-(RL/RLc x RLic/RLi)] x 100 = [1-(RL/RLi x RLic x RLc)] x 100, where RL is the average number of replete larvae recovered per mouse in test groups, RLc is the average number of replete larvae recovered per mouse in controls, RLic is the average number of larvae attached per mouse in controls, and RLi is the average number of larvae attached per mouse for each test group. The inhibition of molting (M) for each test group was calculated with respect to controls as [1-(ML/MLc x RLc/RL)] x 100 = [1-(ML/RL x RLc/MLc)] x 100, where ML is the average number of nymphs per mouse for each test group and MLc is the average number of nymphs per mouse for the control group. The protection efficacy of the vaccine was calculated as E = 100 x [1-(RI x RM)] where RI (average reduction in tick infestation) = 1-I/100 and RM (average reduction in molting) = 1-M/100. [a] Significantly different from controls ($P<0.05$).

Table 2. Results of vaccination with recombinant tick protective antigens on *I. scapularis*, *D. variabilis* and *A. americanum* nymphs.

Tick species	Inhibition of tick nymphal infestation (%)[a]				Reduction in the weight of engorged nymphs (%)[b]			
	4D8	4F8	4E6	All[c]	4D8	4F8	4E6	All[c]
I. scapularis	35	39	0	63	0	0	0	0
D. variabilis	22	0	5	8	32	0	27	0
A. americanum	17	9	29	12	3	1	0	16

[a]Determined with respect to the number of engorged nymphs recovered from the control group as described above for tick larval infestations.

[b]Average weight per engorged nymph with respect to the weight of nymphs collected from the control group.

[c]Rabbit in this group was immunized with a combination of 4D8, 4F8 and 4E6 antigens.

Table 3. Effect of capillary feeding with rabbit immune sera prepared against recombinant protective antigens on adult female *I. scapularis*.

Immune serum	Volume of serum ingested by CF (µl) (N=20)	Weight of ticks (mg) after 13 days of feeding (N=20)	No. dead ticks during the 13 days of feeding	Ratio of dead/fed ticks (%)[a]	Weight of engorged ticks (mg) that survived after 13 days of feeding	Weight of egg mass (mg) after 40 days of oviposition	No. dead ticks during the 40 days of oviposition	Ratio of total dead/fed ticks[b]
4F8	4.9±0.8[c]	183.8±93.7	2	10	208.9±72.0	105±39	1	15
4D8	4.7±0.6[c]	141.7±97.5[c]	5	25[d]	188.9±57.8[c]	103±24[c]	4	45[d]
4E6	4.5±0.7[c]	164.5±105.9[c]	5	25[d]	219.3±48.4	103±34	1	30
4F8+4D8+4E6	4.2±0.8	212.9±102.7	3	15[c]	250.5±50.3	116±39	2	25
Control[f]	4.2±0.5	219.9±90.5	1	5	231.4±76.3	120±28	3	20

[a]Ticks that died during feeding with respect to the 20 ticks that were fed.

[b]Ticks that died during the entire experiment (feeding + oviposition) with respect to the 20 ticks that were fed.

[c]Different from control ($P<0.05$; Student's t-test).

[d,e]Different from control ([d]$P<0.01$, [e]$P<0.05$; $\chi 2$–test).

[f]Control ticks were capillary fed on rabbit preimmune serum.

