DEVELOPMENT OF A VACCINE AGAINST AIDS BASED ON SIV TAT

by

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of the requirements for
the degree of Doctor of Philosophy

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DECLARATION

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1. ABSTRACT

Tat protein (Trans Activator of Transcription) is essential for efficient lentiviral transcription and completion of a lytic infection. During lentiviral infection, Tat is found both inside cells and in the extracellular medium, and extracellular Tat is responsible for some disease symptoms and toxicity during lentiviral infection. The mode of action of Tat makes it a good target for immunisation that would generate both humoral and cellular immune responses, while specific interdiction of the extracellular protein could alleviate some deleterious phenomena attributed to Tat. In the course of this study, the immunogenic potential of recombinant Tat protein from SIVmac32H(J5) was evaluated in BalbC mice and in Rhesus macaque monkeys.

Recombinant Tat was initially expressed in very low amounts in *E. coli*, but optimisation of the Tat coding sequence for translation in the bacterial host significantly improved protein expression. Aiming to purify Tat on the basis of the affinity tag Pk (engineered on the recombinant Tat protein), a method for Pk-affinity purification was successfully developed and is available for general use.

When evaluated in animal immunisation studies, Tat protein was shown to be immunogenic in the absence of a carrier or adjuvant. Furthermore, Tat generated immune responses to poorly immunogenic proteins physically linked to it, with evidence for T_H1-type responses in mice. There was even a suggestion that Tat was modifying the established function of the adjuvant alum towards the development of antigen-specific T_H1-type responses.

Tat showed potential as an antigen in a lentiviral vaccine that would induce antibody and T_H1 responses. Furthermore, Tat being able to generate T_H1-type responses to co-administered antigens would be a very useful antigen carrier to generate immunity against pathogens that are cleared by T_H1-based immunity.

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ABBREVIATIONS EMPLOYED IN THIS REPORT

Ab(s)	antibody(ies)
Ag	antigen
amp	ampicillin
AP	Adenovirus Protease
APC	antigen-presenting cell
APS	ammonium persulphate
ATP	adenosine 5' triphosphate
CIAP	calf intestine alkaline phosphatase
CTL	cytotoxic T lymphocyte
cDNA	complementary DNA
DAPI	4,6-diamino-2-phenylindole
DMSO	dimethyl sulfoxide
DNA	deoxy ribonucleic acid
dNTP	2'-deoxynucleotide 5'-tris phosphate
E. coli	*Escherichia coli*
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot assay
FITC	fluorescein isothiocyanate
GST	glutathione s-transferase from *Scistostoma*
HIV	Human Immunodeficiency virus
His	histidine (the amino acid) or the 6xHis affinity tag
HLA	human lymphocyte antigen (the human MHC locus)
HRP	horse-radish peroxidase
IFN	interferon
Ig#	immunoglobulin class #
IPTG	isopropyl-β-D-thiogalactopyranoside
LB agar/broth	Luria-Bertani agar/broth
LTB	B subunit of the heat labile enterotoxin of *E.coli*
LTR	long terminal repeat
mAb(s)	monoclonal antibody (ies)
MHC	major histocompatibility complex
mRNA	messenger RNA
MW	molecular weight

NaCl	sodium chloride
NiNTA	Nitriloacetic acid
$OD_{\#}$	optical density at #nm
oligo(s)	oligonucleotide(s)
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pH	pondus Hydrogen ($-\log_{10}[H^+]$) (measure of acidity)
Pk tag	affinity tag originating from SV5
Protein A	Protein A component of *Staphylococcus aureus*
RO H_2O	water purified by reverse osmosis
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulphate
SIV	Simean Immunodeficiency virus
SSC	salt-sodium citrate buffer
SV5	Simian Virus type 5
Tat	trans-activator of lentiviral transcription
TBE	tris-boron-EDTA
T_C	T Cytotoxic cell
TCR	T-cell receptor
TE	Tris-EDTA buffer
TEMED	N, N, N',N'-tetramethylethylenediamine
tet	tetracyclin
T_H	T Helper cell
$T_H\#$	T Helper cell, subset #
TLNI	targeted lymph node immunisation
TMB	3,3',5,5'-tetramethylbenzidine
TNF	tumour necrosis factor
Tris	tris-hydroxymethyl amino methane
Triton	t-octyphenyloxpolyethoxyethanol
tRNA	transfer RNA
Tween 20	polyoxyethylene sorbitan monolaurate
UV	ultraviolet light
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
3-D	three-dimensional (structure)

LIST OF PHYSICAL UNITS AND TABLES OF UNIT PREFIXES

Units

Symbol	Name	To measure:
°C	degrees Celsius	temperature
g	gram	mass
m	metre	length
l	litre	volume
mol	mole	quantity
M	molar concentration	moles / ltr
sec	second	time
min	minute	time
h	hour	time
D	Dalton	relative molecular mass
Ci	Curie	radioactivity
V	Volt	electrical potential
A	Ampere	electric current
b	base pairs	DNA fragment length
u	units	concentration / activity
rpm	revolutions per minute	centrifugal speed
g	gravitational acceleration	centrifugal speed

Order Prefixes

c	centi	10^{-2}
m	milli	10^{-3}
μ	micro	10^{-6}
n	nano	10^{-9}
p	pico	10^{-12}

k	kilo	10^{3}
M	mega	10^{6}
G	giga	10^{9}
T	tera	10^{12}

AMINO ACIDS AND THE GENETIC CODE

Amino acids

Alanine	Ala	*or*	ala	A
Arginine	Arg	*or*	arg	R
Aspargine	Asn	*or*	asn	N
Aspartate	Asp	*or*	asp	D
Cysteine	Cys	*or*	cys	C
Glutamine	Gln	*or*	gln	Q
Glutamate	Glu	*or*	glu	E
Glycine	Gly	*or*	gly	G
Histidine	His	*or*	his	H
Isoleucine	Ile	*or*	ile	I
Leucine	Leu	*or*	leu	L
Lysine	Lys	*or*	lys	K
Methionine	Met	*or*	met	M
Phenylalanine	Phe	*or*	phe	F
Proline	Pro	*or*	pro	P
Serine	Ser	*or*	ser	S
Theonine	Thr	*or*	thr	T
Tryptophan	Trp	*or*	trp	W
Tyrosine	Tyr	*or*	tyr	Y
Valine	Val	*or*	val	V

Genetic code

TTT	phe	TCT	ser	TAT	tyr	TGT	cys
TTC	phe	TCC	ser	TAC	tyr	TGC	cys
TTA	leu	TCA	ser	TAA	stop	TGA	stop
TTG	leu	TCG	ser	TAG	stop	TGG	trp
CTT	leu	CCT	pro	CAT	his	CGT	arg
CTC	leu	CCC	pro	CAC	his	CGC	arg
CTA	leu	CCA	pro	CAA	gln	CGA	arg
CTG	leu	CCG	pro	CAG	gln	CGG	arg
ATT	ile	ACT	thr	AAT	asn	AGT	ser
ATC	ile	ACC	thr	AAC	asn	AGC	ser
ATA	ile	ACA	thr	AAA	lys	AGA	arg
ATG	met	ACG	thr	AAG	lys	AGG	arg
GTT	val	GCT	ala	GAT	asp	GGT	gly
GTC	val	GCC	ala	GAC	asp	GGC	gly
GTA	val	GCA	ala	GAA	glu	GGA	gly
GTG	val	GCG	ala	GAG	glu	GGG	gly

2. INTRODUCTION

AIDS has been known for over 20 years and today it is the most important infectious disease, being the most common cause of death in Africa and the forth most common worldwide (Esparza & Bhamarapravati, 2000). Current anti-retroviral drugs are too expensive for the developing countries and there are major problems of adherence, resistance and toxicity (Peters, 2002). A safe, highly effective and affordable preventive vaccine offers the best long-term hope to control the pandemic, especially in less-developed countries. However, despite intense international efforts and extensive funding of vaccine research, a vaccine for HIV is not at hand. The scientific research applied towards an HIV vaccine is reviewed in part 2.3, but before that, the traits of the immune system that are important in vaccination are discussed in part 2.1, followed by a brief presentation of vaccination strategies employed to prevent viral infection, in part 2.2.

2.1 AN OVERVIEW OF THE IMMUNE FUNCTIONS WITH PARTICULAR REFERENCE TO ISSUES RELEVANT TO VACCINATION

The collective system of defences in vertebrates contains non-specific and specific components. While the non-specific responses (innate immunity) delay the entry and spread of a pathogen, the more complex, specific immune responses (adaptive immunity) have time to develop. Specific immune responses are directed against particular and defined biochemical structures termed antigens. Every encounter is recorded and can be recalled upon re-emergence of the same antigen.

2.1.1 Specific immune responses

The specific immune responses are divided into the humoral (Figure 1) and the cell-mediated responses (Figure 2). The main cells of the immune system are the lymphocytes B and T and the antigen presenting cells. Each type of response employs cells and components of both the innate and adaptive immunity. B cells are primarily involved in the humoral immune response. They recognise their specific antigen (Ag) through their surface receptors called antibodies (Abs) or immunoglobulin (Ig). T cells participate in both the humoral and the cellular immune responses. They recognise their specific Ag via the T cell receptor (TCR) that requires that the Ag be presented on a specific type of proteins, the products of the major histocompatibility complex (MHC) (Zinkernagel & Doherty, 1974).

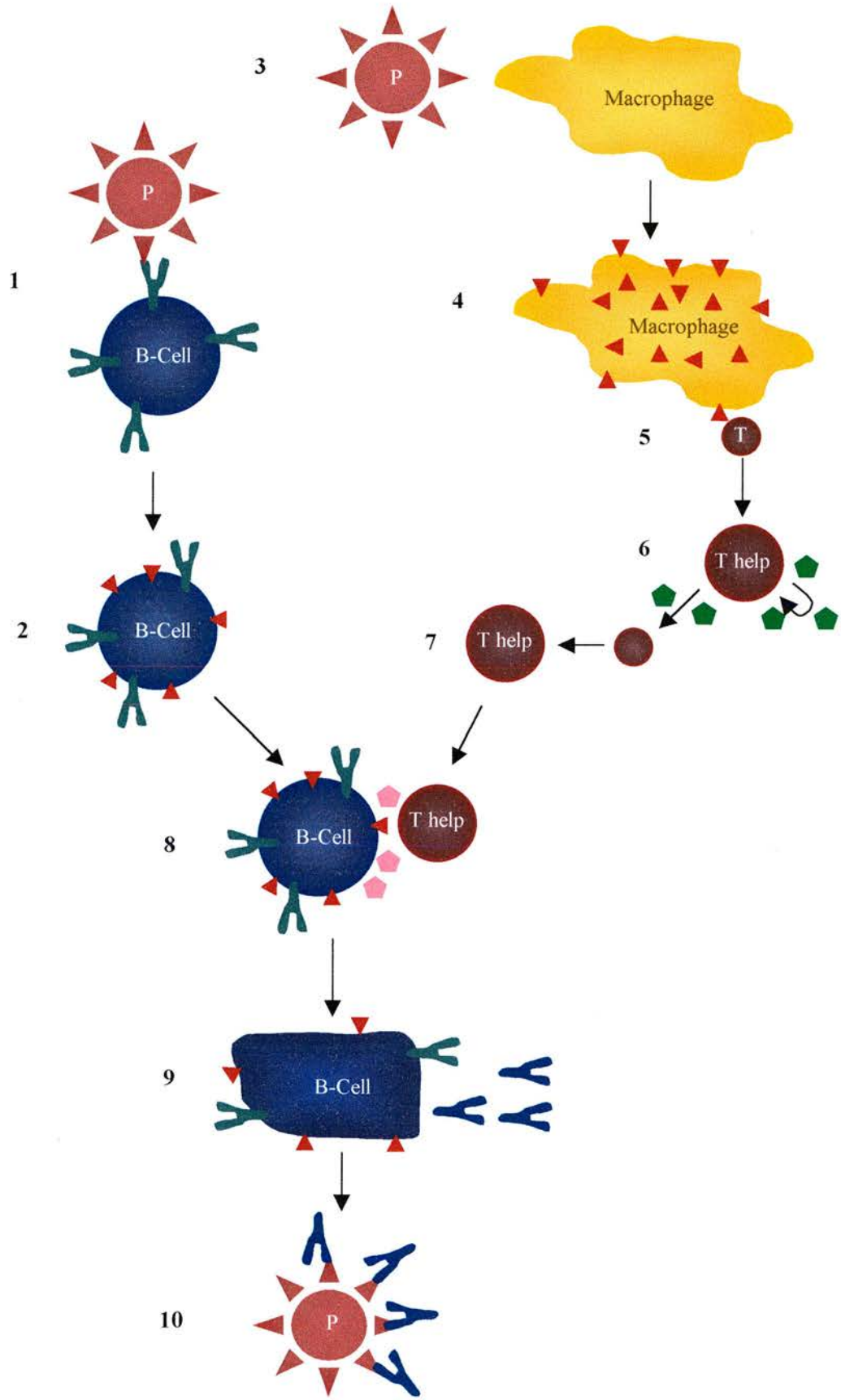
MHC proteins come into two major classes designated I and II. MHC I proteins are present on the surface of practically all nucleated cells, while MHC II molecules are only found on the cell membrane of macrophages, B lymphocytes and dendritic cells, which are collectively referred to as antigen-presenting cells (APCs). The TCR with accessory molecule CD8 (usually on T_C cells) recognises Ag in the context of MHC I proteins, while Ag presented by MHC II molecules is bound by the TCR on CD4+ T cells (see Figure 3). In order for a T cell to become activated, the interaction between its TCR and the MHC-Ag complex has to be of a minimum duration (single encounter model; Kupfer & Singer, 1989) or of a minimum frequency (serial encounter model; Friedl & Gunzer, 2001). T cell activation initiates the cascade of specific immune responses (Figures 1 and 2).

Figure 1: Schematic representation of the phases of a humoral immune response.

A pathogen circulating in the body fluids, depicted in red and denoted 'P', will eventually meet with a B cell whose antigen specificity matches the pathogen's surface antigens. The interaction between the antigen and the antibody bound on the B cell (step 1) induces ingestion of the pathogen by the B cell, resulting in the expression of the foreign antigens on its surface MHC II molecules (step 2).

Pathogenic moieties will also be ingested by APC cells such as macrophages (step 3) allowing the presentation of those antigens on the MHC II molecules of the APC (step 4). A T_H cell with an appropriate specificity will then interact via its TCR with the antigen-MHC II complex (step 5) and will get activated (step 6). As a result, the T_H cell proliferates and secretes cytokines that act upon other naïve T_H cells and activate them (step 7).

Once an activated T_H cell detects its specific antigen displayed on the surface of a B cell, (step 8), the interaction between TCR and antigen- MHC II molecule generates a signal that activates the B cell. The activated B cell then proliferates and creates a clone of cells producing antibody of the same specificity (step 9). That antibody can coat the pathogen (step 10) and precipitate it out of solution or opsonise it for ingestion by phagocytic cells.


3
P
Macrophage
P
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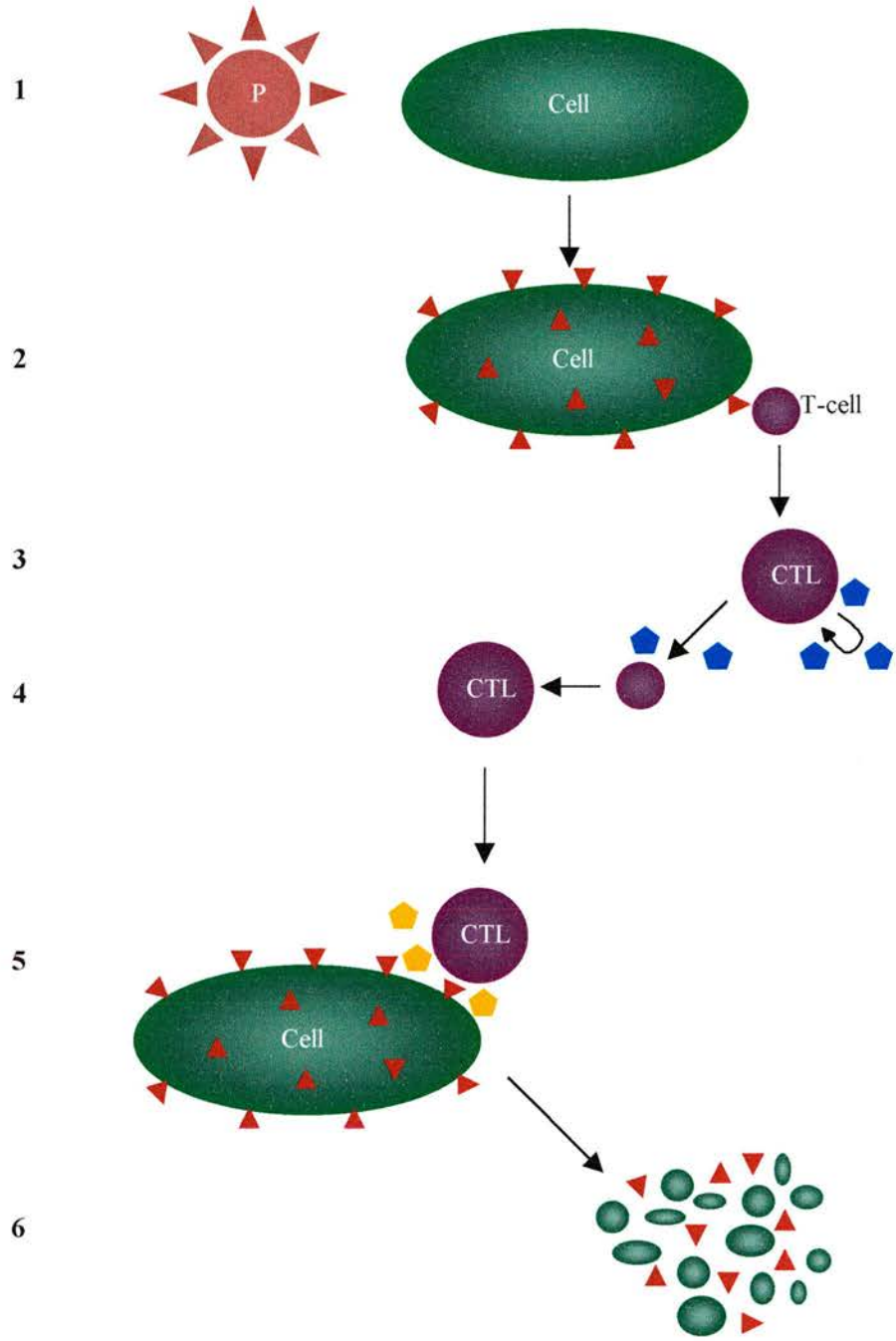


Figure 2: Illustration of the sequence of events comprising the cellular immune response.

Starting from the top: in step 1 the pathogen (P) invades a host cell to replicate within. Consequently, parts of the pathogen's newly synthesised proteins are displayed by MHC class I molecules on the surface of that cell, as shown in step 2.

A T_C cell with a suitable TCR specificity interacts with the antigen-MHC I complex displayed and gets activated, part 3. The activated T cell then secretes cytokines that act on itself and on naïve T_C cells nearby, inducing their activation and proliferation (step 4).

Such an activated T_C cell (CTL) can interact with an infected cell displaying the antigenic determinants of the pathogen, seen in part 5, and through the action of cytokines, trigger the destruction of that cell, shown in part 6.

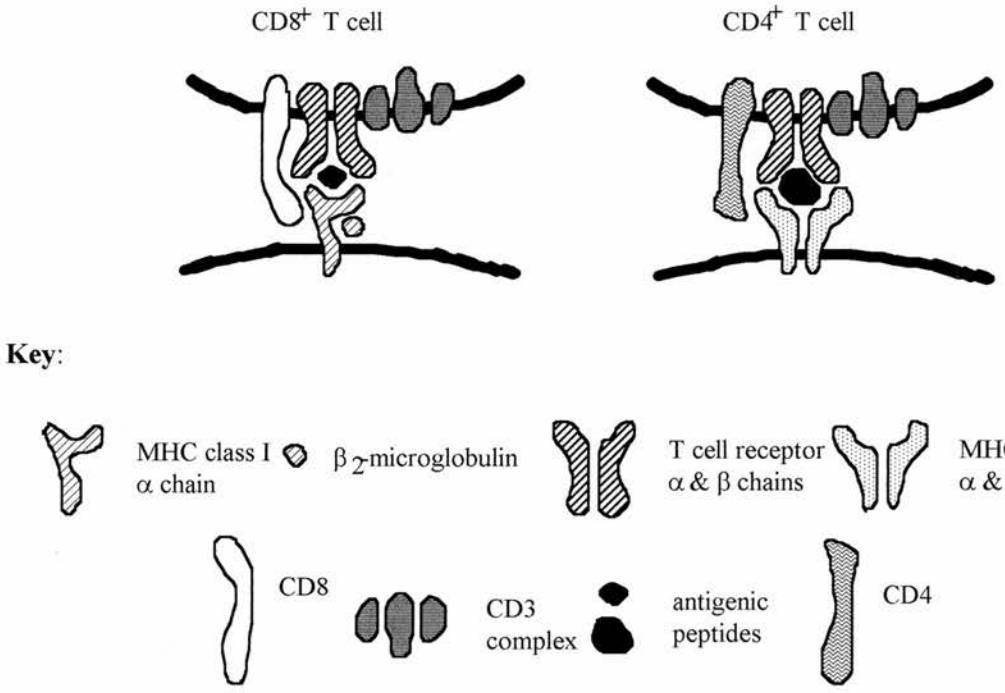


Figure 3: Molecular interactions between the T cell and MHC-presented antigen.

CD8+ T cells recognise their specific antigen in the context of MHC I proteins (seen above, in the left side of the panel). T cells that express the CD4+ glycoprotein interact with Ag presented by MHC II dimers (see right side of panel).

Both types of T cells interact with their specific Ag through their T cell receptor (TCR) that bind both Ag and MHC. The TCR is closely associated with the CD3 complex and either a CD8 or CD4 molecule.

The CD8 and CD4 molecules are co-receptors that recognise the peptide-MHC complex. CD4 co-receptors can increase the avidity of T cell binding to a peptide-MHC molecule. All three CD molecules are involved in signal transduction across the plasma membrane following TCR engagement.

Diagram adapted from Randall & Hanke, 1994, *Reviews in Medical Virology*, vol. 4, pp. 47-61.

2.1.2 Primary and secondary immune responses

The first time that an immunogenic Ag is encountered, specific immune responses are initiated that are collectively called the primary immune response. In the course of an immune response, lymphocyte clonal expansion gives rise to a large number of cells to eliminate the Ag currently present (effector cells) and a small proportion of lymphocytes destined for the future protection of the host against the same Ag (memory cells). If the same Ag were encountered again, memory lymphocytes would initiate a more rapid, more potent and more efficient immune response referred to as the secondary immune response. The secondary response comprises the same events and phases of the primary immune response, but with accelerated kinetics (Ahmed & Sprent, 1999).

The persistence of memory cells protects the host in a variety of ways. Firstly, memory lymphocytes specific for a given Ag are present in higher numbers than their respective non-stimulated counterparts. While memory lymphocytes are alert to the re-emergence of their specific Ag, pre-existing Ab provides the host with the first line of defence upon a second encounter (Ahmed & Sprent, 1999). Memory cells generate a quicker and more efficient immune response, due to their lower activation requirements, their increased efficiency once activated, and their acquired morphological and physiological characteristics that make them better suited to detect and interact with re-emerging Ag. For example, while naïve T cells are restricted to the lymph nodes, blood and spleen, thus relying on dendritic cells for their initial interaction with Ag (Moser & Murphy, 2001), memory T cells can enter tissues more readily and scrutinise vulnerable sites (Goldrath & Bevan, 1999). Between the first and second

encounter, the Ab produced by memory B cells has time to develop a higher affinity for the re-occurring Ag (through affinity maturation) and to change its class (through class switching) to isotypes that support quick elimination of the Ag (IgG isotypes in the secondary response, as opposed to mainly IgM during the primary immune response).

The development of a secondary immune response, results in more rapid and highly efficient elimination of Ag compared to the first time it was encountered. The property of the immune system to remember and recall every encounter with Ag is termed immunological memory and it forms the basis of protective vaccination.

2.1.3 Vaccination: aims and requirements

Spontaneous immune responses triggered by a random encounter with an Ag can be substituted by controlled responses to that Ag induced by vaccination. The basis of protective vaccination lies with the ability of the Ags presented to the immune system to generate a primary immune response that ultimately creates immunological memory. Vaccination involves a lower risk than natural encounter with the Ag, because the Ags employed are chosen so that they will not harm the host. Whilst immunity to every distinct Ag involves a separate natural encounter, appropriately designed vaccine formulations can induce protection against a range of distinct Ags.

The goal of vaccination is to induce long-lived memory cells (Fearon *et al.*, 2001) that will be best suited to protect the host against the particular pathogen. To achieve efficient protection, a number of considerations have to be

made when designing the vaccine formulation. The type of Ag to be employed, the processing pathway the Ag will likely enter for MHC presentation, which type of immune responses to target, are some important decisions. Furthermore, it may be beneficial for the Ag to be presented with some kind of adjuvant, or in conjunction with signals that induce a particular type of T_H responses. Lastly, for a vaccine to achieve broad protection, constraints imposed by MHC restriction have to be considered. These issues are addressed in the following sections.

2.1.4 Antigen processing and presentation

Ag presented in conjunction with MHC proteins is the outcome of a process that first converts protein structures to peptide fragments suitable for TCR recognition and subsequently loads them onto empty MHC molecules. The pathways of Ag-processing and presentation are diagrammatically illustrated in Figure 4. Two distinct pathways exist, namely the cytosolic that results in Ag presentation by MHC class I proteins and the endocytic that presents Ag associated with MHC class II molecules.

2.1.4.1 The cytosolic pathway

Proteins processed through the cytosolic pathway are mainly synthesised within the cell. This includes normal cellular (host) proteins and viral products expressed during viral infection (Cresswell & Lanzavecchia, 2001), as well as abnormal proteins that accompany oncogenic transformation (Yewdell *et al.*, 1999). Cytosolic proteins marked for degradation by ubiquitin get proteolytically cleaved

by the proteasome (Rock & Goldberg, 1999), a ubiquitous, abundant and rather complicated protease that serves the function of destroying damaged or unwanted proteins in all eukaryotic cells (Yewdell *et al.*, 1999).

The proteasome processes proteins to small peptides, which are reduced by exopeptidases to single amino acids (Cox & Coulter, 1997). However, a small proportion of peptides is rescued by binding TAP proteins (Transporter of Antigen Processing) and getting transported into the endoplasmic reticulum (ER) (Cox & Coulter, 1997). In the ER, the protein tapasin loads the peptides on newly synthesised MHC I molecules and the MHC I-Ag complexes are transported through the Golgi apparatus to the cell surface (Neefjes & Momburg, 1993).

In the presence of IFN-γ, the three catalytic subunits of vertebrate proteasomes are replaced by their homologous subunits to form so-called immunoproteasomes (Tanaka & Kasahara, 1998). While the majority of class-I-restricted epitopes can be presented by cells carrying standard proteasomes (Van den Eynde & Morel, 2001), the immunoproteasome has an increased ability to produce peptides with a proper motif for efficient MHC-I binding (Akiyama *et al.*, 1994). Finally, destabilisation of proteins and other factors can result in the generation of peptides suitable for MHC-I presentation, without processing through a proteasome (Yewdell *et al.*, 1999).

Cellular proteins are thus sampled on MHC I molecules for CD8+ T cell screening. CTLs recognise non-self Ags and kill cells that appear abnormal, or infected. Foreign ingested material processed through the cytosolic pathway would result in mistaken recognition by CTLs and killing of normal cells, but this is avoided, as most cells do not process extracellular Ags for presentation by MHC I molecules.

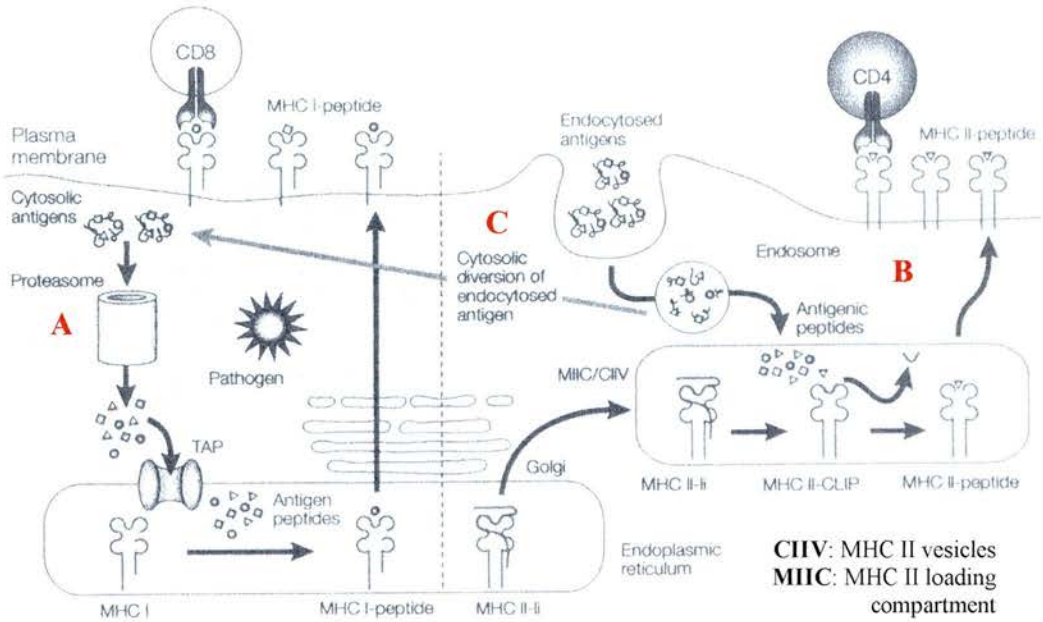


Figure 4: Pathways of Antigen Processing and MHC Presentation.

A. The cytosolic pathway of antigen presentation: Cytosolic Ags are processed through the proteasome and peptides are transported by TAP into the ER where they are loaded onto newly synthesised MHC I molecules. The complexes are transported to the cell surface where they can interact with CD8+ T cells.

B. Extracellular Ags are internalised and targeted to the vesicles of the endocytic pathway where they are reduced to peptides. MHC class II molecules synthesised in the ER contain the invariant chain (Ii) that transports them though the Golgi to the endocytic vesicles, where Ii gets replaced by CLIP. Within the vesicles, CLIP is removed and a peptide is loaded onto each MHC II dimer. MHC II-peptide complexes are transported to the cell membrane where they can be recognised by CD4+ T cells.

C. In dendritic cells, extracellular Ags can escape the endocytic pathway and get localised in the cell cytoplasm. The exact mechanism is unclear, but this escape from the endocytic vesicles allows processing of non-cytosolic Ags through the cytosolic pathway, and presentation on MHC class I molecules by DCs.

Diagram reproduced from Heath & Carbone, 2001, *Nature Reviews*, vol. 1, pp. 126-134.

2.1.4.2 The endocytic pathway

Extracellular Ags are normally processed through the endocytic pathway (Figure 4, pathway B). The endocytic pathway consists of a series of lysosomes and endosomes. APCs capture foreign material from the extracellular matrix, they ingest it (through phagocytosis, or by receptor-mediated translocation) and direct it to the endocytic vesicles (Cox & Coulter, 1997). Within the vesicles, the acidic environment and the presence of proteases reduce proteins into peptides suitable for presentation by MHC class II proteins.

MHC II proteins are also synthesised in the ER, but they do not interact with cytosolic Ags. In newly synthesised MHC II molecules, the Ag-binding groove is occupied by the chaperone protein invariant chain (Ii). Ii stabilises the MHC II molecules and prevents premature loading of peptides (Mellman *et al.*, 1998). It also targets the MHC II molecules from the Golgi to the endocytic vesicles (Pieters *et al.*, 1993; Odorizzi *et al.*, 1994) where Ii is digested and replaced by CLIP (class II -associated invariant chain peptide) (Cresswell, 1996). CLIP is removed within the endocytic compartment, by a class II-like protein that loads antigenic peptides onto the MHC (Mellman *et al.*, 1998). Newly formed MHC II-Ag complexes leave the lysosome and get transported to the cell membrane. The cytosolic pathway thus results in MHC II presentation of extracellular Ag to CD4+ T cells.

2.1.4.3 Cross-presentation of antigens

In their classical form, the cytosolic and endocytic pathways direct endogenous

Ag for MHC I presentation on practically all cells, and exogenous Ag for MHC class II presentation on APCs. However, endogenous Ag can be specifically targeted to the endocytic compartment and therefore presented on MHC II complexes. For example, signals associated with resident lysosomal membrane proteins such as LAMP-1 (lysosomal associated membrane protein) can target endogenously synthesised Ags to the endosomal system (Koch *et al.*, 2000).

More importantly, some APCs are able to process ingested material (extracellular Ag) for presentation on MHC I molecules (Ploegh, 1998; Buseyne *et al.*, 2001). This pathway is often referred to as 'cross-presentation' (pathway C in Figure 4). It allows MHC I presentation of exogenous Ag by CD8+ dendritic cells (Heath & Carbone, 2001) and it could be playing an important role in the generation of CTL immune responses. The exact mechanisms for the cross-presentation pathway remain unclear, but some suggestions have been proposed.

Extracellular Ag that enters the cell by phagocytosis or receptor-mediated endocytosis gets targeted to the endocytic compartment. MHC class II molecules contain a 'coated pit' localisation signal (Pinet *et al.*, 1995; Zhong *et al.*, 1997) and it has been suggested that they can bind extracellular Ags and translocate them across the cell membrane (Gromme *et al.*, 1999) through the formation of coated pits and a vesicle termed the receptosome that shuttles MHC-Ag complexes to the cell interior (Pastan & Willingham, 1981). This translocation would allow extracellular Ag to escape the endocytic pathway and get localised in the cytosol where it could be processed for MHC-I presentation.

Dendritic cells (DCs) express equal amounts of proteasomes and immunoproteasomes when not activated, but only immunoproteasomes when activated (Macagno *et al.*, 1999). MHC I presentation of extracellular Ag is

effective in mature but not in immature DCs (Inaba *et al.*, 2000) and the immunoproteasome is more competent than the proteasome in producing class-I-binding peptides that correspond to the immunodominant epitopes of infectious organisms, but it does not process efficiently a number of epitopes derived from self-proteins (Van den Eynde & Morel, 2001). These observations could suggest a possible involvement of immunoproteasomes in the cross-presentation pathway.

2.1.5 MHC polymorphism

The MHC proteins employed in Ag-presentation to T cells are encoded by a cluster of genes, termed H-2 in mice, or HLA in man. These genes are highly polymorphic (they have many alternative alleles) so each individual, in an outbred population, displays an almost unique combination of MHC proteins. The most polymorphic residues in the MHC molecules protrude into the groove, thus altering the spectrum of peptides that can be bound (Bjorkman *et al.*, 1987). The MHC molecules expressed by an individual essentially determine which parts of a protein can act as T-cell antigens (Hanke & Randall, 1994).

MHC polymorphism has evolved to maximise the likelihood that, for a given pathogen, at least some peptides can be presented by the MHC alleles found in the population (Ploegh, 1998). However, as each peptide can usually bind only one MHC molecule, only a few peptides in any protein are suitable for MHC-presentation by each individual, and these peptides may be different between individuals within an outbred population. For vaccination purposes, to ensure that a reasonable proportion of individuals would respond to a vaccine, the formulation should contain a repertoire of peptides that can bind to the five or ten commonest

MHC alleles in the population (Bangham & Phillips, 1997). This could be delivered as a cocktail of epitopes (Hanke & Randall, 1994) or as multiple minimal epitopes forming a 'polytope' (Leitner *et al.*, 2000).

To achieve optimal function of a vaccine formulation, many epitopes can be made more immunogenic by a process termed epitope enhancement (Berzofsky *et al.*, 2001) and they can be tailored to the MHC makeup of the target population. Antigenic proteins can be maximally truncated, leaving only defined epitopes for B or T cells (Leitner *et al.*, 2000), but bearing in mind that the induction of T_H responses is required both for B cell-based memory (Ahmed & Gray, 1996) and for the generation of effective CTL responses (Kalams & Walker, 1998).

2.1.6 T_H1 and T_H2 responses

T cells are classified as T helper (T_H) or T cytotoxic (T_C) for displaying membrane glycoproteins CD4 or CD8 respectively. Upon activation, T_C cells give rise to Cytotoxic T Lymphocytes (CTLs), while activated T_H cells start secreting growth factors collectively known as cytokines. All activated T_H cells secrete IL-3 and GM-GSF (Mosmann & Coffman, 1987; Bottomly, 1988) and IL-10 (Sornasse *et al.*, 1996), but there exist differences in the pattern of cytokine secretion between different types of activated T_H cells. The two main types are the T_H1 and T_H2 cells (Cherwinski *et al.*, 1987; Romagnani, 1991): the T_H1 clones produce IL-2, IFN-γ and TNF-β, whilst T_H2 clones synthesise IL-4, IL-5, IL-6 and IL-13. Not all cells generated by T_H activation produce cytokines characteristic of either subset (Lanzavecchia & Sallusto, 2000). Such cells could either represent uncommitted

progenitors, or transition intermediates in a pathway leading from one subset to the other (Firestein *et al.*, 1989).

2.1.6.1 Polarisation of the T_H response

The differentiation of a T_H cell into the T_H1 or T_H2 phenotype is determined by the presence of particular cytokines and signals in its environment. IL-12 and IL-4 play a major part (IL-4 promoting the T_H2 phenotype, while IL-12 driving the development into T_H1; Morris *et al.*, 1994; McKnight *et al.*, 1994) but the final outcome of T-cell polarisation is also influenced by other cytokines (Sallusto *et al.*, 1998). Polarisation to T_H happens in a progressive and stochastic manner (Lanzavecchia & Sallusto, 2000), requiring repeated stimulation of the TCR, each new encounter with Ag restarting cytokine synthesis in the T_H cell (Reiner, 2001). The panel of cytokines released by each sub-population of T_H cells promotes the growth and activation of the same subset (Lichtman *et al.*, 1987) while hindering the action of the other subset (Mosmann & Moore, 1991; Fitch *et al.*, 1993). Once a T_H response begins to develop along one pathway, either T_H1 or T_H2, it tends to become progressively polarised to that direction (Abbas *et al.*, 1996).

2.1.6.2 Immune responses augmented by each type of T_H response

Differences in the cytokine secretion patterns among T_H-cell subsets determine the type of immune response made to a particular antigenic challenge (Sallusto *et al.*, 1998). T_H1 clones induce Ab class switching to IgG classes that support phagocytosis and complement fixation (through the action of IFN-γ) and they

promote cell-mediated effector responses such as delayed type hypersensitivity (DTH) and the activation of CTLs (Snapper & Paul, 1987).

On the other hand, T_H2 clones can augment humoral responses (Clerici & Shearer, 1993). IL-5 stimulates eosinophil activation and differentiation, while IL-4 triggers the production of IgE. The coordinated action of IgE and eosinophils helps clear helminthic infections. More importantly, T_H2 clones provide help to B cells (Abbas *et al.*, 1996). Through the action of IL-4 and IL-5 they promote activation and development of B cells (Singh & O' Hagan, 1999) thus inducing proliferation, affinity maturation and Ab class switching to IgE (see above) and IgG isotypes that do not support complement activation (Abbas *et al.*, 1996).

The type of immune responses generated during a T_H response make the T_H1 subset particularly suited to respond to viral infections and intracellular pathogens (Pulendran *et al.*, 2001), while the T_H2 subset is better in eliminating extracellular pathogens (Lanzavecchia & Sallusto, 2000).

2.1.6.3 Factors influencing the T_H polarisation

There is growing evidence that the information needed to polarise the T_H response is carried by dendritic cells (Lanzavecchia & Sallusto, 2000). DCs have the unique capacity to present exogenous Ags on MHC class I molecules (see Figure 4C) and they are probably the most relevant source of IL-12, (Lanzavecchia & Sallusto, 2000). However, IL-12 production in DCs is controlled by a variety of factors including the nature of the maturation stimulus. For example, LPS, some bacteria, and viruses induce IL-12 (thus augmenting T_H1-type responses) while TNF-α, IL-1 and cholera toxin do not (Lanzavecchia & Sallusto, 2000).

A variety of cytokines and immunostimulatory molecules have been shown to tilt the balance towards a particular T_H response. For example, IL-4 and IL-10 favour the development of T_H2-like responses (Mosmann & Sad, 1996; Abbas *et al.*, 1996), whereas IL-12 enhances T_H1-like responses (Trinchieri, 1993; Trinchieri, 1995), and almost always increases CTL responses (Berzofsky *et al.*, 2001). Cytokines such as IL-2, IL-7 or IL-12 can even convert a T_H2-type response to a T_H1 one (Leitner *et al.*, 2000). Including T_H-polarising signals in a vaccine formulation can drive the immune responses towards the T_H pathway that will generate efficient protection against a particular antigen.

2.1.7 Employing adjuvants

Adjuvants have traditionally been defined as substances used in combination with a specific antigen to elicit more potent immunity compared to that induced by the Ag alone (Nabel, 2001). They can be used to improve the immune response to vaccine Ags in many different ways (Singh & O' Hagan, 1999). The mechanisms behind the action of adjuvants are not fully understood, but suggestions include: specific targeting of antigen to APCs or to the MHC class I or II pathways, slow release of Ag and stabilisation of epitopes (Nabel, 2001), better Ag presentation for Ab responses, and preferential induction of either type of T_H responses (Cox & Coulter, 1997).

These effects may be assisted by increased production of co-stimulatory signals, cytokines, or MHC molecules (Singh & O' Hagan, 1999) or by increased cellular infiltration, inflammation and trafficking to the injection site, which is in fact the case for DC precursors (Lanzavecchia & Sallusto, 2000). Adjuvants differ

significantly in their ability to prime DCs for IL-12 production (Sprent & Tough, 2001), but those that induce IL-12 production directly prime the generation of T_H1 responses (Lanzavecchia & Sallusto, 2000). The most notable polarisations of the immune responses are produced by aluminium salts (T_H2) and bacterial endotoxins (T_H1) (Cox & Coulter, 1997). These two different types of adjuvants are briefly reviewed below:

2.1.7.1 Aluminium salts (Alum)

Despite considerable research over many years, aluminium-based mineral salts (generically called 'alum') remain the only adjuvants approved for use with vaccines by the US Food & Drug Administration (Rockville, MD) (O' Hagan, 1997). Aluminium compounds precipitate any Ag mixed with them, through electrostatic interactions (Levine *et al.*, 1955) thus increasing the size of the Ag and consequently the likelihood of ingestion by APCs (Buseyne *et al.*, 2001). Additionally, in the presence of alum, the Ag is released much slower than if it were injected on its own (Cox & Coulter, 1997), thus generating more sustained responses which can create immunological memory. Alum has an excellent safety record, but comparative studies show that it is a relatively weak adjuvant for Ab induction and a poor adjuvant for the induction of cell-mediated immunity (Gupta & Siber, 1995; Gupta, 1998).

2.1.7.2 Bacterial toxins CT and LT

The heat-labile enterotoxin from *Escherichia coli* (LT) and its close relative found

in *Vibrio cholerae* (CT) are both capable of potentiating responses to non-related antigens (O' Hagan, 1997). They are both potent mucosal adjuvants (Lycke & Holmgren, 1986; Clements *et al.*, 1988; Simmons *et al.*, 1999). In fact, CT and LT were evaluated as the most potent mucosal adjuvants available (Klein, 1999), but their toxicity is unacceptable for use in vaccines (O' Hagan, 1997). Intensive research has produced some genetically altered endotoxin derivatives that display reduced toxicity while retaining adjuvantic properties (Singh & O' Hagan, 1999).

Both toxins are composed of an A subunit that confers the toxicity and a B subunit that is not toxic (Rappuoli *et al.*, 1999) and with which the adjuvant function of the toxin is associated (O' Dowd *et al.*, 1999). It has therefore been possible to employ the non-toxic B subunit of each toxin (LTB and CTB) as vaccine adjuvants. The main function of LTB and CTB that makes them particularly suited as adjuvants for mucosal immunisations lies with their ability to attach to epithelial cells lining the mucosal tracts. This specific binding is mediated by the GM1 receptor (Ribi *et al.*, 1988) and allows Ag transport across the mucosal cell layer, making the Ag-toxoid complex available for presentation by macrophages and dendritic cells to B and T cells (Takahashi *et al.*, 1996).

2.1.8 Generating effective vaccine formulations

Taking into account the issues addressed above, it is possible to generate immunity that is best suited for eliminating a particular type of antigen by appropriately designing and administering a vaccine formulation. A wide range of vaccine formulations is currently available for general use, or at various stages of

the preparation and evaluation process. The main vaccine types used in prophylactic immunisation against viral pathogens are presented below.

2.2 Prophylactic Vaccination against Viral Infection

Vaccines designed to protect from viral disease, largely protect the host from the establishment of viral infection. Vaccines that cannot completely abolish infection can still alleviate the disease symptoms brought about by infection. Viral vaccine strategies have traditionally employed whole viral particles (live attenuated, or killed) and purified viral proteins (mainly surface antigens). In the last 15 years, new approaches to vaccine development have emerged, including subunit vaccines that contain recombinant proteins (produced in mammalian cells, yeast, bacteria or baculovirus) and synthetic peptides representing important epitopes, as well as DNA vaccines that administer, to the host, antigen-encoding sequences.

2.2.1 Whole virus vaccines

The original and still most common immunogens used in viral vaccines are whole viral particles (Nabel, 2001). The viral particles employed in vaccine formulations are either inactivated (killed) or live but attenuated (avirulent) to reduce the risk of harming the host, but they contain important antigenic determinants to elicit immune responses that cross-react immunologically with the live virulent virus. Vaccines using whole virus particles can efficiently present multiple antigens from the same virus.

2.2.1.1 Live, attenuated virus vaccines

Vaccination with live attenuated viruses evokes immunity comparable to that generated in naturally occurring infection with wild-type virus (Letvin, 1998). The attenuated virus replicates in the host cells so it generates virus-specific cell-mediated responses. Furthermore, this replication allows prolonged exposure of the host to the vaccine antigens, providing greater immunogenicity that can lift the requirement for booster immunisations. Viruses attenuated from a pathogenic agent have proven safe and effective in many widely used vaccines, including Sabin poliovirus and chickenpox vaccine (Nabel, 2001). Live attenuated vaccines are routinely used to prevent mumps, rubella, measles and poliomyelitis (Hilleman, 2000).

However, live attenuated viruses can revert to a virulent phenotype and cause infection and disease, and in fact, some live attenuated vaccines have caused disease in immunosuppressed individuals, or, with low incidence, to the general population (O' Hagan, 1997). The concerns are even greater when considering the use of a live attenuated retrovirus. A retroviral vaccine component will integrate into the human genome thus posing a higher risk of reversion to virulence by genetic recombination (Klein, 1999), and generating deleterious consequences associated with insertional mutagenesis.

