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I*n vivo* Expression and *in vitro* Regulation of Salivary Histatin 3

A thesis submitted to the University of Dublin in fulfilment of the requirements for the degree Doctor of Philosophy by

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September 2006

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Declaration

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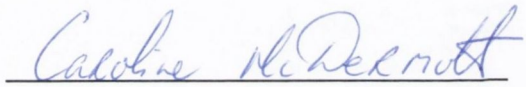

Caroline McDermott

This thesis is dedicated with great affection to my parents

ARTHUR: The Lady of the Lake, her arm clad in the purest shimmering samite, held aloft Excalibur from the bosom of the water signifying by Divine Providence that I, Arthur, was to carry Excalibur. That is why I am your king!

DENNIS: Listen, strange women lying in ponds distributing swords is no basis for a system of government. Supreme executive power derives from a mandate from the masses, not from some farcical aquatic ceremony. You can't expect to wield supreme executive power just 'cause some watery tart threw a sword at you! I mean, if I went 'round saying I was an emperor just because some moistened bint had lobbed a scimitar at me, they'd put me away!

Monty Python and the Quest for the Holy Grail, 1975.

Summary

Human salivary glands and the oral mucosa secrete a wide spectrum of antimicrobial agents which are believed to be essential for the protection and maintenance of a healthy oral environment. Histatins are believed to play a key role in controlling opportunistic fungal pathogens, such as *Candida albicans*, in the oral cavity. An increase in the immunocompromised population over the last few decades has established *Candida* spp. as clinically important oral fungal pathogens. Widespread use of the limited numbers of antifungal agents available to treat candidal infections has led to the rapid development of drug-resistant strains, which are the main cause for antifungal treatment failures. Histatins are naturally occurring antimicrobial peptides which have potent anticandidal activity at physiological concentrations, are non-toxic to mammalian cells, and do not induce resistance. Their mechanism of action is distinct from that of the azole-based antifungal drugs, and they are fungicidal against both azole-susceptible and azole-resistant *Candida* strains. Therefore, they represent a promising therapeutic alternative to current antifungal agents in the treatment of oral candidiasis.

An extensive amount of work has been performed on assessing the *in vitro* antifungal activity of histatins against *C. albicans*. However, there is no direct evidence which proves that histatins are essential to oral health. One important objective of this study was to generate transgenic mice which constitutively express histatin in their salivary glands. Only humans and Old World primates secrete histatin in their saliva, therefore mice provide an ideal model in which to investigate the effects of histatin on oral yeast carriage rates *in vivo*, and to investigate whether the expression of salivary histatin will prevent or attenuate the development of oral candidiasis. The cytomegalovirus promoter was used to direct expression of the transgene to mouse salivary glands. Transgenic mice expressing histatin 3 protein in their salivary glands were successfully generated in this project. Transgene expression was found in all of the positive transgenic mouse tissue-types examined, with highest expression levels observed in the submandibular/sublingual glands, liver, and lung. The histatin-positive transgenic mice generated in this study may be useful for assessing the therapeutic potential of histatins against a range of *Candida* spp., including azole-resistant candidal isolates.

Several small clinical studies performed to date in humans suggest a dynamic relationship between salivary histatin levels and factors such as oral yeast carriage rates, the immune status of the patient, oral candidiasis, patient age, or certain medications. However, there is no direct evidence to prove that histatins are differentially regulated during health and disease. Investigation into some of the external stimuli and *cis*-elements, which may be responsible for histatin gene regulation, was performed in this study. This study presents evidence that the *HIS2* promoter is regulatable *in vitro*. It was found that the 4 kb 5′-flanking region of the *HIS2* promoter contains inducible promoter and enhancer elements after stimulation by microbial products, proinflammatory cytokines, and phorbol myristate acetate *in vitro*. Deletional analysis has located the responsive promoter regions, which include several putative NF-κB, AP-1, and Elk-1 binding sites. This suggests histatins may be regulated by multiple signaling pathways, including NF-κB, protein kinase C, and MAPK pathways. These *cis*-elements have been implicated in the regulation of several other innate antimicrobials involved in mucosal immunity. HIV targets several parts of the NF-κB pathway, which may explain why histatins appear to be down-regulated in HIV-infected individuals. This suggests that histatins may be key constituents of the oral innate immune system, and may play an important role in protecting the oral cavity from fungal infection. Further investigation is required to elucidate the exact molecular mechanisms responsible for regulation of the *HIS2* gene.

In conclusion, transgenic mice which express histatin in their salivary glands were generated in this study. These histatin-positive transgenic mice represent an invaluable tool for studying the efficacy of histatin against *Candida* in the oral cavity. These mice will enable investigation into the therapeutic potential of histatin in preventing or attenuating the development of oral candidiasis, treating azole-resistant oral and systemic candidiasis, its synergistic activity with other antifungal agents, and its efficacy against other medically important bacteria and fungi. This study is the first to report transcriptional regulation of the *HIS2* promoter *in vitro*, and identified several stimuli responsible for up-regulation of the promoter. Identification of stimuli and *cis*-elements which up-regulate the *HIS2* gene may lead to therapies which increase endogenous levels of histatins in saliva, and thus, the resolution of oral candidal infection.

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Abbreviations

A_{260}, A_{280}, A_{600}	absorption at 260 nm, 280 nm, 600 nm
AIDS	acquired immunodeficiency syndrome
azole	synthetic N-substituted azoles, including the imidazole and triazole antifungal antibiotics
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
CE	capillary electrophoresis
CFU	colony forming unit
CMV	cytomegalovirus
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	dideoxynucleoside
ED_{50}	molar concentration required to kill half the maximum number of cells
EDTA	ethylenediaminetetraacetic acid
e.g.	for example
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
ES	embryonic stem
et al.	and others
g	gram(s)
g	gravity

GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GMMs	genetically modified microorganisms
GMOs	genetically modified organisms
h	hour(s)
H3	histatin 3 peptide
HIS1	histatin 1 gene
HIS2	histatin 3 gene
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
HSG	human salivary gland cell line
IC_{50}	concentration required for 50% cell inhibition
ICM	inner cell mass
i.e.	that is
IFN-γ	interferon gamma
IL-1β	interleukin 1 beta
IRES	internal ribosomal entry site
kb	kilobase pair
kDa	kilodalton
kg	kilogram
l	litre(s)
L agar	Luria-Bertani agar
L broth	Luria-Bertani broth
LC_{50}	lethal concentration required for 50% cell killing
LPS	lipopolysaccharide
M	molar

µM	micromolar
mg	milligram
µg	microgram
ml	millilitre
µl	microlitre
min	minute(s)
mRNA	messenger ribonucleic acid
MAIDS	murine acquired immune deficiency syndrome
MCS	multiple cloning site
MMTV-LTR	mouse mammary tumour virus-long terminal repeat
MTE	multiple tissue expression
ng	nanogram
nm	nanometre
Ni-NTA	nickel nitriloacetic
no.	number
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PGN	peptidoglycan
PMA	phorbol myristate acetate
rDNA	ribosomal DNA
RNA	ribonucleic acid
RNase	ribonuclease

rpm	revolution per minute
RT-PCR	reverse transcriptase polymerase chain reaction
s	second(s)
SCID	severe combined immune deficiency
SDS	sodium dodecyl sulphate
SMSL	submandibular/sublingual
spp.	species
TLRs	Toll-like receptors
TNF-α	tumor necrosis factor alpha
tris	tris (hydroxymethyl) aminoethane
U	unit
UV	ultra violet
v/v	% volume in volume: expresses the number of ml of an active constituent in 100 ml of solution
w/v	% weight in volume: expresses the number of grams of an active constituent in 100 ml of solution
YEPD agar	yeast extract peptone agar
YEPD broth	yeast extract peptone broth
>	greater than
<	less than

Chapter 1

General Introduction

1.1 Oral innate immune system

1.1.1 Protective effects of saliva and the oral mucosa

Human salivary glands and the oral mucosa secrete a wide spectrum of antimicrobial agents which are believed to be essential for the protection and maintenance of a healthy oral environment (Oppenheim *et al.*, 1988; Weinberg *et al.*, 1998). Whole saliva consists of a mixture of molecules derived in large part from the secretions of the major salivary glands, consisting of the parotid, submandibular and sublingual glands, with contributions from minor salivary glands, gingival crevicular fluid, and the mucosal epithelium (Oppenheim *et al.*,1988). Antimicrobial peptides present in saliva include histatins, lactoferrin, lysozyme, cystatins, defensins, mucins, agglutins, secretory leukocyte proteinase inhibitor, tissue inhibitors of proteinases, chitinase, peroxidase, and calprotectin (Schenkels *et al.*, 1995). Histatins are believed to play a key role in controlling opportunistic fungal pathogens, such as *Candida albicans*, in the oral cavity (Pollock *et al.*, 1984; Xu *et al.*, 1991). Saliva also provides protection by constantly flushing non-adhered microbes, their toxins and nutrients out of the mouth (Schenkels *et al.*, 1995). The oral mucosa is a moist layer of semi-permeable tissue lining the mouth, and includes epithelial cells, fibroblasts, neutrophils, macrophages, lymphocytes, and dendritic cells. The mucosae provides a barrier to microbial invasion through physical interference, phagocytosis, secretion of defensins, cytokines, chemokines, and proteases, and selective exudation of serum components.

The functional importance of saliva is evident in patients with reduced salivary flow (Schenkels *et al.*, 1995). Hyposalivation results in a painful tongue and mucosa, problems with taste, swallowing, chewing, and phonation, tooth decay and tooth loss, and increased risk for oral infection (Mandel *et al.*, 1989; Axell *et al.*, 1992; Schenkels *et al.*, 1995). This reduced saliva volume can be caused by diseases such as AIDS, Sjogren's syndrome, radiation therapy for head/neck cancers, or certain medications (antihypertensives, antidepressants, and antihistamines) (Valdez *et al.*, 1993).

1.1.2 Oral candidiasis

Oral candidiasis is an opportunistic fungal infection caused primarily by *C. albicans*, a dimorphic organism that is part of the normal microflora of the mucosal surfaces of the oral cavity, gastrointestinal and reproductive tracts (Samaranayake, 1992). *C. albicans* is the

most common and pathogenic of the *Candida* species isolated from the oral cavity, but other *Candida* spp. are also frequently recovered including *C. tropicalis*, *C. glabrata*, *C. pseudotropicalis*, *C. guillierimondii*, *C. krusei*, *C. lusitaniae*, *C. parapsilosis*, and *C. stellatoidea* (Akpan and Morgan, 2002). The prevalence of *C. albicans* is 45%-65% in healthy children and 30%-45% in healthy adults. Despite these high reported carriage rates, this commensal yeast generally causes no problems among the immunocompetent population (Akpan and Morgan, 2002).

1.1.2.1 Predisposing factors

Oral candidiasis is the most common human fungal infection, and occurs when the normal microflora is altered by local or systemic factors, leading to overgrowth of *Candida* species and invasion of mucosal tissues. Oral infection with *Candida* may lead to local discomfort, an altered taste sensation, dysphagia from oesophageal overgrowth resulting in poor nutrition, slow recovery, and often prolonged hospital stay (Akpan and Morgan, 2002). There are four main clinical variants of oral candidiasis: pseudomembranous, hyperplastic, angular cheilitis, and erythematous (Fig. 1.1) (Axell *et al.*, 1997; Ellepola and Samaranayake, 2000; Akpan and Morgan, 2002). Pseudomembranous candidiasis (thrush) is characterized by extensive white pseudomembranes consisting of desquamated epithelial cells, fibrin, and fungal hyphae. These white patches occur on the surface of the labial and buccal mucosa, hard and soft palate, tongue, periodontal tissues, and oropharynx. Hyperplastic candidiasis characteristically occurs on the buccal mucosa or lateral border of the tongue as speckled or homogenous white lesions. This condition can progress to severe dysplasia or malignancy and is sometimes referred to as candidal leukoplakia. Angular cheilitis is an erythematous fissuring at one or both corners of the mouth. (Akpan and Morgan, 2002). Erythematous candidiasis occurs on the dorsum of the tongue, palate, buccal mucosa, or gingivae. Lesions on the dorsum of the tongue present as depapillated areas (Ellepola and Samaranayake, 2000). Predisposing factors to the development of oral candidiasis include the use of broad-spectrum antibiotics, inhaled steroids, immunosuppressive agents in organ transplant recipients, radiation therapy for head/neck cancers, denture prostheses, drugs that reduce salivary secretions, smoking, human immunodeficiency virus (HIV), diabetes mellitus, Sjogren's syndrome, Cushing's

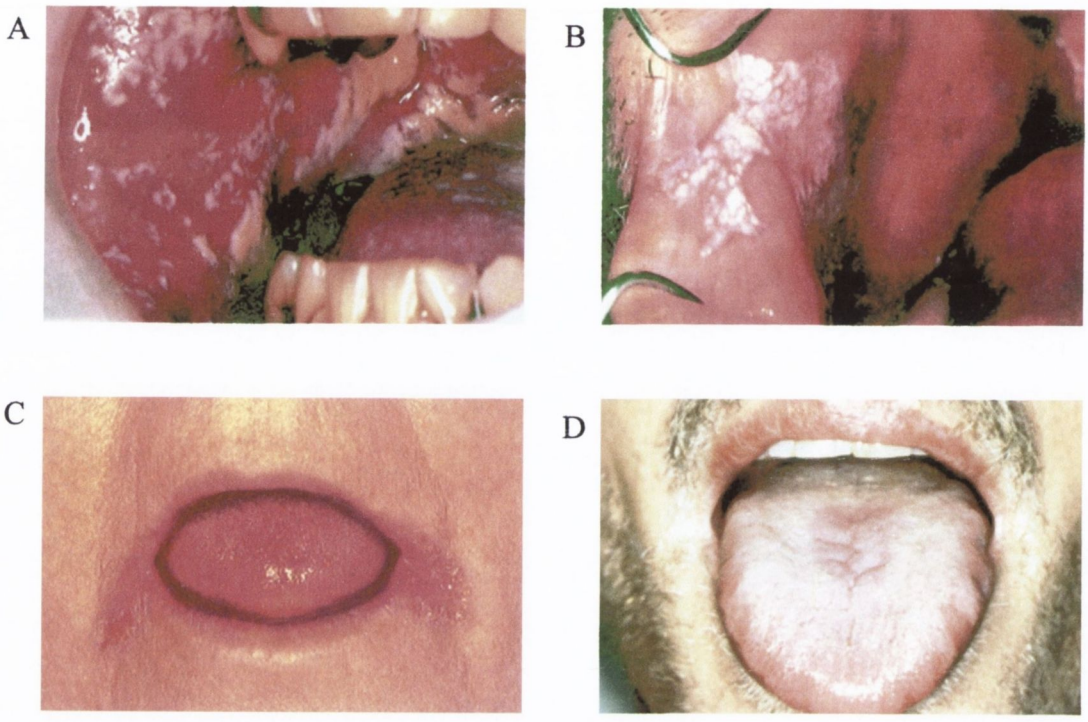


Figure 1.1 Different types of oral candidiasis. **A**, acute pseudomembranous candidiasis, **B**, chronic hyperplastic candidiasis, **C**, angular cheilitis (Akpan *et al.*, 2002), and **D**, erythematous candidiasis (Copyright©1996-2000 David Reznik, DDS).

syndrome, malignancies such as leukaemia, extremes of age, and iron and vitamin deficiencies (Akpan and Morgan, 2002).

1.1.2.2 HIV-infected population

Oral *Candida* infections have received much attention due to the advent of HIV. Up to 90% of HIV-infected individuals suffer from oropharyngeal candidiasis at some stage during their disease (Samaranayake, 1992), with relapse rates between 30% and 50% on completion of antifungal treatment in severe immunosuppression (Phillips *et al.*, 1996). In severe cases, candidal infections can spread to the bloodstream, which can lead to dissemination to the brain, heart, kidneys, eyes, and other tissues, leading to significant morbidity and mortality. Systemic candidiasis carries a mortality rate of 71% to 79% (Akpan and Morgan, 2002). *C. albicans* is the most common of the *Candida* spp. isolated from candidal lesions, with *C. dubliniensis*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei* also isolated at lower frequency (Samaranayake and Samaranayake, 1994; Coleman *et al.*, 1997a,b).

1.1.3 Emerging resistance to antifungals

Antifungal agents currently available for the treatment of candidal infections consist of the polyenes (amphotericin B, nystatin), the imidazoles (clotrimazole, econazole, ketoconazole, miconazole), the triazoles (fluconazole, itraconazole, voriconazole, posaconazole), and the enchinocandins (caspofungin, micafungin, anidulafungin). Many drug-resistant strains are emerging in response to widespread and prolonged use of these antifungal agents, primarily the azoles, making the clinical management of oral candidiasis increasingly difficult (Situ and Bobek, 2000). Resistance to amphotericin B has been observed in *C. albicans* clinical isolates (Helmerhorst *et al.*, 1999a). Fluconazole-resistance has also been observed in clinical isolates of *C. albicans* (Helmerhorst *et al.*, 1999a; Situ and Bobek, 2000), and *C. glabrata* (Situ and Bobek, 2000). Although *C. dubliniensis* is susceptible to most antifungal agents, Moran *et al.* (1997) has reported isolates with reduced fluconazole susceptibilities taken from AIDS patients with prior exposure to fluconazole. These authors also demonstrated that unlike *C. albicans*, *C. dubliniensis* could rapidly develop stable resistance to fluconazole following direct exposure to the drug *in vitro*. These findings may

have implications for antifungal drug treatment regimens, and suggest that antifungal resistance may be a factor in the emergence of *C. dubliniensis* infection. *C. albicans* is by far the most common cause of candidal infection, but the incidence of candidiasis caused by other species, such as *C. glabrata, C. tropicalis*, and *C. krusei*, has also increased (Pfaller *et al.*, 1996). These latter species tend to be less susceptible to commonly used antifungal agents, such as fluconazole, and it has been suggested that this might account for their emergence as significant pathogens (Johnson *et al.*, 1995; Berrouane *et al.*, 1996). Resistance to voriconazole, a new triazole agent, has recently been described in *C. parapsilosis* (Moudgal *et al.*, 2005). The enchinocandins are a new class of β-glucan synthase inhibitors, and have proved to be extremely safe in comparison to the other classes of antifungal agents (Kauffman, 2006). However, resistance to the only current member in therapeutic use, caspofungin, has already been reported in *C. albicans*, *C. glabrata*, and *C. parapsilosis* isolates (Hernandez *et al.*, 2004; Moudgal *et al.*, 2005; Krogh-Madsen *et al.*, 2006).

1.2 Human salivary histatins

1.2.1 Discovery and biochemical isolation

The histatin family of proteins was initially detected in human saliva by several groups of researchers using electrophoretic and chromatographic methods (Steiner *et al.*, 1968; Bonilla, 1969), and was of particular interest since it displayed a highly basic nature. Using an acid-urea starch gel electrophoresis system and a stain specific for arginine-rich basic proteins, Azen (1973) identified a family of five proteins that he named the Parotid basic proteins (Pbs) that were rich in basic amino acids and had a low molecular weight. Hay (1975) examined parotid saliva using anion exchange chromatography and isolated components that had adsorped selectively to hydroxyapatite. One of these components displayed a high content of histidine upon chemical analysis, and was mistakenly identified as histones. Baum *et al.* (1976) used a four-step chromatographic procedure and cationic polyacrylamide gel electrophoresis to isolate five cationic histidine-rich proteins from parotid secretions. MacKay *et al.* (1984) modified the cationic electrophoresis system of Baum and detected seven major and seven minor histidine-rich proteins (HRPs). Oppenheim *et al.* (1986) used anionic, cationic, and gel filtration chromatographic methods

to purify the neutral HRP from parotid saliva. Initial attempts to purify the HRPs to homogeneity using conventional ion-exchange chromatography and gel filtration chromatography proved difficult. These methods provided fractions rich in HRPs but not in pure form and yielded minimal recovery. Final purification of individual HRPs was achieved using reverse-phase high performance liquid chromatography (HPLC) providing high yields of purified, individual HRPs. Cationic electrophoresis was used to monitor purity (Oppenheim *et al.*, 1988). Oppenheim's group proposed to name these proteins "histatins", based on their histidine content and antimicrobial properties.

1.2.2 Histatin antimicrobial function

1.2.2.1 Initial characterisation of antifungal activity

The potent antifungal properties of salivary histatins against *C. albicans* were first reported by Pollock *et al.* (1984). This group used a mixture of histatins purified from parotid saliva, and based on an average molecular weight of 7,500 for the mixture, the histatins were found to be active at 10^{-5} M, which is within the range of effective candidacidal concentration of the imidazole antibiotics miconazole and clotrimazole (Pollock *et al.*, 1984). Pollock *et al.* reported the histatins were antifungal in that they inhibited the growth and viability of *C. albicans*. Xu *et al.* (1991) carried out a more detailed functional characterisation of the anticandidal activities of histatin 1, histatin 3, and histatin 5 investigating the ability of these peptides to kill blastoconidia and germinated cells, and to inhibit germination (Table 1.1). While histatin 5 is the most potent of the histatins in killing the blastospore and germinated forms of *C. albicans*, histatin 3 is the most efficient at inhibiting germ tube formation. The conversion of blastospores to the germinated form is considered an important factor in the development of oral candidal infections (Xu *et al.*, 1991). The levels of histatins required to exert their anticandidal activity *in vitro* fall within the physiological range of histatin concentration (15 to 30 μM) (Xu *et al.*, 1991; Gusman *et al.*, 2004).

1.2.2.2 In vitro measurement of histatin anticandidal activity

The measurement of histatin anticandidal activity used by most researchers is a modification of the method previously described by Xu *et al.* (1991). This method is

Table 1.1 Comparison of the anticandidal activities for histatin 1, histatin 3, and histatin 5 against *C. albicans* (Xu *et al.*, 1991)

Activity	LC_{50} (μM)		
	Histatin 1	**Histatin 3**	**Histatin 5**
Killing of blastoconidia	6.3 ± 0.2	4.2 ± 0.8	2.0 ± 0.2
Killing of germ tubes	72 ± 5	12 ± 1.2	9.7 ± 1.3
Inhibition of germination	52 ± 4	39 ± 3.7	49 ± 9.6

LC_{50}, lethal concentration required for 50% cell killing.

considered the gold standard for the measurement of histatin antimicrobial activity. It has several advantages over the microtitre plate assay used for the measurement of the anticandidal activity of antifungal drugs. The histatin candidacidal assay involves incubation of *Candida* cells with a range of histatin concentrations for 1 h. Killing activity is calculated as the number of colony forming units (CFU) on test plates as a percentage of CFU on control plates (cells incubated in the absence of histatin). The microtitre plate assay involves incubation of *Candida* cells with a range of antifungal drug concentrations over a 24 h period. Histatins are quickly degraded, and therefore a short incubation is preferable to measure histatin anticandidal activity. After 1 h, histatins are degraded, and *Candida* cells would recover. Antifungal drugs are more stable at 37°C for a longer period of time. Also, the histatin assay measures cell viablility by plating, whereas the microtitre plate assay measures optical density of the culture.

1.2.2.3 Antifungal activity against azole-susceptible and -resistant Candida spp.

Widespread use of the limited numbers of antifungal agents available to treat candidal infections has led to the rapid development of drug-resistant strains, which are the one of the main causes for antifungal treatment failures (Situ and Bobek, 2000). As most of the currently available drugs are directed against the ergosterol moiety in the fungal membrane (polyene antimycotics), or against enzymes involved in the biosynthesis of ergosterol (azole antimycotics), there is a threat of cross-resistance (White *et al.*, 1998), and a clear demand for a new class of antifungals with a mode of action against *Candida* spp. distinct from that of azole-based antifungal drugs (Helmerhorst *et al.*, 1999a).

The three main histatins, histatin 1, histatin 3, and histatin 5, have been shown to kill *C. albicans* at low, micromolar concentrations, with histatin 5 having the most potent activity (Pollock *et al.*, 1984; Oppenheim *et al.*, 1988; Raj *et al.*, 1990; Xu *et al.*, 1991). Histatin 5 and its derivatives have demonstrated *in vitro* antifungal activity against both azole-susceptible and -resistant *Candida* spp., and other medically important fungi, at physiological concentrations (Tsai *et al.*, 1997a,b; Helmerhorst *et al.*, 1999a; Situ and Bobek, 2000; Rothstein *et al.*, 2001). Histatin 5 possesses potent antifungal activity against azole-susceptible and -resistant strains of *C. albicans* (ED_{50} values of 6.7 μM and 6.4 μM, respectively), against amphotericin B-susceptible and -resistant *C. neoformans* strains (3.7

μM and 3.8 μM, respectively), and against *C. krusei* (6.4 μM) (Situ and Bobek, 2000). Azole-susceptible and -resistant strains of *C. glabrata* showed reduced susceptibilities to histatin 5 (ED_{50} values of 38.7 μM and 85.4 μM, respectively) compared to that of *C. albicans* (Situ and Bobek, 2000). However, the histatin 5 derivative R12I/H21L was more active against both these strains (ED_{50} values of 31.6 μM and 17.2 μM, respectively), indicating that histatin 5 variants may be effective therapeutic agents against azole-resistant *Candida* strains (Situ and Bobek, 2000). Histatin 5 and its derivatives have also shown antifungal activity against *C. tropicalis* (Rothstein *et al.*, 2001), *C. pseudotropicalis*, *C. parapsilosis*, and *A. fumigatus* (Helmerhorst *et al.*, 1999a).

Studies investigating histatin 3 antifungal activity have demonstrated this protein is also potent against azole-susceptible and -resistant *Candida* spp. O'Connell *et al.* (1996) constructed recombinant adenovirus vectors containing histatin 3 cDNA, which were capable of directing histatin 3 expression in the saliva of rats. The adenovirus-directed histatin 3 demonstrated a 90% candidacidal effect in a timed-kill assay against both fluconazole-susceptible and -resistant *C. albicans* strains, and inhibited germination by 45% in these same strains (O'Connell *et al.*, 1996). Histatin 3 shows potent *in vitro* antifungal activity against *C. dubliniensis*, a recently described *Candida* species. The concentration of histatin 3 giving 50% killing ranged from 0.043 to 0.196 mg/ml among different strains of *C. dubliniensis* (Fitzgerald *et al.*, 2003). Histatin 3 also showed anticandidal activity against a fluconazole-resistant *C. dubliniensis* clinical isolate, and an *in vitro*-generated fluconazole-resistant derivative of a *C. dubliniensis* clinical isolate (IC_{50} values of 0.075 mg/ml and 0.057 mg/ml, respectively) (Fitzgerald *et al.*, 2003).

Together, these studies suggest that the mode of action of histatin antifungal activity against *Candida* spp. is distinct from that of azole-based antimycotics, since histatin 5, histatin 5 derivatives, and histatin 3 kill both azole-susceptible and azole-resistant strains (O'Connell *et al.*, 1996; Tsai *et al.*, 1997b; Helmerhorst *et al.*, 1999a; Situ and Bobek, 2000; Fitzgerald *et al.*, 2003).

1.2.2.4 Antibacterial and other activities

Other activities ascribed to histatin proteins include a role in formation of the enamel pellicle of teeth (Jensen *et al.*, 1992), inhibition of hemagglutination (Murakami *et al.*, 1990, 1992), coaggregation (Murakami *et al.*, 1991), and neutralisation of LPS (Sugiyama *et al.*, 1993). The growth-inhibitory and bactericidal effects of histatin on the oral pathogen *S. mutans* have also been described (MacKay *et al.*, 1984). Periodontal disease is a chronic inflammatory disorder characterised by bone resorption, loss of tooth attachment, and formation of periodontal pockets populated with a flora composed of a specific spectrum of bacteria (Gusman *et al.*, 2001). Many studies have shown that gingivitis and periodontitis lead to increased levels of both host and bacterial proteolytic enzymes in oral inflammatory exudates, which can enter the oral cavity as gingival crevicular fluid and become constituents of whole saliva (Sorsa *et al.*, 1992; Ingman *et al.*, 1994; Makela *et al.*, 1994; Gusman *et al.*, 2001). Among these proteinases, host-derived matrix metalloproteinases (MMPs) are considered key initiators of extracellular matrix degradation associated with periodontal and other oral diseases (Gusman *et al.*, 2001). The levels of at least two of these enzymes, MMP-2 and MMP-9, are elevated in the saliva of patients with periodontal disease (Ding *et al.*, 1994; Ingman *et al.*, 1994; Gusman *et al.*, 2001). Salivary histatin 5 inhibits the activity of MMP-2 and MMP-9 with IC_{50} values of 0.57μM and 0.25 μM, respectively (Gusman *et al.*, 2001). *Porphyromonas gingivalis* is an anaerobic, Gram-negative bacterium which is present in the microflora of subgingival plaque, and has been strongly implicated in the etiology of periodontal disease (Gusman *et al.*, 2001). Several physiologically important proteins, including collagen, fibrin and fibrinogen, fibronectin, plasma protease inhibitors, immunoglobulins, and complement factors, are degraded by proteases from *P. gingivalis* (Gusman *et al.*, 2001). Arg-gingipain and Lys-gingipain are *P. gingivalis*-derived cysteine proteases with strict specificities for cleavage at either arginine or lysine residues, respectively. Histatin 5 inhibits Arg-gingipain and Lys-gingipain activities with IC_{50} values of 22.0 μM and 13.8 μM, respectively (Gusman *et al.*, 2001). The inhibitory activity of histatin 5 against host and bacterial proteases at physiological concentrations points to yet another potential biological function of histatin in the oral cavity (Gusman *et al.*, 2001).

1.2.3 Structure/ function relationship

1.2.3.1 Primary structure

Histatins constitute a family of low molecular weight, cationic, histidine-rich, potent, antimicrobial proteins (Oppenheim *et al.*, 1988). The major members are histatins 1, 3, and 5 and these comprise about 80% of all histatins present in parotid and submandibular/sublingual secretions. Sequence analysis of these proteins indicate that histatins 1, 3, and 5 comprise 38, 32, and 24 amino acids and have molecular weights of 4929, 4063, and 3037, respectively. Each of these histatins contain seven histidine residues and display strong sequence homology. The sequence data also shows that histatins 2, 4, 5, 6, 7, 8, 9, 10, 11, and 12 are proteolytic cleavage products of either histatin 1 or histatin 3 (Table 1.2). Histatins do not display significant sequence similarities with any other proteins, and therefore represent a unique group of salivary proteins (Oppenheim *et al.*, 1988).

1.2.3.2 Functional domain

Studies of the anticandidal activies of histatin 3 fragments have identified the functional domain of histatin 3 responsible for candidacidal activity to reside in the middle portion (amino acids 13-24) of the molecule (Zuo *et al.*, 1995; Raj *et al.*, 1990). All major salivary proteins occur as polymorphic forms and examples of salivary proteins with tandem repeats are proline-rich proteins (PRPs) (Maeda *et al.*, 1985) and mucins (Bobek *et al.*, 1993; Zuo *et al.*, 1995). Since histatins are evolutionarily young proteins, it is possible they have not been afforded sufficient time and the requisite evolutionary pressures to duplicate and thereby generate tandemly repeated sequences (Zuo *et al.*, 1995). Zuo *et al.* (1995) used the pRSET bacterial expression system to produce biologically active functional histatin 3 (reHst3) with an LD_{50} value towards *C. albicans* comparable to that of native histatin 3 (Hst3) purified from salivary secretions (6.8 μM and 7.2 μM, respectively) (Table 1.3). In an effort to anticipate evolution, Zuo *et al.* (1995) used this expression system, recombinant DNA technology, and gene splicing by overlap extension to duplicate the functional domain of histatin 3 in tandem to create reHst3rep (Table 1.3). Duplication of the functional domain was found to significantly enhance candidacidal activity (LD_{50} value of 4.1 μM). At the low end of the dose response curve the enhanced activity of reHst3rep

Table 1.2 Amino acid sequence homology of the major and minor human salivary histatins (Oppenheim *et al.*, 1988)

Peptide	Sequence
Histatin 1	DSHEKRHHGYRRKFHEKHHSHREFPFYGDYGSNYLYDN
Histatin 2	RKFHEKHHSHREFPFYGDYGSNYLYDN
Histatin 3	DSHAKRHHGYKRKFHEKHHSHRGYRSNYLYDN
Histatin 4	RKFHEKHHSHRGYRSNYLYDN
Histatin 5	DSHAKRHHGYKRKFHEKHHSHRGY
Histatin 6	DSHAKRHHGYKRKFHEKHHSHRGYR
Histatin 7	RKFHEKHHSHRGY
Histatin 8	KFHEKHHSHRGY
Histatin 9	RKFHEKHHSHRGYR
Histatin 10	KFHEKHHSHRGYR
Histatin 11	KRHHGYKR
Histatin 12	KRHHGYK

Table 1.3 Sequences of histatin derivatives and their candidacidal activities against *C. albicans* blastoconidia

Peptide	Sequence	LC_{50} (μM)	Reference
reHst3	DSHAKRHHGYKRKFHEKHHSHRGYRS NYLYDN	6.8	Zuo *et al.*, 1995
reHst3rep	DSHAKRHHGYKRKFHEKHHSHRGYKF HEKHHSHRGYRSNYLYDN	4.1	Zuo *et al.*, 1995
reHst5	DSHAKRHHGYKRKFHEKHHSHRGY	2.5	Driscoll *et al.*, 1996
reHst5ΔHis	DSHAKRHHGYKRKF**G**EK**GGSG**RGY	17.0	Driscoll *et al.*, 1996
reHst5ΔGlu	DSHAKRHHGYKRKFH**G**KHHSHRGY	6.0	Driscoll *et al.*, 1996
reHst5ΔLys/Arg	DSHAKRHHGYK**GG**FHE**G**HHSH**G**GY	16.0	Driscoll *et al.*, 1996
m1	DSHAKRHHGYK**I**KFHEKHHSHRGY	4.9	Tsai *et al.*, 1996
m2	DSHAKRHHGYK**I**KFHE**N**HHSHRGY	5.5	Tsai *et al.*, 1996
m12	DSHAKRHHGYK**I**KFHEKHHS**L**RGY	3.6	Tsai *et al.*, 1996
m21	DSHAKRHHGYKR**T**FHEKHHSHRGY	14.7	Tsai *et al.*, 1996
m68	DSHAKRHHGYKR**E**FHEKHHSH**G**GY	49.6	Tsai *et al.*, 1996
m70	DSHAKRHHGYKRKFHEKH**PSR**RGY	4.6	Tsai *et al.*, 1996
m71	DSHAKRHHGYKR**E**FHEKHHSHRGY	19.4	Tsai *et al.*, 1996
F14A/H15A	DSHAKRHHGYKRK**AA**EKHHSHRGY	67	Tsai and Bobek, 1997
H18A/H19A	DSHAKRHHGYKRKFHEK**AA**SHRGY	149	Tsai and Bobek, 1997
P-113	AKRHHGYKRKFH-NH_2	2.3	Rothstein *et al.*, 2001
113-F4.5.12	AKR**FF**GYKRKF**F**- NH_2	2.2	Rothstein *et al.*, 2001
113-Y4.5.12	AKR**YY**GYKRKF**Y**- NH_2	2.5	Rothstein *et al.*, 2001
113-L4.5.12	AKR**LL**GYKRKF**L**- NH_2	3.7	Rothstein *et al.*, 2001
113-Q2.10	A**Q**RHHGYKR**Q**FH- NH_2	>80	Rothstein *et al.*, 2001
113-Q3.9	AK**Q**HHGYK**Q**KFH- NH_2	31.7	Rothstein *et al.*, 2001
113-Q2.3.9.10	A**QQ**HHGYK**QQ**FH- NH_2	>80	Rothstein *et al.*, 2001
113-K6	AKRHH**K**YKRKFH- NH_2	5.6	Rothstein *et al.*, 2001
113-H8	AKRHHGY**H**RKFH- NH_2	3.0	Rothstein *et al.*, 2001
113-K6H8	AKRHH**K**Y**H**RKFH- NH_2	2.6	Rothstein *et al.*, 2001

Underlined sequences refer to functional domains.
Bold letters represent substituted amino acids.
LC_{50}, lethal concentration required for 50% cell killing.

became much more prominent. At 3.1 μM activity was enhanced more than 2-fold, at 1.6 μM activity was enhanced nearly 3-fold, and at 0.8 μM activity was enhanced more than 5-fold over the activity of native Hst3 or reHst3. At even lower concentrations, reHst3rep displayed significant candidacidal activity while native Hst3 and reHst3 were inactive (Zuo *et al.*, 1995).

1.2.3.3 Recombinant histatin structural variants

In order to investigate the role of specific amino acids located within the functional domain, several groups have produced recombinant histatin 5 variants with specific amino acid substitutions. Driscoll *et al.* (1996) used the pRSET bacterial expression system to produce functionally active recombinant histatin 5 (reHst5) and several recombinant variants generated by site-directed mutatgenesis. ReHst5 variants had His, Glu or Lys/Arg amino acids substituted with Gly residues (Table 1.3). Activity of reHst5 and variants were tested for candidacidal activity against *C. albicans*. At all test concentrations, the activity of reHst5 was nearly identical to that of native histatin 5 and exhibited a comparable LD_{50} value (2.5 μM and 2.0 μM, respectively) (Driscoll *et al.*, 1996). ReHst5 variants with either Glu or Lys/Arg substitutions demonstrated significantly lower candidacidal activity, while the variant with His substituted for Gly showed negligible activity at physiological concentrations. These results indicate that the Glu and Lys/Arg residues contribute to activity, while the His residues may be essential for candidacidal activity (Driscoll *et al.*, 1996).

Tsai *et al.* (1996) further investigated the role of specific amino acid residues within the functional domain by replacing His, Lys and/or Arg residues with combinations of Ile, Asn, Leu, Pro or Arg (Table 1.3). The histatin 5 variants m1, m2, m12 and m70 possess candidacidal activity comparable to that of reHst5. The m1, m2 and m12 sequences have Ile in the place of Arg at position 12. In addition to this change, Lys at position 17 is replaced by Asn in m2, whereas in m12, His at position 21 is changed to Leu. The m70 has Pro at position 19 and Arg at position 21 instead of His in the original sequence (Tsai *et al.*, 1996). These changes do not significantly affect the candidacidal activity of these molecules, suggesting that cationic Arg-12 and Lys-17 and imidazole rings of His-19 and

His-21 may not be essential for candidacidal activity of Hst5 (Tsai *et al.*, 1996). The Hst5 variants m21, m68 and m71 are less effective in killing *C. albicans* than reHst5. Variants m21 and m71 have Thr and Glu in place of Lys-13, respectively. The sequence of m68 has Glu and Gly instead of Lys-13 and Arg-22, respectively. The candidacidal activities of these variants are much lower than that of reHst5. The replacement of Lys-13 by Thr or Glu results in a 3-fold reduction in activity, suggesting that a positive charge at this position is important for candidacidal activity (Tsai *et al.*, 1996). The substitution of the negatively charged Glu in the place of Lys-13 and Gly in the place of Arg-22 in m68 significantly lowers the candidacidal potency by almost 10-fold compared to that of reHst5. Collectively, these results suggest that Lys-13 and Arg-22 are important functional elements, while Arg-12, Lys-17, His-19, and His-21 may not be essential for eliciting high candidacidal activity (Tsai *et al.*, 1996).

It was once postulated that the mechanism of action of histatin 5 may resemble those of azole-based antifungal molecules, which act through inhibition of sterol 14α-demethylase, a cytochrome P-450 enzyme involved in the biosynthesis of ergosterol, a major sterol component of the fungal cell membrane (Tsai *et al.*, 1997b). This hypothesis was substantiated by molecular modeling of the Hst5 C-terminal 16 amino acid fragment and one of the azole antifungal drugs, miconazole, which suggested structural similarity between the Phe^{14}-His^{15} and His^{18}-His^{19} dipeptide sequences and that of miconazole (Ramalingam *et al.*, 1996). Tsai *et al.* (1997b) produced two variants of Hst5 in which Phe^{14}-His^{15} or His^{18}-His^{19} dipeptides were replaced by Ala-Ala (F14A/H15A and H18A/H19A, respectively) (Table 1.3) to eliminate the phenyl and imidazole rings of the side chains, and then assessed their activities against *C. albicans*. Analysis of the candidacidal activities of the Hst5 variants indicated that they were were significantly less effective than the unaltered Hst5 at killing *C. albicans*. The ED_{50} values for F14A/H15A and H18A/H19A were ~67 μM and ~149 μM, respectively, compared to ~8 μM for the unaltered Hst5. This suggests that these two dipeptide sequences are important for the candidacidal activity of Hst5 (Tsai *et al.*, 1997b).