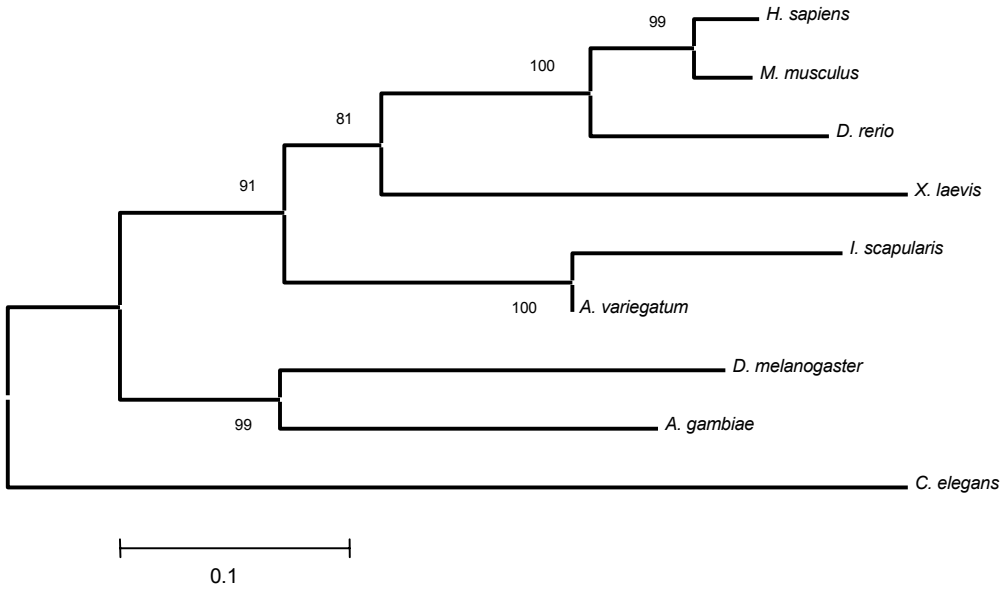

Figure 1. Phylogenetic relationships of 4D8 protein sequences using the minimum-evolution criterion. Bootstrap values for 1000 replicates are shown.

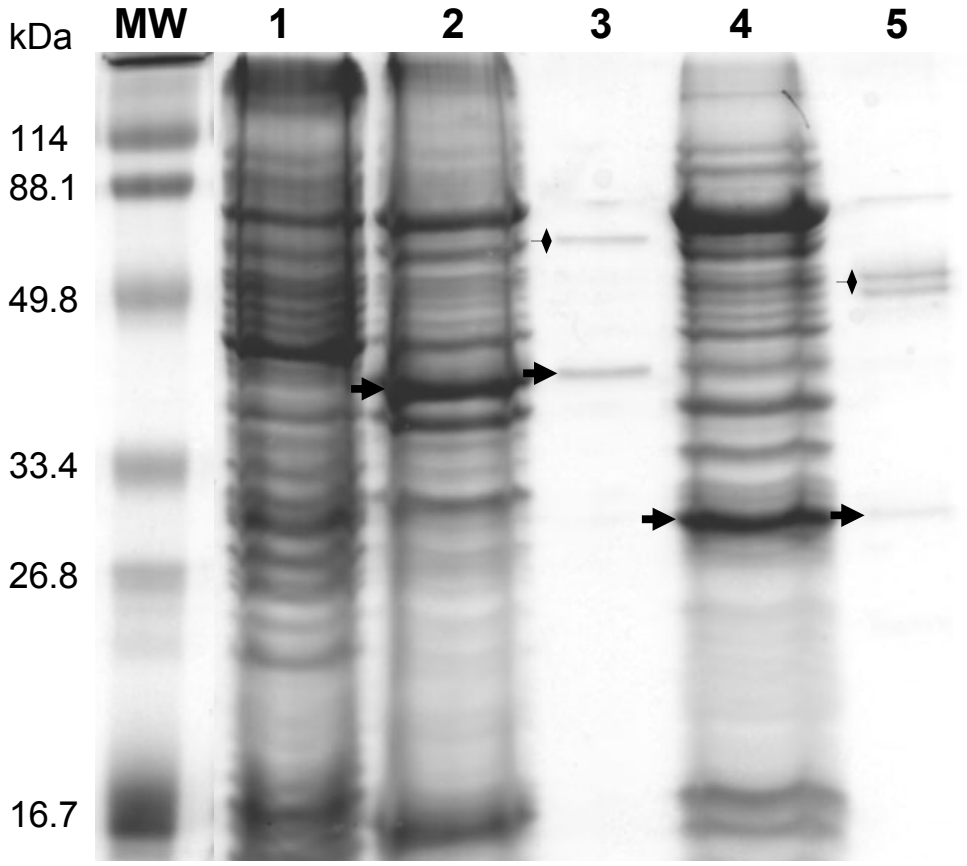

Figure 2. PAGE of recombinant 4F8 and 4D8 proteins. Protein samples were loaded on 12.5% polyacrylamide gels that were stained with Coomassie Brilliant Blue. Lane 1, induced *E. coli* cells (control); lane 2, induced *E. coli* expressing 4F8; lane 3, purified 4F8; lane 4, induced *E. coli* expressing 4D8; lane 5, purified 4D8. Arrows indicate the size of the proteins while diamonds indicate the presence of higher molecular weight products. MW, molecular weight markers (TriChromRanger marker, Pierce Biotechnology, Inc., Rockford, IL, USA).

