Expression of Catalytic Antibody C3 Esterase scFv in *Escherichia coli*

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ABSTRACT

This thesis describes the construction of a vector for the expression of an antibody light chain variable region gene, (V_l), from the monoclonal catalytic antibody C3 in *Escherichia coli.* The antibody C3 was developed by Khalaf, Suckling, Stimson *et al*(1992) at the University of Strathclyde, and catalysed the hydrolysis of 4-nitrophenyl 5-(3-methoxyphenyl)-pentanoate. The binding site of the C3 abzyme was cloned as an scFv by the University of Leicester into an expression vector pPANG1 developed at Leicester. The V_H sequence in the C3scFv construct was found to be an aberrantly rearranged myeloma V_H sequence, and not the genuine C3 V_H sequence. Expression of the C3 scFv sequence from pPANG1 failed to produce any protein. The genuine C3 V_l sequence was cloned into a novel expression vector, pQR627, based on pBluescript II. The novel expression vector carried the $C3V_l$ gene as a fusion to a antibody binding domain of *Staphylococcus aureus* protein A. The $C3V_l$ gene was expressed in *E.coli* from vector pQR627. The results of the expression experiments with vector pQR627 indicated that the $C3V_l$ gene product was cytotoxic to the host *E.coli* cells. This cytotoxicity was the probable cause of the low expression titre of the $C3V_l$ gene product in pQR627 cultures. The SpA-$C3V_l$ protein was probed for determination of its binding properties and its catalytic properties. The SpA-$C3V_l$ failed to exhibit the binding properties of the parent monoclonal antibody C3, it did however exhibit a catalytic function. The SpA-$C3V_l$ protein was found to have 5.9% of the specific activity of the whole C3 IgG as determined by Khalaf, Suckling, Stimson *et al* (1992).

To all my loved ones, but most of all to Alison

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GLOSSARY

Ab	Antibody, abbreviation
Abzyme	Term for a catalytic antibody
BSA	Bovine serum albumin
C3	A catalytic antibody with esterase activity, created at the University of Strathclyde, Dept. of Immunology and Dept. of chemistry
C_H	Constant regions of the heavy chain of an antibody
C_L	Constant region of the light chain of an antibody
CDR	Complementary determining region
Cm	Chloramphenicol
DMF	N,N-Dimethylformamide
dNTP	Deoxy nucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assey
FCS	Foetal calf serum
Fv	Dimer of variable regions of antibody varible regions
HAT	Tissue culture medium containing, hypoxanthine, Aminopterin and thymidine
HGPRT	Enzyme hypoxanthine-guanine phosphroibosyl transferase
HRP	Horse radish peroxidase
IPTG	Isopropyl-β-D-thiogalactopyranoside
Ig	Immunglobulin
LB	Luria broth, bacterial medium
NB	Nutrient broth no.2, bacterial medium supplied by Oxoid
NB agar	Nutrient broth agar, bacterial medium containing agar for plates, supplied by Oxiod
NBCS	New born calf serum
NMR	Nuclear magnetic resonance
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol

scFv	Single chain Fv, a single protein containing the V_H and V_l domains of an antibody joined together by a linker sequence
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SpA	Staphyloccoccal protein A, an antibody binding protein
TAE	Tris acetate eletrophoresis buffer
TB	Terrific broth, bacterial medium
TBE	Tris borate electrophoresis buffer
TBS	Tris buffered saline
TE	Tris-EDTA buffer
TMB	Tetramethylbenzidine
V_H	Variable region of the heavy chain of an antibody
V_l	Variable region of the light chain of an antibody
X-gal	5-bromo, 4-chloro, 3 indolyl-β -D galactopyranoside

1 INTRODUCTION

The catalytic antibody C3 developed at the University of Strathclyde by Khalaf *et al*(1992). acts as an esterase, with the substrate 4-nitrophenyl 5-(3-methoxyphenol)-pentanoate, releasing 4-nitrophenol.

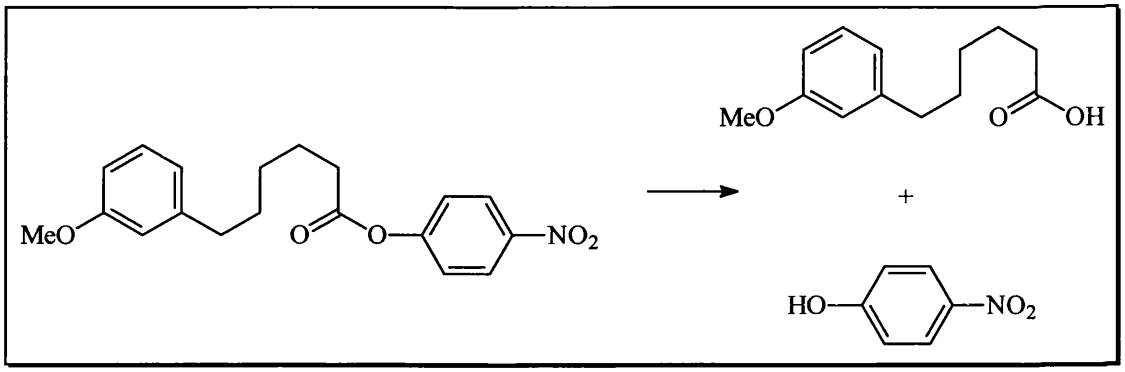

fig. 1 C3 catalysed hydrolysis of 4-nitrophenyl 5-(3-methoxyphenyl)-pentanoate

The release of 4-nitrophenol can be determined by spectrophotometery, and so the catalytic properties of C3 can be determined. The aim of this project was to compare the activity of two forms of the catalytic antibody C3, as a whole IgG3 obtained from hybridoma cultures, and as a scFv or as a scFv-spA fusion produced in *E.coli* cells, in both small and large scale expression systems. To determine any detectable changes in the specificity or selectivity of the esterase activity and so determine whether different methods of antibody production affect the structure of the binding site and the specificity and strength of the antibody:antigen binding.

Catalytic antibodies, abzymes (Tramontano *et al* 1986, Benkovic 1992), are antibodies that express a predetermined catalytic function. The abzymes posses a binding site which has a greater affinity for the transition state of a reaction, than either substrate or product. In the presence of the substrate this affinity of the antibody stabilises the transition state of the reaction and so catalyses the reaction. The binding site of an antibody is located in the variable region of an antibody, the Fv region. A wide range of catalytic antibodies has been generated, using hybridoma technology, with varying degrees of catalytic power some of which have acted as remarkably efficient catalysts (Green and Tawfik 1989).

Antibody variable region genes can be cloned from hybridoma mRNA,(Orlandi *et al* 1989, Huston *et a*l 1988). Either whole antibodies, Fab, Fv or scFv, single chain Fv, fragments can then be produced by bacterial expression systems, such as *E.coli*. Production of antibody fragments and scFvs in *E.coli* offers increased yield and greater cost effectiveness than production from hybridoma cultures Both antibodies and antibody fragments have a wide range of applications such as, tumour imaging, antibody based therapeutic proteins, tailor made catalysts and immunoreagentsfor *in vitro* and *in vivo* assay and diagnosis. Cloning of antibody variable regions means that *E.coli* based production systems can express fusion proteins, antibody fragments conjugated to a wide variety of other proteins, such as toxins, enzymes for immunoassays and other antibody fragments to form bivalent or bispecific antibody fragments (Carter *et al* 1997).

Single chain antibody variable regions scFv,(Bird *et al* 1988) are recombinant proteins that comprise the variable regions of both the heavy and light chains of an antibody joined together with a polypeptide linker. The use of these scFv proteins has a number of advantages over whole antibodies, notably a decrease in non specific binding *in vivo* compared to whole antibodies, improved tumour penetration(Yokota *et al* 1992, King *et al* 1992), increased stability to thermal denaturation, and dissociation over Fvs(Glockshuber *et al* 1990) and because of their lower relative molecular mass an increase in specific activity per gram. The lower relative molecular mass of scFvs makes them preferable to whole IgGs, as a subject for NMR(Freund *et al* 1993&1994)and X-ray studies of protein structure, and antibody antigen interactions(Eigenbrot *et al* 1994, Charbonnier *et al* 1995).