Through the analysis of a series of 25 peptides composed of various portions of the histatin 5 sequence, P-113 (amino acids 4-15) has been identified as the smallest fragment that retains anticandidal activity comparable to that of the parent compound (Table 1.3)

(Rothstein *et al.*, 2001). Amidation of the P-113 C-terminus increased the anticandidal activity of P-113 ~ 2-fold. Modifications of the P-113 sequence were performed to investigate if anticandidal activity of the molecule could be enhanced (Rothstein *et al.*, 2001). In the first group of modifications, to test the importance of hydrophobic residues at positions 4, 5, and 12 of P-113, the His residues were replaced by either Phe, Tyr, or Leu residues (113-F4.5.12, 113-Y4.5.12, and 113-L4.5.12, respectively) (Table 1.3) all of which are unequivocally hydrophobic in nature. Each of the three modified peptides retained potent antimicrobial activity against *C. albicans*. It appeared that these His residues were all dispensible when replaced by hydrophobic residues. However, the His residues of P-113 may play a role *in vivo* if aggregation of microorganisms, tissue binding, and the stability of the peptide, or other factors are important (Rothstein *et al.*, 2001). A second group of modifications were designed to test for the importance of cationic charges. The substitution of Glu residues for either Lys or Arg resulted in reduced activity for peptides with substitutions at positions 3 and 9 (113-Q3.9), or a loss of activity for those with substitutions at positions 2 and 10 against *C. albicans* (113-Q2.10). The replacement of all four cationic residues by Glu resulted in a complete loss of antimicrobial activity (113-Q2.3.9.10). The third group of substitutions was designed to increase the amphipathicity of the peptide, as a guide to optimizing antimicrobial activity. The Gly residue at position 6 was replaced by Lys residue, and the Lys residue at position 8 was replaced by a His residue (113-K6 and 113-H8, respectively). 113-K6H8 contains both a Lys substitution at position 6 and a His substitution at position 8 (Table 1.3). However, none of these peptides exhibited improved killing activities against *C. albicans* compared to that of P-113. These results suggest that the amphipathicity of the putative P-113 α-helix is not the overriding factor that defines the activity of P-113 (Rothstein *et al.*, 2001).

1.2.4 Mechanism of action

Histatin 5 is the most potent antimicrobial peptide in the histatin family, therefore most of the research on histatin mode of killing has been performed with histatin 5. Histatin 5 adopts a random coil structure in aqueous solvents and an amphipathic α-helix structure in non-aqueous solvents (Raj *et al.*, 1998). The mode of action of most α-helical antimicrobial

peptides involves attachment to the anionic microbial surface through their net positive charge, insertion of the peptides into the microbial membrane, which results in permeabilisation of the plasma membrane, loss of cell integrity, and lysis of the microorganism. Extensive research has shown that the mode of action of histatin 5 is different from that of most other α-helical peptides. Fungicidal activity of histatin 5 involves binding to cell surface receptors on *C. albicans*, uptake of the peptide into the cell, targeting of the peptide to specific intracellular structures, cell cycle arrest, efflux of ATP out of the cell, and production of reactive oxygen species (Kavanagh and Dowd, 2004). Loss of cell integrity appears to be a secondary effect following cell death, rather than the result of primary disruption of the yeast cell membrane (Edgerton *et al.*, 1998).

Recent evidence suggests that the molecular mechanism of histatin 5-induced killing in *C. albicans* involves binding to a 67 kDa plasma membrane protein (Edgerton *et al.*, 1998), later identified as Ssa1p and/or Ssa2p members of the *C. albicans* heat shock 70 protein family (Li *et al.*, 2003b). Further investigation into these histatin binding proteins has identified *C. albicans* Ssa2p as the main protein involved in binding and intracellular translocation of histatin 5, whereas Ssa1p appears to have a lesser functional role in histatin 5 toxicity (Li *et al.*, 2006). After binding to Ssa2p , the peptide is internalised and targeted to the mitochondria (Helmerhorst *et al.*, 1999b). *C. albicans* respiratory mutants are resistant to killing by histatin 5 (Gyurko *et al.*, 2000). Once internalised, histatin 5 induces the efflux of ATP and potassium ions from the cell. Loss of ATP and small ions appear to induce G1 cell cycle arrest and dysregulation of cell volume homeostasis, and these events are closely coupled with the loss of intracellular ATP (Koshlukova *et al.*, 1999; Baev *et al.*, 2002). Recently, the TRK1 potassium transporter protein has been identified as providing the essential pathway for ATP loss and being the critical effector for histatin 5 killing of *C. albicans* (Baev *et al.*, 2004). The histatin 5-induced ATP release occurs while the cells are still metabolically active and before any cell lysis occurs (Koshlukova *et al.*, 1999). It has been suggested that the extracellular ATP interacts with a purinergic receptor, which in turn may induce cell death after activation (Koshlukova *et al.*, 2000).

1.2.5 Stability in saliva

Histatins are very susceptible to proteolysis by salivary enzymes (Xu *et al.*, 1992; O'Connell *et al.*, 1996; Yamagishi *et al.*, 2005). Incubation of synthetic histatins with human parotid saliva has revealed a series of peptide degradation products, and sequence analysis has suggested that the activities involved include trypsin-like and chymotrypsin-like enzymatic activities, representing the most active salivary proteases, and also alanine-lysine endopeptidase and histidine peptidase activities (O'Connell *et al.*, 1996; Yamagishi *et al.*, 2005). Furthermore, serine-protease and acidic-protease activities in the oral cavity may originate from the microflora or blood leukocytes (Yamagishi *et al.*, 2005). Histatin proteolytic degradation products are less effective in killing *C. albicans* (Xu *et al.*, 1992).

The high susceptibility of histatins to proteolytic degradation in whole saliva has made comparative analysis of histatin concentrations between various research groups difficult, due to the different ways salivary samples are handled after collection (Tsai *et al.*, 1998). It was reported that the purification yields of histatin from human parotid saliva were significantly improved if histatin proteolysis was delayed by acidification (adjusted to pH 4.5) and boiling of the saliva (for 2.5 min) immediately after collection (Lal *et al.*, 1992).

Yamagishi *et al.* (2005) studied the candidacidal activity of histatin 3 in assays containing rabbit submandibular saliva. Rabbit saliva does not contain histatins, and is more similar to human saliva in terms of pH, ion concentration, glycoprotein and proteolytic environment than the non-organic buffers used in most candidacidal assays (Yamagishi *et al.*, 2005). Exogenously added histatin 3 was degraded almost completely over a 2 h period in the presence of rabbit submandibular saliva (Yamagishi *et al.*, 2005). Therefore rabbit submandibular saliva may also contain protease activities arising from salivary secretions (Yamagishi *et al.*, 2005). The candidacidal activity of histatin 3 reached a maximum after 60 min incubation in the presence of rabbit saliva, and decreased moderately thereafter up to 120 min incubation. This reduction in candidacidal activity may be explained by the degradation of the peptide in saliva. Addition of fresh histatin 3 to rabbit submandibular saliva resulted in increased candidacidal activity (Yamagishi *et al.*, 2005).

1.2.6 Immunochemical properties

Many researchers have encountered difficulties with the preparation of antibodies to human histatins. The reasons for this are unclear, but possible explanations for the failure of an animal's immune system to recognise these as foreign proteins include: low molecular weight, tertiary structure (Tsai *et al.*, 1998), and rapid degradation of histatins in serum (Situ *et al.*, 2000). Rabbit and goat polyclonal antibodies have been generated against histatin 5 (Atkinson *et al.*, 1990; Shrestha *et al.*, 1994). These antibodies were used for the localisation of histatin expression in normal salivary glands (Takano *et al.*, 1993; Ahmad *et al.*, 2004). The effectiveness of the histatin 5-specific antibody for the detection of histatin 3 in saliva was examined in this laboratory using an enzyme-linked immunosorbent assay (ELISA) method. Although the antibody cross-reacts with histatin 3, detecting up to 1 mg/ml pure histatin, histatins were not detectable in human saliva using this antibody. Furthermore, spiking saliva with histatin 3 resulted in masking of the signal (D. Fitzgerald, personal communication). While the histatin 5-specific antibody may be suitable for other applications, its use in detecting histatins in heterogenous mixtures of proteins may be limited and may require consideration of factors such as protease-mediated degradation of histatin, the need to dissociate proteins in complex solutions, and non-specific antibody binding.

1.2.7 Detection in saliva

1.2.7.1 ELISA, capillary electrophoresis, and reverse-phase HPLC

The accurate measurement of histatin concentrations in salivary secretions has represented major technical obstacles as demonstrated by the fact there is no one reliable method used by investigators, and that these studies have yielded varying estimations of histatin concentrations (Atkinson *et al.*, 1990; Mandel *et al.*, 1992; Lal *et al.*, 1992; Sugiyama *et al.*, 1993; Jensen *et al.*, 1994). Each group utilized different quantitation methods: ELISA (Atkinson *et al.*, 1990; Jensen *et al.*, 1994), capillary electrophoresis (Lal *et al.*, 1992), and reverse-phase HPLC (Sugiyama *et al.*, 1993), and different stimulators: 2% (w/v) citric acid (Atkinson *et al.*, 1990; Mandel *et al.*, 1992; Sugiyama *et al.*, 1993), sour lemon drops (Lal *et al.*, 1992), 0.5%, 1.5%, and 5% (w/v) citric acid (Jensen *et al.*, 1994), and silicone tubing mastication (Jensen *et al.*, 1994). After collection, samples were either: immediately frozen

at -70°C, and when thawed, treated with ethylenediaminetetraacetic acid (EDTA) (Atkinson *et al.*, 1990), immediately frozen at -70°C (Mandel *et al.*, 1992; Jensen *et al.*, 1994), immediately adjusted to pH 3 and frozen at -20°C (Sugiyama *et al.*, 1993), or immediately adjusted to pH 4.5, then boiled for 2.5 min (Lal *et al.*, 1992). Lal *et al.* (1992) determined that histatin 3, the most abundant histatin found in saliva, had a mean value of 60 μg/ml (range 8.3-190.7 μg/ml) in stimulated parotid secretions, and 18.8 μg/ml (range 6.3-37.2 μg/ml) in stimulated submandibular/sublingual secretions. The concentrations determined by Lal *et al.* (1992) and Sugiyama *et al.* (1993) are higher than those determined by the other investigators. This may be due to the different quantitation methods, capillary electrophoresis and reverse-phase HPLC as opposed to immunochemical methods, and also to the different handling procedures immediately after saliva collection (Tsai *et al.*, 1998). Lal *et al.* (1992) reported that the purification yields of histatins from parotid saliva were significantly improved if histatin proteolysis was delayed by acidification and boiling of the saliva immediately after collection.

1.2.7.2 Zinc precipitation and reverse-phase HPLC

Gusman *et al.* (2004) recently developed a novel technique for the isolation and purification of histatins from saliva, which is based on their ability to selectively associate and precipitate in the presence of zinc. They showed this method to be rapid, efficient, and to provide excellent separation of histatins from all other salivary proteins except statherin. Used in conjunction with reverse-phase HPLC, this method could be used as a new histatin quantitation method (Gusman *et al.*, 2004). Mean daily histatins concentrations were almost three times higer in submandibular/sublingual secretions than in parotid secretions, demonstrating that there is a differential glandular expression pattern for histatin, and that the submandibular/sublingual glands are the major contributors of histatin in saliva (Gusman *et al.*, 2004). Mean daily concentrations of histatin 1, histatin 3, and histatin 5 in parotid saliva were 1.8 mg% (range 0.7-2.8 mg%), 2.2 mg% (range 0.6-4.3 mg%), and 2.5 mg% (range 1.0-4.3 mg%), respectively (mg histatin per 100 ml of saliva). Mean daily concentrations of histatin 1, histatin 3, and histatin 5 in submandibular/sublingual saliva were 6.4 mg% (range 2.8-12.2 mg%), 3.8 mg% (range 1.5-7.5 mg%), and 4.5 mg% (range

2.6-9.0 mg%), respectively (Gusman *et al.*, 2004). Both parotid and submandibular/sublingual secretions displayed daily variations in histatin concentrations. Parotid values showed a maximum at mid-day and submandubilar/sublingual samples showed a maximum in the morning. This study demonstrated a hitherto unidentified daily variation in histatin concentrations in parotid and submandibular/sublingual secretions, which may impose the need to specify the collection times when reporting results of estimations of histatin concentration in salivary samples (Gusman *et al.*, 2004).

1.2.8 Salivary gland-specific expression

Histatins are produced and secreted specifically by the parotid and submandibular/sublingual glands (Oppenheim *et al.*, 1988; Sabatini *et al.*, 1989; vanderSpek *et al.*, 1989). They are not detectable in other mucosal fluids such as human tear fluid, nasal, bronchial, or cervical mucus, seminal fluid, sweat, or blood plasma. This is in contrast to other salivary proteins, including the statherins, PRPs, cystatins, mucins, defensins, amylase, lysozyme, and kallikrein, which are distributed in mucosal secretions throughout the body (Schenkels *et al.*, 1995; Ganz *et al.*, 1999). Histatin mRNA has not been found in any other tissues examined to date, such as the lung, stomach, duodenum, proximal ileum, distal ileum, cecum, colon, pancreas, kidney, liver, bladder, skin, ovary, endometrium, uterine cervix, fallopian tube, lactating adenoma of breast, prostate, seminal vesicle, or testicle (Sabatini *et al.*, 1989). However, low level expression of histatin mRNA has been found in skeletal muscle, lymph node, trachea, and thyroid gland using a human Multiple Tissue Expression (MTE) array in this laboratory (D. Fitzgerald, personal communication). This data contradicts previous studies and suggests histatins are not exclusively produced by the salivary glands and may protect other sites of the body from microbial assault. This technique may simply be more sensitive or more specific than the methods used in other studies examing histatin mRNA expression. However, the hybridisation experiment using this human MTE array must be repeated several times before it can be said with confidence that histatin mRNA is expressed in tissues other than the salivary glands.

Immunocytochemical studies with anti-histatin antibodies have shown that histatins are present in the serous acinar cells of the parotid and submandibular glands (Takano *et*

al., 1993). More detailed immunocytochemical analysis on the cellular and subcellular distribution of histatins has been performed recently (Ahmad *et al.*, 2004). This study confirmed localisation of histatins in the serous acinar cells of the parotid and submandibular glands, but also in the serous demilune cells of the submandibular and sublingual glands, and in occasional intercalated duct cells. At the subcellular level, immunoreactivity was associated with the rough endoplasmic reticulum and Golgi complex, and in secretory granules of serous acinar and demilune cells. The granules of parotid acinar cells exhibited relatively uniform labeling of their content, whereas the granules of serous cells in the submandibular and sublingual glands showed variable labeling of the dense and light regions of their content. A few intercalated duct cells adjacent to the acinar cells also exhibited labeled granules. These results suggest that the serous cells of the parotid and submandibular glands are the main source of histatins in saliva (Ahmad *et al.*, 2004).

1.2.9 *HIS1/HIS2* gene structure and evolution of the *STATH/HIS* gene family

Histatins are expressed only in saliva of humans and Old World monkeys, but not in the saliva of New World monkeys, dogs, rats, mice, or hamsters (Sabatini *et al.*, 1993). Human histatins are encoded by two highly homologous genes, *HIS1* and *HIS2*, which have been located to chromosome 4, band q13 (vanderSpek *et al.*, 1989). Histatin 1 and histatin 3 are the primary gene products of the *HIS1* and *HIS2* genes, respectively, while histatin 2 and histatins 4 to 12 are their proteolytic cleavage products. The *HIS1* and *HIS2* genes exhibit 84% overall sequence identity, with exon sequences exhibiting 90% identity (Fig. 1.2). It is thought that these two loci arose from a gene duplication event, which is presumed to have occurred in hominoids after their divergence from cercopithecoids and prior to the hylobatid-pongid/hominid divergence. This would place the *HIS1/HIS2* gene duplication event at 15-30 million years ago, making them evolutionarily young proteins (Sabatini *et al.*, 1993).

While statherin and histatin proteins exhibit little similarity in amino acid sequence or function, statherin cDNAs exhibit high similarity to histatin cDNAs in the 5′ and 3′ untranslated and signal peptide sequences, and have also been localised to chromosome 4, band q11-13 (Sabatini *et al.*, 1989; vanderSpek *et al.*, 1989). Genomic sequence analysis

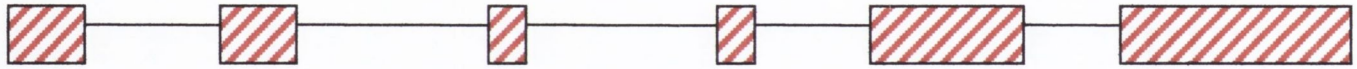
	Exon 1	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6
HIS Gene Arrangements						
HIS1 exon size (nt):	52	66	19	32	103	264
HIS2 exon size (nt):	56	67	18	32	85	266
HIS exons encode:	5′ untranslated region	signal sequence	hydroxyapatite binding domain	antimicrobial domain		3′ untranslated region and polyadenylation sequence
Nucleotide identity in exon regions (%)						
	89.3	92.5	84.2	96.9	76.7	94.0

Figure 1.2 Comparison of the *HIS1* and *HIS2* gene structures. Structural features of the *HIS1* and *HIS2* gene products are based upon results from Troxler *et al.*, 1990; Xu and Oppenheim, 1993; Sabatini *et al.*, 1993; Driscoll *et al.*, 1995.

has indicated ~80% sequence identity in intron DNA, 80-88% sequence identity in certain exons, and only ~50% sequence homology in protein-coding exons 4 and 5, suggesting a possible evolutionary relationship. The reasons for this divergence in exons 4 and 5 of the *HIS* and *STATH* genes is currently unknown. Based upon the estimated mutation rate in the noncoding regions of the *HIS1* and *HIS2* genes and a comparable rate in the analogous *STATH* region, it is estimated an initial gene duplication event occurred 40-50 million years ago which yielded the *HIS* and *STATH* genes from a common ancestral gene (Sabatini *et al.*, 1993).

Parotid-specific regulatory elements were first identified in the salivary amylase gene, *AMY1C* (Ting *et al.*, 1992). Salivary and pancreatic amylase are encoded by distinct but closely related genes. The human genome contains three salivary and two pancreatic amylase genes (Ting *et al.*, 1992). During the evolution of this gene family, insertion of a processed γ-actin pseudogene in the proximal promoter region of the ancestral amylase gene was followed by a retroviral insertion (Ting *et al.*, 1992). The 5′-flanking regions of the salivary amylase genes contain both γ-actin and retroviral sequences, and insertion of the retroviral element is correlated with a switch from pancreatic to parotid expression (Samuelson *et al.*, 1990). Comparison of the 5′-flanking region of AMY1C and other salivary-specific genes have identified three short promoter elements common to salivary gland-specific genes (Ting *et al.*, 1992). The 5′-flanking regions of the *HIS1*, *HIS2*, and *STATH* genes also contain these three parotid-specific promoter elements (Table 1.4).

Primate specificity of the *STATH/HIS* gene family at the DNA level has been questioned in recent genomic studies on the casein gene cluster (Rijnkels *et al.*, 2003). The *STATH/HIS* gene family is located between the *CSN2* and the *CSN1S2*-like genes in the cluster. Comparative analysis indicates the presence of at least two *STATH/HIS*-like genes in cattle, and remnants of these genes are present in mice and rats at the genomic DNA level. mRNA transcripts were detected in bovine salivary gland tissue which have a translated protein with 63% identity to human statherin. The exact number, position, and orientation of *STATH/HIS*-like genes in the bovine genome cannot be assessed until more complete sequences become available. To date, no mouse transcripts have been identified (Rijnkels *et al.*, 2003).

Table 1.4 Common promoter elements in human salivary gland-specific genes

Gene		I		II		III
AMY1C	-849	TTTCCTACC	-802	AGAGTCCCTG	-784	TGAGGGATGC
HIS1	-276	TTTCCT**C**C**A**	-870	AGAG**TTT**TT**A**	-916	TGAGG**AA**C**AA**
HIS2	-293	TTTCCT**C**C**A**	-733	**TGGG**C**C**CCTG	-935	TGAGG**AA**C**AA**
STATH	-767	TTTCCTA**GG**	-602	AGAGTCATT**T**	-887	TGAGG**AA**C**AA**
CST1	-48	T**C**TCCT**G**CC	-173	C - AGTCCC**CA**	-245	- - AGGGA**GAG**
CSTP1	-48	T**C**TCCTA**AT**	-173	AGAG - CCC**C**G	-245	**A**GAGGGA**CGG**
PRP I	-212	T**A**TCCTACC	-183	AGAGTCCC**AA**	-167	TGAGGGAT**A**C
PRP II	-212	**CA**TCCT**C**CC	-183	AGAGTCCC**A**G	-167	TGAGGGAT**A**C

Note. A total of 251 bp of the human salivary amylase gene (*AMY1C*, Ting *et al.*, 1992) was compared with 1 kb of the 5'-flanking sequences of *HIS1*, *HIS2*, and *STATH* genes, approx. 360 bp of the human salivary cystatin genes (*CST1* and *CSTP1*) and 1 kb each of the human proline-rich proteins (*PRP I* and *PRP II*) (Shaw and Chaparro, 1999). **Bold** letters represent nucleotides which differ from the those in the promoter elements found in the *AMY1C* 5′-flanking region, and - indicates the absenceof a nucleotide at this location.

1.3 Mammalian antimicrobial gene regulation

1.3.1 Inducible expression of antimicrobial peptides in mucosal epithelium

1.3.1.1 Regulation of β-defensins

The mucosal epithelium produces a range of antimicrobial proteins which are believed to be essential for host defence against fungal, bacterial and viral infection. Recent investigations support the hypothesis that these antimicrobials are up-regulated in response to microbial stimulation and inflammatory mediators. The β-defensins are small cystine-rich, cationic peptides expressed at numerous mammalian epithelial sites, including the salivary glands, tongue, gingiva, buccal mucosa, nasal, lung and small intestine (Jones *et al.*, 1992; Schonwetter *et al.*, 1995; Huttner *et al.*, 1998; Mathews *et al.*, 1999). Defensins exhibit broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, and enveloped viruses *in vitro* (Ganz, 1999).

Tracheal antimicrobial peptide (TAP) and lingual antimicrobial peptide (LAP) are two members of the β-defensin family. TAP is expressed in bovine respiratory mucosa, and is inducible in cultured tracheal epithelial cells upon challenge with bacterial LPS. LAP is expressed in both bovine tongue and tracheal mucosa, and elevated levels of LAP mRNA have been detected in squamous epithelial cells of the tongue near sites of tissue injury and inflammation (Schonwetter *et al.*, 1995). Increased LAP mRNA expression has also been demonstrated in cultured tracheal epithelial cells after exposure to LPS or TNF-α (Russell *et al.*, 1996).

Two human defensins, human β-defensin 1 (hBD-1) and human β-defensin 2 (hBD-2), were discovered in 1995 and 1997, respectively. hBD-2 is highly effective in killing Gram-negative bacteria such as *P. aeruginosa* and *E. coli*, yeasts such as *C. albicans*, and bacteriostatic against the Gram-positive bacterium *Staphlyococcus aureus* (Harder *et al.*, 1997). One study investigated the expression of hBD-1 and hBD-2 in the oral cavity. hBD-1 mRNA expression was detected in the gingiva, parotid gland, buccal mucosa, and tongue. Expression of hBD-2 mRNA was detected only in the gingival mucosa and was most abundant in tissues with associated inflammation. Levels of β-defensin expression were investigated using cultured gingival keratinocyte cells treated with bacterial LPS or IL-1β.

hBD-1 expression was constitutive, while hBD-2 expression was induced significantly by LPS or by the proinflammatory cytokine (Mathews *et al.*, 1999).

Mouse β-defensin 3 (mBD-3), a murine homologue of human β-defensin 2 is expressed at low levels in the lung, salivary glands, intestine, liver, and pancreas. It exhibits antimicrobial activity against *P. aeruginosa* and *E. coli in vitro* (Bals *et al.*, 1999). After infection of mouse airways with *P. aeruginosa*, mBD-3 mRNA was upregulated significantly in the lung, suggesting that β-defensin contributes to innate host defence. However, mBD-3 mRNA was also significantly increased in the intestine and liver following intratracheal infection, suggesting systemic regulation of defensin genes by diffusible molecules (Bals *et al.*, 1999). It has been suggested elsewhere that by acting as signaling molecules, defensins could increase resistance to microbial infection by activating other defensins at distant body sites, or other host defences (Ganz, 1999).

1.3.1.2 Regulation of mucins

Membrane-associated MUC1 is ubiquitously expressed in epithelial cells lining the gastrointestinal, respiratory, and reproductive tracts, and by all the major salivary glands and oral epithelial cells (Liu *et al.*, 2002). Recently, regulation of MUC1 was investigated in an oral epithelial cell line (Li *et al.*, 2003a). When cells were treated with the pro-inflammatory mediators IL-1β, IL-6, TNF-α, or IFN-γ, real-time PCR demonstrated that treatment with each of these cytokines alone caused ~2-fold increase in MUC1 mRNA levels, whereas treatment with combinations of mediators nearly doubled this effect. The additive effect of mediators suggests that MUC1 gene expression is up-regulated by one or more different signal transduction pathways (Li *et al.*, 2003a). Cytokine treatment resulted in ~5 to 10-fold increases in MUC1 protein levels, suggesting control at both the transcriptional and translational levels (Li *et al.*, 2003a). These same cytokines are produced in oral tissues in response to infection with the periodontal pathogens *P. gingivalis* (Imatini *et al.*, 2000) and *A. actinomycetemcomitans* (Uchida *et al.*, 2001) the cariogenic dental pathogen *S. mutans* (Hahn *et al.*, 2000), and the pathogenic yeast *C. albicans* (Steele and Fidel, 2002; Li *et al.*, 2003a). Increased MUC1 synthesis may be a key element in the host response to infection with oral pathogens (Li *et al.*, 2003a).

Clinically, the onset of *P. aeruginosa* infection in the lungs of cystic fibrosis patients presages airway mucus obstruction and an overall deterioration of lung function (Li *et al.*, 1997). Li *et al.* (1997) hypothesised that the pathogenesis of cystic fibrosis occurs in two stages : (i) the induction of *P. aeruginosa* infection as a direct consequence of the gene mutation in the cystic fibrosis transmembrane conductance regulator, and (ii) the overproduction of, and airway plugging by, mucin as a consequence of *P. aeruginosa* infection. This group used *in situ* hybridization to determine that MUC2 mRNA expression was significantly elevated in the lung explants of cytstic fibrosis patients, as compared to those of non-cystic fibrosis patients. *P. aeruginosa* culture supernatant significantly up-regulated MUC2 mRNA levels in human airway epithelial and human colon epithelial cell lines (Li *et al.*, 1997). To determine whether transcriptional control mechanisms were involved in the observed MUC2 up-regulation, epithelial cell lines were transfected with an expression vector containing 2.8 kb of the MUC2 5′-flanking region fused to a luciferase reporter gene (Li *et al.*, 1997). *P. aeruginosa* culture supernatant increased the luciferase activity up to ~11-fold. It was also observed that *P. aeruginosa* LPS, and specifically the lipid A component of LPS, increased luciferase activity ~ 7-fold (Li *et al.*, 1997). This suggests that lipid A from diverse bacterial species could potentially up-regulate MUC2 expression (Li *et al.*, 1997).

1.3.2 Evidence for regulation of salivary histatins

1.3.2.1 Histatin levels and oral yeast carriage

Several small clinical studies performed to date suggest that salivary histatins are subject to dynamic regulation under normal and pathological conditions. However, the stimuli and molecular pathways involved in the regulation of histatin secretion under these different conditions are currently unknown. Jainkittivong *et al.* (1998) studied the relationship between salivary histatin levels and oral yeast carriage in healthy individuals. Thirty subjects were divided into two groups based on the presence (yeast positive) or absence (yeast negative) of yeast on oral mucosal surfaces. An inverse relationship was found between levels of histatin in parotid and submandibular/sublingual secretions and yeast colony forming units. For example, in the yeast-positive group, levels of histatin 3 were 7.72 μg/ml (range 4.97-23.86 μg/ml) in parotid saliva, and 3.20 μg/ml (range 2.25-6.50

μg/ml) in submandibular/sublingual saliva. However, in the yeast-negative group, levels of histatin 3 were 12.22 μg/ml (8.73-17.94 μg/ml) in parotid saliva, and 6.63 μg/ml (range 5.18-11.63 μg/ml) in submandibular/sublingual saliva. Levels of histatin 1, histatin 5, and total histatin in parotid and submandibular/sublingual secretions followed a similar pattern in yeast-positive and yeast-negative groups. These results suggest that oral yeast status may be influenced by salivary histatin concentration (Jainkittivong *et al.*, 1998).

1.3.2.2 Histatin levels in recurrent oral candidiasis

Bercier *et al.* (1999) evaluated the levels of salivary histatins in 20 patients with recurrent oral candidaisis (ROC) and in age- and sex-matched healthy controls with no history of oral yeast infections (HC). These authors found that levels of histatins were elevated in the saliva of otherwise healthy patients with a history of recurrent oral candidiasis. The mean total histatin concentration was 16.8 ± 7.5 mg% (range 4.6-31.3 mg%) in the ROC group, and 11.1 ± 6.7 mg% (range 2.4-23.3 mg%) (mg histatin per 100 ml of saliva) for the HC group. Patients with a history of recurrent oral candidiasis had ~5.7 mg% increase in the mean total salivary histatin level, which was statistically significant ($p = 0.016$) (Bercier *et al.*, 1999). These results suggest that the salivary glands of patients with recurrent oral candidiasis express higher levels of histatins than individuals with no history of oral candidiasis. However, despite the increase in the levels of histatin, these patients did not appear to overcome the infection or to be protected from further recurrent infection (Bercier *et al.*, 1999). There are several possibilites that may contribute to the progression of candidal infection in the ROC population. Firstly, patients with recurrent oral candidiasis exhibited a significantly lower salivary pH than the healthy controls (5.9 vs 6.3, respectively, $p = 0.002$). Despite the fact that individuals of the ROC group produce adequate quantities of histatin, the acidic environment of their saliva may modulate or inhibit its antifungal activity (Bercier *et al.*, 1999). This suggestion is supported by previous studies that showed that the efficiency of histatin antifungal activity against *C. albicans* is pH dependant (Santarpia *et al.*, 1990). Secondly, saliva contains several histatins that each exhibit qualitative differences in their antifungal activity. However, this study only compared the levels of total histatin. It is possible that patients with recurrent

oral candidiasis have higher quantities of the less biologically active histatins, while the healthy controls have more of the biologically potent histatins (Bercier *et al.*, 1999). A further possibility is that the higher levels of histatins are not sufficient to counteract the underlying cause of recurrent oral candidiasis.

1.3.2.3 Histatin levels in AIDS patients

Lal *et al.* (1992) conducted a study comparing salivary histatin concentrations in 12 healthy adult controls with those of 12 AIDS patients. The study found that the histatin levels in AIDS patients showed either statistically significant decreases or a decreasing trend compared with that of healthy controls (Lal *et al.*, 1992). In healthy adult controls, levels of histatin 3 were 60 μg/ml (range 8.3-190.7 μg/ml) in parotid saliva, and 18.8 μg/ml (range 6.3-37.2 μg/ml) in submandibular/sublingual saliva. However, in AIDS patients, levels of histatin 3 were 20.8 μg/ml (range 0-67.1 μg/ml) in parotid saliva, and 7.7 μg/ml (range 0-12.2 μg/ml) in submandibular/sublingual saliva (Lal *et al.*, 1992). Levels of histatin 1, histatin 5, and total histatin also showed decreased levels in AIDS patients compared with those of healthy adult controls. Two of the 12 AIDS patients had histatin levels that fell within the normal range of healthy adults, and exhibited maximal killing of test strain *C. albicans* GDH 2023 blastospores by one but not both of their glandular salivas (Lal *et al.*, 1992). However, both of these AIDS patients had oral candidiasis. Lal *et al.* (1992) suggested a number of possibilities to explain this: (i) the *C. albicans* strains in these patients are either more resistant to the levels of histatins present or more invasive than the test strain *C. albicans* GDH 2023, (ii) the oral cavities of the AIDS patients contain large concentrations of bacteria or yeasts that bind up the histatins, leaving insufficient quantities to exhibit their antifungal activities against the *Candida* strains present, (iii) the saliva of AIDS patients contains serum components that complex the histatins and prevent them from inhibiting germ tube formation and blastospore viability, or (iv) the AIDS patients have relatively higher quantities of the less biologically active histatins (Lal *et al.*, 1992). These results contradict an earlier study by Atkinson *et al.* (1990) which found that histatin levels were elevated in HIV-positive and AIDS patients. Another group found that HIV-positive patients secreted normal or elevated levels of histatins, while a subgroup exhibited

diminished saliva flow over time, an increasing tendency to oral candidiasis, and a diminution in output of histatins (Mandel *et al.*, 1992).

1.3.2.4 Relationship between histatin levels and increasing age

Johnson *et al.* (2000) investigated the effect of increasing age on the concentration and secretion of salivary histatins. Parotid and submandibular/sublingual saliva was collected from 80 individuals divided into four age groups having approximately equal numbers of males and females: 35-44 years; 45-54 years; 55-64 years; and 65-76 years. In the youngest age group, the concentrations (μg/ml) of histatin 1, histatin 3, and histatin 5 in parotid saliva were 10.1 ± 1.5, 7.3 ± 2.2, and 13.0 ± 2.1, respectively, while the concentrations in submandibular/sublingual saliva were 34.7 ± 4.0, 10.2 ± 1.6, and 17.4 ± 1.7, respectively (Johnson *et al.*, 2000). On comparing the youngest to the oldest group, the decrease in total histatin concentration (defined in this study as histatin 1 + histatin 3 + histatin 5) in parotid saliva was 45%, and in histatin secretion (μg/min) a 57% decrease with aga was found. Similarly, in submandibular/sublingual saliva, a 38% decrease in total histatin concentration was found, and in histatin secretion a 57% decrease (Johnson *et al.*, 2000). Both saliva types showed a significant decrease ($p < 0.0001$) in the histatin concentration per mg of total protein, suggesting a preferential decrease in salivary histatins with age compared to total salivary protein (Johnson *et al.*, 2000). These results suggest that the salivary histatin component of the oral defence system is compromised with increasing age, which may contribute in part to the increased susceptibility of older adults to oral candidiasis (Johnson *et al.*, 2000). Candidal infections increase with age (Lockhart *et al.*, 1999), and with the wearing of dentures (Marsh *et al.*, 1992). Factors which may predispose the wearer to oral candidiasis are (i) the wearing of a maxillary denture that limits the accessibility of saliva to the denture, and (ii) the reduced salivary flow in denture wearers (Johnson *et al.*, 2000). The number of medications taken typically increases with age, and numerous investigations suggest that many of these medications reduce salivary flow, which in turn could further compromise the secretion of salivary histatins (Johnson *et al.*, 2000). Thus, in addition to the overall decrease in salivary histatins with increasing age,

other factors such as dentures and medications can further compromise salivary secretion and the ability of the host to resist oral candidal infections (Johnson *et al.*, 2000).

1.4 Murine model of oral candidiasis

1.4.1 Rationale for an animal model of oral candidiasis

In order to determine the role of histatins in the protection of the oral cavity from candidal infection, a defined oral environment in which the levels of histatin and the type of organism present can be controlled is required. The complexity and poorly-understood nature of the mucosal system precludes the use of simulations or computer models. *In vitro* systems do not reflect the structured, regulated and multicellular environment of the oral cavity (Samaranayake and Samaranayake, 2001). No human populations have been described that do not express histatin. Even if patients were found with different levels of histatin, it would be unethical to inoculate them with *Candida* strains, or to subject them to oral mucosal and salivary gland biopsies. Also, humans are dissimilar in terms of their dietary and social habits, their immune status, and oral physiology such as salivary gland function. Animals, however, can serve as a standardised tool which can be controlled and manipulated to derive universally comparable data (Samaranayake and Samaranayake, 2001). Therefore, the efficacy of histatin antifungal treatment against mucosal infection is best elucidated using a pre-clinical animal model of oral candidiasis.

1.4.2 Clinical relevance and advantages of mouse model

Mice are widely used by groups investigating oral candidiasis since they have several attributes which make them excellent models of infection (Samaranayake and Samaranayake, 2001). *Candida* spp. are not constituent resident oral microbes of laboratory mice, which is ideal for researchers wishing to investigate infection by particular species and strains of *Candida* (Lacasse *et al.*, 1990). The bacterial flora in mice has been well characterised, and consists of fewer than 20 species (Trudel *et al.*, 1986), so the relationship between commensal oral bacteria and *Candida* can be evaluated. Transient oral carriage of yeast in mice can be readily established by topical inoculation of oral mucosal surfaces with 10^8 *Candida* blastospores per ml (Samaranayake and Samaranayake, 2001). The development of oral candidiasis can be encouraged by mildly immunosuppressing the mice

with subcutaneous injections of cortisone acetate or prednisolone (Takakura *et al.*, 2004), or by the addition of chlortetracycline or hydrocortisone to the drinking water (Holbrook *et al.*, 1983). Different sites of the mouse oral cavity (dorsal surface of tongue, buccal mucosa, and gingival mucosa) are preferentially colonised by yeast, but the mid-posterior lingual dorsum appears to be the most favoured site of infection, as in humans (Samaranayake and Samaranayake, 2001). The histopathological changes of the oral mucosa in rat with candidal infection are highly consistent with those of human lesions (Fig. 1.3) (Samaranayake and Samaranayake, 2001). Their small size is an added advantage since it facilitates routine daily monitoring, especially when large numbers are required (Samaranayake and Samaranayake, 2001). However, the small size of the murine oral cavity can be considered a distinct drawback due to the difficulty in monitoring mucosal changes by naked-eye examination. It can be difficult to study the interactions between the artifically introduced exogenous yeasts and the host in the presence of the population pressure exerted by commensal bacteria, so germ-free gnotobiotic mice have been used by some researchers. The commensal flora regulate yeast numbers by inhibiting the adherence of yeasts to oral surfaces by competing for sites and nutrients. It has been shown *in vivo* in gnotobiotic mice and *in vitro*, that candidal colonisation of epithelia could be suppressed by steptococci, which are the predominant resident commensals of oral mucosal surfaces (Liljemark and Gibbons, 1973; Samaranayake *et al.*, 1994; Nair and Samaranyake, 1996).