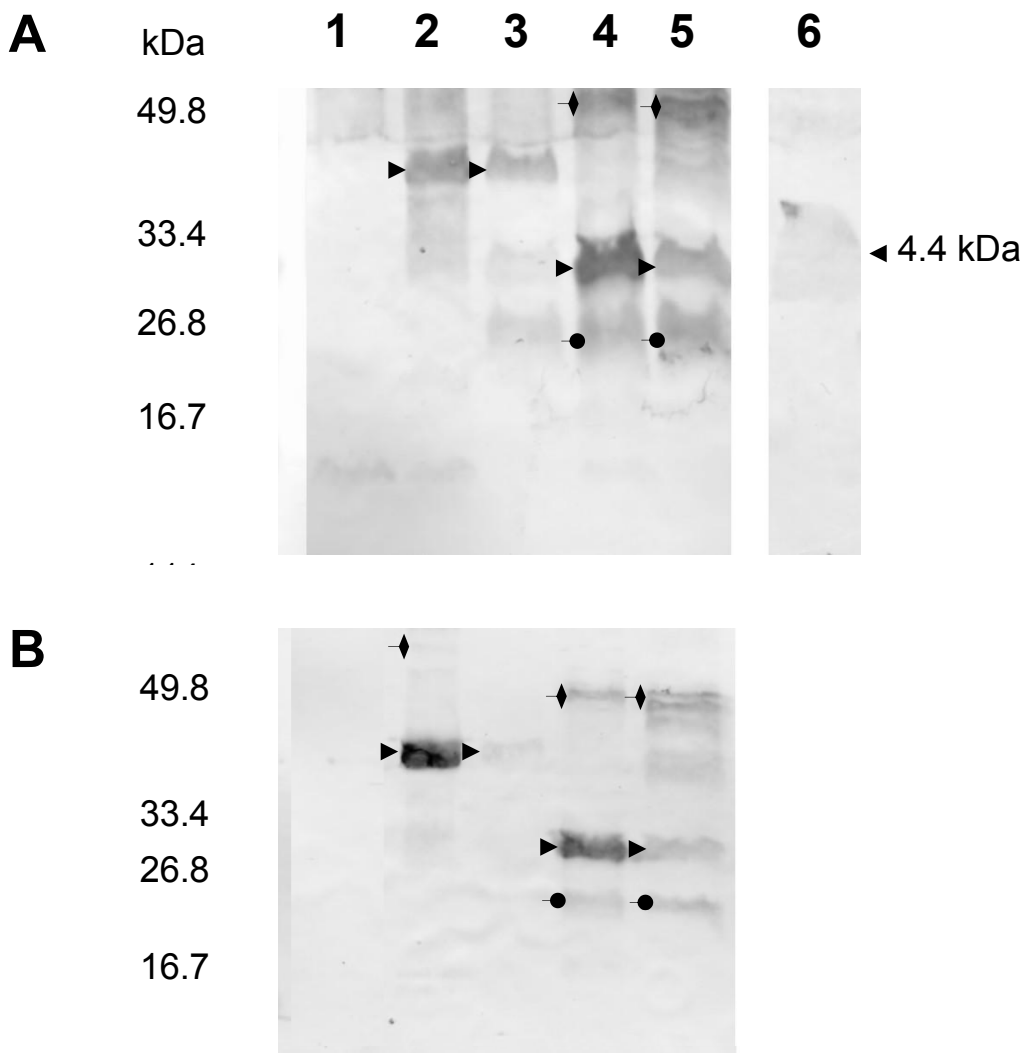

Figure 3. Western blot analysis of recombinant 4F8 (lanes 2, 3) and 4D8 (lanes 4, 5) proteins and synthetic peptide 4E6 (lane 6). Samples of induced *E. coli* proteins, recombinant proteins expressed in *E. coli* lanes 2, 4) and after purification (lanes 3, 5) and the synthetic peptide were separated by SDS-PAGE and reacted with (A) monospecific rabbit immune sera or (B) anti-FLAG M2 monoclonal antibody. Arrows indicate the size of the proteins while diamonds and circles indicate the presence of higher and lower molecular weight products, respectively. MW, molecular weight markers (TriChromRanger marker, Pierce).

