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Two-hybrid analysis and attempted expression of elongation factor 1α from the cattle tick, *Rhipicephalus microplus*.

by

Mariëtte Botha

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Summary

Control of *Rhipicephalus microplus* is predominantly mediated by the application of acaricides, but the rapid acquisition of resistance by this species and environmental pollution resulting from discarded acaricides, necessitates the discovery of new control measures. Due to the fact that *Rhipicephalus* spp. are genetically diverse and often have more than one host, it has been difficult to identify a common protective vaccine candidate able to target all species of this genus. Only one anti-tick antigen, Bm86, has been commercialized to date and is sold as GAVAC® and GAVAC$^{Plus®}$ in South America.

In an attempt to identify protective antigens, a protein termed subolesin was identified using expression library immunisation. RNAi studies showed that subolesin knockdown causes the degeneration of tick guts, salivary glands, reproductive tissues and embryos. Subolesin additionally mediates tick gene expression, impacts the innate immune response and affects tick infection by *Anaplasma*, *Ehrlichia*, *Rickettsia*, *Babesia* or *Theileria* spp. The *R. microplus* EF-1α homolog was identified as a subolesin-interacting protein via yeast two-hybrid and co-affinity purification experiments. RNAi experiments have suggested that EF-1α is another possible anti-tick vaccine candidate since it exhibits a similar phenotype as subolesin upon knockdown.

The aim of the present research was to express *R. microplus* EF-1α in the yeast, *Pichia pastoris* and to exploit the yeast two-hybrid system in an attempt to identify its protein-binding partners. This will provide insight into understanding the translational machinery of this species and of ixodid ticks. Recombinant EF-1α was expressed as a 24 kDa protein, validated by western blotting. A highly representative cDNA library was produced from *R. microplus* mixed lifestages mRNA, fractionated and cloned into a two-hybrid prey vector. No definitive hits were obtained during the two-hybrid screen of reporter genes, as E-values attained after tblastx and PSI-BLAST analysis were higher than the required limit of 1×10^{-4}.

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List of Abbreviations

AA	Amino acid
aa-tRNA	aminoacyl transfer ribonucleic acid
Abs	Antibodies
ADE2	Adenine 2 reporter gene
AD	Activation domain
Amp^r	Ampicillin resistance gene
AP	Affinity purification
APS	Ammonium persulphate
ARS4	Autonomous replication sequence
3-AT	3-aminotriazole
AUR1-C	Reporter for Aureobasidin A resistance.
BD	Binding domain
BLAST	Basic Local Alignment Search Tool
BMGY	Buffered glycerol-complex medium
BMMY	Buffered methanol complex medium
BmPRM	*Boophilus microplus* paramyosin
BRET	Bioluminescence resonance energy transfer
CAPS	N-cyclohexyl-3-aminopropanesulfonic acid
cDNA	Complimentary deoxyribonucleic acid
CEN6	Centromeric sequence
CHAPS	N'N'-methylenebisacrylamide-3-1-propane
CK2	Casein Kinase II
4-CN	4-Chloro-1-Naphtol
CYH2	Cycloheximide resistance marker
DC	Decoding centre
DDO	Double dropout (-His/-Trp)
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DO	Dropout
ds	Double-stranded
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EF-1α	Elongation factor 1 alpha
EGF	Epidermal growth factor
ELI	Expression library immunization
EM	Electron microscopy

ER	Endoplasmic reticulum
EST	Expressed sequence tag
FITC	Fluorescein isothiocyanate
FRET	Fluorescence resonance energy transfer
GAP	Glyceraldehyde-3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GPI	Glycosylphosphatidylinositol
GSP	Gene specific primer
GTP	Guanosine triphosphate
HA	Hemagglutinin
HBP	Histamine binding protein
HIS3	Histidine 3 reporter gene
HSP	Heat shock protein
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
IGBMC	Immunoglobulin binding protein
IPTG	Isopropyl-β-D-thiogalactopyranoside
IREC	Instituto de Investigación en Recursos Cinegéticos
LB	Luria-Bertani
LD-PCR	Long distance polymerase chain reaction
LEU2	Leucine 2 reporter gene
MCS	Multiple cloning site
MDH	Minimal medium with dextrose
MEL1 (lacZ)	β-galactosidase reporter gene
MHC	Major histocompatibility complex
MMH	Minimal medium with histidine
MMLV	Moloney Murine Leukemia Virus
MRC	Medical Research Council
mRNA	messenger ribonucleic acid
MS	Mass spectrometry
MSP-1a	*Anaplasma marginale* surface protein
MWCO	Molecular weight cut-off
ng	Nanogram
Ni-NTA	Nickel-nitriloacetic acid
Ni-TED	Nickel-triscarboxymethyl ethylene diamine
NLS	Nuclear localisation signal
NPCP	Nuclear pore complex proteins
OD	Optical density

ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PKC	Protein kinase C
PMSF	Phenylmethylsulfonyl fluoride
PTC	Peptidyl transferase centre
PTM	Post-translational modifications
PVDF	Polyvinylidene difluoride
QDO	Quadruple dropout (-Trp/-Leu/-His/-Ade)
RACE	Rapid amplification of cDNA ends
rDNA	Recombinant deoxyribonucleic acid
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RT	Reverse transcriptase
RT-PCR	Reverse transcription polymerase chain reaction
SAP	Shrimp alkaline phosphatase
SAT	Sterile acarine techniques
SD	Single dropout (-Trp)
SDS	Sodium dodecyl sulphate
SNF	Sucrose non-fermentable
SOS genes	Derived from international distress signal
SSH	Suppressive subtractive hybridization
TBS	Tris buffered saline
TCR	T-cell receptor
TDO	Triple dropout (-Trp/-Leu/-His)
TEMED	N,N,N’,N’-tetramethyl-ethylenediamine
tHRF	Tick histamine release factor
TMB	3,3’,5,5’-Tetramethylbenzidine
TNE	Tris, Sodium chloride and EDTA
TRP1	Tryptophan 1 reporter gene
tRNA	transfer RNA
UAS	Upstream activation sequence
UCLM	Universidad de Castilla-La Mancha
UK	United Kingdom
URA3	Orotidine 5-phosphate decarboxylase
US	University of Stellenbosch
USA	United States of America

VTDCE	Vitellin degrading cysteine endopeptidase
Y2H	Yeast two-hybrid
YNB	Yeast nitrogen base
YPD	Yeast extract, Peptone, Dextrose
YPDA	Yeast extract, Peptone, Dextrose, Adenine
YPDS	Yeast extract, Peptone, Dextrose, Sorbitol

Chapter 1

Literature review

Control of *Rhipicephalus microplus* is predominantly mediated by the application of acaricides, but the rapid acquisition of resistance by this species and associated environmental pollution necessitates the discovery of new control measures (De la Fuente and Kocan, 2006). Due to the fact that *Rhipicephalus* spp. are genetically diverse, have multiple life stages and often have more than one host, it has been difficult to identify a common vaccine able to target all species of this genus. Only one anti-tick antigen (Bm86) has been commercialized to date as GAVAC®. TickGARD® (Bm86) and TickGARD®Plus (Bm86 and Bm95) have been discontinued due to inefficacy concerns (Pengally, 1999). Bm86 however, fails to protect against all geographical strains of *R. microplus,* exhibiting strain to strain and geographical variations in efficacy (Canales *et al.*, 2009a; Merino *et al.*, 2011).

1.1. *Rhipicephalus microplus* global impact

Identification and taxonomy

Hard ticks are classified into the family Ixodidae, order Ixodida, suborder Parasitiformes, class Arachnida and subclass Acari that it shares with other 8-legged arachnids (Oliver, 1989; Sonenshine, 1991) A hard sclerotized shield (scutum) on the dorsal surface, a leathery integument and the position of the mouthparts, identifies the hard ticks from their soft tick counterparts in the Argasidae family (Sonenshine, 1991). *Rhipicephalus* spp. present with a hexagonal basis capitulum, a rounded spiracular plate and compressed palps that are laterally and dorsally ridged. The anal groove is absent in females, whilst the males have an adanal plate and accessory shields (CFSPH, 2007). Currently, the estimated number of ixodid tick species is 665 (Kolonin, 2009).

The genus *Rhipicephalus* consists of more than 70 species (Kolonin, 2009). *R. decoloratus*, *R. appendiculatus*, *R. evertsi evertsi* and *R. microplus* are the key members of this genera found on cattle in South Africa, whilst *R. annulatus* and *R. geigyi* are more prominent in northern Africa (Walker *et al.*, 2003). Whilst its common names include the cattle tick, southern cattle tick and the Asiatic blue tick, the taxonomic classification of *R. microplus* was widely disputed throughout the years (Horak, 2009).

R. microplus was initially classified as *Boophilus microplus* within the genus *Boophilus*, adjacent to the *Rhipicephalus* genus. Mating data however, suggested that the *Boophilus* genus lays within the *Rhipicephalus* genus. As a result, *R. (B) microplus* was recently re-classified as *R. microplus* after the re-allocation of the *Boophilus* sub-genus into *Rhipicephalus* (Horak, 2009). Morphological data suggests that *R. microplus* is more closely related to *R. decoloratus* and *R. annulatus*, but phylogenetic data suggest differently. Mitochondrial 12S ribosomal phylogenetic data show that *R. microplus* and *R. annulatus* are grouped as sister species, whilst *R. decoloratus* is designated as an outgroup of the genus (Beati and Keirandst, 2001).

Distribution

R. microplus is widely distributed throughout most regions of southern, eastern and western Africa, as well as Asia and South America, having colonized most tropical and subtropical regions between 32°S and 40°N (Sonenshine, 1991; Pipano *et al.*, 2003). Figure 1.1 illustrates the global distribution of *R. microplus*. This species is thought to have been introduced to Africa, Australia and South America from the Indian subcontinent (Cutulle *et al.*, 2010). Other studies indicate that it might have originated from the African continent, as it is most closely related to other *Rhipicephalus* ticks of African origin (Murrell *et al.*, 2000; Barker and Murrell, 2004).

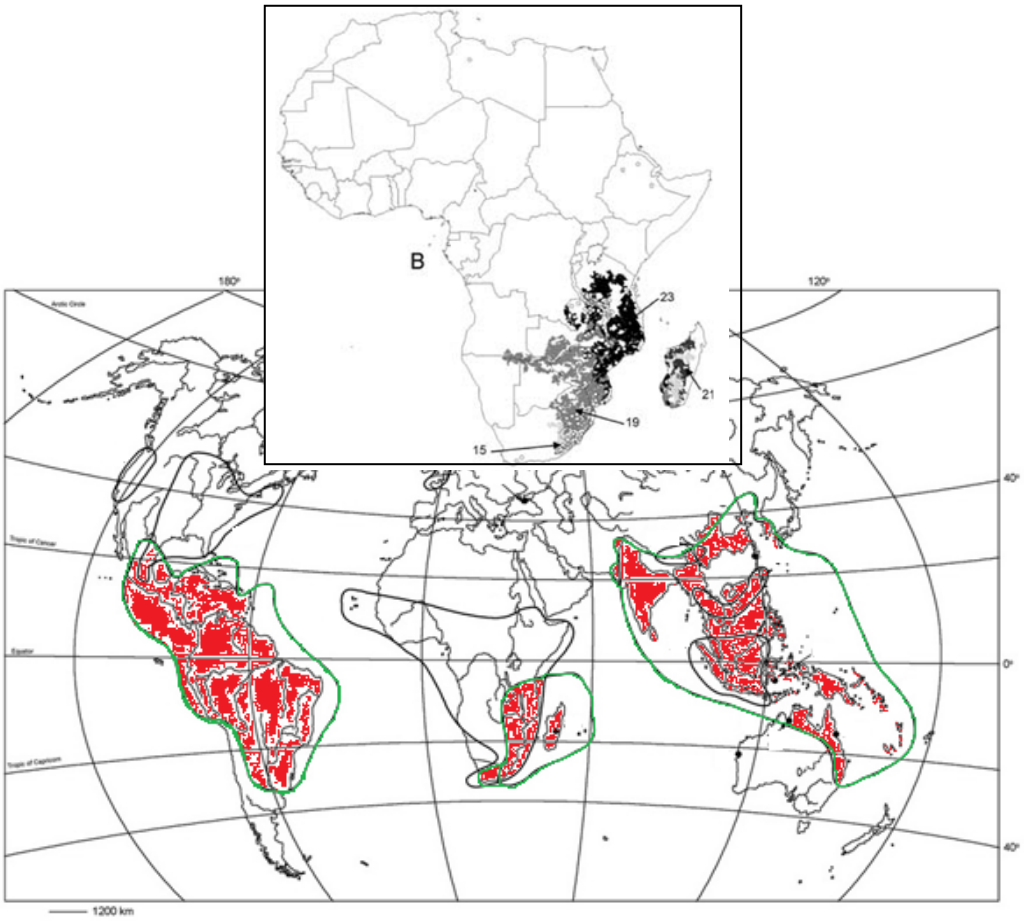

Figure 1.1: Global and African distribution maps of *R. microplus*. *Adapted from (Kolonin, 2009) and (Estrada-Pena et al., 2006).* ***(A)*** *Global areas where R. microplus has been identified are indicated with red and green.* ***(B)*** *The distribution map of Africa is enlarged. Black shaded areas show confirmed records of R. microplus. Grey shaded areas indicate dissimilar vegetation areas where R. microplus is found.*

Much of the current African habitat of *R. microplus* was previously inhabited by native *R. decoloratus* (Lynen *et al.*, 2008; De Matos *et al.*, 2009). Since the introduction of competitively reproducing *R. microplus* to areas such as the eastern parts of South Africa and Tanzania, *R. decoloratus* has steadily been displaced. This encroachment of habitat is evident from studies conducted in Tanzania, Zambia, Swaziland and Ivory Coast in southern Africa, as well as the Free State, Eastern Cape and Limpopo provinces of South Africa (Horak, 2009; Madder *et al.*, 2012).

The reproductive success and spread of *R. microplus* is evident in the Ivory Coast. This tick was identified for the first time in this region in 2007 and has since displaced all indigenous *Rhipicephalus* species, annihilating conventional tick control measures (Madder *et al.*, 2010). Whereas *R. microplus* was previously only present in the eastern and northern provinces of Zambia, it has since spread west towards the central parts of the country (Simuunza *et al.*, 2011b). Range

expansion of *R. microplus*, and its associated pathogens is due to its rapid life cycle, changes in climate as well as increases in host population and migration (Lynen *et al.*, 2008; Merino *et al.*, 2011).

Life cycle, host specificity and reproduction strategy

Ixodid ticks are obligate, hematophagous ecto-parasites with life cycles that comprise of four stages: eggs, larvae, nymphs and adults (Sonenshine, 1991). Being a one-host tick, *R. microplus* completes the larval, nymphal and adult stages on a single host, therefore being considered as monotrophic (Walker *et al.*, 2003). Larvae molt twice before becoming nymphs and then adults (CFSPH, 2007). Mating occurs on the host during feeding where after fertilized, engorged females drop from the host. A large egg mass (3000 – 5000) is deposited and hatched in the hosts' surrounding environment (Oliver, 1989; Sonenshine, 1991). Figure 1.2 depicts the life cycle of *R. microplus*.

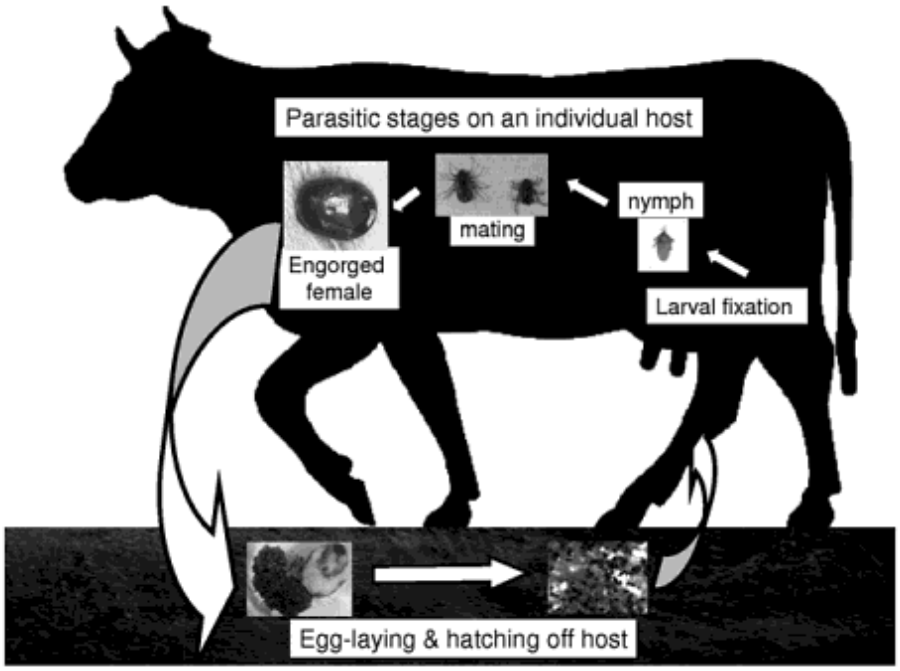

Figure 1.2: Life cycle of *R. microplus* (Morand *et al.*, 2011). *After dropping from the single host, fully engorged females oviposit on the ground and perish. Larvae hatched from eggs survive up to 15 days whilst seeking a host. Larvae, nymphs and adult females are all parasitic blood feeders.*

Female *R. microplus* ticks have a circadian feeding cycle interposed with periods of ingestion, salivation and resting. Diapause occurs in all Ixodid ticks and is synchronised with biological and environmental conditions in the circadian feeding cycle (Oliver, 1989). Ixodid tick diapause is behavioural and is influenced by host availability. Examples include the delay of engorgement of

attached ticks, morphogenetic delays such as embryogenesis, moulting delays of immature ticks and delays of oviposition by engorged females (Oliver, 1989; Sonenshine, 1991).

R. microplus primarily parasitizes cattle and other ruminants such as deer and buffalo, but will occasionally infest dogs, goats, horses, sheep and wildlife (CFSPH, 2007; Tonetti *et al.*, 2009). Preferred bovine hosts are from the *Bos* subgenus (e.g. *Bos taurus* and *Bos indicus*) located on farmland in South Africa (Cutulle *et al.*, 2009; Cutulle *et al.*, 2010). Alternative hosts often come into contact with domestic cattle, where they can act as reservoirs for ticks and tick-borne diseases (Horak, 2009). *R. microplus* life cycle, reproduction and feeding strategy are greatly influenced by photoperiod, temperature, moisture and abundance of hosts and mates (Oliver, 1989).

Ixodid genomes and genetic diversity

The *R. microplus* genome is estimated at 7.1 Gbps (giga base pairs) in length, approximately 2.5 times larger than the human genome. Tick genomes appear analogous to vertebrate genomes, and include a high occurrence (up to 70%) of repetitive sequences which includes unknown short repeats (5% of the *I. scapularis* genome), SINE retroposons in *R. appendiculatus* and Type I and Type II transposable elements in *R. microplus* (Guerrero *et al.*, 2010; Kamau *et al.*, 2011; Bellgard *et al.*, 2012). It is because of this repetitive nature that the *R. microplus* genome has not been completed yet. Currently, 1.8 Gbp's of unique sequence (obtained via Cot sequencing) is available on the CattleTickBase website (Bellgard *et al.*, 2012). The unfinished *R. microplus* mitochondrial genome displays similar characteristics such as low GC content, abundant tandem repeats and propensity towards rearrangement (Campbell and Barker, 1999). Genome data hold the key to understanding strain-to-strain differences.

As mentioned, there are morphological and genetic differences between *R. microplus* strains. This suggests that biogeographical and ecological separation may be responsible for the divergence of different species from dissimilar geographical regions. In 1998, Stephen Barker investigated whether South African and Australian *R. microplus* could be considered different species. Although exploration of ITS 2 rRNA sequences did not yield definitive evidence for speciation, maximum likelihood analyses showed significant bootstrap support for close relationships between Australian and Brazilian *R. microplus* (98%), as well as Kenyan and South African *R. microplus* (84%) (Barker, 1998). Divergence can likewise be studied by investigating the reproductive performance of geographical crosses.

Labruna et al. (2009) tested the reproductive performance and genetic diversity from crosses of *R. microplus* from Africa, Asia, America and Australia. Crosses between Argentinean and

Mozambican (*R. microplus* - Canestrini, 1887) individuals were fertile, whereas crosses of these geo-species with Australian-Indonesian-Caledonian counterparts (*R. australis* – Fuller 1899) were infertile. Genetic analysis of mitochondrial 16S and 12S rDNA confirmed the genetic diversity (1.6 – 2.4%) of Australian *R. microplus* in comparison to the other strains investigated. Complete speciation could not be proved as these hybrids exhibited higher egg production, albeit they were sterile (Labruna *et al.*, 2009).

Disease transmission

R. microplus is the main vector of the rickettsial bacteria *A. marginale* and *A. phagocytophilum*, endemic worldwide (Estrada-Pena *et al.*, 2009). By applying bacterial 16S–tag encoded pyrosequencing, pathogenic genera such as *Wolbachia* and *Borrelia* were recently identified as part of the *R. microplus* microbiome, but disease transmission remains to be proven (Andreotti *et al.*, 2011). It additionally vectors *Babesia bovis* and *Babesia bigemina*, the causative agents of bovine babesiosis (Canales *et al.*, 2009a). *B. bovis* is responsible for the more severe Asiatic babesiosis in cattle (Nyangiwe and Horak, 2007). *Babesia* is transmitted to cattle when ticks take a blood meal. *Babesia* replicates within the host erythrocytes leading to lysis and cell death (Chauvin *et al.*, 2009). Transovarial transmission of the disease occurs between hosts (cattle) and tick vectors (Simuunza *et al.*, 2011a). Due to the economic losses incurred by the lethality and drug resistance of *Babesia* spp., new acaricides and anti-tick vaccines are required to control the parasite through its tick vector.

Economic burden of R. microplus infestation

The Southern cattle tick, *R. microplus*, is regarded as the most important tick species affecting the economical sustainability of the livestock industry. *R. microplus* not only transmits the causative organisms of babesiosis (*B. bovis* and *B. bigemina*) and anaplasmosis (*A. marginale* and *A. phagocytophilum*), but also effects weight loss, meat and milk production (De la Fuente and Kocan, 2006). Damage to hides also occurs due to scar tissue (granuloma) formation at tick feeding sites. Physical indications of *R. microplus* infestation result in general loss of condition. Together, these culminate in direct capital losses for the global and South African livestock industry.

Before its eradication in 1943, babesiosis cost the US an estimated 130.5 million dollars in direct and indirect annual losses (CFSPH, 2007). In 1980, South African cattle losses amounted to 70 – 200 million rand per year (Bigalke, 1980). According to the South African Animal Health Association (SAAHA), farmers currently expend around R195 million on the eradication of ticks per year: R15 million on the treatment of redwater (babesiosis) and another R20 million on the treatment of heartwater (*Ehrlichia ruminantium)* and anaplasmosis (Landbouweekblad, 2012).

1.2. Tick control

1.2.1. Current methods of tick control

Tick control is mediated by integrated pest management, which involves the adaptation of control methods to a specific geographical area. The main control method up to date has been the use of acaricides, but these chemicals have resulted in the widespread development of resistance and contamination of the environment (De la Fuente and Kocan, 2006). Added drawbacks of acaricides include the necessity of repeated administration, cost, contamination of meat and milk products and the duration of new acaricide development against resistant species. Acaricidal and other less promising control methods are discussed below.

Acaricides

Treatment of *R. microplus* requires the use of single, multiple and rotational rounds of acaricide treatment (Baffi *et al.*, 2007; Guerrero *et al.*, 2007). The main acaricides in use include amitraz, pyrethroids (pyrethrins), formamidines, acetylcholine esterase inhibitors such as the organophosphates and cyclodiene organochlorines (Li *et al.*, 2007; Budeli *et al.*, 2009; Beugnet and Franc, 2012). Acaricide resistance hampers the effective control of tick populations.

R. microplus and other ticks gain acaricide resistance within a few generations due to their high genetic diversity and pangamy mating structure (Cutulle *et al.*, 2010). Resistant ticks subsequently confer this attribute to their offspring. Cattle movement, in turn, results in the flow of resistance genes to other farms and provinces (Budeli *et al.*, 2009; Cutulle *et al.*, 2009). At the USA - Mexico border, tick acaricide resistance was so severe that individuals were noted to survive the dipping procedure, endangering the USA toward re-introduction of *R. microplus* following its eradication in 1943 (Madder *et al.*, 2010).

Genetic control

The breeding of naturally immune hosts was initiated with the Bonsmara breed in Africa (Fivaz and De Waal, 1993). Cattle breeds like the Australian Friesian Sahiwal was bred by combining the milk yield and drought resistance of *Bos taurus taurus* with the natural tick immunity of *Bos taurus indicus* (Walker, 2011). Manifestations of natural immunity is evident from a recent investigation. The clotting time of blood collected from tick feeding lesions of naturally resistant (*B. taurus indicus*) and susceptible (*B. taurus taurus*) cattle differed significantly. Clotting times were correlated to the expression profiles of anti-haemostatic proteins in the ticks. It was found that these molecules were over expressed in ticks fed on susceptible cattle (Carvalho *et al.*, 2010).

It should however be noted that both humoral and cellular immunity are important mechanisms of natural tick resistance. Cellular immunity is evident by an enhanced cutaneous basophil hypersensitivity reaction, i.e. the hyperplastic epidermis being infiltrated by basophils, neutrophils, macrophages, eosinophils and mast cells in rabbits and cattle (Trimnell *et al.*, 2005; Carvalho *et al.*, 2010). Degranulated basophils and mast cells release histamine which causes severe vascular permeability resulting in oedema and ultimately causing a reduction of nutritive blood flow to tick feeding sites (Trimnell *et al.*, 2005).

Humoral immunity (acquired resistance) to ticks develops slowly, is more variable, is subject to immunosuppression and includes T and B lymphocytes, plasma cells, macrophages and dendritic cells (Trimnell *et al.*, 2005). Studying the humoral response by transferring serum from immune hosts to non-immune hosts, indicated that antibodies are involved in natural resistance to ticks (Roberts and Kerr, 1976). The mechanism by which acquired immunity is expressed, varies greatly and is dependent on the host and tick species concerned (Akhtar *et al.*, 2010).

Sterile acarine techniques (SATs)

During the application of sterile acarine techniques, ticks are sterilized by hybridization (Hilburn *et al.*, 1991), irradiation (Galun *et al.*, 1972), chemical treatment (Hayes and Oliver, 1981) or RNAi (Merino *et al.*, 2011). SATs can be improved by the development of transgenic ticks, allowing the subsequent large-scale production and release of sterile individuals. These techniques are currently used successfully for the control of vectors such as mosquitoes of the *Anopheles* genera (Munhenga *et al.*, 2011; Nolan *et al.*, 2011). SATs remain at proof of concept stage for acarines, due to the difficulties in rearing the large amounts of ticks required and the ethical hurdles for tick release into the field.

Phytotherapeutic control

A wide variety of plants have shown repellent or acaricidal activity as well as suitability to organic cattle farming industries. Essential oils and plant extracts such as *Cuminum cyminum* (cumin), *Pimenta dioica* (allspice), *Ocimum basilicum* (sweet basil), preparations from *Allium sativum* (garlic) and Citrus (*Rutaceae*) peel exudates, have become popular as acaricides and repellents (Costa-Junior and Furlong, 2011; Martinez-Velazquez *et al.*, 2011; Weldon *et al.*, 2011). Only a few of these extracts have been used successfully for the control of *R. microplus*.

Plants from the following families have been tested against *R. microplus* and the active compounds identified: Annonaceae (custard apple), Lamiaceae (mint), Leguminosae (legumes), Liliaceae (lily), Meliaceae (mahogany), Myrtaceae (cloves, eucalyptus), Piperaceae (pepper), Phytolaccaceae

(pokeweed), Poaceae (true grasses), Rutaceae (citrus) and Winteraceae (Borges *et al.*, 2011). Despite promising *in vitro* results, most of the extracts have not been validated due to limitations in the use of phytotherapeutics for *R. microplus* control. Progress is hampered by proper formulation preparation, differences in the chemical composition of plants of the same species and a lack of information regarding currently used phytotherapeutics (Borges *et al.*, 2011).

Predators

African birds such as the yellow and red-billed oxpeckers (*Buphagus africanus* and *B. erythrorhynchus*) often feed on ectoparasites, with single birds being able to eat as much as 14 grams of ticks per day. Oxpecker populations have decreased in recent years, due to the decrease in tick numbers caused by the use of acaricides (Bezuidenhout and Stutterheim, 1980). Other bird species such as the domestic chicken (*Gallus gallus*) and the helmeted guinea fowl (*Numida meleagris*) are opportunistic predators of ticks (Samish *et al.*, 2004). Alternative predators of ticks that have a slight impact on tick populations include ants, beetles and spiders (Jemal and Hugh-Jones, 1993; Samish and Rehacek, 1999).

Parasitoids

An alternative biological control agent is the parasitic wasp, *Ixodiphagous hookeri*. Female wasps parasitize hard ticks by ovipositing in the body of the tick, where after wasp development is initiated by the nymph's first blood meal. Infestation is fatal to the tick, since the *I. hookeri* larvae will consume the body cavity contents prior to tick molting. In-field *I. hookeri* release (150,000 specimens) resulted in the reduction of 44 to 2 ticks per cow in Kenya (Mwangi *et al.*, 1997). Large-scale release of wasps however, seems inconceivable due to high breeding costs and *I. hookeri* susceptibility to insecticides. Their predatory success is additionally limited by geographical area, presence of host tick species, their preference of nymphal stages and periods of active parasitism (Collatz *et al.*, 2011). Finally, predation by nematodes has been exploited, but is also habitually and environmentally restricted.

Juvenile stage nematodes locate and enter mainly engorged female ticks via natural orifices. Ticks are not killed by the nematode, but by the symbiotic bacteria released into the body cavity (Samish *et al.*, 2004). Nematodes actively parasitic against *R. microplus* include *Steinernema glaseri* (Santa Rosa strain) and *Heterorhabditis bacteriophora* (CCA strain), exhibiting 90% and 80% reduction in oviposition at 5000 and 1500 infective juveniles (i.j), respectively (De Oliveira Vasconcelos *et al.*, 2004). *R. microplus* mortality (40%) was achieved nine days after exposure to *Steinernema diaprepesi* (Colimense strain) (Molina-Ochoa *et al.*, 2009), whereas *Heterorhabditis bacteriophora* (strain HP88) had deleterious effects on oviposition and hatching at doses higher than 75 i.j.

(Monteiro *et al.*, 2010). Similar results were observed for *Steinernema carpocapsae* and *Heterorhabditis indica* (Maru *et al.*, 2011; Da Silva *et al.*, 2012).

Pathogens

Very few fungal species that are entomopathogenic towards ticks have been classified, though commercial products have been developed with specific focus on myco-acaricides. The conidia of *Metarhizium anisopliae* and *Beauveria bassiana* are suggested to germinate on contact with the tick cuticle, after which it penetrates the cuticle with the aid of chitinases and proteases. Lethality is based on nutrient depletion and release of mycotoxins, making this a time dependent control method (Samish and Rehacek, 1999).

Other disadvantages of fungal tick control include susceptibility to environmental conditions such as humidity and degree of sunlight and slow killing rate. Improvements have been made by selecting for highly pathogenic strains with little off-target effects and by improving deployment strategies (Samish *et al.*, 2004). Significant research is required towards understanding the host skin micro-environmental factors that may affect fungal pathogenicity (Polar *et al.*, 2008).

1.2.2. Immunological control: anti-tick vaccine development

Vaccines are composed of either molecular (e.g. a protein antigen or poly-epitope) or supramolecular (whole organism; antigen mixtures) agents that can confer protective immunity against pathogens and their disease symptoms (Bagnoli *et al.*, 2011). Protective immunity involves a precise and adaptive immune response by re-infection of the same or a related organism. Host adaptive immunity is typically induced by the innate immune system (mediated by macrophages, dendritic cells and mobile phagocytic cells in tissue) which is activated upon infection by other organisms in a non-specific manner. The latter results in the activation and proliferation of B- and T cells which aid in the development of immune memory (Flower *et al.*, 2010; Zepp, 2010). Innate and adaptive immunity thus have to interact dynamically via antigen-presenting cells, in order to confer protective immunity. Current anti-tick vaccine selection is based on *in vivo* immune response elicitation, preferably involving mediators of adaptive immunity.

Ticks have however developed mechanisms to counteract the activation of both the innate and adaptive (cellular) immune responses. Host immune modulation (evasion) by ticks is primarily mediated by salivary proteins. The saliva of *I. scapularis*, *I. ricinus*, *R. appendiculatus*, *I. uriae* and *I. hexagonus* was shown to inhibit the activation of the alternative (complement) pathway in human, rat, mouse, guinea pig and hamster sera, as well as the release of anaphylatoxins and chemokines (Vancova *et al.*, 2010a). Histamine blockers and soluble histamine receptors (lipocalins) have been

found to be common components of tick saliva. Furthermore, tick saliva has also been found to contain various modulators of cells (basophils, neutrophils, leukocytes) involved in host innate immunity (Schoeler and Wikel, 2001). These bioactive, immunomodulatory molecules are futher reviewed in (Ribeiro, 1987), (Gillespie *et al.*, 2000), (Wang *et al.*, 2001) and (Vancova *et al.*, 2010a).

Supramolecular (whole organism; antigen mixtures) anti-tick vaccines have been evaluated. The first evidence for whole-fraction induced immunity was provided by Opdebeeck *et al* in 1988. The authors indicated that fractions from *R. microplus* midgut conferred 91% protection to cattle against tick challenge. Additionally, the levels of IgG1 and complement fixing antibodies were comparable to the levels induced by single-antigen vaccination (Opdebeeck *et al.*, 1988; Jackson and Opdebeeck, 1990; Jackson and Opdebeeck, 1995). Another example includes the immunization of BALB/c female mice with whole midgut membrane protein isolates. Serum was collected post immunization, pooled and its reactivity towards *R. microplus* (Mozambique strain) *in silico* predicted antigenic membrane peptides, tested. Three peptides exhibited better recognition (p value < 0.001) by the polyclonal antisera, than the peptides derived from the positive control Bm86 (Maritz-Olivier *et al.*, 2012). The benefits of using whole fraction formulations include low costs incurred and ease of preparation.

Existing and previously commercially available vaccines (Gavac® and TickGARD®) have exhibited overall efficacies that are far below the needs of agricultural producers, require three monthly re-vaccinations and the concomitant use of acaricides to improve efficiency (De la Fuente *et al.*, 2005). The main limitation to the development of new anti-tick vaccines is the identification of antigens that are able to protect the host from tick infestation and reduce tick feeding and reproduction. This is restricted by current approaches during which individual candidates are investigated. Fresh approaches are thus needed to screen large repertoires of antigens at once, whilst simultaneously reducing animal experimentation (De la Fuente *et al.*, 2005). Due to limitations in genomic data availability, conventional vaccine design strategies are still the norm for anti-tick vaccine research. The recent progress in sequencing tick genomes and the rise of bioinformatics have resulted in the application of novel vaccine design strategies termed reverse vaccinology.

Novel approaches for anti-tick vaccine discovery

Genome based vaccine development (reverse vaccinology) provides access to all proteins encoded by an organism using its genome and transcriptome data in combination with computational analysis. This approach bypasses physical investigations into the organism itself. Reverse vaccinology is thus the computational discovery of vaccine candidates by scanning genomes for open reading frames (ORFs), followed by selection of proteins via bioinformatic prediction

methodologies based on accessibility to immune system scrutiny supported by genomic annotation, immunotransciptomics (functional genomics) and computer based epitope prediction (Flower *et al.*, 2010). Reverse vaccinology is further supported by the public availability of multiple genomes and proteomes (Bagnoli *et al.*, 2011). The recently established CattleTickBase Database will be an invaluable resource for the annotation of the *R. microplus* genome and concomitant antigen discovery by current strategies (Maritz-Olivier *et al.*, 2012).

Several factors contributing towards protective immunity need to be considered when planning a genome-wide vaccine screening strategy. This includes host factors such as T- and B-cell epitope presence and type of immune response elicited, parasite (pathogen) factors such as expression level and subcellular localization of antigens, as well as antigen factors such as chemical and physical properties, post-translational modifications (PTMs) and aggregation status. Accessibility of such antigens to the host immune system is mediated by protein expression during the life cycle, as well as secretion or presentation on membranes or external surfaces of the parasite. Membrane antigen topology also needs to be considered so that exoplasmic regions may be selected for antigen preparation (Maritz-Olivier *et al.*, 2012).

As mentioned, anti-tick vaccine antigen discovery is predominantly limited by a lack of genome data, but transcriptome data is proving valuable to date. The genome of *I. scapularis* is already available (http://extension. entm.purdue.edu/igp/ vectorbase.ht ml), whereas that of *R. microplus* is underway. There are two obstacles to the elucidation of tick genomes, namely the large size of these genomes (even larger than the human genome), and the repetitive nature of sequences. Reduced representation sequencing techniques such as Cot filtration, are being used to sequence low-copy regions such as promoters, introns and non-expressed genes (Guerrero *et al.*, 2010), whilst techniques such as phage display simplifies tick mimotope identification (Prudencio *et al.*, 2010a). Currently for *R. microplus*, transcriptome (via DNA microarray or RNAseq) and proteome analyses will allow for the identification of relevant expression patterns in the absence of complete genome data (Maritz-Olivier *et al.*, 2012). Current tick transcriptomic data remains mostly un-annotatable and contains a large amount of truncated transcripts. Proteomic data to verify protein ORFs and subcellular localization is also insufficient. Additionally, *in silico* epitope predictors are less efficient for anti-tick vaccine epitope prediction, due to the fact that it uses human or murine major histocompatibility alleles (Maritz-Olivier *et al.*, 2012). Although cumbersome, reverse vaccinology is beneficial to anti-tick vaccine discovery.

Maritz-Olivier *et al*., (2012) recently applied a functional genomics and reverse vaccinology approach to identify a total of 791 candidates from *R. microplus*. Using a pipeline of *in silico*

prediction of subcellular localization and antigenicity, 176 antigens were identified as membrane-associated and 86 as secreted, soluble proteins (Maritz-Olivier *et al.*, 2012).

Ideal anti-tick vaccine characteristics

The following are optimal features for new anti-tick vaccine candidates: Activity against all life stages, availability to immune detection, possession of host recognizable epitopes, long-lasting immunity, deterring of tick attachment, reduction of disease occurrence, minimal resistance development and cost effectiveness. Since even highly conserved antigens such as subolesin and Bm86 have not exhibited significant results as cross-protective vaccines, combinatorial or cocktail vaccines with synergistic and/or additive effects are currently being considered.

Combinatorial vaccines are achieved by combining two or more tick vector and/or pathogen derived antigens, allowing increased immunogenicity and tick control efficacy (De la Fuente and Kocan, 2006). Prerequisite characteristics of such combinations include that antigens must elicit a similar immune response, or use the same avenue of immune response development without eliciting antagonistic responses (Nuttall *et al.*, 2006). Due to the unpredictability of the immune response elicited by such an antigen combination, either of the following interactions can be expected: synergy, enhanced but unpredictable efficacy (different immunological targets), enhanced but not additive response and an overall reduction in efficacy (Willadsen, 2008). Two types of antigens have been considered for the development of novel, combinatorial anti-tick vaccines i.e. exposed- and concealed antigens.

1.2.3. Exposed antigens

Exposed antigens are naturally exposed to the host immune system, such as the protein antigens present in tick saliva. Exposed antigens are secreted by the tick at the feeding lesion and are able to induce a severe immunological response by priming the immune system. Consequently, every time the tick feeds (and secretes the antigen into the host) it will act as a booster immunization culminating in an anamnestic response. One disadvantage of using exposed antigens, is that ticks have co-evolved with the host and rapidly evolved mechanisms to counteract any possible host defense responses, thus ensuring that exposed antigens no longer elicit a response. Furthermore, the gene family grouping of such immunomodulators suggests that exposed antigen-derived vaccines might fail due to the functional redundancy within such families (Nuttall *et al.*, 2006; Guerrero *et al.*, 2012). Table 1.1 includes the characteristics and vaccine efficacies of exposed antigens described to date.

Table 1.1: Ixodid exposed antigens described to date. *Vaccination efficacy is indicated by the following for antigens therewith evaluated: (n/d), not determined; (-), no significant effect; (+), >25% efficacy; (++), 25 – 50% efficacy; (+++), 50 – 75% efficacy; (++++), 75 – 100% efficacy.*

Antigen	Tick species	Characteristics/putative function	Vaccination efficacy	Reference
Exposed Antigens				
Calreticulin	*Amblyomma americanum, Dermacentor variabilis, R. microplus.*	Endoplasmic reticulum (ER) calcium binding protein. Lacks ER retention (KDEL) sequence. Role in feeding. Low immunogenicity in cattle.	n/d	(Jaworski *et al.*, 1995) (Ferreira *et al.*, 2002b)
Immunoglobulin binding protein (IGBMC).	*R. appendiculatus*	Male-specific salivary protein with an immunomodulatory function. Slight effect on female feeding performance on vaccinated guinea pigs.	n/d	(Wang and Nuttall, 1999) (Wang *et al.*, 1998)
Histamine binding protein (HBP).	*R. appendiculatus*	Lipocalin-like fold. Suppresses host inflammation. Diverse reaction in guinea pig model.	n/d	(Paesen *et al.*, 1999)
P29	*Haemaphysalis longicornis*	Salivary gland putative extracellular matrix protein. Collagen-like. Effective against all tick stages.	++	(Mulenga *et al.*, 1999)
HL34	*H. longicornis*	Salivary protein. Adverse effect on tick feeding on rabbits.	+	(Tsuda *et al.*, 2001)
HLMP1	*H. longicornis*	Salivary proteins with fibrinogenolytic and gelatinase activities and function in blood feeding. Contains a signal sequence, metalloprotease characteristic zinc-binding motif, and cystein-rich region.	+	(Imamura *et al.*, 2009)
RIM36	*R. appendiculatus*	Cement protein. Antigenic but not protective in cattle.	+	(Bishop *et al.*, 2002) (Havlíková *et al.*, 2009)
64P (64TRP).	*R. appendiculatus* *I. ricinus* *R. sanguineus*	Cement protein. Similar composition to vertebrate keratin and collagen. Dual action by cross reacting with concealed (midgut), and exposed (salivary) antigens. Potentially "universal" affecting many different tick species. Exibited average 35% mortality of *I. ricinus* immunized rabbits.	+++	(Trimnell *et al.*, 2002) (Trimnell *et al.*, 2005) (Havlíková *et al.*, 2009)
Tick histamine release factor (tHRF).	*I. scapularis*	Secreted in tick saliva. Stimulates histamine release. Silencing (RNAi): impaired feeding and decreased *Borrelia burgdorferi* infection in mice.	n/d	(Dai *et al.*, 2010)
HL-p36	*H. longicornis*	Immunosuppressor. Inhibited proliferation of mitogen-stimulated immune cells; associated decrease in interleukin-2 messenger RNA (mRNA) levels. rHL-p36-inoculated mice: lower proliferative response of splenocytes *in vivo* and downregulation of immunomodulating genes.	n/d	(Konnai *et al.*, 2008)

Haemangin (Kunitz-type inhibitor)	*H. longicornis*	Disrupted angiogenesis and wound healing (induces apoptosis). Inactivated trypsin, chymotrypsin and plasmin (anti-proteolytic). RNAi: Decreased engorgement success and failed angiogenesis disruption.	n/d	(Islam *et al.*, 2009)
Evasin-3	*Amblyomma.variegatum* *R. appendiculatus* *Dermacentor reticulatus*	7 kilo Dalton (kDa) Salivary protein. Binds neutrophil chemoattractants, CXCL8 and CXCL1. Prevents influx of neutrophils to feeding site.	n/d	(Vancova *et al.*, 2010b)
Sialostatin L/L2	*I. scapularis*	Salivary C1-type cysteine protease inhibitors. Facilitates blood feeding and transmission of *B. burgdorferi*.	++	(Kotsyfakis *et al.*, 2006) (Kotsyfakis *et al.*, 2010)
Cystatin (HISC-1)	*H. longicornis*	Protease inhibitors of Papain-like Cysteine proteases. Expressed in type II acini of salivary glands during early blood feeding.	n/d	(Yamaji *et al.*, 2009)
Longistatin	*H. longicornis*	Anticoagulant, activates plasminogen. EF-hand Ca^{2+} binding domains. Silencing diminished blood pool formation, feeding.	n/d	(Anisuzzaman *et al.*, 2011)
Hq05	*H. qinghaiensis*	Novel gene with ORF of 540 bp. Expressed in salivary glands of nymphal and adult stages. Significant effect on oviposition.	++	(Gao *et al.*, 2009)
HqCRT	*H. qinghaiensis*	ORF of 1,233 bp and identified as calreticulin. Ubiquitously expressed in different tissues and life stages.	+	(Gao *et al.*, 2008b)
HqTnT	*H. qinghaiensis*	Troponin-T is involved in Ca^{2+}-sensitive molecular switching of muscle contraction.	-	(Gao *et al.*, 2008a)
IRACs	*I. ricinus*	Paralogous anti-complement proteins co-expressed in salivary glands.	-	(Gillet *et al.*, 2009)
IRIS	*I. ricinus*	Ubiquitous serpin family of proteins with high affinity for human leukocyte elastase. Interferes with hemostasis and immune response of host. Also inhibits lymphocyte proliferation and the secretion of interferon-γ and/or tumor necrosis factor-α, platelet adhesion, coagulation and fibrinolysis.	++	(Prevot *et al.*, 2009)
Metis 1	*I. ricinus*	Metalloprotease putatively involved in tissue alterations through digestion of its structural components, thereby interfering with homeostasis.	++	(Decrem *et al.*, 2008)

1.2.4. Concealed antigens

Concealed antigens are normally veiled from the host immune mechanisms and are typically those antigens found on the tick gut wall that interact with the host immune cells and immunoglobulins ingested with the blood meal (Nuttall *et al.*, 2006). These antigens do not naturally induce the host's immune response, but are immunogenic when injected into the host as an extract or recombinant protein (De la Fuente and Kocan, 2006).

Vaccination with a concealed antigen results in the induction of specific immunoglobulins that are protective once ingested with the blood meal during feeding. These antibodies interact with surface gut antigens, leading to the activation of T cells and subsequent gut cell damage and leakage of gut content into the body cavity (El-Kammah *et al.*, 2006; Nuttall *et al.*, 2006). For example, anti-Bm86 antibodies were thought to bind to the surface of epithelial cells in the midgut, thereby disrupting endocytosis and resulting in cell lysis (Riding *et al.*, 1994; Cunha *et al.*, 2012). The effectivity of tick infestation/feeding prevention is therefore limited by a time delay between tick ingestion of immunoglobulin and the onset of pathological effects (Liu and Kang, 2011).

Disadvantages of using concealed antigens include the fact that repeated immunizations are needed, since the immune system of the host is not naturally activated by the presence of feeding ticks (Nuttall *et al.*, 2006; Jonsson, 2008). Other difficulties include a lack of consistent effect, unsatisfactory activity when these antigens are used in single-antigen vaccine preparations and the possible elicitation of an autoimmune response (Nuttall *et al.*, 2006).

Because concealed antigens are often part of orthologous protein families, they may have the potential to induce autoimmunity. Since tick proteins lack obvious sequence similarity to sequences found in databases, they are most likely products of divergent or convergent evolution (Maritz-Olivier *et al.*, 2012). It is thus expected that the immune response to intracellular, concealed antigens will mainly be directed at tick-specific epitopes (Hajduseka *et al.*, 2010). Table 1.2 includes the characteristics and vaccine efficacies of concealed antigens described to date.