2.2.1.2 Whole inactivated virus vaccines

Inactivated viruses induce primarily humoral responses. They do not replicate in the host, so they are cleared by the immune system and therefore require multiple

booster immunisations for the development of long-term immunity. Since the vaccine virus does not synthesise any new proteins in the host, these vaccines cannot elicit CTLs (Bangham & Phillips, 1997). However, when delivered with suitable adjuvants, or as part of a prime-boost strategy, inactivated whole viruses can provide long-lasting protection in humans against a number of viruses, including influenza (Letvin, 1998) and Hepatitis A (Hilleman, 2000). Lastly, whole inactivated vaccines can contain reactogenic components that can cause undesirable side effects (Singh & O' Hagan, 1999).

2.2.2 Protein-based vaccines

The use of proteins from a pathogen is safer than using the pathogen itself. However, this strategy reduces the number of antigens presented to the immune system. Proteins that are employed in this strategy include surface antigens and exotoxins that are released during natural infection with a pathogen. Anamnestic responses to surface antigens can assist viral elimination from the host, while immunity generated against inactivated exotoxins can alleviate deleterious effects that occur upon release of the active toxin during natural infection.

Surface antigens and toxins may be purified from preparations of the pathogen or produced as recombinant proteins. For expression of recombinant proteins, the bacterial system presents the advantage of protein production in large quantities, while proteins that require post-translational modifications can be successfully and relatively inexpensively produced in eukaryotic cells. A protein made in this manner is used in the successful hepatitis B vaccine (Letvin, 1998).

Recombinant antigens employed in vaccination strategies can generate humoral immunity in the vaccinated hosts, but in most cases, recombinant proteins have poor immunogenicity (Singh & O' Hagan, 1999). These vaccines do not normally enter the host cells, so they are ineffective in eliciting CTL responses (Bangham & Phillips, 1997). Adjuvants and antigen carriers can be employed to drive the slow and sustained release of the proteins and transport them within the host cells. Antigen carriers that promote fusion with cellular membranes, interaction of encapsulated Ags with APCs, or localisation to the lymph nodes can induce antigen-specific cellular immunity.

A minimalistic approach to protein vaccines employs synthetic peptides. The peptides used in this vaccine type have to be very carefully selected and tested, as it is difficult to predict if they will induce immune responses. Peptides have to be tailored for recognition by B or T lymphocytes, bearing in mind that induction of T_H memory cells is instrumental to both humoral and cellular immunity. Synthetic peptides are simple to manufacture and purify but they tend to be poorly immunogenic (Klein, 1999). They can elicit CTL responses, but they are greatly confounded by MHC restriction (Bangham & Phillips, 1997).

2.2.3 DNA vaccines

This type of vaccine provides the host with the information to make a protein that contains antigenic determinants of the pathogenic organism. Compared to recombinant protein vaccines produced in bacteria or yeast, the proteins expressed from a DNA vaccine are more likely to assume a native conformation (Nabel, 2001). As a consequence, antibody responses generated against these immunogens

are more likely to recognise the native proteins of the pathogen. DNA vaccines can circumvent problems associated with protein-based vaccines such as high costs of production, difficulties in purification, and poor induction of CD8 cells (Leitner *et al.*, 2000). They are considered to be safer than live or killed whole virus vaccines (Baca-Estrada *et al.*, 2000) and they enable the inclusion of many antigens simultaneously (O' Hagan, 1997). The DNA sequence coding for the antigens is delivered to the host either as part of a recombinant vector vaccine or simply as naked DNA.

2.2.3.1 Recombinant vector vaccines

In this type of vaccine, the DNA coding for the antigenic protein is engineered in a non-pathogenic vector, unrelated to the particular pathogen. Viral vectors employed in this type of vaccine include Vaccinia, Canarypox, Semlinki Forest Virus (SFV), Sindbis virus and Venezuelan Equine Encephalitis Virus (VEE). Constrains on this approach are imposed by the size of the gene that can be carried by the parental vector and by the genetic stability of the recombinant organism (Letvin, 1998). Infection of the host with such recombinant viruses leads to immune responses against both the parental organisms and to the products of the inserted genes (Letvin, 1998), but the immune responses can be focused on the antigen rather than the vector if the same antigen is delivered using carriers with little or no immunogenic cross-reactivity (Leitner *et al.*, 2000).

The extent of replication of the recombinant vector largely influences its immunogenicity and its pathogenicity in the host. Non-replicating vectors are safer but show lower efficiency (Nabel, 2001), while vectors that encode their

own replicase tend to be more efficient but present higher risks (Letvin, 1998; Leitner *et al.*, 2000). Avian poxviruses that initiate protein synthesis but do not complete a replication cycle in humans, can elicit immune responses and they present an excellent safety profile (Letvin, 1998).

Self-replicating vectors induce both antibody (Baca-Estrada *et al.*, 2000) and CTL responses (Bangham & Phillips, 1997). Replicating vector vaccines have generated long-lasting T_H1 and T_H2 responses against several pathogens in experimental animal models (Klein, 1999) and they can polarise the immune response towards strong T_H1 responses (Baca-Estrada *et al.*, 2000). The persistence of these vectors results in continuous exposure of the host to their antigens, thus eliminating the need of a booster immunisation (Klein, 1999).

2.2.3.2 Naked DNA vaccines

This approach uses genetically engineered plasmid DNA to direct the synthesis of an immunogen within the host cells. Expression of the immunogen is placed under the regulation of a eukaryotic promoter, enhancer and polyadenylation signals that can be readily manipulated to optimise the level and duration of expression and the potency of the immunogen (Nabel, 2001). Translational efficiency of foreign DNA in mammalian cells can be improved through modifications to human codon sequences (Andre *et al.*, 1998). Plasmid DNA vectors are simple to manufacture and purify (Bangham & Phillips, 1997).

Methods for efficient delivery of naked DNA have been developed, such as gene gun technology that ensures the DNA reaches the cell nucleus before it is degraded (Haynes *et al.*, 1996). Naked DNA usually integrates to chromosomal DNA or persists in episomal form for long periods. Continuous expression of the

antigens can then stimulate strong and persistent humoral and cellular immune responses (Letvin, 1998). Naked DNA vaccines present advantages over recombinant viruses, as they do not induce anti-vector immunity (Letvin, 1998) and they present no danger of a pathogenic infection (Nabel, 2001). However, the genomic integration of DNA raises significant safety concerns (O' Hagan, 1997).

Immune responses induced by DNA constructs tend to be rather weak, but they can be improved by the use of molecular adjuvants, such as co-stimulatory molecules, pro-inflammatory cytokines, T helper cytokines and immunostimulatory sequence motifs such as the dinucleotide CpG (cytosine-phosphorothioate-guanine) (Mooij & Heeney, 2002). Additionally, even naked DNA vaccines that display only relatively weak immunity when delivered on their own, are extremely efficient at priming the immune system in 'prime-boost' vaccine strategies (Nabel, 2001).

2.3 Developing a Vaccine against HIV

Despite the advances in vaccine technology and the furthering of knowledge and understanding of the immune system functions, over 20 years since the discovery of AIDS, no vaccine is available to halt the epidemic proportions of HIV infections worldwide (Piot *et al.*, 2001). HIV vaccine development is hindered by a variety of factors (Ada, 2000). During lentivirus infection, viral epitopes are presented to the immune system (see Figure 5) and induce both antibody and CTL responses. However, the huge diversity in immunogenic viral epitopes, combined with the rapid mutational variations that occur within and between individuals (Malim & Emerman, 2001), render the development of a universal vaccine for

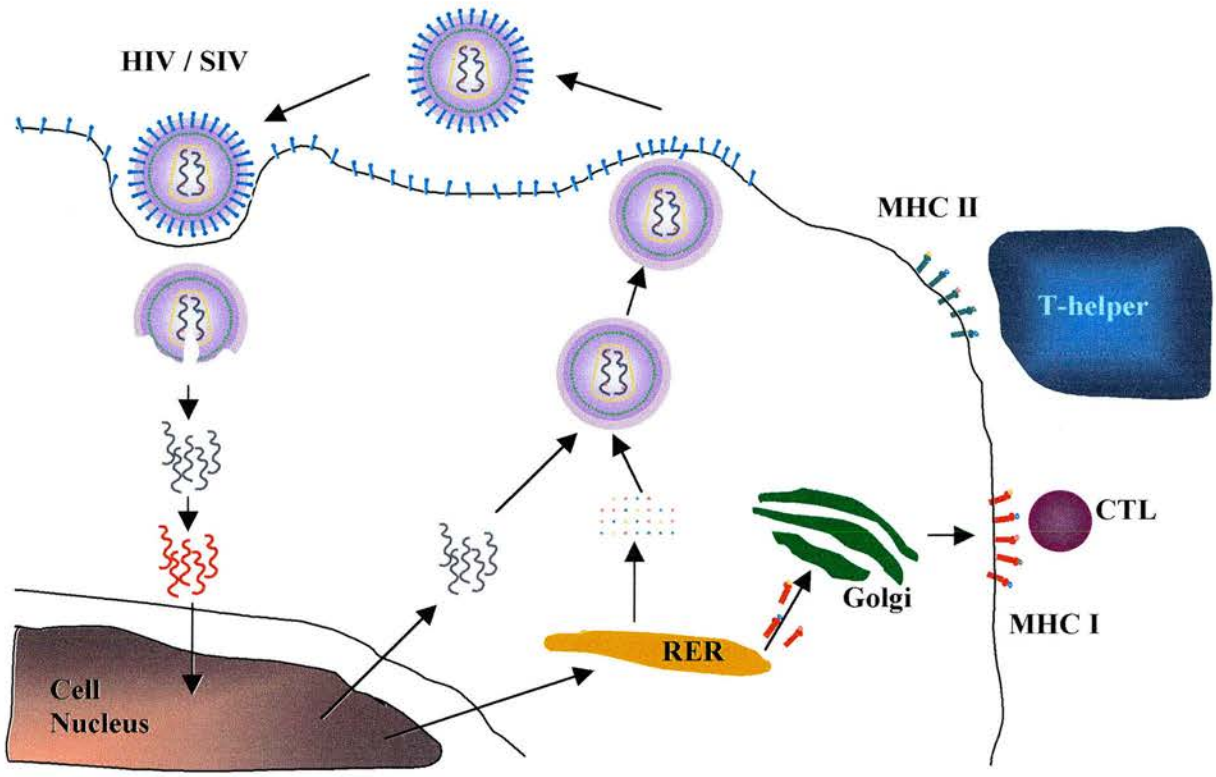


Figure 5: The sequence of events during lentiviral infection and MHC presentation of viral antigens.

The lentivirus (denoted HIV / SIV) binds onto cells that display appropriate receptors. The viral coat fuses with the membrane of the cell and the uncoated virus is released within the cell. The surface antigens of the viral coat get dispersed on the cell membrane.

Once inside the cell, the viral (RNA) genome integrates into the host cell genome as a cDNA copy (RNA depicted in grey, DNA shown in red). From the cell nucleus, the viral cDNA drives the transcription of genomic RNA, and mRNA for the synthesis of the viral proteins. RNA molecules and newly synthesised viral proteins get assembled into viral particles that exit the cell. Newly formed particles acquire their coat from the host cell membrane by budding, thus re-using some of the paternal surface antigens.

During protein synthesis and passage through the rough endoplasmic reticulum (RER), viral proteins are captured by MHC I molecules, transported through the Golgi and presented on the surface of the infected cell for interaction with CD8+ T lymphocytes (mainly Cytotoxic T Lymphocytes - CTLs).

Antigen presenting cells (APCs), regardless of their own infection status, can present viral antigens on their MHC class II proteins. During an acute infection, APCs acquire such antigens from the extracellular milieu, they process them through the endosomal pathway (not shown) and present them on MHC II molecules for recognition by CD4+ T cells (usually T helper).

all HIV-1 strains rather unlikely. The lack of a good animal model for AIDS disease progression also poses limitations on vaccine development. In the most suitable animal model, the macaque monkey, animals die sooner after infection with SIV than do humans after infection with HIV-1 (Letvin, 1998).

Humans develop immune responses following acute HIV infection, but these responses do not clear the virus and they are unlikely to protect against subsequent infection with another lentiviral strain. Neutralising Abs as well as cell-mediated responses play a role in conferring sterilising immunity and preventing progression to AIDS (Klein, 1999). CTLs are thought to control the early HIV viraemia (Matano *et al.*, 1998; Schmitz *et al.*, 1999; Kaul *et al.*, 2000), while CD4+ cells provide help to B cells and CD8+ T cells (Kalams *et al.*, 1999), and they maintain HIV-specific CTL memory (Klein, 1999). A desirable vaccine would induce both cellular and humoral immunity to one or more lentiviral gene products (seen in Figure 6). The progress of AIDS vaccine research and the current status of lentiviral vaccines are presented below.

2.3.1 Current AIDS vaccine status

The first phase I trial of an HIV-1 candidate vaccine was undertaken in the USA in 1987. Since then, several candidate HIV vaccines have been tested for their immunoprotective ability in primates, and evaluated for their immunogenicity in monkeys and man. HIV vaccine candidates have so far entered over 70 phase I, five phase II, and two phase III clinical trials (Nabel, 2001). While earlier candidate vaccines progress to clinical trials, new vaccine strategies are being developed and assessed in pre-clinical studies. Some of these approaches to HIV vaccine development are reviewed here.

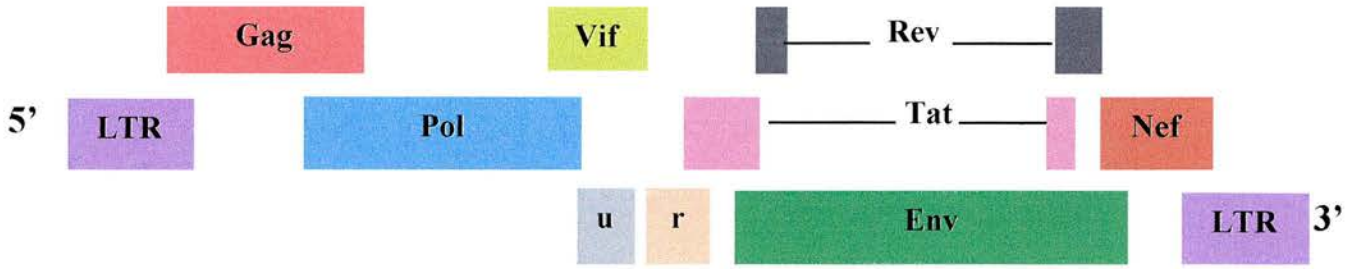


Figure 6: Genomic organisation of HIV-1.

Gag encodes the nucleocapsid proteins (p17, p24, p9 and p7), Env encodes the envelope glycoproteins gp120 and gp41 and Pol encodes the precursor protein that gives rise to the viral enzymes (p32: integrase, p10: protease that cleaves the gag precursor, p51: reverse transcriptase activity and p64: reverse transcriptase and RNAse activity). The remaining proteins are encoded by splicing and frame-shifting from the viral cDNA template. The regulatory proteins Tat, Rev and Nef, Vif are involved in viral protein expression, Vif and Vpu (denoted 'u') are important for maturation of the particle and Vpr ('r') encodes a weak transcriptional transactivator. LTR: long terminal repeat.

The genomes of HIV-2 and SIV are very similar to that of HIV-1, but they encode Vpx instead of Vpu.

2.3.2 Whole lentivirus vaccines

Among the first attempts to develop a lentiviral vaccine, a formulation of inactivated SIV was shown to protect immunised macaques against challenge with homologous pathogenic SIV (Murphey-Corb *et al.*, 1989), but the responses induced were later shown to be directed against the MHC molecules incorporated in the SIV envelopes rather than antigenic determinants of SIV (Stott *et al.*, 1991).

Observations that nef-deficient HIV strains caused milder, asymptomatic infections in rhesus macaques (Kestler *et al.*, 1991) inspired the development of nef- deficient lentiviral vaccines (Daniel *et al.*, 1992). However, even triple deletion mutants of SIV that protected adult macaques (Wyand *et al.*, 1996), induced AIDS-like disease in neonates (Baba *et al.*, 1995) and in some cases in adult macaques, over time (Baba *et al.*, 1999). More recently, infection of monkeys with a nef-deleted SIV was shown to protect against challenge with a pathogenic SIV strain and SHIVs expressing an HIV-1 envelope (Wyand *et al.*, 1999). Unfortunately, individuals in the human cohorts (e.g. the Sydney cohort) that were infected with nef-deficient viruses and remained asymptomatic for a period of up to 10 years, eventually developed disease symptoms (Learmont *et al.*, 1999) after about 12 years of infection.

Among the AIDS vaccine candidates assessed so far, live attenuated lentiviruses have been the most effective yet evaluated in clinical settings (Johnson & Desrosiers, 1998; Mooij & Heeney, 2002). However, it is difficult to retain efficacy while sufficiently attenuating a lentivirus (Cohen, 1997) so safety concerns preclude the use of a replicating lentiviral vaccine in humans. On the other hand, HIV-1 viruses that no longer replicate, but retain their fusogenic

potential, can induce CTL responses by cross-presentation (Buseyne *et al.*, 2001) so they present attractive vaccine candidates.

2.3.3 Naked DNA vaccines encoding lentiviral antigens

DNA immunisation driving the expression of lentiviral proteins was shown to induce antigen-specific cellular and humoral immune responses that conferred protection from limited HIV-1 infection in chimpanzees (Boyer *et al.*, 1997). However, a subsequent phase I study employing a Env- and Rev- encoding DNA plasmid induced only modest and sporadic T cell responses in vaccinated volunteers (Boyer *et al.*, 2000). DNA vaccines enhanced by the presence of CpG motifs have induced T_H1 type responses to gp160 in rodents (Deml *et al.*, 1999) and in primates, a CpG-rich Tat DNA vaccine candidate (Cafaro *et al.*, 2001) conferred protection upon SHIV infection. A study in macaques employing codon-modified DNA plasmids encoding Gag or Env, augmented by the action of an IL-2-Ig fusion protein, generated strong and sustained immune responses and conferred marked protection against infection with a pathogenic SHIV strain (Barouch *et al.*, 2001), but the safety of this approach was questioned when a test animal developed disease symptoms (Barouch *et al.*, 2002).

2.3.4 Replicating vector vaccines encoding lentiviral antigens

Various attenuated poxviruses with different replication potential have been developed to express either SIV or HIV-1 antigens. Immunisation studies with recombinant MVA (Modified Vaccinia virus Ankara) have demonstrated reduced

viraemia, preserved CD4+ counts and improved clinical outcomes in vaccinated macaques upon SIV or SHIV challenge (Seth *et al.*, 2000; Ourmanov *et al.*, 2000). Canarypox vaccines expressing HIV-1 clade B antigens were shown to elicit broad CTL reactivities capable of recognising viruses belonging to genetically diverse HIV-1 clades (Ferrari *et al.*, 1997). In humans, phase I studies have established the safety of vaccinia and canarypox vectors. A canarypox vaccine candidate encoding multiple HIV-1 genes is currently undergoing phase II testing in the USA as part of a prime-boost regime.

Alphavirus and adenovirus constructs have been evaluated in preclinical models, due to their ability to target APCs (Mascola & Nabel, 2001). A single injection of an adeno-associated vector expressing HIV-1 Env, Tat and Rev genes induced strong production of IgG, IgA and CTL activity in BalbC mice (Xin *et al.*, 2001). Intranasal immunisation with a replication-competent adenovirus coding for HIV products induced systemic and mucosal immunity in macaques and in chimpanzees (Buge *et al.*, 1999; Lubeck *et al.*, 1997), demonstrating that replication was necessary to stimulate immunity. More recently, immunisation of rhesus macaques with a replication-defective adenovirus expressing SIV gag was shown to attenuate subsequent infection with SHIV, proving a promising vaccine vector for the development of an HIV-1 vaccine (Shiver *et al.*, 2002).

Alphaviruses such as SFV, VEE and Sindbis have been engineered to express HIV-1 antigens, and alphavirus replicons were shown to induce humoral and cellular responses in mice, primarily of the T_H1 type (Berglund *et al.*, 1998; Hariharan *et al.*, 1998). Macaques immunised with a SFV vaccine were believed to be protected from acute disease following SIV challenge with PRJ14, an acutely lethal variant of SIV (Mossman *et al.*, 1996). However, this virus induces

an unusual acute symptomology, and long-term follow-up of the animals revealed that they progressed to develop AIDS (Mooij & Heeney, 2002). A VEE vector expressing SIV proteins induced immunity and partial protection against SIV in macaques (Davis *et al.*, 2000).

Moreover, immunisation with live attenuated poliovirus encoding SIV genes elicited both systemic and mucosal anti-SIV responses in macaques (Crotty *et al.*, 1999). Attenuated HSV (Herpes Simplex Virus) strains (with and without replicative capacity) expressing SIV proteins were shown to induce anti-SIV antibody and CTL responses in rhesus macaques (Murphy *et al.*, 2000) that protected strongly or partially against challenge with a pathogenic SIVmac virus.

2.3.5 Lentiviral vaccine prime-boost strategies

These strategies include a 'prime' immunisation, usually with a DNA vaccine, followed by recombinant viral vector or recombinant protein vaccines for the 'boost' immunisation (Ramshaw & Ramsay, 2000). This heterologous prime-boost strategy (where the same antigen is presented in a different form in each of the prime and boost immunisations) is the only strategy that has consistently induced both humoral and cellular immunity in man (Klein, 1999). Several groups that employed the heterologous prime-boost regimen in macaques have observed improved HIV-specific immune responses (Letvin *et al.*, 1997; Fuller *et al.*, 1997; Cherpelis *et al.*, 2001; Nilsson *et al.*, 2001).

Early studies in the SIV-macaque model indicated that a recombinant vaccinia virus expressing the SIV Env gene, boosted by soluble envelope protein, could completely protect against a homologous pathogenic SIV strain (Hu *et al.*,

1992). However, subsequent studies employing poxvirus vectors only achieved partial protection (Daniel *et al.*, 1994; Hirsch *et al.*, 1996). Prime-boost regimes employing adeno-associated viral vectors expressing HIV and SIV antigens induced only partial protection in rhesus macaques (Buge *et al.*, 1997; Buge *et al.*, 1999) but conferred long-lasting protection in chimpanzees (Lubeck *et al.*, 1997).

In phase I /II clinical trials, a live recombinant Canarypox virus vaccine expressing HIV-1 Env, Gag and protease genes, boosted with HIV-1 recombinant gp120, induced a broader cytokine response than the vector alone (Gorse *et al.*, 2001) and cross-clade antibody responses (Verrier *et al.*, 2000). Another live recombinant Canarypox vector expressing HIV-1 antigens induced both T_H1- and T_H2- type responses to HIV-1 envelope antigens in humans, as part of a prime-boost approach (Sabbaj *et al.*, 2000). An HIV-encoding plasmid that is attached to microparticles of polylactide coglycolide (PLG), developed by Chiron corp., generated vigorous cell-mediated immunity and Ab responses after a single inoculation in mice and it will be employed in human trials as the prime inoculation, subsequently boosted with a gp120 protein (Cimons, 2002).

A different prime-boost vaccination strategy involves priming with naked DNA and boosting with recombinant vector expressing the same antigens. The DNA prime / MVA boost schedule was shown to induce high levels of antigen-specific CD8+ T lymphocytes in mice and in non-human primates (Hanke *et al.*, 1999; Allen *et al.*, 2000) and to lower viraemia in monkey challenge studies with SHIV (Amara *et al.*, 2001; Rama *et al.*, 2001). Immunisation of rhesus macaques with three different vector systems (DNA, MVA and SFV) showed protection in two out of four immunised animals (Heeney *et al.*, 2000). The Oxford Vaccine Initiative is currently testing a Gag-based naked DNA prime /

MVA boost vaccine candidate (Hanke & McMichael, 2000) in the UK and Kenya, and a DNA / poxvirus candidate vaccine developed by Yerkes Primate Regional Research Centre is planned to be tested in West Africa (Cimons, 2002).

2.3.6 Lentiviral proteins as HIV vaccine candidates

HIV encodes more than 12 gene products (seen in Figure 6), any of which might serve as targets for immune recognition. Protein synthesis is regulated by viral transactivators. It includes proteins derived from messenger RNAs synthesised from highly spliced viral RNA, made early in the course of infection, and those derived from unspliced viral RNA, produced late in the viral life cycle. Both the proteins found on the exterior of the viral particle (envelope proteins) and the internal proteins of the virus have been evaluated as potential vaccine targets.

2.3.6.1 Envelope proteins

Several efforts to develop AIDS vaccines have focused on HIV envelope proteins and peptides derived from them. The envelope proteins are safe for immunisation purposes, but recombinant subunit proteins are poorly immunogenic on their own. Even though passive administration of Ab specific to envelope antigens has illustrated the importance of Ab protection and the potential to block viraemia and disease progression (Mascola *et al.*, 1996, 1999 & 2000; Baba *et al.*, 2000), the Ab levels employed in these studies are unlikely to be induced by vaccination (Nabel, 2001).

The first candidate HIV-1 vaccine to be evaluated in humans was recombinant HIV envelope gp120 protein formulated with alum (Nathanson *et al.*, 1999). Recombinant forms of gp120 and gp160 have since been extensively tested in phase I/II studies (McElrath *et al.*, 2000). However, the limited potency and breadth of neutralising antibodies elicited limits their ability to neutralise primary HIV-1 strains and confines protection only to homologous or very closely related viral strains (Mooij & Heeney, 2002). More recent vaccine candidates employing recombinant proteins that more closely mimic the native structure of the HIV-1 glycoprotein, or immunogens that present neutralisation epitopes that are not immunogenic on the native virus, still did not succeed to generate widely neutralising antibodies (Mascola & Nabel, 2001).

The delivery of HIV-1 envelope-based antigens with more potent adjuvant systems has improved their relatively poor and narrow immunogenicity (Voss & Villinger, 2000; Peter *et al.*, 2001). Strong T_H1 and T_H2-like responses in monkeys were generated by the use of ISCOMs (Bruck *et al.*, 1994) and AS2 (Mooij *et al.*, 1998). In a HIV-1 gp120 phase I/II clinical trial, a MF59 formulation successfully induced neutralising Ab responses against homologous and certain heterologous virus strains (Nitayaphan *et al.*, 2000), while a pre-clinical study also showed modest cellular immune responses in rhesus macaques (Verschoor *et al.*, 1999) and partial protection against infection with SHIV. The oral and intranasal routes are also being explored, employing mucosal adjuvants for the induction of mucosal immunity (Kaneko *et al.*, 2000; Lian *et al.*, 2000; Morris *et al.*, 2000).

A gp120 monomer expressed in mammalian cells and delivered on alum (Francis *et al.*, 1998) is being currently evaluated in phase III trials. However,

during phase II of the human trials, this candidate developed little or no CTL responses (Cohen, 1999) and the Abs generated have little if any ability to neutralise most wild-type HIV isolates (Nathanson & Mathieson, 2000). Moreover, immunised individuals who later became infected with HIV-1 reached similar viral loads to non-vaccinated individuals (Mooij & Heeney, 2002).

2.3.6.2 Regulatory proteins

Regulatory proteins are more conserved than the envelope proteins, so they could induce broadly neutralising immune responses. Nef Tat and Rev are expressed early in the virus life cycle (Emerman & Malim, 1998), so immune responses to these proteins may serve to limit the viral infection before it gets established. Tat, Rev and Nef proteins have been studied as potential vaccine candidates for several years (Miller & Sarver, 1997; Calarota *et al.*, 1998; Calarota *et al.*, 1999; Ayyavoo *et al.*, 2000; Tahtinen *et al.*, 2001) and there are indications that CTLs specific to the early regulatory proteins are important for eliminating infected cells before they produce high levels of mature virions (vanBaalen *et al.*, 1997; Ensoli *et al.*, 2000; Addo *et al.*, 2001). Tat and Rev are the only proteins produced before Nef downregulates the MHC I molecules (Collins *et al.*, 1998) and compromises the ability of the immune system to cope with viral infection. They are interesting candidates because of their potent regulatory activity (Nabel, 2001).

Rhesus macaques immunised with a recombinant vaccinia vector expressing Tat and Rev proteins showed protection and ability to control SIVmac infection (Osterhaus *et al.*, 1999). Immunisation with biologically active Tat showed specific humoral and cell-mediated responses in the absence of toxicity

(Cafaro *et al.*, 1999; Ensoli *et al.*, 2000) and controlled acute viraemia by SHIV infection in cynomolgus monkeys for at least 19 months (Cafaro *et al.*, 1999; Cafaro *et al.*, 2001). Another study with inactivated Tat toxoid in rhesus macaques (Pauza *et al.*, 2000) did not show protection against SHIV infection, but did alleviate symptoms of disease (lowered serum viral load, IFN-γ and chemokine expression on CD4+ cells, reduced CD4+ T cell decline). The difference between complete and partial protection in the two studies could be a reflection of differences in the two animal models: cynomolgus monkeys show viraemia of $1x10^6$ viral copies per ml as opposed to up to $1x10^9$ copies per ml in rhesus macaques (Peters, 2002). Recently, a CTL epitope was identified on HIV-Tat (Morris *et al.*, 2002) that could be employed instead of the full-length protein to generate protective immunity.

In the work presented here, Tat from SIVmac251 was studied for its potential to generate protective immune responses in vaccinated animals. A review of the functions of Tat protein and the reasons that make it a good candidate for a lentiviral vaccine are presented below.

2.4 Lentiviral Protein Tat

Tat protein (TransActivator of viral Transcription) is a small (14 kd) nuclear protein product. It is expressed early after lentiviral infection through transcription of complex spliced mRNAs (Gallo, 1999). Tat comprises five regions that are conserved across the lentivirus family: the N-terminal region (aa 1-21), the cysteine-rich domain (22-37), the core domain (38-48), the basic or arginine-rich region (48-57) and the C-terminal domain (of variable lengths) (Taube, 1999; Liu

et al., 2000). The N' terminal and cysteine-rich domains are important in transactivation (Ruben *et al.*, 1989). The basic region mediates in vitro viral internalisation (Rodman, 1999) and uptake of Tat by cells (Frankel & Pabo, 1988; Brake *et al.*, 1990). The presence of a nuclear localisation signal (Ruben *et al.*, 1989; Truant & Cullen, 1999) localises Tat to the cell nucleus.

2.4.1 The importance of Tat in lentivirus infection and disease

Tat is essential for viral gene expression (Fisher *et al.*, 1986). It is involved in the initiation of transcription and RNA chain elongation by complex processes involving interaction with cellular proteins and a specific region (TAR) of the viral RNA (Jones, 1997; Garber & Jones, 1999). Tat is also a free protein product of the virus (Goldstein, 1996) that exits the infected cells and binds and enters into neighbouring cells.

This uptake of extracellular Tat by both infected and uninfected cells is a key element in Tat-mediated lentiviral infectivity. Tat affects a range of host genes (Gallo, 1999), upregulating or downregulating their products. In infected cells, Tat activates virus replication by activating latent lentiviruses to establish successful lytic infection (Jeang *et al.*, 1999). In uninfected cells, it induces the expression of CCR5 and CXCR4 co-receptors (Huang *et al.*, 1998; Secchiero *et al.*, 1999) that render the cells susceptible to productive viral infection with macrophage-tropic and T cell-tropic HIV-1 strains.

Tat is known to induce cellular apoptosis (Li *et al.*, 1995; Westendorp *et al.*, 1995) and is believed to be involved in the neural degeneration leading to AIDS-related dementia (Rodman, 1999; Kaul *et al.*, 2001). Extracellular Tat

induces a state of immunosuppression (Brand *et al.*, 1997; Reinhold *et al.*, 1999), including functions such as downregulation of MHC I expression (Mhaskalikar *et al.*, 1996) and suppression of antigen-induced (but not mitogen induced; Viscidi *et al.*, 1989) proliferation of T-cells (Zauli *et al.*, 1996; Zagury *et al.*, 1998a). Tat also plays a direct role in cancer by stimulating the cell growth of Kaposi's sarcoma cells (Vogel *et al.*, 1988; Ensoli *et al.*, 1990).

Tat is essential for the productive viral infection and for the massive initial replication of virus through activation of latent viruses in infected cells and priming of uninfected cells for lentiviral infection, possibly facilitating viral escape (Goldstein, 1996). The extracellular presence of Tat also induces a variety of deleterious phenomena. If Tat protein could be stopped and contained at the initial stages of infection, it would be possible to control the spread of the viral infection and avoid the toxic effects and symptoms of infection that lead to AIDS.

2.4.2 HIV-Tat versus SIV-Tat

Replication of the HIV and SIV viruses depends critically on Tat. HIV-Tat efficiently transactivates the HIV and SIV promoters, whereas SIV Tat transactivates the HIV promoter with 5-fold lower activity (Viglianti & Mullins, 1988; Colombini *et al.*, 1989). HIV Tat with an SIV basic region, created during domain swapping studies, transactivates the HIV LTR identically to intact SIV-Tat, 3 times less efficient than HIV-Tat does (Tao & Frankel, 1994).

Tat is largely conserved in the genomes of primate lentiviruses (Jeang *et al.*, 1999), possibly due to constraints imposed by overlapping reading frames (Kusumi *et al.*, 1992). HIV-Tat is highly conserved among HIV isolates

(Goldstein 1996). Its immunogenic epitopes are conserved among the different viral subtypes (with the exception of the O subtype: Cafaro *et al.*, 1999).

On the other hand, Tat from SIV strains shows a higher degree of variability. An 8 amino acid peptide epitope on SIV-Tat was clearly shown to mutate and escape CTL recognition in SIV-infected rhesus macaque monkeys (Allen *et al.*, 2000). Comparison of SIV genomes also proved the high variability in that epitope of Tat and showed SIV-Tat to be the least conserved of the nine encoded products, at the amino acid level (Hughes *et al.*, 2001). This was true even when Tat was compared to Vpr with which it shares a reading frame.

2.4.3 Immunity specific to Tat

Tat is immunogenic and immune responses to Tat are naturally found both in infected individuals and in experimental systems. There is evidence for a protective role of Tat-specific responses against disease progression. Low or absent Tat-specific antibodies correlates with progression to AIDS (Goldstein, 1996). Anti-Tat Abs control disease progression by inhibiting both the effect of extracellular Tat on HIV replication and its immunosuppressive effects on T cells (Cafaro *et al.*, 1999). There is also an inverse correlation between rapid progression to AIDS and the presence of Tat-specific CTLs (vanBaalen *et al.*, 1997; Zagury *et al.*, 1998b), particularly in the initial phase of infection.

Tat protein, unlike most other proteins, can generate specific CD8+ T cell-mediated CTL responses, because it is efficiently taken up by cells and enters the MHC I pathway (Fawell *et al.*, 1994; Kim *et al.*, 1997). Lastly, the use of Tat

in a vaccine formulation would not generate responses that would cross-react with the surface antigens employed in current seroconversion assays (Goldstein, 1996).

In this study, Tat was selected to be evaluated for its immunogenicity and protective potential. The protein employed originated from a SIVmac strain to facilitate assessment in the rhesus macaque model. Recombinant SIV-Tat was engineered so that it could be purified by affinity purification. The principles of affinity purification techniques are presented below.

2.5 Protein Purification Employing Affinity Tags

Each protein can be purified on the basis of its individual chemical and biological properties. Protein purification options include separation on the basis of protein size (gel filtration, membrane exclusion), charge (ion exchange chromatography, electrophoresis) or solubility (reverse phase chromatography, ammonium sulphate precipitation, hydrophobic interactions).

The biological interactions of a protein with other molecules can be employed for its purification by affinity chromatography. This technique employs interactions between antigen and antibody, enzyme and substrate agonist, and receptor and ligand molecules. It relies on the selectivity and tightness of binding between the required protein and the other molecule.

However, establishing the optimal purification strategy for each new protein can be time consuming. Recombinant proteins can be engineered to contain additional amino acid sequences with unique properties that can be exploited for purification purposes (Smith *et al.*, 1988). Such sequences are called

affinity tags and they can be attached to each new protein synthesised, thus providing a universal system for purification of recombinant proteins.

2.5.1 Employing Affinity Tags

Affinity tags are short or longer amino acid sequences that can be engineered as part of a protein of interest. Antibodies that recognise parts of the affinity tags are very useful. Detection of an affinity tag by its specific antibody creates the possibility to follow the whereabouts of an affinity-tagged protein in a dynamic environment where a variety of other proteins may be present. Ab specific to an affinity tag can be immobilised on a solid matrix and serve as a specialised magnet to attract proteins tagged with that affinity tag. If suitable elution conditions are defined, an affinity tag can be employed for affinity purification of affinity-tagged proteins (refer to Figure 7).

The use of affinity tags provides a means of visualising, capturing and purifying a wide variety of distinct proteins, regardless of their individual properties, potentially eliminating the need for separate purification schemes for each individual genetically-engineered protein (Smith *et al.*, 1987). During the course of this work, three affinity tags were employed in protein expression and purification studies, namely GST, His and Pk. Each tag was recognised by its specific antibody(ies) and could be employed in a different form of isolation or purification of tagged protein.

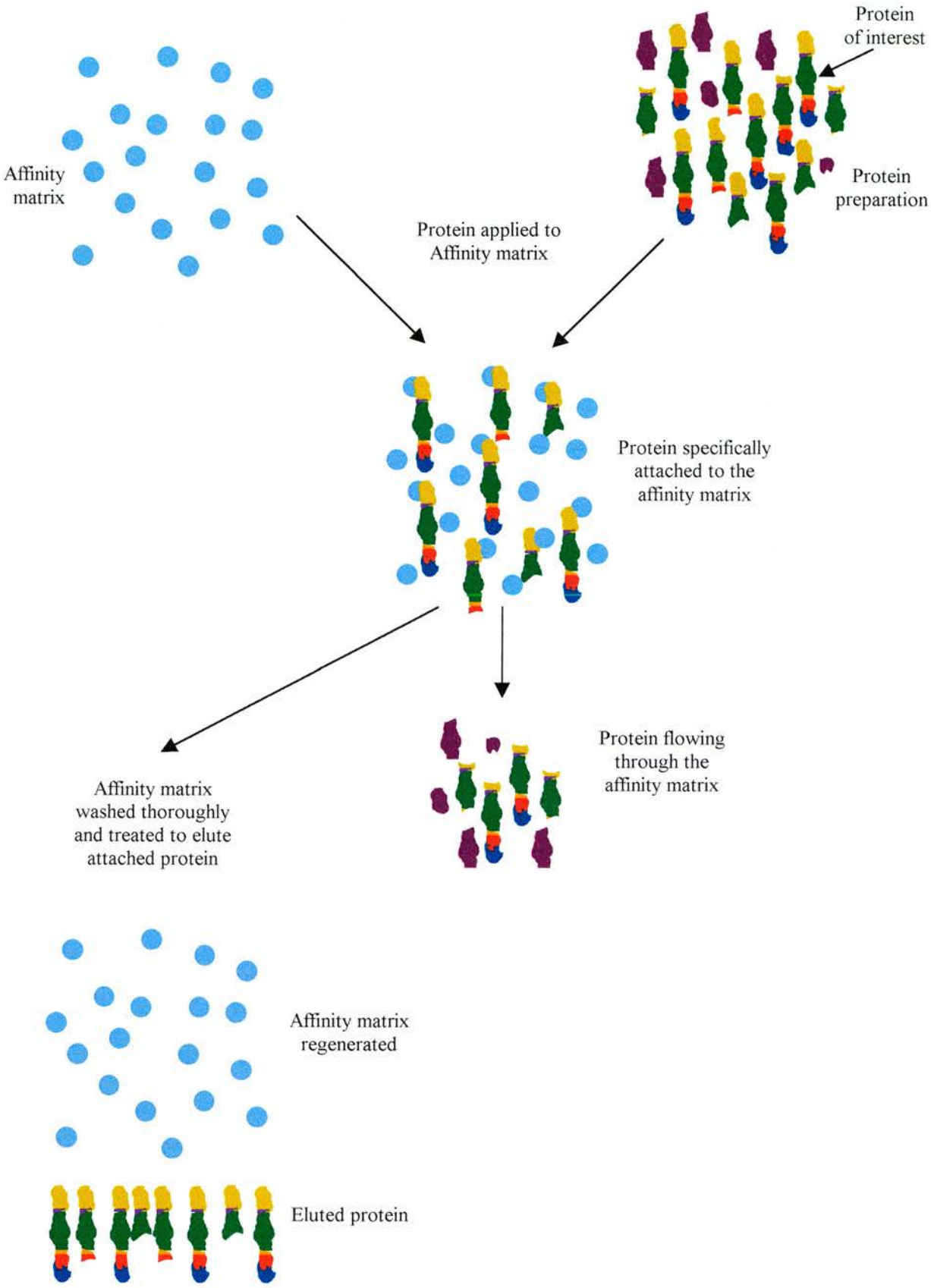
Figure 7: The principle of protein purification on affinity matrix.

An affinity tag can be employed to purify affinity-tagged proteins on solid support. The affinity tag must strongly interact with the matrix at a given set of conditions (to achieve specific binding of the tagged protein to the matrix) while it must no longer interact with the matrix in another set of conditions (so that the protein can be eluted off the matrix).

The affinity matrix is shown at the top left of the diagram, depicted as blue beads. The protein to be purified is found in a protein solution, seen on the right. The protein of interest is represented by a multicoloured drawing, the full-length protein containing 6 sections from yellow to blue. The protein solution contains contaminants (shown in purple) and abrogated proteins (containing some but not all the parts of the full-length protein). The affinity of the protein for the selection matrix lies with the yellow portion of the sequence.

When the protein preparation is allowed to interact with the affinity matrix (middle of diagram), only protein molecules with an intact yellow end bind to the matrix, while all unrelated proteins and shorter fragments of the protein of interest flow through and are discarded.

After extensive washing, the affinity matrix is treated to elute the specifically bound proteins (bottom part). All the eluted proteins contain an intact yellow end, specific to the affinity matrix employed. Note however, that this selection does not eliminate abrogated parts of the protein with an intact yellow end.


Protein
of interest
Affinity
matrix
Protein
preparation
Protein applied to
Affinity matrix
Protein specifically
attached to the
affinity matrix
Protein flowing
through the
affinity matrix
Affinity matrix
washed thoroughly
and treated to elute
attached protein
Affinity matrix
regenerated
Eluted protein

2.5.2 Affinity Tag GST and the pGEX expression vectors

The GST (Glutathione-S-Transferase) technology (Pharmacia) provides a system for protein expression and purification. The pGEX vectors (Smith & Johnson, 1988) drive the expression of the protein of interest in a GST-fusion form. pGEX vectors place protein expression under the control of an IPTG-inducible promoter, while they contain the over-expressed lacIq allele of the *lac* repressor to repress protein expression in the absence of IPTG in all *E. coli* hosts. IPTG (Isopropyl-β-D-ThioGalactopyranoside) triggers high levels of GST-fusion protein expression. GST-fusion proteins can be purified under non-denaturing conditions by affinity chromatography on immobilised glutathione (Smith & Johnson, 1988) and a high degree of purity can be achieved by single-step GST-affinity purification. A protease cleavage site is situated between GST and the engineered protein, so when the GST tag is no longer required, it can be removed from the protein by proteolytic cleavage. Antibodies specific to GST are commercially available.

2.5.3 Affinity Tag His

The His tag is a short affinity tag composed of six histidine residues. His residues have a high affinity for metal cations, thus enabling purification of His-tagged proteins by chelating immobilised metal ions (Smith *et al.*, 1988; Smith *et al.*, 1987). Proteins tagged with the His tag selectively bind to NiNta-agarose columns and they can be eluted by competition with Imidazole, or by stripping the matrix using EDTA (EthyleneDiamineTetraAcetic acid). A series of expression vectors,

commercially available by QiaGen, provides a system for the expression of His-tagged proteins. A range of antibodies specific to the His tag is also available.

His-affinity purification can also be employed for the purification of insoluble proteins. The application of denaturating agents such as urea can facilitate the solubilisation of insoluble proteins, and denaturating conditions do not disturb the purification process on Ni-Nta-agarose (Lilie *et al.*, 1998). Denaturated His-tagged protein is first bound on NiNta-agarose, then the protein is washed with renaturation buffer while bound on the matrix, and finally eluted from the column.

2.5.4 The Pk affinity tag

The Pk tag is a 14-amino acid-long affinity tag that originates from part of the gene encoding proteins P and V of the paramyxovirus Simian Virus 5 (SV5). The monoclonal antibody Pk1 that specifically recognises the Pk tag was isolated (Randall *et al.*, 1987) and its specificity determined (Southern *et al.*, 1991) in our laboratory. Pk-tagged proteins are successfully traced and visualised both in bacterial environments and in tissue culture cells, via the Pk-Pk1 interaction that is both specific and strong (Hanke & Randall, 1995). Pk1 is also successfully employed to isolate Pk-tagged proteins (immune precipitation) and for protein capture on a Pk-affinity solid matrix.

The Pk tag was previously employed as the last purification step for Pk-tagged proteins to be included in SMAA complexes (Hanke, 1993). Those SMAA complexes were employed in animal immunisations and they were shown to generate immunity that was not directed primarily against the Pk tag (Hanke *et al.*,

1992, Randall *et al.*, 1993). However, the Pk-Pk1 interaction is too strong to be disrupted by mild elution conditions (Southern *et al.*, 1991; Dunn *et al.*, 1999), thus precluding its use in a Pk-based purification system. The wide application of the Pk technology in our laboratory would greatly benefit from an affinity purification method for Pk-tagged proteins.

2.5.5 Two-step affinity purification of tagged proteins

If an affinity tag is engineered on either side of the protein of interest, that protein can be purified by a two-step affinity purification. The principle of this double purification is demonstrated in Figure 8: the expressed protein is first selected on the basis of its N' terminal tag, a process that eliminates breakdown products lacking an intact N' terminus. In the second step, the protein positively selected through the first screening is further applied onto a system that selects for the presence of the C' terminal tag, thus removing the shorter translation products that lack a functional C' terminal domain.

Two-step purification of affinity-tagged proteins can yield a cleaner preparation and select for full-size proteins. In our laboratory, two-tag purification has been successfully employed to prepare antigens for use in immunisation studies (Hanke *et al.*, 1992; Randall *et al.*, 1994; Hanke *et al.*, 1994). The two affinity tags employed, His and Pk, were not particularly immunogenic, even when attached to small proteins (such as p17) (Randall *et al.*, 1993) and thus it may not be necessary to remove either of the tags for vaccination purposes (Randall *et al.*, 1994).