The focus of this project was to have been on expression systems for recombinant antibody fragments. The original aim of this project was the comparison of C3 binding sites, the original binding site present in the parent monoclonal as produced by C3 hybridoma tissue culture, and that of the recombinant scFv in *E.coli.* Such comparisons have been made before, the novel aspect of the work was to utilise the catalytic activity of the C3 abzyme as a measure of the fidelity of the recombinant C3scFv binding site. This catalytic activity was to be a tool to probe for any changes there may be between the whole IgG form of the abzyme and the scFv, and so from changes in esterase activity infer a variation in the specificity and selectivity of the C3 binding site.

The project was to develop over three core phases. Firstly the measurement of the esterase properties of the parent monoclonal C3. The esterase activity of C3 would act as a baseline against which all other activities would be compared. In order for that baseline to be validated the same batch of substrate must be used in any catalysis measurement.

The second phase would consist of the expression, purification and assay for catalytic activity of scFv produced in *E.coli.* by the existing scFv containing plasmids developed by the Dept of Botany, University of Leicester. The Dept of Immunology at Strathclyde at the time did not possess suitable molecular biology facilities and so asked the Dept of Botany at Leicester University, who had previously collaborated with the Dept of Immunology at Strathclyde University for help in the cloning of the scFv and the construction of an expression vector for it. The C3 scFv was cloned from the C3 hybridoma cell line and cloned into an existing antibody expression vector pPANG1(Gandecha *et al* 1994).The vector that was created at Leicester was pPANG1C3scFv. This plasmid was to form the basis of all the recombinant DNA aspects of this project.

The third phase was to be the examination of the production of the C3scFv in large scale *E.coli* cultures. For this the pPANG1C3scFv vector would be suitably re engineered for expression of scFv in large scale cultures. Again the scFv produced in these large scale cultures would be assayed for esterase activity and compared to the baseline value for the IgG from hybridoma culture. The third phase would allow for comparison of the efficacy of expression of recombinant antibody fragments from both the small and large scale systems, the esterase activity of the respective antibody fragments being compared to that of the baseline IgG measurement. Thus the esterase activity could be used as a tool for the determination of the suitability of the large scale expression system for other recombinant antibody fragments. The reaction catalysed by C3 was in itself trivial but could have been used to verify the efficacy of a large scale expression system that could be used to synthesise other more scientifically and industrially interesting antibody fragments. The ultimate goal of the project would have been the expression in a large scale bacterial culture of an scFv that possessed esterase activity indistinguishable from that of the parent monoclonal C3.

The starting points of the work were the pPANG1C3scFv plasmid and the C3 hybridoma culture itself. After the project commenced it became clear that these two keystones of the work were not performing as expected, and in the case of

the pPANG1C3scFv plasmid it was clearly defective. As the work progressed it became clear that the original goals of the project were unobtainable. In this situation it became necessary to re target the work towards a series of goals that were achievable, although removed from original intentions. As a result this thesis is divided into two stages. The first half, chapters 3 and 4, describe the failure of the hybridoma culture and pPANG1C3scFv *E.coli* culture to produce the expected results and in the case of the pPANG1C3scFv plasmid why. As a result of the unexpected difficulties described in the first half of the thesis a new direction was adopted. This new direction would involve the salvaging of what genuine material associated with the project existed and using it in a new way to achieve different goals. The second half of the thesis, chapters 5-7, details the use of the plasmid pPANG1C3scFv as the basis of a new direction for the work. This new direction was to examine the expression, and to characterise the properties of the C3 V_l gene product and compare its properties to those of the whole C3 IgG as published by Khalaf *et al.*(1992) This revised strategy would still carry an echo of the original in the use of the catalytic properties of a recombinant antibody fragment with those of the IgG from which it was derived.

1.1 THE IMMUNE RESPONSE

The immune system is a highly complex biological defence system that has evolved to protect host organisms from parasites, bacteria and viruses. The immune system distinguishes between "self" and "nonself", that which is part of the organism and that which is not and attacks that which it does not recognise as "self". When a host organism is exposed to a foreign protein, or molecule an immune response is triggered. This immune response involves T cells, B cells, macrophages, cytokines and other factors. One of the elements of the immune response is the production of antibodies, immunoglobulins (Roitt *et al* 1996).

The presence of a foreign "nonself" molecule, antigen, stimulates the development and expansion of cell lines that produce antibodies. Antibodies are proteins that bind to antigens and the immune response is driven to produce antibodies of the highest antigen affinity, by antigen selection. Antibodies are also highly specific in their binding to antigen epitopes. The immune system produces a number of different immunoglobulin, Ig, protein classes, IgG, IgA, IgM, IgD and IgE. Each class of antibody has its own role in the immune system, but shares a common basic structure. This common structure consists of two heavy polypeptide chains and two light polypeptide chains, which forms a flexible y-shaped molecule with two antigen binding site and a constant region

that defines the antibody sub-class. The human immune response system can produce over 200 million different antibodies in response to different antigens(O'kennedy and Roben 1991).

The primary immune response involves B lymphocyte cells being exposed to antigen, as a response antibody germline DNA gene sequences for both heavy and light chains are rearranged, transcribed and translated as antibody protein. The primary immune response produces a vast repertoire of polyclonal antibodies each with a unique binding site. The primary immune response produces antibodies of the IgM class that are expressed on the surface of B cells. The highest affinity antibodies from this primary antibody library are selected by antigen binding and subsequently undergo a process of affinity maturation to develop antibodies of the highest antigen affinity. The highest antigen affinity antibody producing B cells differentiate and the IgM antibodies undergo somatic hypermutation, this hypermutation of the original germ line antibodies results in a second antibody library. The highest antigen affinity IgM are antigen selected and the B cells multiply and differentiate into plasma cells. The IgM antibodies undergo a genetic class switch, the expressed antibody changes Ig class and is excreted by the plasma cells, but retains the same binding properties as the highest affinity IgM produced by the B cells. The diversity of antibody binding sites arises from the diversity of the germline DNA genes, the recombination of antibody germline DNA, and somatic hypermutation of the expressed IgM antibody DNA sequence (Pattern *et al* 1996).

The secreted antibodies will then bind to antigen in tissue fluids and will mediate effector functions. Different antibody classes performing different effector functions, for example phagocytes will recognise antigens, by IgG antibodies binding to the antigens and phagocytes having receptors for IgG antibodies on their cell surface, which allows them to absorb the antigen. When activated by T cells, via the release of cytokines the phagocytes will destroy the antigen pathogen that they have absorbed. The antibody mediated recognition of antigens by phagocytes forms the basis of the adaptive immune response.

1.1.1 Antibody structure

Antibodies produced by the immune system all have a common basic structure and function. This comprises two identical heavy and two identical light polypeptide chains, linked together by disulphide bridges, two bridges linking the heavy chains together and two bridges linking each of the light chains to one of

the heavy chains (*fig.* 1.1.1). The light chains having a molecular weight of 26kDa and the heavy chains having molecular weights of 55 or 70 kDa depending on antibody class. The heavy and light chains are constructed from smaller domains which are composed of β-pleated sheets. Antibody heavy chains consists of four domains, one variable and three constant, and light chains two, one variable and one constant. The variable region domains of antibodies contain the vast majority of antibody sequence variability and also comprise the antigen binding site. Treatment of antibodies with the proteases, pepsin and papain, produces the antibody fragments $F(ab)_2$ and Fab respectively. The $F(ab)_2$ molecule contains both the light chains and two domains of the heavy chains and retains both of the antibody binding sites. Treatment with papain gives the Fab fragment that contains one light chain and two heavy chain domains comprising one antibody binding site. Fv fragments can also be produced which contain only the variable regions of a light and a heavy chain. The heavy chains contain a hinge region, which allows for conformal changes in the antibody when circulating in the blood stream, or binding to an antigen. The constant regions of antibodies, known as C_l, of which there are two types λ and κ, and C_H, are identical within the same antibody subclass and serve as a backbone structure for the variable regions of the antibody. The C_H regions determine the Ig class and isotype of the antibody and have their own specific roles in secretion and define the antibody's effector function in the immune system, for example IgG C_H regions are responsible for binding to receptors on phagocytes. The C_H regions of IgG antibodies will also bind to staphylococcal protein A and streptococcal protein G.

