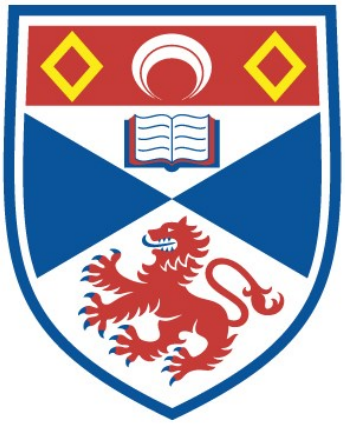
# ISOLATION AND CHARACTERISATION OF MONOCLONAL ANTIBODIES RAISED AGAINST THE PK PEPTIDE TAG

Claire Dunn

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1998

# Isolation and characterisation of monoclonal antibodies raised against the Pk peptide tag

by

Claire Dunn, B.Sc (Hons)

A thesis submitted in partial fulfilment
of the requirements for the
degree of Doctor of Philosophy

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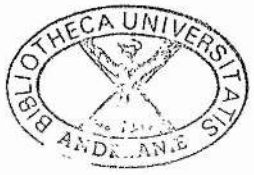

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I would like to dedicate this thesis to the memory of my father, Michael Dunn.

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# ABSTRACT

In this project, a number of monoclonal antibodies were developed which recognised an oligopeptide tag known as the Pk tag. These antibodies were used for the development of an affinity purification protocol in order to purify Pk tagged recombinant proteins. These proteins, in turn, were to be used for the development of multiple epitope vaccines.

The Pk tag is a 14 amino acid oligopeptide which was originally mapped to the binding epitope of the monoclonal antibody SV5-P-k, raised to the Paramyxovirus Simian Virus 5 (SV5) phospho- (P) protein. The tag has been cloned into the bacterial expression vector pQ9cPk so that when expressed, any recombinant protein produced will have a Histidine tag at the N terminus and the Pk tag at the C terminus. These two tags are the basis of a two step purification system, utilising a nickel affinity column for the Histidine tag capture, and monoclonal antibody (mAb) SV5-P-k immobilised on Sepharose beads for Pk tag capture.

The genes encoding the Simian Immunodeficiency Virus (SIV) precursor protein $Pr55^{gag}$ and the auxiliary protein Nef were cloned into pQ9cPk and the recombinant proteins produced purified using the current two step purification protocol. However, the affinity of the mAb SV5-P-k for the Pk tag is so strong, that denaturing conditions were needed to separate the antibody from the tagged protein. Due to this it was decided to develop a number of mAb's to the Pk tag with the aim of creating antibodies whose binding affinities varied from that of SV5-P-k.

A number of hybridoma clones expressing mAb's which recognise the Pk tag were created and after initial characterisation it was demonstrated that of the twenty four clones produced, only four recognised Pk tagged recombinant proteins or the SV5 P protein. The epitopes and binding affinities of these four mAb's were determined, and when compared to SV5-P-k, it was observed that all five mAb's had similar affinities for the Pk tag. Therefore, antibodies to the Pk tag which had lower binding affinities were not obtained.

In order to utilise the five anti-Pk mAb's in the two step purification system, the original primary sequence of the Pk tag was modified, creating three new tags. On further analysis of the binding of the five anti-Pk mAb's to the modified tags, elution conditions were determined which allowed the elution of Pk tagged recombinant proteins from a mAb bound affinity column.

# List of Abbreviations

| | |
|---|---|
| A | Adenine |
| AIDS | aquired immune deficiency syndrome |
| Amp | ampicillin |
| APS | ammonium persulphate |
| | |
| BIA | Biomolecular interaction analysis |
| BSA | bovine serum albumin |
| | |
| C | cytosine |
| C- | carboxy terminus |
| CA | capsid protein |
| $Ca^{2+}$ | calcium |
| CM | carboxymethylated dextran |
| $CO_2$ | carbon dioxide |
| CoA | coenzyme A |
| ConA | concanavalin A |
| CPI | canine parainfluenza virus |
| CTL | cytotoxic T cell |
| | |
| DAPI | 4,6 - diamino-2-phenylindole |
| dATP | 2'-deoxyadenosine 5'-trisphosphate |
| DEAE | diethylaminoethyl |
| dCTP | 2'-deoxycytosine 5'-trisphosphate |
| dGTP | 2'-deoxyguanosine 5'-trisphosphate |
| DMSO | dimethyl sulphoxide |
| DNA | deoxyribonucleic acid |
| dsDNA | double stranded DNA |
| dTTP | 2'-deoxythymidine 5'-trisphosphate |
| | |
| ECL | enhanced chemi-luminescence |
| EDC | N-ethyl-N'(3-dimethylaminopropyl)carbodiimide hydrochloride |
| EDTA | ethylenediaminetetraacetic acid |
| ELISA | enzyme linked immunosorbant assay |
| env | envelope |
| ER | endoplasmic reticulum |

| | |
|---|---|
| Fab | antigen binding fragment of antibody |
| Fc | crystalisable fragment of antibody |
| FCS | Foetal calf serum |
| | |
| G | guanosine |
| gag | group specific antigen |
| gp | glycoprotein |
| GST | glutathione S transferase |
| GTE | glucose, tris, EDTA buffer |
| | |
| HAT | hypoxanthine, aminopterin, thymidine media supplement |
| HBS | HEPES buffered saline |
| HEL | hen egg white lysozyme |
| HGPRT | hypoxanthine guanosine phosphoribosyl transferase |
| His | Histidine tag |
| HIV | human immunodeficiency virus |
| HRP | horseradish peroxidase |
| HSV | Herpes Simplex Virus |
| | |
| IFC | integrated micro-fluidics cartridge |
| Ig | immunoglobulin |
| Int | integrase |
| IPTG | isopropyl-β-D-thiogalactopyranoside |
| | |
| $k_a$ | apparent association rate constant |
| kb | kilobases |
| $k_d$ | apparent dissociation rate constant |
| Kd | Equilibrium rate dissociation constant |
| | |
| LB | Luria Broth |
| LCA | lentil lectin |
| LTB | heat labile enterotoxin subunit B |
| LTR | long terminal repeat |
| | |
| MA | matrix protein |
| mAb | monoclonal antibody |
| MBP | maltose binding protein |
| MHC | major histocompatibility complex |
| $Mn^{2+}$ | manganese |

| | |
|---|---|
| Mr | relative molecular mass |
| mRNA | messenger RNA |
| | |
| N- | amino terminus |
| NaAc | sodium acetate |
| $NAD^+$ | nicotinamide adenine dinucleotide |
| NaSCN | sodium thiocyanate |
| NBCS | new born calf serum |
| NC | nucleocapsid protein |
| NHS | H-hydroxysuccinimide |
| Ni-NTA | nickel nitrilo-tri-acetic acid resin |
| Nef | negative protein |
| NP-40 | Nonident P-40 |
| nuc | nuclease |
| | |
| $OD_{\#}$ | optical density at #nm |
| ORF | open reading frame |
| | |
| P | phospho protein |
| PAG | polyacrylamide gel |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PEG | polyethylene glycol |
| PFHM | protein free hybridoma media |
| pH | Pondus hydrogen ($-\log_{10}[H^+]$) |
| PIV-2 | Parainfluenza virus 2 |
| pK | $-\log_{10}K$ |
| PNA | peanut agglutinin |
| PrA | Staphylococcus protein A |
| PrG | Streptococcus protein G |
| prot | protease |
| | |
| RNA | ribonucleic acid |
| rpm | revolutions per minute |
| RRE | rev response element |
| rt | reverse transcriptase |
| rtTA | reverse tetracycline transactivator protein |
| RU | resonance units |

| | |
|---|---|
| SAIDS | simian AIDS |
| SDS | sodium dodecyl sulphate |
| SH3 | Src homology region - 3 |
| SIV | simian immunodeficiency virus |
| SMAA | solid matrix antibody antigen complexes |
| SPR | surface plasmon resonance |
| SSC | salt - sodium citrate buffer |
| ssDNA | single stranded DNA |
| SV5 | simian virus 5 |
| | |
| T | thymine |
| TBE | tris-borate-EDTA buffer |
| TAR | *trans*-acting response element RNA motif |
| Tat | transactivator protein |
| TBS | Tris buffered saline |
| TCL | Total cell lysate |
| TCR | T cell receptor |
| TE | tris-EDTA buffer |
| TEMED | N,N,N',N'- tetramethylethylenediamine |
| tet | tetracycline |
| TFIID | transcription factor IID |
| $T_H$ | T helper cell |
| TMB | 3,3',5,5'-tetramethylbenzidine |
| TNF | tumour necrosis factor |
| Tris | 2-amino-2(hydroxymethyl)propane-1,3-diol |
| tRNA | transfer RNA |
| tTA | tetracycline transactivator protein |
| | |
| UV | ultraviolet |
| | |
| V | variable genes |
| $V_H$ | variable region of antibody heavy chain |
| $V_L$ | variable region of antibody light chain |
| Vpr | viral protein R |
| Vpu | viral protein U |
| Vpx | viral protein X |
| v/v | volume per volume ratio |
| v/w | volume per weight ratio |

WGA wheat germ agglutinin

## ABBREVIATIONS FOR AMINO ACIDS

| | | |
|---|---|---|
| alanine | ala | A |
| arginine | arg | R |
| asparagine | asn | N |
| aspartic acid | asp | D |
| cysteine | cys | C |
| glutamine | gln | Q |
| gluatamic acid | glu | E |
| glycine | gly | G |
| histidine | his | H |
| isoleucine | ile | I |
| leucine | leu | L |
| lysine | lys | K |
| methionine | met | M |
| phenylalanine | phe | F |
| proline | pro | P |
| serine | ser | S |
| threonine | thr | T |
| tryptophan | trp | W |
| tyrosine | tyr | Y |
| valine | val | V |

## GENETIC CODE

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| TTT | phe | F | TCT | ser | S | TAT | tyr | Y | TGT | cys | C |
| TTC | phe | F | TCC | ser | S | TAC | tyr | Y | TGC | cys | C |
| TTA | leu | L | TCA | ser | S | TAA | OCH | Z | TGA | OPA | Z |
| TTG | leu | L | TCG | ser | S | TAG | AMB | Z | TGG | trp | W |
| CTT | leu | L | CCT | pro | P | CAT | his | H | CGT | arg | R |
| CTC | leu | L | CCC | pro | P | CAC | his | H | CGC | arg | R |
| CTA | leu | L | CCA | pro | P | CAA | gln | Q | CGA | arg | R |
| CTG | leu | L | CCG | pro | P | CAG | gln | Q | CGG | arg | R |
| ATT | ile | I | ACT | thr | T | AAT | asn | N | AGT | ser | S |
| ATC | ile | I | ACC | thr | T | AAC | asn | N | AGC | ser | S |
| ATA | ile | I | ACA | the | T | AAA | lys | K | AGA | arg | R |
| ATG | met | M | ACG | thr | T | AAG | lys | K | AGG | arg | R |
| GTT | val | V | GCT | ala | A | GAT | asp | D | GGT | gly | G |
| GTC | val | V | GCC | ala | A | GAC | asp | D | GGC | gly | G |
| GTA | val | V | GCA | ala | A | GAA | glu | E | GGA | gly | G |
| GTG | val | V | GCG | ala | A | GAG | glu | E | GGG | gly | G |

UNITS

| | |
|---|---|
| °C | degrees Celcius |
| g | gram |
| m | metre |
| mol | mole |
| s | second |
| Ci | Curie [$3.7 \times 10^{10} s^{-1}$ (disintegration per second)] |
| Da | Dalton |
| l | litre |
| M | molar concentration |
| min | minute |
| U | unit of enzymatic activity |

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# INTRODUCTION

The work to be presented in this thesis is part of a long term effort to develop a multiple subunit vaccine against the Human Immunodeficiency Virus (HIV), using the infection of Macaques by the Simian Immunodeficiency Virus macaque strain (SIVmac) as an animal model. To develop the most efficient vaccine to HIV (and SIV), proteins of high purity are required, in a format which allows the immune system to produce a strong humoral and cellular response. This is to reduce any potential interference by contaminating proteins. To achieve this, a two step purification protocol was developed. This protocol utilised fusion proteins containing peptide tags at both the amino (N) and carboxy (C) termini. The tags currently used for this protocol are the hexahistidine peptide tag at the N terminus and the oligopeptide Pk tag at the C terminus. This protocol ensured the affinity purification of full length recombinant proteins. Both of these tags can be used for the affinity purification of tagged recombinant proteins; the hexahistadine tag by nickel chelate affinity chromatography; and the Pk tag by immunoaffinity chromatography.

Section one of the Introduction gives a brief overview of the most commonly used protein purification protocols currently in use in biochemistry today. This gives an insight into the many different ways of purifying recombinant proteins derived from either eukaryotic or prokaryotic expression systems. In addition to this, Section one also reviews the most commonly used affinity tag purification systems, and provides a basis with which to introduce the Pk affinity peptide tag developed by our research group.

Much of the work presented in this thesis concentrates on the development and characterisation of a number of monoclonal antibodies (mAb) which were raised against the Pk tag. The recognition of the Pk tag by the mAbs forms the second stage of the two step affinity purification system, and is also a way of presenting the antigenic vaccine to an animals immune system in the form of solid matrix antibody antigen (SMAA) complexes. Section two of the Introduction details the alternative ways that mAbs can be developed and the most efficient methods for purifying mAbs.

Section three of the Introduction concentrates on the molecular aspects of HIV and SIV, detailing some of the more recent progresses in the understanding of the retroviral life cycle and how this has helped in the development of a vaccine strategy. Section three also details some of the difficulties experienced when trying to develop a vaccine to HIV or SIV.

The final part of the Introduction states the objectives of the work to be presented in this thesis.

# 1. PROTEIN AFFINITY PURIFICATION

The purification of proteins is a prerequisite for many biochemical applications. Thus, a large number of protocols have been devised over recent years to obtain maximum yields of purified proteins, with minimal effort. As there are a wide range of purification protocols available, before choosing which to use, a single question must be addressed : what is the purified protein needed for?

The intended use of the protein can have a significant effect on how well the protein needs to be purified. For example, if the protein is to be used for structural analysis then it must be more than 95% pure, as any impurities can effect the results obtained. However, if the protein is needed for protein activity assays, the purity of the protein is not as important as obtaining sufficient amounts of protein with minimal loss of protein activity (Phizicky & Fields 1995, Nilsson *et al* 1996, Scopes 1996).

Other considerations need to be addressed when choosing a method of protein purification, for example, the properties of the protein. Knowledge of the proteins location within a cell; the presence of any post-translational processing e.g. phosphorylation, the addition of carbohydrates or lipids; the solubility of the protein; and whether it is membrane bound or located within a subcellular organelle, are some of the properties which need to be determined before deciding which purification protocol to use. The more that is known about the protein, the easier it becomes to determine the most efficient method of purification. For example, if the protein is extracellular, then centrifugation of the cells may

be the best way of obtaining pure proteins. However, if the protein is glycosylated then affinity purification on a lectin column may be satisfactory for obtaining relatively pure protein (Scopes 1996).

A way of simplifying the purification protocol and eliminating the necessity of determining the properties of the protein is to express the protein *in vitro* from a controlled expression system. The most common organism used for the expression of recombinant proteins is *Escherichia coli*. (*E.coli*) The full length genome of *E. coli* has now been sequenced, making it one of the most studied organisms to date. By designing expression plasmids with the optimal signals for transcription and translation, yields of up to 40% total cell mass of recombinant protein can be obtained.

One disadvantage of using *E. coli* for the expression of recombinant proteins, is that the proteins are not secreted and are usually found in inclusion bodies. To obtain secreted recombinant proteins, the *Bacillus subtilis* expression system can be used. Although the genetics of this bacterial species are not as well known as those of *E. coli*, high yields of secreted recombinant protein have been obtained. However, a disadvantage to using *B. subtilis*, is that highly active proteases are also secreted. Both of these bacterial systems can be used for the expression of eukaryotic and prokaryotic genes.

One of the fundamental differences of proteins produced in eukaryotic and prokaryotic systems is post-translational processing. Many eukaryotic proteins undergo post-translational modifications such as glycosylation or phosphorylation, processes which do not occur in prokaryotes. To overcome this, if a protein is known to be post-translationally processed, it can be expressed in the yeast *Saccharomyces cerevisiae*. This is a single cell yeast which exhibits all the characteristics of a eukaryote, ensuring that when eukaryotic genes are expressed, post-translational modifications can be added. A disadvantage of using *S. cerevisiae* occurs if protein secretion is required, as this yeast only usually secretes small proteins or peptides.

Regardless of how the protein has been produced, there are four fundamental ways of purifying a protein: size dependent separation (gel-filtration and membrane exclusion); charge dependent separation (ion exchange chromatography and electrophoresis); solubility

dependent separation i.e. the interaction between the surface of the protein and the solvent (reverse phase chromatography, hydrophobic interactions and ammonium sulphate precipitation); and affinity chromatography (Nilsson *et al* 1996, Scopes 1996).

Part 1 of the Introduction describes some of the protein purification methods which are available, concentrating specifically on affinity chromatography.

Affinity chromatography is a system based on the molecular interactions of a protein to its specific ligand (Nilsson *et al* 1996). This technique was first used for the determination of phage and host proteins which interacted with the *E. coli* RNA polymerase (Phizicky & Fields 1995). The efficiency of affinity purification depends on the selectivity and tightness of binding of the required protein to its ligand. Proteins purified in this way tend to have high affinities for their ligands (Scopes 1996). If all conditions are optimised, affinity chromatography is a highly specific technique in which large yields of highly purified proteins can be obtained in a single purification step. This makes affinity chromatography a valuable tool for the detection, purification and immobilisation of proteins.

The different ways of affinity purifying a protein fall into four main categories : (i) the built in affinity of a fusion protein, (ii) the affinity of a protein for its natural ligand, (iii) the use of immobilised antibodies (immuno-affinity purification), and (iv) the unnatural interactions observed between individual proteins and organic molecules such as dyes and pharmaceutical drugs. Examples of each of these categories, with the exception of immuno-affinity purification (see section 2.2), will be discussed in turn.

## 1.1 Affinity chromatography using fusion proteins

The affinity chromatography of fusion proteins is one of the most efficient ways of purifying a protein. The production of fusion proteins is completely dependent upon recombinant DNA technology and the expression of recombinant proteins from a controlled expression system.

There are a number of well defined fusion proteins which are commonly used. They can consist of small peptide affinity tags / epitopes or full length proteins which are then recognised by either an antibody, an inorganic ligand or another biological ligand.

The development of affinity peptide tags is currently one of the fastest growing areas of biochemistry. Affinity tags tend not to be more than 20 amino acids in length and they can consist of the consensus sequence of antibodies, or other biological ligands, or be completely synthetically designed.

If an affinity tag is to be useful for protein purification and detection, it must adhere to a number of requirements. Firstly, the tag should not interfere with the folding, or the activity, of the protein to which it is fused. An affinity tag should be soluble and retain a high degree of exposure in aqueous solution. If it is to be useful in affinity purification, the tag should not have special requirements for binding and should allow elution under relatively mild conditions. Finally, if necessary, it should be easy to remove in a such a way that does not alter the target protein in any way. This final consideration is necessary if untagged target proteins are required (Nilsson *et al* 1996).

In addition to peptide affinity tags, complete proteins can be fused to target proteins for affinity purification. In most cases the binding ligand and the conditions for separation are well defined.

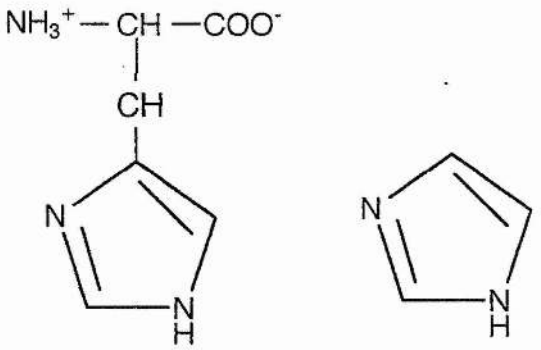
There are many commercially available plasmid vectors which are used for the production of fusion proteins. This section details some of the most commonly used affinity tags and fusion proteins and states the advantages and disadvantages of each one.

*i. Histidine / nickel affinity purification*

One of the most common affinity tags used for protein affinity purification is the polyhistidine tag. Histidine is a basic amino acid (figure 1a), with a highly polarised side chain. Due to the chemical nature of the imidazole ring in its side chain, histidine can be charged or uncharged depending upon its external environment (Stryer 1988).

Histidine has a natural affinity for cations and binds Nickel with a high affinity. For efficient affinity purification, a number of consecutive histidine residues and a nickel

containing resin, such as nickel - nitrilo - tri- acetic acid (Ni-NTA) from Qiagen, are required (Hoculi *et al* 1988). The number of histidines in the tag vary from 6 histidine residues, the most commonly used number, to 10 or 11 residues. The interaction between the histidine residues and the Ni-NTA resin is pH dependent, but tends to be stable between pH 6.0 - 8.0 (Takacs *et al* 1991).




a. Histidine b. Imidazole

**Figure 1 : Structures of histidine and imidazole**

Elution of the proteins from the column can be achieved by one of three methods; by the reduction of the buffer pH; competitive elution using imidazole (figure 1b); and the addition of ethylenediaminotetra-acetic acid (EDTA; Paborsky *et al* 1996). A reduction of the pH to pH 4.0 is sufficient for tagged protein elution. Due to the stability of the histidine - nickel interaction at neutral pH, non-specifically bound proteins can be removed by washing with high salt concentrations and detergents with no affect to binding. To elute tagged proteins by competition with imidazole, concentrations of 0.2 - 0.5 M are required. By washing the column with lower concentrations of imidazole (e.g. 25-50 mM), weak and non-specifically bound proteins can be removed. The addition of EDTA to the elution buffer acts to chelate the nickel cations, making them inaccessible for histidine binding. Histidine tagged protein eluted from nickel chelate columns using any of these elution methods may be >90% pure (Takacs *et al* 1991).

One advantage of the histidine tag over the use of other affinity tags is its stability under denaturing conditions. This ensures that this system can be used to affinity purify

insoluble proteins to a similar purity as soluble proteins. This also means that denaturing compounds such as guanidine hydrochloride can be used to ensure the removal of all non-specifically bound proteins from the resin (Paborsky *et al* 1996).

The histidine affinity tag has been adapted for use in a number of expression vectors including *E. coli* (Takacs *et al* 1991, Waine *et al* 1994, Rasmussen *et al* 1996), yeast (Zhao *et al* 1997), *Dictyostelium discoideum* (Manstein *et al* 1995), insect cells (Kuusinen *et al* 1995) and mammalian cell lines (Caldecott *et al* 1994), for the purification of a range of gene products, including eukaryotic (Takacs *et al* 1991, Dang *et al* 1996, Kuusinen *et al* 1995, Zhao *et al* 1997), plant (Rasmussen *et al* 1996), and bacterial (Sriwanthana *et al* 1996, Abramochkin & Shrader 1995) proteins.

Initially, the major disadvantage to using the histidine tag was its unsuitability for detection assays. Now, however, there are two ways of detecting histidine tagged recombinant proteins. A method devised by Piesecki & Hochuli (1994), and modified by Botting & Randall (1995), conjugated the enzyme alkaline phosphatase to the Ni-NTA complex. In this system, when the conjugate is charged with nickel ions, it can be used for the direct detection of histidine tagged recombinant proteins, and in immunological assays to detect antibodies which have been labelled with a histidine tag (Piesecki & Hochuli 1994, Botting & Randall 1995). There is also a mAb commercially available from Qiagen which recognises the epitope Met. Arg. Gly. His. His. His. His. His. His. This mAb can be used for the detection of a histidine tag, only if the three amino acids Met, Arg and Gly directly preceed it (Qiagen).

*ii. Strep tag affinity purification*

The Strep tag is a decameric peptide (figure 2) which was selected from a genetic fusion peptide library for its ability to bind specifically and reversibly to the tetrameric biotin binding protein Streptavidin (the biotin / avidin system is discussed in more detail in section 1.1.2). It was also selected on the basis of its resistance to host cell proteases and on its hydrophilicity (Wojczyk *et al* 1996). It is the hydrophilicity of the tag which ensures

that it will be expressed as a surface epitope, thus allowing it to be used for both the purification and detection of tagged recombinant proteins (Schmidt *et al* 1996).

Ser. Ala. Trp. Arg. His. Pro. Gln. Phe. Gly. Gly

AGC GCT TGG CGT CAC CCG CAG TTC GGT GGT

**Figure 2 : the amino acid and nucleotide sequences of the Strep tag.**

Tagged proteins can be affinity purified on immobilised recombinant core streptavidin affinity columns. Core Streptavidin is an N and C terminally truncated form of the mature Streptavidin polyprotein. This truncated form of Streptavidin is used as it has been shown that the Strep tag does not efficiently bind to native Streptavidin when used in affinity chromatography (Wojczyk *et al* 1996). Elution from a core streptavidin affinity column is achieved by the addition of 50 mM diaminobiotin to the wash buffer. Native biotin or other biotin-related compounds such as iminobiotin and lysoic acid can also be used for elution (Schmidt *et al* 1996).

The Strep tag can, like the His tag, also be used for the detection of tagged recombinant proteins. The binding partner of the Strep tag, Streptavidin, can be radio- or chemically-labelled and used in immunoblots and other assays (Wojczyk *et al* 1996).

There are, however, a number of disadvantages to using this system. The most important being that the presence of any biotin in the cell extract significantly reduces the efficiency of the interaction between Streptavidin and the Strep tag (Schmidt *et al* 1996). This is because the streptavidin binds to biotin with a higher affinity than the Strep tag. To obtain efficient protein purification, all biotin must be removed from the protein extract by chelating with avidin. The Strep tag does not bind to avidin, ensuring only the biotin is removed (Skerra 1994).

Another disadvantage to using this system is that the tag is only permissible if expressed from a prokaryotic expression system. Attempts have been made to express the Strep tag from a eukaryotic expression system but the tag does not recognise Streptavidin

and so cannot be used for affinity purification. Also, the Strep tag can only be fused to the C terminus of the target protein, because for Streptavidin binding to occur, there needs to be a free C terminal glycine residue (Skerra 1994, Schmidt *et al* 1996).

*iii. FLAG affinity purification*

Another small peptide which is useful for both the affinity purification and detection of tagged recombinant proteins is the FLAG™ tag (figure 3). This is an octameric, hydrophilic peptide which was specifically designed to ensure that it is exposed for detection. This tag was also designed so that the five C terminal amino acids in its primary sequence were the consensus sequence for cleavage by the protease enterokinase, ensuring easy removal of the peptide, if required (Hopp *et al* 1988, Georgiev *et al* 1996).

The FLAG™ tag is recognised by three different mouse mAbs; M1, M2 & M5 (Sloostra *et al* 1996). M1 is the mAb which was originally developed to recognise the FLAG™ tag sequence. The binding of M1 is $Ca^{2+}$-dependent and requires a free $\alpha$ amino group. This signifies that the tag needs to be after a cleavage site or N terminal to the target protein. The chelation of the $Ca^{2+}$ ions by EDTA, destroys binding. The first three amino acids in the tag are essential for antibody binding (Sloostra *et al* 1996).

Asp. Tyr. Lys. Asp. Asp. Asp. Asp. Lys

GAC TAC AAA GAC GAT GAC GAT AAA

**Figure 3 : the amino acid and nucleotide sequences of the FLAG™ tag.**

The binding of the mAbs M2 and M5 are $Ca^{2+}$-independent and they can recognise the FLAG™ tag when fused to either end or within the target protein. Elution from immunoaffinity columns using these two mAbs is obtained by the addition of 0.1 M glycine pH 3.0 to the column. As with mAb M1, the first three amino acids in the tag are essential for antibody binding (Sloostra *et al* 1996).

The FLAG™ tag has been used for the purification of both eukaryotic and prokaryotic proteins produced from mammalian (Chu *et al* 1995), yeast (Hopp *et al* 1988), insect (Sloostra *et al* 1996) and bacterial expression systems (Hopp *et al* 1988, Mazundar *et al* 1996). Since this tag is recognised by mAbs, it can also be used to study the target protein in other applications such as immunoprecipitation, immuno-blotting and immunofluorescence.

*iv. Other examples of affinity tags*

The affinity tags described in detail in this section of the Introduction are by no means the only affinity tags which are available. Many others are the binding epitopes of mAbs to specific proteins, for example, the c-myc tag, the influenza haemagglutinin tag, and the Pk tag.

The Pk tag is a peptide of 14 amino acids which was developed by our laboratory (figure 4; Southern *et al* 1991, Randall *et al* 1993, 1994), and is the sequence of a synthetic peptide which was used to compete with the binding of the mAb SV5-P-k to the Paramyxovirus Simian Virus 5 (SV5) Phospho (P) protein. The mAb SV5-P-k binds to the Pk tag with a very high affinity, which makes this tag useful for immunoblotting, immunofluorescence and immunoprecipitation (Randall *et al* 1987, Southern *et al* 1991). However as denaturing conditions are required for the separation of the mAb from the tag, it is generally not used for affinity purification.

```
        pro  gly  lys  pro  ile  pro  asn  pro  pro  leu  leu  gly  leu
A ATT CCA GGA AAG CCG ATC CCA AAC CCT TTG CTG GGA TTG GAC
      GGT CCT TTC GGC TAG GGT TTG GGA AAC GAC CCT AAC CTG

        ser  thr  OPA
       TCC ACC TGA
       AGG TGG ACT TTAA
```

**Figure 4 : A DNA linker encoding the Pk tag sequence.**
The oligonucelotides encoding the positive and negative strand DNA sequences of the linker coding for the Pk tag were designed to give after annealing *Eco*RI compatible ends.

The c-myc tag is a peptide of 12 amino acids (figure 5). This tag is an epitope recognised by a mAb which was initially raised against the full length c-myc protein. Elution of tagged proteins is obtained by the reduction of pH or by an increase of the buffer salt concentration.

Met. Glu. Gln. Lys. Leu. Ile. Ser. Glu. Glu. Pro. Leu. Lys.

**Figure 5 : Amino acid sequence of the c- myc affinity peptide tag**

The influenza haemagglutinin affinity tag was also the epitope of a mAb originally raised against the haemagglutinin protein of an Influenza Virus. It is a peptide of 9 amino acids (figure 6). The tag can be used for immunoaffinity purification with the elution of tagged proteins being controlled by an increase of the salt concentration and/or a reduction in the pH of the wash buffer.

Tyr. Pro. Tyr. Asp. Val. Pro. Asp. Tyr. Ala.

**Figure 6 : The amino acid sequence of the influenza haemagglutinin affinity tag.**

Another interesting affinity tag is the Bio tag (Wang *et al* 1996). This works in a different way to the other tags as it contains the consensus sequence for the targeting of protein biotinylation. Once the tagged protein has been translated, it is targeted for the addition of a biotin molecule, which then allows the purification of the tagged protein on a streptavidin affinity matrix. The avidin / biotin affinity system is discussed in more detail in section 1.1.2 (i) of the Introduction.

*v. Glutathione S-transferase affinity chromatography*

Glutathione S-Transferase is the most common full length protein to be used for fusion protein affinity purification. Glutathione S-Transferase is derived from *Schistosoma*

*japonicum* and expressed from many expression vectors, including pGEX-2T and pGEX-3X (Smith & Johnson 1988). Glutathione S-Transferase is a 26 kiloDalton (kDa) enzyme which binds to a glutathione affinity matrix. Glutathione S-Transferase can be fused to the N or C termini of the target protein with no adverse effect on its ability to bind to glutathione. Fusion protein elution is obtained by the addition of excess reduced glutathione (~ 5 mM) to the column (Smith & Johnson 1988).

Glutathione S transferase fusion proteins have been used for the purification of a number of eukaryotic proteins, and in most cases the GST increases the solubility of the target protein [e.g. *Plasmodium falciparium* antigens (Smith & Johnson 1988), the core antigens of the human and simian immunodeficiency viruses (Mills *et al* 1992), human papillomavirus type 6 E7 protein (Romanos *et al* 1995)]. The soluble fusion proteins are purified under non-denaturing conditions (Smith & Johnson 1988). In addition, GST can be expressed in many systems including bacteria (Mills *et al* 1992. Ghosh *et al* 1995), yeast (Ward *et al* 1994, Romanos *et al* 1995) and insect cells (Boublik *et al* 1995), and has been shown to have no effect on the post-translational modifications (e.g. glycosylation or phosphorylation) which can be added to eukaryotic target proteins (Ward *et al* 1992. Romanos *et al* 1995). This ensures that the post-translational modifications of a protein can be studied from GST purified proteins.

As the GST fusion system is a popular expression system, a number of commercially available expression plasmids have been adapted for use in bacteria, yeast, and insect cells. As with most fusion proteins, *E. coli* is the most commonly used system for GST fusion protein expression, with yields of up to 15 μg of recombinant protein per ml of bacterial culture. The two expression vectors pGEX-2T and pGEX-3X have been specifically designed for the production of large quantities of GST fusion proteins in *E. coli*. These two plasmids are identical except that pGEX-2T contains genetic sequence for a thrombin cleavage site and pGEX-3X contains the genetic sequence for a blood coagulation factor Xa protease cleavage site (Smith & Johnson 1988). Both of these cleavage sites are situated between the GST and the target protein. The two cleavage sites allow the separation of the GST from the target protein, should untagged target protein be required.

To obtain highly purified untagged target protein, after the initial affinity purification step, the fusion protein undergoes cleavage by the relevant protease. The digestion mixture is then applied to the affinity column again, but this time only the GST or uncleaved fusion proteins are retained, and the target protein passes through for collection. A schematic of this protocol can be seen in figure 7.

With GST being a complete protein, monoclonal and polyclonal antibodies have been raised to it. This allows the tag to be used for a number of other applications including immunoassays (e.g. immunoprecipitation, immunoblotting and immunoaffinity purification) and detection. Glutathione S-Transferase tags are also being used for the study of a target proteins function, the purification of interacting factors to the target proteins and to generate antibodies against the fusion proteins (Bar-Pelad & Raikhel 1996, Hengen 1996).

*vi. Maltose binding protein affinity chromatography*

The maltose binding protein (MBP) is encoded by the gene *malE* in *E. coli*. Tagged proteins are affinity purified on a crosslinked amylose agarose column and fusion protein elution is obtained by the addition of maltose (5-1 mM) to the column. Affinity purification must be carried out under non-denaturing conditions (Maina *et al* 1988, Yang *et al* 1994).

To obtain the purified protein without the fused MBP, the consensus sequence for the blood coagulation factor $X_a$ protease (ile. Glu. Gly. Arg) can be cloned into the expression plasmid between the genes of MBP and the target protein. After cleavage, the affinity purification step is repeated, but this time only the MBP and uncleaved fusion protein is retained. The target protein flows through the column (Maina *et al* 1988). This is identical to the method described for GST (figure 7).

*vii. Protein A / Protein G affinity chromatography*

One of the first proteins to be used for the purification of target proteins was the bacterial immunoglobulin G (IgG) binding protein, Protein A from *Staphylococcus aureus*. This protein binds to the Fc fragment of IgG molecules from many species of animals, and

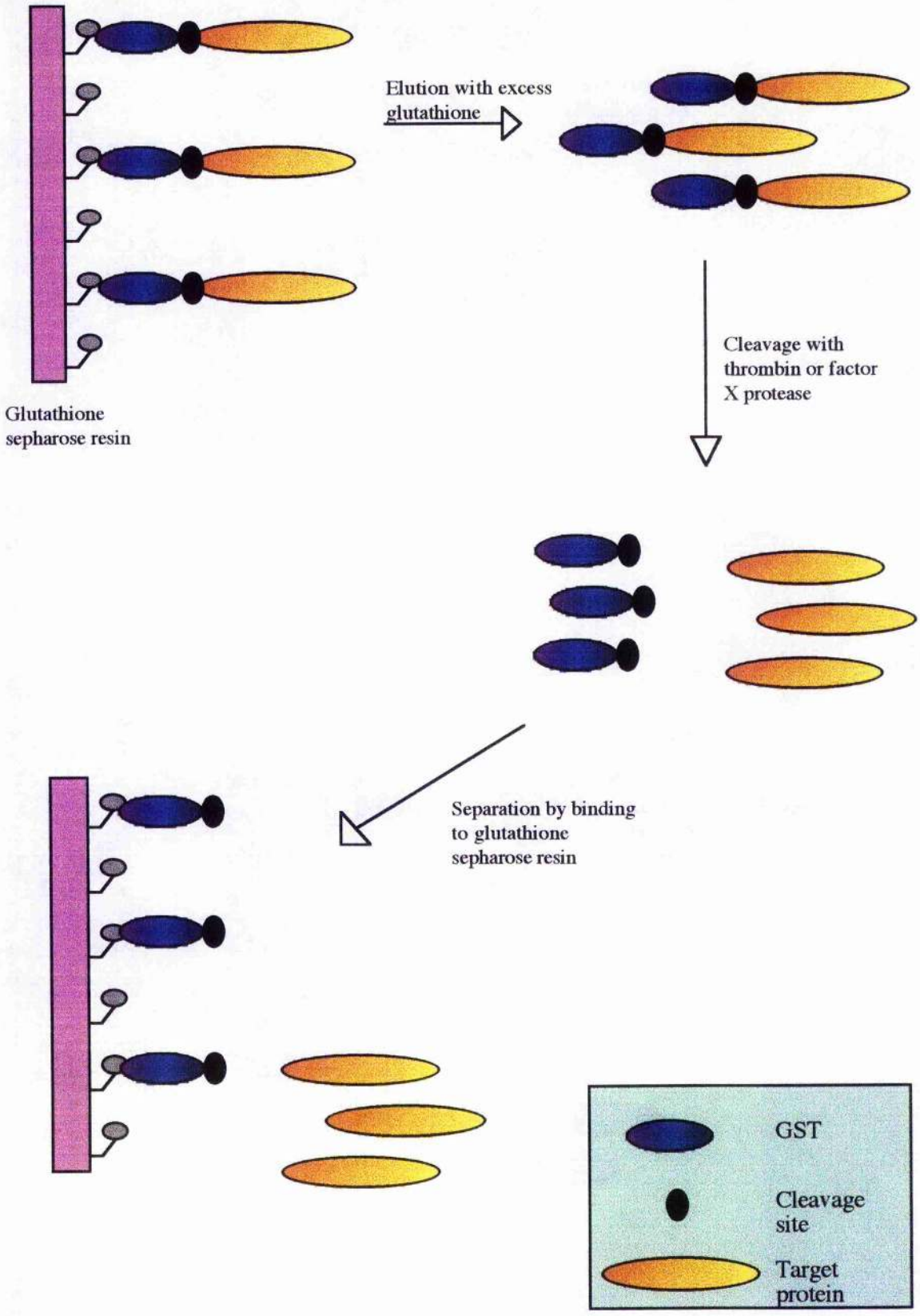



**Figure 7 : Schematic diagram of the two step protocol for the purification of GST fusion proteins**
Adapted from Maina *et al* 1988

has a differing affinity for the different sub-types of IgG (see section 2.2.1; Harlow & Lane 1988).

There are a number of advantages to using Protein A as a fusion protein; it is proteolytically stable and hydrophilic in nature making it ideal for external display; it is highly soluble and appears to increase the solubility of most of the proteins to which it is fused; and finally it has the targeting signals required for secretion into the bacterial periplasmic space.

Protein A has been expressed and purified from many expression systems including *E. coli*, *Saccharomyces cerevisiae*, plants, insect cells and mammalian cells (Stahl & Nygren 1997). Tagged recombinant proteins are purified on an immobilised IgG affinity column and elution is achieved using a low pH buffer (e.g. pH3). This can be harmful to the target protein, and is the main disadvantage of using Protein A affinity tags. However, from this purification system, 90% purity of proteins has been obtained (Stahl & Nygren 1997).

Streptococcal Protein G is similar to Protein A but it can bind to serum albumin in addition to IgG. One characteristic of Protein G is that it binds strongly to most of the IgG subtypes which Protein A binds weakly to (see section 2.2.1). This protein is also proteolytically stable, highly soluble (it has a structure similar to Protein A) and can be produced in high yields from a bacterial expression system. Protein G is less well studied than Protein A, and so far has only been expressed in *E. coli* and mammalian cell lines. Tagged recombinant proteins are purified using either an IgG affinity column or an immobilised human serum albumin affinity chromatography. Like Protein A, elution is also achieved by lowering the pH of the column (Stahl & Nygren 1997).

Both proteins, and their affinity matrices, are resistant to 0.5 M guanidine hydrochloride, which means they can be used for the purification of insoluble proteins, as well as soluble proteins (Goding 1986, Harlow & Lane 1988, Stahl & Nygren 1997).

Owing to the ability of Proteins A & G to bind to immunoglobulins, they can also be used for the purification of monoclonal and polyclonal antibodies (Harlow & Lane

1988). This characteristic also means that these tags can be used for immunodetection as well as purification purposes.

## 1.2 Affinity chromatography of a protein using its natural ligand

Although the use of fusion proteins is the most popular way of affinity purifying proteins, it is not always practical. The production of fusion proteins depends on the use of recombinant DNA technology and the expression of recombinant proteins from a controlled expression system. In many cases this is not the most efficient method of obtaining the target protein. For example if the protein is a membrane protein, the gene of the protein may not have been cloned, or the levels of protein obtained from an expression system may be very low (e.g. HIV gp40 in an *E. coli* expression system; Scopes 1996). If these problems arise, it may be more efficient to obtain the target protein from its natural source, and to purify it in its native form. If this is the case then in depth knowledge of the properties of the protein becomes very important.

In many situations, the target protein interacts with other proteins or biological molecules. The most common biological interactions are antibody - antigen complexes, enzyme - substrate interactions and agonist - receptor complexes, but these are not the only interactions which occur naturally. Described in more detail in this section are the most common ways that natural protein interactions have been exploited for the affinity purification of target proteins.

*i. Biotin / Avidin*

The binding of biotin to the egg white protein avidin, is one of the strongest non-covalent interactions found in nature. It has been shown that if the biotin molecule is coupled covalently to other macromolecules, it's binding to avidin is unaffected. This makes the interaction between these two molecules useful for a number of biological assays (Wilchek & Bayer 1990).

Biotin is a small, water soluble vitamin (figure 8) required in small quantities for a number of cellular processes. Biotin is also the prosthetic group, essential for enzyme function, for the carboxylase and transcarboxylase enzyme families, by acting as a covalently bound carbon dioxide ($CO_2$) carrier (Dakshinamurti & Chauhan 1990, Wilchek & Bayer 1990). In bacteria such as *E. coli*, biotin is not essential for the function of these enzymes. A deficiency in biotin in the diet can cause a number of diseases.

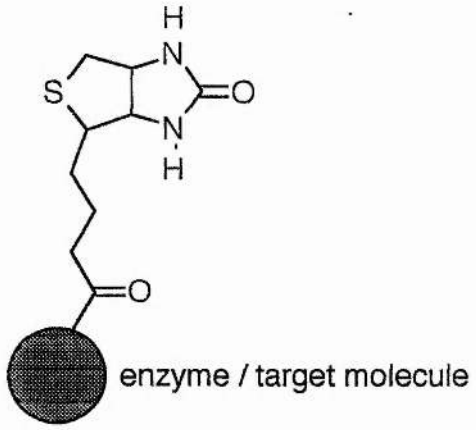



**Figure 8 : the structure of biotin.**

Avidin was shown in hen egg whites to have a bacteriostatic effect, protecting the embryo from bacterial infection. It is a highly specialised glycoprotein which is rarely expressed. It is found as a tetramer, with each subunit containing a single biotin binding site. Each subunit binds to biotin through four tryptophan residues and a lysine residue (Green 1990).

The strong binding of biotin to avidin, and the ability of avidin to continue to recognise the biotin even when coupled to a larger protein, has made it an invaluable tool in molecular biology. By labelling one or both of the molecules with substances such as gold, horseradish peroxidase, alkaline phosphatase, radioactive or fluorescent labels, this interaction has been exploited for a number of assays including cytochemical localisation studies, protein blotting, immunoassays and gene probing as well as affinity chromatography (Wilchek & Bayer 1990a).

Affinity chromatography using the biotin / avidin system was originally restricted to the enzymes which used biotin as a prosthetic group or coenzyme (e.g. Acetyl - CoA carboxylase, sodium transport enzyme), the enzymes involved in the metabolism of biotin (e.g. biotinilase, biotin holocarboxylase synthetase), proteins which interacted with biotin (e.g. avidin, egg-yolk biotin binding protein 1 & 2, nuclear biotin binding protein), or proteins which interacted with avidin (Bayer & Wilchek 1990).

The main disadvantage to using the biotin / avidin system for the affinity purification of proteins is the high affinity that avidin has for biotin. This makes protein elution from an affinity column extremely difficult. Conditions such as 1 M guanidine hydrochloride pH2.0, Sodium dodecyl sulphate (SDS) or acetate pH4.0, sometimes with the addition of heating, are usually required for elution. However, there are cases, such as the purification of acetyl CoA carboxylase, where the addition of excess biotin is sufficient for efficient protein elution (Wilchek & Bayer 1990, 1990a).

These highly denaturing elution conditions can be overcome by using Streptavidin, another biotin binding protein. Streptavidin is an avidin homologue derived from the bacteria *Streptomyces avidinii* (Green 1990). This avidin homologue has not been found in any other bacterial species. Streptavidin has 33% homology with avidin and like avidin it is found as a tetramer, with each subunit having a single biotin binding site. Also like avidin, streptavidin binds to biotin through four tryptophan residues. One major difference between these two protein is that streptavidin is not glycosylated. This, combined with its slightly lower affinity for biotin, makes it ideal for use in biotin protein affinity chromatography (Bayer *et al* 1990, Green 1990).

Biotin affinity chromatography can be used for the direct and indirect purification of proteins. Both purification methods involve the use of a streptavidin - Sepharose affinity column. For the direct purification of proteins (figure 9a), the target protein is either a biotin derivative (e.g. 2-iminobiotin) or biotinylated. The biotin molecule is directly coupled to the protein of interest. The biotin moiety then interacts with the affinity column. Elution of the biotinylated protein is by competition with free biotin molecules (Krachenbuhl & Bonnard 1990).