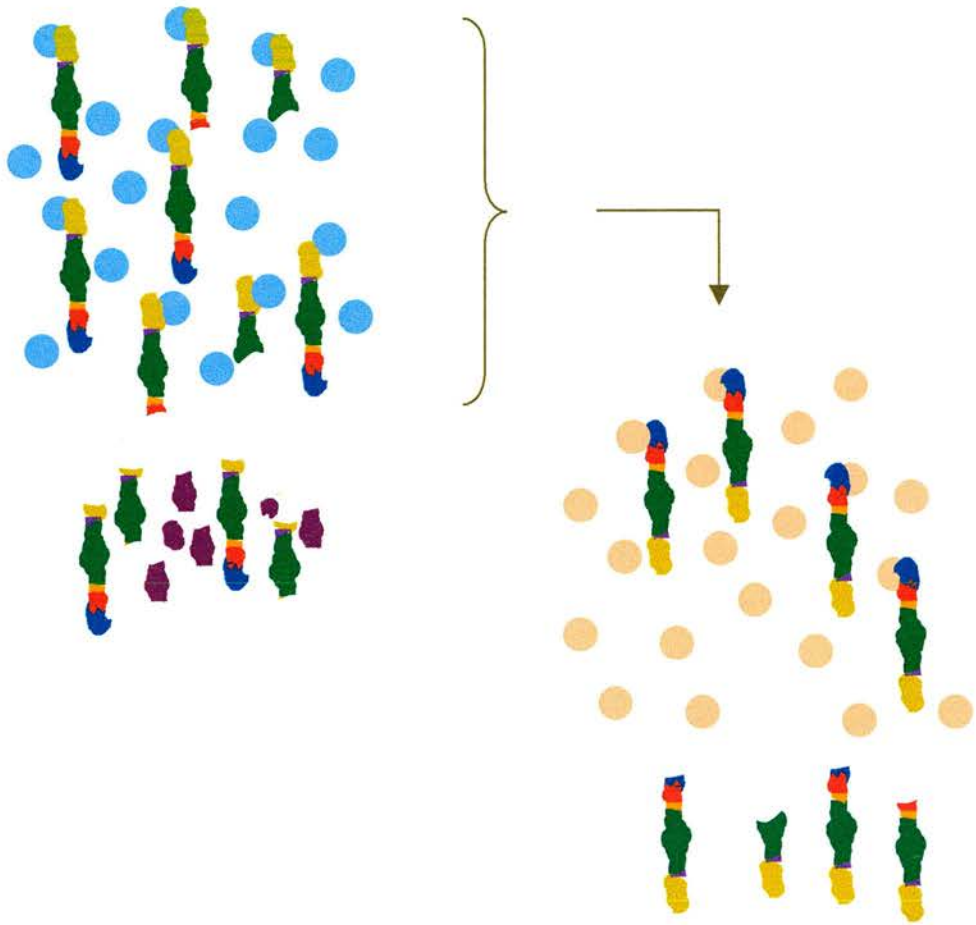


Figure 8: Schematic illustration of the principle of two-step affinity purification.

The left part of the drawing shows affinity purification of protein containing an affinity tag, as already presented in Figure 7: the yellow end of the protein shows affinity for the blue bead matrix, so all contaminants and proteins lacking a functional yellow end are lost through washing.

The specifically bound protein, containing both full-length and abrogated molecules, is then eluted and applied onto a second affinity matrix. The second matrix (depicted as pink beads) is selected for affinity purification on the basis of a tag found at the opposite end of the molecule – the blue end.

Application of the partially purified protein onto the second affinity matrix (right side of drawing) allows specific retention of molecules with an intact blue end, while all protein with an abrogated blue end flows through.

Elution of the protein specifically retained on the second affinity matrix results in a clean preparation of full-length protein. Two-step affinity purification can be employed for the selection of full-length molecules protein found between two affinity tags.

2.6. AIM AND OBJECTIVES OF THIS WORK

2.6.1 Work that led to the present study

In our research group there is an ongoing effort to develop and improve molecules and methods for efficient immunisation. During previous studies, a variety of proteins from SIV was incorporated in SMAA (Solid Matrix Antigen-Antibody) complexes. One approach involved the capture of Pk-tagged antigens on solid matrix that was saturated with a Pk-specific antibody (Randall *et al.*, 1994). Another approach included an LTB-derivative protein whereby the antigens were linked to the LTB adjuvant/carrier through an antibody bridge (Green *et al.*, 1996). The LTB-carrier system was later developed further to allow specific chemical coupling of antigens (O' Dowd *et al.*, 1999) and an experimental LTB-antigen complex induced good antibody responses when delivered intranasally to mice (O' Dowd *et al.*, 1999). The potential generation of CTL responses by this molecule was not investigated.

In a step further, it was desirable to test the efficacy of the LTB carrier in generating immunity to a medically important antigen and to determine whether the LTB-antigen complexes were capable of inducing specific CTL responses. The SIV-proteins that were previously incorporated in SMAA complexes and successfully employed in animal immunisations, were available to us and they presented a good choice for testing the LTB carrier. As we were particularly interested in the generation of cellular immunity, the regulatory proteins of SIV presented more suitable candidates than its structural proteins. Tat protein from SIVmac was therefore selected, to be tested on the LTB carrier, for the reasons

presented in part 2.4. However, as it will be discussed later, recombinant Tat protein dispensed the need for an antigen-carrier, so it was instead evaluated in immunisation studies in a soluble form, or precipitated on the adjuvant alum.

2.6.2 Aim and Objectives of this work

The aim of this study was to assess the immunogenicity of Tat protein from SIVmac251(J5) and to investigate whether it could generate protective immune responses against lentiviral infection in vaccinated animals.

In order to prepare Tat protein for animal immunisations, the protein would first be engineered in a suitable expression system that allows high levels of expression and ease of purification. Subsequently, the recombinant protein would have to be purified to a grade acceptable for use in immunisation studies. Work towards that goal would include the development of a Pk-affinity purification method for Pk-tagged recombinant proteins.

Recombinant Tat protein would then be dissociated from any affinity tags employed for its purification, and it would be delivered to experimental animals in a suitable form (such as coupled to the antigen-carrier LTB) to generate immune responses. The immunogenicity of Tat would then be assessed in mice and in monkeys, with particular interest in the generation of antigen-specific CTL immune responses.

3. METHODS

The various proteins utilised in this study were engineered to contain affinity tags, such as the Glutathione-S-Transferase (GST, Pharmacia), a 6-Histidines tag (His) and the 14-amino acid-long affinity tag Pk (characterised in our lab (Southern *et al.*, 1991; Dunn *et al.*, 1999)). The affinity-tagged proteins were produced in suitable bacterial cultures and they were isolated on a suitable affinity-matrix.

The following sections describe how the protein expressing vectors were engineered (3.1) and how the proteins were isolated and characterised (3.2) and further employed in animal immunisations (3.4) or towards an affinity purification strategy employing Pk (3.3).

3.1 DNA CLONING AND PROTEIN EXPRESSION

The proteins required during this work were engineered using standard procedures (Sambrook *et al.*, 1989), of which some selected methods are described below. In each cloning strategy, the cloning vector and the potential insert were prepared (sections 3.1.2 & 3.1.3), ligated to each other (3.1.8) and the new DNA plasmid was used to transform competent bacterial cells (3.1.10). Potential transformant clones were screened (3.1.11) and the DNA they contained was sequenced (3.1.12). The successfully engineered proteins were then expressed in bacterial hosts, as described in section 3.1.13. For a brief description of all the proteins employed in this work, refer to Appendix 7.1.

3.1.1 Materials

The vectors used were either pGEX-2T vector (Pharmacia; Smith & Johnson, 1988), or its derivative pGEXcPk vector, engineered by Tom Hanke (Hanke, 1993). In those vectors, protein expression lies under the control of the Lac promoter (based on the Lac operon of *Escherichia coli*). IPTG treatment of the bacterial culture harbouring such plasmids yields high levels of the protein of interest, expressed as a GST-fusion. These vectors are retained in the bacterial populations by ampicillin (amp) selection.

For the different protein engineering projects, DNA sequences coding for the polypeptides or proteins of interest were inserted into the pGEX-based vectors using one of the strategies described in section 3.1.3. Detailed descriptions of each cloning strategy accompany the relevant diagrams in the Results chapter, while the exact sequences of vectors and inserts are included in the Appendix. The sequencing primers and all the single-stranded oligonucleotides (oligos) used in cloning were obtained from Oswel DNA Service (Southampton, UK). Their full sequences are presented in Appendix 7.10. The enzymes used for recombinant DNA work (restriction enzymes, T4 DNA-ligase, Alkaline Phosphatase) and the relevant reaction buffers were purchased from New England Biolabs or Promega.

The microorganisms used in the transformation experiments were *Escherichia coli* (*E.coli*) strains DH5α, XL1-blue and B834. DH5α and B834 cells (no antibiotic resistance) were mainly used for cloning and for protein expression. B834 cells were preferred for expression studies as they lack some proteases normally encoded by *E.coli*. XL1-blue cells (tet resistant) were used for

long-term storage (10% glycerol bacterial stocks) since they protect the transfected DNA from accumulation of mutations.

Liquid bacterial cultures were grown at 37 °C, shaking at 200 rpm in Luria Bertani (LB) medium [per 1 l: 10 g bacto-tryptone (Difco), 5 g yeast extract (Difco) and 10 g NaCl, at pH7.5]. Selection plates were prepared using LB agar (as above, supplemented with 1.5% agar (Difco) and 10M $MgSO_4$). Where appropriate, ampicillin (amp) was employed at 100 μg/ml concentration and tetracycline (tet) at 50 μg/ml.

3.1.2 Preparation of cloning vector

Plasmid DNA was obtained from the bacterial hosts using the QiaGen mini-prep extraction kit, according to the manufacturer's instructions. DNA vector was cut open with the appropriate restriction enzyme(s) to create cohesive ends with the respective insert. Typically, 1-10 μg of DNA was digested in a 10 μl reaction with 0.2-5 u of each restriction enzyme in the presence of the appropriate buffer. The restriction reaction was allowed to progress for 4 h. During the last 1 h, the vector was dephosphorylated to avoid self-ligation, by the addition of Alkaline Phosphatase (CIAP): 0.5 u every 15 min.

3.1.3 Preparation of insert

Depending on the cloning strategy, the potential insert was produced or obtained in one of the following ways:

- **Insert obtained from plasmid vector**

Plasmid DNA containing the fragment of interest was isolated from bacterial cell cultures. The DNA fragment was excised by restriction digestion using the appropriate enzymes (as above) but it was not dephosphorylated.

- **Insert produced by PCR amplification**

DNA vector containing the fragment required was submitted to amplification by Polymerase Chain Reaction (PCR) using appropriate amplification primers (details on PCR conditions are given in 3.1.4). It was then treated with restriction enzymes as described above.

- **Insert constructed by hybridisation of single-stranded oligos**

Suitable oligonucleotides (oligos) were designed and obtained. 60 μg of forward and 60 μg of reverse oligo were mixed in the presence of 0.1M NaCl. The mixture was incubated at 95 °C for 5 min and allowed to slowly cool down to room temperature (RT). Once hybridisation was established on a 1.5% agarose electrophoresis gel, the double stranded DNA molecules were digested to form cohesive ends with the linearised vector.

Both linearised vectors and DNA fragments to be used as inserts were purified by Gel Extraction (3.1.6) prior to ligation (3.1.8).

3.1.4 PCR and overlapping PCR

PCR reaction mixtures of 100 μl consisted of DNA polymerase buffer (containing 1.5mM $MgCl_2$), 0.2mM of each dNTP, 2-10 μM of each primer (forward and reverse) and 1 u of DNA polymerase enzyme. When replication accuracy was important, rTth (Perkin Elmer) polymerase was employed due to its proofreading activity, while DyNAzyme (Finnzymes) polymerase was used when the focus was on speed and quantity. Approximately 1 μg of DNA template was used per reaction. For overlapping PCR, the two templates were added at 1μg amounts each.

The PCR conditions used almost invariably were 30 cycles of 94 °C for 30 sec, 55 °C for 1 min and 72 °C for 30 sec. A 10 min preheating at 94 °C and a 7 min final extending at 72 °C were routinely included. For overlapping PCR, the annealing temperature used was 61 °C.

The PCR products to be used as templates in overlapping PCR were first purified on QiaGen columns and subsequently treated with DpnI (1 u for every 10 μl reaction for 1h at 37 °C) to destroy any persisting paternal DNA. Lastly, fragments were purified by Gel Extraction (section 3.1.6) and suspended in Reverse Osmosis (RO) water.

3.1.5 Agarose gel electrophoresis

DNA samples were analysed by horizontal agarose gel electrophoresis on $10cm^2$ minigels (Bioscience 101). Agarose gels were prepared as indicated by Sambrook and colleagues (1989) using agarose powder in electrophoresis buffer TBE (Tris-

Borate 45mM / EDTA 10mM) to agarose contents of 0.5-2%. Ethidium bromide was added to the positive pole of the electrophoresis tank at a final concentration of 0.5 μg/ml). Samples were mixed with 6x DNA loading dye (Promega) and loaded into the wells. The DNA marker used was the Promega 1 kb ladder. The DNA samples were electrophoresed at constant voltage of 70V and visualised under Ultra Violet light.

3.1.6 Gel extraction

In order to purify cut DNA fragments, the digestion reactions were first run on 1% agarose (see above). The band of interest was then excised with a clean scalpel and recovered from the gel using a QiaGen Gel Extraction kit according to the vendor's instructions. DNA was eluted in RO water and stored at -20 °C until required.

3.1.7 DNA precipitation

To optimise the function of restriction endonucleases and DNA modifying enzymes, DNA preparations were incubated in the buffers considered most suitable for each treatment. Thus, at various stages, a change in buffers or salt content was required. To that end, DNA fragments were precipitated by adding to the DNA solution 2.5 volumes of absolute alcohol, 0.1 volumes NaOAc 3M pH5 and 0.1 volumes glycogen (Boehringer-Mannheim). After 1 h incubation at -70 °C and 30 min centrifugation at 10000xg, 4 °C, the DNA pellets were air dried and resuspended in the required volume of RO water or buffer.

3.1.8 Ligation

Aliquots of linearised vector and extracted insert(s) were first analysed on 1% agarose to determine the relative DNA amounts to be used in the ligation reactions. Each 10μl ligation reaction contained about 400 ng vector and 40, 200 or 400 ng insert, mixed with 1000 u T4 DNA ligase and 10x ligase buffer. Ligation reactions were incubated at 14 °C for 16 h.

3.1.9 Preparation of transformation competent cells

E.coli strains DH5α, XL1-blue and B834 were prepared to incorporate the DNA constructs by Heat Shock transformation or Electroporation in the following ways:

- **For heat-shock transformation**

100 ml culture of *E.coli* was grown to optical density of 0.5 at 600 nm. It was then chilled on ice for 15 min and the cells were pelleted by centrifugation at 800xg for 10 min, 4 °C. Pellets were resuspended in ice cold, TFB1, subsequently incubated on ice for 30 min and centrifuged again. Cell pellets were then resuspended in 2-4 ml TFB2, were freeze-dried in 100 μl aliquots, and stored at -70 °C.

Solutions: TFB1: 30mM KOAc / 10mM $CaCl_2$ / 50mM $MnCl_2$ / 100mM PbCl / glycerol 15% / pH adjusted to 5.8 with AcOH 1M. TFB2: 10mM MOPS pH6.5 / 75mM $CaCl_2$ / 10mM RbCl / glycerol 15% / pH adjusted to 6.5 with 1M KOH. Both solutions were filter-sterilised before use and stored at 4 °C.

- **For electroporation**

1 l culture of *E.coli* was grown to optical density of 0.5 at 600 nm. The culture was chilled on ice for 30 min and the cells were pelleted by centrifugation at 6500xg for 15 min, 4 °C. Pellets were resuspended in 250 ml of ice cold, sterile Hepes buffer 1mM, pH7, and centrifuged as before. They were then resuspended in 125 ml of Hepes (as above) and re-centrifuged. The new pellets were resuspended in 20 ml of cold, sterile 10% glycerol and centrifuged at 1000xg for 15 min. Pellets were finally resuspended in 2-3 ml of 100% glycerol, were freeze-dried in 250 µl aliquots and stored at -70°C.

3.1.10 Transformation

Transformation-competent *E.coli* cells were treated to incorporate the foreign DNA plasmids as described below:

- **Heat-shock transformation**

100 µl of *E. coli* Heat Shock competent cells were mixed with each ligation reaction and kept on ice for 30 min. They were heat shocked at 42 °C for 1 min and chilled on ice for 2 min. They were supplemented with 1 ml LB broth and incubated at 37 °C shaking for 1 h. Using a glass spreader, about $1/10^{th}$ of each reaction was plated on LB agar plates containing the appropriate antibiotics for selection. The plates were incubated at 37 °C for 16 h.

- **Transformation by electroporation**

The ligation reactions were spun briefly and the DNA was precipitated as described in section 3.1.7. Each precipitated ligation reaction was mixed with 40µl electrocompetent *E.coli* cells in a chilled 2 mm electroporation cuvette (Flowgen). The cuvette was placed in the electroporator chamber (EasyJect Plus - Flowgen) and given a 5 msec pulse at 2.5 kV. The cells in each cuvette were supplied with 1 ml LB and approximately $1/10^{th}$ of each ligation reaction was plated on selection plates as previously described. Colonies were allowed to grow on the plates for 16 h at 37 °C.

3.1.11 Screening of transformants

Colonies obtained on plates were screened for successful incorporation of insert using one of the following strategies:

- **Plasmid extraction and digestion**

Colonies growing on selection plates were amplified in liquid cultures and their plasmid DNA was extracted (QiaGen Mini-Prep kit). The DNA was treated with appropriate restriction enzymes to release the insert employed, or a suitable fragment that would confirm the incorporation of the insert into the cloning vector.

- **PCR amplification**

Colonies were picked and the DNA fragment of interest was amplified by PCR. Each colony was directly added to a 100 μl PCR reaction mix containing DNA polymerase, buffer, primers and dNTPs (refer to section 3.1.4.) and the DNA stretch of interest was amplified using standard conditions (3.1.4). The PCR products were digested with appropriate restriction enzymes and analysed by agarose gel electrophoresis.

- **Mini expression gel & blot**

In those cases where agarose gel analysis of the plasmids could not distinguish between successful recombinants and untransformed vectors, liquid cultures of the colonies were induced to express the protein encoded by the plasmid they harboured. The total cell protein of each culture was then analysed by SDS gel electrophoresis. The presence of the protein of interest was detected by Coomassie staining of the gel, or by Western Blotting, using suitable antibodies.

- **Radiolabelling**

The GST-PkHis mutants were screened for the presence of the Pk tag using a radiolabelled DNA probe. Colonies and appropriate controls were streaked on an LB agar amp plate and on a Hybond Membrane placed onto LB-agar amp medium, following the same streaking pattern between the two plates. The plates were incubated for 16 h at 37 ^{o}C for the colonies to grow.

The cells on the Hybond membrane were lysed by treatment with NaOH 0.5M / NaCl 1.5M for 5 min and equilibrated with Tris-HCl 0.5M, pH8.5 / NaCl 1.5M for 10 min. When the membrane was dried, the DNA was fixed by UV-irradiation for 2 min. The membrane was then placed in the hybridisation chamber at 65 °C in 25 ml pre-hybridisation solution (6x SSC / 5x Denhards solution / SDS 0.5% / $Na_4P_2O_7$ 0.05%). After 30 min the solution was replaced by 25 ml hybridisation solution (6x SSC / 5x Denhards solution / $Na_4P_2O_7$ 0.05%). [SSC: Salt-Sodium Citrate buffer. For 1 l of 20x SSC: 175.3 g NaCl and 88.2 g $C_6H_5Na_3O_7$, at pH 7.]

DNA probe was end-labelled with ^{32}P by incubating 1 µl probe DNA, 1 µl T4 polynucleotide kinase (Promega), 1 µl of the supplied 10x enzyme buffer, 2 µl RO water and 5 µl (50 µCi) γ-d-ATP for 1 h at 37 °C. The probe mixture was dispensed into the hybridisation roller and was left to hybridise for 16 h.

Excess probe was removed with two 5x SSC / SDS 0.01% and one 2x SSC / SDS 0.01% washes. The membrane was then incubated with a blanked phosphorimager screen for 1h, and the screen was read using MacBas Image Reader (Fuji Film).

- **Blue-white selection for pGEM-T-easy**

pGEM-T-easy vector (Promega) was employed to directly clone a PCR product. Following transformation, the cell suspension was plated on LB-Agar plates containing 0.5mM IPTG and 80 µg/ml X-Gal. When the colonies were fully-grown, it was possible to distinguish colonies containing the insert (appearing white) from those that contained self-ligated vector (blue). The plasmids were

extracted and the DNA sequence was further examined using one of the techniques described above.

3.1.12 DNA sequencing

Following the initial screening of transformant clones, plasmid DNA from successful transformants was sequenced to ensure it contained the expected DNA sequence. The sequencing reactions contained 1 μg of the DNA template to be analysed and 1μM of the sequencing primer in a total volume of 12 μl. Forward and reverse sequencing primers were designed for the pGEX vector family (see Appendix 7.2). Double-stranded DNA templates were analysed by automated sequencing (Perkin-Elmer ABI Prism 377 DNA sequencer) and interpreted using EditView, DNAStrider and GeneJockey computer software and the BLAST/ NCBI homology database.

3.1.13 Protein expression and cell disruption

Bacterial cultures harbouring the plasmid of interest were grown at 37 °C shaking, to optical density of 0.7 at 600 nm. They were then induced with 0.1mM IPTG for 3 to 4 h at 25 °C. Bacteria were pelleted by a brief centrifugation at 6500×g and pellets were stored at -20 °C for 16 h, or until required.

Pellets were resuspended in a small volume of purification Wash Buffer (see section 3.2.1). Lysozyme was added to a final 10 mg/ml if cells were disrupted by sonication, whereas it was omitted if the cells were burst by passing through the cell disruptor ("French Press"). Triton-X was added to the total cell

extract at 1% final concentration and the suspension was centrifuged at 20000xg for 30 min.

The spin supernatant, representing the soluble antigen, was subsequently applied to the appropriate affinity column. Depending on the affinity tag included in the protein of interest, the appropriate purification strategy (section 3.2.1) or affinity capture (3.2.2) was employed.

3.2 Protein Purification and Characterisation

Following expression in the bacterial hosts, the engineered proteins were captured (3.2.2) or purified (3.2.1) on suitable affinity matrices. Where appropriate, proteins were cleaved with Thrombin (3.2.3) or the Adenovirus Protease (3.2.4). At various stages of the preparations, the protein samples were visualised by SDS-electrophoresis (3.2.6) and immunoblotting (3.2.7). Specific binding properties of proteins were assessed by ELISA (3.2.8).

3.2.1 Affinity-matrix protein purification

For affinity purification of Glutathione-S-transferase (GST) tagged proteins, Glutathione immobilised on cross-linked 4% beaded agarose (Sigma) was used. For proteins bearing the 6-Histidines tag (His), NiNta-Agarose (sepharose CL-6B, cross-linked 6% agarose) (QiaGen) was employed.

The required volume of affinity matrix was first washed in Wash Buffer. For GST-affinity purification the Wash Buffer was PBS / NaCl 0.5M, while it was

Tris 20mM pH 7.8 / NaCl 0.3M / imidazole 10mM, for the His-affinity purification. [PBS: Phosphate Buffered Saline, Tris: 2-amino-2-2(hydroxymethyl) propane-1,3-diol]. The washed affinity matrix was cast in a disposable column and equilibrated with Wash Buffer, ensuring it was kept moist at all times.

The soluble antigen obtained as described before was then applied to the affinity column twice, and the column was extensively washed with the appropriate Wash Buffer to remove all non-specifically bound proteins.

The fraction of specifically bound proteins was then eluted with the relevant Elution Buffer. The Elution Buffer for GST purification was: Tris 0.05M, pH8 / NaCl 0.5M with 0.2 g NaOH and 1.54 g glutathione (Sigma) for every 500 ml of buffer. The Nickel-column Elution Buffer was Tris-HCl 20mM, pH 7.8 / NaCl 0.3M / imidazole 50mM.

The eluted fractions containing concentrated protein were pooled and transferred into dialysis tubing with a 12k-14k MW cut-off point. Protein was dialysed for 3 to 4 h with frequent changes, in either GST Elution Buffer lacking glutathione or in Nickel purification Wash Buffer accordingly.

Dialysed protein was spun to eliminate aggregates at 800xg for 10 min. It was then filter sterilised through a 32mm Acrodisc 0.2 μm filter and stored in 500μl aliquots at -20 °C.

When a single affinity purification step was not adequate, a second purification step was added, employing a different affinity tag. Proteins were first purified on the basis of one affinity tag and the partially purified protein was subsequently applied to a second affinity matrix, either full-length, or after an intermediate step of thrombin cleavage (see later, section 3.2.3).

3.2.2 Immune precipitation

Pk-tagged protein was mixed with an excess of anti-Pk monoclonal antibody (mAb) and incubated at 4 °C; mixing for 1 h. Unbound mAb was removed with 3 washes by resuspension in Phosphate-Buffered Saline (PBS) and centrifugation at $1400_{x}g$ for 3 min.

Staphylococcus aureus (Cowan A strain) in the form of a fixed suspension was added to the protein-mAb solution and incubated at 4 °C mixing for 1 h. *S. aureus* A, coated with antibody and antigen, was pelleted by a 3min centrifugation at $1400_{x}g$. The cell pellet was washed as above with Immune Precipitation Buffer (IPB: NaCl 0.65M / Tris-HCl pH7.8, 20mM / EDTA 1mM / NP40 0.5% / NaN_3 1%) and was finally resuspended in a small volume of IPB.

Alternatively, *S. aureus* was substituted by an appropriate amount of Protein A immobilised on cross-linked 4% beaded agarose (Sigma).

3.2.3 Thrombin cleavage

Generally, 480 μg of a GST-fusion protein is cleaved with 1 u of Thrombin (Sigma) at room temperature (RT) over the period of 3 h. Adjustments were made for the different GST-fusion proteins: GST-Tat-APCS-His-Pk required 1 u for full cleavage of 15 μg at RT, 1 h. The protein was usually cleaved in GST Wash Buffer or in GST Elution Buffer (as described earlier, in 3.2.1).

3.2.4 Adenovirus protease cleavage

The buffer for the Adenovirus Protease cleavage was prepared fresh on the day to ensure full activity of the β-mercaptoethanol. GST-Tat-APCS-His-Pk protein was cleaved in 50 µl of freshly prepared Protease Buffer (Tris-HCl 50mM / EDTA 10mM / β-mercaptoethanol 2mM) for up to 2 h at 37 °C. For every 21 µg of protein, 20 µl of Adenovirus Protease and 20 µl of the Activating Peptide (135 µg/ml stock) were required.

3.2.5 Protein quantification

Most protein preparations contained only partially purified protein. The presence of protein contaminants did not allow for protein quantification by conventional methods (direct absorbance measurement, Bradford's assay etc.). Therefore, in some protein solutions, an estimate of the concentration of the protein of interest could only be obtained by gel electrophoresis analysis: by comparing the intensity of the band of interest to that of protein bands of known concentration.

3.2.6 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

In order to visualise the protein preparations, samples were electrophoresed on SDS-Polyacrylamide gels cast into Mini Protean System gel rigs (BioRad).

The separating gel mix consisted of 0.75M Tris (2-amino-2-2 (hydroxymethyl) propane-1,3-diol), 0.1% SDS (Sodium Dodecyl Sulphate) and the appropriate volume of 30% acrylamide-bisacrylamide solution (Severn

Biotech) for the acrylamide content required. The stacking gel contained 0.25M Tris, 0.1% SDS and 5% polyacrylamide. 4.2 μg APS (ammonium persulphate, Fisher) and 5 μl TEMED (N,N,N',N'-TEtraMethylEthyleneDiamine, Sigma) were added to every 6 ml of separating or stacking gel mix.

Prior to loading, the polypeptide samples were disrupted by boiling for 5 min in SDS-Lysis Buffer (Tris-HCl 50mM / SDS 0.2% / β-mercaptoethanol 5% /glycerol 3% / bromophenol blue 0.1%). Samples were electrophoresed in SDS-Electrophoresis Buffer (Tris 25mM / glycine 192mM / SDS 0.1%) at 222 V for 40-65 min.

Gels were stained with Coomassie blue (methanol 20% / acetic acid 10% / Coomassie Brilliant Blue R-250 1% (BDH)) and de-stained with methanol 20% / acetic acid 10%. If they were prepared for Immunoblotting, gels were instead immersed in Transfer Buffer (see section 3.2.7).

3.2.7 Immunoblotting

Protein samples were first ran on SDS-PAGE gels. After a short immersion in Transfer Buffer (Tris 25mM / glycine 192mM / 20% methanol) those gels were assembled in Trans-Blot Cell (BioRad) transfer units. The transfer units were then submerged in gel tanks filled with Transfer Buffer. The samples were transferred to nitrocellulose membrane by applying constant current of 200 mA for 1 h, or 40 mA for 16 h.

The nitrocellulose membrane was then rinsed with RO water and soaked in Blocking Solution (5% skimmed milk powder in PBS) for 1h. The presence of the protein of interest was detected with a specific anti-protein antibody (Ab). The

membrane was incubated in an appropriate dilution of that Ab in PBS / 1% milk for 1h, at RT, agitation. A detailed account of the Abs used can be found later, section 3.2.9.

Non-specifically bound Ab was eliminated by three washes with PBS / 0.05% Tween 20 (Polyoxyethylene Sorbitan Monolaurate, Sigma). The reaction between the protein and the primary Ab was detected with a HorseRadish Peroxidase (HRP) conjugate secondary Ab. The membrane was incubated in the secondary Ab solution as described above for the primary Ab.

After three washes with PBS / Tween 20, the HRP-linked Ab was detected in an Enhanced ChemiLuminescence (ECL) reaction (equal volumes of ECL1 and ECL2 solutions, see below). The membrane was exposed to X-ray film (Fuji) for a period of a few seconds to several minutes and was then developed through a Kodak M35 X-OMAT autoradiography processor.

ECL solutions were made as follows: i) 100 ml ECL1: 1 ml Luminol (250mM in DMSO), 0.44 ml p-Coumaric acid (90mM in DMSO), 20 ml 0.5M Tris pH8.5, RO water; ii) 100 ml of ECL2: 64µl 30% hydrogen peroxide, 20 ml 0.5M Tris pH8.5, RO water.

3.2.8 Enzyme-Linked ImmunoSorbent Assay (ELISA)

This assay was performed in flat-bottom 96-well microtitre plates (Dynex Technologies). All incubations were performed at room temperature (RT) for 1h. Solutions were added to the plates at a standard volume of 100 µl per well. The sequence of steps was usually the following: plate coating, washing, blocking of

excess binding sites, incubation with primary antibody (Ab), washing, addition of secondary Ab, washing and developing.

Plates were coated with protein diluted in Phosphate Buffered Saline (PBS) at 4 °C, 16 h. Wells were blocked with 5% skimmed milk powder in PBS by flooding the plate. Proteins, sera and antibodies were added at appropriate dilutions in PBS/ 1% milk.

Washes were performed by flooding the plate in PBS or PBS / 0.5% Tween 20 and inverting and vigorously shaking to empty wells. The usual practice was three PBS / Tween 20 washes followed by two PBS washes. Excess liquid was drained on tissue.

Plates were developed using a mixture of equal amounts of TMB (3,3',5,5'-Tetra MethylBenzidine) Peroxidase substrate and Peroxidase solution (KPL). The reaction was stopped with 1M HCl and the plates were read at 450 nm in a Dynatech MR 5000 ELISA plate reader.

3.2.9 Antibodies

In methods described above (Immune precipitation, Immunoblotting, ELISA) and in methods detailed later (Immunostaining, protein dotting on Nitrocellulose membrane) a range of antibodies was employed. Details of the origin and working dilutions of those antibodies are given below:

The monoclonal anti-Pk tag antibodies, Pk1, Pk2, Pk3, Pk4 and Pk5 were previously generated in our laboratory (Southern *et al.*, 1991; Dunn *et al.*, 1999). The mouse monoclonal anti-GST antibody was obtained from Sigma. Purified mouse monoclonal penta-His specific antibody came from QiaGen.

Antibody concentrations were previously optimised in our laboratory, so they were empirically used at the following dilutions for Immunoblotting: Pk1 at 1/10k, Pk2 and Pk5 at 1/2k, Pk3 and Pk4 at 1/100. Adjustments were made for use in ELISA. Antibodies anti-His and monoclonal anti-GST were used at concentrations recommended by their manufacturers.

The peroxidase conjugate antibodies employed as secondary antibodies were all supplied by Sigma: goat anti-mouse IgG, rabbit anti-goat IgG, rabbit anti-monkey IgG. They were used at 1/2k in ELISA and 1/1k in Immunoblotting.

3.3 Pk-Affinity Purification

In an attempt to devise an affinity purification schedule using the Pk tag, the binding of antibodies Pk1-Pk5 to GST-fused modified Pk tags was examined. A multitude of binding and elution conditions was tested by ELISA (section 3.3.1) and on Protein-coated Nitrocellulose membranes (3.3.2).

3.3.1 ELISA for anti-Pk antibodies binding to modified Pk tags

In general terms, the assay was performed as described in section 3.2.8. More specifically, the GST, GST-Pk or GST-PkHis proteins were bound on the ELISA plates in carbon / bicarbonate buffer, pH 9.6 for 16 h at 4 °C. The plates were washed and blocked with 5% milk in Phosphate-Buffered Saline (PBS). Primary Ab (Pk1-Pk5) was added at 1/2k in PBS / 1% milk (for antibody details refer to

section 3.2.9). The plates were washed again and coated with secondary Ab (goat anti-mouse IgG peroxidase conjugate) 1/2k in PBS /milk as above. They were washed thoroughly and developed. The following two variations were introduced for reasons inherent to the experiments:

- **Looking for disruption of binding**

Primary Abs were bound in PBS / milk 1% but the washes from that step onwards were performed with a variety of solutions. The secondary Ab was added in the same solutions used in the washes, supplemented with 1% milk.

- **Looking for prevention of binding**

Primary Abs were added in a variety of solutions, supplemented with 1% milk. Plates were washed with PBS, and the secondary Ab was added diluted in PBS / 1% milk.

3.3.2 Pk Abs and modified Pk tags interactions on nitrocellulose

A large number of binding and eluting conditions was tested by binding the GST-Pk and GST-PkHis proteins on nitrocellulose membrane and probing with the Pk1-Pk5 antibodies in a similar way as described for Immunoblotting (section 3.2.7). In order to contain GST-Pk and the three GST-PkHis proteins on the same piece of nitrocellulose membrane, two approaches were taken:

- **Multiple loading on SDS-PAGE followed by immunoblotting**

A single-slot 10% acrylamide gel was prepared and immersed in SDS-Electrophoresis Buffer. GST-Pk protein was loaded in the single well at a final volume of 200 µl. Voltage of 170 V was applied for 15-20 min. Then GST-PkHis4,10 was similarly applied to the single well of the gel and electrophoresed for another 15-20 min. The same was repeated for 200 µl of GST-PkHis9 and 200 µl of GST-PkHis11. The gel was then submitted to wet transfer onto nitrocellulose membrane (as detailed in section 3.2.7). The membrane was briefly rinsed in RO water and sliced into 4mm strips that were stored at -20 °C until required.

- **Direct protein dotting on nitrocellulose membrane**

GST-Pk, and the three GST-PkHis proteins were prepared in solutions of similar concentrations and 3 µl of each protein was applied on a dry 4mm strip of nitrocellulose membrane. Dots were kept about 1 cm apart. The strips were left to dry and either used directly as described below, or stored at -20 °C until later.

The nitrocellulose strips were first immersed in 5% milk in PBS for 1h. Subsequently, strips were incubated with the monoclonal anti-Pk Abs (1/2k) under various conditions of salt content and pH, and in the presence of chaotropic agents. They were washed under similar conditions, and secondary Ab (goat anti-mouse IgG peroxidase conjugate) was added at 1/2k in PBS / milk 1%. The strips were developed an hour later by Chemiluminescence, as described in part 3.2.7.

3.4 ANIMAL IMMUNISATIONS

Prior to immunisation, the protein constructs were tested on monkey cells and visualised by Immunostaining (section 3.4.1). Mouse immunisations included intraperitoneal injection of a GST-Tat test protein and a GST control (3.4.2). Monkey immunisations were performed by injection of GST-Tat-APCS-His-Pk protein (3.4.3). The specific antibodies present in the immunised animals' sera were detected (section 3.4.4) and further characterised (3.4.5) using ELISA. The presence of Tat-specific Abs was further confirmed by Immunoblotting (3.4.6).

3.4.1 Immunostaining

For assessing the adherence and internalisation of constructs intended for immunisations, animal cells were employed: Vero cells, a continuous cell line of African Green monkey kidney cells, obtained from Flow Laboratories and a mouse epithelial cell line: BF cells. Cells were routinely grown in tissue culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) from GibcoBRL, supplemented with 10% foetal calf serum (FCS), 50 u/ml penicillin and 50 μg/ml streptomycin, at 37 °C, in the presence of 5% CO_2.

10mm glass coverslips were first coated with cells: coverslips were placed in individual wells of a 24-well plate and incubated with a DMEM / FCS suspension containing 10^5 cells, for 16 h at 37 °C, 5% CO_2. Then, each cell-coated coverslip was treated with 1 μg/ml of a Pk-tagged protein, in DMEM / FCS.

Following a 90 min incubation at 37 °C, 5% CO_2, the cells were supplied with fresh medium (lacking the proteins). After 30-120 min (different time-points

taken), the cell monolayer on the coverslips was fixed by immersing into fixing solution (formaldehyde 5% / sucrose 2%) for 10 min, RT. Coverslips were then washed in PBS / FCS 1% and stored at 4 °C in PBS.

When all time-points were collected, cells were permeabilised with PBS / FCS 1% / NP-40 0.5% / sucrose 10% for 5 min, and washed with PBS / FCS. Each coverslip was incubated with 15 μl of Pk1 antibody (1/500 to 1/1k) for 1 h and washed twice with PBS / FCS.

Coverslips were then treated for 1h with 15 μl of secondary antibody: sheep-anti-mouse IgG conjugated with Texas Red at 1/500, supplemented with DNA-binding fluorochrome DAPI (4,6, diamino-2-phenylindole) at 0.5 μg/ml. Excess Ab was washed off as before. Specifically bound secondary Ab was then fixed onto the cells by treating coverslips with fixing solution as described above.

After three PBS / FCS washes and a brief rinse in RO water, coverslips were mounted (face down) onto a drop of citifluor AF-1 (Citifluor Ltd.) and visualised under a Nikon Microphot-FXA immunofluorescence microscope.

3.4.2 Mouse immunisations

Balb-C mice were injected intraperitoneally with protein suspensions in Phosphate-Buffered Saline (PBS). Each mouse received three doses, each consisting of 500 μl of protein suspension containing 10 μg of protein soluble, or precipitated on alum.

The samples containing alum were prepared as follows: The amount of protein required was suspended in PBS to a final volume of 500 μl. To this suspension, 50 μl of 1M $NaHCO_3$ was added and mixed well. Subsequently, 100

μl of 10% aluminium potassium sulfate (alum) was added and the mixture was centrifuged at 6500xg for 2 min. The pellet formed was resuspended in 3 ml of PBS and was adequate to immunise 6 mice (500 μl per animal, as described above).

Blood was drawn from the mice by making a small incision in the tail, three and six weeks after the first immunisation. Blood samples were chilled at 4 °C for 1 h and were centrifuged at 10000xg for 10 min to isolate the serum fraction. Sera obtained from each of the two bleeds were subsequently tested for the presence of specific antibodies (section 3.4.4). These antibodies were further examined to determine their antibody isotypes (3.4.5).

3.4.3 Monkey immunisations

Monkey immunisations were kindly arranged by Dr Martin Cranage at the Centre for Applied Microbiology & Research (CAMR) in Salisbury. The monkeys employed were Indian rhesus macaques expressing the Mamu A*01 rhesus macaque class I MHC type.

Six male monkeys were given 100 μg GST-Tat-APCS-His-Pk per dose, three doses, by Targeted Lymph Node Immunisation (TLNI) – which is in fact deep subcutaneous injection in a region of interior iliac nodes, as defined by Lehner and colleagues (Lehner *et al.*, 2000).

Blood samples were taken 10 weeks after the first immunisation and were analysed against pre-immunisation control samples, for the presence of specific antibodies to the injected protein, as detailed in sections 3.4.4 and 3.4.6.

3.4.4 ELISA for detection of specific antibody

The ELISA assays were generally performed as described earlier, in 3.2.8. In more detail, plates were first coated for 16 h with 10 μg/ml of protein. Plates were washed and blocked with blocking solution. Two-fold serial dilutions of mouse or monkey sera (initial dilution 1:50) were performed in the plates. The necessary controls were added and the plates were incubated for 1 h, RT. After washing thoroughly, wells received anti-monkey or anti-mouse HRP conjugate antibody (see 3.2.9) for another hour at RT. After extensive washing, the plates were developed and read at 450 nm, as already described in part 3.2.8.

3.4.5 ELISA for typing specific antibody

For Antibody Typing ELISA, in addition to the Abs detailed in section 3.2.9, a panel of mouse monoclonal antibody isotyping reagents was also employed. Heavy chain specific Abs: anti-mouse IgG1, anti-mouse IgG2a, anti-mouse IgG2b and anti-mouse IgG3, α chain specific anti-mouse IgA and μ chain specific anti-mouse IgM, all developed in goat, were obtained from Sigma. These antibodies were employed in ELISA at 1/1k dilution.

Plates were coated with GST-Pk, washed and blocked as detailed earlier (3.2.8) and mouse sera were applied at 1:50 and diluted two-fold as described in 3.4.4. The wells were washed and primary Ab was added: anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM and IgA Abs were used to distinguish between different Ab isotypes of specific mouse Abs in sera. After 1h incubation, non-specifically bound Ab was removed by careful and extensive washing. Anti-goat HRP

conjugate Ab was added for 1h, RT and washed off. The plates were then developed and read as already described.

3.4.6 Immunoblotting for detection of Tat-specific antibody

To confirm the presence of Tat-specific Ab in the serum of immunised monkeys, Tat protein was bound on Nitrocellulose membrane and the membrane was probed with the animal serum. Serum Abs that specifically bound to the membrane were detected with a polyclonal anti-mouse or anti-monkey Ab.

200 μl of GST-Tat-His-Pk or His-Tat-Pk protein solution was applied to a 12.5% single-slot polyacrylamide gel and electrophoresed at 222 V for 55 min. The gel was then submitted to wet transfer onto nitrocellulose membrane (as detailed in section 3.2.7). The membrane was briefly rinsed in RO water and sliced into 4mm strips that were stored at -20 °C until required.

Nitrocellulose strips were blocked with PBS / 5% milk for 1h, RT. Each strip was incubated with a 1/500 dilution of monkey serum for 1h, RT. Strips were washed with PBS / Tween 20 and incubated in HRP-conjugate anti-monkey antibody (1/2k) for another hour, RT. Strips were finally aligned and developed using Chemiluminescence, as described in section 3.2.7.

The presence of Tat-specific Abs in the monkey serum was confirmed by a light signal on the autoradiography film, indicating the presence of antibodies specific to GST-Tat-APCS-His-Pk or His-Tat-Pk protein.

4. RESULTS

The development of suitable antigen carriers for vaccination purposes has been a long-term project in our laboratory. A variety of antigens have been tested on a range of antigen carriers and adjuvants, to assess their ability to induce specific responses in the immunised animals. Among the antigens employed in these studies, proteins of SIV (Simian Immunodeficiency Virus) were previously included in subunit vaccines. As already explained in the Introduction, protein Tat (Trans-Activator of viral Transcription) is considered a good candidate to be included in a prophylactic vaccine against HIV. The work presented here investigates the potential of Tat protein from SIVmac251 to generate immune responses that could offer protection against lentiviral infection.

A. PREPARING TAT PROTEIN FOR USE IN IMMUNISATIONS

During the course of this work, the sequence encoding SIV-Tat was engineered in a plasmid vector that allows high levels of protein expression and ease of protein purification. Further modifications aiming to improve the yield of protein expression and the degree of purity of SIV-Tat are all presented in Part A of the Results. Once an adequate amount of Tat protein in a suitable form was obtained, a series of immunisations was performed on mice and monkeys as presented in the Results Part B.

4.1 Expression of SIV-Tat as a GST-Fusion Protein

In order for Tat protein to be employed in immunisation studies, it needed to be produced in a form that supported purification of large amounts of protein to a high grade of purity. The DNA sequence coding for SIV-Tat protein from SIVmac32H(J5) was therefore inserted in a pGEX-2T–based vector as detailed in Figure 9. The resulting vector was driving the expression of Tat protein, tagged at the N' terminus with GST protein and at the C' terminus with the affinity tag Pk (details of the affinity tags are included in Introduction 2.5; for the full DNA and protein sequences refer to Appendix 7.6). Following affinity purification, GST-Tat-Pk protein would be treated with the protease thrombin to release the GST portion (Smith & Johnson, 1988) and Tat-Pk would be employed in immunisation studies. In previous immunisations employing Pk-tagged proteins, the immunity generated was not directed primarily against the Pk tag (Hanke *et al.*, 1992; Randall *et al.*, 1993).

Following expression of the protein in bacterial hosts, soluble antigen containing GST-Tat-Pk was applied to a) GST-affinity matrix or b) Pk-affinity matrix. As seen in Figure 10, the protein was equally captured on GST (lane 4) and on Pk (lane 5) affinity matrix. The first observation from this preliminary experiment was that GST-Tat-Pk was being produced in very small amounts. The second observation was that the protein preparation retained on GST matrix contained some proteins other than GST-Tat-Pk, that were absent from the protein sample captured on anti-Pk. It was possible that those proteins represented breakdown products of the full-length protein with an intact N' terminus (GST) that lacked a functional C' terminus (Pk tag).

Figure 9: Engineering DNA coding for SIV-Tat in a pGEX-2T–based vector, to drive the expression of Tat as a GST-fusion protein.