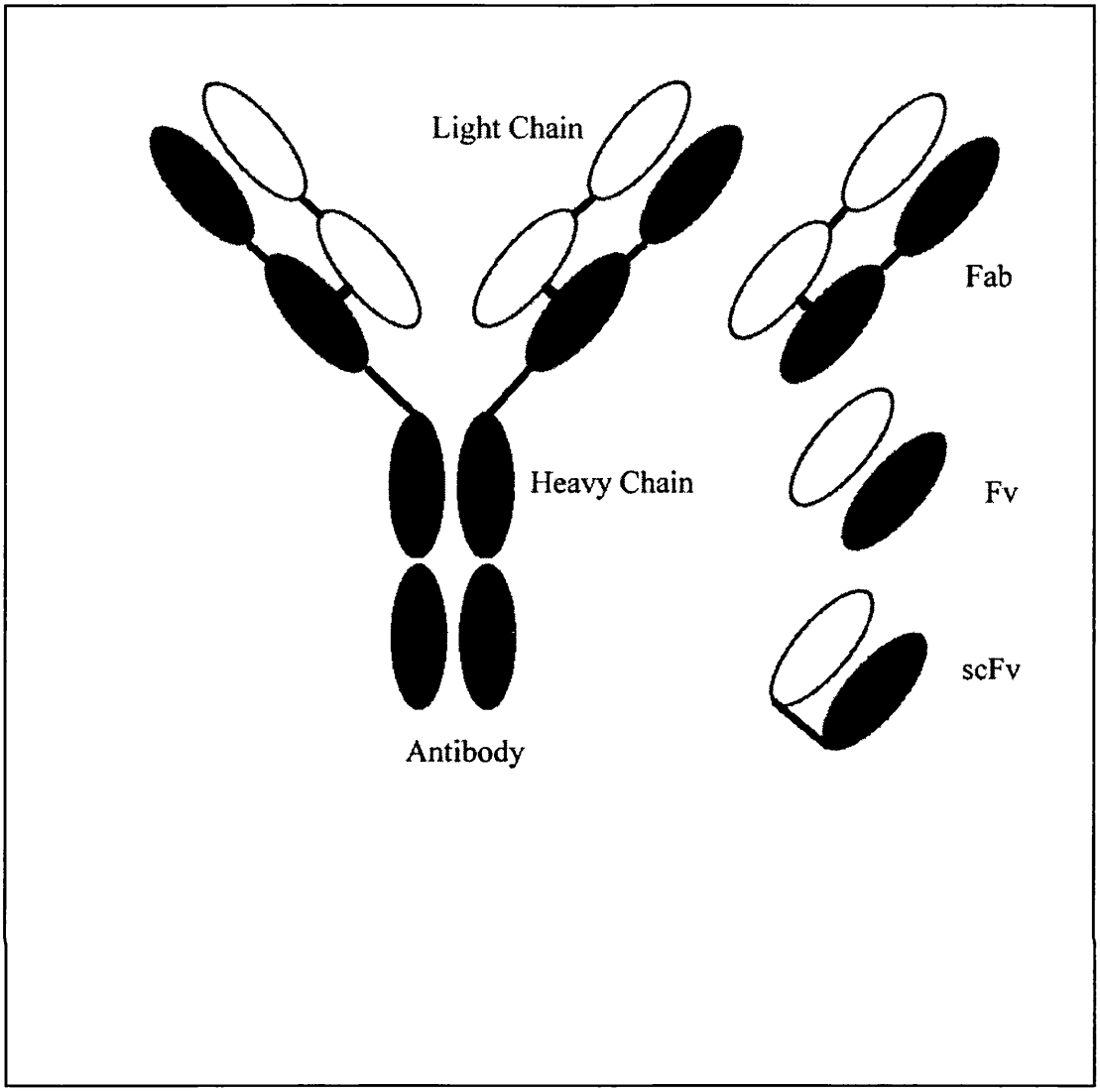

fig. 1.1.1 Antibody and antibody fragment structures

The differences between different antigen binding antibodies is contained in the antibody variable regions, Fv, which contain the antigen binding site. The binding site of antibodies is at the NH_2 terminus of both the light and the heavy chain variable regions of an antibody, known as V_l and V_H respectively. By amino acid sequence comparison Kabat and Wu (1987) demonstrated that the V_l and V_H regions are constructed from conserved framework regions, FR, with less frequent variation between antibodies, joined together by hypervariable loops, which vary greatly in amino acid composition and length. Both V_H and V_l domains contain three hypervariable loops which in the native state form the tip of the antibody molecule. X-ray crystal studies (Eigenbrot *et al* 1994) have confirmed that the binding site of the antibody is formed by the three dimensional folded structure of the hypervariable loops, also known as complementary determining regions, CDRs, in both the V_l and V_H domains of the antibody. The

specificity of antigen binding depends on the geometry of these CDR loops, and the amino acid residues present in the hypervariable loops of the CDRs. The framework regions are β-pleated sheets, the amino acid sequence of these FR β-pleated sheets is highly conserved, with little variation in the amino acid sequence between different light chains or heavy chains. These framework regions serve as a scaffold structure for the support of the hypervariable loops.

The binding of antigen by an antibody depends on the formation of multiple non-covalent bonds between the amino acid residues of the antibody binding site and the antigen, to give a high net attractive effect. Antibody-antigen interactions are dependant on hydrogen bonding, electrostatic interactions, van der Waals forces and hydrophobic interactions between the antigen and the antibody binding site. All of these forces are dependant on the distance between the interacting groups, the closer the interacting groups the greater the antigen affinity of the antibody. Steric interference between antigen and binding site will decrease the net attractive effect, and so a high affinity binding site will form a highly complementary three dimensional fit with the antigen, to maximise the overall binding (Standfield 1994). As the immune system is driven to produce the highest affinity antibodies it will also produce the most antigen complementary antibody binding sites (Standfield *et al* 1994 Rees *et al* 1994). Antibody sequence comparison, X-ray crystallography and binding studies of antibody antigen complexes have produced a great deal of data regarding antibody:antigen binding. From various studies the V_H appears to contribute more to the overall binding activity of the Fv than the V_l, (Ward *et al* 1989) as several different specificity antibodies have been shown to possess the same V_l and differing V_H where as the reverse is only rarely observed. The six CDR loops of the antibody comprise the antigen binding site, which can take one of three forms, cavity, groove and planar. Antibodies that bind haptens form cavity type binding sites where the antigen is buried within the cavity, polypeptide binding antibodies have groove type binding sites and protein binding antibodies form planar binding sites (Chotia *et al* 1989).

The major factor in antigen binding appears to be the third hypervariable loop of the heavy chain, CDRH3, which is also the most variable of the CDR loops in both aminoacid composition and length(Chotia *et al* 1989). Structural evidence shows that the V_l CDRs, CDR H1 and CDR H2 loops have a small repertoire of canonical structures of similar sequence and length, the canonical structures being determined by key residues at certain positions in both the hypervariable

loops and in the framework regions. Variation in the hypervariable loops, away from the key residues, can modify the canonical structure to increase antigen binding by modifying the surface residues in the canonical structure that are in contact with antigen (Chotia *et al* 1989). The third heavy chain hypervariable loop appears to have no canonical structures, the CDR H3 aminoacid sequence variability producing a greater repertoire of three dimensional structures than that available for the other hypervariable regions. Structural studies of uncomplexed and complexed antibodies have shown that antibodies exist in multiple conformation states and that the structure of the complexed antibody is different to that of the uncomplexed antibody, which suggests that antibody antigen binding takes place via an induced fit mechanism (Standfield 1994). The differences between bound and unbound antibody conformation can include rearrangement of the V_l-V_H domains and movements of the CDR loops.