Table 1.2: Ixodid concealed antigens discribed to date. *Vaccination efficacy is indicated by the following for antigens therewith evaluated: (n/d), not determined; (-), no significant effect; (+), >25% efficacy; (++), 25 – 50% efficacy; (+++), 50 – 75% efficacy; (++++), 75 – 100% efficacy.*

Antigen	Tick species	Characteristics/putative function	Vaccination efficacy	Reference
Concealed Antigens				
Bm86	*R. microplus*	Gut cell surface protein (glycoprotein). Commercial vaccine: Gavac®. Possible function in the regulation of gut digest cell growth.	+++ (recombinant) ++++ (native).	(De la Fuente and Kocan, 2003) (Willadsen, 2004) (Tellam *et al.*, 1992) (Willadsen *et al.*, 1989)
Ra86	*R. appendiculatus*	Two homologues identified (654 and 693 amino acids with 80% amino acid identity. The 654 amino acid homologue is transcriptionally dominant. This represents an example of tick allelic exclusion (different alleles/variants at single locus).	-	(Kamau *et al.*, 2011)
Ba86	*R. annulatus*	Immunization of cattle with rBa86: reduced tick infestation, weight, oviposition, and hatching for *R. microplus* and *B. annulatus.* Better efficacy for *B. annulatus* – possibly due to specific antigenic epitopes.	++++	(Canales *et al.*, 2009a)
Bm86	*A. variegatum*	Gut cell surface protein (glycoprotein).	-	(de Vos *et al.*, 2001)
Bm86	*Hy. anatolicum*		++	(de Vos *et al.*, 2001)
Bm86	*Hy. dromedarii*		++++	(de Vos *et al.*, 2001)
Bm86	*R. decoloratus*		+++	(de Vos *et al.*, 2001; Odongo *et al.*, 2007)
Bm86	*R. sanguineus*		++	(Perez-Perez *et al.*, 2010)
Haa86	*Hy. anatolicum*		+++	(Azhaniahambi *et al.*, 2009)
Bm91	*R. microplus*	Carboxy dipeptidase. Supposed to increase efficacy of Bm86.	++	(Willadsen *et al.*, 1996)
Bm95	*R. microplus*	Gut protein of unknown function. Bm86 allelic variant cloned from Argentinean A strain (64 kDa) used in Australian vaccine, TickGARD® Plus. Protects cattle against infestations by *R. microplus* strains from different geographical areas.	++++	(De la Fuente and Kocan, 2003) (Willadsen, 2004) (Kumar *et al.*, 2009) (Garcia-Garcia *et al.*, 2000)
Bm95	*Rhiphicephalus haemaphysaloides*	Protected *Bos indicus* cattle against *R. haemaphysaloides* larval, nymphal and adult infestation.	++++	(Sugumar *et al.*, 2011)
Vitellin	*R. microplus*	Abundant egg protein. Product of vitellogenin. Ovine vaccination with native protein: increased mortality, reduced weight and oviposition. 45% Engorged females displayed damaged, red phenotype.	++	(Tellam *et al.*, 2002)
VTDCE (vitellin	*R. microplus*	Vitellin hydrolysis in eggs during tick development. Enzyme activity in ovary, gut, fat	+	(Seixas *et al.*, 2008)

degrading cysteine endopeptidase)		body, salivary gland and haemolymph. Within female gut: localized to areas of protein synthesis and trafficking with haemolymph.		(Seixas *et al.*, 2010)
BmPRM (paramyosin).	*R. microplus*	Multi-functional, conserved invertebrate muscle protein. rBmPRM binds IgG and Collagen. Roles in immunomodulation.	n/d	(Ferreira *et al.*, 2002a)
HLS1/HLS2	*H. longicornis*	Serine protease inhibitor (Serpin). Rabbit vaccination with rHLS1: 43.9% mortality of nymphs.	+/++	(Sugino *et al.*, 2003) (Imamura *et al.*, 2005)
Voraxin	*Amblyomma hebraeum*	Male engorgement factor; impairs feeding (<74%) of females in a rabbit model; salivary gland degeneration, partial ovary development.	n/d	(Weiss and Kaufman, 2004)
Voraxinα	*R. appendiculatus*	Expression induced by blood feeding. Vaccination of rabbits: elicited humoral and protective immunity against female ticks and reduced weight.	+++	(Yamadaa *et al.*, 2009)
P27/30 (troponin-1 like protein).	*H. longicornis*	Troponin I-like protein. Impairs tick feeding on immunized mice. Low larval attachment: 31.1%.	n/d	(Myung-Jo, 2005)
BmTI	*R. microplus*	Trypsin and serine proteinase inhibitor. Reduction in population (69.7%) and egg weight (71.3%).Functions in larval attachment and feeding.	+++	(Andreotti *et al.*, 2002)
4F8	*I. scapularis*	Nucleotidase.	++	(Almazan *et al.*, 2005a) (Almazan *et al.*, 2005b)
Subolesin (4D8).	*I. scapularis*	Putative function in gene regulation, tick feeding and reproduction. Detailed section follows.	+++	(Almazan *et al.*, 2003c) (Almazan *et al.*, 2003b) (Almazan *et al.*, 2005a) (Almazan *et al.*, 2005b) (Almazan, 2005)
4E6	*I. scapularis*	Unknown function. Glutamine and Alanine rich protein.	-	(Almazan *et al.*, 2005a) (Almazan *et al.*, 2005b)
(Elongation factor 1α)	*R. microplus* *R. microplus* *B. annulatus*	Involved in feeding and reproduction, function still to be proven. RNAi: increased mortality and reduced oviposition. Could not produce sufficient rEF-1α for vaccination.	n/d	(De la Fuente *et al.*, 2008c) (Almazan *et al.*, 2010)
Serine proteinase (HISP, HISP2, HISP3).	*H. longicornis*	Digestion (blood feeding), immune defense. Transcribed in the midgut, lumen. Silencing: Reduced engorgement weight.	n/d	(Miyoshi *et al.*, 2008)

Varisin (defensin)	*Dermacentor variabilis* *I. scapularis* *Amblyomma americanum* *Amblyomma hebraeum* *R. microplus*	Small cationic peptides that provide immunity against gram +/- organisms and *Babesia* spp. Silencing varisin – reduced *A. maginale* numbers.	n/d	(Johns *et al.*, 2001) (Kocan *et al.*, 2008)
5'-Nucleotidase	*R. microplus*	Ectoenzyme bound by a glycosyl phosphatidylinositol (GPI) anchor to plasma membrane. Degrades nucleotide mono, di-and triphosphates for purine salvage. Located in the malphigian tubules. Sheep (challenged with freshly moulted adults) and cattle (challenged with larvae) vaccinated with r5'-nucleotidase. Significant difference in antibody titre between cattle and sheep. No significant protection.	-	(Hope *et al.*, 2010)
ATAQ	*R. microplus* *Amblyomma variegatum* *Dermacentor reticulatus* *Haemaphysalis elliptica* *H. longicornis* *Hyalomma anatolicum anatolicum* *Hyalomma marginatum* *Ixodes ricinus* *I. scapularis* *R. appendiculatus* *Rhipicephalus evertsi evertsi*	Orthologue of Bm86 with conserved signature peptide YFNATAQRCYH ; signal peptide; epidermal growth factor (EGF) -like domains. Silencing provides weak phenotype.	n/d	(Nijhof A.M. *et al.*, 2010)
Rhipilin-1	*R. microplus*	Kunitz-type anticoagulant. Silencing: significant decrease in attachment rate and body weight.	n/d	(Gao *et al.*, 2011)
Vitellogenins (Vg1, Vg2, *HIV*g-1, *HIV*g-2, *HIV*g-3).	*D. variabilis* *H. longicornis*	Major yolk protein (vitellin) precursor (phosphoglycoprotein). N-terminal lipid binding domain, von Willebrand factor. RNAi results shows importance for egg development and oviposition.	n/d	(Sullivan *et al.*, 1999) (Boldbaatar *et al.*, 2010b) (Khalil *et al.*, 2011)

I. scapularis antifreeze glycoprotein (IAFGP).	*I. scapularis*	Binds and restricts ice crystal growth enhancing tick survival in extreme cold. Induced by *Anaplasma phagocytophilum* infection	n/d	(Neelakanta *et al.*, 2010)
Amblyomin-X (Kunitz type inhibitor).	*Amblyomma cajennesse*	Kunitz type protease inhibitor. Inhibitor of Factor Xa (blood coagulation).	n/d	(Batista *et al.*, 2008)
Small heat-shock protein (sHSPII).	*B. annulatus*	Alpha crystalline, small heat shock proteins superfamily. Highly immunogenic. Confers thermal protection to other proteins (molecular chaperones).	n/d	(Shahein *et al.*, 2010)
Class B scavenger receptor (CD36)	*H. longicornis*	Cell surface proteins. Recognition of microbial surface antigens. Silencing led to reduction in engorged female weight.	n/d	(Aung *et al.*, 2011)
Lipocalins (e.g. LIPERs).	*I. ricinus* *I. persulcatus* *R. appendiculatus*	Low molecular weight. Immunomodulatory functions: cellular homeostasis and inhibition of platelet aggregation and complement. Vaccination of mice resulted in delayed engorgement and reduced weight.	-	(Konnai *et al.*, 2011)
Legumain (aspariginyl endopeptidases) HILgm1 and HILgm2. IrAE	*H. longicornis* *I. ricinus*	Midgut specific. Haemoglobin digestion cascade. Silencing (RNAi) of HILgm1 and HILgm2: Failure to reach repletion, reduced engorgement weight, damage to midgut tissue, reduced oviposition, aberrant egg development, and feeding- subsequent cellular remodeling.	n/d	(Alim *et al.*, 2009) (Sojka *et al.*, 2007)
Kynurenine aminotransferase (HIKAT).	*H. longicornis*	Enzyme of tryptophan pathway (kynurenine pathway). Transcribed in all tissues. *In vitro* inhibition of *Babesia caballi* growth.	n/d	(Battsetseg *et al.*, 2009)
GATA factor	*H. longicornis*	Transcribed in midgut and fat body. Transcriptional activator of Vg gene. Knockdown: Inhibition of Vg and significantly reduced oviposition.	n/d	(Boldbaatar *et al.*, 2010a)
Aquaporin (IrAQP1), (*Rs*AQP1).	*R. sanguineus* *I. ricinus*	Water channel; expressed in gut and salivary glands. Reduced blood ingestion and engorgement weight. Affects gut to saliva water flux and haemolymph osmolarity.	n/d	(Campbell *et al.*, 2010)
HlYKt6. Soluble N-ethylmaleimide sensitive fusion protein attachment protein receptors (SNAREs). Synaptobrevin (VAMP2).	*H. longicornis* *A. americanum*	Functions in signaling, exocytosis. Silencing (RNAi): Significant decrease in engorgement weight and increase in mortality. Decreased salivation and salivation protein repertoire. Knockdown: decrease in engorgement rate and body weight.	n/d	(Gong *et al.*, 2009)
Ferritin 2 (IrFER2 and RmFER2).	*I. ricinus* *R. microplus* and *R. annulatus*	Iron storage/homeostasis proteins. No vertebrate orthologs. Vaccination: Reduced weight and fertility and increased mortality – significantly better results in *I. ricinus*.	+++	(Hajduseka *et al.*, 2010)

Leucine aminopeptidase (HlLAP).	*H. longicornis*	Localized in ovarian cells. Regulatory role in vitellogenesis. HlLAP knockdown: Oocytes with abnormal morphology, reduced oviposition.	n/d	(Hatta *et al.*, 2010)
Hemelipoglycoprotein (HeLp).	*D. marginatus* *R. microplus*	Carrier protein of heme in hemolymph (290 kDa): Able to agglutinate red blood cells. Complexes with fibrinogen related proteins.	n/d	(Dupejova *et al.*, 2011)
Mimotopes generated by Phage Display.	*R. microplus*	Cattle vaccination: causes hemorrhagic events in the gastrointestinal and–reproductive tracts.	+	(Prudencio *et al.*, 2010a)
Chitinase (CHT1)	*H. longicornis*	Degrades older chitin during molting. Induced by ecdysteroids. Vaccination: Longer feeding and molting periods (76.7% increased molting rate) and reduced egg weight.	+	(Fujisaki and You, 2009)
BMA7	*R. microplus*	Mucin-like membrane glycoprotein (63 kDa). Similarity to vertebrate mucins and unknown function.	+	(McKenna *et al.*, 1998)
BYC (Boophilus Yolk Cathepsin)	*R. microplus*	Aspartic proteinase involved in embryogenesis. rBYC used to vaccinate cattle: overall protection of 25.24%. Dose-dependent decrease in oviposition.	+ (native protein) ++ (recombinant protein)	(da Silva Vaz *et al.*, 1998) (Leal *et al.*, 2006)
RAS-3 (*Rhipicephalus appendiculatus* Serpin 3), RAS-4 and RIM36.	*R. appendiculatus*	Serine protease inhibitors with important roles in blood coagulation, fibrinolysis and complement activation. Assessed as anti-tick cocktail vaccine in cattle. Resulted in tick mortality rate of 39.5%, and 48.5% in *Theileria parva* infected female ticks.	++	(Imamura *et al.*, 2008)
Ubiquitin	*R. annulatus*	Ubiquitin (GenBank Accession number: AA257892) was used for RNAi and vaccination experiments. Inconclusive results and low immunogenicity.	+	(Almazan *et al.*, 2010)
Ubiquitin	*R. microplus*		+++	

It can be deduced from the data in Tables 1.1 and 1.2 that an extensive variety of tick antigens have been identified and that fewer than 5% have been evaluated in vaccination trials. In addition, a broader scope of concealed antigens is characterized with higher efficacy than their exposed counterparts. One of these promising concealed antigens, Bm86, will be discussed below.

Bm86: the only commercialized antigen

Bm86 is the only commercialized anti-tick vaccine to date. It is available as GAVAC® (in South America), whilst TickGARD® (Bm86) and TickGARD®Plus (Bm86 and Bm95) have been discontinued due to inefficacy concerns (Legalforce, 1997; Pengally, 1999). Bm86 is a glycoprotein found on the surface of midgut digestive cells, with an unidentified function to date (Nijhof *et al.*, 2007). Epidermal growth factor (EGF)-like domains present in the structure of Bm86 and its homologues, and its localization on the surface of midgut microvilli, suggests a role in midgut cell growth and endocytosis (Willadsen *et al.*, 1989; Liao *et al.*, 2007).

The most characteristic phenotype of ticks feeding on Bm86 vaccinated cattle is lysis of the tick midgut, resulting in blood meal leakage into the hemocoel (Almazan *et al.*, 2003c; Almazan *et al.*, 2005a). Histopathological evaluation of affected tissues confirmed that tick gut cells were shed into the caecal lumen and the gut epithelium was reduced to thin strips (Agbede and Kemp, 1986). Bm86 based vaccines were able to protect against infestations of *R. decaloratus*, *R. annulatus*, *Hyalomma anatolicum anatolicum* and *Hy. dromedarii* and *Rhipicephalus* spp., but failed to protect against *Amblyomma* spp, *R. appendiculatus* and some geographical strains of *R. microplus* (de Vos *et al.*, 2001; Almazan *et al.*, 2005b). Additionally, recombinant Bm86 reduced the transmission of *B. bovis* and *A. marginale* (De la Fuente and Kocan, 2006).

Strain to strain variations of *R. microplus* susceptibility to Bm86 vaccination have been observed, suggesting that sequence or physiological differences might be a factor, requiring that vaccines are adapted to geographic regions as per local *R. microplus* strain (Goto *et al.*, 2008). Studies have shown the presence of divergent homologues of Bm86 within a single tick species. Two divergent homologues of Ra86 were discovered from *R. appendiculatus,* sharing only 80% identity. Only one dominant genotype was transcribed in female ticks, however both allelic variants were detected in the genome (Nijhof *et al.*, 2009). Sequences comprising two distinct variants of the gene were isolated from east African *R. decoloratus* (Bd86) and South African *R. appendiculatus* (Ra86), exhibiting 95% and 93% identity (Odongo *et al.*, 2007). Homologous identities do not, however, seem to influence the inter-and intraspecies efficacy of Bm86-based vaccines.

Recently it appeared that the ability/disability of the Bm86 vaccine to cross-protect heterologous tick species was not linked to the extent of sequence identity amongst homologues. Despite a high degree of homology, Bm86-based vaccines are not effective against *R. appendiculatus* and conferred more protection against species where lower homologies were observed. Using tick EF-1α as reference gene, the expression profiles of Bm86 and Ra86 were compared and found to be more constitutive for Bm86 than for Ra86, thus suggesting a basis for *R. appendiculatus* reaction to Bm86/Ba86 vaccination (Nijhof A.M. *et al.*, 2009). Insights into the transcriptional control of Ra86 have suggested reasons for its poor antigenic performance.

Reverse transcription polymerase chain reaction (RT-PCR) and 3' untranslated region (UTR) PCR of Ra86, exhibited transcriptional control usually redolent of multicopy gene family members such as the olfactory receptor genes in mammals and the Pfemp1 (var) genes from *Plasmodium falciparum* (Kamau *et al.*, 2011). This suggests a mechanism by which transcript expression is controlled at the Ra86 (Bm86) loci. The lack of a dominant transcript (for Ra86 and Pfemp (var) genes) suggests that the expression of these gene families is random. The high variability and recombination/mutation possibilities for these gene families simplifies antigenic evasion of immune barriers, suggesting that these type of antigens are less effective anti-tick vaccine candidates (Chen, 2007).

Subolesin: putative Bm86 successor

Subolesin was discovered in 2003 by cDNA expression library immunization (ELI) of mice. The cDNA library was created from a continuous IDE8 cell line derived from *I. scapularis* embryos (Almazan 2003a). Immunohistochemistry of gut sections revealed that subolesin is present in the tick gut and that it is an intracellular protein. In addition, rabbit anti-subolesin antibodies were able to cross the plasma membrane into tick gut cells (Figure 1.3). This suggested that subolesin is neither an exposed nor a concealed antigen, but an intracellular concealed antigen, contributing to its intrigue as a vaccine candidate (De la Fuente *et al.*, 2006a).

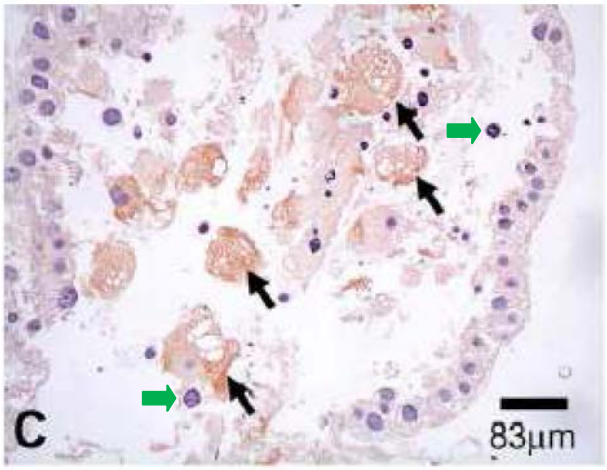

Figure 1.3: Immunohistochemistry on gut sections of female *I. scapularis* ticks (De la Fuente *et al.*, 2008b). *Nuclei are indicated by green arrows. Though plasma membranes are not clearly visible, individual cells can be distinguished. Regions where subolesin was detected by antibody are stained orange and indicated by black arrows.*

In 2006 subolesin was cloned from 6 different hard tick genera represented by the species *I. scapularis, Amblyomma americanum, Dermacentor variabilis, R. sanguineus, D. marginatus, H. punctata, Hy. m. marginatum, I. ricinus* and *R. microplus.* The subolesin nucleotide and protein sequences were found to be conserved amongst all species with the identities/similarities ranging between 65 – 98% and 60 – 98%, respectively (De la Fuente *et al.*, 2006a). Apart from being highly conserved amongst invertebrates and vertebrates, subolesin is expressed in all developmental stages and adult tissues of a variety of tick species (Almazan *et al.*, 2005a). It was proposed that knockdown of such a conserved antigen would have deleterious effects on vital tick metabolic functions.

RNAi studies in various tick species suggests that subolesin functions in tick feeding, reproduction and development, as degeneration of the gut, salivary glands and reproductive tissues is invariably observed. Successive knockdown and immunization studies in *R. microplus, R. annulatus, R. sanguineus, A. americanum* and *I. scapularis* reiterated the abovementioned phenotypic effects and decreased the transmission of both *A. marginale* and *A. phagocytophilum* (De la Fuente *et al.*, 2006b; De la Fuente *et al.*, 2007; Kocan *et al.*, 2007; Canales *et al.*, 2009c). Recently, *Amblyomma hebraeum* females were injected with subolesin (or subolesin + voraxin) dsRNA. Voraxin, a concealed antigen, was previously identified from *A. hebraeum* (Weiss and Kaufman, 2004). These ticks displayed the phenotype described above (Smith *et al.*, 2009). In 2008, silencing of both *R. microplus* EF-1α (a subolesin binding protein) and subolesin transcripts had significant effects on tick blood digestion, reproduction and egg development. The eggs of both EF-1α and

subolesin dsRNA injected females showed aberrant development (figure 1.4) (De la Fuente *et al.*, 2008b).

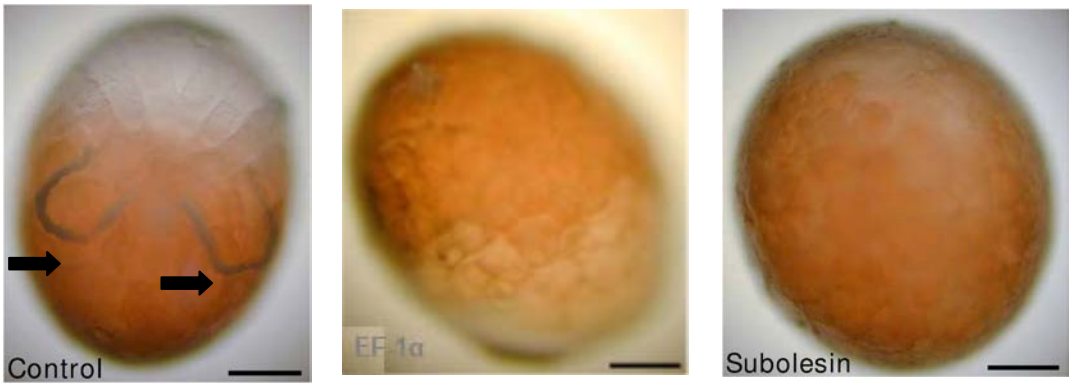

Figure 1.4: Development of *R. microplus* eggs after saline (control), GII (EF-1α) and subolesin dsRNA injection of ticks. *(Adapted from De la Fuente* et al*., 2008b and (Sonenshine, 1991). Whilst embryonic development can be observed for the negative control (black arrow indicating malpighian tubule formation), only undifferentiated egg masses are visible for the eggs of both subolesin and EF-1α dsRNA injected replete females.*

Transovarial silencing of subolesin was proven for the three-host tick species *A. americanum, D. variabilis* and *I. scapularis.* Egg and larval morphological and developmental aberrations were observed (Kocan *et al.*, 2007). A subsequent subolesin RNAi experiment resulted in reduced oviposition and hatching, as well as aberrant larval development (Nijhof *et al.*, 2007). These findings suggested that transovarial silencing by RNAi might be a familiar mechanism amongst ixodid ticks, and may serve as a method to characterize genes involved in embryonic development (Kocan *et al.*, 2007). Characterization of subolesin's involvement in disease transmission is as paramount as understanding embryogenesis.

Subolesin mRNA levels were determined by RT-PCR in uninfected and *A. marginale*-infected *Dermacentor variabilis* guts and salivary glands as well as cultured IDE8 tick cells. The same determinations were made for uninfected and *A. phagocytophilum*-infected *I. scapularis* nymphs, ISE6-cultured tick cells and the human cell line HL-60. Subolesin mRNA levels were increased in response to *A. marginale* infection in *D. variabilis* salivary glands, but not increased in gut and *A. phagocytophilum* infected ISE6 cells. RNAi was used to observe the effect of subolesin silencing during *Anaplasma* spp. infection/multiplication in the abovementioned tick tissues and cultured tick or human cells. Knockdown of subolesin only reduced *Anaplasma* multiplication in cells in which subolesin expression was increased concomitantly due to infection, i.e. *I. scapularis* salivary glands and IDE8 cells. (De la Fuente *et al.*, 2008a). A subsequent, similar study suggested that subolesin is involved in salivary gland innate immunity .

The objective of a recent study was to determine the expression level of subolesin in different tick species infected with *Anaplasma, Ehrlichia, Rickettsia, Babesia* or *Theileria* spp., as well as the effect of RNAi mediated subolesin knockdown on infection levels in male *D. variabilis.* Expression levels varied significantly between tick species and pathogen infection when analyzed in whole ticks, but were reduced overall. In *A. marginale* infected ticks, subolesin expression increased in the salivary glands but not the gut. When non-tick-borne pathogens were capillary fed to subolesin silenced ticks, it did not result in lower infection levels. It was thus proposed that subolesin knockdown affects infection levels (by some pathogens) directly by decreasing innate immunity resulting in higher infection levels (Zivkovic *et al.*, 2010). Seeing as disease burden evokes stress responses in ticks, the involvement of subolesin during environmental stresses were investigated. A study was performed to investigate the role of subolesin and heat shock proteins (*hsp20* and *hsp70*) in *I. scapularis* stress responses to heat shock, blood-feeding, pathogen infection and questing. By silencing *I. scapularis* subolesin and HSPs, the authors determined that *I. scapularis* were protected from stress and *A. phagocytophilum* infection during feeding. It was suggested that HSPs and subolesin are overexpressed at physiological pressures (feeding; higher temperatures) and that this confers immunological protection and higher questing speeds (Busby *et al.*, 2011). The aforementioned involvement of subolesin in innate immunity was confirmed by examining its association with NF-κB.

Evolutionary conservation was suggested by high sequence homology between tick subolesin and insect akirins, a group of proteins that function in *Drosophila* spp. and murine transcription and innate immunity. This suggested a function for subolesin in NF-κB-dependent and independent transcriptional regulation. RNAi mediated NF- κB knockdown resulted in silencing of both subolesin and EF-1α, suggesting that subolesin and EF-1α mediate transcriptional regulation by NF- κB. The suggested functions are plausible in light of the physiological effects of their silencing in ticks, mice and *Drosophila* spp. (Galindo *et al.*, 2009). Such functions suggested by RNAi results, can be confirmed or rebutted by immunization with protein antigen.

As previously mentioned, subolesin was identified by cDNA expression library immunization (ELI) (Almazan *et al.*, 2003a). Murine immunization with subolesin prevented molting of challenge-exposed *I. scapularis* larvae to nymphs. Larvae presented with extended guts and a red phenotype (figure 1.5), later identified as a symptom of subolesin immunization (Almazan *et al.*, 2003a). Three of the antigens identified 4D8 (subolesin), 4F8 and 4E6, were used to vaccinate sheep for challenge-exposure of adult *I. scapularis.* This resulted in a 58%, 12% and 20% reduction in infestation and a 22 – 49% decrease in oviposition for all immunized groups (Almazan *et al.*, 2005b). Various attempts have been made to improve subolesin vaccination efficacies.

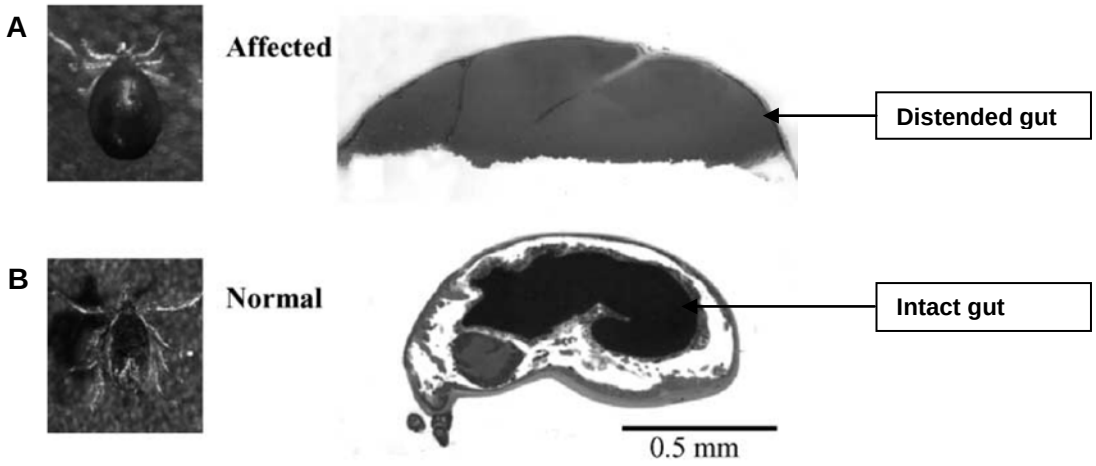

Figure 1.5: Normal and affected *I. scapularis* after challenge-exposure to subolesin immunized mice (Almazan *et al.*, 2003a). *Affected larvae presented with a red phenotype (A), whilst normal larvae did not (B). Microscopic analysis of larval longitudinal cross-sections indicated that the guts were severely distended in affected individuals (A), whilst normal morphology can be seen in (B).*

In an attempt to increase the protective vaccine efficacy of subolesin, a SAT was combined with vaccination. Cattle were vaccinated with recombinant subolesin and infested with subolesin dsRNA injected, replete *R. microplus* females. Autocidal control of the tick population was achieved, but only significantly increased if the tick population on vaccinated cattle included > 80% subolesin knock-down individuals (Merino *et al.*, 2011). This investigation rendered sterile acarine techniques not feasible in large-scale, field-based situations.

In order to improve the quality and multi-target efficacy of subolesin based vaccines, the conserved protective epitopes in tick and mosquito subolesin orthologs were identified. Three epitope types were recognized, using phage and peptide display to rabbit and ovine pre-immune serum followed by computational modelling. Linear B-cell epitopes, conformational epitopes and conformational discontinuous epitopes were recognized (Prudencio *et al.*, 2010b). This can be used for epitope specific antigen design towards more effective, targeted vaccines.

1.3. Eukaryotic elongation factor 1 alpha (EF-1α)

RNAi and immunization approaches have suggested a function for subolesin in disease transmission (e.g. *Anaplasma*, *Ehrlichia*, *Rickettsia*, *Babesia* or *Theileria* spp.), protein- and nucleic acid metabolism, energy regulation, immunity, signal transduction and transport (De la Fuente *et al.*, 2008b; Busby *et al.*, 2011). The *R. microplus* EF-1α homolog was identified as a subolesin-interacting protein via yeast two-hybrid and co-affinity purification experiments. RNAi experiments

suggested that *R. microplus* EF-1α is another possible anti-tick vaccine candidate, since it exhibits a similar phenotype as subolesin upon knockdown and might thus be involved in similar metabolic processes. Below follows a short discussion on eukaryotic elongation factors as an insight into the basal function of EF-1α.

1.3.1. Elongation factors and the ribosome

Ribosomal translation consists of three phases i.e. initiation, elongation, and termination and a fourth phase termed ribosome recycling suggested recently (Rodnina and Wintermeyer, 2009). Translation is performed on the ribosome in a 5' to 3' direction, with the ribosomes arranged in an end-to-end (polyribosome) fashion. The polyribosome enables the translation of multiple copies of the same peptide from a single mRNA.

Briefly, during polypeptide synthesis amino acid residues are added to the C-terminal of the ribosomally attached growing polypeptide strand. Single amino acids are transported to the new strand attached to a tRNA molecule known as aminoacyl tRNA (aa-tRNA). After peptide bond formation, the C- terminal residue is invariably esterified to a tRNA residue and the peptide known as peptidyl-tRNA (Voet and Voet, 2004). The complete translation process is very complex with many phases and contributing factors involved. Table 1.3 lists the factors involved in the four phases of translation. Eukaryotic EF-1α is the focus of this discussion.

EF-1α (EF-Tu) functions as follows during the elongation phase: EF-1α is activated by GTP binding, where after a ternary complex is formed with aa-tRNAs. Directly on the ribosome, the ternary complex formation of EF-1α leads to the decoding of genetic information via a Watson-Crick interaction, which occurs between the mRNA (A-site) and the tRNA anticodon. aa-tRNA is thus accommodated on the A-site of the ribosome, prior to release by EF-1α and subsequent to GTP hydrolysis (Nilsson and Nissen, 2005). This results in GTP hydrolysis induction on the ribosome and release of EF-1α-GDP. EF-1α is recycled to its EF-1α- GTP form by another elongation factor, EF-1β (Andersen *et al.*, 2003). *R. microplus* EF-1α is the focus of the remainder of the discussion.

Table 1.3: Translation factors active in the three kingdoms of life (Rodnina and Wintermeyer, 2009).

Translation step	Bacteria	Archaea	Eukarya
Initiation	IF1 IF2 IF3	aIF1A aIF5B aIF1 aIF2α aIF2β aIF2γ aIF2Bα aIF2Bδ aIF4A aIF5 aIF6	eIF1A eIF5B eIF1 eIF2α eIF2β eIF2γ eIF2Bα eIF2Bβ eIF2Bγ eIF2Bδ eIF2Bε eIF3 eIF4A eIF4B eIF4E eIF4G eIFH eIF5 eIF6 PABP
Elongation	EF-Tu EF-Ts SelB EF-G	aEF-1α aEF-1β SelB aEF-2	eEF-1α eEF-1β (2 or 3 subunits) eEF-Sec SBP2 eEF-2
Termination	RF1 RF2 RF3	aRF1	eRF1 eRF3
Recycling	RRF EFG		 eIF3 eIF3j eIF1A eIF1

1.3.2. Protein-protein interactions of eukaryotic EF-1α

In order to elucidate the function of *R. microplus* EF-1α, a yeast two-hybrid assay will be performed using an EF-1α BD/bait construct and *R. microplus* AD-fusion library. This approach will lead to the identification of EF-1α binding partners and clarification of EF-1α function in tick gene expression and metabolism. In order to predict such *R. microplus* EF-1α–acarine protein interactions, other interactions of eukaryotic EF-1α and implied functions thereof are tabulated below (Table 1.4)

Table 1.4: Biological partners and implied functions of EF-1α in eukaryotes. *Proteins marked by * are experimentally determined interacting partners of ef-1α. Unmarked biological partners are only listed for their implied function of EF-1α.*

Interacting partner	Organism / cell type	Implied function of EF-1α	Reference
*Rho kinase and Myosin phosphatase (myosin binding subunit).	Rat liver cytosol extract.	Upon EF-1α phosphorylation by Rho kinase, binding affinity and bundling of EF-1α for F-actin is decreased. Rho kinase and Myosin phosphatase regulates the phosphorylation state of EF-1α. Phosphorylation of EF-1α by Rho kinase reduces F-actin affinity and increases aa-tRNAs affinity (protein synthesis).	(Izawa *et al.*, 2000)
*Ca^{2+}/Calmodulin (CaM)	*Tetrahymena* spp. and *Paramecium caudatum* cilia.	Co-location of EF-1α and Ca^{2+}/Calmodulin (CaM) in membrane /matrix of axonemal microtubules. Movement of cilia and flagella. CaM, Ca^{2+} dependent activation of guanylate cyclase and cyclic nucleotide phosphodiesterase. Kinase/phosphatase activation.	(Ueno *et al.*, 2003)
*Akt protein kinase 2 (serine threonine kinase).	Chinese hamster ovary (CHOT) cells overexpressing insulin receptors and HA-tagged Akt.	EF-1α bound to C-terminal 75 amino acid residues through a complex with β-tubulin. Control of the cytoskeleton and cell motility. Directing of protein synthesis to sites of cellular activity.	(Lau *et al.*, 2006)
*β-actin mRNA and F-actin (simultaneous).	Cellular protrusions of chicken embryo fibroblasts. Co-sedimentation with β-actin mRNA and F-actin *in vitro*.	Localization of β-actin mRNA on the cytoskeleton in the lamella prior to translation. Stabilizes actin filament and influences rate of polymerization. Involved in the identification of EF-1α domain regions. See Rho kinase and Myosin phosphatase.	(Liu *et al.*, 2002)
*Split pleckstrin homology domain (PH domain) of *Phospholipase C – γ1 (PLC-γ1) a.k.a. PI-4 kinase activating protein.	Recombinant murine (rat) GST-PH fusion protein and detergent lysate of fibroblast (NIH 3T3) cells.	Role in phospholipid (phosphoinositide) metabolism. Promote production of phosphatidylinositol 4- phosphate (PIP) and phosphatidylinositol 4,5-biphosphate (PIP_2) by activation of PI-4-kinase and subsequent increase in PIP_2 hydrolysis rate via PLC-γ1 activation.	(Chang *et al.*, 2002)
*Tyrosine phosphatase-1 (SHP-1).	*Leishmania donovani* promastigote lysates from infected Syrian hamsters or murine macrophage cell line RAW 264.7. *In vitro*. Co-immunoprecipitation of *Leishmania* EF-1α and SHP-1 *in vivo* (infected cells).	Blocks/deactivates macrophage – cell signaling. *Leishmania* EF-1α is exported from phagosome to host cytosol. Addition of *Leishmania* EF-1α to macrophages activates SHP-1 and blocks inducible nitric oxide synthase expression in response to interferon-γ.	(Nandan *et al.*, 2002)
*Thioredoxin 1 (Trx1).	*Dictyostelium discoideum* (Amoeba).	Trx modulates enzymes and other cellular targets using disulfide-dithiol changes in pro and-eukaryotes (Redox reaction). EF-1α undergoes the mixed heterosulfide mechanism of Trx1 – might play a role in the control of ribosomal translation rate.	(Brodegger *et al.*, 2004)
*CDA14	Human prostate carcinoma (PC11) cells and hepatocellular carcinoma (HepG2) cell lysates.	Cell proliferation and carcinogenesis. Function of CDA14 not fully resolved.	(Yang *et al.*, 2008)

*Endothelium derived nitric oxide synthase (eNOS).	TNF-α stimulated fraction of human umbilical vein endothelial cells (HUVECs).	EF-1α (domain III) was found to be a binding partner of the eNOS 3'-UTR upon TNF-α inhibition. TNF-α increased expression of EF-1α. Regulation of vascular function suggested.	(Yan *et al.*, 2008)
*Txk (Tec family of non-receptor tyrosine kinases) binding partner. Also binds INF-γ gene promoter with Txk.	Human Th1/Th0 cells.	Th1 transcriptional regulation in the nucleus. Actin cytoskeletal reorganization and cell signaling.	(Maruyama *et al.*, 2006)
*Bni1p (G-protein)	*S. cerevisiae* cytosol.	Rho1p targets Bni1p. EF-1α binds a 186 amino acid stretch present between formin homology (FH1) and FH2. Bni1p – EF-1α binding inhibits F-actin bundling. Function: Reorganization of actin cytoskeleton for yeast bud formation. Overexpression of EF-1α leads to aberrant fission yeast morphology. Bni1p might localize EF-1α. See Rho kinase and Myosin phosphatase.	(Umikawa *et al.*, 1998)
*Zinc finger protein ZPR1.	*S. cerevisiae*	EF-1α interacted with ZPR1 after mitogen (epidermal growth factor) treatment of cells. Subsequent translocation of complex to nucleus. Disruption of complex – accumulation in G2/M phase. EF-1α – ZPR1 communicates mitogenic signals.	(Gangwani *et al.*, 1998)
*Elongation factor 3 (fungal origin exclusively).	*Saccharomyces* spp.	Binding enhanced by ADP (ATP hydrolysis). Interaction results in delivery of aa-tRNA at the ribosomal A-site after ATP hydrolysis. Signal for POST to PRE state transition of ribosome. Overexpression of EF-3 shifts EF-1α function to protein synthesis.	(Anand *et al.*, 2006)
*ATPase subunit Rpt1 of the proteasome.	Yeast expressed human proteasome subunit and yeast EF-1α interaction.	EF-1α interacts with damaged proteins ligated to multi-ubiquitinated chains and binds to nascent polypeptide chains, unfolded proteins and hydrophobic peptides. Promotes degradation of Nα-acetylated proteins *in vitro.*	(Chuang *et al.*, 2005)
*Leucine-rich repeat kinase 2 (LRRK2).	LRRK2 expressed in High-Five insect cells and EF-1α from human embryonic kidney 293 cells.	Inhibits autophosphorylation of LRRK2 – GTPase activity unchanged. Impairs microtubule assembly of tubulin polymerization by EF-1α *in vitro*. Loss of LRRK2 kinase activity (by e.g. PARK8 gene mutation) responsible for 5% Parkinsons cases.	(Gillardon F., 2009)
*Subolesin. Intracellular antigen.	*I. scapularis* *R. microplus* *Amblyomma americanum, Dermacentor variabilis, Rhipicephalus sanguineus,Dermacentor marginatum, Ixodes ricinus, H. punctata, Hy. m. marganatum. Amblyomma hebraeum.* *A. andersoni*	Identified from *I. scapularis* ELI. Functions in protein and nucleic acid metabolism, energy regulation, immunity, signal transduction and transport. Has been identified as a tick protective antigen in *I. scapularis* and other species. RNAi studies showed that subolesin knock-down had a profound effect on tick biology, causing the degeneration of tick tissues, including guts, salivary glands, reproductive tissues and embryos. Subsequent studies indicated that subolesin controls tick gene expression, impacts the innate immune response, and affects tick infection by *Anaplasma,*	(De la Fuente *et al.*, 2008c), (De la Fuente *et al.*, 2006d), (De la Fuente *et al.*, 2006b), (De la Fuente *et al.*, 2006c), (Almazan *et al.*, 2003c), (Almazan *et al.*, 2003b), (Almazan *et al.*, 2003a), (De la Fuente *et al.*, 2008c), (Almazan *et al.*, 2005b), (Nijhof *et al.*, 2007), (Kocan *et al.*, 2007), (De la Fuente *et al.*, 2006d),

		Ehrlichia, Rickettsia, Babesia or *Theileria* spp:.	(De la Fuente *et al.*, 2007), (Smith *et al.*, 2009), (Canales *et al.*, 2009c), (Galindo *et al.*, 2009), (Prudencio *et al.*, 2010b), (Merino *et al.*, 2011),(Zivkovic *et al.*, 2010)
Interleukin 3 (IL-3; growth factor).	HeLa (human cervica cancer) cells.	HeLa cDNA expression library screened for genes able to inhibit apoptosis upon IL-3 withdrawal. Surviving clones contained full-length EF-1α, suggesting it protects against ER stress, creating a mutagen friendly environment, and increasing cancer development. *I. ricinus* salivary gland extract reduced IL-10 and CD69 production in mitogen stimuted murine splenocytes, and decreased B-lymphocyte apoptosis.	(Talapatra *et al.*, 2002) (Hannier *et al.*, 2003)
Ubiquitin (Ubq).	Human epidermoid cell line.	Inhibition of proteasomal activity by lactacystin or IFN-α results in accumulation of ubq-EF-1α complexes which stimulate degradation. Suggestion: EF-1α acts as Ubq C-terminal hydrolase leaving protein substrates vulnerable. Microarray performed after *R. microplus* Ubiquitin-63E knockdown: EF-1α EST not identified, but might still be affected.	(Lamberti *et al.*, 2004) (Lew-Tabor *et al.*, 2011)
Tumor inducing gene 1 (PTI-1); truncated EF-1α.	Human prostate carcinoma cells.	EF-1α has oncogenic potential due to overexpression in metastatic compared to non-metastatic cells, and loss of EF-1α actin affinity in metastatic cells. EF-1α upregulates p53.	(Ejiri, 2002; Lamberti *et al.*, 2004)

1.4. Tick EF-1α: A possible paradigm in tick vaccines

R. microplus EF-1α was found to be nearly identical (94%; 100%) to the EF-1α ESTs obtained from *Amblyomma* spp. (De la Fuente *et al.*, 2008b). BLAST analysis revealed that EF-1α was also present in a soft tick species, *Ornithodoros coriaceus* (Table 2.3). Interestingly, this discovery of EF-1α in the *Ornithodoros* spp. proves that this protein is present in both hard and soft ticks, increasing its potential as an important target for further studies. The EF1-αII and EF1-αIII domains, characteristic of vertebrate and invertebrate elongation factors, were present in all transcripts compared (Figure 1.6). *R. microplus* EF-1α additionally exhibited N-myristoylation, Casein Kinase II (CK2) and PKC phosphorylation sites (Figure 1.6), which also suggested a role for this protein in gene regulation (De la Fuente *et al.*, 2008b). Together, these domains implicate EF-1α in a variety of tick gene regulatory and metabolic functions.

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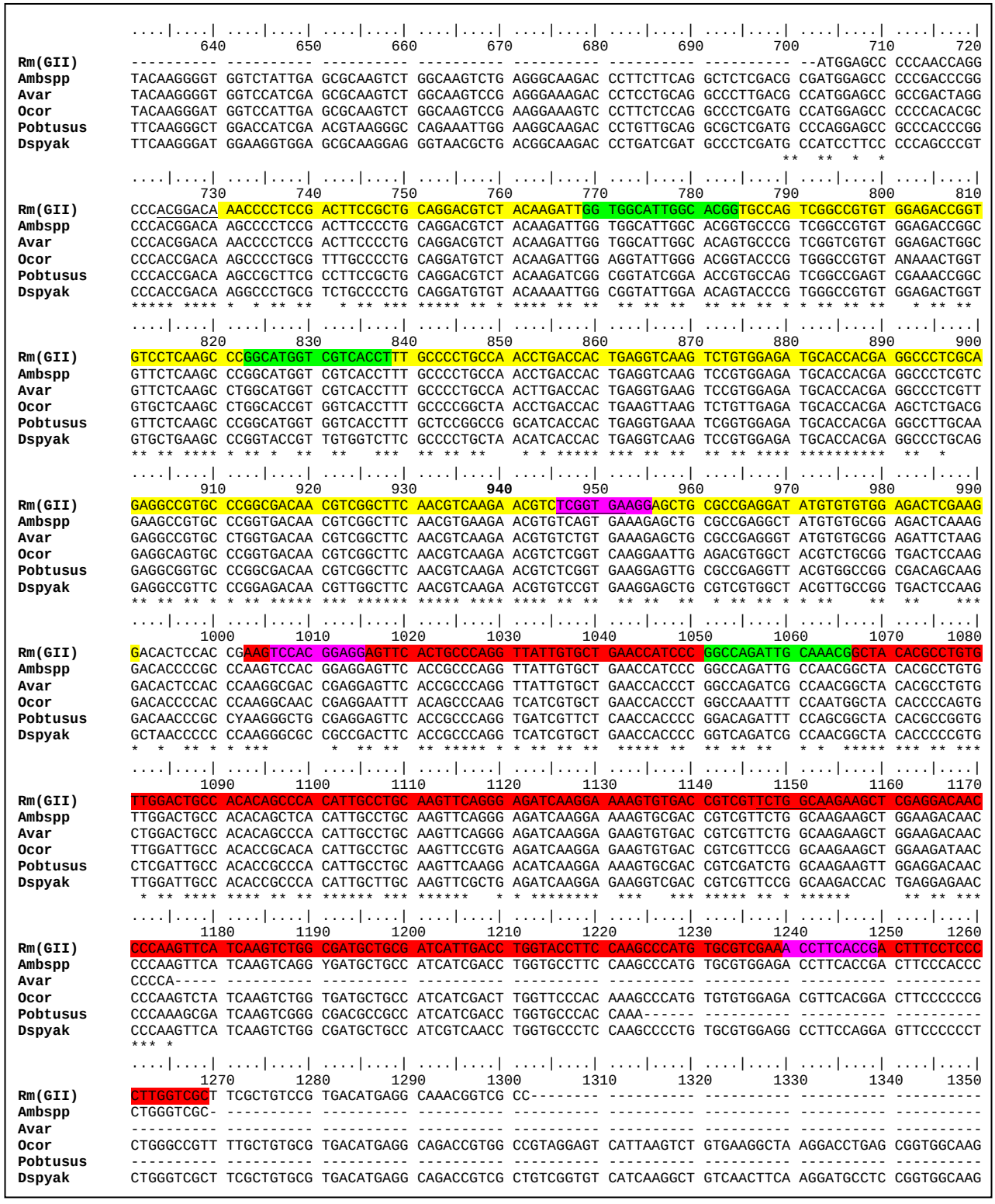

Figure 1.6: Nucleotide sequence of *R. microplus* EF-1α. *Adapted from De la Fuente et al. (2008b). The boxed regions indicate the EF1-α II (yellow) and EF1-α III (red) domains. N-myristoylation sites are indicated in green, CK2 sites in pink and PKC phosphorylation sites are underlined. BmGII* = R. microplus, *Ambspp* = *A. maculatum*, *Avar* = *A. variegatum*, *Ocor* = *O. coriaceus*, *Pobtusus* = *Pterolichus obtusus* and *Dspyak* = *Drosophila yakuba*.

To investigate its biological functions, RNA interference (RNAi) was performed to explore the effects of EF-1α knockdown on *R. microplus* and *R. annulatus*. Knockdown in unfed and replete adult females resulted in reduced tick survival, feeding, oviposition and hatching. EF-1α knockdown also produced a similar phenotype as that obtained with silencing of subolesin expression (De la Fuente *et al.*, 2008b; Almazan *et al.*, 2010). Due to the promising results obtained with RNAi, the authors aimed to evaluate its immunological properties in cattle. They were however, unable to produce sufficient amounts of rEF-1α for vaccination (Almazan *et al.*, 2010). These results suggested that EF-1α plays a role in vital tick metabolic processes required for the development and feeding of ticks.

Since both subolesin and EF-1α knockdown have significant effects on various regulatory processes, and were proven to be interactive proteins, it was hypothesized that this interaction might be essential to gene expression regulation. Further, the intracellular location of subolesin implies that EF-1α is itself an intracellular, concealed antigen. A brief discussion therefore follows on the dynamics of vaccination with intracellular antigens, as immunization was a long-term goal of the research proposed in this document.

1.4.1. Vaccination with intracellular antigens

Due to the highly conserved nature of subolesin across vertebrate and invertebrate eukaryotes, it was proposed that immunization with this antigen might lead to the development of autoimmunity (De la Fuente *et al.*, 2006a). Recent experiments with mosquito subolesin orthologs predicted that the antibody response will be directed primarily towards non-self epitopes, reducing the possibility of damage to host tissues (Canales *et al.*, 2009c). EF-1α, like subolesin, is an intracellular antigen and highly conserved amongst vertebrates and invertebrates, thus posing the same immunological questions and possible complications as subolesin upon vaccination.

Table 1.5 lists intracellular antigens which have been evaluated as potential vaccine candidates, together with their efficacies. From the data, it is evident that several of the antigens displayed similar non-target effects. Additionally, just as subolesin vaccination resulted in a decrease in *Anaplasma* spp. infection of ticks (De la Fuente *et al.*, 2006b), these antigens were able to reduce pathogen infections in other organisms. Significant autoimmune responses were not noted. A key mechanism involved in intracellular antigen efficacy is the transport of antibodies across plasma membranes.

Table 1.5: Results of immunizations with various intracellular proteins.