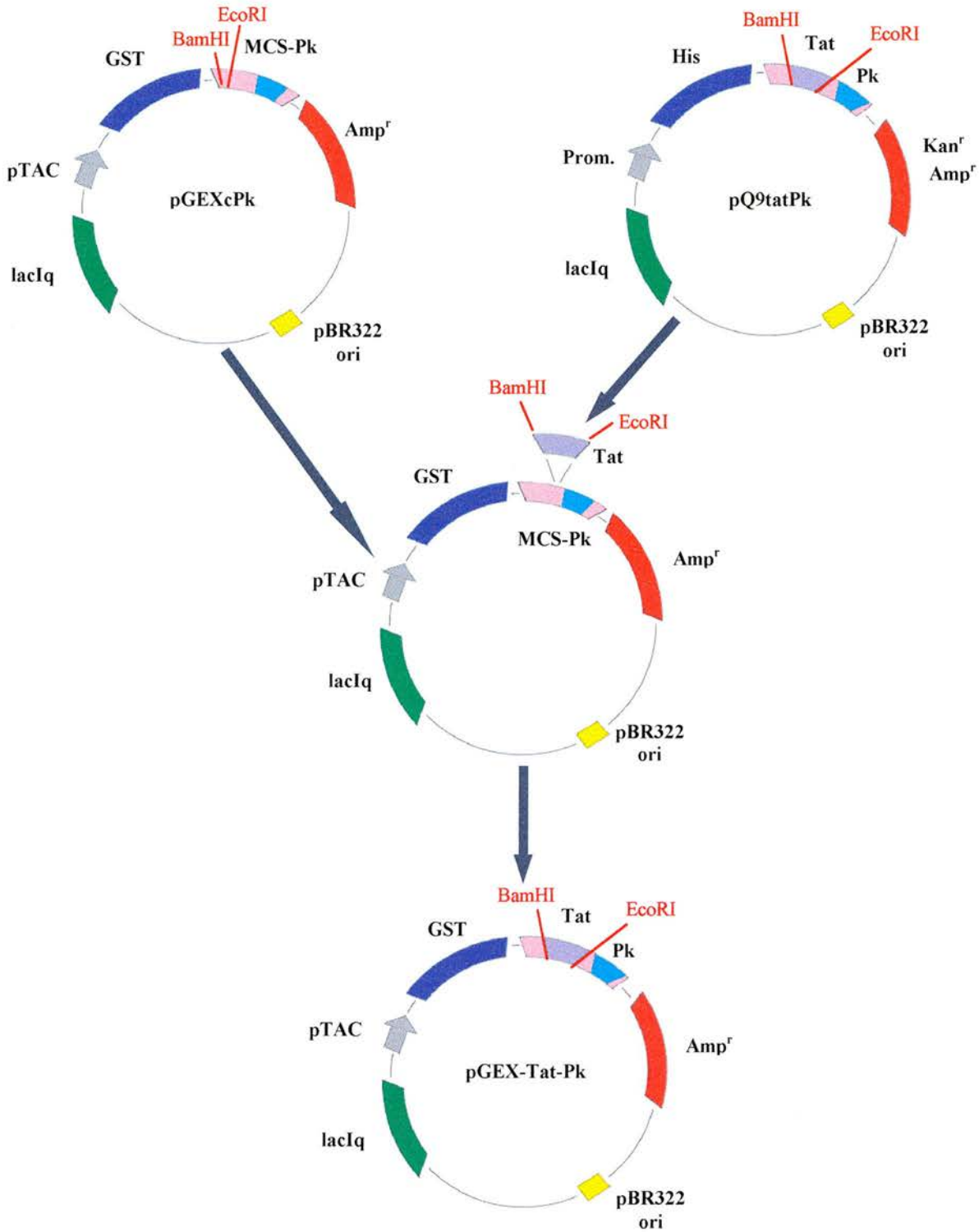
The SIV-Tat insert
The DNA sequence coding for 131 amino acids of SIV-Tat originated from SIVmac32H (pJ5) proviral DNA. It was PCR amplified from an already cloned and spliced gene in pBluescript (Stratagene) (Kornfield *et al.*, 1987) and cloned into a derivative of the pQE9 (QiaGen) vector (Hanke *et al.*, 1994). For the purposes of this work, the insert encoding Tat was isolated from the pQ9tatPk vector (Hanke *et al.*, 1994; top right vector) by double BamHI and EcoRI digestion.

The pGEX-2T –based vector
A pGEX-2T (Pharmacia) vector was previously modified to include the coding sequence for the Pk affinity tag downstream of the cloning site (Hanke *et al.*, 1994). This pGEXcPk vector (top left) drives the expression of a GST protein tagged at the C' terminus with the Pk affinity tag.

Construction of vector pGEX-Tat-Pk
The SIV-Tat encoding sequence was obtained by BamHI and EcoRI treatment of pQ9tatPk vector (top right). pGEXcPk vector (top left) was linearised by digestion with the same restriction enzymes. Both the linearised vector and the fragment coding for Tat were analysed on 1% agarose gel electrophoresis and they were gel purified prior to ligation (Methods, 3.1.5 and 3.1.6). The purified Tat-containing fragment was inserted into the MCS of pGEXcPk (middle) to create vector pGEX-Tat-Pk (bottom), driving the expression of GST-Tat-Pk protein. Full sequences of the DNA and protein encoded are included in Appendix 7.6.

Screening of transformants
Potential transformants were screened by BamHI and EcoRI double digestion to release the insert (as described in 3.1.11) and were analysed on 1% agarose gel electrophoresis alongside a pQ9tatPk vector treated to release the Tat fragment. Further DNA sequencing (part 3.1.12) confirmed the sequence for Tat as a Pk-affinity tagged GST-fusion protein.

Protein expression
The pGEX-2T derivative vector retained the ability to suppress the expression of the GST-fusion protein, unless the pTAC promoter was induced with Isopropyl-β-D-Thiogalactopyranoside (IPTG). Cultures of transformed clones were grown and IPTG-induced to express GST-Tat-Pk protein (detailed in 3.1.13).



Key: VECTORS: pBR322 ori: origin of DNA replication, lacIq: gene coding for lac repressor protein, pTAC: promoter of transcription (IPTG inducible), GST: Glutathione-S-Transferase protein, MCS: Multiple Cloning Site, Amp^r: gene conferring Ampicillin resistance. Prom.: IPTG-inducible promoter, Kan^r: gene conferring Kanamycin resistance, His: 6-Histidines affinity tag, Pk: 14 amino acid affinity tag.

INSERT: Tat: TransActivator of Transcription protein of SIV.

RESTRICTION SITES: BamHI, EcoRI.

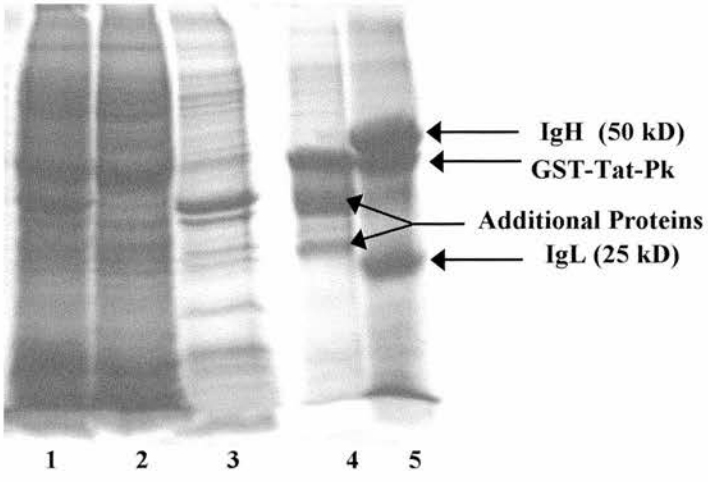


Figure 10: SDS-PAGE analysis (11.5%) of GST-Tat-Pk protein expression in bacterial hosts and its subsequent capture on GST-affinity matrix, or immune precipitation with anti-Pk antibody.

A bacterial culture harbouring vector pGEX-Tat-Pk vector (see Figure 9) was grown and induced to express GST-Tat-Pk protein. The cells were harvested by centrifugation and burst by lysozyme treatment and sonication (Methods 3.1.13). The total cell fraction can be seen in lane 1. The soluble protein fraction (lane 2) was separated from the insoluble fraction (lane 3) by centrifugation (section 3.1.13).

The soluble cell fraction was divided in two equal parts. One part was incubated with GST-affinity matrix (section 3.2.1), the outcome of which is shown in lane 4, while the other was immune precipitated with anti-Pk antibody (section 3.2.2), seen here in lane 5. GST-Tat-Pk protein is detected as a strong band co-migrating in both lanes.

In lane 5, the heavy and light chains of the anti-Pk antibody can be clearly distinguished (IgH and IgL respectively). The presence of additional proteins, other than the full-length GST-Tat-Pk is evident in the sample captured on GST affinity matrix (lane 4), most of which were absent from the preparation that was retained on the anti-Pk antibody (lane 5).

It therefore seemed that GST-Tat-Pk protein could be isolated to a higher degree of purity by Pk-affinity purification. Furthermore, the presence of the two affinity tags on either end of Tat could be used to select for the full-length GST-Tat-Pk protein by two-step affinity purification (on GST and on Pk; as represented diagrammatically in Figure 8). The following sections present the work undertaken to develop a suitable Pk-affinity purification strategy.

4.2 Pk Affinity Purification

Due to the vast application of the Pk technology in our laboratory, there has been a continuous effort to develop an affinity purification strategy for Pk-tagged proteins. The Pk tag was previously successfully employed as the last purification step for Pk-tagged proteins to be included in SMAA complexes (Hanke *et al.*, 1992; Randall *et al.*, 1994; Hanke *et al.*, 1994). However, the interaction between the Pk tag and its specific mAb (Pk1) cannot be disrupted by mild conditions that could be employed for eluting a Pk-tagged protein from a Pk-affinity matrix.

4.2.1 The quest for suitable elution conditions in a Pk-purification protocol

Aiming to determine the conditions where a Pk-specific antibody (Ab) would dissociate from the Pk tag, four anti-Pk mAbs, additional to Pk1, were previously isolated and characterised (Dunn *et al.*, 1999). Unfortunately, the interactions between the Pk tag and the four new antibodies (Pk2, Pk3, Pk4 and Pk5) are also very strong and they cannot be disrupted by mild elution conditions (Dunn 1998; Dunn *et al.*, 1999).

During those studies, the minimal binding epitope for each of the five anti-Pk mAbs was defined (refer to Table 1, part 2). An amino acid (a.a) substitution at positions of the Pk tag that are important but not essential for Ab binding, could reduce the binding of the mAbs to that modified Pk tag. In order to test this possibility, the aliphatic a.a at positions 4, 8 and 11 of the Pk tag (Ile, Leu, Leu respectively, Table 1, part 1) were substituted with Ala, an aliphatic a.a with a small side chain. Ala was selected with the view that it would incur only a small change in the 3-D conformation of Pk.

In fact, the mAbs still bound strongly the Ala-modified tags, suggesting that Ala was not causing an alteration significant enough to reduce Ab recognition (Dunn 1998). It was then proposed that a.a that can change their charge with different pH conditions would be better candidates for substitution studies (Dunn 1998). By altering the pH conditions, the change of charge on such an a.a from neutral to charged would affect the local conformation of Pk (Dunn 1998). It would be thus possible to determine a set of conditions in which the Ab would no longer bind the modified Pk tag, while it would still retain the ability to bind that tag in another set of conditions.

The favourable a.a for such a study was histidine, because of its property to change its charge around the neutral pH range (pK=6.5, Stryer 1995). It was expected that the His substitution would not interfere with Ab binding when His was neutral (pH<6.5), while when charged (pH>6.5) it would distort the Ab epitope and allow the modified Pk tag to elute. During the course of the present study, this hypothesis was tested by substituting a.a found at positions 4, 9, 10 and 11 of the Pk tag with His, as presented below.

Table 1: Amino acid sequences of the original Pk tag and the three His-modified Pk tags. The minimal binding epitopes of the mAbs Pk1-Pk5 on the original Pk sequence are also included.

Part 1 presents the amino acid sequence of the original Pk tag as previously determined in our laboratory (Southern, *et al.*, 1991).

Part 2 displays the minimal binding epitopes for each of the five mAbs to the native Pk tag as determined by Cys and Ala substitution studies (Dunn *et al.*, 1999). The five mAbs raised against the Pk tag (referred to as Pk1-Pk5) recognise slightly different epitopes on the Pk tag. Amino acid residues indicated in bold phase were shown to be required for mAb binding, while amino acids featuring in italic form were thought to be important but not essential for binding. Residues towards the C' and N' termini of the Pk tag were not necessary for Pk1-Pk5 mAb binding.

If amino acids that were important but not essential for Ab binding were substituted in the Pk sequence, the binding regions would be altered in a way that could affect the binding affinity of the mAbs to the modified-Pk tags. By examining the minimal requirements for binding of the five Abs, amino acids found at positions 4, 9, 10 and 11 were selected to be substituted.

In **Part 3**, the original Pk tag sequence is included, the amino acids chosen to be replaced featuring in bold font. Leucine at positions 9 and 11, isoleucine at position 4 and glycine at position 10 were substituted by histidine as detailed in Figure 11.

1. Sequence of Pk Affinity Tag

Position	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Pk tag	gly	lys	pro	ile	pro	asn	pro	leu	leu	gly	leu	asp	ser	thr

2. Minimal Binding Epitopes of mAbs Pk1-Pk5 on the Pk Tag

Pk1 *ile* **pro asn pro leu leu** *gly leu*

Pk2 *ile* **pro asn pro leu** *leu*

Pk3 *ile* **pro asn pro leu leu** *gly* **leu** *asp*

Pk4 **ile** *pro* **asn pro leu leu** *gly leu*

Pk5 *pro ile* **pro asn pro leu leu gly leu**

3. His Substitutions of Amino Acids of the Pk Tag sequence

Pk tag	gly	lys	pro	**ile**	pro	asn	pro	leu	**leu**	**gly**	**leu**	asp	ser	thr
His 9	gly	lys	pro	ile	pro	asn	pro	leu	**his**	gly	leu	asp	ser	thr
His 11	gly	lys	pro	ile	pro	asn	pro	leu	leu	gly	**his**	asp	ser	thr
His 4,10	gly	lys	pro	**his**	pro	asn	pro	leu	leu	**his**	leu	asp	ser	thr

4.2.1.1 Generation and properties of the His-modified Pk tags

Three modified Pk tags were generated to bear His substitutions at positions 9, 11 and 4,10 (Table 1, part 3). Each of the modified tags was engineered into vector pGEX-2T (Pharmacia) as it is detailed in Figure 11. These vectors expressed the modified tags as GST-fusion proteins that were subsequently purified on a glutathione matrix. GST (expressed from unmodified vector pGEX-2T), as well as GST-Pk (where Pk was the native affinity tag, expressed by construct pGEXcPk, as seen in Figure 9) were also available and employed as control proteins for the modified Pk tags. All five proteins can be seen in Figure 12.

Once all three modified Pk tags were obtained, the binding of the five mAbs to the modified Pk tags was assessed by comparing relative absorbance values as measured by ELISA (detailed in 3.2.8).

Table 2: Interaction between the five anti-Pk mAbs and the His-modified Pk tags.

	GST-Pk	GST-PkHis4,10	GST-PkHis9	GST-PkHis11	GST
Pk1	++++	+	–	+++	–
Pk2	+++	++	++	+++	–
Pk3	++	–	–	++	–
Pk4	+	–	–	+	–
Pk5	+++	+++	+++	+	–

Key: -: no binding, +: weak binding, ++: strong binding, +++: very strong binding, and ++++: extra strong binding.

As seen in Table 2, the Pk tag bearing a His modification at position 11 was strongly bound by antibodies Pk1 and Pk2, while Pk5 mAb best recognised the tags modified at positions 9 and 4,10. Antibodies Pk3 and Pk4 displayed only much weaker affinities to the His11-modified tag, so they were used at a much higher concentration (dilution of 1/50 to 1/100) in all subsequent studies.

Figure 11: Schematic representation of the construction of expression vectors pGEX-PkHis: pGEX-PkHis9, pGEX-PkHis11 and pGEX-PkHis4,10.

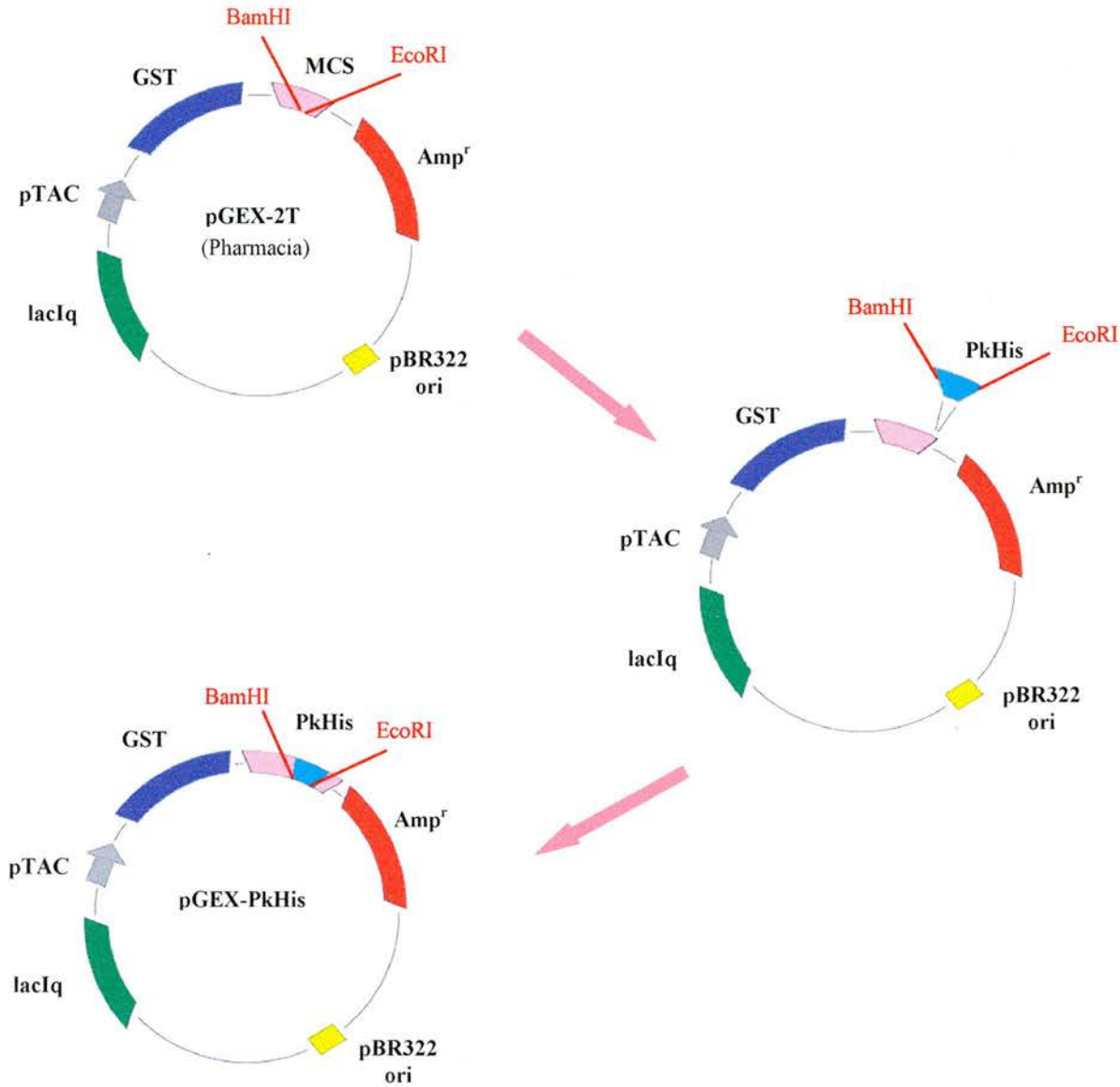
Cloning Strategy
As seen in Table 1, it was planned for the Pk tag to be modified by His substitutions at positions 9, 11 and 4&10. To this end, the full sequence of each His-modified Pk tag was inserted in the pGEX-2T vector (Pharmacia) to generate GST-fusions of the His-modified Pk tags.

Preparation of His-modified Pk tag inserts
Single stranded oligonucleotides (s.s. oligos) for each His-modified Pk tag were designed and obtained (for full sequences refer to Appendix 7.10). Each s.s. oligo contained the required sequence for each DNA strand (forward or reverse) of the modified Pk tags, flanked by BamHI and EcoRI sites. Hybridisation of each set of complementary oligos (Methods 3.1.3) produced a full-length Pk tag, containing the appropriate His substitution(s), between restriction sites BamHI and EcoRI.

Cloning of inserts into pGEX-2T vector
For each of the three His-modified Pk tags, both the double stranded oligo and the Pharmacia pGEX-2T vector (top of Figure) were treated to create cohesive ends for BamHI and EcoRI. Each fragment was then ligated into the linearised vector (middle) to create plasmids pGEX-PkHis (bottom). The three plasmids thus generated: pGEX-PkHis9, pGEX-PkHis11 and pGEX-PkHis4,10, were encoding GST-PkHis9, GST-PkHis11 and GST-PkHis4,10 proteins respectively.

Screening of transformants
Potential transformants were screened by hybridisation with a radiolabelled probe, as described in the Methods, section 3.1.11. The probe consisted of a mixture of three single stranded oligos (one from each pair) employed in the construction of the modified Pk tags in a final concentration of 40 μg/ml. The clones that indicated incorporation of the insert were selected and their plasmid DNA was extracted and sequenced.

Protein expression
Once the DNA sequence of all three plasmids was established, GST-PkHis9, GST-PkHis11 and GST-PkHis4,10 proteins were expressed by IPTG induction (refer to section 3.1.13) and purified on GST-affinity matrix (section 3.2.1) as presented in Figure 12.



Key: VECTOR: pBR322 ori: origin of DNA replication, lacIq: gene coding for lac repressor protein, pTAC: promoter of transcription (IPTG inducible), GST: Glutathione-S-Transferase protein, MCS: Multiple Cloning Site, Amp^r: gene conferring Ampicillin resistance.

INSERT: Pk His: His-modified Pk affinity tag.

RESTRICTION SITES: BamHI, EcoRI.

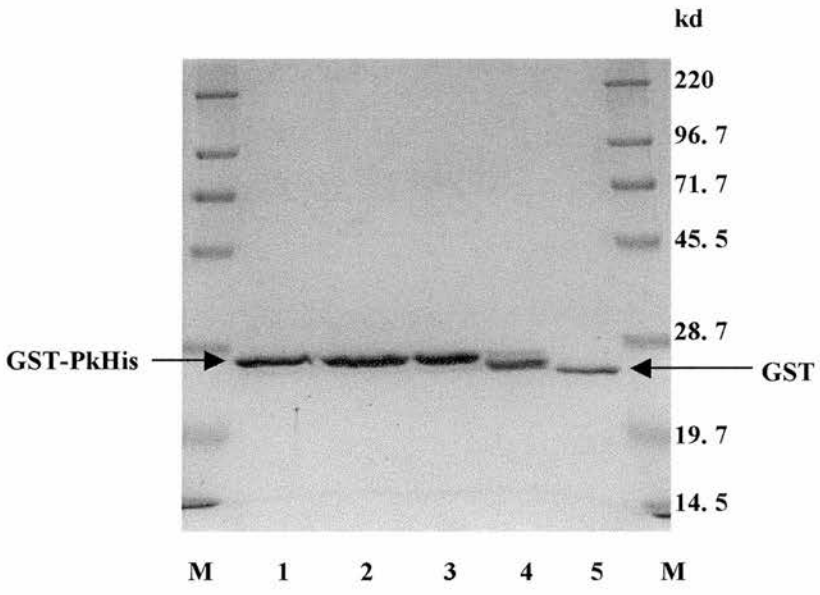


Figure 12: 13.5% acrylamide gel electrophoresis of the three GST-PkHis proteins, along with GST and GST-Pk control proteins, following purification on GST-affinity solid support. Lanes 1, 2 and 3, contain purified proteins GST-PkHis4,10, GST-PkHis9 and GST-PkHis11 respectively. GST-Pk unmodified (original Pk tag) is shown in lane 4 and GST protein alone can be seen in lane 5. M denotes the Molecular Weight Markers (GibCo) used for protein size determination and to minimise the possibility of gel distortion.

For all five proteins, one-step purification on GST-affinity matrix resulted in very high protein purity. The GST protein lacking the 14 aa-long Pk tag (lane 5) is expectedly migrating faster than the GST-fusion proteins. The GST-Pk proteins bearing histidine substitutions would have a higher MW than the original tag: GST-PkHis9 and GST-PkHis11 proteins: 24d higher, and GST-PkHis4,10: 104d higher [Pk tag sequences are shown in Table 1, part 3, while the molecular weights (MW) of the amino acids at Pk positions 4, 9, 10 and 11 are: MW_{His}: 155d, MW_{Leu}: 131d, MW_{Ile}: 131d and MW_{Gly}: 75d]. Indeed, the GST-Pk (original Pk tag, lane 4) migrated faster than the His-modified Pk tags that co-migrated (lanes 1, 2, and 3).

However, the MW difference of 80d between GST-PkHis4,10 and GST-PkHis9 or GST-PkHis4,10 and GST-PkHis11 proteins was not detected on the gel, even though the smaller, just 24d difference between GST-PkHis9 and GST-Pk or GST-PkHis11 and GST-Pk was noticeable. It is likely that the difference in the electrophoretic properties of the Pk tags was not caused by their molecular weight difference, but perhaps by a difference in charge. While Leu, Ile and Gly (found in the original tag) are aliphatic amino acids and display no charge, His (substituted in the modified tags) is basic and can be positively charged. This positive charge on the GST-PkHis proteins could result in their slight retardation compared to GST-Pk on the SDS-PAGE.

4.2.1.2 Attempts to disrupt the anti-Pk mAbs binding to the modified Pk tags

Having established the binding affinities of all five anti-Pk antibodies to the modified Pk tags, the effect of pH on those interactions was investigated. As initially envisaged, the antibody-to-modified-tag interactions were tested in a pH range from 4 to 10 using ELISA. However, similar to observations for the original Pk tag and Pk tags bearing Cys or Ala substitutions (Dunn 1998) the binding properties of the five antibodies to the modified Pk tags remained unvaried across the pH 4 to 10 spectrum (data not shown).

Failing to reduce the binding of any of the mAbs to the modified Pk tags by simply altering the pH conditions, other treatments, expected to disrupt the binding of Ab to its specific protein (Harlow & Lane 1999), were applied. A large number of different elution conditions were tested, both by ELISA and on nitrocellulose strips, employing salts, detergents and chaotropic agents. A few selected examples are included in Figures 13, 14 and 15.

In Figure 13, Pk5 binding to the Pk tags was tested by the addition of mild disruptive agents at neutral pH (lanes 2-5). Neither the strong interaction of Pk5 with PkHis9 and PkHis4,10 nor the weak binding of Pk5 to GST-PkHis11 were gravely affected by the presence of 3.5M $MgCl_2$, 0.1M NaCl, 2M urea or 3M sodium thiocyanate at pH 7.4.

Figure 14 presents the effect of high pH on the antibody-to-tag interactions. Antibodies Pk2-Pk5 retained their binding affinity at high pH conditions (pH11, lanes '1'). The combination of high pH and detergent (0.2% Triton X at pH 12.5, seen in lanes '2') severely affected binding of the mAbs to the His-modified Pk tags, but again, binding was not completely abolished.

Figures 13 and 14: Various conditions of antibody binding and subsequent washing employed, aiming to disrupt the interaction between Pk-specific mAbs Pk2-Pk5 and the Pk tags (original and His-modified Pk tags, expressed as GST-fusion proteins).

Experimental procedures
Strips containing GST-Pk, GST-PkHis4,10, GST-PkHis9 and GST-PkHis11 were produced by simply dotting 3 μl of each protein on a 4mm-wide strip of nitrocellulose membrane. Each strip was probed with primary antibody under various binding conditions and washed in similar conditions lacking Ab. Abs Pk2 and Pk5 were employed at 1/2k, while Abs Pk3 and Pk4 were used at a dilution of 1/100. The binding and washing conditions used for each strip are indicated in the short text legend below each Figure. Membranes were immersed in secondary Ab solution (goat anti-mouse IgG peroxidase conjugate in PBS / 1% milk) and rinsed extensively. Strips were then placed on solid support, aligned and developed by Chemiluminescence (for details refer to section 3.3.2).

Comments and observations
Pk5 binding to the native Pk tag and the modified tags PkHis4,10 and PkHis9 was not reduced in any of the conditions presented in Figure 13. The much weaker binding of Pk5 to PkHis11 was affected by the presence of $MgCl_2$, urea or sodium thiocyanate (strips 2, 4 and 5). However, binding was not entirely prevented in any of those conditions.
In Figure 14, binding of all four antibodies (Pk2 to Pk5) was not abolished at high pH conditions (pH11, strips '1'), but the combination of high pH and the presence of detergent (0.2% Triton X at pH 12.5, strips '2') severely affected binding of the mAbs to all four Pk tags.

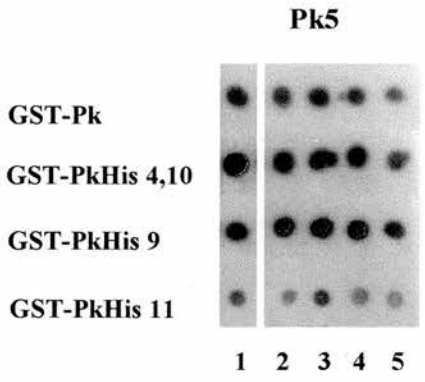


Figure 13: Addition of disruptive agents at neutral pH in an attempt to find suitable elution conditions of Pk5 antibody from the Pk tags. Strip 1: control binding at pH 7.4. Strips 2-5: treatment with 3.5M $MgCl_2$ (2), 0.1M NaCl (3), 2M urea (4) and 3M sodium thiocyanate (5) at pH 7.4.

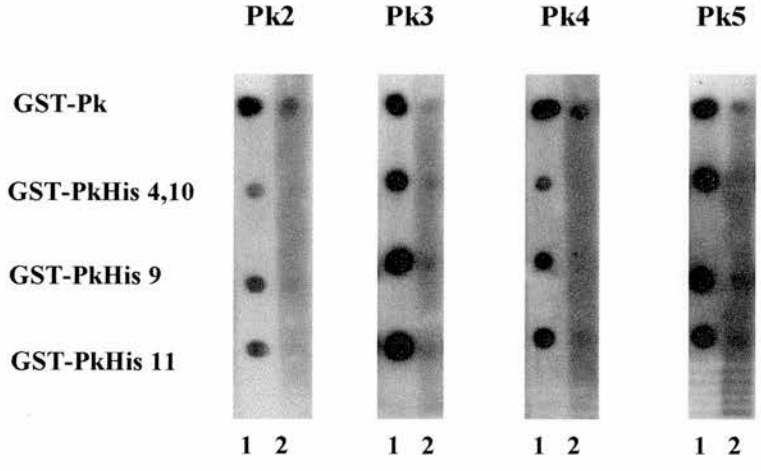


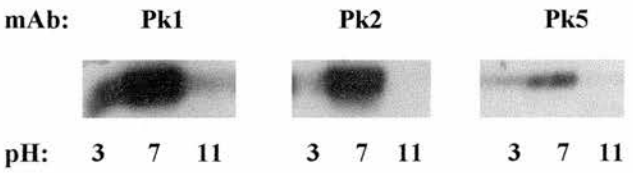
Figure 14: Interactions between each of the anti-Pk mAbs (Pk2, Pk3, Pk4 and Pk5) and each of the Pk tags (native and with His substitutions) under conditions of high pH (pH11: strips '1') and in a combination of high pH and the presence of detergent (pH 12.5 / Triton X 0.2%: strips '2').

The only conditions that resulted in complete loss of binding are those presented in Figure 15. Chaotropic agents urea and sodium thiocyanate (Harlow & Lane, 1999) were expected to disrupt the binding of Pk1, Pk2 and Pk5 to GST-PkHis11. Even so, 2M urea was well tolerated at neutral pH. Addition of 2M urea or 3.5M sodium thiocyanate at low pH, successfully reduced binding but did not abolish it. Pk2 and Pk5 binding to GST-PkHis11 was only prevented by the presence of either of these chaotropic agents at pH 11.

4.2.1.3 Conclusions from the His-modified Pk tags study

This part of the work aimed to define a combination of mAb and modified Pk tag that would interact with high affinity at one set of conditions and with much lower affinity at another set of conditions. These conditions would respectively provide the binding and elution steps in a purification system employing the Pk technology. However, the interactions of the five mAbs with the three modified Pk tags were too strong to be disrupted by mild elution conditions. The only conditions that disrupted the mAb to Pk tag binding were extreme pH values (high or low) in combination with either 2M urea or 3.5M sodium thiocyanate. At that point, those potential elution conditions were considered a threat to protein stability and functionality, so they were not tested in an experimental system.

2M Urea



3.5M Sodium Thiocyanate

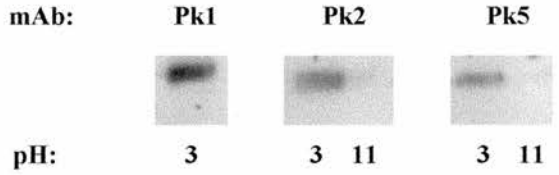


Figure 15: Treatment with 2M urea or 3.5M sodium thiocyanate at pH3, pH7 and pH11, aiming to disrupt the interaction of mAbs Pk1, Pk2 and Pk5 to protein GST-PkHis11.

Outline of experimental procedures

GST-PkHis11 protein was analysed on a single-slot 10% polyacrylamide gel and was subsequently transferred on nitrocellulose membrane by Western blotting. The membrane was cut into 4mm strips that were blocked with 5% milk in PBS. Each strip was further treated with a primary antibody solution containing 1% milk and supplemented with either 2M urea or 3.5M sodium thiocyanate at different pH conditions. Pk1, Pk2 or Pk5 antibodies were used at 1/2k. Strips were washed in the same solutions and incubated in secondary antibody (goat anti-mouse IgG peroxidase conjugate 1/2k in PBS / 1% milk). After thorough washing, strips were aligned and developed using Chemiluminescence.

Conclusions

In the presence of 2M urea, Pk1 binding to GST-PkHis11 was very strong at pH7 and still strong at pH3, but significantly reduced at pH11. Pk2 binding was strong at pH7, but it was severed at pH3 and completely abolished at pH11. The same was true for Pk5, although Pk5 was binding GST-PkHis11 with lower affinity than Pk1 or Pk2.

In the presence of 3.5M sodium thiocyanate, Pk1 was still binding GST-PkHis11 significantly at pH3, while Pk2 and Pk5 bound weakly at pH3, but not at all at pH11.

Among the multitude of conditions tested, the combination of high pH and the presence of either 2M urea or 3.5M sodium thiocyanate were the only conditions that completely abolished binding of the mAbs to the modified tags.

4.2.2 Elution by clipping of the Pk tag

It was quite obvious that the Pk tag (original or modified) could not be dissociated from its monoclonal antibody(ies) under mild elution conditions. As a result, Pk-tagged proteins would not elute from a Pk-affinity matrix. Since the interaction between the Pk-affinity matrix and the Pk tag on the tagged proteins could not be disrupted, a different solution to the elution step was considered: By creating the means to dissociate the Pk tag from the remainder of the protein, it would be possible to first attach the Pk-tagged protein to the affinity matrix and then remove the tag from the protein. As a result of that detachment, the Pk tag would remain strongly bound to the matrix, while the protein of interest (now untagged) would be free to elute off.

4.2.2.1 Technology employed for the Pk-tag clipping

The gist of this approach was to first selectively bind GST-Tat-Pk protein on Pk-affinity matrix and then clip the Pk tag off: the Pk tag would remain on the matrix, allowing GST-Tat to flow through and be collected. The means selected for removing the Pk tag from the GST-Tat-Pk protein was by proteolytic cleavage. If the recognition sequence for a protease were engineered between the Tat and Pk sequences, the proteolytic action of that protease on GST-Tat-Pk would clip the Pk tag off GST-Tat.

To that end, the protease from human adenovirus serotype 2 was employed. The adenovirus protease (AP) was selected because of the ongoing study of its function by the research group of Dr. Graham Kemp, St. Andrews

University. This fact meant easy access to information and components of the AP technology. AP (reviewed by Krausslich & Wimmer, 1988) proteolytically processes six virion precursors to mature components (Anderson, 1990) in adenoviral particles. Its function depends upon recognition of a cleavage motif on its substrate (i) and on the presence of a particular oligopeptide of highly conserved sequence, its activating peptide (sequence: GVQSLKRRRCF) (ii) (Webster *et al.*, 1993), as depicted in Figure 16. The activating peptide and the protease itself were produced and kindly provided by Dr G. Kemp.

The substrate requirements for AP are unusual and highly specific (Tremblay *et al.*, 1983; Webster *et al.*, 1989). The recognition sequence selected for our purposes was leucine, alanine, glycine, glycine (LAGG), referred to as APCS for: Adenovirus Protease Cleavage Site. The APCS sequence was therefore inserted upstream of Pk in the expression vector encoding GST-Tat-Pk. The sequence coding for a 6-Histidine (His) tag was included downstream of APCS to create the possibility of another affinity purification step. The engineering steps are illustrated in Figure 17, while the DNA and amino acid sequences of the final product are presented in Appendix 7.7. The action of AP would remove both the Pk and His tags from the GST-Tat protein, as schematically shown in Figure 18.

4.2.2.2 Obtaining protein GST-Tat-APCS-His-Pk

Bacterial hosts transformed with the modified vector (pGEX-Tat-APCS-His-Pk, Figure 17) were shown to successfully express full-length GST-Tat-APCS-His-Pk protein (confirmed by immunoblotting, data not included). It was possible to isolate the full-length protein on a GST-affinity matrix (seen later, in Figure 21).

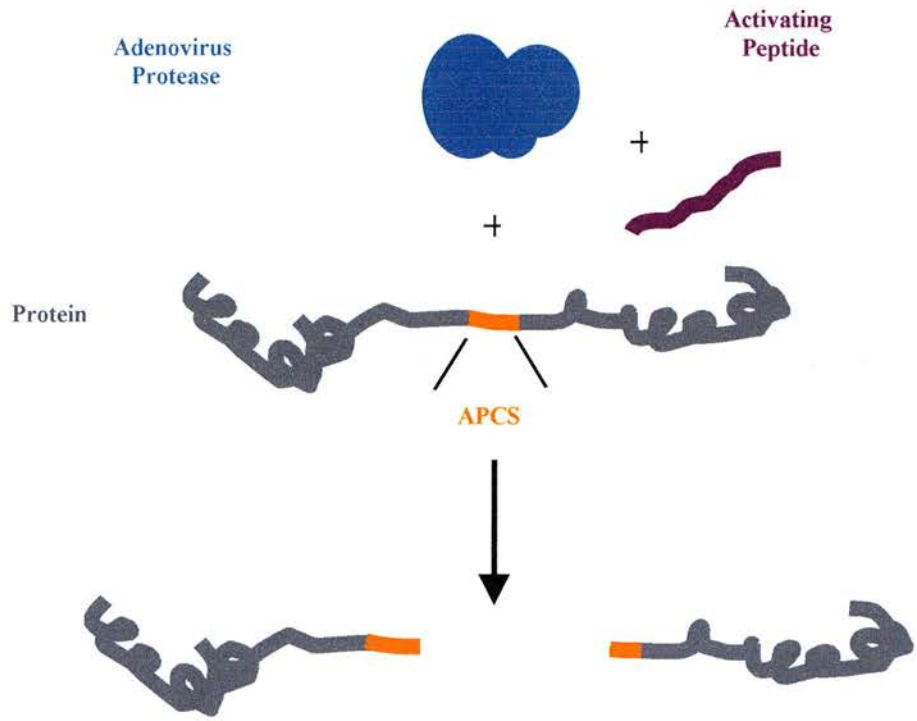


Figure 16: The principle of protein cleavage by the Adenovirus Protease.

Adenovirus Protease is found in human adenovirus type 2 (Ad2) and it is essential for the development of infectivity in newly synthesised adenovirus particles. It cleaves its substrate upon recognition of a cleavage motif on the protein sequence (Adenovirus Protease Cleavage Site: APCS) and on the presence of a particular oligopeptide, its Activating Peptide (sequence: GVQSLKRRRCF).

The protease, depicted in blue (top), recognises the amino acid sequence found in the APCS, shown in orange, located on a protein (drawn in grey, middle). In the presence of the Activating Peptide, appearing here in purple, the Adenovirus Protease cleaves the protein right on the cleavage recognition site (bottom of diagram).

Figure 17: Engineering the pGEX-Tat-APCS-His-Pk construct by inserting the APCS-His cloning fragment into vector pGEX-Tat-Pk.

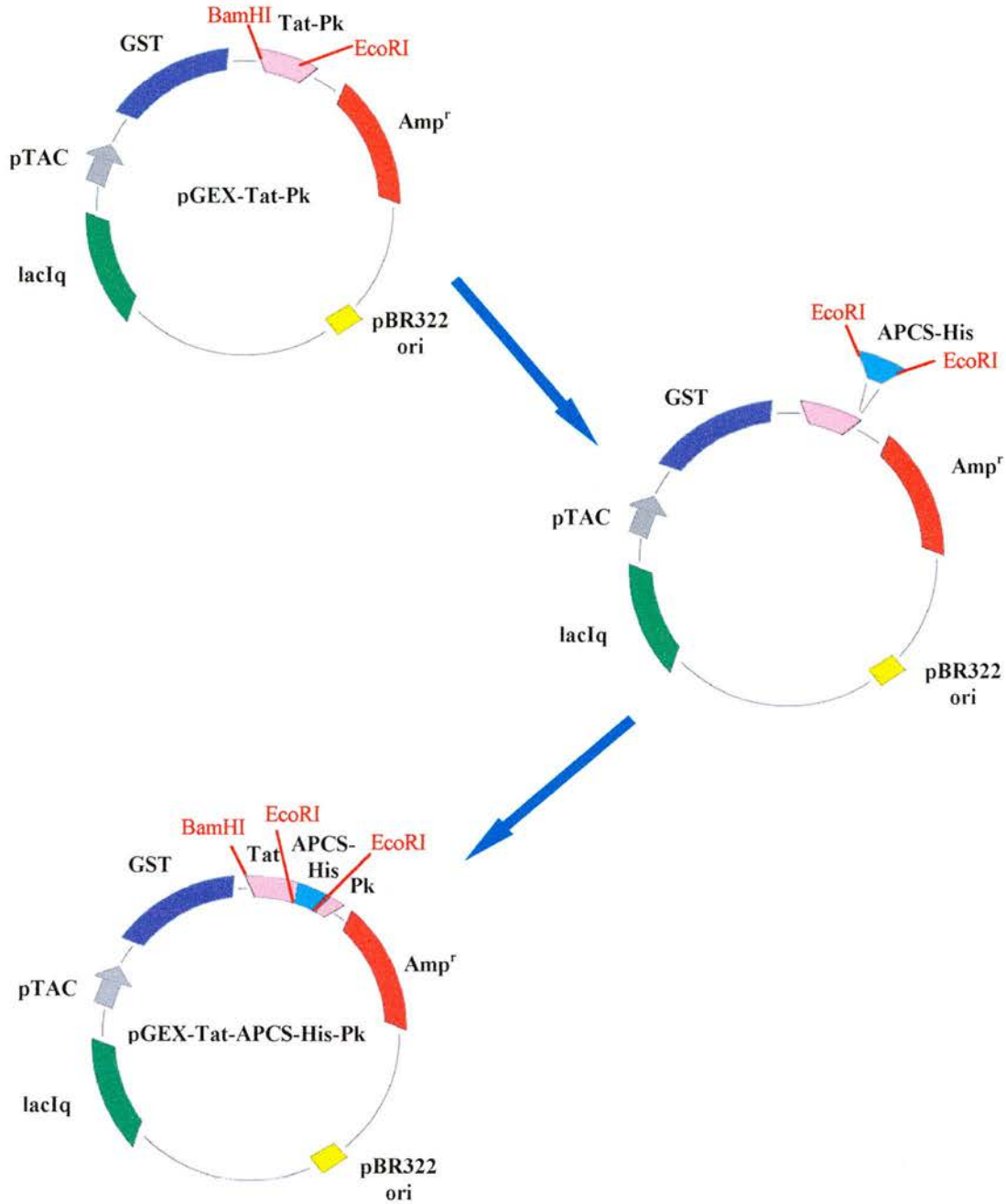
In order to devise a means of clipping the Pk tag off the GST-Tat-Pk protein, the recognition sequence for the viral protease Adenovirus Protease (AP) was engineered between the Tat and Pk tag sequences. The sequence coding for a 6-Histidine (His) tag was also inserted just upstream of the Pk tag.

The APCS-His cloning fragment
Single stranded oligos (forward and reverse) containing the protease recognition sequence (APCS) and the His affinity tag, flanked by EcoRI sites, were designed and obtained from Oswel DNA service (for detailed sequences refer to Appendix 7.10). The double stranded APCS-His cloning fragment was prepared by hybridisation of the single stranded oligos (section 3.1.3) and digested with restriction enzyme EcoRI.

Cloning of the insert into the vector
Expression vector pGEX-Tat-Pk (top of Figure) was first linearised with EcoRI treatment. The APCS-His insert (displaying EcoRI-compatible ends) was ligated between Tat and the Pk tag of the vector, as shown in the middle diagram (for details see Methods, 3.1.8). The vector generated, pGEX-Tat-APCS-His-Pk, is depicted at the bottom of the diagram. Its complete sequence is included in Appendix 7.7.

Screening of transformants
The engineered vector was transfected into competent *E. coli* cells (section 3.1.10) and the clones obtained were screened by MscI digestion. The restriction site MscI was present in the APCS-His insert and was also found once in the vector. Digestion with MscI would result in linearisation of an empty vector, or in the release of a 1 kb fragment when the insert was successfully incorporated. The correct orientation of insertion of the APCS-His fragment was confirmed by DNA sequencing (section 3.1.12).

Protein expression
Under the control of the pTAC promoter, vector pGEX-Tat-APCS-His-Pk encoded protein GST-Tat-APCS-His-Pk. Protein expression and isolation of GST-Tat-APCS-His-Pk on GST-affinity matrix is shown later in Figure 21.



Key: VECTOR: pBR322 ori: origin of DNA replication, lacIq: gene coding for lac repressor protein, pTAC: promoter of transcription (IPTG inducible), GST: Glutathione-S-Transferase protein, MCS: Multiple Cloning Site, Amp^r: gene conferring Ampicillin resistance, Pk: 14 amino acid affinity tag, Tat: TransActivator of Transcription, protein of SIV.

INSERT: APCS: Adenovirus Protease Cleavage recognition Site, His: 6-Histidines affinity tag..

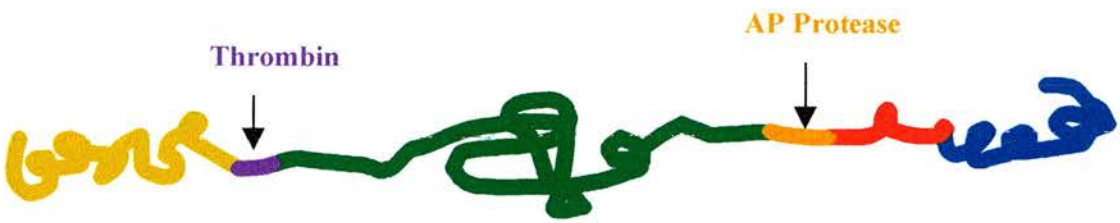
RESTRICTION SITES: BamHI, EcoRI.

1.

2.

GST-Tat-APCS-His-Pk

3.



4.

Figure 18: Schematic representation of the protein-coding part of vector pGEX-Tat-APCS-His-Pk (1), protein GST-Tat-APCS-His-Pk (2) and cleavage of full-length protein with the two proteases (3) to release protein Tat (4).

P: Promoter, GST: Glutathione-S-Transferase, TCS: Thrombin Cleavage Site, SIV: Simian Immunodeficiency Virus, Tat: Trans Activator of Transcription (lentiviral protein), APCS: Adenovirus Protease Cleavage Site, His: Affinity Tag made of 6 His, Pk: 14-amino acid- long Affinity Tag, S: Stop codons.

However, as previously noted for GST-Tat-Pk protein, it was immediately noticed that protein GST-Tat-APCS-His-Pk was produced in very low amounts, less than 0.1 mg/l. When the protein was expressed in different strains of *E. coli* (such as B834 cells that lack a bacterial protease and can generally provide a higher yield of full-length protein) the expression yield for GST-Tat-APCS-His-Pk protein remained low (data not shown).