Single chain Fvs are novel proteins, (Bird *et al* 1988, Glockshuber *et al* 1990) derived from Fv fragments, that comprise the V_H and V_l sequences fused into a single protein, which has a molecular weight of approximately 27 kDa. The β pleated sheet framework structures of the heavy and light chain variable regions can be joined together by a linker sequence between the NH_2 terminus of one chain and the COOH terminus of the other. The linker does not however interfere with the correct folding of the variable regions, so that its antigen binding properties are not altered (Freund *et al* 1994). Disulphide stabilised Fv fragments, dsFv are another type of engineered Fv (Brinkmann *et al* 1993), the light and heavy V regions bound together by the introduction of a disulphide bond. Fv and scFv antibody fragments are the smallest antibody fragments that contain the whole of the antibody's antigen binding site, Fvs and scFvs demonstrate equivalent antigen binding characteristics to their parent whole antibodies.

1.1.2 Genetics of antibody production

The primary immune response is characterised by the expression of IgM antibodies on the surface of B cells. The IgM antibody structure is derived from rearranged germline DNA, the germline DNA encodes for separate light and heavy chain sequences. Both heavy and light chains are transcribed and translated separately. The heavy and light chains are transported to the endoplasmic reticulum, where the polypeptide chains are folded and assembled together to form the expressed antibody.

It has been shown that there are two kinds of light chain, κ and λ, λ chains having three different types. Both κ and λ light chain gene loci have a number of exons, separated by non-encoding introns, which will be rearranged and joined together to form the expressed antibody mRNA. There are approximately three hundred and fifty variable regions, Vκ, each proceeded by a leader sequence. These are followed by five Jκ, joining regions and one Cκ, constant region exon. Once a B cell is stimulated by an antigen a recombination event takes place, bringing Vκ and Jκ exons together. The Vκ and Jκ exons joined together will encode for the V_l region of the κ light chain. This recombination event gives rise to approximately 1400 different possible κ light chain variable regions. The join between Vκ and Jκ exons will vary, giving greater diversity. The Jκ3 exon is a pseudogene and is never expressed. The resulting V-J section along with the Cκ, constant region exon, is then transcribed to RNA, which is in turn spliced bringing the locus together. The resulting RNA encodes a leader sequence, V-J and C regions, and a polyA tail to give a mature mRNA transcript which is then translated as light chain precursor. The NH_2 leader sequence is responsible for transportation of the light chain to the endoplasmic reticulum and is proteolytically cleaved, after translation to give the mature light chain (Fudenberg *et al* 1984).

The process for the λ types of light chain is similar but with less potential for diversity. As with the κ light chains the V_l region is encoded by Vλ and Jλ exons that are recombined in antigen stimulated B cells. There only three Vλ genes and four Jλ genes each with its own constant Cλ region, one of which Jλ4 is a pseudogene and not expressed. These three Jλ- Cλ gene segments giving rise to the three λ light chain types. The recombined λ gene locus is transcribed, spliced and translated in the same way as the κ light chain.

The heavy chains are constructed in a similar way to the light chains, but the heavy chains posses a third D (diversity) exon as well as the V (variable) and J (joining) exons, giving even greater diversity in the expressed antibody heavy chain variable region . The heavy chain germline DNA consists of several hundred V exons, thirty identified D exons, six J exons and three J pseudogenes. Two recombinant events are needed for heavy chain rearrangement because of the extra D exon. The first recombination event gives a D-J section, which in turn is recombined with the V region to produce a V-D-J section which will encode for the V_H heavy chain sequence. The D exons are highly variable gene segments, and the recombined D-J gene sequence encodes for the third hypervariable loop of the heavy chain, which is the most variable sequence in immunoglobulins. The

V-D-J locus is then transcribed along with the constant region to give the heavy chain RNA primary transcript. The C, constant region, exons encode for the C_H domains and so will determine the expressed antibody class. The RNA transcript is then spliced to bring the loci together and form the leader sequence-V-D-J-C mature mRNA transcript that is subsequently translated to heavy chain precursor. The leader sequence being responsible for the transportation of the heavy chain precursor to the endoplasmic reticulum and as with the light chain precursor the NH_2 leader sequence is post-translationally cleaved to give the expressed heavy chain (Fudenberg *et al* 1984).

After encountering antigen the B cells differentiate in a number of ways, the differentiation is mediated by cytokines released from T cells. One of the differentiation pathways induces somatic hypermutation of the expressed IgM antibodies, by an unknown mechanism. The V_H and V_l regions are hypermutated and the highest affinity antibodies generated by the somatic hypermutation are antigen selected from the mutated variants. The somatic mutation may produce higher affinity antibodies from germline antibodies by stabilising the binding site formed by the hypervariable loops of the CDR's, rather than producing a binding site that was more complementary to the antigen than the germline antibody. Patten *et al*(1996) have compared the antigen affinity of somatically mutated antibodies to that of the germline antibodies from which they were derived. In this study the mutations occurred in the CDR's at residues that were not in direct contact with the hapten, but that were involved with inter or intra CDR loop interactions. The increase in antigen affinity due to the somatic mutations was found to 14,000 fold for the antibody studied by Patten *et al.* The highest affinity antibody producing B cells then advance to plasma cell stage, when they will secrete soluble antibodies. The antibodies will undergo a genetic class switch, the V_H region being rearranged to combine with a new C_H region, changing the antibody type from IgM to other Ig classes. After the class switch the antibody is secreted by the plasma cells.

1.1.3 Hybridoma technology

Splenocytes from immunised animals have short *in vitro* life spans and so are unsuitable for tissue culture, and the production of antibodies *in vitro*. In order to create a continuous cell line that generated antibodies with predefined specificity Kohler and Milstein (1975) fused splenocytes with myelomas.

Myeloma cells (Potter 1972) are cell lines of lymphocyte tumours, one lymphocyte dividing uncontrollably producing monoclone cells. Tumours of lymphocytes can be induced in laboratory animals such as mice, by inoculation with a mineral oil, such as paraffin oil, or other irritants such as pristane. These tumour cells produce an antibody of unknown specificity but will grow *in vitro* indefinitely (Horibata 1970).

Kohler and Milstein fused the spleen cells of a mouse immunised against sheep red blood cells (SRBC), with a myeloma cell line (P3-X63Ag8) using inactivated sendai virus to produce a cell line with an indefinite *in vitro* life span and that produced monoclonal antibodies, the presence of which was detected by a plaque assay technique. The hybridomas produced by the fusion were monoclone and produce a homogeneous population of antibody molecules, known as monoclonal antibodies. Monoclonal antibodies are specific for one antigen.

Kohler and Milstein used a myeloma cell line (P3-X63Ag8) that would not survive in the culture conditions used for the fusion, so that only the fused progeny of both cell lines would be viable under the selective culture conditions. The selective culture medium used by Kohler and Milstein was HAT (Littlefield 1964). Cellular DNA synthesis can be blocked by the use of an antagonist called aminopterin (A). Most cells have an alternative or salvage DNA synthesis pathway which utilises hypoxanthine (H) and thymidine (T) using the enzyme hypoxanthine - guanine phosphoribosyl transferase (HGPRT). Mutant cells that are HGPRT-ve will die in medium that contains hypoxanthine, aminopterin and thymidine (HAT), as the main DNA synthesis pathway will be blocked and they are unable to utilise the salvage pathway. The myeloma line used by Kohler and Milstein was HGPRT-ve and so after the fusion of the splenocyte and myeloma cells culturing cells in HAT medium ensured that only the hybridomas, which inherited *in vitro* stability from the myeloma cell line and the ability to utilise the salvage DNA synthesis pathway from the splenocytes would be viable.

Harris and Watkins (1965) reported that it was possible to fuse human and mouse cells to produced an artificial animal cell heterokaryon using inactivated sendai virus. The resulting hybrid cells produced protein and both the human and murine nuclei synthesised RNA. Kohler and Milstein subsequently used sendai virus as the fusing agent in their splenocyte-myeloma fusions. More recently polyethylene glycol, PEG, has become the most widely used fusing agent (Pontecorvo 1976). PEG can be used as a chemical fusing agent to induce fusion of cell lines at less

cost than sendai virus, fusion using PEG is also less time consuming than fusing using sendai virus. The closer the genetic relationship between the splenocyte and myeloma cell lines used in fusions the more stable hybridoma will be with respect to chromosome loss. For example hybridomas from the fusion of myelomas and splenocytes both derived from BALB/c mice.