Intracellular protein	Vaccination target	Results of vaccination	References
Heat shock protein (HSP65).	Murine infection with H37Rv *Mycobacterium leprae.*	rHSP65 produced in monocyte-like tumor cell line J774. Significant protection from intraperitoneal and intravenous infection. Bacterial count was 100x lower after 5 weeks.	(Silva, 1999)
Trichophyton verrucosum crude ribosomal fraction (CRF).	Calves post-immunization infected with *T. verrucosum*.	Significant reduction in period of visible dermatophyte infection (9.5 to 3.7 weeks). Same immunoprotective properties for *Candida albicans* and *Microsporum canis*.	(Elad and Segal, 1995)
Brucella melitensis recombinant ribosome recycling factor-homologous protein (CP24).	Murine infection with *B. melitensis.*	rCP24 expressed in *E. coli*. Vaccination induced INF-γ, IL-10, and IL-2. No protection against infection.	(Cassataro *et al.*, 2002)
MUC-1 like tandem repeat proteins.	Human metastatic melanoma patient vaccinated with irradiated, autologous tumor cells engineered to secrete granulocyte-macrophage colony stimulating factor (GM-CSF).	Antigen detected: putative opoid growth factor receptor with MUC-1 like tandem repeats. 21% of lung cancer, 16% of prostate cancer, and 5/6 breast cancer patients produced Abs.	(Mollick *et al.*, 2003)
14-3-3 proteins of *Echinococcus* spp. E14ζ	Canine alveolar/cystic echinococcosis caused by *E. granulosus*. Oral challenge of mice with 2000 *E. granulosus* eggs post immunization.	Anti-14-3-3 Abs detected in infected dogs. Vaccination resulted in 97.35% parasitic load reduction.	(Siles-Lucas *et al.*, 2008)
Microtubule associated proteins (p52) co-purified with glycosomal enzymes (aldolase and GAPDH).	Murine (rats and mice) infection with *Trypanosoma brucei.*	Significant protection against infection. Sera caused *in vitro* aggregation of trypanosomes. Anti-p52 Abs located in trypanosome cytoplasm, thus able to cross plasma membrane.	(Balaban *et al.*, 1995)

1.4.2. Transport of antibodies across the plasma membrane

As noted in section 1.2.2 (Figure 1.3), anti-subolesin antibodies derived from subolesin immunized animals and used during immunohistochemistry, were able to cross the plasma membrane of tick gut cells. The antigens listed in Table 1.5 were similarly able to elicit responses from antibodies with the same membrane passage abilities. It is assumed that this is also possible for anti-EF-1α antibodies. A few methods have been suggested for the trafficking of whole antibodies across plasma membranes.

Anti-DNA antibodies (especially those associated with autoimmune diseases) have the ability to cross plasma membranes, localize in the nucleus of mammalian cells and lead to the development

of cytotoxicity. The authors proposed that non-specific electrostatic interactions between anti-DNA antibodies and negatively charged molecules in the plasma membrane such as heparin-sulphate proteoglycans might be involved in this process. Alternatively, endocytic pathways such as macropinocytosis, clathrin-mediated endocytosis, and caveolae/lipid-raft mediated endocytosis, have been suggested (Jang *et al.*, 2009).

Jang *et al*. (2009) reported intracellular trafficking routes as well as cytotoxicity in HeLa cells for a single chain variable fragment (scFv) anti-DNA antibody, 3D8, able to hydrolyze DNA and RNA. Monoclonal 3D8 was isolated from autoimmune-prone MRL-lpr/lpr mice (Kim *et al.*, 2006). Intracellular trafficking of the antibody occurred via caveolae/lipid-raft endocytosis and was dependent on the presence of caveolin-1 and dynamin (figure 1.7). Time course chasing experiments indicated that 3D8 scFvs were able to escape directly from the caveosome into the cytosol without ever passing into the endosomes, lysosomes, ER, Golgi or nucleus, whilst maintaining nuclease activity (Jang *et al.*, 2009). These lipid raft mechanisms were later used for the design of an artificial antibody transport system.

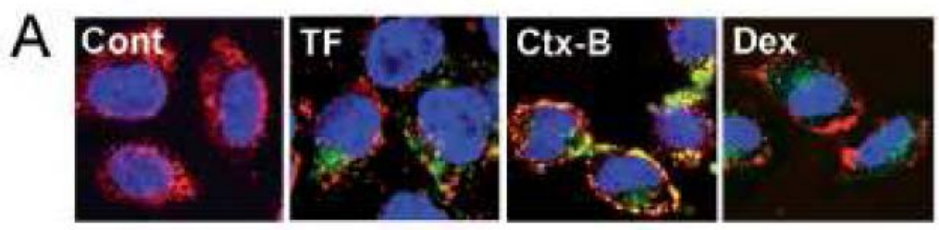

Figure 1.7: 3D8 trafficking occurs via the caveolae / lipid-raft mediated endocytosis (Jang et al., 2009). *Co-localization of 3D8 scFv with endocytic markers of the caveolae / lipid-raft mediated pathway. HeLa cells were treated with both 3D8 scFv (red), Alexa 488-transferrin (green), Alexa-488 choleratoxin- B (green) and FITC-dextran (green) for 2h and analysed by confocal microscopy. Nuclei were stained with Hoechst 33342 (blue).*

1.5. Study aims.

The significant effects observed upon vaccination with recombinant subolesin, proves that subolesin is a promising anti-tick vaccine candidate. The vaccine potential of EF-1α however, remains to be tested. It is therefore proposed that EF-1α and subolesin may lead to the development of a cocktail vaccine formulation that will result in a more severe disruption of feeding and fecundity. The aims of the study were therefore as follows:

1. To compare recombinant EF-1α protein expression in both prokaryotic (*Escherichia coli*) and eukaryotic (*Pichia pastoris*) systems for future cattle vaccination trails.
2. To perform a yeast two-hybrid assay using EF-1α BD/bait constructs and *R. microplus* AD-fusion library to identify EF-1α binding proteins and further elucidate the function of EF-1α in *R. microplus* ticks.

Chapter 2

Evaluation of prokaryotic- and eukaryotic systems for recombinant protein expression of EF-1α (rEF-1α).

Introduction: Recombinant protein expression

New proteins from a multitude of organisms are discovered daily. The obstacles however remain to understand their biological functions and their possible application as therapeutics. A variety of organisms of bacterial, yeast, plant and animal origin, as well as cell-free systems, have been created for heterologous protein expression (Reyes-Ruiz and Barrera-Saldanya, 2006). Suitable expression and purification techniques are essential to the large-scale production of pure and often functionally active proteins for therapeutic use. The development of microbioreactors and large-scale bioreactors has also significantly increased yield towards this purpose (Kensy *et al.*, 2009). One example is insulin, of which large quantities are needed commercially for diabetic patients. Insulin production has been extensively optimized and various analogues expressed in order to overcome such issues (Heller *et al.*, 2007). Approaches to improve protein production also include lowering culturing temperature, promoter variation, modified growth media, solubility enhancing tags, vector variation, host variation, fusion protein partners and improved refolding techniques (Baneyx and Mujacic, 2004; Roodveldt *et al.*, 2005). The choice of expression system is influenced by the origin of the protein (prokaryotic or eukaryotic, soluble or membrane bound) and the study purpose (such as elucidation of kinetics, antibody production or drug development).

Alternative hosts for recombinant protein expression

Alternative hosts for protein expression include insect cells, plant and animal cells as well as cell-free systems. Insect cells, in some cases, are able to produce higher yields of protein than conventional systems. For example, cells from *Spodoptera frugiperda* larvae that are infected with *Baculovirus* are able to produce high yields of protein with the necessary eukaryotic post translational modifications (PTMs). *Trichoplusia ni* and *Drosophila* spp. cells are also employed with success (Reyes-Ruiz and Barrera-Saldanya, 2006). Improvements on insect cell expression include the engineering of new vectors and optimisation of transfection (Kost *et al.*, 2005).

Cell-free extracts (obtained from eukaryotic and prokaryotic cells) include all the machinery necessary for transcription and translation under *in vitro* conditions. Advantages of the system

include avoidance of recombinant protein toxicity and aggregation of protein in inclusion bodies, as well as the absence of intracellular proteases.

The main advantage of plant systems is low production costs, whilst drawbacks include time from transformation to protein production and the deposition of protein in specific plant organs. More rare is the use of animals, for example protein expression in the milk of livestock to levels as high as 1 $g.l^{-1}$, and use of the ovalbumin promoter to express proteins in chicken eggs (Reyes-Ruiz and Barrera-Saldanya, 2006). Drawbacks to the use of animal models are mostly economical as well as logistical.

2.1. Prokaryotic protein expression: *Escherichia coli*

E. coli is the most common prokaryotic expression host due to its low culturing costs, the multitude of strains and vectors available, ease of maintenance and average recombinant protein yield of 0.5–50 mg/l of culture (Reyes-Ruiz and Barrera-Saldanya, 2006). Its characterized physiology and genetics, as well as its susceptibility to genetic manipulation also made it a very popular expression host (Baneyx 2004). Drawbacks include its inability to perform certain PTMs on eukaryotic proteins, its non-eukaryotic codon usage profile and the fact that the recombinant proteins are often found as part of aggregates or inclusion bodies (De Bernardez Clark, 1998; Reyes-Ruiz and Barrera-Saldanya, 2006). Inclusion bodies are defined as intracellular foci into which aggregated proteins are deposited. These aggregated proteins are usually poorly soluble in aqueous or detergent solvents and have abnormal sub– or extracellular localizations as well as non-native structure. All this contributes to the increased mass of these inclusion bodies, which results in them being precipitated along with cellular debris upon centrifugation (Kopito, 2000). New bacterial strains have been produced in an effort to circumvent these problems.

2.1.1. The *E. coli* JM109 strain

A number of *E. coli* strains are available for protein expression. As initial expression of EF-1α was attempted in *E. coli* JM109 with some success (Dr. M. Canales, Spain), this strain will be used in this study and therefore be described. The full genotype of the *E. coli* JM109 strain is listed in Table 2.1. JM109 is $recA^-$, which means that this strain will not restriction digest the cloned deoxyribonucleic acid (DNA), or recombine the cloned DNA with its own genomic DNA. This strain lacks the *E. coli* K restriction system (endonuclease A mutation), resulting in higher yield of quality plasmid DNA (i.e. plasmid DNA is not partly digested). JM109 has deletions in the genomic and episomal copies of the *lacZ* gene, making it deficient in β-galactosidase activity and disabling metabolism of lactose derivatives. This deletion in the episomal F' factor copy of the *lacZ* gene

however, can be replaced by the α-peptide of a suitable vector. These vectors will thus equip the bacterium with the ability to metabolize lactose derivatives thus enabling the use of blue/white screening procedures.

Table 2.1: Genotype of *E. coli* JM109 (Chalenko *et al.*, 2012).

Strain	Genotype
E. coli JM109	*endA*1, *recA*1, *gyrA*96, *thi*, *hsdR*17 (r_K-, m_K+), *relA*1, *supE*44, Δ(*lac-pro*AB), [F', *traD*36, *proAB*, $lacI^qZ\Delta M15$]

2.1.2. *E. coli* JM109 culturing conditions

E. coli is a facultatively anaerobic, gram negative bacterium generally used for the expression of a vast array of proteins from various sources. *E. coli* can be grown on different, relatively inexpensive carbon sources. In addition, it can quickly be grown to high cell densities in large scale preparations. Its ability to grow on simple, defined media enables higher biosynthetic capabilities of all its organic cellular constituents from a single carbon source such as glucose (Madigan *et al.*, 2003). Strain JM109 should ideally be maintained on M9 minimal medium supplemented with 1 mM thiamine-HCl, in this way selecting for the presence of the F' episome which provides the growth nutritional requirement of proline biosynthesis, subsequently reducing the frequency of false positives (Promega Corporation, 1996).

2.1.3. Choice and selection of vector

The pQE-60 vector was chosen for *R. microplus* EF-1α expression because of its strongly inducible promoter and the fact that it had been used previously for the expression of a variety of other tick proteins. pQE-60 additionally provides only the Shine-Delgarno sequence for ribosome binding, allowing translation of the coding fragment to initiate at the original start codon without fusion of additional amino acids to the N-terminal (Qiagen, 2003). The native pQE-60 vector confers the α-peptide necessary for β-galactosidase activity of clones absent of inserts, simplifying the selection of insert containing clones due to the disruption of this activity. pQE-60 provides the Amp^R (ampicillin resistance) gene. Since ampicillin is an unstable antibiotic due in part to the β-lactamase secreted by resistant cells, the plasmid should be maintained by examining the cells on plates with and without ampicillin. If stability is an issue, the concentration of ampicillin can be increased (200 μg/ml) or the cells can be grown in the presence of a more stable β-lactam such as carbenicillin (http://www1.qiagen.com/ literature/pqesequences/pqe60.pdf).

2.1.4. Promoters

The pQE-60 vector contains a strongly inducible T5 phage *lac* operator promoter. The strength of this promoter allows the expressed protein to accumulate to high levels within the bacterial cell cytoplasm. Since the T5 promoter is under the control of the *lac* operator, the promoter can be switched on by inducers such as lactose or related β-galactosidase substrates such as isopropyl-β-D-thiogalactopyranoside (IPTG) (Madigan *et al.*, 2003). The bacteriophage-derived promoters T5 and T7, are strongly inducible by IPTG, and sometimes indirectly induced in the absence IPTG leading to the "auto induction" of protein expression. This is because the T3, T5 and T7 RNA polymerases are often erroneously placed under the control of a *lac* promoter within the host chromosome (Baneyx, 2004).

2.1.5. Inducers and terminators

Fusion protein synthesis is stalled until a suitable cell density is achieved at which point an inducer is added. IPTG is a lactose analog and therefore not a hydrolysable substrate of β-galactosidase. Optimization of induced and non-induced expression by time-course analysis of the expression level commences after clones have been selected for efficient expression of the construct. This analysis aids in identifying the balance between the amount of soluble protein in the cells, the formation of inclusion bodies and protein degradation (Qiagen, 2003). If the vector is present at high copy numbers in the cell, the chromosomal encoded repressor might be inadequate to keep the promoter from functioning, leading to a process called auto induction (Madigan *et al.*, 2003). Using the JM109–pQE-60 system, translation is terminated by three stop codons provided in all three possible reading frames.

2.1.6. His6 Tag

pQE-60 codes for a poly-histidine tag which is fused to the C-terminal of the expressed protein enabling binding of the recombinant protein to nickel-nitriloacetic acid (Ni-NTA), simplifying detection and purification of recombinant protein. This affinity tag is poorly immunogenic, small, uncharged at pH 8.0, and should not affect secretion, compartmentalisation and folding of the fusion protein inside the bacterial cell (Qiagen, 2003).

2.2. Eukaryotic protein expression: Using yeast (*Pichia pastoris)* as a vehicle for protein expression

Yeast is the next most common expression host. These eukaryotes offer the advantages of eukaryotic PTMs with the maintenance ease of prokaryotes, as well as the availability of both intracellular and extracellular expression options. Although *Saccharomyces cerevisiae* is the first

choice, it does tend to hyperglycosylate proteins. Consequently, new strains were introduced such as *P. pastoris, Hansenula polymorpha, Schizosaccharomyces pombe, Kluyveromyces lactis* and *Yarrowia lypolytica*. *P. pastoris* is the most widely used strain to date due to high protein production yields and lack of hyperglycosylation (Cregg *et al.*, 1993; Reyes-Ruiz and Barrera-Saldanya, 2006).

Other advantages of *P. pastoris* as an expression system include eukaryotic protein processing, folding, gene-targeting, high frequency DNA transformation, cloning via functional complementation, stability at high cell densities and similar molecular manipulation possibilities and growth characteristics to bacterial hosts (Macauley-Patrick *et al.*, 2005). Use of the *P. pastoris* system enables high protein production yield, the ability to scale up to bioreactor fermentations, and the subsequent control of parameters such as pH, aeration and concentration of carbon source (usually methanol during induction) (Macauley-Patrick *et al.*, 2005).

2.2.1. Methanol induced expression

P. pastoris is a methylotrophic yeast, meaning it can metabolize methanol as its only source of carbon (Invitrogen Corporation, 2001). Three other genera share this phenotype namely *Hansenula, Candida* and *Torulopsis* (Macauley-Patrick *et al.*, 2005). Methanol metabolism entails the oxidation of methanol to formaldehyde utilizing molecular oxygen and the enzyme alcohol oxidase. The process predominantly takes place within the peroxisome where alcohol oxidase, catalase and dihydroxyacetone synthase are produced. The latter also simplifies the removal of the toxic byproduct, hydrogen peroxide (Invitrogen Corporation, 2001). Subsequent steps of methanol metabolism take place in the cytoplasm (Macauley-Patrick *et al.*, 2005). The *AOX* promoter is naturally a very strong promoter *in vivo,* and produces large amounts of alcohol oxidase needed by the cell due to its low affinity for oxygen. The strength of the promoter is therefore exploited to drive foreign protein expression (Invitrogen Corporation, 2001).

Two *P. pastoris* genes encode alcohol oxidase, *AOX1* and *AOX2*. Most of the enzyme activity is due to production of alcohol oxidase from the *AOX1* gene, with the enzyme making up as much as 30% of total soluble protein. Regulation of expression of the gene is tightly regulated, as the promoter is only induced by the addition of methanol to the growth media (Invitrogen Corporation, 2001). Expression of *AOX1* leads to the Mut^+ phenotype. These cells display high rates of methanol degradation and grow as fast as native cells (Macauley-Patrick *et al.*, 2005). The *AOX1* promoter can therefore be used to induce high-level expression of recombinant proteins, even in the presence of a single integrated expression cassette. Beneficial of the *AOX1* promoter is its ability to switch off when methanol is replaced by alternative carbon sources such as glucose or glycerol. This additionally enables Mut^+ clones to be used either as non-induced controls, or for the

expression of toxic proteins. Even though *AOX2* is 97% homologous to *AOX1*, growth in the presence of this homologue is much slower (Daly and Hearn, 2005). Expression of *AOX2* results in the Mut^S phenotype which is sometimes more suitable to protein production, and occurs due to the disruption of *AOX1* (Invitrogen Corporation, 2001).

Alternative promoters and carbon sources have been investigated and patented in the attempt to evade the use of methanol, a toxic, flammable substance (Balamurugan *et al.*, 2007). One example includes the use of the Glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter instead of P_{AOX1} (Bollok *et al.*, 2009). Other carbon sources used during expression induction in Mut^- strains include alanine, sorbitol, trehalose and mannitol. These were found to increase the expression of recombinant β-galactosidase in comparison to glucose or glycerol as carbon source (Daly and Hearn, 2005; Macauley-Patrick *et al.*, 2005). Mixed substrate feedings of methanol and glycerol or methanol and sorbitol applied to fermentations, are being investigated in an attempt to fix heat and oxygen transfer limitations (Bollok *et al.*, 2009). Additionally, it was found that ethanol and acetate are by-products of glucose and glycerol metabolism, which may interfere with subsequent induction by methanol ($P_{AOX1/AOX2}$ regulation), necessitating the continuous removal of these metabolites (Poutou-Piñales *et al.*, 2010).

2.2.2. Homologous recombination

Transformation of *P. pastoris* occurs via homologous recombination between the exogenous DNA and the yeast genome at sites of homology such as the *aox1* locus (Invitrogen Corporation, 2001). Recombinant constructs exhibit stability in non-selective media due to the presence of multiple copies. Single insertions are also more likely to occur than double crossovers (Daly and Hearn, 2005). Multiple insertions (in tandem) occur at about 1 – 10% the frequency of single insertions. These insertions can occur at either of two loci for the pPICZ vectors: the *aox1* locus or the *aox1* transcription termination region (Figure 2.1). One or multiple copies of the vector will then be inserted upstream or downstream of the *AOX1* or *AOX1:arg4* genes. Phenotypically, the transformants will be Mut^+ (X-33 or GS115) or Mut^S (KM71H) if constructs are linearized prior to transformation (Cregg *et al.*, 1993). Replacement (single copy clones) can be induced by digestion of the vector to produce yeast identical 5' and 3' regions of *AOX1* and thus targeted replacement of the AOX gene (Daly and Hearn, 2005).

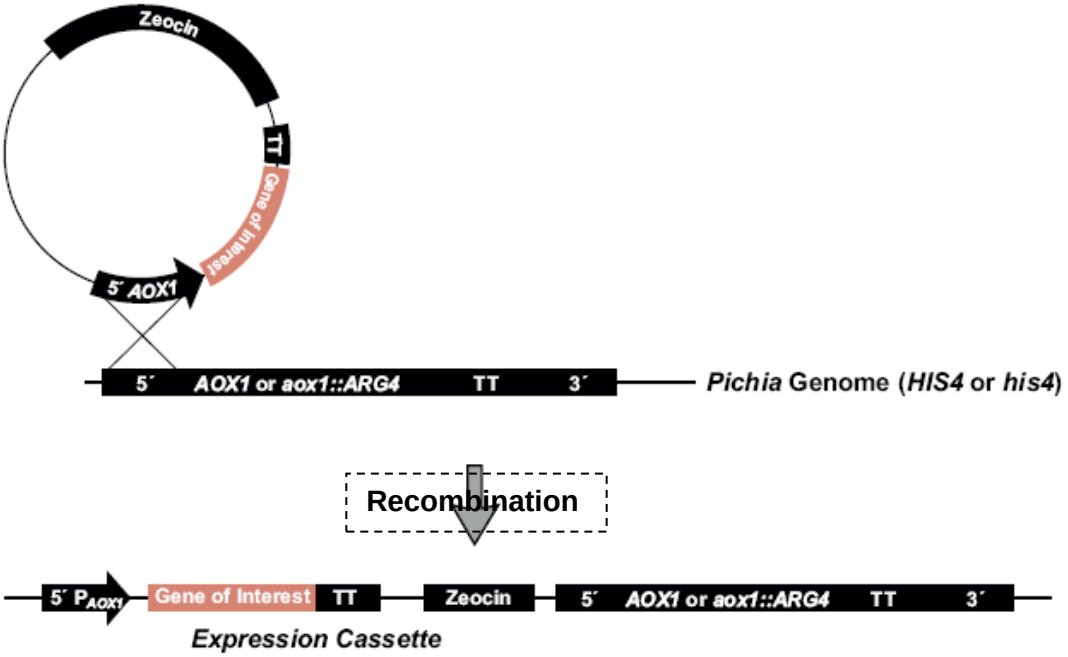

Figure 2.1: Insertion of plasmid 5' to intact aox1 locus (Invitrogen Corporation, 2001). *Insertion by recombination results in the gain of PAOX1, the fragment of interest, and the Zeocin resistance gene. Non-linearized vectors will recombine at much lower regularity.*

2.2.3. The EasySelect™ *Pichia* expression system

The EasySelect™ Pichia expression system was used to express EF-1α during the course of this study. The system includes three *P. pastoris* strains namely X-33, GS115, and KM71H. X-33 is a wild-type strain commonly used as a control for growth on Zeocin™ or simply in YPD or minimal media. GS115 (Mut^+) grows in complex media or media supplemented with histidine due to its mutation in the histidinol dehydrogenase (*his4*) gene. KM71H cannot grow in the absence of arginine due to a mutation in the argininosuccinate lyase (*arg4*) gene which was purposefully inserted or cloned into the *AOX1* gene in order to result in Mut^S native cells (Invitrogen Corporation, 2001). This strain thus relies on *AOX2* for its metabolism of methanol (Daly and Hearn, 2005). One advantage of using KM71H is that no selection for Mut phenotype is necessary as all the transformants will be Mut^S. GS115 can exhibit a Mut^S phenotype if the recombination site is the 3' terminus of *AOX1*, resulting in the disruption of the *AOX1* gene. Controls for the EasySelect™ system include un-transformed GS115 (Mut^S and Mut^+), a positive control for secreted expression (GS115/His^+ Mut^S Albumin) and a positive control for intracellular expression (GS115/pPICZ/*lac*Z Mut^+ β-galactosidase) (Table 2.2.) (Invitrogen Corporation, 2001). To test the Mut phenotype of transformants, cells are plated onto minimal medium with dextrose (MDH) or minimal medium with histidine (MMH) plates and the growth on these plates monitored.

Table 2.2: Genotypes and phenotypes of *P. pastoris* strains. ***Adapted from*** *(Daly and Hearn, 2005) and (Stutzer, 2008).*

Strain / construct	Genotype	Phenotype	Application
GS115	*his4*	Mut$^+$, His$^-$	Slow expression with Mut^S.
KM71H	*his4, aox1 : ARG4,arg4*	Mut^S, His$^-$	Faster growth, lower expression level. Mut$^+$ phenotype.
X-33	Wild type	–	Fast (large-scale) growth. Native selection on ZeocinTM. Control for background expression.
GS115/Albumin	*HIS4*	Mut^S	Control for secreted expression and Mut^S phenotype.
GS115/pPICZ/*lacZ*	*his4*	Mut$^+$, His$^-$	Control for antibiotic resistance, intracellular expression, Mut$^+$ phenotype and tag purification.

Intracellular and extracellular expression: vector selection

Once the gene of interest has been identified and cloned, a suitable vector has to be selected that complies with the expression needs of the user. The EasySelect™ kit contains vectors that utilize the P_{AOX1} promoter for the expression of the protein of interest (Figure 2.2). Both vectors, pPICZ (for intracellular expression) and pPICZα (for secreted expression) are provided in three reading frames to assist in the in-frame cloning of the desired insert with either the N-terminal (pPICZ) or C-terminal (pPICZα) c-myc and His_6-tags. To facilitate positive antibiotic selection, both vector systems contain a Zeocin™ resistance gene. pPICZ and pPICZα additionally contain *Bgl*II and *Bam*HI sites that allow the preparation of constructs with multiple copies of insert (Invitrogen Corporation, 2001).

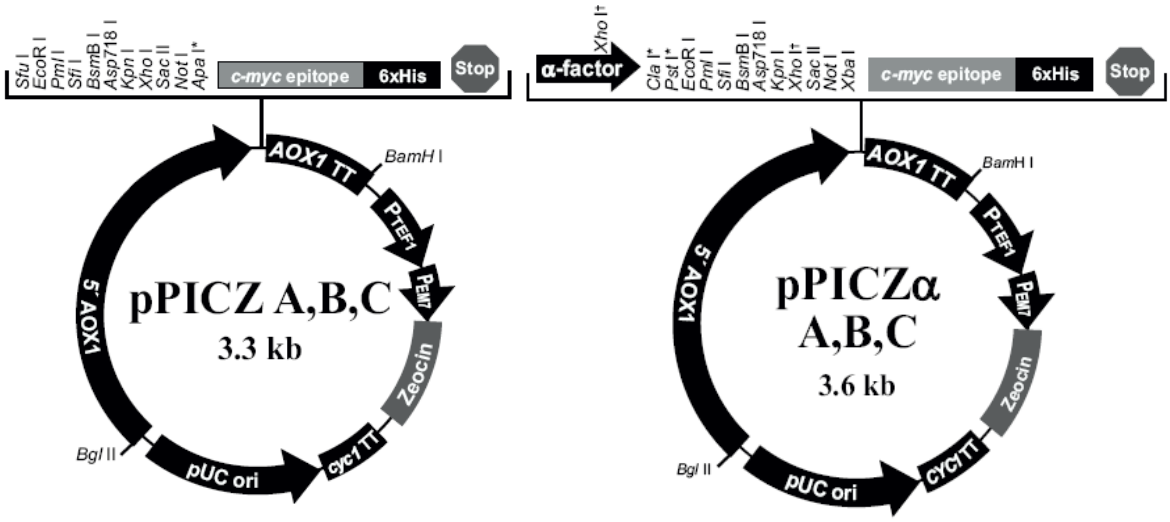

Figure 2.2: Vector maps of pPICZ A, B, C and pPICZα A, B, C (Invitrogen Corporation, 2001). *PTEF1 refers to the TEF promotor, PEM7 to the EM7 promoter, pUC ori to the pUC origin of replication, cyc1TT to the cyc1 transcription termination region and Zeocin to the Zeocin® antibiotic resistance gene.*

Initial manipulations and amplification of the plasmid can be done in *E. coli* strains that are recombination (recA) and endonuclease (*endA*) deficient as well as deficient of the Tn*5* transposable element. The Tn*5* transposable element encodes the bleomycin resistance gene, which confers to the cells Zeocin™ resistance. The bleomycin gene is regulated in yeast by the transcription elongation factor 1 gene promoter (*TEF1*) of *S. cerevisiae* origin and in *E. coli* by the *EM7* promoter. Although both vectors contain the pUC origin of replication which allows replication in *E. coli*, they do not contain any yeast origins of replication so that Zeo^R transformants can only be selected if recombination occurred between the recombinant construct and the *P. pastoris* genome (Invitrogen Corporation, 2001). Linearization of pPICZ and pPICZα can be achieved via digestion with *Sac*I, *Pme*I or *BstX*1 to facilitate integration of the plasmid into the genome at the *AOX1* locus.

When selecting intracellular or extracellular expression, it is important to consider the native location and processing of the protein. If the protein is natively cytosolic and non-glycosylated, intracellular expression might be the best option. If the protein of interest is naturally secreted, glycosylated and directed towards intracellular organelles, extracellular expression will generate better results (Invitrogen Corporation, 2001). During *P. pastoris* intracellular expression, the N-terminal methionine of intracellularly expressed proteins is usually removed by methionine amino-peptidase. N-terminal acetylation is also common. Disadvantages include lack of glycosylation and purification difficulties as heterologous protein usually makes up only 1% of total cytosolic protein (Daly and Hearn, 2005). The pPICZα vectors contain the α-factor pre-pro peptide from *S. cerevisiae* that targets the recombinant protein to the yeast secretory pathway (Macauley-Patrick *et al.*, 2005). The major advantage of utilizing secreted expression is that *P. pastoris* secretes very few native proteins and thus the secreted heterologous protein will make up most of the protein in the medium, significantly simplifying purification (Balamurugan *et al.*, 2007). Unwanted glycosylation at recognized glycosylation sites and the lack of other essential PTMs can be a drawback to extracellular expression (Daly and Hearn, 2005). In this study, the pPICZ A vector was used.

2.2.4. Culturing conditions for *Pichia pastoris*

Yeast cultures are most often maintained in Erlenmeyer type flasks in shaking incubators, unless large-scale expression is attempted in bioreactors. The concentration of recombinant protein is often up to 10 times lower in these flask systems, compared to that of bioreactors. This may be due to limitation in cell densities obtained, as well as the inability to control parameters such as aeration (Daly and Hearn, 2005). Fermentation-based, large-scale protein production has proven to be very successful with the use of *P. pastoris*. Secretion signals and glycosylation patterns have also been investigated in an effort to improve the quality of recombinant proteins produced in these systems (Balamurugan *et al.*, 2007).

The initial use of complex media (containing yeast extract, peptone and glycerol/glucose as carbon source) to dramatically increase cell density prior to induction has been suggested (Macauley-Patrick *et al.*, 2005). For large-scale protein production however, a defined medium containing trace salts and vitamins is suggested. Methanol concentrations are usually in the range of 0.5 - 1.0 %, but optimal methanol concentration needs to be determined for each protein of interest. In order to curb the effects of proteases, it has been suggested to maintain the pH between 3 and 6, and to add casamino acids, L-arginine hydrochloride and ammonium salts (Daly and Hearn, 2005). Other factors that should be optimised include induction time and incubation temperature, however generally temperatures are maintained at 28 – 30°C.

2.3. Tick proteins recombinantly expressed in *Pichia pastoris*

2.3.1. Bm86 and orthologs

Various tick proteins have been expressed in the yeast, *P. pastoris,* of which Bm86 is probably the best described. Bm86 , a 86 kDa glycosylphosphatidylinositol (GPI) anchored protein, was first characterized and expressed in *E. coli* in 1989 (Rand *et al.*, 1989). Since then, expression of Bm86 in both eukaryotic and prokaryotic systems has been extensively investigated and modified. Currently, Bm86 produced in *P. pastoris* has been commercialized as the anti-tick vaccine, Gavac® (Canales *et al.*, 1997). The first expression of Bm86 in *P. pastoris* was performed in 1993 on both conventional and bioreactor level. The authors were able to produce high levels (up to 1.5 $g.l^{-1}$) of protein with 95% purity (Rodriguez *et al.*, 1994). In another fermentation experiment, the recombinant protein was found to strongly associate with the membrane (insoluble) fractions of the yeast, and consequently a method of washing, harvesting, solubilisation and ultrafiltration was employed to isolate the protein from these fractions. In this way, sufficient quantities of the recombinant protein could be produced (Buxadó *et al.*, 2004). Although mostly expressed intracellularly, Bm86 was also expressed as a secreted protein in *P. pastoris* X-33 with similar success (Canales *et al.*, 2010).

Bm86, Ba86 (from *R. annulatus*) and Bd86 (from *R. decoloratus*) was successfully expressed in a *P. pastoris* bench top fermentation system for vaccination purposes (Canales *et al.*, 2008; Canales *et al.*, 2009a). rHaa86, the Bm86 analogue from *Hyalomma anatolicum anatolicum* was expressed in *P. pastoris* as the first recombinantly produced protein from this tick species. The immunoprotective capacity of rHaa86 was subsequently tested in calves infested with *Hy. a. anatolicum* (Azhaniahambi *et al.*, 2009).

2.3.2. Other tick proteins

Another prominent tick antigen that has been successfully expressed in *P. pastoris* is Bm95. Bm95 was produced in a bioreactor system with supplemented saline medium, harvested under reducing conditions and purified via diafiltration (Boue *et al.*, 2004). The authors later determined that the expressed protein is both N-and O-glycosylated (Gonzalez *et al.*, 2004).

Asparaginyl endopeptidase (legumain IrAE), derived from *Ixodes ricinus*, was active post secreted expression and isolation from *P. pastoris* X33 (Sojka *et al.*, 2007; Canales *et al.*, 2009b). Secreted expression was used to express a 5'-nucleotidase isoform 1 from the soft tick, *Ornithodoros savignyi* (Stutzer *et al.*, 2008). An enzymatically active pyrethroid metabolizing esterase, Czest9, from a Mexican population of *R. microplus* was expressed as a secreted protein in GS115 (Guerrero and Nene, 2008). Recently, a Kunitz-BPTI domain protease inhibitor, rBmTI-6, was expressed with the recombinant protein maintaining its inhibitory properties (Sasaki and Tanaka, 2008).

2.4. The *R. microplus* EF-1α transcript

As mentioned in chapter 1, *R. microplus* EF-1α was identified as the subolesin interacting protein EF-1α, as part of a study to elucidate the role of subolesin in tick gene expression. EF-1α was identified using yeast two-hybrid and subsequently exposed to co-affinity precipitation and RNA interference for verification of the interaction (De la Fuente *et al.*, 2008b). For the purpose of elucidating the cloning strategy and characteristics of the fusion protein recombinantly expressed during this study, it should be mentioned that the EF-1α fragment identified only represents the partial coding sequence (cds) of EF-1α. The transcript identified contains the EF-1α II and EF-1α III domains and multiple N-myristoylation, CK2 and PKC phosphorylation sites (De la Fuente *et al.*, 2008b).

Blastp was used to identify proteins with identity to the *R. microplus* EF-1α fragment from (De la Fuente *et al.*, 2008b). The first eight hits with the lowest E values are shown in Table 2.3. Interestingly, four of the hits are tick derived (*A. maculatum, I. scapularis* and *O. coriaceus*), whilst the other four are from other arthropods (spider and scorpion). E-values were significantly low, indicating considerable similarity amongst query and result sequences. Residues 280 – 474 (corresponding to the *R. microplus* fragment) of the eight hits were subjected to multiple sequence alignment using CLUSTALW (http://www.genome.jp/tools/clustalw/). Identities of 90 – 99% were obtained, with the *A. maculatum* fragment being 99% identical to the fragment under investigation.

Table 2.3: Blastp analysis of the *R. microplus* EF-1α fragment. *The eight results with the highest E values are shown, as well as their identies to R. microplus EF-1α (residues 280 – 474).*

***R. microplus* EF-1α amino acid fragment.**			
RPTDKPLRLPLQDVYKIGGIGTVPVGRVETGVLKPGMVVTFAPANLTTEVKSVEMHHEALAEAVPGDNVG FNVKNVSVKELRRGYVCGDSKDTPPKSTEEFTAQVIVLNHPGQIANGYTPVLDCHTAHIACKFREIKEKCD RRSGKKLEDNPKFIKSGDAAIIDLVPSKPMCVETFTDFPPLGRFAVRDMRQTVA			
Accession number	**Description**	**E value**	**Identity* to *R. microplus* EF-1α (%).**
AEO32591.1	hypothetical protein [*Amblyomma maculatum*]	$7e^{-140}$	99
XP_002411147.1	translation elongation factor EF-1 alpha/Tu, putative [*Ixodes scapularis*] >gb|	$3e^{-138}$	97
ACB70375.1	translation elongation factor EF-1 alpha/Tu [*Ornithodoros coriaceus*]	$2e^{-137}$	96
BAI83413.1	elongation factor 1 alpha [*Parasteatoda tepidariorum*]	$6e^{-134}$	93
AAK12647.1	elongation factor-1alpha [*Amblyomma* sp. 'Amb2']	$5e^{-132}$	93
AAK12670.1	elongation factor-1alpha [*Stenochrus portoricensis*]	$2e^{-128}$	90
AAC03151.1	elongation factor-1 alpha [*Mastigoproctus giganteus*]	2e-^{128}	90
AAC03144.1	elongation factor-1 alpha [*Aphonopelma chalcodes*] >gb|ADG27871.1| elongation factor 1 alpha [Lycosa sp. KS-2010]	$4e^{-128}$	90

The alignment in Figure 2.3 shows the full-length amino acid sequences of EF-1α from *I. scapularis, A. maculatum*, and *S. cerevisiae*, as well as partial sequences (fragments) of *R. microplus*, *O. coriaceus, P. tepidariorum* and *Amblyomma* spp. The *S. cerevisiae* sequence was added to the alignment because it is a well-defined full-length sequence. It is clear that the available *R. microplus* EF-1α sequence encodes a functional195 amino acid C-terminal fragment, approximately starting at residue number 280 and ending at 474.

```
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50         60         70
BmGII           ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ocoriaceus      ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ptepidaror      ---------- ---------- ---------- ---------- ---------- ---------- ----------
Amblyomma       ---------- ---------- ---------- ---------- ---------- ---------- ----------
Iscapulari      ---------- ---------- ---------- ---------- MGKEKTHINI VVIGHVDSGK STTTGHLIYK
Amaculatum      GSSFSVGCVG KLRSLFIRVV PVLALRSPSV VLTGTRKTAT MGKEKTHINI VVIGHVDSGK STTTGHLIYK
Scerevis        ---------- ---------- ---------- ---------- MGKEKSHINV VVIGHVDSGK STTTGHLIYK


                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        80         90        100        110        120        130        140
BmGII           ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ocoriaceus      ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ptepidaror      ---------- ---------- ---------- ---------- ---------- ---------- ----------
Amblyomma       ---------- ---------- ---------L DKLKAERERG ITIDITLWKF ETPKYYVTVI DAPGHRDFIK
Iscapulari      CGGIDKRTIE KFEKEAQEMG KGSFKYAWVL DKLKAERERG ITIDITLWKF ETPRYYVTVI DAPGHRDFIK
Amaculatum      CGGIDKRTIE KFEKEAQEMG KGSFKYAWVL DKLKAERERG ITIDITLWKF ETPKYYVTVI DAPGHRDFIK
Scerevis        CGGIDKRTIE KFEKEAAELG KGSFKYAWVL DKLKAERERG ITIDIALWKF ETPKYQVTVI DAPGHRDFIK


                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       150        160        170        180        190        200        210
BmGII           ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ocoriaceus      ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ptepidaror      ---------- ---------- ---------- ---------- --------IV GVNKMDTTEP PFSESRFEEI
Amblyomma       NMITGTSQAD CAVLIVAAGT GEFEAGISKN GQTREHALLA YTLGVKQMIV GVNKMDTTEP PFSQSRFEEI
Iscapulari      NMITGTSQAD CAVLVVAAGT GEFEAGISKN GQTREHALLA YTLGVKQMIV GVNKMDTTEP PFSQTRFEEI
Amaculatum      NMITGTSQAD CAVLIVAAGT GEFEAGISKN GQTREHALXA YTLGVKQMIV GVNKMDTTEP PFSQSRFEEI
Scerevis        NMITGTSQAD CAILIIAGGV GEFEAGISKD GQTREHALLA FTLGVRQLIV AVNKMDS--V KWDESRFQEI


                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       220        230        240        250        260        270        280
BmGII           ---------- ---------- ---------- ---------- ---------- ---------- ---------R
Ocoriaceus      ---------- ---------- ---------- -LDASPNMPW YKGWSIERKS GKSEGKSLLQ ALDAMEPPTR
Ptepidaror      KKEVSAYVKK IGYNPATVPF VPISGWNGDN MLEPSSNMPW YKGWSIERKG NKSDGKTLLQ ALDAMEPPSR
Amblyomma       QKEVSAYIKK IGYNPATVPF VPISGWNGDN MLEPSQNMPW YKGWSIERKS GKSEGKTLLQ ALDAMEPPTR
Iscapulari      QKEVSAYIKK IGYNPATVPF VPISGWNGDN MLDASPNMGW YKGWTIERKS GKSEGKSLLQ ALDAMEPPTR
Amaculatum      QKEVSAYIKK IGYNPATVPF VPISGWNGDN MLEPSTNMPW YKGWSIERKS GKSEGKTLLQ ALDAMEPPTR
Scerevis        VKETSNFIKK VGYNPKTVPF VPISGWNGDN MIEATTNAPW YKGWEKETKA GVVKGKTLLE AIDAIEQPSR
                                                                                           *


                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       290        300        310        320        330        340        350
BmGII           PTDKPLRLPL QDVYKIGGIG TVPVGRVETG VLKPGMVVTF APANLTTEVK SVEMHHEALA EAVPGDNVGF
Ocoriaceus      PTDKPLRLPL QDVYKIGGIG TVPVGRVXTG VLKPGTVVTF APANLTTEVK SVEMHHEALT EAVPGDNVGF
Ptepidaror      PLDKPLRLPL QDVYKIGGIG TVPVGRVETG ILKPGTVVTF APANITTEVK SVEMHHESLT EAVPGDNVGF
Amblyomma       PTDKPLRLPL QDVYKIGGIG TVPVGRVETG VLKPGMVVTF APANLTTEVK SVEMHHEALV EAVPGDNVGF
Iscapulari      PTDKPLRLPL QDVYKIGGIG TVPVGRVETG LLKPGMVVTF APANITTEVK SVEMHHEALT EAVPGDNVGF
Amaculatum      PTDKPLRLPL QDVYKIGGIG TVPVGRVETG VLKPGMVVTF APANLTTEVK SVEMHHEALV EAVPGDNVGF
Scerevis        PTDKPLRLPL QDVYKIGGIG TVPVGRVETG VIKPGMVVTF APAGVTTEVK SVEMHHEQLE QGVPGDNVGF
                * ******** ********** ******* ** ::*** **** ***.:***** ******* *  :.********


                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       360        370        380        390        400        410        420
BmGII           NVKNVSVKEL RRGYVCGDSK DTPPKSTEEF TAQVIVLNHP GQIANGYTPV LDCHTAHIAC KFREIKEKCD
Ocoriaceus      NVKNVSVKEL RRGYVCGDSK DTPPKATEEF TAQVIVLNHP GQISNGYTPV LDCHTAHIAC KFREIKEKCD
Ptepidaror      NVKNVSVKEL RRGFVCGDSK DTPPKSTEKF TAQVIVLNHP GQICNGYTPV LDCHTAHIAC KFLEIKEKCD
Amblyomma       NVKNVSVKEL RRGYVCGDSK DTPPKSTEEF TAQVIVLNHP GQIANGYTPV LDCHTAHIAC KFREIKEKCD
Iscapulari      NVKNVSVKEL RRGYVCGDSK DSPPKSTEEF TAQVIVLNHP GQISNGYTPV LDCHTAHIAC KFREIKEKCD
Amaculatum      NVKNVSVKEL RRGYVCGDSK DTPPKSTEEF TAQVIVLNHP GQIANGYTPV LDCHTAHIAC KFREIKEKCD
Scerevis        NVKNVSVKEI RRGNVCGDAK NDPPKGCASF NATVIVLNHP GQISAGYSPV LDCHTAHIAC RFDELLEKND
                *********: *** ****:* : ***.  .* .* ******* ***. **:** ********** :* *: ** *
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```
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       430        440        450        460        470        480        490
BmGII           RRSGKKLEDN PKFIKSGDAA IIDLVPSKPM CVETFTDFPP LGRFAVRDMR QTVA------ ----------
Ocoriaceus      RRSGKKLEDN PKSIKSGDAA IIDLVPTKPM CVETFTDFPP LGRFAVRDMR QTVAVGVIKS VKAKDLSGGK
Ptepidaror      RRSGKKLEDN PKFIKSGDAA IVELVPSKPM CVETFTDFPP LGRFAVRDMR QTVAVGVIKS VTPKDLSGGK
Amblyomma       RRSGKKLEDN PKFIKSGDAA IIDLVPSKPM CVETFTDFPP LGR------- ---------- ----------
Iscapulari      RRSGKKLEDN PKFIKSGDAA IIDLVPSKPM CVETFTDFPP LGRFAVRDMR QTVAVGVIKS VKPKEASGGK
Amaculatum      RRSGKKLEDN PKFIKSGDAA IIDLVPSKPM CVETFTDFPP LGRFAVRDMR QTVAVGVIKS VKPKEASGGK
Scerevis        RRSGKKLEDH PKFLKSGDAA LVKFVPSKPM CVEAFSEYPP LGRFAVRDMR QTVAVGVIKS VDKTEKAAKV
                *********: ** :****** ::.:**:*** ***:*:::** ***


                ....|....| ..
                       500
BmGII           ---------- --
Ocoriaceus      VTKAAEKAQK KK
Ptepidaror      VTKAAEKAQK KK
Amblyomma       ---------- --
Iscapulari      VTKAAEKAQK KK
Amaculatum      VTKAAEKAQK KK
Scerevis        TKAAQKAAKK --
```

Figure 2.3: Amino acid alignment of EF-1α from different tick species. *Sequences correspond to R. microplus (BmGII), O. coriaceus (Ocoriaceus), P. tepidariorum (Ptepidaror), A. americanum (Amblyomma), I. scapularis (Iscapulari), A. maculatum (Amaculatum) and S. cerevisiae (Scerevis) full-length sequences. Identical residues are indicated by (*). Conserved substitutions are indicated by (:) and semi-conserved substitutions by (.).*

2.5. Hypothesis

- *R. microplus* EF-1α can be expressed successfully in both prokaryotic and eukaryotic systems.

2.6. Aims

- Construction and transformation of a pQE60-EF-1α construct into the appropriate prokaryotic expression host (*Escherichia coli* JM109).
- Subcloning of *R. microplus* EF-1α into an appropriate vector for intracellular expression in *Pichia pastoris*.
- Comparing expression of recombinant EF-1α (rEF-1α) in prokaryotic and eukaryotic systems using western blotting.