In order to test the Pk-affinity purification strategy employing the cleavage with AP, considerable amounts of the test protein were required. Therefore, before testing the function of AP, it was essential to generate adequate amounts of GST-Tat-APCS-His-Pk protein. The following sections present the work that led to an increase in protein yield through optimisation of the induction and expression conditions.

4.3 Increasing the Low Expression Yield of GST-Tat-APCS-His-Pk

When expressing a heterologous protein in a bacterial host, large amounts of heterologous mRNA are produced, increasing the likelihood of the translational machinery to encounter heterologous mRNA rather than the host mRNA (Kane, 1995). However, some heterologous proteins do not achieve high expression levels. Possible reasons for low-level expression include toxicity of the gene product and induction of heat-shock response (Brinkmann *et al.*, 1989). At the translational level, the particular structure at the 5' end of the mRNA molecule can interfere with ribosome binding and initiation of translation (Tessier *et al.*, 1984; Looman *et al.*, 1986), while certain amino acids present immediately after the initial Met can reduce the half-life of the expressed protein (Tobias *et al.*,

1991; Furlong *et al.*, 1992). Additionally, if the heterologous protein contains codons that are not frequently used by the host, slower translation rates are observed (Pedersen *et al.*, 1984; Sorensen *et al.*, 1989; Varenne *et al.*, 1984), while clusters of rare codons are presumed to cause ribosomes to pause (Bonekamp *et al.*, 1985).

In the course of this and previous work, proteins expressed in a GST*protein*Pk conformation were produced in the bacterial system at much higher amounts than those observed here. Therefore the C' and N' termini of GST-Tat-Pk could not be causing the reduction in protein expression yield. Additionally, previous attempts to express Tat protein from SIVmac251 in a bacterial expression system were characterised by very low expression yield (Hanke, 1993; Dunn, 1998). It was therefore clear that the expression of the Tat gene was causing the reduction in the levels of protein expression.

4.3.1 The Tat-coding sequence contains rare Arg codons

Since the Tat encoding sequence had not been optimised for expression in bacterial hosts, it was possible that it contained codons that are rarely used by *E. coli*. Among all codons found in *E. coli* genes, the arginine codons AGG and AGA are the least used (Zhang, 1991) and it so happens that these two codons are most commonly employed by primates (Zhang, 1991).

The DNA sequence of SIV-Tat from SIVmac32H (J5) (GenBank entry number: D01065) contains eleven arginines (positions 6, 18, 45, 80, 82, 83, 84, 100, 105, 107 and 131) seven of which are encoded by AGA and AGG codons (see Appendix 7.7). These rare arginine codons correspond to 9% of the Tat

sequence. Furthermore, the arginines at positions 82, 83 and 84 are all encoded by rare triplets, thus forming a cluster of consecutive rare codons. The presence of these rare Arg codons in the Tat gene, and especially that cluster of rare codons, could be responsible for the low levels of protein expression observed in *E. coli*.

4.3.2 Approaches to minimise the effect of rare codons in a protein sequence

When rare codons are found in a protein-coding sequence, the rate and efficiency of translation is reduced due to limited availability of tRNA molecules for those rare codons (Hu *et al.*, 1996; Ikemura, 1981). Indeed, arginine residues AGA and AGG are read by a tRNA encoded by the *argU* gene in *E. coli* (Dieci *et al.*, 2000) and the concentration of those tRNA molecules is among the lowest in *E. coli* (Ikemura, 1981).

This shortage of tRNA molecules can be overcome in one of the three following ways: i) by directly supplying the bacterial host with the limiting tRNA species, ii) by engineering the gene that encodes that tRNA species in the bacterial expression system so the host can make the limiting tRNA, or, iii) by substituting the rare codons in the sequence of the heterologous protein, so that it can be translated by tRNA molecules that are more abundant in the bacterial host.

4.3.3 Choosing a method to overcome the effect of the rare arginine codons

Previous work in our laboratory, as well as published studies of other workers helped us decide between the three available technologies. Previously in our laboratory, a range of SIV proteins was expressed in *E. coli* transformed with the

argU gene (Dunn, 1998). Among the proteins studied, only a few benefited from the *argU* gene product, and the maximum impact in expression yield was a five-fold increase (Dunn, 1998). Furthermore, Dieci and colleagues reported that tRNA supplementation did not increase protein expression more that two-fold when the protein contained a cluster of rare codons (Dieci *et al.*, 2000), while Hu and colleagues achieved a 40-fold increase in expression yield by replacing two clusters of rare arginines, corresponding to just one third of the total rare arginine codon content of their protein of interest (Hu *et al.*, 1996).

Since tRNA supplementation had not dramatically increased the expression yield of the SIV proteins tested, and due to the presence of a cluster of rare arginines in the Tat coding sequence, it seemed more profitable to attempt to overcome the problem of rare codons by substituting the three consecutive rare arginine triplets with codons more frequently used in *E. coli*. If the protein yield were indeed increased, it would allow for universal expression of the engineered Tat protein, rather than confinement of protein expression in a system that requires tRNA supplementation.

4.3.4 Replacement of a cluster of rare arginines in the Tat sequence

The region of consecutive rare R codons at position 82-84 of the Tat coding sequence was altered in the GST-Tat-APCS-His-Pk-expressing construct by PCR-based mutagenesis. As part of this cloning strategy, an unwanted EcoRI site was destroyed [this site was created by previous insertion of the APCS-His fragment in the EcoRI site of the vector pGEX-Tat-Pk (Figure 17)]. The cloning strategy is outlined in Figure 19, while the steps of PCR generation of the modified fragment

are presented in Figure 20. The final plasmid, termed pGEX-Tat-APCS-His-Pk-2, was different from its predecessor, vector pGEX-Tat-APCS-His-Pk, by three Arg triplets at positions 308, 309 and 310 and by the absence of an EcoRI site at position 358-360 (for full sequences refer to Appendices 7.7 & 7.8).

4.3.5 Protein expression yield after the Arg codon replacement

Protein GST-Tat-APCS-His-Pk was expressed from clones transformed with pGEX-Tat-APCS-His-Pk-2 and a yield improvement was immediately noticed. The expression yield was then compared to that of protein expression before replacing the three rare R codons: Bacterial cultures harbouring either pGEX-Tat-APCS-His-Pk or pGEX-Tat-APCS-His-Pk-2 plasmids were grown and induced in parallel, under the same conditions. Comparable samples at different purification stages for GST-Tat-APCS-His-Pk originating from each culture are presented in Figure 21: GST-Tat-APCS-His-Pk expressed from pGEX-Tat-APCS-His-Pk-2 was by far the strongest band in the preparation, which was not the case for protein expressed from pGEX-Tat-APCS-His-Pk.

The replacement of the sequence encoding the three rare arginines resulted in an estimated increase of 20 to 50- fold in protein expression for GST-Tat-APCS-His-Pk. Without interfering with the amino acid sequence of Tat protein a massive increase in protein expression yield was achieved. In subsequent protein preparations of GST-Tat-APCS-His-Pk, the estimated expression and purification protein yield ranged from 3 to 5 mg/l.

Figure 19: Schematic representation of the cloning strategy for the replacement of the rare arginine codons and the destruction of the second EcoRI site.

Template and Primers

A diagram of the construct coding for (GST-)Tat-APCS-His-Pk is presented in section A. The position of the three rare Arg codons is depicted in black, while the positions of important restriction sites are shown in red. The primers designed for this cloning strategy are presented in section B, where the direction of each primer and the position of its hybridisation on the construct are indicated.

Primers 2 and 3, designed to change the arginine codons, included part of the SIV-Tat sequence with the Arg triplets CGT CGC CGT replacing the codons AGA AGA AGA. Those primers were complementary at their 5' ends, but they each extended a 3' end overhang.

This cloning strategy included the destruction of the unwanted EcoRI site, created by previous insertion of the APCS-His fragment in the EcoRI site of the pGEX-Tat-Pk (Figure 17). To that end, Primer 3 was designed to contain an MfeI restriction site instead of the second EcoRI site (position 1073-1078: nucleotide base count in the pGEX-Tat-APCS-His-Pk sequence, Appendix 7.7).

Finally, sequencing primer pGEX FWD (Primer 1) was also employed. Primer 1 hybridises on the pGEX vectors, 50 bp upstream of the BamHI site. Full sequences of all primers are included in section 7.10 of the Appendix.

Cloning Strategy

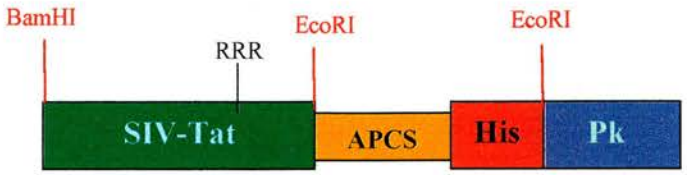
Primers were designed to anneal to pGEX-Tat-APCS-His-Pk (Part A) as shown in Part B, and they were employed in overlapping PCR (as detailed in Figure 20) to produce the fragment shown in Part C.

That fragment was first ligated into vector pGEM-T easy (Promega) and transfected into competent *E.coli* cells (Methods, 3.1.10). Transformants were identified by white/blue colony selection on LB-Agar / 0.5 mM IPTG / 80 μg/ml X-Gal plates (section 3.1.11) followed by BamHI & MfeI double digestion.

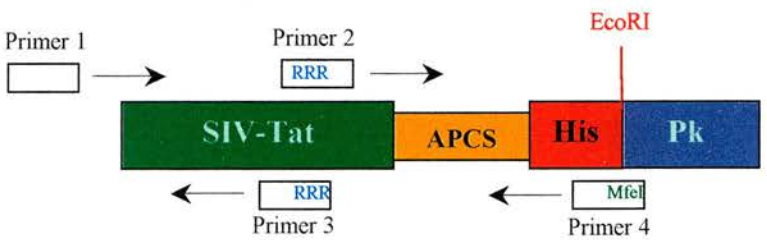
The modified Tat-APCS-His fragment released by treatment with BamHI and MfeI (shown in D) was then inserted between the BamHI and EcoRI sites of pGEXcPk vector (a diagram of this vector is included in Figure 9).

In the final construct (shown in Part E) the Arg codons have been replaced (now depicted in blue) and the second EcoRI site has been destroyed by creating an MfeI / EcoRI hybrid restriction site, as confirmed by plasmid DNA sequencing. The newly synthesised expression vector, pGEX-Tat-APCS-His-Pk-2 (seen in Appendix 7.8) encoded protein GST-Tat-APCS-His-Pk, like its predecessor vector pGEX-Tat-APCS-His-Pk.

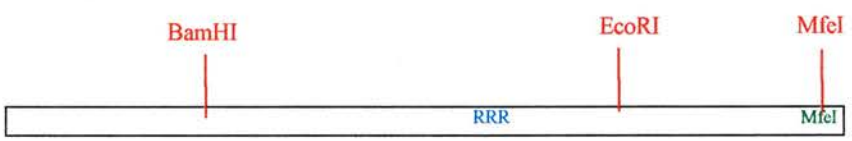
A. Original expression vector coding for GST-Tat-APCS-His-Pk



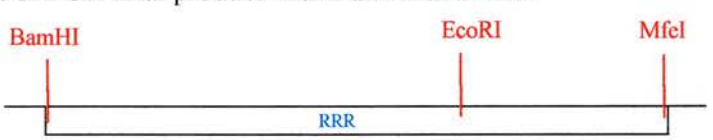
B. Design of PCR amplification primers for Overlapping PCR



C. Replacement of RRR codons and EcoRI site by PCR (Figure 20)



D. Treatment of PCR final product with BamHI and MfeI



E. Insertion of digested fragment between BamHI and EcoRI in pGEXcPk

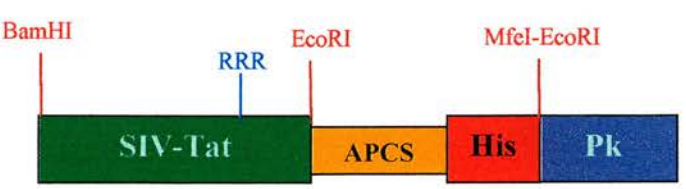


Figure 20: Diagrammatic illustration of the PCR amplification steps employed to generate the modified Tat-APCS-His-Pk fragment, required for replacing the rare arginine codons (codons 82, 83 and 84 of Tat from SIVmac251(J5)) and the second EcoRI site in the pGEX-Tat-APCS-His-Pk construct (region 1073-1078 bp).

The PCR template, coding for Tat-APCS-His-Pk, along with the primers employed, are shown in section A (for primer and template details, see text accompanying Figure 19, or refer to Appendix sections 7.10 and 7.7).

Part B presents the first step of the PCR amplification where part of the template (GST-Tat-APCS-His-Pk construct) was amplified using primers 1 and 3. That PCR product thus contained the first part of the construct up to the three arginine residues, now replaced with the new codons.

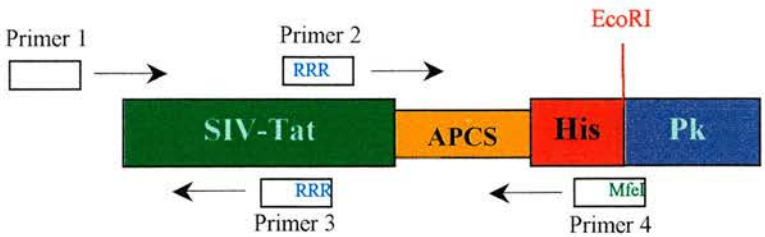
In the same manner, primers 2 and 4 were used for PCR amplification of the second part of the GST-Tat-APCS-His-Pk construct as shown in Part C. The product of this step contained the sequence of the modified arginines and MfeI restriction site at position 1073-1078 (instead of EcoRI).

The products of those two PCR amplification steps were used in an overlapping PCR (section D) where primers 1 and 4 drove the synthesis of the full-length modified construct.

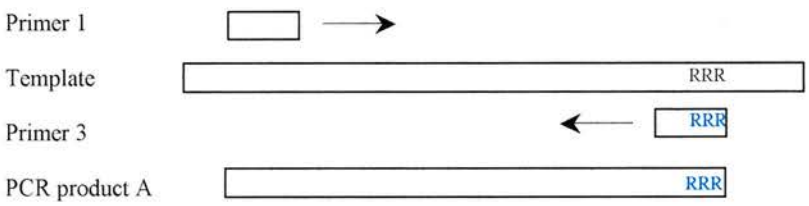
The diagram in section E depicts the differences between the original construct coding for Tat-APCS-His-Pk and the product of the overlapping PCR. The final PCR amplification product contained the modification replacing the rare arginine codons and an MfeI restriction site instead of the EcoRI.

The newly amplified Tat-APCS-His-Pk fragment was then inserted in a pGEM-T easy PCR-cloning vector (Promega) and retrieved by BamHI / MfeI double digestion, as detailed in the legend of Figure 19.

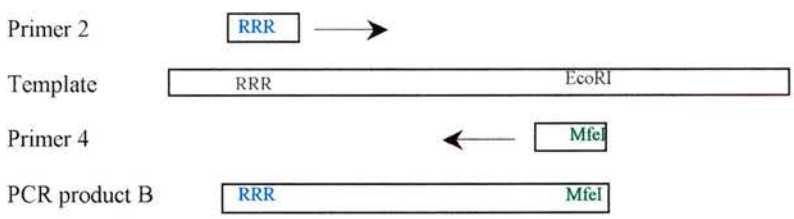
A.


Primer 1
Primer 2
RRR
EcoRI
SIV-Tat
APCS
His
Pk
RRR
MfeI
Primer 3
Primer 4

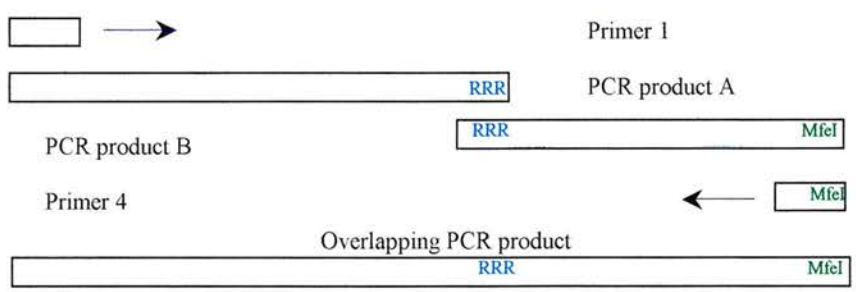
B.


Primer 1
Template
RRR
Primer 3
RRR
PCR product A
RRR

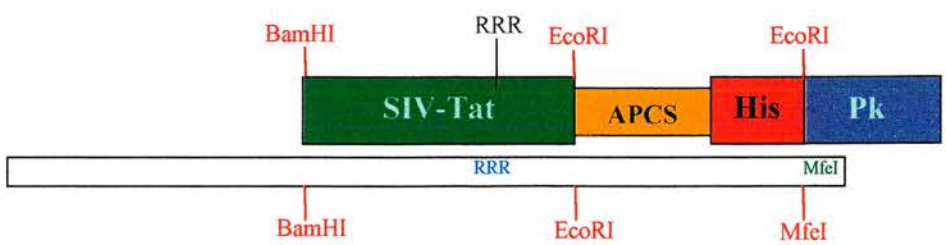
C.


Primer 2
RRR
Template
RRR
EcoRI
Primer 4
MfeI
PCR product B
RRR
MfeI

D.


Primer 1
RRR
PCR product A
RRR
MfeI
PCR product B
Primer 4
MfeI
Overlapping PCR product
RRR
MfeI

E.


BamHI
RRR
EcoRI
EcoRI
SIV-Tat
APCS
His
Pk
RRR
MfeI
BamHI
EcoRI
MfeI

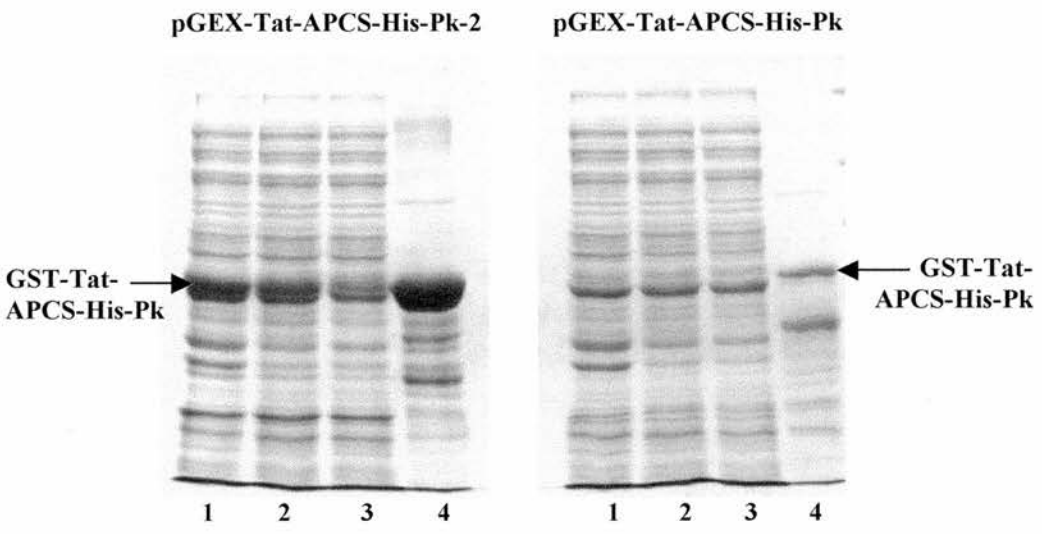


Figure 21: Comparison of yield for GST-Tat-APCS-His-Pk expressed from vector pGEX-Tat-APCS-His-Pk (right panel) and vector pGEX-Tat-APCS-His-Pk-2 (left panel).

The difference between the two vectors lies in the coding sequence for a string of three arginines at positions 82, 83 and 84 of the SIV-Tat sequence. pGEX-Tat-APCS-His-Pk vector codes for the three Arg with triplets not favoured by *E. coli*, while pGEX-Tat-APCS-His-Pk-2 encodes those three Arg with codons optimised for use in the bacterial host (full sequences are available at Appendices 7.7 and 7.8). pGEX-Tat-APCS-His-Pk-2 vector was engineered from vector pGEX-Tat-APCS-His-Pk as detailed in Figures 19 and 20.

Two bacterial cultures, each harbouring one of the vectors pGEX-Tat-APCS-His-Pk and pGEX-Tat-APCS-His-Pk-2, were grown and IPTG-induced in parallel, under the same conditions. GST-Tat-APCS-His-Pk protein expressed in each culture was isolated from the rest of the bulk of the proteins present, by GST-affinity purification on Glutathione-Agarose. Comparable samples at different stages of purification were analysed on 11.5% SDS electrophoresis as presented in lanes 1 to 4 of the panels above.

The total cell contents, equivalent to 1% of each 500 ml bacterial culture, are seen in lanes '1'. The soluble antigen fractions appear in lanes '2' and the column flow through for each protein is shown in '3'. Partially purified GST-Tat-APCS-His-Pk protein, shown in lanes '4', represents 5% of the total purification amount for each culture.

In the left panel, GST-Tat-APCS-His-Pk was by far the main band in the preparation, which was not the case for the right panel, where GST-Tat-APCS-His-Pk protein was not detectable in the relevant total cell or soluble antigen samples (lanes 1 and 2). GST-Tat-APCS-His-Pk in the left panel was even saturating the affinity matrix and leaking in the flow-through fraction (lane 3).

It is evident that the replacement of the codons for the three arginines resulted in a dramatic increase of yield for GST-Tat-APCS-His-Pk: an estimated 20 to 50-fold increase in protein yield form the right panel to the left panel.

4.3.6 Optimisation of induction conditions

Having attained a very significant increase of protein expression for GST-Tat-APCS-His-Pk, the conditions of culture growth and protein induction were further optimised to ensure the maximum amount of protein was obtained. In order to test a variety of induction conditions, mini-cultures harbouring vector pGEX-Tat-APCS-His-Pk-2 were grown and induced under slightly different conditions and the total cell contents were compared by 13.5% SDS-PAGE analysis. The conditions tested were different induction temperatures and durations, as well as a range of IPTG concentrations.

Temperature of induction

When studying the effect of induction temperature on protein expression yield, similar amounts of protein were produced when induction took place at 25°, 32° or 37° C (Figure 22, lanes 8-10). It was therefore concluded that the temperature of induction did not influence the amount of protein produced during induction.

Concentration of IPTG

Induction of mini-cultures with a range of IPTG concentrations from 0.01 mM to 10 mM (Figure 22, lanes 1-5) revealed that IPTG was required at a minimum final concentration of 0.1 mM (lane 2). IPTG concentrations higher than 0.1 mM (lanes 3-5) did not further improve the protein yield.

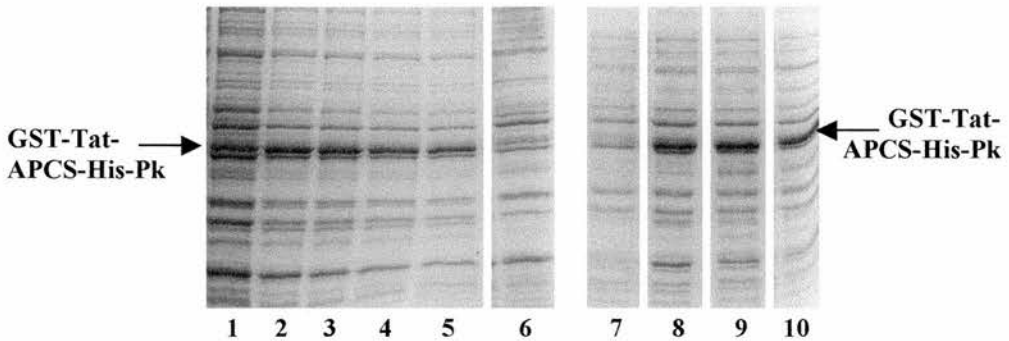


Figure 22: GST-Tat-APCS-His-Pk protein expression at different induction conditions.

Experimental Procedures
An overnight culture of *E. coli* harbouring vector pGEX-Tat-APCS-His-Pk-2 was used to set up identical mini-cultures (1 ml) that were further grown for 3 h at 37 °C shaking. Each culture was subsequently incubated in the presence or absence of IPTG for another 3 h (for precise induction conditions see below). Following induction, the volume of each mini-culture was reduced to 100 µl by brief centrifugation at 6500_xg and a sample equivalent to $1/40^{th}$ of the initial culture was supplemented with SDS-Lysis Buffer (Methods 3.2.6) and analysed by 13.5% SDS PAGE as shown above.

Induction Conditions
The total cell protein samples presented in lanes 1 to 5 originate from cultures induced at 25 °C with a final IPTG concentration of 0.01 mM, 0.1 mM, 1 mM, 10 mM and 5 mM respectively. Mini-cultures in lanes 8-10 were induced with 1 mM IPTG at 25°, 32° or 37° C respectively. Samples in lanes 6 and 7 represent cultures that were incubated without the addition of IPTG.

Results and Observations

GST-Tat-APCS-His-Pk protein appeared as a bold band of comparable intensity across lanes 1 to 5 and 8 to 10, while it was faintly present in the uninduced controls due to leaky expression (lanes 6 and 7).

In lanes 2 to 5, GST-Tat-APCS-His-Pk protein corresponded to the major band in the total protein fraction. However, in lane 1, that band matched the intensity of other unrelated proteins, suggesting that IPTG final concentration of 0.01 mM was not enough for preferential expression of the GST-fusion protein. GST-Tat-APCS-His-Pk protein was synthesised by the bacterial host in larger amounts than any other protein when IPTG was used at a final concentration of 0.1 mM (lane 2) or higher.

GST-Tat-APCS-His-Pk protein was produced at comparable amounts when the temperature of induction was 25, 32 or 37 °C (lanes 8, 9 and 10 respectively). It was therefore concluded that the temperature of induction did not significantly influence the protein expression yield.

Duration of induction

When monitoring the rate of protein synthesis during the course of induction, the amount of protein produced was shown to rise steadily in the first 3 h of induction. However, past the first 3 h, the protein concentration did not significantly increase with time (data not included).

Timing of induction

A series of timepoints was tested for the initiation of induction. It was concluded that induction at a higher O.D. (corresponding to an $O.D._{600}$ in the bacterial culture as high as 2) produced higher amounts of protein in the 3h induction period (results not shown).

Having gathered information on the parameters that affect the protein expression yield for GST-Tat-APCS-His-Pk, the growth and induction conditions for the optimal protein expression were determined. In the following studies, all bacterial cultures harbouring a Tat-encoding plasmid were grown at 37° C to an O.D. of 1.5 or higher, and induced at 25° C for 3 h with a final concentration of 0.1 mM IPTG.

4.3.7 Conclusions on yield improvement & optimal induction conditions

The low protein expression yield initially observed for GST-Tat-APCS-His-Pk was corrected by replacing three consecutive rare Arg triplets in the Tat sequence with Arg codons more readily used by *E. coli*. This codon optimisation resulted in

an increase in protein expression up to 50- fold, allowing the production of as much as 5 mg of GST-Tat-APCS-His-Pk protein per litre of bacterial culture. The use of the optimal induction conditions, as defined above, further increased the protein expression yield, to a maximum yield of 7 mg/l. Through the Arg codon replacement and the optimisation of induction conditions, a significant increase in the expression yield of Tat protein was achieved, that resulted in the production of substantial amounts of GST-Tat-APCS-His-Pk.

4.4 Purification Employing the Adenovirus Protease

Having obtained adequate amounts of protein, it was then possible to study the potential of a Pk-affinity purification strategy including an AP cleavage step. It was important to establish whether AP would pick out the recognition site in the 3-D structure of a large unrelated protein and induce cleavage at that particular site. Additionally, given the high specificity of AP, in the absence of any other AP recognition sites in the full-length protein, it was important to demonstrate that the protease was inducing a single clean cut. To that end, GST-Tat-APCS-His-Pk was treated with AP, both in solution and whilst attached to a solid matrix.

4.4.1 Adenovirus protease cleavage in solution

For cleavage in solution, protein GST-Tat-APCS-His-Pk was incubated with AP and the activating peptide that the protease requires (Figure 16) in a reducing buffer, the exact composition of which is described in Methods (3.2.4). The reaction was allowed to proceed at 37 °C. An example of AP cleavage in solution

is presented in Figure 23: samples from the cleavage reaction were removed at regular intervals and analysed by 11.5% SDS PAGE. In that study, most of the GST-Tat-APCS-His-Pk protein was cleaved to produce GST-Tat in the first 60 min of incubation with AP. It was later established that 1 μl of AP was sufficient for complete cleavage of 1 μg of GST-Tat-APCS-His-Pk at 37° C for 1 h, in solution.

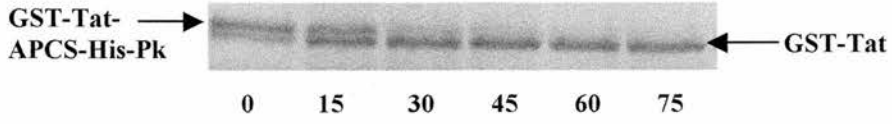
4.4.2 Adenovirus protease cleavage on solid matrix

Once the conditions of AP cleavage in solution were established and standardised, AP cleavage was attempted when GST-Tat-APCS-His-Pk protein was captured on a solid matrix. The cleavage experiment presented in Figure 24 shows GST-Tat-APCS-His-Pk protein, attached onto Protein A-agarose, getting partially cut in the presence of AP, yielding a lower band that corresponds to GST-Tat.

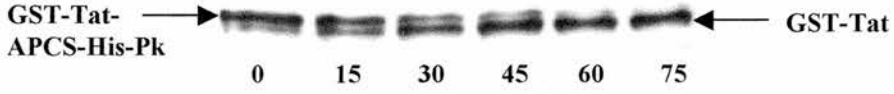
However, even in experiments yielding 100% cleavage, the cut GST-Tat portion (lacking a Pk sequence) did not detach from the Pk-specific solid matrix. Various washing conditions, including 0.05% Tween 20, 0.1% SDS and pH lower than 5, were applied to elute GST-Tat from the affinity support (data not shown). GST-Tat would only elute from the matrix when the anti-Pk antibody itself was eluted.

Protein A is naturally found on *Staphylococcus aureus*. When fixed *S. aureus* was employed instead of the Protein A-agarose matrix, it was again impossible to dissociate the GST-Tat protein from the His-Pk portion under mild elution conditions (data not shown). On the other hand, GST protein alone was shown to flow through an anti-Pk-ProteinA-agarose column (results not included).

1. Coomassie Stained Gel



2. Anti-GST Immunoblot



3. Anti-His Immunoblot

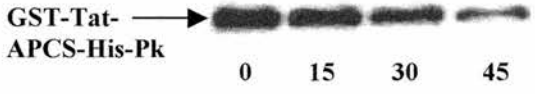


Figure 23: Time course of cleavage of GST-Tat-APCS-His-Pk protein to GST-Tat by the Adenovirus Protease (AP).

35 μg of GST-Tat-APCS-His-Pk protein was incubated with 20 μl AP at 37 °C for up to 75 min. Time-points were taken, samples were disrupted in SDS Lysis Buffer and analysed by 11.5% gel electrophoresis on three identical gels. Samples were removed at times zero (before AP addition, uncut protein), 15, 30, 45, 60, and 75 min, as indicated underneath each lane.

From the three gels ran, one was stained with Coomassie blue (top) while the other two were transferred on nitrocellulose membrane and probed with anti-GST (middle) or anti-His (bottom) antibodies. Antibody anti-GST detected both the full-length GST-Tat-APCS-His-Pk protein and the cleaved GST-Tat protein. Antibody anti-His marked the uncut GST-Tat-APCS-His-Pk protein only. The cleaved fragment APCS-His-Pk was too small to be seen on the gels.

The cleavage of GST-Tat-APCS-His-Pk to GST-Tat protein was observed by the disappearance of the band corresponding to the full-size protein and the appearance (or enhancement) of the GST-Tat band with time. In the Coomassie stained gel and the GST immunoblot, a faint band representing GST-Tat was evident at time 0: probably a small percentage of the GST-Tat-APCS-His-Pk protein was losing its C' terminal end even in the absence of AP.

From all three gels, it can be deduced that most of GST-Tat-APCS-His-Pk protein was cleaved to GST-Tat within the first 60 min of the incubation. This observation is most clear from the immunoblots: in the anti-GST blot, the protein at time 0 gradually shifts to the lower band, resulting in a single band by 60 min and in the anti-His blot, full length GST-Tat-APCS-His-Pk protein was fully absent by 60 min.

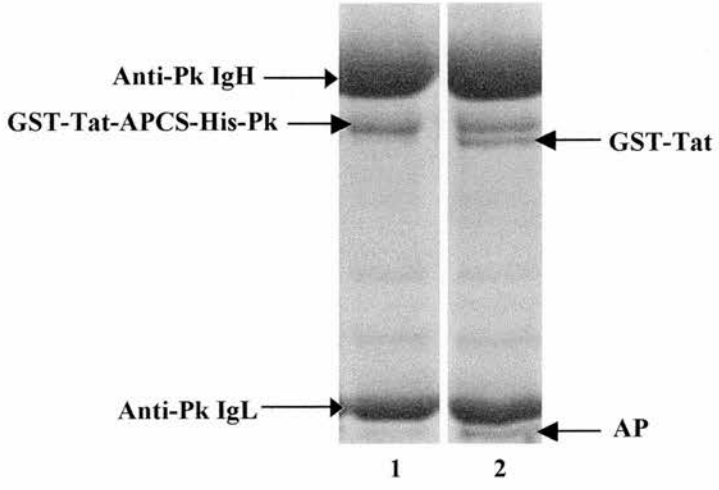


Figure 24: GST-Tat-APCS-His-Pk protein captured on anti-Pk saturated Protein A-agarose, untreated (1) or treated (2) with Adenovirus Protease (AP).

Outline of Experimental Procedures

Protein A-agarose was pre-coated with anti-Pk antibody and was then allowed to interact with GST-Tat-APCS-His-Pk protein.
All protein molecules with an intact C' terminal domain bound (via their Pk tag) to the anti-Pk antibody on the matrix, while all residual protein was washed away (Methods section 3.2.2).
After the last wash, the agarose beads were resuspended in the required volume of Protease Buffer (Methods 3.2.4) and supplemented with Activating Peptide.
The preparation was divided in two identical samples, only one of which received 4 μl of Adenovirus Protease (seen in lane 2).
Both samples were incubated at 37° C, for 3 h. Samples were disrupted by addition of SDS-Lysis Buffer (Methods 3.2.6), were boiled for 5 min and finally analysed on 11.5% gel electrophoresis as presented above.

Experimental Outcome

GST-Tat-APCS-His-Pk in lane 1 (sample lacking AP) remained uncut, while the sample in lane 2 (containing AP) was partially cut, generating the lower band corresponding to GST-Tat protein.
GST-Tat did not contain a sequence that was recognised by the anti-Pk antibody, so it was expected to detach from the Pk-affinity solid support. However, despite thorough washing of the Protein A beads, GST-Tat protein remained bound to the anti-Pk matrix.

4.4.3 Conclusions for Pk-affinity purification employing AP

During the course of this work, it was possible to demonstrate a specific, clean cleavage with the adenovirus protease in a large protein structure. The AP cleavage thus provided a means to capture Pk-tagged proteins on Pk-affinity solid support and remove the Pk tag to allow their elution and collection.

Due to time constraints, it was not possible to test the AP-based Pk-purification with proteins other than Tat. However, the construct encoding GST-Tat-APCS-His-Pk protein was engineered so that the Tat protein region could be replaced with any other insert displaying BamHI and EcoRI compatible ends. This construct would then drive the expression of other proteins in the form of GST-*protein*-APCS-His-Pk. The technology of C' terminal purification (Pk or His) followed by AP cleavage is therefore available for use with other proteins.

For the purposes of this study, however, the persistence of cleaved GST-Tat on the anti-Pk matrix did not allow the use of Pk-affinity purification for Tat protein. It was concluded that this stickiness of GST-Tat was caused by the presence of Tat protein. The underlying reasons for this phenomenon will be discussed later.

4.5 Alternative Purification Procedures

Since the AP approach did not produce the expected means of purification that was initially envisaged for GST-Tat-APCS-His-Pk, other purification methods were explored for this protein. During initial isolation on a GST-affinity matrix (presented in lane 4 of Figure 21) GST-Tat-APCS-His-Pk was the major band in

the partially purified preparation. Still, the protein preparation contained a series of additional bands. In order to improve the purity of the protein preparation, various purification steps were tested for their ability to eliminate the additional protein bands that persisted through GST-affinity purification.

4.5.1 Clarification by precipitation

In an attempt to isolate the GST-Tat-APCS-His-Pk protein from the large number of bacterial proteins present in the total cell fraction, treatments with ammonium sulfate (AS) and with polyethyleneimine (PEI) were employed. AS precipitates hydrophobic proteins and Tat contains some highly hydrophobic domains. PEI precipitates nucleic acids along with molecules that may be attached on them (PEI was employed to purify T7 polymerase, by precipitating nucleic acids and associated proteins from a crude cell extract, by Joyce & Grindley, 1983) and Tat binds RNA, so it could be found complexed with RNA in the bacterial cell and total cell fraction.

Both PEI and AS precipitation were employed as initial protein isolation steps for recombinant HIV Tat (Frankel & Pabo, 1988). However, protein precipitation with a final concentration of 10% to 70% AS, or 0.1% to 0.6% PEI, did not result in a situation where GST-Tat-APCS-His-Pk was isolated in a protein fraction away from the bulk of the proteins in the soluble antigen (data not shown). Therefore, precipitation with either AS or PEI did not provide a beneficial initial step in a multi-step GST-Tat-APCS-His-Pk protein purification process. Attempts to further purify the protein were thus limited to affinity purification on the basis of the GST and His affinity tags.

4.5.2 Single-step purification of full-length protein

The quality and yield of GST- and His- affinity purification of GST-Tat-APCS-His-Pk protein was tested by parallel purifications on glutathione-agarose (GST tag) and NiNta-agarose (His tag) matrices, as presented in Figure 25. Equal amounts of bacterial culture were employed in each purification, and all conditions were kept identical. However, the protein samples electrophoresed were adjusted to contain approximately equimolar amounts of GST-Tat-APCS-His-Pk protein, so the protein band intensities in Figure 25 do not convey information about the quantitative yields of the protein purifications.

4.5.2.1 GST- and His- affinity protein purification

GST-Tat-APCS-His-Pk protein isolated on glutathione-agarose (Figure 25, lane 2) contained a number of additional bands that were not seen in the protein preparation partially purified on NiNta-agarose (lane 3). However, purification on NiNta-Agarose was characterised by lower yield of purified protein. His-affinity purification was estimated to produce 40 times less GST-Tat-APCS-His-Pk than the GST-affinity partial purification. In an attempt to determine the reason for the low yield of purification on His-affinity matrix, and aiming to investigate the nature of the additional protein bands in the protein preparation isolated on glutathione agarose, both protein preparations were analysed by immunoblotting with mAbs anti-GST and anti-His, as presented in Figure 26.

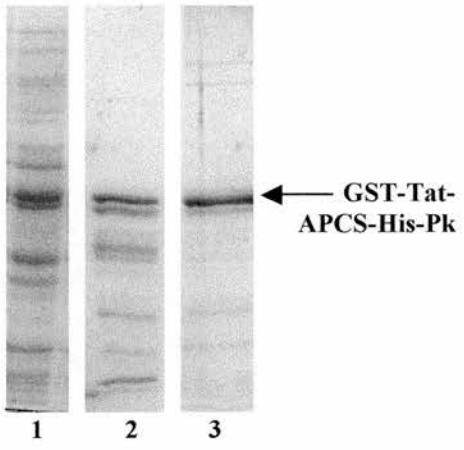


Figure 25: Parallel purification of GST-Tat-APCS-His-Pk on GST and His affinity matrices. Analysis performed on 11.5% acrylamide gel electrophoresis.

1 ltr of culture of *E. coli* host harbouring vector pGEX-Tat-APCS-His-Pk-2 was grown at 37 °C shaking. When it reached cell density corresponding to absorbance of 1.5 at 600 nm, it was induced with 0.1 mM IPTG at 25 °C for 3 h. Cells were harvested by centrifugation at 6500_xg and the cell pellets were resuspended in PBS / NaCl 0.5M to a total volume of approximately 15 ml. The cell suspension was passed twice through the cell disruptor (French Press) and Triton X was added to a 1% final concentration. A sample of the cell suspension, at that stage, representing about 3% of the bacterial culture, is included above in lane 1.

The total cell fraction was then centrifuged for 30 min at 20000_xg and the soluble cell fraction was divided into 2 equal parts and incubated with appropriate volumes of Glutathione-agarose matrix or His-affinity matrix (NiNta-agarose). Both affinity matrices were thoroughly washed with their respective Wash Buffers (Methods 3.2.1) and specifically bound protein was eluted from the GST- and His- affinity columns, using the respective Elution Buffers (3.2.1).

The eluted protein fractions from GST-agarose and NiNta-agarose are presented above in lanes 2 and 3 respectively. Note that the analysis presented here is qualitative and not quantitative: the samples in lanes 2 and 3 are adjusted to give approximately equimolar amounts of protein; they do not correspond to equal volumes of protein preparation or bacterial culture.

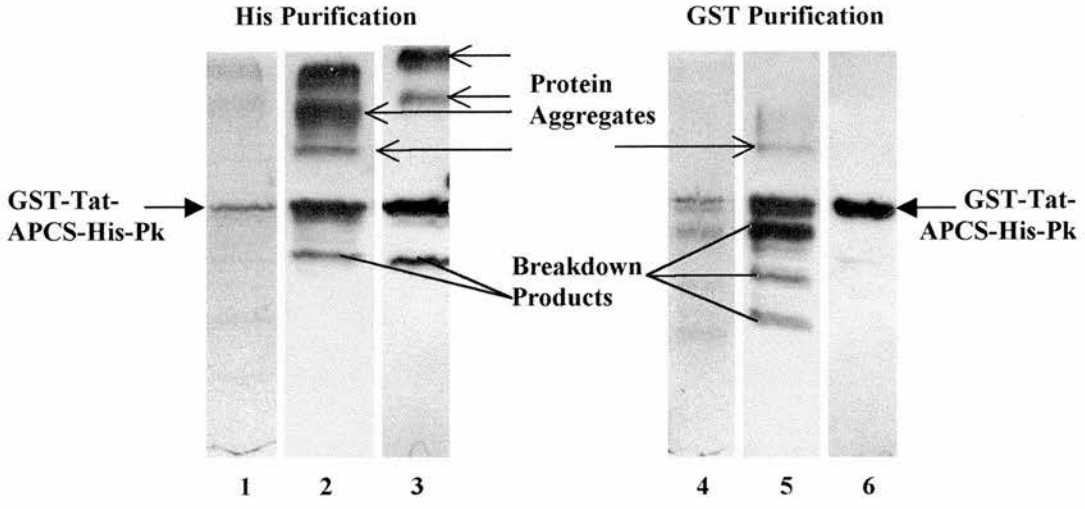


Figure 26: GST-Tat-APCS-His-Pk from Figure 25, purified on GST matrix or on His matrix, was analysed on 11.5% gel electrophoresis (lanes 1 and 4) and subsequently transferred onto nitrocellulose membrane and probed with antibodies anti-GST (lanes 2 and 5) or anti-His (lanes 3 and 6).

Anti-GST and anti-His antibodies were mouse monoclonal Abs, visualised with a mouse-specific HRP conjugate Ab. For comparison purposes, an identical Coomassie-stained gel is also included (lanes 1 and 4). The band indicated by bold arrows represents GST-Tat-APCS-His-Pk protein in the partially purified preparations.

Additional bands present in the protein preparations could represent contaminants carried over from the bacterial host, or breakdown products and aggregates of proteins of GST-Tat-APCS-His-Pk origin. Since these additional proteins were recognised by the anti-GST and anti-His Abs, they must represent breakdown products of the full-length protein (bands with lower molecular weights than GST-Tat-APCS-His-Pk protein) and products of aggregation of GST-Tat-APCS-His-Pk full-length protein with itself and with its breakdown products (proteins running slower than GST-Tat-APCS-His-Pk).

4.5.2.2 Investigating the origin of the additional protein bands

As seen in Figure 26, the additional bands resulting from GST selection were all recognised by the anti-GST antibody (lane 5). They evidently contained an intact N' terminus (GST) driving their purification on GST-affinity matrix and resulting in recognition by the anti-GST antibody. Therefore, these bands did not represent bacterial protein contaminants that persisted from expression in the bacterial system. Most likely they were breakdown products originating from the GST-Tat-APCS-His-Pk protein, or they represented incomplete protein products as a result of early termination of translation.

These abrogated GST-fusion proteins were expected to lack the C' terminal portion of GST-Tat-APCS-His-Pk protein and indeed, they were not recognised by the anti-His mAb (lane 6) and they were absent from the preparation on NiNta-affinity matrix (lanes 2 and 3). It was therefore concluded that the majority of the additional protein bands present in the GST preparation could be potentially removed by subsequent His-affinity selection.

4.5.2.3 Suggestions for the low protein yield for His-affinity purification

There are two possible reasons that could cause this low protein yield for His-affinity purification: First the C' terminus of GST-Tat-APCS-His-Pk protein might be cleaved off from a percentage of protein molecules. The single protein band obtained through His-affinity purification would then represent the percentage of GST-Tat-APCS-His-Pk protein molecules with an intact C' terminus. Alternatively, in a proportion of GST-Tat-APCS-His-Pk molecules, the

His tag could be somehow hidden in the 3-D structure of the protein. That would then make the His tag unavailable for His-affinity purification and the protein band isolated on NiNta-agarose would correspond to the portion of full-length protein with an exposed His tag.

If the His tag was being cleaved off from a percentage of GST-Tat-APCS-His-Pk protein molecules, then the protein molecules isolated on the basis of GST-affinity would not all have a functional C' terminus. In Figure 26, when equimolar amounts of GST- and His- purified GST-Tat-APCS-His-Pk protein were probed with anti-His mAb, they produced signals of similar intensity (lanes 3 and 6, Figure 26). This observation precludes the possibility that the His tag was clipped off from a proportion of the GST-Tat-APCS-His-Pk protein isolated through GST-affinity purification. Furthermore, the fact that the anti-His mAb recognises the His-tag equally in lanes 3 and 6, further strengthens the argument that the tag is still intact, and it is available for Ab-binding when the protein is denaturated by SDS-PAGE analysis, while it may be concealed when the protein assumes its 3-D conformation.

4.5.3 GST and His two-step purification of GST-Tat-APCS-His-Pk

In order to remove the GST-fusion proteins that lacked a C' terminus, the protein preparation isolated by GST-affinity purification was subjected to a His-affinity selection. Unfortunately, GST-Tat-APCS-His-Pk was detected in the NiNta-agarose column flow through and the resulting protein yield was again very low. It was thus concluded that passage through a GST-affinity column did not make the His tag more accessible for His-purification. It seemed possible though that the

His tag might become more exposed following cleavage of the GST tag from the full-length protein. This possibility was subsequently investigated.