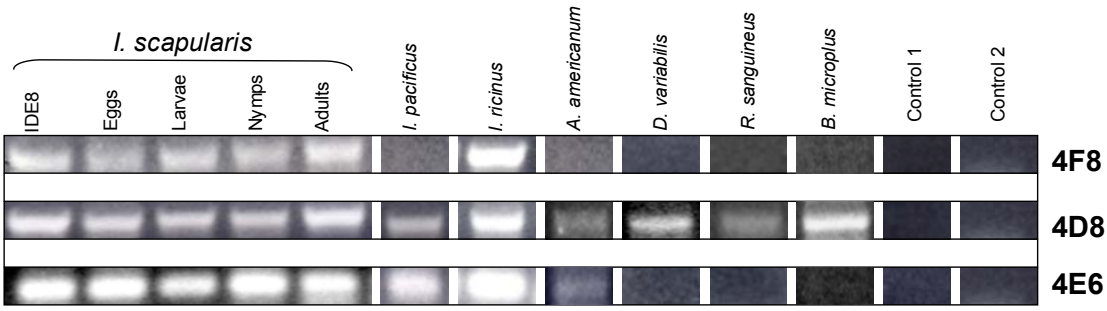

Figure 4. Expression of tick protective antigen mRNAs in *I. scapularis* developmental stages and adult tissues and in other tick species. RT-PCR reactions were done with primers specific for 4F8, 4D8 and 4E6 *I. scapularis* sequences with conditions described in Materials and Methods. Control reactions were performed using the same procedures but without RT to control for DNA contamination in the RNA preparations (Control 1) and without RNA added to control contamination of the PCR reaction (Control 2).