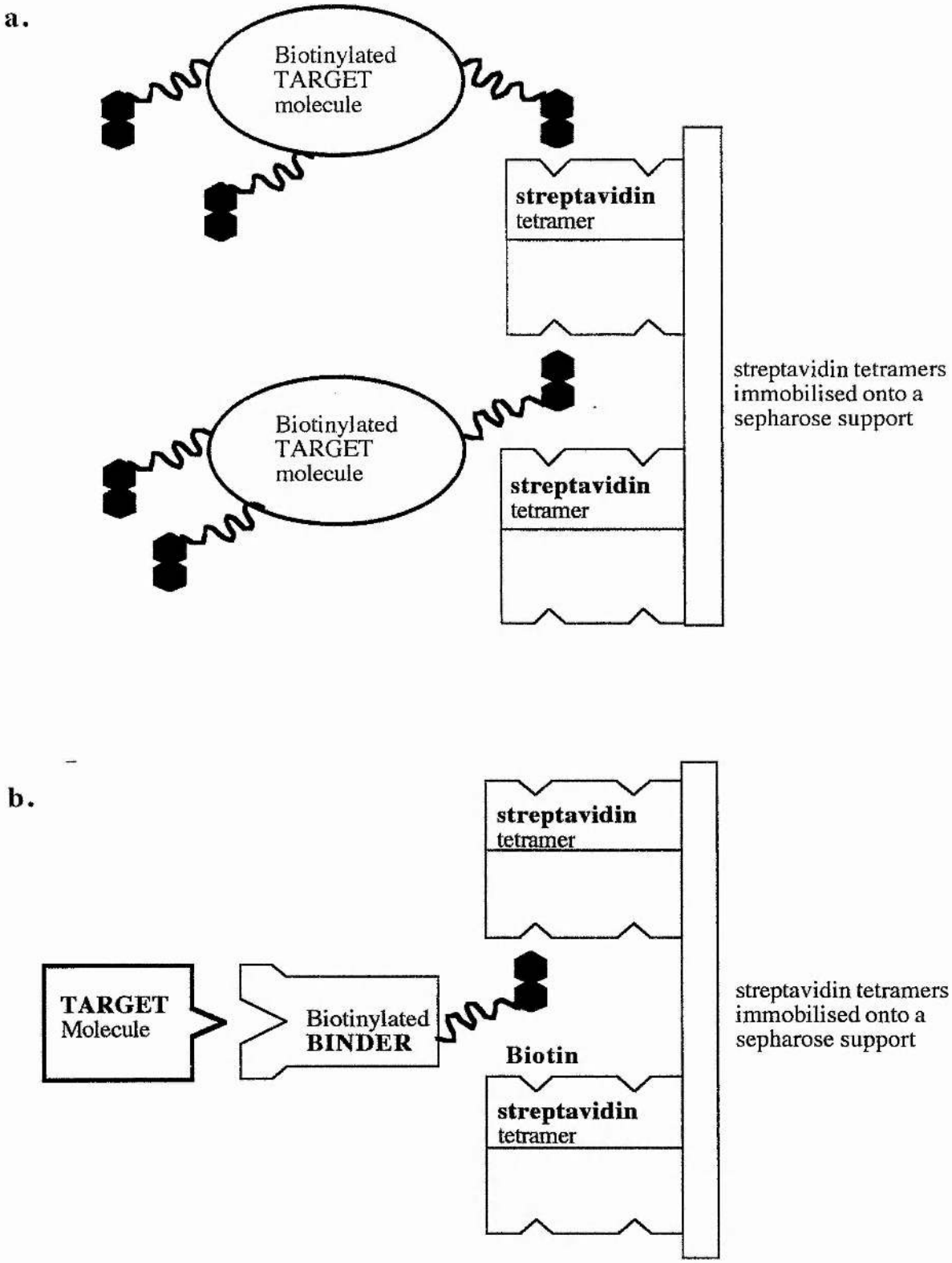



**Figure 9 : a schematic diagram of the biotin - streptavidin affinity purification systems.**
a. a directly biotinylated target protein is captured by the immobilised streptavidin. b. indirect purification using the biotin-streptavidin system. A natural binding partner of the target protein is biotinylated and captured by the immobilised streptavidin. The target protein is then captured by its binding partner.

Indirect affinity purification (figure 9b) is achieved using a biotinylated intermediary which recognises and binds the protein of interest. This could be any substance which is known to bind to the protein of interest, e.g. lectins for glycoproteins, antibodies for antigens, receptors for specific ligands. In this case, elution of the protein from the affinity column is dependent upon the type of interaction shown between the biotinylated molecule and the protein of interest (Wilchek & Bayer 1990a).

*ii. Lectins*

The most commonly used method for the affinity purification of glycoproteins is lectin affinity chromatography. Lectins are a group of proteins which recognise a specific sequence of sugar residues. They were originally derived from plant seeds (e.g. Concanavalin A, wheat germ agglutinase) and slime moulds (*Dictyostelium discoideum* discoidin-1) but animal lectins have now been discovered. Lectins were originally discovered due to their ability to agglutinate the different human erythrocyte types. They are now thought to have many different functions, including the initiation of the proliferation of lymphocytes, to facilitate cell-to-cell interactions, and the polarisation and capping of phagocytes and other motile cells (Sutton 1993).

Lectins are composed of one or more subunits in the form of multimers. The number of subunits, and therefore the size of the molecule, differ for each lectin. A summary of the number of subunits found in some of the more commonly used lectins can be seen in table 1. A lectin can be composed of either homologous or heterologous subunits. If the lectin contains homologous subunits, it may have multiple binding sites for a single saccharide. However, if the subunits within the lectin are heterologous, the lectin may be able to bind two or three different saccharides (Sutton 1993).

The affinity of lectins for saccharides is weaker than antibody - antigen complexes or agonist - receptor complexes (Persson & Jergil 1995), but of sufficient strength for the affinity purification of glycoproteins, proteoglycans and glycolipids. For the purpose of this review, only glycoproteins will be discussed.

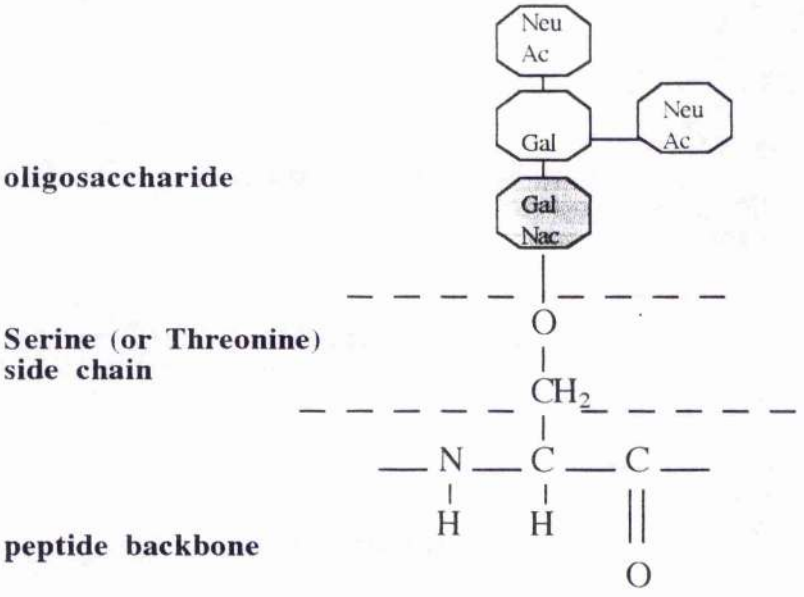
| Lectin (abbreviation) | molecular weight | number of subunits | simple sugar specificity | metal ions required |
|---|---|---|---|---|
| Concanavalin A (ConA) (Jack bean) | 55 000 | 2<br>4 | α-D-man > α-D-glu > α-D-GlcNAc | $Ca^{2+}$, $Mn^{2+}$ |
| Lentil Lectin (LCA) | 49 000 | 2 | α-D-man > α-D-glu > α-D-GlcNAc | |
| Soybean Lectin (SBA) | 110 -120 000 | 4 | α-D-GalNAc > β-D-GalNAc > α-D-Gal | $Ca^{2+}$, $Mn^{2+}$ |
| Wheatgerm agglutinin (WGA) | 36 000 | 2 | $(\beta\text{-D-GlcNAc})_3$> $(\beta\text{-D-GlcNAc})_2$ | $Ca^{2+}$, $Mn^{2+}$, $Zn^{2+}$ |
| Peanut agglutinin (PNA) | 120 | 4 (αβ) | β-D-Gal-(1-3)-D-GalNAc > D-$GalNH_2$ | |

**Table 1 : Biochemical characteristics of some of the more common lectins used for affinity purification.** This table also shows the sugar specificity for each lectin, from the simple structures recognised by LCA to the more complex oligosaccharides recognised by PNA. The table also highlights the different divalent cations necessary for the binding of some lectins to the specific saccharides. ConA has a dimer structure under conditions below pH5.5, as a tetramer between pH5.6 and 7.0 and as aggregates above pH7.0 (Adapted from Sutton 1993).

The choice of a lectin for the affinity purification of glycoproteins is dependent upon the oligosaccharide-protein linkage. The sugar - moiety is usually bound to the protein through one of two linkages; a O-glycosylation linkage and a N-glycosylation linkage.

The O-glycosylation linkage is usually an N-acetylgalactosamine bound to the side chains of either serine or threonine residues (figure 10a; Stryer 1988). This form of linkage is usually found in mucins and of the common lectins, peanut agglutinin (PNA) and

**a.**




**b.**

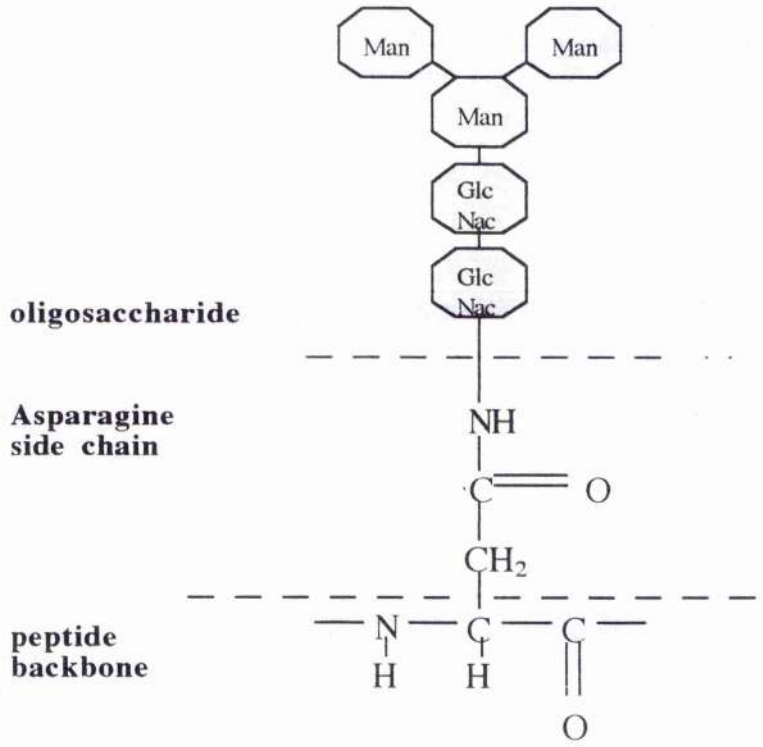



**Figure 10 : a schematic representation of the two different glycosidic linkages.**
**a.** an O linked glycoprotein; **b.** an N linked glycoprotein, the asparagine is usually only glycosylated if part of the sequence Asp-X-Ser of Asp-X-Thr.

wheatgerm agglutinin (WGA) preferentially bind this form of glycoprotein. The oligosaccharide tends to be no longer than four sugar units long.

The N-glycosylation linkage is usually between N-acetylglucosamine and the side chain of the amino acid asparagine (figure 10b; Stryer 1988). The oligosaccharides bound to proteins using this linkage tend to be longer, usually twelve sugar units, and more complex with a high mannose content. Of the commonly used lectins, Concanavalin A (ConA) and lentil lectin (LCA) bind this form of glycoprotein. The specific affinities each lectin has for the specific sugar moiety allows them to be exploited for the purification of different groups of glycoproteins.

A number of physiological conditions are important for lectin binding to saccharides. For example, the binding is pH dependent and needs to kept at physiological levels (pH 6.8 - 7.5). Also in many cases a specific divalent cation is needed for lectin binding, usually $Mn^{2+}$ or $Ca^{2+}$ (table 1). As binding is not an ionic interaction, high salt concentrations do not interfere with the lectin-oligosaccharide interaction. It is beneficial, therefore, to use a wash buffer of high salt concentration as this reduces non-specific interactions which can occur (Sutton 1993).

The main advantage of using lectins for the affinity purification of glycoproteins is the relatively mild conditions which can be used for protein elution. One way of eluting the glycoprotein from the affinity column is by chelating the divalent cation necessary for lectin binding. Another is by changing the buffer pH to either mildly acidic or mildly alkaline conditions. The most commonly used method for glycoprotein elution is competition with free monosaccharides. For example, when using a ConA affinity column, the addition of 0.5 M α-D-methyl mannoside to the equilibration buffer is sufficient for glycoprotein elution (Sutton 1993).

## 1.3 Affinity chromatography using organic compounds

One of the most recent methods devised for the purification of target proteins is the use of reactive textile dyes. It was originally observed that the triazine dye Cibacron Blue

F3G-A, from blue dextran, bound to the liver enzyme alcohol dehydrogenase (Lowe *et al* 1992, Cusack & Beynen 1993). This interaction was thought to be due to the structural similarities exhibited between the reactive dye and the enzyme interactant $NAD^+$. Since that time a number of other proteins have been purified using Cibacron Blue F3G-A, including the ricin A-chain, albumin, numerous coenzyme-dependent enzymes, decarboxylases, nucleases, polymerases and hydrolases (Lowe *et al* 1992). From this original discovery, a variety of triazine dyes have been purposely constructed to produce high yields of highly purified protein (Garg *et al* 1996). A summary of the most commonly used dyes and the proteins which they have been used to purify can be seen in table 2.

| Protein | Dye | Average Yield (%) | Eluent |
|---|---|---|---|
| Alcohol dehydrogenase | Procion Red HE3B | 44 | eluted in flow through |
| | Procion Blue MX4GD | 94 | $NAD^+$ |
| Ricin A chain | Procion Red H3B | all 95 | pure NaCl |
| | Procion Red HE3B | | |
| | Procion Red HE7B | | |
| | Procion Yellow HE4R | | |
| inositol 1,3,4,5,6-phosphokinase | Cibacron Blue 3GA | 93 | $NAD^+$ |
| Phosphatidic acid phosphohydrolase A | Cibacron Blue F3GA | 75 | NaCl |
| Phosphatidic acid phosphohydrolase B | Cibacron Blue F3GA | 3 | NaCl |
| luciferase | Orange A | 100 | eluted in flow through |
| glucose-6-phosphate dehydrogenase | Blue A | 71 | KCl |
| | Red A | 57 | KCl |
| | Red A | 47 | KCl |
| placental protein 12 | Cibacron Blue | 92 | eluted in flow through |

**Table 2 : A selection of proteins purified using dye affinity chromatography**
Table 2 also shows the dyes used for the purification of each protein and the average protein yields obtained from the purification process. (Adapted from Garg *et al* 1996)

The choice of a lectin for the affinity purification of glycoproteins is dependent upon the oligosaccharide-protein linkage. The sugar - moiety is usually bound to the

protein through one of two linkages; a O-glycosylation linkage and a N-glycosylation linkage.

The reactive dyes usually consist of a chromophore, which give the dye its distinctive colouration, and a reactive group capable of effecting a nucleophilic substitution, to allow binding to the protein. The majority of the dyes are anionic although there are cationic dyes available, such as the 6-aminohexyl analogue which has been used to purify pancreatic kallikrein (Garg *et al* 1996).

The separation of the proteins from the dyes is achieved in three ways; by competition with polymers which form stable complexes with the dyes; using very high or very low pH buffers; and by the addition of chaotropic ions to the buffer (Lowe *et al* 1992. Garg *et al* 1996).

There are a number of advantages to using dye affinity chromatography including, the moderate to high selectivity for enzymes, low cost, high reactivity of the dyes making them easier to couple to matrices, and their resistance to chemical and biological degradation. However, there are two main disadvantages for using dye affinity chromatography the cross-reactivity of the dyes to other proteins present in the crude extract; and they are generally unsuitable for immobilisation on a resin for column chromatography. Triazine dyes are more suited to other forms of partition, such as two phase aqueous partition (Garg *et al* 1996).

## 2. MONOCLONAL ANTIBODIES

One of the most widely used tools in molecular biology today are antigen - specific monoclonal antibodies (mAbs). Monoclonal antibodies have been used in many ways to expand the understanding of a number of biological processes. Their usefulness in many techniques is because of three properties; their specificity of binding; their homogeneity; and their ability to be produced in large quantities. The production of unlimited quantities of mAbs is a definite advantage over the use of anti-sera, as is the fact that mAbs can be raised

against an impure antigen. To produce antigen specific anti-sera, a highly purified antigen must be used (Goding 1986, Harlow & Lane 1988).

Any antigen which elicits a humoral immune response can be used for the production of mAbs. This means that not only can mAbs be raised against proteins, but also against carbohydrates, lipids and nucleic acids (Harlow & Lane 1988). The type and purity of the antigen defines the specificity of the mAb and the affinity of the mAb for its antigen. The selection of high or low affinity mAbs can usually be controlled during the screening process. The use of mAbs also reduces the potential of obtaining cross-reactivity between unrelated proteins, although cross-reactivity will occur if a large enough population of antigens are screened. It is very rare that an unexpected cross-reaction occurs (Goding 1986).

This section of the Introduction details the main protocols for the development of antigen - specific mAbs, and also gives details of the most common methods in which mAbs are purified.

## 2.1 Methods of Monoclonal Antibodies Development

Monoclonal antibodies can be developed in a number of ways. The traditional method involves the isolation of mAb secreting hybridoma clones. The isolation, care and screening of the hybridoma clones is very time consuming. An alternative method involves the cloning of the immunoglobulin (Ig) variable (V) genes, and the displaying of the gene products on the surface of bacteriophage. Again the screening and expansion of the antigen specific mAbs is time consuming. Consequently, it is essential that the requirement of mAbs is weighed against the effort made in developing them. The traditional and alternative methods of developing antigen - specific mAbs are discussed in more detail in this section.

### 2.1.1 Traditional Method

This protocol was developed by Kohler and Milstein in 1975. They also determined that this method could be adapted for the production of mAbs which were specific for any antigen. Kohler and Milstein fused the antibody producing cells of mice to MOPC-21 myeloma cells, and produced a successful hybrid cell line which produced antigen specific mAbs. The most common myeloma cell type used today is the SP2/0 cell type. The SP2/0 myeloma cell type was chosen for its inability to salvage purine nucleotides through the salvage pathway, due to it's lack of the purine salvage enzyme hypoxanthine guanosine phosphoribosyl transferase (HGPRT). The salvage of nucleotides is one method that a cell has of producing nucleic acids, the other is by *de novo* synthesis. By exploiting this salvage pathway deficiency, a highly effective way of selecting for hybrid cells is developed. After cell fusion, if the *de novo* synthesis pathway is blocked by drugs, such as aminopterin or azaserine, then the cell is forced to use the salvage pathway. However, in HGPRT deficient cells, this pathway is also blocked, so the cells die. The fused hybrids, however, have the intact HGPRT gene obtained from the fused antibody secreting cells, so they can grow in the media containing the *de novo* synthesis pathway blocking drug. The unfused mAb secreting cells are mortal and die in tissue culture after a few days. A summary of this selection can be seen in figure 11.

The cell fusion process was originally mediated by Sendai virus. Now, however, it is easier to use polyethylene glycol (PEG) mediated cell fusion. Polyethylene glycol is commercially available and inexpensive to use. It is usually used in a final concentration of 40 - 50% as below 30% little cell fusion occurs, and above 50% the PEG become toxic to the cells (Goding 1986, Harlow & Lane 1988). This method of fusion is still not very efficient, with only about 1% of the starting cells becoming fused, with only about 1 in $10^5$ of those forming viable hybridomas (Harlow and Lane 1988). This means that a lot of hybridoma colonies need to be screened in order to guarantee obtaining the required amount of mAb - secreting hybridoma cells. Hybridoma screening is the most labour intensive part of the whole procedure.

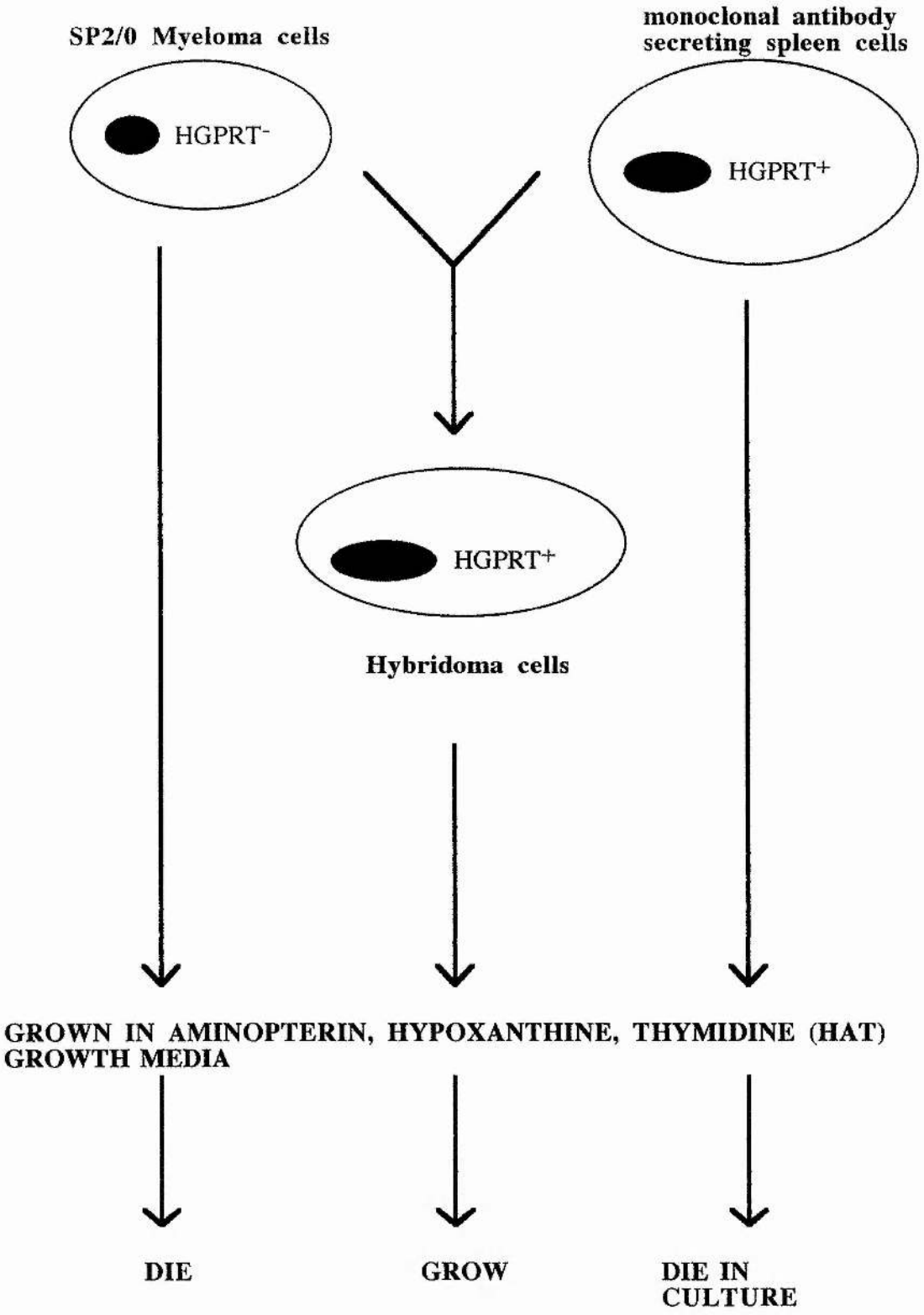



**Figure 11: Schematic diagram of the procedure used for the selection of hybridoma cells**
The myeloma cells are HGPRT deficient and can not grow in the HAT media. The spleen cells contain an intact HGPRT gene but are mortal and so die after a few days. The hybridoma cells have the HGPRT gene from the spleen cells and their immortality from the myeloma cells, so they survive in the HAT media.

The hybridoma clones are usually plated into 96 well microtitre plates. Due to the low plating efficiency of the hybridoma clones, this process is usually done in the presence of feeder cells such as macrophages. Although the cells attach to the surface of the wells. they are not adhesive. The advantage of using hybridoma clones as a way of making mAbs is that they secrete the antigen specific mAbs into the tissue culture media in which they are grown. This allows easy screening for positive clones. The most popular way of screening for positive hybridoma colonies is by antibody capture, usually ELISA. The antigen with which the animals were immunised is immobilised on the surface of a microtitre plate. The growth media, containing the mAbs, is incubated with the antigen and the mAbs allowed to bind. A labelled anti-Ig mAb is then used to probe for antibody-antigen complexes.

Once the positive mAbs have been detected, the hybridoma clones need to be subcloned. This is to ensure that the antigen specific mAbs are homologous and were secreted from a colony which originated from a single cell. The clones are subcloned by the limiting dilution of the hybridoma cell suspension. As with the original round of screening, the subcloned colonies need to be screened to eliminate any cells which do not secrete antigen specific mAbs. Limiting dilution is not the only method available for subcloning the hybridoma clones, but it is the most commonly used as it is quick, convenient and allows the direct testing of the tissue culture supernatants. Other methods, such as cloning by growth into soft agar, are time consuming and require additional practical steps.

There are two ways of expanding the growth of the hybridoma clones. One is by expansion in tissue culture and the other is by injection of the hybridoma clones into the peritoneal cavity of same strain mice. Hybridomas grown in tissue culture media produce up to 10 $ugml^{-1}$ of specific mAbs, whereas hybridomas grown in the ascitic fluid produce about 5 - 20 $mgml^{-1}$. Although the ascitic fluid method of expansion produces vast quantities of mAbs, the mAb containing solutions will also contain other proteins and small amounts of host immunoglobulins and so is not suitable for some practical applications.

The protocol described is highly efficient for the production of antigen specific mAbs. However, it is time consuming and requires the sacrifice of at least two animals for each fusion. The mAbs obtained from this system are highly specific, with binding

affinities which vary from relatively low affinity (e.g. $10^6$M) to exceedingly high (e.g. $10^{12}$M). The desired affinity of a mAb to its antigen can be selected during the screening process. This method of isolating antigen specific mAbs is currently the most popular method used. A summary of the stages involved in the whole process can be seen in figure 12.

### 2.1.2 Phage Display

Phage display utilises recombinant gene technology to mimic the natural immune response. This technique involves the alteration of the pairing between heavy and light chain variable domains ($V_H$ and $V_L$ respectively) using V gene libraries.

Initially the $V_H$ and $V_L$ genes were amplified, using degenerate primers, by the Polymerase Chain Reaction (PCR) from hybridoma clones. The resulting variable pairs were manipulated and cloned into a bacterial expression vector to create more stable hybridoma clones (McCafferty *et al* 1990, Winter & Milstein 1991). The soluble antibody fragments produced from these pairings were then secreted from the bacteria and screened. This method was eventually adapted to include genes directly amplified from the B cells of immunised animals.

The screening of the bacteria however, was time consuming as each positive bacterial colony had to be individually grown, screened and characterised. To overcome this, McCafferty *et al* (1990) displayed the $V_H$ and $V_L$ antibody fragments on the surface of the Fd bacteriophage, which allowed the phage particles to be screened by antigen affinity chromatography. Rare phage particles (one in $10^6$) can be isolated in this way. If multiple rounds of affinity screening are performed then mAbs of differing affinities can be produced. From the first round, a number of antigen binding activities can be detected, but few bind with high affinity. After the second round of affinity screening, the binding affinities are enriched with strong binders, at the expense of the weak binders.

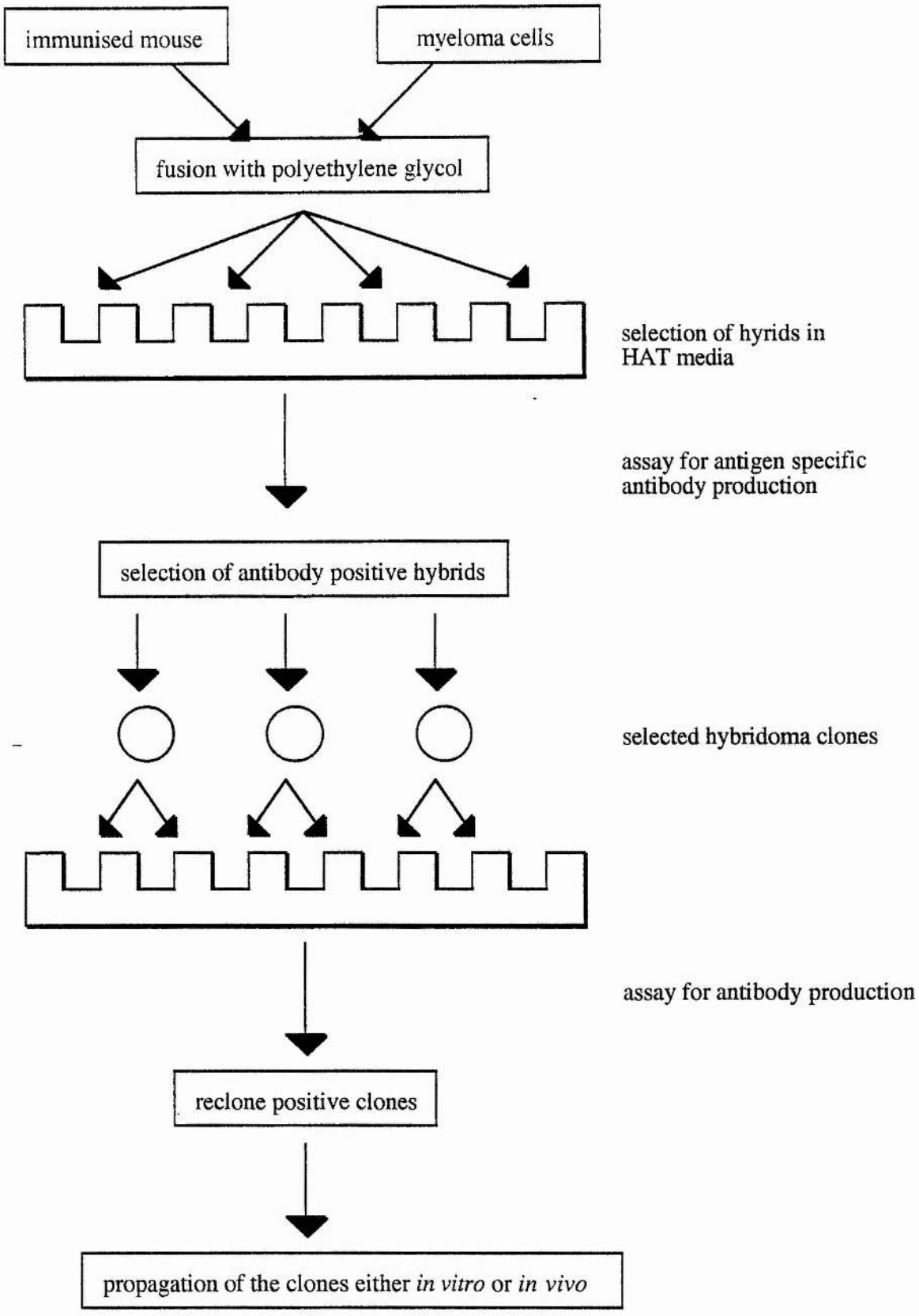



**Figure 12 : The experimental protocol of the production of antibody producing hybridoma clones**

There are two ways of displaying the $V_H$ and $V_L$ antibody fragments of the surface of bacteriophage Fd, and both involve the fusion of the antibody fragments to the N terminus of one of the coat proteins. McCafferty *et al* fused the $V_H$ and $V_L$ fragments to the gene III protein product. There are approximately four copies of this protein found within the virus particle. However. for cell attachment, a single native copy is required. The fusion of the $V_H$ and $V_L$ fragments to this coat protein allows the antibody fragments to be selected on the basis of affinity. Clackson *et al* (1991) and Söderlind *et al* (1992) used a different coat protein, the Gene VIII gene product. There are estimated to be approximately 2000 copies of this coat protein in a virus particle. This system is not efficient for the selection of antibody fragments on the basis of affinity but is useful for the selection of multiple fragments of low affinity which when combined have a high affinity for a specific antigen (multivalency). Antibodies displayed on the surface of the phage particles provide a faster and more efficient method of selection than bacterial screening.

Phage display technology is a powerful selection system which still needs a source of rearranged and affinity matured immunoglobulin genes (Söderlind *et al* 1992), which can only be obtained from immunised or seropositive animals. If artificial combinatorial libraries are used, e.g. the expression of $V_H$ and $V_L$ gene libraries in a phagemid vector where they can be randomly combined, the size of the libraries becomes restrictive. Only $10^7$ clones are available using this method, which is a small proportion of the natural antibody repertoire available ($10^{12}$).

One way of overcoming the size of the library, and the use of immunised animals, is to use *in vitro* site specific recombination. Geoffrey *et al* (1994) developed a system of using both phagemid and plasmid technology and the site specific recombination sites found within *E. coli*. Their method involves *E. coli* being transformed with a plasmid encoding a repertoire of $V_L$ genes (about $10^6$ clones). These transformed bacteria are then infected with a phagemid encoding a repertoire of $V_H$ genes (about $10^6$ clones). For recombination to occur, both the plasmid and the phagemid need distinct replicons and *att* sites. The plasmid also needs to be transformed into a bacterial strain which is susceptible to phage infection, and which is capable of expressing the heat-inducible *Int* recombinase.

Once recombination occurs, the number of clones available are equivalent to the natural immune system. In addition, the produced recombined vector is stable and capable of infecting other phagemid susceptible bacteria for the construction of multi-combinatorial antibody libraries.

Phage display is a highly versatile process. The antibodies produced can be used in the cloning and rescue of hybridomas, and for the screening of other large combinatorial libraries (McCafferty *et al* 1990). It can be also used to change and refine the properties of other proteins or peptides which can then be selected by display on the surface of phage particles (Clackson *et al* 1991, Courtney *et al* 1995). The main advantage to this method of producing antigen specific mAbs over the traditional method is that the selection process. As selection can be performed using an affinity column, this allows a more specific mode of selection, and allows the selection of lower affinity mAbs. The main disadvantage to using phage display for the production of mAbs is when large scale production of mAbs for further study are required. The bacteriophage are not as efficient as hybridoma cells at producing large amounts of mAb.

## 2.2 Monoclonal antibody purification

Most mAbs are secreted into a growth media where other proteins are present. In order to ensure that there are no unwanted protein - protein cross-reactions when using the antibodies in various immunological techniques, the mAbs need to be purified. The method of purification depends on the species of animal in which the mAbs were raised, the class and subclass of the immunoglobulin from which the mAb is composed and the methods used to expand the growth of the hybridomas e.g. ascitic fluids or tissue culture media. The homogeneity of the mAbs makes them easier to purify than polyclonal antibodies, and as such standard purification protocols can be used (Goding 1986. Harlow & Lane 1988). This section of the introduction describes some of the most common ways of purifying mAbs.

### 2.2.1 Affinity Chromatography

The immobilisation of the antigen, to which the mAb was raised, on Sepharose / agarose beads is the simplest way of affinity purifying a mAb. Monoclonal antibodies bind to the affinity column between pH 4.0 - 7.0, and in a salt concentration of less than 1 M (Perry & Kirby 1990, Cusack & Beynon 1993). The elution of the purified mAbs from the affinity columns is dependant upon the affinity of the mAb for its antigen. Those mAbs with low affinity elute from the column by increasing or decreasing the buffer pH, or by increasing the buffer salt concentration. The high affinity mAbs are more difficult to elute from the affinity column, needing denaturing conditions such as 8 M urea or 6 M guanidine hydrochloride (Harlow & Lane 1988, Perry & Kirby 1990).

Sometimes the harsh conditions used for the elution of the mAbs from the antigen affinity column does not make this an ideal way of purifying mAbs. If the mAb comprises of immunoglobulin G (IgG), then an alternative method is available.

In section 1.1 (iv) of the Introduction, a method was described for the purification of recombinant proteins tagged with either Staphylococcal Protein A or Streptococcal Protein G. The affinity column used for the purification of the tagged proteins was an IgG affinity column. This can also work in reverse. Protein A or Protein G affinity columns can be used for the purification of mAbs containing the IgG subtypes. One problem with this is the different binding affinities that the IgG subtypes have for the two bacterial proteins (Harlow & Lane 1988). A summary of the variation in binding to Protein A / Protein G exhibited by the IgG subtypes can be seen in Table 3.

The binding of the IgG containing mAbs to Protein A or Protein G can be enhanced by the physiological conditions of the affinity column. Those subtypes which have a relatively high affinity for Protein A can bind under physiological conditions. However, mAbs with a relatively low affinity for the bacterial proteins require high salt conditions if mAb binding is to occur. The high salt concentration acts to strengthen the hydrophobic interactions needed for mAb binding to the bacterial proteins, and therefore, increases the strength of binding. Elution of the purified mAbs is achieved by lowering the pH of the

wash buffer. Purification using this method produces highly pure mAbs (Goding 1986. Harlow & Lane 1988, Perry & Kirby 1990, Cusack & Beynon 1993).

| Species | Affinity for Protein A | Affinity for Protein G |
|---|---|---|
| human IgG1 | ++++ | ++++ |
| human IgG2 | ++++ | ++++ |
| human IgG3 | - | ++++ |
| human IgG4 | ++++ | ++++ |
| rat IgG1 | - | + |
| rat IgG2a | - | ++++ |
| rat IgG2b | - | ++ |
| rat IgG2c | + | ++ |
| mouse IgG1 | + | ++++ |
| mouse IgG2a | ++++ | ++++ |
| mouse IgG2b | +++ | +++ |
| mouse IgG3 | ++ | +++ |

**Table 3 : The Protein A and Protein G affinities for various monoclonal antibody types.**

key: ++++ - high affinity; + - low affinity; - no binding. (Harlow and Lane 1988)

An alternative method of affinity purifying mAbs is by the immobilisation of anti-immunoglobulin (anti-Ig) antibodies. The anti-Ig antibodies are covalently bound to activated beads (e.g. cyanogen bromide activated Sepharose beads) and the mAb containing solution is applied to the column. Elution is obtained by washing the column with a buffer of low pH followed by a buffer of high pH (Cusack & Beynon 1993).

The affinity purification of mAbs is recommended for the purification of mAbs which are found in low amounts, such as those found in tissue culture media. The highly specific nature of affinity chromatography purifies the mAbs efficiently, and without loss of the mAb molecules.

### 2.2.2 Ammonium sulphate precipitation

The precipitation of immunoglobulins using this method is one of the most commonly used methods of non-specifically purifying mAbs. It is more commonly used for the purification of the high concentrations of mAbs found in ascitic fluid or serum (Harris 1993). The concentration of ammonium sulphate used is species dependent. Rabbit mAbs precipitate in a 40% saturated ammonium sulphate solution whereas mouse mAbs need a 45-50% saturated ammonium sulphate solution before precipitation occurs. To overcome these differences, immunoglobulins are precipitated in a standard 50% saturated ammonium sulphate solution (Harris 1993).

An advantage of using immunoglobulin precipitation as a method of mAb purification is that unlike Protein A / Protein G affinity chromatography, ammonium sulphate precipitation is not immunoglobulin specific. A disadvantage to using this method, however, is that other high-molecular weight molecules (e.g. proteins) will also precipitate out of solution under these conditions, contaminating the mAb solution. Therefore, the ammonium sulphate precipitation of mAbs is usually used as a single step in an overall purification protocol.

### 2.2.3 Ion - exchange chromatography

This purification method works on the principle that antibodies have a more basic isoelectric point than serum proteins (Scopes 1996). For example, in anion exchange chromatography, if the salt concentration of the buffer is raised then the mAbs will be the first molecules to be eluted. This property of mAbs has been exploited in two ways. First by keeping the pH below the isoelectric point so that the mAb molecules will not bind to an anion exchange column (e.g. DEAE-Sepharose) but the serum proteins will. Secondly, by keeping the buffer above the pH of the isoelectric point of the mAbs so that they will bind to an anion exchange column. By raising the pH of the buffer, the mAbs elute first, leaving the other serum proteins bound (Harris 1993, Scopes 1996).

Like ammonium sulphate precipitation there are likely to be serum proteins with a similar isoelectric point to the immunoglobulins, and so ion exchange chromatography can

also not be used for the single step purification of mAbs. When ammonium sulphate precipitation is followed by ion - exchange chromatography, highly pure mAbs are obtained (Harris 1993).

## 2.4 Conclusions

Monoclonal antibodies are being used for an ever growing number of practical and clinical applications, in both medical and molecular biological research, and as such, have become an important tool in biological and immunological techniques.

With their ability to be labelled in numerous ways, their uses have become boundless. Monoclonal antibodies can be radiolabelled; labelled with enzymes such as horseradish peroxidase, alkaline phosphatase or β-galactosidase; labelled with biotin; or labelled with fluorochromes (Harlow & Lane 1988). All of these labelling techniques have allowed mAbs to be used for the detection of many proteins, carbohydrates or nucleic acids, in many different expression systems. Owing to this, much of the information currently known about a number of biological processes would not have been determined if not for mAbs.

# 3. HUMAN IMMUNODEFICIENCY VIRUS (HIV)

Since their discovery in the 1980's, the human immunodeficiency viruses type 1 and 2 (HIV-2 & HIV-2) have become two of the most studied viruses in medical and molecular biological research. The first of the HIV types to be discovered was HIV-1 in Los Angeles in the United States of America in 1981. HIV-2 was isolated later in 1986 in West Africa and has been shown to be less pathogenic than HIV-1. Also, individuals infected with HIV-2 tend to develop AIDS later than those individuals infected with HIV-1 (Hirsch & Curran 1996). Both of these viruses cause the same depletion of circulating helper T lymphocytes, and cause a similar spectrum of opportunistic illnesses in an infected individual (e.g. *Pneumocystis carinii* pneumonia, Kaposis sarcoma, mucosal candidiasis

and disseminated cytomegalovirus infection). One of the major undertakings involving HIV-1 and HIV-2 is the development of a protecting vaccine against HIV infection. To achieve this, the structure of the virus and its life cycle need to be fully understood (Hirsch & Curran 1996).

The human immunodeficiency viruses, and the related simian immunodeficiency viruses (SIV), are members of the lentivirus genus of the Retroviridae viral family. They are single stranded positive sense RNA viruses which have enveloped virions. The genetic material of the virus consists of two identical strands of positive sense RNA closely associated to the nucleocapsid protein, derived from the *gag* gene. Like most eukaryotic RNA strands, the genome is capped at its 5' end and polyadenylated at the 3' end. There is also evidence of the internal methylation of selected adenine residues within the RNA sequences (Luciw 1996).

The virions are spherical in shape and are approximately 110 nm in diameter. The virion consists of a lipid bilayer envelope surrounding a cone shaped nucleocapsid core (figure 13a). The envelope is composed of the lipid membrane from the host cell from which the virus budded, and viral envelope glycoprotein "spikes". Each virion particle has approximately 72 spikes, which are composed of the two envelope glycoproteins. Each spike is 9 - 10 nm long and has a bulbous tip with a diameter of 14 - 15 nm. The spikes are used for the attachment of the virus to the cellular receptor of the next target cell to be infected (see section 3.2 *env*). Within the virion shell is the nucleocapsid which contains the genetic material of the virus. The nucleocapsid structure is conical in shape and at its longest runs the length of the virion. Its width varies from 40 - 60 nm at its widest point to 20 nm at its narrowest point (Luciw 1996).

## 3.1 Life Cycle

The infectious life cycle of HIV and SIV can be divided into two distinct phases (figure 14). Phase one involves the attachment of the virus to the target cell, uncoating and entry of the nucleocapsid into the cytoplasm of the cell (figure 14 (i)). Once in the cell the

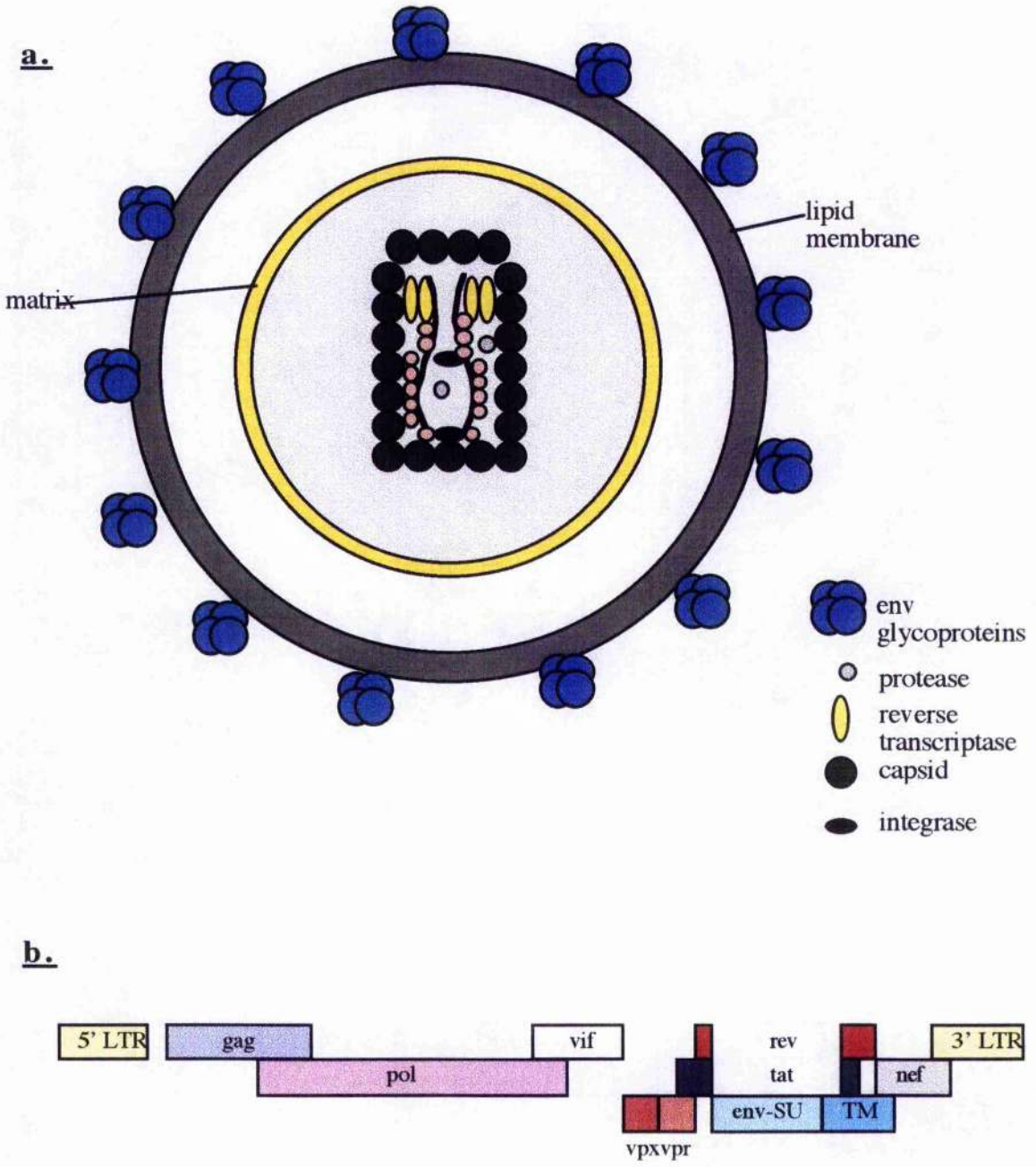



**Figure 13 : Schematic representation of the structure of the virus particle (a) and the genomic organisation (b) of HIV-2 and SIV**

There are small differences between the genomic organisation of HIV-2 / SIV and HIV-1. In the HIV-1 genome, the long terminal repeats (LTR's) are smaller than those in the HIV-2 / SIV genomes and the HIV-1 genome does not contain the gene *vpx* but instead has the gene *vpu* .