Co-expression of antibody chains from both fusion partners in the hybridoma can lead to the secretion of mixed species antibody molecules, containing chains of myeloma and splenocyte antibody chains together. To prevent this, myeloma cell lines that do not produce antibody chains, such as X63/Ag8.653 are used as fusion partners. These non Ig producing myeloma cell lines do not produce antibody, but do produce antibody mRNA. The antibody mRNA produced by these cell lines is aberrantly rearranged at the V-J recombination site, or the D-J recombination site and is not translated into protein. It has been demonstrated that hybridomas formed using these non Ig producing myeloma cell lines will also produce an aberrant mRNA transcript, (Carroll *et al* 1988, Nicholls *et al* 1993)that is not translated into protein. In these hybridomas the amount of aberrantly rearranged mRNA produced can vary. In certain hybridomas the levels of aberrant mRNA can be high and may be higher than the levels of the translated antigen specific antibody mRNA.

To create hybridoma cell lines antigen is usually injected into mice at repeated intervals of approximately 3-5 weeks. Approximately 10 days after injection a tail bleed is taken from the immunised animals, and the blood used to test for the presence of desired antigen specific antibodies. The animals giving the best results are used for the fusion. The resulting splenocytes are fused with the chosen myeloma using PEG. The hybrids are then grown in the selection medium HAT. Approximately 10 days later the myeloma cells will be dead. Once the myeloma cells are dead then the surviving hybrids can be cultured in HT medium which lacks aminopterin. Aminopterin inhibits many normal cell functions and so should be removed from the culture medium as soon as possible. The surviving hybrids are screened by assaying culture supernatant for desired antibody production. Positive hybridomas, which are producing antibodies which bind to the desired antigen, are cloned, usually by limiting dilution in suspension culture, although cloning in soft agar is also used. Again the clones are screened and the positive, antibody producing, clones are recloned. The monoclonal hybridomas can then be characterised and desired clones selected for further study (Galfre and Milstein 1981).

These hybridomas are then grown *in vitro*, with stocks of the desired hybridomas stored in liquid N_2, expanded either for *in vitro* or *in vivo* antibody production. For *in vivo* antibody production mice are primed with pristane then inoculated, intra peritoneum, with the hybridoma cell line. The antibody is then purified from the resulting ascities fluid. The mice used should be compatible with the hybridomas used, hybridomas produced from BLAB/c mouse spleen/myeloma fusions should be inoculated into BLAB/c mice. Antibodies are present in high concentrations in ascities fluids, but are contaminated with other immunoglobulins and plasma proteins.

Screening of hybridoma clones is usually carried out using indirect binding assays. Antibody in the spent medium from hybridoma tissue culture is allowed to bind to antigen and the amount of antibody is determined by a second labelled antibody binding to the antibody from the culture medium. Antigen is bound to a solid support, most commonly polyvinyl or polystyrene 96 well plates, which are coated to encourage protein antigen binding. The antigen is absorbed onto the solid surface, but the plates are not saturated with antigen. Once the antigen is bound any other sites on the solid surface can be blocked with bovine serum albumin, new born calf serum, gelatin or bovine globulin in a buffered solution to prevent antibody in the spent medium from binding to the plate. The spent medium is added to the plate and incubated, so that the antibody binds to the surface bound antigen. This bound antibody is detected with a second antibody, that binds the antibody to be detected, which is either radio labelled or enzyme labelled.

The system most commonly used is the enzyme labelled system, enzyme linked immunosorbent assay (ELISA). Sheep antimouse Ig or goat antimouse Ig antibodies are used as second antibodies to detect murine hybridoma produced antibodies. For radio labelled second antibodies radioactive iodine is bound to the protein. For enzyme labelled second antibodies an enzyme with a chromagenic substrate is covalently linked to the antibody, the enzymes used are usually alkaline phosphatase, β-galactosidase or horse radish peroxidase (HRP) (McCullough and Spier 1990).

Hybridoma technology allows for the exploitation of the immune system to generate monoclonal antibodies that will bind to natural products or synthetic molecules, and so form the basis of a useful tool for biology and medicine. The

resulting antibodies will be specific for one antigen and will form a high affinity complex with that antigen. Hybridoma technology does however suffer from several drawbacks. The first is that the use of monoclonal antibodies from murine hybridoma cultures in human patients is hampered by the human antimouse antibody reaction, (HAMA) (Winter *et al* 1991, Chester *et al* 1995). The mouse antibodies illicit an immune response in humans so that any therapeutic action they possess will be diminished by the antimouse antibody immune response. Human hybridoma cultures could counter the HAMA response, but human hybridoma culture has a number of practical and ethical obstacles, least of all the immunisation of humans to generate immunised splenocytes as fusion partners. The second drawback is that hybridomas cannot be raised against self antigens.

1.2 CATALYTIC ANTIBODIES

1.2.1 Enzyme catalysis and kinetics

Chemical reactions proceed from reactants, substrates, through any intermediates, to products via a transition state, the transition state being the most energetic, least stable, species along the reaction pathway. The reactants, intermediates and products are all species in which the chemical bonds are either fully formed or fully broken, in the transition state however the molecular bonds are partially formed. The difference in free energy, between the reactants and the transition state of the reaction is the free energy of activation of the reaction. The energy of activation acts as a barrier to the formation of the products, as the reactants need to possess at least the activation energy to form the transition state, before the products are formed. The rate of formation of the products will depend on the energy of activation of the reaction, the relative amounts of the products formed will be determined by the relative thermodynamic stability of reactants and products (Fersht 1984).

Enzyme catalysis utilises the binding interaction in enzyme-substrate complexes to reduce the free energy of activation on the chemical reaction pathway, by stabilising the transition state of the reaction such that,

$$[E]+[S]\underset{}{\overset{k_1,k_{-1}}{\longleftrightarrow}}[ES]\xrightarrow{k_{cat}}[E]+[P] \qquad eq.\ 1.2.1$$

Pauling proposed that the active site of an enzyme was most complementary to the transition state of the reaction, rather than the substrate itself. The transition state would then be stabilised by the high affinity of the enzyme-transition state complex, and the energy of activation on the reaction pathway would be reduced, catalysing the reaction. Therefore a substance that was chemically stable with similar geometry to the theoretical transition state of the reaction, a transition state analogue, would bind tightly to the enzyme that catalyses that reaction. The concept of transition state analogue is now established as a tool for the design of enzyme inhibitors.

In the analysis of enzyme kinetic data it is assumed that the concentration of enzyme-substrate complex is constant as the rate of formation of the enzyme-substrate complex equals the rate of its decomposition. This assumption is known as the steady state approximation. A reaction catalysed by an enzyme follows Michaelis-Menten kinetics such that,

$$V = \frac{V_{max}[S]}{([S] + K_M)} \qquad eq.\ 1.2.2$$

where K_M is the Michaelis constant,

$$K_M = \frac{(k_{-1} + k_{cat})}{k_1} \qquad eq.\ 1.2.3$$

V = rate of reaction

V_{max} = maximum rate of reaction

K_M is a measure of enzyme-substrate affinity, a relatively high K_M indicating a low enzyme-substrate affinity and a relatively low K_M a high enzyme-substrate affinity. The specificity of an enzyme can be described by k_{cat}/K_M. The rate of reaction of an enzyme catalysed reaction from Michaelis-Menten kinetics is,

$$V = k_{cat} / K_M [E][S] \qquad eq.\ 1.2.4$$

in which k_{cat}/K_M is an apparent second order rate constant. The rate of reaction for two competing substrates, S_1 and S_2 will be,

$$V_{S1} = (k_{cat} / K_M)_{S1}[E][S_1] \qquad eq.\ 1.2.5$$

$$V_{S2} = (k_{cat} / K_M)_{S2}[E][S_2] \qquad eq.\ 1.2.6$$

and the ratio of these rates of reaction will be,

$$\frac{V_{S1}}{V_{S2}} = \frac{(k_{cat} / K_M)_{S1}[E][S_1]}{(k_{cat} / K_M)_{S2}[E][S_2]} \qquad eq.\ 1.2.7$$

at equal concentrations of S_1 and S_2 the rate of reaction will be determined by the relative values of k_{cat}/K_M thus k_{cat}/K_M can be used to describe the specificity of an enzyme for a substrate, relative to a second substrate (Price and Stephens 1989).