Materials and Methods

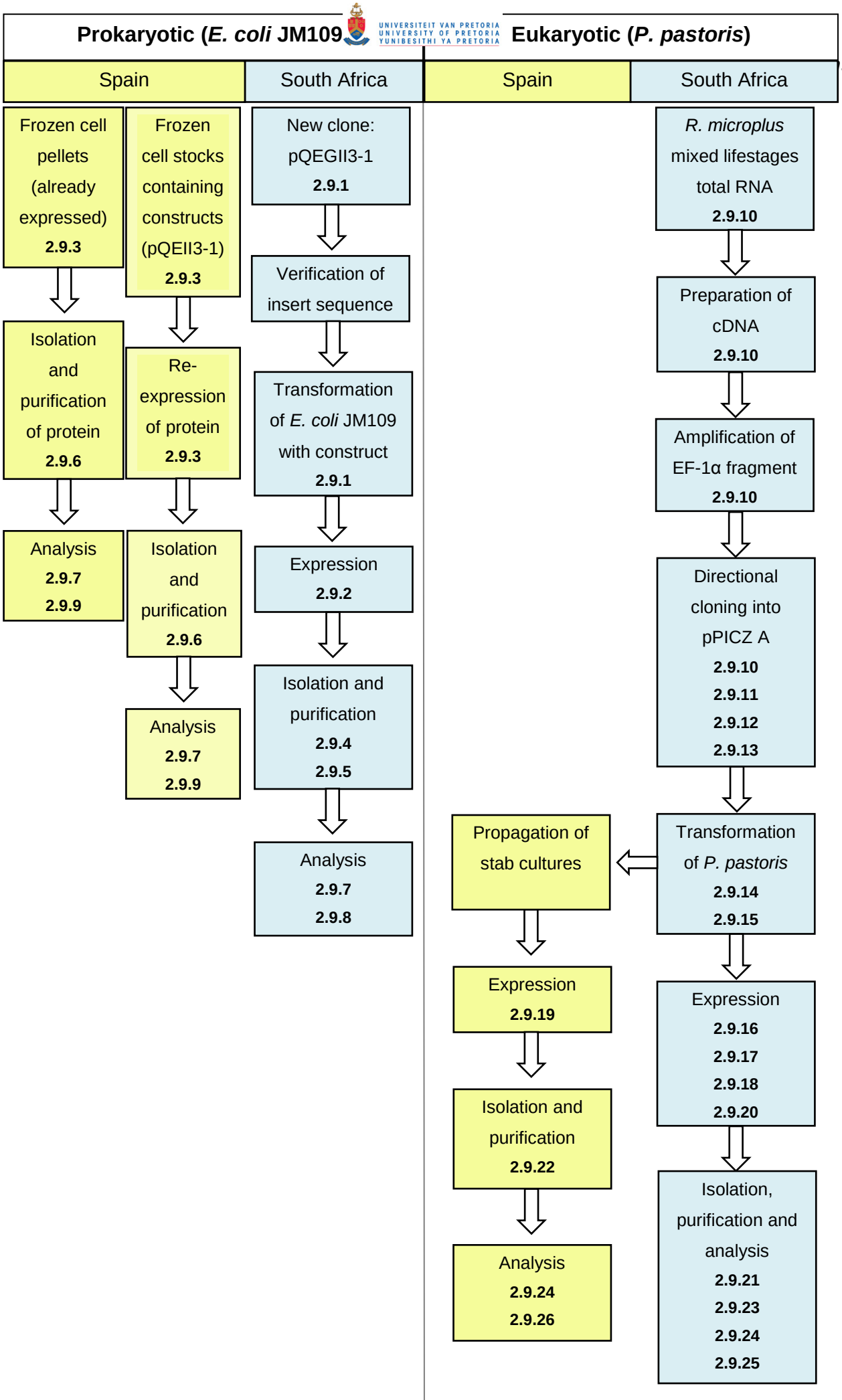
2.7. Materials

The 6 X His ladder and the Qiagen Ni-NTA kit were obtained from Qiagen (CA, USA) Kapa Taq Readymix® was obtained from Kapa Biosystems, MA, USA. Ethanol, methanol (analytical grade and HPLC grade), glacial acetic acid, glycerol, glucose (dextrose) and iso-propanol were obtained from Merck chemical company (Darmstadt, Germany). Tris (hydroxymethyl) aminomethane, sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride ($MgCl_2$), calcium chloride ($CaCl_2$), silver nitrate ($AgNO_3$), sodium carbonate (Na_2CO_3) were obtained from Merck chemical company (Darmstadt, Germany). Acrylamide, sodium thiosulfate pentahydrate ($Na_2S_2O_3.5H_2O$), sodium dodecyl sulphate (SDS), N'N'-methylenebisacrylamide, 3-1-propane (CHAPS), di-potassium hydrogen orthophosphate (K_2HPO_4), manganese chloride ($MnCl_2.4H_2O$), magnesium chloride ($MgCl_2.6H_2O$), potassium acetate (KCO_2H), sorbitol, histidine, 4-Chloro-1-naphtol, glass beads, Tricine, Triton X-100, hydrogen peroxide (H_2O_2), biotin and the Colorburst™ Electrophoresis marker were obtained from Sigma-Aldrich (Steinheim, Germany). Yeast extract, tryptone, agar and peptone was obtained from Oxoid Ltd. (Basingstoke, Hampshire, UK). Yeast nitrogen base without amino acids and ammonium sulphate (YNB) was obtained from Difco (Laboratoria). PVDF membrane (Immobolin P, Millipore Corporation, USA), filter paper and 0.22 µm filters were obtained from Merck (Darmstadt, Germany). SuperSignal West Pico chemiluminescent Substrate and Nitrocellulose membrane was obtained from Pierce Biotechnology Inc., Rockford, USA (Separations). Quick Start™ Bradford Dye Reagent was obtained from Bio-Rad Laboratories Inc. (California, USA). ECL X-ray film was obtained from Amersham Biosciences (Pittsburgh, USA). Universal developer and HIPAM universal fixer was obtained from Illford (Illford imaging UK Ltd., Cheshire, UK). Agarose was obtained from Promega, Wisconsin, USA (Anatech, SA). The EasySelect™ *Pichia* expression kit and the antibiotic Zeocin® were obtained from Invitrogen, USA. The NucleoSpin® Extract II and NucleoSpin® Plasmid isolation kits were obtained from Macherey-Nagel, Düren, Germany (Separations). TaKaRa *ExTaq*™ and TaKaRa *Taq*™ DNA polymerase were obtained from Takara Shuzo Co., Japan (Separations). T4 DNA ligase and restriction enzymes *Eco* I, *Sac* II, and *BstX*I were obtained from Promega, USA (Anatech). Restriction enzyme *Pme* I was obtained from New England BioLabs, USA. Shrimp alkaline phosphatase was obtained from Fermentas (Canada). MicroPulser® electroporation cuvettes (0.2 cm) were obtained from BioRad, USA. Primers were either synthesised by Integrated DNA Technologies, USA (Whitehead Scientific) or Inqaba biotec (South Africa). Ampicillin was obtained from Roche, South Africa. Isopropyl β-D thiogalactopyranoside (IPTG) was from MG Biochemicals, South Africa. N,N,N',N'-tetramethyl-ethylenediamine (TEMED) and Ammonium persulphate (APS) was obtained from ICN (Separations, SA). Bacterial cell lysis was achieved by incubating cells in Bugbuster® Protein extraction reagent (Novagen Merck, Darmstadt, Germany). Protease inhibitor cocktail was obtained from Sigma

Aldrich, Germany and Protease Inhibitor mix from GE Healthcare (UK). DNase I and the PageRuler® prestained protein ladder was obtained from Fermentas (Canada). 5% Skim milk was obtained from Asturiana, Spain. The Macherey Nagel Protino® Ni-TED kit was used as an alternative for His_6-Tag purification of recombinant protein and obtained from Macherey-Nagel, Düren, Germany (Separations). Monoclonal anti-His_6 antibodies and horseradish peroxidase (HRP) conjugated goat anti mouse IgG was obtained from KPL Protein Research Products Inc., Maryland USA. Superscript® Reverse Transcriptase III (MMLV RT) was obtained from Clontech, USA. A 5X First strand buffer and DTT was included with the enzyme. RNasin™ RNase inhibitor and the Maxwell® Polyhistidine protein purification kit was obtained from Promega, Wisconsin, USA (Anatech). Pre-cast gels (PAGE*gel*™-SDS cassette gel), sample buffer, SDS-PAGE running buffer, and reducing reagent, were obtained from PAGE*gel*™ Inc, San Diego, CA, USA). Protoblue™ Safe staining solution was obtained from National Diagnostics, USA.

2.8. Flow diagram of methodology

Prokaryotic and eukaryotic expression was done both in South Africa (University of Pretoria (UP)) and Spain (Instituto de Investigación en Recursos Cinegéticos (IREC), Universidad de Castilla la Mancha (UCLM)). In order to clarify the course of both approaches, a flow diagram is included.
The diagram includes references to specific units of the methods section.


Prokaryotic (E. coli JM109
Eukaryotic (P. pastoris)
Spain
South Africa
Spain
South Africa
Frozen cell pellets (already expressed) 2.9.3
Isolation and purification of protein 2.9.6
Analysis 2.9.7 2.9.9
Frozen cell stocks containing constructs (pQEII3-1) 2.9.3
Re-expression of protein 2.9.3
Isolation and purification 2.9.6
Analysis 2.9.7 2.9.9
New clone: pQEGII3-1 2.9.1
Verification of insert sequence
Transformation of E. coli JM109 with construct 2.9.1
Expression 2.9.2
Isolation and purification 2.9.4 2.9.5
Analysis 2.9.7 2.9.8
R. microplus mixed lifestages total RNA 2.9.10
Preparation of cDNA 2.9.10
Amplification of EF-1α fragment 2.9.10
Directional cloning into pPICZ A 2.9.10 2.9.11 2.9.12 2.9.13
Transformation of P. pastoris 2.9.14 2.9.15
Expression 2.9.16 2.9.17 2.9.18 2.9.20
Isolation, purification and analysis 2.9.21 2.9.23 2.9.24 2.9.25
Propagation of stab cultures
Expression 2.9.19
Isolation and purification 2.9.22
Analysis 2.9.24 2.9.26

2.9. Methods

Prokaryotic expression of EF-1α using *Escherichia coli* JM109

2.9.1. Transformation, growth and selection of *Escherichia coli* JM109 containing the recombinant construct (performed at UP)

The recombinant construct, pQEGII3-1, was prepared using the plasmid pQE-60 by Dr. Mario Canales (Instituto de Investigación en Recursos Cinegéticos (IREC), University of Castilla La Mancha (UCLM), Spain). The *R. microplus* EF-1α partial coding sequence was directionally ligated using *Nco*I and *Bgl*II recognition sites (Table 2.4). Upon receival of the construct, the presence of the EF-1α insert was re-confirmed subsequent to transformation, employing the gene specific primers pQE-60R and pQE-60F (Table 2.4) used for polymerase chain reaction (PCR) and DNA sequencing.

Table 2.4: Primers used to amplify the EF-1α insert for pQEGII3-1 construct preparation and subsequent colony screening of transformants.

Melting temperatures of primers were always confirmed by using the equation (Tm = 69.3 + 0.41(% G/C) – (650/primer length) (Rychlik et al., 1990). Restriction enzyme cut sites are underlined.

Primer	Sequence	Melting temperature (Tm)
QEGII5	5'-GGCCATGGCCTCAACCAGGCCCACGGACAAACCCCTC-3' *Nco*I	79.0°C
QEGII3	5'-GGAGATCTGGCGACCGTTTGCCTCATGTC-3' *Bgl*II	71.7°C
pQE-60F	5'-GTGTGAAATTGTTATCCGCTCAC-3'	61.0°C
pQE-60R	5'-TGGACTCCTGTTGATAGATCC-3'	60.6°C

E. coli JM109 was transformed with pQEGII3-1 via electroporation. Electroporation cuvettes (Bio-Rad) were placed at –20°C one hour prior to use. A 1:10 dilution of the recombinant construct, pQEGII3-1 (20 ng/µl), was prepared. Some 10 µl was mixed with 90 µl of electrocompetent *E. coli* JM109. The mixture was pipetted into the slit of an electroporation cuvette and the cuvette placed in a MicroPulser electroporator (Eppendorf, Germany). Cells were exposed to 1.5 – 2 kilovolts (kV) for 5 milliseconds. Cells were placed in Luria-Berthani (LB) - Glucose (0.02 M glucose) and incubated for 60 minutes at 37°C. Various dilutions of the cell suspension were plated onto LB-Amp plates and incubated overnight. LB-Amp plates were prepared by melting 2% (w/v) of bacteriological Agar in 100 ml LB broth in a microwave oven. Once this had cooled, ampicillin was added to a concentration of 100 µg/ml. The molten LB-Amp was decanted into Petri dishes and allowed to cool and set before use.

Single colonies were screened for insert with the pQE-60F and pQE-60R primers (Table 2.4). Colony PCR reactions were prepared containing: 1 µl of cells, 0.4 µM pQE-60F forward and 0.4 µM pQE-60R reverse primers (Table 2.4.), 12.5 µl of KapaTaq® Readymix, and 10.5 µl of double distilled de-ionized water ($dddH_2O$). This was performed with the following cycling parameters: 94° C for 7 minutes, followed by 30 cycles of 94°C for 30s, 72°C for 30s, and 72°C for 2 minutes. The last two holds were 72°C for 5 minutes (final extension) and 4°C indefinitely. PCR amplification was performed in the Gene Amp PCR System 2100 (Perkin Elmer Applied Biosystems, USA). All samples were run on an agarose gel stained with ethidium bromide (EtBr), to analyze the size of the transcripts amplified from the transformed cells.

Expression of recombinant EF-1α in *E. coli* JM109

2.9.2. First attempt at prokaryotic expression from previously constructed clones (performed at UP)

Colonies with EF-1α cloned in-frame (from Spain) were cultivated in 10 ml of LB broth containing 50 µg/ml ampicillin and 0.4% glucose, incubated overnight at 200 rpm and 37°C and subsequently used to inoculate 250 ml cultures. The 250 ml cultures were grown at the same conditions until OD_{600} = 0.4 was reached. IPTG was added as inducer (to a final concentration of 0.5 mM) and cell growth resumed for 3.5 hours. A negative control (non-induced cells) was included.

2.9.3. Second attempt at prokaryotic expression from previously constructed clones (performed at IREC, Spain)

Due to the expression difficulties encountered at UP and the initial success attained by Dr. M. Canales with this clone, it was decided to attempt large-scale expression of EF-1α from pQEGII3-1 at IREC, Spain. Initially, frozen pellets (stored at -70°C) were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western Blot. A fresh expression was also performed by propagating frozen cell stocks. Briefly, 10 ml Luria-Berthani (LB) broth was inoculated with stocks of clone pQEGII3-1, grown overnight at 37°C and 200 rpm and used to inoculate 200 ml of LB broth. Following growth to an OD_{600} = 0.4, expression was induced with IPTG (0.5 mM).

Isolation and purification of recombinant EF-1α from *E. coli* JM109

2.9.4. First attempt at prokaryotic expression from previously constructed clones (performed at UP): Recovery of protein from the soluble fraction

Each 250 ml culture (induced and non-induced) was divided into 5 fractions of 50 ml each. These fractions were centrifuged for 15 minutes at 2,000 x g. The supernatant was discarded, and the mass of each cell pellet determined. Protease inhibitor cocktail (10 µl/ml) and DNase I (10 µg/ml)

was added according to the volume of Bugbuster® Protein Extraction Reagent added (5 ml reagent per gram of wet cell paste). Lysates were transferred to 10 ml culture tubes and incubated overnight at 4°C on an orbital shaker. In order to remove cellular debris, lysates were centrifuged for 20 minutes at 16,000 x g (4°C). The supernatant (soluble fraction) was saved and subjected to His-tag purification. Pellets were kept for inclusion body purification.

Soluble fractions were filtered through a 0.45 µm filter in order to remove any remaining cell debris. His-Tag purification of EF-1α was performed as in the Macherey Nagel Protino® Ni-TED 150 kit manual. Briefly, 1X LEW (50 mM NaH_2PO_4, 300 mM NaCl, adjusted to pH 8.0) buffer and 1X elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, adjusted to pH 8) was prepared. The column was equilibrated with 320 µl LEW buffer and the clarified lysate loaded. The column was washed with 2 x 320 µl LEW buffer, where after the protein was eluted with 3 x 240 µl elution buffer and collected in three separate fractions.

2.9.5. First attempt at prokaryotic expression from previously constructed clones (performed at UP): Recovery of protein from the insoluble fraction

Previous expression studies by M. Canales indicated that the recombinant protein was present in inclusion bodies. Proteins were isolated from the pellets (cellular debris obtained after soluble protein recovery) by following the guidelines in the Macherey Nagel Protino® Ni-TED kit. Briefly, pellets were resuspended in 2 ml LEW buffer and centrifuged at 10,000 x g for 30 minutes at 4°C. The supernatant was discarded and the pellets resuspended in 2 ml denaturing solubilisation buffer (50 mM NaH_2PO_4, 300 mM NaCl, 8 M urea, adjusted to pH 8). Thereafter, the suspension was sonified for 10 x 16 pulses of 0.25 minutes at 30% duty cycle and output control of 3 using a Branson sonifier cell disruptor B-30 (Branson Sonic Power Co., USA). Sonified samples were then incubated on an orbital shaker for 60 minutes at 4°C and centrifuged at 10,000 x g for 30 minutes at 20°C to remove insoluble materials. The supernatant obtained after this step was filtered through a 0.45 µm filter before commencing with His-Tag purification as described previously for soluble proteins, with the exception of elution buffer being replaced with denaturing elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 8 M urea, 250 mM imidazole, adjusted to pH 8).

2.9.6. Second attempt at prokaryotic expression from previously constructed clones (performed at IREC, Spain): Recovery of protein from the insoluble fraction

Protein isolation commenced with the addition of 5 ml disruption buffer to 3.54 g pellet, followed by the addition of 10 µl/ml of protease inhibitor mix. The resuspended cell suspension was sonicated at 20 kHz and 70 kW with a duty cycle of 0.5 seconds using the Bandelin Sonopuls MS73

(BANDELIN electronic, Berlin, Germany). The process was repeated three times. Disrupted cells were centrifuged at 5,000 x g for 20 minutes using the Beckman Allegra X-22R. Protein was isolated from the insoluble phase under reducing conditions using the Qiagen Ni-NTA kit (principal similar to Macherey Nagel Protino® Ni-TED kit). Protein was additionally isolated from frozen cell stocks of a previous expression performed by M. Canales, and used as a positive control for SDS-PAGE.

2.9.7. Protein concentration determination by Bradford method (UP and IREC, Spain)

Protein concentration was determined using Quick Start™ Bradford Dye Reagent (Bio-Rad Laboratories Inc., CA, USA). Standard bovine serum albumin (BSA) dilutions (50 µl) were prepared from a BSA stock solution (100 mg/ml). This, together with sample (50 µl) and dye reagent (150 µl), were pipetted into the wells of a microtitre plate. All reactions were performed in triplicate. The plate was incubated for 5 minutes and the absorbance at 595 nm determined with the Multiskan Ascent 354 multiplate scanner (Thermo Labsystems, Finland). A standard curve was prepared from the serial BSA dilutions and used to determine sample protein concentration.

Analysis and detection of recombinant EF-1α expressed in *E. coli* JM109

2.9.8. First attempt at prokaryotic expression from previously constructed clones (performed at UP): Analysis

Expression of recombinant EF-1α was analysed by Tricine SDS – PAGE. The gel consisted of a 4% stacking gel and a 12% separating gel that were prepared from an acrylamide stock solution (49.5% Acrylamide, 3% N'N' – Bis-methylene- acrylamide) and a gel buffer (3 M Tris-HCl, 0.3% SDS, pH 8.45). Gel solutions were degassed prior to polymerisation. Polymerisation was initiated by adding 0.7 mg/ml ammonium persulphate (APS) and 5 µl TEMED to both stacking and separating solutions. The gels were cast and run in the Hoefer® mini VE vertical gel system (Amersham Pharmacia biotec, USA).

Sample fractions collected (soluble and insoluble) were diluted 3:1 in SDS reducing sample buffer (60 mM Tris-HCl, 2% SDS w/v, 0.1% glycerol v/v, 0.05% mercapto ethanol v/v, 0.025% bromophenol blue, pH 6.8) and denatured at 70°C for 10 minutes. Samples were loaded alongside the PageRuler® prestained protein ladder. Electrophoresis was performed with anode buffer (0.2 M Tris – HCl, pH 8.9) in the lower chamber and cathode buffer (0.1 M Tris, 0.1 M Tricine, 0.1% SDS, pH 8.2) in the upper chamber. Gels were run initially at 60 V for +/- 30 minutes and then at 100 V for +/- 2 hours. Gels were visualized by silver staining or subjected to western blot analysis.

The silver staining method followed was adapted from (Blum *et al.*, 1987) and (Shevchenko *et al.*, 1996). Gels were incubated in fixing solution (30% ethanol, 10% acetic acid) for 30 minutes on a rocking platform, followed by 30 minutes incubation in sensitizing solution (30% ethanol, 0.5 M sodium acetate, 0.5% gluteraldehyde, 0.2% $Na_2S_2O_3$). Gels were washed three times for 10 minutes with $dddH_2O$, followed by 30 minute incubation in silver reaction solution (0.1% silver nitrate, 0.25% formaldehyde). This was followed by two brief washing steps with $dddH_2O$ and development in developing solution (2.5% Na_2CO_3, 0.01% formaldehyde). Termination was performed with the addition of 0.05 M EDTA.

2.9.9. Second attempt at expression from previously constructed clones (performed at IREC, Spain): Analysis

Pre-cast gels (PAGE*gel*™-SDS cassette gel, PAGE*gel*™ Inc, San Diego, CA, USA) were used for protein separation. Samples were prepared by combining 10 µg of sample, 25% v/v of PAGE*gel*™ LDS sample buffer, 10% v/v reducing agent (PAGE*gel*™ Inc) and $dddH_2O$ to a final volume of 50 µl. Samples were placed in boiled water for 10 minutes for denaturation. PAGE*gel*™ SDS-PAGE run buffer was prepared as per manufacturers' instruction, 200 ml placed in the inner chamber (cathode) and 400 ml in the outer chamber (anode). The system was then connected to the electrophoresis power supply EPS601 (Amersham Biosciences). The following parameters were set: 200 VDC voltage, a starting current of 90 mA/gel, an end current of 40 mA/gel and run time of 40 - 70 minutes. Gels were briefly washed with $dddH_2O$ prior to overnight staining with Protoblue™ Safe staining solution (10% v/v in ethanol). Background staining was removed by washing the gel in $dddH_2O$.

Western Blot electrophoretic transfer was achieved using the Mini-Genie® Electroblotter semi-dry transfer unit (Idea Scientific, Corvallis, OR, USA) as per manufacturers' instruction. Blots were incubated in 5% skim milk (Asturiana, Spain) in TBS buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.4) for 1 hour, washed three times for 10 minutes in TBS buffer (pH 7.4), incubated with a 1/1000 dilution of the Ni-NTA conjugate in TBS-Tween (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.05% Tween-20) followed by three 5 minute washes with TBS buffer. Colour development with TMB stabilized substrate for horseradish peroxidase was preceded by a single wash with $dddH_2O$. The substrate was washed away with $dddH_2O$ once colour development was satisfactory and the membrane left to dry.

Eukaryotic: Recombinant expression of EF-1α using the methylotrophic yeast, *Pichia pastoris*

2.9.10. In-frame cloning of EF-1α into pPICZ A

Because of the difficulties encountered during prokaryotic expression (low expression; insoluble), a new clone was created for intracellular expression of *R. microplus* EF-1α in the methylotrophic yeast, *P. pastoris*.

Primer design

GSPs (Table 2.5) were designed for the in-frame sub-cloning of the EF-1α fragment into pPICZ A for subsequent intracellular expression in *Pichia pastoris*. The EF-1α transcript was screened for the presence of restriction enzyme cut sites using the program pDRAW32 version 1.0, revision 1.1.97 (Acaclone software, USA), to ensure that the restriction enzymes chosen, did not cut within the insert. The forward primer (GIIEcopPICAF1) contains an *Eco*RI restriction enzyme cut site and the reverse primer (GIISacpPICAR1) a *Sac*II restriction enzyme cut site. Primer characteristics were analyzed using the Oligo 6 primer analysis software version 6.71 (Molecular Biology Insights Inc. USA).

Table 2.5: Primers used in the amplification and subcloning of EF-1α, as well as the preparation of *R. microplus* first strand cDNA. *Restriction enzyme sites are underlined. Melting temperatures of primers were always confirmed by using the equation (Tm = 69.3 + 0.41(% G/C) – (650/primer length) (Rychlik et al., 1990).*

Primer	Sequence (5' – 3')	Tm (°C)
GIIEcopPICAF1	CCGGAATTCTATAATGGAGCCCCCAACCAGGCCCACGGACAAAC *Eco*RI	72.4
GIISacpPICAR1	TCCCCGCGGGGGGCGACCGTTTGCCTCATGTCACGGACAGC *Sac*II	77.0
5'SMART Oligo IIA	AAGCAGTGGTATTAACGCAGAGT T(6)	71.0

cDNA synthesis

Total RNA was isolated from a mixed lifestages population of *R. microplus* obtained from Clinvet International, Bloemfontein, South Africa. Isolation was performed with TRI-REAGENT®, following manufacturers' instructions. The quantity and purity of the RNA was determined by measuring the 260/280 nm ratio spectrophotometrically with the GeneQuant pro RNA/DNA calculator™ (Amersham Pharmacia Biotech™, Biochrom Ltd., Cambridge, England). First strand cDNA synthesis was performed using 0.5 µM of a poly thymine (T) primer (5'SMART Oligo IIA, Table 2.5), 1 µg of *R. microplus* mixed lifestages RNA (2000 ng/µl) and 0.5 mM dNTPs. The volume of the reaction was adjusted to 12 µl with dddH_2O and subsequently denatured at 70°C for 10 minutes. The reaction

mixture was immediately chilled on ice for 2 minutes, followed by the addition of 4 µl of 5x First strand buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$, pH 8.3), 2 µl of 0.1 M DTT and 1 µl RNAse inhibitor (40 U/ µl, Roche Diagnostics GmbH, Germany). The reaction mixture was incubated at 42°C for 2 minutes, before the addition of 1 µl (200 U) of Superscript™III reverse transcriptase (Invitrogen corporation), and incubated at 42°C for a further 60 minutes to ensure full-length synthesis, before heat inactivating the enzyme at 70°C for 10 minutes.

Amplification of EF-1α

PCR amplification of EF-1α was performed in the Gene Amp PCR System 2100 (Perkin Elmer Applied Biosystems, USA). Reactions were prepared as follows: 0.5 µl of cDNA template, 0.4 µM GIIEcopPICAF1 forward and 0.4 µM GIISacpPICAR1 reverse primers, 4 µl of 10x ExTaq™ buffer (containing 20 mM $MgCl_2$) and 4 µl dNTPs (2.5 mM of each) was added and adjusted with double distilled de-ionized water ($dddH_2O$) to a final volume of 40 µl. A hot-start protocol (94°C for 2 minutes, 80°C for 1 minute) was followed, where after 10 µl of enzyme mix was added. This mixture contained: 0.5 µl ExTaq™ polymerase, 1.0 µl ExTaq buffer and 8.5 µl $dddH_2O$. Amplification commenced with 30 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 2 minutes. The PCR ended in a final extension at 72°C for 5 minutes and an infinite incubation at 4°C.

PCR products were analyzed via DNA gel electrophoresis stained with EtBr and purified using the Macherey Nagel Extract II kit (Macherey Nagel, Germany). Briefly, the samples were mixed with two volumes of NT buffer, loaded onto the NucleoSpin® Extract II spin column, centrifuged at 11,000 x g for 1 minute and the flow through discarded. The column was washed with 600 µl buffer NT3 (100 mM Tris-H_3PO_4, 15% ethanol, 1.15 M KCl, pH 6.3), centrifuged at 11,000 x g for 1 minute and the column dried by centrifugation at 11,000 x g (2 minutes). Templates were eluted in 50 µl pre-warmed $dddH_2O$. The concentration and purity of the PCR products was determined using the GeneQuant pro RNA/DNA calculator™.

2.9.11. Preparation of the pPICZ A intracellular expression vector

The intracellular expression vector, pPICZ A (500 ng), was transformed into *E. coli* TOP10F' using electroporation (see section 2.5.1). Since Zeocin™ is sensitive to high salt concentrations, all culturing was done using low salt LB (1% tryptone, 0.5% NaCl, 0.5% yeast extract, pH 7.5). Cells were plated onto low salt LB-agar plates (1.5% agar in low salt LB-Broth) containing Zeocin™ (12.5 µg/ml final concentration) and incubated overnight at 30°C. Positive colonies were selected, grown overnight at 30°C with shaking in 50 ml low salt LB-Broth containing 12.5 µg/ml Zeocin™ and

plasmids isolated using the Macherey Nagel Plasmid isolation kit (Macherey Nagel). Plasmid concentration was determined using the GeneQuant pro RNA/ DNA calculator™.

2.9.12. Directional cloning of EF-1α into pPICZ A

Both the pPICZ A plasmid and the EF-1α transcript were subjected to sequential restriction enzyme digestion with *Sac*II and *Eco*RI. Firstly *Sac*II digestion was performed in reaction mixtures containing 1 µg of pPICZ A plasmid or 1 µg of insert, 1.5 µl of *Sac*II restriction enzyme (10 U/µl), 6 µl of 10x restriction enzyme buffer C (100 mM Tris-HCl, 500 mM NaCl, 100 mM $MgCl_2$, 10 mM DTT, pH 7.9) and adjusted with double distilled deionised water to a final volume of 60 µl. Reactions were incubated at 37°C overnight and the enzyme inactivated with incubation at 70°C for 10 minutes. The digested templates were purified utilising the NucleoSpin® Extract II kit and yields determined spectrophotometrically with the GeneQuant pro RNA/DNA calculator™. To analyse digestion efficiency, DNA gel electrophoresis was performed with 150 ng of digested plasmid.

Similar reaction mixtures were prepared for the sequential digestion with *Eco*RI utilizing the total purified *Sac*II digested products for plasmid or insert as templates. A reaction mixture was prepared containing template (1000 ng), 1.5 µl of *Eco*RI restriction enzyme (10 U/µl), 6 µl of 10x restriction enzyme buffer H (60 mM Tris-HCl, 1.5 M NaCl, 60 mM $MgCl_2$, 10 mM DTT, pH 7.9) and adjusted with double distilled de-ionized water to a final volume of 60 µl. Following incubation at 37°C overnight, the enzyme was inactivated by incubation at 70°C for 15 minutes. Digested products were purified as before, eluted in 50 µl $dddH_2O$ and the concentrations determined as described previously. The double-digested pPICZ A plasmid was dephosphorylated with the addition of 5 U shrimp alkaline phosphatase (SAP) and 10X SAP buffer in a total volume of 50 µl. This was followed by incubation at 37°C for 1 hour and heat-inactivation at 65°C for 15 minutes.

Ligation was performed at an insert: vector ratio of 3:1 and 5:1, utilizing 50 ng of dephosphorylated pPICZ A plasmid. An overnight ligation reaction was performed at 16°C containing 2 µl of a 10x T4 ligation buffer (300 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP, 10% PEG, pH 7.8) and 2 µl T4 DNA ligase (3 U/µl) in a final reaction volume of 20 µl. Ligase was heat-inactivated by incubation at 70°C for 20 minutes, the ligation mixture precipitated with the addition of 3 volumes of ethanol and 1/5 volumes of sodium acetate (3M; pH 5.2), centrifuged at 11,000 x g for 45 minutes, the supernatant decanted and the pellet washed with 70% ethanol. The centrifugation step was repeated for 30 minutes and the supernatant decanted again. The pellet was finally dried *in vacuo*, and re-dissolved in 20 µl of $dddH_2O$. The ligation reaction (10 µl) was used to transform *E. coli* TOP10F' via electroporation (see section 2.5.1.).

2.9.13. Selection and DNA sequencing of recombinant *E. coli* TOP10F' clones

Various dilutions of the transformed TOP 10F' cells were plated onto low salt LB-agar plates containing 12.5 µg/ml Zeocin™ and incubated overnight at 30°C. A total of 30 colonies were selected for colony PCR screening using 0.4 µM of 5' AOX1 and 3' AOX1 primers (Table 2.6), 12.5 µl 2x KapaTaq® Readymix and 10.5 µl ddH_2O to a final volume of 25 µl per reaction. The reactions were performed with the following cycling parameters: 94° C for 7 minutes, 30 cycles of 94°C for 30s, 56°C for 30s, and 72°C for 2 minutes. The last two holds were 72°C for 5 minutes (final extension) and 4°C indefinitely. All samples were analysed using agarose gel electrophoresis stained with EtBr.

Table 2.6: Primers used in colony PCR to determine positive clones containing the EF-1α insert.

Melting temperatures of primers were always confirmed by using the equation (Tm = 69.3 + 0.41(% G/C) – (650/primer length) (Rychlik et al., 1990).

Primer name	Primer Sequence (5' – 3')	Tm(°C)
5'AOX	GACTGGTTCCAATTGACAAGC	57.87
3'AOX	GCAAATGGCATTCTGACATCC	57.87

Clones displaying the correct insert size were grown overnight for 16 hours at 30°C with shaking in 50 ml flasks containing 10 ml low salt LB-Broth with Zeocin™ (12.5 µg/ml). Glycerol stocks were prepared by adding 350 µl of a 50% (v/v) glycerol solution to 700 µl culture for storage at -70°C. From the remaining culture, plasmids were isolated with the NucleoSpin® plasmid kit. DNA concentrations were confirmed with the GeneQuant pro RNA/DNA calculator™ and sequences confirmed via automated nucleotide sequencing.

DNA sequencing of the pPICZA-EF-1α construct was performed by combining 5 pmol 5'AOX primer (Table 2.6), 2 µl 5X BigDye buffer (400 mM Tris-HCl, 10 mM $MgCl_2$, pH 9), 550 ng plasmid and ddH_2O in a final reaction volume of 20 µl. The cycling parameters were preceded by a 94°C denaturation step for 2 minutes and a 80°C "hot-start" step for 1 minute. The sequencing PCR was performed in the Gene amp PCR system 2100 (Perkin Elmer, CA, USA), and included a denaturation step of 94°C for 30 seconds, an annealing temperature of 56°C for 30 seconds and extension at 60°C for 4 minutes repeated for 26 cycles. Data analysis was performed using BioEdit, ClustalW and the Pubmed BLAST functionality.

2.9.14. Linearization of the recombinant construct and transformation into *Pichia pastoris*

A linearization reaction using 6 µg of pPICZA-EF-1α and 6 µg of native pPICZ A control vector was prepared according to the EasySelect™ *Pichia* expression kit manual (Invitrogen Corporation). pPICZA-EF-1α was linearized using *Pme*I (New England laboratories, USA) and pPICZ A with *Bst*XI (New England laboratories, USA). Reaction mixtures were prepared containing the vector(s), 10x restriction enzyme buffer 3 or 4, 5 µl of *Bst*XI (1 U/µl) or *Pme*I (1 U/µl) and adjusted to a final volume of 200 µl with sterile $ddd H_2O$. Following incubation at 37°C for 16 hours overnight, the enzymes were heat-inactivated by incubation at 65°C for 15 minutes. Linearization efficiency was determined using DNA gel electrophoresis prior to precipitation. Linearized plasmids were precipitated with the addition of 1/10 the volume of NaOAc (3 M; pH 5.2) and 2.5 volumes of 100% ethanol, followed by centrifugation at 11,000 x g for 45 minutes, where after the pellets were washed with 70% ethanol. Residual ethanol was decanted, the recombinant constructs dried *in vacuo* and re-dissolved in 50 µl sterile $ddd H_2O$.

2.9.15. Transformation of *P. pastoris* with linearized plasmids

Linearized pPICZA-EF-1α and native plasmid were transformed into the GS115 and KM71H strains of the methylotrophic yeast, *P. pastoris*. Electrocompetent yeast cells were prepared as per EasySelect™ *Pichia* expression kit manual specifications (Invitrogen Corporation, 2001). Overnight cultures were prepared for GS115 and KM71H yeast cells in 5 ml YPD (1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose) with shaking at 250 rpm and 30°C. These cultures (0.5 ml) were used to inoculate 500 ml YPD and grown overnight to OD_{600} = 1.3 to 1.5. Thereafter, the cells were collected by centrifugation at 1,500 x g (5 minutes, 4°C) and resuspended in 500 ml ice-cold sterile $ddd H_2O$. Cells were washed in 250 ml ice-cold sterile $ddd H_2O$ and resuspended in 20 ml ice-cold 1 M sorbitol. Following centrifugation at 1,500 x g (5 minutes, 4°C), the pellet was resuspended in 1 ml of 1 M sorbitol.

Electrocompetent yeast cells were transformed by electroporation using the MicroPulser® electroporator (Eppendorf, Germany). A mixture of 80 µl electrocompetent GS115 or KM71H cells and 1 µg linearized plasmid was loaded into a pre-chilled electroporation cuvette (0.2 cm gap, Bio-Rad, USA). This was incubated on ice (5 minutes) and pulsed at 1.5 kilovolts for 3-5 milliseconds. The transformed cells were immediately transferred into 1 ml ice-cold 1M sorbitol in a 15 ml tube and incubated without shaking at 30°C for 1 - 2 hours. The cells were spread on YPDS plates (1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose, 1M sorbitol) containing 100 µg/ml Zeocin™. Plates were incubated at 30°C for 4 days or until colonies formed. Zeocin™ resistant colonies were chosen for EF-1α to determine the Mut phenotype and confirm the presence of insert by colony PCR.

2.9.16. Determination of the GS115 Mut-phenotype

To determine the growth phenotypes of transformed GS115 cells, Zeocin™ resistant colonies were selected from YPDS plates and their growth assessed on methanol media by replica-plating. Control strains for Mut^+ (GS115/pPICZ/*lac*Z Mut^+ -galactosidase) and Mut^S (GS115/His+ Mut^S Albumin) were included to distinguish between phenotypes. Plates with minimal media containing dextrose (MDH) and methanol (MMH) were prepared for phenotype distinction. Each MDH (1.34% w/v Yeast Nitrogen Base (YNB), 0.00004% w/v biotin, 0.004% w/v histidine, 2% w/v dextrose) and MMH (1.34% w/v YNB, 0.00004% w/v biotin, 0.004% w/v histidine, 0.5% w/v methanol) agar plate (1.5% w/v) was divided into a grid to simplify replica-plating. Zeocin™ resistant colonies, including Mut^+ and Mut^S controls, were spread on MDH media with sterile wooden sticks and incubated at 30°C for 2 days. Colonies were replica-plated onto fresh MDH and MMH media and incubated for 2 days at 30°C. Mut^S transformants were then assessed according to growth on MMH versus MDH, relative to the controls.

2.9.17. Colony PCR screening of recombinant GS115 and KM71H clones

The presence of the EF-1α transcript in *Pichia pastoris* GS115 and KM71H cells was determined directly from cells on MDH plates without prior genomic DNA isolation, based on a method developed by (Abath *et al.*, 2002). Colony PCR was performed using vector specific primers 5' – and 3'AOX (Table 2.6) and the products analyzed by DNA gel electrophoresis. Positive colonies were inoculated into 5 ml of buffered glycerol-complex medium BMGY (1% w/v yeast extract, 2% w/v peptone, 100 mM potassium phosphate pH 6, 1.34% w/v YNB, 0.00004% biotin w/v, 1% v/v glycerol) and incubated overnight with vigorous shaking (200 - 300 rpm) at 30°C. Glycerol stocks of selected positive colonies were prepared by adding 350 µl 50% glycerol to 700 µl cells in cryovials and stored at -70°C.

Expression of rEF-1α in GS115 and KM71H cells

2.9.18. Preliminary expression of EF-1α in *P. pastoris* (performed at UP)

After determining the Mut phenotype and selecting the positive clones using colony PCR for both KM71H and GS115, expression was commenced. Controls included an un-induced GS115 clone (negative control) as well as GS115/pPICZ/lacZ Mut^+ (positive control). BMGY medium (25 ml) was inoculated with each clone in 250 ml Erlenmeyer flasks. Cells were grown at 30°C and 300 rpm until the OD_{600} was 2 - 6. Cells were harvested by centrifugation (3,000 x g for 5 minutes at room temperature) and the pellet resuspended into 200 ml of BMMY (1% w/v yeast extract, 2% w/v peptone, 100 mM potassium phosphate pH 6, 1.34% w/v YNB, 0.00004% biotin w/v, 0.5% v/v methanol) medium in a 1 liter flask to an OD_{600} of 1, in order to induce expression. Flask mouths were plugged with cotton wool and covered with aluminium foil in order to allow proper aeration

during expression. Methanol (100%) was added to the medium to a final concentration of 0.5% every 24 hours to initialize and maintain the inductive effect. Expression cultures (10 ml) were collected at 24 hours and 48 hours in order to monitor expression at these post induction time points. Aliquots were pelleted and stored at -20°C.

2.9.19. Small-scale expression of EF-1α in *P. pastoris* (performed at IREC, Spain)

After performing the initial time-point analysis expressions, the intracellular expression of EF-1α in both GS115 and KM71H was attempted on small scale. Appropriate clones were once again chosen for both strains, inoculated into 5 ml of YP (1% w/v yeast extract, 2% w/v peptone) medium, and grown (200 rpm, 30°C) for 5 days with addition of 1% (v/v) methanol every 24h in order to induce expression. After 5 days, the cells from the different time points were pelleted (4,990 x g for 15 minutes, Beckman Allegra X-22R) and the pellets disrupted (mechanically using glass beads – Sigma Aldrich) for protein analysis.

2.9.20. Time course study of expression of EF-1α in *P. pastoris* (performed at UP)

After obtaining putative results with the initial expressions, an additional experiment was conducted to optimize the recombinant protein yield, by harvesting samples at different time points after expression. The methodology was similar to the first set of initial expressions, the only difference being the volume of culture medium used. BMMY (100 ml) was used for the time course experiment.

Solubilisation and Nickel-affinity purification

2.9.21. Preliminary expression of EF-1α in *P. pastoris* (performed at UP): Solubilisation and purification

Isolation of recombinant EF-1α from the initial small-scale expression was performed using the *Pichia* EasySelect™ kit method (Invitrogen Corporation, 2001). Briefly, cells were collected via centrifugation (5,000 x g for 5 minutes) and placed on ice. Thereafter, 100 µl of breaking buffer (50 mM sodium phosphate, pH 7.4, 1 mM protease inhibitor cocktail, 1 mM EDTA and 5% glycerol) was added for every 1 ml of pellet. An equal volume of glass beads were added and 8 cycles of a 30 second vortex and 30 second incubation on ice repeated. This was followed by a 10 minute centrifugation at 5,000 x g in order to separate the clear supernatant (cell lysate) and pellet (cellular debris, membrane fraction).

2.9.22. Small-scale expression of EF-1α in *P. pastoris* (performed at IREC, Spain): Solubilisation and purification

Prior to disruption, the pellets were washed twice with 5 ml of distilled water and centrifuged at 4,990 x g for 15 minutes (Beckman Allegra X-22R). 2.5 ml Disruption buffer (100 mM Tris pH 7.5, 150 mM NaCl, 1 mM PMSF, 5 mM $MgCl_2.6H_2O$, 0.1% Triton X-100), and 10 µl/ml of Protease inhibitor cocktail mix (AEC Amersham) were added and the pellets resuspended. Round-bottom Eppendorf tubes (2 ml) were filled with 800 µl glass beads and 1ml of the disruption buffer cell suspension. All samples underwent 6 cycles of 1 minute vortexing, followed by 1 minute incubations on ice. Supernatants were removed and centrifuged to remove all remaining debris, and protein concentration determined using the Nanodrop ND-1000 before and after purification using Qiagen Ni-NTA spin columns under reducing conditions.

2.9.23. Time course study of expression of EF-1α in *P. pastoris* (performed at UP): Solubilisation and purification

Protein isolation was performed as for the small-scale expression, with the exception of the use of 10 µl/ml Protease inhibitor cocktail (Sigma Aldrich).

2.9.24. Protein concentration determination by Bradford method (UP and IREC, spain)

Protein concentration was determined using Quick Start™ Bradford Dye Reagent (Bio-Rad Laboratories Inc., CA, USA). Standard bovine serum albumin (BSA) dilutions (50 µl) were prepared from a BSA stock solution (100 mg/ml). This, together with sample (50 µl) and dye reagent (150 µl), were pipetted into the wells of a microtitre plate. All reactions were performed in triplicate. The plate was incubated for 5 minutes and the absorbance at 595 nm determined with the Multiskan Ascent 354 multiplate scanner (Thermo Labsystems, Finland). A standard curve was prepared from the serial BSA dilutions and used to determine sample protein concentration.

Tricine SDS-PAGE, western blot and dot-blot analysis

2.9.25. Preliminary and time course expression of EF-1α in *P. pastoris* (performed at UP): Analysis

Tricine SDS-PAGE and silver staining was performed as in section 2.5.4.

For western blotting, proteins were transferred from the acrylamide gel onto polyvinylidene fluoride (PVDF) membrane. Electrophoretic transfer was performed with the semi-dry Trans-Blot electrophoretic transfer cell system (Bio-Rad, USA). The membrane was activated in 100% methanol for 5 seconds and soaked in 10mM CAPS (3-(cyclohexylamino)-1-propanesulfonic acid,

pH 9) for another 5 seconds. Six Layers of filter paper used for electrophoretic transfer (20 V for 25 minutes) were soaked in CAPS. Membranes were placed in sealed plastic bags and incubated in TBS blocking buffer (20 mM Tris-HCl, 150 mM NaCl, 5% skim milk powder, pH 7.4) for a minimum of 1 hour at room temperature, or 4°C overnight. Membranes were washed three times for 15 minutes, prior to being incubated with gentle agitation at 37°C for 1 hour in TBS buffer (pH 7.4) containing a 1:500 dilution of monoclonal anti-His_6 antibody. Blots were then washed three times for 15 minutes at 37°C in TBS buffer, followed by incubation with a 1:1000 TBS buffer dilution of horseradish peroxidase (HRP) conjugated goat anti-mouse IgG for 1 hour.

A colourimetric method utilizing 4-chloro- 1-naphtol (4-CN) was employed to visualize the antibody-protein complexes on the membranes. Two separate solutions were prepared in light-protected (foil covered) containers. 4-CN (60 mg) was dissolved in 20 ml methanol and 600 µl 3% Hydrogen peroxide (H_2O_2) in 100 ml TBS buffer. Following the final wash steps, the two solutions were mixed and the membrane covered. The membrane was incubated at room temperature until colour developed, washed with $dddH_2O$ and left to dry.

2.9.26. Small-scale expression of EF-1α in *P. pastoris* (performed in Spain): Dot-blot analysis

Representative protein samples were blotted onto nitrocellulose membrane by pipetting small volumes (2 µl) at a time. Dots were allowed to dry in between pipetting repeats. The blot was developed by incubation in 5% skim milk in TBS buffer (pH 7.4) for 1 hour, washed three times for 10 minutes in TBS buffer (pH 7.4), incubated with a 1/1000 dilution of Ni-NTA conjugate in TBS-Tween (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.05% Tween-20) and followed by three 5 minute washes with TBS buffer. Colour development with TMB stabilized substrate for horseradish peroxidase was preceded by a single wash with $dddH_2O$. Once colour development was satisfactory, the membrane was washed with $dddH_2O$ and left to dry.

2.10. Results and discussion

Prokaryotic expression of EF-1α

2.10.1. Transformation of *E. coli* JM109 with the pQEGII3-1 recombinant construct

Following transformation of *E. coli* JM109 with the pQEGII3-1 recombinant construct (received from Dr. M. Canales, IREC, Spain) via electroporation, single colonies were screened for the EF-1α insert with the pQE-60F (forward) and pQE-60R (reverse) primers (Figure 2.4). All clones were found to be positive, containing the 590 bp band. Both a positive control (induced culture), as well as a negative control (un-induced culture), were used for expression.

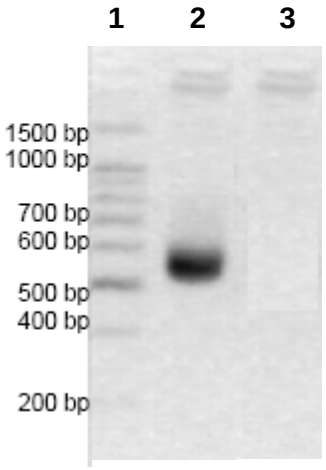


Figure 2.4.: Representative colony PCR screen of transformed *E. coli* JM109. *PCR amplification of inserts directly from JM109 E. coli cells. Visualisation was aided by EtBr. Lane 1: 100 bp molecular size marker. Lane 2: Representative positive clone exhibiting the 590 bp band indicated. Lane 3: Negative (no template) control.*

Tricine SDS-PAGE and western blot analysis of the prokaryotically expressed recombinant proteins

The predicted size of the *R. microplus* EF-1α recombinant protein fragment was determined by submitting the amino acid sequence to the Protein Calculator V3.3 software (http://www.scripps.edu/~cdputnam/protcalc.html). The isotopically averaged molecular weight of the protein was determined as 21337.5625 Da. With a further 0.84 kDa contributed by the C-terminal 6xHis-tag, the expected band size is thus 22 kDa (Qiagen, 2003).

2.10.2. First attempt at expression from previously constructed clones (performed at UP): SDS-PAGE and western blot analysis

After the initial expression in *E. coli* JM109 and the subsequent His-Tag purification of expressed proteins under reducing conditions from the insoluble fractions (inclusion bodies), three fractions of both induced and uninduced cells were obtained (Figure 2.5). Each sample (10 µg) was loaded into

the wells of the Tricine SDS-PAGE gel. Samples were assessed for the presence of a 22 kDa band corresponding in size to EF-1α using Tricine SDS-PAGE. Gels were visualized using silver staining.

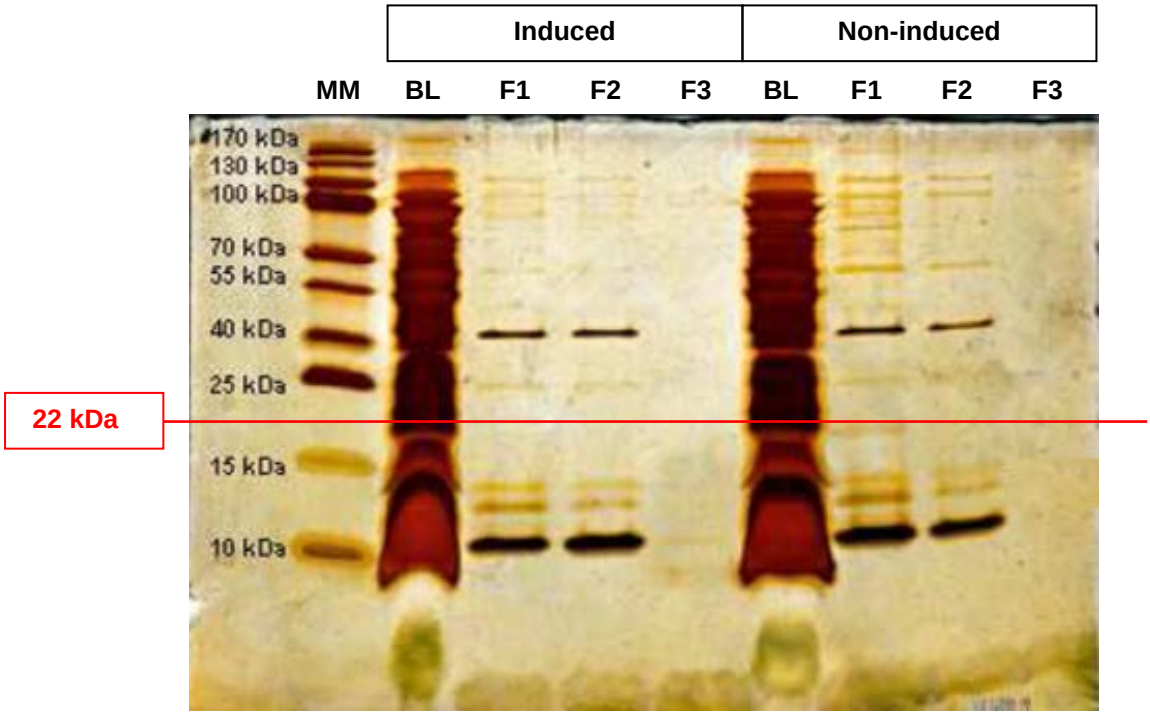


Figure 2.5: Tricine SDS-PAGE profile of the induced and non-induced unpurified Bugbuster® lysate and purified insoluble fractions from expression with *E. coli* JM109. *Tricine SDS-PAGE analysis of induced and non-induced fractions from an expression of EF-1α in E. coli JM109. Expression was induced with 0.5 mM IPTG for 3.5 hours. The gel was visualised with silver staining. Abbreviations correspond to: MM: Molecular weight marker (in kDa), BL = Bugbuster lysate and F1 - F3 = Eluted fractions 1 - 3. The expected size range (22 kDa) for EF-1α is indicated by the straight red line.*

As can be seen in Figure 2.5, rEF-1α was not obtained. No 22 kDa band could be detected for the induced fractions, and the expression profiles of both induced and non-induced fractions were identical. A western blot was performed due to its higher detection sensitivity (1 – 10 pg), but failed to detect any recombinant protein. His-tagged *Plasmodium falciparum* lactose dehydrogenase (*Pf* LDH) was included as a positive control during western blotting (results not shown).