4.5.3.1 Thrombin cleavage of GST from GST-Tat-APCS-His-Pk

GST-Tat-APCS-His-Pk protein contained the recognition sequence for cleavage by the protease thrombin between the GST and Tat sequences (diagram 18). Full-length GST-Tat-APCS-His-Pk, obtained by GST-affinity partial purification, was therefore treated with thrombin to remove the GST tag.

An example of the action of thrombin on GST-Tat-APCS-His-Pk with time is presented in Figure 27. In the study presented here, all GST was cleaved off from Tat-APCS-His-Pk in the first 90 min of thrombin treatment. Optimisation of the GST removal by thrombin treatment resulted in the use of 1 unit of the protease for complete cleavage of 15 μg of GST-Tat-APCS-His-Pk at RT, 1 h.

4.5.3.2 Two-step protein purification including a thrombin cleavage step

Once the conditions for thrombin cleavage were optimised, protein preparations treated with thrombin were applied to a His-affinity matrix to examine whether the His-tag was now adequately exposed to drive the purification of Tat-APCS-His-Pk. Figure 28 presents the steps of the procedure.

Even though the His-affinity column was thoroughly washed to the point where no protein was flowing through (Figure 28, lane 4), GST protein was still present in the protein fraction eluted from the column (lane 5).

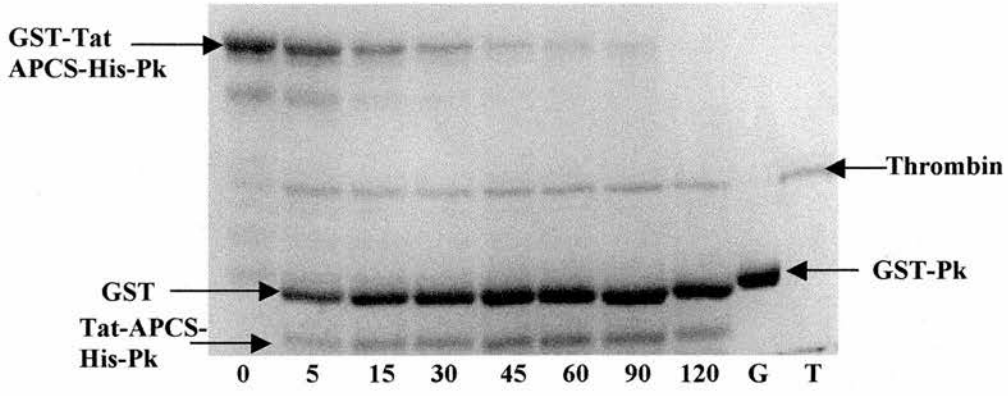
Figure 27: Time course of GST-Tat-APCS-His-Pk protein cleavage with thrombin.

15 μg of GST-Tat-APCS-His-Pk protein was incubated with 1.2 units of the protease thrombin for a total of 3 h, at room temperature. Samples were removed after 5, 15, 30, 45, 60, 90 and 120 min as indicated under each lane. A sample of GST-Tat-APCS-His-Pk protein before the addition of thrombin was included, marked 0.

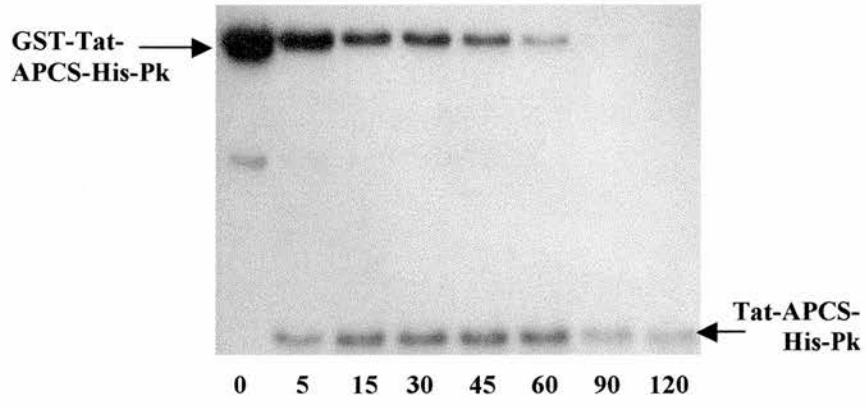
The samples removed from the developing reaction were immediately supplemented with SDS-Lysis Buffer and when all time-points were collected, samples were analysed on three identical 13.5% polyacrylamide gels. One gel was stained with Coomassie Blue, shown here at the top, while the other two gels were transferred on nitrocellulose membrane and probed with anti-His (middle) or anti-GST antibody (bottom part). [The immunoblots were treated with anti-GST or anti-His antibodies, to detect each of the two cleaved parts of GST-Tat-APCS-His-Pk]. In the Coomassie stained gel, samples of thrombin alone (T) and protein GST-Pk (G) were included as a molecular weight guide.

GST-Tat-APCS-His-Pk protein was completely cleaved by 90 min, as indicated by the absence of the band representing full-length protein at 90 min, both in the Coomassie stained gel and in the two immunoblots. A large proportion of the protein was already cleaved at just 15 min as seen both in the gel and in the anti-His blot. The intensity of the band representing cleaved GST remained constant across the gel and the anti-GST blot. The Tat-APCS-His-Pk cleaved portion however, reached a peak of signal intensity (protein concentration) at 60 min (best observed in the anti-His immunoblot) after which the band intensity weakened, probably due to protein degradation.

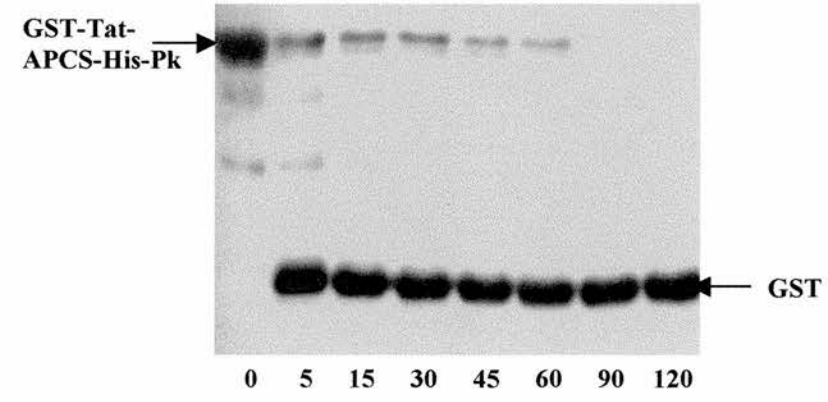
1. Coomassie Stained Gel



2. Anti-His immunoblot



3. Anti-GST immunoblot



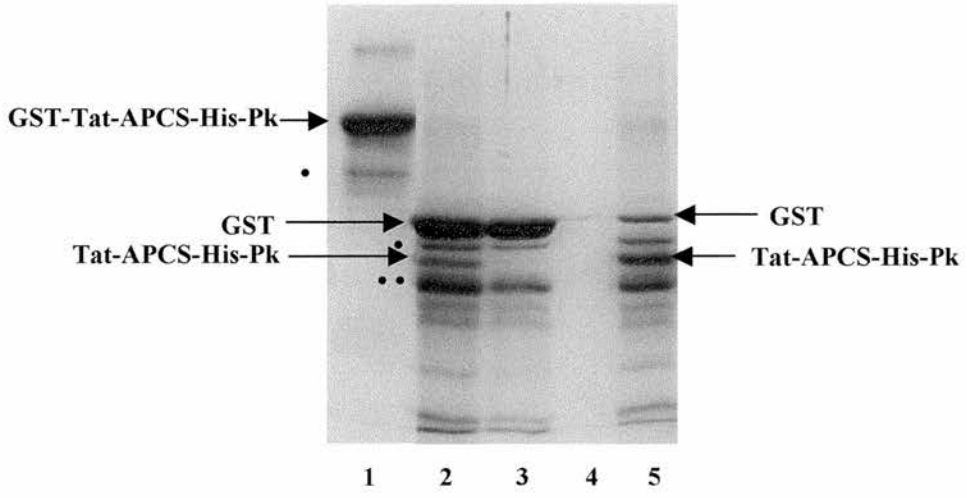


Figure 28: GST-affinity isolation of protein GST-Tat-APCS-His-Pk, followed by cleavage with thrombin and application of Tat-APCS-His-Pk to His-affinity matrix.

Full-length GST-Tat-APCS-His-Pk protein, isolated on GST-affinity matrix (lane 1) was treated with thrombin to remove the GST part. Cleaved Tat-APCS-His-Pk and GST mixture after thrombin treatment (lane 2) was applied to His-affinity support. Material that did not bind to the column is shown in lane 3, and the final wash of the column before elution of specifically bound proteins can be seen in lane 4. The protein sample eluted from the column is presented in lane 5. It is by no means pure, as it still contains a large number of contaminating proteins, including a considerable amount of GST protein.

The presence of additional proteins in the full-length GST-Tat-APCS-His-Pk sample caused the appearance of multiple cleavage products following treatment with thrombin. Protein preparations of untreated GST-Tat-APCS-His-Pk protein routinely contained a band of slightly lower molecular weight than the full-length protein, responsible for the appearance of the protein as a doublet in SDS-PAGE (barely noticeable in lane 1). That band is thought to represent protein with a severed C' terminus. Upon treatment with thrombin and loss of the GST part, that protein could generate the band observed between GST and Tat-APCS-His-Pk (indicated with a dot in lane 2). The considerable band seen below Tat-APCS-His-Pk (marked with two dots in lane 2) could be the product of thrombin cleavage of the breakdown product represented by a strong band in the GST-Tat-APCS-His-Pk protein preparation (lane 1: indicated by a dot).

Furthermore, the protein preparation eluted from the NiNta column contained a series of additional protein bands.

This observation was consistent in subsequent experiments: The presence of multiple contaminants in the protein preparation obtained by Ni-Nta-purification of Tat-APCS-His-Pk could not be circumvented through the application of mild elution conditions. This phenomenon was not due to a malfunction of the NiNta-agarose (different batches of beads were tested: 'home-made' beads (Botting & Randall, 1995) and matrix supplied by QiaGen).

Moreover, the cleaved protein sample was dialysed and applied onto a second GST-affinity column, aiming to retain all the cleaved GST molecules. The column flow through was subsequently applied onto a His-affinity matrix, extensively washed and eluted. Unfortunately, some GST protein was still present in the column eluate and the additional protein bands persisted in the protein preparation obtained after all three purifications (data not shown).

4.5.3.3 Difficulties in NiNta purification of the thrombin-cleaved protein

Treatment with thrombin was shown to successfully cleave off the GST part of GST-Tat-APCS-His-Pk protein (Figure 27). In protein preparations denaturated by SDS-Lysis Buffer and SDS gel electrophoresis, GST was clearly detached from Tat-APCS-His-Pk (detected by probing with anti-GST and anti-His monoclonal antibodies, Figure 27). However, the persistence of the cleaved GST portion following purification on Ni-Nta-agarose (Figure 28) implied that, in non-denaturing conditions, cleaved GST remained physically attached to Tat-APCS-His-Pk protein.

This interaction must be of high affinity, as cleaved GST complexed with Tat-APCS-His-Pk was not selectively retained by application on a second GST column. Similar observations following cleavage with the adenovirus protease (part 4.4.3), where protein GST-Tat remained attached to the cleaved portion His-Pk, strengthen the hypothesis that the presence of Tat protein encourages non-specific interactions with proteins (GST) and polypeptidic sequences (His-Pk). Possible reasons for these non-specific interactions will be discussed later.

4.5.4 Conclusions on the purification work for GST-Tat-APCS-His-Pk

Following the observation that the AP-cleaved Tat (lacking a Pk tag) persisted on the Pk-affinity column, alternative methods of isolating GST-Tat-APCS-His-Pk protein were tested. Initial attempts to precipitate the protein with polyethyleneimine or ammonium sulphate did not provide an early isolation step for GST-Tat-APCS-His-Pk protein. In order to further purify GST-Tat-APCS-His-Pk, the selective retention of the protein on GST and His affinity matrices was investigated.

When GST-Tat-APCS-His-Pk protein was isolated by GST-affinity purification, some GST-fusion proteins lacking an intact C' terminus persisted in the protein preparation. It was envisaged that these additional proteins would be removed by subsequent purification on the basis of the His tag (C' terminal tag). However, His-affinity purification resulted in low protein yield, both when employed as a first purification step and when following initial isolation by GST selection.

Furthermore, when full-length protein was treated with thrombin to lose the GST tag, His-affinity purification did not successfully separate Tat-APCS-His-Pk protein from the cleaved GST tag. Moreover, the thrombin-treated protein isolated on His-affinity matrix contained some additional proteins that could not be eliminated under mild elution conditions. Even though the additional bands present in the protein preparation (seen in lane 5 of Figure 28) could be accounted for, this 3-step purification, providing a rather low yield for Tat-APCS-His-Pk protein, did not present a satisfactory alternative to the single step GST-affinity purification.

On the other hand, the protein preparation obtained by selection on a GST-affinity matrix did not contain any bacterial contaminants; all additional protein bands were shown to represent GST-fusion proteins containing shorter sequences of GST-Tat-APCS-His-Pk. Further purification on the basis of the C' terminal tag His did not provide the means to eliminate those proteins while obtaining large amounts of GST-Tat-APCS-His-Pk. Since these additional proteins were not of bacterial origin and the purification methods available to us did not provide the means to further improve the protein preparation, it was decided at that point that the GST-Tat-APCS-His-Pk protein preparation isolated on a GST matrix was satisfactory for use in animal immunisations.

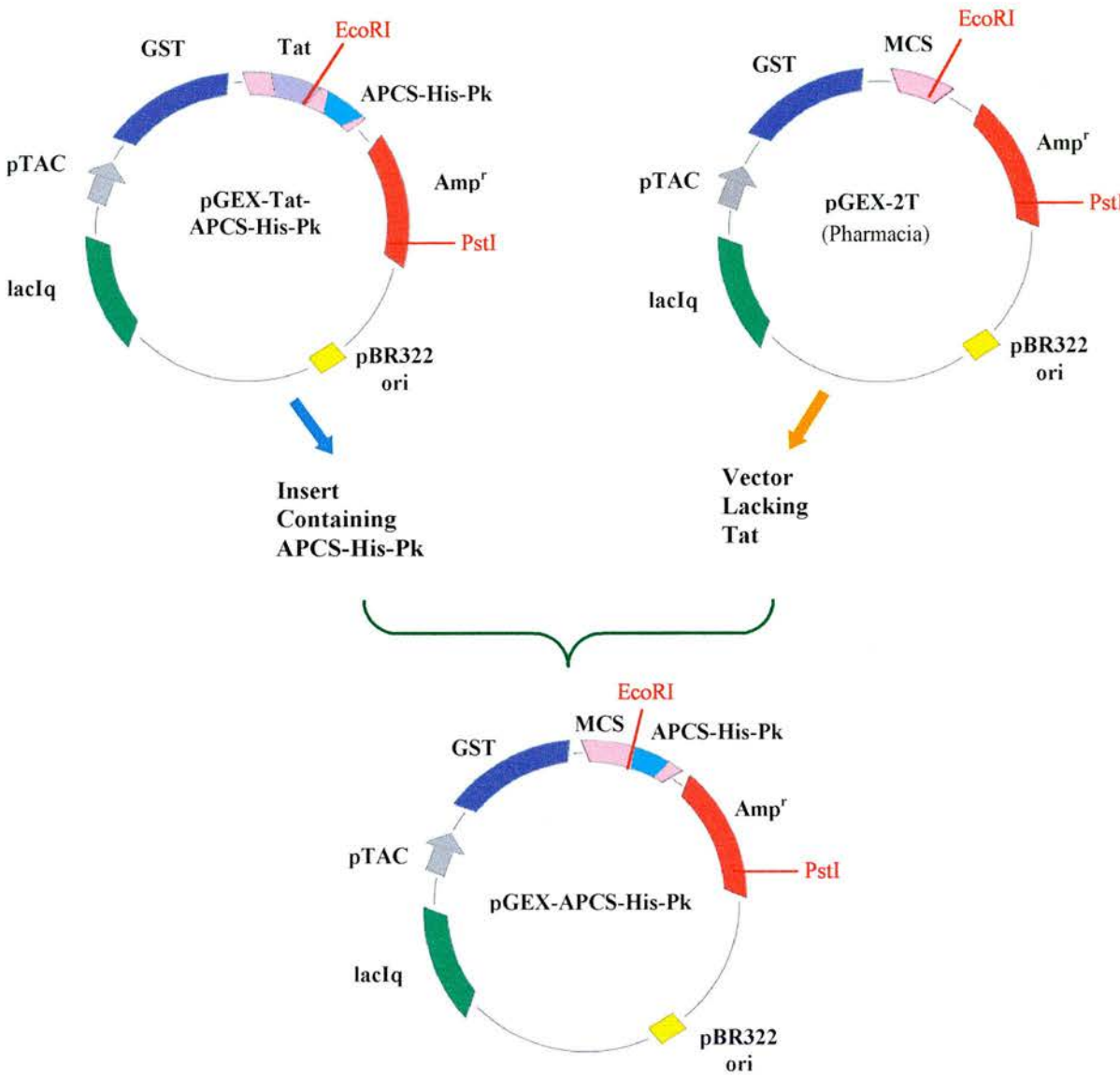
It is worth mentioning here that it was initially envisaged for Tat to be tagged with the three affinity tags purely for purification purposes and that all tags be removed for Tat protein alone to be used in immunisation studies. Since this was not the case and the GST-Tat-APCS-His-Pk protein was tagged with all three tags, it was necessary to include an immunisation control protein lacking Tat but containing the affinity tags sequences. To that end, protein GST-Pk was included

in the first mouse immunisation study, while the improved GST-APCS-His-Pk protein (engineered as shown in Figure 29) was used as a control in the second mouse immunisation study. The proteins employed in the two studies are shown in Figure 30.

4.6 Choice of Adjuvant-Carrier System

Prior to injection into the animals, GST-Tat-APCS-His-Pk and the control protein would have to be incorporated in a suitable antigen carrier system, such as SMAA complexes or the adjuvant LTB, both successfully employed in our laboratory in previous animal immunisation studies. However, Tat lentiviral protein has been documented to attach to the cellular membrane and to enter cells (Frankel & Pabo, 1988, Mann & Frankel, 1991; Vives *et al.*, 1997), a property that allows delivery of Tat in the absence of an antigen carrier. If Tat in the GST-Tat-APCS-His-Pk conformation retained this ability, then no carrier system would be required for immunisation with GST-Tat-APCS-His-Pk.

To investigate this possibility, GST-Tat-APCS-His-Pk was allowed to interact with tissue culture animal cells. When cell monolayers were incubated with GST-Tat-APCS-His-Pk in solution, the protein was clearly attaching itself to the cell membranes, with possible internalisation. On the other hand, neither GST-Pk (seen in Figure 31), nor GST-APCS-His-Pk (data not shown) displayed a similar pattern of attachment, so this phenomenon is clearly attributed to the presence of Tat. It was concluded that this function of Tat was not compromised by incorporation of Tat in a larger protein.



Key: VECTOR: pBR322 ori: origin of DNA replication, lacIq: gene coding for lac repressor protein, pTAC: promoter of transcription (IPTG inducible), GST: Glutathione-S-Transferase protein, MCS: Multiple Cloning Site, Amp^r: gene conferring Ampicillin resistance.

INSERT: APCS: Adenovirus Protease Cleavage recognition Site, His: 6-Histidines affinity tag, Pk: 14 amino acid affinity tag.

RESTRICTION SITES: EcoRI, PstI.

Figure 29: Construction of pGEX-APCS-His-Pk vector, to encode an immunisation control for GST-Tat-APCS-His-Pk.

Previously constructed vector pGEX-Tat-APCS-His-Pk (top left) and unmodified pGEX-2T (top right) were treated with EcoRI and PstI to release a 1kb fragment. The fragment removed from pGEX-2T was replaced with the APCS-His-Pk–containing insert from pGEX-Tat-APCS-His-Pk, generating vector pGEX-APCS-His-Pk (bottom).The resulting vector coded for protein GST-APCS-His-Pk, seen in Figure 30. Complete DNA and protein sequences for GST-APCS-His-Pk can be found in Appendix 7.4.

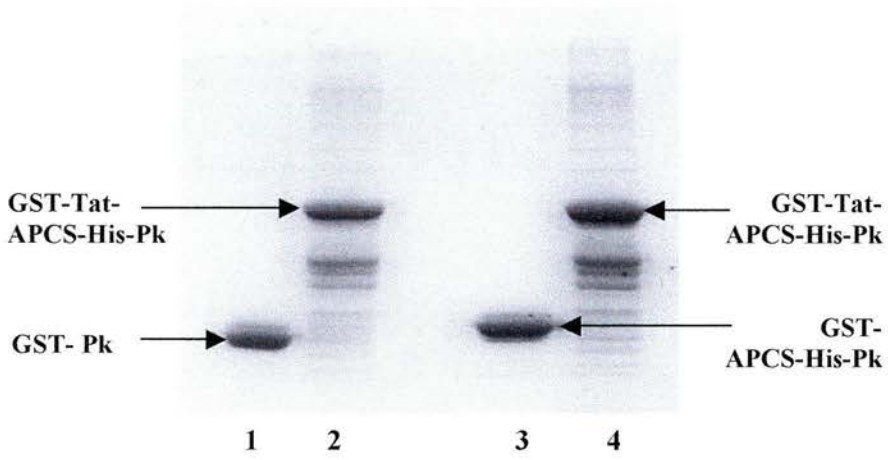
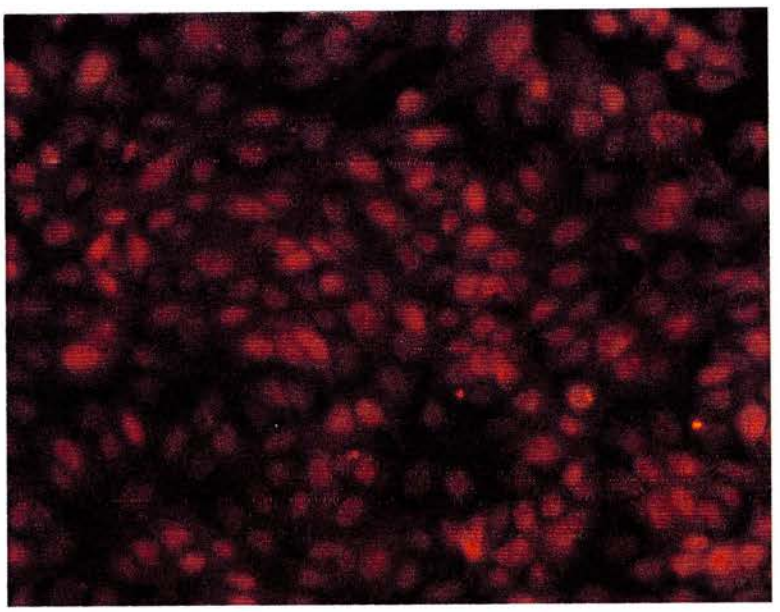


Figure 30: Test and control proteins employed in immunisation experiments. All four GST-fusion proteins were isolated on GST-affinity matrix.

The first mouse immunisation included proteins GST-Tat-APCS-His-Pk (lane 2) and GST-Pk (lane 1). In the repeat mouse immunisation, animals received (a different batch of) GST-Tat-APCS-His-Pk protein, shown in lane 4, or GST-APCS-His-Pk (lane 3). Due to the presence of a range of additional protein bands, the protein of interest in each protein sample is indicated with an arrow. The additional bands present in the GST-Tat-APCS-His-Pk protein preparation were previously shown to be of a GST-fusion nature, containing shorter sequences of protein GST-Tat-APCS-His-Pk.

A.



B.

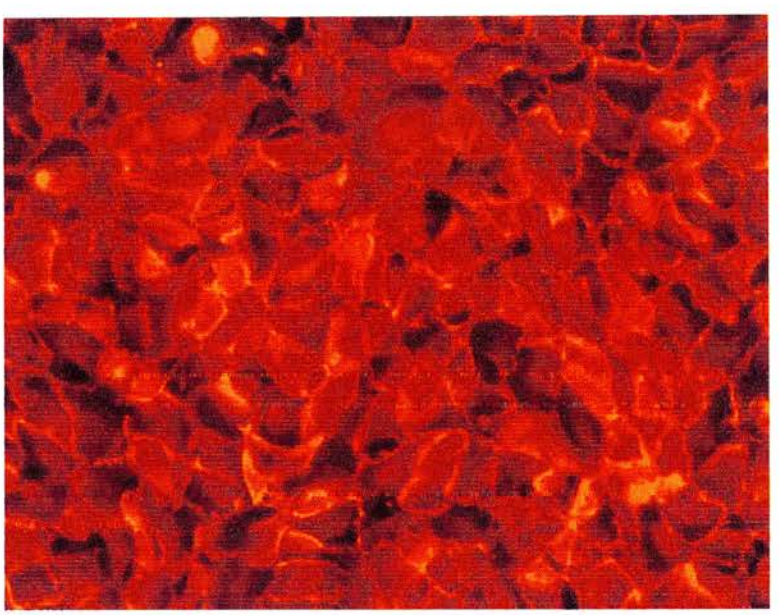


Figure 31: Monolayer of Vero tissue culture cell line incubated with protein GST-Pk (A) or GST-Tat-APCS-His-Pk (B).

A monolayer of Vero cells (Tissue Culture continuous cell-line of monkey kidney cells) was incubated with 1 μg/ml GST-Tat-APCS-His-Pk or GST-Pk control for 90 min (details in Methods: 3.4.1). The cells were thoroughly washed and fixed with a 5% formaldehyde solution. Monolayers were examined for specific attachment of the Pk-tagged proteins by anti-Pk antibody probing. The fluorescence pattern was generated by rhodamin-conjugated secondary antibody (section 3.4.1).

GST-Tat-APCS-His-Pk specifically attached itself to the cell membranes as seen in panel B, the fluorescence pattern outlining the cell boundaries. In panel A though, GST-Pk did not attach to the cells, generating only a minimal, background signal.

This pattern of specific attachment of GST-Tat-APCS-His-Pk onto tissue culture cells was repeatedly obtained in cell lines of monkey origin (Vero cells, a kidney cell line, Figure 31) and of mouse origin (BF cells, an epithelial cell line, data not shown). It was therefore decided that GST-Tat-APCS-His-Pk did not require a carrier or transporter system to be employed in animal immunisations.

B. Animal Immunisations with Tat Protein

Even though GST-Tat-APCS-His-Pk protein, isolated on GST-affinity matrix, was considered pure enough for use in animal immunisations, the difficulties encountered during proteolytic cleavage of the full-length protein with proteases thrombin and AP, excluded the possibility of obtaining Tat on its own, detached from all affinity tags. As a result, Tat-specific responses in the immunised animals could not be measured directly. Instead, it was planned for the immunogenicity of Tat to be assessed indirectly, by assaying responses directed to parts of the molecule other than Tat. GST protein delivered on its own was previously shown to be poorly immunogenic, and since GST-Pk was included as an immunisation control, GST-Pk was chosen as a suitable marker for immunisation-induced immune responses. The immunogenicity of Tat as part of the GST-Tat-APCS-His-Pk molecule was therefore investigated by immunisation studies in mice (section 4.7) and in monkeys (section 4.8).

4.7 Immunisation Studies in Mice

During mouse immunisation studies, GST-Tat-APCS-His-Pk or a GST-fusion protein lacking Tat was injected intraperitoneally to mice, either soluble, or precipitated on the adjuvant alum. A prime immunisation was given at week 0 and two booster immunisations at weeks 2 and 4, each immunisation dose containing 10 μg of protein. Blood samples were collected at the end of weeks 4 ('Bleed 1') and 6 ('Bleed 2') and the serum fractions were isolated and tested for the presence of specific antibody responses to GST-Pk by ELISA (details in 3.4.4 and 3.4.5).

Sera were applied on microtitre plates at an initial dilution of 1:50 and serially diluted two-fold. A mouse-specific anti-Ig HRP-conjugate Ab was employed to detect total specific Ab (to GST-Pk) in serum, while to determine the isotype of the Abs present, anti-mouse isotype-specific Abs developed in goat were used, visualised by a goat-specific anti-Ig HRP-linked Ab.

Plates were developed and read, and average absorbance values for all animals within each treatment were calculated for Bleed 1 and Bleed 2. A cut-off point of twice the value of the background readings was used to determine the end-point titres of specific Ab. The validity and reproducibility of the data presented here was confirmed by multiple assays.

Note: It was shown that assaying serum samples from each individual mouse in a group and averaging their Ab responses would generate the same results as assaying a sample of pooled sera. Pooled sera samples were generally assayed in the following studies, but sera from individual mice were always tested to ensure there was no deviation from the general trend of responses.

4.7.1 Findings from the first mouse immunisation study

This study included four mice per group and the immunisation control employed for GST-Tat-APCS-His-Pk was GST-Pk. Table 3 contains the end-point titres of total specific serum Ab to GST-Pk, as calculated in the vaccinated mice.

In agreement with the action of alum, both GST-Pk and GST-Tat-APCS-His-Pk proteins triggered strong immune responses when presented precipitated on alum. In the absence of alum, GST-Pk did not generate any detectable responses, while in striking contrast, soluble GST-Tat-APCS-His-Pk induced specific antibody.

Table 3: Detection of antibody specific to protein GST-Pk, in the sera of mice immunised with soluble, or alum-precipitated GST-Pk or GST-Tat-APCS-His-Pk after two or three immunisations (Bleed 1 and Bleed 2 respectively).

Protein Injected	Antibody Titre	
	Bleed 1	Bleed 2
GST-Pk	50	<50
GST-Tat-APCS-His-Pk	400	3200
GST-Pk & Alum	1600	13 k
GST-Tat-APCS-His-Pk & Alum	13 k	50 k

GST-Tat-APCS-His-Pk was clearly immunogenic in the absence of an adjuvant or carrier. Even though specific Abs to GST-Pk were only assayed, since GST was physically linked to Tat, it is reasonable to believe that Tat-specific Abs were also generated. Furthermore, since soluble GST-Tat-APCS-His-Pk but not GST-Pk, triggered GST-Pk–specific Abs, it was concluded that the immunogenicity of GST-Pk was increased when it was presented in the context of Tat. The presence of Tat in the immunisation protein resulted in better presentation of antigens on the immunisation protein outside the sequence of Tat.

The different isotypes making up the total Ab generated by immunisation were detected with a range of mouse-isotype-specific Abs. The results presented here correspond to serum samples collected at the end of the immunisation study (Bleed 2). A similar pattern of Ab responses was observed during analysis of the sera collected after just two immunisation doses (Bleed 1, data not shown). Out of the six Ab isotypes assayed, specific Ab titration for IgM, IgG1 and IgG2a isotypes are presented in Figure 32, while the end-point titres for all six Abs tested are compared in Table 4.

Table 4: Characterisation of specific antibody to protein GST-Pk in the serum of mice immunised with protein GST-Pk or GST-Tat-APCS-His-Pk, soluble or precipitated on alum.

Protein Injected	Antibody Titre					
	IgM	**IgG1**	**IgG2a**	**IgG2b**	**IgG3**	**IgA**
GST-Pk	50	<50	<50	50	100	50
GST-Tat-APCS-His-Pk	400	3200	200	400	100	50
GST-Pk & Alum	400	50 k	100	100	50	50
GST-Tat-APCS-His-Pk & Alum	50	13 k	1600	800	<50	<50

GST-Pk was poorly immunogenic in the absence of alum: it failed to generate specific Ab responses with an end-point titre over 100. There was no suggestion for the ability of GST-Pk without alum to trigger Ab-based immunological memory. On the other hand, immunisation with soluble GST-Tat-APCS-His-Pk generated good levels of IgG1 and lower levels of IgG2b, IgG2a and IgG3. The induction of specific antibody of the IgG isotypes suggested that the Tat-fusion protein was inducing immunological memory in the absence of alum.

Alum increased the immunogenicity of GST-Pk, reflected by a vigorous IgG1 response. Alum-precipitated GST-Tat-APCS-His-Pk also triggered a very

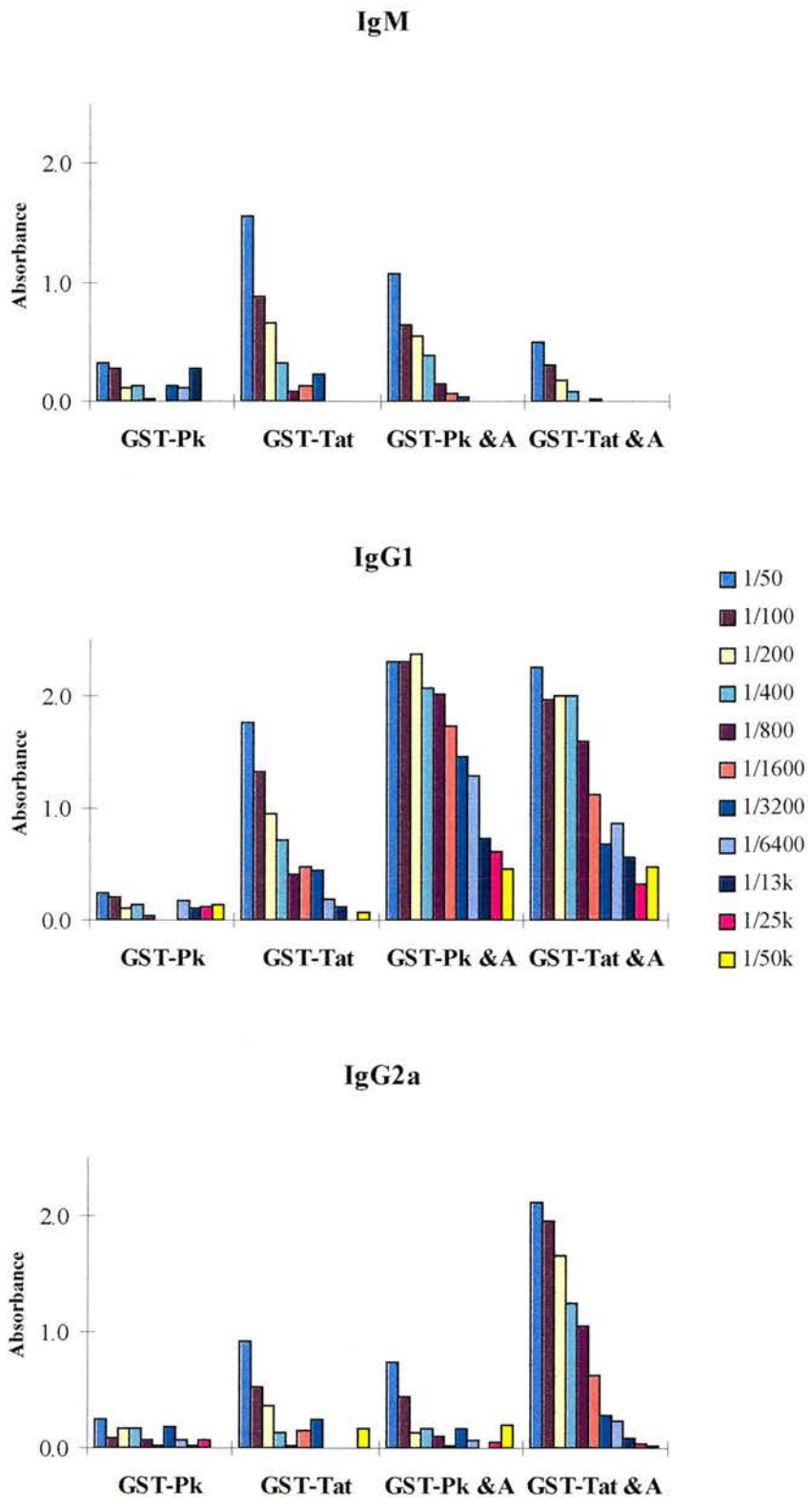


Figure 32: Titration of antibodies specific to GST-Pk, of the IgM, IgG1 and IgG2a isotypes, as detected in the sera of immunised mice. The animals received three intraperitoneal injections of GST-Pk or GST-Tat-APCS-His-Pk protein (denoted 'GST-Tat'), in the absence or in the presence of alum (& A). Serial two-fold dilutions of the sera were tested, starting at an initial dilution of 1/50.

potent IgG1 response, far superior to that induced by soluble GST-Tat-APCS-His-Pk, as well as notable IgG2a and IgG2b responses. Since the established function of alum does not include the generation of IgG2a Ab, it is possible that this shift in the adjuvantic action of alum was caused by the presence of Tat in the immunisation protein.

In mice, the abundance of IgG1 Ab isotype is largely associated with T_H2-type immune responses, while the production of IgG2a Ab is the hallmark of T_H1-type responses. Immunisation with alum-precipitated GST-Pk triggered responses with an antibody type ratio of IgG1 over IgG2a suggesting the induction of a strong T_H2-type response, with no evidence of a T_H1 response. Immunisations with GST-Tat-APCS-His-Pk with and without alum generated Ab responses with IgG1 to IgG2a ratios indicating that a T_H1 response was developing alongside the strong T_H2 response.

To summarise the findings of this first immunisation, soluble Tat protein was shown to be immunogenic, as GST-Tat-APCS-His-Pk induced Ab responses that were not matched by GST-Pk. The immunogenicity of GST-Tat-APCS-His-Pk was further increased by alum precipitation, while the presence of Tat appeared to transform the type of immune response usually induced in the presence of alum, towards the production of IgG2a and the generation of T_H1-type immunity. Additionally, Tat was shown capable of augmenting the immunogenicity of an unrelated co-administered protein.

The results obtained during this first immunisation were very encouraging, so GST-Tat-APCS-His-Pk was employed in a second immunisation study to confirm these observations.

4.7.2 Findings from the second immunisation study

The repeat immunisation included groups of five mice, and the immunisation control employed was GST-APCS-His-Pk. The same immunisation schedule was followed, and specific Ab was assayed as already described. Table 5 presents the end-point titres for total specific Ab to GST-Pk, as detected in the animal sera.

During this immunisation study, soluble GST-Tat-APCS-His-Pk induced an overwhelming response after just two immunisations, with an end-point titre of 50k after all three immunisations. On the other hand, soluble GST-APCS-His-Pk generated only a moderate response after all three immunisations.

Table 5: End-point titres of serum samples taken after two immunisations (Bleed 1) and three immunisations (Bleed 2). Mice were injected with soluble or alum-precipitated GST-APCS-His-Pk or GST-Tat-APCS-His-Pk.

Protein Injected	Antibody Titre	
	Bleed 1	Bleed 2
GST-APCS-His-Pk	50	1600
GST-Tat-APCS-His-Pk	13 k	50 k
GST-APCS-His-Pk & Alum	1600	3200
GST-Tat-APCS-His-Pk & Alum	3200	6400

Alum-precipitated GST-APCS-His-Pk produced a specific Ab response higher than that generated by the soluble GST-APCS-His-Pk, but the improvement was not as staggering as that observed during the first immunisation study for GST-Pk (Table 3). Surprisingly, precipitated GST-Tat-APCS-His-Pk triggered Ab responses far inferior to those induced when the protein was injected in a soluble form, in complete disagreement to the findings of the previous study and the

established action of alum. Even so, GST-Tat-APCS-His-Pk still triggered marginally better Ab responses than the control protein in the presence of alum.

The observation that alum increased the immunogenicity of GST-APCS-His-Pk (even slightly), but decreased that of GST-Tat-APCS-His-Pk, was highly surprising. We were hoping to gain some insight from the isotyping analysis of the total specific Ab.

Table 6 contains the end-point titres for all six Ab isotypes tested on serum samples collected at the end of the immunisation study (Bleed 2), which were much in accord with the data obtained when assaying serum samples from Bleed 1 (after the first two immunisations, data not shown).

Table 6: End-point titres for different Ab isotypes specific to protein GST-Pk in sera of immunised mice. Animals received three doses of soluble or alum-precipitated GST-APCS-His-Pk or GST-Tat-APCS-His-Pk.

Protein Injected	Antibody Titre					
	IgM	**IgG1**	**IgG2a**	**IgG2b**	**IgG3**	**IgA**
GST-APCS-His-Pk	400	1600	100	<50	<50	<50
GST-Tat-APCS-His-Pk	3200	6400	6400	13 k	3200	50
GST-APCS-His-Pk & Alum	400	6400	50	50	50	<50
GST-Tat-APCS-His-Pk & Alum	800	6400	50	50	50	<50

In agreement with the observations from the first immunisation study, immunisation with GST-Tat-APCS-His-Pk, with or without alum, generated good IgG responses to GST-Pk, thus confirming the immunogenicity of the molecule and the ability of Tat to act as an antigen carrier. However, in the first immunisation, higher IgG responses were observed for both proteins in the presence rather than in the absence of alum, which was completely reversed here (for ease of comparison, refer to Figure 33).

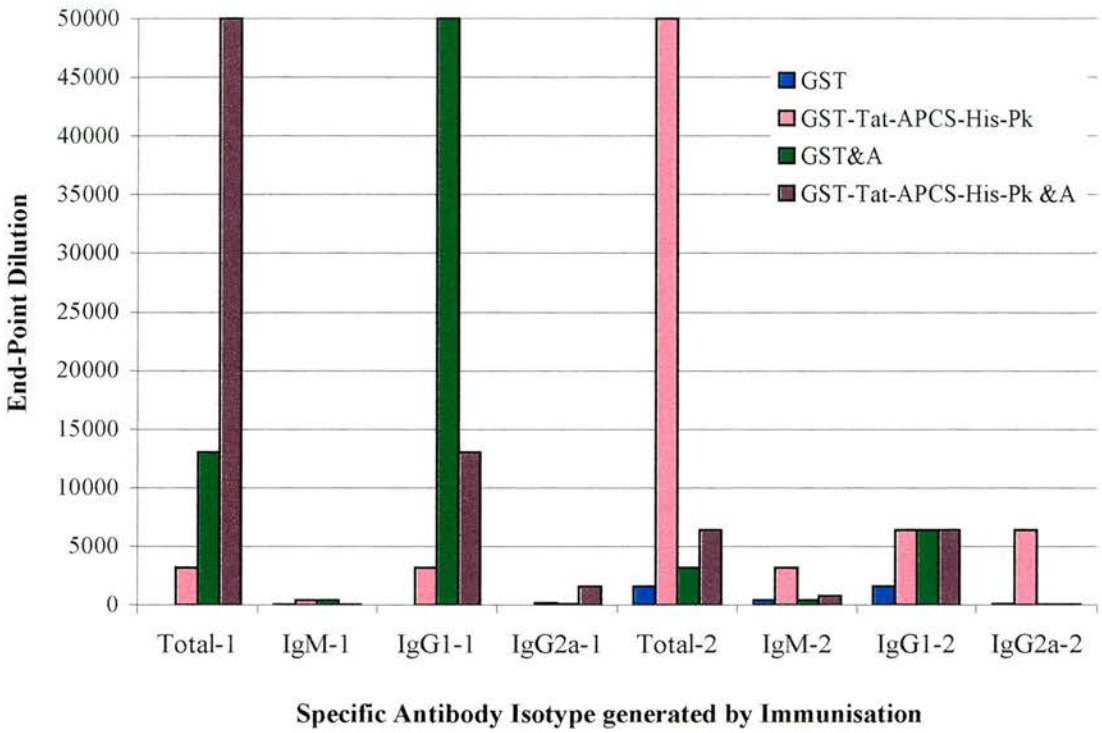


Figure 33: Specific Ab responses to GST-Pk, as assayed in the sera of mice immunised with three doses of either soluble or alum-precipitated GST-Tat-APCS-His-Pk or GST control (GST-Pk for Immunisation 1 and GST-APCS-His-Pk for Immunisation 2).

The end-point titres for total Ab (Bleed 2) and for isotypes IgM, IgG1 and IgG2a, as already presented in Tables 3, 4, 5 and 6 are compared here. The number next to each isotype denotes the study to which those titres belong: '1' for the first immunisation and '2' for the repeat immunisation. Similarly, total Ab is marked 'Total-1' and 'Total-2' respectively. The values of the Y-axis represent the end-point dilution of serum that contained specific Ab to protein GST-Pk.

In the second immunisation study, alum-precipitated GST-Tat-APCS-His-Pk induced poorer responses than the soluble protein, for five out of six Ab isotypes tested. This decrease in the immunogenicity of GST-Tat-APCS-His-Pk was not mirrored in GST-APCS-His-Pk, which was more immunogenic when precipitated than when soluble (reflected by a stronger IgG1 response). The isotype analysis thus confirmed the trend observed by total Ab responses (Table 5): the presence of alum increased the immunogenicity of GST-APCS-His-Pk, but decreased that of GST-Tat-APCS-His-Pk.

Soluble GST-Tat-APCS-His-Pk induced a good IgG2a response, which was an encouraging observation for the ability of Tat to drive T_H1 responses. In fact, soluble GST-Tat-APCS-His-Pk induced an IgG1 to IgG2a ratio suggesting the development of a stronger T_H1 response compared to the first immunisation. However, GST-Tat-APCS-His-Pk precipitated on alum did not generate an IgG2a response, unlike the findings of the first immunisation study. Therefore, the repeat immunisation did not suggest a Tat-induced switch in the function of alum.

It is worth mentioning that, due to the inconsistencies between the two immunisation studies, the mice sera were also assayed individually and confirmed the trend observed when assaying the pooled sera.

4.7.3 Cellular immunity induced by Tat

The two mouse immunisation studies established that soluble GST-Tat-APCS-His-Pk was highly immunogenic and that it was triggering T_H1-type immune responses. It was therefore possible that immunisation with GST-Tat-APCS-His-Pk could induce specific CTL responses. As pure Tat protein was not available to

employ for antigen-stimulation in CTL assays, two specific CTL epitopes were selected to enable the detection of immunisation-induced CTLs in the spleens of immunised mice. The one epitope was derived from gp120 of HIV and restricted to BalbC H2Dd mice, while the other originated from OVA and was restricted to mice expressing MHC haplotypes H2-kb and C57BL/6.

The sequence encoding these two epitopes was inserted in the vectors coding for proteins GST-Tat-APCS-His-Pk and GST-APCS-His-Pk, as detailed in Figures 34 and 35. Newly produced proteins GST-CTLs-Tat-APCS-His-Pk and GST-CTLs-APCS-His-Pk were then expressed in bacterial hosts as presented in Figure 36.

However, the two proteins were being produced in very low amounts (see Figure 36). It is possible that the hydrophobic CTLs sequence was rendering the proteins insoluble, as overexpression of hydrophobic proteins often results in the formation of inclusion bodies (Lilie *et al.*, 1998).

For that reason, testing of those proteins in immunisation studies could not be completed within the frame of this work.