Jencks (1969) suggested that if the active site of an enzyme was most complementary to the transition state of a reaction, and that the binding interaction in the enzyme-substrate complex was utilised, by transition state stabilisation, to catalyse the reaction then other proteins that could complex with the transition state of a reaction could express a catalytic function. If a stable

analogue of a theoretical transition state of a reaction is used, as a hapten, to generate an immune response system, then the binding site of the resulting antibodies could express a catalytic function for the reaction represented by the transition state analogue The antigen affinity of the antibody would then be utilised, to stabilise the transition state of the reaction in a similar way to the binding energy of enzyme-substrate complexes, and so catalysing the desired reaction. Such a catalytic antibody should catalyse a reaction according to Michaelis-Menten kinetics, and be inhibited by the transition state analogue itself.

A second method for the generation of catalytic antibodies would be to produce an antibody binding site that mimicked the active site of an enzyme, by an antigen internal image (Friboulet *et al* 1994). If antibodies are raised to an enzyme, then some of the polyclonal antibodies will be anti-active site antibodies, complementary to the active site of the enzyme and act as inhibitors of the enzyme by binding to the active site. A complementary, inhibitory antibody would be a mould of an enzyme site, and if antibodies were in turn raised to the anti-active site antibodies, some of them would bind to the anti-active site Fv portion of the anti-active site antibodies. These antibodies would possess a binding site that would be an internal image of the active site of the enzyme the inhibitory enzymes were raised to, an express the same catalytic function as the original immunising enzyme.

1.2.2 The development of catalytic antibodies

Catalytic antibodies were developed using Jencks' and Paulings' enzyme catalysis mechanism of transition state stabilisation. The first catalytic antibody research programmes studied antibodies that would catalyse carboxylic ester hydrolysis.

Carboxylic ester hydrolysis reactions proceed via a tetrahedral transition state. The transition state analogue for carboxylic esterolysis, is the equivalent phosphonate ester, which possess the same substituents, (R), as the ester (*fig.* 1.2.2). The phosphonate ester mimics the tetrahedral geometry of the esterolysis transition state, and also the partially charged oxygens of the transition state. The support for these analogues being similar to reaction transition states comes from the fact that these phosphonate esters act as inhibitors of enzymes that catalyse ester hydrolysis, given that the phosphonate esters are a more stable species than the transition state and so will not change.

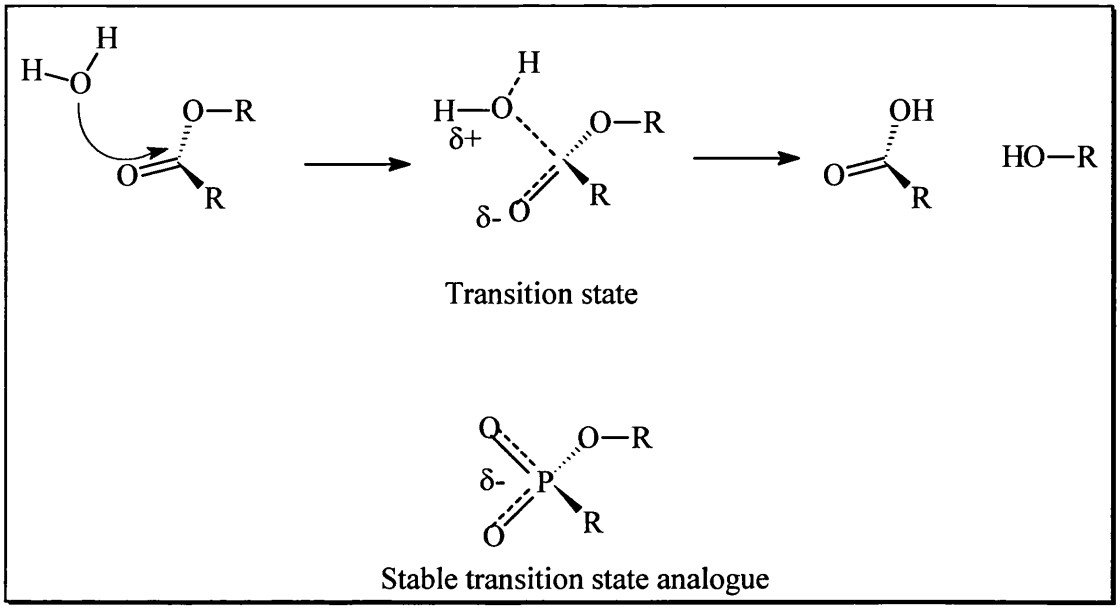

fig. 1.2.2 Carboxylic ester hydrolysis and phosphonate transition state analogues

Pollack *et al* (1986) studied the antibody MOPC167 which is a member of a class of antibodies specific for phoshorylcholine mono and diesters. MOPC167 was known to bind 4-nitrophenylphosphorylcholine, which is a transition state analogue for the hydrolysis of 4-nitrophenyl-N-trimethyaminoethylcarbonate. Pollack *et al.* used 4-nitrophenyl-N-trimethyaminoethylcarbonate, as a substrate, to test MOPC167 for catalytic activity. The rates of hydrolysis of 4-nitrophenyl-N-trimethyamino-ethylcarbonate in the presence and absence of MOPC167 were determined, as a function of substrate concentration. MOPC167 was found to hydrolyse 4-nitrophenyl-N-trimethyaminoethylcarbonate in agreement with Michaelis-Menten kinetics.

The value of the catalytic constant k_{cat} was found to be $0.4mins^{-1}$ and K_M was found to be 208μM. The MOPC167 catalysed hydrolysis of 4-nitrophenyl-N-trimethyaminoethylcarbonate was found to be 770 times greater than the uncatalysed hydrolysis reaction. MOPC167 was denatured and found to have lost catalytic activity.

The MOPC167 catalysed hydrolysis of 4-nitrophenyl-N-trimethyamino-ethylcarbonate was inhibited by the addition of 4-nitrophenylphoshocholine, the transition state analogue. MOPC167 was also cleaved with pepsin to give Fab

fragments, which also catalysed the hydrolysis of 4-nitrophenyl-N-trimethyaminoethylcarbonate to the same degree as the whole antibody.

Tramontano *et al*(1986) were the first group to successfully induce catalytic antibodies, using hybridoma technology with phosphonate ester transition state analogues as haptens. The resulting antibodies hydrolysed a carboxylic ester substrate at a 960 fold increase in the rate of reaction over the uncatalysed reaction. Also these first catalytic antibodies were highly selective with respect to the substrates catalysed. As with enzymes the reactions catalysed by these antibodies followed Michaelis-Menten kinetics K_M was found to be 1.90 $\times 10^{-6}$M, and k_{cat} 2.7 $\times 10^{2}$ sec^{-1}. The original transition state analogue used to generate these antibodies also acted as an inhibitor of the antibody. Tramontano proposed the term abzymes for antibodies that expressed a catalytic function.

Since then a number of reactions have been catalysed using catalytic antibodies, including amide hydrolysis (Janda *et al* 1988), redox reactions (Shokat *et al* 1988), and Diels-Alder reactions(Gouvener *et al* 1993, Suckling *et al* 1993). The catalysis of the Diels-Alder reaction by an antibody was an important step as this reaction is not catalysed by any enzyme, and the reaction proceeded at room temperature and pressure. Iverson and Lerner (1989) successfully created monoclonal antibodies that would catalyse the specific hydrolysis of a Gly-Phe bond, at neutral pH with a metal cofactor. The catalysis of amide bond hydrolysis is important, as it could result in a series of catalytic antibodies that would cleave at specific points in a unique sequence of several amino acids, as opposed to a protease that is specific for only one or two residues in a protein sequence. The resultant catalytic antibody would act as the protein chemistry equivalent of restriction enzymes in molecular biology (Smithrud *et al* 1997, Benkovic 1992).