Due to the expression difficulties encountered at UP and the initial success attained by Dr. M. Canales with this clone, it was decided to attempt large-scale expression of EF-1α from pQEGII3-1 at IREC, Spain.

2.10.3. Second attempt at expression from previously constructed clones (performed at IREC, Spain): SDS-PAGE and western blot analysis

Expression was performed with a validated clone (pQEGII3-1 in *E. coli* JM109) from Dr. M. Canales (De la Fuente *et al.*, 2008b). Protein was also isolated from frozen cell stocks of a previous successful expression performed by Dr. M. Canales and used as a positive control for SDS-PAGE. Using SDS-PAGE and western blot, protein was detected in the insoluble fractions of both the frozen pellets and repeated expression (Figure 2.6).

It was evident that a band was present on western blot (lane 3, Figure 2.6). Its molecular mass was however, too high to be positively identified as rEF-1α, for which the expected molecular mass is 22 kDa. Because the western blot detection was performed with a Ni-NTA HRP conjugate, the band could not be positively identified as rEF-1α. This high molecular mass band (45 kDa) can be due to the Anti-His_6 antibodies potentially binding to a protein (other than rEF-1α) with a relatively long stretch of histidines. It is alternatively possible that the rEF-1α protein formed intracellular aggregates that were not sufficiently denatured and therefore represents the 45 kDa band. The long-term freezing of the pellets likely caused this aggregation, as environmental stresses often incur structural deviations from the native (soluble) state. When refolding is attempted, the proteins are not converted to the native structure, but instead assume a partially folded (aggregated) state (Hamada *et al.*, 2009). This 45 kDa band will be analysed using LC-MS-MS, prospectively.

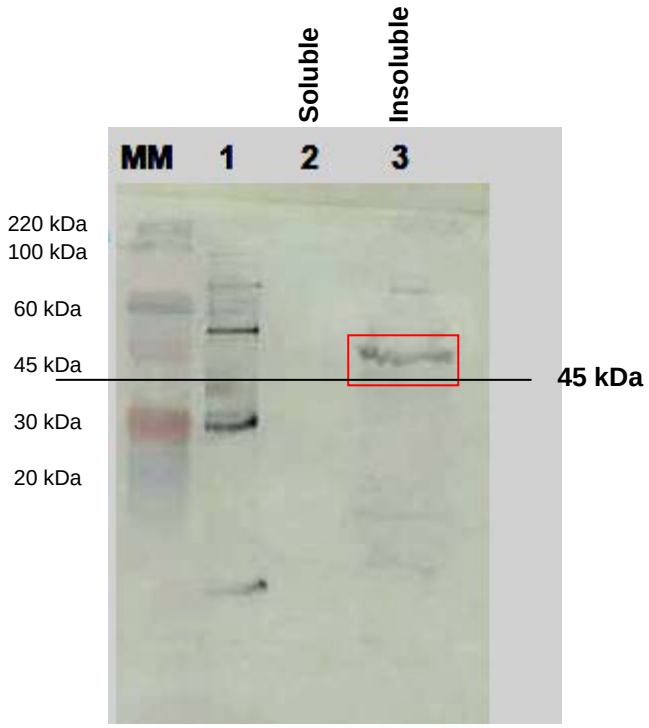


Figure 2.6: Western blot of nickel-purified soluble and insoluble fractions isolated from frozen cell pellets. *Western immunodetection of EF-1α with anti-His antibody (Qiagen) and TMB substrate (Promega). The large band (45 kDa) identified by His6 detection, is indicated in the red block. Lane MM: Sigma Colorburst™ molecular weight marker. Lane 1: 6X His tagged ladder (Qiagen) Lane 2: Soluble fraction Lane 3: Insoluble fraction. Soluble fraction (15 µg) was loaded in lane 2, whereas a set volume (15 µl) of insoluble fraction was loaded in lane 3. This is because the concentration could not be determined due to the fact that it was a crude suspension containing insoluble material.*

The insoluble fraction from both the frozen cell stocks as well as the repeated expression, were subsequently submitted to His-tag purification using the Qiagen Ni-NTA kit and all samples concentrated using Centricon® centrifugal devices. Protein (15 µg) was loaded into each SDS-PAGE well. Bands obtained in Figure 2.7 (lanes 3 and 7) were very clear, and putatively identified as rEF-1α, due to the fact that it corresponds to the expected band size of 22 kDa. Unfortunately, the result could not be verified by western blot. We therefore concluded that the *E. coli* JM109-pQE-60 system is not optimal for EF-1α expression. Due to its insoluble nature (association with inclusion bodies), insufficient quantities of rEF-1α would have been produced for small-scale vaccination trials on cattle. Optimization of parameters such as temperature, time and IPTG concentration did not improve yields. Intracellular expression of EF-1α in *Pichia pastoris* was therefore chosen due to EF-1α's mostly cytosolic location *in vivo*.

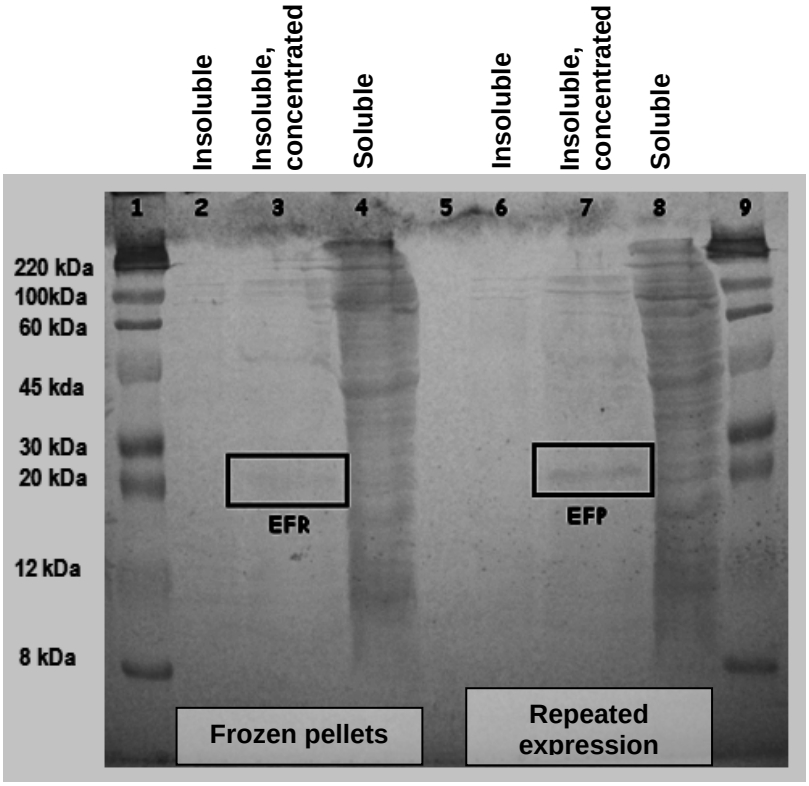


Figure 2.7: SDS-PAGE of soluble and insoluble fractions from frozen pellets (EFP) and repeated expression (EFR) following Ni purification. *SDS-PAGE analysis of insoluble and soluble fractions from both frozen pellets and repeated expression. Expression was induced at 0.5 mM IPTG for 4 hours. Lane 1: Sigma Colorburst™ molecular weight marker. Lane 2: Insoluble fraction from frozen pellet, concentration undetermined. Lane 3: Insoluble fraction from frozen pellet, concentrated. Lane 4: Soluble fraction from frozen cell pellet. Lane 6: Insoluble fraction from repeated expression, concentration undetermined. Lane 7: Insoluble fraction from repeated expression, concentrated. Lane 8: Soluble fraction from repeated expression. Lane 9: Sigma Colorburst™ molecular weight marker. The gel was visualised by Coomassie staining.*

Eukaryotic expression of EF-1α in *P. pastoris*

2.10.4. Primer design and preparation of the EF-1α – pPICZ A recombinant construct

Primer pairs were designed for the in-frame cloning of EF-1α into pPICZ A. The nucleotide sequence of the C-terminal EF-1α fragment was used for primer design. The primers (GIIEcopPICAF1 and GIISacpPICAR1), their sequences and Tm values are listed in the Table 2.5. Figure 2.8 contains the vector map of pPICZ A which incorporates both the vector and insert sequences, indicating the position of the primers and reading frame. The yeast "TATA" consensus sequence aids the yeast translation machinery in the recognition of the start-codon (Invitrogen Corporation, 2001). It is situated downstream of the *Eco*RI site and precedes the start-codon. The orientation of these consensus sequences additionally placed the EF-1α transcript in the correct reading frame for addition of the His_6-tag upon expression. Translated, it can be seen that the EF-

1α sequence begins with a Methionine which initiates expression, is followed by the rest of the yeast sequence and eventually the His_6 tag which is followed by a stop-codon that terminates translation.

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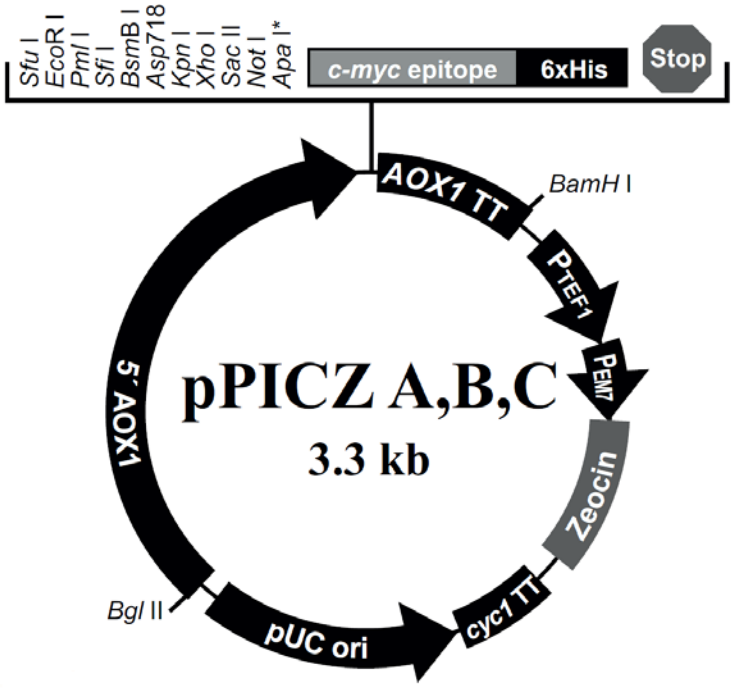
A



B

pPICZ A multiple cloning site

ATG ATC AAA ATT TAA CTG TTC TAA CCC CTA CTT GAC AGC AAT ATA TAA ACA GAA GGA AGC TGC CCT GTC TTA AAC CTT TTT TTT TAT CAT CAT TAT TAG CTT ACT TTC ATA ATT GCG ACT GGT TCC AAT TGA CAA GCT TTT GAT TTT AAC GAC TTT TAA CGA CAA CTT GAG AAG ATC AAA AAA CAA CTA ATT ATT CGA AAC GAG GAA TTC TATA ATG GAG CCC CCA ACC AGG CCC ACG GAC AAA CCC CTC CGA CTT CCG CTG CAG GAC GTC TAC AAG ATT GGT GGC ATT GGC ACG GTG CCA GTC GGC CGT GTG GAG ACC GGT GTC CTC AAG CCC GGC ATG GTC GTC ACC TTT GCC CCT GCC AAC CTG ACC ACT GAG GTC AAG TCT GTG GAG ATG CAC CAC GAG GCC CTC GCA GAG GCC GTG CCC GGC GAC AAC GTC GGC TTC AAC GTC AAG AAC GTC TCG GTG AAG GAG CTG CGC CGA GGA TAT GTG TGT GGA GAC TCG AAG GAC ACT CCA CCG AAG TCC ACG GAG GAG TTC ACT GCC CAG GTT ATT GTG CTG AAC CAT CCC GGC CAG ATT GCA AAC GGC TAC ACG CCT GTG TTG GAC TGC CAC ACA GCC CAC ATT GCC TGC AAG TTC AGG GAG ATC AAG GAA AAG TGT GAC CGT CGT TCT GGC AAG AAG CTC GAG GAC AAC CCC AAG TTC ATC AAG TCT GGC GAT GCT GCG ATC ATT GAC CTG GTA CCT TCC AAG CCC ATG TGC GTC GAA ACC TTC ACC GAC TTT CCT CCC CTT GGT CGC TTC GCT GTC CGT GAC ATG AGG CAA ACG GTC GCC CCC CGC GGC GGC CGC CAG CTT GGG CCC GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG AAT AGC GCC GTC GAC CAT CAT CAT CAT CAT CAT TGA

C

> pPICZ A multiple cloning site TRANSLATED

M I K I * L F * P L L D S N I * T E G S C P V L N L F F Y H H Y * L T F I I A T G S N * Q A F D F N D F * R Q L E K I K K Q L I I R N E E F Y T **M** E P P T R P T D K P L R L P L Q D V Y K I G G I G T V P V G R V E T G V L K P G M V V T F A P A N L T T E V K S V E M H H E A L A E A V P G D N V G F N V K N V S V K E L R R G Y V C G D S K D T P P K S T E E F T A Q V I V L N H P G Q I A N G Y T P V L D C H T A H I A C K F R E I K E K C D R R S G K K L E D N P K F I K S G D A A I I D L V P S K P M C V E T F T D F P P L G R F A V R D M R Q T V A P R G G R Q L G P E Q K L I S E E D L N S A V D H H H H H H * V L A L D M T V P Q F K L G T Y E K T G L A R F * S R G C Q N A I C L R D A G F I F D T F L F V T Y I V * D F F C H F

Figure 2.8: Map of the pPICZ A,B,C vectors indicating restriction enzyme cut sites and cloning strategy of EF-1α into pPICZ A (adapted from (Invitrogen Corporation, 2001). ***(A):*** *Schematic representation of the pPICZ A, B, C vectors.* ***(B)****: Nucleotide sequence of the multiple cloning site including the EF-1α insert in-frame of the C-terminal His_6-tag. Plasmid sequence is indicated in blue, insert sequence is underlined, gene specific regions used in primer design indicated in grey, synthetic amino acid (aa) sequence (with integrated start codon) in green, EcoRI site in pink, SacII site in dark blue and the stop-codon in red or indicated by *. The nucleotides flanking the EcoRI and SacII RE sites (indicated in yellow) are part of additional consensus sequences that aid in the recognition of these RE recognition sites by the respective restriction enzymes.* ***(C)****: Translated amino acid sequence of (B). The translated insert sequence is underlined. The starting methionine is indicated in bold and the three in-frame stopcodons by (*).*

The EF-1α transcript was amplified from *R. microplus* mixed lifestages cDNA using the GIIEcopPICAF1 and GIISacpPICAR1 primers (Table 2.5). A 600 bp product was obtained (Figure 2.9), including the restriction enzyme sites used for directional cloning. Both the insert and native pPICZ A plasmid were then subjected to sequential RE digestion with *Eco*RI and *Sac*II.

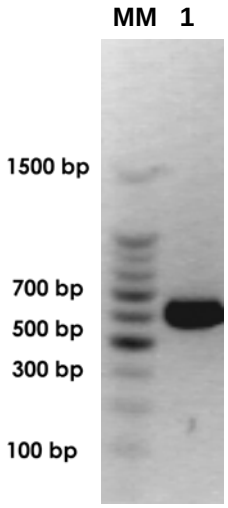


Figure 2.9: Amplified transcript of EF-1α from *R. microplus* mixed lifestages cDNA. *Visualisation was aided by EtBr. Lane MM: 100 bp Molecular mass marker. Lane 1: EF-1α transcript amplified with GIIEcopPICAF1 and GIISacpPICAR1 primers.*

2.10.5. Transformation and screening of *E. coli* TOP10F' cells containing the pPICZA-EF-1α recombinant construct

Following ligation, electroporation of competent *E. coli* TOP10F' and screening of colonies by PCR, a number of positive clones were identified (Figure 2.10). Plasmids were isolated from liquid culture of selected clones and subjected to automated DNA sequencing of the insert (Figure 2.11 and Figure 2.12). Clone number 5 (Figure 2.10, encircled) was selected for integration into *P. pastoris* and subsequent expression as it encoded for the correct amino acid sequence (Figure 2.11 and Figure 2.12). No single nucleotide polymorphisms (SNPs) were observed (Figure 2.11).

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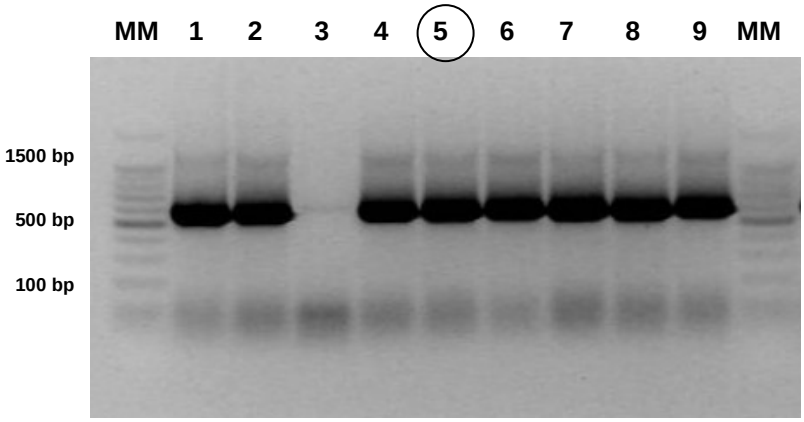


Figure 2.10: Representative colony PCR screen of transformed *E. coli* TOP10F' cells. *PCR amplification of inserts directly from TOP10F' E. coli cells. Visualisation was aided by EtBr. Lanes MM: 100 bp Molecular mass marker. Lanes 1 – 19: All indicated positive clones representing the 600 bp band. Clone number 5, used for integration into P. pastoris, is encircled.*

```
                10        20        30        40        50        60        70
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
BmGII           ATGGAGCCCC CAACCAGGCC CACGGACAAA CCCCTCCGAC TTCCGCTGCA GGACGTCTAC AAGATTGGTG
EF-1aC5         ATGGAGCCCC CAACCAGGCC CACGGACAAA CCCCTCCGAC TTCCGCTGCA GGACGTCTAC AAGATTGGTG

                80        90        100       110       120       130       14
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
BmGII           GCATTGGCAC GGTGCCAGTC GGCCGTGTGG AGACCGGTGT CCTCAAGCCC GGCATGGTCG TCACCTTTGC
EF-1aC5         GCATTGGCAC GGTGCCCGTC GGCCGTGTGG AGACCGGTGT CCTCAAGCCC GGCATGGTCG TCACCTTTGC

                150       160       170       180       190       200       21
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
BmGII           CCCTGCCAAC CTGACCACTG AGGTCAAGTC TGTGGAGATG CACCACGAGG CCCTCGCAGA GGCCGTGCCC
EF-1aC5         CCCTGCCAAC CTGACCACTG AGGTCAAGTC TGTGGAGATG CACCACGAGG CCCTCGCAGA GGCCGTGCCC

                220       230       240       250       260       270       28
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
BmGII           GGCGACAACG TCGGCTTCAA CGTCAAGAAC GTCTCGGTGA AGGAGCTGCG CCGAGGATAT GTGTGTGGAG
EF-1aC5         GGCGACAACG TCGGCTTCAA CGTCAAGAAC GTCTCGGTGA AGGAGCTGCG CCGAGGATAT GTGTGTGGAG

                290       300       310       320       330       340       35
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
BmGII           ACTCGAAGGA CACTCCACCG AAGTCCACGG AGGAGTTCAC TGCCCAGGTT ATTGTGCTGA ACCATCCCGG
EF-1aC5         ACTCGAAGCA CACTCCACCA AAGTCGACGG AGGAGTTCAC TGCCCAGGTT ATTGTGCTGA ACCATCCCGG

                360       370       380       390       400       410       42
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
BmGII           CCAGATTGCA AACGGCTACA CGCCTGTGTT GGACTGCCAC ACAGCCCACA TTGCCTGCAA GTTCAGGGAG
EF-1aC5         CCAGATCGCA AACGGCTACA CGCCTGTGTT GGACTGCCAC ACAGCCCACA TTGCCTGCAA GTTCAGGGAG

                430       440       450       460       470       480       49
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
BmGII           ATCAAGGAAA AGTGTGACCG TCGTTCTGGC AAGAAGCTCG AGGACAACCC CAAGTTCATC AAGTCTGGCG
EF-1aC5         ATCAAGGAAA AGTGTGACCG TCGTTCTGGC AAGAAGCTCG AGGACAACCC CAAGTTCATC AAGTCTGGCG

                500       510       520       530       540       550       56
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
BmGII           ATGCTGCGAT CATTGACCTG GTACCTTCCA AGCCCATGTG CGTCGAAACC TTCACCGACT TTCCTCCCCT
EF-1aC5         ATGCTGCGAT CATTGACCTG GTACCTTCCA AGCCTATGTG CGTCGAAACC TTCACCGACT TTCCTCCCCT

                570       580       590       600
                ....|....| ....|....| ....|....| ....|....|
BmGII           TGGTCGCTTC GCTGTCCGTG ACATGAGGCA AACGGTCGCC
EF-1aC5         TGGTCGCTTT GCTGTCCGTG ACATGAGGCA AACGGTCGCC
```

Figure 2.11: Nucleotide alignment of clone number 5 (EF-1aC5) and native EF-1α (BmNative). *Transcripts (in FASTA format) were aligned using GeneDoc sequence alignment editor and analyser.*

```
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50         60         70
BmNative        RPTDKPLRLP LQDVYKIGGI GTVPVGRVET GVLKPGMVVT FAPANLTTEV KSVEMHHEAL AEAVPGDNVG
EF-1aC5         RPTDKPLRLP LQDVYKIGGI GTVPVGRVET GVLKPGMVVT FAPANLTTEV KSVEMHHEAL AEAVPGDNVG
                ********** ********** ********** ********** ********** ********** **********

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        80         90        100        110        120        130        140
BmNative        FNVKNVSVKE LRRGYVCGDS KDTPPKSTEE FTAQVIVLNH PGQIANGYTP VLDCHTAHIA CKFREIKEKC
EF-1aC5         FNVKNVSVKE LRRGYVCGDS KDTPPKSTEE FTAQVIVLNH PGQIANGYTP VLDCHTAHIA CKFREIKEKC
                ********** ********** ********** ********** ********** ********** **********

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|
                       150        160        170        180        190
BmNative        DRRSGKKLED NPKFIKSGDA AIIDLVPSKP MCVETFTDFP PLGRFAVRDM RQTVA
EF-1aC5         DRRSGKKLED NPKFIKSGDA AIIDLVPSKP MCVETFTDFP PLGRFAVRDM RQTVA
                ********** ********** ********** ********** ********** *****
```

Figure 2.12: Alignment of translated DNA sequencing of clone number 5 (EF-1aC5) and native EF-1α (BmNative). *Transcripts (in FASTA format) were aligned using BioEdit sequence alignment editor. Identical residues are indicated by *.*

2.10.6. Transformation of *P. pastoris* GS115 and KM71H cells

Restriction enzyme digestion to linearize the recombinant construct and native pPICZ A was performed with *Pme*I and *Bst*XI. The result of this linearization is not shown, but was successful for both plasmids.

Following linearization of native pPICZ A and pPICZA-EF-1α, the recombinant constructs were transformed into GS115 and KM71H cells, Zeocin™ resistant colonies selected and streaked onto MDH (Minimal Dextrose with Histidine) plates. Included on extra plates, were the two control constructs, GS115/His$^+$ Mut^S Albumin and GS115/pPICZ/*lac*Z Mut$^+$ galactosidase. Subsequent replica-plating of the obtained cells onto fresh MDH and MMH (Minimal Methanol with Histidine) plates, allowed the determination of the growth phenotypes of the obtained GS115 clones. Most GS115 transformants exhibited similar growth rates on MDH and MMH plates compared to the Mut$^+$ control and thus confirmed as Mut$^+$ (wild type), whilst a single clone displayed similar growth as the Mut^S control.

Once transformed with pPICZA-EF-1α, the presence of the construct in GS115 and KM71H cells was assessed via PCR screening using the AOX5' and AOX3' primers directed at the *AOX* flanking regions of the integrated plasmid (Table 2.6). Positive clones exhibited a 600 bp band corresponding to the EF-1α transcript and MCS of pPICZ A (Figure 2.13). Only results from a screen of GS115 cells is included, as KM71H screens displayed identical profiles.

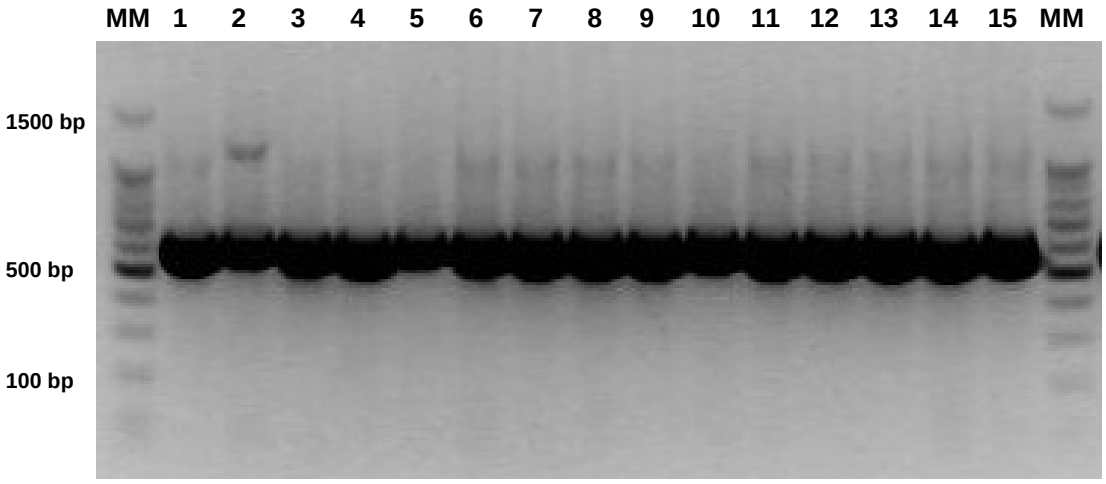


Figure 2.13: Colony PCR screen of clones from the transformation of *P. pastoris* GS115 with pPICZA-EF-1α clone 5. *PCR amplification of inserts directly from GS115 P. pastoris cells. Visualisation was aided by EtBr. Lane MM: 100 bp Molecular mass marker. Lanes 1 – 15: Individual clones subjected to colony PCR screening.*

Expression of EF-1α and western blot analysis

2.10.7. Preliminary expression of EF-1α in *P. pastoris* (performed at UP): SDS-PAGE and western blot analysis

Expression in both GS115 and KM71H was monitored for 24 hours post induction. Only GS115 clones exhibited bands that could represent rEF-1α (Figure 2.14). A distinct band was observed at 20 kDa for the GS115 clones (blocked red) that was not observed in either the KM71H clones (not shown), or the un-induced control (Figure 2.14). This band will be further evaluated using LC-MS-MS, prospectively.

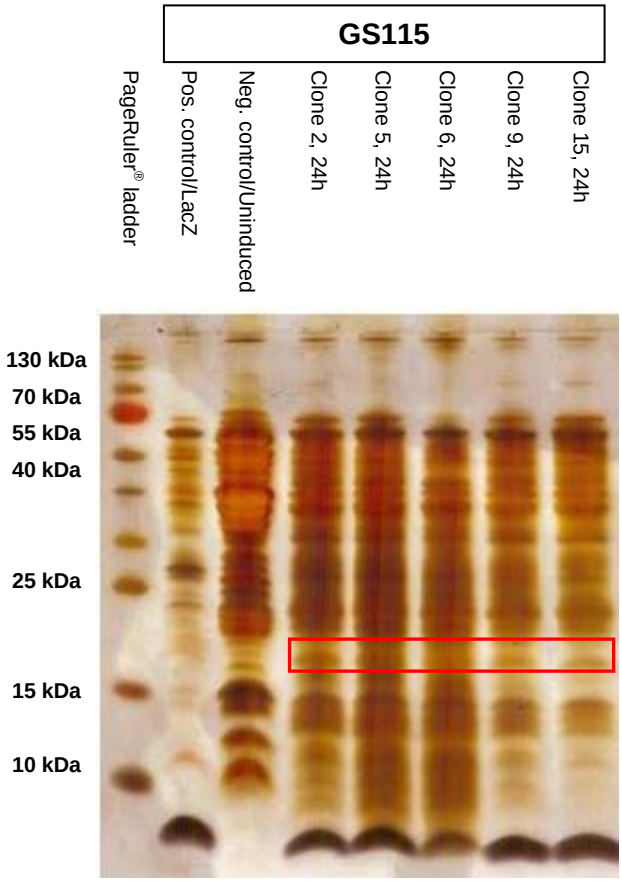


Figure 2.14: Small scale expression analysis of GS115 clones. *Tricine SDS-PAGE analysis of GS115 clones. Expression was continued for 24 hours after induction with 5% methanol. The gel was visualised by silver staining. Molecular weight (in kDa) is indicated.*

2.10.8. Small-scale expression of EF-1α in *P. pastoris* (performed in Spain): Dot blot analysis

Positive signals were obtained for all the insoluble samples, whilst very weak signals were observed for the soluble fractions (Figure 2.15) using the anti-His_6 Abs. The signal detected for the soluble fraction of KM71 clone 7 (K7) can be attributed to background expression of native proteins or residual cell debris when compared to the X-33 control for insoluble fractions. The apparent absence of soluble protein, as well as previous results obtained during prokaryotic expression, suggested that rEF-1α was contained within the insoluble fraction. GS115 clones (G3, G5 and G6) seemed to have overall higher amounts of rEF-1α. This correlates well with the results in Figure 2.14, suggesting that strain GS115 is more suitable for rEF-1α expression.

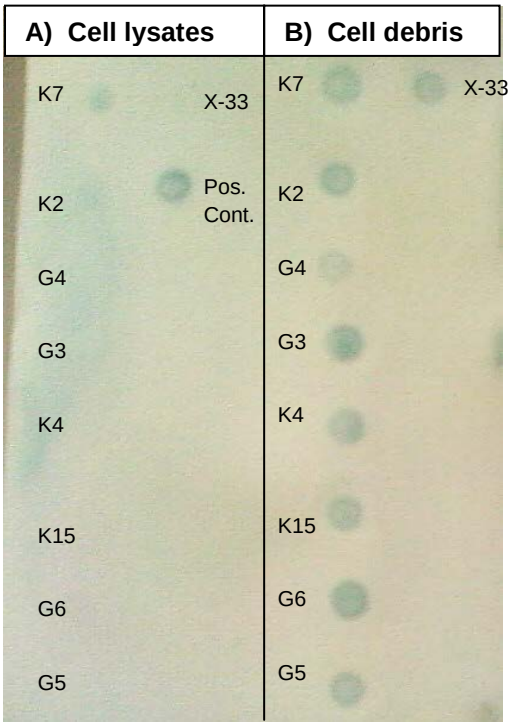


Figure 2.15: Dot-blot analysis of small-scale expression of GS115 and KM71H clones. *GS115 and KM71H cell lysates (soluble fraction) and cellular debris (insoluble fraction) were applied directly to the nitrocellulose membrane. Immunodetection was performed with anti-His antibody and visualisation with TMB substrate. K refers to the KM71H and G to the GS115 strains of P. pastoris. P. pastoris X-33 was included as a control for background expression. Qiagen His-tagged ladder (see Figure 2.6) was used as a positive control for colour development.*

The fact that crude, unpurified samples were used complicated protein concentration determination and as a result, the comparison is volume based. A quantitative comparison would have been optimal and therefore this served simply as a guide to identify the clones that expressed recombinant protein. The crude fractions might also have resulted in the background signal observed for the *P. pastoris* X-33 negative control. Since no signal was expected, it is suspected that the unknown protein content of this crude fraction, resulted in cross-reactive binding by antibodies.

Due to the initial success obtained during the small-scale expressions, it was decided to perform a time course study in an effort to optimize the amount of rEF-1α produced.

2.10.9. Time course study of expression of EF-1α in *P. pastoris* (performed at UP): Dot-blot, SDS-PAGE and western blot analysis

Expression was performed for 5 days (120 hours) with induction every 24 hours. The time frame was based on the advice of Dr. M. Canales who has extensive experience of protein expression in

P. pastoris. Dot-blot results suggested that rEF-1α is overall more prevalent in the insoluble fractions, and that the highest concentration of recombinant protein is present after 120 hours for both GS115 clone 1 and KM71H clone 2 (Figure 2.16). This result contradicts slightly with the lesser strength of the *AOX2* promoter (KM71H) compared to the AOX1 promoter (GS115), leading to the expectation of lesser recombinant protein for KM71H.

From Figure 2.15, the GS115 clones G3 and G6 were subjectively identified as expressing high concentrations of recombinant protein. It is not certain why these same clones were not identified during this round of expression, but it is suggested that the dissimilar reaction conditions may have played a role. Although GS115 clone 15 seemed to contain a high concentration of soluble protein, it was decided to focus on the insoluble fractions. Previous results suggested that rEF-1α is present in the insoluble fractions.

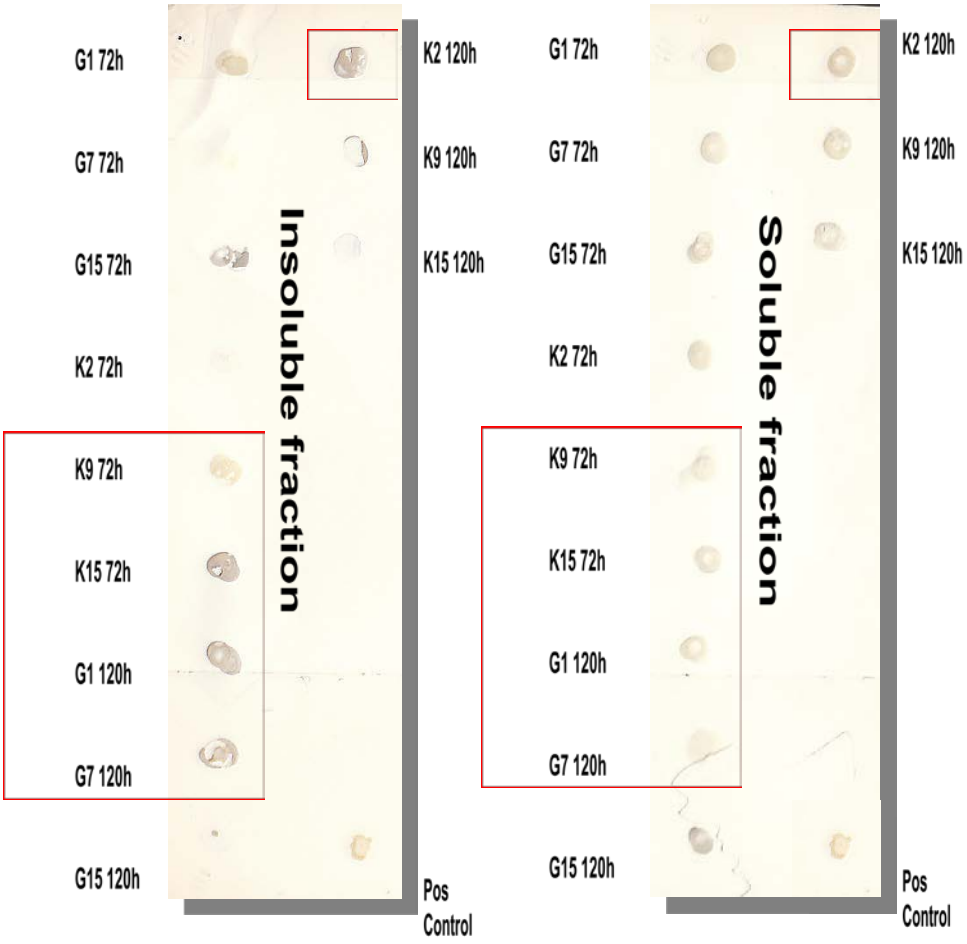


Figure 2.16: Dot-blot analysis of time-point expression of GS115 and KM71H clones. *A total volume of 5 µl (containing 2 µg of protein) was loaded per spot on PVDF membrane. Immunodetection was performed with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and visualisation with 4-chloro-1-naphtol substrate. The blocked regions indicate the intensity difference between insoluble and soluble fractions. K refers to the KM71H and G to the GS115 strains of P. pastoris.*

The samples blocked in red (Figure 2.16) were analyzed with Tricine SDS-PAGE and western blot in subsequent steps (Figure 2.17). These clones seemed to contain large amounts of recombinant protein in the insoluble fraction (Figure 2.16).

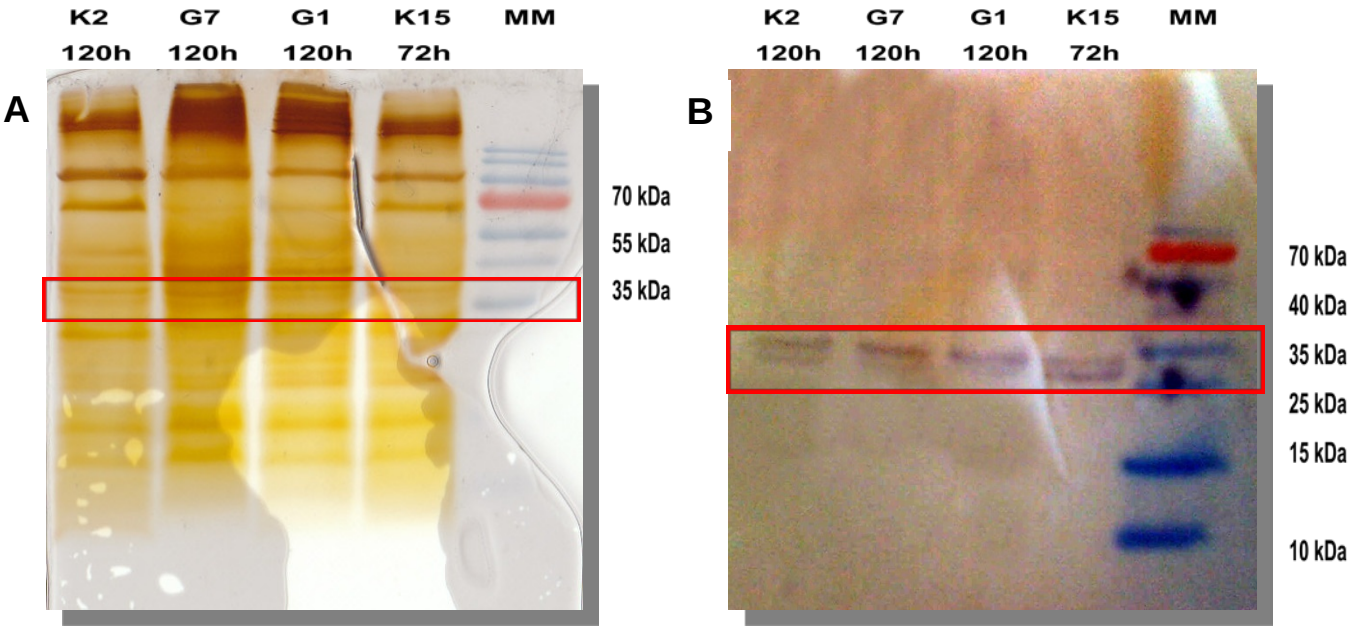


Figure 2.17: SDS-PAGE (A) and western blot (B) analysis of time-point expression of GS115 and KM71H clones. *Indicated are SDS-PAGE and western blot analysis of fractions collected over 5 days of methanol (0.5%) induced expression. Unpurified protein (10 µg) was loaded into each well. K refers to the KM71H and G to the GS115 strains of P. pastoris. The gel in (A) was silver stained and the blot in (B) developed colourimetrically using 4-chloro-naphtol. Red blocks indicate the size of rEF-1α. Molecular weight (in kDa) is indicated.*

Two very distinct bands of very similar sizes were detected on the western blot developed with Anti-His_6 Ab (Figure 2.17 B). These bands are not clearly visible on the silver stained SDS-PAGE gel, possibly due to background expression of native proteins and residual cellular debris, or the low concentration of rEF-1α not detectable by silver staining. A 24 kDa band was expected for rEF-1α which would include the recombinant protein (21 kDa) and a further 2.5 kDa contributed by the C-terminal histidine- and *c-myc* tags. Bands were observed at the different time points at 27-30 kDa, and seemed most intense for strain KM71H (clone K15) at 72 hours after induction. This result coincided with the dot-blot results in Figure 2.16. Slight protein degradation was observed throughout induction (2 bands, Figure 2.17 B) which was likely due to proteases in the cytoplasm of lyzed cells, or due to glycosylation, creating a modified form of rEF-1α which has a slightly higher molecular weight.

2.11. Conclusion

Prokaryotic expression of EF-1α from *E. coli* JM109 was unsuccessful. A 45 kDa band was identified from frozen cell pellets by western blot (Figure 2.6), but was much larger than the expected 22 kDa for rEF-1α and putatively attributed to the formation of aggregates or multimers of the recombinant protein. Alternatively, the detection of this high molecular weight band could be due to non-specific binding of the anti-His_6 antibody. After fresh expression using a stock clone, a 22 kDa band was identified on SDS-PAGE (Figure 2.7). Even though the size of this band was comparable to results previously obtained by M. Canales, the results of this and previous expressions, were generally not repeatable and the prokaryotic expression of EF-1α was then discontinued. If time allowed, the expression may have been optimized by alternately changing parameters such as time, temperature, inducer (IPTG) concentration or *E. coli* strain used.

Expression of EF-1α in *P. pastoris* was subsequently evaluated due to its eukaryotic translation profile. Expression of *R. microplus* EF-1α was obtained from both GS115 and KM71H strains of *P. pastoris*. Dot-blot of the time course samples indicated that the recombinant protein was present in highest percentage in the insoluble fraction, although some protein was also detected in the soluble fractions from individual clones (Figure 2.15). A 20 kDa band was identified from the soluble fraction during the preliminary expression of EF-1α (Figure 2.14). This corresponded well with the results in Figure 2.17, although soluble protein could not be detected during the small-scale experiments (Figure 2.15). Four representative clones were therefore chosen from Figure 2.16, the insoluble fractions analyzed by Tricine SDS-PAGE and western blot and two bands of similar size positively identified at 27 – 30 kDa (Figure 2.17). The higher molecular weight band might be the result of glycosylation of rEF-1α.

Future optimization may include a secreted expression approach, due to the fact that intracellular expression is limited by purification difficulties, as heterologous protein is usually 1% of total cytosolic protein (Daly and Hearn, 2005). With intracellular expression, the expressed protein is exposed to all the cytosolic proteases, whereas during secreted expression the protein is secreted into the growth medium, thus evading the protease onslaught. Isolation and purification is also greatly simplified with the heterologous protein in the growth medium and making up 30% of all secreted proteins. Optimization of either intracellular – or secreted expression may have included determining the optimal state of parameters such as induction time, methanol concentration, temperature and the pH of the growth medium. Methanol toxicity can be considered, as higher substrate methanol concentrations were found to have a greater negative impact on the production of heterologous protein than on the growth rate of cells (Kupcsulik and Sevella, 2004). Once small-scale expression has been optimized, bioreactor fermentation can be considered for the large-scale

production of rEF-1α. This system allows for the real-time control of parameters such as pH, aeration and concentration of carbon source.

Several difficulties were encountered during both the prokaryotic and eukaryotic expression of EF-1α. The identities of the bands obtained during prokaryotic expression could never be unequivocally proven, and even though the identity of the protein expressed in *P. pastoris* could be verified, the location of the recombinant protein in both hosts created hurdles. During expression in both *E. coli* JM109 and *P. pastoris* the protein of interest was found in insoluble fractions (i.e. associated with membrane fractions). Purification would have entailed a vigorous method of washing, harvesting, solubilisation and ultrafiltration under strongly denaturing conditions (6 – 8 M urea), followed by refolding to isolate the protein from these fractions. The effects of such complicated purification steps would have been particularly severe since the initial purpose of EF-1α expression was for it to be used as a recombinant vaccine. As a result of the abovementioned constraints, the expression of EF-1α was abandoned and the company GenScript® entrusted to produce the recombinant protein. It was later established that GenScript® had to create various constructs, before successfully expressing the protein for isolation and refolding from inclusion bodies.

Chapter 3

Determination of protein-protein interactions of *Rhipicephalus microplus* EF-1α by means of the GAL4 two-hybrid system.

Introduction

An important property of intricate biological systems is the existence of protein interaction networks. In order to understand the biological function of a protein, it is valuable to know the protein-protein interactions it is involved in. Proteins function as stable or ephemeral complexes with other proteins and it has been estimated that 80% of proteins function in this manner (Berggård *et al.*, 2007; Lalonde *et al.*, 2008). These interactions are crucial for various cellular functions including architecture, metabolism, signalling, deoxyribonucleic acid (DNA) replication, transcription, translation, cell cycle control, intermediary metabolism and the liberation of cellular energy (Mukherjee *et al.*, 2001). Proteins further function to mediate the specificity of interactions between enzymes and substrates, shield proteins from their physiological environments, facilitate substrate channelling or in the assembly of molecular machines (Lalonde *et al.*, 2008).

The detection of protein interactions is often complicated by the fact that proteins are chemically distinct entities with variant charges, numerous secondary and tertiary folds and many different post-translational modifications (Howell *et al.*, 2006). Classical biochemical methods for the study of protein-protein interactions include copurification, immunoprecipitation and affinity purification. These techniques are limited by low sensitivity and bias toward interactions with high affinity (Mukherjee *et al.*, 2001; Brückner *et al.*, 2009). Contemporary methods include mass spectrometry (MS), *in situ* hybridisation, immunohistochemistry, immunocytochemistry, protein chip arrays, fluorescent detection in live cells, fluorescence resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET) and confocal microscopy for intracellular localisation of interactions (Howell *et al.*, 2006; Berggård *et al.*, 2007). These days MS is coupled to affinity purification (AP), and has since been improved by the development of an incorporated workflow that couples rapid generation of bait expressing cell lines with increases in protein complex purification using a double affinity strategy (Brückner *et al.*, 2009).

Tandem affinity purification (TAP-MS) is the most successful dual method. The TAP tag consists of a calmodulin binding peptide (CBP) and Protein A tag (AC-TAP) connected by a short linker

containing a cleavage site for tabacco etch virus (TEV). The TAP system allows expression in homologous cells, assembly under physiological conditions, purification of complexes from all subcellular compartments and identification of a single tagged protein of interest (Gavin *et al.*, 2011). One major disadvantage of these classical and contemporary applications, is that they require the availability of a protein binding partner (bait) as these techniques are performed on a protein level. These techniques additionally do not reflect the *in vivo* interactions that are usually much more complex.

Multi-protein complexes are increasingly recognized as the molecular basis of cellular fluxes of molecules, signals and energy. Technologies which enable us to interpret cellular interactions between biomolecules (interactomics) together with those measuring metabolite movements (metabolomics, fluxomics) and signalling cascades (phosphoproteomics) are of utmost importance for systems biology (Brückner *et al.*, 2009). For genome-scale studies, more advanced techniques are required that can run in parallel and are automated. Yeast two-hybrid and affinity purification coupled to MS (AP/MS) can be applied in this regard (Brückner *et al.*, 2009). Yeast two-hybrid interactions are deemed reliable based on the reproducibility of interactions, the promiscuous nature of the interactors, topology of the network, comparisons with peripheral data and evolutionary conservation of the interacting partner (Koegl and Uetz, 2008). Since no data is available on the protein interactions of *R. microplus* EF-1α, we aimed to use the yeast two-hybrid system to enhance our understanding of EF-1α function in ticks. Since the principles of the Matchmaker™ GAL4 Two-Hybrid Systems 2 and 3 were used for this study, it forms the basis of subsequent discussions.

3.1. The yeast two-hybrid system

S. Fields and O. Song were the first to describe a novel genetic system for the investigation of protein-protein interactions. This system takes advantage of the GAL4 protein of the yeast, *Saccharomyces cerevisiae* (Fields and Song, 1989). The authors investigated SNF1, SNF4 and the GAL4 protein which is active as a transcriptional activator necessary for the expression of the genes required for galactose metabolism. GAL4 consists of two divisible domains that are both functionally important. Firstly, the DNA-binding domain (DNA-BD) which binds to specific DNA sequences such as a 17-mer consensus upstream activating sequence (UAS_G). Secondly, the C-terminal activation domain (AD) which is required for transcription activation via directing of the RNA polymerase II complex to the transcription of the reporter gene downstream of the UAS. Using this property of transcription factors, Fields and Song suggested that protein interaction could be detected if potential interactors were expressed as chimeras with the BD and AD domains, respectively (Mukherjee *et al.*, 2001). A bait gene is expressed as a fusion to the GAL4 DNA binding domain (DNA-BD), and another gene or cDNA library (prey) as a fusion to the GAL4 activation

domain (AD). The binding domain (BD) and activation domain (AD) of yeast two-hybrid transcription factors are thus physically separable, but the function of the assembled module is maintained. The AD can function by simply binding a promoter bound BD-protein. By generating the two-hybrid proteins GAL4(1-147)-SNF1 and GAL4(768-881)-SNF4, transcription of reporter genes was achieved (Fields and Song, 1989). At present time, it is known that many eukaryotic trans-acting transcription factors are made up of physically separable and functionally dependant domains. Thus in principle, it should be possible to pair any DNA-BD with any AD for the mediation of downstream reactions (Fields and Song, 1989; Mukherjee *et al.*, 2001).