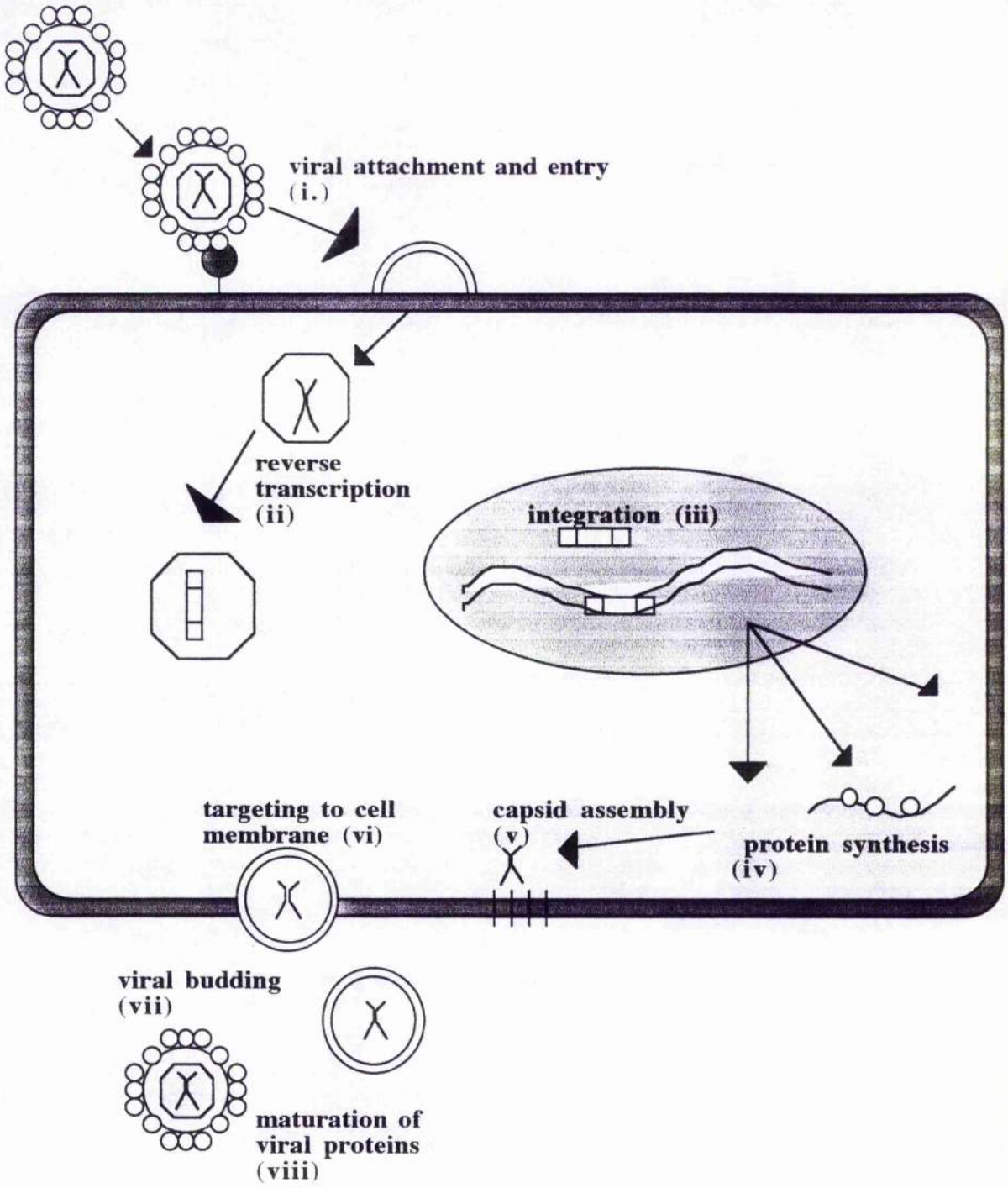



**Figure 14: An overview of the life cycle of the the human and simian immunodeficiency viruses.**

The life cycle of retroviruses involves a number of processes including (i) targeting of the virus to the cellular receptor and viral entry; (ii) uncoating of the viral particle and reverse transcription; (iii) proviral DNA synthesis and integration into the host genome; (iv) viral mRNA and protein synthesis; (v) viral capsid assembly; (vi) targeting of the envelope glycoproteins and the assembled capsid to the cell membrane; (vii) viral budding through the cell membrane; and (viii) cleavage and maturation of the viral proteins to form the infectious viral particle.

nucleocapsid disperses, exposing the viral RNA, which is then used as a template for the synthesis of, first negative sense DNA and then double stranded (ds) DNA by reverse transcription (ii). The dsDNA is then transported to the nucleus where it can either by used as a template for the synthesis of viral mRNA or genomic viral RNA, or it can integrate into the genome of the cell as a provirus and lay dormant for many years (iii). This first phase of the virus replicative life cycle occurs using only those proteins which are present in the virion. No viral gene expression is needed (Luciw 1996).

Phase two of the life cycle involves the synthesis and processing of the viral genome, viral mRNA's and finally viral proteins (iv). This is accomplished using the host cell's transcription / translation machinery. Once all viral proteins have been synthesised, the new virions begin to assemble (v). The two viral RNA strands are encapsidated by the *gag* gene products, the Nucleocapsid, Matrix, and Capsid proteins. Also found within the capsid are the viral Protease, Polymerase and Reverse Transcriptase proteins, and within the nucleocapsids of HIV-2 and SIV, the accessory proteins Vpr and Vpx. Simultaneously with the formation of the nucleocapsid, is the targeting and accumulation of the envelope glycoproteins at the plasma membrane (vi). The completed nucleocapsid migrates to the inner surface of the cell membrane where it buds out of the cell, through the plasma membrane which becomes integrates as part of the virion envelope (vii). Once out of the cell, the viral proteins undergo proteolytic cleavage to produce the fully infectious viral proteins (viii), allowing the virus particles to infect other cells, and start the cycle again (Luciw 1996).

## 3.2 Genomic Organisation

The genomic organisation of HIV and SIV is similar to most other members of the Retrovirus family (figure 13b). They have the three genes common to all retroviruses, *gag*, *pol* and *env*, which encode the viral particle proteins, enzymatic proteins and envelope proteins. However, in addition to these three genes are a number of other genes. Other Retroviruses have accessory genes, e.g. the *tax* and *rex* genes of human T-cell lymphoma

viruses and B-cell lymphoma viruses, but no retrovirus has the same number as HIV and SIV. Both the HIV and SIV group of viruses have six additional genes, one is a transactivator protein which increases proviral replication (*tat*), one is a regulator of virus expression and gene splicing (*rev*) and the other four are known as accessory proteins. The function for some accessory gene products has yet to be determined (Luciw 1996). The functions of all HIV/SIV genes are discussed in more detail.

*gag*

The *gag* gene encodes the structural capsid proteins which are used to package the viral RNA genome into mature virion particles. It is translated late in the life cycle and its production is Rev - dependent (see *rev*). The *gag* gene is translated as a $Pr55^{gag}$ polyprotein, which then undergoes cleavage by the viral protease into p17 matrix (MA), p24 capsid (CA), p2, p7 nucleocapsid (NC) and the C terminal p6 protein (Luciw 1996).

The MA protein is produced from the 5' end of the *gag* gene and resolves at 17 - 18 kDa when analysed by SDS-PAGE. The protein is localised between the virion capsid and the envelope of the mature virus particles, and within an infected cell, targets to the cell membrane (Luciw 1996). The methionine at the start of the protein is cleaved soon after translation and a myristic acid moiety is added, by the cellular enzyme N myristyl transferase, to the glycine which is now the N terminal amino acid. If this glycine is removed then myristylation does not occur. Myristylation is important for the targeting of the protein to the cell membrane, as are the first 31 amino acids (Luciw 1996). In addition to myristylation, MA is also phosphorylated on serine residues. The purpose of this post-translational modification is not known, but it appears to have no effect of protein function.

The MA protein is thought to be involved in viral infectivity. If deletions are made in the first two thirds of the MA protein then $Pr55^{gag}$ polyprotein processing and capsid formation are unaffected. However, the incorporation of the Env glycoproteins into the virion particles is impaired and the virus particles are not infectious. This suggests an interaction with one of the Env glycoprotein subunits, possibly gp41 (Bukrinsky *et al* 1993).

The MA protein also functions during the early stages of viral infection. For this protein function, an intact C terminus is required. Within the C terminus are a number of basic amino acids which act as a nuclear localisation signal to direct the viral pre-initiation complex to the cell nucleus (Luciw 1996). If this signal is removed or mutated, the virus can integrate and replicate but only in cells undergoing cell division, not in growth arrested cells (Bukrinsky *et al* 1993).

The capsid (CA) protein is produced from the central portion of the *gag* gene. When resolved by SDS PAGE, the protein appears to be 24 - 27 kDa in size. Capsid protein is the major subunit of the viral capsid shell. Within its structure are a large amount of hydrophobic residues and like MA, CA is also phosphorylated on serine residues. The role for this phosphorylation is unknown (Coffin *et al* 1996, Luciw 1996).

Within the C terminus of CA is a region of 20 amino acids known as the Major Homology Region. This region is conserved in the CA proteins of many diverse Retroviruses. Mutations to this area affect virion particle assembly, and deletions to this area affect the formation of precursor nucleocapsid. The Major Homology Region is thought to be important for viral particle assembly and the infectivity of the virus, not only in HIV-1 & 2 and SIV but also in Mason-Pfizer Monkey Virus (Reicin *et al* 1995).

The N terminus is not well defined, but deletions to this area inhibit virus replication. Mutant viruses, with an N terminal deletion in CA, assembled into particles and were released as wild type but were not infectious. This lack of viral replication may be due to the N terminus of the protein being involved in the interaction with cyclophilins A and B. This interaction is unique to HIV-1 and SIVchimpanzee (SIVcpz), no other primate lentiviruses have this interaction (Braaten *et al* 1996, Thall *et al* 1994).

The cyclophilins are peptidyl-prolyl isomerases which catalyse a rate limiting step in protein folding, and protect the cell from heat shock. Both cyclophilin A and B are incorporated into the virions of HIV-1 (and SIVcpz), but not the virions of other primate lentiviruses (Franke *et al* 1994). A proline rich region of 25 amino acids found in the centre of the protein is required for cyclophilin binding and incorporation (Franke *et al* 1994), specifically a Gly-Pro motif (Colgan *et al* 1996, Reimer *et al* 1997). If this Proline residue

is mutated then the cyclophilin A - CA interaction is blocked, and cyclophilin A is not incorporated into the virions (Franke *et al* 1994). The multimerisation of the CA protein is also a required for cyclophilin binding and incorporation, as is the interaction of CA with genomic RNA. The incorporation of the genomic RNA is necessary for cyclophilin incorporation.

The interaction of CA and the cyclophilins can be blocked by the immunosuppressant cyclosporine A or its non-immunosuppressant analogue SDZ NIM 811 (Thall *et al* 1994, Franke & Luban 1995, Steinkasserer *et al* 1995, Franke *et al* 1995). If the interaction is blocked then HIV-1 replication is inhibited, nuclear localisation is prevented and proviral integration is inhibited. In contrast, these two drugs had no effect on the replication of the viruses SIVmac and HIV-2. These results suggest that the inclusion of the cyclophilins into the virion particle is essential for HIV-1 and SIVcpz replication.

The p2 protein is found between the CA and nucleocapsid (NC) proteins of the $Pr55^{gag}$ polyprotein. The location of this protein is conserved in all primate lentiviruses, but not the sequence and length. A deletion of this domain from the $Pr55^{gag}$ polyprotein results in the release of particles which are less infectious than the wild type. When the rates of cleavage for the CA-p2 and p2-NC cleavage sites were studied *in vitro*, the cleavage at the CA-p2 site was significantly slower (approximately 40 times slower) than that observed at the p2-NC site. This gave a potential function for p2 in the control of the sequential processing *in vitro*, and in infected cells, of the $Pr55^{gag}$ polyprotein cleavage products (Pettit *et al* 1994).

p2 has an alternative function when still fused to the CA protein. If the CA-p2 fusion from HIV-1 was substituted for the CA-p2 proteins in SIVmac and HIV-2, then a sensitivity to SDZ NIM 811 was conferred, and cyclophilin A was packaged into the virions (Dorfman & Göttlinger 1996). This supports the evidence for p2 having a role in virion assembly (Pettit *et al* 1994).

The NC protein is found at the C terminus of the *gag* gene, and is formed from two rounds of protease processing. First a protein of 15 kDa is produced from the processing of the $Pr55^{gag}$ polyprotein. This 15 kDa protein is then cleaved again to form the p7 NC

protein and the C terminal p6 protein (see later). NC is a basic, hydrophilic protein which binds to the viral RNA genome. Each molecule of NC covers 4-6 nucleotides on the RNA strand. The main function of NC may be to condense the viral RNA for packaging into the mature capsids during virion morphogenesis. Nucleocapsid is not required for the production of virion particles (Luciw 1996).

Within the NC structure of HIV-1, there is a central globular domain flanked by flexible N and C termini. Within the central globular domain there are two copies of the Cys-His motif: Cys-$X_2$-Cys-$X_4$-His-Cys. Within SIV and HIV-2 there is only a single copy of this motif. The Cys-His motif is similar to the metal binding domains of many proteins which interact with nucleic acids, and a zinc ion has been found within mature virion particles. The first Cys-His motif has been shown to be required for RNA packaging, but the second is dispensible (Luciw 1996).

The NC protein has been shown to interact with the major packaging site, *psi*, on viral RNA. The binding of NC to the *psi* sites on two genomic RNA molecules may have an influence on the formation and stability of the dimeric form of the viral RNA genome. In a similar vein, NC has also been implicated to be involved in the annealing of the $tRNA^{lys}$ primer to viral RNA (Luciw 1996).

In addition to the protein functions mentioned, there is preliminary evidence to show that NC is involved in the uncoating of the virus during entry, and has an influence on the reverse transcription process which occurs in the preinitiation complex (Colgan *et al* 1996).

The C terminal p6 protein is the least understood of the proteolytic products of the $Pr55^{gag}$ polyprotein. It is thought to be involved in the mediation of viral budding from infected cells (Reicin *et al* 1995).

A complete summary of the proteolytic processing of the $Pr55^{gag}$ polyprotein is shown in figure 15.

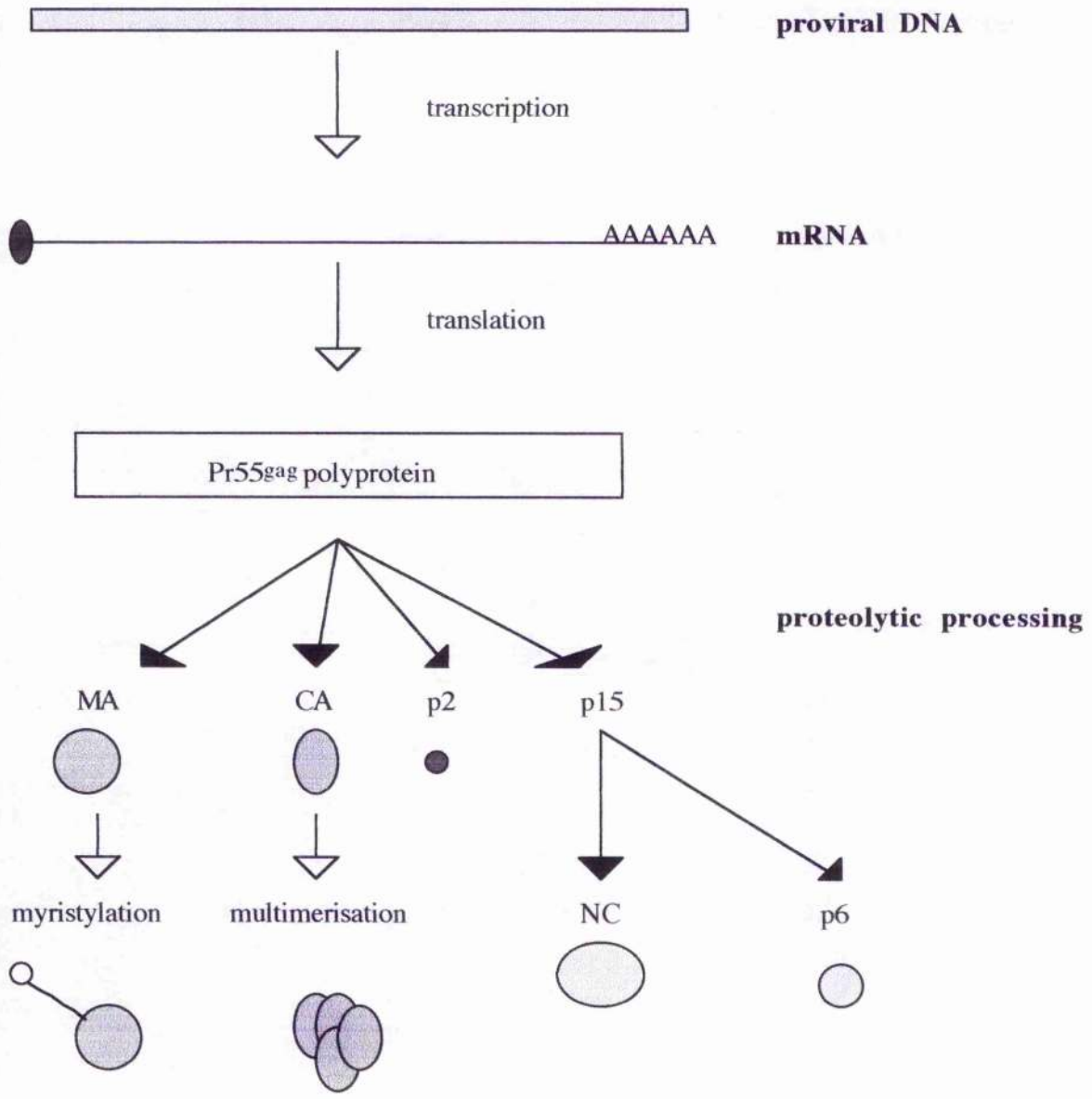



**Figure 15 : The products obtained from the proteolytic processing of the Pr55gag polyprotein.**

The cleavage of Pr55gag polyprotein by the viral protease produces the mature structural proteins required for the assembly of the virion particles.

*pol*

This gene encodes the enzymatic proteins, Protease, Reverse Transcriptase (including RNaseH activity) and Integrase, needed for viral infection (Luciw 1996). These proteins are translated as the Pr160 Gag-Pol polyprotein from mRNA, formed by frameshifting of the RNA polymerase at the 3' end of *gag*. Once translated the polyprotein is cleaved by the viral protease into its protein constituents. There are four protease cleavage sites in the Gag polyprotein and seven protease cleavage sites in the Gag-Pol polyprotein (Hirsch & Curran 1996, Luciw 1996).

The first cleavage of the polyprotein releases the Protease protein. It is an autocatalytic cleavage that occurs *in trans*. The Protease protein is about 10 kDa in size, and is related to cellular aspartate proteases. The viral protease needs dimerisation for function activity, whereas the other aspartate proteases function as monomers. The dimerisation of the protease is also essential for viral replication. The active site has been shown by X-ray crystallography to be able to accommodate a substrate seven amino acids long. However, the cleavage sites can be variable, and are determined by both the primary sequence and conformational structure of the protein. A loose specificity for a specific primary sequence has been elucidated which involves a hydrophobic residue four amino acids upstream of the cleavage site (usually a tyrosine or a phenylalanine) and a proline residue three amino acids downstream of the cleavage site. All of the information obtained on the method of action and the structure of the protease, and its targets, has helped in the development of protease inhibitors for the treatment of infected patients (Hirsch & Curran 1996, Luciw 1996).

The Reverse Transcriptase (RT) protein contains two enzymatic activities : the RNA dependent DNA polymerase activity, and an RNaseH activity. This protein is cleaved from the Pr160 Gag-Pol polyprotein by Protease as a protein with a molecular weight of 66 kDa. The enzyme dimerises soon after protease cleavage to form a homodimer. One of the 66 kDa subunits is then C terminally cleaved, again by Protease, to form the mature RT heterodimer consisting of a 66 kDa subunit and a 51 kDa subunit. The p66-p66 homodimer is catalytically active as is the mature p66-p51 heterodimer, but a p51-p51 homodimer has no catalytic activity (Luciw 1996). Reverse transcriptase acts to synthesise the dsDNA

provirus from an RNA template. For this, it needs a primer sequence, which the RNaseH activity provides. The RNaseH then digests the RNA part of the RNA-DNA hybrid produced so that the full length viral DNA can be synthesised. Both of these enzymatic activities are needed for viral replication.

Reverse transcriptase also plays a large part in the diversity of the HIV and SIV viruses due to its high error rate. This is attributed to the protein having no 3' - 5' exonuclease activity to proof-read for mutations. The error rate of RT in HIV types 1 & 2 and SIV is 1 error per 1700 - 4000 nucleotides, and even for Lentiviruses this error rate is very high. The errors that occur can be due to misincorporation of a nucleotide to give a single base mutation, slippage along the strands at repetitive sequences to give deletions or insertions, or frameshifts caused by a misalignment of the template primer. All of these processes cause the genetic sequence, and sometimes the amino acid sequence, of the virus to be constantly changing (Hirsch & Curran 1996, Luciw 1996).

Integrase is a protein 32 kDa in size which mediates the insertion (and excision) of the viral provirus into (and out of) the host cell's genome. For this it has both DNA cleavage and DNA ligation activities. This protein is processed by the Protease from the Pr160 Gag-Pol polyprotein, and it also acts as a dimer (Luciw 1996). The dimerisation of the protein is essential for integrase activity and also incorporation of the protein into the viral particles. Integrase also appears to be required for viral replication in some cell lines. Within its structure are two conserved Histidine residues and two conserved cysteine residues which are thought to be involved in DNA binding as they can form a secondary structure similar to the metal binding domain of some DNA binding proteins. It is not known what the recognition sequences are within the host cell genome but the integrase protein recognises the 6 - 9 nucleotides *att* sequences found at either end of the linear viral DNA (Hirsch & Curran 1996, Luciw 1996).

A complete summary of the proteolytic processing observed for the Gag-Pol precursor can be seen in figure 16.

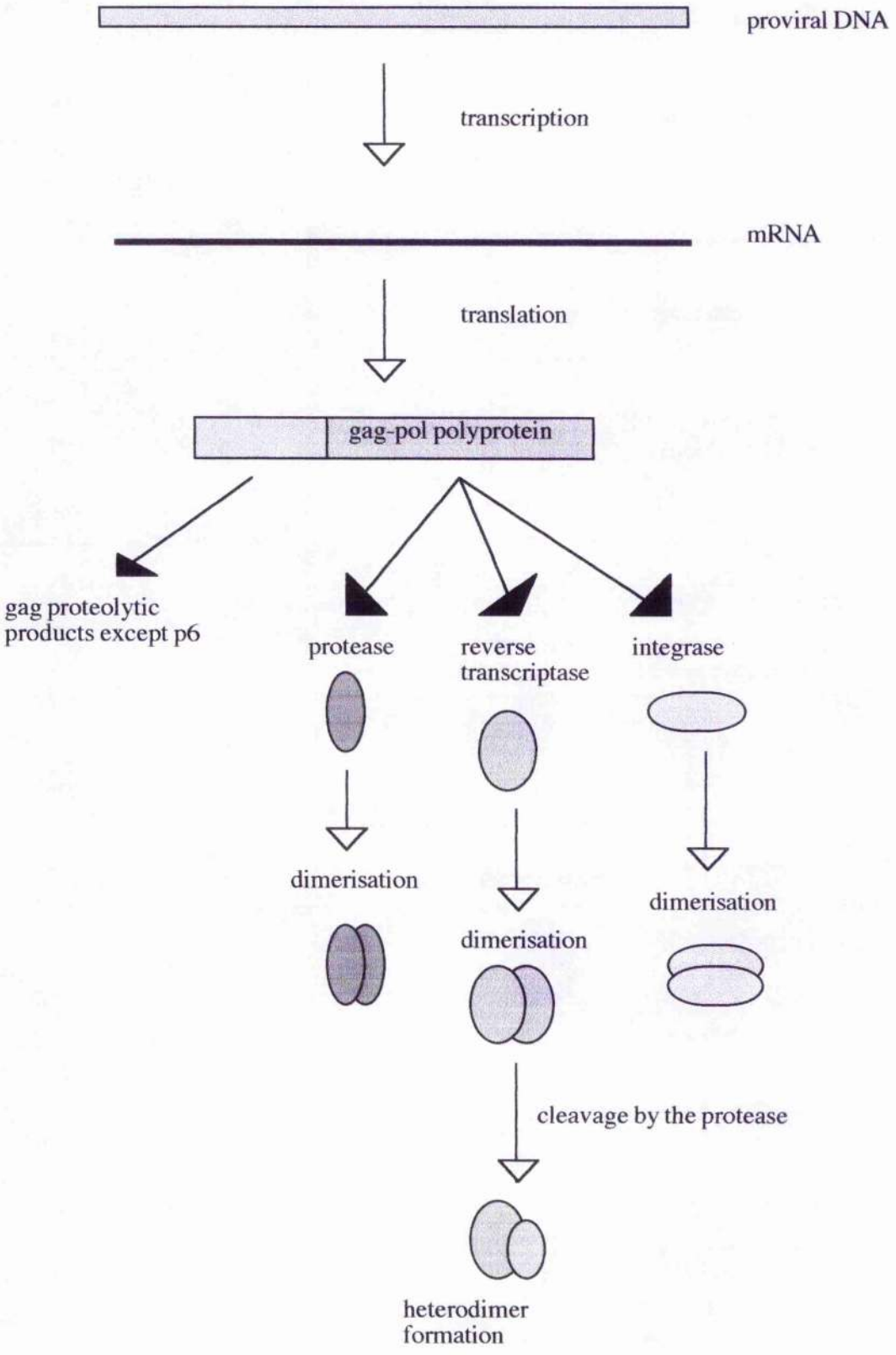



**Figure 16 : the cleavage products obtained from the proteolytic processing of the Gag-pol polyprotein**

*env*

The *env* gene encodes the envelope glycoproteins found on the surface of the virion particle and the plasma membrane of infected cells. The function of the envelope glycoproteins (env gp) is the attachment of the virus particle to target cell membrane receptors, and to facilitate the entry of the virus particles into the cells by the fusion of the virus membrane to the plasma membrane of the targeted cell. The most commonly targeted cellular receptor is the CD4 receptor found on the surface of $CD4^+$ T lymphocytes, monocytes, macrophages and dendritic cells (Luciw 1996). However, recent research has shown that the CD4 molecule alone is insufficient for the promotion of viral fusion. In addition to CD4, co-receptors have been discovered which are necessary for viral entry. These co-receptors are members of the seven - transmembrane - spanning receptor family fusin (Moore *et al* 1997). In T-lymphocyte - tropic virus strains the co-receptor is principally CXCR4 (Chen *et al* 1997, Moore *et al* 1997), and for macrophage - tropic virus strains the co-receptor is principally CCR5 (Yang *et al* 1997, Moore *et al* 1997). This is a potential area for further study as it was also determined that the co-receptor function of these proteins is inhibited by their natural $\alpha$ - and $\beta$- chemokine ligands (Moore *et al* 1997).

The *env* gene is transcribed and translated as a polyprotein of unknown size. The gene product undergoes extensive glycosylation which occurs soon after translation and which creates the glycoprotein gp160. This large glycoprotein is then cleaved by the viral Protease to give the N terminal glycoprotein gp120 (gp120), and the C terminal glycoprotein 41 (gp41; Luciw 1996).

Glycoprotein 120 is known as the surface (SU) protein. This is a highly glycosylated hydrophilic protein which is found on the external surface of the virion membrane and the plasma membrane of infected cells. It is non-covalently attached to gp41 and it contains the domain for binding to the CD4 receptor. This domain is highly conserved and requires the conservation of both the primary sequence (e.g. the conservation of amino acids in non-contiguous regions) and the conformational structure (e.g. discontinuous epitopes; Luciw 1996, Fox *et al* 1997). The overall sequence of gp120 falls into five variable regions with interspersed conserved regions. These regions are

prone to attack by neutralising antibodies, with the most targeted being the V3 loop region. The V3 region is important for the cytopathicity and cell tropism of the virus and if it is mutated, viral infectivity is abolished. As expected there is no sequence homology between the V3 loops of HIV-1 and HIV-2. This region is less important for SIV infection (Rizvi 1992, Fox *et al* 1997).

Another function of gp120 is the down-regulation of CD4 from the surface of cells. This protein acts in conjunction with, but independently of the other viral proteins, Nef, and in HIV-1, Vpu. The exact mechanism of gp120 induced CD4 down regulation is unknown, but gp120 is thought to bind to CD4 within the golgi apparatus to prevent its transport to the cell surface (Sanfridson *et al* 1994, Luciw 1996).

Glycoprotein 41 is known as the transmembrane (TM) protein and it has three main structural motifs. An N terminal hydrophobic region required for the fusion of the viral and cellular lipid membranes during virus particle entry. A second, more C terminal, hydrophobic region which anchors the protein in the viral membrane or plasma membrane of infected cells. The third region is found between these two regions, and outside of the cellular / viral membrane. It is a highly conserved hydrophilic region which is thought to be involved in protein - protein interactions with other viral proteins, but not necessarily only gp120. Glycoprotein 41 is thought to be directly involved in the budding of the virus from the infected cell as well as viral entry (Luciw 1996).

The env glycoproteins of HIV-1, HIV-2 and SIV are all similar. As the envelope glycoproteins are exposed to attack from an infected hosts immune system, the virus has introduced a high degree of variation into the *env* gene nucleotide sequence (Luciw 1996). Within HIV-1 and HIV-2, there are a number of subtypes or clades, determined by the variations which have been observed in the *env* gene. To date, there are nine clades for HIV-1 and five clades for HIV-2. Within each clade there is also intrasubtype diversification. This viral diversification is due, in the most part, to recombination between the different subtypes found in an individual, or to host transfer. The more hosts the virus comes into contact with, the higher the potential diversification (Daniel *et al* 1992, Cranage *et al* 1995, Luciw 1996).

*tat*

Tat is a 10 - 12 kDa protein which is produced from a series of multiply spliced mRNA moieties, and is one of the earliest proteins to be produced in the viral cell cycle. It is a *trans*-activator protein which is found in all Lentiviruses. It is a powerful stimulator of long terminal repeat (LTR) directed gene expression, and it is essential for viral growth. Tat has been localised to the nucleus, specifically the nucleolus, of infected cells (Kao *et al* 1991, Luciw 1996).

Within the structure of the protein there are three essential domains. One is an acidic N terminus containing two glutamate residues and one aspartate residue. These residues are found on a single face of an amphipathic α-helix, and are essential for Tat's *trans*-activation function (Steffy & Wong-Staal 1991). The second domain consists of a stretch of seven cysteine residues. This domain is thought to be involved in the zinc ion mediated dimerisation of Tat. All seven residues, bar one, are essential for Tat function and cannot be mutated (Steffy & Wong-Staal 1991). The final domain is found in the C terminus and consists of a number of basic amino acid residues. This domain has the ability to act as a nuclear localisation signal and is thought to be the part of the protein which interacts with the *trans*-acting response element (TAR) RNA motif (Steffy & Wong-Staal 1991, Luciw 1996).

The TAR element is an RNA element which is located in the 5' LTR, in a region which is transcribed. In HIV-1 and HIV-2, TAR has been mapped to the nucleotides +1 to +111, although only nucleotides +1 to +42 are essential for a Tat response by forming a hair-pin loop secondary structure. The position of the TAR element cannot be moved from its current position or altered (e.g. inverted) if Tat function is to be maintained (Steffy & Wong-Staal 1991). Within the TAR structure there are two areas that specifically can not be altered. One is the sequence CUGGG at position +30 to +34 and the other is the trinucleotide bulge found at the base of the hairpin. If any of these structures are altered then Tat function is abolished. An alteration in any of the other parts of TAR (as long as the secondary structure is not effected) decreases the effect of Tat, but does not abolish its activity.

The TAR element of SIV is slightly different. It is longer and has been mapped to sequences +1 to +124 (Viglianti *et al* 1992). Instead of a single hair-pin stem loop, SIV TAR forms three. The first loop is similar in sequence and structure to that formed in both HIV-1 and HIV-2. In fact they are partially functionally interchangeable, with only a slight decrease in HIV or SIV Tat efficiency (Viglianti *et al* 1992).

The mechanism of action of Tat has been well studied but is far from being completely understood. It is thought that it acts at both transcriptional and post-transcriptional levels. At the transcriptional level Tat acts as an anti-terminator of RNA elongation and also as a promoter of the rate of transcription initiation (Steffy & Wong-Staal 1991, Kao *et al* 1991, Brother *et al* 1996). This has been supported by the fact that in the absence of Tat the rate of initiation is lower than that seen in cells where Tat is present, and viral transcripts are prematurely terminated after 60 nucleotides (figure 17).

The mechanism of Tat dependent RNA elongation is not full understood, but Tat's ability to bind to a number of host factors is thought to be involved. One possible explanation could be due to a direct interaction between Tat and the TATA binding protein (TBP) subunit of Transcription Factor IID (TFIID). The direct effect of Tat on TFIID is unknown, but Tat has been shown to stabilise the interaction of TFIID to another transcription factor, TFIIA, on the HIV TATA box. TFIIA is necessary for the initiation of HIV and SIV transcription (Kashanchi *et al* 1996). Another explanation is concerned with the interaction of Tat to a functional TFIIH containing complex. Once Tat binds to this complex, it phosphorylates the C terminal domain of RNA polymerase II, thereby enhancing the polymerases ability to move further down the DNA, and produce longer RNA transcripts (Parada *et al* 1996).

As stated earlier, Tat also appears to act on a post-transcriptional level by stabilising the TAR containing mRNA and allowing it to be translated more efficiently (Kao *et al* 1991, Steffy & Wong-Staal 1991). Another interesting function of Tat is it's ability to *trans*-activate the gene expression of cellular genes as well as viral genes. One gene whose expression has been conclusively proved to be *trans*-activated by Tat is that which encodes Tumour Necrosis Factor (TNF). Within TNF mRNA is a TAR-like secondary structure.

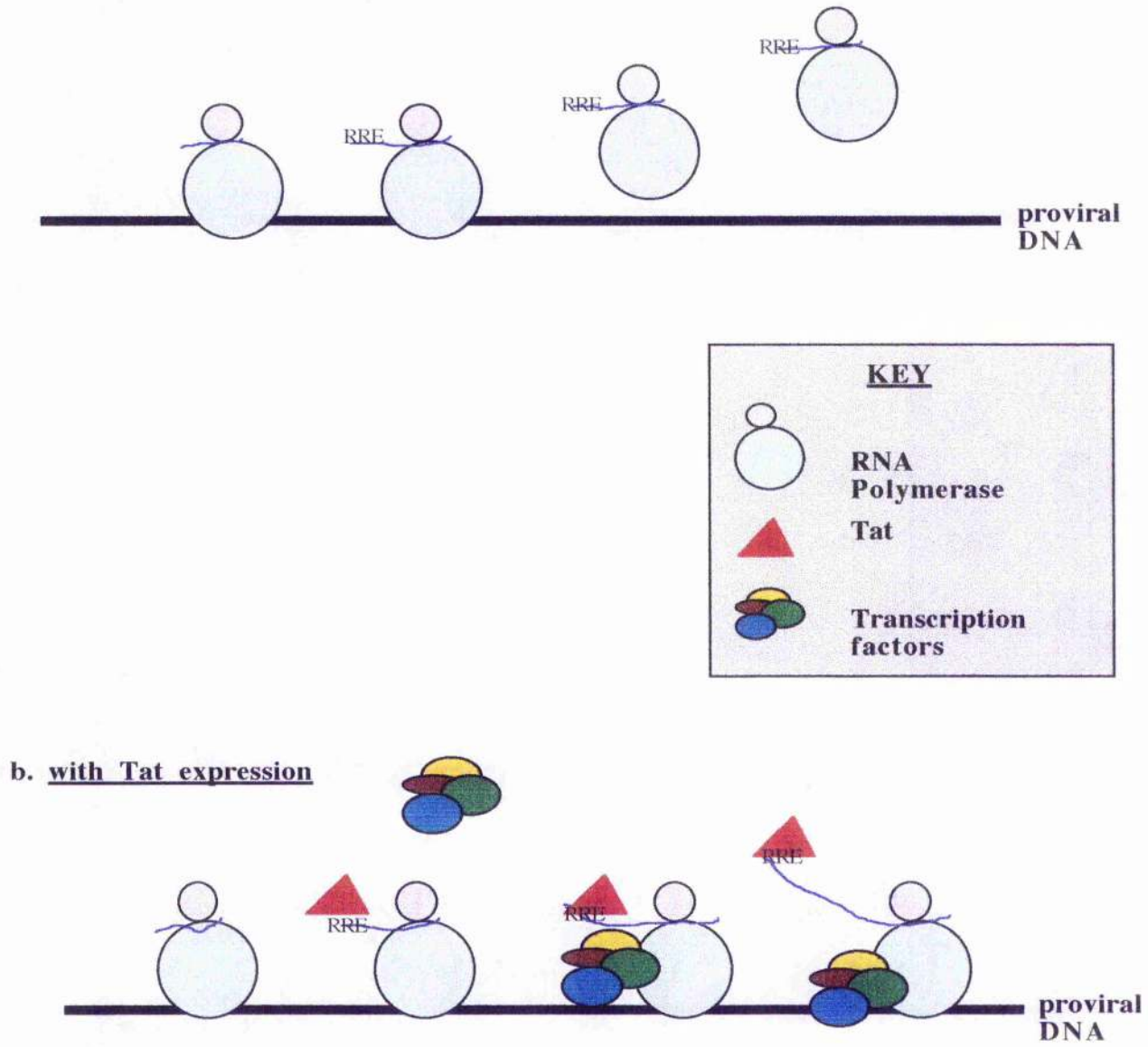



**Figure 17 : Possible mechanism of Tat induced gene expression**
Without the binding of Tat to the Rev response element (RRE), the RNA polymerase stalls and falls off the DNA after transcribing approximately 60 nucleotides. When Tat is present, it binds to the RRE and in some way promotes the association of transcription factors to the TATA box of the promoter region, promoting the elongation of mRNA synthesis.

This structure could be the reason why Tat can enhance the expression of this gene (Brother *et al* 1996). Tumour Necrosis Factor is found in increasing concentrations in infected individuals and has the ability to activate HIV-1 gene expression, and also to rescue Tat defective HIV-1 proviruses. Tumour necrosis factor has been shown to effect the vascular, immune and central nervous systems of an infected individual. This is a potential example of the exploitation of cellular proteins by the virus to increase its own gene expression and infectivity, and to induce the onset of disease (Brother *et al* 1996). In addition to this, Tat has been implicated to be involved in T cell death by inducing apoptosis, either directly or indirectly.

*rev*

Viral genes are expressed either early or late in the viral life cycle. Those which are expressed early in the life cycle are the non-structural genes *tat*, *rev* and *nef*. These are produced as small multiple spliced mRNA moieties of 1 - 2 kb (Luciw 1996). The late genes are the structural genes (*gag* and *env*), the accessory genes (*vif*, *vpr*, *vpx* and *vpu*) and the *pol* gene. These genes are produced as singly spliced 4 kb mRNA moieties (Luciw 1996). In addition to the production of mRNA for protein production, the virus also needs to produce genomic RNA from the DNA provirus. This is produced as an unspliced 9 kb mRNA moiety (Steffy & Wong-Staal 1991, Unger *et al* 1991). The Rev protein controls the differential expression of the viral proteins at the post-translational level by allowing the accumulation and utilisation of unspliced and singly spliced viral mRNA (Steffy & Wong-Staal 1991).

Rev is a 19 kDa phosphoprotein which is localised to the nucleolus of infected cells. There are two motifs within the protein structure which are critical for protein function. The first is a short stretch of basic arginine amino acids, situated in the N terminus of Rev. These amino acids are important for nuclear localisation and for the sequence specific binding of Rev to the *cis* -acting Rev response element (RRE) RNA motif (Steffy & Wong-Staal 1991). The second motif is a leucine rich region found in the C terminus. This motif is believed to be a nuclear export signal. The nuclear export signal has

also been found in the other Lentiviruses Visna Virus and Equine Infectious Anaemia Virus (Meyer *et al* 1996). Within the protein there are two serine residues which are phosphorylated, but this phosphorylation does not appear to be necessary for protein activity.

The RRE is a 200 nucleotide sequence found within the *env* gene. It's sequence allows the formation of a complex secondary structure of five stem loops and it is this secondary structure which is important for Rev binding. The second stem loop is the most important for Rev responsiveness, and if altered abolishes Rev function (Unger *et al* 1991). However, if an RNA structure of similar sequence is substituted, then Rev function is restored. The Rev / RRE function can also be replaced *in vitro* by the *cis* - acting constitutive transport element from both the Simian type D Retrovirus and Mason-Pfizer Monkey Virus (Rizvi *et al* 1996). Rev interacts with the RRE as a multimer but it is not known if this multimerisation is essential for Rev function (Steffy & Wong-Staal 1991).

Rev also functions to stabilise the transport of RRE containing mRNA molecules from the nucleus. This is supported by evidence of decreasing levels of *gag*, *pol* and *env* mRNA transcripts in the cytoplasm when Rev is absent. This is also supported by the presence of the RRE in unspliced and singly spliced mRNA transcripts (Steffy & Wong-Staal 1991, O'Reilly *et al* 1995). As with all viral proteins, the full function, and the mechanism of function, of Rev has not been fully determined.

*nef*

Nef is one of the least understood proteins of the lentiviruses. It is found in all primate lentiviruses, though Nef of HIV-2 / SIV is slightly larger than that of HIV-1. Nef is formed from the multiply spliced early mRNA transcripts which are produced in a Rev - independent manner. If Nef is deleted, a number of contradictory observations have been made which have not been explained. For example, Huang *et al* (1995) observed that if Nef was deleted, then there was a low level of viraemia and a lack of disease progression in Rhesus Macaques. However, to contradict this, Miller *et al* (1995) observed that Nef

deficient viruses were more infectious and had an increased infectivity to a wild type control virus.

Nef is a 23 - 27 kDa protein which is found in the cytosol and associated with the inner surface of the cell membrane. Like the $Pr55^{gag}$ polyprotein. Nef is also myristylated on a glycine residue once the first methionine amino acid is removed. Removal of the glycine residue abolished myristylation and the attachment of Nef to the cell membrane (Luciw 1996). In addition to myristylation, Nef is also phosphorylated on serine and threonine residues. In HIV-1 the phosphorylated residues are a threonine at position 15, a serine between positions 1 - 35 and a threonine between positions 36 - 86 (Bodéus *et al* 1995). The kinase thought to be responsible for this phosphorylation is Protein Kinase C (Coates & Harris 1995, Luciw 1996).

In the centre of Nef is a conserved leucine zipper like motif which has a core of hydrophobic residues. Within the C terminus of Nef is a highly acidic region stretching for over 40 amino acid residues. Within this structure there are two predicted α-helices which are separated by a β-linker sequence. This has homology to the activation domains of acidic transcriptional activation factors, suggesting that Nef may be part of a class of non-DNA binding leucine zipper acidic transcription activation factors (Samuel *et al* 1991). This has now been supported by the discovery that Nef can bind RNA *in vitro* (Echarri *et al* 1996). The Nef proteins from HIV-1 & -2 and SIV all have this activity.

Within the N terminus of Nef is an acidic region followed by, in HIV-1, many repeats of the proline rich motif Pro Xa Xa Pro. HIV-2 and SIV have only a single copy of this repeat. This proline rich region is found in proteins which interact with the Src-homology region-3 (SH3) domain of cellular proteins which are involved in the mediation of signal transduction. The binding of Nef to SH3-containing proteins is specific, e.g. Nef has been shown to bind to the cellular proteins Hck and Lyn but not to others of similar sequence and structure such as Lck (Saksela *et al* 1995).

The functions of Nef have yet to be fully understood, but to date at least two activities have been described. One is the down regulation of surface CD4 and the other is the promotion of viral growth. The effect of Nef on the down regulation of surface CD4

occurs in lymphoid and non-lymphoid cells. As the levels of CD4 mRNA are not effected, Nef must exert its effect post-translationally. The steady state level of CD4 in Nef expressing cells is significantly reduced compared to control cells. One hypothesis on Nef mode of action is that it promotes the degradation of newly synthesised CD4. As the biosynthesis and oligosaccharide processing of CD4 is normal then the effect of Nef must occur later in the biosynthetic pathway, possibly in an acidic compartment (Sanfridson *et al* 1994). It is thought that Nef induces the sorting of CD4 into a cellular compartment where it can be degraded. For the down-regulation of CD4, the Nef protein needs to be myristylated (Saksela *et al* 1995). There are many reasons why the virus would want to down-regulate CD4, and with there being three viral proteins which have this function (Env, Nef and Vpu), it implies that this is an important prerequisite for viral infection. All three proteins act independently of each other, possibly as a fail-safe system so if one protein is inactivated there are still two others to complete the task.

For the promotion of viral growth the PxxP motif needs to be active. This sequence is dispensible for CD4 down regulation implying that the two functions of Nef are entirely separate (Saksela *et al* 1995). The promotion of viral growth could be due to the interaction of Nef to a number of cellular proteins via the PxxP motif. Nef binding proteins have been found in both the cytosol and to be membrane associated. Again the myristylation of the glycine residue is necessary for the binding of Nef to the membrane associated proteins, however the un-myristylated form can still bind to the cytosolic proteins (Harris & Coates 1993).

Nef may also effect viral growth by having an effect on the initiation of reverse transcriptase. Nef defective viruses were shown, in several cell types, to be limited in their ability to perform reverse transcription once internalised. This activity could be fully rescued if Nef was given *in trans*. The Nef proteins of HIV-2 and SIVmac could only partially rescue Nef defective HIV-1 viruses suggesting that the stimulation of the synthesis of the DNA provirus is partially species specific (Aiken and Traso 1995).

The full function of Nef is unknown, but one suggestion is that Nef may have multiple functions within the viral life cycle. This has become more accepted due to the

discovery of a cleavage derivative of Nef ( a protein of 18 kDa) in virus particles (Bukovsky *et al* 1997). However, as Nef has shown no specific interaction with a virion component, Nef may simply be packaged because of its association with the cell membrane (Bukovsky *et al* 1997).

*vif*

Vif is one of the accessory proteins found only within the Lentivirus genus of the Retroviridae family, and is found within all Lentiviruses except the Equine Infectious Anaemia Virus. The *vif* gene is transcribed late in the viral cell cycle as singly spliced mRNA. Its production, like all late genes, is dependent on the function of Rev. The Vif protein is a non-glycosylated protein of 23 kDa. It has been shown that 90% of the Vif protein produced associates with the cytoplasmic membranes of an infected cell, with the other 10% lying within the cytosol (Simon *et al* 1997). However, Vif is not an integral membrane protein. Within the structure of Vif there are a number of conserved cysteine residues, the number of cysteines vary, depending on the virus. For example, within HIV-1 there are 2 conserved cysteine residues and within HIV-2 and SIV there are 4 or 5 conserved cysteine residues. These cysteines are essential for Vif protein function.

The function of Vif, and indeed all accessory proteins, has yet to be completely determined. One of the first observations made was that in the H9 $CD4^+$ T-cell line there was a 1000 fold reduction in viral infectivity. This gave rise to the theory that Vif was involved in virion maturation in a natural infection (Sova *et al* 1995).

One of the difficulties in the determination Vif function was the different phenotypes exhibited by Vif deletion mutant viruses in different cell lines. In some cell lines such as the H9 T cell line, Peripheral Blood Lymphocytes (PBL) and Macrophages, the Vif deficient mutant virus would not replicate. These cell lines were deemed non-permissive. In other cell lines, such as HeLa and COS cell lines, the Vif deficient mutant viruses would replicate. These cell lines were deemed permissive. To complicate matters further, there are now semi-permissive cell lines (e.g. SupT1 & some CEM) where partial viral replication is observed (Sova *et al* 1995, Borman *et al* 1995). For the purpose of

studying the function of Vif, only the infection of non-permissive cell lines by viruses containing deletion mutants have been studied.

Vif deletion mutants in non-permissive cell lines have an abnormally shaped virion. Instead of the cone shaped virion, it now appears condensed. Initially it was shown that the quantity of the Gag, Pol and Env polyproteins within the virion were altered (Borman *et al* 1995, Simm *et al* 1995), but this has since been disproved (Fouchier *et al* 1996, Bouyac *et al* 1997, Simon *et al* 1997 & Kotler *et al* 1997). The presence of an abnormal virion in Vif deficient viruses has led to the theory that Vif is involved in the formation of the virus particles. This theory has been strengthened by the discovery that the Vif protein has been shown, using double fluorescent labelling, to colocalise with the Gag polyprotein at the cellular membrane as virion assembly and budding takes place (Simon *et al* 1997).