The mouse model has been exploited by various researchers wishing to elucidate the clinical, therapeutic, and immunological features of oral candidiasis. Several studies have used the mouse model to observe candidal colonisation patterns and the inflammatory response in chronic recurrent candidal infection (Lacasse *et al.*, 1990, 1993). The therapeutic activity against oral candidiasis of orally administered bovine lactoferrin , and the influence of lactoferrin on immune responses relevant to this therapeutic effect have been examined in immunosuppressed mice (Takakura *et al.*, 2003, 2004). Flattery *et al.* (1996) developed a murine model of chronic oropharyngeal and gastrointestinal mucosal colonisation by *C. albicans* specifically for the evaluation of antifungal drugs. To simulate the immune deficiency observed in AIDS patients, DBA/2 mice were specifically depleted of CD4+ T lymphocytes by treatment with a rat immunoglobulin G2 monoclonal antibody secreted by GK1.5 hybridoma cells, which is specific for mouse CD4+ T cells. The

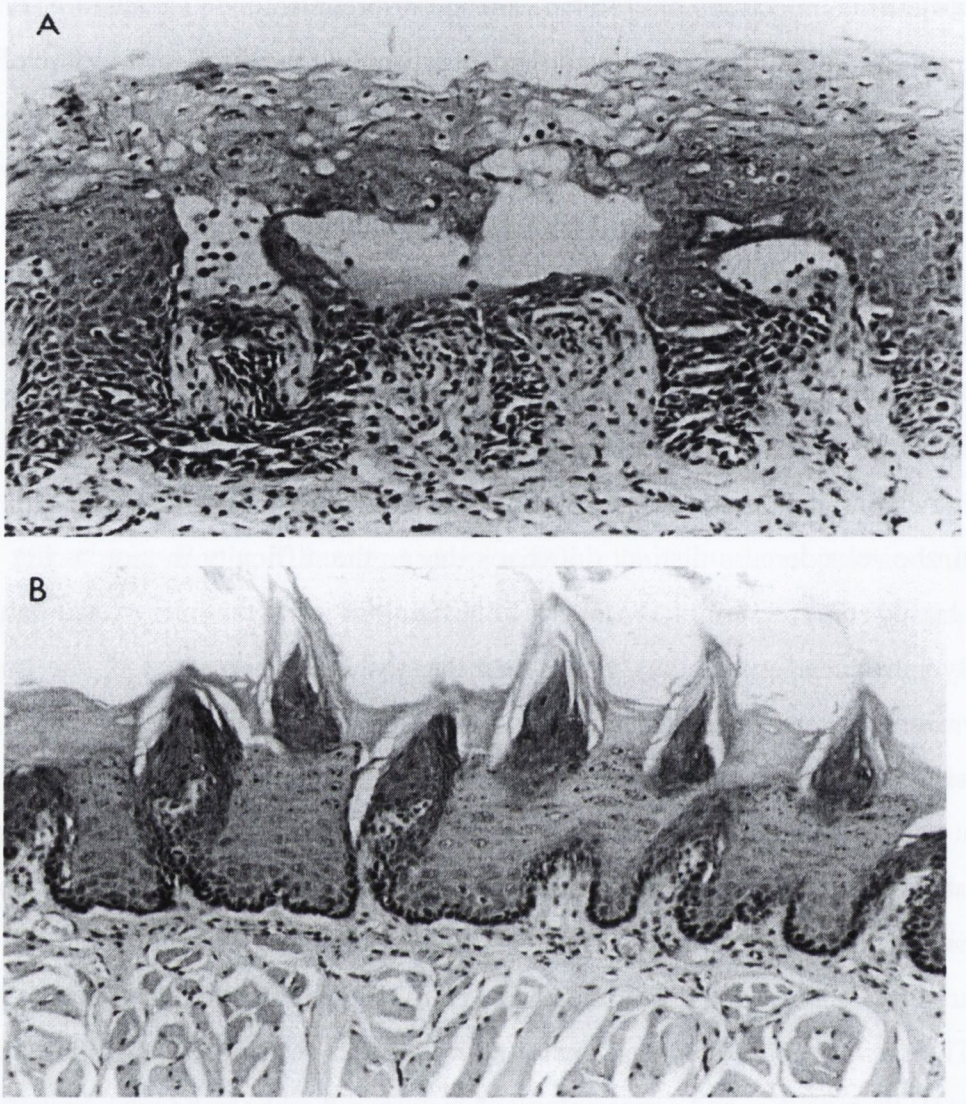


Figure 1.3 A, Histopathologic section of the dorsal tongue of a rat infected with *C. albicans*. Note the loss of filiform papillae and the flat-surfaced, parakeratotic, edematous and acanthotic lingual epithelium. **B**, Histological section of the lingual mucosa from the posterior dorsal tongue of a rat demonstrating uninfected epithelium. Note the normal filiform papillae covered by a layer of thick acellular orthokeratin (Samaranayake and Samaranayake, 2001).

efficacies of the commercially available antifungal agents fluconazole, ketoconazole, and nystatin, and the lipopeptide antifungal agents L-693989 and L-733560 were evaluated in this model of mucosal colonisation.

The mouse model and its variants with immune abnormalities are suited to evaluation of the humoral and cellular immune responses associated with candidal infection. Cantorna and Balish (1990) introduced a mouse model with a low CD4+ T-cell count and defective phagocytic function. This congenitally immunodeficient germ-free mouse model (*bg/bg nu/nu*) appears to be naturally susceptible to oral and esophageal candidiasis without supplements of antibiotics, immunosuppressives, or cytotoxic drugs. This group investigated the ability of athymic (*nu/nu*), euthymic (*nu/+*), beige (*bg/bg*), black (*bg/+*), beige athymic (*bg/bg nu/nu*), and beige euthymic (*bg/bg nu/+*) germ-free mice to contract superficial and systemic candidiasis (Samaranayake and Samaranayake, 2001). An autosomal recessive mutation responsible for severe combined immune deficiency (SCID syndrome) has been reported in mice (Bosma *et al.*, 1983). SCID is a rare congenital syndrome of humans that results in loss of both B-cell and T-cell immunity, and SCID mice are also severely deficient in these lymphocytes (Samaranayake and Samaranayake, 2001). SCID mice have provided valuable information on the roles of both the B and T cells in mucosal candidiasis (Balish *et al.*, 1993). Mice infected with the Du5H(G6T2) mixture of mouse leukemia viruses develop a syndrome, MAIDS, that has many of the immune abnormalities found in HIV infection. Data from studies using this model are consistent with a role for Th1 cells in host resistance to mucosal candidisis (Deslauriers *et al.*, 1997), but suggest that CD4+ and/or T cells, or NK cells, may also contribute either through the production of gamma interferon (Samaranayake and Samaranayake, 2001), or through the direct antifungal activity of CD8+ cells against *C. albicans* (Coleman *et al.*, 1997). Together, the SCID and MAIDS mouse models may be useful in the future for studying oral candidiasis in HIV infection.

1.4.3 Transgenic animal technology

More is known about the genome of the mouse than any other mammalian species, which is one reason for its popularity as a research animal. Several inbred strains are widely available, which is useful when a genetically homologous background is required. The

C57Bl/6 strain is a popular inbred mouse strain used by many commercial transgenetic facilities, and has been used in previous studies as a mouse model of oral candidiasis (Deslauriers *et al.*, 1997). Transgenic animal technology is routinely used to express human genes in mice, so the function of the gene can be assessed in a controlled environment (Wolfensohn and Lloyd, 2003). There are two main methods used to produce transgenic animals: pronuclear microinjection and embryonic stem (ES) cell-mediated gene transfer. Gene transfer by microinjection is the predominant method used to produce transgenic mice. The transgene is microinjected into the male pronuclei of fertilised eggs, and the eggs implanted into pseudopregnant females. Typically 10-30% of the mice that are born from the injected embryos have incorporated the transgene into their genomes (Pinkert, 1994). Integration is random, usually occurs at a single site, and can include from one to hundreds of copies of the transgene. Integration normally occurs before the first zygotic cleavage event, which results in all the tissues of the mouse, including the germ cells, containing the same transgene insert (Samuelson, 1996). Founders are bred with wild type mice to ensure the transgene can be stably inherited. Positive offspring are identified by the polymerase chain reaction method. Brother-sister breeding pairs are established to derive mice homozygous for the transgene. Homozygosity generally yields mice with more copies of the transgene, and consequently, higher levels of transgene expression. Homozygous mice are identified using Southern blot hybridisation, and breeding homozygotes with wild type mice should result in transmission of the transgene to 100% of its offspring (Pinkert, 1994).

Another technique for producing transgenic mice has been developed using pluripotent mouse ES cells. This technique exploits the unique ability of these cultured cells to contribute to the germline of a developing mouse, together with homologous recombination techniques, to generate mice with defined genetic modifications. ES cells are isolated from the inner cell mass (ICM) of preimplantation blastocysts and can be grown continuously in culture in an undifferentiated, pluripotent state and genetically manipulated using somatic cell genetic techniques (Pinkert, 1994; Samuelson, 1996). Specific gene mutations are introduced into an endogenous gene copy by homologous recombination with a DNA construct containing an engineered mutation. Once a targeted ES cell line has been identified and characterised, the cells are microinjected into a preimplantation embryo where the ES cells mix with the host ICM and contribute to all the tissues of the developing

chimera, including the germ cells. Breeding chimeras allows the transmission of the engineered gene mutation and the establishment of a mutant mouse line (Samuelson, 1996).

After transfer into an experimental animal, the expression of a transgene is dependent on a number of factors. Strong promoter/enhancers from simian virus 40, Rous sarcoma virus, and cytomegalovirus (CMV) are active in the majority of cell types, including the salivary glands, because they contain a number of binding sites for ubiquitous transcription factors. The mouse mammary tumor virus-long terminal repeat drives expression in salivary, mammary, and other secretory epithelium. Cell/tissue specific promoters can be used to restrict transgene expression to one or more cell/tissue types (Samuelson, 1996).

Several groups have generated transgenic mice that express human antimicrobial proteins to investigate the *in vivo* function of these proteins in different models of disease. Transgenic mice expressing human lactoferrin and lysostaphin have been used in models of *S. aureus* infection (Kerr *et al.*, 2001; Guillen *et al.*, 2002), while transgenic mice expressing human intestinal defensin have been used in a model of enteric salmonellosis (Salzman *et al.*, 2003). These studies offer compelling support for the emerging role of mammalian antimicrobial peptides in host defence against microbial challenge.

Mice do not have the histatin gene, and therefore wild type strains make an ideal negative control group. Transgenic technology can be applied to mice to express histatin in their saliva, and thus determine the efficacy of histatin in a mouse model of oral candidal infection. Mice breed relatively quickly: female mice cycle every 4-5 days, gestation lasts 19-21 days, pups are weaned at 3 weeks, male mice reach puberty at 7 weeks, and females at 6 weeks (Wolfensohn and Lloyd, 2003). This is an important consideration when using transgenic animals, since it may take four generations before a stable transgenic line is established. Directive 90/219/EEC on the contained use of genetically modified micro-organisms (GMMs) provides common rules througout the EU for the use of GMMs both in research laboratories and inductrial facilities and to provide appropriate measures to protect human health and the environment from any risk from activities arising using GMMs. On March 15th 2001 the Genetically Modified Organisms (contained use) Regulations, 2001, S.I. No. 73 of 2001 were transposed into Irish law giving effect to Directive 98/81/EC amending Directive 90/219/EEC on the contained use of GMOs. In accordance with article

5 of the GMOs (contained use) Regulations S.I. No. 73 of 2001, the onus is on the user carrying out a contained use activity, to ensure that all appropriate measures are taken to avoid adverse effects on human health or the environment, and to apply the principles of Good Animal House Practice.

1.5 Aims of this project

Fungal infection of the oral and gastrointestinal tract is a potentially life-threatening problem, with systemic candidiasis carrying a mortality rate of up to 79%. Candidiasis affects a wide variety of patients, including those infected with HIV, organ transplant recipients, cancer patients, diabetics, and denture wearers. A paper published by the Health Protection Surveillance Centre in November 2006, reports that there are currently 4,251 known cases of HIV infection in Ireland. According to the latest figures published in the UNAIDS/WHO 2006 AIDS Epidemic Update, an estimated 39.5 million people are living with HIV globally. There were 4.3 million new infections in 2006, with 2.9 million people dying of AIDS-related illnesses. Up to 90% of HIV-infected individuals suffer from oropharyngeal candidiasis at some stage during their disease, with relapse rates between 30% and 50% on completion of antifungal treatment in severe immunosuppression. A major concern with the management of candidiasis is the increasing frequency of drug resistant organisms. Approximately 5% of *C. albicans* and 35% of *C. glabrata* infections are azole-resistant, with a limited range of antifungal drugs available. Consequently, there is an urgent need for new treatment alternatives for oral candidiasis.

Human saliva contains a range of potent antimicrobial proteins, including lysozyme, lactoferrin, and histatins. Histatins demonstrate potent antimicrobial activity against various *Candida* spp. and other pathogenic yeasts and bacteria *in vitro*. They are also non-toxic to mammalian cells and do not induce resistant microorganisms. These attributes suggest that histatins may be a promising alternative therapeutic agent for the treatment of oral candidiasis. However, there is no direct evidence to support the hypotheses that histatins are essential to oral health and are regulated by illness. The role histatins play in the maintenance of oral health, and the extent to which they are regulated, are currently unknown. This project seeks to help define the role of this salivary antifungal agent in

controlling the oral population of *Candida* during health and disease, and thus, its therapeutic potential in the management of mucosal candidiasis.

The main objectives of this project were:

- To design a suitable transgene cassette capable of expressing recombinant human histatin 3 in the saliva of transgenic mice. The pIRES2-EGFP vector was chosen for use in this study since it contains both a CMV promoter and an enhanced green fluorescent (EGFP) reporter gene. The CMV promoter has been shown to drive high levels of recombinant protein expression in mammalian salivary glands. The EGFP protein is useful for locating transgene expression in cells. Histatin 3 cDNA was tagged with an internal polyhistidine tag to simplfy purification and detection of recombinant histatin in cultured cell media and tissue samples since there are no useful antibodies available to detect histatin in heterogenous mixtures of proteins.
- To generate histatin-positive transgenic mice which may then be used in a mouse model of oral candidiasis. This requires the generation of transgenic mice which constitutively express histatin in their saliva. Only humans and Old World primates secrete histatin in their saliva, therefore mice provide an ideal model in which to investigate the effects of histatin on oral yeast carriage rates *in vivo*, and to investigate whether the expression of salivary histatin will prevent or attenuate the development of oral candidiasis. Transgenic mice must first be fully characterised regarding stable inheritance and intactness of the transgene. Tissue-specific expression of the transgene, and levels of trasngene expression, must also be characterised, using RT-PCR and semi-quantitative RT-PCR techniques, respectively. It must be confirmed that the transgenic mice are expressing the histatin protein in their salivary glands before these mice can be used in candidal infection experiments.
- To identify some of the external stimuli and *cis*-elements which may be responsible for histatin gene regulation *in vitro.* Several small clinical studies performed to date in humans suggest a dynamic relationship between salivary histatin levels and factors such as oral yeast carriage rates, the immune status of the patient, oral candidiasis, patient age, or certain medications. However, there is no direct evidence to prove that histatins are differentially regulated during health and disease. In order to identify which

region(s) of the *HIS2* promoter are responsive to stimuli, deletional analysis of the histatin promoter must be performed, and the relative activities of each of the deletion constructs investigated under different conditions. Identification of stimuli and *cis*-elements which up-regulate the *HIS2* gene may lead to therapies which increase endogenous levels of histatins in saliva, and thus, the resolution of oral candidal infection.

Chapter 2

Materials and Methods

2.1 Chemicals, enzymes, and oligonucleotides

All chemicals used were of analytical-grade or molecular biology-grade and were purchased from Sigma-Aldrich Ireland Ltd. (Tallaght, Dublin, Ireland), BDH (Poole, Dorset, UK) or from Roche (Lewes, East Sussex, UK). Enzymes for molecular biology procedures were purchased from the Promega Corporation (Madison, Wisconsin, USA), Roche or New England Biolabs Inc. (Beverley, Massachusetts, USA). DNA molecular weight markers were purchased from GibcoBRL Life Technologies (Gaithersburg, Maryland, USA) and Promega. Oligonucleotide primers were custom synthesised by Sigma-Genosys Biotechnologies Ltd. (Cambridge, UK).

2.2 Human submandibular gland cell line

The human submandibular gland-derived (HSG) cell line (supplied by Prof. Brian O'Connell, Trinity College Dublin, Ireland) was maintained in a 37℃ static incubator with 5% CO_2 in Eagle's minimal essential medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, and routinely passaged every 7 days using trypsin EDTA (O'Connell *et al.*, 1996). All cell culture reagents were purchased from Sigma.

2.3 Small and large scale isolation of plasmid DNA from *E. coli*

Small scale preparations of plasmid DNA for sequencing, and large scale preparations of plasmid DNA for transfections, were prepared using Quantum Prep miniprep and maxiprep kits from Bio-Rad (Hercules, California, USA), according to the manufacturer's instructions.

2.4 Purification of restriction endonuclease-digested DNA fragments from agarose gels

Restriction endonuclease-digested DNA fragments were purified from agarose gels using NA45 DEAE membranes (Schleicher and Schuell, Dassel, Germany). Fragments were electrophoresed on agarose gels and viewed on a UV transilluminator (345 nm) (Ultra-Violet Products, Cambridge, UK). Using a clean scalpel blade, a small rectangular trough was excised from the gel immediately ahead of the fragment of interest, and a piece of

NA45 DEAE paper was placed adjacent to it. The excised fragment of gel was replaced and the electrophoresis was allowed to continue until the fragment had run onto the paper, which could be verified by the fluorescent staining of the paper with ethidium bromide. The paper was then placed in 0.5 ml 1 M NaCl and placed in a water bath at 37°C for at least 1 h to elute the fragment. The DNA solution was then extracted three times with iso-butanol to remove the ethidium bromide, and once with phenol:chloroform (1:1). The DNA was precipitated with two volumes of ice-cold ethanol, pelleted at 10,000 x *g* in a microfuge and resuspended in 10 µl sterile distilled water.

2.5 Ligation of DNA fragments

Purified DNA fragments were ligated to vectors digested with the appropriate restriction endonucleases. Ligation of PCR products to these vectors was carried out *via* restriction sites which had been designed within the oligonucleotide primers used in the amplification reactions. Gel purified DNA fragments were ligated directly into the appropriate restriction endonuclease generated site in the cloning vector. Ligation reactions were carried out in a 20 µl volume, with a 3:1 ratio of insert to vector DNA in 1x ligase buffer, with 1 U of T4 DNA ligase (Promega). Reactions were carried out at room temperature for 18 h.

2.6 Preparation of competent *E. coli* using $CaCl_2$

Transformation of *E. coli* with $CaCl_2$ was carried out by the method of Sambrook *et al.* (Sambrook, 2001). *E. coli* DH5α was inoculated from an overnight broth culture into 100 ml LB and grown at 200 rpm in a Gallenkamp orbital incubator (New Brunswick Scientific Company Inc., Edison, New Jersey, USA) at 37°C for 3 h to an A_{600} of ~0.5. The culture was then decanted into ice-cold 50 ml Falcon tubes and chilled on ice for 10 min. Cells were then pelleted by centrifugation at 5,000 x *g* in a Sorvall SS34 rotor (Dupont Co., Denver, Colorado, USA) at 4°C for 10 min. Each pellet was resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and centrifuged as before. The pellets were then resuspended in a volume of 2 ml 0.1 M $CaCl_2$ for each 50 ml of original culture.

2.7 Transformation of competent *E. coli*

A 0.2 ml aliquot of competent *E. coli* cell-suspension was transferred to a sterile microfuge tube on ice for each transformation experiment. Plasmid DNA (up to 50 ng) was added to each tube and incubated on ice for 30 min. A known amount of a standard plasmid preparation was added to a separate tube as a positive control, and a second control tube was also included which contained no plasmid DNA at all. The tubes were then heat shocked at 42°C for exactly 90 s and rapidly transferred to an ice bath. The cells were then incubated at 37°C for 1 h in a water bath in the presence of 0.8 ml LB medium to allow the cells to recover and express the antibiotic resistance marker. A 0.1 ml aliquot of this suspension was then spread on LA plates containing the appropriate antibiotic and incubated at 37°C overnight.

2.8 DNA sequence analysis

DNA sequencing was performed commercially by Lark (Saffron Walden, Essex, UK) using the dideoxy chain termination method of Sanger *et al.* (1977), and an automated Applied Biosystems 373A DNA sequencer (Foster City, California, USA) and dye-labelled terminators. Chromatograms were analysed using the 373A Data Analysis program version 1.2.0 (Applied Biosystems). Sequence analysis was carried out using the DNA Strider 1.3f11 software for DNA and protein analysis (CEA/Saclay, Gif-sur-Yvette, France). Alignments of nucleotide and amino acid sequences were carried out using CLUSTAL W sequence alignment computer program (Higgins, 1988).

Chapter 3

Construction and Functional Analysis of the 6xHisH3 Transgene

3.1 Introduction

Salivary histatins are believed to play a key role in protecting the oral cavity from opportunistic fungal infections. Extensive research has shown that histatins have potent antimicrobial activity against *C. albicans* and other microbes *in vitro*. However, there is no direct evidence which demonstrates the *in vivo* role or significance of histatins in the oral cavity. Mice do not secrete salivary histatins, and therefore provide an ideal model in which to investigate the effects of histatins on the oral flora. The purpose of this part of the study was to design an expression cassette capable of driving high levels of constitutive histatin expression to the salivary glands of transgenic mice.

Transgenic expression studies in mice and rats have identified several promoters which can direct transgene expression to the salivary glands (Samuelson, 1996; O'Connell *et al.*, 1996; Zheung *et al.*, 2001; Zheung and Baum, 2005). Strong promoters from simian virus 40, Rous sarcoma virus, and cytomegalovirus (CMV) are active in the majority of cell types, including the salivary glands, because they contain a number of binding sites for ubiquitous transcription factors. The mouse mammary tumor virus-long terminal repeat (MMTV-LTR) drives expression in salivary, mammary, and other secretory epithelium. However, the MMTV-LTR can be influenced by the different hormones levels expressed in male and female mice. Cell/tissue specific promoters can be used to restrict transgene expression to one or more cell/tissue types (Samuelson, 1996). The human salivary amylase and kallikrein promoters are active in acinar and ductal cells, respectively, while the CMV is active in most cell types. However, a recent study evaluating viral and mammalian promoters for use in gene delivery to salivary glands found that in adenoviral vectors, the CMV promoter showed highest activity in the rat submandibular A5 cell line *in vitro* and in rat submandibular glands *in vivo* (Zheng and Baum, 2005). The CMV promoter has been shown to drive high levels of histatin expression to rat salivary glands both *in vitro* and *in vivo* (O'Connell *et al.*, 1996).

In the present study, histatin 3 (H3) cDNA was amplified and cloned into the pIRES2-EGFP vector. This vector contains several features which made it suitable for use in the design of a transgene cassette capable of expressing histatin in the salivary glands of transgenic mice. It contains the human immediate early CMV promoter which has been shown to drive expression of transgenes in salivary glands. It also contains an internal

ribsomal entry site (IRES) of the encephalomyocarditis virus between the multiple cloning site (MCS) and the enhanced green fluorescent protein (EGFP) reporter gene. This permits translation of both H3 and EGFP from a single bicistronic mRNA. EGFP is a red-shifted variant of wild type GFP which has been optimised for brighter fluorescence and higher expression in mammalian cells. EGFP may also be used to localise transgene protein expression in transgenic mice using confocal microscopy and/or immunohistochemistry. SV40 polyadenylation signals downstream of the EGFP gene direct proper processing of the 3′-end of the bicistronic mRNA. All these elements comprise the expression cassette which was used in the generation of histatin-positive transgenic mice later in this project.

3.2 Materials and Methods

3.2.1 Amplification and cloning of histatin 3 cDNA into pIRES2-EGFP vector

Histatin 3 (H3) cDNA was amplified with forward primer HTN3F2 and reverse primer HTN3R2 using pCMV.SV2H3 vector as the template (supplied by Prof. Brian O'Connell, Trinity College Dublin, Ireland) (Table 3.1). PCR was carried out in a 20 µl volume containing 1 U of *Taq* Polymerase (Promega). PCR cycling conditions for each round were as follows: 1 cycle at 94°C for 1 min, 35 cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min, and 1 cycle at 72°C for 10 min. The amplicon was cloned into pIRES2-EGFP (pEGFP) on a *Bgl*II/*Eco*RI fragment. The resulting vector was termed pEGFP-H3. Digested vector and PCR fragments were purified from agarose gels using NA45 DEAE membranes.

3.2.2 Transient transfection of HSG cell line using calcium phosphate method

Transfections were performed using the calcium phosphate transfection kit from Invitrogen (Carlsbad, California, USA) according to the manufacturer's instructions. For large scale expression of H3, HSG cells were seeded at a density of 2 x 10^7 cells/plate (dimensions: 245 mm x 245 mm x 25 mm). pEGFP-H3 (100 µg) were used to transfect each plate. Negative controls used consisted of a no-plasmid control and cells transfected with pEGFP alone. Supernatant was harvested 24 h post-transfection.

3.4.3 Dialysis and concentration of HSG supernatant

Tissue culture supernatant (800 ml) was collected from cells transfected with pEGFP-H3. The supernatant was placed in SpectraPor 7 dialysis tubing (m.w. cut off 1,000) (Spectrum Laboratories Inc., Rancho Dominguez, California, USA) and dialysed against 5 l of dialysis buffer (5% (v/v) acetic acid in water pH 2.7) 3 times over a 6 h period. The sample was concentrated by placing the dialysis bags directly onto polyethylene glycol (PEG) (nominal m.w. 8,000) overnight at 4°C (O'Connell *et al.*, 1996). When the sample volume was reduced to approximately 5 ml (a 160-fold concentration of the original 800 ml volume), the liquid was removed, centrifuged briefly to remove debris, and stored at -80°C until required. Negative controls consisted of supernatant from non-transfected cells and cells transfected with pEGFP alone.

Table 3.1 Primers used to amplify H3 cDNA, generate an extra 4 histidine residues in H3 cDNA, and complete the 5′-end of the 6xHisH3 gene

Primer	Nucleotide sequence 5′-3′	Restriction site
Amplify H3 cDNA		
HTN3F2 primer	CGCTCTAGATCTAGCCAACTATGAAG	*Bgl*II
HTN3R2 primer	CGGGTATCGAATTCACTAAATCGTGG	*Eco*RI
Generate 6xHis tag in H3 cDNA		
HTN3FP1	GCAAAGAGA**CATCATCATCAT**CATCATGGGTATA	N/A
HTN3FP2	TCCATGACTGGAGCTGATTCACATGCAAAGAGACA	N/A
6xHis F1	ACTCAGATCTAGCCAACTATGAAGTTTTTTGTTTTT GCTTTAATCTTGGCTCTCATGCTTTCCATGAC	*Bgl*II
HTN3R2	CGGGTATCGAATTCACTAAATCGTGG	*Eco*RI

Underlined sequences refer to positions of restriction sites.
Bold letters refer to the 4 CAT codons in the HTN3FP1 forward primer used to generate the polyhistidine tag in H3 cDNA.
N/A, not applicable.

3.2.4 Cationic polyacrylamide gel electrophoresis (PAGE)

Cationic PAGE was performed using a modification of the system as described by Baum *et al.* (1977). Samples were electrophoresed in a cationic, 15% (v/v) acrylamide gel system, with the exception that the gels were stained overnight.

3.2.5 Zinc precipitation of cationic proteins from HSG supernatant

Zinc precipitation of H3 from the supernatant prepared in section 3.4.3 was performed using the method described by Flora *et al.* (2001). Briefly, zinc chloride was added to the samples to give a final concentration of 500 μM zinc chloride. The pH of the samples were raised to pH 9.0 with 0.1 M NaOH and incubated at 4°C for 20 min, followed by centrifugation at 16, 000 x *g* for 10 min. The supernatant was removed and the pellets containing the histatin-metal complexes were washed once with water containing 500 μM zinc chloride, centrifuged again and stored at 4°C until required.

3.2.6 Generation of a 6xHis tag in H3 cDNA by PCR

H3 cDNA was labelled with an internal polyhistidine tag through 3 rounds of PCR, using pEGFP-H3 as the template. The polyhistidine tag consisted of 6 consecutive histidine residues. The first PCR product was amplified using forward primer HTN3FP1 and reverse primer HTN3R2 (Table 3.1) which generated an extra 4 CAT codons immediately upstream of the region which codes for the 2nd and 3rd histidine residues in the mature H3 peptide. The 5′ end of the gene was generated through 2 more rounds of PCR using forward primers HTN3FP2, 6xHis F1 and reverse primer HTN3R2 (Table 3.1). PCR reactions were carried out in 20 μl volumes containing 1 U of *Taq* Polymerase. PCR cycling conditions for each round were as follows: 1 cycle at 94°C for 1 min, 35 cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min, and 1 cycle at 72°C for 10 min. The final PCR product was cloned into pEGFP on a *Bgl*II/*Eco*RI fragment (Fig. 3.1). The resulting vector was termed pEGFP-6xHisH3. Digested vector and PCR fragments were purified from agarose gels using NA45 DEAE membranes. The presence of the internal polyhistidine tag was confirmed by DNA sequence analysis. Since the tag is positioned away from the

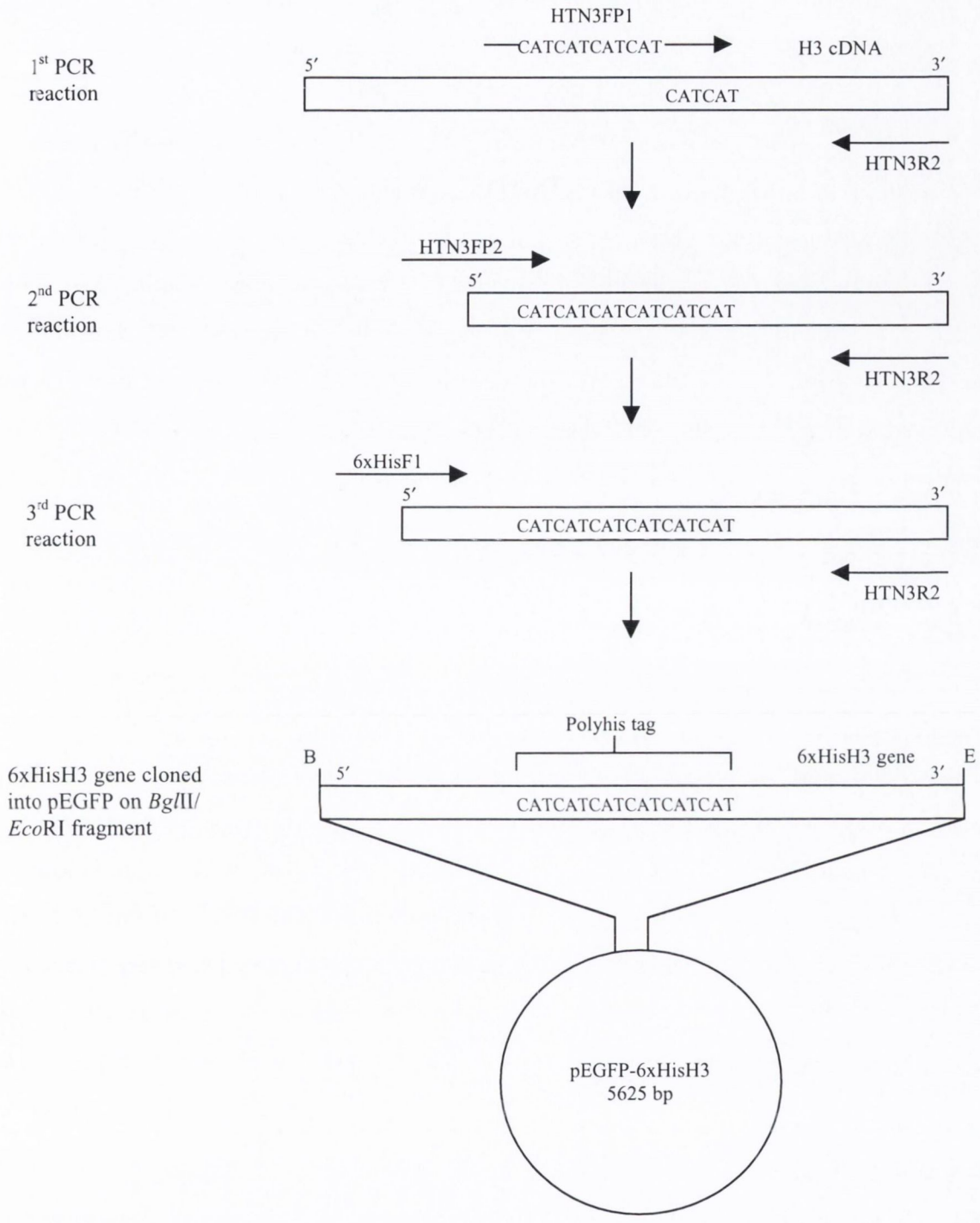


Figure 3.1 Schematic diagram showing the generation of an internal polyhistidine tag in H3 cDNA by PCR. The first PCR reaction introduced 4 additional CAT codons immediately upstream of the region which codes for the 2nd and 3rd histidine residues in the mature H3 peptide, using forward primer HTN3FP1. This resulted in 6 consecutive histidine residues (6xHis tag) within the H3 gene. The 5′-end of the gene was generated through 2 more rounds of PCR using forward primers HTN3FP2 and 6xHisF1. The amplicon, 6xHisH3, was cloned into the pEGFP vector on a *Bgl*II/*Eco*RI fragment, and the resulting plasmid termed pEGFP-6xHisH3. B, *Bgl*II; E, *Eco*RI.

antimicrobial domain, it should not interfere with histatin candidacidal activity (Fig. 3.2). A schematic diagram of the pEGFP-6xHisH3 plasmid is shown in Fig. 3.3.

3.2.7 Selective purification of 6xHisH3 using nickel-nitrilotriacetic (Ni-NTA) silica

Purification of 6xHis-tagged H3 (6xHisH3) from supernatant transfected with pEGFP-6xHisH3 was performed on Ni-NTA spin columns (Qiagen, Crawley, West Sussex, UK) under native conditions, according to the manufacturer's instructions. Briefly, 6xHisH3 was selectively bound to the Ni-NTA columns using binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, 1 mM imidazole, pH 8.0). Any non-tagged contaminating proteins were removed with a low stringency wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM imidazole, pH 8.0). The 6xHisH3 protein was eluted twice in 100 µl aliquots of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). Supernatant from non-transfected and pEGFP transfected cells were also run on Ni-NTA spin columns as negative controls.

3.2.8 Functional analysis of 6xHisH3 by candidacidal assay

The candidacidal assay used was a modification of the method previously described by Xu *et al.* (1991). *C. albicans* 132A (Gallagher *et al.*, 1992) was grown on potato dextrose agar (PDA) (Oxoid, Dorset, United Kingdom) plates at 30°C for 48 h. A single colony was inoculated into 10 ml of yeast extract-peptone-dextrose (YEPD) medium and grown overnight at 30°C in a Gallenkamp orbital incubator set at 200 rpm. Cells were washed with 0.9% (w/v) NaCl, counted using a hemocytometer, and resuspended at 10^5 cells/ml. Candidacidal assays were performed in 100 µl of 10 mM potassium buffer, pH 7.4, and contained 10^4 cells and a range of histatin concentrations (0.01 to 0.04 mg/ml). The reaction was incubated at 37°C for 1 h with vigorous mixing. The mixture was then diluted 1/10 by adding 900 µl of 0.9% (w/v) NaCl, and 100 µl of each dilution was spread onto PDA plates, which was followed by incubation at 30°C for 48 h. The number of single colonies on each plate was then counted. Killing activity was calculated as the number of CFU on test plates as a percentage of CFU on control plates (cells incubated in the absence

A **H3 sequence**

DSHAKR**HH**GYKRKFHEKHHSHRGYRSNYLYDN

B **H3 sequence with internal polyhistidine tag (6xHisH3)**

DSHAKR**HHHHHH**GYKRKFHEKHHSHRGYRSNYLYDN

Figure 3.2 Sequences of the mature H3 peptide A, before, and B, after generation of an internal polyhistidine tag. An extra 4 histidine residues were incorporated immediately upstream of the region which codes for the 2nd and 3rd histidine residues in the mature H3 peptide to yield a recombinant peptide with an internal 6xHis tag. Since the tag is positioned away from the antimicrobial domain (highlighted in grey) of the H3 peptide, it should not interfere with the candidacidal activity of the 6xHisH3 recombinant peptide.

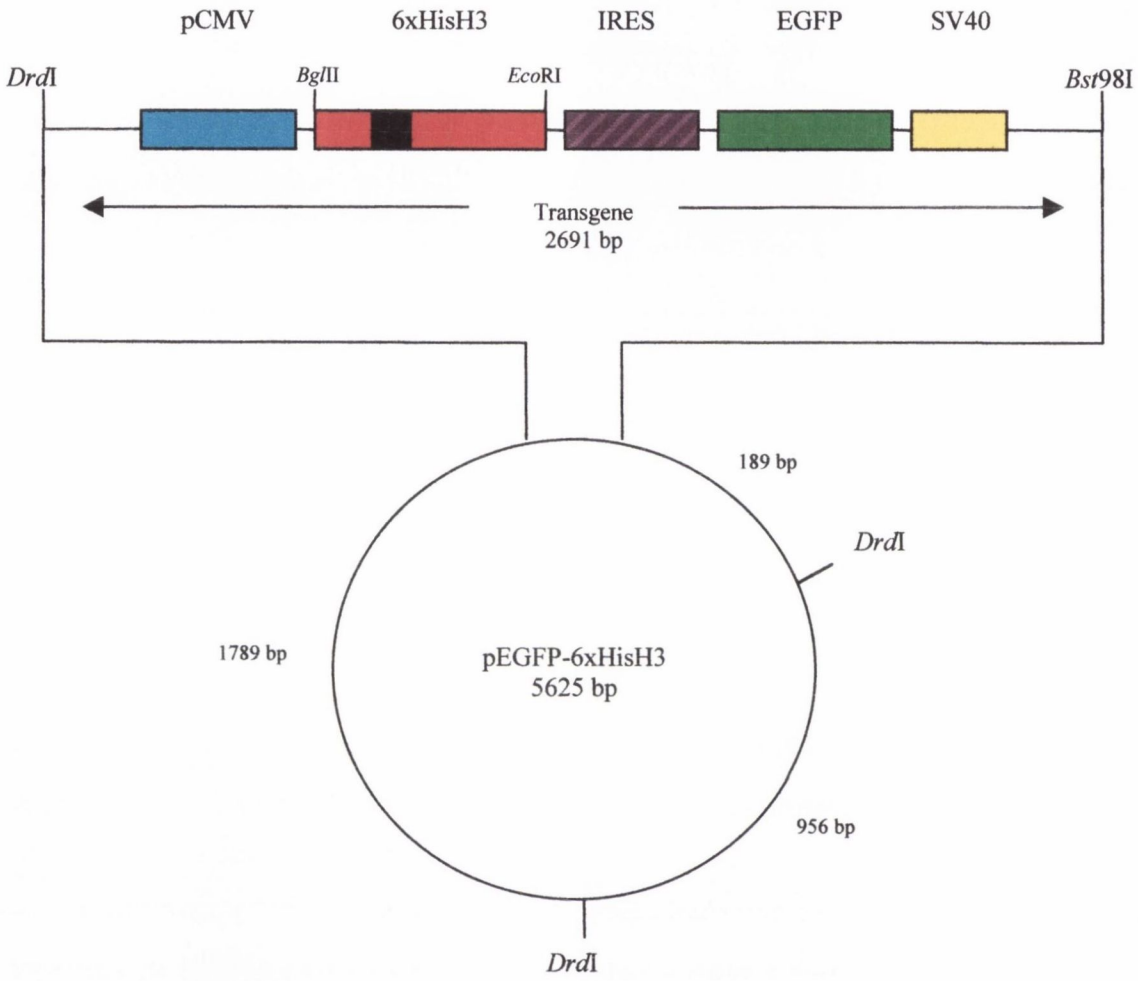


Figure 3.3 Schematic diagram of the pEGFP-6xHisH3 plasmid. The 6xHisH3 gene was cloned into the pEGFP plasmid on a *Bgl*II/*Eco*RI fragment. Double digestion of pEGFP-6xHisH3 yields 4 fragments, 2691 bp, 1789 bp, 956 bp, and 189 bp in length. The 2691 bp fragment representing the 6xHisH3 transgene was used to generate transgenic mice expressing the 6xHisH3 protein in their salivary glands.

of H3). Anticandidal activity of 6xHisH3 was compared to non-tagged synthetic H3 (Albachem, University of Edinburgh, Edinburgh, Scotland).

3.2.9 Detection of 6xHisH3 protein by dot blot

Positope protein and anti-6xHisG alkaline phosphatase-conjugated antibody (AP) were purchased from Invitrogen. Positope is a recombinant protein specifically engineered to contain seven different tags, including a 6xHisG tag, for detection with seven different antibodies. It is intended for use as a positive control for antibody function in immunodetection experiments. A nitrocellulose hybridization membrane (0.2 μm) was cut into a 8 cm x 4 cm strip. Positope Control Protein 6xHisG (100 ng), 2 μl of pEGFP-6xHisH3 cell lysate, pEGFP-6xHisH3 supernatant, pEGFP cell lysate, pEGFP supernatant, non-transfected HSG cell lysate, and non-transfected HSG supernatant were allowed to dry for 1 h onto one pre-cut membrane (6xHisH3 membrane). The strip was placed in a 50 ml Falcon tube and incubated with 5 μl of anti-6xHisG (1:1000 dilution) in 5 ml blocking buffer (1% (w/v) BSA and 0.1% (v/v) Tween-20 in TBS) overnight. The membrane was washed twice with 5 ml of wash buffer (0.1% (v/v) Tween-20 in TBS) for 5 min. One tablet of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) alkaline phosphate substrate (Sigma) was dissolved in 10 ml of distilled water. Alkaline phosphate substrate was incubated with the membrane until signals developed.