Upon interaction of bait and prey proteins, the DNA-BD and AD are brought into closer proximity, targeted to the yeast nucleus, a novel transcriptional activator with binding affinity for GAL4 responsive UAS assembled and the activation of four reporter genes initiated (Figure 3.1). The activation of these reporter genes makes an interaction phenotypically detectable (Clontech, 1998). The sensitivity of Matchmaker™ GAL4 Two-Hybrid Systems is attributable to the *in vivo* amplification of positive signals. These include transcriptional, translational and enzymatic signals (Clontech, 2007a). Yeast two-hybrid systems are sufficiently sensitive to detect weak and transient interactions that may be critical for the functioning of cellular systems.

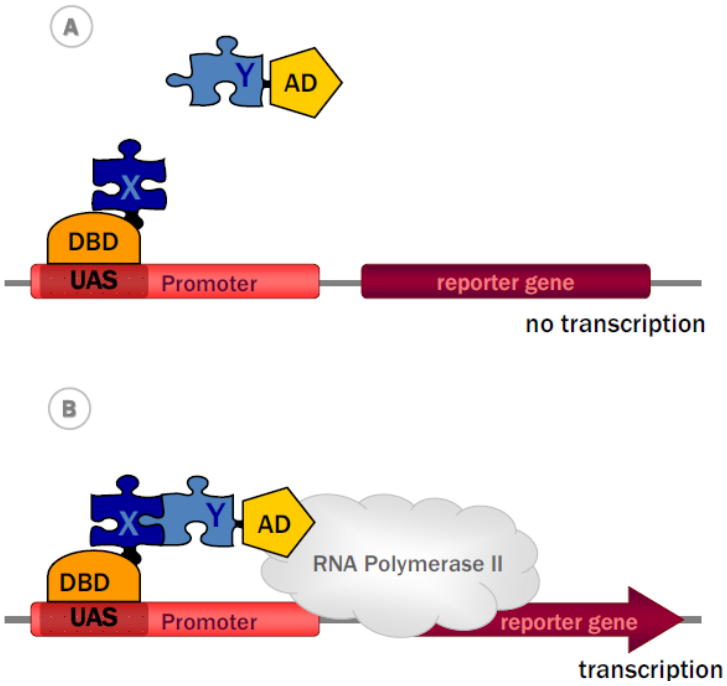


Figure 3.1: The classical yeast two-hybrid principal (Clontech, 2007b; Brückner *et al.*, 2009). *Protein X (of interest; bait) is fused to the DNA-BD, and the potential interaction protein Y (prey) to the activation domain. DNA-BD-X binds the UAS of the promoter. The interaction of bait (X) with prey (Y) recruits AD, resulting in the reconstitution of a functional transcription factor. The recruitment of RNA polymerase II follows, which leads to the transcription of a reporter gene.*

3.2. Yeast strains, promoters, phenotypes and reporter genes

3.2.1. Strains

Common two-hybrid yeast strain genotypes and associated promoter systems are summarized in Table 3.1. Strains relevant to this study, are further discussed in this section and characterized in Table 3.2. The Matchmaker™ GAL4 two-hybrid systems 2 and 3 utilize amongst others, the AH109 strain of the yeast *S. cerevisiae,* a derivative of the PJ69-2A strain (James *et al.*, 1996). In system 3, the Y2HGOLD strain eliminates false positives by the usage of three reporters *ADE2, HIS3* and *MEL1* (lacZ) that are under the control of the GAL4 upstream activating sequences and TATA boxes, all of which specifically responds to GAL4. Two types of false positives are subsequently eliminated; those interacting with sites flanking GAL4 and those that interact with transcription factors bound to TATA boxes.

Table 3.1: Common yeast two-hybrid yeast strains, their promoter systems and reporter genes. *Adapted from (James, 2001) and (Clontech, 2010)*.

Yeast strains	Promoter system	Reporter gene(s)
PJ69-4A	Gal 4	*ADE2, HIS3, lacZ, MEL1*
PJ69-4α	Gal 4	*ADE2, HIS3, lacZ, MEL1*
SFY526	Gal 4	*lacZ*
YPB2	Gal 4	*HIS3, lacZ*
HF7c	Gal 4	*HIS3, lacZ*
Y153	Gal 4	*HIS3, lacZ, MEL1*
Y187	Gal 4	*lacZ, MEL1*
Y19D	Gal 4	*HIS3, lacZ, MEL1*
YD116	Gal 4	*URA3, lacZ*
YD119	Gal 4	*URA3, lacZ*
PCY2	Gal 4	*lacZ*
MaV52	Gal 4	*HIS3, lacZ, MEL1*
MaV95, MaV96, MaV97, MaV99	Gal 4	*HIS3,URA3, lacZ, MEL1*
CL9	Gal 4	*CYH2, lacZ*
CBY12a, CBY12α	Gal 4	*LEU2, lacZ*
CBY14a, CBY14α	Gal 4	*HIS3, lacZ*
AH109	Gal4	*ADE2, HIS3, lacZ, MEL1*
Y2HGold	Gal4	*AbA^r, ADE2, HIS3, MEL1*
CTY10-5d	LexA	*lacZ, MEL1*
L40	LexA	*HIS3, lacZ*
EGY48, EGY191, EGY195	LexA	*LEU2*
EGY40	LexA	*Negative control: None*
SKY48, SKY191, SKY473	LexA, cI	*LEU2, LYS2*
PL1, PL3	ER	*URA3*

The yeast two-hybrid Y2HGold yeast strain is based on the AH109 strain, except that the *lacZ* reporter has been replaced by an *AUR1-C* reporter. Its expression (*AbA*^{r}) in *S. cerevisiae* confers resistance to Aureobasidin A (Clontech, 2010). Due to the fact that no methodological changes were made after resuming the two-hybrid screen in this strain, the remainder of this document refers to the Y2HGold strain.

Table 3.2: Matchmaker™ yeast strain genotypes.

Yeast strain	Genotype	Use/additional information	Reference
AH109	*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200,*; *gal4Δ, gal80Δ, LYS2 : : GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-HIS3, GAL2*$_{UAS}$*-GAL2*$_{TATA}$*-ADE2, URA3 : : MEL1*$_{UAS}$*-MEL1* $_{TATA}$*-lacZ*	Screen AD/library with *HIS3, ADE2, and MEL1*.	(Clontech, 2007a)
Y187	*MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met–, gal80Δ, URA3 : : GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-lacZ*	Testing for interactions between two host proteins using *lacZ* reporter (β-gal assays). As mating partner (CG-1945, Y190) for interaction confirmation.	(Clontech, 2007a)
CG-1945	*MATa, ura3-52, his3-200, ade2-101, lys2-801,trp1-901, leu2-3, 112, gal4-542, gal80-538, cyhr2, LYS2 : : GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-HIS3, URA3 : : GAL4* $_{17\text{-}mers(x3)}$*-CYC1*$_{TATA}$*-lacZ*	Separation of DNA-BD and AD plasmids via cycloheximide counter selection. Selection with *HIS3* reporter.	(Clontech, 2007a)
Y2HGold	*MATa, trp1-901, leu2-3, 112,ura3-52, his3-200, gal4Δ, gal80Δ, LYS2 : : GAL1*$_{UAS}$*–Gal1*$_{TATA}$*–His3, GAL2*$_{UAS}$*–Gal2*$_{TATA}$*–Ade2 URA3 : : MEL1*$_{UAS,}$ *Mel1*$_{TATA}$ *AUR1-C MEL1*	Screen AD/library with *HIS3, ADE2, and AUR1C* (Aureobasidin A resistance).	(Clontech, 2010)

3.2.2. Promoters

All yeast structural genes are preceded by a loosely conserved sequence that determines the transcription start site. Many other genes are associated with *cis*-acting elements (DNA sequences to which transcription factors and other trans-acting regulatory proteins bind) that affect transcription levels. "Promoter" refers to the TATA box and its associated *cis*-regulatory elements. The TATA region minimal promoter of yeast is situated 25 base pairs upstream of the transcription start site. An example of a *cis*-acting transcription element in yeast is the UASs that are recognized by specific transcriptional activators and that increase transcription from adjacent downstream TATA regions. This enhancing function of UASs is sensitive to orientation effects if moved more than a few hundred bps from the TATA region. Reporter genes are usually under the control of artificial promoter constructs comprised of a TATA and UAS sequence derived from another gene. In GAL4-

based systems, either a native GAL UAS or a synthetic UAS_G 17-mer consensus sequence provides the binding site for GAL4 DNA-BD (Clontech, 1998).

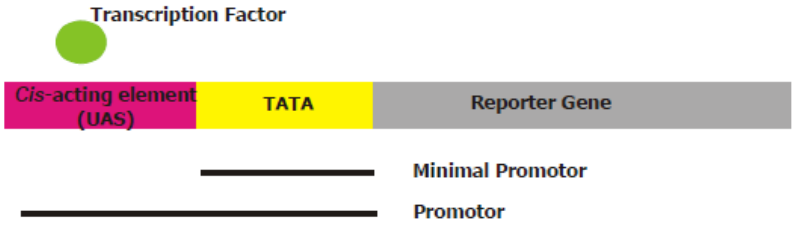


Figure 3.2: Schematic representation of a yeast promoter (Maritz-Olivier, 2005).

3.2.3. Phenotypes

Table 3.2 contains a list of the four yeast strains available in the Matchmaker™ 2, 3 and Y2HGold yeast two-hybrid systems. All of these strains use the GAL4 promoter system. Strain Y2HGold was used during this study, due to the availability of an extra reporter gene (*ADE2),* allowing for the selection on not only TDO (Triple dropout) i.e. /-Trp/-Leu/-His, but also QDO (Quadruple dropout) i.e. /-Trp/-Leu/-His/-Ade media. The *AUR1-C* reporter provides antibiotic selection using Aureobasidin A.

3.2.4. Reporter genes

An appropriate reporter gene must encode a protein whose function provides a simple readout, rendering it easily distinguishable. Familiar reporter genes provide a colourimetric or fluorescent readout, or allow growth on nutritionally selective media (Koegl and Uetz, 2008). Examples are the *LEU2, HIS3, ADE2, URA3* and *LYS2* reporter genes of *S. cerevisiae* (Figure 3.3). The selection of two or more reporter genes increases the stringency of the assay by requiring strong transcriptional activation, but simultaneously limits the detection of weak or transient interactions (Brückner *et al.*, 2009). The *HIS3* reporter is often leaky, and subsequently requires the addition of 3-aminotriazole (3-AT), a histidine analogue inhibitor of the *HIS3* gene product. This is needed to lessen background colony growth during selection (McAlister-Henn *et al.*, 1999).

The Y2HGold strain approaches elimination of false positives by the usage of three reporters: *ADE2, HIS3, MEL1* and *AUR1-C* that are under the control of the GAL4 UASs and TATA boxes and that specifically responds to GAL4 (Figure 3.4). With the *ADE2* reporter providing strong nutritional selection, the *HIS3* reporter provides the user with additional control over selection stringency (James *et al.*, 1996). *MEL1* or *lacZ* can be used as additional nutritional selectors, encoding α-and β-galactosidase, respectively. The *MEL1* gene is endogenous and because the enzyme is

secreted, the assay can be performed directly on plates containing X-α-GAL (Clontech, 2007a). The yeast two-hybrid Y2HGold yeast strain is almost identical to AH109, except that the *lacZ* reporter has been replaced by an *AUR1-C* reporter. Its expression (*AbA*^{r}) in *S. cerevisiae* confers resistance to Aureobasidin A (Clontech, 2010). This reporter results in lower background activity in comparison to the *HIS3* reporter alone, consequently resulting in fewer false positives.

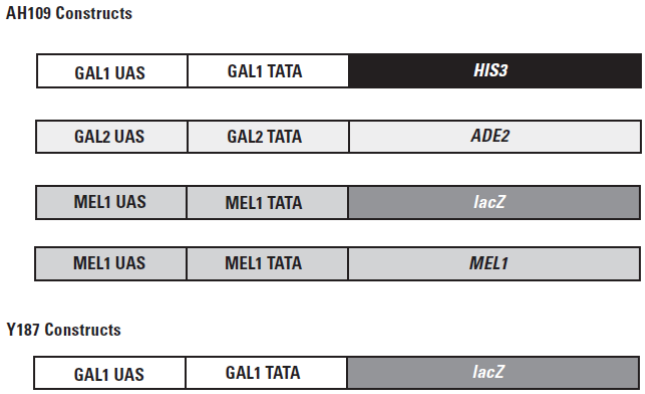


Figure 3.3: Reporter constructs of strains AH109 and Y187 of *S. cerevisiae* (Clontech, 2007b). *HIS3, ADE2, and MEL1/lacZ genes are under the control of three heterologous GAL4 responsive UASs, as well as promoters GAL1, GAL2 and MEL1.*

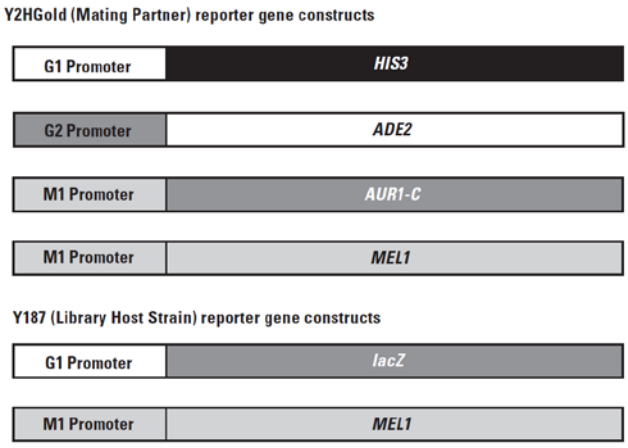


Figure 3.4: Reporter constructs of the Y2HGold strain of *S. cerevisiae* (Clontech, 2010). *In the Y2HGold strain HIS3, ADE2, and MEL1/AUR1-C reporter genes are under the control of three completely heterologous Gal4-responsive promoter elements—G1, G2, and M1, respectively. Protein-binding sites within the promoters are different, although related to the 17-mer consensus sequence recognized by Gal4.*

3.3. DNA-BD and AD vectors

In this study the bait protein, *R. microplus* EF-1α, was cloned into the pAS2-1 vector from Matchmaker™ GAL4 Two-Hybrid System 2 (Figure 3.5). This vector contains both the bacterial

(Col E1) and yeast (2µ) origins of replication, as well as a phenotypic marker (*TRP1*) used for selection in the chosen yeast strain, Y2HGold. This vector additionally contains a cycloheximide resistance marker (*CYH2*) useful for the elimination of false positives that have lost the bait plasmid and an ampicillin resistance gene (Amp^r) for selection in bacteria.

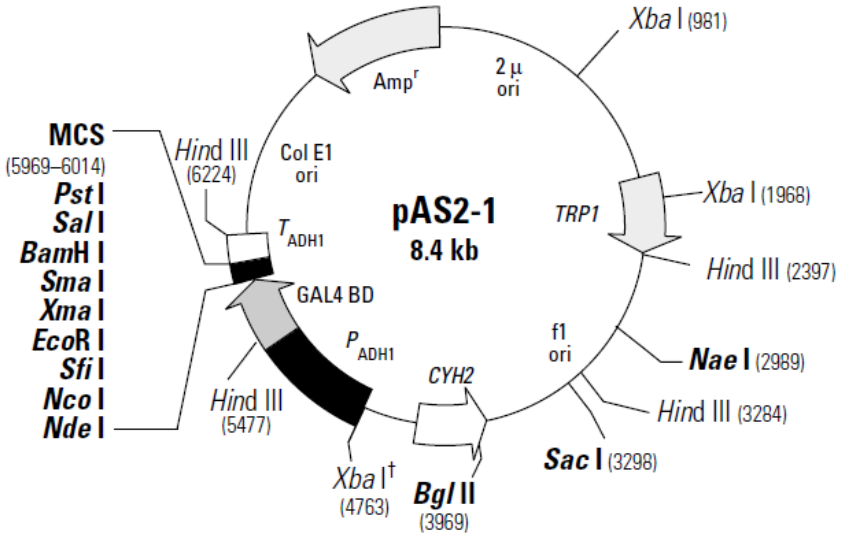


Figure 3.5: Vector map of pAS2-1 (Clontech, 1997a). *Unique restriction enzyme cut sites are in bold. pAS2-1 is a cloning vector used to generate fusions of a bait protein with the GAL4 DNA-BD (amino acids 1–147). pAS2-1 is derived from pAS2 and from pAS1CHY2 and carries the CYH2 gene for cycloheximide sensitivity. The hybrid protein is expressed at high levels in yeast host cells from the full-length ADH1 promoter (P_{ADH1}). The hybrid protein is targeted to the yeast nucleus by nuclear localization sequences. The Xba I site at bp 4763 is methylation sensitive (†).T_{ADH1} refers to the ADH1 transcription termination signal.*

The pGADT7 vector from Matchmaker™ GAL4 Two-Hybrid System 3 was specifically designed for high-level protein expression of prey proteins and the facilitation of protein interaction confirmation (Figure 3.6). Prey inserts are expressed as fusions to GAL4, and contain c-Myc and hemagglutinin (HA) tags. These tags provide for easy antibody-mediated identification of fusion proteins. pGADT7 additionally contains T7 promoter sequences downstream of the GAL4 coding sequences that allow fusion proteins to be transcribed and translated *in vitro,* e.g. during co-immunoprecipitation. T7 also serves as a priming site for DNA sequencing. The vector expresses an antibiotic selection marker (ampicillin; Amp^r) and a pUC origin of replication (Clontech, 2007a). The *LEU2* marker of pGADT7 can be used for selection in Leu$^-$ auxotrophic yeast strains, and in *E. coli* strains containing a *leu6* mutation. This distinguishes between the bait and prey plasmids during plasmid (prey) rescue (Clontech, 1998).

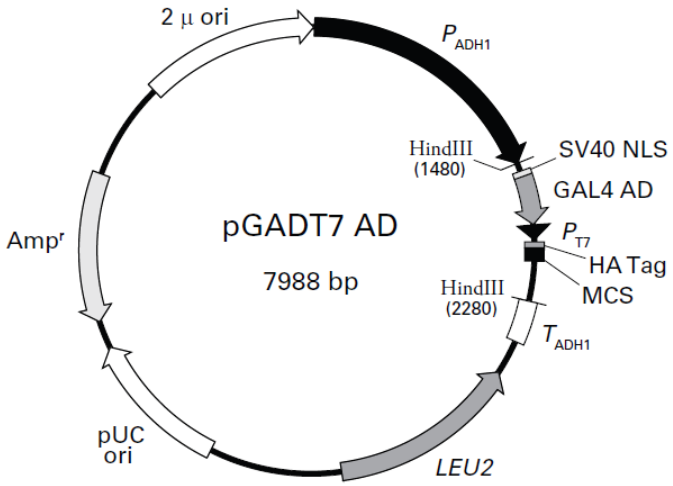


Figure 3.6: Vector map of pGADT7-AD (Clontech, 2007c). *pGADT7 is used to express a protein of interest fused to the GAL4 domain. Transcription of the fusion is driven by the ADH1 promoter (P_{ADH1}) and terminated at the ADH1 transcription termination signal (T_{ADH1}). The GAL4-AD fusion additionally contains an N-terminal SV40 nuclear localisation signal (SV40 NLS) responsible for targeting the protein to the yeast nucleus.*

3.3.1. Promoters, terminators and copy number

Most two-hybrid vectors utilize the ADH1 promoter to drive the expression of fusion proteins, whilst transcription termination occurs at the ADH1 transcription termination signal. This promoter is available in full-length and truncated (ADH1*) forms that yield lower expression levels. The pAS2-1 plasmid however contains the full-length ADH1 resulting in high levels of expressed DNA-BD-bait fusion-protein (Clontech, 1997a). The detection of weak interactions is thus enabled, but toxicity and background expression of reporter genes simultaneously increased (MacDonald, 2001).

Most two-hybrid vectors make use of the 2μ origin of replication which maintains plasmids at high copy number (15 – 30 plasmids per cell). This is advantageous as bait protein expression is increased, but disadvantageous as copy number for bait and prey may vary depending on the amount of preys expressed. This clarifies why cells need extended amounts time to amplify plasmids before more stringent reporters (such as *ADE2*) can be activated (MacDonald, 2001).

3.3.2. Fusion domains, dimerization and protein folding

Even though most fusion domains function in a similar manner, there are several reasons for switching to alternative fusion domains (AD or BD). These reasons include poor stability, the incorrect folding of the fusion protein or the obstruction of binding sites. These problems are difficult to identify and therefore choosing a domain is often matter of trial and error. Two fusion domains are most often used namely the GAL4 and LexA proteins that both bind DNA as dimers. GAL4

contains a SV40 nuclear localisation sequence (NLS), whereas the LexA fusion is able to enter the nucleus via an unknown mechanism. LexA normally functions as a repressor of the SOS genes (derived from international distress signal) in *E. coli* by binding to LexA operator sequences, but in the LexA yeast two-hybrid system it acts like a DNA-BD. In these systems the reporter gene to be induced must have a LexA operator sequence for the binding of LexA (Fairbanks and Andersen, 1999; Mukherjee *et al.*, 2001).

An advantage of the LexA system is the galactose inducible promoter present in the activation domain plasmid, which enables the controlled expression of chimeric proteins that would normally be toxic to the yeast cells. This is deemed the "interaction trap" mechanism (McAlister-Henn *et al.*, 1999). GAL4 and LexA have been shown to exhibit increased efficiency upon the presence of multiple binding sites in the reporter gene (MacDonald, 2001). Most two-hybrid vectors result in the fusion of the bait protein to the carboxyl terminus of the transcription factor domain (DNA-BD). This can be detrimental if the N-terminal domain is required by the bait protein for interaction. The user can then switch to an amino-terminal fusion approach such as LexA, or opt for utilising smaller fractions of the bait protein if the domain structure thereof is known (MacDonald, 2001).

3.3.3. Constructing DNA activation domain prey constructs

The impact of the two-hybrid system is largely based on its ability to screen AD-fusion libraries for novel genes that interact with a protein of interest (bait). AD-fusion libraries have various important properties, of which the first is that each clone encodes a fusion of AD to the peptide/protein encoded by the insert. AD-libraries are usually composed of cDNA as only protein coding sequences are relevant, and fusions must be expressed in yeast cells for the assay to be functional. Lastly, AD-fusion libraries must be constructed in plasmid vectors allowing co-transformation and easy selection.

Insert length, priming and cloning

AD fusion libraries (unlike conventional cDNA libraries) do not require full-length cDNA. For studies of yeast or prokaryotic protein interactions, fragmented genomic DNA can also be used (Koegl and Uetz, 2008). If the aim is to investigate multiple interactions, or if the interactions require complex protein folding, longer inserts are required.

Priming methods greatly influence the quality of cDNA libraries. The first priming method uses an oligo-(dT)25(A/G/C) primer, resulting in the enrichment of clones with carboxy-terminal domains (Clontech, 1997b). This is because oligo(dT) priming is highly biased toward the 3'-end of expressed transcripts. The second uses random priming (hexamers or nonamers) that will ensure

that the library contains 5'-enriched clones that encode N-terminal domains and even full-length proteins. In both approaches, the size range for cloning must be analysed to control insert length using size fractionation.

Directional cloning can greatly increase the complexity of a library (usually 2-fold) and can be achieved by two methods. The first involves the ligation of an adapter to the double stranded (ds) cDNA followed by restriction digestion, phosphorylation and ligation into a dephosphorylated vector (Clontech, 1997b). In the second method, primers containing directional cloning sites are used during SMART™ cDNA synthesis with directional cloning achieved after restriction enzyme digestion and ligation (Clontech, 2001).

After library preparation, the bait and prey plasmids are either sequentially or co-transformed into a single yeast strain or separately transformed into haploid yeast strains of different mating types. The haploid yeast cells are mated to give rise to diploid cells, in which interactions might occur. Both approaches can be used to screen single or multiple baits with a cDNA library (Mukherjee *et al.*, 2001).

Library complexity

The complexity of the library can be explained as the number of independent clones present in the original, unamplified library prior to library amplification. It is the single most important factor that determines whether a screen of an AD-fusion library will be successful. The more independent clones present, the higher the complexity of the library and the greater the chances of finding interactions. A library should ideally consist of at least 1 x 10^6 independent clones and the titer must be >10^8 (Bartel and Fields, 1997).

The sequence representation or quality of the library can be determined by the use of probes against household genes. This is only viable if sufficient information about the organism studied is available. An example is the β-actin probe used for mammalian libraries, which cross-reacts with all mammalian β-actin cDNA. These libraries must exhibit a minimum β-actin frequency of 0.10% and 0.05% in other mammalian libraries. Non-mammalian libraries can also be analysed with a constitutively expressed species-specific probe. Another quality control measure is restriction digestion of library cDNA. Southern blot analysis or polymerase chain reaction (PCR) can be used to determine if the library contains the cDNA encoding the bait protein, with actin acting as a control transcript (McAlister-Henn *et al.*, 1999).

3.4. Limitations of the yeast two-hybrid system

Yeast two-hybrid systems have been used successfully to identify many types of protein interactions from yeasts, prokaryotes, plants, model organisms such as *Drosophila* and mammals. Despite these successes, there are still various limitations to the system (Clontech, 2007a).

All yeast two-hybrid assays have the disadvantages of false positives and negatives. False negatives are the result of interactions not being detected due to limitations of the screening method. An important cause of false negatives is that the DNA-BD or AD site may impede the normal interaction site via steric hindrance, or prevent the correct folding of the protein (Clontech, 2007a; Brückner *et al.*, 2009). This issue can be overcome by cloning both prey and library transcripts into both DNA-BD and AD vectors, and choosing the right combination capable of identifying interactions (McAlister-Henn *et al.*, 1999). Conditions inside yeast cells might not be optimal for correct protein folding as well as the application of post-translational modifications (PTMs) that are required for the interaction of some eukaryotic proteins. The latter problem can be corrected by co-expressing the necessary modifying enzyme in the yeast. Some proteins will fail to interact in the environment of the yeast nucleus, e.g. proteins of secretory systems that need oxidative conditions or glycosylation for proper folding (Koegl and Uetz, 2008). Some interactions might not be detectable in a GAL4 system, but may be detectable using a LexA based two-hybrid system. The success/failure thereof cannot be predicted prior to testing either system (Clontech, 2007a). Additionally, the interactions of membrane proteins are often undetectable in conventional yeast two-hybrid approaches.

False positives are interactions detected by a two-hybrid system that are not detectable with any other independent system. These can be separated into two types, namely technical false positives, where an interaction is generated by reactions other than protein-protein interactions, or biological false positives where proteins do interact, but only when they are co-expressed in non-native environments (Koegl and Uetz, 2008). False positives can be generated by inherent overexpression and is dependent on the system used. Bait proteins can have inherent DNA binding or transcriptional activating properties, whilst prey proteins might be able to interact with reporter proteins or membrane anchors. Deletion of these regions can be used to abolish these reactions prior to a yeast two-hybrid screen (Clontech, 2007a). Fusion proteins may also not be stably expressed in yeast or transferred to the yeast nucleus, therefore other systems such as Phage display and novel membrane-localised two hybrid systems (Table 3.3), may be used to identify interactions that take place on the cell surface (Clontech, 2007a). Finally, the expressed protein might be incorrectly folded or “sticky” resulting in false interactions. A list of recurring false positives can be found at http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html (Brückner *et al.*, 2009).

The occurrence of false positives can be decreased by carefully considering the molecular aspects of the method, such as the reporter gene constructs and expression vectors used for hybrid protein design. Von Mehring *et al.* (2002) suggested that the accuracy of a classic yeast two-hybrid screen is less than 10%. Therefore, the quality of a data set (list of interactions) should be verified by testing the interactions with other methods (see section 3.1) in order to confidently publish data. It is advisable to use more than one such method for validation (Brückner *et al.*, 2009).

3.5. Advantages and novel applications of the yeast two-hybrid system

The fact that only a bait protein and a prey cDNA library is needed to initialize a yeast two-hybrid assay, is a great advantage above classical methods which require the availability of purified proteins and/or antibodies (Mukherjee *et al.*, 2001). The sensitivity of the yeast two-hybrid can identify specific residues involved in an interaction and evaluate different protein variants for their relative strengths of interaction. A GAL4 based two-hybrid assay performed by Yang *et al*., indicated that protein interaction with dissociation constants (K_d) of 70 µM can be detected, although lower K_ds of 1 µM have been observed (Young, 1998; Puertollano *et al.*, 2001). The LexA system has been shown to detect binary interactions with K_d values of 1 nM to 1 µM (McAlister-Henn *et al.*, 1999). Another advantage of the yeast two-hybrid system includes that the assay is performed in live cells, better mimicking a cellular environment.

The yeast two-hybrid assay can easily be automated for high-throughput studies of protein interactions on a genome-wide scale. This has been done successfully for bacteriophage T7, *S. cerevisiae*, *Drosophila melanogaster, Ceanorhabditis elegans* and *Homo sapiens* (Brückner *et al.*, 2009). Several interaction networks for human proteins have been created for specific areas of study such as signal transduction, biochemical pathways, protein families, subcellular structures or virus-host interactions (Koegl and Uetz, 2008). Yeast two-hybrid is much more accessible than methods like MS, and a large amount of data is generated in a single screen. In excess of 5600 interactions have been reported for yeast, involving 69% of yeast proteins and roughly 6000 interactions for humans (Parrish *et al.*, 2006; Brückner *et al.*, 2009).

The yeast two-hybrid system has been utilised extensively to investigate the protein-protein interactions in a variety of organisms and molecular systems in recent years. Figure 3.7 below indicates the number of interactions in specific organisms per year, since the development of the yeast two-hybrid by Fields and Song in 1989. Recently, high throughput yeast two-hybrid technologies have become commonplace, increasing the understanding of protein networks, and is expected to result in fast progression in interactome mapping in the fields of botany, virology, metabolism and disease (Mohr and Koegl, 2012; Stynen *et al.*, 2012).

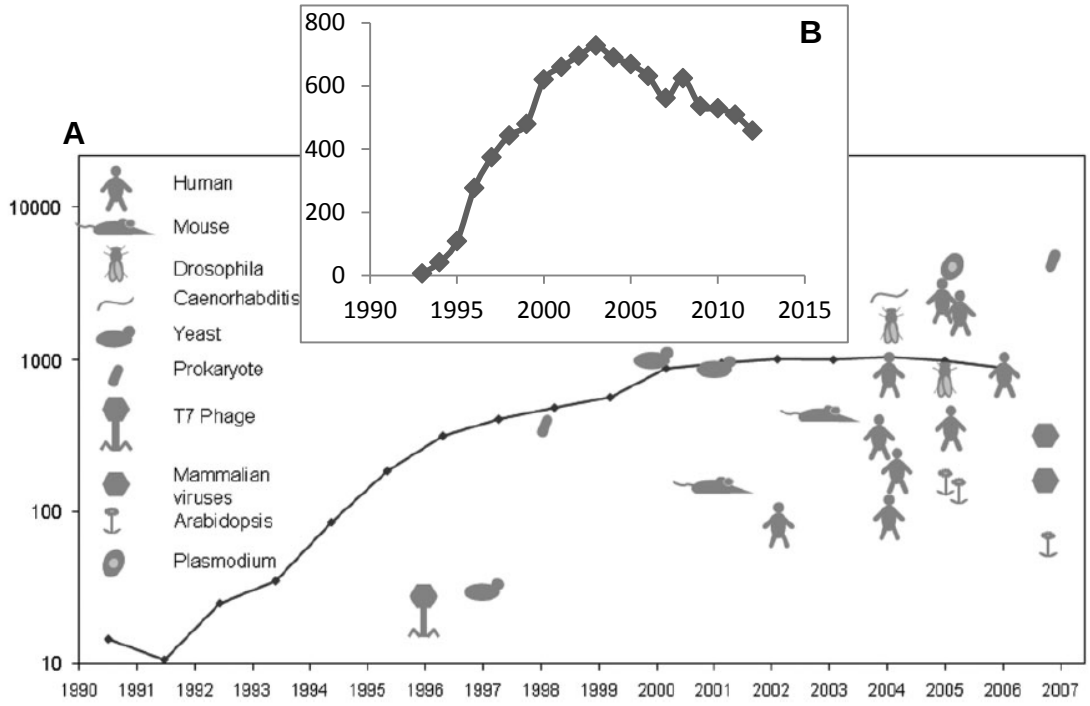


Figure 3.7: A chronological representation of yeast two-hybrid data published up until 2012 (Koegl and Uetz, 2008), Scopus search results). ***A)*** *The solid line represents the number of papers found in Pubmed, in any field, per year. The symbol represents large systematic interaction studies using yeast two-hybrid, and their position on the Y-axis represents the number of interactions reported for that investigation.* ***B)*** *Since 2008, the number of published yeast two-hybrid articles has decreased slightly.*

3.6. Characteristics of classical and novel yeast two-hybrid systems

In their original publication, Fields and Song proposed the following prerequisites for a successful screen: “The system requires that the interaction can occur within the yeast nucleus, that the Gal4-activating region is accessible to the transcription machinery and that the Gal4 (1-147)-protein-X hybrid is itself not a potent activator”. This limitation excludes a lot of proteins from being assayed and explains the need for developing novel two-hybrid systems (Fields and Song, 1989; Brückner *et al.*, 2009). As an example, polymerase II transcription activators can not be used as baits in the classical yeast two-hybrid, as these have a tendency to stimulate reporter gene expression in the absence of interaction (Bunker and Kingston, 1995).

Almost all of the yeast two-hybrid systems rely on a similar principle, namely the modular structure of the protein reporting the interaction. Similar to DNA-BD and AD reconstituting a transcription factor in the original yeast two-hybrid system, these employ proteins containing two structural domains which can fold correctly independently of each other and which reconstitute the functional reporter system if brought together via bait-prey interaction. An exception of this principle is the recruitment-based yeast two-hybrid, where the reporter cascade is activated by forced membrane

localization of the bait-prey complex (Brückner *et al.*, 2009). Table 3.3 below contains an overview of the yeast two-hybrid systems developed to date, their characteristics and the year they were developed. These approaches offer promising alternatives to explore interactions of proteins that cannot undergo nuclear localization, or as alternatives to conventional selection/reporter systems.

Table 3.3: Characteristics of classical and novel yeast two-hybrid applications. *# Indicates that the system has been used to screen a cDNA library. Adapted from (Brückner et al., 2009).*

Year	Yeast two-hybrid method	Possible baits screened	Response	Cellular compartment of interaction	Reference
1989	Classic yeast two-hybrid system	Non-transactivating proteins capable of entering nucleus	Transcriptional activation	Nucleus	(Fields and Song, 1989)
1994	SOS recruitment system (SRS). "Cyto-trap" system	Transactivating, cytosolic proteins	#Ras signalling	Membrane periphery	(Aronheim *et al.*, 1997) (McAlister-Henn *et al.*, 1999)
1994	Split-ubiquitin system	Nuclear, membrane and cytosolic proteins	#Uracil auxotrophy and 5-fluoro-orotic acid (5-FoA) resistance	Cytosolic	(Johnsson and Varshavsky, 1994)
1998	Membrane split-ubiquitin System (MbY2H)	Membrane proteins	#Transcriptional activation	Membrane periphery	(Stagljar *et al.*, 1998)
1998	Ras recruitment system (RRS).	Transactivating, cytosolic proteins	#Ras signalling	Membrane periphery	(Broder *et al.*, 1998)
1999	Dual bait system	Two non-transactivating proteins capable of entering nucleus	#Transcriptional activation. Each bait is directed to different operators and reporter genes	Nucleus	(Serebriiskii *et al.*, 1999) (Brückner *et al.*, 2009)
2000	G-protein fusion system	Membrane proteins	Inhibition of protein G signalling	Membrane periphery	(Ehrhard *et al.*, 2000)
2001	RNA polymerase III based two-hybrid (Pol III)	Transactivating proteins (in the RNA polymerase II pathway)	#Transcriptional activation	Nucleus	(Petrascheck *et al.*, 2001)
2001	Repressed transactivator system (RTA)	Transactivating proteins capable of entering nucleus	#Inhibition of transcriptional activation. Can screen for protein interaction inhibitors	Nucleus	(Hirst *et al.*, 2001) (Brückner *et al.*, 2009)
2001	Reverse Ras recruitment system (rRRS)	Membrane proteins	#Ras signalling	Membrane periphery	(Hubsman *et al.*, 2001)
2003	SCINEX-P system	Extracellular and transmembrane proteins	Downstream signalling and transcriptional activation	Endoplasmic reticulum (ER)	(Urech *et al.*, 2003)
2004	Split-Trp system	Cytosolic, membrane proteins	#Trp1p activity	Cytosolic	(Tafelmeyer *et al.*, 2004)
2007	Cytosolic split-ubiquitin System (cytoY2H)	Transactivating, cytosolic proteins	#Transcriptional activation	ER membrane periphery	(Mockli *et al.*, 2007)
1997	Mammalian	n/a	n/a	n/a	(Tsan *et al.*, 1997)
1995	Prokaryotic	Interactions involving dimerization of protein	#Transcriptional activation (inactivation of the cI repressor)	n/a	(Bunker and Kingston, 1995)

3.7. Yeast two-hybrid screens involving elongation factors as bait or prey

There are various examples in literature where elongation factors were either used as baits in the search of its binding partners, or during which it was identified as a binding partner of other bait proteins. Table 3.4 below includes examples of cross-species screening and the use of EF-1α as various BD/baits and preys.

Table 3.4: The use/identification of elongation factors in yeast two-hybrid assays.

Bait	Prey	Proteins identified by yeast two-hybrid assay and implications thereof.	Reference
Calcium binding protein 1 (CBP-1).	*Dictyostelium* cDNA library (mixture of mRNAs isolated from vegetative 8 and-16h starved AX2 cells).	Protovillin, EF-1α. CBP-1 appears prior to cell aggregation, constant throughout development. CBP-1 forms Ca^{2+} dependent associations with the cytoskeleton, possible function in reorganization thereof.	(Dharamsi *et al.*, 2000)
Trichosanthin (TCS) variant E160A – E189A (1000 old reduction *in vitro* ribosome inactivating activity) from the root tuber of *Trichosanthes kirilowii.*	Matchmaker™ cDNA library of human placenta and T-cell leukaemia virus HTLV transformed cell line SLB-1.	Ribosomal phosphoproteins P0 and P1, putative mitotic checkpoint protein MAD28.	(Chan *et al.*, 2001)
Ribosomal phosphoproteins P0 of *Plasmodium falciparum (*PfP0) and *Saccharomyces cerevisiae* (ScP0).	*S. cerevisiae* cDNA library.	P0 has various functions. Interactants were ribosomal proteins, proteins of nucleotide binding, integral membrane proteins, such as RNA helicase, EF-2.	(Aruna *et al.*, 2004)
3rd Cytoplasmic loop of Dopamine D_3 receptor.	Human brain cDNA library.	EF-1Bγ. Immunocytochemical studies of interactants: clusters on plasma membrane. EF-1Bγ is phosphorylated by PKC.	(Choa *et al.*, 2003)
Translationally controlled tumour protein (TCTP) - human. Histamine releasing factor (HrHRF) a.k.a. TCTP/p23/fortilin.	cDNA library from human monocytic leukemia U937 cells. Jurkat T-cell cDNA library.	EF-1α and EF-1Bβ. TCTP stabilizes the GDP bound form of EF-1α and impaired GDP exchange by EF-1Bβ. Partial EF-1δ cDNA. Confirmed with co-immunoprecipitation. Possible intracellular role for HrHRF.	(Cans *et al.*, 2003) (Langdon *et al.*, 2004)
Kinectin (lacking kinesin anchor domain).	Matchmaker™ human foetal brain cDNA library.	EF-1δ. Proved by co-immunoprecipitation. EF-1δ provides guanine nucleotide exchange function of EF-1α. Kinectin anchors EF-1δ on ER membrane.	(Ong *et al.*, 2003)f
Sex hormone-binding globulin (SHBG).	Human prostate cDNA library.	EF-1α, flotillin-1, PRV-1, cathepsin D, kallikrein 4, acid phosphatase, various metallothioneins. EF-1α previously associated with prostatic carcinoma tumor inducing gene 1	(Pope and Lee, 2005)

		and TCTP – both putative oncogenes.	
Murine EF-1α-2.	Matchmaker™ pre-transformed 9–12 week-old mouse brain cDNA library.	Peroxiredoxin I (Prdx-I). Correlated with co-immunoprecipitation. Increases resistance to peroxide induced cell death with activation of caspase 3 and-8, and Akt.	(Chang and Wang, 2007)
Human Promyelocytic leukemia zinc finger protein (PLZF). Acts as transcriptional repressor.	Human ovary cDNA library.	Cervical cancer suppressor 3 (CCS-3) – isoform of EF-1α. Down regulated in cervical tumors/cell lines. Over expression induces apoptosis and suppresses Cyclin A2.	(Rho *et al.*, 2006)
Sphingosine kinase (SK1 and SK2).	Human leukocyte cDNA library.	EF-1α. Confirmed via microscopy and co-immunoprecipitation. EF-1α increases activity/substrate affinity of SK1 and SK2 *in vitro*. Knockdown of EF-1α – reduced SK1 and SK2 transcript levels.	(Leclercq *et al.*, 2008)
Nucleocapsid (N) of Severe acute respiratory syndrome coronavirus (SARS-CoV).	Human fetal liver cDNA library.	EF-1α. N protein induces aggregation of EF-1α, inhibits translation and cytokinesis.	(Zhou *et al.*, 2008)
Apple aminopropyl transferase (MdACL5).	cDNA library from young fruits (19 days after full bloom) of *Malus sylvestris* and *M. domestica.*	EF-1α and S-adenosyl-L-methionine synthetase (SAMS). Confirmed by bait-prey vector swapping and co-immunoprecipitation. NB for the ethelene cascade – roles in plant development/tissue differentiation.	(He *et al.*, 2008)
Human Testis-specific protein Y-encoded (TSPY). Putative gene for gonadoblastoma.	Murine gonodal cDNA library.	EF-1α. Bound at the Se translocation/Nucleosome assembly protein (SET/NAP) domain. Co-localized in tumour germ cells (seminoma).	(Kido and Lau, 2008)
Blood POZ containing gene type 2 (BPOZ-2). Adapter protein for E3 ubiquitin ligase scaffold protein CUL3. Ubiquitous in human organs. Potential oncogene for leukemia.	Matchmaker™ human Thymus cDNA library.	EF-1α-1. Confirmed by pull-down and immunoprecipitation. Binding mediated by akirin repeats and BTB/POZ domains. BPOZ-2 promoted EF-1α-1 (substrate) ubiquitylation and degradation. BPOZ-2 inhibits EF-1α-1 GTP binding.	(Koiwai *et al.*, 2008)
Drosophila EF-1γ (full-length and C-terminal).	*Drosophila* third instar cDNA library.	Darkener of apricot locus (Doa) which encodes the Lammer/Clk protein kinase. Doa responsible for embryonic and adult cuticular development. Phosphorylation site on EF-1γ C-terminal. EF-1γ has vital cellular functions.	(Fan *et al.*, 2010)
West-Nile virus/Dengue virus capsid proteins (C-proteins).	Human brain/liver cDNA library.	Sec3 exocyst protein (an elongation factor). Binds to N-terminal of C-protein.	(Bhuvanakantham *et al.*, 2010)

		Sec3 modulates viral transcription/translation through sequestration of EF-1α.	
Pleckstrin homology (PH) domain of Phospholipase C (PLC-γ1).	EF-1α	Only used to confirm this interaction after identification by GST pull-down and immunoprecipitation assays.	(Chang *et al.*, 2002)

3.8. Hypothesis

- Identification of EF-1α binding partners by means of the Y2H system will provide insight into its biological function in *R. microplus* ticks.
- Vaccination of cattle against both EF-1α and its binding partners will improve the efficiency of an EF-1α based vaccine.

3.9. Aims

- Construction of a EF-1α-BD fusion construct.
- Construction of a full-length poly-$(A)^+$ *R. microplus* mixed lifestages plasmid AD-fusion library.
- Two-hybrid screen to identify EF-1α binding proteins.
- Data analyses.

Materials and methods

3.10. Materials

The Matchmaker™ GAL4 two-hybrid system 2 (pAS2-1 and pACT2) was a gift from Dr. A. Dhugra, University of Pennsylvania, USA. Matchmaker™ GAL4 two-hybrid system 3 (pGADT7) and the Super SMART™ cDNA synthesis kit was purchased from Clontech Laboratories Inc. (Southern Cross Biotech). KC8 *E. coli* cells were provided by Dr. H. Moolman-Smook, University of Stellenbosch, South Africa. The NucleoSpin® Plasmid isolation kit, NucleoSpin® Extract II kit, and the NucleoBond® PC100 maxiprep kit, were purchased from Macherey Nagel, Düren, Germany (Separations). *Sfi*I restriction enzyme and PCR nucleotide mix (10 mM each) were purchased from Roche Diagnostics. Shrimp Alkaline Phosphatase (SAP) was obtained from Fermentas, Canada. Yeast extract, Tryptone and Peptone were obtained from Oxoid Ltd., England. Yeast nitrogen base (without amino acids and ammonium sulphate) was obtained from Difco (Labretoria). Agar was purchased from Whitehead Scientific. Deoxyribonucleic acid sodium salt from Salmon sperm, PEG-4000, glass beads (425 – 600 µm), Triton X-100, amino acids, lithium acetate (LiOAc), sodium dodecyl sulphate (SDS), phenol:chloroform:isoamylalcohol (25:24:1), and cycloheximide were

purchased from Sigma Aldrich, Germany. RNase inhibitor, DNA polymerase I (Klenow fragment), Proteinase K, the Promega Wizard® SV Gel and PCR Clean-UP System, *Eco* RI, *Hind* III, *Bam* HI, *Nco* I, Lambda marker and RNasin™ ribonuclease inhibitor were obtained from Promega, Wisconsin, USA (Whitehead Scientific). L-adenine hemisulphate, ammonium acetate and ampicillin were obtained from ICN (Separations). rTaq (5 U/µl), ExTaq (5 U/µl) and T4 DNA ligase (350 U/µl) were from Takara Bio Inc., Japan (Separations). All primers were synthesized by Inqaba Biotech, South Africa. Glucose, sodium chloride (NaCl), ethelene diamine tetra-acetic acid (EDTA), Tris (hydroxymethyl) aminomethane, methanol, ethanol, isopropanol and chloroform were obtained from Merck, Darmstadt, Germany. Electroporation cuvettes were purchased from Bio-Rad Laboratories Inc. The AccuBlock™ dry block heater was from Labnet International Inc. Superscript® reverse transcriptase, LR Clonase™ II and the pCR®8/GW/TOPO® vector were obtained from Invitrogen Corporation. The GeneQuant™ Pro was from Biochrom Ltd., Cambridge (Applied Biosystems). The pDEST-GADT7 vector was a kind gift from Dr. Max Bush, Department of Crop Genetics, John Innes Centre, UK. The pGADT7-Rec2 vector was obtained from Nicky Creux, Department of Genetics, University of Pretoria, South Africa. The Zyppy plasmid isolation kit and the Frozen-EZ yeast transformation II™ kit were purchased from Zymo Research (Inqaba Biotec). Petri dishes (90 mm and 150 mm) were from Sterilin® Limited. KapaTaq® readymix was a product from Kapa Biosystems. The Perkin Elmer GeneAmp PCR system 2700 was supplied by Applied Biosystems™. The yeast two-hybrid Y2HGold strain (Clontech) was a gift from Jomien Mouton (US/MRC Centre for Molecular and Cellular Biology, University of Stellenbosch).

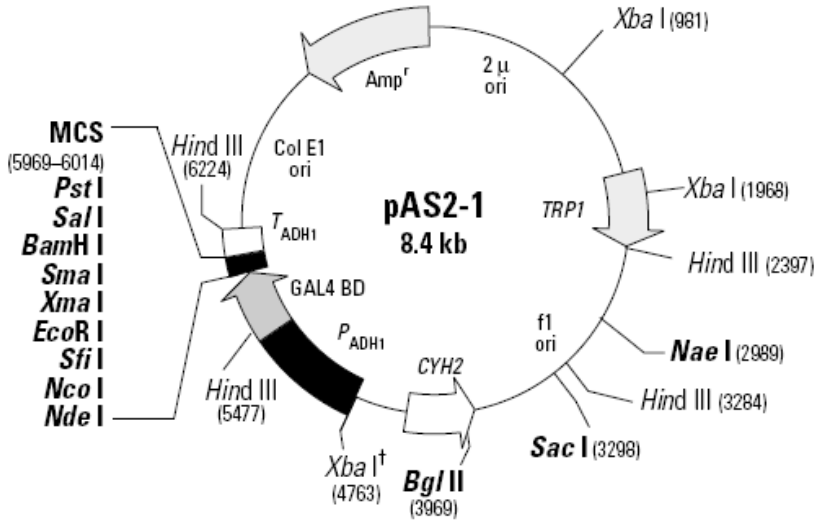
Methods

3.11. GAL4 DNA-BD/bait construction

3.11.1. Amplification and cloning of EF-1α (bait)

The sequence of EF-1α was obtained from Dr. C. Maritz-Olivier, University of Pretoria. EF-1α was prepared from R. *microplus* first strand cDNA (mixed lifestages) using gene-specific primers. Primers were designed to directionally clone the insert in the correct reading frame into the pAS2-1 plasmid (Table 3.5 and Figure 3.8). Primer (10 pmol of each) was used for PCR amplification, using the same parameters as in chapter 2. Amplified inserts were analyzed using 2% agarose gel electrophoresis and purified using the NucleoSpin® Extract II kit. The Extract II system utilizes the principle of conventional DNA silica binding in the presence of chaotropic salts. Sequential digestions with the appropriate restriction endonucleases (12 U per 1 µg DNA) were performed on both the inserts and the pAS2-1 plasmid. Digested inserts and plasmids were purified using the NucleoSpin® Extract II kit. Plasmid (1 µg) was dephosphorylated using 5 U of Shrimp alkaline phosphatase (SAP) by incubation at 37°C for one hour, followed by inactivation at 65°C for 15

minutes. Plasmid and insert were ligated overnight at 16°C using T4 DNA Ligase (3 U), followed by inactivation at 70°C for 10 minutes. DNA was subsequently precipitated with 20 µg transfer RNA (tRNA), 1/5 volumes of NaOAc (3 M, pH 5.2) and 3 volumes of 100% ethanol, before electroporation into DH5α *E. coli* cells.