However, there is also evidence that the Vif protein may have another function which occurs earlier in the virus life cycle. Camaur and Trono (1996) have shown that the Vif protein is present in virus particles. It has been estimated that there are between 7 and 80 molecules of Vif protein present within the virion, significantly less than the amounts present in infected cells. This is unusual as virion - associated proteins tend to be found in higher concentrations within the virion than in the infected cell. This discovery implies that the Vif protein is needed in the early phase of the life cycle, before gene expression is initiated. One possible function of Vif is the stabilisation of the preinitiation (nucleoprotein) complex immediately after viral entry. In Vif deficient mutants, proviral DNA and genomic RNA can not be detected. One explanation is that the preinitiation complexes are disassembling prematurely, leaving the exposed DNA and RNA open for degradation (Borman *et al* 1995, Simon *et al* 1996). This is not seen in viruses where Vif is intact.

The involvement of Vif in the assembly of virus particles has also come a step closer to being proved. The Vif protein is not present in the virions of Vif deficient viruses, hence the abnormally shaped viral cores.

The actual function of the Vif protein has yet to be fully determined but it has been implicated in the assembly and stabilisation of the viral core, the stabilisation of the viral

preinitiation complex, and recently in the regulation of the viral protease (Kotler *et al* 1997), although more work needs to be done to confirm this.

*vpr*

The *vpr* gene is a produced late in the viral life cycle as a single spliced mRNA. Vpr is a 15 kDa virion - associated protein which accumulates late in infection (Luciw 1996). Vpr deficient mutants have no effect on the replication of HIV-1, HIV-2 and SIV. It is localised to cytoplasmic membranes and to the nucleus, inferring that it has a role in the targeting of the preinitiation complex to the infected cell's nucleus (Lang *et al* 1993, Luciw 1996). Its structure has three distinguishing features, a single conserved cysteine residue, the potential for an amphipathic α-helix in the N terminus and an arginine rich region in the C terminus (Luciw 1996).

The Vpr protein has been shown to bind to the Gag polyprotein, an interaction which is necessary for Vpr incorporation into the virion. For this interaction to occur the Gag polyprotein does not need to be processed but an intact p6 protein domain is required (Lavallee *et al* 1994). The acidic amino acids and the leucine residues within the N terminus region are also necessary for this interaction to occur. Any mutations to the leucine residues abolishes the incorporation of Vpr into the virion and seriously effects the stability of the protein (Mahalingham *et al* 1995, 1995a).

Vpr is thought, therefore, to have a number of functions including; the targeting of the viral preinitiation complex to the nucleus (Luciw 1996); the promotion of maximal viral replication; for the persistence of a high level of viral infection; the progression of disease (Lang *et al* 1993); and the reactivation of the HIV provirus from latently infected T and B cell lines (Levy *et al* 1995, Rogel *et al* 1995). The complete function of the Vpr protein has not been fully determined or understood.

*vpx*

The *vpx* gene is highly homologous to the *vpr* gene (Luciw 1996). Only HIV-2 and some SIV strains (e.g. SIVmac) have this gene. It is produced as singly spliced mRNA late

in the virus life cycle. Vpx is a 14 - 16 kDa virion - associated protein. Like Vpr it has a predicted amphipathic α-helix as its N terminal. Within the cell, it is found on the inner surface of the cellular membrane (Yu *et al* 1991, Luciw 1996). For virion incorporation, Vpx needs the $Pr55^{gag}$ polyprotein or the CA protein. Its presence in the virus particle implies that it has a function early in the viral life cycle, before gene expression occurs (Yu *et al* 1993). Within the virus particle, most of the Vpx protein is localised to the outside of the virus core indicating an involvement in viral entry (Yu *et al* 1993). Vpx is also thought to be needed for efficient viral replication.

*vpu*

Vpu is a protein of about 15 kDa which is produced late in the virus life cycle from a singly spliced mRNA. It is found only in HIV-1 and SIVcpz. Within the N terminus there are 27 hydrophobic residues, with the rest of the protein being hydrophilic. This makes it a potential type I integral membrane protein. Vpu is localised to the cellular membrane, and has two phosphorylation sites, Ser-52 and Ser-56, where it is phosphorylated by casein kinase II (Luciw 1996).

The function of Vpu is one of the best understood of the accessory proteins. It has two distinct functions, to facilitate the budding of the virus particles from the infected cell (Bour *et al* 1996) and the down modulation of the cellular receptor CD4 from the cell surface (Luciw 1996). Unlike the Env glycoprotein which binds to the CD4 receptor in the endoplasmic reticulum (ER), Vpu causes the rapid and selective degradation of CD4 within the ER. It causes a reduction in the life span of the CD4 receptor from 6 hours to 12 minutes. The down modulation of CD4 receptors is dependent on the phosphorylation of Vpu, whereas the mediation of the release of viral particles is only partially dependent on protein phosphorylation.

The down modulation of CD4 receptors by Vpu occurs independently of the mode of action of the Env glycoprotein and the Nef protein. However, the action of all three proteins are needed for the clearance of all CD4 molecules from the cell surface (Chen *et al* 1996, Fujita *et al* 1996).

A complete summary of the localisation within an infected call and the virion particle, and the known functions of all viral proteins is shown in table 4.

## 3.3 Problems for the development of a vaccine to HIV

Over recent years, the development of a vaccine to HIV has progressed slowly, and with little success. Much of information on the method of transmission of HIV, the progress of the immunodeficiency disease and the molecular basis of HIV has been determined, and yet no vaccine is available. Some of the main difficulties encountered when designing a vaccine to HIV are discussed in more detail.

The first problem is the ability of the HIV DNA provirus to integrate into the genome of a host cell where it can lie dormant for many years. This makes the provirus invisible to the immune system. Also, with HIV being able to pass directly from cell to cell, viral transmission can occur without the virus ever being detected by either neutralising antibodies or cytotoxic T cells (Tijhaar 1997). To overcome this, a method must be found to target integrated proviral containing cells without harming normal uninfected cells (Sabin 1992).

In addition to proviral integration, HIV and SIV have a high mutation rate due to the inaccuracy of reverse transcriptase (RT) during DNA synthesis. This inaccuracy is caused by RT having no proofreading activity to correct any mistakes. Owing to this, the genomic sequence of HIV and SIV, and the amino acid sequence of the viral proteins, constantly change, with the most variable region being the V3 region of gp120. In addition, HIV-1 and HIV-2 have a number of subtypes. If using the variability of the *env* gene to determine viral sub-type, for HIV-1 there are nine, and for HIV-2 there are five. A major problem is that these subtypes are non- or minimally cross-neutralising (Daniel *et al* 1992, Cranage *et al* 1995). As this constant changing can not be prevented, an ideal vaccine needs be able to be modified or mutate at a similar rate.

Another problem is the location of the initial infection. Most HIV infected individuals contracted the virus through the rectal mucous membrane, where the virus can

| Gene | Gene Product | Location within viral particle | Cellular location | Protein Function |
|---|---|---|---|---|
| *gag* | MA | viral matrix | cell membrane associated | (i) viral particle assembly: (ii) nuclear localisation of the pre-initiation complex: (iii) viral infectivity |
| | CA | viral capsid | cytosolic | (i) virus particle assembly: (ii) incorporation of cyclophilin A into HIV-1 virion particle |
| | p2 | unknown | unknown | (i) control of the rate of proteolysis of the gag precursor (ii) virion particle assembly and incorporation of cyclophilin A into virion particles |
| | NC | nucleocapsid | cytosolic | (i) packaging and condensation of the viral genomic RNA into virion particles (ii) possibly virus uncoating |
| | p6 | unknown | viral envelope (?) | viral budding |
| *pol* | Protease | nucleocapsid | cytosol | maturation of viral proteins |
| | Reverse transcriptase | nucleocapsid | nucleus | synthesis of proviral DNA |
| | Integrase | nucleocapsid | nucleus | intergation and excision of the proviral DNA from the host cell genome |
| *env* | Gp120 | envelope | extracellular to cell membrane | (i) attachment of virus particle to CD4 cellular receptor (ii) surface CD4 down regulation |
| | Gp41 | envelope | integral cell membrane protein | Entry and budding of mature virus particles |

| | | | | |
|---|---|---|---|---|
| *tat* | Tat | not virion associated | nucleolus | (i) stimulation of LTR directed gene expression by promotion of the elongation of mRNA synthesis<br>(ii) stabilisation of TAR containing mRNA for translation |
| *rev* | Rev | not virion associated | nucleolus | (i) promotion of mRNA splicing<br>(ii) nuclear export of mRNA |
| *nef* | Nef | not virion associated (cleavage product found in matrix) | cellular and cell membrane associated | (i) down regulation of surface CD4<br>(ii) promotion of viral growth |
| *vif* | Vif | matrix | cell membrane associated although a small proportion found in cytosol | assembly of virus particle |
| *vpr* | Vpr | nucleocapsid | nucleus and cell membrane associated | targeting of pre-initiation complex to nucleus |
| *vpx* | Vpx | matrix | inner surface of cell membrane | viral entry and replication |
| *vpu* | Vpu | not virion associated | perinuclear region of the cell membrane | (i) facilitate budding of virus<br>(ii) down regulation of surface CD4 |

**Table 4 : A summary of the cellular and viral localisation, and the function of the viral proteins of the human immunodeficiency virus (HIV).**

penetrate into the surrounding tissues (e.g. dendritic cells) where the virus can replicate. In addition, a number of HIV infected individuals have been found to have HIV proviral DNA within the cells of the colon (Joag *et al* 1996). To prevent viral replication in this initial stage of infection, an effective immune response is necessary. However, most current vaccine strategies do not induce a protective mucosal immune response (Joag *et al* 1996, Tijhaar 1997).

The final problem encountered when trying to control the replication of HIV and SIV, is that they selectively target T helper cells, a cell type which has an important role in the immune system. One interesting observation made was that if the immune response of a newly infected animal was studied, then a strong humoral and cellular response was elicited. This initial immune response, however, did not have the ability to totally eradicate the viral infection before it became persistent. In many cases where an individual became infected, i.e. by blood transmission or *in utero* transmission from a mother to the child, neutralising antibodies were present which recognised viral proteins, and yet infection still occurred. This makes the design of a vaccine more difficult as it is not known what type of immune response is needed before protection is obtained. One recent observation is that if a $T_H1$ type helper T cell response is elicited, rather than a $T_H2$ type helper T cell response then the infection can be prevented. However, if $T_H2$ type helper T cell response is elicited, then infection is not prevented to the same extent (Clerici & Shearer 1993, Ahlers *et al* 1997). The direction of the initial immune response towards a $T_H1$ type helper T cell response could aid in the development of a vaccine which gives protection to a cell - associated HIV infection.

## 3.4 SIV infection of Rhesus macaques as a vaccine model for HIV

The infection of Rhesus macaques (*Macacamullata*) with SIVmac is one of the most commonly used animal models for the study of HIV pathogenesis and the testing of potential vaccines. Although HIV-1 has been shown to infect chimpanzees and the gibbon, these animals do not succumb to an AIDS-like immunodeficiency disease (Tijhaar 1997).

This makes it impossible to observe the pathogenesis of the disease and to test any potential vaccines which are developed.

SIVmac was discovered by Daniel *et al* in 1985 in captive macaques at the New England Primate Research Centre (McClure 1991). As yet no Asian macaques in the wild have been found to be infected with SIVmac, and is thought that SIVmac is a derivative of the SIV found in Sooty Mangabeys (SIVsmm). When comparing SIVmac to HIV-1, they were found to share morphological, biological, serological and molecular properties.

The genomic organisation of SIVmac is similar to HIV-1, but more so to HIV-2. In addition there is strong sequence homology. For the whole genomic sequence, SIVmac has 75% homology to HIV-2 and 40% homology to HIV-1. Like HIV-1 and HIV-2, SIVmac also has the ability to undergo genetic variation. When looking at the microscopic morphology of SIVmac, it is impossible to distinguish SIVmac from HIV-1 or HIV-2. SIVmac has a restricted tropism to $CD4^+$ cells *in vitro* and has been shown to exhibit similar cytopathic effects such as the formation of syncytia. Finally, and most important of all, SIVmac is highly virulent and causes a disease in the Rhesus macaque similar to AIDS in humans. This is known as simian AIDS or SAIDS (McClure 1991, Joag *et al* 1996). With the macaques succumbing to an AIDS like disease, it allows the study of viral pathogenesis and the testing of potential vaccines for effectivity.

All of the properties described make the infection of macaques by SIVmac an ideal model for the study of vaccines against HIV-1 and HIV-2.

## 4. Objectives of the work

Many of the vaccines developed against HIV and SIV have made use of the structural proteins and envelope glycoproteins for the production of an immune response. Their location within the viral particle, and their function, make them natural targets for the host's immune system. However, although a vaccine incorporating these proteins does produce a strong immune response, to date no protection has been attained. The emphasis of vaccine design has therefore turned towards using other viral proteins, such as the

accessory proteins or the enzymatic proteins, in a vaccine in order to obtain an immune response capable of protecting the host from an infectious viral challenge. A number of vaccines have been devised which incorporate viral accessory proteins. For example, the use of live attenuated virus vaccines (Kestler *et al* 1990, Daniel *et al* 1992, Cranage *et al* 1997), DNA vaccines (Lu *et al* 1996, Tijhaar 1997) and multiple epitope vaccines (Hanke 1993, Kent *et al* 1996). The current vaccine methods favoured by our research group are DNA vaccines and multiple epitope vaccines, both of which utilise recombinant DNA technology.

The multiple subunit vaccine favoured by the research group are solid matrix antibody antigen (SMAA) complexes. A SMAA complex is composed of a antibody-antigen complex immobilised on a solid matrix support, usually agarose beads. In view of the method in which SMAA complexes are constructed, they can be homogenous (i.e. contain many molecules of a single antigenic epitope) or heterogenous (i.e. contain many antigenic epitopes; Hanke 1993). This allows the presentation of a number of viral proteins within a single multiple epitope vaccine.

For the construction of SMAA complexes, a two step purification system was devised. Viral genes were cloned into an expression vector which, when expressed, tagged the recombinant protein produced at both termini. The affinity tags most commonly used were the hexahistidine tag and the oligopeptide Pk tag, devised by the research group (Southern *et al* 1991, Randall *et al* 1993). A monoclonal antibody was available which recognised the Pk tag. The Pk tag - mAb interaction was used as the final step of the purification protocol, and as the first stage in the production of the SMAA complexes.

The mAb which recognises the Pk tag binds with an unusually high affinity such that denaturing conditions are required to separate them. In order to utilise the two step purification system for the purification of Pk tagged recombinant proteins, which are not required for incorporation into SMAA complexes, the mAb-Pk tag interaction needed to be studied more closely.

The aims of this project were the efficient purification of Histidine and Pk tagged recombinant non-glycosylated SIV proteins for incorporation into a multiple epitope

vaccine other than SMAA complexes. In order to achieve this, a range of mAbs which recognised the Pk tag were developed and characterised. In addition, the Pk tag was also modified to allow further study of the mAb - Pk tag interaction. Using the newly developed anti-Pk mAbs and the modified Pk tag, the original two step purification protocol was modified to allow Pk tagged recombinant protein purification from an affinity column.

# Materials And Methods

## 1. Plasmids

Plasmid pQ9cPk was derived from the plasmid pQE-9 (Qiagen, Hybaid, Middlesex, U.K.). Plasmid pUBS 501 is derived from the pFDX500 (Brinkmann *et al* 1989). Plasmid pBluescript is copyright of Stratagene (Cambridge, U.K.).

## 2. Recombinant DNA preparation

DNA was isolated from bacterial cells using the lithium chloride alkaline lysis method devised by Griff *et al* (1990). Pelleted cells were resuspended in ice cold GTE (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA), and lysed with a freshly prepared solution of 0.2 M NaOH, 1% SDS. Cell debris was precipitated using a solution of KCl (29.4g potassium acetate plus 11.5ml glacial acetic acid in 100ml). The mixture was centrifuged at 4000rpm for 10 min and the supernatant removed. DNA was precipitated by the addition of 1 volume of propan-2-ol, and pelleted by centrifigation at 7000rpm for 30 min. After the DNA was resuspended in TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA), 4.4M LiCl was added and left on ice to precipitate any RNA present. The mixture was centrifuged at 4000rpm for 15 min and the supernatant removed. DNA was precipitated by the addition of 2.5 volumes of ethanol and centrifuged at 7000rpm for 30 min. The pelleted DNA was resuspended in TE and 4 units of RNase (Boehringer Mannheim, Lewes, East Sussex) were added. After the addition of 10% SDS, the mixture was heated to 70°C for 10 min before extraction with 1 volume of phenol:chloroform:isoamyl alcohol (100:96:4) and precipitation with NaAc and ethanol. The pelleted DNA was then resuspended in TE or water to an approximate solution of 1mg/ml.

## 3. DNA linkers and Primers

nefFOR 5' C TAG CTG AAT TCC GCC TTC TTC TAA CCT CTT CCT C-3'

nefBACK 5' GTG CAG TGA TCA ATG GGT GGA GCT ATT TCC ATG AGG-3'

p17FOR 5' C TAG CTG AAT TCC GTA ATT TCC TCC TCT GCC GCT AGA TGG-3'
p15BACK 5' GTG CAG GGA TCC ATG GCA GAA GCC CTG AAA GAG GCC-3'

pGEXc-Pk(**I4A**) FORWARD : 5'AATT CCA GGA AAG CCG GCC CCA AAC CCT TTG CTG GGA TTG GAC TCC ACC TGA-3'
pGEXc - Pk(**I4A**) BACK : 5'AATT TCA GGT GGA GTC CAA TCC CAG CAA AGG GTT TGG GGC CGG CTT TCC TGG-3'
pGEXc - Pk(**L8A**) FORWARD : 5'AATT CCA GGA AAG CCG ATC CCA AAC CCT GCG CTG GGA TTG GAC TCC ACC TGA-3'
pGEXc - Pk(**L8A**) BACK : 5' AATT TCA GGT GGA GTC CAA TCC CAG CGC AGG GTT TGG GAT CGG CTT TCC TGG-3'
pGEXc - Pk(**L11A**) FORWARD : 5' AATT CCA GGA AAG CCG ATC CCA AAC CCT TTG CTG GGA GCG GAC TCC ACC TGA-3'
pGEXc - Pk(**L11A**) BACK : 5' AATT TCA GGT GGA GTC CGC TCC CAG CAA AGG GTT TGG GAT CGG CTT TCC TGG-3'

For sequencing :
pQ9gagPk : 5' AGG ACC CAG CTG TGG ATC T-3'

**4. DNA Digestions**

Restriction enzymes used for recombinant DNA work were purchased from New England Biolabs (NEB, Bishop's Stortford, Herts) or Promega (Southampton), and used according to manufacturers recommendations.

**5. Agarose Gel Electrophoresis**

DNA samples were analysed by horizontal agarose gel electrophoresis. The agarose (Flowgen Instruments Ltd., Lichfield, Staffordshire, U.K.) was reconstituted in TBE (90mM Tris-borate, 2mM EDTA pH8.0) buffer to a final percentage of 1% (w/v). The size of the gel was 10 x 10 x 0.5 cm. The DNA samples were mixed 1:5 (v/v) with X6 loading

buffer ( 15% Ficoll, 0.25% Bromophenol blue - type 400) and then loaded into the wells. The DNA was separated at 65 - 85 volts for varying lengths of time, and was stained with 1 $\mu g.ml^{-1}$ ethidium bromide in the gel and buffer and visualised using an Ultra Violet (UV) trans-illuminator.

**6. Recovery of DNA from agarose gels**

The relevant band of DNA, visualised on a UV transilluminator, was excised from the agarose gel. The DNA was isolated from the gel using the Qiaquick Gel Extraction Kit (Qiagen ), according to the manufacturers recommendations.

**7. DNA ligations**

50-100ng of cut plasmid vector was mixed with 100-200ng of DNA insert in a total of 50$\mu$l ligation buffer containing 20U T4 DNA Ligase (Promega). The mixture was incubated at 37°C for 1 hour. The ligation mix was used directly in the transformation of competent bacterial cells

**8. Competent Bacteria Transformations**

20 - 25$\mu$l of the ligation mix was combined with 50 - 100$\mu$l of *E. coli* competent cells, XL1 Blue strain (Stratagene), and stored on ice for 1 hour. The bacteria were heat shocked by heating at 42°C for 90 seconds before returning the mixture to ice for a further 90 seconds. 1 ml Luria broth (LB) was added and the transformation mix placed in a 37°C incubator for 1 hour. The bacteria were plated onto L-agar plates containing the relevant antibiotic(s) for selection.

**9. Competent Bacteria Preparation**

*Escherichia coli* strain XL1-Blue were made competent using the $MgCl_2$ / $CaCl_2$ method. A 50 ml culture was grown to an $OD_{600}$ = 0.4. The culture was placed on ice and split between two 30ml Corex tubes. The cells were pelleted by centrifugation at 5000rpm for 5 min. The pellets were resuspended in 5 ml sterile 0.1M $MgCl_2$ and placed on ice for 2 min.

The suspensions were centrifuged again at 5000rpm for 10 min and the pellets resuspended in 1 ml sterile 0.1M $CaCl_2$. The freshly made cells were left for at least 30 min before use.

### 10. Hybridisation

60-70 colonies were streaked onto an agar plate and incubate at 37°C overnight. The following day a nitrocellulose filter was placed onto the plate for 5 min. The cells which had stuck to the nitrocellulose filters were lysed by moistening the filters with lysis buffer (0.5M NaOH, 1.5M NaCl), and the NaOH was neutralised by the addition of 0.5M Tris/HCl pH 7.5, 1.5M NaCl. The filters were dried and the DNA crosslinked to the filter by exposure to UV light. The filters were washed with X3 Sodium chloride /sodium citrate (SSC), 1% SDS at 65°C for 1 hour, and then incubated with pre-hybridisation buffer (6X SSC, 5X Denhardt's solution, 0.05% sodium pyrophosphate, 0.5% SDS) at 37°C for 1 hour. The filters were then mixed incubated in hybridisation buffer (6X SSC, 1X Denhardt's, 0.05% sodium pyrophosphate) containing the radioactive probe at 65°C overnight. The DNA template used was dependent upon the hybridisation performed. The filters were washed in reducing salt concentrations of SSC/0.05% sodium pyrophosphate, and exposed to a phosphorimaging plate for varying lengths of time. Any radioactive signal obtained was visualised using a Fujix Bas 1000 Phosphorimager (Fuji).

### 11. Radiolabelling of probes for hybridisation

The radioactive probe was made using the T7QuickPrime™ Kit (Boehringer Mannheim) to manufacturers recommendations.

### 12. Induction of pQ9*nef*Pk and pQ9*gag*Pk Gene Expression

Cultures of LB containing the relevant antibiotic(s) were inoculated with the bacteria containing either pQ9*nef*Pk or pQ9*gag*Pk and incubated at 37°C overnight. The overnight cultures were used as the inoculum for a larger LB cultures, which were incubated at 37°C until an $OD_{600}$ = 0.4 - 0.6 was reached. Gene expression was induced by the addition of IPTG to a final concentration of 1 mM, unless otherwise specified, and the bacterial

cultures left at 37°C for varying lengths of time. Samples were taken and the cells pelleted. The pellets were resuspended in Sodium Dodecyl Sulphate - Polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and analysed by SDS-PAGE.

### 13. SDS-PAGE and Immunoblot analysis

Samples were diluted 3 :1 in SDS-PAGE loading buffer (X4 concentration : 20% glycerol, 200 mM Tris/HCl pH 7.0, 20% 2-mercaptoethanol, 8% sodium dodecyl sulphate (SDS), Bromophenol blue type 400) and boiled for 15 min before loading onto a polyacrylamide gel (PAG). The polypeptides were separated on a polyacrylamide gel cross-linked by *bis* -acrylamide and TEMED, in the Bio-rad mini gel system. The polypeptides were either stained by Coomassie Brilliant Blue or transferred to nitrocellulose filters (BDH Laboratory Supplies, Poole Dorset, U.K.) using a semi-dry gel electroblotter. The filters were blocked in 5% Marvel (Premier Beverages, Adbaston, Staffordshire, U.K.) and incubated with monoclonal antibodies in 5% blocking solution ( 5% Marvel, 0.1% Tween 20, 0.14 mM NaCl, 0.02 mM Tris/HCl pH 7.6). The bound antibodies were detected by incubation with a horseradish peroxidase (HRP) conjugated anti - mouse Ig mAb (Scottish Antibody Production Unit, Carluke, Lanarkshire, U.K.) and enhanced chemiluminescence (ECL).

### 14. Expression and Nickel affinity purification of His-Nef-Pk

*nef* gene expression was induced with 1 mM IPTG at 37°C for 1 hour. The bacteria were pelleted and the pellet resuspended in TBS buffer (0.3 M Tris HCl, pH 8.0, 0.02 M NaCl). Lysozyme was added to a final concentration of 1 $mg.ml^{-1}$, and incubated on ice for 15 min. Triton X-100 was added to a final concentration of 1% (v/v), the lysates were sonicated and centrifuged at 12000 rpm for 30 min. The supernatant was removed and incubated with $Ni^{2+}$-NTA beads for one hour. The resulting heterogeneous mixture was used to pack a column. The column was washed with 10 volumes of TBS and the recombinant protein eluted with TBS containing 250 mM imidazole. Protein elution was detected by spotting a 1µl sample from each fraction collected onto nitrocellulose filters and

staining with napthalene black. To confirm the purification of His-Nef-Pk, samples were analysed on 12.5% SDS-polyacrylamide gels (PAG's).

### 15. Immunisation schedule for development of an immune response to the $Pk_{TH}$ peptide

Six BALB/c mice were injected intraperitoneally with 10μg of a branched peptide containing the Pk tag and a $T_H$ cell epitope (see figure 28). After 3 - 4 weeks the immunisation was repeated. Over the following 21 days samples of blood were taken from the mice, by making a small incision in the tail, and the specific serum antibody responses studied by ELISA. After the 21 days, the immune response in two of the mice was boosted by an intravenous injection into the tail vein. Two to three days later the mice were sacrificed by cervical dislocation, and their spleens removed.

### 16. Development of monoclonal antibody secreting hybridoma clones

The two freshly removed spleens were washed with PBS before the tissue was broken up by grinding over sterile wire gauze. The cell suspension obtained was centrifuged at 1500 rpm for 15 min, and the majority of the supernatant removed by pouring. To lyse any red blood cells, 0.83% ammonium chloride was added and left to stand for 3 - 5 min. A layer of new born calf serum (NBCS) was carefully added to the bottom of the tube, centrifuged at 1500 rpm for 5 min and the supernatant removed. The cells were washed with PBS and centrifuged at 1500 rpm for 5 min to remove the NBCS. This process was repeated three times. As the splenocytes were being washed, the SP2/0 myeloma cells were prepared by pelleting and washing with PBS, and a 50% solution of polyethylene glycol (PEG) prepared.

For the fusion. the splenocyte and SP2/O cells were mixed together in a ratio of 3:1 (splenocytes : SP2/0), and centrifuged at 1500 rpm for 15 min. All of the supernatant wa removed before the 0.5 ml of the 50% PEG solution was added. After a short 45 second centrifugation, the cells were once again resuspended in the PEG solution and protein free hybridoma media (PFHM) was added over a period of 2 - 3 min. The cells were pelleted by

centrifugation at 1500 rpm for 15 min before being resuspended in PFHM (+/- foetal call serum) + HAT media supplement to an approximate concentration of 2 - 4 x $10^4$ SP2/0 cells (not including splenocytes) per 100 ml of PFHM. 100 µl of the fused cells suspension was plated into each well of a microtitre plate containing macrophages (one week prior to the fusion, forty 96 well microtitre plates were seeded with macrophages from BALB/c mice) and incubated at 37°C in a humidified incubator in the presence of 5% carbon dioxide ($CO_2$).

**17. Screening of monoclonal antibody secreting hybridoma clones**

Immunlon-4 ELISA plates (Dynatech Laboratories Inc., Chantilly, Virginia, U.S.A.) were coated with PBS containing 1% gluteraldehyde and incubated at 37°C for 5 hours. The plates were washed with PBS, coated with PBS containing 50 µg of the $Pk_{TH}$ peptide, and incubated at 4°C overnight. The wells were blocked with PBS + 10% (w/v) Marvel at 37°C for 1 hour, and incubated at 37°C for 1 hour, with tissue culture supernatant taken from wells in which the hybridoma colonies were confluent. After washing with PBS, positive binding was detected using a HRP conjugated anti mouse Ig mAb and a solution of TMB (3,3',5,5'- tetramethylbenzidine; Kirkegaard & Perry Laboratories Inc, Gaithersburg, MD 20879).

**18. Immunofluorescence analysis**

BALB/c cells stably expressing the SV5 phospho (P) protein were plated onto immunofluorescence slides, with the expression repressor tetracycline removed, and incubated at 37°C overnight. The slides were washed with PBS and fixed for 10 min in PBS containing 5% formaldehyde and 2% sucrose. After washing with PBS containing 1% calf serum and 0.1% azide, the cells were permeabilised by incubating in a solution of PBS containing 0.5% NP-40, 10% sucrose and 1% calf serum for 10 min. After further washing with PBS + 1% calf serum, the wells were incubated with mAb containing tissue culture supernantant for 1 hour at room temperature. After washing, the wells were incubated, for 1 hour at room temperature, with a Texas Red conjugated anti-mouse Ig

mAb, and DAPI (4,6-diamino-2-phenylindole) for DNA staining. After washing, the slides were incubated once again in the fixing solution, before mounting in the anti fading agent AF-1(Citifluor Products, Canterbury, Kent, U.K.).

### 19. Isotyping of anti-$Pk_{TH}$ monoclonal antibodies

The isotypes of the anti-$Pk_{TH}$ mAbs were determined using a isotyping kit for mouse mAbs from Serotec (Oxford, U.K.), using the retailers test procedure.

### 20. SPOT's analysis

Membranes containing synthesised peptides (Genosys Biotechnologies, Cambridge, U.K.) were blocked for 1 hour at room temperature with the commercially available Blocking solution (Genosys Biotechnologies, Cat. No. SU-07-250). The membranes were then incubated for 1 hour at room temperature with tissue culture supernatant containing the anti-Pk mAbs. After washing, a positive result was developed by incubating the membranes with a HRP conjugated anti- mouse Ig mAb and ECL. Once a result was obtained, the membranes were regenerated by a 30 min incubation of a solution of 8 M urea, 1% SDS and 0.1% β-mercaptoethanol, followed by a 30 min incubation with a solution of Ethanol. water and acetic acid in a ratio of 5:5:1. Finally the membranes were rinsed in PBS + 1% Tween 20 and stored at 4°C in blocking solution.

### 21. Covalent coupling of the $Pk_{TH}$ peptide to a CM5 sensor chip

The $Pk_{TH}$ peptide was concentrated on the carboxylated dextran matrix by an ion exchange effect at pH below the isoelectric point of the peptide, and covalently coupled via primary amine groups. The immobilisation run was performed at a flow of $5\mu l.min^{-1}$ in HBS, pH 7.4 (10 mM Hepes, 0.15 M NaCl, 3.4 mM EDTA, 0.05% surfactant 20). The carboxylated matrix was activated with 30 - 35 µl of a mixture of N-hydroxysuccinimide (NHS) and N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). After washing with HBS, $Pk_{TH}$ peptide (50µl) at a concentration of 50 $\mu g.ml^{-1}$ in HBS buffer was injected. Unreacted groups were blocked by the injection of 30µl of 1 M ethanolamine

hydrochloride / NaOH, pH 8.5, followed by 15 µl of 50 mM NaOH to remove covalently bound peptide.

## 22 Biosensor analysis of the interaction between the anti-Pk mAbs and the $Pk_{TH}$ peptide

All experiments were carried out on the BIALite (BIACORE AB, Stevenage, Herts, U.K.) system. The five anti-Pk mAbs at concentrations ranging from 1.4 nmol to 140 nmol in HBS was allowed to interact with sensor surface on which the $Pk_{TH}$ peptide was immobilised. The runs were performed at 37°C at a flow rate of 5µl.min$^{-1}$ during 8 min (40 µl injection), and a sensorgram recorded all time points and resonance units (RU). After the run, the surface of the sensor chip was regenerated using 0.2 M glycine pH 2.8 over 6 min (30 µl injection) and 50 mM HCl over 6 min (30 µl injection).

The apparent association and dissociation rate constants and the Equilibrium rate constant were calculated according to equilibrium reaction kinetics using BIAevaluation software (BIACORE AB). For a full review see Altschuh *et al* 1992.

## 23. Dot Blot analysis

Nitrocellulose filters were incubated with 100 µg.ml$^{-1}$ of His-Nef-Pk or SV5 P protein in PBS for 1 hour at room temperature. After blocking with 5% Marvel in PBS the nitrocellulose sheets were sandwiched between 84-well Terasaki plates, with 13µl of anti-Pk mAb containing tissue culture supernatant per well. After this 1 hour incubation at room temperature, the filters were cut into lengthwise strips so that each strip contained an area which had been incubated with each of the anti-Pk mAbs. Each nitrocellulose strip was then incubated for 1 hour at room temperature, with mixing, with one of a number of elution buffers. After this time, the strips were washed with PBS before being developed using a HRP conjugated anti-mouse Ig mAb and ECL.

## 24. Determination of conditions of recombinant protein elution using ELISA analysis

ELISA plates were coated with PBS containing 100 $mg.ml^{-1}$ of one of the four GST fusion proteins and stored at 4°C overnight. The plates were blocked with PBS containing 10% Marvel, at 37°C for 1 hour. Each row of wells was then incubated at 37°C for 1 hour with a different anti-Pk mAb. After washing with PBS, each column of wells was then washed a number of times with a different elution buffer, ensuring that each mAb was subjected to the same conditions. The wells were washed again with PBS before being developed with a HRP conjugated anti- mouse Ig mAb and TMB. After a set time, usually 5 min after the addition of TMB, the reaction was stopped by the addition of 1 M phosphoric acid.

## 25. Coupling of anti-Pk monoclonal antibodies to protein G Sepharose beads

Previously swelled PrG-Sepharose beads (Sigma-Aldrich,Co. Ltd., Poole Dorset, U.K.) were incubated with supernatant containing one of the five anti-Pk mAb for 2 hours at room temperature. After two washes with 0.2 M borate / 3 M NaCl, pH 9.0, the beads were then incubated for 30 min with the borate / NaCl buffer containing 0.02 M dimethylpimelidate, the coupling agent. After a further two washes with the borate / NaCl buffer, and a single wash with 200 mM ethanolamine, pH8.0, the beads were incubated for an hour with 200 mM ethanolamine. The coupled beads were then subjected to two washes with phosphate buffered saline (PBS), two washes with 0.2 M glycine pH 2.5 and then a further two washes with PBS. The mAb coupled beads were then stored in PBS + 0.1% sodium azide.

## 26. Batch purification of Pk tagged recombinant proteins

100 µl of anti-Pk monoclonal antibody coupled Protein G Sepharose bead slurry were washed with PBS incubated with one of the GST fusion proteins for 1 hour at room temperature. The beads were then washed with PBS before aliquoting into 20µl samples, which were washed 3 - 4 times with one of a number of elution buffers. The aliquots were

washed with PBS before being resuspended in SDS-PAGE loading buffer and analysed on a 10% SDS-PAG. The PAG`s were stained with Coomassie Brilliant blue type R-250

# RESULTS

## 1. CLONING, EXPRESSION AND PURIFICATION OF RECOMBINANT SIV PROTEINS

Previously in our laboratory, recombinant Simian Immunodeficiency Virus (SIV) proteins have been used for the production of multiple epitope subunit vaccines against SIV. The recombinant SIV proteins which were incorporated into the subunit vaccines were tagged at both ends with small oligopeptide tags, an N terminal Histidine (His) tag and a C terminal Pk tag. Together, these two tags allow tagged recombinant proteins to be detected and efficiently affinity purified (Hanke *et al* 1994). Section 1 of the Results describes the production and purification of two nonglycosylated SIV proteins, termed Nef and $Pr55^{gag}$.

### 1.1 The construction of pQ9*nef* Pk

All the nonglycosylated SIV genes, derived from the SIVmac251 clone BK28, had previously been cloned into the expression vector pQ9cPk (figure 18; Hanke *et al* 1994). When expressed, the recombinant proteins were tagged with a His tag and a Pk tag. The His tag consisted of 12 amino acids, six of which were histidine residues. The expressed His tag has a size of 1.4 kilodaltons (kDa). The Pk tag is a peptide of 14 amino acids which when expressed has a size of 2 kDa (see section 1.1 of the Introduction for more details).

New Medical Research Council (MRC) directives on vaccine development stated that all vaccines for SIV were to be derived from the infectious J5 molecular clone of SIVmac32H. The difference in the primary sequence between the J5 and BK28 molecular clones lies exclusively in the last 1.2kb of their genomes. This involves changes to the C

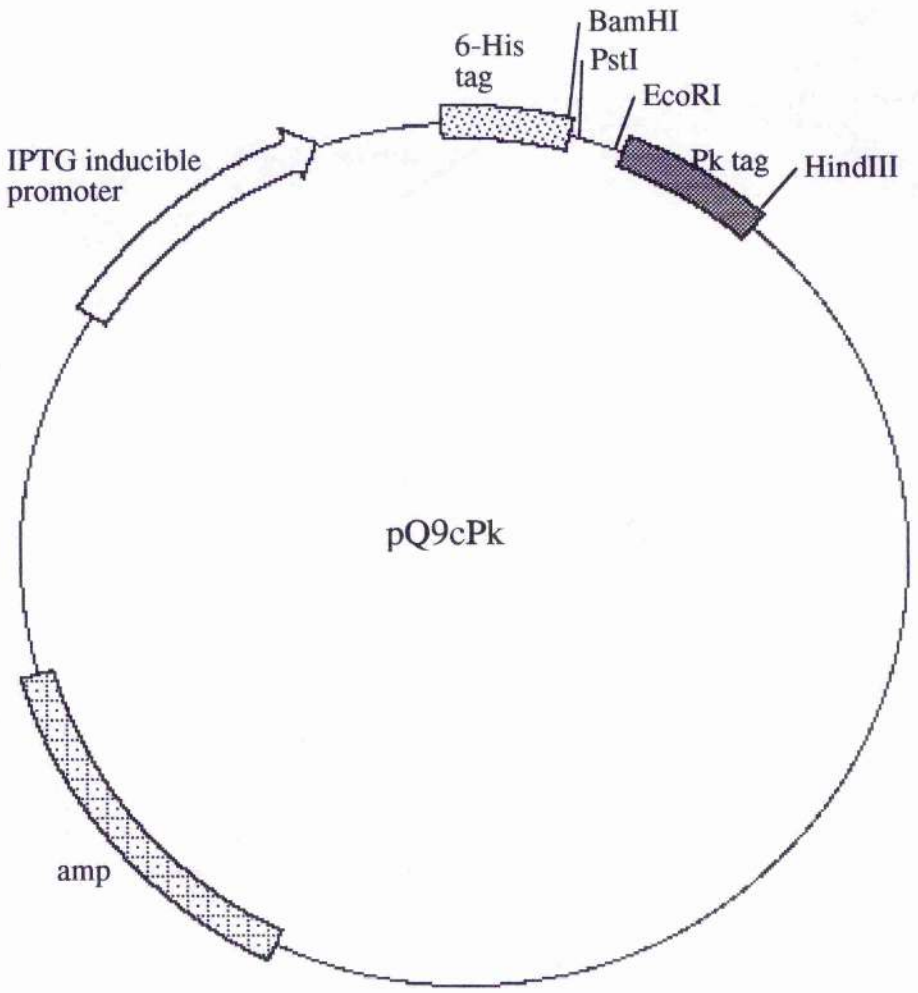



**Figure 18 : plasmid map of the expression vector which produces tagged recombinant proteins**

termini of the *env* and *rev* genes, the whole of the *nef* gene and all of the 3' long terminal repeat (LTR; Rud *et al* 1994). For future vaccine studies, the *nef* gene from the J5 molecular clone was cloned into the pQ9cPk expression vector to facilitate the purification of the J5 Nef protein.

The *nef* gene was amplified by the Polymerase Chain Reaction (PCR) from the J5 SIV molecular clone genomic sequence, using the oligonucleotide primers NefFOR and NefBACK (see Material and Methods). The oligonucleotide primers were designed to produce an amplified insert with *Bam*HI and *Eco*RI compatible restriction sites at the 5' and 3' ends respectively. The amplified *nef* gene was initially ligated into *Bam*HI and *Eco*RI restriction sites of the expression vector pBluescript to facilitate cloning. The *nef* insert was then excised from pBluescript and ligated into the pQ9cPk expression vector. Figure 19 shows the digestion pattern of pQ9*nef*Pk when cut with the restriction endonucleases *Bam*HI and *Eco*RI, which confirmed that *nef* had been cloned.

## 1.2 Expression and purification of His-Nef-Pk

The recombinant Nef protein was expressed as a fusion protein with a His tag at the N terminus and the oligopeptide Pk tag at the C terminus (His-Nef-Pk). Both tags have been shown to have little effect on the immunogenicity of the recombinant proteins they are fused to (Takacs & Gerard 1991; Randall *et al* 1993; Randall *et al* 1994). This ensures that any significant immune response elicited by a multiple epitope subunit vaccine which contains recombinant fusion proteins, is due for the most part to the proteins and not the oligopeptide tags.

### 1.2.1 Optimisation of expression of pQ9*nef*Pk

To confirm that recombinant Nef was produced from pQ9*nef*Pk, *E. coli* were transformed with the plasmid and expressed by induction at 37°C with 1 mM isopropyl-

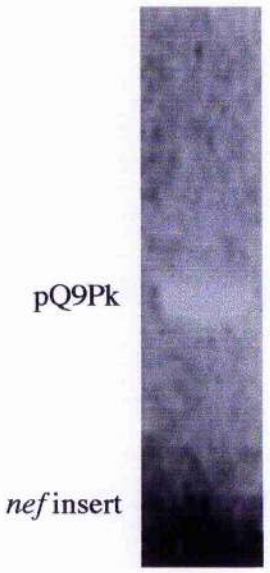



**Figure 19 : The restriction pattern obtained when digesting pQ9*nef*Pk with the restriction enzymes *Bam*HI and *Eco*RI.**

pQ9*nef*Pk was digested with the restriction enzymes *Bam*HI and *Eco*RI at 37°C for 1 hour. The mixture was loaded onto a 1% agarose - TBE gel. The DNA was visualised using ethidium bromide and UV light

β-D-thiogalactopyranoside (IPTG), for 2 hours. Total cell lysate (TCL) samples were loaded onto a 12% polyacrylamide gel (PAG) and electrophoresed. After transfer to nitrocellulose filters, the separated proteins were probed with the anti-Pk mAb SV5-P-k. Detection was by enhanced chemi-luminescence (ECL). The result can be seen in figure 20, lane a. This confirmed that the *nef* gene had been cloned and that recombinant His-Nef-Pk was being produced.

To ensure that sufficient amounts of recombinant protein were produced for protein purification and vaccine production, a number of experimental conditions were investigated to determine the optimal conditions for the expression of *nef*.

In many cases, unexpectedly low protein yields have been obtained even with the optimisation of the transcription and translation signals. This is especially true for many eukaryotic genes which are expressed from prokaryotic systems (Brinkman *et al* 1989; Gutierrez *et al* 1996). One of the main differences between the genetic sequences of eukaryotic and prokaryotic genes is the usage of codons. Some codons which eukaryotes target as "major" (i.e. are the most frequently used within many eukaryotic genes), prokaryotes target as "minor" (i.e. are the least used codons in prokaryotic genes; Brinkman *et al* 1989; Gutiérrez *et al* 1996). As prokaryotes only have surplus amounts of the tRNA molecules which are frequently used in host cell gene expression, the supply of charged tRNA's molecules corresponding to the major eukaryotic codons may be limited (Gutiérrez *et al* 1996). If the eukaryotic genes to be expressed contain a large percentage of codons which the bacterial expression system determine as "minor", or if many of these codons run concurrently within the gene, translation could stall owing to an exhaustion of the tRNA supply. This in turn causes a reduction in the amount of full length recombinant protein produced (Chen *et al* 1990; Gutiérrez *et al* 1996).

An example of codons considered by eukaryotes to be major, and prokaryotes to be minor, are the arginine codons AGG and AGA (Chen *et al* 1990; Schenk *et al* 1995). These two codons are considered to be the most minor in *E. coli*, and yet in many

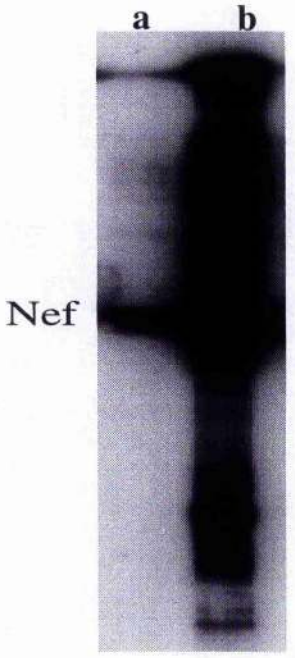



**Figure 20 : A Western Blot showing the relative amounts of His-Nef-Pk recombinant protein which was produced when various conditions were tried**

5ml cultures of *E. coli* previously transformed with pQ9*nef*Pk +/- pUBS520, were grown at 37°C until an OD600 of 0.5 - 0.7 was reached. The cultures were induced with 1 mM IPTG for 2 hours. 1 ml samples were removed and the cells pelleted. The pelleted cells were resuspended in SDS-PAGE loading buffer and loaded on to a 12.5% SDS-PAG. The separated proteins were transferred to nitrocellulose filters and probed with the anti-Pk mAb SV5-P-k. The blots were developed by ECL and visualised on photographic film.

key : a - pQ9*nef*Pk alone; b - pQ9*nef*Pk + pUBS520.

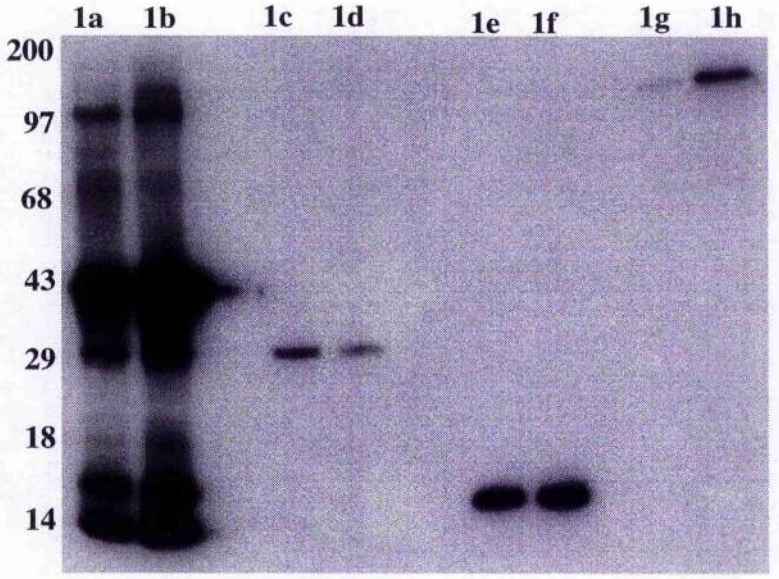
eukaryotic genes, such as HIV gp40, a high percentage of the AGG/AGA arginine codons are found. When these genes are expressed in *E. coli*, the amount of protein obtained is very small (Brinkman *et al* 1989). With SIV and HIV being genetically similar, SIV genes may also use codons which would make them susceptible to the production of low protein yields in prokaryotes.

To determine whether this was the case. *E.coli* strain XL1Blue bacteria were transformed with the expression vector pUBS520. This plasmid contains the *dnaY* (or *argU*) gene which encodes the $tRNA_{AGA/AGG}$ molecules (Brinkman *et al* 1989; Schenk *et al* 1995). Bacteria containing pUBS520 were made competent and transformed with pQ9*nef*Pk to produce double transformants. Bacterial cultures containing both plasmids were induced with 1 mM IPTG at 37°C for 2 hours and samples taken for TCL analysis by Western blotting (figure 20).