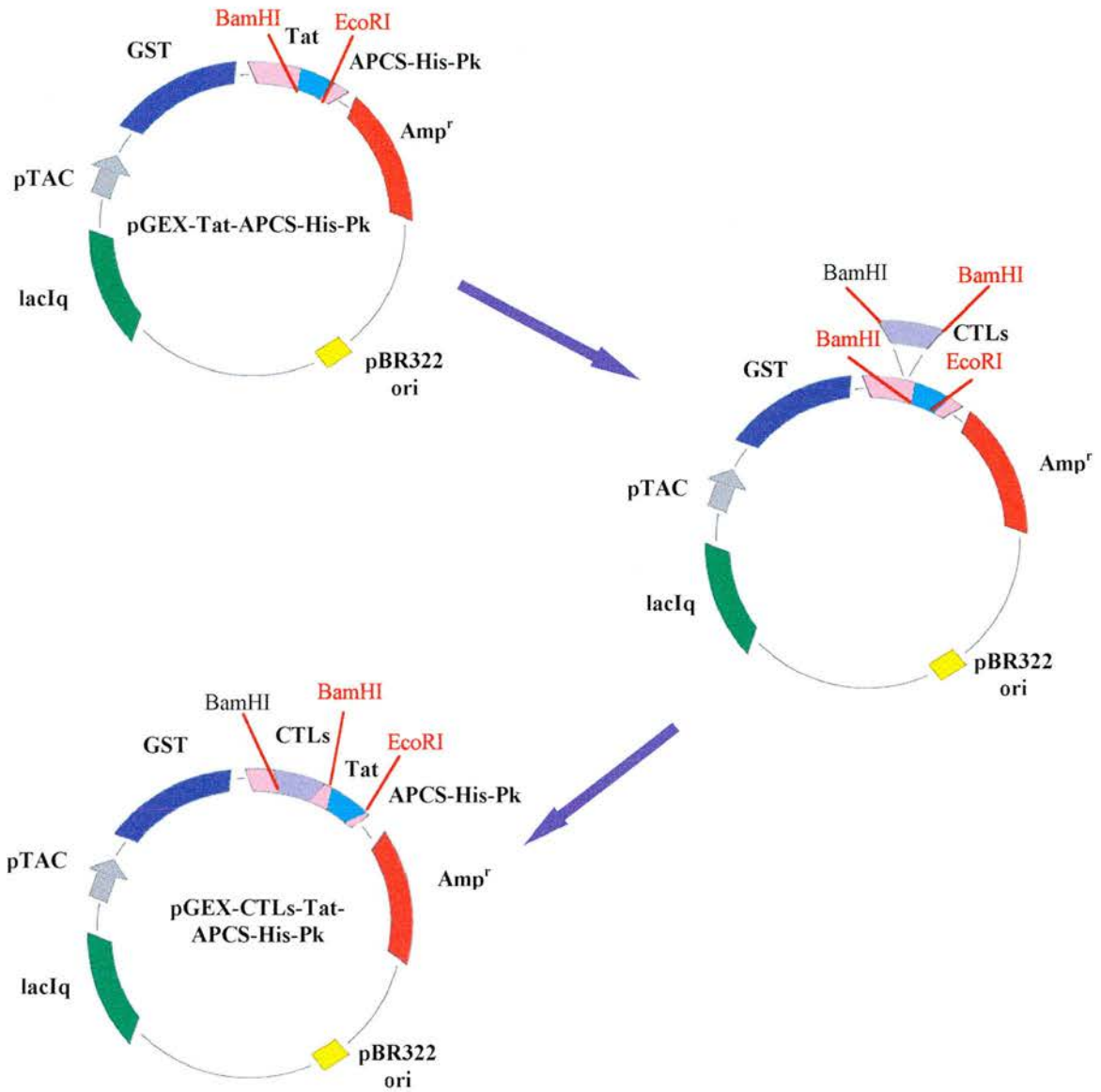
Even though it was not possible to test whether the Tat-containing protein would generate CTL responses in mice, the Ab responses observed were very encouraging. We were very interested to investigate whether the protein would generate Ab and CTL responses in monkeys; and as the window of opportunity arose, GST-Tat-APCS-His-Pk was tested in monkeys to assess its immunogenicity and its potential to offer protection against lentivirus infection.

Figure 34: Insertion of the CTLs sequence in the GST-Tat-APCS-His-Pk vector.

The CTLs fragment
Complementary single stranded oligos (forward and reverse) coding for epitopes derived from gp120 of HIV (for mice BalbC carrying the MHC haplotype H2Dd) and from OVA (for mice expressing MHC haplotypes H2-kb and C57BL/6) were designed and obtained. When the two complementary oligos were hybridised (as described in Methods 3.1.3) the double stranded DNA molecule (see middle diagram) contained an intact BamHI site -depicted in red- and a BamHI-compatible (but not cohesive) overhang -shown in black (for exact sequences see Appendix 7.10).

The cloning strategy
Vector GST-Tat-APCS-His-Pk (top of diagram) was linearised by BamHI digestion and the CTLs fragment was inserted into the BamHI restriction site as indicated in the middle diagram. The construct generated was coding for the CTL epitopes (bottom), while the BamHI site at the start of SIV-Tat (shown in red) was retained without generating an additional BamHI restriction site (the full sequence is included in Appendix 7.9).

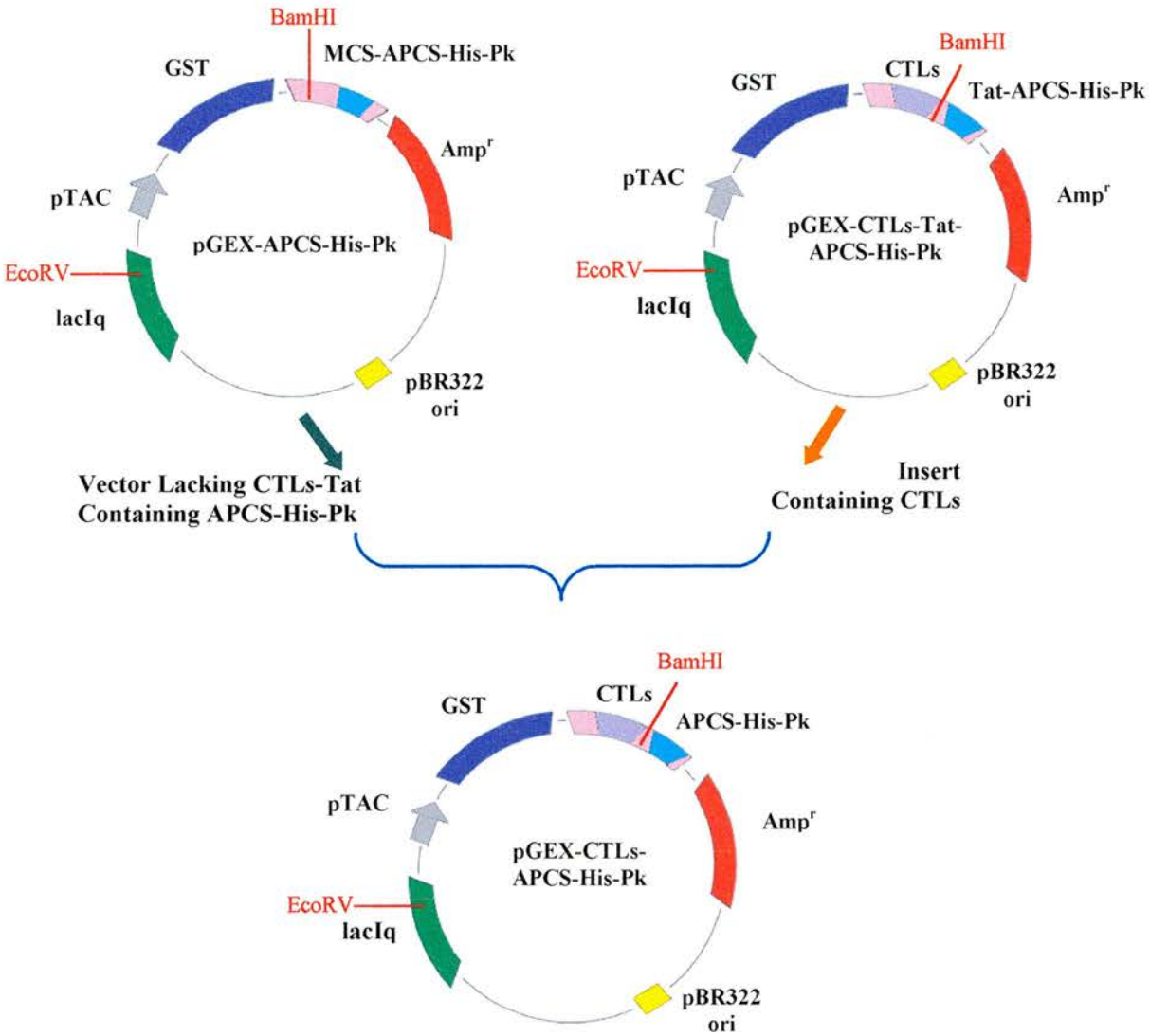
Screening of transformants
The resulting plasmid was transfected into competent *E. coli* cells (Methods 3.1.10) which were plated on selective media. Transformant clones were screened for a combination of in-frame protein expression and increase in protein molecular weight resulting from the addition of the 100-bp long CTLs insert. Minicultures of clones were grown and induced with IPTG and their total cell fractions were subjected to immunoblotting with His-specific antibody as described in Methods 3.1.11. Plasmid DNA was subsequently sequenced and confirmed to be driving the expression of GST-CTLs-Tat-APCS-His-Pk protein. Selection of the protein on the basis of GST affinity can be found later, in Figure 36.



Key: VECTOR: pBR322 ori: origin of DNA replication, lacIq: gene coding for lac repressor protein, pTAC: promoter of transcription (IPTG inducible), GST: Glutathione-S-Transferase protein, MCS: Multiple Cloning Site, Amp^r: gene conferring Ampicillin resistance, Tat: Transactivator of Transcription, protein of SIV, APCS: Adenovirus Protease Cleavage recognition Site, His: 6-Histidines affinity tag, Pk: 14 a.a. affinity tag.

INSERT: CTLs: Cytotoxic T-Lymphocyte epitopes sequence.

RESTRICTION SITES: BamHI, EcoRI.



Key: VECTOR: pBR322 ori: origin of DNA replication, lacIq: gene coding for lac repressor protein, pTAC: promoter of transcription (IPTG inducible), GST: Glutathione-S-Transferase protein, MCS: Multiple Cloning Site, Amp^r: gene conferring Ampicillin resistance, Tat: Transactivator of Transcription, protein of SIV, APCS: Adenovirus Protease Cleavage recognition Site, His: 6-Histidines affinity tag, Pk: 14 a.a. affinity tag.
INSERT: CTLs: Cytotoxic T-Lymphocyte epitopes sequence.
RESTRICTION SITES: BamHI, EcoRV.

Figure 35: Engineering the pGEX-CTLs-APCS-His-Pk construct to encode an immunisation control for GST-CTLs-Tat-APCS-His-Pk protein.

Previously constructed vectors pGEX-CTLs-Tat-APCS-His-Pk (top right, refer to Figure 34) and pGEX-APCS-His-Pk (top left, shown in Figure 29) were digested with EcoRV and BamHI to release a 2kb fragment. The fragment obtained from pGEX-CTLs-Tat-APCS-His-Pk (containing the CTLs coding sequence) was ligated into the opened pGEX-APCS-His-Pk. The newly engineered vector pGEX-CTLs-APCS-His-Pk (bottom diagram - for full sequence, refer to Appendix 7.5.) was therefore driving the expression of protein GST-CTLs-APCS-His-Pk, that can be seen in Figure 36.

Figure 36: Purification of proteins GST-CTLs-Tat-APCS-His-Pk (A), GST-CTLs-APCS-His-Pk (B) and GST-APCS-His-Pk (C) on GST-affinity matrix.

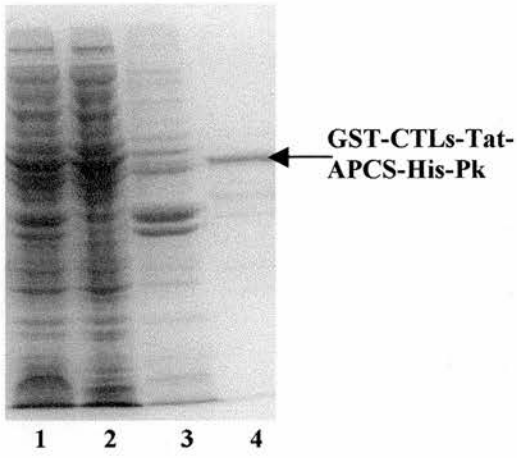
Proteins containing the CTL epitopes were generated to be used in a third mouse immunisation. The vectors encoding the two proteins were engineered as described in Figures 34 and 35. Protein GST-APCS-His-Pk was employed in the second mouse immunisation and was generated as described in Figure 29.

Purification on GST-affinity matrix produced the proteins seen in lanes 4 of the three panels. Purification stages for GST-CTLs-Tat-APCS-His-Pk protein are presented in Panel A, for GST-CTLs-APCS-His-Pk in Panel B and for GST-APCS-His-Pk in Panel C: Total cell fraction in lane 1, soluble antigen in lane 2, insoluble antigen in lane 3 and GST-purified protein in lane 4. The proteins were obtained to high purity by single-step GST purification.

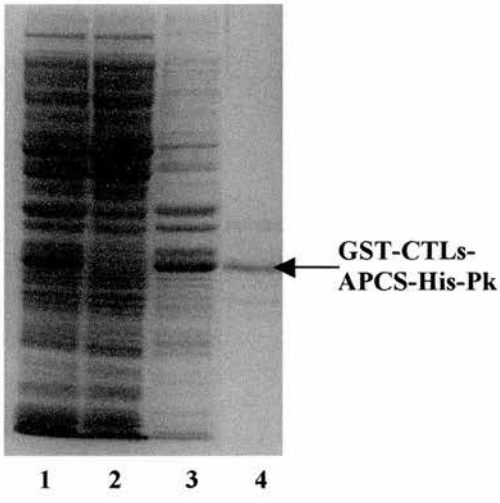
However, for the two proteins containing the CTL epitopes sequence, a very large amount of each protein was lost to the insoluble antigen fraction. The yield of protein purification from a litre of bacterial culture was just 0.8 mg for GST-CTLs-APCS-His-Pk and less than 0.025 mg for GST-CTLs-Tat-APCS-His-Pk protein.

The purification of GST-APCS-His-Pk protein is included here for the sake of comparison: the total cell fraction and soluble antigen represent 1‰ of the bacterial culture, yet the band corresponding to GST-APCS-His-Pk is clearly visible, with a typical protein purification yield of 10 mg per litre of culture. The addition of the CTLs sequence caused a decrease of at least ten-fold in protein expression (panels B and C).

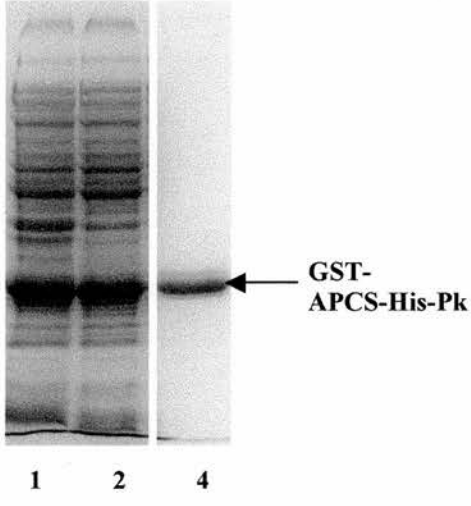
A. GST-CTLs-Tat-APCS-His-Pk



B. GST-CTLs-APCS-His-Pk



C. GST-APCS-His-Pk



4.8 Immunisation of Monkeys with GST-Tat-APCS-His-Pk

This study was performed in collaboration with the research group of Dr Martin Cranage at the Centre for Applied Microbiology and Research (CAMR) Institute in Salisbury, UK. Six male Mamu A*01 Indian rhesus macaque monkeys were available for immunogenicity studies, even though they were not suitable for challenge studies, as they had received SIV-specific vaccine formulations previously. Mamu A*01 are CTL-restricted for the epitope STPESANL, found in SIV-Tat (Allen *et al.*, 2000). Even though Tat from mac251(J5) contains the epitope TTPESANL instead (S substituted for T at the anchor point), Mamu A*01 recognise that epitope in Tat from SIV251 (Todd M. Allen, Univ. of Wisconsin, USA, unpublished observations). Therefore, if Tat had the potential to generate cellular immune responses, any CTL responses generated by immunisation with GST-Tat-APCS-His-Pk could be assayed in the monkeys.

Immunisations were carried out at the CAMR. The animals were bled before the first injection (week 0) and those samples were employed as a pre-immunisation control. Monkeys were subsequently given three doses of GST-Tat-APCS-His-Pk, 100 μg per dose, by targeted lymph node injection (see Methods 3.4.3). Injections were given in weeks 0, 4 and 8 (one priming and two booster immunisations) and a final bleed was performed in week 10.

The six immunised monkeys were analysed at the CAMR for the presence of Tat-specific cellular immune responses. By week 10, IFN-γ responses were assayed by ELISPOT and peripheral blood mononuclear cells (PBMC) were tested for specific CTL activity. Some weak transient IFN-γ responses to two twentymer Tat peptide pools were recorded, but no responses to the specific

epitope peptide were detected. Furthermore, it was not possible to detect any CTL activity specific to the peptide epitope in PBMC and the IFN-γ responses did not persist after the second boost in PBMC (Dr M. Cranage, personal communication).

The serum fraction of the monkeys' blood samples was isolated and analysed in the St. Andrews laboratory. Serum samples from week 0 and week 10 were assayed by ELISA for the presence of specific Ab. The absorbance values measured for serum samples from week 10 were corrected for any non-specific responses by subtracting the background signal in sera from week 0 for each individual monkey, and the end-titres of the total Ab responses specific to GST-Tat-APCS-His-Pk and GST-Pk were determined. The ELISA assays were standardised so that the results from GST-Pk – and GST-Tat-APCS-His-Pk – coated plates would be comparable, as presented in Appendix 7.11.

As seen in the first column of Table 7, four out of six monkeys generated strong specific Ab responses to protein GST-Tat-APCS-His-Pk, with titres from 13k to 50k. The remaining two animals showed lower, but considerable Ab responses to the protein, with titres of 3200 and 6400.

Table 7: End-titre of specific antibody to proteins GST-Tat-APCS-His-Pk and GST-Pk, as detected in the post-immunisation serum of each of the six monkeys injected with three doses of GST-Tat-APCS-His-Pk protein.

Monkey Code	Antibody Titre to Protein:	
	GST-Tat-APCS-His-Pk	GST-Pk
421	50 000	3200
427	25 000	800
437	13 000	800
459	3 200	200
X37	13 000	800
X47	6 400	800

GST-Pk assays produced the results presented in the second column of Table 7. The total Ab specific to GST-Pk ranged from an end-point titre of 200 to a titre of 3200, establishing the action of Tat as an antigen carrier, also in the simian hosts.

By comparing the end-point titres of specific Ab to proteins GST-Tat-APCS-His-Pk and GST- Pk, it was obvious that not all Ab responses detected by the GST-Tat-APCS-His-Pk assay were directed against the GST and Pk parts. This was indirectly implying that some Tat-specific Abs could be present in the monkey sera.

To investigate that possibility, serum samples were employed in immunoblotting, using nitrocellulose strips with immobilised protein His-Tat-Pk (produced and kindly provided by H.Y. Chen, Univ. of St. Andrews) and GST-Tat-APCS-His-Pk, as seen in Figure 37.

The pattern obtained by immunoblotting largely conforms to the Ab titres presented in Table 7. Post-immunisation sera from monkeys 421, 427, X37 and X47 contained high levels of Ab specific to GST-Tat-APCS-His-Pk and they were indeed shown to react with that protein on the nitrocellulose strips.

Animal 459 had a much lower Ab titre and in fact, serum Ab from this animal did not vigorously bind protein GST-Tat-APCS-His-Pk in Figure 37.

Monkey 437 however, displayed a high titre of GST-Tat-APCS-His-Pk–specific Ab, which was not reproduced on the nitrocellulose strip.

It is possible that the Ab titrated in the serum of this monkey (Table 7) was mainly directed against conformational epitopes of GST-Tat-APCS-His-Pk that were lost through SDS-PAGE analysis of the protein.

Figure 37: Detecting antibodies specific to GST-Tat-APCS-His-Pk and His-Tat-Pk proteins, in the serum samples of six monkeys immunised with GST-Tat-APCS-His-Pk protein.

Outline of Experimental Procedures

Proteins GST-Tat-APCS-His-Pk and His-Tat-Pk were electrophoresed on single-slot 12.5% acrylamide gels and were transferred onto rectangular pieces of nitrocellulose membrane. Each membrane piece was subsequently sliced into 4mm-wide strips.

Samples taken before immunisation (Week 0) and two weeks after the last immunisation with GST-Tat-APCS-His-Pk (Week 10) were tested on such strips. For each immunised animal, a 1/500 dilution of the serum sample for Week 0 and Week 10 was incubated with a strip bearing either protein GST-Tat-APCS-His-Pk or protein His-Tat-Pk.

Panel A comprises of strips coated with protein GST-Tat-APCS-His-Pk, while panel B contains strips bearing protein His-Tat-Pk. The numbers underneath each strip represent monkey code names to indicate the monkey from which the serum sample originated.

To create a set of controls, a strip bearing protein GST-Tat-APCS-His-Pk and a strip coated with His-Tat-Pk were both incubated with a GST-specific Ab (a-GST), while an identical set of strips was treated with an Ab specific to the His tag (a-His).

Specific binding to GST-Tat-APCS-His-Pk or His-Tat-Pk protein on the nitrocellulose strip was detected by anti-monkey polyclonal Ab, HRP conjugate (for detailed procedures see section 3.4.6).

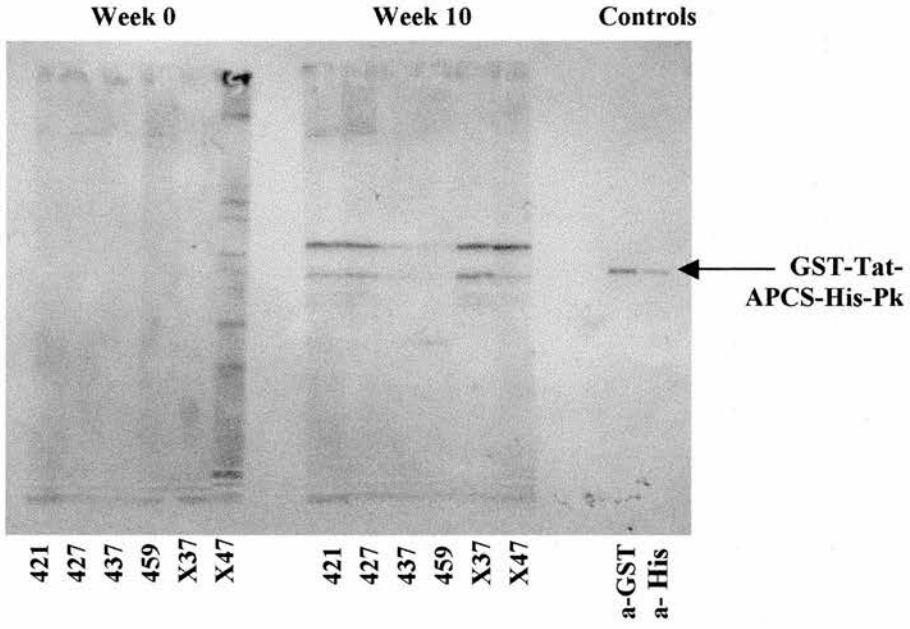
Results and Observations

Four out of six animals contained antibody in their post-immunisation serum that recognised GST-Tat-APCS-His-Pk and His-Tat-Pk proteins. Monkeys 437 and 459 showed some very weak antibody responses to protein GST-Tat-APCS-His-Pk (Panel A), but no detectable responses to His-Tat-Pk protein (Panel B).

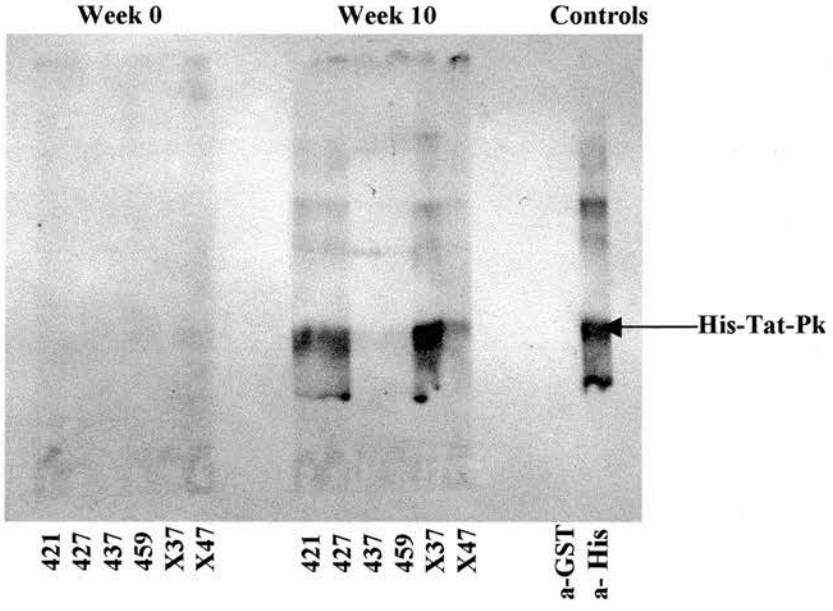
The specific recognition of protein His-Tat-Pk by four monkeys indicated that a fraction of their Ab response to GST-Tat-APCS-His-Pk was specific to parts of the protein other than the GST portion.

Note: X47 strip for GST-Tat-APCS-His-Pk Week 0 was coated not with purified GST-Tat-APCS-His-Pk, but with the total cell fraction of a culture expressing GST-Tat-APCS-His-Pk protein –hence the different banding pattern.

Panel A: GST-Tat-APCS-His-Pk



Panel B: His-Tat-Pk



More importantly, monkeys 421, 427, X37 and X47 that contained low levels of GST-Pk–specific Ab in their sera (Table 7) were strongly binding His-Tat-Pk protein on the strip (Figure 37), confirming that they recognised parts of the protein other than GST. It is clear from these results that at least some Ab was directed against the Tat moiety of the immunisation protein.

To summarise the findings of the monkey immunisation study, immunisation with GST-Tat-APCS-His-Pk generated specific Ab responses to GST-Tat-APCS-His-Pk in all six immunised animals. These responses were exclusive to the post-immunisation serum samples and in four out of six monkeys they were of a great magnitude. Four out of six animals were further shown to contain Ab specific to parts of the protein other than GST, giving reason to believe that some of the Ab responses generated were specific to Tat.

Even though the monkeys were restricted for an epitope found in the Tat protein employed, no cellular responses to that epitope were detected. Possible reasons for that observation are proposed later.

4.9 Summary of the Findings for Tat from Animal Immunisations

During the course of the immunisation studies in mice and monkeys, it became evident that immunisation with GST-Tat-APCS-His-Pk protein in the absence of a carrier or adjuvant was inducing specific immune responses. All three immunisation studies (in monkeys and in mice) demonstrated the induction of specific Ab responses in animals injected with GST-Tat-APCS-His-Pk. Even though it was not possible to directly assay immunisation-induced Tat-specific Abs, the affinity tags Pk and His have been employed before in immunisations and were shown to be not primarily immunogenic (Hanke *et al.*, 1992; Randall *et*

al., 1993). There is therefore reason to believe that at least some of the Ab responses induced were specific to the Tat moiety.

Additionally, the presence of Tat in the immunisation protein clearly enhanced the immunogenicity of unrelated proteins physically attached to it (as witnessed by the presence of GST-Pk–specific Ab both in mice and in the monkeys). Moreover, the first immunisation study in mice implied a shift towards a T_H1-type immune response when Tat was co-administered with alum. Even though this observation was not confirmed by the second immunisation study, and while the reason for this inconsistency is under investigation in our laboratory, the presence of Tat in the immunisation protein, when administered in a soluble form (without alum), clearly induced T_H1-type responses to the co-administered antigens.

5. DISCUSSION

At the outset of the present study, lentiviral vaccine research was shifting towards the regulatory viral proteins as potential targets for immunisation. In the study presented here, Tat protein from SIVmac32H(J5) was assessed in BalbC mice and in Rhesus macaque monkeys for its immunogenic and protective potential. Recombinant Tat was overexpressed in *E. coli* as a GST-fusion protein. During expression and purification stages of the fusion protein, a series of issues had to be addressed before Tat was obtained in sufficient quantities for animal immunisations.

5.1 EXPRESSION AND PURIFICATION OF SIV-TAT

Expressing the recombinant Tat in the bacterial host presented the first hurdle. The protein expression yield was very low, much in agreement with previous observations for SIVmac32H(J5) Tat expression in *E. coli*, (Hanke, 1993; Dunn, 1998). The coding sequence for Tat from SIVmac32H(J5) contains, within its arginine-rich region, three consecutive Arg codons that are unfavourable for expression in *E. coli*. While some researchers chemically synthesise lentiviral Tat, or parts of it, (Vogel *et al.*, 1993; Kim *et al.*, 1997; Goldstein *et al.*, 2000) there exist reports of recombinant Tat expression (Frankel & Pabo, 1988; Cafaro *et al.*, 1999; Tyagi *et al.*, 2001). Among these, there is a study mentioning high-levels of protein expression for recombinant Tat from HIV-1 in *E. coli* (Aldovini *et al.*, 1986). Importantly, the sequence encoding the Tat employed in that study contains only a single rare R codon in the RRR cluster of the R-rich region

(Aldovini *et al.*, 1986). During the present study, by replacing all three Arg triplets in the SIVmac32H(J5) sequence with codons preferred by *E. coli*, an increase in protein expression as much as 50-fold was achieved for the Tat-fusion protein.

Having overcome the initial problem of protein expression yield for the Tat-fusion protein, GST-Tat-APCS-His-Pk expressed in a bacterial culture was isolated using affinity purification. The affinity tag Pk allows specific retention on a matrix coated with anti-Pk Abs. Since the Pk tag cannot be dissociated from the anti-Pk Ab under mild elution conditions (Dunn, 1998 & observations during this study), a method was devised so that the protein could be eluted by losing the Pk tag. The recognition sequence for protease AP was engineered upstream of the C' terminal Pk tag so that the Pk tag would be removed by the action of AP. Indeed, the protease induced a single cut, clipping off the Pk-containing portion and allowing the protein of interest to elute through the Pk-affinity matrix.

Disappointingly, when GST-Tat-APCS-His-Pk protein was treated with AP to release the GST-Tat portion, cleaved GST-Tat protein (cleavage verified by electrophoretic analysis) did not flow through and elute from the affinity matrix. It is possible that GST-Tat was somehow complexed with the Pk-containing part, or with the solid support. A similar phenomenon was observed when thrombin was employed to remove the GST portion of the protein. Treatment with thrombin cleaved GST off Tat-APCS-His-Pk, but GST persisted through a His-affinity support, and co-purified with Tat-APCS-His-Pk through a GST-specific matrix.

These difficulties encountered when trying to separate Tat from cleaved parts of the fusion protein are likely caused by Tat. Tat has been reported to be 'sticky' (Ensoli *et al.*, 1993): the highly basic region of Tat, believed to mediate

binding to cellular membranes (Mann & Frankel, 1991, Kim *et al.*, 1997; Schwarze *et al.*, 1999) is also thought to be responsible for the sticky nature of the protein (Tyagi *et al.*, 2001). It is likely that the 'stickiness' of Tat was responsible for the presence and persistence of contaminants through purification procedures, as well as for hindering the dissociation of cleaved parts of the protein from each other. This phenomenon could be further aggravated by the fact that GST protein can readily form dimers. A combination of the 'sticky' Tat protein and the pairing of GST-fusion protein molecules could generate a complex meshwork of proteins binding to each other and retaining cleaved counterparts.

It had been envisaged for Tat protein alone to be employed in animal immunisations, but the persistence of cleaved parts of the protein following protease treatments precluded the purification of untagged Tat protein. Therefore, the full length GST-Tat-APCS-His-Pk protein was isolated using His- and GST-affinity purification. Purification on His-affinity support was plagued by considerable protein losses, possibly because the His tag was buried in the protein structure. GST-affinity purification of the protein provided a reasonably clean protein preparation, but it contained a range of shorter GST-fusion proteins. Reports of GST-purification of a GST-Tat protein include a GST fusion of HIV Tat purified up to 90% homogeneity (Brand *et al.*, 1996). However, these observations are true for sequences shorter than the full-length Tat protein.

Even though the particular nature of Tat did not allow its purification on the basis of the Pk tag, the technology developed during this study sets the ground for affinity purification of Pk-tagged proteins. Isolation of a Pk-tagged protein on a Pk-specific matrix, followed by proteolytic cleavage of the Pk tag from the protein of interest to allow its elution from the matrix, presents the first ever Pk-

affinity purification protocol. The method was shown to work in principle and it could be employed for the purification of proteins other than Tat.

In order to prepare Tat protein in adequate amounts for immunisation studies, SIV-Tat was expressed in a bacterial system. The low expression yield observed initially was significantly improved by optimising the Tat sequence for translation in a bacterial host. Due to the difficulties encountered when proteolytically removing the affinity tags from Tat protein, Tat was finally employed in animal immunisations tagged with all three affinity tags.

5.2 Animal Immunisations Employing SIV-Tat

5.2.1 Antibody responses

The immunisation studies conducted during this work demonstrated the immunogenicity of Tat protein in the GST-Tat-APCS-His-Pk conformation in the absence of a carrier or adjuvant. Immunisation with soluble GST-Tat-APCS-His-Pk induced specific Ab responses in mice and in monkeys, some of which could be indirectly attributed to the Tat moiety. The immunogenicity of soluble Tat protein, delivered in the absence of adjuvants, has been observed by others (Pauza *et al.*, 2000). Moreover, Tat has been reported to act as an antigen carrier, potentiating Ab responses to unrelated antigens physically attached to it (Fawell *et al.*, 1994; Vives *et al.*, 1997; Schwarze *et al.*, 1999). In the present study, injection with soluble GST-Tat-APCS-His-Pk induced GST-Pk–specific Ab responses. Soluble GST proteins lacking Tat, showed negligible immunogenicity in mice.

The reason for the immunogenicity and adjuvanticity of Tat is thought to lie with its ability to bind cell surfaces and translocate inside cells. Heterologous proteins conjugated to Tat protein or Tat peptides can get delivered within cells, even if they could not enter the cells by themselves (Vives *et al.*, 1997). While the cysteine-rich domain of Tat plays a role in the attraction of monocytes and macrophages (Albini *et al.*, 1998), specific uptake of the Tat fusion protein by phagocytic cells is thought to induce efficient cross-presentation on MHC class I molecules. Presentation of antigens in the context of Tat has been shown to prime CD8+ responses and the induction of CTLs (Kim *et al.*, 1997).

5.2.2 T_H responses

In the course of the mouse immunisations, soluble GST-Tat-APCS-His-Pk generated both IgG2a and IgG3 Ab, suggesting the development of T_H1-type responses (Coffman *et al.*, 1993) in the vaccinated mice. Firstly, Tat induces the production of IL-2 (Mhashilkar *et al.*, 1996, Goldstein, 1996), a cytokine known for tilting the balance towards T_H1 responses. Additionally, the ability of Tat to transport antigens across the cell membrane resembles the mode of action of bacterial enterotoxins employed as vaccine adjuvants, and they too generate T_H1-type responses specific to the antigens they deliver (Cox & Coulter, 1997). Soluble Tat could therefore be used as an antigen carrier to induce specific T_H1 responses to unrelated proteins. Such usage of Tat would require the physical attachment of the potential antigen to Tat, as simple co-administration of antigen with Tat can in fact have the opposite effect (immunosuppressive action of Tat; Cohen *et al.*, 1999).

The immunogenicity and adjuvanticity of GST-Tat-APCS-His-Pk observed during immunisation in the absence of any carrier or adjuvant, was not necessarily increased by the adjuvant alum. While GST-Tat-APCS-His-Pk protein was more immunogenic in the presence of alum in the first mouse immunisation study, alum-precipitation decreased the immunogenic potential of the protein in the second immunisation study. At the same time, alum increased the immunogenicity of the control proteins employed in both studies, even if the increase in the second study was not of the magnitude observed in the first study. The protein formulations were prepared and administered in exactly the same way in both studies. It is unclear why this discrepancy was brought about, and it remains to be resolved.

During the first mouse immunisation study, alum-precipitated GST-Tat-APCS-His-Pk protein (but not GST-Pk) induced specific IgG2a Ab, suggesting the generation of T_H1-type responses, much in disagreement with the established function of alum. This transformation of the function of alum is clearly attributed to the presence of Tat and it could be linked to the ability of Tat to actively enter cells. The increased IgG2a responses observed for alum-precipitated protein, compared to soluble protein, could reflect larger amounts of antigen per cell delivered when the antigen was precipitated.

The observation that the combination of Tat and alum was also inducing T_H1 immune responses to an unrelated antigen was potentially of great value as it would provide an easier and quicker way to produce a vaccine formulation that induced T_H1-type immunity to any co-precipitated antigen without the need for physical attachment to Tat. However, the observation that alum-precipitated GST-Tat-APCS-His-Pk induced a T_H1-type immune response was not reproduced in

the second mouse immunisation study. As already discussed, the function of alum in that study was questioned, as alum was in fact decreasing the immunogenicity of GST-Tat-APCS-His-Pk.

Additionally, the GST-Tat-APCS-His-Pk proteins employed in the two mouse immunisation studies, originated from different preparations. This could have influenced the immunisation outcome in two possible ways. Firstly, if Tat protein in the first immunisation were fully functional, while the functionality of Tat protein employed in the repeat immunisation were compromised, each protein could have generated a distinct pattern of immune responses. Functional Tat induces the production of IL-2 that preferentially triggers T_H1 responses. This phenomenon could be attributed to efficient activation of DCs and macrophages by active Tat, and perhaps inactive Tat could not activate those APCs. The absence of IL-2 could have allowed the generation of T_H2-type responses.

Tat-containing proteins tend to degrade, as well as aggregate, over time. Tat protein when aggregated, no longer binds to cellular membranes (H.Y. Chen, Univ. of St. Andrews, personal communication). A comparative immunisation study employing Tat protein fully soluble (i) or aggregated and precipitated (ii) revealed that soluble protein clearly induces better immune responses (H.Y. Chen, unpublished observations). For a Tat-based protein to be employed in vaccine formulations, it would be of interest to define the conditions that preserve the protein in a soluble and functional form, as well as to develop an assay for testing its activity and conformation to ensure it would prime the desirable immune responses.

The fact that the two GST-Tat-APCS-His-Pk proteins employed in the mouse immunisations originated from different protein preparations could have

influenced the immunisation outcomes in yet another way. A miscalculation when estimating the concentration of each protein sample could result in the use of unequal amounts of protein between the two immunisations. The balance between T_H1 and T_H2 subsets in immune responses is influenced by the dose or concentration of antigen (Abbas *et al.*, 1996). With few exceptions, low antigen concentrations tend preferentially to induce T_H1 responses, whereas high doses induce T_H2 development (Bretscher *et al.*, 1992; Clerici & Shearer, 1993; Hosken *et al.*, 1995; Leitner *et al.*, 2000). It has been suggested that the principal APCs at low concentrations of antigen are DCs or macrophages, both types of cells producing IL-12 that would tilt the balance towards T_H1 differentiation, while higher amounts of antigen may be presented by APCs that do not secrete IL-12, thus favouring T_H2 development (Abbas *et al.*, 1996).

5.2.3 CTL responses

The prevalent opinion in the scientific community seems to be that protection against lentivirus infection will require both antibody and CTL responses (Bangham & Philips, 1997; Nabel, 2000; Peters, 2002).

SIV-Tat from mac251(J5) contained a CTL epitope restricted to Rhesus macaques, but assaying the IFN-γ levels and CTL activity did not provide evidence for the generation of specific CTL responses in the immunised monkeys by week 10. There exist reports where anti-Tat CTL activity was not detectable before 28 weeks post immunisation (Cafaro *et al.*, 1999) so it is possible that significant CTL responses might not have had the time to develop in the immunised monkeys. However, a more plausible explanation would be that Tat

protein in the immunisation molecule had lost its functionality, as already discussed for the second immunisation study in mice, thus being unable to enter cells and get presented on MHC I molecules to generate CTL responses.

Immunisation studies to assess whether Tat was generating specific CTL responses in mice were not performed due to practical reasons. Although it was not possible to directly monitor CTL responses in mice, the isotype analysis of the specific Ab responses to the Tat-containing protein indicated the presence of T_H1-type responses, that could imply a possible generation of CTL responses. A different combination of BalbC-restricted CTL epitopes has been recently included in a Tat-fusion protein in our laboratory, and is currently being assessed for its potential to trigger specific CTL responses.

5.3 Concluding Remarks

To summarise the important findings of the work presented here, Tat protein from SIVmac251(J5) was shown to be immunogenic when injected in a soluble form in BalbC mice and in Rhesus macaque monkeys. Tat induced antibody responses specific to the immunisation molecule both in mice and in monkeys, with evidence for generation of T_H1-type responses in mice. Further immunisation studies will be required to determine whether Tat from SIVmac251(J5) can induce CTL responses.

Tat protein has been proposed as a target for a potential vaccine to control lentivirus infection. The codon optimisation that increased the protein expression levels for Tat from SIVmac251(J5) in *E. coli* allows the purification of adequate amounts of active Tat protein for use in immunisation studies. Tat

protein from SIVmac presents a good candidate for immunogenicity and viral challenge studies in the rhesus macaque animal model.

The generation of immune responses to Tat does not ensure on its own right that the immunised animals will be protected against lentiviral infection. In fact, two recent studies have shown generation of potent immune responses to Tat, but the immunised monkeys were not protected against challenge with the virulent lentiviral strains of SIVmac239 (Allen *et al.*, 2001) or SHIV89.6p (Silvera *et al.*, 2002). However, the generation of strong immune responses specific to Tat, as well as the evolutionary pressure that drives SIV-Tat to escape recognition by CTLs (Allen *et al.*, 2000) make Tat a good component of a potential HIV vaccine, as part of a combination of regulatory and structural proteins of HIV.

Further to the immunogenicity of SIV-Tat, immunisation studies in mice and in monkeys also confirmed its adjuvanticity and antigen-carrier function. Tat was shown to generate specific Ab responses to unrelated proteins physically linked to it, even though these proteins were unable to induce specific Ab in the absence of Tat. The isotypes of the specific Ab generated implied the induction of a mixture of T_H2 and T_H1-type responses. Proteins are generally considered to be the safest components of vaccine formulations, but protein-based vaccines that induce T_H1-type responses and class-I restricted CTL responses have proved difficult to design (Kim *et al.*, 1997). For that reason, Tat being able to generate T_H1-type responses to co-administered antigens would be very useful for particular types of pathogens, such as intracellular parasites and viruses, that are cleared by T_H1-based immunity.

To ensure reproducibility of results when working with Tat, it would be important to develop and standardise methods to assay the activity and trans-activating ability of Tat proteins, before they were employed in vaccine formulations as antigens or indeed as antigen carriers. The activity of Tat can be measured in HeLa cells containing the lacZ gene under the control of the lentiviral LTR (Vives *et al.*, 1994). The transactivation assay could also provide information about the ability of Tat to translocate across the cell membrane, as the two functions have been shown to be inseparable (Subramanian *et al.*, 1991).

Lastly, this study presented evidence for Tat altering the established function of alum towards the generation of antigen-specific T_H1-type responses. Even though more work will be required to confirm this observation, this is a very exciting finding. If the co-precipitation of Tat, alum and antigen can induce antigen-specific T_H1-type responses in immunised hosts, this strategy would provide a quicker and easier way to produce vaccine formulations containing a wide variety of antigens, without the need to physically attach the antigens to Tat.

The inconsistency observed between the immunogenicity of alum-precipitated proteins from different immunisation studies could be resolved by conducting some detailed studies on the translocating abilities of Tat. It would be interesting to see whether in fact alum-precipitated Tat-protein enters the cells and how quickly it translocates in various compartments of the cytoplasm. Studies employing confocal microscopy could reveal slice by slice the components of the cell to allow visualisation of the exact localisation of soluble or alum-precipitated Tat protein.

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7. APPENDIX

This Appendix contains a table of all the proteins used in this study (7.1), along with their DNA and amino acid sequences (7.3-7.9). The full DNA sequence of vector pGEX-2T is also included (7.2), as well as the sequences of the primers and oligonucleotides employed in engineering these proteins (7.10). Lastly, a sample calculation of protein amounts to be employed in ELISA assays is shown in 7.11.