3.2.10 Detection of EGFP expression using fluorescence microscopy

HSG cells were seeded at a density of 9 x 10^5 cells/well in 6-well tissue culture plates containing sterile coverslips. pEGFP-6xHisH3 and pEGFP alone (5 μg each) were transfected into each well. Non-transfected cells were used as negative controls. The culture medium was removed 24 h post-transfection and the cells were washed 3 times with PBS. To fix the cells, 2 ml of freshly prepared 4% (w/v) paraformaldehyde in PBS was added to each coverslip, incubated at room temperature for 30 min, and washed twice with PBS. The coverslips were mounted on glass microscope slides using PBS and allowed to dry for 30 min. Fixed cells were viewed under the 40X lens of a Nikon (Eclipse ε600) (Chiyoda-ku,

Tokyo, Japan) microscope with a fluorescent light source (Nikon Super High Power Mercury Lamp).

3.2.11 Detection of EGFP by dot blot

Recombinant EGFP (rEGFP) protein and anti-EGFP antibody were obtained from Clontech (Mountain View, California, USA). Alkaline phosphatase-conjugated anti-rabbit IgG (AP) was purchased from Sigma. A nitrocellulose hybridization membrane (0.2 μm) was cut into a 8 cm x 4 cm strip. rEGFP (1 μg), 2 μl of p6xHisH3 cell lysate, p6xHisH3 supernatant, pEGFP cell lysate, pEGFP supernatant, non-transfected HSG cell lysate, and non-transfected HSG supernatant were allowed to dry for 1 h onto a pre-cut membrane (EGFP membrane). The strip was placed in a 50 ml Falcon tube and incubated with 25 μl of anti-EGFP (1:200 dilution) in 5 ml blocking buffer (1% (w/v) BSA and 0.1% (v/v) Tween-20 in TBS) overnight. The membrane was washed twice with 5 ml of wash buffer (0.1% (v/v) Tween-20 in TBS) for 5 min. Anti-rabbit IgG (AP) (10 μl) (1:500 dilution) was incubated with the EGFP membrane for 6 h, and washed twice with wash buffer. One tablet of BCIP/NBT alkaline phosphate substrate was dissolved in 10 ml of distilled water. Alkaline phosphate substrate was incubated with the membrane until signals developed.

3.3 Results

3.3.1 Analysis of H3 expression in HSG cels by cationic PAGE

H3 cDNA was amplified from a pCMV.SV2H3 vector by PCR, and cloned into the MCS of the pIRES2-EGFP vector on a *Bgl*II/*Eco*RI fragment. The H3 cDNA was positioned downtream of the CMV promoter, and upstream of the IRES. The resulting vector was termed pEGFP-H3. DNA sequence analysis confirmed the H3 cDNA was intact, and that no errors had occurred during amplification. In order to express the H3 protein for analysis, HSG cells were transiently transfected with the pEGFP-H3 vector using the $CaPO_4$ transfection method. HSG cells transfected with pEGFP alone, and non-transfected cells, were used as negative controls. The HSG supernatant was collected 24 h post-transfection and electrophoresed on a cationic gel. No bands were present in any of the supernatants examined (Fig. 3.4). This method proved an unsuccessful way to detect H3 directly in the supernatant. In order to concentrate the H3 protein, a large number of HSG cells were transfected with the pEGFP-H3 vector. The supernatant (800 ml) was subjected to dialysis to remove salts and other impurities, and the volume reduced to 5 ml (160-fold concentration of the original volume) with PEG to concentrate histatin in the supernatant. The samples were then electrophoresed on a cationic gel. Despite the 160-fold concentration of proteins present in the supernatant, the H3 protein was not detected in the supernatant of cells transfected with the pEGFP-H3 vector (same result as Fig. 3.4).

3.3.2 Zinc precipitation of H3 and analysis by cationic PAGE

In order to selectively isolate and precipitate the H3 protein from HSG supernatant, samples were processed using the zinc precipitation method, as described by Flora *et al.* (2001). This method is based on the ability of histatins to selectively associate and precipitate in the presence of zinc. Zinc chloride was added to all the concentrated supernatants, and the resultant pellets were then resuspended and separated by cationic PAGE. However, while a band was present in the lane containing supernatant from cells transfected with the pEGFP-H3 vector, bands of a similar size were also present in the lanes containing supernatants from the empty pEGFP vector and non-transfected cells (Fig. 3.5).

Figure 3.4 Cationic PAGE of supernatants from HSG cells transfected with pEGFP-H3, pEGFP alone, and a no-plasmid control. HSG cells were transiently transfected with pEGFP-H3, pEGFP alone, and a no-plasmid control using the calcium phosphate transfection method, and supernatants collected 24 h later. Supernatants were dialysed, and concentrated using PEG. Lane 1, synthetic H3; lane 2, supernatant from pEGFP-H3-transfected cells; lane 3, supernatant from pEGFP-transfected cells; lane 4, supernatant from non-transfected cells. Synthetic H3 has a molecular weight of 4063.

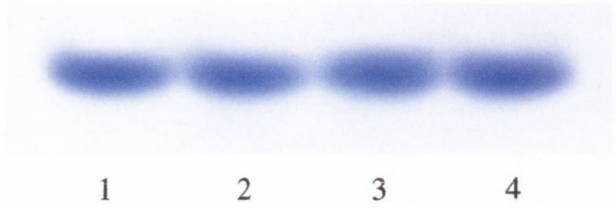


Figure 3.5 Cationic PAGE of zinc chloride precipitation of cationic proteins from HSG cell supernatants. HSG cells were transiently transfected with pEGFP-H3, pEGFP alone, or a no-plasmid control using the calcium phosphate method, and supernatants collected 24 h later. Supernatants were dialysed, and concentrated using PEG. Cationic proteins were precipitated from cell supernatants using zinc chloride. Lane 1, synthetic H3; lane 2, precipitate from pEGFP-H3-transfected cells; lane 3, precipitate from pEGFP-transfected cells; lane 4, precipitate from non-transfected cells. Synthetic H3 has a molecular weight of 4063.

3.3.3 Generation of internal polyhistidine tag in H3 cDNA and purification of 6xHisH3 from transfected HSG supernatant using Ni-NTA silica

In order to simplify the expression, purification and detection of the H3 protein in transfected HSG cells, H3 cDNA was labeled with an internal polyhistidine tag. This internal polyhistidine tag was generated in H3 cDNA by three rounds of PCR (Fig. 3.1). This generated an extra four histidine residues immediately upstream of the region which codes for the 2nd and 3rd histidine residues in the mature H3 peptide, and results in an internal tag of six consecutive histidine residues (Fig. 3.2). The amplicon was cloned into the pIRES2-EGFP vector on a *Bgl* II/*Eco* RI fragment, and the resulting vector termed pEGFP-6xHisH3 (Fig. 3.3). The presence of the tag was confirmed by DNA sequence analysis. A large number of HSG cells were transiently transfected with the pEGFP-6xHisH3 vector, and the supernatants collected 24 later. Negative controls consisted of HSG cells transfected with pEGFP alone, and non-transfected cells. Supernatants were again subjected to dialysis and PEG to concentrate the proteins and reduce the volume of the media. Once the supernatants were reduced from 800 ml to approximately 5 ml, the supernatants were centrifuged with Ni-NTA columns (concentrated a further 5-fold), which selectively bind 6xHis-tagged proteins. The eluates were then separated using cationic PAGE. A band was only detected in the lane representing the eluate from cells transfected with the pEGFP-6xHisH3 vector (Fig. 3.6). No bands were present in lanes representing eluates from cells transfected with pEGFP alone, and non-tranfected cells. The 6xHisH3 protein was successfully purified and eluted with this method, while no proteins of a similar charge and size were eluted from supernatants from empty pEGFP and non-transfected cells. The band representing the 6xHisH3 protein ran slightly further down the cationic gel than the synthetic H3 control (Fig. 3.6). The total yield of 6xHisH3 protein from 800 ml of pEGFP-6xHisH3-transfected HSG cell supernatant was calculated as approx. 80 μg.

3.3.4 Candidacidal activity of 6xHisH3

To ensure the purified 6xHisH3 protein was functional and the internal polyhistidine tag did not interfere with antifungal activity, the candidacidal activity of 6xHisH3 against *C. albicans* 132A was compared to that of synthetic histatin 3. The 6xHisH3 and synthetic H3 proteins were incubated with *C. albicans* blastoconidia at various concentrations for 1 h,

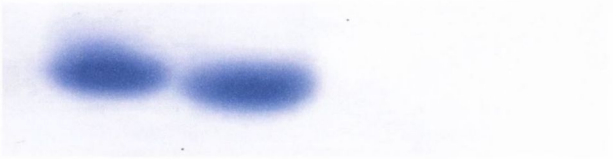


Figure 3.6 Cationic PAGE of the selective purification of 6xHisH3 protein from transfected-HSG cells using Ni-NTA silica. HSG cells were transiently transfected with pEGFP-6xHisH3, pEGFP alone, or with a no-plasmid control using the calcium phosphate transfection method, and supernatants collected 24 h later. Supernatants were dialysed, and concentrated using PEG. The 6xHisH3 protein was selectively purified from pEGFP-6xHisH3-transfected cells using Ni-NTA silica. Lane 1, synthetic H3; lane 2, eluate from pEGFP-6xHisH3-transfected cells; lane 3, eluate from pEGFP-transfected cells; lane 4, eluate from non-transfected cells. Synthetic H3 has a molecular weight of 4063.

and killing activity was calculated as the number of CFU on test plates as a percentage of CFU on control plates (cells incubated in the absence of H3). The 6xHisH3 protein demonstrated potent antifungal activity against *C. albicans*, comparable to the antifungal activity shown by synthetic H3 (Fig. 3.7). This indicates that the internal polyhistidine tag has no adverse effects on 6xHisH3 antifungal activity.

3.3.5 Detection of 6xHisH3 using anti-6xHisG antibody

In order to confirm that the 6xHisH3 protein was detectable with antibodies raised against the polyhistidine tag, cell lysates and supernatants from transfected HSG cells were dried on nylon membranes and probed with alkaline phosphatase-conjugated anti-6xHisG antibody. The presence of 6xHisH3 was detected only in the cell lysates and supernatants of cells transfected with the pEGFP-6xHisH3 vector. No signal was detected in the cell lysates or supernatants of HSG cells transfected the pEGFP alone or non-transfected cells (Fig. 3.8).

3.3.6 EGFP detection using fluorescence microscopy and anti-EGFP antibody

Transfected HSG cells were examined for EGFP expression using fluorescence microscopy initially. HSG cells were transfected with pEGFP-6xHisH3 or pEGFP alone. Non-transfected HSG cells were used as a negative control. HSG cells transfected with pEGFP-6xHisH3 and empty pEGFP were positive for EGFP expression when exposed to UV light, while non-transfected cells did not fluoresce, as expected (Fig 3.9). EGFP expression was visualized at the cell surface only, and not in the surrounding medium. Cell lysates and supernatants from transfected and non-transfected cells were probed with an anti-EGFP primary antibody, and then an alkaline phosphatase-conjugated anti-rabbit IgG secondary antibody. Cell lysates from pEGFP-6xHisH3 and empty EGFP demonstrated the presence of EGFP, whereas the supernatants from cells transfected with these two vectors were not positive for EGFP (Fig. 3.10). This was expected since EGFP is expressed at the cell surface only, and is not secreted into the cell medium. Cell lysate and supernatant from the non-transfected cells did not demonstrate the presence of EGFP protein.

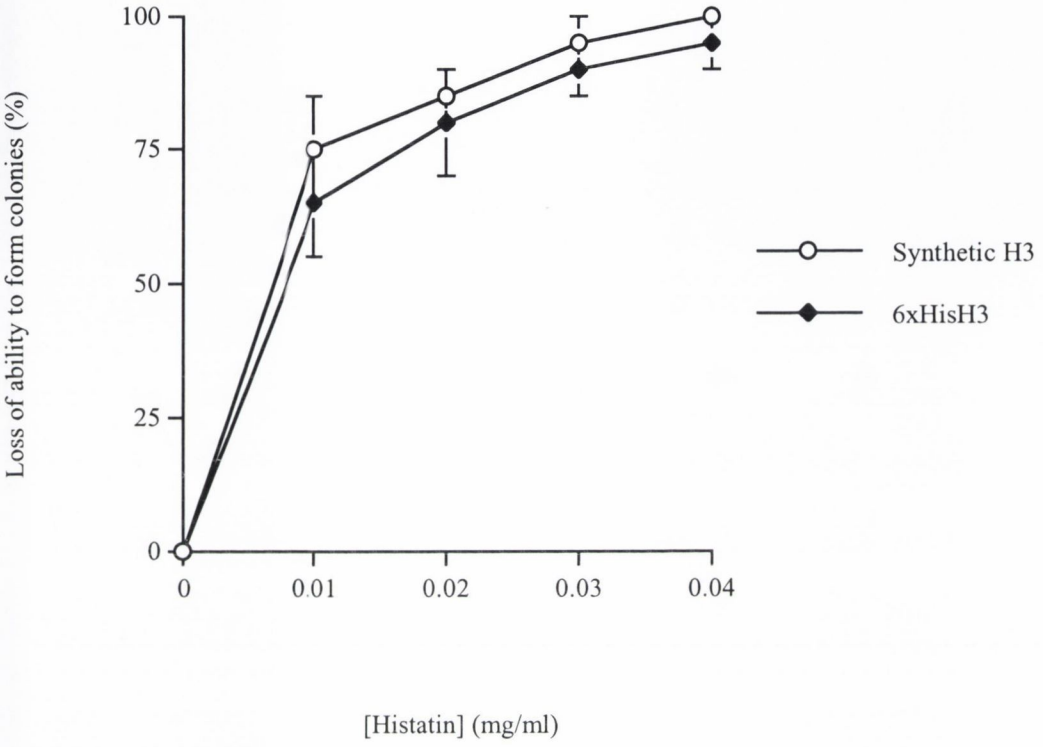


Figure 3.7 Candidacidal activity of synthetic H3 and 6xHisH3 against *C. albicans* 132A. Synthetic H3 and 6xHisH3 were incubated with *C. albicans* blastoconidia at various concentrations. Killing activity was calculated as the number of CFU on test plates as a percentage of CFU on control plates (cells incubated in the absence of H3). Results are means ± standard deviations (error bars) of three independent assays. Where error bars are not visible, they are smaller than the data symbol.

Figure 3.8 Immunodetection of 6xHisH3 by dot blot. HSG cells were transiently transfected with pEGFP-6xHisH3, pEGFP, and a no-plasmid control. The presence of 6xHisH3 was examined in transfected cell lysates and supernatants using an anti-6xHisG alkaline phosphatase-conjugated antibody. The 6xHisH3 protein was only detected in the cell lysate and medium of HSG cells transfected with pEGFP-6xHisH3. Lane **1**, Positope, lane **2**, pEGFP-6xHisH3 cell lysate, lane **3**, pEGFP-6xHisH3 supernatant, lane **4**, pEGFP cell lysate, lane **5**, pEGFP supernatant, lane **6**, non-transfected cell lysate, lane **7**, non-transfected cell medium. Positope protein is a recombinant protein which contains the 6xHis tag, and was used as a positive control for 6xHisH3 expression.

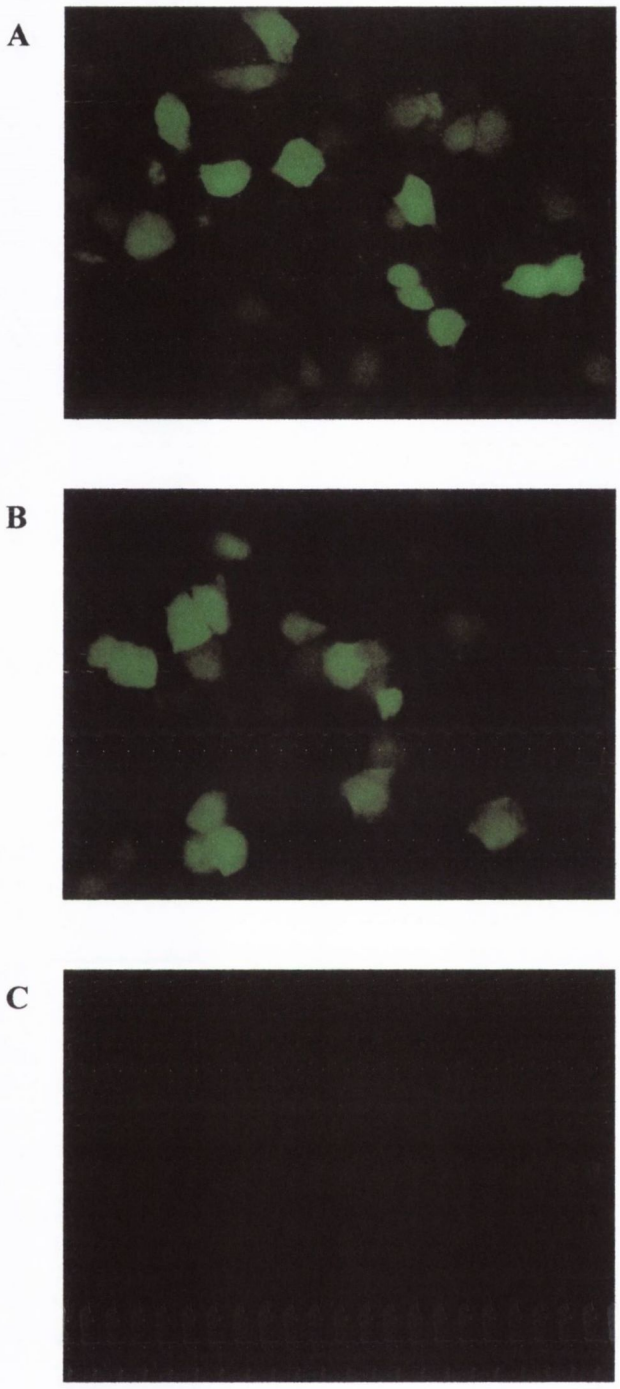


Figure 3.9 Fluorescence microscopy of HSG cells transfected with A, pEGFP-6xHisH3, B, pEGFP alone, and C, a no-plasmid control. EGFP expression was detected in HSG cells transfected with pEGFP-6xHisH3 and pEGFP alone, but not in non-transfected cells. Non-transfected HSG cells were used as a negative control for EGFP expression.

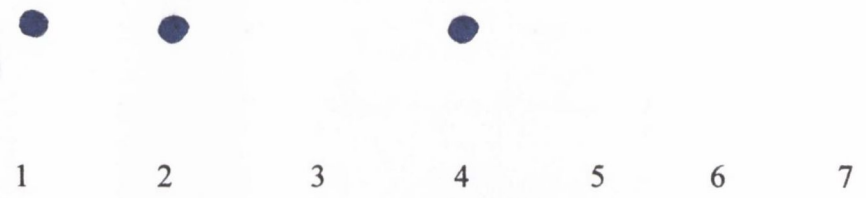


Figure 3.10 Immunodetection of EGFP by dot blot. HSG cells were transiently transfected with pEGFP-6xHisH3, pEGFP, and a no-plasmid control. The presence of EGFP was examined in transfected cell lysates and supernatants using an anti-EGFP primary antibody. Alkaline phosphatase-conjugated anti-rabbit IgG was used as the secondary antibody. The EGFP protein was only detected in the cell lysate of HSG cells transfected with pEGFP-6xHisH3 and pEGFP alone. Lane **1**, Positope, lane **2**, pEGFP-6xHisH3 cell lysate, lane **3**, pEGFP-6xHisH3 supernatant, lane **4**, pEGFP cell lysate, lane **5**, pEGFP supernatant, lane **6**, non-transfected cell lysate, lane **7**, non-transfected cell medium. rEGFP was used as a positive control for EGFP expression.

3.4 Discussion

Salivary histatins exhibit potent antifungal activity against *C. albicans in vitro* which suggests that histatins may play an important role in protecting the oral cavity from candidal infection. However, there is no direct evidence to support this hypothesis. An animal model is required to study the *in vivo* role of histatins in the oral cavity. Mice do not secrete salivary histatins, and therefore provide an ideal model in which to investigate the effects of histatins on the oral yeast population. The purpose of this part of the study was to design and analyse an expression cassette for use in the generation of transgenic mice expressing histatin in their salivary glands. The pIRES2-EGFP vector contains several features which made it appropriate for use in construction of the transgene. The human CMV promoter drives high levels of transgene expression in the salivary glands of rats (O'Connell *et al.*, 1996; Zheung and Baum, 2005). The pIRES2-EGFP vector contains the human immediate early CMV promoter upstream of the MCS. It contains an EGFP reporter gene which may be useful for localizing transgene protein expression. It also contains an IRES which enables translation of the H3 cDNA and EGFP from a single bicistronic mRNA. SV40 polyadenylation signals downstream of the EGFP gene direct proper processing of the 3′-end of the bicistronic mRNA.

Preliminary studies were performed to ensure integrity and proper functioning of the transgene cassette. H3 cDNA was cloned into the MCS of the pIRES2-EGFP vector. HSG cells were transfected with the pEGFP-H3 vector, and expression of the H3 protein was investigated using cationic PAGE. Detection of the H3 protein with this method proved difficult. The H3 peptide may have been expressed at levels too low to be detected directly on a cationic gel. Histatins must be present at a concentration of at least 1 μg per 20 μl of sample loaded on a cationic gel in order to be detected. Histatins are very susceptible to degradation, and may have been proteolysed in the cell medium by fetal bovine serum. A large number of HSG cells were transfected with the pEGFP-H3 vector. The supernatants were dialysed, and concentrated using PEG. Despite the 160-fold concentration of the proteins in the cell medium, the H3 protein was still below the level of detection by cationic PAGE.

The mesurement of histatin concentrations in salivary secretions has represented major technical obstacles reflected in the large variations in mean histatin concentrations in published reports, and the fact that there is no one standard, reliable method used by different research groups for the isolation, purification, and quantitation of histatins (Gusman *et al.*, 2004). A novel technique was developed by Flora *et al.* (2001) for the isolation and purification of histatins, which is based on their ability to selectively associate and precipitate in the presence of zinc. This method was used in the present study to attempt to precipitate H3 from the supernatant of HSG cells transfected with the pEGFP-H3 vector. A protein of similar size and charge to synthetic H3 was detected by cationic PAGE in the pEGFP-H3 supernatant. However, the presence of proteins of a similar size and charge to synthetic H3 in the supernatant of negative controls, i.e. HSG cells transfected with pEGFP alone and non-transfected cells, indicated this method was not selective enough to precipitate pure H3 from the cell medium. Small cationic proteins present in fetal bovine serum may also have associated and precipitated in the presence of zinc. This method was also unsuitable for purifying the H3 peptide for downstream analysis of H3 function in candidacidal assays, since zinc forms an irreversible complex with histatin, rendering it inactive.

As mentioned previously, the isolation and measurement of histatins in complex mixtures of proteins has represented significant technical difficulties to different research groups. In order to simplify the purification and detection of the H3 peptide in cell supernatant, PCR mutagenesis was employed to engineer a polyhistidine tag within the H3 cDNA sequence. An extra four histidine residues were inserted immediately upstream of the 2^{nd} and 3^{rd} histidine residues in the mature H3 peptide, to yield six consecutive histidine residues. The resulting vector was termed pEGFP-6xHisH3. Since the tag was positioned away from the active site of the protein, it was thought it should not interfere with H3 antifungal activity. The tag is much smaller than other commonly used tags, and is uncharged at physiological pH. Proteins which have been labeled with the polyhistidine tag demonstrate a selectivity and affinity for Ni-NTA silica. A large number of HSG cells were transiently transfected with the pEGFP-6xHisH3 vector. The supernatant was dialysed, amd concentrated with PEG. The samples were centrifuged through Ni-NTA metal-affinity chromatography matrices, and the eluates analysed by cationic PAGE. The 6xHisH3

protein ran slightly further down the cationic gel than the synthetic H3. This may be due to the extra 4 positively charged histidine residues within the 6xHisH3 peptide. The polyhistidine tag enabled the selective purification and detection of the 6xHisH3 peptide in pEGFP-6xHisH3 supernatant. No proteins were detected in the eluates from cells transfected with the empty pEGFP vector, or from non-transfected cells. The total yield of 6xHisH3 was 80 μg from 800 ml of supernatant, which was below the limit of detection by cationic PAGE alone. The histatin purified with Ni-NTA silica was in a pure form, allowing it to be used in downstream applications.

In order to ensure the polyhistidine tag had no adverse effects on the antifungal activity of the 6xHisH3 peptide, *C. albicans* blastoconidia was incubated with various concentrations of the 6xHisH3 and synthetic H3 peptides in a candidacidal assay. The 6xHisH3 peptide demonstrated potent antifungal activity comparable to that of synthetic H3 against *C. albicans*. It was expected that the tag would have no adverse effect on histatin antifungal activity, since it was positioned away from the antimicrobial domain of the peptide.

Immunodetection of the 6xHisH3 peptide in pEGFP-6xHisH3 cell lysates and supernatants was performed with the dot blot method, using an anti-6xHisG alkaline phosphatase-conjugated antibody. No signal was detected in the cell lysates and supernatants of negative controls. The dot blot method is a relatively simple, rapid, and sensitive technique for the qualitative detection of specfic peptides. It enables the detection of antigen levels of 100 ng or lower. This antibody may be useful for the detection and quantitation of the 6xHisH3 peptide in the saliva and tissues of histatin-positive transgenic mice.

Investigation of the expression of EGFP was performed using fluorescence microscopy. EGFP expression was detected at the surface of cells transfected with pEGFP-6xHisH3 and empty pEGFP vectors, but not in the surrounding cell medium. This was expected since EGFP is expressed only at the cell surface, and is not secreted. No EGFP was detected at the surface of non-transfected cells, or in the surrounding cell medium. EGFP was also detected in pEGFP-6xHisH3 and empty pEGFP cell lysates by dot blot, but not in the cell lysates of non-transfected cells, or in any of the supernatants examined.

Purified 6xHisH3 was successfully detected using cationic gel electrophoresis, and dot blots with an antibody raised against the 6xHis tag. No histatin was detected in any of the cells or supernatants of empty pEGFP- and non-transfected HSG cells on cationic gels or on dot blots. The 6xHisH3 protein was found to have antifungal activity against *C. albicans* 132A comparable to that of synthetic histatin 3 in an anticandidal assay, indicating the tag had no adverse effect on histatin antifungal activity. EGFP expression was successfully detected by fluorescence microscopy and dot blots. All these experiments indicated that the transgene cassette was completely functional.

In conclusion, the transgene cassette designed in this part of the study was proven to express functional histatin 3 and EGFP in HSG cells. The recombinant 6xHisH3 protein was detected using cationic gel electrophoresis and dot blot, and was active against *C. albicans* 132A. EGFP expression was detected by fluorescence microscopy and dot blot. The transgene cassette was considered suitable for use to generate transgenic mice expressing histatin in their salivary glands.

Chapter 4

Generation of Transgenic Mice Expressing Histatin 3 cDNA

4.1 Introduction

Salivary epithelial cells secrete a range of antimicrobial proteins, such as histatins, lysozyme, lactoferrin, mucins, and β-defensins, which are believed to play an important role in protecting the oral cavity from bacterial and fungal infection. Salivary histatins are believed to play a key role in controlling the opportunistic yeast pathogen *C. albicans*. Mucosal candidal infections have received much attention due to the large increase in the immunocompromised population. Antifungal agents currently available for the treatment of candidal infections consist of the polyenes, the imidazoles, the triazoles, and the enchinocandins. Many drug-resistant strains are emerging in response to widespread and prolonged use of these antifungal agents, making the management of oral candidiasis increasingly difficult (Situ and Bobek, 2000). Histatins are naturally occurring antimicrobial peptides which have potent anticandidal activity at physiological concentrations, are non-toxic to mammalian cells, and do not induce resistance. Therefore, they represent a promising therapeutic alternative to current antifungal agents in the treatment of oral candidiasis.

An extensive amount of work has been performed on assessing the *in vitro* antifungal activity of histatins against *C. albicans* (Tsai and Bobek, 1998; Calderone and Fonzi, 2001). However, there is no direct evidence which proves the efficacy of histatin against fungi in an *in vivo* model of oral candidiasis. Transgenic mice have been used to investigate the *in vivo* efficacy of several other antimicrobial agents. Transgenic mice expressing human lactoferrin and lysostaphin have been used in models of *S. aureus* infection (Kerr *et al.*, 2001; Guillen *et al.*, 2002), while transgenic mice expressing human intestinal defensin have been used in a model of enteric salmonellosis (Salzman *et al.*, 2003). Other studies have used murine models of mucosal candidiasis to evaluate the *in vivo* efficacy of exogenously-added antifungal agents (Flattery *et al.*, 1996; Petraitis *et al.*, 2001; Intini *et al.*, 2003). One of the main objectives of this study was to generate transgenic mice which constitutively express histatin in their salivary glands. Only humans and Old World primates secrete histatin in their saliva, therefore mice provide an ideal *in vivo* model in which to investigate the efficacy of histatin against *Candida* spp. These mice will be an invaluable tool for defining the *in vivo* role of histatin in the oral cavity, and for assessing the therapeutic potential of histatins in the management of oral candidiasis.

4.2 Materials and Methods

4.2.1 Preparation of the 6xHisH3 transgene for microinjection

Double digestion of the pEGFP-6xHisH3 vector with restriction endonucleases *Drd*I and *Bst98*I yielded 4 fragments, 2691 bp, 1789 bp, 956 bp, and 189 bp in length (Fig. 3.3). The 2691 bp fragment representing the 6xHisH3 transgene was purified using NA45 DEAE membranes, and used in the microinjection of C57Bl/6 mouse zygotes. Routine methods in the generation of transgenic mice were used as described in Hogan *et al.* (1986).

4.2.2 Pronuclear microinjection of the 6xHisH3 transgene into C57Bl/6 zygotes

Microinjection was performed by PolyGene Transgenetics, Riedmattstrasse 9, CH-8153 Rumlang, Switzerland. Transgenic mice were generated by pronuclear microinjection of the transgene into C57Bl/6 mouse zygotes. The purified 2.7 kb transgene fragment was microinjected into 243 mouse zygotes of C57Bl/6 background. Following microinjection, 152 embryos survived and were transferred into 8 pseudopregnant females.

4.2.3 Purification of genomic DNA from mouse tail biopsies

Genomic DNA was purified from mouse tail biopsies according to the method of Hogan *et al.* (1986), with some modifications. Tail biopsies were performed when the pups were weaned (3 weeks). Briefly, 0.5 cm of each tail was placed into 0.5 ml of tail buffer (50 mM Tris-HCl pH 8.0; 100 mM EDTA pH 8.0; 100 mM NaCl; 1% (w/v) SDS) with proteinase K added to a 0.5 mg/ml final concentration. The tissues were incubated at 55°C for 18 h with gentle agitation, 1 µl of RNase A (10 mg/ml) was added, and the samples were incubated at 37°C for 2 h. After addition of 0.7 ml of neutralised phenol/chloroform/isoamyl alcohol (25:24:1), samples were mixed vigorously for 1 h using a clinical rotator, and centrifuged at 18,000 x *g* for 5 min. Approximately 0.5 ml of upper aqueous phase was transferred to a fresh microfuge tube, 1 ml of 100 % ethanol added, and the tube inverted until a DNA precipitate formed. The samples were centrifuged at 18,000 x *g* for 5 min, and the supernatant removed and discarded. The pellet was washed by the addition of 0.5 ml of 70% (v/v) ethanol (-20°C), and the tubes inverted several times. The samples were centrifuged again at 18,000 x *g* for 5 min, and the supernatant removed and discarded. The pellets were dried, resuspended in 100 µl of TE buffer, and incubated at 65°C for 15 min to

resuspend DNA. The samples were incubated at 95°C for 5 min to inactivate proteinase K, and the DNA concentration determined spectrophotometrically at 260 nm. Samples were stored at 4°C until required.

4.2.4 Screening of 6xHisH3 transgenic founder mice

4.2.4.1 Identification of 6xHisH3 transgene cassette by PCR

H3 transgenic founder mice were identified by PCR of genomic DNA from mouse tail biopsies using forward primer H3miceF and reverse primer H3miceR (Table 4.1). PCR cycling conditions were as follows: 1 cycle at 94°C for 90 s, 35 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 3 min, and 1 cycle at 72°C for 10 min.

4.2.4.2 Identification of intact 6xHisH3 transgene cassette by PCR

Forward primer DrdF2 and reverse primer Bst98R2 were used to investigate if the transgene, when present, was fully intact (Table 4.1). When the transgene is present, it should yield a PCR product 2548 bp in length. See Fig. 4.1 for positions of these primers. PCR conditions were as follows: 1 cycle at 94°C for 90 s, 35 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 3 min, and 1 cycle at 72°C for 10 min.

4.2.5 Generation and screening of 6xHisH3 transgenic F1 generation

Positive transgenic founder mice were mated with wild-type mice to investigate stable inheritance of the transgene. Presence and integrity of the transgene in the offspring (F1 generation) were investigated as described in sections 4.2.4.1 and 4.2.4.2, respectively.

4.2.6 Generation and screening of 6xHisH3 transgenic F2 generation

Investigation of the presence of the transgene in the F1 generation was investigated as described in section 4.2.4.1. Integrity of the transgene was confirmed using the method described in section 4.2.4.2. Brother-sister breeding pairs were established among transgene positive mice to generate mice homozygotic for the transgene (F2 generation).

Table 4.1 Primers used to screen transgenic mice for the 6xHisH3 transgene, investigate 6xHisH3 mRNA expression levels, amplify the 18S rRNA gene, ensure 6xHisH3 transgene is intact, and investigate GAPDH mRNA expression levels

Primer	Sequence 5′-3′	Amplicon size (bp)
Screen transgenic mice, investigate 6xHisH3 mRNA expression		
H3miceF	TTGGCTCTCATGCTTTCCATGAC	237
H3miceR	CAAAAAGCAGTGGTAGTGTGATG	
Amplify 18S rRNA gene		
18S sense	CGGCTACCACATCCAAGGAA	190
18S antisense	GCTGGAATTACCGCGGCT	
Intact 6xHisH3 transgene		
DrdF2	CTGATTCTGTGGATAACCGTATTACC	2548
Bst98R2	GACAAACCACAACTAGAATGCAGTGA	
GAPDH mRNA expression		
GAPDH F2	TTCCAGTATGACTCCACTCACGG	168
GAPDH R2	TGAAGACACCAGTAGACTCCACGAC	

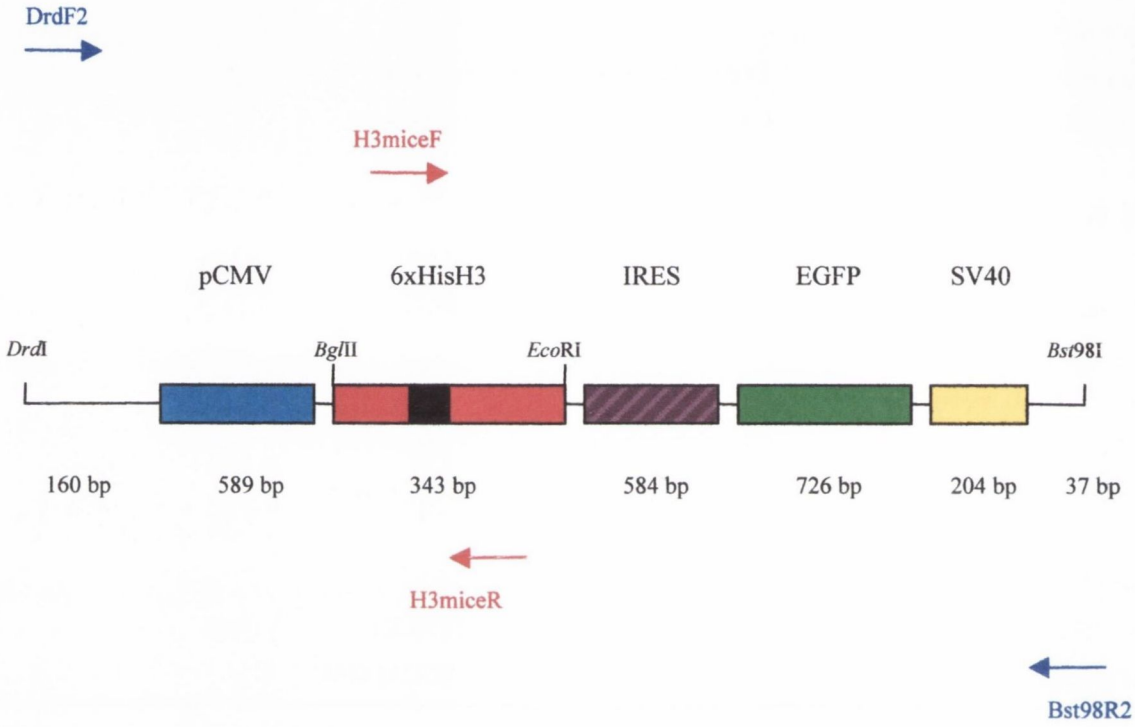


Figure 4.1 Schematic diagram showing the approximate location and direction of the primer sequences used to investigate transgenic mice. Forward primer H3miceF and reverse primer H3miceR were used to screen for the presence of the 6xHisH3 transgene in transgenic mice genomic DNA, and to amplify 6xHisH3 mRNA. Forward primer DrdF2 and reverse primer Bst98R2 were used to investigate whether the 6xHisH3 transgene was intact. Primer sequences designed in this study are shown in Table 4.1. The black box in the 6xHisH3 gene respresents the polyhis tag. **pCMV**, cytomegalovirus promoter, **6xHisH3**, polyhis tagged-histatin 3 cDNA, **IRES**, internal ribosomal entry site, **EGFP**, enhanced green fluorescent protein, **SV40**, polyadenylation tail.

4.2.7 Tissue expression of 6xHisH3 mRNA

4.2.7.1 Harvesting of mouse tissues

The tissues harvested from each mouse were the submandibular/sublingual glands, brain, heart, kidney, liver, lung, skeletal muscle, and small intestine. Mouse tissues were submerged in 3 ml of RNAlater RNA Stablization Reagent (Qiagen) immediately after harvesting. This reagent ensured immediate stabilisation and protection of RNA, allowed processing of the samples at room temperature, and removed the risk of RNA degradation after multiple freeze-thaw cycles. Samples were stored at -80°C until required.

4.2.7.2 Isolation and preparation of total RNA

Total RNA was isolated from mouse tissues using the RNeasy kit (Qiagen), according to the manufacturer's instructions. Briefly, 30 mg of each tissue was placed in 600 µl of lysis buffer RLT, and simultaneously disrupted and homogenised using an Omni rotar-stator homogeniser (Marietta, Georgia, USA) at top speed until the sample was uniformly homogeneous. The tissue lysate was centrifuged at 12,000 x *g* for 3 min, and the supernatant transferred to a fresh tube. One volume of 70% (v/v) ethanol was added to the cleared lysate, and mixed immediately by pipetting. The sample was applied to an RNeasy column placed in a 2 ml collection tube, and centrifuged at 8,000 x *g* for 15 s. The column was washed several times with supplied buffers RW1 and RPE. To elute total RNA, 50 µl of RNase-free water was added to the RNeasy column, and centrifuged at 8,000 x *g* for 1 min. RNA concentration was quantified spectrophotometrically at 260 nm, and RNA purity was estimated by the ratio of the absorbance measurements at 260 nm and 280 nm (A_{260} /A_{280}). RNA quality was analysed by electrophoresis through a 1% (w/v) agarose gel. Samples were stored at -80°C until required.

4.2.7.3 DNase treatment of RNA samples

Contaminating DNA was removed from RNA samples using the TURBO DNA-free kit from Ambion (Foster City, California, USA). Briefly, 10 µg of each RNA sample was treated with 2 U of TURBO DNase in a 50 µl reaction volume containing 1x TURBO DNase buffer, and incubated at 37°C for 30 min. DNase inactivation reagent (5 µl) was

added to each sample to remove DNase and divalent cations, and incubated at room temperature for 2 min, with occasional mixing. Samples were centrifuged at 10,000 x *g* for 90 s to pellet the DNase inactivation reagent, and supernatants containing DNA-free RNA were transferred to fresh tubes.

4.2.7.4 Generation of first-strand complementary DNA

Complementary DNA (cDNA) was generated using the Reverse Transcription System (Promega), according to the manufacturer's instructions. DNA-free total RNA (1 µg) was incubated at 70°C for 10 min, centrifuged briefly, and placed on ice. Each cDNA synthesis reaction contained a final concentration of 5 mM $MgCl_2$, 1x reverse transcription buffer, 1 mM of each dNTP, 1 U/µl recombinant RNasin ribonuclease inhibitor, 15 U of AMV reverse transcriptase, 0.5 µg of Oligo $(dT)_{15}$ Primer, and 1 µg of total RNA per 20 µl reaction volume. Each reaction was incubated at 42°C for 60 min to produce more abundant transcripts. Each sample was then incubated at 95°C for 5 min to inactivate the AMV reverse transcriptase, and incubated at 4°C for 5 min to prevent it from binding to the cDNA.