When a comparison of the cultures induced in the presence or absence of the extra tRNA molecules was made, approximately a fivefold increase in the amount of protein produced was observed in those cultures where the extra tRNA molecules had been present (figure 20, lanes a & b). In addition, there were a number of other protein bands in the TCL sample where the extra tRNA molecules had been present, which were not observed in the sample where the extra tRNA molecules were absent. These bands were thought to be degradation and aggregation products of recombinant His-Nef-Pk, due to the large amounts of protein being produced.

In addition to the *nef* gene, other nonglycosylated SIV genes had been cloned into the pQ9cPk expression vector and the conditions for the optimisation of gene expression determined (Hanke *et al* 1994). However, at that time, the use of the extra tRNA molecules to increase protein production had not been tested.

The expression of the other nonglycosylated SIV genes was performed using identical conditions to those used for His-Nef-Pk and the results can be seen in figure 21. For some genes the presence of the extra arginine tRNA molecules did not effect the amount of protein produced, e.g. *rev* (lanes 1c & 1d) and *Npol* (1e & 1f). For others the effect was negligible, only increasing protein production 1 or 2 fold, e.g. *rt* (lanes 1a & 1b)



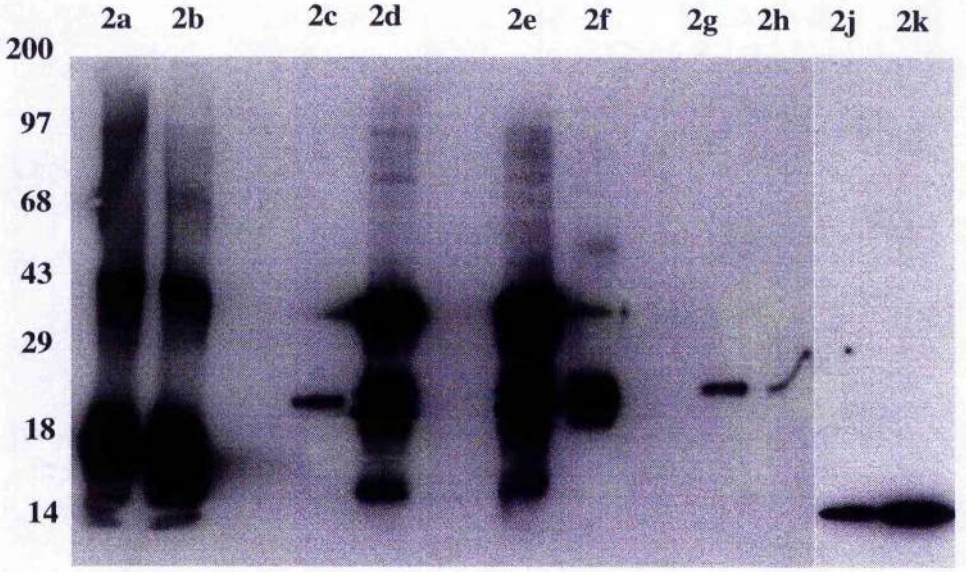




**Figure 21 : Western blot analysis showing a comparison between the levels of protein produced from bacteria transformed with pQ9SIVPk and pQ9SIVPk plus pUBS520.**
Small scale cultures of bacteria containing either pQ9SIVPk or pQ9SIVPk + pUBS520 were induced with 1 mM IPTG, harvested and the pellets obtained resuspended in SDS-PAGE loading buffer. Samples were then loaded onto a 12.5% SDS-PAG. The separated polyproteins were transferred onto nitrocellulose filters, and the filters probed with the mAb SV5-P-k. The protein bands were visualised by ECL.
Key: **1a** - RT; **1b** - RT + tRNA's; **1c** - Rev; **1d** - Rev + tRNA's; **1e** - Npol; **1f** - Npol + tRNA's; **1g** - Nuc; **1h** - Nuc + tRNA's; **2a** - Vif; **2b** - Vif + tRNA's; **2c** - Vpx; **2d** - Vpx + tRNA's; **2e** - Vpr; **2f** - Vpr + tRNA's; **2g** - Tat; **2h** - Tat + tRNA's; **2j** - Prot; **2k** - Prot + tRNA's

and *vif* (lanes 2a & 2b) However, *nuc* showed an approximate tenfold increase in protein production when the extra arginine tRNA molecules were present (lanes 1g & 1h).

The length of induction can also effect the levels of protein produced. The longer the induction, the more time bacterial proteases have to degrade the recombinant proteins once maximal translation has been reached. For the *nef* gene of the SIV molecular clone BK28, the optimal length of induction was 30 min (Hanke *et al* 1994). After this time, the levels of recombinant protein produced did not increase. For the newly cloned J5 *nef* gene, it was important to determine if this was also the case.

Small scale bacterial cultures previously transformed with both pQ9*nef*Pk and pUBS520 were induced with 1 mM IPTG at 37°C, and samples taken at 0, 30, 60, 120 and 180 minutes. The TCL samples were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS - PAGE) and analysed by Western blotting (figure 22i).

When comparing the levels of protein obtained at each time point, it was observed that the protein yield increased with time [figure 22 (i)]. However, it was also clear that the longer the time of induction, more degradation products were obtained (compare 30 min and 180 min lanes). It was also observed that recombinant protein was produced when IPTG had not been present (see 0 min lane). This may have been due to insufficient repression of the promoter by the LacI repressor protein. However, as the growth of the bacteria was unaffected by this "background" expression, steps to rectify it were unnecessary.

Initially, *nef* was induced with 1 mM IPTG. This concentration of IPTG may have been far in excess of what was necessary to achieve maximal induction. Small scale bacterial cultures containing pQ9*nef*Pk and pUBS520 were induced at 37°C for 2 hours with the following final concentrations of IPTG : 1 mM, 0.5 mM, 0.25 mM, 0.1 mM and 25 $\mu$M. Total cell lysate samples for each IPTG concentration were separated by SDS - PAGE and analysed by Western blotting [figure 22 (ii)]. A comparison was made of the amount of protein obtained for each IPTG concentration. All final concentrations of IPTG

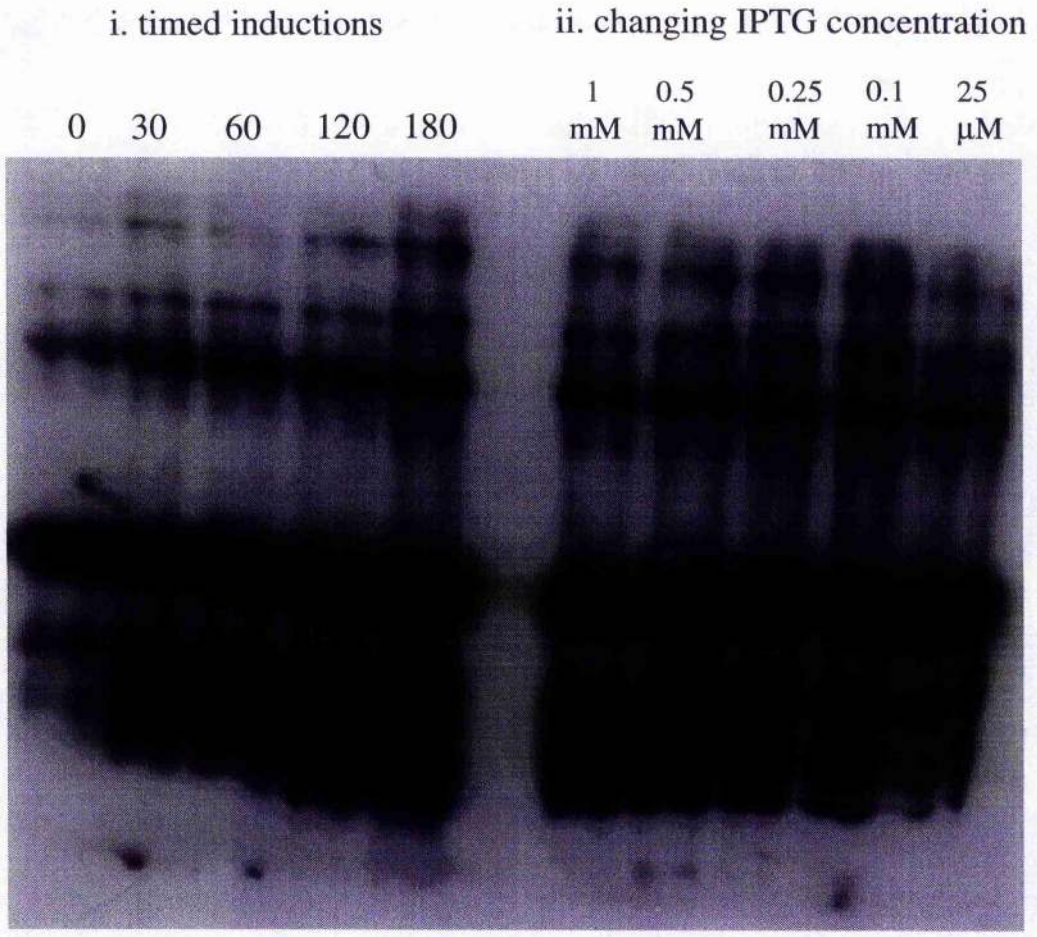



**Figure 22 : Western blot analysis of bacterial total cell lysates containing the His-Nef-Pk fusion protein**

Bacterial cultures were previously transformed with pQ9*nef*Pk and pUBS520. These were then induced with IPTG, harvested and resuspended in SDS-PAGE loading buffer. Samples were loaded onto a 12.5% SDS-polyacrylamide gel. Once transferred to nitrocellulose filters, the blots were probed with the mAb SV5-P-k and developed by ECL.

Key : i. timed inductions with samples taken at 30, 60, 120 and 180 minutes; ii. inductions using various concentrations of ITPG; 1 mM, 0.5 mM, 0.25 mM, 0.1 mM & 25 µM.

gave the same yields of protein. For all subsequent inductions, a concentration of 25 $\mu$M IPTG was used.

From the preceding data, it was determined that the optimal induction of *nef* gene expression occurred in the presence of the *dnaY* gene product, at 37°C for 30 min, and with a final concentration of 25 μM of IPTG. These optimal conditions were applied to all subsequent inductions.

### 1.2.2 Purification of His-Nef-Pk

The expressed recombinant Nef protein was tagged at the N terminus by a His tag and at the C terminus by the oligopeptide Pk tag. These two tags together provided the basis of a two step affinity purification protocol which ensured that only full length recombinant proteins were purified (Randall *et al* 1993; Randall *et al* 1994). This two step purification protocol was initially developed for the production of antibody - antigen complexes, specifically solid matrix antibody antigen (SMAA) complexes, to be used for immūnisation purposes.

The first step in the purification protocol involved the histidines within the His tag. The His tag can bind reversibly to nickel (Hochuli *et al* 1987), even when the nickel is immobilised on agarose beads (e.g. a $Ni^{2+}$ - NTA resin). The use of a nickel affinity column ensured that only His tagged recombinant proteins bound, and that non-tagged proteins were washed through. Elution from the nickel column was achieved by competition with relatively high concentrations of imidazole, e.g. 250 mM (see section 1.1.1a of the Introduction for more details).

The second part of the two step purification protocol uses the mAb SV5-P-k, which recognises the Pk tag, immobilised on Sepharose - Protein A beads. When the eluant from the nickel affinity purification step was passed through the immuno-affinity column, only full length recombinant proteins containing both the N terminal His tag and the C terminal Pk tag were retained. Elution of recombinant proteins from this column is difficult because SV5-P-k binds to the Pk tag with a high affinity (Randall *et al* 1993; Randall *et al* 1994). This makes the two step purification protocol ideal for the production of antibody - antigen

complexes which can then be used as SMAA complexes. However, as this experiment did not require antibody - antigen complexes, the immuno-affinity purification step was not used to purify His-Nef-Pk recombinant protein.

Bacterial cultures expressing His-Nef-Pk were optimally induced with IPTG, as described in section 1.2.1. The cells were lysed using lysozyme and Triton-X100, sonicated and centrifuged. The soluble protein fraction was mixed at room temperature with $Ni^{2+}$-NTA beads and the resulting heterologous mixture was used to pack a column. After washing with PBS, the bound proteins were eluted with a solution containing 250 mM imidazole. Samples were taken at each stage of this procedure and analysed by Western blotting (figure 23). As can be seen in lane c, His-Nef-Pk recombinant protein was eluted from the nickel affinity column. However, when comparing to the TCL sample shown (lane a), not all of the protein which was bound to the column was eluted. This was confirmed when studying a sample of the nickel beads after protein elution (lane d). From this analysis, it was estimated that approximately 70% of the bound protein was eluted.

## 1.3 The Construction of pQ9*gag* Pk

Previously, the genetic sequences for the structural proteins p15, p17 and p27 had been cloned into the expression vector pQ9cPk (Hanke *et al* 1994). In order to simplify the purification of the structural proteins, the *gag* gene was cloned into the expression vector pQ9cPk. The *gag* gene encodes the $Pr55^{gag}$ polyprotein from which the structural proteins are derived. This meant that in order to obtain purified structural proteins, only a single recombinant protein needed to be synthesised and purified, instead of three.

The *gag* sequence was amplified by PCR from the SIVmac32H J5 molecular clone using the primers 17FOR and 15BACK (see Materials and Methods). The oligonucleotide primers were designed such that 17FOR contained a *Bam*HI compatible restriction site and 15BACK contained an *Eco*RI compatible restriction site. This created an amplified insert with a 5' *Bam*HI compatible restriction site and a 3' *Eco*RI compatible restriction site. To aid cloning, the insert was ligated into the *Bam*HI and *Eco*RI restriction sites of the vector

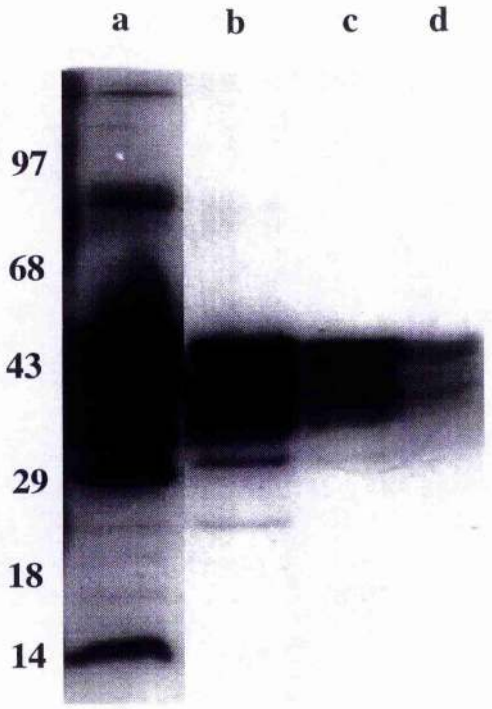



**Figure 23 : Western blot analysis of the protein fractions collected during the Nickel chelate purification of His-Nef-Pk.**

500 ml bacterial cultures which had previously been transformed with pQ9*nef*Pk & pUBS520, were induced at 37°C for 30 min with 25 µM IPTG. The cells were lysed and centrifuged, and the soluble protein fraction incubated with Ni-NTA beads for 1 hour. After washing with PBS the bound protein was eluted with 250 mM imidazole. Samples were collected at each step of the procedure and loaded onto a 12.5% PAG. The separated proteins were transferred to nitrocellulose filters and probed with the mAb SV5-P-k

**a** - total cell lysate; **b** - nickel beads before elution; **c** - eluted recombinant protein; **d** - nickel beads after elution

pBluescript. However, when excising the *gag* insert from pBluescript, it was noticed that the insert contained an internal *Bam*HI site. This made it impossible for the second ligation into pQ9cPk to be carried out in a single step. The transfer of the *gag* gene from pBluescript to pQ9cPk was, therefore, carried out in two stages. Initially, the 3' *Bam*HI / *Eco*RI fragment was ligated into the relevant restriction sites of pQ9cPk, and then using a partial digest from the pBluescript expression vector, the 5' *Bam*HI / *Bam*HI fragment was ligated into the *Bam*HI restriction site of pQ9cPk, and in front of the previously ligated 3' fragment. However, when sequencing was performed to verify the orientation of the 5' insert, it was discovered that the insert had been ligated in the incorrect orientation (see figure 24). To rectify this, the second step of the ligation was repeated. To ensure the correct orientation of the insert, the plasmid vector was dephosphorylated prior to the ligation of the insert. Restriction digestion of the gene confirmed that the gene had been cloned (see figure 25), and sequencing of the *gag* gene confirmed the orientation (data not shown).

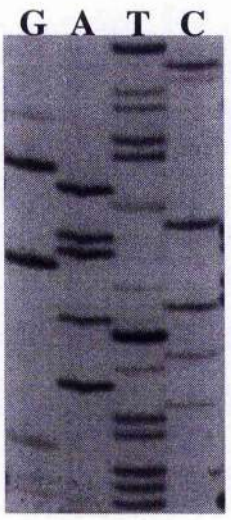
## 1.4 Expression and purification of the His-Pr55$^{gag}$-Pk polyprotein

As with *nef*, the recombinant protein produced from the *gag* gene was tagged at the N terminus with a His tag and the C terminus with the Pk tag. This protein was known as His-Pr55$^{gag}$-Pk.

### 1.4.1 Optimisation of the expression of pQ9*gag*Pk

In order to confirm the orientation of the *Bam*HI / *Bam*HI insert and to ensure that recombinant protein was being produced from the pQ9*gag*Pk expression vector, *E. coli* were transformed with pQ9*gag*Pk. Expression was induced with 1 mM IPTG, at 37°C for 3 hours. Total cell lysate samples were loaded onto a 12% PAG and analysed by Western blotting. The nitrocellulose filters were probed with the anti-Pk mAb SV5-P-k, and detection was by ECL (figure 26).

**i.**




**ii.**

5'- GATATTGCAGGAACAACTAGTTCAGTAGATGAACAAATCCAGTGGATGT

3' - CTATAACGTCCTTGTTGATCAAGTCATCTAC**TTGTTTAGGTCACCTACA**

AAGACAACAGAACCCCATACCAGTAGGCAACATTTACAGGAGATGGATCC -3'

**TTCTGTTGTCT**TGGGGTATGGTCATCCGTTGTAAATGTCCTC TACCTAGG - 5'

**Figure 24 : a sequencing gel (i) and corresponding DNA sequence (ii) showing that initial attempts to clone *gag* resulted in the 5' *Bam*HI / *Bam*HI insert being ligated in the incorrect orientation.**

The nucleotides shown in bold indicate those shown on the sequencing gel. The nucleotides underlined correspond to the *Bam*HI restriction site

*Bam*HI/*Eco*RI insert
*Bam*HI/*Bam*HI insert

**Figure 24 : The restriction pattern obtained when digesting pQ9*gag*Pk with the restriction enzymes *Bam*HI and *Eco*RI.**

pQ9*gag*Pk was digested with the restriction enzymes *Bam*HI and *Eco*RI at 37°C for 1 hour. The mixture was loaded onto a 1% agarose - TBE gel. The DNA was visualised using ethidium bromide and UV light.

As can be seen recombinant protein was produced, and because the protein produced was of the correct size, it was determined that the 5' *Bam*HI / *Bam*HI insert had been ligated into the plasmid vector in the correct orientation.

As with His-Nef-Pk, a number of experimental conditions were tested to determine optimal conditions for the induction of *gag*. From the experiments performed, it was determined that optimal gene expression was obtained when pQ9*gag*Pk was induced with 1 mM IPTG, at 26°C, for 3 hours. The presence of extra tRNA molecules had no effect on protein yield.

### 1.4.2 Purification of His-Pr55$^{gag}$-Pk

The induction culture volume was increased, and gene expression induced using the conditions described in section 1.4.1. The pelleted cells were lysed with lysozyme and Triton X-100, sonicated and centrifuged. The soluble protein fraction was removed for further analysis.

The soluble protein fraction thought to contain His-Pr55$^{gag}$-Pk was mixed with $Ni^{2+}$-NTA beads for 1 hour and the heterologous mixture used to pack a column. As with His-Nef-Pk, 250 mM imidazole was used for elution. Unfortunately, no protein elution was detected (data not shown). One unusual observation made was that protein was detected binding to the column, but once elution was attempted, this protein was no longer detectable. The reason for this was unclear, and has not yet been explained.

As insufficient protein was obtained from this purification step for either immunisation studies, or the second purification step, it may therefore be more efficient to express and purify the structural proteins individually rather than to try and obtain sufficient expression and purification from the whole *gag* gene.

## 1.5 Conclusions

The purified His-Nef-Pk was used, in collaboration with E. Tijhaar, to produce anti-sera in rabbits. Figure 27 shows a Western blot in which untagged SIV Nef was

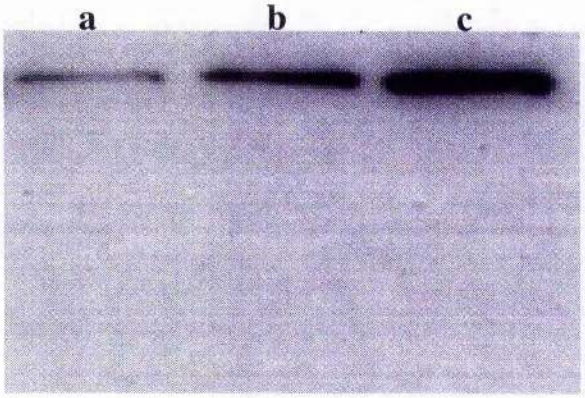



**Figure 26 : Western blot analysis showing the production of His-Pr55$_{gag}$Pk after the induction of pQ9*gag*Pk**

Small cultures of *E. coli* which had previously been transformed with pQ9*gag*Pk were induced with 1 mM IPTG at 37°C. Samples were taken after 0, 1 and 2 hours. The pelleted cells were resuspended in SDS-PAGE loading buffer and loaded onto a 12.5% PAG. After transfer to a nitrocellulose filter, the proteins were probed with the anti-Pk mAb SV5-P-k and visualised by ECL.

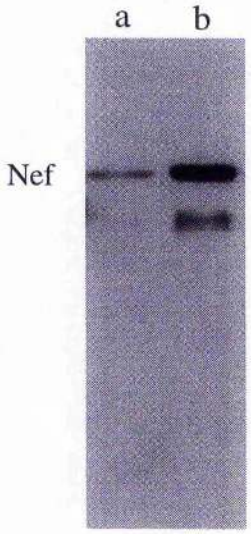



**Figure 27 : Western blot showing the detection of untagged recombinant SIV Nef by the developed Nef anti-sera**

Bacteria containing the expression vector pQ9*nef*7Pk were induced with 1 mM IPTG at 37°C for 1 hour. Samples were taken and the cells pelleted. The pelleted cells were resuspended in SDS-PAGE loading buffer and loaded onto a 12.5% PAG. After transfer to a nitrocellulose filter, the protein was probed with the Nef anti-sera. Protein was detected using and anti-rabbit conjugate as the secondary antibody and ECL.

Key : a - uninduced sample; b - sample induced with IPTG.

probed with the rabbit anti-sera obtained. Both the purified His-Nef-Pk, and the Nef anti-sera, that were generated in this project have been used for a number of different purposes by a variety of research groups. Samples of purified protein and Nef anti-sera have been sent to the Medical Research Council to be used for a number of purposes.

The purified Nef has been used to generate mAbs to Nef (K. Kent, personal communication), and as an antigen in T lymphocyte proliferation assays to produce an immune response in SIV infected macaques.

In addition, the recombinant Nef was incorporated into multivalent SMAA complexes for immunisation / protection studies. Unfortunately, although the animals made an antibody and cell mediated immune response to the recombinant SIV proteins within the vaccine, they were not protected upon challenge (J. Stott, personal communication).

## 2. DEVELOPMENT, SCREENING AND CHARACTERISATION OF MONOCLONAL ANTIBODIES RAISED TO THE Pk TAG

The failure to induce protective immunity in macaques was disappointing. However, it was felt that perhaps recombinant proteins presented with a different adjuvant may be more successful. The problem was that the two tag purification system developed was only capable of producing antibody - antigen complexes because of the high affinity of the SV5-P-k monoclonal antibody for its tag. Consequently, it was decided to develop a number of monoclonal antibodies with differing binding affinities to the Pk tag, in the expectation that the resulting mAbs may be used in immuno-affinity column chromatography. This would enable Pk tagged recombinant proteins to be used for a number of procedures, for which SV5-P-k had been unsuitable. This section of the Results chapter describes the generation of additional mAbs to the Pk tag, their characterisation, and initial attempts to further develop the Pk tag for purification purposes.

## 2.1 Immunisation, development and screening of mAb-secreting hybridomas

**2.1.1 Immunisation schedule for the development of an anti-$Pk_{TH}$ immune response** Six BALB/c mice were primed by injection with 10μg of a peptide containing the Pk tag. If a peptide as small as the Pk tag is to generate an immune response, without the aid of a carrier protein, it must contain a helper T cell epitope (McLean *et al* 1992). A peptide complex was constructed which contained the Pk tag amino acid sequence fused to a class II restricted T (helper T) cell epitope, derived from sperm whale myoglobin. This peptide was designated the $Pk_{TH}$ peptide (figure 28). The complex was constructed with a branched structure of eight peptide molecules surrounding a lysine core. The branched nature of the peptide enhances the immune response signal produced by the immunised animal (McLean *et al*1992; Marsden *et al* 1992). The helper T cell epitope fused to the Pk tag sequence, was known to be active in BALB/c mice (McLean *et al* 1992, Tam 1988).

Gly. Lys. Pro. Ile. Pro. Asn. Pro. Leu. Leu. Gly. Leu. Asp. Ser. Thr. Asn. Lys. Ala. Glu. Leu. Phe. Arg. Lys. Asp. Ile. Ala. Ala. Lys. Tyr. Lys. Glu

**Figure 28 : the amino acid sequence of the $Pk_{TH}$ peptide used for the immunisation of BALB/c mice.**
The amino acid sequence of the original Pk tag is shown underlined.

The soluble peptide was injected intraperitoneally. After 3 - 4 weeks, the mice were injected intraperitoneally a second time with the $Pk_{TH}$ peptide and over the next 21 days, blood samples were taken and the specific serum antibody responses observed. After 21 days, the immune response was boosted by an intravenous injection into the tail vein. Three to four days later, the two mice were sacrificed and their spleens removed.

### 2.1.2 Development and care of anti-$Pk_{TH}$ mAb secreting hybridoma clones

The monoclonal antibody-secreting hybridomas were made in the classical way by the fusion of splenocytes, from the immunised mice, to SP2/0 melanoma cells. SP2/0 melanoma cells were used for a number of reasons: they grow rapidly and are relatively easy to maintain; they do not constitutively produce any globulin chains; and they do not have the purine salvage enzyme hypoxanthine guanosine phosphoribosyl transferase (HGPRT; see section 2.1.1 of the Introduction for further details). In the now standard protocol, adapted from that devised by Kohler and Milstein in 1975, the fused cells are grown in the presence of foetal calf serum (FCS). To obtain mAbs with few contaminating proteins, the FCS must be removed. This is carried out by "weaning" the hybridoma cells from the FCS at a later stage in the protocol. To evaluate to effect of FCS on the growth and development of mAb secreting hybridoma clones, and also to remove the weaning stage in the protocol, half of the fused cells were grown in Protein Free Hybridoma Media (PFHM), and half in PFHM containing 10% FCS. Each fused cell suspension was plated into 96 well microtitre plates.

A comparison was made between those plates with hybridoma colonies grown in the presence of FCS and those where FCS was absent. The following characteristics were observed. As expected, the presence of FCS caused the hybridoma cells to grow at a faster rate and to form larger colonies than the cells grown in media without FCS. However, when studying the individual wells in more detail it was noted that there was more cell debris present within wells where FCS was present. This could have been due to the FCS used, or to the FCS having a detrimental effect on the macrophage feeder cells present. When a comparison was made of the relative number of hybridoma clones visible in both sets of plates, at the time of counting, there were more hybridoma colonies visible in, and therefore, potentially more colonies screened from, the 96 well plates where FCS was present in the media. A comparison of the number of hybridoma clones screened for each condition can be seen in table 5.

| | Average number of wells per plate containing hybridoma colonies | Potential number of wells available for screening | Total number of wells screened |
|---|---|---|---|
| with FCS | 58.3 | 1340 | 407 |
| without FCS | 38.6 | 598 | 423 |

**Table 5 : a numerical comparison between hybridoma clones grown in the presence and absence of foetal calf serum.**

More hybridoma clones were screened from those plates where FCS was absent due to a fungal infection contaminating the plates where FCS had been present. The plates without FCS eventually succumbed to the fungal infection 2 -3 weeks after those where FCS had been present.

### 2.1.3 Screening of the hybridoma clones produced for anti-$Pk_{TH}$ mAbs

The hybridoma clones secreted mAbs into the tissue culture media in which they were grown. To select anti-$Pk_{TH}$ mAb positive clones, 70% of the tissue culture media from wells containing confluent hybridoma colonies was removed, and screened by Enzyme-linked Immunosorbent Assay (ELISA). The ELISA plates were coated with the $Pk_{TH}$ peptide antigen. Those colonies which screened positive were transferred to 24 well plates and allowed to expand. The tissue culture media was screened again, to confirm that the clones were continuously secreting anti-$Pk_{TH}$ mAbs, before the clones were sub-cloned. Sub-cloning was performed in order to ensure that the hybridoma clones secreting the anti-$Pk_{TH}$ mAbs originated from a single hybridoma cell, and were therefore homogeneous. From this fusion, 24 anti-$Pk_{TH}$ mAb secreting hybridoma clones were produced. They were numbered according to the order in which they were screened.

## 2.2 Characterisation of anti-$Pk_{TH}$ monoclonal antibodies

The 24 positive hybridoma clones obtained were grown in large culture volumes and the growth media, into which the mAbs were secreted, collected. The media containing the secreted mAbs were subjected to a number of tests in order to define the characteristics of each mAb.

### 2.2.1 ELISA analysis of the anti-$Pk_{TH}$ mAbs

The hybridoma colonies had originally been screened by ELISA where the plates had been coated with the $Pk_{TH}$ peptide antigen used to immunise the mice. From this analysis, 24 hybridoma clones which secreted anti-$Pk_{TH}$ mAbs were obtained. The next step was to determine whether the 'new' mAbs would recognise either the Pk tag when fused to recombinant proteins, or the native P and V proteins of SV5, from which the Pk tag was derived (Randall *et al* 1987; Southern *et al* 1991). ELISA plates were coated with either purified His-Nef-Pk, or BALB/c cell extracts containing the P and V proteins of SV5, and probed with each of the mAbs. The secondary antibody used for detection was an anti- mouse Ig HRP conjugate. The results are shown in table 6.

The results of the ELISA studies were disappointing. It appeared that of the 24 mAbs, only four (No's 2, 46, 196, & 337) recognised both the native P and V proteins and the His-Nef-Pk recombinant protein. There were three additional mAbs (no's 287, 523, & 727) which showed weak binding to extracts of P and V expressing cells, but no binding to His-Nef-Pk. The reason for this may have been that these three mAbs recognised a cross-reacting epitope within the BALB/c cell extracts rather than the Pk epitope itself.

To ascertain whether this was the case, the V gene, which had previously been cloned into a bacterial expression vector pET11C, was expressed in *E. coli* and the protein produced used to coat ELISA plates. Again the V protein was not purified and the cell lysate was used to coat the ELISA plates. No mAb binding was shown. This confirmed that mAbs no 287, 523, & 727 cross-reacted with an epitope within BALB/c cells.

| Clone Number | $Pk_{TH}$ peptide | Nef - Pk | P protein (balb/c expressed) | V protein (balb/c expressed) | V protein (*E. coli* expressed) |
|---|---|---|---|---|---|
| SV5-P-k | +++ | +++ | +++ | +++ | +++ |
| 1 | +++ | - | - | - | - |
| 2 | +++ | +++ | +++ | +++ | +++ |
| 3 | +++ | - | - | - | - |
| 36 | +++ | - | - | - | - |
| 46 | +++ | +++ | +++ | +++ | +++ |
| 146 | +++ | - | - | - | - |
| 187 | +++ | - | - | - | - |
| 196 | +++ | +++ | +++ | +++ | +++ |
| 287 | +++ | - | ++ | ++ | - |
| 337 | +++ | +++ | +++ | +++ | +++ |
| 467 | +++ | - | - | - | - |
| 523 | +++ | - | ++ | ++ | - |
| 534 | +++ | - | - | - | - |
| 537 | +++ | - | - | - | - |
| 551 | +++ | - | - | - | - |
| 579 | +++ | - | - | - | - |
| 622 | +++ | - | - | - | - |
| 638 | +++ | - | - | - | - |
| 652 | +++ | - | - | - | - |
| 727 | +++ | - | ++ | ++ | - |
| 757 | +++ | - | - | - | - |
| 759 | +++ | - | - | - | - |
| 768 | +++ | - | - | - | - |
| 812 | +++ | - | - | - | - |
| SV5-HN-4a | - | - | - | - | - |

**table 6 : A summary of the ELISA data obtained on the recognition of all 24 anti-$Pk_{TH}$ mAb's to various Pk epitope containing recombinant proteins.**

ELISA plates were coated with either His-Nef-Pk, SV5-Pprotein or SV5-V protein. Each of these plates was then probed with the anti-$Pk_{TH}$ mAb's developed. The binding patterns of all the mAb's was shown by the relative colour change when the assay was developed by TMB.

Key :- +++ strongly positive reaction; ++ weakly positive reaction; - no reaction

### 2.2.2 Isotyping

The isotype of an antibody is the antigenic determinant of the constant - region of the immunoglobulins within the antibody. These determinants define each heavy-chain class and subclass and each light-chain type. The class and sub-class of an antibody defines its properties and to a certain degree its method of action.

A range of anti-isotype antibodies are commercially available for the determination of the class, and sub-class, of mAbs. In this study, an isotyping kit from Serotec was used to isotype mAbs in tissue culture supernatants, and was based on the principle of red blood cell agglutination. Sheep anti-isotype antibodies were coupled to red blood cells. When the anti-isotype antibody came into contact with a mAb of the relevant isotype, the red blood cells agglutinated and formed a lattice. When the antibody coated red blood cells were added to a supernatant containing a class of antibody which the antibody did not recognise, no agglutination was observed. The red blood cells settled to the bottom of the well, forming a small circle or "button". The isotyping results for the 24 anti-Pk mAbs are shown in table 7.

Most of the antibodies were from the IgG class of immunoglobulins. This was not surprising as IgG is the most common immunoglobulin produced. The fact that four were IgA was slightly surprising, because although IgA is found in high concentrations in external secretions, it is found in relatively small concentrations in serum.

### 2.2.3 Immunoblotting of His-Nef-Pk

To confirm that only four of the 24 anti-$Pk_{TH}$ mAbs recognised the Pk tag (or the epitope within the P/V proteins), all 24 mAbs were used to probe an immunoblot of His-Nef-Pk.

A sample of purified His-Nef-Pk recombinant protein was electrophoresed on a 12.5% PAG and transferred to nitrocellulose filters. The filters were cut into strips and each strip probed with one of the 24 mAbs. SV5-P-k was used as a positive control, and the mAb SV5-HN-4a (a mAb which recognises the SV5 HN protein) was used as a negative control. The secondary antibody was an anti-mouse Ig HRP conjugated mAb, and

| Clone Number | Isotype | Clone Number | Isotype |
|---|---|---|---|
| SV5-P-k | IgG2a | 534 | IgG1 |
| 1 | IgG1 | 537 | IgG1 |
| 2 | IgG1 | 551 | IgG1 |
| 3 | IgG1 | 579 | IgM |
| 36 | IgG1 | 622 | IgG1 |
| 46 | IgG1 | 638 | IgA |
| 146 | IgG1 | 652 | IgG1 |
| 187 | IgG1 | 727 | IgG1 |
| 196 | IgG1 | 757 | IgA |
| 287 | IgG1 | 759 | IgA |
| 337 | IgG1 | 768 | IgG1 |
| 467 | IgG1 | 812 | IgA |
| 523 | IgG1 | | |

**Table 7 : The isotypes of the newly developed anti-$Pk_{TH}$ monoclonal antibodies**
The isotype of the mAb's were defined using the Serotec isotyping kit. 100µl samples of each mAb-containing supernatant were incubated with 100µl of each of the anti-isotype red blood cell conjugated mAb's. The resulting mixtures were left to stand for 1 hour to allow agglutination to occur, or the red blood cells to settle to the bottom of the wells. The results were observed by studying the bottom of the wells from underneath.

detection was by ECL (figure 29). Lanes 1 and 10 are the positive and negative controls respectively. This immunoblot shows the binding of three of the four mAbs which had recognised the Pk tag in all previous characterisation experiments (figure 29, lanes 3, 6 & 8). The immunoblot also shows that one of the mAbs which cross-reacted with a balb/c cellular epitope, did not recognise the Pk tag when it was fused to a recombinant protein (figure 29, lane 7).

### 2.2.4. Immunofluorescence studies in a SV5 Phospho (P) protein expressing cell line

Within our research group, a number of cell lines which express the P and V proteins of SV5 have been developed. Gene expression, within this system, is controlled by the tetracycline transactivator protein (tTa). The tetracycline transactivator protein consists of the TetR protein fused to the transcriptional activator domain of Herpes Simplex Virus (HSV; Gossen & Bujard 1992). The tetracycline transactivator protein stimulates transcription from the mammalian promoter controlling the gene of interest, for example the *P* or *V* genes of SV5, when it combines with upstream *tet* operators. The presence of tetracycline reversibly represses gene expression (Gossen & Bujard 1992). There is also a reverse phenotype to the system described, where the addition of tetracycline, or a tetracycline derivative, stimulates the binding of the reverse tTa (rtTa) to the operator (Gossen *et al* 1995). In this case tetracycline needs to be present for transcription to occur. For the purposes of the immunofluorescence data presented here, a BALB/c P expressing cell line with transcription activated by the removal of tetracycline was used.

BALB/c P expressing cells were washed with phosphate buffered saline (PBS) to remove the tetracycline, and plated onto immunofluorescence slides. The slides were placed at 37°C overnight to allow for the accumulation of the expressed P protein. After fixing and permeabilisation, each well of the slide was incubated with one of the mAbs to be tested. For detection, the secondary antibody was an anti-mouse Texas Red conjugate and the fluorescence was visualised under green light (figure 30).

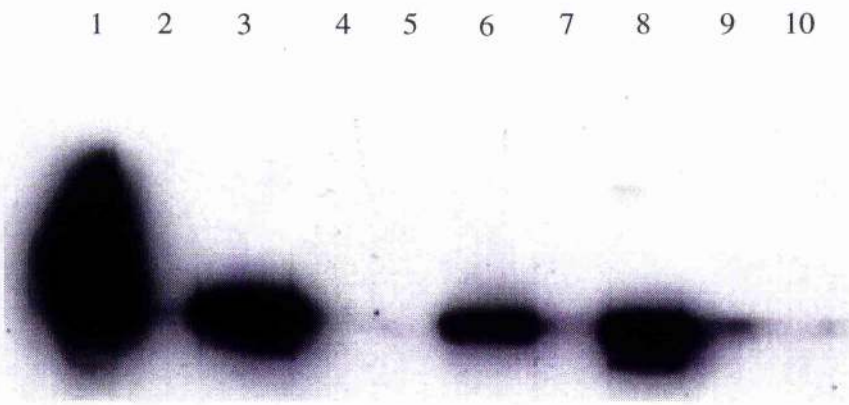



**Figure 29 : Western blot analysis of His-Nef-Pk**

Nickel purified His-Nef-Pk was loaded onto a 12.5% SDS-PAG. The fusion protein was transferred onto a nitrocellulose filter, and the filter cut into strips. Each strip was probed with a different anti-Pk$_{TH}$ mAb. Detection was by ECL.

Key : **1** - SV5-P-k; **2** - 36; **3** - 46; **4** - 146; **5** - 187; **6** - 196; **7** - 287; **8** - 337; **9** - 467; **10** - SV5-HN-4a

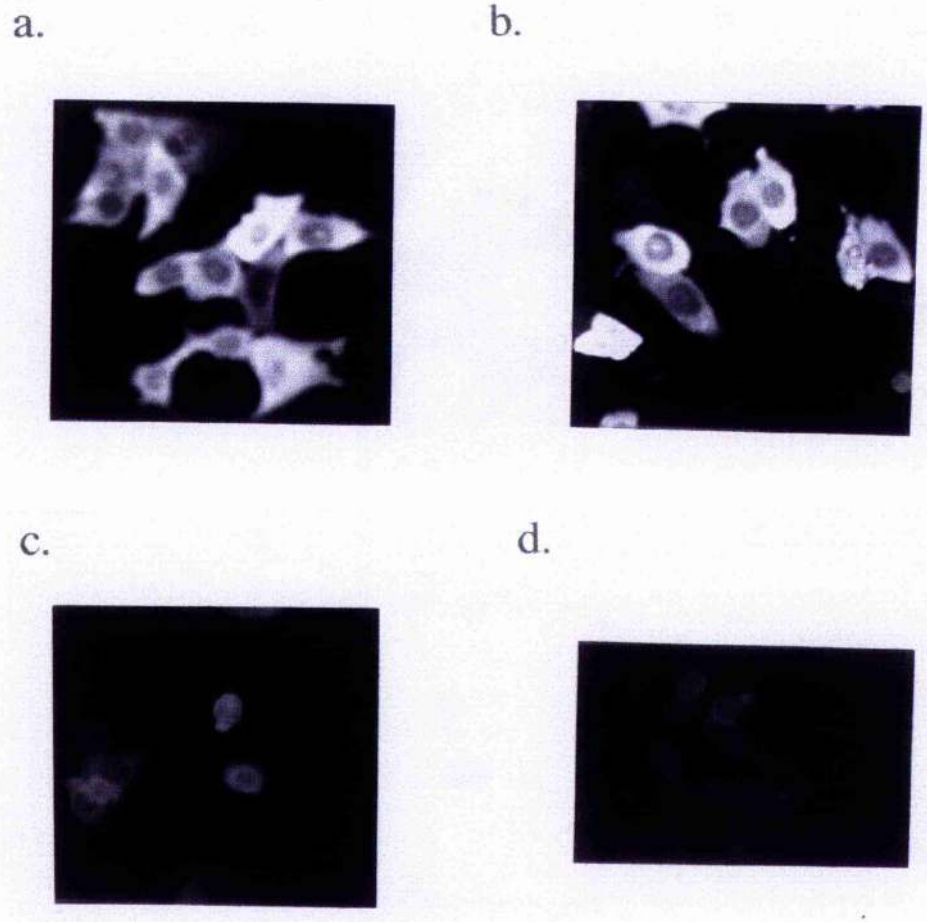



**Figure 30 : Immunofluorescence assay showing the four different results obtained for the anti-$Pk_{th}$ mAb's.**

BALB/c cells were plated onto immunofluorescence slides in the absence of the gene expression repressor tetracycline and incubated over night at 37°C. This allows the P protein which is expressed to accumulate within the cells. After fixing and permeabilisation, the cells were incubated with the anti-$Pk_{TH}$ mAb's. Detection was using a Texas Red conjugated anti mouse Ig mAb as a secondary probe.

Of the 24 mAbs tested by immunofluorescence, four exhibited strong fluorescence equivalent to that shown by mAb SV5-P-k (figure 30b). They were the four mAbs which had shown strong binding in the ELISA assays described in section 2.2.1 (i.e. no's 2, 46, 196 & 337). In addition there were three other mAbs which showed weak fluorescence (figure 2.2c). They were the three which had given a positive result to only the P and V proteins only in the ELISA experiments described in section 2.2.1 (no's 287, 523 & 727). The other 17 mAbs did not give any fluorescence at all. A complete summary of this data can be seen in Table 8.

## 2.3 Conclusions

From the experiments described in this section, the following conclusions were drawn. From the ELISA studies and the immunoblots, of the 24 anti-$Pk_{TH}$ mAbs developed, only four (no's 2, 46, 196, & 337) recognised the Pk tag when it was fused to a recombinant protein. In the ELISA studies and immunofluorescence assays it was observed that there were three other mAbs (no's 287, 523 & 727) which showed weak binding to BALB/c extracts containing the P and V proteins. On closer examination, it transpired that these three mAbs were cross-reacting to a cellular epitope within the BALB/c cell extracts. From all of the experiments, it became clear that the remaining 17 mAbs only recognised the $Pk_{TH}$ peptide antigen with which the mice had been immunised.

Although the results were disappointing, the fusion process had created hybridoma clones which produced a further four mAbs which recognised the Pk tag, now termed anti-Pk mAbs. Further analysis was necessary to determine whether these four mAbs could be used for the affinity purification of Pk tagged recombinant proteins.

| Clone Number | Fluorescence Intensity | Clone Number | Fluorescence Intensity |
|---|---|---|---|
| SV5-P-k | +++ | 534 | - |
| 1 | - | 537 | - |
| 2 | +++ | 551 | - |
| 3 | - | 579 | - |
| 36 | - | 622 | - |
| 46 | +++ | 638 | - |
| 146 | - | 652 | - |
| 187 | - | 727 | ++ |
| 196 | +++ | 757 | - |
| 287 | ++ | 759 | - |
| 337 | +++ | 768 | - |
| 467 | - | 812 | - |
| 523 | ++ | SV5-HN-4a | - |

**table 8 : a summary showing the levels of fluorescence obtained for each mAb when used to probe for SV5 P protein in a BALB/c cell line.**

BALB/c cells were grown overnight on an immunofluorescence slide to allow the accumulation of P protein. The cells were then fixed, permeabilised and incubated with each of the anti-Pk mAb's. The levels of fluorescence were visualised under green light and the relative amounts compared to SV5-P-k.

Key :- +++ levels of fluorescence equivalent to SV5-P-k; ++ distinct fluorescence visible but much weaker than SV5-P-k; - no fluorescence visible.

# 3. BINDING AFFINITY STUDIES FOR ANTI - Pk MONOCLONAL ANTIBODIES

The four anti-Pk mAbs (described in section 2 of the Results) were further characterised, as was SV5-P-k, and all five were renamed. Section 3 details the determination of the binding epitopes and binding affinities of the four anti-Pk mAbs and SV5-P-k. Section 3 also details preliminary data on buffer conditions, which may be useful for the elution of Pk tagged recombinant proteins from mAb coupled affinity columns.

## 3.1 Determination of antibody binding epitopes

The five mAbs which were used for further characterisation studies were renamed :

| | | |
|---|---|---|
| SV5-P-k | - | SV5-Pk1 |
| 2 | - | SV5-Pk2 |
| 46 | - | SV5-Pk3 |
| 196 | - | SV5-Pk4 |
| 337 | - | SV5-Pk5 |

To determine the binding epitopes, SPOT's technology (Genosys) was used. SPOT's is a technique where a number of different peptides can be synthesised in a format which is suitable for the systematic analysis of antibody epitopes. This involved a method where peptides, of known sequence, were synthesised onto derivatised cellulose sheets and then assayed for antibody binding (Geysen *et al* 1984). As well as epitope mapping, this technology has been used for a number of other applications; e.g. peptide combinatorial library studies; the study of protein - protein interactions; phosphorylation studies; and enzyme - substrate interactions (Böldicke *et al* 1988; Scott & Smith 1990) .

A number of peptides were synthesised by SPOT technology. Their sequences were derived from the Pk tag 14 amino acid sequence (figure 31). The peptides in the first membrane were essentially N terminal deletions (figure 31a). However, in the event that the binding epitopes of the mAbs mapped across the C terminus of the Pk tag, and into the helper T cell epitope, those amino acids found in the helper T cell epitope were added to the C terminus of the Pk tag sequence as an amino acid was deleted from the N terminus of the peptide. This meant that the N terminal deletion peptides remained the same length. The second membrane contained C terminal deletions (figure 31b).