7.1 TABLE OF PROTEIN INFORMATION FOR THE PROTEINS EMPLOYED IN THIS STUDY

7.2 DNA SEQUENCE OF CLONING VECTOR PGEX-2T (PHARMACIA) AND ENGINEERED PRIMERS: FORWARD AND REVERSE

7.3 CODING SEQUENCE OF VECTOR PGEX-2T & AMINO ACID SEQUENCE OF GST

7.4 DNA SEQUENCE OF VECTOR PGEX-APCS-HIS-PK & AMINO ACID SEQUENCE OF GST-APCS-HIS-PK

7.5 DNA SEQUENCE OF VECTOR PGEX-CTLS-APCS-HIS-PK & AMINO ACID SEQUENCE OF GST-CTLS-APCS-HIS-PK

7.6 DNA SEQUENCE OF VECTOR PGEX-TAT-PK & AMINO ACID SEQUENCE OF GST-TAT-PK

7.7 DNA SEQUENCE OF VECTOR PGEX-TAT-APCS-HIS-PK & AMINO ACID SEQUENCE OF GST-TAT-APCS-HIS-PK

7.8 DNA SEQUENCE OF VECTOR PGEX-TAT-APCS-HIS-PK-2 & AMINO ACID SEQUENCE OF GST-TAT-APCS-HIS-PK

7.9 DNA SEQUENCE OF VECTOR PGEX-CTLS-TAT-APCS-HIS-PK & AMINO ACID SEQUENCE OF GST-CTLS-TAT-APCS-HIS-PK

7.10 PRIMERS AND OLIGONUCLEOTIDES EMPLOYED

7.11 ELISA STANDARDISATION FOR COMPARATIVE ASSAYS OF MONKEY SAMPLES WITH PROTEINS GST-PK & GST-TAT-APCS-HIS-PK

7.1 TABLE OF PROTEIN INFORMATION FOR THE PROTEINS EMPLOYED IN THIS STUDY

Protein Name	Expressed by Vector	Main Function	Brief Description
GST	pGEX-2T (Pharmacia)	Control	Unmodified protein
GST-Pk	pGEXcPk (Tom Hanke)	Imms. Control	GST tagged with Pk
GST-PkHis9	pGEX-PkHis9	Pk-purification	His substitutions
GST-PkHis11	pGEX-PkHis11	Pk-purification	on Pk tag at positions:
GST-PkHis4,10	pGEX-PkHis4,10	Pk-purification	9, 11 or 4 and 10
GST-Tat-Pk	pGEX-Tat-Pk	Intermediate	Tat with GST and Pk tags
GST-Tat-APCS-His-Pk	pGEX-Tat-APCS-His-Pk	Intermediate	His tag and APCS added
GST-Tat-APCS-His-Pk	pGEX-Tat-APCS-His-Pk-**2**	Immunisations	3 rare R replaced, no EcoRI
GST-APCS-His-Pk	pGEX-APCS-His-Pk	Imms. Control	GST tagged with Pk & His
GST-CTLs-Tat-APCS-His-Pk	pGEX-CTLs-Tat-APCS-His-Pk	Immunisations	CTLs addition to Tat
GST-CTLs-APCS-His-Pk	pGEX-CTLs-APCS-His-Pk	Immunisations	CTLs addition to GST
His-Tat-Pk	Engineered by Hsiang Yun-Chen	Western blot.	Tat protein lacking GST

7.2 DNA SEQUENCE OF CLONING VECTOR PGEX-2T PHARMACIA) AND ENGINEERED PRIMERS: FORWARD AND REVERSE

ORIGIN OF REPLICATION

1 acgttatcga ctgcacggtg caccaatgct tctggcgtca ggcagccatc ggaagctgtg
61 gtatggctgt gcaggtcgta aatcactgca taattcgtgt cgctcaaggc gcactcccgt
121 tctggataat gtttttgcg ccgacatcat aacggttctg gcaaatattc tgaaatgagc
181 tgttgacaat taatcatcgg ctcgtataat gtgtggaatt gtgagcggat aacaatttca
241 cacaggaaac agtattcatg tcccctatac taggttattg gaaaattaag ggccttgtgc
301 aacccactcg acttcttttg gaatatcttg aagaaaaata tgaagagcat ttgtatgagc
361 gcgatgaagg tgataaatgg cgaaacaaaa agtttgaatt gggtttggag tttcccaatc
421 ttccttatta tattgatggt gatgttaaat taacacagtc tatggccatc atacgttata
481 tagctgacaa gcacaacatg ttgggtggtt gtccaaaaga gcgtgcagag atttcaatgc
541 ttgaaggagc ggttttggat attagatacg gtgtttcgag aattgcatat agtaaagact
601 ttgaaactct caaagttgat tttcttagca agctacctga aatgctgaaa atgttcgaag
661 atcgtttatg tcataaaaca tatttaaatg gtgatcatgt aacccatcct gacttcatgt
721 tgtatgacgc tcttgatgtt gttttataca tggacccaat gtgcctggat gcgttcccaa
781 aattagtttg ttttaaaaaa cgtattgaag ctatcccaca aattgataag tacttgaaat
841 ccagcaagta tatagcatgg cctttgcagg gctggcaagc cacgtttggt ggtggcgacc
901 atcctccaaa atcggatctg gttccgcgtg gatccccggg aattcatcgt gactgactga
961 cgatctgcct cgcgcgtttc ggtgatgacg gtgaaaacct ctgacacatg cagctcccgg
1021 agacggtcac agcttgtctg taagcggatg ccgggagcag acaagcccgt cagggcgcgt
1081 cagcgggtgt tggcgggtgt cggggcgcag ccatgaccca gtcacgtagc gatagcggag
1141 tgtataattc ttgaagacga aagggcctcg tgatacgcct atttttatag gttaatgtca
1201 tgataataat ggtttcttag acgtcaggtg gcacttttcg gggaaatgtg cgcggaaccc
1261 ctatttgttt atttttctaa atacattcaa atatgtatcc gctcatgaga caataaccct
1321 gataaatgct tcaataatat tgaaaaagga agagtatgag tattcaacat ttccgtgtcg
1381 cccttattcc ctttttgcg gcattttgcc ttcctgtttt tgctcaccca gaaacgctgg
1441 tgaaagtaaa agatgctgaa gatcagttgg gtgcacgagt gggttacatc gaactggatc
1501 tcaacagcgg taagatcctt gagagttttc gccccgaaga acgtttttcca atgatgagca
1561 cttttaaagt tctgctatgt ggcgcggtat tatcccgtgt tgacgccggg caagagcaac
1621 tcggtcgccg catacactat tctcagaatg acttggttga gtactcacca gtcacagaaa
1681 agcatcttac ggatggcatg acagtaagag aattatgcag tgctgccata accatgagtg
1741 ataacactgc ggccaactta cttctgacaa cgatcggagg accgaaggag ctaaccgctt
1801 ttttgcacaa catgggggat catgtaactc gccttgatcg ttgggaaccg gagctgaatg
1861 aagccatacc aaacgacgag cgtgacacca cgatgcctgc agcaatggca acaacgttgc
1921 gcaaactatt aactggcgaa ctacttactc tagcttcccg gcaacaatta atagactgga
1981 tggaggcgga taaagttgca ggaccacttc tgcgctcggc ccttccggct ggctggttta
2041 ttgctgataa atctggagcc ggtgagcgtg ggtctcgcgg tatcattgca gcactggggc
2101 cagatggtaa gccctcccgt atcgtagtta tctacacgac ggggagtcag gcaactatgg
2161 atgaacgaaa tagacagatc gctgagatag gtgcctcact gattaagcat tggtaactgt
2221 cagaccaagt ttactcatat atactttaga ttgatttaaa acttcatttt taatttaaaa
2281 ggatctaggt gaagatcctt tttgataatc tcatgaccaa aatcccttaa cgtgagtttt
2341 cgttccactg agcgtcagac cccgtagaaa agatcaaagg atcttcttga gatccttttt
2401 ttctgcgcgt aatctgctgc ttgcaaacaa aaaaaccacc gctaccagcg gtggtttgtt
2461 tgccggatca agagctacca actcttttc cgaaggtaac tggcttcagc agagcgcaga
2521 taccaaatac tgtccttcta gtgtagccgt agttaggcca ccacttcaag aactctgtag
2581 caccgcctac atacctcgct ctgctaatcc tgttaccagt ggctgctgcc agtggcgata

2641 agtcgtgtct taccgggttg gactcaagac gatagttacc ggataaggcg cagcggtcgg
2701 gctgaacggg gggttcgtgc acacagccca gcttggagcg aacgacctac accgaactga
2761 gatacctaca gcgtgagcta tgagaaagcg ccacgcttcc cgaagggaga aaggcggaca
2821 ggtatccggt aagcggcagg gtcggaacag gagagcgcac gagggagctt ccagggggaa
2881 acgcctggta tctttatagt cctgtcgggt ttcgccacct ctgacttgag cgtcgatttt
2941 tgtgatgctc gtcaggggggg cggagcctat ggaaaaacgc cagcaacgcg gccttttttac
3001 ggttcctggc cttttgctgg ccttttgctc acatgttctt tcctgcgtta tcccctgatt
3061 ctgtggataa ccgtattacc gcctttgagt gagctgatac cgctcgccgc agccgaacga
3121 ccgagcgcag cgagtcagtg agcgaggaag cggaagagcg cctgatgcgg tattttctcc
3181 ttacgcatct gtgcggtatt tcacaccgca taaattccga caccatcgaa tggtgcaaaa
3241 cctttcgcgg tatggcatga tagcgcccgg aagagagtca attcagggtg gtgaatgtga
3301 aaccagtaac gttatacgat gtcgcagagt atgccggtgt ctcttatcag accgtttccc
3361 gcgtggtgaa ccaggccagc cacgtttctg cgaaaacgcg ggaaaaagtg gaagcggcga
3421 tggcggagct gaattacatt cccaaccgcg tggcacaaca actggcgggc aaacagtcgt
3481 tgctgattgg cgttgccacc tccagtctgg ccctgcacgc gccgtcgcaa attgtcgcgg
3541 cgattaaatc tcgcgccgat caactgggtg ccagcgtggt ggtgtcgatg gtagaacgaa
3601 gcggcgtcga agcctgtaaa gcggcggtgc acaatcttct cgcgcaacgc gtcagtgggc
3661 tgatcattaa ctatccgctg gatgaccagg atgccattgc tgtggaagct gcctgcacta
3721 atgttccggc gttatttctt gatgtctctg accagacacc catcaacagt attattttct
3781 cccatgaaga cggtacgcga ctgggcgtgg agcatctggt cgcattgggt caccagcaaa
3841 tcgcgctgtt agcgggccca ttaagttctg tctcggcgcg tctgcgtctg gctggctggc
3901 ataaatatct cactcgcaat caaattcagc cgatagcgga acgggaaggc gactggagtg
3961 ccatgtccgg ttttcaacaa accatgcaaa tgctgaatga gggcatcgtt cccactgcga
4021 tgctggttgc caacgatcag atggcgctgg gcgcaatgcg cgccattacc gagtccgggc
4081 tgcgcgttgg tgcggatatc tcggtagtgg gatacgacga taccgaagac agctcatgtt
4141 atatcccgcc gttaaccacc atcaaacagg attttcgcct gctggggcaa accagcgtgg
4201 accgcttgct gcaactctct cagggccagg cggtgaaggg caatcagctg ttgcccgtct
4261 cactggtgaa aagaaaaacc accctggcgc ccaatacgca aaccgcctct ccccgcgcgt
4321 tggccgattc attaatgcag ctggcacgac aggtttcccg actggaaagc gggcagtgag
4381 cgcaacgcaa ttaatgtgag ttagctcact cattaggcac cccaggcttt acactttatg
4441 cttccggctc gtatgttgtg tggaattgtg agcggataac aatttcacac aggaaacagc
4501 tatgaccatg attacggatt cactggccgt cgttttacaa cgtcgtgact gggaaaaccc
4561 tggcgttacc caacttaatc gccttgcagc acatcccccc ttcgccagct ggcgtaatag
4621 cgaagaggcc cgcaccgatc gcccttccca acagttgcgc agcctgaatg gcgaatggcg
4681 ctttgcctgg tttccggcac cagaagcggt gccggaaagc tggctggagt gcgatcttcc
4741 tgaggccgat actgtcgtcg tcccctcaaa ctggcagatg cacggttacg atgcgcccat
4801 ctacaccaac gtaacctatc ccattacggt caatccgccg tttgttccca cggagaatcc
4861 gacgggttgt tactcgctca catttaatgt tgatgaaagc tggctacagg aaggccagac
4921 gcgaattatt tttgatggcg ttggaatt
//

KEY: Forward Primer, Multiple Cloning Site, Reverse Primer.

7.3 CODING SEQUENCE OF VECTOR pGEX-2T & AMINO ACID SEQUENCE OF GST

```
1/1                                                 31/11
atg tcc cct ata cta ggt tat tgg aaa att aag ggc ctt gtg caa ccc act cga ctt ctt
M   S   P   I   L   G   Y   W   K   I   K   G   L   V   Q   P   T   R   L   L
61/21                                               91/31
ttg gaa tat ctt gaa gaa aaa tat gaa gag cat ttg tat gag cgc gat gaa ggt gat aaa
L   E   Y   L   E   E   K   Y   E   E   H   L   Y   E   R   D   E   G   D   K
121/41                                              151/51
tgg cga aac aaa aag ttt gaa ttg ggt ttg gag ttt ccc aat ctt cct tat tat att gat
W   R   N   K   K   F   E   L   G   L   E   F   P   N   L   P   Y   Y   I   D
181/61                                              211/71
ggt gat gtt aaa tta aca cag tct atg gcc atc ata cgt tat ata gct gac aag cac aac
G   D   V   K   L   T   Q   S   M   A   I   I   R   Y   I   A   D   K   H   N
241/81                                              271/91
atg ttg ggt ggt tgt cca aaa gag cgt gca gag att tca atg ctt gaa gga gcg gtt ttg
M   L   G   G   C   P   K   E   R   A   E   I   S   M   L   E   G   A   V   L
301/101                                             331/111
gat att aga tac ggt gtt tcg aga att gca tat agt aaa gac ttt gaa act ctc aaa gtt
D   I   R   Y   G   V   S   R   I   A   Y   S   K   D   F   E   T   L   K   V
361/121                                             391/131
gat ttt ctt agc aag cta cct gaa atg ctg aaa atg ttc gaa gat cgt tta tgt cat aaa
D   F   L   S   K   L   P   E   M   L   K   M   F   E   D   R   L   C   H   K
421/141                                             451/151
aca tat tta aat ggt gat cat gta acc cat cct gac ttc atg ttg tat gac gct ctt gat
T   Y   L   N   G   D   H   V   T   H   P   D   F   M   L   Y   D   A   L   D
481/161                                             511/171
gtt gtt tta tac atg gac cca atg tgc ctg gat gcg ttc cca aaa tta gtt tgt ttt aaa
V   V   L   Y   M   D   P   M   C   L   D   A   F   P   K   L   V   C   F   K
541/181                                             571/191
aaa cgt att gaa gct atc cca caa att gat aag tac ttg aaa tcc agc aag tat ata gca
K   R   I   E   A   I   P   Q   I   D   K   Y   L   K   S   S   K   Y   I   A
601/201                                             631/211
tgg cct ttg cag ggc tgg caa gcc acg ttt ggt ggt ggc gac cat cct cca aaa
W   P   L   Q   G   W   Q   A   T   F   G   G   G   D   H   P   P   K
```

7.4 DNA Sequence Of Vector pGEX-APCS-His-Pk & Amino Acid Sequence Of GST-APCS-His-Pk

```
1/1                                                 31/11
atg tcc cct ata cta ggt tat tgg aaa att aag ggc ctt gtg caa ccc act cga ctt ctt
M   S   P   I   L   G   Y   W   K   I   K   G   L   V   Q   P   T   R   L   L
61/21                                               91/31
ttg gaa tat ctt gaa gaa aaa tat gaa gag cat ttg tat gag cgc gat gaa ggt gat aaa
L   E   Y   L   E   E   K   Y   E   E   H   L   Y   E   R   D   E   G   D   K
121/41                                              151/51
tgg cga aac aaa aag ttt gaa ttg ggt ttg gag ttt ccc aat ctt cct tat tat att gat
W   R   N   K   K   F   E   L   G   L   E   F   P   N   L   P   Y   Y   I   D
181/61                                              211/71
ggt gat gtt aaa tta aca cag tct atg gcc atc ata cgt tat ata gct gac aag cac aac
G   D   V   K   L   T   Q   S   M   A   I   I   R   Y   I   A   D   K   H   N
241/81                                              271/91
atg ttg ggt ggt tgt cca aaa gag cgt gca gag att tca atg ctt gaa gga gcg gtt ttg
M   L   G   G   C   P   K   E   R   A   E   I   S   M   L   E   G   A   V   L
301/101                                             331/111
gat att aga tac ggt gtt tcg aga att gca tat agt aaa gac ttt gaa act ctc aaa gtt
D   I   R   Y   G   V   S   R   I   A   Y   S   K   D   F   E   T   L   K   V
361/121                                             391/131
gat ttt ctt agc aag cta cct gaa atg ctg aaa atg ttc gaa gat cgt tta tgt cat aaa
D   F   L   S   K   L   P   E   M   L   K   M   F   E   D   R   L   C   H   K
421/141                                             451/151
aca tat tta aat ggt gat cat gta acc cat cct gac ttc atg ttg tat gac gct ctt gat
T   Y   L   N   G   D   H   V   T   H   P   D   F   M   L   Y   D   A   L   D
481/161                                             511/171
gtt gtt tta tac atg gac cca atg tgc ctg gat gcg ttc cca aaa tta gtt tgt ttt aaa
V   V   L   Y   M   D   P   M   C   L   D   A   F   P   K   L   V   C   F   K
541/181                                             571/191
aaa cgt att gaa gct atc cca caa att gat aag tac ttg aaa tcc agc aag tat ata gca
K   R   I   E   A   I   P   Q   I   D   K   Y   L   K   S   S   K   Y   I   A
601/201                                             631/211
tgg cct ttg cag ggc tgg caa gcc acg ttt ggt ggt ggc gac cat cct cca aaa tcg gat
W   P   L   Q   G   W   Q   A   T   F   G   G   G   D   H   P   P   K   S   D
661/221                                             691/231
ctg gtt ccg cgt GGA TCC CCG GGA ATT CCG GCG GAA ATT CTG GCG GGT GGC CAT CAC CAT
L   V   P   R   G   S   P   G   I   P   A   E   I   L   A   G   G   H   H   H
721/241                                             751/251
CAC CAT CAC GCA ATT CCA GGA AAG CCG ATC CCA AAC CCT TTG CTG GGA TTG GAC TCC ACC
H   H   H   A   I   P   G   K   P   I   P   N   P   L   L   G   L   D   S   T
781/261
TGA
*
```

Key: GST, pGEX, BamHI, EcoRI, spacer, APCS, His, Pk.

7.5 DNA Sequence Of Vector pGEX-CTLs-APCS-His-Pk & Amino Acid Sequence Of GST-CTLs-APCS-His-Pk

```
1/1                                                 31/11
atg tcc cct ata cta ggt tat tgg aaa att aag ggc ctt gtg caa ccc act cga ctt ctt
M   S   P   I   L   G   Y   W   K   I   K   G   L   V   Q   P   T   R   L   L
61/21                                               91/31
ttg gaa tat ctt gaa gaa aaa tat gaa gag cat ttg tat gag cgc gat gaa ggt gat aaa
L   E   Y   L   E   E   K   Y   E   E   H   L   Y   E   R   D   E   G   D   K
121/41                                              151/51
tgg cga aac aaa aag ttt gaa ttg ggt ttg gag ttt ccc aat ctt cct tat tat att gat
W   R   N   K   K   F   E   L   G   L   E   F   P   N   L   P   Y   Y   I   D
181/61                                              211/71
ggt gat gtt aaa tta aca cag tct atg gcc atc ata cgt tat ata gct gac aag cac aac
G   D   V   K   L   T   Q   S   M   A   I   I   R   Y   I   A   D   K   H   N
241/81                                              271/91
atg ttg ggt ggt tgt cca aaa gag cgt gca gag att tca atg ctt gaa gga gcg gtt ttg
M   L   G   G   C   P   K   E   R   A   E   I   S   M   L   E   G   A   V   L
301/101                                             331/111
gat att aga tac ggt gtt tcg aga att gca tat agt aaa gac ttt gaa act ctc aaa gtt
D   I   R   Y   G   V   S   R   I   A   Y   S   K   D   F   E   T   L   K   V
361/121                                             391/131
gat ttt ctt agc aag cta cct gaa atg ctg aaa atg ttc gaa gat cgt tta tgt cat aaa
D   F   L   S   K   L   P   E   M   L   K   M   F   E   D   R   L   C   H   K
421/141                                             451/151
aca tat tta aat ggt gat cat gta acc cat cct gac ttc atg ttg tat gac gct ctt gat
T   Y   L   N   G   D   H   V   T   H   P   D   F   M   L   Y   D   A   L   D
481/161                                             511/171
gtt gtt tta tac atg gac cca atg tgc ctg gat gcg ttc cca aaa tta gtt tgt ttt aaa
V   V   L   Y   M   D   P   M   C   L   D   A   F   P   K   L   V   C   F   K
541/181                                             571/191
aaa cgt att gaa gct atc cca caa att gat aag tac ttg aaa tcc agc aag tat ata gca
K   R   I   E   A   I   P   Q   I   D   K   Y   L   K   S   S   K   Y   I   A
601/201                                             631/211
tgg cct ttg cag ggc tgg caa gcc acg ttt ggt ggt ggc gac cat cct cca aaa tcg gat
W   P   L   Q   G   W   Q   A   T   F   G   G   G   D   H   P   P   K   S   D
661/221                                             691/231
ctg gtt ccg cgt GGA TCA CGT ACG CGT GGC CCG GGT CGT GCG TTT GTG ACC ATC TCC ATT
L   V   P   R   G   S   R   T   R   G   P   G   R   A   F   V   T   I   S   I
721/241                                             751/251
ATC AAT TTT GAA AAA CTG GGA TCC CCG GGA ATT CCG GCG GAA ATT CTG GCG GGT GGC CAT
I   N   F   E   K   L   G   S   P   G   I   P   A   E   I   L   A   G   G   H
781/261                                             811/271
CAC CAT CAC CAT CAC GCA ATT CCA GGA AAG CCG ATC CCA AAC CCT TTG CTG GGA TTG GAC
H   H   H   H   H   A   I   P   G   K   P   I   P   N   P   L   L   G   L   D
841/281
TCC ACC TGA
S   T   *
```

KEY: GST, pGEX, CTLs, BamHI, EcoRI, spacer, APCS, His, Pk.

7.6 DNA Sequence Of Vector pGEX-Tat-Pk & Amino Acid Sequence Of GST-Tat-Pk

```
1/1                                                 31/11
atg tcc cct ata cta ggt tat tgg aaa att aag ggc ctt gtg caa ccc act cga ctt ctt
M   S   P   I   L   G   Y   W   K   I   K   G   L   V   Q   P   T   R   L   L
61/21                                               91/31
ttg gaa tat ctt gaa gaa aaa tat gaa gag cat ttg tat gag cgc gat gaa ggt gat aaa
L   E   Y   L   E   E   K   Y   E   E   H   L   Y   E   R   D   E   G   D   K
121/41                                              151/51
tgg cga aac aaa aag ttt gaa ttg ggt ttg gag ttt ccc aat ctt cct tat tat att gat
W   R   N   K   K   F   E   L   G   L   E   F   P   N   L   P   Y   Y   I   D
181/61                                              211/71
ggt gat gtt aaa tta aca cag tct atg gcc atc ata cgt tat ata gct gac aag cac aac
G   D   V   K   L   T   Q   S   M   A   I   I   R   Y   I   A   D   K   H   N
241/81                                              271/91
atg ttg ggt ggt tgt cca aaa gag cgt gca gag att tca atg ctt gaa gga gcg gtt ttg
M   L   G   G   C   P   K   E   R   A   E   I   S   M   L   E   G   A   V   L
301/101                                             331/111
gat att aga tac ggt gtt tcg aga att gca tat agt aaa gac ttt gaa act ctc aaa gtt
D   I   R   Y   G   V   S   R   I   A   Y   S   K   D   F   E   T   L   K   V
361/121                                             391/131
gat ttt ctt agc aag cta cct gaa atg ctg aaa atg ttc gaa gat cgt tta tgt cat aaa
D   F   L   S   K   L   P   E   M   L   K   M   F   E   D   R   L   C   H   K
421/141                                             451/151
aca tat tta aat ggt gat cat gta acc cat cct gac ttc atg ttg tat gac gct ctt gat
T   Y   L   N   G   D   H   V   T   H   P   D   F   M   L   Y   D   A   L   D
481/161                                             511/171
gtt gtt tta tac atg gac cca atg tgc ctg gat gcg ttc cca aaa tta gtt tgt ttt aaa
V   V   L   Y   M   D   P   M   C   L   D   A   F   P   K   L   V   C   F   K
541/181                                             571/191
aaa cgt att gaa gct atc cca caa att gat aag tac ttg aaa tcc agc aag tat ata gca
K   R   I   E   A   I   P   Q   I   D   K   Y   L   K   S   S   K   Y   I   A
601/201                                             631/211
tgg cct ttg cag ggc tgg caa gcc acg ttt ggt ggt ggc gac cat cct cca aaa tcg gat
W   P   L   Q   G   W   Q   A   T   F   G   G   G   D   H   P   P   K   S   D
661/221                                             691/231
ctg gtt ccg cgt gga tcc ATG GAG ACA CCC TTG AGG GAG CAG GAG AAC TCA TTA GAA TCC
L   V   P   R   G   S   M   E   T   P   L   R   E   Q   E   N   S   L   E   S
721/241                                             751/251
TCC AAC GAG CGC TCT TCA TGC ATT TCA GAG GCG GAT GCA ACC ACT CCA GAA TCG GCC AAC
S   N   E   R   S   S   C   I   S   E   A   D   A   T   T   P   E   S   A   N
781/261                                             811/271
CTG GGG GAG GAA ATC CTC TCT CAA CTA TAC CGC CCT CTA GAG GCG TGC TAT AAC ACA TGC
L   G   E   E   I   L   S   Q   L   Y   R   P   L   E   A   C   Y   N   T   C
841/281                                             871/291
TAT TGT AAA AAG TGT TGC TAC CAT TGC CAG TTT TGT TTT CTT AAA AAG GGA TTG GGG ATA
Y   C   K   K   C   C   Y   H   C   Q   F   C   F   L   K   K   G   L   G   I
901/301                                             931/311
TGT TAT GAG CAG TCA CgA AAG AGA AGA AGA ACT cCG AAA AAG GCT AAG GCT AAT ACA TCT
C   Y   E   Q   S   R   K   R   R   R   T   P   K   K   A   K   A   N   T   S
961/321                                             991/331
TCT GCA TcA AAC AAC AgA CCC ATA TCC AAC AGG ACC CGG CAC TGC CAA CCA GAG AAG GCA
S   A   S   N   N   R   P   I   S   N   R   T   R   H   C   Q   P   E   K   A
1021/341                                            1051/351
AAG AAG GAG ACG GTG GAg AAG GCG GTG GCA ACA GCT CCT GGC CTT GGC AGA GGA ATT cCA
K   K   E   T   V   E   K   A   V   A   T   A   P   G   L   G   R   G   I   P
1081/361                                            1111/371
GGA AAG CCG ATC CCA AAC CCT TTG CTG GGA TTG GAC TCC ACC TGA
G   K   P   I   P   N   P   L   L   G   L   D   S   T   *
```

KEY: GST, pGEX, BamHI, TAT, EcoRI, PK.

7.7 DNA Sequence Of Vector pGEX-Tat-APCS-His-Pk & Amino Acid Sequence Of GST-Tat-APCS-His-Pk

```
1/1                                                                 31/11
atg tcc cct ata cta ggt tat tgg aaa att aag ggc ctt gtg caa ccc act cga ctt ctt
M   S   P   I   L   G   Y   W   K   I   K   G   L   V   Q   P   T   R   L   L
61/21                                                               91/31
ttg gaa tat ctt gaa gaa aaa tat gaa gag cat ttg tat gag cgc gat gaa ggt gat aaa
L   E   Y   L   E   E   K   Y   E   E   H   L   Y   E   R   D   E   G   D   K
121/41                                                              151/51
tgg cga aac aaa aag ttt gaa ttg ggt ttg gag ttt ccc aat ctt cct tat tat att gat
W   R   N   K   K   F   E   L   G   L   E   F   P   N   L   P   Y   Y   I   D
181/61                                                              211/71
ggt gat gtt aaa tta aca cag tct atg gcc atc ata cgt tat ata gct gac aag cac aac
G   D   V   K   L   T   Q   S   M   A   I   I   R   Y   I   A   D   K   H   N
241/81                                                              271/91
atg ttg ggt ggt tgt cca aaa gag cgt gca gag att tca atg ctt gaa gga gcg gtt ttg
M   L   G   G   C   P   K   E   R   A   E   I   S   M   L   E   G   A   V   L
301/101                                                             331/111
gat att aga tac ggt gtt tcg aga att gca tat agt aaa gac ttt gaa act ctc aaa gtt
D   I   R   Y   G   V   S   R   I   A   Y   S   K   D   F   E   T   L   K   V
361/121                                                             391/131
gat ttt ctt agc aag cta cct gaa atg ctg aaa atg ttc gaa gat cgt tta tgt cat aaa
D   F   L   S   K   L   P   E   M   L   K   M   F   E   D   R   L   C   H   K
421/141                                                             451/151
aca tat tta aat ggt gat cat gta acc cat cct gac ttc atg ttg tat gac gct ctt gat
T   Y   L   N   G   D   H   V   T   H   P   D   F   M   L   Y   D   A   L   D
481/161                                                             511/171
gtt gtt tta tac atg gac cca atg tgc ctg gat gcg ttc cca aaa tta gtt tgt ttt aaa
V   V   L   Y   M   D   P   M   C   L   D   A   F   P   K   L   V   C   F   K
541/181                                                             571/191
aaa cgt att gaa gct atc cca caa att gat aag tac ttg aaa tcc agc aag tat ata gca
K   R   I   E   A   I   P   Q   I   D   K   Y   L   K   S   S   K   Y   I   A
601/201                                                             631/211
tgg cct ttg cag ggc tgg caa gcc acg ttt ggt ggt ggc gac cat cct cca aaa tcg gat
W   P   L   Q   G   W   Q   A   T   F   G   G   G   D   H   P   P   K   S   D
661/221                                                             691/231
ctg gtt ccg cgt GGA TCC ATG GAG ACA CCC TTG AGG GAG CAG GAG AAC TCA TTA GAA TCC
L   V   P   R   G   S   M   E   T   P   L   R   E   Q   E   N   S   L   E   S
721/241                                                             751/251
TCC AAC GAG CGC TCT TCA TGC ATT TCA GAG GCG GAT GCA ACC ACT CCA GAA TCG GCC AAC
S   N   E   R   S   S   C   I   S   E   A   D   A   T   T   P   E   S   A   N
781/261                                                             811/271
CTG GGG GAG GAA ATC CTC TCT CAA CTA TAC CGC CCT CTA GAG GCG TGC TAT AAC ACA TGC
L   G   E   E   I   L   S   Q   L   Y   R   P   L   E   A   C   Y   N   T   C
841/281                                                             871/291
TAT TGT AAA AAG TGT TGC TAC CAT TGC CAG TTT TGT TTT CTT AAA AAG GGA TTG GGG ATA
Y   C   K   K   C   C   Y   H   C   Q   F   C   F   L   K   K   G   L   G   I
901/301                                                             931/311
TGT TAT GAG CAG TCA CgA AAG AGA AGA AGA ACT cCG AAA AAG GCT AAG GCT AAT ACA TCT
C   Y   E   Q   S   R   K   R   R   R   T   P   K   K   A   K   A   N   T   S
961/321                                                             991/331
TCT GCA TcA AAC AAC AgA CCC ATA TCC AAC AGG ACC CGG CAC TGC CAA CCA GAG AAG GCA
S   A   S   N   N   R   P   I   S   N   R   T   R   H   C   Q   P   E   K   A
1021/341                                                            1051/351
AAG AAG GAG ACG GTG GAg AAG GCG GTG GCA ACA GCT CCT GGC CTT GGC AGA GGa att cCG
K   K   E   T   V   E   K   A   V   A   T   A   P   G   L   G   R   G   I   P
1081/361                                                            1111/371
gcg gaa att ctg gcg ggt ggc cat cac cat cac cat cac GgA ATT cCA GGA AAG CCG ATC
A   E   I   L   A   G   G   H   H   H   H   H   H   G   I   P   G   K   P   I
1141/381                                                            1171/391
CCA AAC CCT TTG CTG GGA TTG GAC TCC ACC TGA
P   N   P   L   L   G   L   D   S   T   *
```

Key: GST, pGEX, BamHI, Tat, EcoRI, spacer, APCS, His, EcoRI, Pk.

7.8 DNA Sequence Of Vector pGEX-Tat-APCS-His-Pk-2 & Amino Acid Sequence Of GST-Tat-APCS-His-Pk

```
1/1                                                                 31/11
atg tcc cct ata cta ggt tat tgg aaa att aag ggc ctt gtg caa ccc act cga ctt ctt
M   S   P   I   L   G   Y   W   K   I   K   G   L   V   Q   P   T   R   L   L
61/21                                                               91/31
ttg gaa tat ctt gaa gaa aaa tat gaa gag cat ttg tat gag cgc gat gaa ggt gat aaa
L   E   Y   L   E   E   K   Y   E   E   H   L   Y   E   R   D   E   G   D   K
121/41                                                              151/51
tgg cga aac aaa aag ttt gaa ttg ggt ttg gag ttt ccc aat ctt cct tat tat att gat
W   R   N   K   K   F   E   L   G   L   E   F   P   N   L   P   Y   Y   I   D
181/61                                                              211/71
ggt gat gtt aaa tta aca cag tct atg gcc atc ata cgt tat ata gct gac aag cac aac
G   D   V   K   L   T   Q   S   M   A   I   I   R   Y   I   A   D   K   H   N
241/81                                                              271/91
atg ttg ggt ggt tgt cca aaa gag cgt gca gag att tca atg ctt gaa gga gcg gtt ttg
M   L   G   G   C   P   K   E   R   A   E   I   S   M   L   E   G   A   V   L
301/101                                                             331/111
gat att aga tac ggt gtt tcg aga att gca tat agt aaa gac ttt gaa act ctc aaa gtt
D   I   R   Y   G   V   S   R   I   A   Y   S   K   D   F   E   T   L   K   V
361/121                                                             391/131
gat ttt ctt agc aag cta cct gaa atg ctg aaa atg ttc gaa gat cgt tta tgt cat aaa
D   F   L   S   K   L   P   E   M   L   K   M   F   E   D   R   L   C   H   K
421/141                                                             451/151
aca tat tta aat ggt gat cat gta acc cat cct gac ttc atg ttg tat gac gct ctt gat
T   Y   L   N   G   D   H   V   T   H   P   D   F   M   L   Y   D   A   L   D
481/161                                                             511/171
gtt gtt tta tac atg gac cca atg tgc ctg gat gcg ttc cca aaa tta gtt tgt ttt aaa
V   V   L   Y   M   D   P   M   C   L   D   A   F   P   K   L   V   C   F   K
541/181                                                             571/191
aaa cgt att gaa gct atc cca caa att gat aag tac ttg aaa tcc agc aag tat ata gca
K   R   I   E   A   I   P   Q   I   D   K   Y   L   K   S   S   K   Y   I   A
601/201                                                             631/211
tgg cct ttg cag ggc tgg caa gcc acg ttt ggt ggt ggc gac cat cct cca aaa tcg gat
W   P   L   Q   G   W   Q   A   T   F   G   G   G   D   H   P   P   K   S   D
661/221                                                             691/231
ctg gtt ccg cgt GGA TCC ATG GAG ACA CCC TTG AGG GAG CAG GAG AAC TCA TTA GAA TCC
L   V   P   R   G   S   M   E   T   P   L   R   E   Q   E   N   S   L   E   S
721/241                                                             751/251
TCC AAC GAG CGC TCT TCA TGC ATT TCA GAG GCG GAT GCA ACC ACT CCA GAA TCG GCC AAC
S   N   E   R   S   S   C   I   S   E   A   D   A   T   T   P   E   S   A   N
781/261                                                             811/271
CTG GGG GAG GAA ATC CTC TCT CAA CTA TAC CGC CCT CTA GAG GCG TGC TAT AAC ACA TGC
L   G   E   E   I   L   S   Q   L   Y   R   P   L   E   A   C   Y   N   T   C
841/281                                                             871/291
TAT TGT AAA AAG TGT TGC TAC CAT TGC CAG TTT TGT TTT CTT AAA AAG GGA TTG GGG ATA
Y   C   K   K   C   C   Y   H   C   Q   F   C   F   L   K   K   G   L   G   I
901/301                                                             931/311
TGT TAT GAG CAG TCA CgA AAG cGt cGc cGt ACT cCG AAA AAG GCT AAG GCT AAT ACA TCT
C   Y   E   Q   S   R   K   R   R   R   T   P   K   K   A   K   A   N   T   S
961/321                                                             991/331
TCT GCA TcA AAC AAC AgA CCC ATA TCC AAC AGG ACC CGG CAC TGC CAA CCA GAG AAG GCA
S   A   S   N   N   R   P   I   S   N   R   T   R   H   C   Q   P   E   K   A
1021/341                                                            1051/351
AAG AAG GAG ACG GTG GAg AAG GCG GTG GCA ACA GCT CCT GGC CTT GGC AGA GGa att cCG
K   K   E   T   V   E   K   A   V   A   T   A   P   G   L   G   R   G   I   P
1081/361                                                            1111/371
gcg gaa att ctg gcg ggt ggc cat cac cat cac cat cac GcA ATT gCA GGA AAG CCG ATC
A   E   I   L   A   G   G   H   H   H   H   H   H   A   I   A   G   K   P   I
1141/381                                                            1171/391
CCA AAC CCT TTG CTG GGA TTG GAC TCC ACC TGA
P   N   P   L   L   G   L   D   S   T   *
```

Key: GST, pGEX, BamHI, Tat, EcoRI, spacer, APCS, His, Pk.

7.9 DNA Sequence Of Vector pGEX-CTLs-Tat-APCS-His-Pk & Amino Acid Sequence Of GST-CTLs-Tat-APCS-His-Pk

```
1/1                                                                 31/11
atg tcc cct ata cta ggt tat tgg aaa att aag ggc ctt gtg caa ccc act cga ctt ctt
M   S   P   I   L   G   Y   W   K   I   K   G   L   V   Q   P   T   R   L   L
61/21                                                               91/31
ttg gaa tat ctt gaa gaa aaa tat gaa gag cat ttg tat gag cgc gat gaa ggt gat aaa
L   E   Y   L   E   E   K   Y   E   E   H   L   Y   E   R   D   E   G   D   K
121/41                                                              151/51
tgg cga aac aaa aag ttt gaa ttg ggt ttg gag ttt ccc aat ctt cct tat tat att gat
W   R   N   K   K   F   E   L   G   L   E   F   P   N   L   P   Y   Y   I   D
181/61                                                              211/71
ggt gat gtt aaa tta aca cag tct atg gcc atc ata cgt tat ata gct gac aag cac aac
G   D   V   K   L   T   Q   S   M   A   I   I   R   Y   I   A   D   K   H   N
241/81                                                              271/91
atg ttg ggt ggt tgt cca aaa gag cgt gca gag att tca atg ctt gaa gga gcg gtt ttg
M   L   G   G   C   P   K   E   R   A   E   I   S   M   L   E   G   A   V   L
301/101                                                             331/111
gat att aga tac ggt gtt tcg aga att gca tat agt aaa gac ttt gaa act ctc aaa gtt
D   I   R   Y   G   V   S   R   I   A   Y   S   K   D   F   E   T   L   K   V
361/121                                                             391/131
gat ttt ctt agc aag cta cct gaa atg ctg aaa atg ttc gaa gat cgt tta tgt cat aaa
D   F   L   S   K   L   P   E   M   L   K   M   F   E   D   R   L   C   H   K
421/141                                                             451/151
aca tat tta aat ggt gat cat gta acc cat cct gac ttc atg ttg tat gac gct ctt gat
T   Y   L   N   G   D   H   V   T   H   P   D   F   M   L   Y   D   A   L   D
481/161                                                             511/171
gtt gtt tta tac atg gac cca atg tgc ctg gat gcg ttc cca aaa tta gtt tgt ttt aaa
V   V   L   Y   M   D   P   M   C   L   D   A   F   P   K   L   V   C   F   K
541/181                                                             571/191
aaa cgt att gaa gct atc cca caa att gat aag tac ttg aaa tcc agc aag tat ata gca
K   R   I   E   A   I   P   Q   I   D   K   Y   L   K   S   S   K   Y   I   A
601/201                                                             631/211
tgg cct ttg cag ggc tgg caa gcc acg ttt ggt ggt ggc gac cat cct cca aaa tcg gat
W   P   L   Q   G   W   Q   A   T   F   G   G   G   D   H   P   P   K   S   D
661/221                                                             691/231
ctg gtt ccg cgt GGA TCA CGT ACG CGT GGC CCG GGT CGT GCG TTT GTG ACC ATC TCC ATT
L   V   P   R   G   S   R   T   R   G   P   G   R   A   F   V   T   I   S   I
721/241                                                             751/251
ATC AAT TTT GAA AAA CTG GGA TCC ATG GAG ACA CCC TTG AGG GAG CAG GAG AAC TCA TTA
I   N   F   E   K   L   G   S   M   E   T   P   L   R   E   Q   E   N   S   L
781/261                                                             811/271
GAA TCC TCC AAC GAG CGC TCT TCA TGC ATT TCA GAG GCG GAT GCA ACC ACT CCA GAA TCG
E   S   S   N   E   R   S   S   C   I   S   E   A   D   A   T   T   P   E   S
841/281                                                             871/291
GCC AAC CTG GGG GAG GAA ATC CTC TCT CAA CTA TAC CGC CCT CTA GAG GCG TGC TAT AAC
A   N   L   G   E   E   I   L   S   Q   L   Y   R   P   L   E   A   C   Y   N
901/301                                                             931/311
ACA TGC TAT TGT AAA AAG TGT TGC TAC CAT TGC CAG TTT TGT TTT CTT AAA AAG GGA TTG
T   C   Y   C   K   K   C   C   Y   H   C   Q   F   C   F   L   K   K   G   L
961/321                                                             991/331
GGG ATA TGT TAT GAG CAG TCA CGA AAG CGT CGC CGT ACT CCG AAA AAG GCT AAG GCT AAT
G   I   C   Y   E   Q   S   R   K   R   R   R   T   P   K   K   A   K   A   N
1021/341                                                            1051/351
ACA TCT TCT GCA TCA AAC AAC AGA CCC ATA TCC AAC AGG ACC CGG CAC TGC CAA CCA GAG
T   S   S   A   S   N   N   R   P   I   S   N   R   T   R   H   C   Q   P   E
1081/361                                                            1111/371
AAG GCA AAG AAG GAG ACG GTG GAG AAG GCG GTG GCA ACA GCT CCT GGC CTT GGC AGA GGA
K   A   K   K   E   T   V   E   K   A   V   A   T   A   P   G   L   G   R   G
1141/381                                                            1171/391
ATT CCT GCG GAA ATT CTG GCG GGT GGC CAT CAC CAT CAC CAT CAC GCA ATT CCA GGA AAG
I   P   A   E   I   L   A   G   G   H   H   H   H   H   H   A   I   P   G   K
1201/401                                                            1231/411
CCG ATC CCA AAC CCT TTG CTG GGA TTG GAC TCC ACC TGA
P   I   P   N   P   L   L   G   L   D   S   T   *
```

KEY: GST, pGEX, CTLs, BamHI, Tat, EcoRI, spacer, APCS, His, Pk.

7.10 Primers And Oligonucleotides Employed

- **Primers designed for pGEX-2T vector (Pharmacia):**

pGEX fwd (Primer 1): GCA TGG CCT TTG CAG GGC TGG

pGEX rev: TGT GAC CGT CTC CGG GAG CTG

- **Oligos for GST-PkHis cloning**

Oligos for GST-PkHis4,10:

PkHis4,10 fwd:

CCC TTT GGA TCC GGA AAG CCG CAT CCA AAC CCT CTA TTA CAT CTG GAC TCC ACC GAA TTC CCC TTT

PkHis4,10 rev:

AAA GGG GAA TTC GGT GGA GTC CAG ATG TAA TAG AGG GTT TGG ATG CGG CTT TCC GGA TCC AAA GGG

Oligos for GST-PkHis9:

PkHis9 fwd:

CCC TTT GGA TCC GGA AAG CCG ATC CCA AAC CCT CTA CAT GGT CTG GAC TCC ACC GAA TTC CCC TTT

PkHis9 rev:

AAA GGG GAA TTC GGT GGA GTC CAG ACC ATG TAG AGG GTT TGG GAT CGG CTT TCC GGA TCC AAA GGG

Oligos for GST-PkHis11:

PkHis11 fwd:

CCC TTT GGA TCC GGA AAG CCG ATC CCA AAC CCT CTA TTA GGT CAT GAC TCC ACC GAA TTC CCC TTT

PkHis11 rev:

AAA GGG GAA TTC GGT GGA GTC ATG ACC TAA TAG AGG GTT TGG GAT CGG CTT TCC GGA TCC AAA GGG

- **Engineering GST-Tat-APCS-His-Pk**

Oligos for APCS-His:

fwd APCS-His:

AAT TCC GGC GGA AAT TCT GGC GGG TGG CCA TCA CCA TCA CCA TCA CGG

rev APCS-His:

AAT TCC GTG ATG GTG ATG GTG ATG GCC ACC CGC CAG AAT TTC CGC CGG

Primers for RRR change:

Primer 2 (TATRRR FWD):

GAG CAG TCA CGA AAG CGT CGC CGT ACT CCG AAA AAG GCT AAG GCT

Primer 3 (TATRRR REV):

ACG GCG ACG CTT TCG TGA CTG CTC ATA ACA

Primer for EcoRI destruction:

Primer 4 (TATMEF BCK):

TCC TGC AAT TGC GTG ATG GTG ATG GTG ATG GCC ACC

Oligos for addition of the CTLs sequence:

CTL fwd:

GAT CAC GTA CGC GTG GCC CGG GTC GTG CGT TTG TGA CCA TCT CCA TTA TCA ATT TTG AAA AAC TGG GAT CCG AGCT

CTL rev:

CGG ATC CCA GTT TTT CAA AAT TGA TAA TGG AGA TGG TCA CAA ACG CAC GAC CCG GGC CAC GCG TAC GT

7.11 ELISA STANDARDISATION FOR COMPARATIVE ASSAYS OF MONKEY SAMPLES WITH PROTEINS GST-PK & GST-TAT-APCS-HIS-PK

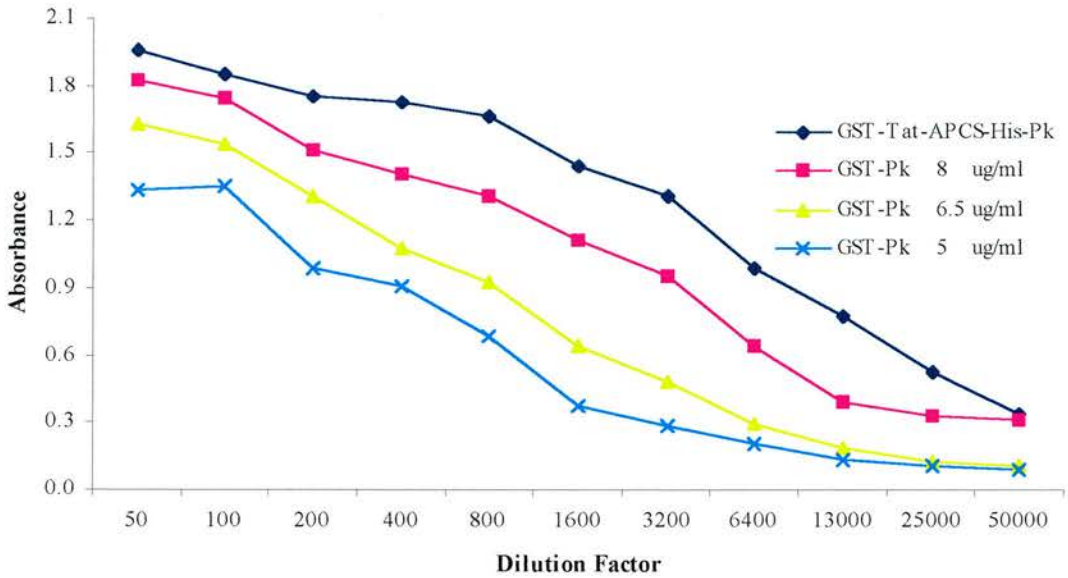

Figure 38: Optimisation of protein concentration on ELISA plates to ensure comparable readings between GST-Pk and GST-Tat-APCS-His-Pk – coated plates.

GST-Tat-APCS-His-Pk was employed at 10 μg/ml, while GST-Pk was tested at 8 μg/ml, 6.5 μg/ml and 5 μg/ml concentrations (GST-Pk was available in larger amounts, so it was employed in different concentrations to match the single concentration of GST-Tat-APCS-His-Pk).

Following coating of the plates and blocking of excess binding sites, the proteins were probed with anti-Pk antibody. Starting at an initial dilution of 1/3000, serial two-fold dilutions of the antibody were performed, and the absorbance readings obtained were plotted as shown above.

It was concluded that the concentration of 8-8.5 μg/ml of GST-Pk protein would provide a comparable result to that obtained on ELISA plates coated with 10 μg/ml of GST-Tat-APCS-His-Pk.