4.2.7.5 Non-quantitative reverse transcription PCR

Reverse transcription PCR (RT-PCR) was performed using the Reverse Transcription System (Promega), according to the manufacturer's instructions. Briefly, the first-strand cDNA synthesis reaction was diluted to 100 µl with nuclease-free water. Each RT-PCR reaction contained a final concentration of 2 ng/µl first-strand cDNA reaction, 200 µM dNTPs, 2 mM $MgCl_2$, 1x reverse transcription buffer, 1 µM of the forward primer (H3miceF for 6xHisH3, GAPDH F2 for GAPDH), 1 µM of the reverse primer (H3miceR for 6xHisH3, GAPDH R2 for GAPDH), and 1.25 U of *Taq* DNA polymerase per 50 µl reaction volume. PCR cycling conditions were as follows: 1 cycle at 94°C for 60 s, 35 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and 1 cycle at 72°C for 10 min.

4.2.7.6 Normalising cDNA stock samples

To equalise the amount of cDNA from each sample, a PCR trial was performed using the internal standard GAPDH. The RT-PCR reaction mixture was prepared as described in section 4.2.7.5 into a total volume of 50 µl then divided into 5 tubes of 10 µl each. PCR cycling conditions were 1 cycle at 94°C for 60 s, followed by 16 to 24 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The 10 µl PCR for GAPDH were amplified 16, 18, 20, 22, and 24 cycles, collecting one tube at the end of the 72°C extension phase. The PCR samples were run on a 1.5% (w/v) agarose gel, and the quantity of GAPDH PCR fragmants were estimated according to band intensity. A minimal signal intensity for 20 cycles was then chosen as the baseline, and the cDNAs adjusted to all give the same signal strength for GAPDH when amplifying 1 µl of the adjusted cDNA stock.

4.2.7.7 Relative semi-quantitative RT-PCR

The linear amplification range for the H3miceF/H3miceR primer pair was predetermined using 1 µl of standardised cDNA per 20 µl PCR assay. At 20 cycles before the end of the last cycle, primers for the housekeeping gene GAPDH were added at the end of the 72°C extension phase. This would then generate PCR products for 6xHisH3 cDNA and GAPDH cDNA both within their linear amplification range. The PCR products were visualized on 1.5% (w/v) agarose gels, and gene expression was determined relative to the internal standard PCR signal. Comparison of 6xHisH3 transgene expression between samples was adjusted according to the internal standards which were previously normalised between samples.

4.2.8 Isolation and analysis of protein in mouse salivary gland tissues

Proteins were extracted from mouse submandibular/sublingual glands by homogenising 100 mg of tissue in 1 ml of extract buffer (10 mM potassium phosphate, pH 7.2). The homogenate was centrifuged at 600 x *g* for 10 min at 4°C to pellet larger cellular debris. Protein concentrations were determined spectrophotometrically at 280 nm, and diluted to 2 mg/ml total protein concentration with extract buffer. Cationic gel electrophoresis was performed as described in section 3.2.4, and 20 µl of each tissue sample (40 µg total

protein) was loaded onto each lane. Each tissue sample was incubated with synthetic histatin 3 for 1 h to investigate possible proteinase activity of the homogenates, and examined by cationic gel electrophoresis. Immunodetection of 6xHisH3 and EGFP proteins in the tissue homogenates was performed using the dot blot method, as described in sections 3.2.9 and 3.2.11, respectively.

4.3 Results

4.3.1 Generation and identification of histatin-positive transgenic mice founders

The 6xHisH3 expression cassette was excised from the pEGFP-6xHisH3 vector on a *Drd*I/*Bst*98I 2.7 kb fragment. The purified fragment was then microinjected into 243 mouse zygotes of C57/Bl6 background. Microinjection was performed by PolyGene Transgenetics, Riedmattstrasse 9, CH-8153 Rumlang, Switzerland. One hundred and fifty two embryos survived and were transferred into 8 foster mothers. Thirty one offspring were born and tail bopsies performed once the pups were weaned at 3 weeks. Genomic DNA was prepared from the mouse tail biopsies, and mice that had incorporated the transgene into their DNA were identified by PCR analysis. Generally, 10-30% of offspring will inherit a transgene following microinjection, so it was expected that 3-9 of the offspring would be positive. Of the 31 mice born, 3 mice tested positive for the 6xHisH3 transgene; mouse numbers F0.9 (female), F0.28 (female), and F0.31 (male) (Fig. 4.2). To ensure no histatin-positive mice tested as false negatives, the presence and quality of genomic DNA was confirmed by performing PCR analysis on each sample using primers to amplify the 18S rRNA gene. All 31 samples tested positive for the 18S rRNA gene. PCR analysis was also used to confirm that the 6xHisH3 expression cassette was fully intact in the 3 positive founder mice. Primers were used which annealed at the 5′- and 3′- ends of the cassette (Fig. 4.3).

4.3.2 Generation and screening of F1 transgenic mice

In order to ensure the 6xHisH3 transgene could be stably inherited, all 3 founder mice were mated with wild type C57/Bl6 mice. Mouse number F0.9 yielded 2 litters, a total of 12 pups, mouse number F0.28 yielded 9 pups, and mouse number F0.31 also yielded 9 pups. Tail biopsies were taken from each of the 30 pups once they were weaned at 3 weeks, and PCR analysis used to test for the presence of the transgene. Of the 30 pups tested, 3 tested positive for the transgene, mouse numbers F1.15 (male), F1.21 (female), and F1.22 (female) (Fig. 4.4). All 3 positive mice were the offspring of mouse number F0.9. None of the offspring from mouse numbers F0.28 and F0.31 tested positive for the transgene. PCR analysis of the tail biopsies using primers for the 18S rRNA gene ensured none of the mice

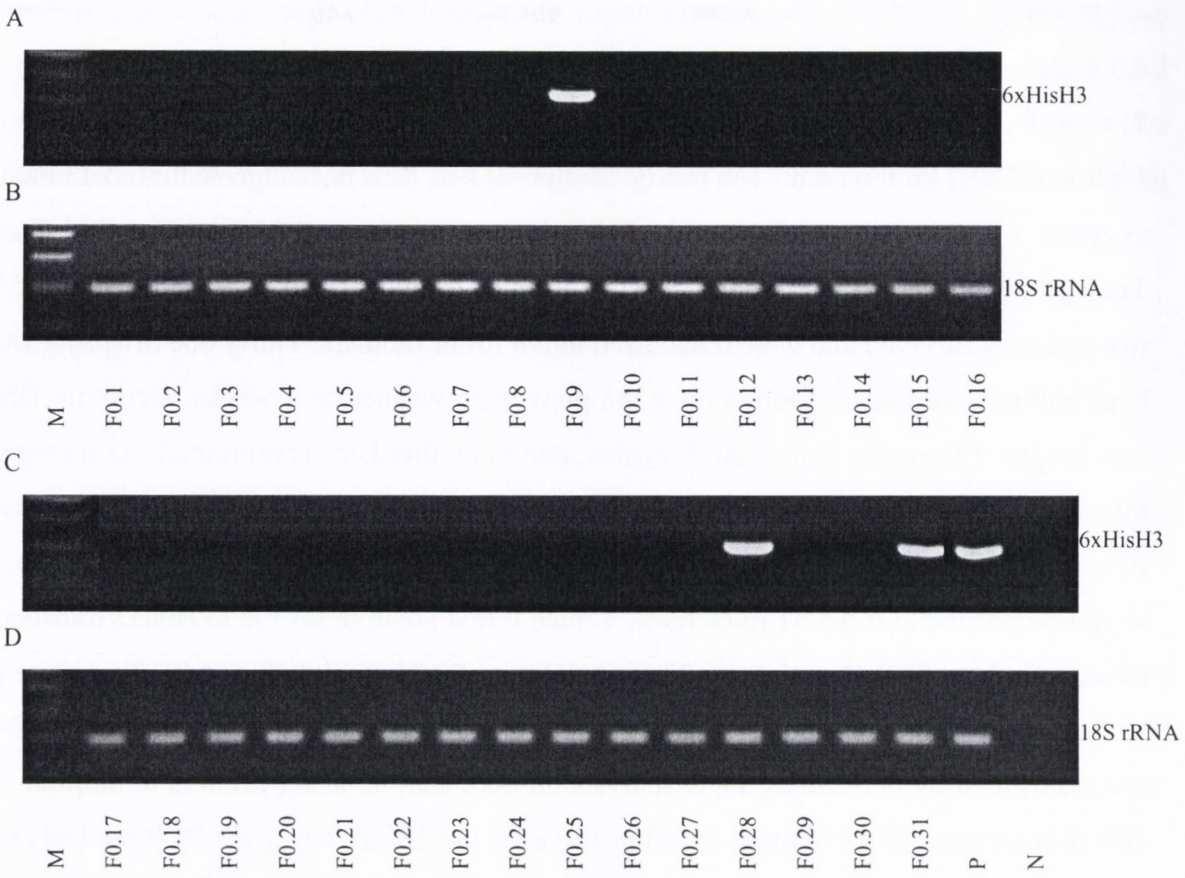


Figure 4.2 Identification of positive 6xHisH3 transgenic founder offspring by PCR analysis of mouse genomic DNA. A, **C**, Detection of the 6xHisH3 transgene. Positive mice gave a 237 bp product using primers H3miceF and H3miceR. Mice F0.9, F0.28, and F0.31 tested positive for the presence of the transgene. **B**, **D**, Detection of the 18S rRNA gene. The 18S rRNA gene was used as a positive internal control for presence and quality of mouse genomic DNA using primers 18S sense and 18S antisense. **M**, 100 bp DNA ladder, **P**, human genomic DNA used as positive control, **N**, no template added to PCR reaction used as a negative control.

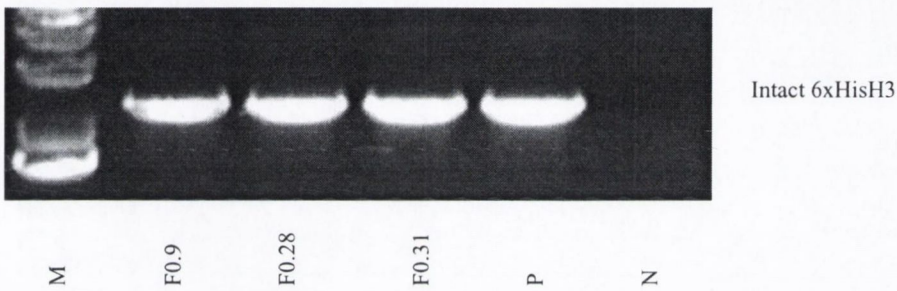


Figure 4.3 Investigation of intactness of the 6xHisH3 transgene in transgenic founder mice by PCR analysis of mouse genomic DNA. The intact transgene gave a 2548 bp product using primers DrdF2 and Bst98R2. **M**, 1 kb DNA ladder, **P**, pEGFP-6xHisH3 vector used as template for positive control, **N**, no template added to PCR reaction as a negative control.

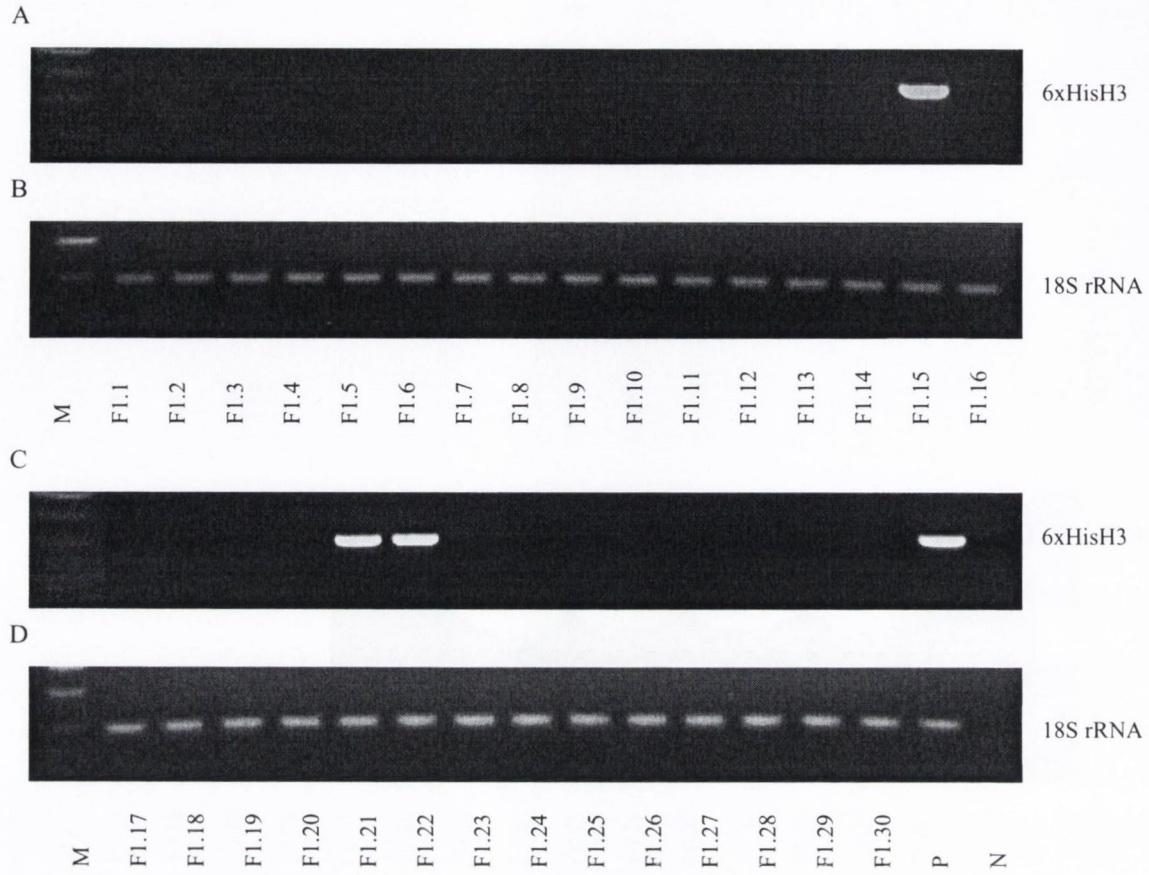


Figure 4.4 Identification of transgenic mice which have stably inherited the 6xHisH3 transgene by PCR analysis of mouse genomic DNA. A, **C**, Detection of the 6xHisH3 transgene. Positive mice gave a 237 bp product using primers H3miceF and H3miceR. Mice F1.15, F1.21, and F1.22 stably inherited the transgene. **B**, **D**, Detection of the 18S rRNA gene. The 18S rRNA gene was used as a positive internal control for presence and quality of mouse genomic DNA using primers 18S sense and 18S antisense. **M**, 100 bp DNA ladder, **P**, human genomic DNA used as positive control, **N**, no template added to PCR reaction used as negative control.

had tested as false negatives (Fig. 4.4). PCR analysis confirmed the transgene was intact in all 3 positive mice (Fig. 4.5).

4.3.3 Generation and screening of F2 transgenic mice

In order to generate homozygote offspring, brother-sister breeding pairs were established between male mouse F1.15 and female mice F1.21 and F1.22. It was expected 25% of the offspring would be homozygotes, 25% would be non-transgenic, and 50% would be hemizygotes. A total of 14 pups were born, and tail biopsies were performed once the mice were weaned at 3 weeks. All mice were screened by PCR analysis, and 10 of the 14 mice born tested positive for the transgene. The transgene was transferred to 71% of the offspring, which should include both homozygote and hemizygote mice. All samples tested positive for the presence of the 18S rRNA gene (Fig. 4.6). The transgene was intact in all 10 mice tested (Fig. 4.7). A schematic diagram showing the breeding and identification of transgenic mice in this study is shown in Fig. 4.8.

4.3.4 The CMV promoter drives histatin mRNA expression in the salivary glands of transgenic mice

A total of 4 histatin-positive transgenic mice (one male and one female from each litter, mouse numbers F2.1, F2.4, F2.5, and F2.8) and one wild type mouse (mouse number F2.7) were culled and their organs harvested for analysis of tissue-specific expression of the transgene. Salivary glands (submandibular/sublingual), brain, heart, kidney, liver, lung, skeletal muscle, and small intestine were harvested from each mouse. RNA was prepared from the salivary glands initially for analysis by reverse transcriptase PCR. It was found that all 4 histatin-positive transgenic mice expressed histatin in their salivary glands, while the wild type mouse did not. Negative controls which had no reverse transcriptase added to the cDNA reaction confirmed that no contaminating genomic DNA was present in any of the samples (Fig. 4.9).

4.3.5 Expression of the histatin transgene is not tissue-specific

RNA was prepared from the remaining tissue samples from each mouse and analysed by RT-PCR. It was found that histatin mRNA was expressed in all the tissue types tested,

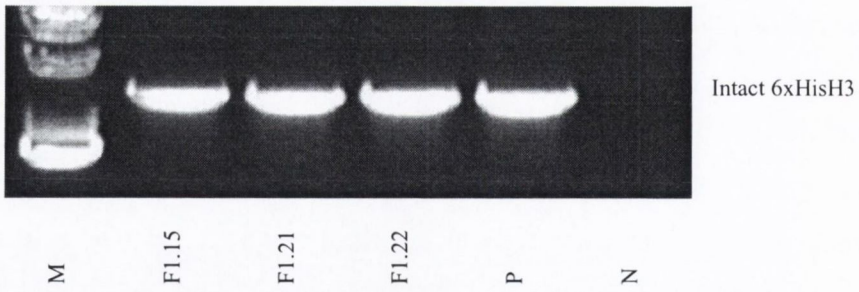


Figure 4.5 Investigation of intactness of the 6xHisH3 transgene in the F1 generation by PCR analysis of mouse genomic DNA. The intact transgene gave a 2548 bp product using primers DrdF2 and Bst98R2. **M**, 1 kb DNA ladder, **P**, pEGFP-6xHisH3 vector used as template for positive control, **N**, no template added to PCR reaction as a negative control.

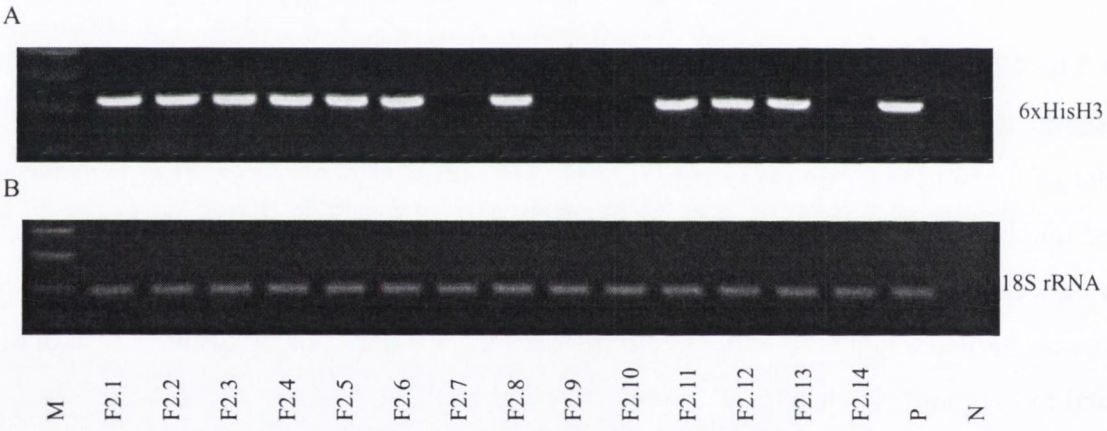


Figure 4.6 Identification of positive 6xHisH3 transgenic mice in the F2 generation by PCR analysis of mouse genomic DNA. A, Detection of the 6xHisH3 transgene. Positive mice gave a 237 bp product using primers H3miceF and H3miceR. Mice F2.1, F2.2, F2.3, F2.4, F2.5, F2.6, F2.8, F2.11, F2.12, and F2.13 tested positive for the presence of the transgene. **B**, Detection of the 18S rRNA gene. The 18S rRNA gene was used as a positive internal control for presence and quality of mouse genomic DNA using primers 18S sense and 18S antisense. **M**, 100 bp DNA ladder, **P**, human genomic DNA used as positive control, **N**, no template added to PCR reaction used as a negative control.

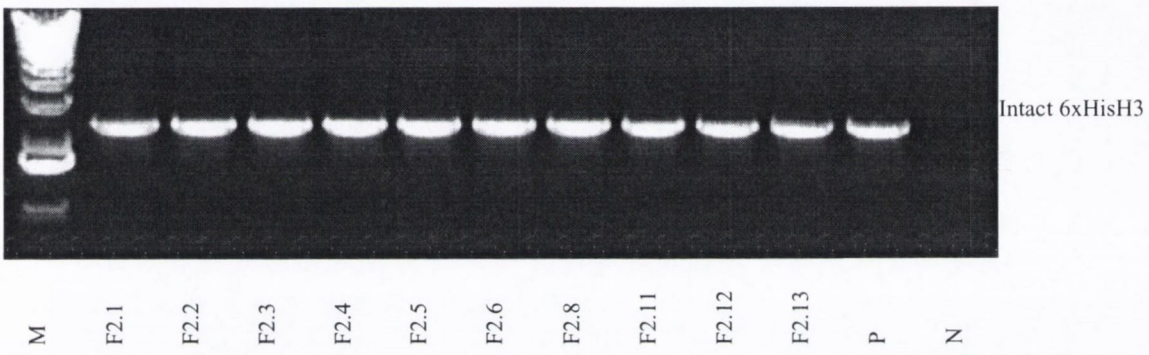
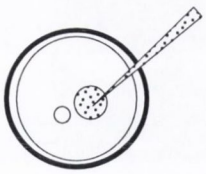
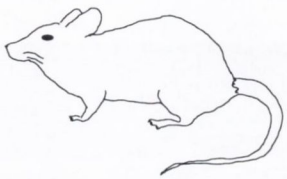


Figure 4.7 Investigation of intactness of the 6xHisH3 transgenic by PCR analysis of mouse genomic DNA. The intact transgene gave a 2548 bp product using primers DrdF2 and Bst98R2. **M**, 1 kb DNA ladder, **P**, pEGFP-6xHisH3 vector used as template for positive control, **N**, no template added to PCR reaction as a negative control.

Breeding **Identification**

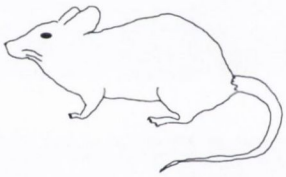
The 6xHisH3 transgene was used in the pronuclear microinjection of 243 C57Bl/6 mouse zygotes. The surviving 152 embryos were transferred into 8 pseudopregnant females.





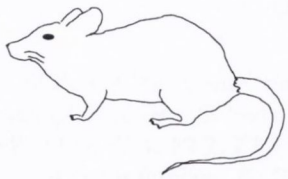
F0 generation: Transgenic founder mice were identified by PCR. Three of the 31 offspring born carried the intact 6xHisH3 transgene. Mice F0.9, F0.28, and F0.31 were positive for the transgene.

F0.9, F0.28, and F0.31 were mated with wild type C57Bl/6 mice to investigate stability of the transgene.



F1 generation: Mice stably inheriting the intact 6xHisH3 transgene were identified by PCR. Three of the 30 offspring born inherited the transgene, and were identified as mice F1.15, F1.21, and F1.22. All three mice were sired by founder mouse F0.9. Mice F0.28 and F0.31 did not sire any positive offspring.

Brother/sister breeding pairs were established between F1.15, F1.21, and F1.22 to generate mice homozygous for the transgene.



F2 generation: Ten of the 14 offspring born tested positive for the intact 6xHisH3 transgene by PCR.

Figure 4.8 Schematic diagram showing the breeding and identification of 6xHisH3 transgenic mice.

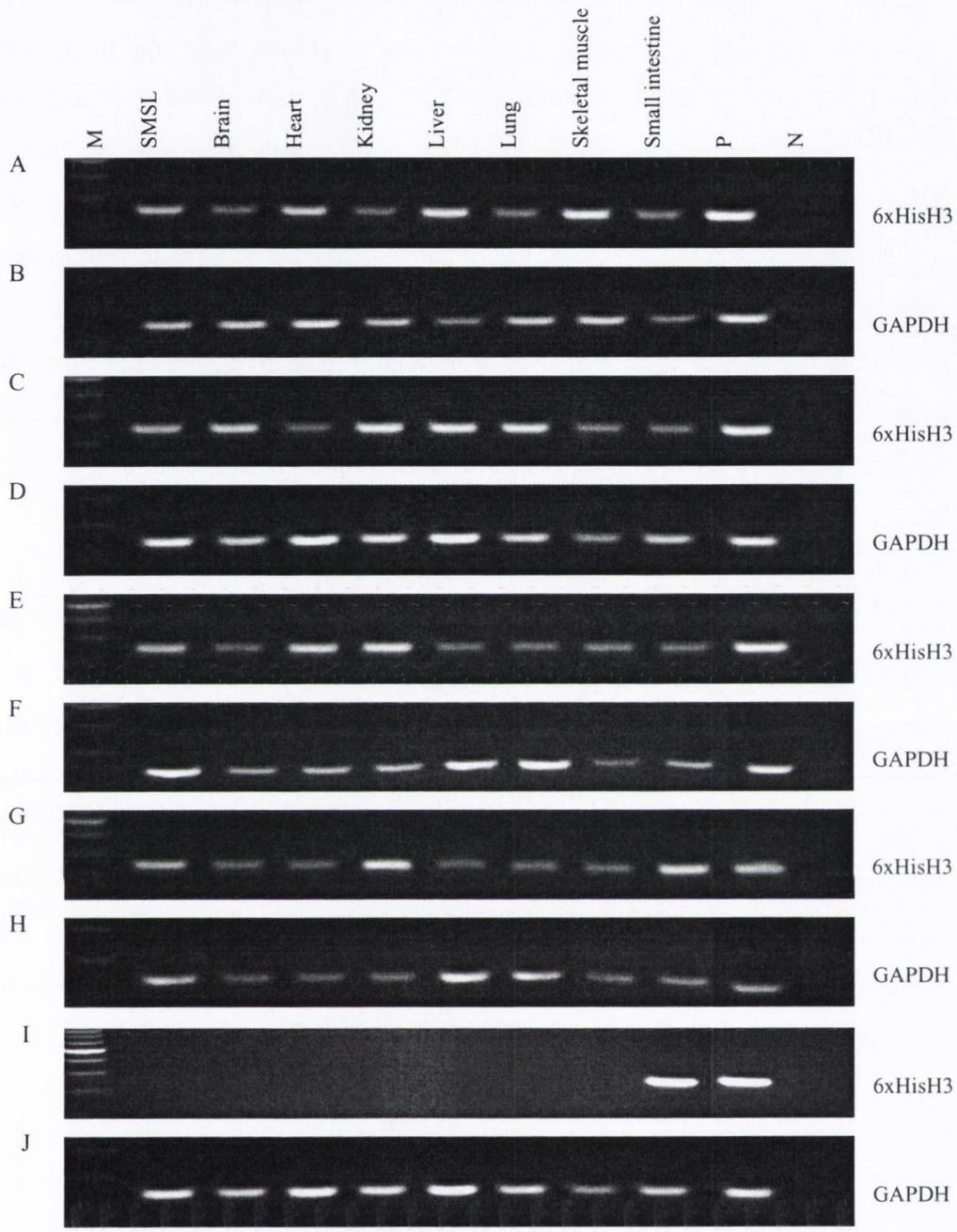


Figure 4.9 Investigation of 6xHisH3 mRNA tissue expression by RT-PCR analysis of mRNA prepared from mouse tissues. Tissues examined were SMSL, brain, heart, kidney, liver, lung, skeletal muscle, and small intestine. **A**, **C**, **E**, and **G**, RT-PCR analysis of 6xHisH3 expression in transgenic mice F2.1, F2. 4, F2.5, and F2. 8, respectively. **I**, RT-PCR anlysis of transgene expression in non-transgenic mouse F2.7, which was used as negative control. Tissues which expressed 6xHisH3 mRNA gave a 237 bp product using primers H3miceF and H3miceR. **B**, **D**, **F**, **H**, and **J**, RT-PCR analysis of GAPDH expression in mice F2.1, F2. 4, F2.5, F2.8, and F2.7, respectively. **M**, 100 bp DNA ladder, **SMSL**, submandibular/sublingual glands, **P**, human salivary gland mRNA as used a positive control, **N**, no reverse transcriptase added to cDNA reaction to ensure no contaminating mouse genomic DNA was present.

indicating that the CMV promoter is not tissue-type specific. Highest levels of 6xHisH3 mRNA expression was found in the submandibular/sublingual glands, lung, and liver, with 6xHisH3 mRNA levels approximately 10-fold that of GAPDH mRNA levels. Moderate mRNA expression levels were found in the brain, heart, kidney, skeletal muscle, and small intestine, with transgene mRNA levels approximately 3-fold that of GAPDH mRNA levels. Levels of 6xHisH3 transgene mRNA expression did not appear to be affected by mouse gender, since male and female mice expressed similar levels of the transgene in all of the tissues tested. None of the tissues tested positive for histatin mRNA in the wild type mouse. Negative controls which had no reverse transcriptase added during the cDNA reaction confirmed that genomic DNA was not present in any of the samples (Fig. 4.10).

4.3.6 6xHisH3 protein expression in transgenic mouse salivary glands

Expression of the 6xHisH3 protein in transgenic mouse salivary glands could not be detected by cationic gel electrophoresis (Fig. 4.11). Tissue samples were spiked with synthetic H3 and electrophoresed through a cationic gel to investigate whether proteinase activity of the homogenates had degraded any of the 6xHisH3 protein present in the samples. No degradation of synthetic H3 was observed in any of the samples (Fig. 4.12). The presence of the 6xHisH3 protein was successfully detected using the dot blot method and an antibody specific for the 6xHisG epitope in the 6xHisH3 peptide (Fig. 4.13). EGFP was also detected in transgenic mouse salivary gland tissue using the dot blot method and an antibody specific for the EGFP peptide (Fig. 4.13).

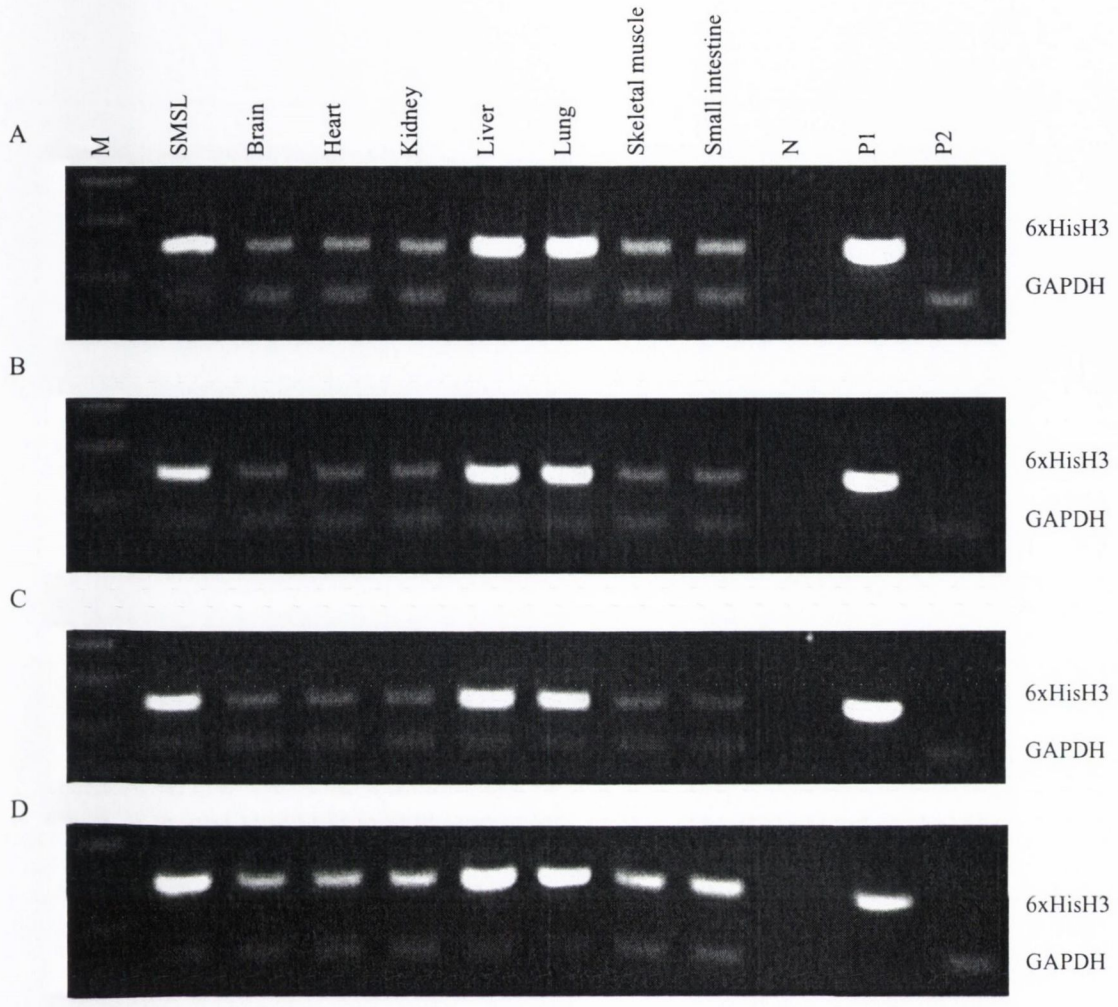


Figure 4.10 Investigation of 6xHisH3 mRNA expression levels by semi-quantitative RT-PCR analysis of mRNA prepared from mouse tissues. **A**, **B**, **C**, and **D**, Semi-quantitative RT-PCR alalysis of mRNA expression in transgenic mice F2.1, F2. 4. F2.5, and F2.8, respectively. Tissues examined were SMSL, brain, heart, kidney, liver, lung, skeletal muscle, and small intestine. Expression levels of 6xHisH3 mRNA is presented relative to GAPDH mRNA expression. SMSL, liver, and lung express the highest levels of 6xHisH3 mRNA. **M**, 100 bp DNA ladder, **SMSL**, submandibular/sublingual glands, **N**, no reverse transciptase added to cDNA reaction to ensure no contaminating genomic DNA was present, **P1**, human salivary gland mRNA used as positive control for H3 mRNA, **P2**, human salivary gland mRNA used as positive control for GAPDH mRNA.

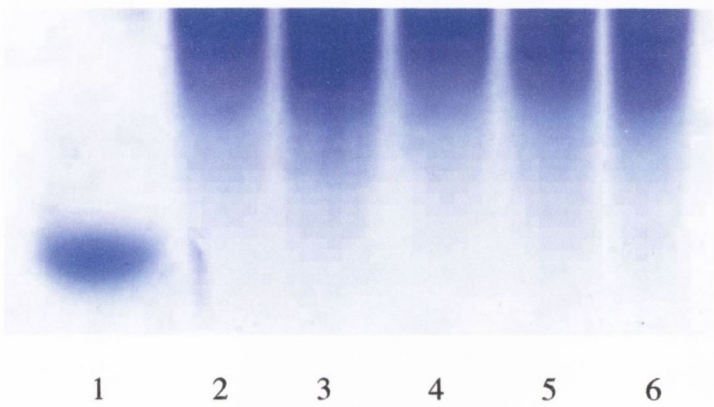


Figure 4.11 Cationic PAGE of protein samples prepared from salivary gland tissue homogenates of transgenic mice. Lane **1**, synthetic H3, lane **2**, transgenic mouse F2.1 salivary gland homogenate, lane **3**, transgenic mouse F2.4 salivary gland homogenate, lane **4**, transgenic mouse F2.5 salivary gland homogenate, lane **5**, transgenic mouse F2.8 salivary gland homgenate, lane **6**, non-transgenic mouse F2.7 salivary gland homogenate (used as a negative control). Synthetic H3 has a molecular weight of 4063.

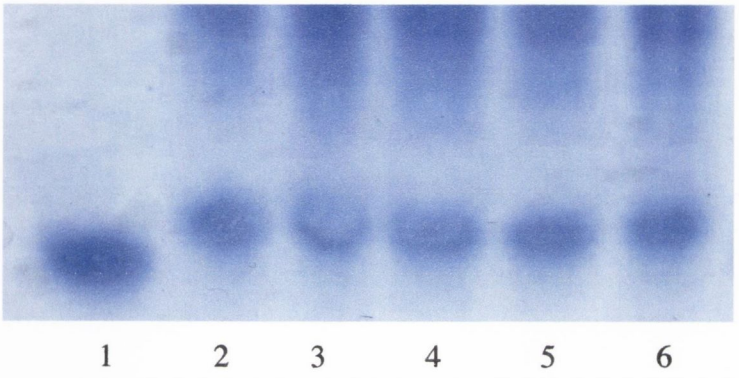


Figure 4.12 Cationic PAGE investigating possible proteolytic degradation of histatin in salivary gland tissue homogenates from transgenic mice. Lane **1**, synthetic H3. All other lanes contained synthetic H3 incubated with salivary gland tissue homogenates from transgenic mice. Lane **2**, mouse F2.1, lane **3**, mouse F2.4, lane **4**, mouse F2.5, lane **5**, mouse F2.8, lane **6**, non-transgenic mouse F2.7. No proteolyic degradation of synthetic H3 was observed in any of the salivary gland tissue homogenates tested. Synthetic H3 has a molecular weight of 4063.

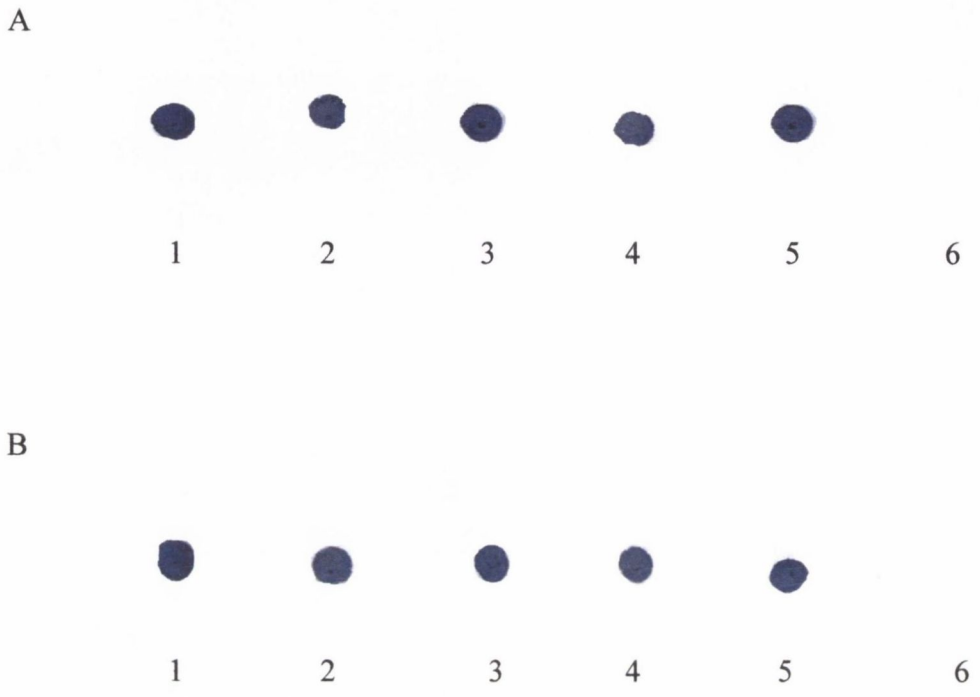


Figure 4.13 Immunodetection of 6xHisH3 and EGFP in the salivary gland tissue homogenates of transgenic mice by dot blot. The 6xHisH3 protein was detected using an anti-6xHisG antibody. Panel **A**, lane **1**, Positope protein, lane **2**, mouse F2.1, lane **3**, mouse F2.4, lane **4**, mouse F2.5, lane **5**, mouse F2.8, lane **6**, non-transgenic mouse F2.7. Positope protein is a recombinant protein which contains the 6xHis tag, and was used as a positive control for 6xHisH3 expression. Non-transgenic mouse F2.7 salivary gland homogenate was used as a negative control for 6xHisH3 expression. The EGFP protein was detected using an anti-EGFP primary antibody, and an alkaline phosphatase-conjugated anti-rabbit IgG secondary antibody. Panel **B**, lane **1**, rEGFP protein, lane **2**, mouse F2.1, lane **3**, mouse F2.4, lane **4**, mouse F2.5, lane **5**, mouse F2.8, lane **6**, non-transgenic mouse F2.7. rEGFP protein was used as a positive control for EGFP expression. Non-transgenic mouse F2.7 salivary gland homogenate was used as a negative control for EGFP expression.