Both membranes were incubated with each of the five mAbs in turn. The results were developed by ECL, and visualised on photographic film. A positive result, i.e. one where the antibody binds to the reduced peptide, is shown by a distinct dark 'spot' on an autoradiograph (figure 32). The results obtained for each mAb are discussed in turn.

*SV5-Pk1* - this mAb recognised and bound to the first five peptides (figure 32a) of the N terminal deletions. This showed that for mAb binding to occur, the proline at position 5 (pro-5) needed to be present. When studying the C terminal deletions, SV5-Pk1 recognised and bound to the first five peptides strongly, and to the sixth and seventh peptides weakly (figure 32b). This suggested that the removal of the leucine at position 11 (leu-11) and the glycine at position 10 (gly-10) reduced the binding efficiency of the mAb, but only with the removal of the leucine at position 9 (leu-9) was mAb binding abolished.

*SV5-Pk2* - this mAb bound to the first four of the N terminal deletion peptides (figure 32a), and the first eight of the C terminal deletion peptides (figure 32b). This indicated that the removal of the isoleucine at position 4 (ile-4) abolished mAb binding, as did the removal of the leucine at position 8 (leu-8).

*SV5-Pk3* - recognised the first five N terminal deletion peptides (figure 32a), and the first four C terminal deletion peptides strongly. SV5-Pk3 also recognised the fifth C terminal deletion peptide weakly (figure 32b). This demonstrated that for mAb binding to occur, pro-5 needed to be present within the tag. The results obtained for the C terminal deletions suggested that the removal of the aspartate residue at position 12 (asp-12) reduced the binding of the mAb to the tag, but binding was not abolished until leu-11 was removed.

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| gly. | lys. | pro. | ile. | pro. | asn. | pro. | leu. | leu. | gly. | leu. | asp. | ser. | thr |

a.

| | |
|---|---|
| 1 | gly.lys.pro.ile.pro.asn.pro.leu.leu.gly.leu.asp.ser |
| 2 | lys.pro.ile.pro.asn.pro.leu.leu.gly.leu.asp.ser.thr |
| 3 | pro.ile.pro.asn.pro.leu.leu.gly.leu.asp.ser.thr.asn |
| 4 | ile.pro.asn.pro.leu.leu.gly.leu.asp.ser.thr.asn.lys |
| 5 | pro.asn.pro.leu.leu.gly.leu.asp.ser.thr.asn.lys.ala |
| 6 | asn.pro.leu.leu.gly.leu.asp.ser.thr.asn.lys.ala.leu |
| 7 | pro.leu.leu.gly.leu.asp.ser.thr.asn.lys.ala.leu. **glu** |
| 8 | leu.leu.gly.leu.asp.ser.thr.asn.lys.ala.leu. **glu.leu** |
| 9 | leu.gly.leu.asp.ser.thr.asn.lys.ala.leu. **glu.leu.phe** |
| 10 | gly.leu.asp.ser.thr.asn.lys.ala.leu. **glu.leu.phe.arg** |
| 11 | leu.asp.ser.thr.asn.lys.ala.leu. **glu.leu.phe.arg.lys** |

b.

| | |
|---|---|
| 1 | lys.pro.ile.pro.asn.pro.leu.leu.gly.leu.asp.ser.thr |
| 2 | lys.pro.ile.pro.asn.pro.leu.leu.gly.leu.asp.ser |
| 3 | gly.lys.pro.ile.pro.asn.pro.leu.leu.gly.leu.asp.ser |
| 4 | gly.lys.pro.ile.pro.asn.pro.leu.leu.gly.leu.asp |
| 5 | gly.lys.pro.ile.pro.asn.pro.leu.leu.gly.leu |
| 6 | gly.lys.pro.ile.pro.asn.pro.leu.leu.gly |
| 7 | gly.lys.pro.ile.pro.asn.pro.leu.leu |
| 8 | gly.lys.pro.ile.pro.asn.pro.leu |
| 9 | gly.lys.pro.ile.pro.asn.pro |
| 10 | gly.lys.pro.ile.pro.asn |
| 11 | gly.lys.pro.ile.pro |
| 12 | gly.lys.pro.ile |

**Figure 31 : The amino acid sequences of the peptides within the SPOT's membranes**

The amino acids highlighted in Bold indicate those NOT derived from the Pk tag.

a. N terminal deletions; b. C terminal deletions.

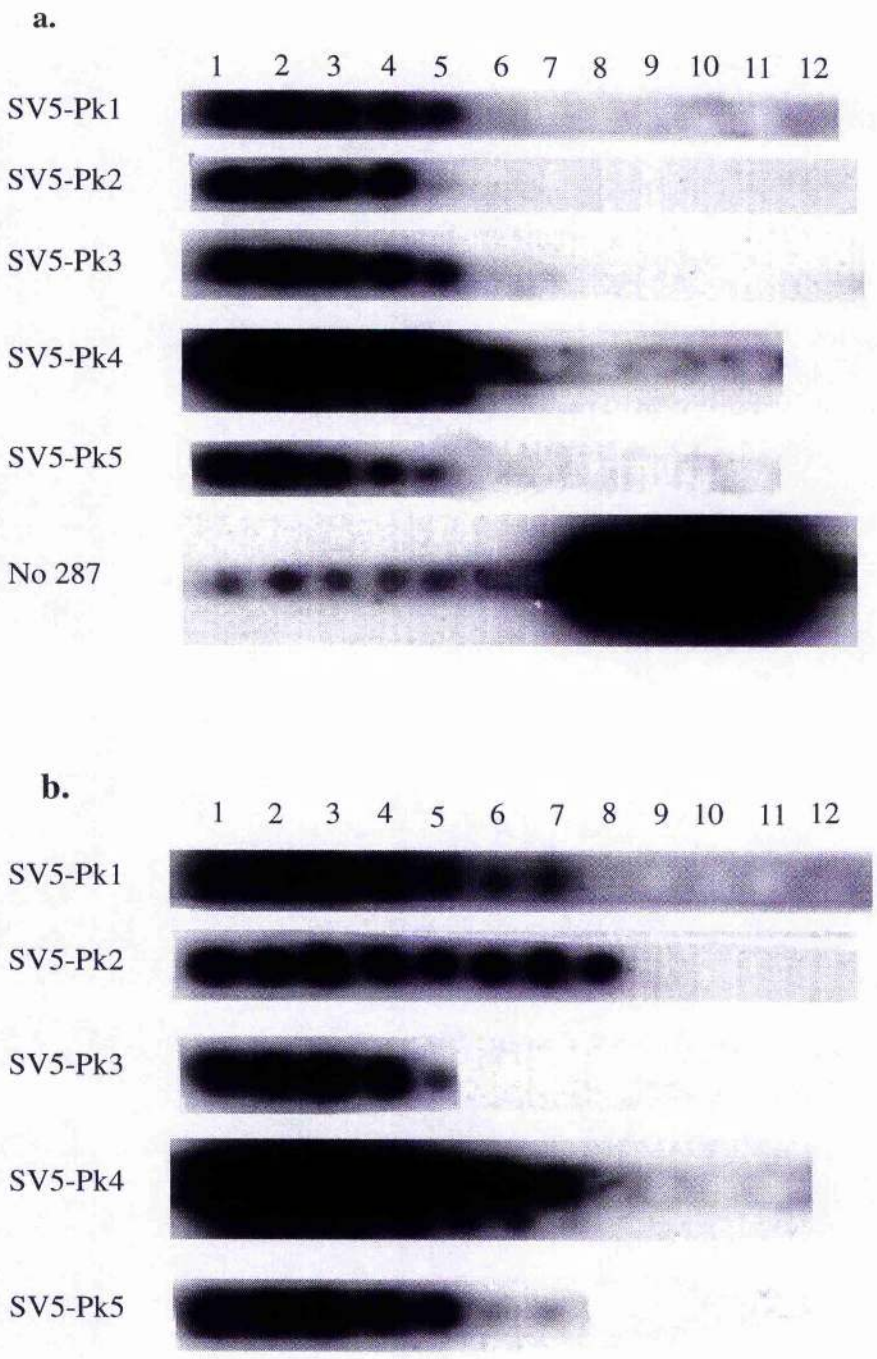



**Figure 32 : Autoradiographs showing the results of the SPOT tests.**

The SPOT membranes were probed with each of the 5 anti-Pk mAb's and mAb No287. These mAb's were probed with an horse radish peroxidase conjugated anti-mouse Ig mAb and a positive result was detected by ECL.

Key : **a.** N terminal deletions; **b.** C terminal deletions

*SV5-Pk4* - bound to the first five N terminal deletion peptides (figure 32a), and to the first eight C terminal deletion peptides (figure 32b). These results showed that for mAb binding to occur, pro-5 and leu-8 needed to be intact.

*SV5-Pk5* - bound to the first three N terminal deletion peptides strongly and to the fourth and fifth peptides weakly (figure 32a). This suggested that the deletion of the proline as position 3 (pro-3) and ile-4 reduced mAb binding, but the removal of pro-5 abolished mAb binding. When studying the C terminal deletion peptides, it was observed that SV5-Pk5 bound to the first five peptides (figure 32b). This showed that the removal of leu-11 abolished mAb binding.

When the information obtained from both the N and C terminal deletion membranes was combined, the minimum binding epitope for each mAb antibody was determined. These are shown in figure 33.

| **Pk tag** | Gly. Lys. Pro. Ile. Pro. Asn. Pro. Leu. Leu. Gly. Leu. Asp. Ser. Thr |
|---|---|
| SV5-Pk1 | Pro. Asn. Pro. Leu. Leu |
| SV5-Pk2 | Ile. Pro. Asn. Pro. Leu |
| SV5-Pk3 | Pro. Asn. Pro. Leu. Leu. Gly. Leu |
| SV5-Pk4 | Pro. Asn. Pro. Leu. Leu. Gly |
| SV5-Pk5 | Pro. Ile. Pro. Asn. Pro. Leu. Leu. Gly. Leu |

**figure 33 : minimum binding epitopes of the anti-Pk mAbs.**
The minimum binding epitopes are the amino acids within the Pk tag sequence which were found to be essential for the binding of the mAbs to the Pk tag. The above figure also shows the position of the minimum binding epitopes relative to the Pk tag sequence.

To confirm that the mAbs which had shown weak immunofluorescence in SV5 P and V expressing BALB/c cells, and which had given a positive result in the P and V protein ELISA tests, were recognising an epitope other than the Pk tag, mAb no. 287 (a mAb which had not been renamed) was also incubated with the N and C terminal deletion

membranes (figure 32a). The mAb only showed a positive result for the last four peptides of the N terminal deletion series, and did not give a positive result to any of the C terminal deletion peptides. From this data, it was deduced that the amino acid sequence this mAb was recognising was **leu.phe.arg.lys**, a sequence which was part of the T helper cell epitope which had been added to the Pk tag for immunisation purposes.

## 3.2 Immunofluorescence studies showing mAb cross reacting to HeLa cells

An interesting observation of SV5-Pk1 was made prior to this work. The mAb SV5-Pk1 showed a specific cross reaction with a cellular epitope within naive HeLa cells when immunofluorescence studies were performed. This cross reaction was found to be confined to the cytoplasmic side of the nuclear membrane, possibly the Golgi stack or rough endoplasmic reticulum (RER) (figure 34a). When looking for the same specific fluorescence in other cell lines e.g. naive Vero cells, this specific binding was not found (figure 34b). This confirmed that the specificity of this fluorescence was confined to HeLa cells. As there were similarities between the new anti-Pk mAbs and SV5-Pk1, experiments were carried out to see if they also showed this specific cross reaction in HeLa cells.

Naive HeLa cells were incubated, after permeabilisation, with each of the mAbs. SV5-Pk1 was used as a positive control and the anti - HN mAb, SV5-HN-4a, as a negative control. At the same time, HeLa cells were infected with SV5 and fixed. Simian virus 5 was used for the immunofluorescence studies as the viral P and V proteins are known to contain the epitope which is recognised by the anti-Pk mAbs. The infected cells also provided a positive control, to ensure the mAbs had gained entry into the permeabilised cells. For detection the secondary antibody was an anti - mouse Ig Texas Red conjugate and the fluorescence was visualised under green light.

SV5-Pk2 (figure 35c) and SV5-Pk3 (data not shown) appeared to cross react with the naive HeLa cells. The cross-reaction was confined to the cytoplasm surrounding the nucleus as it was for SV5-Pk1 (compare figure 35a & 35c). The fluorescence observed in cells incubated with SV5-Pk2 and SV5-Pk3 was not as strong as the fluorescence obtained

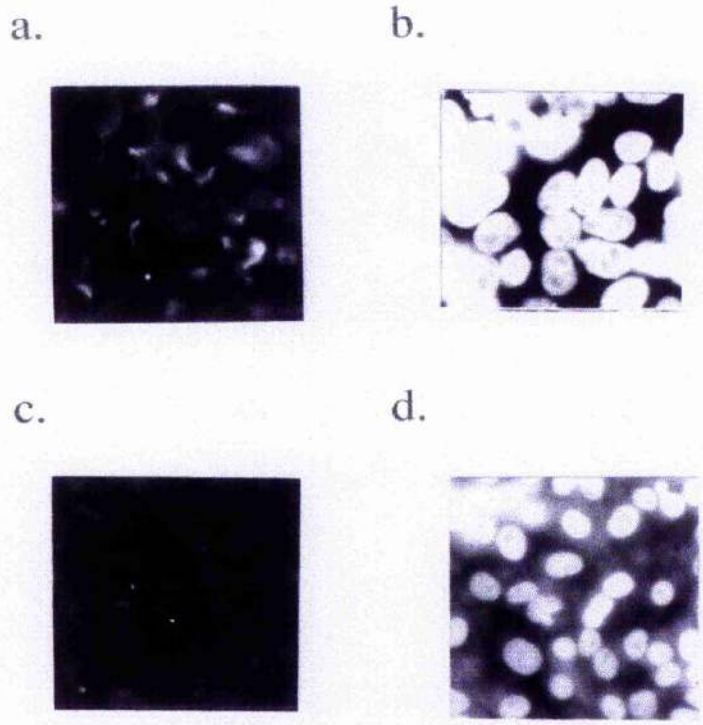



**Figure 34 : Immunofluorescence assay showing the cross reaction exhibited in HeLa cells by SV5-Pk1**

Naive HeLa and Vero cells were plated onto immunofluorescence slides and incubated at 37°C overnight to allow the cells to adhere. After fixing and permeabilisation the cells were incubated with tissue culture supernatant containing SV5-Pk1. Immunofluorescence was detected using a solution of Texas Red conjugated anti mouse Ig mAb and DAPI.

Key : a. - HeLa cells showing background immunofluorescence; b. - DAPI staining of the HeLa cells; c. - Vero cells stained with Texas Red (negative control); d. DAPI staining of Vero cells

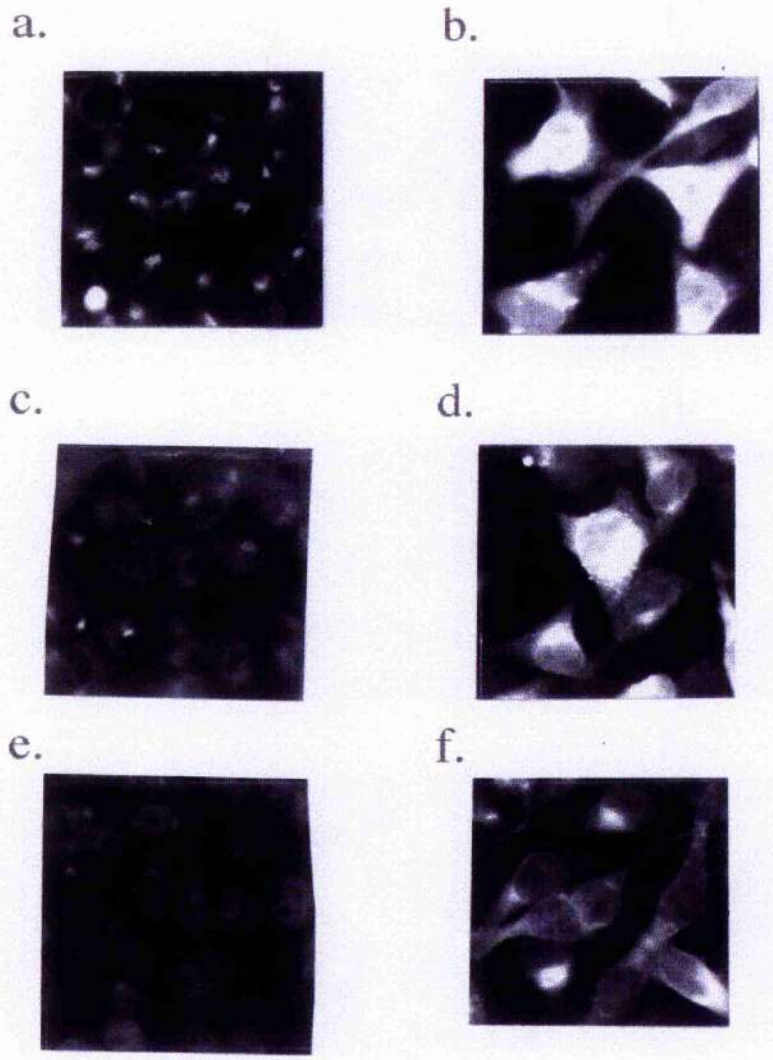



**Figure 35 : Immunofluorescence assay showing the cross reaction exhibited in the HeLa cell line by the newly isolated anti-Pk mAb's.**

Naive HeLa cells were plated onto immunofluorescence slides and incubated at 37°C overnight to allow the cells to adhere. At the same time naive HeLa cells were infected with the W3 strain of SV5, and incubated at 37°C overnight. After fixing and permeabilisation, the cells were incubated with tissue culture supernatant containing the anti-Pk mAb's. Immunofluorescence was detected using a solution of Texas Red conjugated anti mouse Ig mAb.

Key : a. - SV5-Pk1, uninfected; b. - SV5-Pk1 + SV5 infection; c. - SV5-Pk2, uninfected; d. - SV5-Pk2 + SV5 infection; e. SV5-Pk4, uninfected; f. - SV5-Pk4 + SV5 infection

for SV5-Pk1 (compare figure 35a & 35c). SV5-Pk4 (figure 35e) and SV5-Pk5 (data not shown) did not show any cross reaction with the naive HeLa cells. To confirm that the fluorescence observed was restricted to HeLa cells, the mAbs were also incubated with naive Vero and BALB/c cells. No fluorescence was obtained (data not shown).

The infected cells also emphasised that the fluorescence obtained when probing for the SV5 P/V proteins was so strong, that any background fluorescence obtained with SV5-Pk1, SV5-Pk2 and SV5-Pk3 in HeLa cells was masked (figure 35b, d & f).

## 3.3 Analysis of the mAb - Antigen interaction by Biosensor technology

Biomolecular interaction analysis (BIA) is a relatively new technique in the field of biomolecular research. It is a technology which is used for the monitoring of molecular interactions in "real time" without the use of labels. It is a fast and non-invasive method of detecting the affinity and kinetics of an interaction and can be used for the study of the interactions of proteins, peptides, nucleic acids, carbohydrates, lipids and low molecular weight molecules such as pharmaceuticals (Foote & Milstein 1991; Altschuh *et al* 1992; Borrebaeck *et al* 1992; Gruen *et al* 1993).

Detection is based on the principle of Surface Plasmon Resonance (SPR). This is described as "an optical phenomenon arising in thin metal films under conditions of total internal reflection" (figure 36). Surface plasmon resonance detects the changes in the refractive index of a solution close to the surface of a sensor chip. As the refractive index is directly related to the concentration of a solute at the surface layer, a number of questions can be addressed. In the case of the monoclonal antibodies to be tested; how fast do they associate and dissociate from the peptide antigen tag; and how strongly do they interact with the peptide antigen tag?

To perform the analysis, the ligand is immobilised on the surface of a gold covered sensor chip. The sensor chip forms one wall of the micro-flow cell (figure 37) where the interactions are monitored. The analyte is then injected over the surface of the sensor chip in a controlled flow. The flow is controlled by micro-fluidics using a miniaturised

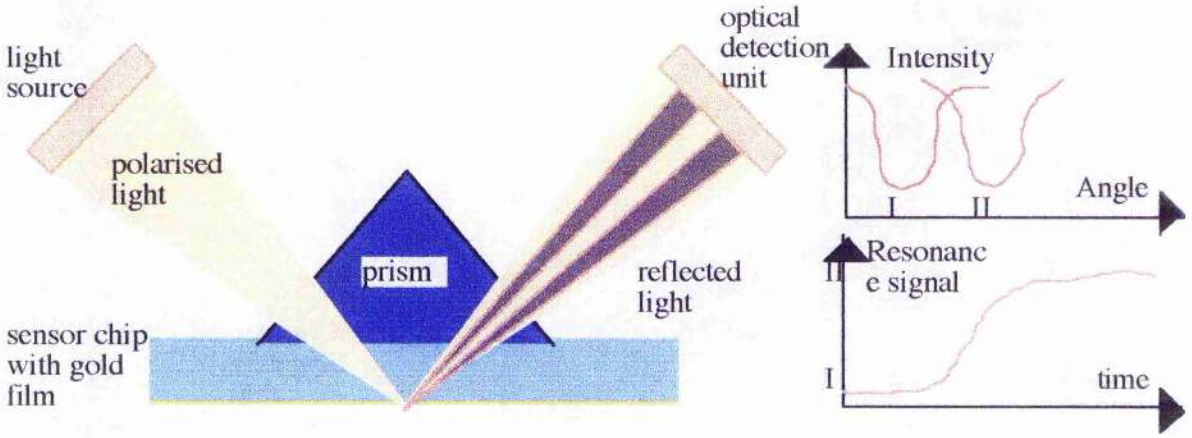



**Figure 36 : The principles of surface plasmon resonance (SPR).**
The SPR angle is sensitive to the mass concentration of molecules close to the sensor chip surface. As this concentration changes, the SPR angle shifts and produces a response. A response of 1000 resonance units (RU) corresponds to a change in surface concentration of 1 $ng.mm^{-2}$.

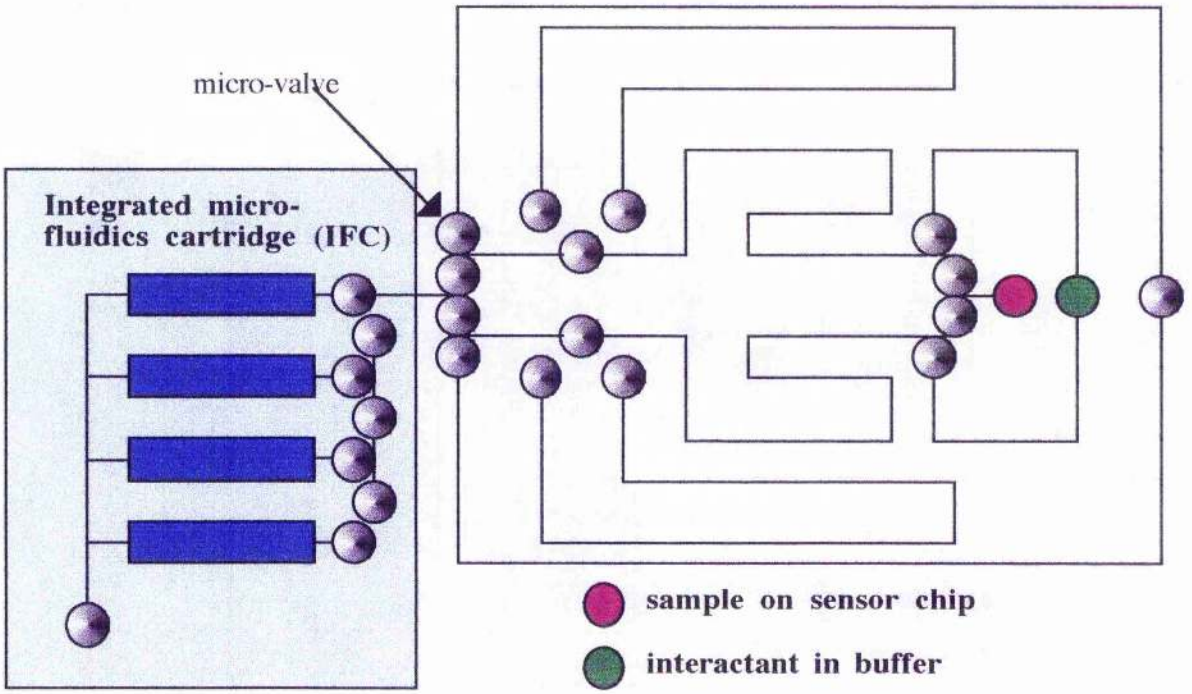



**Figure 37 : A microfluidics flow chart.**
A diagrammatic vertical section through the flow cell shows how the cell is formed by the IFC pressing against the sensor chip.

integrated micro-fluidics cartridge (IFC). The IFC controls the delivery of samples and buffers to the sensor chip through micro-computer controlled pneumatic microvalves. A change in solute concentration at the surface of the sensor chip caused by an interaction between the two interactants is detected as an SPR signal and expressed in resonance units (RU). A continuous display of RU against time is known as a sensorgram (figure 38). A change in response of 1000 RU corresponds to a change in sensor chip surface concentration of 1ng/mm$^2$ (Biosensor Handbook. Pharmacia). Once the experiment is completed the sensor chip must be regenerated so it can be used for the next reaction. The method of regeneration is dependent upon the strength of the interaction between the two interactants used in the experiment.

The $Pk_{TH}$ peptide tag was covalently immobilised on a CM5 sensor chip by amine coupling. The sensor chip is composed of three layers; glass, gold and carboxymethylated (CM) dextran. After the $Pk_{TH}$ tag was covalently coupled to the CM dextran layer of the sensor chip, each mAb was passed over the chip in the flow buffer and the change in the RU studied on the sensorgram (figure 38). A number of different concentrations of each mAb were tested to allow for discrepancies in the association and dissociation phases. After the binding injection was completed, a period of dissociation must occur. This is for the determination of the apparent dissociation rate constant, which is important for the determination of the apparent rate association constant and the Equilibrium constant. A time period of about 400s is usually sufficient for the calculation of the apparent rate dissociation constant. Once the interaction has been left for a suitable length of time to allow for dissociation, the sensor chip was regenerated to be used again for another mAb - $Pk_{TH}$ tag interaction.

The apparent association ($k_a$) and dissociation ($k_d$) rate constants, and the Equilibrium rate dissociation constants (Kd) were calculated using a simple mathematical model (see materials and methods; Table 9).

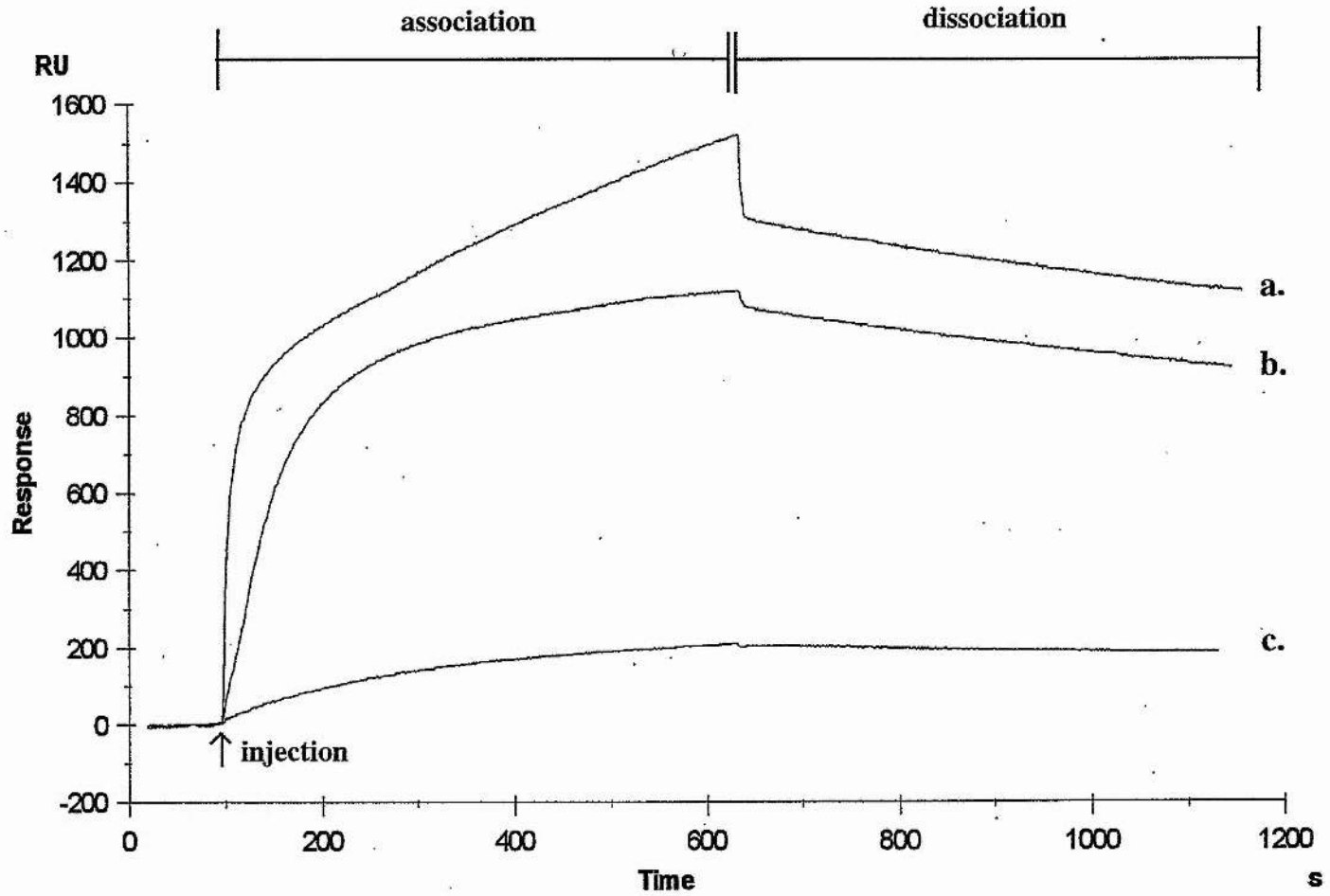



**Figure 38 : A sensorgram showing the binding patterns of three different concentrations of mAb binding to the Pk peptide tag when immobilised on a CM5 sensorchip**
**a - 140 mmol; b - 14 mmol; c 1.4 mmol**

| Monoclonal Antibody | association constant $k_a$ | dissociation constant $k_d$ | Kd for Pk peptide |
|---|---|---|---|
| SV5-Pk1 | $2.98 \times 10^6$ | $7.03 \times 10^{-5}$ | 23.6 pmol |
| SV5-Pk2 | $3.73 \times 10^6$ | $2.27 \times 10^{-4}$ | 60.9 pmol |
| SV5-Pk3 | $5.08 \times 10^6$ | $2.26 \times 10^{-4}$ | 44.5 pmol |
| SV5-Pk4 | $3.54 \times 10^6$ | $1.73 \times 10^{-4}$ | 48.9 pmol |
| SV5-Pk5 | $3.4 \times 10^6$ | $2.13 \times 10^{-4}$ | 62.6 pmol |

**Table 9 : the average apparent association / dissociation constants and the equilibrium dissociation constants obtained for the binding of the five anti-Pk mAbs to the $Pk_{TH}$ peptide.**

The main aim of developing more mAbs was to obtain antibodies which had lower binding affinities for the Pk tag and which could be used for the affinity purification of tagged recombinant proteins. The Biosensor studies gave the first indications of how the binding affinities of the four mAbs developed differed from SV5-Pk1.

There was little difference between the association rate constants obtained for the five anti-Pk mAbs. However, the dissociation rates constants of the four newly developed mAbs showed a 10 fold difference to the dissociation constant obtained for SV5-Pk1. Of the four newly developed anti-Pk mAbs, SV5-Pk4 showed the quickest dissociation from the $Pk_{TH}$ tag, and the largest dissimilarity to SV5-Pk1.

The Biosensor data has shown that there were small differences in the Equilibrium constants obtained for all five mAbs. The Equilibrium constant obtained for SV5-Pk1 showed that of the five anti-Pk mAbs, it still had the strongest affinity for the Pk tag. However, the other four mAbs did not appear to have binding affinities which were sufficiently low enough to allow easy elution of tagged recombinant proteins from affinity columns.

## 3.4 Preliminary analysis of conditions which cause the dissociation of tagged recombinant proteins from the anti-Pk monoclonal antibodies

The Biosensor binding affinity studies in section 3.3, showed that although the four new mAbs developed had strong binding affinities, they did not bind as strongly as SV5-Pk1. However, the affinity of a mAb for its antigen does not necessarily determine the difficulty with which the immunocomplex will be dissociated. In immuno-affinity chromatography the ease with which an antigen can be eluted can also be determined by the rate of dissociation. As the newly developed mAbs had faster dissociation rates, it was decided to compare the conditions required for the elution of the mAbs from tagged recombinant proteins.

An efficient way of investigating a large number of elution conditions was by Dot blotting, a cross between Western blotting and ELISA's. In this assay, nitrocellulose filters were incubated with bacterial TCL containing the SV5 P protein. The protein bound to the nitrocellulose filters, allowing the coated filters to be probed with the anti-Pk mAbs. By using 84 well Terasaki plates (see Materials and Methods), all five anti-Pk mAbs could be assayed simultaneously. The series of dots which are developed on the autoradiograph (see figure 39) represent an area of the nitrocellulose filter which had been incubated with an anti-Pk mAb. Once the filters had been incubated with the anti-Pk mAbs, they were cut into strips and each strip incubated with a different elution buffer. A number of different elution buffers were tested in order to find relatively mild combinations which would elute the mAbs from Pk tagged recombinant proteins. The strips were incubated with an HRP conjugated secondary antibody, and developed in the same way as a Western blot. From these experiments, an efficient elution buffer is one which shows the least detected mAb binding when compared to the PBS positive control (figure 39i, ii, iii, lane a).

Initially relatively mild, standard conditions for mAb elution were tested e.g by varying the pH (figure 39i), the salt concentration of the elution buffer (figure 39ii) and by the addition of various detergents (figure 39iii).

**Figure 39 : Dot blot analysis of SV5 Phospho (P) protein coated filters.**

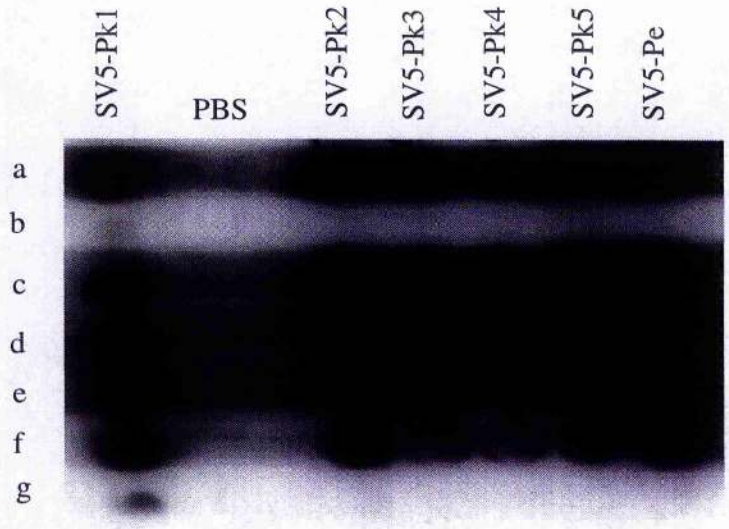
Nitrocellulose filters were coated with SV5-P protein. The filters were probed with the 5 anti-Pk mAb's, and the anti-P protein mAb SV5-Pe as a positive control, using an 84 well Terasaki plate to create the "dot's". The filters were cut into strips and each strip washed with a different elution buffer. Detection of mAb binding was by ECL.

i. Filter strips were washed with elution buffers at different pH. **a.**PBS; **b.** pH2; **c.** pH4; **d.** pH6; **e.** pH8; **f.** pH10; **g.** pH12

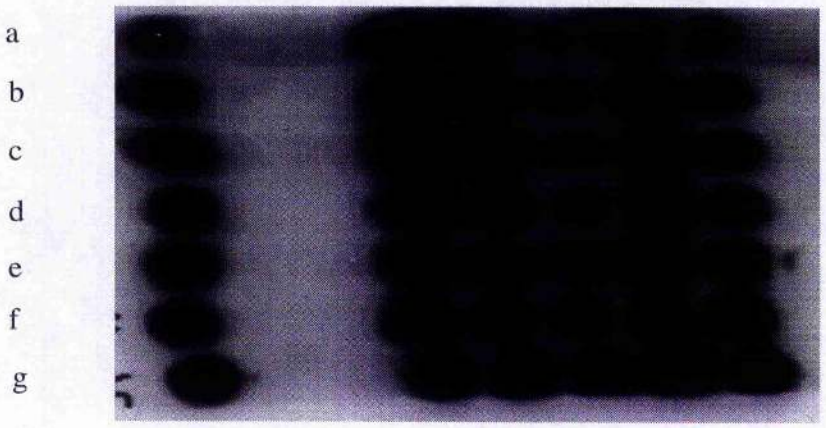
ii. Filter strips were washed with elution buffers containing different NaCl concentrations. **a.** PBS; **b.** 2.5 M; **c.** 3 M; **d.** 3.5 M; **e.** 4 M; **f.** 4.5 M; **g.** 5 M

iii.. Filter strips were washed with elution buffers containing different salt concentrations and different detergents. **a.** PBS; **b.** 0.5 M NaCl + SDS; **c.** 2 M NaCl +SDS; **d.** 5 M NaCl + SDS; **e.** 0.5 M NaCl + NP40; **f.** 2 M NaCl + NP40; **g.** 5 M NaCl +NP40

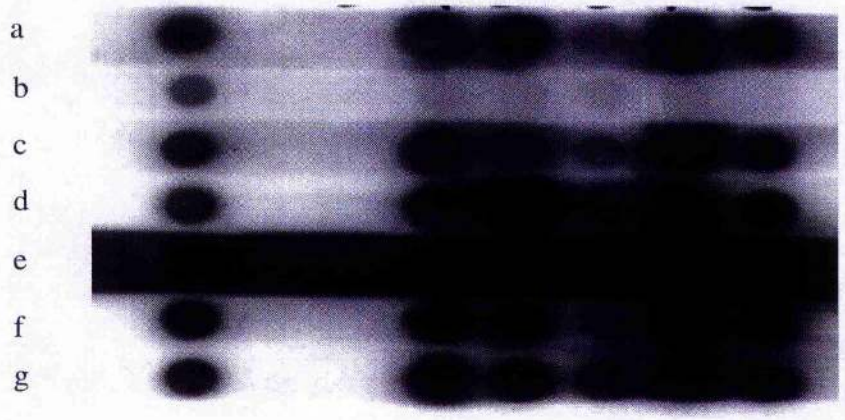
i.


SV5-Pk1
PBS
SV5-Pk2
SV5-Pk3
SV5-Pk4
SV5-Pk5
SV5-Pe
a
b
c
d
e
f
g

ii.


a
b
c
d
e
f
g

iii.


a
b
c
d
e
f
g

Only the most extreme pH's such as pH2 (figure 39i, lane b) or pH12 (figure 39i, lane g) appeared to affect the binding of the anti-Pk mAbs. Although a decrease in mAb binding was seen at pH10 for mAbs SV5-Pk2 and SV5-Pk3 (figure 39i, lane f). With the effective pH's being so extreme, it was unlikely that a change in pH alone would be useful as an elution buffer. Another disadvantage to using very high or very low pH is that the extreme pH could denature the recombinant protein being purified.

A number of salt concentrations and detergents were tested together to see if these combinations would effect antibody binding, as salt concentration alone had no effect (figure 39ii), even with concentrations as high as 5 M (lane g). Initially the detergents Tween 20, Triton X-100, sodium dodecyl sulphate (SDS), and Nonidet p40 (NP40) were tested in combinations with relatively high (5 M), medium (2 M) and low (0.5 M) sodium chloride concentrations. Of those tested, only the SDS and NP40 results are shown (figure 39iii). Of all detergents tested, only SDS showed an effect on antibody binding (lane b) and then only under relatively low salt conditions. Although this appeared promising, SDS is not ideal for use in an elution buffer as it is extremely difficult to remove completely from a column.

Having experimented with changes in pH, salt concentration and the addition of detergents, it became clear that mild elution conditions were not sufficient to elute the P protein or tagged recombinant proteins from the newly developed anti-Pk mAbs (see figure 39). To analyse fully conditions which could be used for protein elution, a number of other elution buffers were used : 0.2 M glycine at various pH's (pH 2.8, 10.1 & 11.5); chaotropic ions [3.5 M magnesium chloride ($MgCl_2$); 3.5 M sodium thiocyanate (NaSCN)]; other ionic detergents (0.1% sodium deoxycholate); mild denaturing conditions (0.1 M diethylamine pH 11), ethylene glycol pH 11.5 (1%, 10% & 50%), and as a last resort strong denaturing conditions such as 6 M guanidine hydrochloride, 8 M Urea and 20 mM hydrochloric acid (HCl). All of these elution buffers were tested on nitrocellulose filters coated with His-Nef-Pk (figure 40).

From figure 40 it was observed that the anti-Pk mAbs SV5-PK3 and SV5-Pk4 did not bind well to the filters. This is a result of the poor quality of tissue culture media

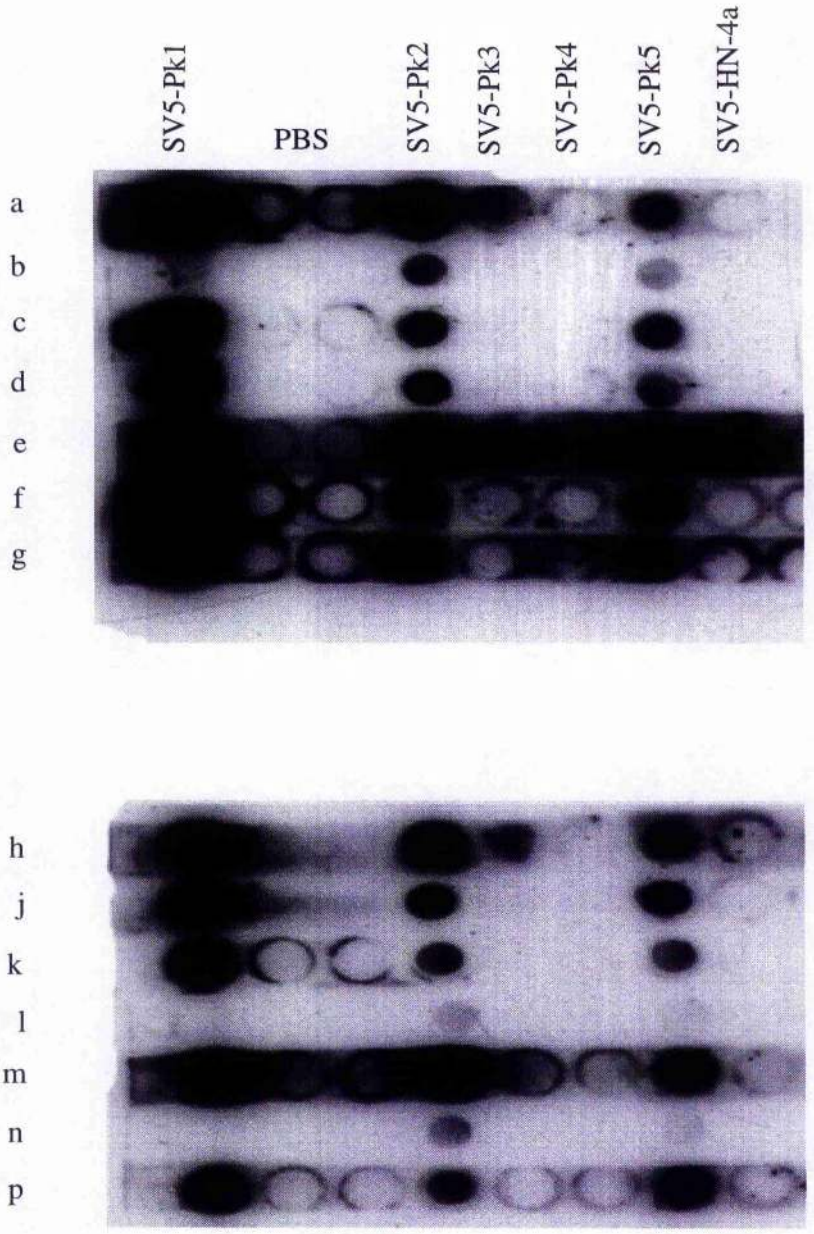



**Figure 40 : Dot blot analysis of His-Nef-Pk coated nitrocellulose filter.**

Nitrocellulose filters were coated with purified His-Nef-Pk and probed with the five anti-Pk mAb's, with SV5-HN-4a as a negative control. The filters were cut into strips, and each strip was incubated with a

different elution buffer. Dectection of mAb binding was by ECL.

Key: **a** & **h** - phosphate buffered saline; **b** - 0.1 M glycine pH 2.8; **c** - 0.1 M glycine pH 10.1; **d** - 0.1 M glycine pH 11.5; **e** - 3.5 M magnesium chloride; **f** - 3.5 M sodium thiocyanate; ; **g** - 0.1% sodium deoxycholate; **j** - 1% ethylene glycol; **k** - 10% ethylene glycol; **l** - 0.1 M diethylamine; **m** - 0.05% sodium acetate pH 4.0; **n** - 20mM hydrochloric acid; **p** - 0.1% sodium dodecyl sulphate

available for use in this experiment. The results stated in this section for these two anti-Pk mAbs are, therefore, from subsequent experiments where the data is not shown. Of all the conditions tested, glycine pH 2.8 (figures 40, lane b), diethylamine (figures 40, lane l) and hydrochloric acid (figures 40, lane n) gave the most significant effect on antibody binding for all five anti-Pk mAbs. Glycine pH 2.8 significantly reduced the binding of SV5-Pk5 to His-Nef-Pk (lane b), but only gave a slight decrease in the binding of SV5-Pk2 to His-Nef-Pk (lane b). In addition, unlike the other anti-Pk mAbs, SV5-Pk2 binding was not abolished by diethylamine (lane l) and hydrochloric acid (lane n), only significantly reduced. The binding of SV5-Pk4 and SV5-Pk5 was reduced by the elution buffer glycine pH11.5 (figures 40, lane d). The strong denaturing conditions, 6 M guanidine hydrochloride and 8 M urea, completely removed mAb binding (data not shown), but this may have been due to the removal of the bound protein from the nitrocellulose filters rather than the antibody from the protein.

## 4. CONSTRUCTION OF MODIFIED Pk TAGS

From the experiments described in Section 3, it was apparent that the four new anti-Pk mAbs did not bind to the Pk tag with a significantly lower binding affinity to that obtained for SV5-Pk1. Also shown in section 3, was that each mAb had a different minimal binding epitope. It was postulated, therefore, that by developing tags which contained a single amino acid change to the original Pk tag, the binding of the existing mAbs may be affected. Section 4 focuses on the determination of which amino acids within the Pk tag sequence were to be modified, and what effect the amino acid modifications had on mAb binding.

## 4.1 Determination of amino acids, outside of the binding epitope, that are essential for antibody binding

### 4.1.1 SPOT substitutions and insertions

As well as the membranes used in section 3.1, two other membranes for SPOT testing were synthesised (figure 41). The peptides on these membranes were also derived from the Pk tag sequence but instead of having amino acid deletions, there were either cysteine substitutions at each position (figure 41a) or the insertion of a cysteine residue between the amino acids at each position (figure 41b). Cysteine was chosen because these two SPOT membranes were originally designed for another project within the group. This was involved with the development of the Pk tag as a spacer arm for chemically linking antigens to the β subunit of the heat labile enterotoxin of *E. coli* (LTB) for vaccine purposes.