Sequence specific abzyme proteases, used in conjunction with protein ligases, as developed by Jackson *et al* (1994) would form the basis of a "cut and paste" technology for the *in vitro* construction of proteins from polypeptide fragments. Jackson *et al.* engineered a subtilisin variant, which they termed subtiligase, which acted as a protein ligase. The subtiligase efficiently ligated esterified peptides, in sequence, in aqueous solution to completely synthesise fully active RNase A. The RNase A was constructed from six peptide fragments each between 12 and 30 residues long. Jackson *et al.* also succeeded in producing a RNase A variant which contained the unnatural amino acid 4-fluorohistidine, in

place of histidine, at positions 12 and 119, by using subtiligase to ligate 4-fluorohistidine containing peptides in the construction of the RNase A variant.

Catalytic antibodies are generated using existing hybridoma technology (Galfre and Milstein 1981), used for the production of other monoclonal antibodies. The transition state analogue hapten is conjugated to a carrier protein, usually bovine serum albumin or keyhole limpet hemocyanin, with a spacer arm long enough to avoid steric interference by the carrier protein in the hapten antibody complex. Coupling strategies have to be compatible with hapten structure and *in vivo* stability. Usually amide bond formation is used, from carboxyl groups on the hapten and the amino groups of lysine residues on the surface of the carrier protein.

The conjugated haptens are used to immunise laboratory mice. The resulting splenocytes are then fused with myeloma cells, using PEG, then cultured in HAT. The hybridomas are cultured and screened. Positive cell lines producing catalytic antibodies specific for the desired reaction.

Many reactions are accelerated by general acid base catalysis and the amino acid residues needed for this can be put into antibodies using a "bait and switch" strategy. The antigenic hapten, "bait", possess a group that does not have an equivalent on the substrate of the desired reaction, this "bait" induces a corresponding residue on the antibody, so that the antigen affinity is increased. When the substrate, "switch", binds to the antibody the specially induced residue or residues take on a different function, facilitating catalysis, such as hydrogen bonding to a certain part of the substrate. Cofactors necessary for catalysis can also be included in the antigen and so produce a binding site for the cofactor in the antibody as well. The resulting antibody would then bind both substrate and cofactor together in a reaction mixture to facilitate catalysis (Smithrud 1997, Benkovic 1992).

The nature of abzyme catalysis has been investigated by comparison of the structures of complexed and uncomplexed Fab of an esterase antibody, which catalysed the esterolysis of p-nitrophenyl acetate, by X-ray crystallography. The topography of the uncomplexed Fab binding site was a groove, but the binding site changed topography to that of a cavity when the Fab was complexed with substrate with the substrate buried within this cavity. This study by Charbonnier *et al*(1995) suggested that antigen binding takes place by an induced fit

mechanism, and this translates to an induced fit mechanism for substrate binding which is comparable with that of enzymes.

The relationship between abzyme amino acid sequence and catalytic mechanism was investigated by Gao *et al*(1995) who demonstrated that a single change in amino acid sequence changed the catalytic mechanism. A single phosphonate transition state analogue was used to generate abzymes and from the same immunisation four separate and distinct abzymes were isolated. Studies of the abzyme 17E3 suggested that its mechanism was similar to that of esterase enzymes that feature the conserved active site Ser-His-Asp and that the catalysed reaction proceeded via a rate limiting acyl intermediate step. The abzyme 29G11 was four fold less active than 17E3 with nine point mutations between the two abzymes. the active site of both abzymes differed by a single residue 29G11 possess a Gly at position 99 and 17E3 possessing a Ser. Gao *et al* concluded, by sequence comparison, that the three dimensional structures of the two abzyme active sites were very similar and that they binded to both hapten and substrate in a similar manner. The crucial difference between the two was the Gly→Ser change in 29G11, the 17E3 Ser acting as a nucleophille to form the acyl-antibody complex which did not happen with the 29G11 antibody. This work also demonstrated the immune systems ability to produce multiple abzymes with different mechanisms.

1.2.3 Development of catalytic antibody C3.

Suckling and Stimson (Khalaf, Suckling, Stimson, *et al* 1992) at the University of Strathclyde became interested in producing an abzyme that would catalyse electrophilic aromatic substitution. Such an abzyme would be of potential synthetic interest and catalyse a reaction that only a few naturally occurring enzymes do, such as phenyltransferase, thymidylate synthetase and tryptophan synthetase.

Suckling and Stimson wanted to develop an abzyme that would catalyse a cyclisation reaction producing benzocycloheptenones from disubstituted phenylpentanoic acids as substrates (*fig.* 1.2.3.1).

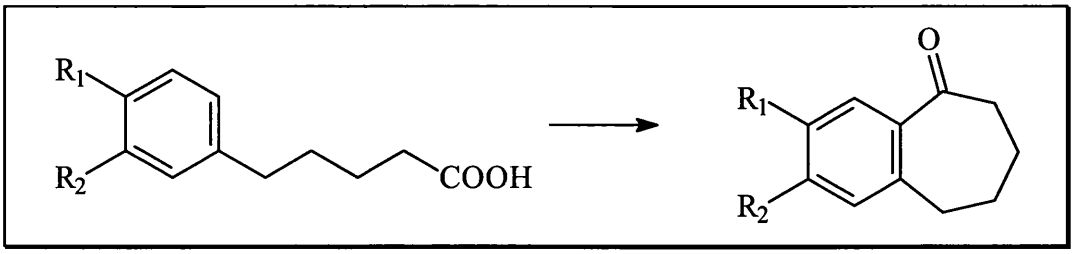

fig. 1.2.3.1 Cyclisation of disubstituted phenylpentanoic acids.

Aromatic substitution is well understood to proceed through cationic intermediates. The group at Strathclyde produced a transition state analogue, the protonated form of which can be compared with the positively charged intermediates in electrophilic aromatic substitution, to use as a hapten for the generation of abzyme producing hybridomas.

A transition state analogue for the reaction, 4-(2,3,4,5-tetrahydro-1-methyl-1 -benzazepin-7-yloxy)butanoic acid, (*fig.* 1.2.3.2) was synthesised, by Khalaf(1992). This transition state analogue was used as a hapten to generate an abzyme which might catalyse an electrophillic aromatic substitution reaction.

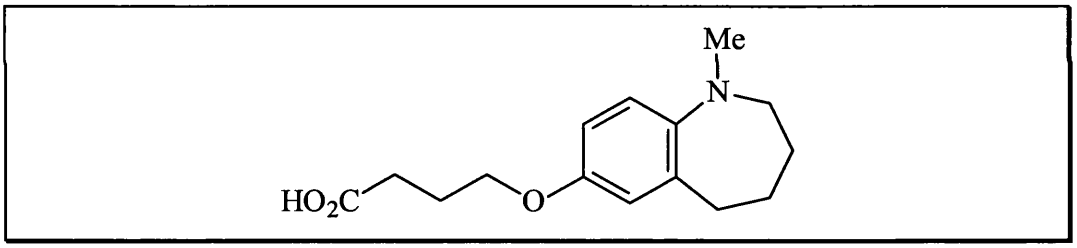

fig. 1.2.3.2 4-(2,3,4,5-tetrahydro-1-methyl-1 -benzazepin-7-yloxy)butanoic acid.

This hapten 4-(2,3,4,5-tetrahydro-1-methyl-1 -benzazepin-7-yloxy)butanoic acid methiodate, was linked via the butanoic acid group to Bovine Serum Albumin (BSA). The hapten conjugate was then emulsified in Freunds Complete Adjuvant (FCA). Male NZB/BALB-C F1 8-12 week old mice were then immunised with 0.5 mg hapten -BSA conjugate in 0.5 ml i.p. , per mouse. Two weeks later the mice were boosted with hapten -BSA conjugate, emulsified in Freund's Incomplete Adjuvant (FIA) with each mouse again receiving 0.5 mg in 0.5 ml i. p. .Ten days later a test bleed from a tail vein was tested by ELISA for antibody production. If a serum titre of >1:250 was observed the mouse spleen cell fusion was carried out the next day. The spleens were removed from the immunised mice (2 per fusion), and the spleen cells were fused with myeloma cells (x63 Ag8 653) and the cell mixtures cultured in flat bottomed 96 well plates in RPMI 1640,

containing 10% foetal calf serum and HAT. The cultures were fed at 7 days with RPMI 1640 containing HAT and afterwards as necessary. After two weeks antibody production was assessed by ELISA and the positive cell lines expanded and cloned by standard procedures .From these hybridoma lines two were chosen for further study. These cell lines were designated C3 and C5 respectively.