4.4 Discussion

Salivary histatins are believed to play a key role in controlling the opportunistic oral yeast pathogen *C. albicans*. Despite the large amount of data collected on the potent antifungal activity of histatin against *Candida* spp. *in vitro*, there is no direct evidence to prove that histatins are essential to oral health. In this study, transgenic mice were successfully generated which express histatin in their salivary glands. Wild type mice do not express histatin, and therefore represent an ideal model in which to study the the efficacy of histatin *in vivo*. The 6xHisH3 transgene cassette was excised from the pEGFP-6xHisH3 vector (see Fig. 3.3 in chapter 3 for plasmid map), and was comprised of the CMV promoter, 6xHisH3 gene, IRES, EGFP reporter gene, and SV40 polyadenylation signal sequence. This cassette was used in the pronuclear microinjection of C57Bl/6 mouse zygotes. It was expected that 10-30% of the founders would be transgenic (Pinkert, 1994). In the F0 generation, 3 of the 31 C57Bl/6 mice born were positive for the histatin gene by PCR analysis (mouse numbers F0.9, F0.28, and F0.31). During the integration of a transgene into the host genome, sequences on both ends of the injected fragment are frequently lost. Therefore, it is imperative to analyse carefully the intactness of the injected transgene at a chromosomal site (Pinkert, 1994). Primers which were complementary to both ends of the cassette were used to confirm intactness of the transgene by PCR analysis. The transgene was fully intact in all 3 founder transgenic mice. However, an intact transgene does not ensure expression, since its expression may be silenced by the surrounding chromatin structure. Confirmation of transgene expression must be assessed by RT-PCR and protein analysis.

The next step in evaluating the transgenic mice was determining whether the transgene could be inherited as a stable genetic element. These 3 founder mice were mated with wild type C57Bl/6 mice to investigate germline transmission of the transgene. Only 1 of the 3 founder mice (mouse F0.9) gave rise to offspring which stably inherited the transgene (F1 generation). Ideally, integration occurs at a single site in the genome during the one-cell stage of embryogenesis, and assuming the transgene does not have a deleterious effect on gametogenesis that induces transmission ratio distortion, half of the F1 offspring should inherit the transgene. In practice, integration at a single site or multiple genomic sites may occur later than the one-cell stage of development, resulting in the generation of a genetically mosaic animal. A mosaic founder animal with a single

integration site will transmit the transgene to less than 50% of its offspring, and the exact percentage will be dictated by the stage at which integration occurs and the contribution of the recipient blastomere to the formation of germ cells during development (Pinkert, 1994). In this study, mouse F0.9 yielded a total of 12 pups, and transferred the transgene to one quarter of its offspring, mouse numbers F1.15, F1.21, and F1.22, which suggests mouse F0.9 may be a mosaic founder animal with a single integration site.

The derivation of homozygous mice was attempted by the establishment of brother-sister breeding pairs between male mouse F1.15 and female mice F1.21 and F2.22. Homozygosity should yield mice with increased gene dosage, and consequently, higher levels of transgene expression. Ideally, breeding homozygous mice with wild type mice should result in transmission of the transgene to 100% of its offspring, which is more efficient than breeding hemizygous mice with wild type mice. In the F2 generation in this study, 10 of the 14 pups born were positive for the transgene. Assuming Mendelian inheritance, this cross should generate 25% homozygous, 50% hemizygous, and 25% non-transgenic offspring. In this study, the transgene was successfully transferred to 71% of the offspring, which should include both homozygous and hemizygous mice.

Several of the histatin-positive transgenic mice of the F2 generation were sacrificed to evaluate transgene expression. Two male (numbers F2.4 and F2.8) and two female (numbers F2.1 and F2.5) positive mice, and one non-transgenic male mouse (F2.7), were sacrificed. Submandinular/sublingual glands, brain, heart, kidney, liver, lung, skeletal muscle, and small intestine were harvested from each animal and analysed for histatin mRNA expression. Non-quantitative RT-PCR analysis indicated that all the tissues of the positive transgenic mice examined expressed histatin mRNA, while those of the wild type mouse did not. This is unsurprising since the CMV promoter is expressed ubiquitously in most cell and tissue types. Importantly, histatin mRNA was detected in the salivary glands of all the positive transgenic mice tested. The present study did not investigate salivary gland cell-type specificity of the transgene. However, another study investigating the cell-specificity of the CMV promoter upstream of a luciferase reporter gene in an adenoviral vector found that luciferase activity was not restricted to either salivary gland cell-type *in vivo* (Zheng *et al.*, 2001). However, they did find that luciferase activity driven by the CMV promoter was ~ 2-fold higher in acinar cells than that in ductal cells. Cell- and

tissue-specificity, and strength of the CMV promoter, was compared to that of the human amylase promoter *in vivo* (Zheng *et al.*, 2001). While the human amylase promoter was more specific in driving luciferase activity in the rat acinar cells, the CMV promoter drove ~ 3000-fold more luciferase activity to the acinar cells compared to that driven by the human amylase promoter (Zheng *et al.*, 2001).

Semi-quantitative RT-PCR analysis was performed to determine levels of transgene expression in each of the histatin-positive transgenic mouse tissues. Transgene expression was relatively high in the submandibular/sublingual glands, liver, and lung, with medium expression levels observed in the brain, heart, kidney, skeletal muscle, and small intestine. These results correlate well with the relative luciferase activities driven by the CMV promoter in an adenoviral vector in rat tissues *in vivo* (Zheng *et al.*, 2001). Most importantly, transgene mRNA expression levels were high in the salivary glands of histatin-positive transgenic mice.

Protein purification and analysis was performed on each of the five submandibular/sublingual gland tissue biopsies to test for 6xHisH3 protein expression. Histatin was not detected in any of the transgenic salivary gland homogenates using cationic gel electrophoresis. Synthetic histatin was incubated with each of the tissue homogenates to investigate if proteolysis had occurred during preparation. However, no degradation of the synthetic histatin incubated with tissue homogenates was observed compared to that of synthetic histatin incubated with protein buffer containing no tissue homogenate. This suggests that the tagged histatin was expressed at levels too low in the salivary glands of these particular transgenic mice to be detected by cationic gel electrophoresis. However, expression of tagged histatin was successfully detected in salivary gland samples using the dot blot method. The polyhistidine tag enabled immunodetection of histatin at low levels in the homogenates. The dot blot is a simple, rapid, and more sensitive method for the detection of low levels of 6xHisH3 than cationic gel electrophoresis. The limit of detection on a cationic gel is 1 μg, whereas dot blots enable the detection of antigen levels of 100 ng or lower.

In conclusion, the 6xHisH3 transgene was used to successfully generate transgenic mice expressing histatin 3 protein in their salivary glands. Transgene expression was found in all the positive transgenic mouse tissue-types examined, with highest expression levels

observed in the submandibular/sublingual glands, liver, and lung. These histatin-positive transgenic mice represent an invaluable tool for studying the efficacy of salivary histatin against *Candida* spp. in the structured, regulated, and multicellular environment of the oral cavity.

Chapter 5

Transcriptional Regulation of the *HIS2* Promoter *in vitro*

5.1 Introduction

Antimicrobial peptides are essential components of the mammalian innate immune system and constitute a rapidly inducible first line of defence against microbial infection. Numerous studies investigating mammalian antimicrobial proteins produced at mucosal surfaces suggest that they are subject to dynamic regulation after exposure to biological stimuli both *in vitro* and *in vivo*. Such studies include regulation of β-defensins (Russell *et al.*, 1996; Krisanaprakornkit *et al.*, 1998, 2000; Harder *et al.*, 2000; Wada *et al.*, 2001), mucins (Li *et al.*, 1997, 1998, 2003), or cathelicidin (Wu *et al.*, 2000) produced by oral, respiratory, intestinal, or reproductive tract epithelial cells after incubation with microbial products, proinflammatory cytokines, and various other biological stimuli.

Salivary histatins are potent antifungal agents and are believed to play a key role in controlling opportunistic fungi such as *C. albicans* in the oral cavity. They are produced by salivary glands adjacent to the oral epithelium, and it is reasonable to speculate that their expression may also be subject to up- or down-regulation after exposure to certain biological stimuli. Several small clinical studies performed to date in humans suggest a dynamic relationship between salivary histatin levels and factors such as oral yeast carriage rates (Jainkittivong *et al.*, 1998), the immune status of the patient (Atkinson *et al.*, 1990; Lal *et al.*, 1992), oral candidiasis (Mandel *et al.*, 1992), recurrent oral candidiasis (Bercier *et al.*, 1999), patient age (Johnson *et al.*, 2000), or certain medications, such as β_1-adrenolytic agents (Jensen *et al.*, 1994). However these studies were all cross-sectional, and therefore may have been self-selecting populations.

To date, no studies have been published investigating histatin regulation under any conditions *in vitro*. Disease states are often associated with increased or reduced expression of critical gene products. The ability to modulate gene expression can provide the basis for therapies. Identifying promoter regions of genes and determining how they interact with transcription factors to control gene expression is an important area of study. The main objective of this part of the study was to investigate the extent to which salivary histatins are regulated *in vitro* in a HSG cell line, and to identify some of the external stimuli, *cis*-element(s), and signaling pathway(s) which may be involved. Since histatin 3 is the most abundant histatin family member found in saliva, the *HIS2* promoter was chosen to investigate histatin gene regulation. Identifying promoter regions responsible for gene

regulation involves generating successive deletions in a promoter sequence, and investigating transcriptional activity of these promoter constructs under various conditions. Generally, most of the important promoter elements are located within 6 kb from the transcriptional start site of a gene. Therefore, initial experiments investigating histatin promoter activity in this project used *HIS2* promoter deletion constructs of 2, 4, and 6 kb in length.

5.2 Materials and Methods

5.2.1 Amplification and cloning of the *HIS2* promoter sequence

5.2.1.1 Generation of 2, 4, and 6 kb HIS2 promoter constructs

Various lengths of the *HIS2* promoter were amplified by PCR from human genomic DNA (GibcoBRL Life Technologies) using the Expand High Fidelity PCR System (Roche), according to the manufacturer's instructions, and cloned into the promoterless pGL2-Basic vector (Promega) upstream of the firefly luciferase reporter gene on *Mlu*I/ *Xho*I fragments. Forward primers HIS2A, HISPro4, and HISPro6, and reverse primer HISRev (Table 5.1), were used to amplify 2, 4, and 6 kb of the *HIS2* promoter to generate promoter constructs p2B, p4B, and p6B, respectively. PCR cycling conditions to amplify the 2 kb fragment were as follows: 1 cycle at 94°C for 2 min, 30 cycles at 94°C for 15 s, 55°C for 30 s, 72°C for 2 min, and 1 cycle at 72°C for 10 min. PCR cycling conditions to amplify the 4 kb and 6 kb fragments were as follows: 1 cycle at 94°C for 2 min, 30 cycles at 94°C for 15 s, 55°C for 30 s, 68°C for 4 min, and 1 cycle at 68°C for 10 min. Inserts of the correct size were screened by PCR using forward primer GLprimer1 and reverse primer GLprimer2 (Table 5.1), which bind either side of the pGL2-Basic multiple cloning site.

5.2.2.2 Generation of 400 bp deletions in the HIS2 4 kb promoter

Initial experiments investigating *HIS2* promoter activity identified the 4 kb promoter construct as the most active deletion construct when stimulated with candidal supernatants. Therefore, it was decided to further investigate activity of the 4 kb promoter using deletional analysis. Successive 400 bp deletions in the 5′-3′ direction were generated in the *HIS2* 4 kb promoter using p4B as a template. PCR was performed using *Pfu* DNA polymerase (Promega). Forward primers H3Pr0.4, H3Pr0.8, H3Pr1.2, H3Pr1.6, H3Pr2.4, H3Pr2.8, H3Pr3.2, H3Pr3.6, and reverse primer HISRev (Table 5.1) were used to generate the *HIS2* promoter constructs pGL0.4, pGL0.8, pGL1.2, pGL1.6, pGL2.4, pGL2.8, pGL3.2, and pGL3.6, respectively. PCR cycling conditions to amplify the fragments were as follows: 1 cycle at 94°C for 1 min, 35 cycles at 94°C for 15 s, 55°C for 30 s, 72°C for 4 min, and 1 cycle at 72°C for 10 min. Deletion inserts of the correct size were screened by PCR using primers GLprimer1 and GLprimer2 (Table 5.1). The 2 kb and 4 kb fragments from vectors p2B and p4B were subcloned into pGL3-Basic vectors.

Table 5.1 Primers used to amplify 2, 4, and 6 kb of the *HIS2* promoter, to generate 400 bp deletions in the *HIS2* 4 kb promoter, and to identify correct size inserts

Primer	Sequence 5′-3′	Amplicon size (bp)
Generate 2, 4, and 6 kb HIS2 promoter constructs		
HIS2A[a]	CAGGACGCGTGGTGCATATCTGTAGC	2019
HISPro4[a]	GCATAACGCGTTTAAACTTCCC	4015
HISPro6[a]	CATCACGCGTCAGGGCCTGTTG	6020
HISRev[b]	GTGAAGTCTCTCGAGATGTCCTTTCC	N/A
Generate HIS2 promoter deletion constructs		
H3Pr0.4[a]	TAAAACGCGTCATAACATGG	419
H3Pr0.8[a]	ATCTACGCGTCATGTTTGTC	822
H3Pr1.2[a]	AGCCACGCGTCCTGATGTGTG	1220
H3Pr1.6[a]	TCAAACGCGTGGGAAATATC	1616
H3Pr2.4[a]	AACAACGCGTTCTTAGATCC	2416
H3Pr2.8[a]	CATGACGCGTCCAAAGAAC	2810
H3Pr3.2[a]	ATAGACGCGTGAGTCACATGTTG	3205
H3Pr3.6[a]	GCTTACGCGTCATACAAACTGC	3619
Identify promoter inserts		
GLprimer1	TGTATCTTATGGTACTGTAACTG	N/A
GLprimer2	CTTTATGTTTTTGGCGTCTTCCA	N/A

Underlined sequences represent restriction sites.
[a]*Mlu*I restriction site.
[b]*Xho*I restriction site.
N/A, not applicable.

5.2.2 Transient transfection of the HSG cell line

5.2.2.1 Calcium phosphate method

Transfections were initially performed with the p2B, p4B, and p6B vectors using the calcium phosphate transfection kit from Invitrogen, according to the manufacturer's instructions. Briefly, 1 μg of each test plasmid and 0.02 μg of internal control *Renilla* luciferase vector was transfected into HSG cells (1.5×10^5 cells/well). Cells were harvested 24 h post-transfection.

5.2.2.2 FuGene method

HSG cells were seeded at a density of 1.5×10^5 cells/well in 24 well plates, and transfections were performed immediately afterward using FuGENE 6 transfection reagent (Roche). A total of 1 μg of test plasmid (pGL3 contructs) and 0.1 μg of reference plasmid phRL-TK (to correct for transfection efficiency) were transfected per well, at a ratio of 1:3 FuGENE 6. Stimulations were performed 24 h post-transfection, and harvested at the indicated time points.

5.2.3 Strains and reagents used

Reference strains *C. albicans* 132A (Gallagher *et al.*, 1992), *C. albicans* SC5314 (Fonzi and Irwin, 1993), and *C. dubliniensis* CD36 (Sullivan *et al.*, 1995) were used in this study. All *Candida* strains were routinely cultured on PDA at 30°C for 48 h. For liquid culture, isolates were routinely grown in YEPD broth at 30°C in an orbital incubator. *E.coli* lipopolysaccharide (LPS), *S. aureus* peptidoglycan (PGN), zymosan, human recombinant tumour necrosis factor alpha (TNFα), and L-arginine were purchased from Sigma. Human recombinant interleukin 1 beta (IL-1β) and interferon gamma (IFNγ) were obtained from Roche. Phorbol myristate acetate (PMA) was purchased from GibcoBRL Life Technologies. L-isoleucine was obtained from Fluka Biochemicals (Buchs, Switzerland).

5.2.4 Stimulations

Candida strains were plated fresh on PDA at 30°C for 48 h prior to experiments. Yeast cells were grown in YEPD broth at 30°C for 16 h at 200 rpm. Transfected HSG cells were

incubated with either live or heat-killed *Candida* (1 x 10^8 cells/ml for 3, 6, 12, and 24 h. Yeast cells were heat-killed by incubation at 60°C for 30 min. Transfected HSG cells were also treated with either TNFα (10 ng/ml), IFNγ (250 U/ml), IL-1β (10 ng/ml), LPS (10 μg/ml), PGN (10 μg/ml), or zymosan (10 μg/ml) for 3, 6, 12, and 24 h. At the end of the incubation period, cell culture medium was removed and the cells washed gently with PBS. Cell lysis buffer (100 μl) was added to each well and plates were shaken gently at room temperature for 20 min. Cells lysates were stored at -80°C until promoter activity was measured using the dual luciferase reporter assay.

5.2.5 Dual luciferase reporter (DLR) assay

DLR assays were performed using the DLR Assay System (Promega), according to the manufacturer's instructions. DLR assays are designed to measure both firefly and *Renilla* luciferase activity from a single sample. Briefly, to measure firefly luciferase activity, 20 μl of luciferase assay reagent was predispensed into each vial, followed by 4 μl of cell lysate, and this was mixed by pipetting 3 times. Firefly luciferase activity was measured in a luminometer (Turner Designs Model TD-20/20, West Maude Avenue, Sunnyvale, California, USA) with a pre-programmed 3 s delay, followed by a 10 s integration period. The vials were removed, 20 μl of Stop & Glo Reagent added to each vial, vortexed briefly to mix, and replaced in the luminometer to measure *Renilla* luciferase activity with a 3 s delay, and 10 s integration period. Experimental firefy luciferase activity was normalised to *Renilla* luciferase activity, and promoter activity was expressed as relative light units.

5.2.6 Transcription factor binding site analysis of *HIS2* promoter sequence

The TRANSFAC database was used to define potential transcription factor binding sites within 4 kb of the *HIS2* promoter sequence. The search was conducted using MatInspector V2.2 software (Mascheroder Weg, Braunschweig, Germany). Cut-offs were employed which minimised false negative error rates.

5.3 Results

5.3.1 Activity of 2, 4, and 6 kb lengths of the *HIS2* promoter

Increased transcriptional activity of the 2, 4, and 6 kb lengths of the *HIS2* promoter were observed after stimulation with *C. albicans* 132A and *C. dubliniensis* CD36 supernatants. Transcriptional activity of the promoter constructs were almost 2-fold over the activity of the unstimulated control. The 2 kb promoter length gave the highest transcriptional activity. No additional increase in promoter activity was observed between the 4 kb and 6 kb promoter lengths. The 4 kb promoter was used in the rest of this study to investigate *HIS2* promoter activity (Fig 5.1).

5.3.2 Optimisation studies for FuGene transfection

5.3.2.1 Ratio of Fugene reagent to pGL3-Control firefly luciferase vector in HSG cells

To investigate the optimum amount of FuGene reagent to luciferase vectors used to transfect HSG cells, 3:1, 3:2, and 6:1 ratios of µl of FuGene reagent to µg of pGL3-Control firefly luciferase vector were used to transfect HSG cells (1.5×10^5 cells/well), with or without penicillin and streptomycin added to the cell culture medium. Luciferase activity of pGL3-Control was measured 24 h and 48 h post-transfection. It was observed that a ratio of 3:1 of µl of FuGene reagent to µg of pGL3-Control gave the highest luciferase activity without penicillin and streptomycin in the media (Fig. 5.2). This ratio gave ~3-fold greater luciferase activity than the 3:2 ratio, and ~4-fold greater luciferase activity than the 6:1 ratio in cells without penicillin and streptomycin after 24 h (Fig. 5.2A). No significant increase in luciferase activity was observed in any of the ratios investigated 48 h post-transfection (Fig. 5.2B). The absence of penicillin and streptomycin in the cell culture media increased the luciferase activity of the 3:1 ratio ~4-fold after 24 h and 48 h. An absence of penicillin and streptomycin was observed to increase the luciferase activity of all the ratios tested.

5.3.2.2 Molar ratio of firefly luciferase vectors to phRL-TK Renilla luciferase vector in HSG cells

Firefly and *Renilla* luciferase vectors can exhibit strong negative trans-acting effects on luciferase activity when co-transfected into different cell types in certain molar ratios.

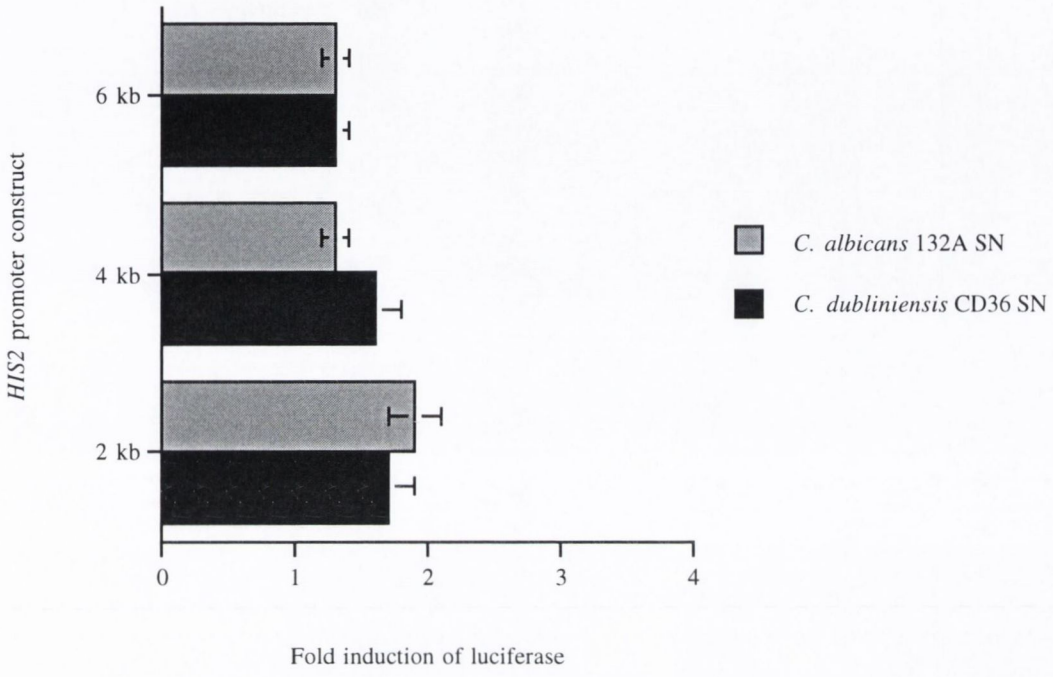


Figure 5.1 Promoter activity of the *HIS2* 2, 4, and 6 kb promoter fragments in HSG cells following incubation with *C. albicans* 132A and *C. dubliniensis* CD36 supernatants. p2B, p4B, and p6B (1 μg each) were transiently transfected into HSG cells (1.5 x 10^5 cells/well). Supernatants were added to HSG cell media at a 1:3 ratio and the cells were harvested 24 h later. Promoter activity is represented as fold induction of luciferase activity of the stimulated promoter compared to that of the unstimulated control. Experimental firefly luciferase activity was normalised to the internal *Renilla* luciferase activity of the co-transfected phRL-TK vector. Results are presented as means ± SD of three independent experiments performed in duplicate. **SN**, supernatant.

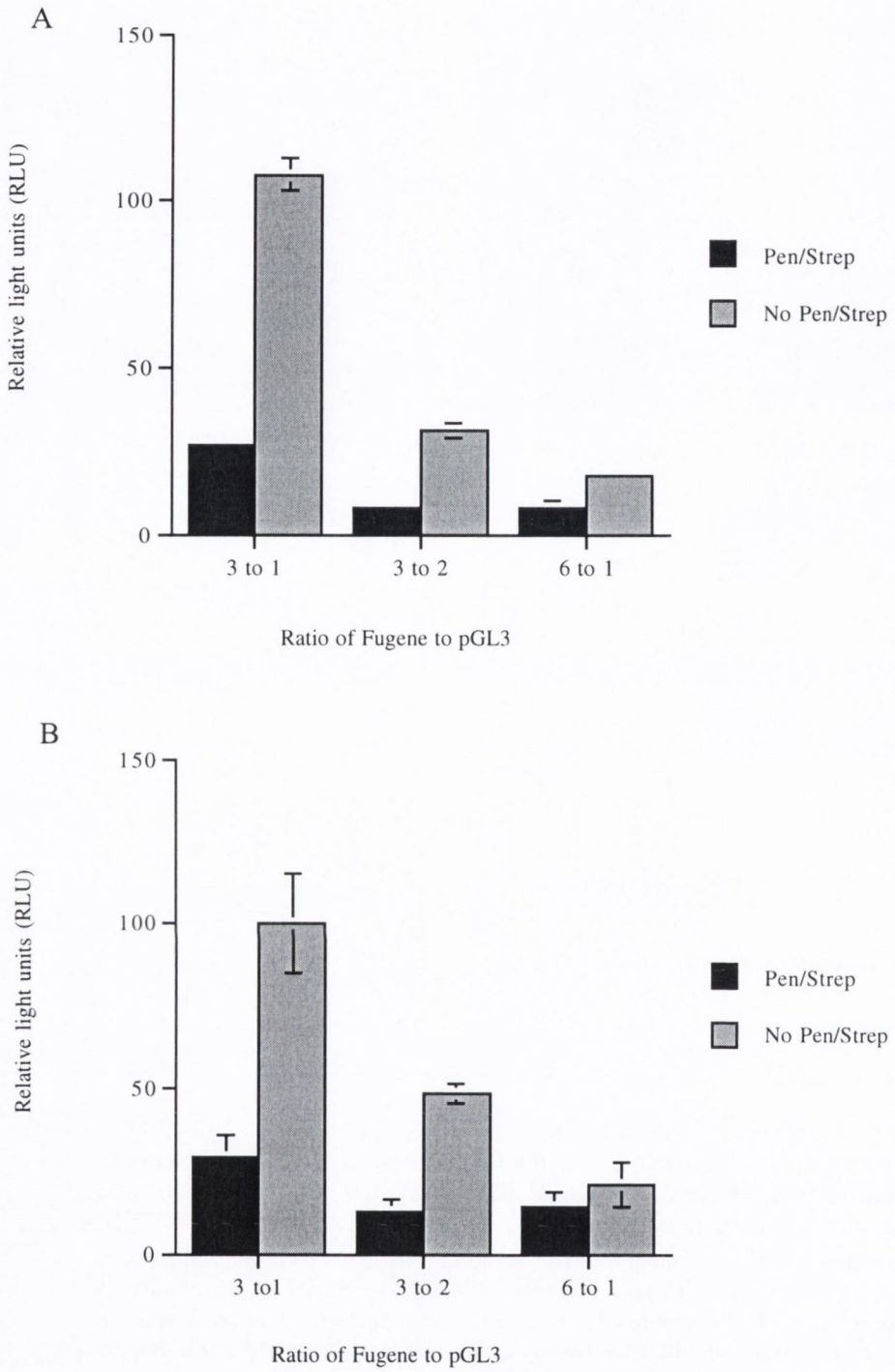


Figure 5.2 Optimisation of the ratio of Fugene to pGL3-Control firefly luciferase vector in the HSG cell line. Ratios of 3:1, 3:2, and 6:1 of µl of Fugene reagent to µg of pGL3-Control vector were used to transfect 1 x 10^5 HSG cells/well in 24 well plates immediately after trypsinisation, with and without penicillin and streptomycin. Luciferase activity was measured (A) 24 h and (B) 48 h post-transfection. Results are expressed as means ± SD of three independent experiments performed in duplicate.

Therefore, the optimum molar ratio of pGL3-Control and pGL4.0 firefly luciferase vectors to the phRL-TK *Renilla* luciferase vector which did not exert negative effects on luciferase activity was assessed in the HSG cell line. It was observed that at ratios of 10:1, 20:1, 30:1, 40:1, and 50:1 of pGL30Control and pGL4.0 firefly luciferase vectors to phRL-TK, the *Renilla* luciferase vector exhibited strong negative trans-acting effects on the firefly luciferase vectors (Fig. 5.3). However, at a ratio of 100:1 of firefly luciferase vectors to phRL-TK, the firefly luciferase activities returned to the levels observed when transfected without *Renilla* luciferase vector. Low-level background activity of *Renilla* luciferase was observed at this ratio, but was ~3-fold higher than the levels in HSG cells with no *Renilla* luciferase vector.

5.3.3 Analysis of *HIS2* 4 kb promoter activity

5.3.3.1 Lipopolysaccharide stimulation

Increased transcriptional activity of the *HIS2* promoter was observed after incubation with 100 ng/ml and 10 μg/ml of the Gram-negative bacterial product lipopolysaccaride (LPS) (Fig. 5.4). Transcriptional activity was at its highest (~2.5-fold) after 3 h incubation with 10 μg/ml LPS, and remained elevated after 12 h stimulation. Promoter activity returned to basal levels after incubation for 24 h. The *HIS2* promoter was up-regulated in a dose-dependent manner in response to the two concentrations of LPS used. LPS at 10 μg/ml induced the greatest up-regulation of the promoter after 3 h, while 100 ng/ml of LPS also induced up-regulation of the *HIS2* promoter, but the levels were 60% less than those observed for 10 μg of LPS at the same time point.

5.3.3.2 Peptidoglycan stimulation

Induction of *HIS2* promoter activity was found after incubation with 100 ng/ml and 10 μg/ml of the Gram-positive bacterial product peptidoglycan (PGN) (Fig. 5.5). Transcriptional activity was at its highest (~2.6-fold) after 6 h incubation with 10 μg/ml PGN. The levels of transcriptional activity remained elevated after 3 h to 6 h incubation with PGN, returning to basal levels after 12 h. A moderate rise in promoter activity was

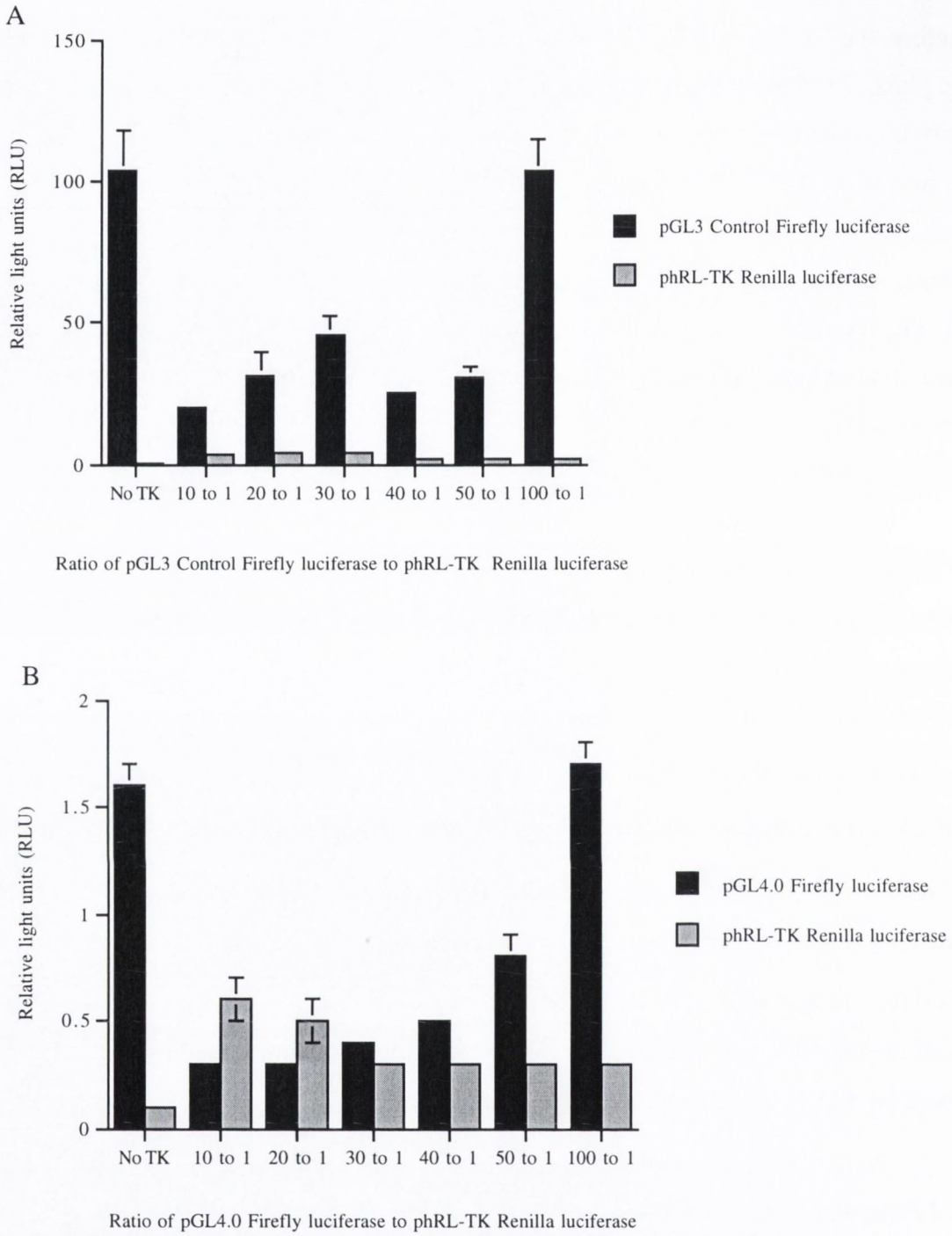


Figure 5.3 Optimisation of the molar ratio of A, pGL3-Control firefly luciferase to phRL-TK *Renilla* luciferase, and of B, pGL4.0 firefly luciferase to phRL-TK *Renilla* luciferase. Molar ratios of 10:1, 20:1, 30:1, 40:1, 50:1, and 100:1 of firefly luciferase vectors to *Renilla* vector were used to transfect 1.5 x 10^5 HSG cells/well in 24 well plates. Luciferase activity was measured 24 h post-transfection. Results are expressed as means ± SD of three independent experiments performed in duplicate.

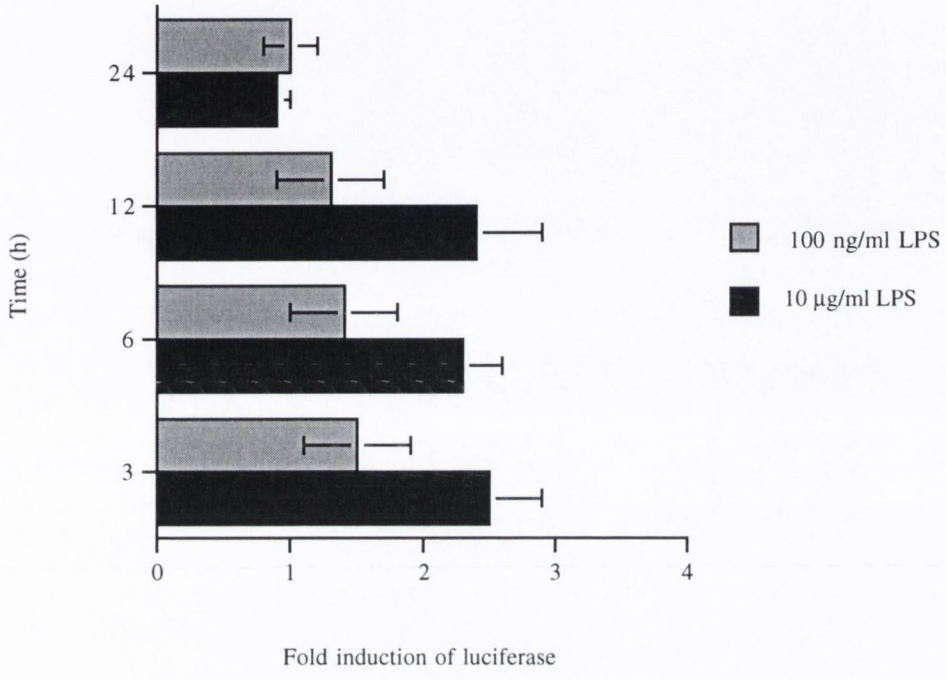


Figure 5.4 Promoter activity of the *HIS2* 4 kb promoter in HSG cells following incubation with lipopolysaccharide (LPS). pGL4.0 (1 µg) containing 4 kb of the 5′-upstream region of the *HIS2* gene was transiently transfected into HSG cells (1.5 x 10^5 cells/well). LPS was added 24 h post-transfection to a final concentration of 100 ng/ml and 10 µg/ml, and the cells harvested after 3 h, 6 h, 12 h, and 24 h. Promoter activity is represented as fold induction of luciferase activity of the stimulated promoter compared to that of the unstimulated control. Experimental firefly luciferase activity was normalised to the internal *Renilla* luciferase activity of the co-transfected phRL-TK vector. Results are presented as means ± SD of three independent experiments performed in duplicate.

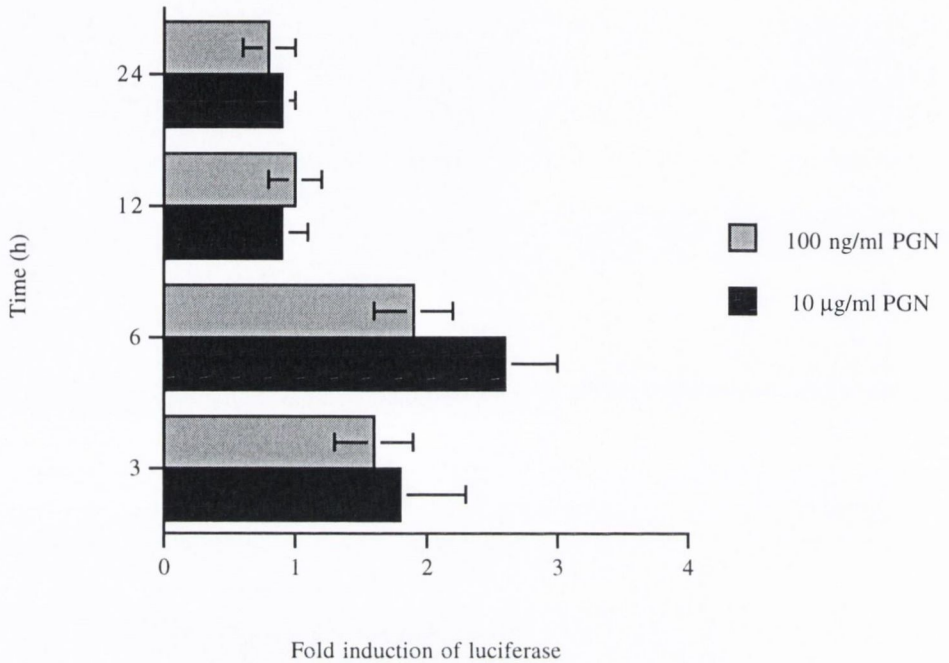


Figure 5.5 Promoter activity of the *HIS2* 4 kb promoter in HSG cells following incubation with peptidoglycan (PGN). pGL4.0 (1 µg) containing 4 kb of the 5′-upstream region of the *HIS2* gene was transiently transfected into HSG cells (1.5 x 10^5 cells/well). PGN was added 24 h post-transfection to a final concentration of 100 ng/ml and 10 µg/ml, and the cells harvested after 3 h, 6 h, 12 h, and 24 h. Promoter activity is represented as fold induction of luciferase activity of the stimulated promoter compared to that of the unstimulated control. Experimental firefly luciferase activity was normalised to the internal *Renilla* luciferase activity of the co-transfected phRL-TK vector. Results are presented as means ± SD of three independent experiments performed in duplicate.

observed after 3 h and 6 h incubation when the concentration of PGN was increased from 100 ng/ml to 10 μg/ml, indicating the *HIS2* promoter responds to the presence of PGN in a dose-dependent manner.

5.3.3.3 Zymosan stimulation

Up-regulation of the *HIS2* promoter was found after incubation with 100 ng/ml and 10 μg/ml of zymosan (Fig. 5.6). Transcriptional activity was at its highest after 3 h and 6 h incubation (~1.7-fold and ~1.8-fold, respectively) with 10 μg/ml zymosan. Promoter activity returned to unstimulated levels after incubation of 12 h. A 100-fold increase in zymosan concentration from 100 ng/ml to 10 μg/ml zymosan showed a minor increase in promoter activity at time points 3 h and 6 h, indicating that the lower concentration of 100 ng/ml zymosan is sufficient to induce *HIS2* promoter transcription. Promoter activity also returned to basal levels of transcriptional activity after 12 h incubation with the lower concentration of zymosan.