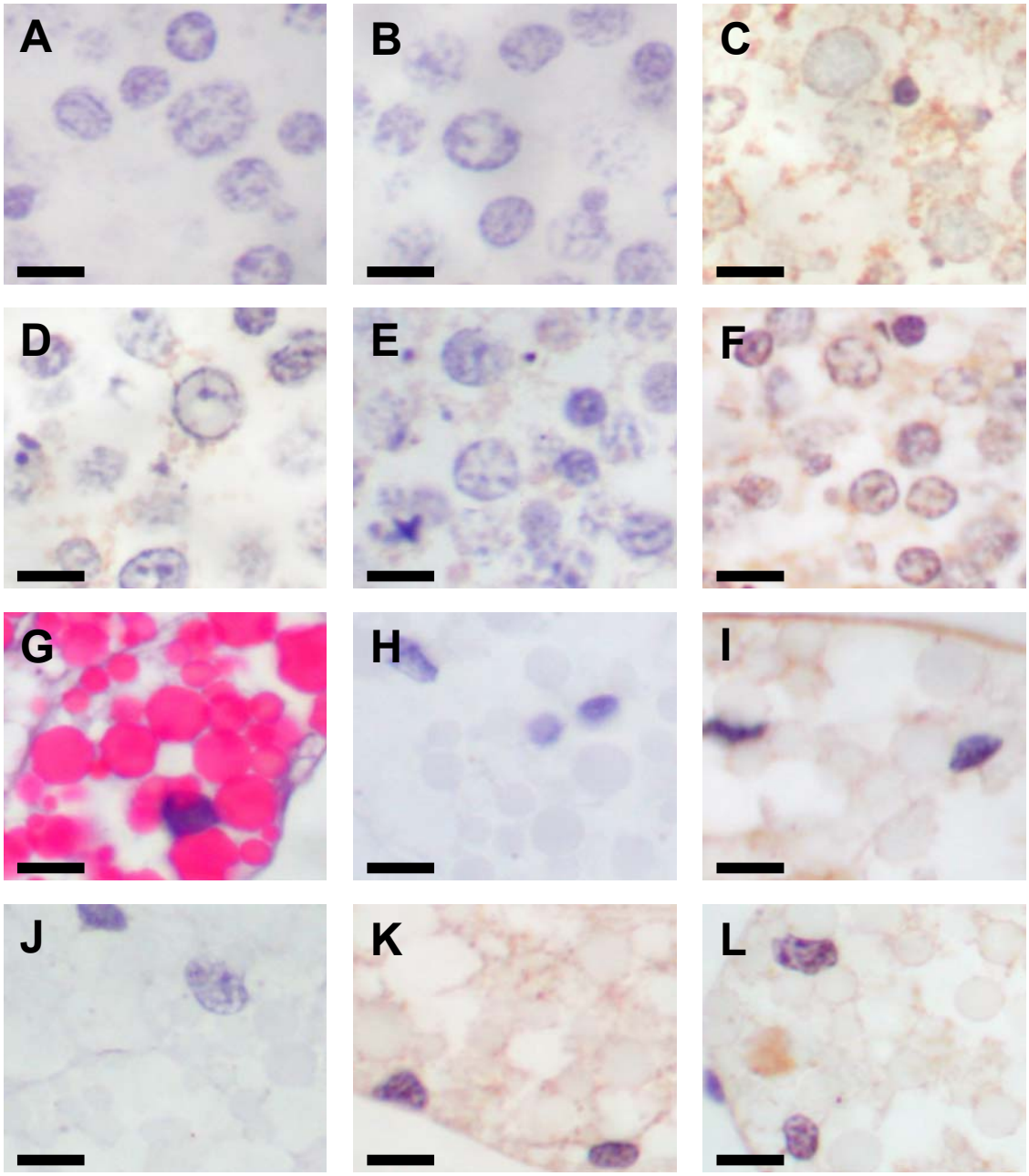

Figure 5. Localization of tick protective antigens by immunohistochemistry in paraffin sections of IDE8 tick cells (A-F) and adult *I. scapularis* guts (G-L). Sections A and G, stained with hematoxylin and eosin (bar 6.7 μm (A) and 6.9 μm (G)); B and H, negative controls reacted with rabbit preimmune serum (bar 6.7 μm (B) and 6.9 μm (H)); C and I, positive

controls reacted with anti-IDE8 antibodies (bar 6.9 μm (C) and 6.7 μm (I)); D and J, reacted with anti-4F8 antibodies (bar 7.0 μm); E and K, reacted with anti-4D8 antibodies (bar 6.8 μm (E) and 6.9 μm (K)); F and L, reacted with anti-4E6 antibodies (bar 6.7 μm (F) and 6.8 μm (L)). Original magnification, 600X. All sections except A and G were counterstained with hematoxylin.