The membranes were incubated with each of the mAbs, and the results developed as stated in section 3.1 (figure 42). The cysteine substitutions indicated which amino acids could not be deleted (or substituted for another amino acid) at a specific position within the primary tag sequence (figure 42i). A number of conclusions could be made from the observations concerning the critical amino acids required for the binding of the mAbs within the Pk tag :

*SV5-Pk1* - substitutions of pro-5, asn-6, and leu-9 significantly reduce the binding of the mAb to the Pk tag (figure 42a). This was expected as these amino acids are all within the minimum binding epitope determined for SV5-Pk1 in section 3.1. However, a substitution of leu-11 also affected the binding of the mAb to the Pk tag (figure 42a). This was unexpected, as from previous experiments this amino acid could be removed with no effect to mAb binding (see section 3.1).

*SV5-Pk2* - substitutions to asn-6 and pro-7 abolish the binding of this mAb to the Pk tag (figure 42b). In addition to these two amino acids, the substitution of ile-4 and leu-8 reduced the binding of the mAb to the Pk tag (figure 42b). The effects of all four amino

a.

| | |
|---|---|
| 1 | **cys**.lys.pro.ile.pro.asn.pro.leu.leu.gly.leu.asp.ser |
| 2 | gly.**cys**.pro.ile.pro.asn.pro.leu.leu.gly.leu.asp.ser |
| 3 | gly.lys.**cys**.ile.pro.asn.pro.leu.leu.gly.leu.asp.ser |
| 4 | gly.lys.pro.**cys**.pro.asn.pro.leu.leu.gly.leu.asp.ser |
| 5 | gly.lys.pro.ile.**cys**.asn.pro.leu.leu.gly.leu.asp.ser |
| 6 | gly.lys.pro.ile.pro.**cys**.pro.leu.leu.gly.leu.asp.ser |
| 7 | gly.lys.pro.ile.pro.asn.**cys**.leu.leu.gly.leu.asp.ser |
| 8 | gly.lys.pro.ile.pro.asn.pro.**cys**.leu.gly.leu.asp.ser |
| 9 | gly.lys.pro.ile.pro.asn.pro.leu.**cys**.gly.leu.asp.ser |
| 10 | gly.lys.pro.ile.pro.asn.pro.leu.leu.**cys**.leu.asp.ser |
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| 13 | gly.lys.pro.ile.pro.asn.pro.leu.leu.gly.leu.asp.**cys** |
| 14 | lys.pro.ile.pro.asn.pro.leu.leu.gly.leu.asp.ser.**cys** |

b.

| | |
|---|---|
| 1 | gly.**cys**.lys.pro.ile.pro.asn.pro.leu.leu.gly.leu.asp.ser |
| 2 | gly.lys.**cys**.pro.ile.pro.asn.pro.leu.leu.gly.leu.asp.ser |
| 3 | gly.lys.pro.**cys**.ile.pro.asn.pro.leu.leu.gly.leu.asp.ser |
| 4 | gly.lys.pro.ile.**cys**.pro.asn.pro.leu.leu.gly.leu.asp.ser |
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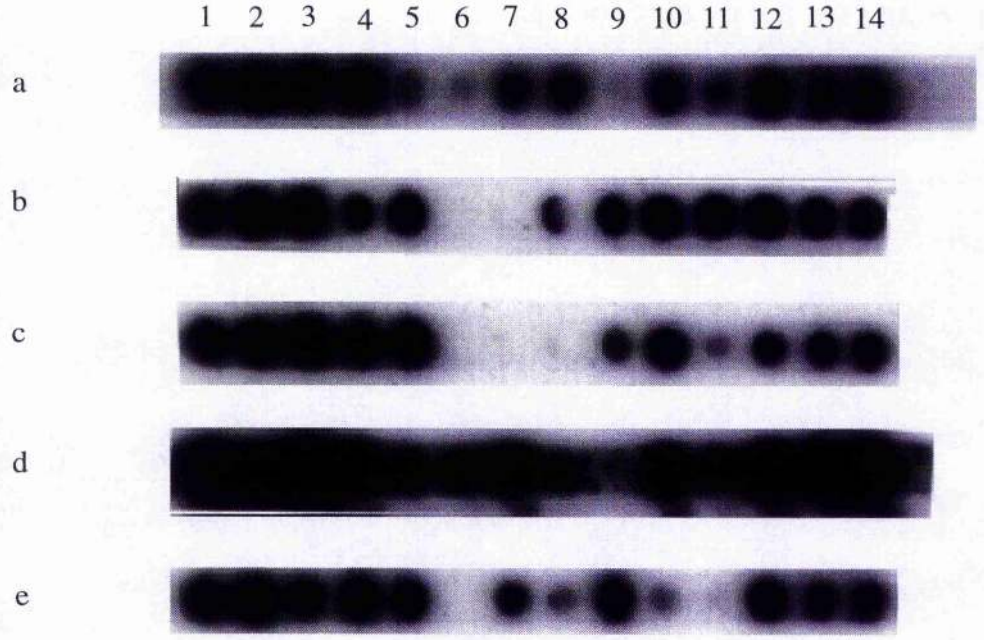
| 10 | gly.lys.pro.ile.pro.asn.pro.leu.leu.gly.**cys**.leu.asp.ser |
| 11 | gly.lys.pro.ile.pro.asn.pro.leu.leu.gly.leu.**cys**.asp.ser |
| 12 | gly.lys.pro.ile.pro.asn.pro.leu.leu.gly.leu.asp.**cys**.ser |
| 13 | gly.lys.pro.ile.pro.asn.pro.leu.leu.gly.leu.asp.ser.**cys** |

**Figure 41 : The amino acid sequences of the peptides on the SPOT membranes**

a. peptides containing cysteine substitutions; b. peptides containing cysteine insertions.

i Cysteine substitutions




ii. Cysteine insertions

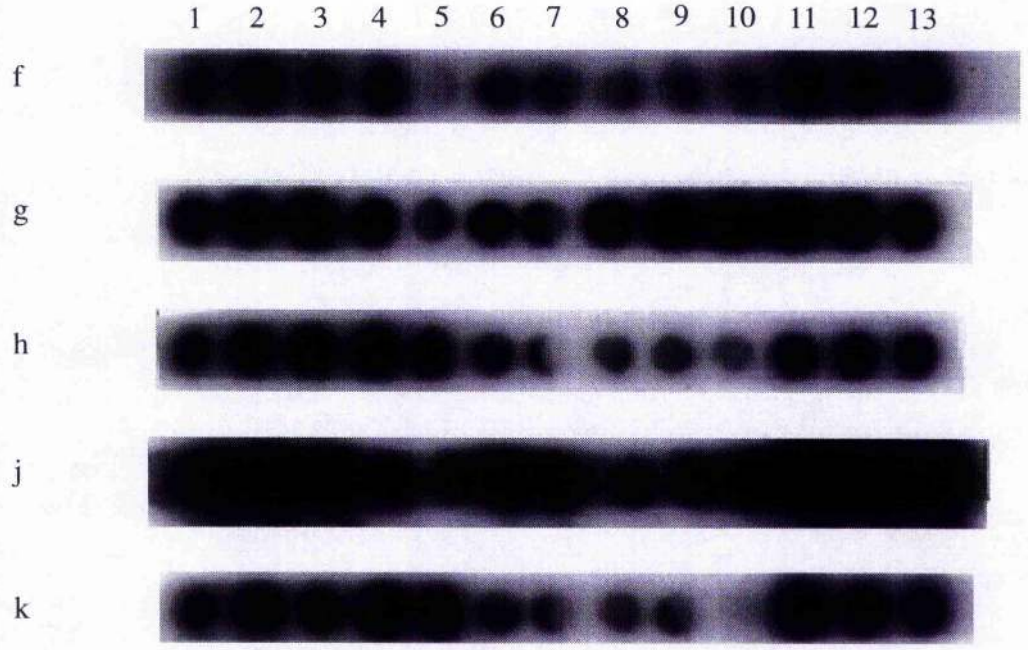



**Figure 42 : Autoradiographs showing the results of the SPOT tests.**

The SPOT membranes were probed with each of the five anti-Pk mAb's. Detection was by ECL. The numbering corresponds to that shown in figure 41.

Key : **a & f** - SV5-Pk1; **b & g** - SV5-Pk2; **c & h** - SV5-Pk3; **d & j** - SV5-Pk4; **e & k** - SV5-Pk5

acids were expected as all four are found within the minimum binding epitope determined in section 3.1.

*SV5-Pk3* - substitution of asn-6, pro-7 and leu-8 completely abolished mAb binding (figure 42c). In addition, substitutions of leu-8 and leu-11 also had the effect of reducing mAb binding (figure 42c). All of these amino acids were found within the minimum binding epitope determined for the mAb (see section 3.1).

*SV5-Pk4* - substitution of leu-9 abolished binding of the mAb completely (figure 42d). Substitutions to pro-5, asn-6, and leu-8 also acted to reduce the binding of the mAb to the Pk tag (figure 42d).All four amino acids were found to be within the minimum binding epitope determined for mAb binding (section 3.1).

*SV5-Pk5* - substitutions to asn-6 and leu-11 abolish mAb binding completely (figure 42e). Substitutions to leu-8 and gly-10 also significantly reduce the binding of the mAb to the Pk tag (figure 42e). All four mAbs are found within the minimum binding epitope determined for mAb binding (section 3.1). However, in addition to the amino acids stated, the substitution of the proline at position 3 (pro-3), the serine at position 13 (ser-13), and the threonine at position 14 (thr-14) also affected mAb binding (figure 42e), though not as significantly as the other substitutions previously mentioned.

The results obtained for the cysteine substitutions have highlighted some of the differences observed on the amino acid requirements of mAb binding. A summary of this information can be seen in figure 43.

The insertion of a cysteine residue between amino acids pairs did not have the same noticeable effect on mAb binding that was observed for the cysteine substitutions. The cysteine insertions (figure 42ii) determined which part of the Pk tag amino acid sequence could not be altered if mAb binding was to occur. As with the cysteine substitutions, a number of conclusions could be drawn from this experiment.

*SV5-Pk1* - a cysteine insertion between pro-5 & asn-6 abolishes mAb binding altogether (figure 42, lane f). However, cysteine insertions between leu-8 & leu-9, leu-9 & gly-10, and gly-10 & leu-11 also had the effect of reducing mAb binding (lane f). The effect of

| | |
|---|---|
| **SV5-Pk1** | pro.asn.pro.leu.leu. |
| **a** | gly.lys.pro.ile.** . ** . * .leu.***.gly. * .asp.ser.thr |
| **b** | gly.lys.pro.ile.n.d.***.***.leu.***.n.d.leu.asp |
| **c** | gly.lys.pro.ile.pro.asn.pro.leu.leu.gly.leu.asp.ser.thr |
| | *** * * ** ** ** |
| **SV5-Pk2** | ile.pro.asn.pro.leu |
| **a** | gly.lys.pro.** . * . ***.***. ** . * . gly.leu.asp.ser.thr |
| **b** | gly.lys.pro.***.n.d.***.***.***.leu.n.d.leu.asp |
| **c** | gly.lys.pro.ile.pro.asn.pro.leu.leu.gly.leu.asp.ser.thr |
| | ** * ** |
| **SV5-Pk3** | pro.asn. pro.leu. leu.gly.leu |
| **a** | gly.lys.pro.ile.pro.***.***.***.** . * .***. * .ser.thr |
| **b** | gly.lys.pro.ile.n.d.***.***.***.leu.n.d.** .asp. |
| **c** | gly.lys.pro.ile.pro.asn.pro.leu.leu.gly.leu.asp.ser.thr |
| | * ** ** ** ** |
| **SV5-Pk4** | pro. asn. pro. leu. leu. gly. |
| **a** | gly.lys.pro. * .** . ** . * . ***. ***.gly. * .asp.ser.thr |
| **b** | gly.lys.pro.** .n.d.***.***.***.(**) n.d.leu.asp. |
| **c** | gly.lys.pro.ile.pro. asn. pro. leu. leu. gly.leu.asp.ser.thr |
| | * ** * * ** ** * |
| **SV5-Pk5** | pro.ile.pro.asn. pro. leu. leu.gly.leu |
| **a** | gly.lys.pro.ile.pro.***. * . **. leu. **.***.asp.ser.thr |
| **b** | gly.lys.pro.ile.n.d.***.***.***.leu.n.d.**. asp. |
| **c** | gly.lys.pro.ile.pro.asn.pro. leu. leu.gly.leu. asp.ser.thr |
| | * ** ** ** *** |

**Figure 43 : A summary of the information obtained from the cysteine substitutions and insertions, and the LTB Pk cysteine substitutions experiments.**

Key : **a.** - SPOT cysteine substitutions; **b** - LTB-Pk-cys substitutions; **c** - SPOT cysteine insertions

*** - no binding visible; ** - an effect on binding strongly visible; * - little effect to mAb binding; n.d. - mutant peptide not developed.

insertions between these last amino acids on mAb binding was surprising as they are not within the minimum binding epitope.

*SV5-Pk2* - no cysteine insertion had the effect of completely disrupting mAb binding to the tag (lane g). However, insertions between pro-5 & asn-6, and pro-7 & leu-8 had the effect of reducing mAb binding.

*SV5-Pk3* - a cysteine insertion between pro-7 & leu-8 abolished mAb binding (lane h). Cysteine insertions between asn-6 & pro-7, leu-8 & leu-9, leu-9 & gly-10, and gly-10 & leu-11 significantly reduce mAb binding.

*SV5-Pk4* - as with SV5-Pk2, no cysteine insertion had the effect of abolishing mAb binding (lane j). However, insertions between ile-4 & pro-5, and leu-8 & leu-9 reduce mAb binding (lane j). The isoleucine at position 4 is not found within the minimum binding epitope determined for the mAb, whereas the other three amino acids are.

*SV5-Pk5* - an insertion between gly-10 & leu-11 abolished mAb binding completely (lane k). The cysteine insertions showed the greatest effect on SV5-Pk5 mAb binding. The binding of the mAb to the tag was affected by cysteine insertions between, gly-1 & lys-2, pro-3 & ile-4, asn-6 & pro-7, pro-7 & leu-8, leu-8 & leu-9, leu-9 & gly-10, and ser-13 & thr-14. The effect of these amino acids on mAb binding and the relatively large minimum binding epitope, indicated that for efficient SV5-Pk5 binding almost the whole of the Pk tag needed to be intact.

The cysteine insertions have shown that the position of the amino acid within the Pk tag sequence can have either a significant effect on mAb binding (e.g. SV5-Pk5) or have little effect on mAb binding (e.g. SV5-Pk2). A complete summary of this information can be seen in figure 43.

### 4.1.2 LTB-Pk-cysteine substitutions

As stated in section 4.1.1, the substitution and insertion of a cysteine residue was due to the SPOT membranes having been designed for another project within our group. This project is concerned with the development of an immunisation system which uses the Pk tag as a spacer arm to form a chemical link between an adjuvant and an antigen. The

adjuvant selected was LTB from *E. coli* (Tsuji *et al* 1997). Within our laboratory, the original Pk tag, and Pk tags containing cysteine substitutions at each position, were fused to the C terminus of LTB (LTB-Pk-cys) and expressed in *Vibrio sp.* The cysteine substituted Pk tags were labelled A - L and the amino acid sequences for the peptide tags can be seen in figure 44. Once expressed the secreted soluble LTB-Pk-cys protein was collected.

The heat labile enterotoxin has been shown to bind to GM1, a sugar found on the external surfaces of most eukaryotic cells (Lindner *et al* 1994). ELISA plates were coated with GM1 and used to capture the LTB-Pk-cys mutants. To ensure the LTB-Pk-cys mutants were captured by GM1, an anti - LTB mAb was used as a positive control (data not shown). The capture of the LTB-Pk-cys mutants allowed all of the anti-Pk mAbs to be screened for their ability to bind to the altered Pk tags. (figure 45). At the time the ELISA experiments were performed, the E and J cysteine substituted tags, shown in figure 44, had not been cloned and are not illustrated in figure 45.

As can be seen in figure 45, the substitution in Pk tag G affected the binding of all five anti-Pk mAbs. This meant that for mAb binding to occur, a substitution of pro-7 was not tolerated. When comparing this to the results obtained for the cysteine substitutions on the SPOT test, there were discrepancies (see figure 42). The SPOT membranes showed that a substitution to pro-7 only affected the binding of SV5-Pk2 and SV5-Pk3 to the Pk tag.

The binding of mAb SV5-Pk1 (figure 45a) was also disrupted by substitutions to asn-6 and leu-9. These two substitutions correlated with the SPOT test results (see figure 42, lane a).

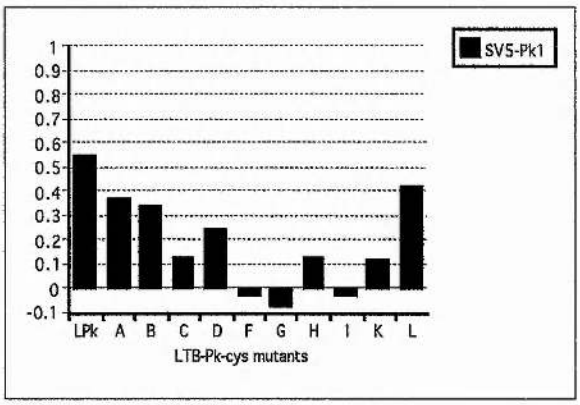
In addition to the disruption to mAb binding shown by the pro-7 substitution, the binding of SV5-Pk 2 (figure 45b) was also disrupted by the substitution of asn-6 and leu-8. These results confirm those obtained for SV5-Pk2 by SPOT testing (figure 42, lane b).

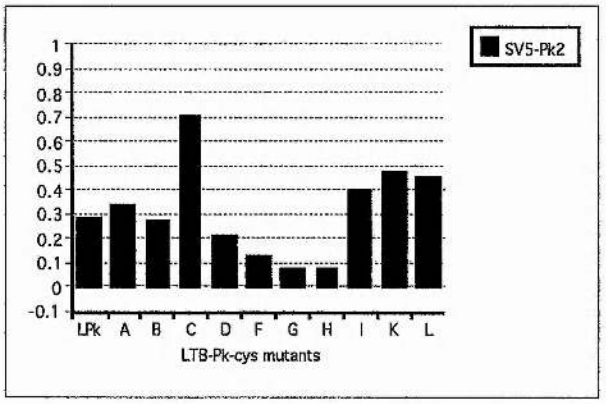
SV5-Pk3 binding was only affected by the substitution of pro-7, as stated earlier.

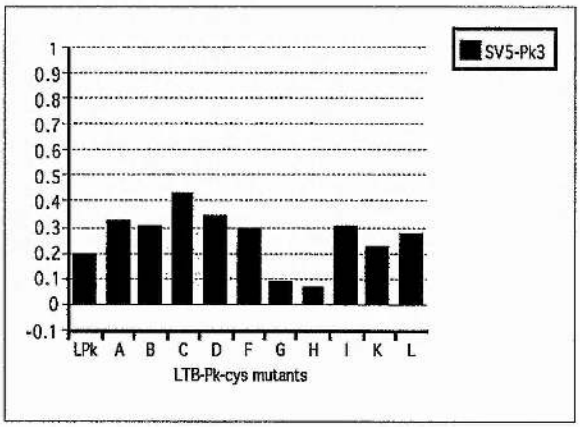
| | |
|---|---|
| **Pk tag** : | leu. gly. lys. pro. ile. pro. asn. pro. leu. leu. gly. leu. asp. ser. thr. |
| **A** (1): | leu. **cys**. lys. pro. ile. pro. asn. pro. leu. leu. gly. leu. asp. ser. thr. |
| **B** (2): | leu. gly. **cys**. pro. ile. pro. asn. pro. leu. leu. gly. leu. asp. ser. thr. |
| **C** (3): | leu. gly. lys. **cys**. ile. pro. asn. pro. leu. leu. gly. leu. asp. ser. thr. |
| **D** (4): | leu. gly. lys. pro. **cys**. pro. asn. pro. leu. leu. gly. leu. asp. ser. thr. |
| **E** (5): | leu. gly. lys. pro. ile. **cys**. asn. pro. leu. leu. gly. leu. asp. ser. thr. |
| **F** (6): | leu. gly. lys. pro. ile. pro. **cys**. pro. leu. leu. gly. leu. asp. ser. thr. |
| **G** (7): | leu. gly. lys. pro. ile. pro. asn. **cys**. leu. leu. gly. leu. asp. ser. thr. |
| **H** (8): | leu. gly. lys. pro. ile. pro. asn. pro. **cys**. leu. gly. leu. asp. ser. thr. |
| **I** (9): | leu. gly. lys. pro. ile. pro. asn. pro. leu. **cys**. gly. leu. asp. ser. thr. |
| **J** (10): | leu. gly. lys. pro. ile. pro. asn. pro. leu. leu. **cys**. leu. asp. ser. thr. |
| **K** (11): | leu. gly. lys. pro. ile. pro. asn. pro. leu. leu. gly. **cys**. asp. ser. thr. |
| **L** (12): | leu. gly. lys. pro. ile. pro. asn. pro. leu. leu. gly. leu. **cys**. ser. thr. |

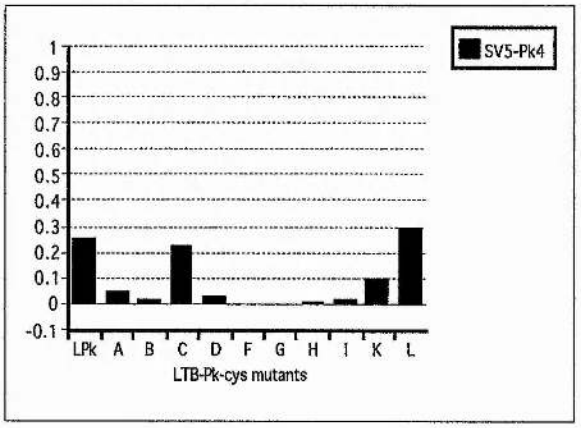
**Figure 44 : the amino acid sequences of the Pk peptide tags and the cysteine substituted Pk tags which were cloned onto the C terminus of LTB and expressed in *Vibrio spp.***

The numbers in parentheses correspond to the peptides on the SPOT membranes shown in figure 41.



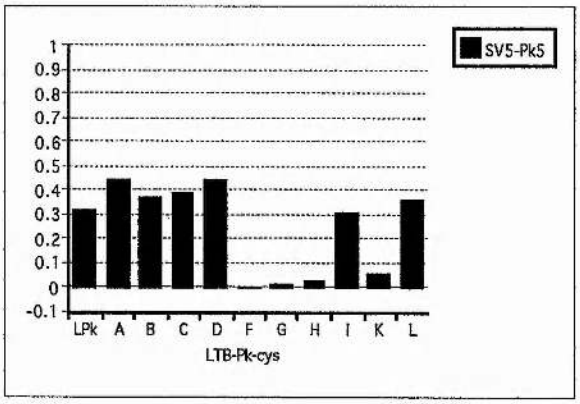




**Figure 45 : Bar graphs to show the effect of cysteine substitutions at each position in the Pk tag on the anti-Pk mAb binding.**
The binding of the five anti-Pk monoclonal antibodies to the LTB-Pk-cys mutants were tested by ELISA. LTB-Pk (LPk) was used as a positive control and the supernatant from *Vibrio sp.* which had not been transformed was used as a negative control (data not shown).

and leu-8 (figure 45c). This corroborates the information obtained from the SPOT tests. However, the LTB-cys substitutions show no effect on mAb binding when asn-6 was substituted. This is a complete contradiction to the data previously obtained from the SPOT tests (figure 42, lane c).

For effective binding to the Pk tag, mAb SV5-Pk4 could not tolerate the substitution of a number of amino acids (figure 45d). In addition to pro-7, the substitution of lys-2, ile-4, asn-6, leu-8, and leu-9 could not be tolerated if mAb binding was to occur. When comparing this information to that obtained from the SPOT test, there were a number of differences. In the SPOT test, substitutions to lys-2 or ile-4 had not affected mAb binding. However, mAb binding was affected by substitutions to asn-6, leu-8 and leu-9 (figure 42, lane d).

SV5-Pk5 (figure 45e) could not tolerate the substitution of asn-6, leu-8 and leu-11. This confirms the results previously obtained from the SPOT test.

A complete summary of the results obtained from the LTB-Pk-cys experiments can also be seen in figure 43. The discrepancies shown between the ELISA testing of the LTB-Pk-cys mutants and the SPOT tests have not been explained. However, it has given an indication of the differences in the requirements of binding exhibited by the five anti-Pk mAbs.

## 4.2 Construction and expression of pGEX-I4APk, pGEX-L8APk and pGEX-L11APk

The main objective for altering the Pk tag was to obtain a modified tag which would reduce the affinity of the mAbs for the Pk tag, and allow antibody - antigen separation to be achieved using relatively mild elution conditions.

From the results obtained by the SPOT's cysteine substitutions and insertions, and the LTB-Pk-cys substitutions, the amino acids at positions 4, 8 and 11 were chosen to be altered (figure 46). The amino acids to be changed were the non-polar leucine or isoleucine residues. The amino acids at all three positions were changed to an alanine residue, instead

**Pk tag:**

pro gly lys pro ile pro asn pro pro leu leu gly leu ser thr OPA

A ATT CCA GGA AAG CCG ATC CCA AAC CCT TTG CTG GGA TTG GAC TCC ACC TGA
GGT CCT TTC GGC TAG GGT TTG GGA AAC GAC CCT AAC CTG AGG TGG ACT TTAA

**I4APk tag:**

pro gly lys pro **ala** pro asn pro leu leu gly leu asp ser thr OPA

A ATT CCA GGA AAG CCG GCC CCA AAC CCT TTG CTG GGA TTG GAC TCC ACC TGA
GGT CCT TTC GGC CGG GGT TTG GGA AAC GAC CCT AAC CTG AGG TGG ACT TTA A

**L8APk tag:**

pro gly lys pro ile pro asn pro **ala** leu gly leu asp ser thr OPA

A ATT CCA GGA AAG CCG ATC CCA AAC CCT GCG CTG GGA TTG GAC TCC ACC TGA
GGT CCT TTC GGC TAG GGT TTG GGA CGC GAC CCT AAC CTG AGG TGG ACG TTA A

**L11APk tag:**

pro gly lys pro ile pro asn pro leu leu gly **ala** asp ser thr OPA

A ATT CCA GGA AAG CCG ATC CCA AAC CCT TTG CTG GGA GCG GAC TCC ACC TGA
GGT CCT TTC GGC TAG GGT TTG GGA AAC GAC CCT CGC CTG AGG TGG ACT TTA A

**Figure 46 : The DNA linkers containing the sequences for the Pk tag and the modified Pk tags.**

The positive and negative sense oligonucleotides were designed so that when annealed an *Eco*RI compatible site was created at each of the ends. The amino acid mutated in each of the Pk tags is shown in bold.

of a cysteine as was performed previously. This was done to preserve the chemical property of the amino acid side chain, as alanine is an amino acid which also has a non-polar side chain. This substitution would not dramatically affect the three-dimensional folding of the Pk tag.

Previously all antibody binding studies had been performed using recombinant His-Nef-Pk or the P protein of SV5. However, to test the modified tags, the model protein glutathione S transferase (GST) was selected instead. This was because large amounts of highly purified GST can be produced using a simple protocol, in a relatively short length of time (see section 1.1 of the Introduction for more details). This makes GST an ideal protein with which to test the modified Pk tags.

The sequence for the original Pk tag had previously been cloned into the pGEX-2T expression vector, creating pGEXcPk (Hanke 1993). From this plasmid, a fusion protein in which the Pk tag was fused to the C terminus of GST was produced, known as GST-Pk.

To produce GST fusion proteins which contained the modified tags, oligonucleotide primers were synthesised which contained the individual 5' and 3' DNA sequences of the three modified tags. When annealed together, the DNA linkers created contained *Eco*RI compatible restriction sites at both ends. The DNA linkers were ligated into the *Eco*RI restriction site of pGEX-2T.

Positive transformants, containing the plasmids with the modified tags, were screened by hybridisation using the 5' oligonucleotide primers as probes. As the DNA linkers had been ligated into a single restriction site, the orientation of the modified tags needed to be confirmed. This was performed by inducing the bacterial transformants at 27°C for 2 hours with 1 mM IPTG, and analysing the TCL samples by Western blotting (figure 47). The nitrocellulose filters were probed with a mixture of all five anti-Pk mAbs. The fusion proteins produced were named: GST-Pk (from pGEXcPk), GST-I4APk, GST-L8APk and GST-L11APk.

To acquire sufficient protein for mAb binding studies, large scale inductions were performed. The resuspended cells were kept in the presence of protease inhibitors at all

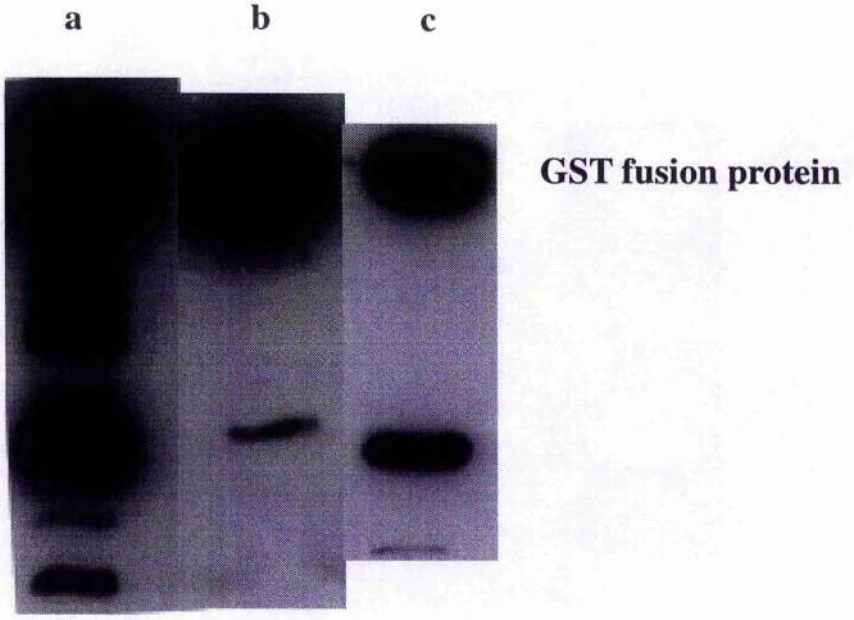



**Figure 47 : Western blot analysis to determine the orientation of GST fusion proteins containing the mutated Pk tags.**

Small scale cultures of bacteria previously transformed with pGEX-2T containing the ligated DNA linkers were induced with 1 mM IPTG for 2 hours. Samples were taken, the pelleted cells resuspended in SDS-PAGE loading buffer, and the total cell lysates were loaded onto a 10% polyacrylamide gel. The separated proteins were transferred to nitrocellulose filters and probed with a mixture of all five anti-Pk mAb's. Detection was by ECL.

Key : **a** - GST-I4APk; **b** - GST-L8APk; **c** - GST-L11APk

times during cell lysis and purification, to ensure that the produced recombinant fusion proteins were not degraded by bacterial proteases. After cell lysis and centrifugation, the soluble protein fractions containing the GST fusion proteins were immediately purified on a glutathione column. The proteins were eluted by the addition of an excess of glutathione to the wash buffer.

## 4.3 Binding studies for GST-I4APk, GST-L8APk and GST-L11APk

To determine if the mAbs recognised all 3 modified tags, ELISA plates were coated with the GST fusion proteins and probed with the five anti-Pk mAbs. The results showed that SV5-Pk1 recognised all 3 modified tags. SV5-Pk2 also bound to all 3 modified tags but appeared to bind GST-I4APk with less affinity than GST-L8APk and GST-L11APk. SV5-Pk3 and SV5-Pk5 only recognised GST-I4APk, whereas SV5-Pk4 recognised GST-L8APk and GST-L11APk but not GST-I4APk (table 10).

To determine if this pattern of mAb binding was obtained when using batch affinity chromatography, the five anti-Pk mAbs were immobilised on PrG Sepharose beads. Each fusion protein was incubated with each of the mAbs bound to the PrG Sepharose beads. The mixture was washed with PBS and loaded onto a 10% PAG. The PAG's were stained with Coomassie Brilliant Blue to visualise the proteins (figure 48).

On the stained PAG there were three bands visible (see figure 48). The very large and very small bands represented the heavy and light chains of the mAbs respectively. The heavy and light chains were visible because the mAbs had not been covalently coupled to the Protein G Sepharose beads, which allowed separation in the SDS-PAGE

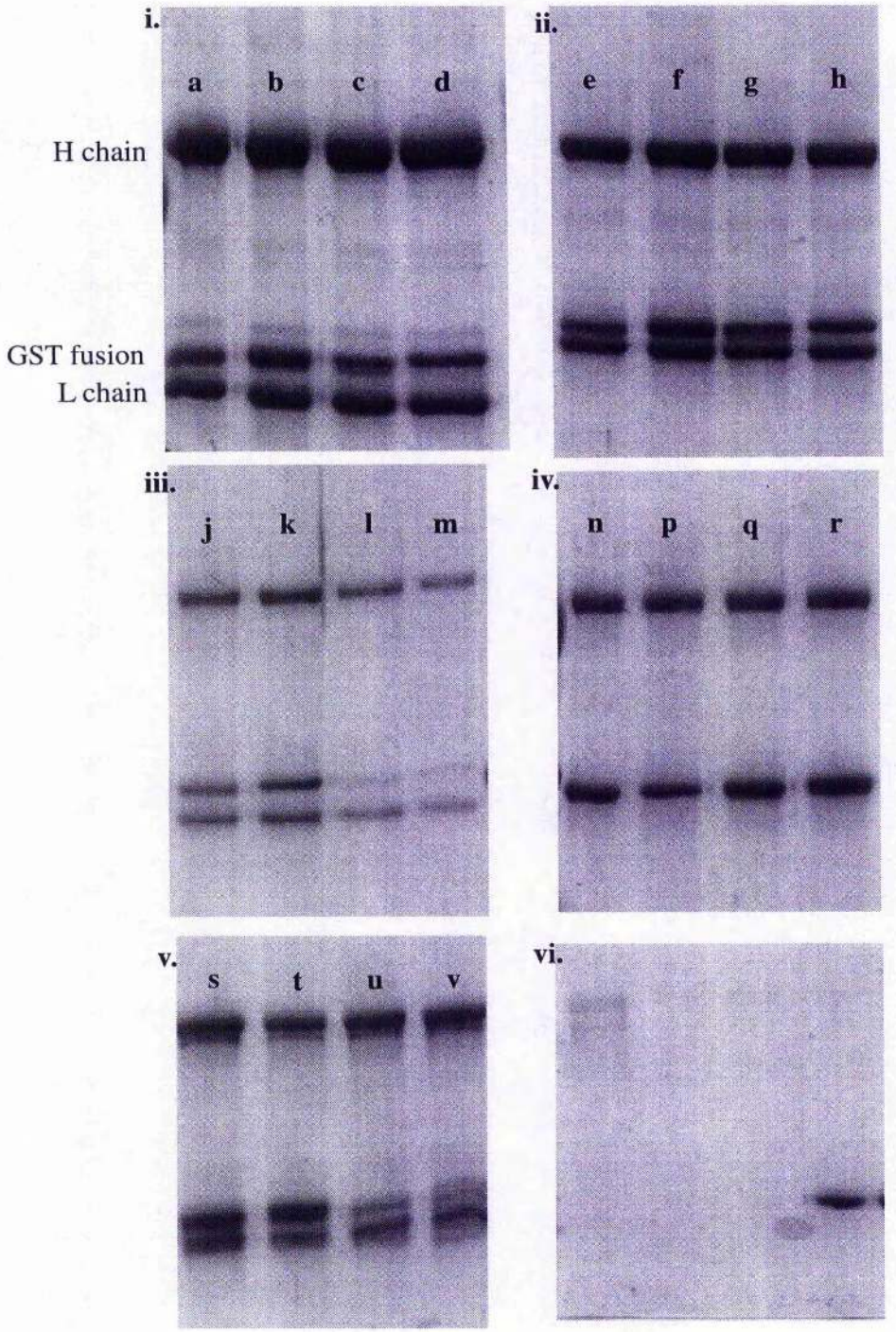



**Figure 48 : Coomassie stained gels showing the patterns of binding of the five anti-Pk mAb's to the modified Pk tags.**

Each of the anti-Pk mAb's were immobilised on PrG-sepharose beads and incubated with each of the GST-Pk fusion proteins for 1 hour. The beads were washed with PBS, resuspended in SDS-PAGE loading buffer and loaded onto a 10% PAG. The protein bands were visualised by Coomassie staining.

Key : i - SV5-Pk1; ii - SV5-Pk2; iii - SV5-Pk3; iv. SV5-Pk4; v - SV5-Pk5; vi. - Protein G beads only (negative control)

**a, e, j, n & s** - GST-Pk; **b, f, k, p & t** - GST-I4APk; **c, g, l, q & u** - GST-L8APk; **d, h, m, r & v** - GST-L11APk

| | SV5-Pk1 | SV5-Pk2 | SV5-Pk3 | SV5-Pk4 | SV5-Pk5 |
|---|---|---|---|---|---|
| GST-Pk | +++ | +++ | +++ | +++ | +++ |
| GST-I4APk | +++ | + | +++ | - | +++ |
| GST-L8APk | +++ | +++ | - | +++ | - |
| GSTL11APk | +++ | +++ | - | +++ | - |

**Table 10 : The binding patterns of the anti-Pk mAbs to GST-Pk and the three modified Pk tags.**

ELISA plates were coated with each of the GST fusion proteins and probed with the anti-Pk mAbs. The pattern of binding was observed by a change in colour when the assay was developed.

Key : +++ - strong binding to the fusion protein; + - weak binding to the fusion protein; - - no binding to the fusion protein.

loading buffer. If a third band was present in the sample, this showed mAb binding to the modified tag. If only two bands were visible, then no, or reduced, binding of the fusion protein containing the modified tag to the anti-Pk mAb was observed. The most obvious example of this is shown in lanes l & m. This indicated that SV5-Pk3 did not bind to the GST-L8APk and GST-L11APk altered tags.

The light chain of SV5-Pk4 separated at exactly the same size as the GST fusion proteins on the Coomassie stained gel (figure 48, lanes n, p, q & r). This made it impossible to determine which of the altered tags SV5-Pk4 recognised. To overcome this the samples were analysed by Western blotting and probed with an anti - GST polyclonal antibody. From this experiment, it became clear that SV5-Pk4 did not bind to, or showed reduced binding to, the GST-I4APk mutant. Binding to the GST-L8APk and GST-L11APk mutants was observed (data not shown).

In general, the results obtained from the batch affinity chromatography experiments confirmed the results of the previous ELISA experiment, however, there was a single contradiction. The ELISA experiments revealed that SV5-Pk2 showed a reduced binding to the GST-I4APk modified tag (see table 10), but the batch affinity chromatography experiments showed that SV5-Pk2 bound to GST-I4APk with equivalent strength to that of the other modified tags (figure 48, lane f). The differences in the two practical techniques

may partially explain the contradictory observations of SV5-Pk2 binding to GST-I4APk. However, the reduction in the binding of SV5-Pk2 to the I4APk modified tag may be a way of producing a protocol for the affinity purification of Pk tagged recombinant proteins.

# 5. THE DEVELOPMENT OF A PROTOCOL FOR THE AFFINITY PURIFICATION OF Pk TAGGED RECOMBINANT PROTEINS

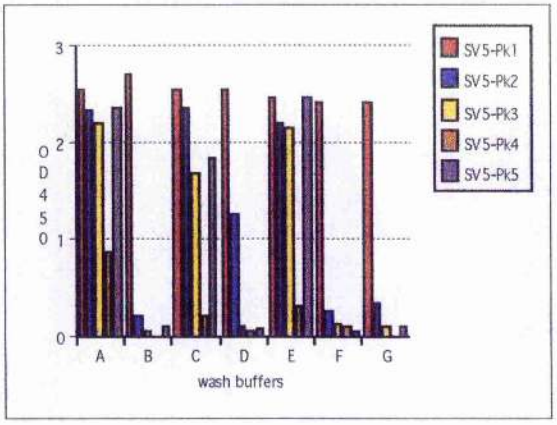
Section 5 concentrates on the determination of buffers which could be used for the elution of purified tagged recombinant proteins from a mAb affinity column.

## 5.1 Determination of wash conditions for the elution of bound monoclonal antibodies from tagged recombinant proteins

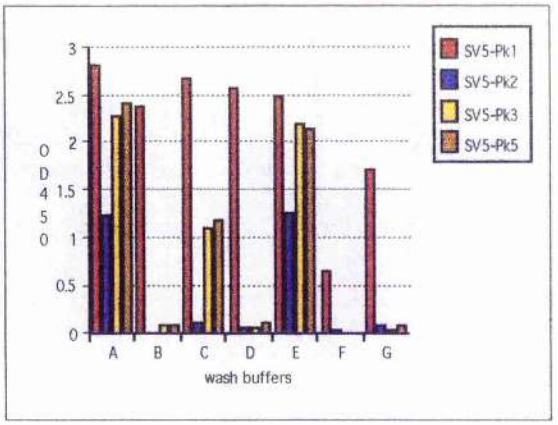
An efficient method of testing a large number of conditions is by using ELISA. To test buffers which can be used for the elution of Pk tagged recombinant proteins from anti-Pk mAbs, or vice versa, ELISA plates were coated with the GST fusion proteins containing the modified tags. The plates were then probed with the five anti-Pk mAbs, using an anti - GST polyclonal antibody as a positive control. The anti-GST polyclonal antibody was used to ensure that the GST fusion proteins were binding to the ELISA plates, and to confirm that a reduction in the amount of mAb bound was not due to the fusion proteins being stripped from the plates when using harsh elution conditions. A number of elution buffers were tested (figure 49).

Figure 49 shows the effect that a number of elution buffers had on antibody binding to GST-Pk (figure 49a), GST-I4APk (figure 49b), GST-L8APk (figure 49c) and GST-L11APk (figure 49d). These experiments were designed to identify any elution buffer which had little effect on mAb binding to the GST-Pk fusion protein, but which showed a drastic effect on the binding of the mAbs to a fusion protein containing a modified tag. As with the ELISA and batch chromatography experiments described in section 4.3, the best

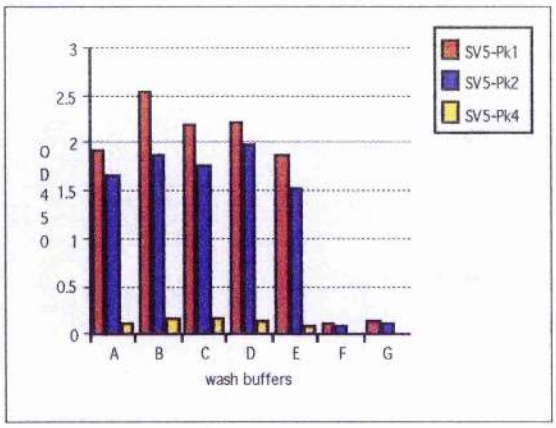
**a. GST-Pk**




**b. GST-I4APk**




**c. GST-L8APk**




**d. GST-L11APk**

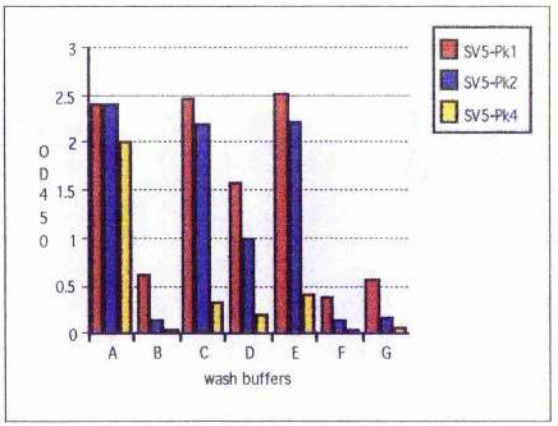



**Figure 49 : Bar charts showing the effect of elution buffers on the binding of the anti-Pk mAbs to GST fusion proteins containing the modified Pk tags.**
Each of the mAb's were incubated on ELISA plates with each relevant GST-Pk fusion proteins. Each ELISA plate was then incubated with a number of elution buffers to determine which has the most significant effect on mAb binding.
Key to elution buffers : A - phoshate buffered saline; B - 0.2 M glycine pH2.8; C - 3.5 M Magnesium chloride; D - 3.5 M sodium thiocyanate; E - 0.1 % Sodium deoxycholate; F - 0.1 M diethylamine; G - 20 mM hydrochloric acid

example observed was the binding of SV5-Pk2 to the GST-I4APk modified tag (compare figure 49a & 49b, lane e). In this example, the elution buffer 3 M magnesium chloride ($MgCl_2$) showed little effect on the binding of SV5-Pk2 to GST-Pk (figure 49a, lane e), yet appeared to completely disrupt the binding of SV5-Pk2 to the GST-I4APk mutant tag (figure 49b, lane e).

A complete summary of all the elution buffers tested on the binding of all four fusion proteins to all five anti-Pk mAbs can be seen in Table 11. Table 11 also shows the effects that a single amino acid change to the Pk tag had on mAb binding for different elution buffers.

The data obtained from these ELISA experiments was used as a basis for the determination of buffers which could be used for the elution of tagged recombinant proteins from mAb coupled affinity columns.

## 5.2 Batch purification of Pk tagged recombinant proteins from PrG-mAb beads

When altering the sequence of the Pk tag to create the three new tags, it was hoped that for each mAb there would be a single modified tag which would change the binding of the mAbs and allow the elution of tagged recombinant proteins using relatively mild elution conditions. From the information obtained from the ELISA experiments described in section 5.1, it was shown that for each mAb there was a single modified tag which allowed protein elution under mild conditions. The modified tag selected for each mAb, and shown in Table 12, was to be used for further affinity chromatography studies.

The five anti-Pk mAbs were covalently coupled to Sepharose-Protein G beads using dimethylpimelidate (Materials and Methods). Small samples of the PrG - mAb coupled Sepharose beads were incubated, for 1 hour, with GST-Pk, or GST-I4APk, GST-L8APk or GST-L11APk, depending on the mAb. After the incubation with the GST fusion proteins, the beads were then incubated with a number of elution buffers. The elution

**Table 11 : A summary of the ELISA data obtained on the effects of elution buffers on the binding of the anti-Pk mAb's to the GST fusion proteins.**

ELISA plates were coated with the four GST fusion proteins and probed with the five anti-Pk mAb's. Each column of wells was then washed with a different elution buffer, using PBS as the positive control. The effectiveness of the buffers were determined by a direct comparison to the PBS control.

| | | PBS | 0.1M glycine pH 2.8 | 0.1M glycine pH 4.0 | 0.1M glycine pH 10.1 | 0.1M glycine pH 11.5 | 3.5M $MgCl_2$ | 3.5M NaSCN | 0.1% deoyx-cholate | 0.1% SDS | 0.1% CHAPS | 1% ethylene glycol | 10% ethylene glycol | 50% ethylene glycol | 0.1M diethyl -amine | 0.1M propio-nic acid | 20mM ethanol -amine pH 8.0 | 20mM HCl |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Pk-1 | Pk | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| | I4A | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | ++ | +++ | +++ | + |
| | L8A | +++ | - | +++ | +++ | ++ | +++ | + | +++ | +++ | +++ | +++ | +++ | +++ | - | +++ | - | - |
| | L11A | +++ | ++ | +++ | +++ | ++ | +++ | ++ | +++ | +++ | +++ | +++ | +++ | +++ | + | +++ | - | + |
| Pk-2 | Pk | +++ | + | +++ | ++ | ++ | +++ | ++ | +++ | +++ | +++ | +++ | +++ | +++ | ++ | ++ | - | + |
| | I4A | ++ | - | ++ | - | - | - | - | +++ | + | +++ | +++ | +++ | ++ | - | - | - | - |
| | L8A | +++ | - | +++ | +++ | + | ++ | + | +++ | +++ | +++ | +++ | +++ | +++ | - | ++ | - | - |
| | L11A | +++ | - | +++ | +++ | ++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | ++ | + | + | - |
| Pk-3 | Pk | +++ | + | +++ | +++ | + | +++ | ++ | +++ | +++ | +++ | +++ | +++ | +++ | + | + | - | - |
| | I4A | +++ | - | +++ | + | - | ++ | - | +++ | + | +++ | ++ | +++ | +++ | - | + | - | - |
| | L8A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - |
| | L11A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - |
| Pk-4 | Pk | +++ | + | +++ | ++ | + | +++ | + | +++ | + | +++ | +++ | +++ | +++ | + | + | - | + |
| | I4A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | L8A | + | - | +++ | ++ | + | ++ | - | +++ | + | +++ | +++ | +++ | +++ | - | + | - | - |
| | L11A | +++ | - | +++ | +++ | + | + | - | +++ | - | ++ | +++ | +++ | +++ | + | ++ | - | - |
| Pk-5 | Pk | +++ | + | +++ | +++ | ++ | +++ | + | +++ | +++ | +++ | +++ | +++ | +++ | ++ | ++ | - | - |
| | I4A | +++ | - | +++ | + | - | ++ | - | +++ | ++ | +++ | +++ | +++ | +++ | - | + | - | - |
| | L8A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | L11A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

## KEY

- No Binding Observed
+ Little Binding Observed
++ Moderate Binding Observed
+++ Strong Binding Observed

buffers were specifically chosen for each mAb on the basis of how well they had disrupted the mAb - antigen complexes in the ELISA studies described in section 5.1 (see Table 11).

| Anti-Pk monoclonal antibody | Fusion protein containing the modified Pk tag chosen for further elution analysis |
|---|---|
| SV5-Pk1 | GST - L8APk |
| SV5-Pk2 | GST-I4APk |
| SV5-Pk3 | GST-I4APk |
| SV5-Pk4 | GST-L8APk |
| SV5-Pk5 | GST-I4APk |

**Table 12 : a summary of the fusion proteins containing the modified tags chosen for each mAb for further affinity chromatography analysis.**
The modified tag chosen for each mAb was based on the information obtained, by ELISA, on the relative effect of elution buffers on the binding of the anti-Pk mAbs to the modified tags.