5.3.3.4 Stimulation with heat-killed C. albicans SC5314

Increased activity of the *HIS2* promoter was observed after incubation with two concentrations of heat-killed *C. albicans* SC5314 blastospores (Fig. 5.7). Transcriptional activity was at its highest (~2.7-fold) after 12 h incubation with 1 x 10^6 cells/ml SC5314. Promoter activity remained elevated for up to 12 h with 1 x 10^6 cells/ml SC5314, with activity returning to basal levels after 24 h stimulation. Increasing the concentration of SC5314 from 1 x 10^4 cells/ml to 1 x 10^6 cells/ml had a moderate effect on *HIS2* promoter activity. The observed increase in promoter activity was similar for both concentrations at 3 h and 6 h, with a moderate increase in promoter activity after 12 h incubation at the higher concentration.

5.3.3.5 Interleukin 1 beta stimulation

The *HIS2* promoter was up-regulated in response to incubation with 10 ng/ml and 100 ng/ml interleukin 1 beta (IL-1β) (Fig. 5.8). Transcriptional activity was increased ~2.0-fold

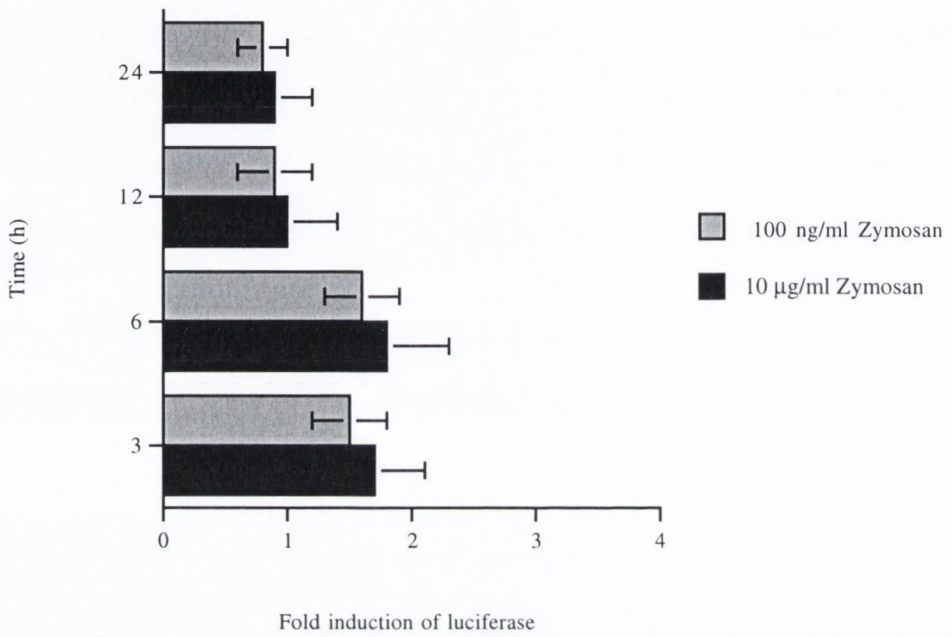


Figure 5.6 Promoter activity of the *HIS2* 4 kb promoter in HSG cells following incubation with zymosan. pGL4.0 (1 µg) containing 4 kb of the 5′-upstream region of the *HIS2* gene was transiently transfected into HSG cells (1.5 x 10^5 cells/well). Zymosan was added 24 h post-transfection to a final concentration of 100 ng/ml and 10 µg/ml, and the cells harvested after 3 h, 6 h, 12 h, and 24 h. Promoter activity is represented as fold induction of luciferase activity of the stimulated promoter compared to that of the unstimulated control. Experimental firefly luciferase activity was normalised to the internal *Renilla* luciferase activity of the co-transfected phRL-TK vector. Results are presented as means ± SD of three independent experiments performed in duplicate.

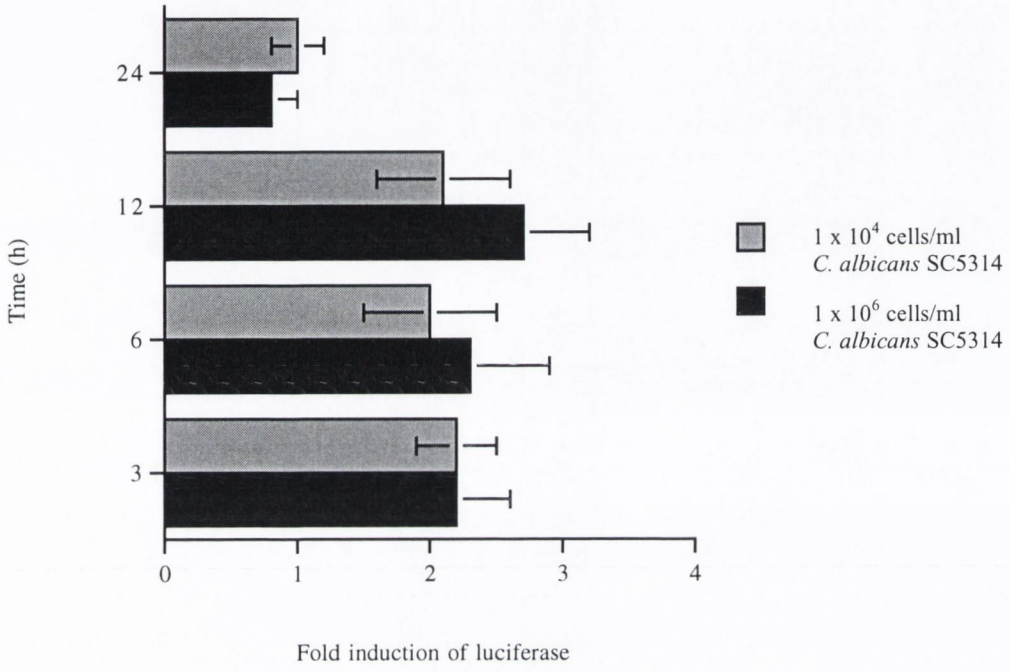


Figure 5.7 Promoter activity of the *HIS2* 4 kb promoter in HSG cells following incubation with heat-killed *C. albicans* SC5314. pGL4.0 (1 µg) containing 4 kb of the 5′-upstream region of the *HIS2* gene was transiently transfected into HSG cells (1.5 x 10^5 cells/well). Heat-killed *C. albicans* was added 24 h post-transfection to a final concentration of 1 x 10^4 cells/ml and 1 x 10^6 cells/ml, and the cells harvested after 3 h, 6 h, 12 h, and 24 h. Promoter activity is represented as fold induction of luciferase activity of the stimulated promoter compared to that of the unstimulated control. Experimental firefly luciferase activity was normalised to the internal *Renilla* luciferase activity of the co-transfected phRL-TK vector. Results are presented as means ± SD of three independent experiments performed in duplicate.

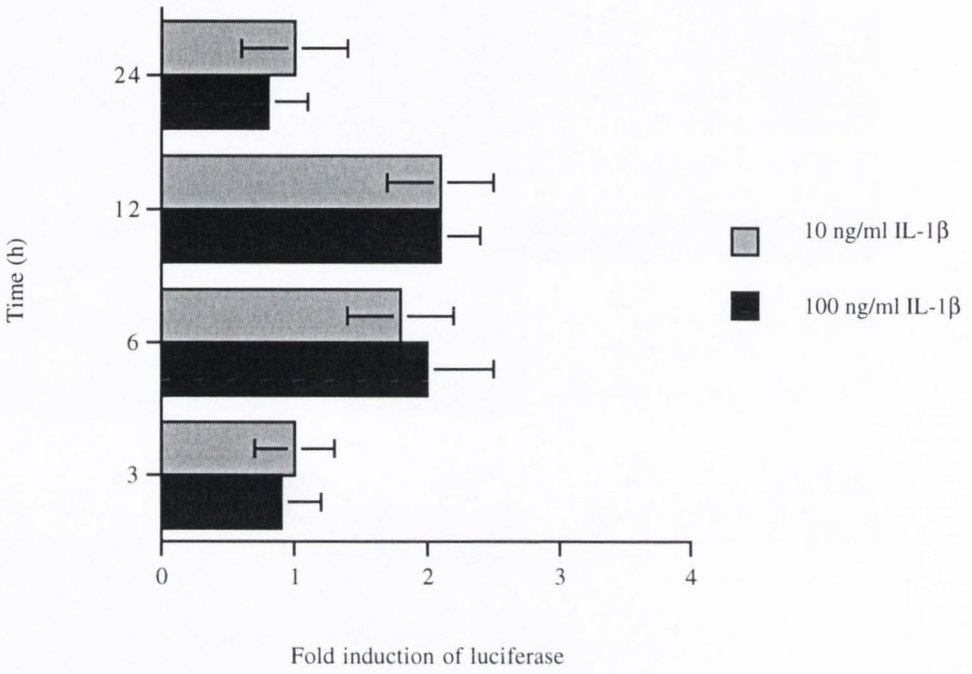


Figure 5.8 Promoter activity of the *HIS2* 4 kb promoter in HSG cells following incubation with interleukin 1 beta (IL-1β). pGL4.0 (1 μg) containing 4 kb of the 5′-upstream region of the *HIS2* gene was transiently transfected into HSG cells (1.5 x 10^5 cells/well). IL-1β was added 24 h post-transfection to a final concentration of 10 ng/ml and 100 ng/ml, and the cells harvested after 3 h, 6 h, 12 h, and 24 h. Promoter activity is represented as fold induction of luciferase activity of the stimulated promoter compared to that of the unstimulated control. Experimental firefly luciferase activity was normalised to the internal *Renilla* luciferase activity of the co-transfected phRL-TK vector. Results are presented as means ± SD of three independent experiments performed in duplicate.

after 6 h incubation with 100 ng/ml IL-1β and ~2.1-fold after 12 h incubation. No increase in promoter activity above background was observed after 3 h and 24 h incubation with 10 ng/ml or 100 ng/ml IL-1β. Increasing the IL-1β concentration from 10 ng/ml to 100 ng/ml produced a minor increase in promoter activity after 6 h incubation, while both IL-1β concentrations increased promoter activity by the same magnitude after 12 h, indicating that 10 ng/ml of IL-1β is sufficient to produce a ~2.0-fold increase in *HIS2* promoter activity.

5.3.3.6 Interferon gamma stimulation

The pro-inflammatory cytokine interferon gamma (IFNγ) has a moderate effect on *HIS2* promoter ativity (Fig. 5.9). Transcriptional activity was elevated ~1.5-fold to ~1.8-fold after 3 h to 12 h incubation with 250 U/ml IFNγ, with levels at their highest after 12 h incubation. Transcriptional activity returned to basal levels after 24 h incubation with IFNγ. Increasing the concentration of IFNγ from 50 U/ml to 250 U/ml caused a minor increase in *HIS2* promoter activity. Transcriptional activity was moderately up-regulated after 3 h, 6 h, and 12 h incubation when IFNγ concentration was increased from 50 U/ml to 250 U/ml, with activity returning to basal levels with both concentrations after the 24 h incubation period.

5.3.3.7 Tumor necrosis factor alpha stimulation

The *HIS2* promoter showed a small increase in promoter activity following incubation with 10 ng/ml and 100 ng/ml of tumor necrosis factor alpha (TNFα) (Fig. 5.10). Promoter activity was at its highest after 6 h incubation with both concentrations of TNFα. A 10-fold increase in TNFα concentration from 10 ng/ml to 100 ng/ml induced only a minor increase in promoter activity (~1.1-fold to ~1.3-fold, respectively). No increase above background promoter activity was observed after 3 h, 12 h, and 24 h incubation with TNFα.

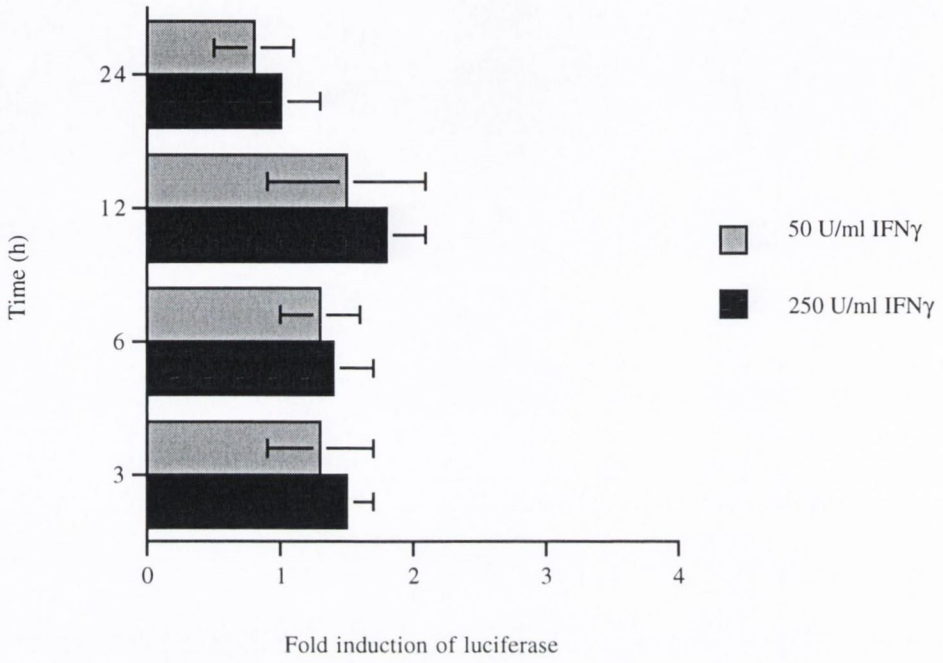


Figure 5.9 Promoter activity of the *HIS2* 4 kb promoter in HSG cells following incubation with interferon gamma (IFNγ). pGL4.0 (1 μg) containing 4 kb of the 5′-upstream region of the *HIS2* gene was transiently transfected into HSG cells (1.5 x 10^5 cells/well). IFNγ was added 24 h post-transfection to a final concentration of 50 U/ml and 250 U/ml, and the cells harvested after 3 h, 6 h, 12 h, and 24 h. Promoter activity is represented as fold induction of luciferase activity of the stimulated promoter compared to that of the unstimulated control. Experimental firefly luciferase activity was normalised to the internal *Renilla* luciferase activity of the co-transfected phRL-TK vector. Results are presented as means ± SD of three independent experiments performed in duplicate.

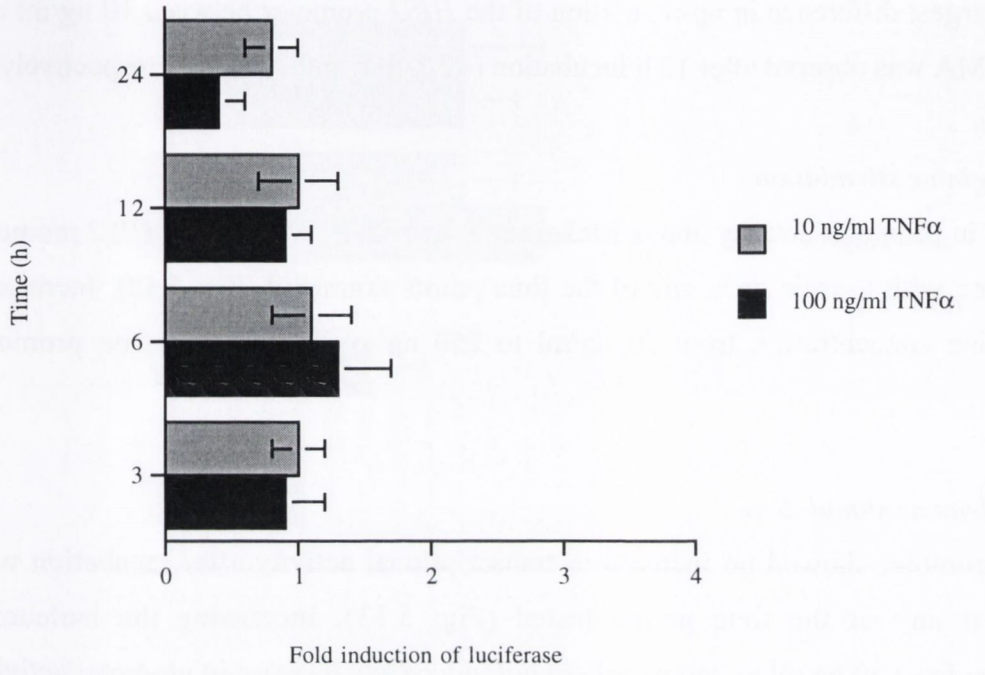


Figure 5.10 Promoter activity of the *HIS2* 4 kb promoter in HSG cells following incubation with tumor necrosis factor alpha (TNFα). pGL4.0 (1 μg) containing 4 kb of the 5′-upstream region of the *HIS2* gene was transiently transfected into HSG cells (1.5 x 10^5 cells/well). TNFα was added 24 h post-transfection to a final concentration of 10 ng/ml and 100 ng/ml, and the cells harvested after 3 h, 6 h, 12 h, and 24 h. Promoter activity is represented as fold induction of luciferase activity of the stimulated promoter compared to that of the unstimulated control. Experimental firefly luciferase activity was normalised to the internal *Renilla* luciferase activity of the co-transfected phRL-TK vector. Results are presented as means ± SD of three independent experiments performed in duplicate.

5.3.3.8 Phorbol myristate acetate stimulation

The *HIS2* promoter demonstrated up-regulation in response to incubation with 10 ng/ml and 100 ng/ml of phorbol myristate acetate (PMA) (Fig. 5.11). Transcriptional activity was elevated after 6 h to 12 h incubation, but was at its highest (~2.8-fold) after 12 h stimulation. Promoter activity was also elevated for 6 h to 24 h incubation with 10 ng/ml of PMA. The largest difference in up-regulation of the *HIS2* promoter between 10 ng/ml and 100 ng/ml PMA was observd after 12 h incubation (~2.2-fold and ~2.8-fold, respectively).

5.3.3.9 L-arginine stimulation

No increase in promoter activity above background was observed after the *HIS2* promoter was incubated with L-arginine at any of the time points examined (Fig. 5.12). Increasing the L-arginine concentration from 50 ng/ml to 250 ng/ml had no effect on promoter activity.

5.3.3.10 Isoleucine stimulation

The *HIS2* promoter showed no increase in transcriptional activity after incubation with isoleucine at any of the time points tested (Fig. 5.13). Increasing the isoleucine concentration from 50 ng/ml to 250 ng/ml did not induce any increase in promoter activity. A summary of the induction profiles of the *HIS2* promoter in response to different stimulants is given in Table 5.2.

5.3.3.11 With and without interferon gamma

Synergistic transcriptional activity of the *HIS2* promoter was examined using combinations of LPS, PGN, zymosan, and *C. albicans* with the proinflammatory cytokine IFNγ. No additional increase in *HIS2* promoter activity was observed when these microbial products were used in combination with IFNγ (Fig. 5.14).

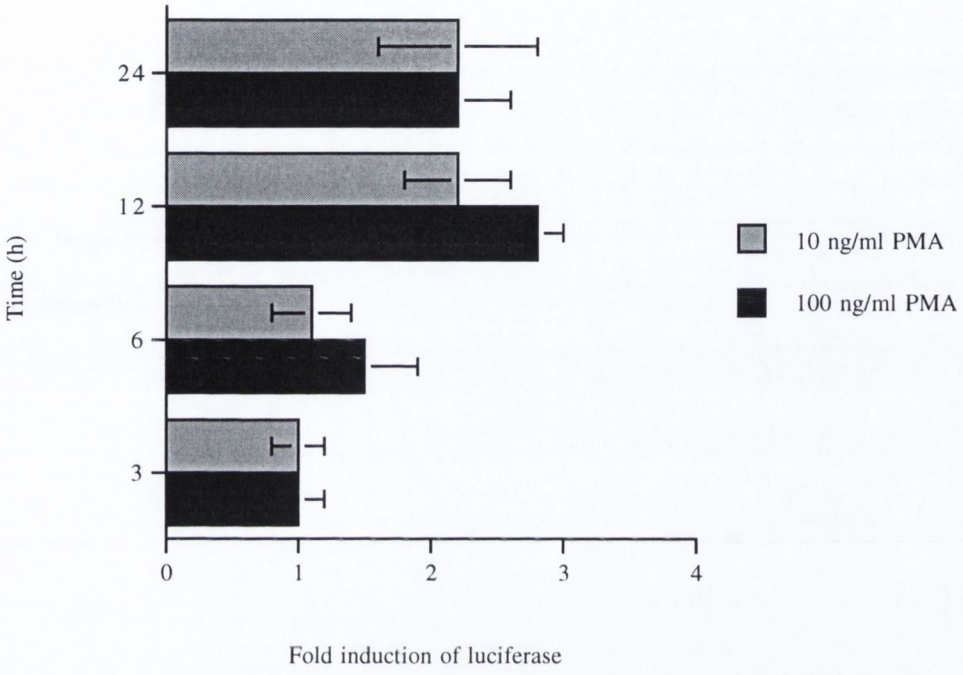


Figure 5.11 Promoter activity of the *HIS2* 4 kb promoter in HSG cells following incubation with phorbol myristate acetate (PMA). pGL4.0 (1 μg) containing 4 kb of the 5′-upstream region of the *HIS2* gene was transiently transfected into HSG cells (1.5 x 10^5 cells/well). PMA was added 24 h post-transfection to a final concentration of 10 ng/ml and 100 ng/ml, and the cells harvested after 3 h, 6 h, 12 h, and 24 h. Promoter activity is represented as fold induction of luciferase activity of the stimulated promoter compared to that of the unstimulated control. Experimental firefly luciferase activity was normalised to the internal *Renilla* luciferase activity of the co-transfected phRL-TK vector. Results are presented as means ± SD of three independent experiments performed in duplicate.

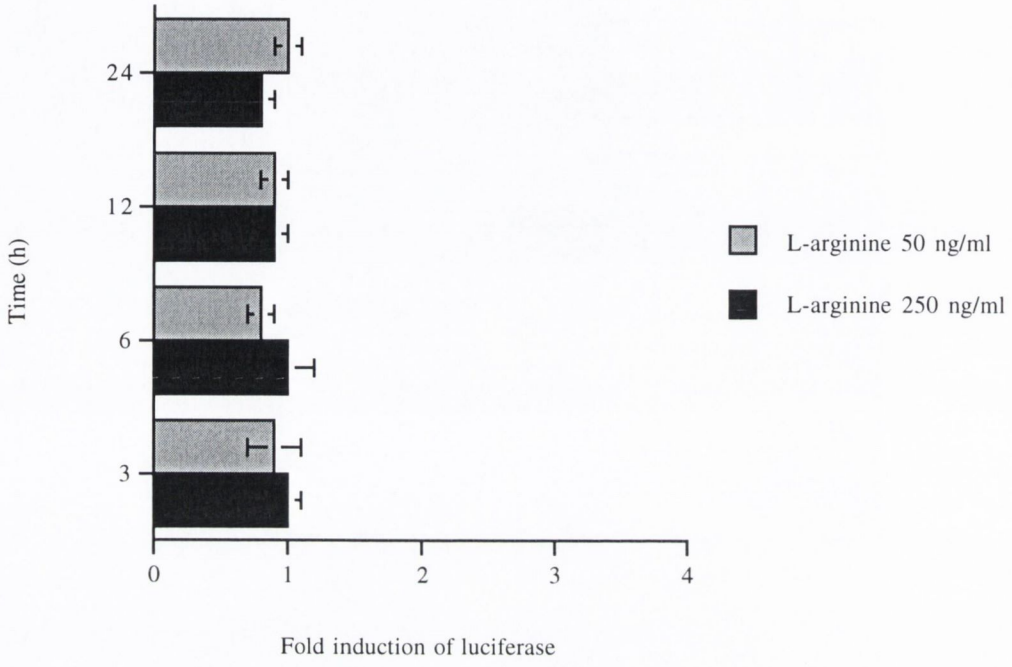


Figure 5.12 Promoter activity of the *HIS2* 4 kb promoter in HSG cells following incubation with L-arginine. pGL4.0 (1 μg) containing 4 kb of the 5′-upstream region of the *HIS2* gene was transiently transfected into HSG cells (1.5 x 10^5 cells/well). L-arginine was added 24 h post-transfection to a final concentration of 50 ng/ml and 250 ng/ml, and the cells harvested after 3 h, 6 h, 12 h, and 24 h. Promoter activity is represented as fold induction of luciferase activity of the stimulated promoter compared to that of the unstimulated control. Experimental firefly luciferase activity was normalised to the internal *Renilla* luciferase activity of the co-transfected phRL-TK vector. Results are presented as means ± SD of three independent experiments performed in duplicate.

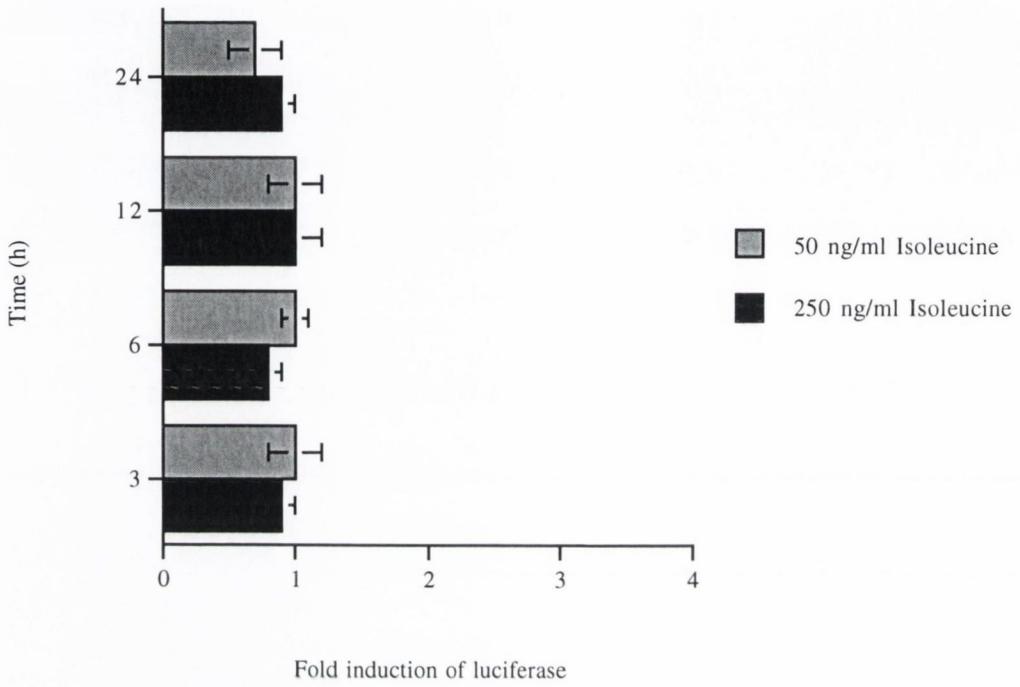


Figure 5.13 Promoter activity of the *HIS2* 4 kb promoter in HSG cells following incubation with isoleucine. pGL4.0 (1 μg) containing 4 kb of the 5′-upstream region of the *HIS2* gene was transiently transfected into HSG cells (1.5 x 10^5 cells/well). Isoleucine was added 24 h post-transfection to a final concentration of 50 ng/ml and 250 ng/ml, and the cells harvested after 3 h, 6 h, 12 h, and 24 h. Promoter activity is represented as fold induction of luciferase activity of the stimulated promoter compared to that of the unstimulated control. Experimental firefly luciferase activity was normalised to the internal *Renilla* luciferase activity of the co-transfected phRL-TK vector. Results are presented as means ± SD of three independent experiments performed in duplicate.

Table 5.2 Summary of the fold induction of *HIS2* 4 kb promoter activity after stimulation with microbial products, proinflammatory cytokines, PMA, and immune-enhancing proteins at the indicated time points

Stimulant	**3 h (± SD)**	**6 h (± SD)**	**12 h (± SD)**	**24 h (± SD)**
LPS (10 μg/ml)	2.5 (0.4)	2.3 (0.3)	2.4 (0.5)	0.9 (0.1)
PGN (10 μg/ml)	1.8 (0.5)	2.6 (0.4)	0.9 (0.2)	0.9 (0.1)
Zymosan (10 μg/ml)	1.7 (0.4)	1.8 (0.5)	1 (0.4)	0.9 (0.3)
C. albicans SC5314 (1×10^6 cells/ml)	2.2 (0.4)	2.3 (0.6)	2.7 (0.5)	0.8 (0.2)
IL-1β (100 ng/ml)	0.9 (0.3)	2 (0.5)	2.1 (0.3)	0.8 (0.3)
IFNγ (250 U/ml)	1.5 (0.2)	1.4 (0.3)	1.8 (0.3)	1 (0.3)
TNFα (100 ng/ml)	0.9 (0.3)	1.3 (0.4)	0.9 (0.2)	0.9 (0.2)
PMA (100 ng/ml)	1 (0.2)	1.5 (0.4)	2.8 (0.2)	2.2 (0.4)
L-arginine (250 ng/ml)	1 (0.1)	1 (0.2)	0.9 (0.1)	0.8 (0.1)
Isoleucine (250 ng/ml)	0.9 (0.1)	0.8 (0.1)	1 (0.2)	0.9 (0.1)

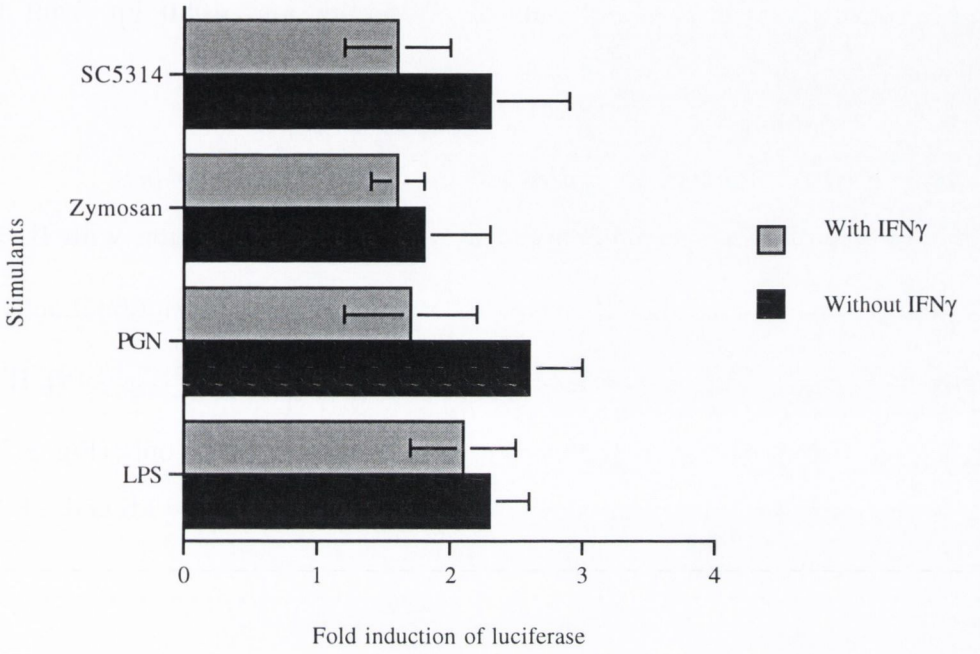


Figure 5.14 Promoter activity of the *HIS2* 4 kb promoter in HSG cells following incubation with LPS (10 μg/ml), PGN (10 μg/ml), zymosan (10 μg/ml), and *C. albicans* SC5314 (1 x 10^6 cells/ml) with or without IFNγ (250 U/ml). pGL4.0 (1 μg) containing 4 kb of the 5′-upstream region of the *HIS2* gene was transiently transfected into HSG cells (1.5 x 10^5 cells/well). The cells were incubated with stimulants 24 h post-transfection, and harvested after 6 h. Promoter activity is represented as fold induction of luciferase activity of the stimulated promoter compared to that of the unstimulated control. Experimental firefly luciferase activity was normalised to the internal *Renilla* luciferase activity of the co-transfected phRL-TK vector. Results are presented as means ± SD of three independent experiments performed in duplicate.

5.3.4 Deletional analysis of the *HIS2* 4 kb promoter

5.3.4.1 Stimulation of HIS2 promoter constructs with microbial products

Transcriptional activity of *HIS2* promoter constructs following incubation with LPS (Fig. 5.15), PGN (Fig. 5.16), zymosan (Fig. 5.17), and heat-killed *C. albicans* (Fig. 5.18) was investigated. Elevated transcriptional activity was generally observed in the regions 800 bp from the transcriptional start site, and between 3600 bp and 4000 bp from the transcriptional start site.

5.3.4.2 Stimulation of HIS2 promoter constructs with proinflammatory cytokines

Transcriptional activity of *HIS2* promoter constructs following incubation with IL-1β, IFNγ, and TNFα was investigated. IL-1β showed some increased transcriptional activity between regions 2.4 kb and 2.8 kb, and between regions 3.6 kb and 4.0 kb (Fig. 5.19). IFNγ showed increased transcriptional activity between region 3.6 kb and 4.0 kb only (Fig. 5.20). TNFα showed some increased transcriptional activity between regions 2.4 kb and 2.8 kb, and between regions 3.6 kb and 4.0 kb (Fig. 5.21).

5.3.4.3 Stimulation of HIS2 promoter constructs with phorbol myristate acetate

Transcriptional activity of the *HIS2* promoter following incubation with PMA was examined. Elevated transcriptional activity was observed between regions 1.2 kb and 1.6 kb, and remained elevated (Fig. 5.22).

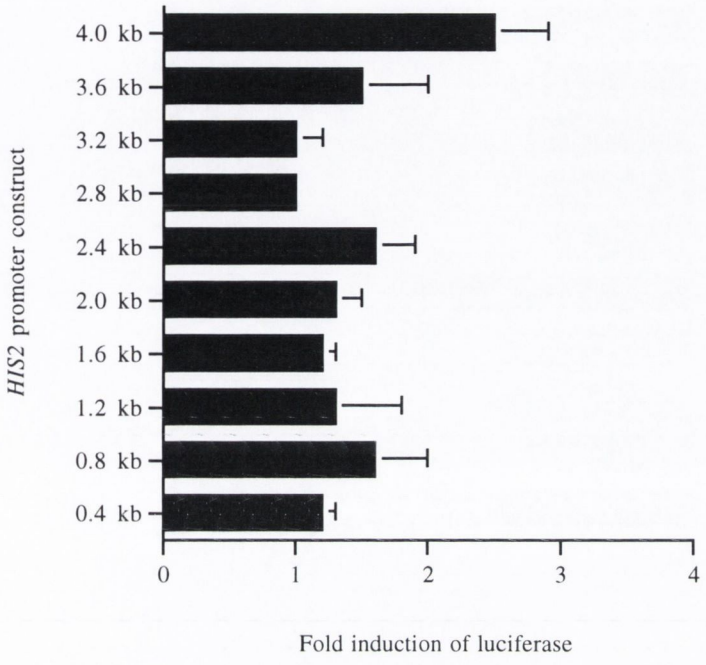


Figure 5.15 Deletional analysis of *HIS2* promoter activity in HSG cells following incubation with lipopolysaccharide (LPS). *HIS2* promoter constructs (1 µg each) were transiently transfected into HSG cells (1.5 x 10^5 cells/well). LPS (10 µg/ml) was added 24 h post-transfection, and the cells were harvested after 3 h. Promoter activity is represented as fold induction of luciferase activity of the stimulated promoter compared to that of the unstimulated control. Experimental firefly luciferase activity was normalised to the internal *Renilla* luciferase activity of the co-transfected phRL-TK vector. Results are presented as means ± SD of three independent experiments performed in duplicate.

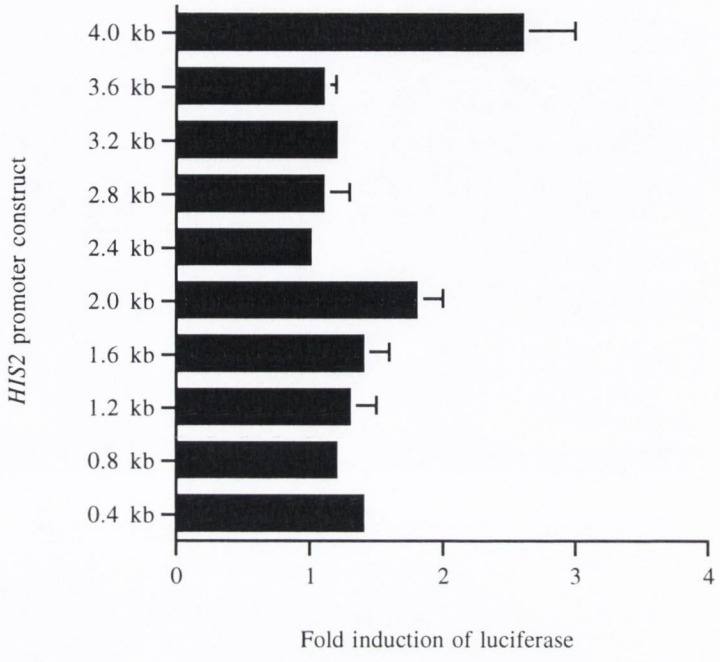


Figure 5.16 Deletional analysis of *HIS2* promoter activity in HSG cells following incubation with peptidoglycan (PGN). *HIS2* promoter constructs (1 μg each) were transiently transfected into HSG cells (1.5 x 10^5 cells/well). PGN (10 μg/ml) was added 24 h post-transfection, and the cells were harvested after 6 h. Promoter activity is represented as fold induction of luciferase activity of the stimulated promoter compared to that of the unstimulated control. Experimental firefly luciferase activity was normalised to the internal *Renilla* luciferase activity of the co-transfected phRL-TK vector. Results are presented as means ± SD of three independent experiments performed in duplicate.

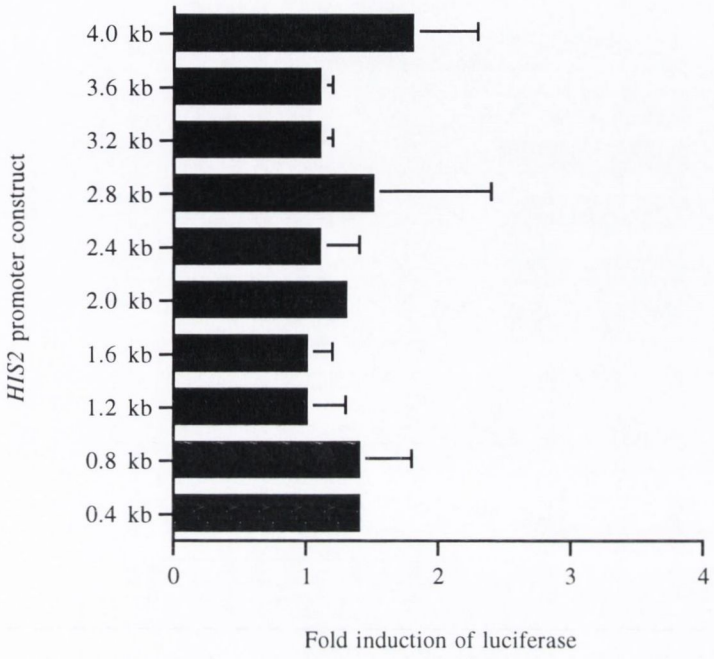


Figure 5.17 Deletional analysis of *HIS2* promoter activity in HSG cells following incubation with zymosan. *HIS2* promoter constructs (1 μg each) were transiently transfected into HSG cells (1.5 x 10^5 cells/well). Zymosan (10 μg/ml) was added 24 h post-transfection, and the cells were harvested after 6 h. Promoter activity is represented as fold induction of luciferase activity of the stimulated promoter compared to that of the unstimulated control. Experimental firefly luciferase activity was normalised to the internal *Renilla* luciferase activity of the co-transfected phRL-TK vector. Results are presented as means ± SD of three independent experiments performed in duplicate.