>pAS2-1 multiple cloning site

TCA TCG GAA GAG AGT AGT AAC AAA GGT CAA AGA CAG TTG ACT GTA TCG CCG GTA TTG CAA TAC CCA GCT TTG ACT CAT ATG GCC ATG GCA AGG CCC ACG GAC AAA CCC CTC CGA CTT CCG CTG CAG GAC GTC TAC AAG ATT GGT GGC ATT GGC ACG GTG CCA GTC GGC CGT GTG GAG ACC GGT GTC CTC AAG CCC GGC ATG GTC GTC ACC TTT GCC CCT GCC AAC CTG ACC ACT GAG GTC AAG TCT GTG GAG ATG CAC CAC GAG GCC CTC GCA GAG GCC GTG CCC GGC GAC AAC GTC GGC TTC AAC GTC AAG AAC GTC TCG GTG AAG GAG CTG CGC CGA GGA TAT GTG TGT GGA GAC TCG AAG GAC ACT CCA CCG AAG TCC ACG GAG GAG TTC ACT GCC CAG GTT ATT GTG CTG AAC CAT CCC GGC CAG ATT GCA AAC GGC TAC ACG CCT GTG TTG GAC TGC CAC ACA GCC CAC ATT GCC TGC AAG TTC AGG GAG ATC AAG GAA AAG TGT GAC CGT CGT TCT GGC AAG AAG CTC GAG GAC AAC CCC AAG TTC ATC AAG TCT GGC GAT GCT GCG ATC ATT GAC CTG GTA CCT TCC AAG CCC ATG TGC GTC GAA ACC TTC ACC GAC TTT CCT CCC CTT GGT CGC TTC GCT GTC CGT GAC ATG AGG CAA ACG GTC GCC CGG ATC CGT CGA CCT GCA GCC AAG CTA ATT CCG GGC GAA TTT CTT ATG ATT TAT GAT TTT TAT TAT TAA ATA AGT TAT AAA AAA AAT AAG TGT ATA CAA ATT TTA AAG TGA CTC TTA GGT TTT AAA ACG

> pAS2-1 multiple cloning site TRANSLATED

S S E E S S N K G Q R Q L T V S P V L Q Y P A L T H **Met** A **Met** A R P T D K P L R L P L Q D V Y K I G G I G T V P V G R V E T G V L K P G **Met** V V T F A P A N L T T E V K S V E **Met** H H E A L A E A V P G D N V G F N V K N V S V K E L R R G Y V C G D S K D T P P K S T E E F T A Q V I V L N H P G Q I A N G Y T P V L D C H T A H I A C K F R E I K E K C D R R S G K K L E D N P K F I K S G D A A I I D L V P S K P **Met** C V E T F T D F P P L G R F A V R D **Met** R Q T V A R I R R P A A K L I P G E F L **Met** I Y D F Y Y **Stop** I S Y K K N K C I Q I L K **Stop** L L G F K T

Figure 3.8: Map of the pAS2-1 vector indicating the restriction enzyme sites used for directional cloning. *Below the vector map, is the nucleotide sequence of the multiple cloning site including the insert cloned in-frame of the C-terminal His tag. Plasmid sequence is indicated in blue, insert sequence is underlined, gene specific regions used in primer design indicated in grey, the NcoI site in pink, the BamHI site in dark blue, additional nucleotides required for in-frame cloning in yellow and the stop-codon in red.*

Table 3.5: Primers used for amplification and directional cloning of EF-1α.

Primer	Sequence (5' – 3')	Tm (°C)
GIIpASNco1F1	CATGCCATGGCAAGGCCCACGGACAAAC *Nco*I	67.4
GIIpASBamH1R1	CGCGGATCCGGGCGACCGTTTGCCTC *Bam*HI	70.8

Restriction enzyme cut sites are underlined.

3.11.2. GAL4 DNA-BD (EF-1α) transformation of yeast

A modified version of the yeast electroporation protocol in the EasySelect *Pichia* expression kit manual was used to transform *S. cerevisiae* Y2HGOLD cells with the bait construct. YPDA media (5 ml) was inoculated with *S. cerevisiae* Y2HGOLD cells and incubated overnight at 30°C and 200 rpm. Fresh YPDA (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose, 0.003% w/v adenine hemisulphate) (500 ml) was inoculated with 5 ml cells and incubated at 30°C with shaking (200 rpm) until an OD_{600} of 1.0 – 1.3 was reached. The cells were subsequently centrifuged at 1,500 x g for 5 minutes at 4°C and the pellet resuspended in 500 ml ice-cold, sterile dddH_2O. The washing step was repeated using 250 ml ice-cold, sterile ddd H_2O. A final wash step using 20 ml ice-cold 1 M sorbitol was followed by centrifugation and resuspension in 1 ml of ice-cold 1 M sorbitol. Cells (80 µl) were mixed with 1 µg of the bait plasmid and the mixture transferred to an ice-cold electroporation cuvette. The cells were pulsed at 1500 mV in an electroporator (Eppendorf), to which 1 ml of ice-cold 1 M Sorbitol was immediately added. Cells were transferred to a 2 ml tube and incubated at 30°C for 1 - 2 hours. Thereafter, cells were plated onto SD/-Trp plates and incubated at 30°C for 2 - 5 days until colonies appeared.

As an alternative, the following small-scale method of Dr. Hanlie Moolman-Smook (University of Stellenbosch) was used. Y2HGOLD yeast cells were streaked onto YPDA plates and incubated for at least 2 days at 30°C. A colony (25 – 50 µl cells) from the plates was resuspended in 1 ml ddd H_2O, vortexed and the cells collected by centrifugation at 16,000 x g for 30 seconds. Cells were made competent by the addition of 1 ml LiOAc (100 mM), followed by stationary incubation at 30°C for 5 minutes and collection of the pellet by centrifugation. To mediate transformation, the following were added to the pellet in this specified order: 240 µl 50% PEG-4000, 36 µl 1 M LiOAc, 25 µl heat denatured salmon sperm DNA (2 mg/ml), and 500 ng DNA-BD/bait plasmid in a final volume of 500 µl. This mixture was vortexed for 1 minute, followed by a 20 minute heat-shock at 42°C in an AccuBlock™ Dry-Bath (Labnet International, Inc.). The transformed cells were collected by centrifugation at 16,000 x g for 30 seconds, resuspended in 250 µl ddd H_2O and spread on SD/-Trp plates using glass beads. Plates were incubated upside down for 2 – 5 days at 30°C until colonies appeared.

3.11.3. GAL4 DNA-BD / bait test for autonomous reporter gene activation

The DNA-BD/bait constructs were tested for autonomous transcriptional activation by plating the bait transformed host strain on SD/-Trp, DDO/-His/-Trp and TDO/-His/-Trp/-Leu. If cells survived on DDO or TDO, the construct was not suitable for screening the library.

3.12. Full-length Gal4 AD/library construction

3.12.1. cDNA synthesis and amplification using the Super SMART System™

R. microplus mixed lifestages total RNA was available. Double strand cDNA (ds cDNA) synthesis and amplification by LD-PCR was performed according to the Super SMART™ PCR cDNA synthesis kit (Clontech). Two sets of primers (sequences derived from the Super SMART™ kit) were used for library construction (Table 3.6). The first set contained a *Sfi*I restriction enzyme cut site, which allows for the directional cloning of transcripts into the pACT2 vector utilizing a single restriction enzyme step. The *Sfi*I site is additionally a low abundance cut site, meaning that digestion of tick transcripts will most likely be limited.

Table 3.6: Primers used for synthesis and amplification of cDNA during library construction.

SfiI cut sites are underlined.

Primer	Sequence	Application
SMART IV	5'-AAGCAGTGGTATCAACGCAGAGTGGCCATGGAGGCCGGG-3'	cDNA synthesis and amplification.
CDS III/ 3'PCR	5'-ATTCTAGAGGCCTCCATGGCCGACATG$(T)_{30}$NN-3'	cDNA synthesis and amplification.
SMART III	5'-AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCCC-3'	cDNA amplification by LD-PCR.
GAL4Recomb	5'-CACCAAACCCAAAAAAAGAGGGAGGCCGGG-3'	cDNA synthesis and amplification – recombination approach.
ADH1Recomb	5'-CATGCCGGTAGAGGTGTGGTGGCCGACATG-3'	cDNA synthesis and amplification – recombination approach.
CDS III	5'-ATTCTAGAGGCCTCCATGGCCGACATGTTTTT-3'	cDNA amplification by LD-PCR.
5'AmplimerGADT7	5'-CTATTCGATGATGAAGATACCCCACCAAACCC–3'	PCR screen of pGADT7 plasmid.
3'AmplimerGADT7	5'-GTGAACTTGCGGGGTTTTTCAGTATCTACGATT–3'	PCR screen of pGADT7 plasmid.
Nested5'pGAD	5'-ATGAACATGGAGGCCAGTGAA–3'	Nested PCR screen of pGADT7 plasmid.
Nested3'pGAD	5'-CAGCTCGAGCTCGATGGATCC–3'	Nested PCR screen of pGADT7 plasmid.
GW1	5'-GTTGCAACAAATTGATGAGCAATGC-3'	PCR screen of TOPO® plasmid.
GW2	5'-GTTGCAACAAATTGATGAGCAATTA-3'	PCR screen of TOPO® plasmid.
TOPO NF	5'-GACTGATAGTGACCTGTTC-3'	Nested PCR screen of TOPO® plasmid.
TOPO NR	5'-GTAATACGACTCACTATAG-3'	Nested PCR screen of TOPO® plasmid.

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The Super SMART™ PCR cDNA synthesis kit offers a PCR-based method for the production of high-quality cDNA from nanogram (ng) quantities of total RNA. The modified oligo-dT and 3'CDS III primers mediate first strand synthesis (Figure 3.9). When the Superscript® reverse transcriptase

(RT) reaches the mRNA 5' end, the enzyme's terminal transferase activity adds a few additional nucleotides (primarily deoxycytidine) to the first strand cDNA 3' end. The SMART IV primer, which contains an oligo-dG sequence at its 3' end, then base-pairs with the deoxycytidine stretch and creates an extended template for replication. Upon template switching by Superscript® RT, replication is continued to the end of the oligonucleotide. The new full-length, single-stranded cDNA contains the complete 5' end of the mRNA, as well as the sequences complementary to the SMART IV primer. The SMART™ anchor sequence as well as the poly (A) sequence serves as priming sites for cDNA amplification by long distance polymerase chain reaction (LD-PCR). cDNA without these anchor sites, such as prematurely terminated cDNAs, contaminating genomic cDNA or cDNA transcribed from poly(A)$^-$ RNA, will not be amplified exponentially (Clontech, 2001). Because the stretch of deoxycytidine was added to the 3' end of the first strand, directional cloning of these fragments is possible, since different reading frames were generated arbitrarily towards protein expression.

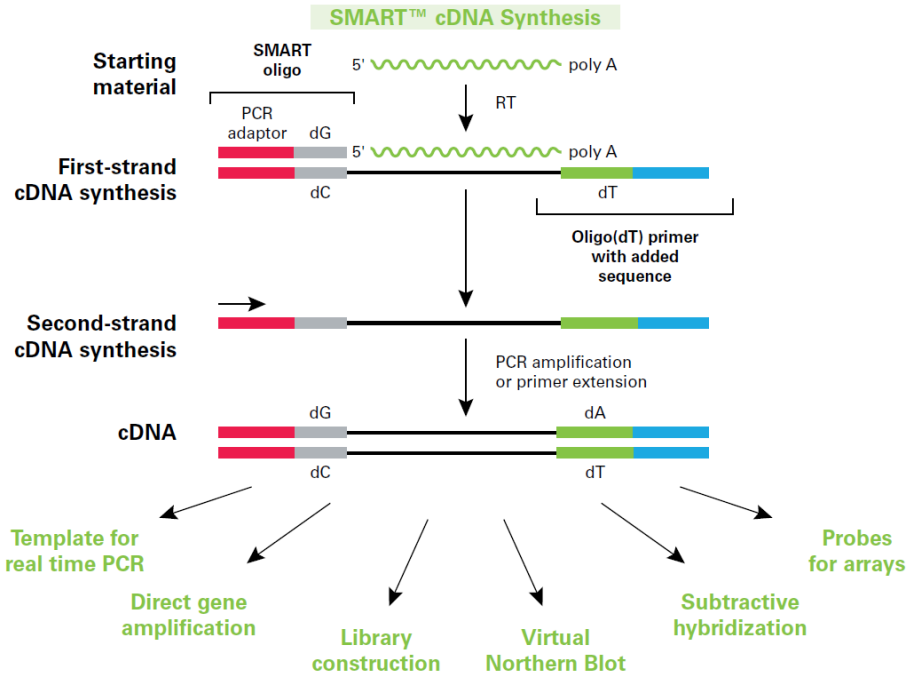

Figure 3.9: Diagrammatical representation of Super SMART™ PCR cDNA synthesis (Clontech, 2007d).

First strand cDNA synthesis was performed by combining the following: 500 ng total RNA (*R. microplus* mixed lifestages), 1 µl CDS III/3'PCR primer (12 µM), 1µl SMART IV primer (12 µM) and ddd H_2O to a volume of 11 µl. The contents was briefly mixed by vortexing and collected by centrifugation. RNA was denatured by incubation at 70°C for 10 minutes, chilled on ice for 5 minutes and pelleted by centrifugation at 16,000 x g for 2 minutes. The following were added: 4µl 5X First strand buffer, 1 µl dNTPs (10 mM each), 2µl DTT (0.1 M) and 1 µl RNasin™ ribonuclease

inhibitor (40 U/µl). This was mixed by centrifugation and incubated for 2 minutes at 42°C. Superscript® III Reverse Transcriptase (1 µl; 200 U) was added and the sample incubated for 90 minutes at 42°C. Inactivation at 70°C for 15 minutes was performed before the cDNA was purified using the NucleoSpin® Extract II kit (Chapter 2). cDNA was stored at –20°C.

Long distance PCR (LD-PCR) was performed based on the guidelines provided by Clontech. Briefly, cDNA (5 µl) was diluted in 79 µl ddd H_2O. ExTaq buffer (10 µl), 2 µl dNTP mix (10 mM each) and 2 µl of the SMART IV and CDS III primers (12 µM each) were added and the mixture briefly vortexed and centrifuged. Thermal cycling was then performed using the Applied Biosystems 2700 with the following parameters: 2 minutes at 94 °C and 1 minute at 80 °C, followed by the addition of 2 µl ExTaq polymerase (5 U/ µl). PCR cycling was performed at 94°C for 15 seconds, 58°C for 30 seconds, and elongation at 60°C for 6 minutes. Aliquots (10 µl) were taken after 18, 20, 22 and 24 cycles for optimisation of PCR cycle number and the remaining volume stored at 4°C until further use. The fractions from cycles 18 to 24 were analysed on 0.8% agarose/ethidium bromide gel alongside the appropriate DNA molecular size markers. Optimal cycle number was determined as one fewer cycle than necessary to reach the plateau, thus ensuring that the DNA remains in the exponential phase of amplification. LD-PCR was then repeated on the remaining sample to reach the optimal number of cycles. Double strand cDNA was purified using the NucleoSpin® Extract II kit (Chapter 2) and the yield determined using the GeneQuant Pro spectrophotometer (Biochrom Ltd., Cambridge – Applied Biosystems).

3.12.2. ds cDNA polishing and size fractionation

Following LD-PCR, the ds cDNA was polished according to the guidelines provided by Clontech. The 100 µl LD-PCR product was divided into two 50 µl samples. Proteinase K (4 µl, 10 µg/µl) was added to each sample and incubated at 45°C for 60 minutes, where after it was inactivated at 90°C for 10 minutes. Both tubes were chilled for 2 minutes in ice water, before the addition of T4 DNA Polymerase (3 µl, 5 U/µl) and subsequent incubation at 16°C for 30 minutes. This allows for synthesis of full-length, blunt ended ds cDNA. The reaction was terminated at 72°C for 10 minutes and purified using the NucleoSpin® Extract II kit (Chapter 2). Yield and purity was determined using the GeneQuant Pro spectrophotometer (Biochrom Ltd., Cambridge – Applied Biosystems).

Size fractionation of the cDNA library was optimized using 3 methods. In the first method, PALL® Nanosep columns of different size exclusion limits (10K, 30K, and 100K) were used. The columns were washed with 70% ethanol and centrifuged at 14,000 x g for 5 minutes. This wash step was repeated with TNE buffer (100 mM Tris, 2 M NaCl, 10 mM EDTA, pH 7.4). Lambda marker (5 µg) or LD-PCR product (in a final volume of 50 µl TNE) was loaded on the first column (100K) and the

column centrifuged at 750 x g for 2 minutes. The retentate (expected > 1000 bp) was dissolved from the membrane using 40 µl TNE buffer. The filtrate (expected < 1000 bp) was loaded onto the 30K column and similarly processed. In order to remove the smaller fractions (< 500 bp), the 30K filtrate was processed with the 10K column. The final retentate and filtrate was purified using the Promega Wizard® SV Gel and PCR Clean-UP System kit to remove residual EDTA. Different centrifugation speeds were tested in an attempt to improve fractionation efficiency.

The second approach entailed the use of self-packed columns consisting of a glass tube (modified Pasteur pipette), glass wool and Sephacryl S-400 resin. The narrowing neck of a Pasteur pipette was plugged with glass wool, and the pipette filled with 3 cm of packed Sephacryl S-400 resin. The column was washed with TNE buffer and allowed to drain by gravity flow. Once the buffer meniscus reached the resin, 5 µg Lambda marker or LD-PCR product (in a final volume of 50 µl TNE) was loaded onto the resin. Fractions (20 µl) were collected drop-wise whilst eluation occurred by gravity flow, DNA precipitated with ethanol and 3 M NaOAc and dried *in vacuo*. Once re-dissolved in 10 µl dddH_2O, the fractions were analyzed on a 0.8% agarose gel.

Lastly, Sephacryl S-400 resin (1000 µl in suspension) was placed inside an empty 2 ml centrifuge column (Promega, Wisconsin, USA). The column was centrifuged for 1 minute at 600 x g to remove the residual suspension buffer. TNE buffer (650 µl) was added to wash the resin and centrifuged again at 600 x g for 30 seconds. This step was repeated six times. The 7th centrifugation step was extended to 90 seconds in order to partially dry the resin (resin receded from column wall). The LD-PCR product was carefully pipetted to the middle of the resin and the column centrifuged at 300 x g for 30 seconds to elute the DNA. After fractionation, the ds cDNA was purified using the Promega Wizard® SV Gel and PCR Clean-UP System as follows. An equal amount of membrane binding solution (4.5 M guanidine isothiocyanate, 0.5 M potassium acetate, pH 5.0) was added to the sample. This sample was placed in a SV Minicolumn inside a collection tube and incubated for 1 minute at room temperature. This was centrifuged at 16,000 x g for 1 minute, where after the liquid was discarded. The column was washed by the addition of 700 µl membrane wash solution (10 mM potassium acetate pH 5.0, 80% ethanol, 16.7 µM EDTA pH 8.0) and centrifuged at 16,000 x g for 30 seconds. This was followed by another wash using 500 µl of membrane wash solution and a 5 minutes centrifugation step. The SV Minicolumn was transferred to a clean 1.5 ml tube, and 50 µl nuclease free water added to the middle of the column. This was incubated at room temperature for 5 minutes and centrifuged at 16,000 x g for 1 minutes to elute the ds cDNA. The sample was stored at -20°C until further use.

3.12.3. Construction of the GAL4/AD plasmid- cDNA library fusion

Three approaches were followed to clone the ds cDNA library into a suitable AD vector. The first was a directional cloning method that made use of the *Sfi*I restriction enzyme cut site within the MCS of the pACT2 plasmid and incorporated into the SMART IV and CDS III oligonucleotides. As the first alternative, the first strand cDNA and the LD-PCR were prepared using two new primers, GAL4Recomb and ADH1Recomb (Table 3.6) and the cDNA library cloning attempted using a homologous recombination approach. The second, successful alternative was to use the Gateway® technology by the Invitrogen Corporation, to first clone the cDNA library into the pCR®8/GW/TOPO® vector via A/T cloning, followed by a homologous recombination approach to subclone the library into the pDEST-GADT7 AD vector. The cDNA library for the Gateway® methodology was also prepared using the SMART IV and CDS III/3'PCR primers (Table 3.6). The first two approaches will be discussed briefly below, whereas the third GateWay® method will receive more attention.

The SMART/SfiI approach

Briefly, polished SMART ds cDNA and pACT2 plasmid were digested with *Sfi*I at 50°C for 2 hours, followed by the addition of 2 µl Proteinase K (20 µg/ml) and subsequent incubation at 45°C for 1 hour. The reaction was purified using the NucleoSpin® Extract II kit. To avoid ligation of the complementary *Sfi*I generated overhangs of the pACT2 plasmid, the digested plasmid was dephosphorylated with the addition of 10 µl SAP(1 U/µl), incubated for 1 hour at 37°C and terminated by heat inactivation at 65°C for 15 minutes. To ensure that pACT2 was successfully dephosphorylated prior to ligation of the library, 100 ng native plasmid was incubated with T4 DNA ligase (3 U) for 1 hour at 16°C, and analyzed by plating out transformed *E. coli* BL21 on LB-Amp and scrutinizing colony growth absence. Both native pACT2, as well as dephosphorylated pACT2 subjected to ligation, were additionally analysed using agarose gel electrophoresis.

In order to optimize library ligation into pACT2, various vector: insert (cDNA library) ratios were tested. pACT2 and cDNA library were combined with 1µl T4 DNA Ligase (100 U/µl), incubated overnight at 16°C and followed by heat inactivation at 70°C for 10 minutes. Ligated products were precipitated in the presence of 20 µg tRNA and 3M NaOAc (see 3.11.1) and electroporated into *E. coli* BL21 cells. Transformation was assessed by plating transformed cells at different dilutions onto 2% agar plates containing 50 mg/ml ampicillin.

The homologous recombination approach

For this method, Matchmaker™ One hybrid (Clontech) primers (SMART III and CDS III, Table 3.6) were modified by shortening the original sequence and adding a partial anchor sequence to both, to ensure the amplification of full-length cDNA as before using the new GAL4Recomb and

ADH1Recomb primers. 2 µg pGADT7-Rec2 vector was linearized with 2 µl *Sma* I (20 U/µl) at 25°C overnight, heat inactivated at 65°C for 20 minutes, purified using the NucleoSpin® Extract II kit and 1 µg of pGADT7-Rec2 and cDNA library co-transformed into *S. cerevisiae* Y2HGOLD. Cellular recombinases were expected to repair the gap created in the pGADT7-Rec2 vector by homologous recombination, resulting in functional expression constructs (Figure 3.10).

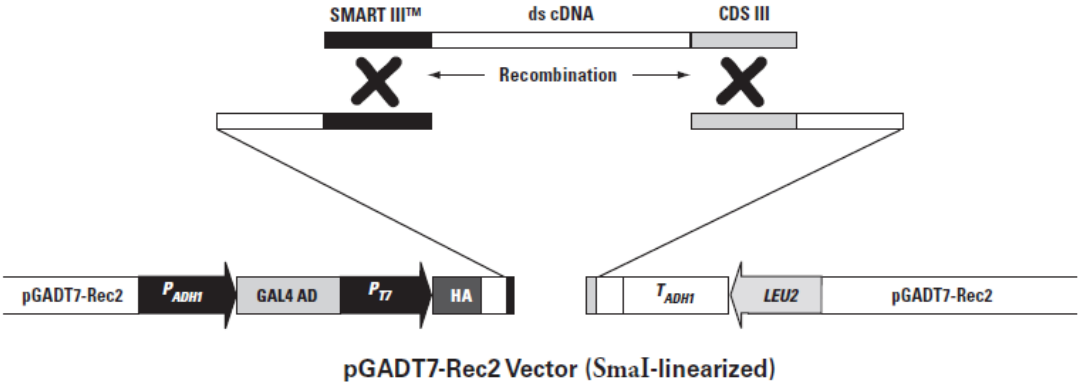

Figure 3.10: Cloning of cDNA into the pGADT7-Rec2 vector by homologous recombination (Clontech, 2009).

The GateWay® approach

The pCR®8/GW/TOPO® TA Cloning® kit combines Invitrogen's TOPO® Cloning and Gateway® technologies to facilitate quick, one-step cloning of *Taq* polymerase- amplified PCR products into a plasmid vector with very high efficiency (> 95%). One-step cloning is possible due to the Topoisomerase covalently bound to the vector, which means that the addition of ligase, or the use of restriction enzymes is unnecessary. Once characterized, these clones can be transferred via a recombination based method from the pCR®8/GW/TOPO® entry vector to a Gateway® compatible destination vector for expression of the gene of interest in virtually any system (Invitrogen, 2006). The pCR®8/GW/TOPO® vector (Figure 3.11) contains the spectinomycin resistance marker for efficient selection in *E. coli*. This allows transfer of the PCR product or cDNA library into ampicillin- or kanamycin-resistant Gateway® destination vectors, via recombination-based transfer mediated by attL1 and attL2 sites on the vector. Primer binding sites within attL1 and attL2 are utilized for sequencing with GW1 and GW2 primers (Table 3.6).

For the GateWay® approach, the same primers were used as for the SMART/*Sfi*I method i.e. SMART IV and CDS III/3'PCR (Table 3.6). The cDNA library was cloned into the pCR®8/GW/TOPO® vector by the following method. Purified LD-PCR product (172 ng), 0.5 µl of the diluted salt solution (provided in the kit), 0.5 µl of the TOPO® vector (5 – 10 ng/µl) and 1 µl ddd H_2O was added together to produce a half-reaction. The reaction was briefly vortexed and centrifuged,

incubated at room temperature for 20 minutes, and put on ice until used for transformation into *E. coli* DH5α via electroporation (Chapter 2). The complexity of this intermediate library was determined via colony PCR using the GW1 and GW2, as well as TOPO NF and TOPO NR nested primers (section 2.9.17). For the purpose of creating a representative, large-scale cDNA library, 4 of these half-reactions were performed simultaneously, and the transformation products of all plated onto 40 LB-Agar plates. Cells from these were pooled and used for large-scale plasmid library isolation.

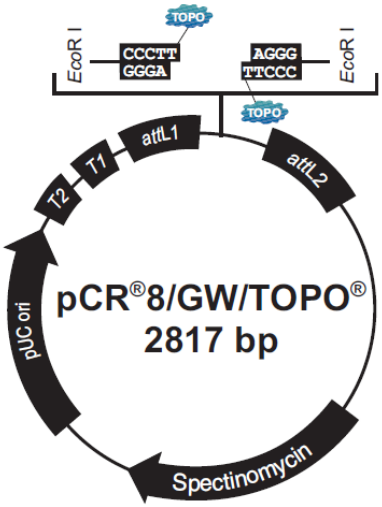

Figure 3.11: Vector map and features of pCR®8/GW/TOPO® (Invitrogen, 2006). *T1 and T2 refer to the transcription termination sequences, attL1 and attL2 to the specific sites for recombination-based transfer of the gene of interest and pUCori to the pUC origin of replication.*

Gateway® methodology is dependent on the presence of attL1 and attL2 sites in the entry vector (pCR®8/GW/TOPO®) and attR1 and attR2 sites in the destination vector (pDEST-GADT7). The L and R sites are complementary. The pDEST-GADT7 destination vector was linearized with *Eco* RI (24 U per 1 µg DNA) at 37°C overnight, after which the reaction was inactivated at 70°C for 10 minutes. Thereafter, 75 ng of pCR®8/GW/TOPO®-cDNA library was combined with 75 ng of linearized pDEST-GADT7 and added to a final volume of 4 µl by the addition of ddd H_2O. LR Clonase™ II enzyme (1 µl) was added, the mixture vortexed, centrifuged and incubated at 25°C in a thermal cycler for 16 hours. Proteinase K (1 µl, 2 µg/µl) solution was added and incubated at 37°C for 10 minutes. The reaction was transformed directly into *E coli* BL21 cells via electroporation. For the purpose of creating a representative, large-scale cDNA library, 4 of these half-reactions were performed simultaneously, and the transformation products of all plated onto 40 LB-agar plates containing 50 µg/ml Ampicillin. The cells from these were pooled for collection of the plasmid library by maxiprep.

3.12.4. Determining the number of recombinant/independent clones

In order to determine the percentage of recombinant clones in the library, at least 15 isolated colonies were randomly picked from near confluent plates. Clones were subjected to colony PCR with the GW1/GW2 primers for the pCR®8/GW/TOPO® vector and the 5'Amplimer and 3'Nested primers for the pDEST-GADT7 vector. The size and uniqueness of each clone (amplified from pGADT7) was verified via restriction mapping using 1 µl each of *Nde* I (12 U/µl) and *Bam* HI (10 U/µl), with incubation at 37°C for 2 hours and subsequent inactivation at 70°C for 10 minutes. Samples were analyzed using 0.8% agarose gel electrophoresis. A transformation mixture was only used for library titering and amplification if at least 10/15 clones contained unique inserts.

3.12.5. Plasmid library titering

A plasmid library has to be tittered in order to ensure its representivity. In general, the titer should be at least 10-fold higher than the number of independent clones and at least 10^8 cfu/ml for long-term storage. To determine the titer, 1 µl of the pooled library (in *E. coli* BL21) was added to 1 ml LB broth in a 1.5 ml microcentrifuge tube (this is dilution A, $1:10^3$) and mixed by vortexing. 1µl of Dilution A was added to 1 ml LB broth to create dilution B ($1:10^6$). For plating the cells, 1 µl of dilution A was diluted in 50 µl LB broth, mixed and the entire mixture plated. Two aliquots (50 µl and 100 µl) from dilution B were also plated. Following overnight incubation at 37°C, the number of colonies was counted and the titer (cfu/ml) calculated according to the following formulas: number of colonies from dilution A x 10^3 x 10^3 = cfu/ml (number of colonies from dilution B / plating volume) x 10^3 x 10^3 x 10^3 = cfu/ml.

3.12.6. Large-scale library plasmid isolation

Cells were scraped off the 40 plates using adequate volumes of LB-broth. Large-scale plasmid isolation was performed using the NucleoBond™ PC100 system on 10 ml of cell suspension. Cells were collected via centrifugation at 6,000 x g for 15 minutes and the supernatant discarded. The pellet was resuspended in 4 ml of buffer S1 (50 mM Tris-HCl, 10 mM EDTA, 100 µg/ml RNase A, pH 8.0), lyzed with the addition of 4 ml buffer S2 (1% SDS, 200 mM NaOH) and incubated at room temperature for 3 - 5 minutes. The released chromosomal DNA was precipitated with the addition of 4 ml pre-cooled buffer S3 (2.8 M potassium acetate, pH 5.1), mixed by inversion and incubated on ice for 5 minutes. The lysate was clarified by centrifugation (6,000 x g for 15 minutes), loaded on a pre-equilibrated NucleoBond® AX100 column (2.5 ml Equilibration buffer N2; 100 mM Tris-HCl, 15% ethanol, 900 mM KCl, 0.15% TritonX100, adjusted to pH 6.3 with H_3PO_4) and allowed to elute by gravity. The column was washed twice with 10 ml of buffer N3 (100 mM Tris-HCl, 15% ethanol, 1.15 M KCl, adjusted to pH 6.3 with H_3PO_4) before eluting the plasmid DNA with 5 ml buffer N5 (100 mM Tris-HCl, 15% ethanol, 1 M KCl, adjusted to pH 8.5 with H_3PO_4). During elution, 1 ml fractions

were collected in 2 ml tubes, which were immediately precipitated by the addition of 800 µl isopropanol and the samples centrifuged at 16,000 x g for 30 minutes at 4°C. Pellets were washed with 70% ethanol, vacuum-dried and dissolved in 50 µl ddd H_2O. Yields were determined spectrophotometrically.

3.12.7. Sequential transformation of Y2HGOLD yeast cells

The sequential transformation of *S. cerevisiae* Y2HGold was performed using two different protocols, of which the second was more successful. Both are discussed below, with the second receiving more attention. The basis composition of dropout media is as follows: 0.67% (w/v) yeast nitrogen base without amino acids, 2% glucose (w/v), 20 mg/l L-adenine hemisulphate, 20 mg/l L-arginine HCl, 20 mg/l L-histidine HCl monohydrate, 30 mg/l L-isoleucine, 100 mg/l L-leucine, 30 mg/l L-lysine HCl, 20 mg/l L-methionine, 50 mg/l L-phenylalanine, 200 mg/l L-threonine, 20 mg/l L-tryptophan, 30 mg/l L-tyrosine, 20 mg/l L-uracil and 150 mg/l L-valine, with pH adjusted to 5.8.

The TRAFO protocol

The 5 X TRAFO protocol described by Agatep R, Kirkpatrick, R.D., Parchaliuk, D.L., Woods, R.A. and Gietz, R.D. was used (http://home.cc.umanitoba.ca/gietz/). Since sequential transformation was performed, Y2HGold yeast cells containing the DNA-BD-EF-1α (pAS2-1-EF-1α) construct were inoculated in 12.5 ml SD/-Trp (single dropout) and grown overnight at 30°C with shaking. The cell titer was determined, and the volume of cell suspension yielding a total of 2.5 x 10^8 cells calculated. This number of cells were collected with centrifugation (3,000 x g, 5 minutes), diluted in 25 ml pre-warmed YPDA medium and incubated at 30°C until the cell titer reached 2 x 10^7 cells/ml. Cells were harvested by centrifugation (3,000 x g, 5 minutes), washed with 12.5 ml water, collected by centrifugation, resuspended in 1.5 ml lithium acetate (100 mM) and incubated for 30 minutes at 30°C without shaking, cells again collected by centrifugation and the supernatant removed. The following were added to the cells in the listed order: 1.2 ml 50% PEG-4000, 180 µl LiOAc (1M), 250 µl heat denatured salmon-sperm DNA (2 mg/ml) and 10 µg DNA-AD/ library pGADT7 plasmid. The mixture was vigorously vortexed for 1 minute until the cell pellet was totally resuspended and incubated at 30°C for 30 minutes. Cells were heat shocked at 42°C for 15 minutes during which it was mixed by inversion for 15 seconds every 5 minutes. Cells were collected by centrifugation, gently resuspended in 5 ml water, and 500 µl aliquots spread on 10 large (150 mm) DDO/-Trp/-Leu (double dropout) plates using glass beads. Plates were incubated at 30°C for 3-5 days until co-transformed colonies appeared. The cell suspension was additionally plated on SD/–Leu and SD/–Trp as positive controls for the transformation of the bait and prey plasmids, respectively.

Transformation with the Zyppy Frozen-EZ Yeast Transformation II™ kit

Y2HGold yeast containing the pAS2-1-EF-1α (bait) construct were inoculated in 20 ml SD/-Trp and grown overnight at 30°C with shaking. The following day, cells were inoculated into fresh YPDA and grown to mid-log phase (2 x 10^7 cells/ml or OD_{600} = 1). EZ1 – 3 solutions were pre-warmed to 30°C before use. Cells (10 ml) were pelleted at 3,000 x g for 5 minutes, after which the supernatant was discarded. EZ1 solution (5 ml) was added to the pellet, cells centrifuged at 3,000 x g for 5 minutes and the supernatant discarded. EZ2 solution (500 µl) was used to resuspend the pellet. This volume was separated into 10 x 50 µl fractions in 1.5 ml tubes and 500 ng pGADT7-library cDNA and EZ3 solution (500 µl) added to each tube. Tubes were vortexed for 30 seconds to mix the contents and incubated at 30°C for 120 minutes with inversion every 20 minutes. The contents of each tube (500 µl) were spread on a 150 mm DDO/-Trp/-Leu plates using glass beads. These were incubated at 30°C for 3 - 5 days until co-transformed colonies appeared. The cell suspension was also plated on SD/–Leu and SD/–Trp as positive controls for the transformation of the bait and prey plasmids individually. Co-transformation efficiency was calculated as follows. The cfu/µg was multiplied with the amount of library plasmid used. As an example for one of the transformations with the Frozen-EZ Yeast Transformation II™ kit, co-transformation efficiency was calculated as 7.2 x 10^4 cfu/µg and the amount of library used was 0.5 µg. The number of clones screened was thus determined as 3.6 x 10^7 (7.2 x 10^4 cfu/µg x 0.5ug = 3.6 x 10^7 cfu). This was sufficient for a yeast two-hybrid screen.

As a positive control for the yeast two-hybrid system, native *S. cerevisiae* Y2HGold was also co-transformed with the control plasmids pGADT7-T and pGBKT7-53 (included in Matchmaker system 3) using the Zyppy Frozen-EZ Yeast Transformation II™ kit and plated onto the selective media.

3.13. Two-hybrid screen for activation of reporter genes

Co-transformed cells were scraped from the plates using DDO/-Trp/-Leu medium, collected by centrifugation, resuspended in 10 ml DDO/-Trp/-Leu and 10 x 500 µl aliquots plated on TDO/-Trp/-Leu/-His (TDO, Triple drop-out). Plates were incubated at 30°C for 2-8 days, or until colonies appeared. The remaining cells were stored in 1 ml aliquots at –70°C in DDO/-Trp/-Leu containing 25% glycerol. TDO positive colonies were transferred to duplicate TDO master plates in a gridded pattern with sterile wooden sticks and incubated at 30°C overnight. One master plate was stored at 4°C as back-up, while colonies from the second master plate were transferred onto QDO/-Trp/-Leu/-His/-Ade (QDO, Quadruple drop-out) for further selection. QDO positive colonies were also transferred to QDO master plates, and re-grown in small volumes of DDO/-Trp/-Leu liquid media in order to amplify positive clones.

3.14. Screening of positive clones using nested PCR

Nested PCR was performed directly on yeast cells, similar to the colony PCR described for prokaryotes in chapter 2. The PCR mixture contained: 10 pmol of both 5' and-3' Amplimer pGAD primers (Table 3.6), 12.5 µl KapaTaq Readymix and ddd H_2O to a final volume of 25 µl. Cells were disrupted at 94°C for 7 minutes in a Perkin Elmer GeneAmp PCR system 2700 and cooled to 80°C for 1 minute. Thirty cycles of denaturation (94°C for 30 seconds), primer annealing (58°C for 30 seconds) and extension (72°C for 2 minutes) were performed. For the nested PCR, 1 µl of the above PCR product was used as template with the Nested5'pGAD and Nested3'pGAD primers (Table 3.6) and identical composition. Nested PCR was performed for 30 cycles of denaturation (94°C for 30 seconds), primer annealing (58°C, 30 seconds) and extension (72°C for 2 minutes). Products were analyzed with agarose gel electrophoresis.

3.15. Isolation of plasmid from yeast cells

QDO positive yeast cells were grown in 1 ml DDO/-Trp/-Leu at 30°C overnight with shaking, before adding 4 ml YPDA medium and growing cells for a further 4 hours. Cells were collected by centrifugation (3,000 x g for 5 minutes), the supernatant removed and cells resuspended in 200 µl Smash-and-Grab buffer (1% SDS, 2% Triton X-100, 100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8). Half the volume of glass beads (425-600 µm) and 200 µl phenol: chloroform: isoamylalcohol (25:24:1) were added, and the mixture vortexed vigorously for 3 minutes to break open the yeast cells. The mixture was centrifuged (16,000 x g for 5 minutes) and the aqueous layer removed. To precipitate the plasmid from the solution, 0.5 volumes of ammonium acetate (7.5 M, pH 5) and 2 volumes of 100% ethanol was added and the mixture centrifuged at 4°C (16,000 x g for 25 minutes). The pellet was washed with 70% ethanol, vacuum dried and dissolved in 20 µl water.

3.16. AD/library clone rescue via transformation in KC8 *E. coli*

In order to rescue the library plasmid from the yeast, the isolated plasmids were transformed into electrocompetent *E. coli* KC8. KC8 *E. coli* cells have a defect in *leuB*, which can be complemented by *LEU2*. Thus, KC8 cells can be used to separate AD/library plasmids (which carry *LEU2*) from yeast co-transformants that also contain a DNA-BD/bait plasmid (Clontech, 2007b). Plasmids isolated from QDO positive colonies were electroporated (see chapter 2) into electro-competent KC8 *E. coli* cells. For selection purposes, cells were plated on M9 minimal medium (0.06 M Na_2HPO_4-$7H_2O$, 0.1 M KH_2PO_4, 0.04 M NaCl, 0.09 M NH_4Cl, 0.02 M $MgSO_4$, 0.02 M $CaCl_2$, 0.4% Glucose, 0.001 M thiamine-HCl, 1X DO stock solution) which contained ampicillin (50 mg/ml) and lacked leucine.

3.17. Sequencing of AD/library inserts

KC8 *E. coli* cells harboring the AD/library plasmid were grown overnight in 10 ml LB-ampicillin (50 mg/ml) medium at 30°C with shaking. Plasmid was isolated from these cells using the Zyppy Plasmid Miniprep Kit (Zymo Research, Inqaba Biotec). Automated nucleotide sequencing of the insert was performed with the GAL4 AD sequencing primer or the 5'Amplimer using the Big Dye v3.1 Sequencing kit on an ABI Prism 377 DNA sequencer (Perkin Elmer Applied Biosystems, USA) as described in chapter 2.

3.18. Sequencing analysis

Preliminary sequence analysis was performed using the BioEdit v. 5.0.9. program (Hall, 1999). DNA and deduced amino acid sequences were analysed using tblastx and position-specific iterated basic local alignment search tool (PSI-BLAST) programs (http://0-blast.ncbi.nlm.nih.gov.innopac.up.ac.za/). The non-redundant sequences (nr) database was used for both approaches (Tables 3.8 and 3.9) and the database analysis limited to *R. microplus* (taxid: 6941) for the second approach (Table 3.9).

Results and Discussion

3.19. Construction of the GAL4 DNA-BD (bait) fusion

EF-1α was amplified from *R. microplus* mixed lifestages first strand cDNA using gene-specific primers. The primers were designed to directionally clone the inserts in the correct reading frame into the pAS2-1 plasmid using the *Bam*HI and *Nco*I sites of the vector. Figure 3.12 shows the PCR amplified fragment of EF-1α. DNA sequencing results indicated that the transcript was correctly cloned in-frame into the pAS2-1 plasmid, and that a single amino acid mutation (histidine (H) to arginine (R)) was present at the C-terminal end (Figure 3.13). When studying the amino acid alignment of EF-1α from different tick species in Figure 2.3, it was observed that all the complete sequences had an arginine in this position. Therefore, it was concluded that the arginine to histidine mutation was not a natural mutation occurring in the tick (such as an isoform), but likely induced during the cloning procedure. Since both histidine and arginine have positively charged side chains, the yeast two-hybrid was performed with this clone.

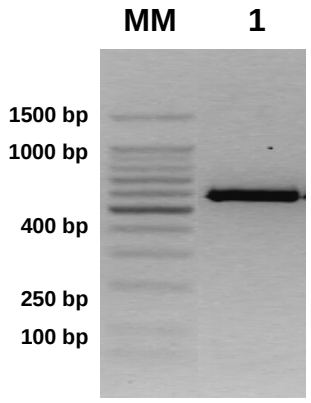

Figure 3.12: PCR amplification of the EF-1α transcript. *PCR amplification of EF-1α transcript from R. microplus mixed lifestages cDNA. Visualisation was aided by EtBr. MM represents the 100 bp DNA ladder (Promega), and lane 1 the EF-1α amplified insert.*

```
CLONE 16        RPTDKPLRLPLQDVYKIGGIGTVPVGRVETGVLKPGMVVTFAPANLTTEVKSVEMHHEAL
Native          RPTDKPLRLPLQDVYKIGGIGTVPVGRVETGVLKPGMVVTFAPANLTTEVKSVEMHHEAL
                ************************************************************

CLONE 16        AEAVPGDNVGFNVKNVSVKELRRGYVCGDSKDTPPKSTEEFTAQVIVLNHPGQIANGYTP
Native          AEAVPGDNVGFNVKNVSVKELRRGYVCGDSKDTPPKSTEEFTAQVIVLNHPGQIANGYTP
                ************************************************************

CLONE 16        VLDCHTAHIACKFREIKEKCDRRSGKKLEDNPKFIKSGDAAIIDLVPSKPMCVETFTDFP
Native          VLDCHTAHIACKFREIKEKCDRRSGKKLEDNPKFIKSGDAAIIDLVPSKPMCVETFTDFP
                ************************************************************

CLONE 16        PLGRFAVHDMRQTVA
Native          PLGRFAVRDMRQTVA
                *******:*******
```

Figure 3.13: Amino acid sequence alignment of the native and cloned EF-1α (bait) sequence. *Transcripts (in FASTA format) were aligned using GeneDoc sequence alignment editor and analyser. The newly amplified EF-1α construct (clone 16) and the native sequence are shown. The single amino mutation is indicated in green.*

3.20. Transformation of the GAL4 DNA-BD (bait) construct into Y2HGold

Using either the modified *Pichia* method or the small-scale protocol, bait constructs were successfully transformed into *S. cerevisiae* Y2HGold and selected on SD/-Trp media. To test for auto activation (self-activation of reporter genes *HIS3* and *ADE2*), transformed cells were plated onto TDO/-Leu/-Trp/-His and QDO/-Trp/-Leu/-His/-Ade. None of the bait clones were found to activate reporter genes (results not shown).

3.21. GAL4-AD/full-length cDNA plasmid library construction

A typical agarose gel profile of ds cDNA synthesized with the SMART™ protocol can be seen in Figure 3.14. A smear was visible around 100 – 3000 bp, with the high-abundance transcripts centering around 500 - 1000 bp. The optimal cycle number was determined as 21, since no additional amplification was visible thereafter and the amplification of smaller, highly abundant transcripts were suppressed (blocked region, Figure 3.14), indicating that the plateau PCR phase was reached. At the optimal 21 cycles, the size range of amplified fragments is satisfactory, with highly abundant fragments visible as bands with increased signal intensity (indicated by arrows).

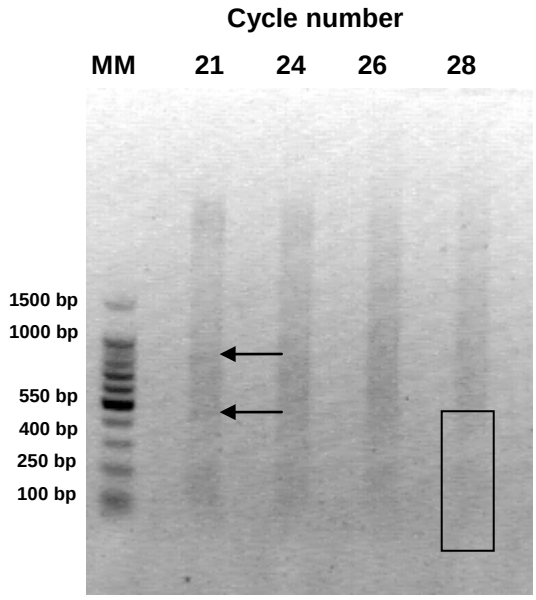

Figure 3.14: Amplification of ds cDNA by LD-PCR using Super SMART™ technology. *LD-PCR amplification of R. microplus mixed lifestages cDNA library. Visualisation was aided by EtBr. The molecular weight marker is indicated, as well as cycle number. The blocked region indicates the suppression of amplification of smaller transcripts (suppression PCR), whilst the arrows indicate the presence of high abundance transcripts.*

After amplification, the ds cDNA library was polished and purified with the NucleoSpin® Extract II kit. Size fractionation was performed with three different methods of which the third was successful. The exclusion of smaller fragments from the ds cDNA library by the three methods was analysed by

agarose gel electrophoresis by submitting the Lambda marker (Promega) to size fractionation. Results of the glass column (Pasteur pipette) method was omitted.