After the incubation with the elution buffers, the beads were washed with PBS and resuspended in SDS-PAGE loading buffer. The samples were loaded onto a 10% PAG for electrophoresis and the protein bands visualised by Coomassie staining. To ascertain how effective each buffer was at eluting the GST fusion proteins containing the various Pk tags, from mAb affinity beads, a comparison was made of the levels of elution obtained from the GST-Pk and the modified GST-Pk beads (see figures 50, 52, & 53). To ensure that a direct comparison could be performed, each of the samples contained an equal amount of PrG-mAb beads.

As stated earlier, the GST-L8APk mutant tag was selected for further study with the anti-Pk mAbs SV5-Pk1 and SV5-Pk4. PrG-mAb Sepharose beads which had been incubated with either GST-Pk or GST-L8APk, were then incubated with the elution buffers 0.2 M glycine pH2.8 (glycine 2.8), 0.2 M glycine pH11.5 (glycine 11.5) and 2 M sodium thiocyanate (NaSCN), as well as PBS as the positive control. An efficient elution buffer is one which gives good protein elution when compared to the PBS control. The results can be seen in figure 50.

The greatest differences observed between the binding of SV5-Pk1 to the modified tag and to the original Pk tag, were shown for the elution buffers glycine 2.8 (compare lanes b & f) and NaSCN (compare lanes d & h). The presence of these two buffers, had a dramatic effect on the binding of SV5-Pk1 to GST-L8APk (lanes f & h), but no effect on mAb binding to GST-Pk (lanes b & d). It appeared that changing the leucine at position eight of the Pk tag, drastically affected the efficiency with which SV5-Pk1 binds the Pk tag, allowing relatively easy separation. Glycine 11.5 showed no protein elution (lanes c & g) and gave protein elution equivalent to the PBS positive control (lanes a & e).

As with SV5-Pk1, glycine 2.8 (figure 50, compare lanes k & o) also showed the most significant effect on the binding of SV5-Pk4 to the modified tag, when compared to the original Pk tag. NaSCN and glycine 11.5 appeared to have no effect on the binding of SV5-Pk4 to GST-Pk or GST-L8APk (compare lanes m & q and l & p respectively), although there was less protein bound than that observed in the PBS positive control (lanes j & n).

From the ELISA data discussed in section 5.1, a number of elution buffers affected the binding of SV5-Pk2 to the I4APk modified tag (figure 49). As the mildest condition to affect SV5-Pk2 binding was 3 M $MgCl_2$, it was thought that other divalent cations may also effect mAb binding.To test this, the elution buffers 2 M calcium chloride ($CaCl_2$), 2 M manganese chloride ($MnCl_2$) and 2 M zinc chloride ($ZnCl_2$) were examined. These elution buffers were originally tested by ELISA to determine which was the most effective at allowing protein elution (figure 51). Of the buffers tested, the most promising were found to be 2 M $MnCl_2$ (lane D) and 2 M $MgCl_2$ (lane B), with the least effective being 2 M $ZnCl_2$ (lane E). Due to the results obtained from this ELISA study, $MnCl_2$ and $MgCl_2$, along with glycine 2.8, were used for the elution of the GST-Pk and GST-I4APk fusion proteins from SV5-Pk2 coupled Protein G - Sepharose beads. As with the previous experiments, PBS was used as a positive control. The results can be seen in figure 52.

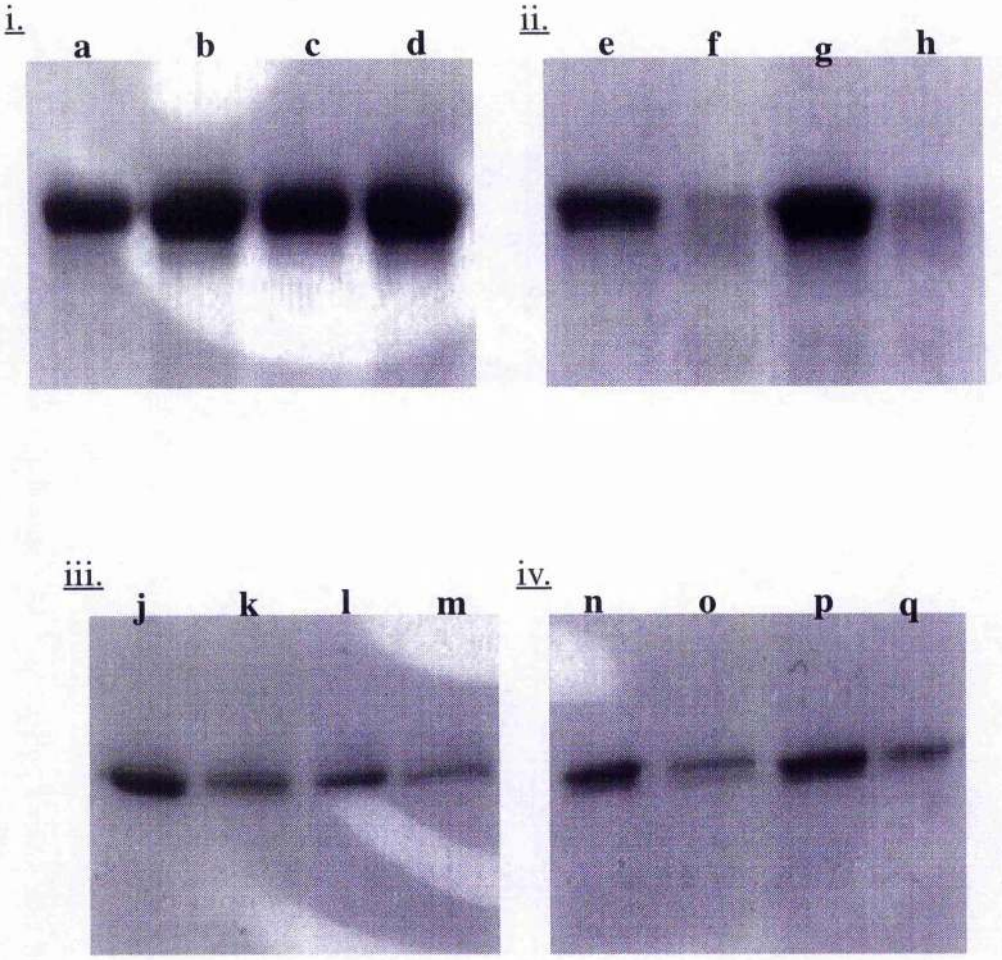



**Figure 50 : Coomassie stained gels showing the effect of elution buffers on the binding of the anti-Pk mAb's to GST-Pk and GST-L8APk.**

The anti-Pk mAb's SV5-Pk1 (i & ii) & SV5-Pk4 (iii & iv) were covalently coupled to PrG-sepharose beads and incubated with the GST-Pk (i & iii) & GST-L8APk (ii & iv) fusion proteins for 1 hour.

Samples of each were then incubated with different elution buffers before being washed with PBS and loaded onto a 10% PAG. The protein bands were visualised by Coomassie blue staining.

Key to elution buffers : **a, e, j, & n -** PBS; **b, f, k, & o -** 0.2 M glycine pH 2.8; c, g, l & **p -** 0.2 M glycine pH 11.5; **d, h, m & q -** 3.5 M sodium thiocyanate

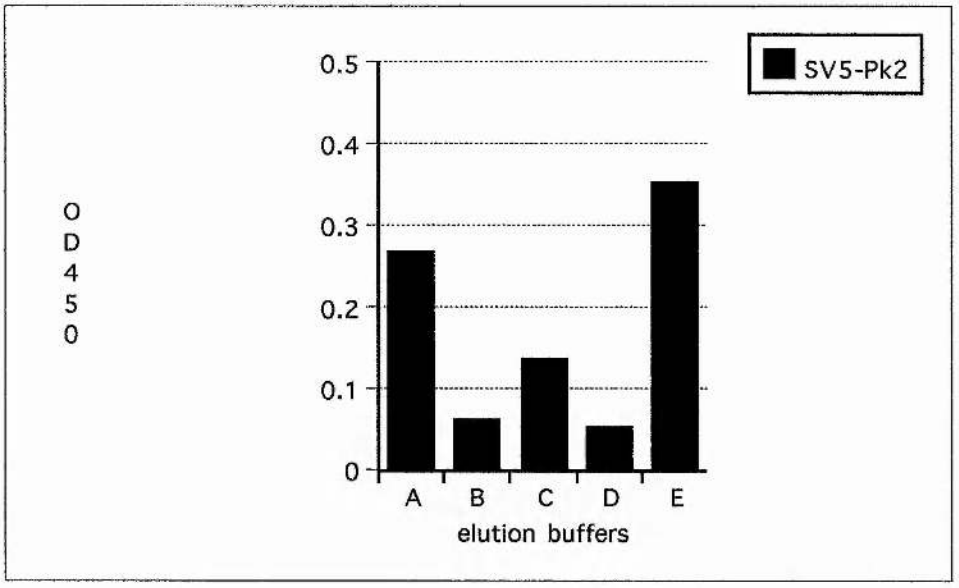



**Figure 51 : bar chart showing the effect of elution buffers on the binding of the anti-Pk mAb SV5-Pk2 to the modified GST-I4APk tag**
The data was obtained by the testing of ELISA plates coated in the GST-I4APk mutant fusion protein, and probing with SV5-Pk2. The plates were then washed with a number of elution buffers and using PBS as the positive control, assayed for their effectivity.
key : A - PBS; B- 2 M $MgCl_2$; C - 2 M $CaCl_2$; D - 2 M $MnCl_2$; E - 2 M $ZnCl_2$.

An effect on mAb binding to the GST-I4APk fusion protein was seen with all three buffers tested, with the largest effect elicited by glycine 2.8 (lane f). However, when comparing this to the protein elution obtained for GST-Pk, the differences were not so distinct. When a comparison was made, the largest difference between the two tags was seen for $MgCl_2$ (compare lanes c & g), although $MnCl_2$ does not show much less of a difference (compare lanes d & h). Unfortunately, it was found that after elution with $MgCl_2$, a precipitate formed on washing the beads with PBS. To overcome this problem, the beads were washed with Tris buffered Saline (TBS) instead of PBS.

As the mAbs SV5-Pk3 and SV5-Pk5 only recognised the I4APk modified tag, this tag was compared to the original Pk tag, and used for further elution studies. Samples of PrG-SV5-Pk3 and PrG-SV5-Pk5 beads were incubated with either GST-Pk or GST-I4APk before being incubated with the elution buffers glycine 2.8, glycine 11.5 and NaSCN. As with the other experiments, PBS was used as a positive control (figure 53).

The differences observed for SV5-Pk3 were not as marked as those observed for the mAbs previously discussed. Figure 53 shows that only slightly less protein elution was obtained from the GST-Pk tag than the modified GST-I4APk tag [compare figure 53 (i) & (ii)]. This was contradictory to the data obtained by ELISA (Table 11). Glycine 2.8 gave the most protein elution for both tags (lanes b & f). However, unlike the anti-Pk mAbs described previously, glycine 11.5 also showed a large effect on protein binding (lanes c & g). Of the elution buffers tested NaSCN gave the least protein elution (lanes d & h), although when compared to the PBS control a considerable amount of protein was still eluted.

When a comparison of the levels of protein eluted from SV5-Pk5 affinity beads was made between the original Pk tag and the modified L8APk tag, there appeared to be very little difference [compare figure 53, (iii) & (iv)]. For both tags, little protein elution was obtained when comparing each of the elution buffers to the PBS positive control (lanes j & n). Of the elution buffers tested, only glycine 11.5 (lanes l & p) gave a significant reduction in protein binding of the GST-L8APk fusion protein containing the modified tag. The other

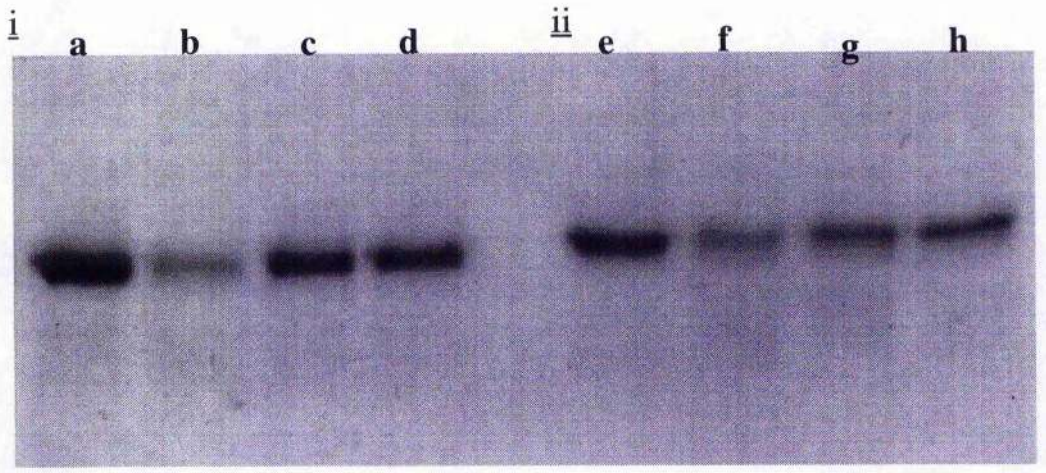



**Figure 52 : Coomassie stained gels showing the effect of different elution buffers on the binding of SV5-Pk2 to GST-Pk and GST-I4APk.**

The SV5-Pk2 anti-Pk mAb was covalently coupled to PrG-sepharose beads and incubated with the GST-Pk (i) and GST-I4APk (ii) fusion proteins for 1 hour. Samples of each were then incubated with different wash buffers before being washed with PBS and loaded onto a 10% PAG gel for Coomassie Brilliant blue staining.

Key to elution buffers : **a & e -** PBS; **b & f -** 0.2 M glycine pH 2.8; **c & g -** 2 M magnesium chloride; **d & h -** 2 M manganese chloride

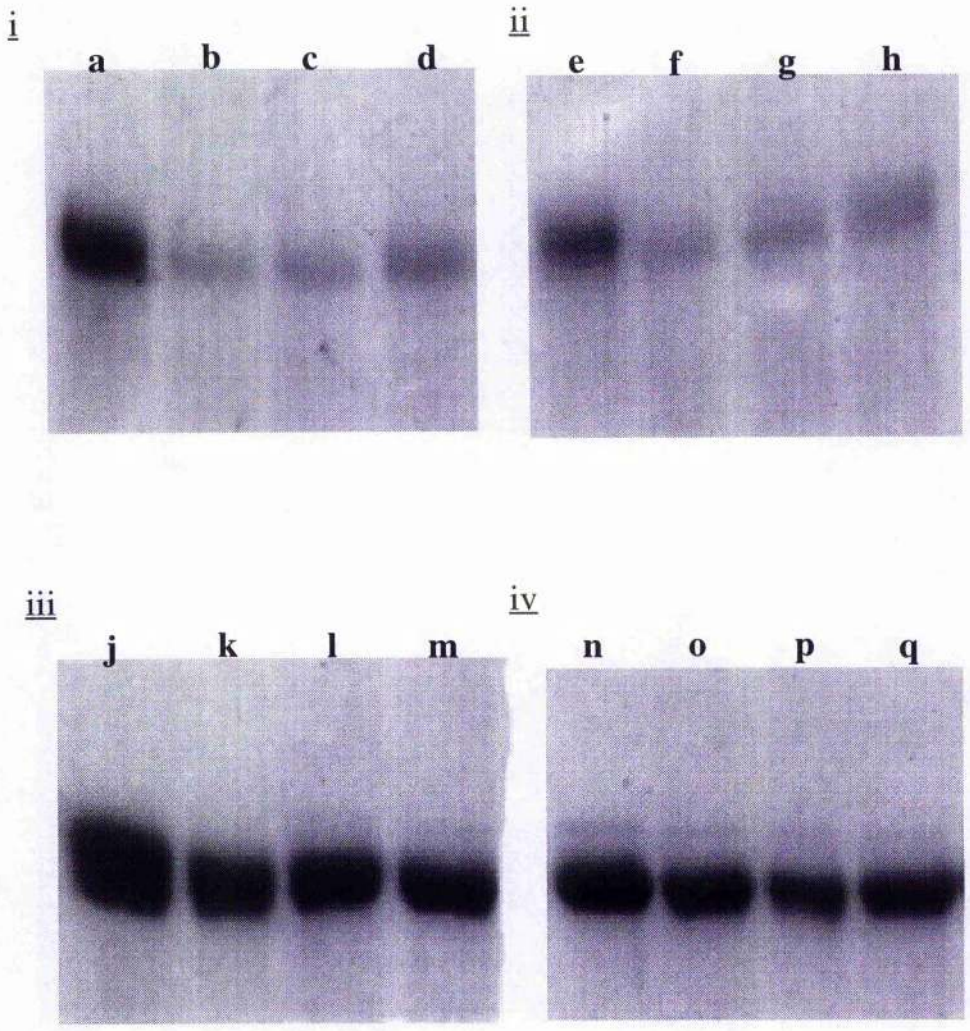



**Figure 53 : Coomassie stained gels showing the effect of elution buffers on the binding of the anti-Pk mAb's SV5-Pk3 and SV5-Pk5 to GST-Pk and GSTI4APk.**

The anti-Pk mAb's SV5-Pk3 (i & ii) & SV5-Pk5 (iii & iv) were covalently coupled to PrG-sepharose beads and incubated with the GST-Pk (i & iii) & GST-I4APk (ii & iv) fusion proteins for 1 hour.

Samples of each were then incubated with different elution buffers before washing with PBS and loading onto a 10% PAG. The proteins were visualised by staining with Coomassie Brilliant Blue. Key to elution buffers : **a, e, j, & n -** PBS; **b, f, k, & o -** 0.2 M glycine pH 2.8; c, g, l & **p -** 0.2 M glycine pH 11.5; **d, h, m & q -** 2 M sodium thiocyanate

elution buffers tested showed little observable protein elution (compare lanes k & o for glycine 2.8, and lanes m & q for NaSCN).

## 5.3 Conclusions

The data described in this section was utilised for further studies into the elution of tagged recombinant proteins from mAb affinity columns. The preliminary data obtained from these experiments showed that the conditions described for each mAb did in fact elute tagged proteins from PrG-mAb affinity chromatography columns, although no quantitative data was obtained on how much protein was eluted. This signified that although the exact protocol for a two - step affinity purification protocol had not been developed, the foundations for one had been laid.

# DISCUSSION

## 1. SIV GENE EXPRESSION AND RECOMBINANT PROTEIN PURIFICATION

In order to have a complete repertoire of cloned and purified SIVmac genes and proteins from the J5 molecular clone, the genes *nef* and *gag* were cloned into, and expressed from, the expression vector pQ9cPk. The conditions required to achieve optimal induction of *nef*, and the subsequent purification of recombinant His-Nef-Pk were determined, and these conditions are outlined in section 1.2 of the Results. This purified recombinant Nef has been used for a number of purposes including, the development of Nef anti-sera, the isolation of anti-Nef monoclonal antibodies (mAbs), and the incorporation of recombinant Nef into multivalent epitope vaccines. However, a number of problems arose when aiming to repeat this process for *gag*.

Like *nef*, the conditions for the optimal induction of the *gag* gene have been determined, and are shown in section 1.4 of the Results. Significant problems arose, however, when trying to purify recombinant His-Pr55$^{gag}$-Pk on a nickel affinity column. His-Pr55$^{gag}$-Pk was observed binding to the nickel beads, but no protein elution was seen when imidazole was applied to the column. In addition, no recombinant protein could be detected on the column by SV5-P-k once protein elution had been attempted. One explanation for this may be that conditions within the column environment may be sufficient to cause recombinant protein aggregation, and therefore, protein precipitation. The *gag* gene encodes the Pr55$^{gag}$ precursor protein, which after proteolysis, produces the viral particle structural proteins. Within an infected cell, this precursor protein is cleaved, almost instantaneously after translation, by the viral protease into the constituent proteins (p15, p17 & p24). It is possible therefore that His-Pr55$^{gag}$-Pk has an unstable configuration, exposing amino acid residues which allow protein aggregation. This change in the configuration of His-Pr55$^{gag}$-Pk may also have caused the Pk tag to be no longer

accessible to SV5-P-k, which would partially explain why no protein was detected within the column once protein elution was attempted.

To determine if the solubility of His-Pr55$^{gag}$-Pk was the problem during affinity purification, both the soluble protein fraction and the insoluble protein fraction, resuspended in 8 M urea, of the bacterial lysate were studied using identical conditions. The results of the soluble protein fraction gave no protein elution from a nickel affinity column (data not shown). The denatured insoluble protein fraction was studied under similar conditions due to the stable nature of the histidine tag and its ability to bind to nickel beads even under denaturing conditions. When eluting the denatured protein from nickel beads using a solution of imidazole and urea, small amounts of purified His-Pr55$^{gag}$-Pk protein were obtained (data not shown). The small percentage of protein eluted (approx 1% of the total protein detected) suggests that the recombinant protein is inherently insoluble in aqueous solution and that only under denaturing conditions will protein elution occur.

In a previous study by Hanke (1993), the cloning and expression of the individual *gag* gene sequences (*p15*, *p17* & *p24*), and the subsequent purification of the gene products, were examined. All three genes gave high yields of recombinant protein, which were nickel affinity purified with little difficulty. Due to the dificulties encounted when trying to purify Pr55$^{gag}$, it will be more efficient to express the *gag* genes individually and to purify each recombinant protein produced, instead of trying to obtain sufficient purified His-Pr55$^{gag}$-Pk recombinant protein.

## 2. THE ISOLATION OF ANTI-Pk$_{TH}$ MONOCLONAL ANTIBODIES

The Pk tag, which is fused to the C terminus of recombinant proteins, was originally the sequence of a synthetic peptide which was used to compete with the binding of the mAb SV5-P-k to its antigen, the P protein of Simian Virus 5 (SV5; Southern *et al* 1991). On further analysis, SV5-P-k was shown to have an unusually high affinity for the Pk peptide (23.6 pmol), making it useful for the detection and analysis of tagged

recombinant proteins, and for the incorporation of recombinant proteins into solid matrix antibody antigen (SMAA) complexes, but not for protein affinity purification (Hanke 1993, Randall *et al* 1994 & 1994a).

Previously developed within the research group was an affinity purification protocol using the Pk tag, and another (either the hexahistidine tag or GST), to obtain full length recombinant proteins (Randall *et al* 1993). As the recombinant proteins were required at that time for the development of SMAA complexes, the separation of the mAb SV5-P-k from the tag was not necessary. However, subsequent evidence showed that SMAA complexes did not give protection against SIV challenge (J. Stott & R.E. Randall, personal communication), suggesting that this was not the way to proceed in SIV vaccine development. This result, however, did not invalidate the use of multiple epitope vaccines as a way of immunising animals to give protection against SIV challenge. This meant that purified full length recombinant proteins were required in which the Pk tag was not complexed to the mAb SV5-P-k. Initial experiments to determine conditions which could be used for the separation of Pk tagged recombinant proteins from SV5-P-k showed that only the most denaturing conditions (e.g. 8 M urea or 6 M guanidine hydrochloride) had an effect, but this could permanently affect the structure of the recombinant proteins to be purified.

In order to obtain a number of mAbs which could potentially be used for the affinity purification of Pk tagged recombinant proteins, a group of mice were immunised with a peptide containing the Pk tag known as $Pk_{TH}$ (figure 28). From the initial immunisation with this peptide, twenty four anti-$Pk_{TH}$ mAbs were isolated. On further analysis by western blotting (figure 29), ELISA (table 6) and immunofluorescence (figure 30), of these twenty four mAbs, only four recognised the Pk tag sequence. The other twenty recognised only the $Pk_{TH}$ peptide.

In addition to the four anti-Pk mAbs which were tested, the binding sites of the twenty anti-$Pk_{TH}$ mAbs were also determined by SPOT testing (data not shown). All were found to recognise an area within the first half of the $T_H$ cell epitope, suggesting that this area within the $T_H$ cell epitope was immunodominant over the Pk tag amino acid sequence.

The inital characterisation of the four anti-Pk mAbs indicated similarities in their behaviour. For example, when experiments were performed to determine conditions which would allow the elution of the mAbs from His-Nef-Pk coated filters (figure 40), all four mAbs needed strong denaturing conditions to separate the antibody-antigen complex. When comparing this information to that initially obtained for SV5-P-k, it was observed that the four new mAbs did not allow protein elution under less restrictive conditions. The data obtained from these experiments was confirmed by Biosensor analysis, which asigned the four new anti-Pk mAbs binding affinities similar to that of SV5-P-k (table 9). All of this data suggests that the method used for the isolation of anti-Pk mAbs was not suitable for the production of low affinity mAbs, and exhibited preferential selection for high affinity mAbs.

In the initial immunisation protocol, the mice were injected twice before the final booster and removal of the spleen. The intial injection primes the immune system to a subsequent challenge with the immunogen. In this initial immune response, low affinity antibodies are generated to the immunogen, and it is only with the second challenge that high affinity antibodies are generated (Goding 1984, Harlow and Lane 1988, Kuby 1997). For the generation of mAbs to the Pk tag, it may be more advantageous to perform a fusion using the spleens from mice which were only challenged once with the immunogen, therefore selecting for low affinity mAbs. A disadvantage to this is that to develop a similar amount of hybridoma clones would require a lot more screening, and also that the mAbs produced may be of such a low affinity that they would be of no practical use.

An alternative method is phage display, which generally gives lower affinity mAbs than the traditional method of hybridoma technology. Phage display also allows a more controlled method of screening, and allows the selection of specific affinity mAbs. However, the disadvantage to using this method is the difficulties encountered when producing large amounts of antibody for further study.

## 3. THE IMMUNOGENICITY OF THE Pk TAG

Using synthetic peptides which were deletions of the Pk tag to determine the minimum binding epitopes of the five anti-Pk mAbs, it was shown that all five needed a common motif to be present in order for binding to occur. This was the tetrameric amino acid sequence Pro (5). Asn (6). Pro (7). Leu (8). (see figure 33 of the results). The apparent immunodominance of these amino acids may be caused by the conformation induced by the spatially rigid proline residues. If the predicted conformation of the Pk peptide is modelled and examined (figure 54), the prolines within this tetrameric sequence force the peptide backbone to undergo a sharp turn to form a loop-like structure. This loop structure corresponds to the predictions made on the shape and properties of mAb binding epitopes (Kuby 1997). Generally a mAb binding epitope tends to be accessible, hydrophilic, have site mobility and can be continuous or non-continuous.

Accessibility and hydrophilicity ensure that the peptide can be recognised by the mAb. The binding epitopes of a number of large proteins, such as neuraminidases, influenza haemagluttinin and GST, are hydrophilic and are found as loops and turns on the surface. The Pk tag contains hydrophilic amino acids which ensure that it is surface expressed when part of a fusion protein. This hydrophilicity is also exhibited by other peptide affinity tags which have been specifically designed for mAb recognition e.g. FLAG™, Strep and c-myc.

Mobility within the binding epitope has also been shown to be important for mAb binding to a number of binding sites within the proteins insulin, myohemerythricin, cytochrome C, myoglobin and haemoglobin (Tainer *et al* 1984). The proposed theory for the importance of mobility is that site mobility maximises the complementarity of the antigen within the mAbs binding site. However, a mAb binding to a flexible site has a lower affinity than mAbs binding to a rigid region (Tainer *et al* 1984, Kuby 1997). The 3-dimensional (3-d) structure of the Pk tag appears to contain mobile ends surrounding a rigid turn/loop structure. This could partially explain the high affinity that the anti-Pk mAbs have for the Pk tag. The mobile ends of the tag (figure 54) may allow the peptide to

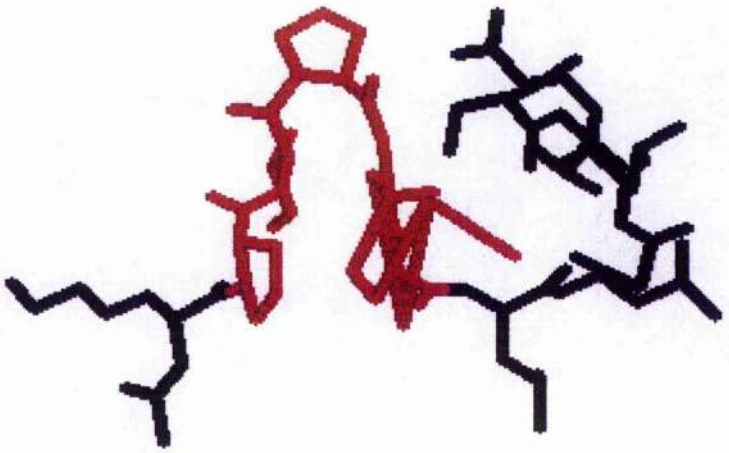


**Figure 54: A representation of the predicted 3 dimensional structure of the Pk tag.**
The amino acids in red represent those which form the rigid loop / turn structure (pro-3 to leu-8) which is required for the binding of the anti-Pk mAbs to the peptide tag. The structure was predicted using the RasMol programme for Macintosh.

be sufficiently flexible to maximise the mAb binding site complementarity. However, the rigidity enforced on the structure by the prolines at positions 3, 5 & 7 would allow for the production of high affinity mAbs. This flexibility is also found within a 14 amino acid epitope of the myelin basic protein (Mendz *et al* 1995). Structural analysis revealed that this 14 amino acid motif forms a loop between amino acids 7 and 12, which is thought to be targeted by mAbs. The N terminal amino acids (1 - 7) appear flexible, whereas the loop itself is rigid. In the case of this tetradecapeptide, mAb binding is further enhanced by the twisting of the peptide backbone within the loop to expose charged amino acid side chains (Mendz *et al* 1995).

Another observation made of the five anti-Pk mAbs was the high affinity with which they bound to the Pk tag. All five require denaturing conditions to separate the antibody-antigen complex. Antibody - antigen interactions are composed of a number of weak non-covalent interactions, such as hydrogen bonds, ionic bonds, hydrophobic interactions and van der Waals interactions. Together these weak forces combine to make the strong, reversible antibody-antigen interaction. These weak interactions act over short distances (about $10^{-7}$ mm), and depend upon the close fit of the epitope within the mAb binding site (Kuby 1997). As discussed earlier, site mobility is important in a mAb binding epitope. When this is combined with the natural flexibility found within a mAb binding site, a highly complementarity interaction is formed between the antibody and the antigen. The strong binding of the mAbs to the Pk tag suggest that there are a large number of these forces acting on the interaction and suggest that the Pk tag has adopted a conformation which allows the optimal number of non-covalent interactions to occur, thus greatly stabilising the antibody-antigen interaction (Tainer *et al* 1984, Regenmortel 1989, Kuby 1997). In order to determine how important the position of an amino acid within the Pk sequence is for mAb binding, cysteine residues were inserted between each amino acid in the tag. The results (figure 42) showed that for SV5-Pk5, the position of the amino acids within the tag were important for mAb binding. Even those amino acids which were found at the edges of the tag (figure 42, lane k). In contrast, the insertion of cysteine residues between amino acids had no effect on the binding of SV5-Pk2 (figure 42, lane g). This

indicates that SV5-Pk5 utilises most of the 14 amino acids within the tag for mAb binding, whereas SV5-Pk2 needs only those found within the minimum binding epitope for successful mAb binding.

When the binding of the anti-Pk mAbs were studied by Biosensor technology, differences were detected between the binding of the mAbs to the $Pk_{TH}$ peptide and to the Pk tag when part of a fusion protein. When the antibodies bound to the $Pk_{TH}$ peptide alone, they showed a normal antibody-antigen interaction. However, when a similar test was performed on the binding of the anti-Pk mAbs to GST-Pk (data not shown), there were discrepancies in the results. The sensorgrams obtained showed a normal 1 : 1 antibody-antigen interaction for the association phase, however there appeared to be a complex interaction for the dissociation phase. This suggested that either the antibody or the tag was multivalent, that either the antibody or the tag was exhibiting cooperativity or that the mAb was re-associating with the immobilised Pk tag. Of the three suggestions, the third is the most probable explanation, although there was insufficient data to completely rule out the other two suggestions (Pathak & Savelkoul 1997). To date this has not been fully explained.

## 4. THE MODIFICATION OF THE Pk TAG

When the binding patterns exhibited by the five anti-Pk mAbs were studied more closely using synthetic peptides containing cysteine substitutions, it was observed that there were subtle differences. The effects of substituting amino acids at certain positions within the tag showed that there were more amino acids involved in the binding of the mAbs to the tag than just those amino acids within the minimum binding epitope. For example, SV5-Pk5 needs a number of amino acids, which range from the proline at position 3 to the threonine at position 14, for binding to occur. Although mAb binding is not abolished if these two amino acids are substituted, it is greatly reduced (see figure 42). In contrast, SV5-Pk2 and SV5-Pk4 have relatively compact binding sites, requiring only those amino acids which are found within the minimum binding epitopes. The other two anti-Pk mAbs,

SV5-Pk1 and SV5-Pk3, require a single amino acid (the leucine at position 11) in addition to those within the minimum binding epitope in order for binding to take place. In order to see how other amino acid substitutions would affect mAb binding, the Pk tag was modified by the substitution of three amino acids to an alanine residue. The positions which were modified were chosen on the basis of the observations made during the cysteine substitution experiments. The substitutions produced three "new" Pk tags; I4APk; L8APk; and L11APk.

Based on the previous substitution experiments described in section 4.1 and 4.2 of the Results, the effects of these modified Pk tags on anti-Pk mAb binding was as expected (see table 10), with a single exception. SV5-Pk4 does not bind to the I4APk modified tag. This was unexpected as the previous cysteine substitution experiments had shown that substituting ile-4 for a cysteine had no effect on the binding of SV5-Pk4. However, SV5-Pk4 did not recognise the modified tag when part of the GST fusion protein. An explanation for this could be ascribed to structural changes in the 3-d structure of the Pk tag, induced by the substitution of the isoleucine for an alanine.

Isoleucine is a non polar amino acid with a side chain composed of an aliphatic chain. Alanine is also a non polar amino acid, however its side chain consists of a single methyl group, making it the second smallest amino acid. From the predicted 3-d structure shown in figure 55a, it can be seen that the side chain of the isoleucine (highlighted) causes the proline at positions 3 and 7 to be pushed close together by a twisting of the peptide backbone. When comparing this to the predicted structure of the I4APk tag (figure 55b), this twisting of the peptide backbone is not as pronounced, and the two proline side chains are not as close together. The overall shape of the loop structure is also flattened. This indicates that for binding to occur, SV5-Pk4 needs the proline at position 7 to be pushed close to the proline at position 3, and that the sides of the loop structure need to be steep. The twisting of the peptide backbone can also be seen in the predicted structures of both the L8APk and L11APk modified tags (figure 55c & 55d respectively), tags to which SV5-Pk4 readily bind. The cause for the lack of binding of the other anti-Pk mAbs to the modified

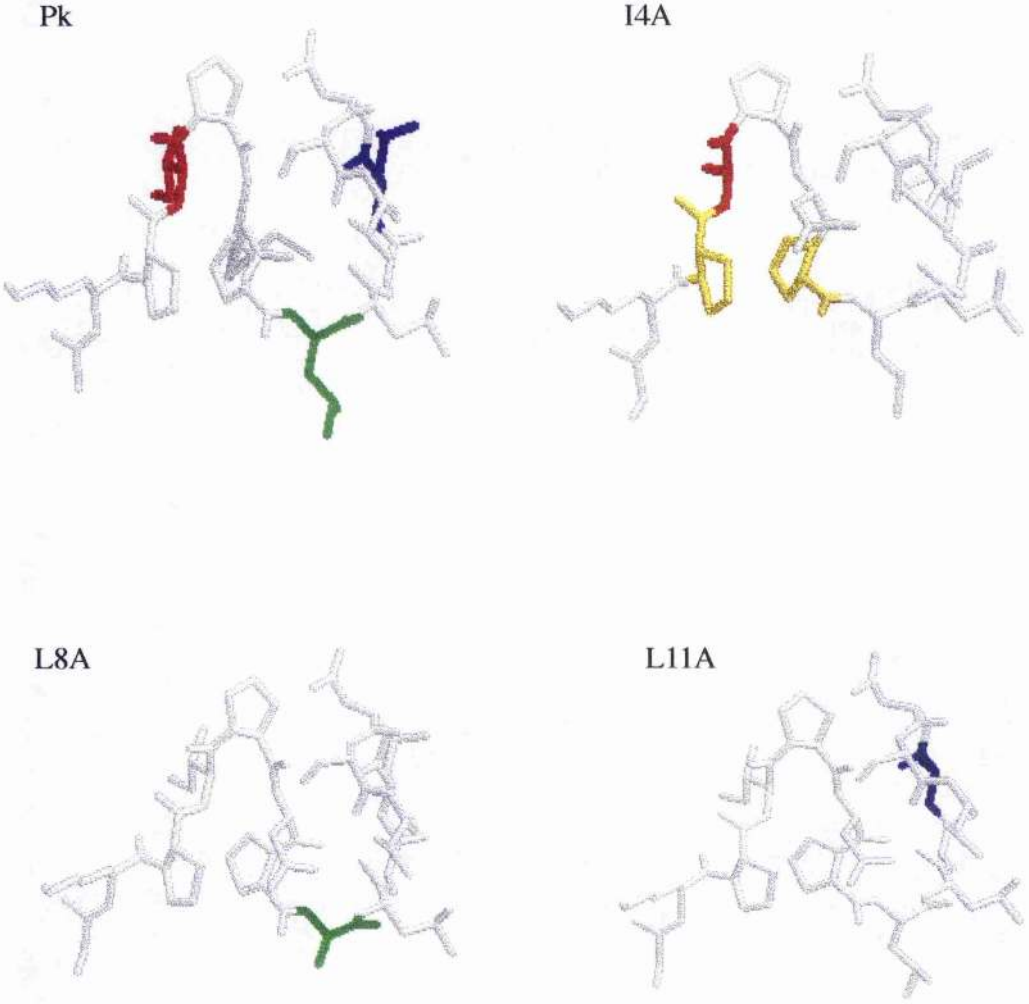



**Figure 55 : The predicted 3 dimensional structures of the original Pk tag and the three modified Pk tags.**

Those amino acids highlighted represent the three which had been modified; the isoleucine at position 3 (red); the leucine at position 8 (green); and the leucine at position 11 (blue). The amino acids highlighted in yellow represent the prolines at position 3 and 7. As can be seen the substitution of the isoleucine at position 4 (b) has the effect of flattening the rigid loop and pushing the prolines at positions 3 and 7 further apart. The other two substitutions to positions 8 (c) and 11 (d) have no significant effect on the rigid loop but do cause the C terminus to twist.

tags is not as apparent and appears to correspond to the data obtained from the insertion and substitution experiments.

The three amino acids which were substituted (ile-4, leu-8, and leu-11) were chosen on the basis of the data obtained from both the SPOT's tests (cysteine insertions and substitutions) and the LTB-Pk-cys substitutions tested by ELISA (see section 4.1 & 4.2 of the Reults). These amino acids were substituted on the basis that they caused an apparent reduction is the affinity of the mAb for the tag. These three amino acids were chosen as they did not completely abolish the binding of any of the anti-Pk mAbs, but did affect the binding of two or three. In addition to the three amino acids chosen for substitution, only the leucine at position 9 or the proline at position 5 were alternatives which were expected to have a similar effect on mAb binding. However, the proline at position 5 starts the loop in the peptide structure which is thought to be necessary for mAb binding, and was therefore discounted due to its position. The leucine at position 9 was also shown to be necessary for the binding of SV5-Pk1 and SV5-Pk4, making it inappropriate for the study of these two mAbs, and for this reason was also discounted for substitution.

The Pk tag was modified in order to determine which of the new tags could be eluted from the anti-Pk mAbs using mild elution conditions. However, when testing the modified tags for elution capability, it was determined that only very low pH or chaotropic ions were effective (table 11). There was a single exception to this, the binding of SV5-Pk2 to the I4APk modified tag. This tag could be eluted from mAb coupled beads using mild buffers such as 3.5 M magnesium chloride. Although the modifications to the tag were deemed successful for protein elution, the small non polar amino acid alanine may not have been the most suitable choice for a substitution which affects protein elution. Alanine forms a compact structure in water (Stryer 1988), and as such has no discernible properties which can be exploited. The decision to chose alanine was based on preserving the 3d structure of the peptide by not introducing a large amino acid side chain or one of a different charge. To allow greater control of protein elution, the substitution of isoleucine or leucine to a more charged amino acid may have been more appropriate. For example, histidine, glutamic acid

or aspartic acid. All three of these amino acids are neutral or negatively charged depending upon the environmental conditions. Of the three, histidine would have been the most suitable choice for the substitution, due to its relatively neutral pK (6.5; Stryer 1988). This means that tagged proteins would be more probably to bind to the mAb column at a pH of below pH 6.5. By increasing the pH to above pH 6.5 the histidine would become uncharged, thus aiding in the elution of the tagged protein from the column.

As stated earlier, the Pk tag was originally derived from the P and V proteins of SV5, as a peptide which competed for the binding of SV5-P-k (SV5-Pk1). Simian Virus 5 is a member of the Paramyxavirus family and is closely related to mumps virus and Parainfluenza virus type 2 (PIV-2; Southern *et al* 1991). The relevant sequence within the P proteins of SV5 and PIV-2 which corresponds to the Pk tag, differ in a number of amino acids but not in the key tetrapeptide sequence (see figure 56). Although the tetrapeptide motif remains intact, SV5-Pk1 does not recognise and bind to the P protein of PIV-2 when tested by immunofluorescence assay. The reason for this is unknown as the amino acids which are different are those which are not effected when substituted by a cysteine residue. One explanation may be that by the substitution of a rigid proline residue between two amino acids needed for mAb binding (leu-9 and leu-11) may effect mAb binding. When testing, by immunofluorescence, the four other anti-Pk mAbs against the P protein of PIV-2, some fluorescence was detected. The results are summarised in table 13. SV5-Pk2 recognised and bound strongly to the P protein of PIV-2 in immunofluorescence assays, as did SV5-Pk3 and SV5-Pk4 though not as strongly. From the data obtained in the previous substitution experiments this was expected. The amino acids in the P protein of PIV-2 which are different to the P protein of SV5 had no effect on the binding of these three mAbs when substituted. However, SV5-Pk5 did not recognise the P protein of PIV-2. This was also expected as this mAb requires the most amino acids for binding to occur, some of which are found on the periphery of the tag, where most of the differences are found between the two P proteins.

Simian virus 5 was originally isolated from rhesus monkey kidney cell culture but has also been isolated from humans and dogs. SV5-Pk1 was tested previously on the P

| | | |
|---|---|---|
| a. | SV5 W3-Pk | gly.lys.pro.ile.pro.asn.pro.leu.leu.gly.leu.asp.ser.thr |
| b. | CPI-Pk | gly.lys.pro.ile.pro.asn.pro.pro.leu.gly.leu.asp.ser.thr |
| c. | PIV2 | asn.thr.pro.ile.pro.asn.pro.leu.leu.pro.leu.ala.arg.pro |

**Figure 56 : the amino acid sequences of the Pk epitope within SV5 (a), the CPI$^-$ isolate (b) and PIV-2 (C).**

| anti-Pk monoclonal antibody | Reaction to the P protein of PIV-2 |
|---|---|
| SV5-Pk1 | - |
| SV5-Pk2 | +++ |
| SV5-Pk3 | ++ |
| SV5-Pk4 | ++ |
| SV5-Pk5 | - |

**Table 13 : The results obtained for immunofluorescence assays on the recognition of the anti-Pk mAb's for the P protein of PIV-2.**

To determine if the anti-Pk mAb's recognised the P protein of PIV-2, BALB/C cells were infected with PIV-2 and probed with the anti-Pk mAb's. Any immunofluorescence produced was detected using a Texas Red conjugated anti- mouse Ig mAb.

Key : - - no fluorescence detected; ++ - fluorescence obviously visible about background; +++ - strong fluorescence detected.

proteins of two canine isolates of SV5 termed CPI$^+$ and CPI$^-$ (Southern *et al* 1991). There are four amino acid differences between the proteins of the two isolates, one of which is found within the Pk motif (figure 56). This is the substitution of a leucine at position 8 for a proline residue. SV5-Pk1 only recognised the isolate which had the original Pk sequence (CPI$^+$) and not the isolate with the proline substitution (Southern *et al* 1991). Subsequent studies have shown that leu-8 is not necessary for SV5-Pk1 binding to the tag, making it surprising that this mAb did not bind to the CPI$^-$ isolate. This again suggests that it is the substitution for a proline which has the detrimental effect on binding and not the loss of the amino acid that was substituted. Although the four other anti-Pk mAbs have not been tested on these two isolates, it would be expected that they would not recognise the CPI$^-$ isolate as leu-8 is required for the binding of all four mAbs to the Pk tag.

In retrospect, the methods used in this thesis may not have been the most effective for the production of reagents that were needed for efficient protein purification. Already discussed in this section are ways in which the project could have been altered. For example, the use of a different method for the production of the mAbs could have produced mAbs which would have more acceptible binding properties for protein elution. If mAbs with a lower affinity had been isolated, this would have removed the necessity for modifying the Pk tag to reduce the affinity of the mAbs. Also, instead of altering the method of isolating the mAbs, with the data obtained from the binding epitope studies, an alternative way of obtaining lower affinity mAbs would be to alter the tag with which the mice were immunised. By reducing the immunogenicity of the Pk tag, there would also be a higher probability of obtaining mAbs which would bind to different parts of the tag instead of the tetrameric sequence discussed in section 2 of this Discussion.

The substitution for the amino acids histidine, glutamate or aspartate when modifying the tag may also have been more beneficial for allowing the use of relatively mild conditions for protein elution. The charged nature of the amino acid side chains would not only alter the affinity of the mAbs for the Pk tag, but also allow pH to affect mAb binding to the tag, and therefore allow protein elution from a immunoaffinity column.

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