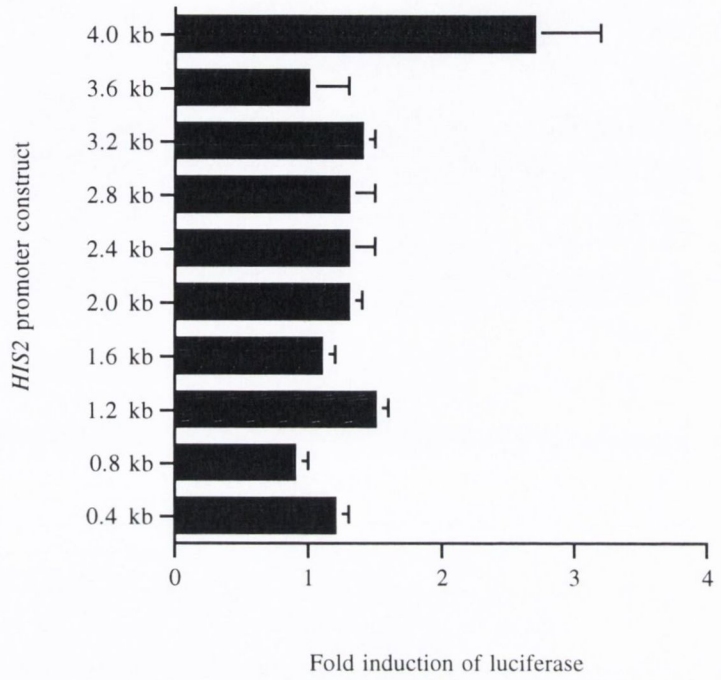


Figure 5.18 Deletional analysis of *HIS2* promoter activity in HSG cells following incubation with heat-killed *C. albicans* SC5314. *HIS2* promoter constructs (1 μg each) were transiently transfected into HSG cells (1.5 x 10^5 cells/well). Heat-killed *C. albicans* (1 x 10^6 cells/ml) was added 24 h post-transfection, and the cells were harvested after 12 h. Promoter activity is represented as fold induction of luciferase activity of the stimulated promoter compared to that of the unstimulated control. Experimental firefly luciferase activity was normalised to the internal *Renilla* luciferase activity of the co-transfected phRL-TK vector. Results are presented as means ± SD of three independent experiments performed in duplicate.

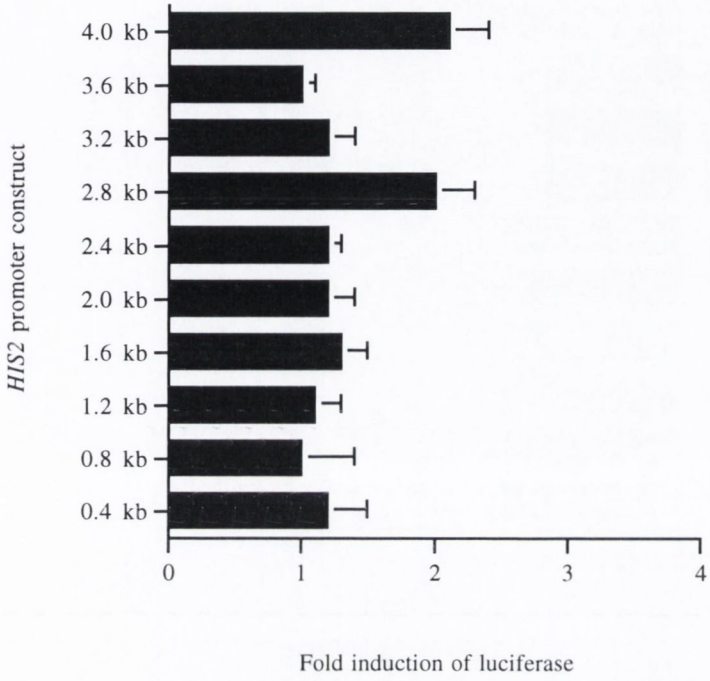


Figure 5.19 Deletional analysis of *HIS2* promoter activity in HSG cells following incubation with interleukin 1 beta (IL-1β). *HIS2* promoter constructs (1 μg each) were transiently transfected into HSG cells (1.5 x 10^5 cells/well). IL-1β (100 ng/ml) was added 24 h post-transfection, and the cells were harvested after 12 h. Promoter activity is represented as fold induction of luciferase activity of the stimulated promoter compared to that of the unstimulated control. Experimental firefly luciferase activity was normalised to the internal *Renilla* luciferase activity of the co-transfected phRL-TK vector. Results are presented as means ± SD of three independent experiments performed in duplicate.

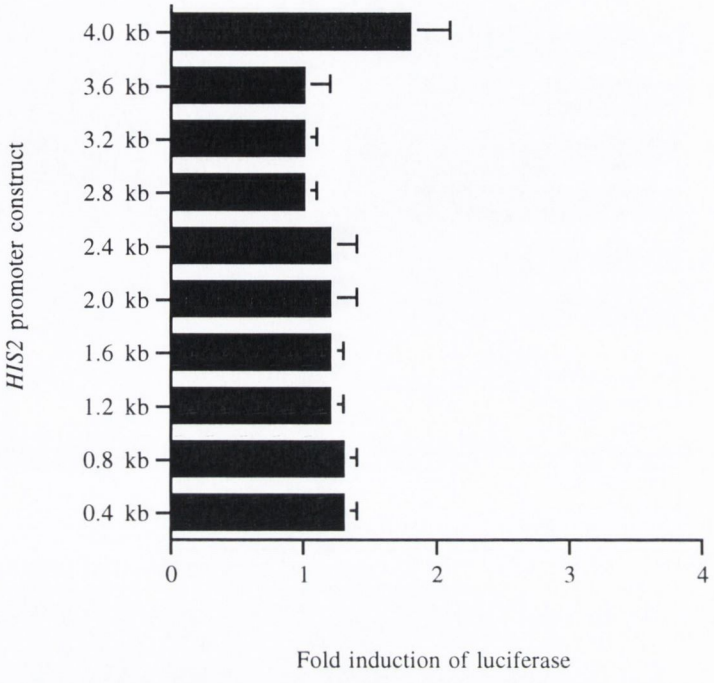


Figure 5.20 Deletional analysis of *HIS2* promoter activity in HSG cells following incubation with interferon gamma (IFNγ). *HIS2* promoter constructs (1 μg each) were transiently transfected into HSG cells (1.5 x 10^5 cells/well). IFNγ (250 U/ml) was added 24 h post-transfection, and the cells were harvested after 12 h. Promoter activity is represented as fold induction of luciferase activity of the stimulated promoter compared to that of the unstimulated control. Experimental firefly luciferase activity was normalised to the internal *Renilla* luciferase activity of the co-transfected phRL-TK vector. Resulats are presented as means ± SD of three independent experiments performed in duplicate.

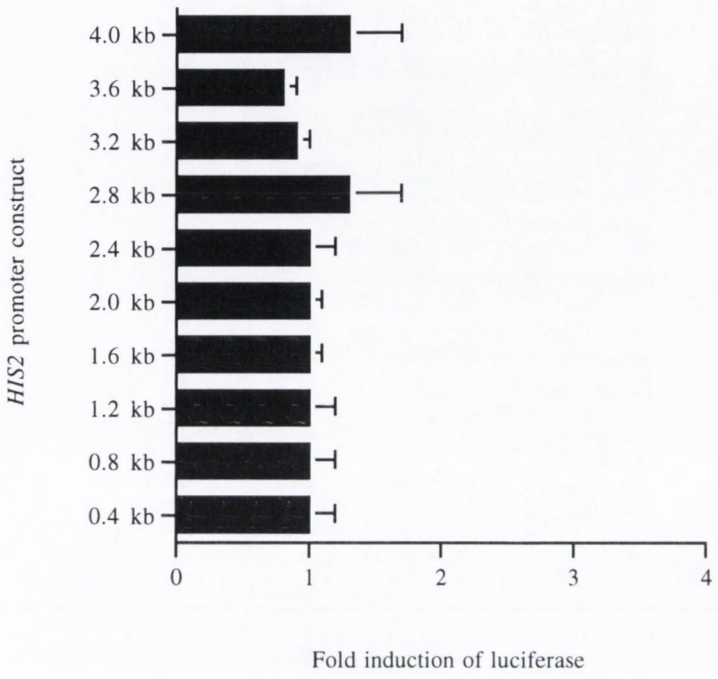


Figure 5.21 Deletional analysis of *HIS2* promoter activity in HSG cells following incubation with tumor necrosis factor alpha (TNFα). *HIS2* promoter constructs (1 μg each) were transiently transfected into HSG cells (1.5 x 10^5 cells/well). TNFα (100 ng/ml) was added 24 h post-transfection, and the cells were harvested after 6 h. Promoter activity is represented as fold induction of luciferase activity of the stimulated promoter compared to that of the unstimulated control. Experimental firefly luciferase activity was normalised to the internal *Renilla* luciferase activity of the co-transfected phRL-TK vector. Results are presented as means ± SD of three independent experiments performed in duplicate.

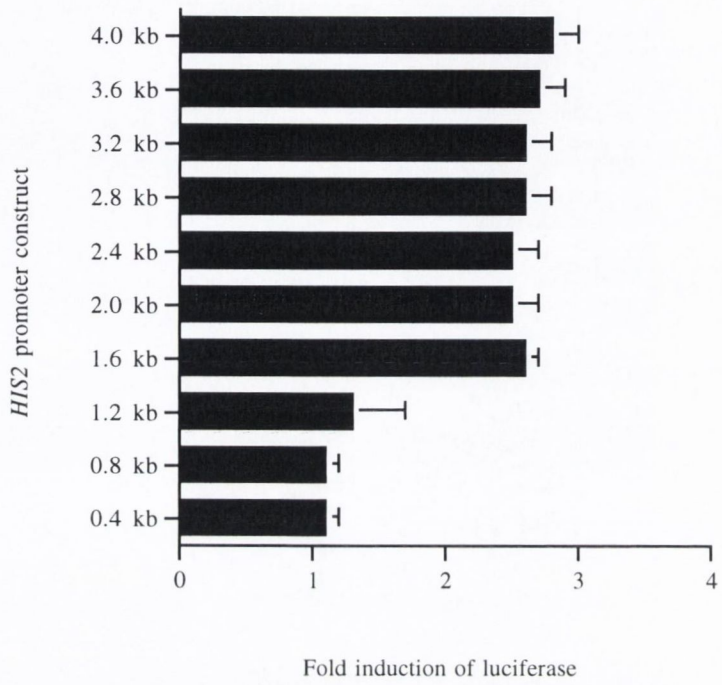


Figure 5.22 Deletional analysis of *HIS2* promoter activity in HSG cells following incubation with phorbol myristate acetate (PMA). *HIS2* promoter constructs (1 μg each) were transiently transfected into HSG cells (1.5 x 10^5 cells/well). PMA (100 ng/ml) was added 24 h post-transfection, and the cells were harvested after 12 h. Promoter activity is represented as fold induction of luciferase activity of the stimulated promoter compared to that of the unstimulated control. Experimental firefly luciferase activity was normalised to the internal *Renilla* luciferase activity of the co-transfected phRL-TK vector. Results are presented as means ± SD of three independent experiments performed in duplicate.

5.4 Discussion

Microbial products are known to increase the transcriptional expression levels of antimicrobial peptides such as β-defensins (Russell *et al.*, 1996; Mathews *et al.*, 1999; Harder *et al.*, 2000; Vora *et al.*, 2004; Pivarcsi *et al.*, 2005), mucins (Li *et al.*, 1997, 1998), and cathelicidin (Wu *et al.*, 2000) after direct contact with the mucosal epithelium both *in vitro* and *in vivo*. In order to investigate if salivary histatin expression is also subject to up-regulation at the transcriptional level after exposure to microbial products *in vitro*, 4 kb of the 5′-upstream region of the *HIS2* promoter was fused to a firefly luciferase reporter gene, transfected into a human salivary gland cell line, and *HIS2* promoter activity measured after incubation with LPS, PGN, zymosan, and heat-killed *C. albicans*. Using heat-killed *C. albicans* allowed stable culture conditions and yeast concentrations, and circumvented the disadvantage of extensive growth of live yeast while in culture. The *HIS2* promoter was found to be up-regulated after exposure to all the microbial products tested. This suggests that the 4 kb 5′-flanking region of the *HIS2* promoter contains inducible *cis*-elements which are responsive to Gram-negative and Gram-positive bacterial products such as LPS and PGN, and to fungal compounds such as zymosan and to *C. albicans*. Deletional analysis of the *HIS2* promoter was performed to localise these responsive elements. The results indicate that these elements are located between -1 and -800 bp, and between -3600 and -4000 bp, from the transcriptional start site. This suggests that the *HIS2* 5′-flanking region contains inducible promoter and enhancer elements. Computer analysis of the *HIS2* promoter sequence using MatInspector V2.2 software indicates the presence of several putative NF-κB and AP-1 binding sites (Fig. 5.23). These transcription factors have been implicated in the regulation of other antimicrobial proteins such as β-defensins (Fehlbaum *et al.*, 2000; Wada *et al.*, 2001; Tsutsumi-Ishii and Nagoka, 2003; Vora *et al.*, 2004; Wehkamp *et al.*, 2004; Pivarcsi *et al.*, 2005) and NGAL (Cowland *et al.*, 2003), and are considered important mediators of the innate immune system. Further work is required to identify exactly which *cis*-elements are involved in *HIS2* promoter regulation, including more detailed deletional analysis of the *HIS2* promoter and mutagenesis of putative *cis*-elements.


Elk-1
NF-κB
AP-1
4.0 kb
3.6 kb
3.2 kb
2.8 kb
2.4 kb
2.0 kb
1.6 kb
1.2 kb
0.8 kb
0.4 kb
luc

Figure 5.23 Schematic diagram of the positions of putative NF-κB, AP-1, and Elk1-1 transcription factor binding sites within 4 kb of the 5′-flanking region of the *HIS2* promoter sequence. The search was conducted using MatInspector V2.2 software. Cut-offs were employed which minimized false negative error rates. Green boxes represent putative NF-κB binding sites, red boxes represent putative AP-1 binding sites, and purple boxes represent putative Elk-1 binding sites. Positions of putative NF-κB and AP-1 binding sites from the transcriptional start site of the *HIS2* gene are as follows: four NF-κB sites at -67, -129, -283, -385, and two AP-1 sites at -268, -394, within 400 bp. One NF-κB site at -725, and five AP-1 sites at -475, -544, -594, -661, -754 within 800 bp. One AP-1 site at -1028, and two Elk-1 sites at -945, 1195 within 1200 bp. Three NF-κB sites at -1203, -1309, -1338, and two Elk-1 sites at -1424, 1473 within -1600 bp. Two NF-κB sites at -1613, 1640, and three AP-1 sites at -1657, -1770, -1926 within 2000 bp. Four NF-κB sites at -2010, 2181, 2355, -2395, and two AP-1 sites at -2083, -2132 within 2400 bp. Two NF-κB sites at -2561, -2644, and three AP-1 sites at -2501, 2635, 2675 within 2800 bp. Three NF-κB sites at -2822, 2845, 3182, two Ap-1 sites at -3108, 3196, and one Elk-1 site at -3024 within 3200 bp. Three NF-κB sites at -3207, -3255, 3293, and four AP-1 sites at -3217, -3267, -3312, -3434 within 3600 bp. Two NF-κB sites at -3899, -3935, one AP-1 site at -3987, and one Elk-1 site at -3651 within 4000 bp.

Up-regulation of the *HIS2* promoter occurred in a time-dependant manner. Generally, increased levels of promoter activity were observed within 3 h of stimulation, and remained elevated for up to 12 h with the higher concentrations of LPS and *C. albicans* tested. Importantly, this suggests that histatin levels may be up-regulated within hours of exposure to microbes in the oral environment. Only a slight increase in *HIS2* promoter activity was observed after concentrations were increased 100-fold from 100 ng/ml to 10 μg/ml for LPS, PGN, and zymosan, and from 1 x 10^4 cells/ml to 1 x 10^6 cells/ml for *C. albicans*. This indicates that the lower concentrations of microbial products used in this study are sufficient to induce the maximum levels of histatin expression possible under these *in vitro* conditions.

Various *in vitro* and *in vivo* models of oral candidiasis have identified an array of proinflammatory cytokines that are generated from the interaction of oral epithelial cells with *C. albicans*, including IL-1β, IFN-γ, and TNFα (Rouabhia *et al.*, 2002; Schaller *et al.*, 2002; Steele and Fidel, 2002; Dongari-Bagtzoglou and Kashleva, 2003; Dongari-Bagtzolou *et al.*, 2004; Lilly *et al.*, 2004; Villar *et al.*, 2005). Up-regulation of β-defensins (Harder *et al.*, 2000; Krisanaprakornkit *et al.*, 2000; Joly *et al.*, 2005;), mucins (Li *et al.*, 2003; Gray *et al.*, 2004), and NGAL (Cowland *et al.*, 2003) have been induced by exogenously-added cytokines *in vitro*. In this study, *HIS2* promoter activity was measured after incubation with IL-1β, IFNγ, and TNFα. The *HIS2* promoter was up-regulated by varying amounts by the proinflammatory cytokines tested. IL-1β and IFNγ were the strongest inducers of promoter activity, while TNFα increased promoter activity to a lesser extent. Deletional analysis of the *HIS2* 5′ promoter sequence localised responsive elements for IL-1β and TNFα between -2400 and -2800 bp, and between -3600 and -4000 bp from the transcriptional start site. This suggests the *HIS2* promoter contains enhancer elements inducible by IL-1β and TNFα. IL-1β and TNFα signal through the NF-κB and mitogen-activated protein kinase (MAPK) pathways depending on the stimuli (Krisanaprakornkit *et al.*, 2002; Chung and Dale, 2004). Computer analysis of the *HIS2* promoter sequence indicates the presence of several putative NF-κB binding sites and one Elk-1 binding site in these regions (Fig. 5.23).

Therefore, these pathways may be involved in transcriptional regulation of the *HIS2* promoter. Deletional analysis located responsive elements for IFNγ between -3600 and -4000 bp from the *HIS2* transcriptional start site. IFNγ signals through the JAK/STAT pathway, but computer analysis of the *HIS2* promoter did not indicate the presence of any STAT binding sites between the -3600 and -4000 bp sequence under the cut-off points used in this study (Fig. 5.23). Further work is required to identify specific *cis*-elements which may be involved in the regulation of the *HIS2* promoter in the presence of proinflammatory cytokines.

PMA is an epithelial cell activator and may regulate genes through protein kinase C and MAPK pathways (Jung *et al.*, 2000). PMA was found to up-regulate the *HIS2* promoter in this study. Deletional analysis localised the responsive elements between -1200 and -1600 bp from the transcriptional start site. Activation of most protein kinase C pathways are thought to ultimately lead to NF-κB activation. MAPK pathways involve activation of the Elk-1 transcription factor, among many others. Computer analysis of the *HIS2* promoter sequence indicates the presence of three putative NF-κB and two putative Elk-1 binding sites between -1200 and -1600 bp from the transcriptional start site (Fig. 5.23). This suggests that the *HIS2* gene may be regulated by several signaling pathways, including protein kinase C and MAPK pathways.

The beneficial activity of immune-enhancing ingredients, such as arginine and isoleucine, which are added to immune-enhancing formulas, has been investigated by several groups (Fehlbaum *et al.*, 2000; Sherman *et al.*, 2006). It is thought that these amino acids mediate their beneficial activity by bolstering the immune system. The effect of L-arginine and isoleucine on *HIS2* promoter regulation was investigated in this study. However, no up-regulation of the *HIS2* promoter was observed after incubation with these two amino acids.

Synergistic expression of antimicrobial peptides by various combinations of microbial products and cytokines, and by various combinations of cytokines, have been observed *in vitro* (Li *et al.*, 2003a; Joly *et al.*, 2005). In this study, synergistic transcriptional activity of the *HIS2* promoter was examined using combinations of LPS, PGN, zymosan, and *C. albicans* with the proinflammatory cytokine IFNγ. However, no

additional increase in *HIS2* promoter activity was observed when these microbial products were used in combination with IFNγ, compared to the promoter activity induced by the microbial products alone. Further investigation into possible synergistic regulation of the *HIS2* promoter would require the use of other combinations of microbial products and the vast array of proinflammatory cytokines found to be up-regulated in the oral mucosa of patients suffering from oral candidiasis (Dongari-Bagtzoglou and Fidel, 2005).

One of the mechanisms by which the mammalian innate immune system recognises the presence of microbes at the mucosal surface is through a family of receptors called Toll-like receptors (TLRs). TLRs recognise conserved motifs called pathogen-associated molecular patterns (PAMPs) which are present in microbial components, and allow the host to distinguish between self and foreign pathogens (Beutler and Rietschel, 2003). TLRs play important roles in innate immunity in mammals (Janeway and Medzhitov, 2002). To date, 10 members have been identified in the TLR family, each of which sense different microbial products. LPS is sensed through TLR4, PGN is sensed through TLR2, while zymosan and fungi such as *C. albicans* are sensed through TLR2 and TLR6 (Joualt *et al.*, 2003; Akira and Hemmi, 2003; Takeda and Akira, 2005). These receptors have a vital role in the induction of antimicrobial genes such as β-defensins. Expression of all 10 TLRs has been detected in salivary gland tissue (Nishimura and Naito, 2005). In the present study, *HIS2* promoter activity in a human salivary gland cell line was up-regulated after stimulation with LPS, PGN, zymosan, and *C. albicans*. This suggests that induction of the *HIS2* promoter by these microbial products may be mediated through TLR-associated signaling pathways in the *in vitro* system. However, the main limitation of *in vitro* single cell systems as models of antimicrobial peptide expression, and microbial infection, is that they lack many characteristics of native mucosae, such as polarised cell phenotype, differentiation level, and metabolic responses to external stimuli. Therefore, the responses of these cultures *in vitro* may not accurately reflect their responses *in vivo* (Dongari-Bagtzoglou and Fidel, 2005). HSG cells used in this study have a poorly-differentiated ductal cell phenotype when cultured on plastic. However, histatins are primarily produced and secreted by acinar cells *in vivo*. HSG cells can develop an acinar cell phenotype *in vitro* when cultured on a reconstituted basement membrane matrix called Matrigel (Zheng *et al.*, 1998). This acinar cell phenotype may more accurately reflect the responses of histatin-

producing acinar cells to microbial products and cytokines *in vivo*, and may be used in future studies investigating *HIS2* promoter regulation.

In conclusion, this study presents evidence for the first time, that the *HIS2* promoter is regulatable *in vitro*. It was found that the 4 kb 5′-flanking region of the *HIS2* promoter contains inducible promoter and enhancer elements after stimulation by microbial products, proinflammatory cytokines, and PMA *in vitro*. Deletional analysis has located the responsive promoter regions which include several putative NF-κB, AP-1, and Elk-1 binding sites. These results suggest histatins may be regulated by multiple signaling pathways, including NF-κB, protein kinase C, and MAPK pathways. HIV targets several parts of the NF-κB pathway, which may explain why histatins appear to be down-regulated in HIV-infected individuals. This suggests that histatins may be key constituents of the oral innate immune system, and may play an important role in protecting the oral cavity from pathogen invasion and from potential pathogen invasion among the commensal flora. Further investigation is required to elucidate the exact molecular mechanisms responsible for regulation of the *HIS2* gene both *in vitro* and *in vivo*.

Chapter 6
General Discussion

6.1 General Discussion

6.1.1 Introduction

Oral candidiasis is an opportunistic fungal infection caused primarily by *C. albicans*, a dimorphic organism that is part of the normal microflora of the mucosal surfaces of the oral cavity, gastrointestinal and reproductive tracts (Samaranayake, 1992). Fungal infection of the oral and gastrointestinal mucosa is a potentially life-threatening clinical problem. Candidal infections are particularly common in immunocompromised patients, such as those with AIDS and those treated with immunosuppressant drugs. Other predisposing factors to the development of oral candidiasis include the use of broad-spectrum antibiotics, inhaled steroids, radiation therapy for head/neck cancers, denture prostheses, diabetes mellitus, and reduced salivary flow (Akpan and Morgan, 2002). Antifungal agents currently available for the treatment of candidal infections consist of the polyenes, the imidazoles, the triazoles, and the enchinocandins. Many drug-resistant strains are emerging in response to widespread and prolonged use of these antifungal agents, making the clinical management of oral candidiasis increasingly difficult (Situ and Bobek, 2000). Amphotericin B-resistance has been observed in *C. albicans* isolates, fluconazole-resistance has been observed in *C. albicans*, *C. glabrata*, and *C. dubliniensis* isolates (Moran *et al.*, 1997; Helmerhorst *et al.*, 1999; Situ and Bobek, 2000), while resistance to a more recent antifungal drug, caspofungin, has already been observed in *C. albicans*, *C. glabrata*, and *C. parapsilosis* isolates (Hernandez *et al.*, 2004; Moudgel *et al.*, 2005; Krogh-Madsen *et al.*, 2006). The importance of the search for new treatment alternatives for mucosal candidiasis cannot be overemphasised.

Salivary histatins are believed to play a key role in protecting the oral cavity from opportunistic fungal infections. The most significant function of histatins may be their potent antifungal activity against *C. albicans* and other *Candida* spp. (Oppenheim *et al.*, 1988). Histatins and their variants display potent antimicrobial activity at physiological concentrations against other medically important fungi and bacteria, such as *C. neoformans*, *P. aeruginosa*, *A. fumigatus*, *S. aureus*, *S. mutans*, and *P. gingivalis* (Tsai and Bobek, 1997a; Helmerhorst *et al.*, 1997; van't Hof *et al.*, 2000; Cirioni *et al.*, 2004; Giacometti *et al.*, 2005). Histatins demonstrate anticandidal activity against both azole-susceptible and azole-resistant *Candida* spp., and their mechanism of action involves

pathways distinct from those involved in the mechanisms of action of azole-based antimycotics (O'Connell *et al.*, 1996; Fitzgerald *et al.*, 2003). Histatins are also non-toxic to mammalian cells and do not induce resistance (Tsai and Bobek, 1998). Therefore, they represent a promising therapeutic alternative to current antifungal agents in the treatment of oral candidiasis.

6.1.2 Transgenic mice expressing salivary histatin

An extensive amount of work has been performed on assessing the *in vitro* antifungal activity of histatins against *C. albicans* (Tsai and Bobek, 1998; Calderone and Fonzi, 2001). However, there is no direct evidence which proves that histatins are essential to oral health. One of the main objectives of this study was to generate transgenic mice which constitutively express histatin in their salivary glands. Only humans and Old World primates secrete histatin in their saliva, therefore mice provide an ideal model in which to investigate the effects of histatin on oral yeast carriage rates *in vivo*, and to investigate whether the expression of salivary histatin will prevent or attenuate the development of oral candidiasis. These histatin-postive transgenic mice represent a way forward to a deeper understanding of the role histatins play in the maintenance of a healthy oral environment.

The generation of transgenic mice expressing histatin in their saliva requires a promoter which will direct transgene expression to salivary glands. The cytomegalovirus is trophic for salivary glands, and has been used in other studies for the expression of foreign genes in rat salivary gland cells both *in vitro* and *in vivo* (Hoque *et al.*, 2000; Zheng *et al.*, 2001, 2005). A recombinant adenovirus containing the CMV promoter has previously been used to direct expression of histatin 3 to rat salivary glands (O'Connell *et al.*, 1996). In this study, the CMV promoter was used to drive expression of histatin 3 and EGFP in a human submandibular gland cell line. The isolation and mesurement of histatins in complex mixtures of proteins has represented major technical obstacles, as reflected in the large variations in mean histatin concentrations in published reports, and the fact that there is no one standard, reliable method used by different research groups for the isolation, purification, and quantitation of histatins (Gusman *et al.*, 2004). In this study, histatin 3 cDNA was labeled with an internal polyhistidine tag to simplify purification and detection of the histatin protein both in HSG cells and in the transgenic mice. The candidacidal assay

showed that histatin antifungal activity against *C. albicans* was unaffected by the tag. Low levels of the protein were detected in HSG supernatants and cell lysates using an antibody specific for the polyhistidine epitope. Other studies involving measurement of histatin may find this tag useful, since the tag does not interfere with antifungal activity, and allows the use of more versatile methods of purification and quantitation of histatin in complex mixtures.

This project has developed a system in which the *in vivo* role of salivary histatin may be better understood. Transgenic mice were generated that express the histatin peptide in their salivary glands. Histatin mRNA expression was found in all the tissue-types examined, with the highest levels detected in the submandibular/sublingual glands, liver, and lung. These histatin-positive transgenic mice will be an invaluable tool in experiments seeking to investigate the efficacy of histatin under various conditions. These transgenic mice may be used to study the effect of different salivary histatin concentrations on oral carriage rates of *Candida*, and to investigate whether the presence of histatin in saliva will prevent or attenuate the development of oral candidiasis. The combined antifungal effects of candidacidal activity and inhibition of germination by histatin may be advantageous in mucosal disease, where germ tube penetration into squamous epithelium is a significant factor. Anticandidal activity must be evaluated both microbiologically and histopathologically. Histatins show potent anticandidal activity against both azole-susceptible and azole-resistant *Candida* isolates *in vitro* (O'Connell *et al.*, 1996; Fitzgerald *et al.*, 2003). The emergence of azole-resistant *Candida* strains are the main cause for antifungal treatment failures. These mice may be used to determine the *in vivo* efficacy of histatin against these azole-resistant strains. Synergistic activity of histatin and histatin analogues with other antimicrobial agents against bacteria and fungi have been shown *in vitro* (van't Hof*et al.*, 2000; Wei and Bobek, 2004; Giacometti *et al.*, 2005). Combination antimicrobial therapy may be used to prevent or delay the development of drug resistance, and reduce the concentration of one or more of the antimicrobial agents, which in turn may reduce the incidence and severity of associated side-effects. These mice may be used to assess the *in vivo* synergistic effects of histatin 3 with different combinations of antimicrobial agents against various pathogenic bacteria and fungi. Phenotypic changes and markers of resistance in the inoculating organisms must be monitored. Changes in the oral

microflora of the mice should also be monitored. These transgenic mice express histatin in many tissue-types including the submandibular/sublingual glands, brain, heart, kidney, liver, lung, skeletal muscle, and small intestine. This may be a useful system to investigate the efficacy of histatin in treating systemic candidiasis. These mice may also be employed to evaluate the potency of histatin against other medically important oral bacteria and fungi, and in models of other systemic diseases, such as *P. aeruginosa* or *S. aureus* infection.

Future experiments investigating the role of histatin in controlling the oral population of *Candida* would involve establishing oral carriage of yeast in these mice. Animals would be restrained and their mouths swabbed daily for several days with cotton applications soaked in a fresh culture of the test organism. In order to facilitate *Candida* measurements and saliva collection, the mice would be anaesthetised with an intramuscular injection of xylazine and ketamine. An intraperitoneal injection of pilocarpine would be given to stimulate salivary flow. At the end of the saliva collection period, an intraperitoneal injection of atropine would be given to reverse the effects of pilocarpine. Salivary histatin levels would be measured using ELISA, Western blot, CE, or cationic gel electrophoresis. Measurements of *Candida* populations would be made by plating serial dilutions of oral swabs on PDA medium. Saliva collection and *Candida* measurements would be performed every second day over a two week period. Mice would then be mildly immunosuppressed by the addition of hydrocortisone to the drinking water, in order to encourage the development of oral candidiasis. Saliva collection and *Candida* measurements would be performed again over a two week period as before. At the end of the experiments, mice would be then be euthanised by an overdose of halothane, and histological sections made of the oral mucosa to investigate the extent of invasion by *Candida* spp. Histological sections would also be made of the salivary glands and other organs to determine the tissue-specificity and levels of histatin expression achieved. In experiments using these mice in a mouse model of systemic candidiasis, establishment of systemic candidal infection would involve injections of candidal suspensions. Quantitative assessment of *Candida* in the fecal pellets would be made throughout these experiments. Histological sections would also be performed on all mouse tissues to investigate the extent of tissue invasion by *Candida.*

The level of candidal infection in the oral and gastrointestinal tracts of these mice may be limited by the population pressure exerted by the commensal bacteria, which may inhibit adherence of the yeast by competing for sites and nutrients. These mice must be kept in microisolator cages to prevent contamination from microbes in the environment. Expression of the histatin transgene in these mice may be influenced by a number of factors, including insertion site of the transgene and transgene copy number, which will produce mice with varying levels of histatin. Experiments using mice expressing low levels of histatin in their saliva would require larger sample sizes, which would be more costly and time-consuming. The most likely adverse effect to occur in these experiments relates to the use as pilocarpine as a secretatogue for saliva collection. In excessive doses, pilocarpine can lead to fluid collection in the airways and asphyxiation. This can be prevented by limiting the dose of pilocarpine to <5 mg/kg, and the administration of atropine should symptoms develop. Breeding transgenic mice for use in experiments is very time-consuming. It may take four generations before a stable transgenic line is established. Each generation requires careful screening and characterisation. Transgenic mice (F2 generation) expressing histatin were generated in this project but time constraints did not allow for experiments investigating oral candidal infection to be undertaken in the present study. However, these histatin-positive transgenic mice will be an invaluable tool in future studies seeking to investigate the efficacy of histatin under various conditions.

Current research efforts are focused on the design of histatin analogues with improved potency and stablity (Zuo *et al.*, 1995; Driscoll *et al.*, 1996; Tsai *et al.*, 1996; Tsai and Bobek, 1997b; Rothstein *et al.*, 2001). Histatins and their analogues may be used to treat azole-resistant candidal infections in immunocompromised patients, or as components of artificial saliva in patients with reduced salivary flow (Tsai and Bobek, 1998). Research efforts are also focused on the efficient and safe transfer of the histatin gene to salivary glands using gene transfer technology (O'Connell *et al.*, 1996, 2003; Baum and O'Connell, 1999). The use of salivary glands as short-term, slow-release devices of histatin is more preferable than intermittent topical delivery. It is desirable to have continuous delivery of histatin to the oral cavity because of its short duration of action and rapid destruction by proteolysis (O'Connell *et al.*, 1996). Findings from histatin-positive

transgenic mice experiments should promote these efforts into the development of histatin antifungal therapy as a promising alternative to the current management of oral candidiasis.

6.1.3 Transcriptional regulation of the *HIS2* promoter

Disease states are often associated with increased or reduced expression of critical gene products. The ability to modulate gene expression can provide the basis for antimicrobial therapies. Identifying promoter regions of genes and determining how they interact with transcription factors to control gene expression is an important area of study. Several small clinical studies performed to date in humans suggest a dynamic relationship between salivary histatin levels and factors such as oral yeast carriage rates (Jainkittivong *et al.*, 1998), the immune status of the patient (Atkinson *et al.*, 1990; Lal *et al.*, 1992), oral candidiasis (Mandel *et al.*, 1992), recurrent oral candidiasis (Bercier *et al.*, 1999), patient age (Johnson *et al.*, 2000), or certain medications, such as β_1-adrenolytic agents (Jensen *et al.*, 1994). The main objective of this part of the thesis was to investigate the extent to which salivary histatins are regulated *in vitro*, and to identify some of the external stimuli, *cis*-element(s), and signaling pathway(s) which may be involved.

This study presents the novel finding that the *HIS2* promoter is regulatable *in vitro*. It was found that the 4 kb 5′-flanking region of the *HIS2* promoter contains inducible promoter and enhancer elements after stimulation by microbial products, proinflammatory cytokines, and PMA *in vitro*. Deletional analysis has located the responsive promoter regions which include several putative NF-κB, AP-1, and Elk-1 binding sites. These results suggest histatins may be regulated by multiple signaling pathways, including NF-κB, protein kinase C, and MAPK pathways. Multiple signaling pathways have been implicated in the regulation of β-defensin 2 (Krisanaprakornkit *et al.*, 2000). The HSG cells used in this study have a poorly-differentiated ductal cell phenotype when cultured on plastic. *In vitro* single cell systems lack many characteristics of native mucosae, such as polarised cell phenotype, differentiation level, and metabolic responses to external stimuli. Therefore, the responses of these cell systems *in vitro* may not accurately reflect their responses *in vivo* (Dongari-Bagtzoglou and Fidel, 2005). Future studies investigating *HIS2* promoter regulation may use differentiated HSG cells cultured on a reconstituted basement

membrane matrix called Matrigel. This matrix encourages differentiation of the HSG cells to an acinar phentype, which may be more biologically relevant. Co-culture of *Candida*-infected oral epthelial cells, such as the TR146 oral epithelial cell line, with HSG cells may enable immunological "cross-talk" between the two cell types in future experiments, and is a step closer to the *in vivo* model than HSG cells alone. The regulation of the *HIS2* promoter must also be determined *in vivo.* Transgenic mice expressing a luciferase reporter gene downstream of the *HIS2* promoter may be used to investigate histatin regulation during health, during oral candidiasis, and after resolution of infection.

Histatins are produced by salivary glands adjacent to the oral epithelium, and it is unlikely that yeast and bacteria come into direct contact with the acinar cells of salivary glands *in vivo*. During oral infection with *Candida*, a large number of proinflammatory cytokines and immunoregulatory cytokines are generated in the oral mucosa. In oral mucosal infections *in vivo*, innate immunity is represented by inflammatory cells such as antigen presenting cells (APCs) (Dongari-Bagtzoglou and Fidel, 2005). APCs include B cells, activated macrophages, dendritic cells, and activated T cells. These cells can migrate from the oral mucosa to the salivary glands. APCs produce proinflammatory cytokines including IL-1β, IFNγ, and TNFα in response to infection with *Candida* spp. These proinflammatory cytokines may be responsible for the up-regulation of antimicrobial proteins such as histatins in the salivary glands, which protect the oral mucosa from invading pathogens. A study evaluating T cell populations in oral lesions from HIV-infected patients with oropharyngeal candidiasis found that the HIV-infected individuals had low levels of CD4+ T cells, and elevated levels of CD8+ T cells, at the lamina propria epithelium interface of the infected sites, compared with uninfected controls (Myers *et al.*, 2003). Inflammatory cells regulate antimicrobial peptides by the production of cytokines which signal through NF-κB pathways. It is now recognised that pathogens have targeted several parts of these pathways, allowing them to interfere with immune response genes. The ability of HIV to interfere with NF-κB signaling is associated with an inhibition of the immune response (Tato and Hunter, 2002). HIV has developed a sophisticated strategy to enlist the nuclear machinery of CD4+ T cells to promote viral replication, and contributes to the loss of T cell-mediated immunity. This decrease in CD4+ T cells during HIV-

infection may explain why histatin levels are decreased in the saliva of HIV-infected patients, and why these individuals are more susceptible to opportunistic mucosal infections such as oral candidiasis.

Identification of stimulants which up-regulate the *HIS2* promoter may lead to the design of drugs which mimic this effect, and may increase a patient's endogenous levels of salivary histatin. Mice containing the *HIS2* promoter fused to a reporter gene, such as firefly luciferase, must be generated in order to provide a better understanding of histatin regulation *in vivo*. One strategy to control fungal infection in severely immunocompromised hosts is to improve the immune effector functions of cells involved in the clearance of microbes by the use of cytokine-based therapeutics as adjuncts to traditional antifungal drugs (Dongari-Bagtzoglou and Fidel, 2005). However, cytokine-based therapeutics would not be suitable for the general treatment of oral candidiasis. The development of optimally designed recombinant adenoviral vectors for the efficient and safe delivery of therapeutic agents to specific tissues is an exponentially-growing field of research (Baum *et al.*, 2000). Higher cell- or tissue-specific promoter activity could result in decreased doses of vector administered, with consequently lower toxicity or side-effects from the vector, restrict unwanted transgene expression in non-targeted cell types, and facilitate persistent transgene expression (Zheng and Baum, 2005). The promoter deletion constructs designed in this study may be used in differentiated HSG cells or in transgenic mice to identify salivary gland-specific elements in the *HIS2* promoter which may be responsible for cell- or tissue-specific expression in the salivary glands. The pGL0.4 construct contains salivary gland element I, the pGL0.8 construct contains salivary gland elements I and II, while the pGL1.2 construct contains salivary gland elements I, II, and III (see Table 1.4 in chapter 1 for position of elements). Results from these experiments may contribute to the design of recombinant adenoviral vectors with specific promoter activity for the salivary glands.

6.1.4 Concluding remarks

The histatin-positive transgenic mice generated in this project represent an invaluable tool for studying the efficacy of salivary histatin against *Candida* in the oral cavity. These mice will enable investigation into whether histatin expression in saliva reduces the density of

oral yeast carriage, and whether histatin prevents or attenuates the development of oral candidaisis. These histatin-positive transgenic mice may be useful for assessing the therapeutic potential of histatins in treating azole-resistant oral and systemic candidiasis, the synergistic activity of histatin with other antifungal agents, and the efficacy of histatin against other medically important bacteria and fungi. This study also presents evidence that the *HIS2* promoter is regulatable *in vitro*. The results suggest histatins may be up-regulated by microbial products, proinflammatory cytokines, and PMA through multiple signaling pathways. Further investigation is required to elucidate the exact molecular mechanisms responsible for regulation of the *HIS2* gene. The findings of this study support continued efforts towards the development of histatin as an alternative therapeutic agent for the treatment of mucosal candidiasis.

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