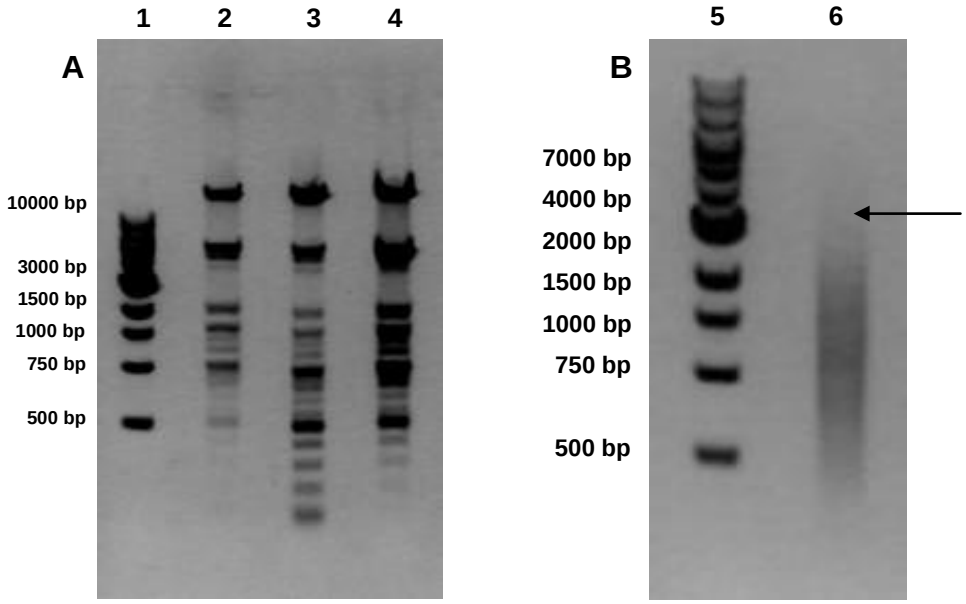

Figure 3.15: Size fractionation of Lambda marker (A) and the amplified ds cDNA (B). *Attempted size fractionation of both the Lambda marker and ds cDNA with PALL Nanosepp 100K columns and Sephacryl S-400. Visualisation was aided by EtBr staining.* ***(A)*** *1: 1000 bp molecular mass marker. 2: Retentate from fractionation of Lambda marker with PALL Nanosepp 100K columns. 3: Eluate from fractionation of Lambda marker with PALL Nanosepp 100K columns. 4: Fractionation of Lambda marker with Sephacryl S-400 in spin columns.* ***(B)*** *5: 1000 bp molecular marker.6: Eluate of ds cDNA fractionated with Sephacryl S-400 in spin columns.*

The retentate from the PALL® Nanosepp 100K column (lane 2, Figure 3.15) only contained fragments larger than 500 bp, which corresponds to its theoretical molecular weight cutoff (MWCO) of 475 – 1450 bp. The 100K eluate however, still contained a large amount of the entire size spectrum of the lambda marker loaded onto the column, indicating that retention was not successful. Attempts were made at improving the ultrafiltration results by varying the centrifugation speed (centrifugal force applied) from the 5,000 x g prescribed, with no significant improvements. Applying the 100K eluate to columns with smaller MWCOs, such as the 30K and 10K columns, also failed to yield any improvements. Alternatively, PALL® Nanosepp columns with higher MWCOs such as 300K (MWCO: 1,450-2,900 bp) or 1000K (MWCO: 4,800-9,500 bp), could have been used. The manufacturers did suggest that ultrafiltration will only accomplish significant separation if molecules to be separated differ by a minimum of 10-times in size. Only 10K, 30K and 100K columns were available at the time and therefore a different fractionation method was investigated.

Fractionation of both lambda marker (Figure 3.15A, lane 4) and ds cDNA library (Figure 3.15B, lane 6) was successful utilizing Sephacryl S-400 resin in a Promega spin column. When comparing lanes 3 and 4, it can be observed that most of the fractions smaller than 500 bp were removed from the eluate. Attempts were made at improving fractionation by varying DNA load and centrifugal force, but results did not improve beyond that observed in Figure 3.15B. Smaller fragments were also removed from the ds cDNA using this method (Figure 3.15B, lane 6), with the high abundance transcripts still centering around 500 - 1500 bp and very high molecular weight (> 3000 bp) transcripts still present (arrow, Figure 3.15B).

3.21.1. Construction of the GAL4/AD plasmid- cDNA library fusion

As mentioned in the methods section, three different methods were followed for the directional cloning of the ds cDNA library into a suitable AD vector. The first SMART method which utilised the *Sfi*I mediated directional cloning into the pACT2 vector could not be mastered. Upon transformation, only empty vector was obtained. If ligation were successful, only a few colonies were obtained. This was thought to be the result of unsuccessful dephosphorylation, but even after the amount of SAP was increased and a new sample thereof purchased from a different company, results were not repeatable. New samples of *Sfi*I were also tried without success. As a last effort to increase the efficiency of ligation, new primers for library amplification were designed. These primers included longer *Sfi*I recognition sequences that would theoretically increase the cutting efficiency, but this approach too, was unsuccessful.

The second approach was based on the Matchmaker™ One hybrid principle of recombination. The *Sma* I linearized pGADT7-Rec2 vector and the amplified cDNA library (using recombination primers) were simply co-transformed into Y2HGold cells and the resulting transformation mixture plated onto SD/-Leu. Even after repeating this procedure, very low transformation efficiencies were obtained.

The Gateway® approach entailed two parts. The first step is an A/T mediated cloning of the AD-cDNA library into the pCR®8/GW/TOPO® vector (containing attL1 and attL2 recombination sites). The second step is a recombination based cloning step into the pDEST-GADT7 vector (containing attR1 and attR2 recombination sites). A/T based cloning into pCR®8/GW/TOPO® was very successful. Single colonies were screened in a colony PCR using the GW1 and GW2 primers, amplified transcripts digested with *Eco*RI and many inserts of different sizes identified (Figure 3.16A). A semi-log plot of molecular size (in bp) vs. migration distance (Figure 3.16B) was used to infer the size of bands outside of the range of the molecular marker used. Because the amplified inserts were digested with *Eco*RI, more than one band was expected. This would include

undigested and digested (insert) fractions. The observation of multiple bands could indicate the presence of more than one recombinant construct in a single clone.

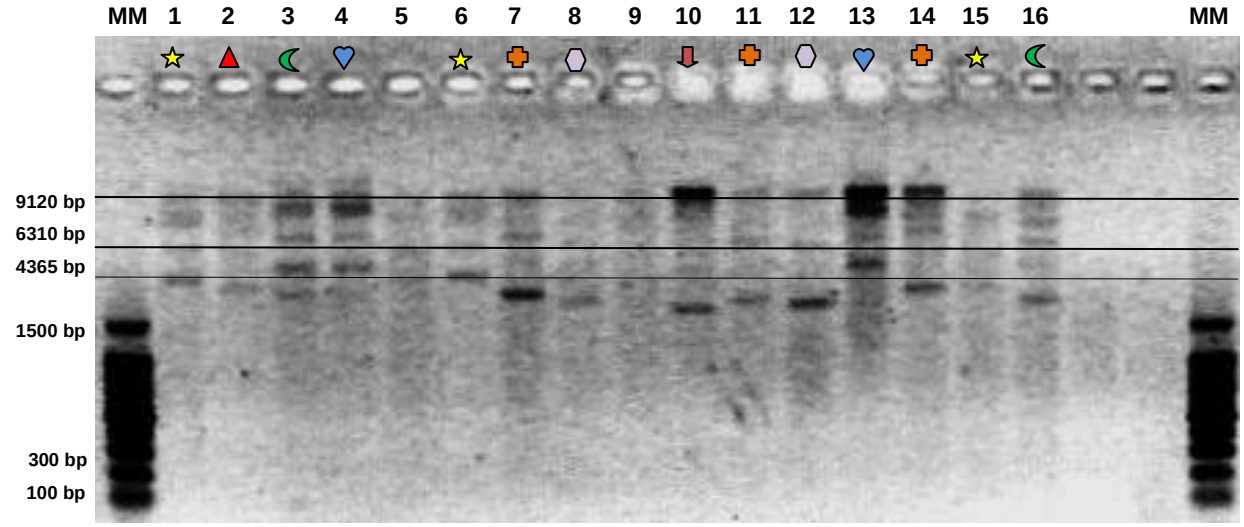

Figure 3.16A: Colony PCR and *Eco*RI digestion of cDNA library inserts from the pCR®8/GW/TOPO® vector. *PCR amplification of inserts directly from DH5α E. coli cells. Products were subsequently digested with EcoRI. Visualisation was aided by EtBr staining. Lane 1: 100 bp Molecular marker (Promega). Lanes 2 - 16: EcoRI digested library inserts amplified from the pCR®8/GW/TOPO® vector. Similar clones are indicated with corresponding symbols.*

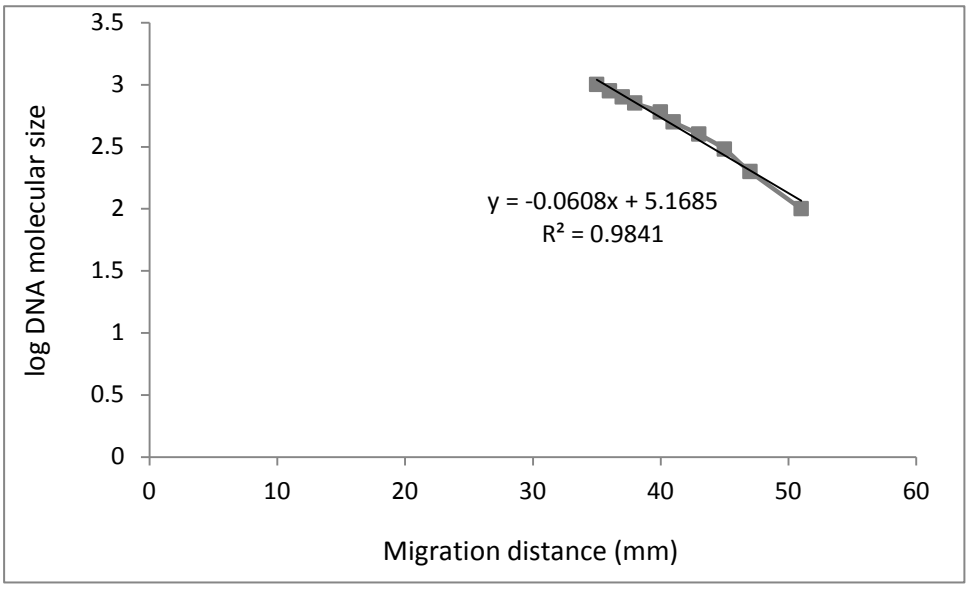

Figure 3.16B: Semi-log curve representing migration size as a function of migration distance. *The R^2 value and function of the trendline are included in the figure.*

Due to the fact that many of the bands were of similar sizes, it was difficult to determine the number of indepent clones. Therefore, it was uncertain whether the number of independent clones

exceeded the recommended 60%. Despite this, it was decided to use this library for recombination into pDEST-GADT7 and subsequent yeast two-hybrid screen. Running the samples on a higher percentage agarose gel may have resulted in improved resolution of similarly sized bands.

After the pCR®8/GW/TOPO® - cDNA library was isolated from *E coli* DH5α using the NucleoBond® PC100 kit, a sample of this DNA was subjected to PCR with the TOPO NF and TOPO NR primers (Figure 3.17). This clearly indicated that a wide size range of inserts was ligated successfully into the pCR®8/GW/TOPO® vector and that these still centered around 500 – 1000 bp (see blocked region). It was also clear that very few smaller (< 500 bp) fragments were available for spontaneous ligation into this vector, again confirming successful fractionation.

The size distribution of bands in Figure 3.16A does not correspond directly with the sizes observed after PCR (Figure 3.17). Figure 3.17 suggests that the most abundant transcripts centre around 900 – 1,000 bp. The large insert sizes however, still correspond to the sizes of transcripts observed in Figure 3.17, even though these transcripts were larger and less abundant. The lack of very large fragments (> 2000 bp) in the PCR amplification (Figure 3.17) can be explained by the more efficient amplification of smaller transcripts during PCR.

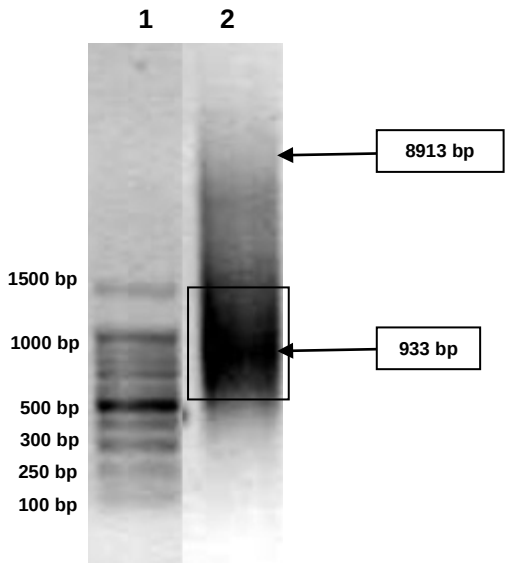

Figure 3.17: Amplification of cDNA library inserts from the pCR®8/GW/TOPO® vector. *PCR amplification of ds cDNA library directly from large-scale TOPO® recombinant construct isolation. Visualisation was aided by EtBr staining. Lane 1: 100 bp Molecular marker (Promega). Lane 2: Library inserts amplified from the pCR®8/GW/TOPO® vector. A semi-log curve was used to infer the size of fragments outside the range of the molecular marker.*

LR recombination was performed with LR Clonase™ II in order to transfer the library inserts from the pCR®8/GW/TOPO® vector to the *Eco*RI linearized pDEST-GADT7 vector via the L-R

recombination sites present in these vectors (Figure 3.18). Figure 3.18 is a graphical representation of the LR recombination reaction performed and the products subsequently produced.

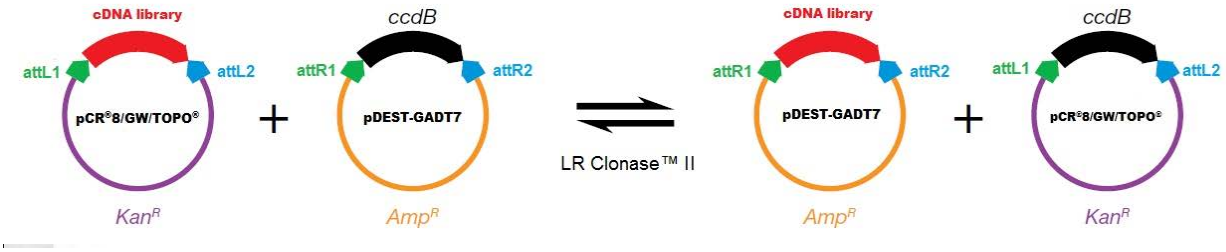

Figure 3.18: Schematical representation of the recombination transfer reaction mediated by LR Clonase™ II (Adapted from (Invitrogen, 2008)**.** *Kan^R refers to kanamycin resistance, and Amp^R to ampicillin resistance.*

The pDEST-GADT7 vector (destination vector) was linearized at the position where the gene of interest (flanked by *att* sites) was to be inserted after recombination. Using linearised vector favours single crossover recombination events (Invitrogen Corporation, 2001). Both cut and uncut vectors are indicated by arrows in Figure 3.19. Extension of the incubation time from the suggested 60 minutes did not have a significant effect on recombination efficiency.

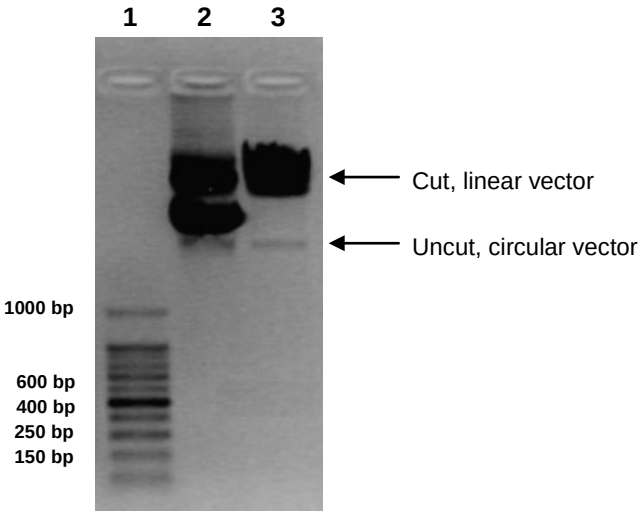

Figure 3.19: Agarose gel electrophoresis of *Eco*RI linearized pDEST-GADT7 vector. *Native pDEST-GADT7 vector was digested with EcoRI prior to recombination. Visualisation was aided by EtBr staining. Lane 1: 100 bp Molecular marker (Promega). Lane 2: Undigested pDEST-GADT7 vector (Intact, coiled). Lane 3: EcoRI digested pDEST-GADT7 (linear).*

The recombination reaction was transformed into *E. coli* DH5α. Single colonies were screened in a colony PCR using the 5'Amplimer and 3'NestedpGAD primers. Inserts of different size ranges were identified (results not shown). The most prevalent sizes for inserts were 500-1,500 bp, although larger fragments were also present. Both bands were verified as ds cDNA inserts, as they were the incorrect size to represent either the amplified MCS of pGADT7 (72 bp) or gateway cassette frame B (1713 bp). The number of independent colonies was deemed sufficient to continue with yeast two hybrid screen.

3.22. Library transformation and two-hybrid screen of reporter genes

The 5x and 10x scale TRAFO transformation protocol was initially followed for the sequential transformation of pGADT7-cDNA library into Y2HGold yeast cells already containing the GAL4 DNA-BD-bait construct. Although successful, transformation efficiencies of no higher than 1 x 10^5 were obtained (efficiencies of 10^6 are a minimum required for a yeast two-hybrid screen). A large-scale version of the electroporation protocol in the EasySelect™ *Pichia* manual was experimentally evaluated, but could not deliver a significant improvement. For this reason, a more efficient protocol was needed.

The Frozen-EZ Yeast Transformation II™ (Zymo Research) was successfully used to obtain high enough transformation efficiencies. Co-transformation efficiency was calculated as follows. The cfu/µg was multiplied with the amount of library plasmid used. As an example for one of the transformations with the Frozen-EZ Yeast Transformation II™ kit, co-transformation efficiency was calculated as 7.2 x 10^4 cfu/µg and the amount of library used was 0.5 µg. The number of clones screened was thus determined as 3.6 x 10^7. These clones were subsequently plated on DDO, followed by plating on TDO and QDO to identify any positive (interacting) clones.

3.23. Nested PCR screen of QDO positive clones

Nested PCR was performed by PCR screening directly from clones on QDO plates. Nested PCR was required due to the high amount of chromosomal DNA present after DNA isolation from yeast. The regions within the MCS of the pGADT7 plasmid used for the design of the two sets of primers and the *Eco*RI linearization site, are indicated in Figure 3.20.

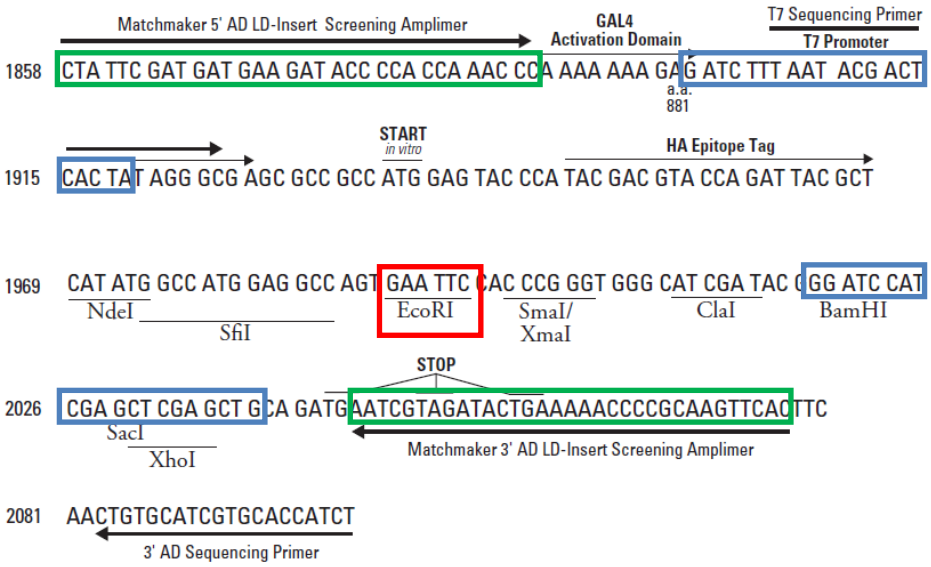

Figure 3.20: Multiple cloning site of the pGADT7 vector. *Indicated are the first (green; 5' and 3'Amplimer) and second (blue; 5' and 3'NestedpGAD) sets of primers required for nested PCR. The EcoRI site used for linearization of the vector prior to recombination, is also indicated (boxed, red).*

Selected colonies were subjected to nested PCR and subsequent restriction enzyme mapping with *Bam*HI and *Nde*I (Figure 3.21). The size range of the clones was between 250 and 1,500 bp, with most transcripts around 800 – 1,000 bp in size.

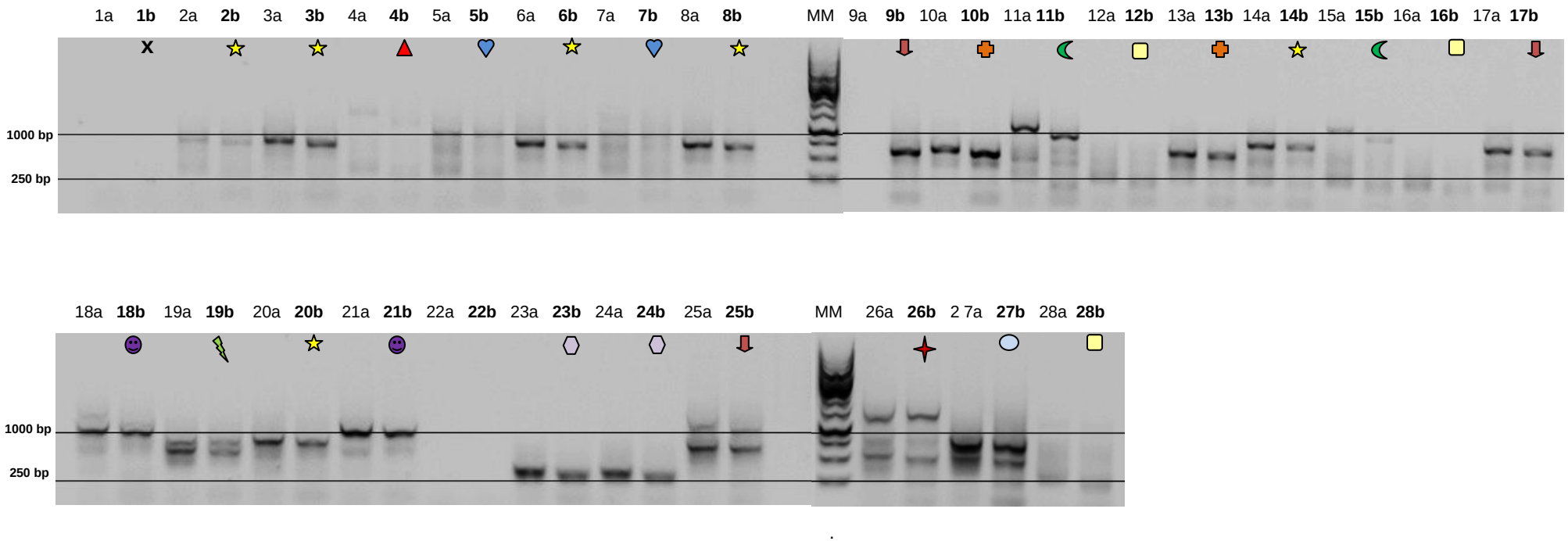

Figure 3.21: *Bam*HI and *Nde*I digestion of amplified fragments from QDO positive colonies. *Nested PCR amplification of inserts directly from QDO positive S. cerevisiae cells. Products were subsequently digested with BamHI and NdeI. Visualisation was aided by EtBr staining. Lane MM: Fermentas GeneRuler® 1 kb DNA ladder. Lanes 1 – 28 correspond to different QDO positive clones. Undigested (a) and BamHI and NdeI digested (b) PCR screen products, are indicated. Similar clones are indicated with corresponding symbols, where x indicates that no unique bands were amplified during nested PCR. Sample 9a was lost during agarose gel loading preparation.*

Unique clones from Figure 3.21 were identified and placed in the table according to size (represented by symbol) and percentage prevalence. Some 12 unique clones were identified, each with very low prevalence amongst the 28 colonies screened. This suggests that a variety of different inserts were identified during the yeast two-hybrid screen. The most prevalent of which seems to be an 800 bp transcript (lanes 2,3,6,8, 14 and 21, Figure 3.21). One clone corresponding to each one of the symbols in Table 3.7 were selected for further analysis.

Table 3.7: Summary of unique clones identified during nested PCR. *The symbols in column 1 correspond to the symbols in Figure 3.21.*

Clone classification	Number of clones	Prevalence (%)
800 bp	6/28	21.4
300 bp; 1,400 bp	1/28	3.6
300 bp; 750 bp	2/28	7.1
600 bp	3/28	10.7
500 bp	2/28	7.1
950 bp	2/28	7.1
200 bp	2/28	7.1
1,000 bp	2/28	7.1
650 bp; 750 bp	1/28	3.6
250 bp	1/28	3.6
500 bp; 1,500 bp	1/28	3.6
450 bp; 650 bp	1/28	3.6
No insert x	2/28	7.1

3.24. Sequencing and analysis of QDO positive clones

The QDO clones contain both the pAS2-1 DNA-BD (bait) plasmids as well as the pGADT7 AD (library) plasmids. *E. coli* KC8 cells have a defect in *leuB* which can be complemented by *LEU2* from the pGADT7 AD (library) plasmid, to select for only the prey plasmid in M9 minimal medium with ampicillin and lacking leucine. Plasmids were isolated from these cells and subjected to sequencing with the 5'AmplimerpGAD primer. No significant identities were attributed to the clones using blastn or blastp and therefore the tblastx and PSI-BLAST programs were used with the BLOSUM62 matrix. As PSI-BLAST is more sensitive to detect weak but biologically relevant sequence similarities between evolutionary more distant proteins, PSI-BLAST analysis were performed using the non-redundant sequences (nr) database for the first approach (Table 3.8). PSI-BLAST and tblastx analysis against the available *R. microplus* (taxid: 6941) non-redundant database, were performed for the second approach (Table 3.9). Hits were only obtained for clones 19, 36 and 45.

PSI-BLAST analysis of the clones using the non-redundant sequences (nr) database identified a few targets of biological relevance (Table 3.8), such as the cell division control protein, alpha 2B adrenergic receptor and C6 transcription factor. The expect (E)-value, which is a parameter that describes the number of hits one can randomly expect to obtain when searching a database of particular size, indicated that similarity was not significant. E- values must be closer to zero (<1 x 10^{-4}) in order to specify a significant match. It should be noted however, that searches with short sequences can have higher E-values, due to the fact that the calculation takes into account the length of the query sequence and size of the database.

Results of both the tblastx and PSI-BLAST runs, performed during the second approach, are shown in Table 3.9. More biologically relevant hits were obtained with lower E-values and greater degrees of confidence, but were still deemed insignificant. It is interesting to note that an adrenergic-like receptor (JN974909.1) was detected for clone 36 during tblastx analysis of the *R. microplus* (taxid: 6941) non-redundant database (Table 3.9), as well as during PSI-BLAST analysis of the non-redundant sequences (nr) database (Table 3.8). *R. microplus* Papilin was detected for both tblastx analysis of clone 19 (E-value = 1.3) and PSI-BLAST analysis of clone 36 (E-value = 5.6). Due to the high E-values, the results are inconclusive and no inferences can be drawn from it.

Table 3.8: PSI-BLAST analysis of clones 19, 36 and 45 using the non-redundant sequences (nr) database.

Clone number	Description	Accession number	E-value	Maximum identity (%)
19	PREDICTED: uncharacterized protein LOC100936722 [*Pongo abelii*]	XP_003776069.1 (scored below threshold on previous iteration)	97	45
	Cell division control protein [*Verticillium albo-atrum* VaMs. 102]>gb/EEY23703.1/	XP_003000093.1 (scored below threshold on previous iteration)	97	45
36	Alpha 2B adrenergic receptor [*Callithrix jacchus*]	CAJ19288.1 (scored below threshold on previous iteration)	14	39
	C6 transcription factor [*Trichophyton tonsurans* CBS 112818]	EGD97428.1 (scored below threshold on previous iteration)	32	53
	PREDICTED: transmembrane protein 22-like [*Oreochromis niloticus*]	XP_003439251.1 (scored below threshold on previous iteration)	37	39
	Inner membrane ABC transporter permease protein YjfF [Burkholderia thailandensis MSMB43]>ref/ZP_18327979.1/	ZP_02467828.1 (scored below threshold on previous iteration)	39	36

Table 3.9: Tblastx and PSI-BLAST analysis of clones 19, 36 and 45 using the non-redundant sequences (nr) database limited to *R. microplus* (taxid:6941).

Clone number	Description	Accession number	E-value	Maximum identity (%)
tblastx				
19	*B. microplus* mRNA for acetylcholinesterase	AJ223965.1	0.039	N/A
	R. microplus papilin (Ppn) gene, partial cds; and pogo transposable element (pogo), hypothetical protein (E3G_000003), hypothetical protein (E3G_000004), endonuclease reverse transcriptase (E3G_000005), and SeRPin family member (srp) genes, complete cds, complete sequence	HM748961.1	1.3	N/A
	R. microplus strain Deutsch clone boom1_13804, complete sequence	HM193853.1	1.3	N/A
36	*B. microplus* paramyosin mRNA, complete cds	AF479582.1	0.004	N/A
	R. microplus strain NRFS beta 2 adrenergic-like octopamine receptor mRNA, complete cds	JN974909.1	0.018	N/A
	B. microplus putative sodium channel mRNA, partial cds	AF134216.2	0.047	N/A
45	*R. microplus* strain Deutsch clone boom1_14607, complete sequence	HM193855.1	8.9	N/A
PSI-BLAST				
19	notch-like protein [*R. microplus*]	AAN06819.1(scored below threshold on previous iteration)	3.9	35
36	Putative secreted protein [*R. microplus*]	ABA55034.1 (scored below threshold on previous iteration)	0.89	64
	papilin, partial [*R. microplus*]	ADK62391.1 (scored below threshold on previous iteration)	5.6	67
	Cytochrome P450 [*R. microplus*]	AAD54000.1 (scored below threshold on previous iteration)	6.7	67

Conclusion

The aim of this investigation was to identify the protein-protein interactions of *R. microplus* EF-1α using the yeast two-hybrid system. After successful cloning of the bait fragment into the appropriate vector, a clone was obtained with a single conservative missense mutation of arginine to histidine. A pre-computed BLAST (BLink) analysis of *R. microplus* EF-1α against the non-redundant sequences (nr) database, revealed that all the transcripts had an arginine in this position. Therefore, it was concluded that the arginine to histidine mutation was not a natural mutation occurring in the tick, but likely occured during the cloning procedure. Because both amino acids have positively charged side chains, this mutation was not thought to have a significant impact on protein conformation and was thus used for subsequent cloning into pAS2-1. The resulting construct was transformed into Y2HGold using either the small-scale TRAFO protocol, or the Zyppy Frozen-EZ Yeast Transformation II™ kit.

A highly representative cDNA library was constructed with transcript size ranging from 100 – 3000 bp, and transcript abundance centering around 500 bp. Three self-developed techniques were undertaken, of which the third (spin columns containing Sephacryl S-400) worked well in removing fragments smaller than 500 bp. The PALL® Nanosepp columns failed, even though the theoretical molecular weight cutoff (MWCO) of the 100K column (475 – 1450 bp) was in the size range required to remove fragments smaller than 500 bp. Even though centrifugation speed was well below that suggested by the manufacturer, the eluate still contained the full size spectrum of dsDNA fragments. The manufacturers however suggest that ultrafiltration will only result in significant separation of molecules with a 10X size difference. Dependency on gravity flow may have negatively influenced separation using the self-packed Sephacryl S-400 column. This may have allowed smaller fragments more time to pass through the resin, resulting in their simultaneous elution with larger fragments in the fraction volumes collected.

For cloning of the cDNA library, the Gateway® approach was successful in delivering a transformation efficiency of 7.2×10^4 cfu/µg. The SMART/*Sfi*I approach may have been impeded by the use of a symmetrical *Sfi*I cut site determined by the *Sfi*I site of the pACT2 vector. This may have resulted in the self-ligation of both the vector (evident by the apparent dephosphorylation failure) and the amplified ds cDNA, explaining the low ligation efficiencies. This could, in turn, have affected the number of independent clones (or representative nature) of the library ligated into the pCR®8/GW/TOPO® and pGADT7 vectors. As an alternative to the SMART/*Sfi*I approach, two unrelated restriction sites could have been introduced as primer adapters.

Upon screening of recombined pGADT7 clones, two prevalent bands were detected at 500 bp and 1500 bp respectively. Although this represented a low number of independent clones, the library

was used for the continuation of the yeast two-hybrid screen. The size of these inserts coincided with the sizes of the most abundant transcripts in the amplified library (Figure 3.16 and Figure 3.17). After the yeast two-hybrid screen of reporter genes, 27 positive clones were identified with nested PCR (Figure 3.23). Inserts ranged in size from 200 bp to >1000 bp, indicating that the library was more representative than previously suggested. Preliminary sequencing results were obtained for 3 of the 27 clones. Hits were obtained for clones 19, 36 and 45 using tblastx and PSI-BLAST analysis of the non-redundant sequences (nr) and *R. microplus* (taxid: 6941) non-redundant databases. E-values were however, too low to make definitive conclusions regarding the function of *R. microplus* EF-1α.

Chapter 4

Concluding discussion

Universally, ribosomal polypeptide synthesis consists of three phases namely chain initiation, chain elongation, chain termination and a possible fourth step termed ribosome recycling (Noble and Song, 2008; Rodnina and Wintermeyer, 2009). Eukaryotic EF-1α functions during the elongation phase as follows: EF-1α is activated by GTP binding where after a ternary complex is formed with aa-tRNAs. Directly on the ribosome, the ternary complex formation of EF-1α leads to the decoding of genetic information via a Watson-Crick interaction which occurs between the mRNA (A-site) and the tRNA anticodon. This results in GTP hydrolysis induction on the ribosome and release of EF-1α-GDP (Andersen *et al.*, 2003; Campbell and Farrell, 2003).

The *R. microplus* EF-1α homolog was identified as a subolesin interacting protein via yeast two-hybrid and co-affinity purification experiments. RNAi experiments have suggested that EF-1α is another possible anti-tick vaccine candidate, since it exhibits a similar phenotype as subolesin upon knockdown and is essential for vital cellular processes such as translation and gene expression (De la Fuente *et al.*, 2008c; Almazan *et al.*, 2010). The mechanisms underlying the translational machinery of ticks and of ixodid ticks in particular, have not been addressed previously. Knowledge regarding the components involved in this vital cellular process is paramount, since these are all potential targets for anti-tick control strategies.

The study under discussion aimed to express *R. microplus* EF-1α in the yeast *P. pastoris* for possible vaccination trials and to investigate its protein-protein interactions using yeast two-hybrid technology for the discovery of new anti-tick vaccine candidates.

4.1. Prokaryotic versus eukaryotic expression of EF-1α

Initially prokaryotic recombinant expression of EF-1α was attempted in *E. coli* JM109. In all experiments, EF-1α (22 kDa) could be identified putatively in both the soluble and insoluble fractions using SDS-PAGE, but this could not be confirmed by western blot. A single band (45 kDa) was later identified with western blot (Figure 2.6), but was deemed too large. It might have been that an intracellular protein aggregate was identified or that the anti-His_6 antibodies used, detected a different protein containing a stretch of histidines. The long-term freezing of the pellets likely caused such aggregation, as environmental stresses often incur structural deviations from the

native (soluble) state. When refolding is attempted, the proteins are not converted to the native structure, but instead assume a partially folded (aggregated) state (Hamada *et al.*, 2009). The 45 kDa band will be analysed using LC-MS-MS. Optimization of prokaryotic expression parameters such as temperature, time and IPTG concentration did not improve yield or solubility. The prokaryotic expression strategy was subsequently abandoned due to repeatability concerns and the putative insoluble nature of the protein.

Almazan *et al*. recently attempted the expression of *R. microplus* EF-1α from the same construct (pQEGII3-1) in *E. coli* JM109 for the purpose of small-scale vaccination trials in cattle. They were unable to produce sufficient quantities of recombinant protein for purification and vaccination and therefore decided to omit EF-1α from the study (Almazan *et al.*, 2010). The same authors were able to express *R. microplus* EF-1α as a chimera to the *A. marginale* surface antigen, MSP1α. Due to the chimeras' association with the bacterial membranes, rigorous treatments with 1% Triton X-100 and 6M guanidine hydrochloride were not successful in extracting the protein. The insoluble fraction after cell disruption was consequently used for vaccination of cattle. Although the protein resulted in high antibody titers in vaccinated cattle, it exhibited low efficacy against *R. microplus* and *R. annulatus* infestations (Almazán *et al.*, 2012). The non-eukaryotic codon usage profile of *E. coli* might additionally have been deleterious to the production of the protein for vaccination (Reyes-Ruiz and Barrera-Saldanya, 2006).

As a result solubility issues experienced during prokaryotic expression, Intracellular expression of EF-1α in *P. pastoris* was attempted. This choice was supported by EF-1α's mostly cytosolic location *in vivo* and the inability of *E. coli* to perform PTMs such glycosylation which contributes to immunogenicity. The *R. microplus* EF-1α transcript was thus cloned in-frame to the pPICZ A vector for eukaryotic, intracellular expression in *P. pastoris.* Mut^+ GS115 and KM71H cells were initially induced for 24 hours with 0.5 % methanol, after which 20 kDa bands could be identified for the GS115 clones on Tricine SDS-PAGE (Figure 2.14). Dot-blot analysis of protein from a small-scale expression study indicated that the recombinant protein was present in the cellular debris (Figure 2.15). Subsequent dot-blot results suggested that the highest concentration of recombinant protein was present for both GS115 and KM71H after 120 hours of 0.5 % methanol induction (Figure 2.16). These clones were subsequently analysed with Tricine SDS-PAGE and western blot during which two bands of 27 – 30 kDa were detected (Figure 2.17). The slightly larger size of these bands can be attributed to the influence of glycosylation when proteins are expressed intracellularly in *P. pastoris* (De la Fuente *et al.*, 2006e). Interestingly, slight protein degradation was observed at 120 hours after induction (in comparison to K15, 72 hours) which was likely due to proteases in the cytoplasm of lyzed cells.

The difference in the results obtained for the small-scale expressions in Spain (5 ml culture, 1 % methanol induction) and locally (25 ml culture, 0.5 % methanol induction), can likely be attributed to the differences in culture volume, as well as the amount of methanol added. The small culture volume used initially might not have been sufficient for the production of detectable amounts of recombinant protein. Additionally, although Mut^+ cells require high feeding rates of methanol, and successful expressions have been performed at levels as high as 3 % (Macauley-Patrick *et al.*, 2005), the 1 % methanol induction used during this experiment could have been detrimental to rEF-1α production in such small culture volumes. The expression strategy reported herein could have been optimized by investigating parameters such as pH of the growth medium (for inactivation of specific proteases), aeration and temperature (Invitrogen Corporation, 2001).

In retrospect, extracellular expression might have been a better choice for EF-1α recombinant expression, due to the fact that intracellular expression is limited by purification difficulties as heterologous protein is usually 1% of total cytosolic protein (Daly and Hearn, 2005). Additionally during intracellular expression, the expressed protein is exposed to all the cytosolic proteases, whereas during extracellular expression the protein is secreted into the growth medium, thus evading the onslaught of these proteases. Isolation and purification is greatly simplified with the heterologous protein in the growth medium and making up to 30% of all secreted proteins. Extracellular expression would additionally have benefited the purpose of EF-1α vaccination, since the protein would have undergone hyperglycosylation in passing through the secretory pathway. Although cytosolic due to its role in translation, Billaut-Mulot *et al*. determined that EF-1α has a cellular distribution that varies between cytosolic and nuclear, meaning that the protein might be directed toward the nucleus when necessary (Billaut-Mulot *et al.*, 1996). Intracellular expression is often chosen for cytosolic proteins, but extracellular is selected for proteins that are transported to other organelles. This further supports the idea of taking the extracellular expression route for EF-1α expression. Furthermore, the group of Prof. J. De la Fuente (IREC, Spain) have successfully expressed various ixodid tick proteins extracellularly in *P. pastoris.* These include soluble proteins such as Bm86 and its homologs, ubiquitin, subolesin, akirin and Bm95, as well as membrane-bound MSP1α chimeras of ubiquitin, subolesin and Bm95 (Almazán *et al.*, 2012; Ben Said *et al.*, 2012).

During expression in both *E. coli* JM109 and *P. pastoris* GS115/KM71H the protein of interest was found in to be associated with membrane (insoluble) fractions. Purification would have entailed a vigorous method of washing, harvesting, solubilisation and ultrafiltration under strongly reducing conditions (6 – 8M Urea) to isolate the protein from these fractions. The effects of such complicated purification steps would have been particularly severe since the initial purpose of the expression of EF-1α was for it to be used as a recombinant vaccine. Such harsh purification steps would result in

the loss of structural epitopes, leaving only linear epitopes available to immune scrutiny (Hammarström *et al*., 2002).

4.2. Yeast two-hybrid identification of EF-1α interacting protein partners

To initiate the Y2H screen with EF-1α, the *R. microplus* EF-1α transcript was cloned in-frame to the pAS2-1 vector. A clone was obtained with a single conservative missense mutation of arginine to histidine. The bait construct was not found to activate reporter genes in the absence of prey transcripts. A highly representative cDNA library was constructed with transcript sizes ranging from 100 – 3000 bps and transcript abundance centering around 500 bps (Figures 3.14 and 3.17).

Due to financial constraints, an appropriate kit for size fractionation of the cDNA library was not available. Resultantly, three self-developed techniques were undertaken, of which the third (Promega spin columns containing Sephacryl S-400) worked well in removing fragments < 500 bp. Ideally it would have been preferable if an established method such as the Chroma Spin™ 400 columns (Clontech) could have been used for the size fractionation of the library. This method is based on the principle of gel filtration chromatography as the columns contain resins designed to exclude contaminating molecules based on pore size. These columns have been found successful in removing fragments <500 bp during the construction of cDNA libraries of various origins (Chen *et al.*, 2009; Shao *et al.*, 2009; Clontech, 2011).

Low transformation efficiencies were obtained with both the one-hybrid recombination approach and the *Sfi*I directional cloning approach previously used by Dr. Christine Maritz-Olivier (De la Fuente *et al.*, 2008c). For the *Sfi*I directional cloning approach, the limiting step was ligation. Colonies obtained after ligation and transformation contained only the empty vector, suggesting that the dephosphorylation of the plasmid was not efficient. This explanation was however, not satisfactory, as SAP was purchased from three different companies without an improvement on ligation success. This method was abandoned, despite its advantages of preventing the loss of the transcript 5' terminal (due to transcripts being selected based on the presence of both unique 5'-and 3' end *Sfi*I sites) and the directional cloning conferred (Zhu Y.Y. *et al.*, 2001). The SMART/*Sfi*I approach may have been impeded by the use of a symmetrical *Sfi*I cut site determined by the *Sfi*I site of the pACT2 vector. This may have resulted in the self-ligation of both the vector (evident by the apparent dephosphorylation failure) and the amplified ds cDNA, explaining the low ligation efficiencies.

The Gateway® approach was successful for cloning of the cDNA library, delivering a transformation efficiency of 7.2×10^4 cfu/µg. Although it was successful in generating a representative library, the

Gateway® system has a few disadvantages. The TOPO® vector utilizes A/T cloning mediated by topoisomerase. As a result, the cDNA library transcripts are not directionally cloned into the entry vector. Seeing as the transcripts were merely transferred to the destination vector (pDEST-GADT7) via recombination, this vector also contained non-directionally cloned inserts. The sub-cloning performed may also have reduced the frequency of occurrence of individual cDNA library transcripts. These facts may have significantly decreased the occurrence of representative cDNA library transcripts and thus possible interacting proteins.

Obstacles were encountered upon the sequential transformation of the Gateway® library into *S. cerevisiae* AH109 using the TRAFO protocol. Transformation efficiencies obtained were always in the range 10^2 – 10^3, except for one occasion when 10^4 was obtained. Similar results were obtained when control plasmids were used for yeast transformation. One explanation is the quality of the PEG-4000 and LiOAc used. Hygroscopic PEG must be prepared to the proper concentration and the accumulation of water prevented to avoid a decrease in effective concentration. Although fresh solutions were prepared, the transformation efficiency remained low. Another problem considered, was the expediency of the AH109 yeast cell stocks initially used, due to time these cells were stored at -70ºC and the fact that they were acquired in 2005. Even when a new transformation method (Frozen-EZ Yeast Transformation II™ kit) was initially tested on *S. cerevisiae* AH109 cells, the transformation efficiency still did not improve. It was only after the Y2H Gold® yeast strain was obtained from the University of Stellenbosch, that sufficient transformation efficiencies were attained.

One reason for the few positive clones detected on QDO, is the occurrence of false negatives due to limitations of the screening method. The DNA-BD or AD site may have impeded the normal interaction site via steric hindrance, or conditions inside the yeast cells may have prevented the correct folding and PTMs of the protein (Brückner *et al.*, 2009). It might also be that the complexity of the library was not sufficient, and that some putative interactors were thus under-represented in the library, preventing positive interactions. Recombinant, ixodid EF-1α was observed to aggregate during expression (chapter 2). This may also have impeded putative interactions of rEF-1α during the yeast two-hybrid screen. The fact that the C-terminal EF-1α fragment cloned into the pAS2-1 vector was the partial coding sequence, may mean that there were interacting domains present in N-terminal end of the protein that were thus neglected during the study (such as domain I of EF-1α). It is therefore advisable to identify the complete coding sequence by e.g. rapid amplification of cDNA ends (RACE) or via the CattleTickBase and B*mi*GI Version 2.1 databases, before attempting another yeast two-hybrid screen.

Despite possible impediments, 27 clones were identified and three successfully sequenced after the yeast two-hybrid screen. Unfortunately, neither the 5'AmplimerpGAD, nor the GAL4 AD sequence could be identified within these sequences, complicating the determination of the reading frame. Hits were obtained for three clones (19, 36 and 45) using tblastx and PSI-BLAST analysis of the non-redundant sequences (nr) database. E-values were however, too high to make any conclusions regarding the function of *R. microplus* EF-1α. Hits obtained that are of biological relevance include a cell division control protein, alpha 2B adrenergic receptor and C6 transcription factor (Table 3.8), as well as acetylcholinesterase, paramyosin, adrenergic-like octopamine receptor and cytochrome P450 (Table 3.9). These hits correspond to the pathway involvements proposed for EF-1α, including cytoskeletal organization, microtubule assembly, cell division, mitosis, signalling cascades, plant development and tissue degeneration as well as vesicular transport (Tables 3.1 and 3.4).

4.3. Future considerations.

If time had allowed it, the system used in this study (intracellular expression, *P. pastoris*) could have been optimised and subsequently scaled-up to bioreactor fermentation for large-scale protein production. However as suggested previously, its membrane-associated nature would have been a significant hindrance to purification from cell lysates.

As mentioned before, extracellular expression in *P. pastoris* can be considered for the production of recombinant EF-1α. Other parameters that can be investigated are: lowering of culture temperature, promoter variation (possible vector variation), modified growth media, solubility enhancing tags, host variation, the inclusion of fusion protein partners and improved refolding techniques subsequent to expression (Baneyx and Mujacic, 2004; Roodveldt *et al.*, 2005). Obtaining the full-length *R. microplus* EF-1α sequence via RACE or the CattleTickBase and B*mi*GI databases might result in the expression of a more stable recombinant protein. Because tick codon usage bias is affected by the high GC-content of the genome, codon optimisation can be employed to better reflect the codon usage bias of the expression host used (in this case *E. coli* and *P. pastoris*). This might improve expressed protein stability and subsequent ease of purification.

Following two-hybrid screening, preliminary sequencing results were obtained for three clones (19, 36 and 45) using tblastx and PSI-BLAST analysis of the non-redundant sequences (nr) database (Tabes 3.8 and 3.9). E-values were however, too high to make any definite conclusions regarding the function of *R. microplus* EF-1α. Hits obtained that exhibit lower E-values, can be verified in a subsequent yeast two-hybrid screen or co-affinity purification experiment. The methodology for this approach would include that transcripts are amplified with the correct primers to facilitate directional

cloning into an expression vector. A good consideration might be to perform 5' and-3' RACE in order to identify the full coding sequences for cloning into an expression vector or yeast two-hybrid vector of choice. Alternatively, with the partial genome data now available on the CattleTickBase and B*mi*GI databases, these databases can be searched for the full-length sequences of potential interactors. These constructs can then be used for yeast two-hybrid screening or attempted expression of recombinant proteins for immuno-precipitation.

In order to improve the yeast two-hybrid results, the following factors might be considered for future screens. Firstly, the amount of library cDNA and heat shock time can be varied to determine the optimum, so as to eliminate transformants that take up more than one plasmid which greatly complicates positive colony analysis. Secondly, the amount of carrier DNA required for the yeast strain used can be tested, as well as the amount of PEG added. The latter is especially important as the concentration of PEG solutions can vary due to the viscosity and hygroscopic nature of the compound (Gietz R.D., 1996). Thirdly, more effort can be invested in the preparation of a more representative library containing longer cDNAs typical of full-length clones and 3'-ends. This can be achieved by utilizing alternative cDNA priming methods. A viable alternative that may be considered in order to avoid the problems with transformation efficiency after sequential transformation, is the separate transformation of bait and prey constructs into haploid yeast strains. These haploid strains are mated to give co-transformed, diploid strains.

It might be valuable to try an alternative yeast two-hybrid promoter method, such as the LexA system which is more sensitive in its detection of binary interactions (K_d of 1nM - 1μM), or the Split-ubiquitin system where the interaction site is the cytosol. The latter might be specifically suited to EF-1α, a mostly cytosolic protein. The ultimate goal is to identify a yeast strain/plasmid combination that results in a successful yeast two-hybrid screen (Gietz R.D., 1996). Many interactions and functions other than translation elongation have however been identified for EF-1α in this manner, including cytoskeletal organization, microtubule assembly, cell division, mitosis, signalling cascades, plant development and tissue degeneration and vesicular transport (Table 3.4).

Chapter 4: Concluding Discussion.

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