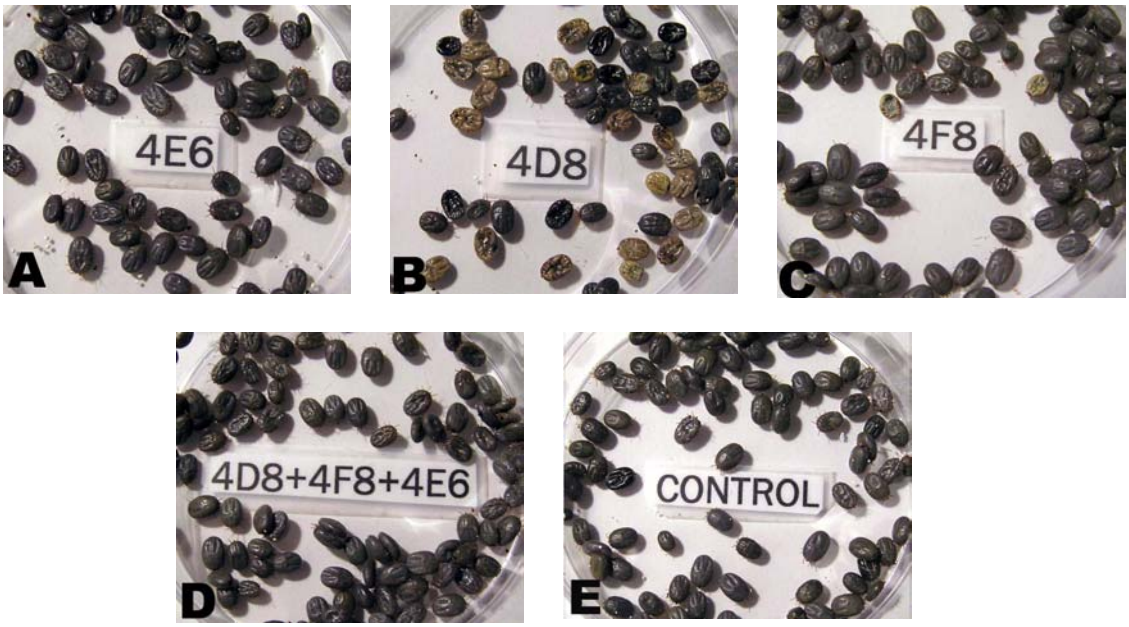

Figure 6. Effect of vaccination with recombinant *I. scapularis* antigens on *D. variabilis* nymphs. Engorged nymphs were collected from rabbits immunized with recombinant tick antigens and inspected for morphological changes in comparison with nymphs recovered from the control group. Nymphs visibly damaged were observed in the group that fed on the 4D8-vaccinated rabbit.

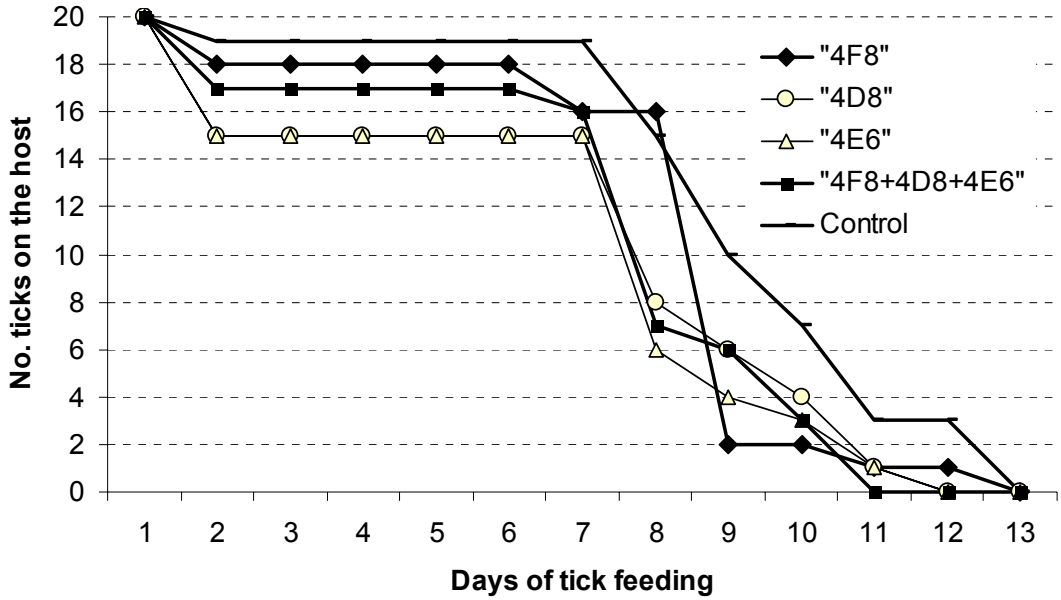

Figure 7. Daily number of female ticks feeding on sheep after capillary feeding on rabbit immune sera prepared against recombinant tick protective antigens.

Chapter 5

SUMMARY

Ticks are ectoparasites of domestic and wild animals and they rank second after mosquitoes as vectors of human diseases. The life cycle of some ticks is developed on one host while in other tick species like *Ixodes* spp. the life cycle requires three hosts, which difficult the control of these ectoparasites. Currently, the most effective method of tick control is the chemical; however, resistance of ticks to acaricides has propitiated the searching for new alternatives of control. Vaccination represents a good alternative for prevent both tick infestations and tick borne diseases. However, despite the efforts for produce vaccines against ticks, only one vaccine against the cattle tick *B. microplus* is available.

This thesis focuses on the identification and characterization of tick protective antigens in the three hosts tick *I. scapularis*. We hypothesized that cDNAs encoding protective antigens against *I. scapularis* infestations can be identified by ELI of a cDNA library constructed from a tick cell line (IDE8) derived from embryonic *I. scapularis*.

The identification of protective antigens against *I. scapularis* infestations was performed by construction of a cDNA ELI of IDE8 ticks cells, derived from embryos of *I. scapularis* in the vector pEXP1. cDNA clones were subjected to several rounds of screening where mice were immunized with cDNA pools and then challenged with *I. scapularis* larvae. The inhibition of tick infestation and inhibition of molting was evaluated. Three screenings were performed and 9 pools containing 351 cDNA clones that induced protection against tick infestations were selected in the first part of this study for further characterization.

The identified sequences were partially sequenced with a 5' vector specific primer (5'-CGACTCACTATAGGGAG-3'). Most of the obtained sequences were larger than 700 nucleotides. The nucleotide sequences were analyzed using the program AlignX. Multiple sequence alignment was performed using an engine based on the Clustal W algorithm. Phylogenetic trees were constructed by using the Neighbor algorithm of Saitou and Nei and searching of NCBI databases was performed by BLAST algorithm. From the 351 cDNAs identified, 316 contained non-redundant sequences and 101 produced a significant identity to sequences reported previously. Vaccination of mice with plasmid DNA and followed of tick challenging resulted in identification of cDNAs that inhibited tick infestation (identical to nucleotidase, heat shock proteins, beta-adaptine, chloride channel, ribosomal proteins, and proteins with unknown function) or promoted tick feeding (identical to beta-amyloid precursor, block of proliferation, mannose-binding lectine, RNA polymerase III, ATPases and a protein of unknown function).

Further experiments on mice allowed the selection of genes 4D8, 4F8, and 4E6. These genes were cloned and proteins expressed in *E. coli*. The expression of these proteins was demonstrated by protein gel electrophoresis and Western blot. The expression profile of proteins 4D8, 4F8 and 4E6 was studied by RT-PCR. Expression of 4F8, 4D8 and 4E6 mRNA was detected in *I. scapularis* eggs, larvae and nymphs and in guts and salivary glands from adult ticks. Expression and sequence conservation of these three genes was detected in *I. ricinus* and in *I. pacificus*. 4E6 was detected in *A. americanum* and 4D8 in *D. variabilis*, *A. mericanum, R. sanguineus* and *B. microplus*. Protein localization was studied by immunohistochemistry in sections of IDE8 cells and in sections of *I. scapularis* adult ticks. The three protective proteins were detected in *I. scapularis* IDE8 cells and 4D8 and 4E6 were detected in *I. scapularis* gut

sections. The protective properties of selected proteins against *I. scapularis* larvae were tested by immunization of 5 mice with 10 μg/dose of purified recombinant 4F8 and 4D8 proteins and with the synthetic 4E6 peptide followed of infestation with 100 *I. scapularis* larvae. The antibody response was evaluated by ELISA and Western blot. The overall efficacy of immunization was calculated for each tick antigen considering the effect on larval infestations and on molting to nymphs and ranged from 62% to 71%. To evaluate the effect of these proteins against nymphs, New Zealand rabbits were immunized with 50 μg/dose of purified 4F8 and 4D8 proteins or 4E6 synthetic peptide, a combination of 4F8+4D8+4E6, followed of infestation with 100 *I. scapularis* nymps on the right ear. In order to evaluate the effect of these proteins against other tick species, the same rabbist were infesteded with 100 nymphs of *A. americanum* and *D. variabilis*. Immunization with protein 4D8 was demonstrated by inhibition of nymphal infestations in the studied tick species.The protective effect of selected proteins against *I. scapularis* adults was evaluated by artificially feeding of ticks with serum from rabbits immunized with 4D8, 4F8, 4E6 and a combination of the 3 proteins. The major effects of tick capillary fed with rabbit immune sera were reduction of the feeding period, reduction of weight of engorged ticks, mortality, and reduction in oviposition.

The results presented herein confirmed the identification and characterization of three protective antigens against *I. scapularis* infestations. The antigens 4F8, 4D8 and 4E6, especially 4D8 appear to be good candidates for the development of tick vaccines. Although 4D8 is expressed in different tick species, further studies are required to identify the biological function and location of this protein in ticks and to test its efficacy against other tick species in experimentally and naturally infested hosts.

VITA

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Thesis: IDENTIFICATION AND CHARACTERIZATION OF PROTECTIVE ANTIGENS FOR CONTROL OF *Ixodes scapularis* INFESTATIONS

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Minor: Veterinary Pathobiology

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