The C3 and C5 hybridoma cell lines were expanded. Male NZB/BALB-C F1 8-12 week old mice were treated with 0.5 ml pristane (2,6,10,14-tetramethyl pentadecane) per mouse. 7-14 days later the mice were injected with the C3 and C5 hybridomas (10^7 cells per mouse). The resulting ascities were collected 10-14 days later and centrifuged. The supernatant was fractionated with 50% (w/v) $(NH_4)_2SO_4$ and then dialysed overnight against 0.01mol dm^{-3} citrate, pH 5.0 . C3 and C5 antibodies were purified by gradient ion exchange chromatography using Sepharose-S Fast Flow columns and 0.01mol dm^{-3} citrate, pH 5.0 buffer containing 0.1mol dm^{-3} to 1mol dm^{-3} NaCl. The purified antibody was dialysed overnight against 0.15mol dm^{-3} NaCl at 4°C and stored at -20°C. The purity of C3 and C5 antibodies was demonstrated by SDS-PAGE gel electrophoresis, and the immunoglobulin class of the monoclonal antibodies C3 and C5 were determined, by ELISA, to be IgG3 and IgG2b respectively.

The catalytic properties of the monoclonal antibodies C3 and C5 were then investigated. 5-(3-methoxyphenyl)-pentanoic acid and its, ethyl, ethylthio, 4-chlorophenyl and 4-nitrophenyl esters were prepared as substrates of the monoclonal antibodies C3 and C5.

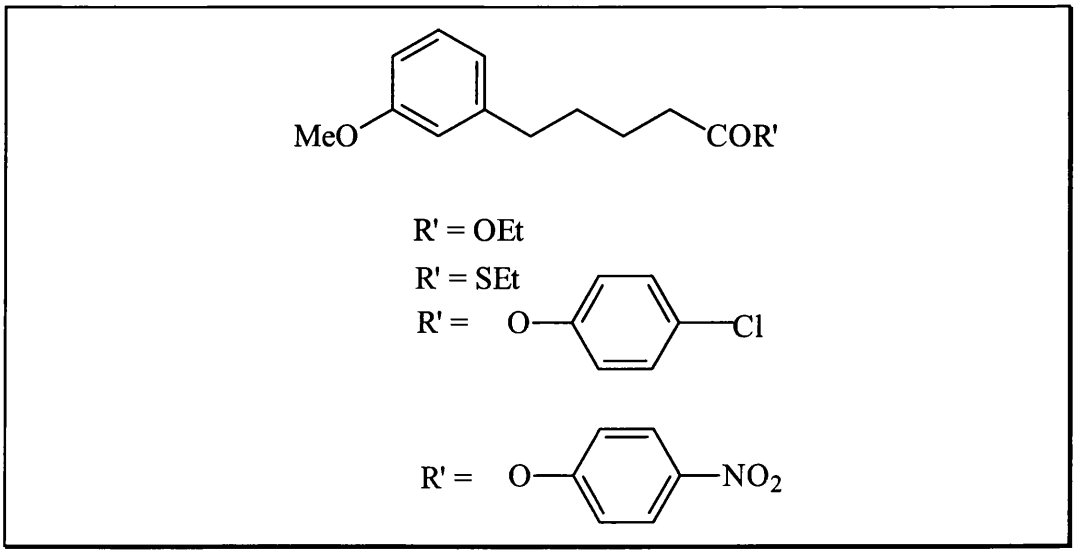

fig. 1.2.3.3 5-(3-methoxyphenyl)-pentanoic acid substrates.

The monoclonal antibodies (4.2μmol dm^{-3}) were incubated with substrate (0.05-0.25mmol dm^{-3}) at pH 8.5. With the monoclonal antibody C5 no reactions of any kind were detected by HPLC. The C3 monoclonal antibody only reacted with 4-nitrophenyl 5-(3-methoxyphenyl)-pentanoate. The C3 monoclonal antibody failed to catalyse the expected cyclisation reaction, but did catalyse the ester hydrolysis reaction, producing 4-nitrophenol.

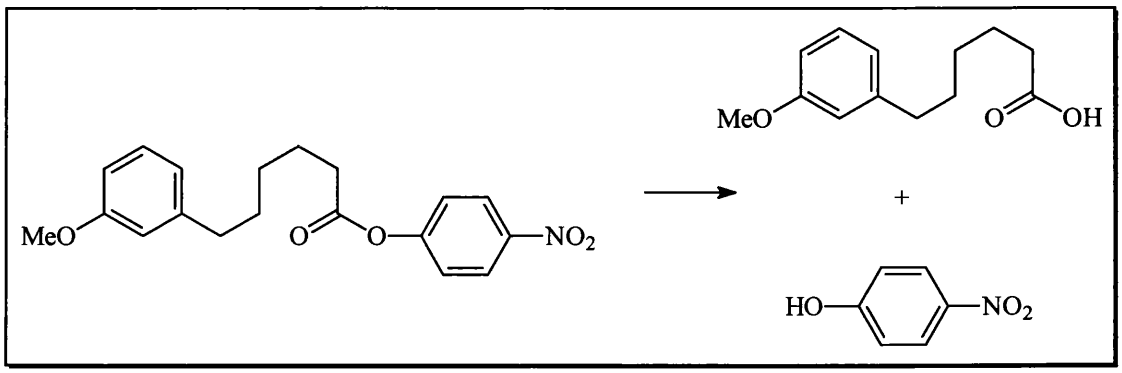

fig. 1.2.3.4 C3 catalysed hydrolysis of 4-nitrophenyl 5-(3-methoxyphenyl)-pentanoate.

The mechanism of the C3 catalysis was investigated. C3 was shown to hydrolyse 4-nitrophenyl 5-(3-methoxyphenyl)-pentanoate according to Michaelis-Menten kinetics. At pH 8.5 k_{cat} was determined to be $2.4s^{-1}$ and K_M 0.4 mmol dm^{-3} giving a value of 6000 dm^3mol^{-1} s^{-1} for k_{cat}/K_M. Under the same conditions the uncatalysed solvolysis reaction gave a rate constant of 6.9×10^{-7} s^{-1} which suggests a rate enhancement of 3.5×10^6 by monoclonal antibody C3, meaning that C3 is acting as an efficient catalyst for this reaction. The reaction was also shown to be pH dependant with a plateau of maximum rate above pH 8, suggesting that an ionisable group on the monoclonal antibody C3 could be involved in the reaction. To identify the significant functional group chemical modification experiments were performed. The only chemical modification that affected the catalytic properties of C3 was incubation of C3 with glycine ethyl ester in the presence of 1-ethyl 3-(3-dimethylaminopropyl)carbodiimide which subsequently completely inhibited C3 catalytic activity. This result, also considering the fact that the reaction is pH dependant, suggests that a carboxylate anion is functioning as a general base in C3.

1.3 ENGINEERED ANTIBODY FRAGMENTS

Antibodies have aroused a great deal of interest as therapeutic proteins due to their nature as high affinity, high specificity binding proteins. The rodent hybridoma technology used to produced antibodies, has a number of disadvantages for the production of antibodies intended as therapeutics. Monoclonals can be generated from the hybridomas *in vitro* or from mouse ascities fluids *in vivo*, both methods can be used to allow large scale production, both process are expensive requiring media and animals and the desired product must be extensively purified. The murine antibodies produced by these methods are also immunogenic when introduced into humans and are attacked by the human immune system because of their murine origins, the human antimouse antibody, HAMA response. The HAMA response vastly reduces the effectiveness of a mouse antibody used as a pharmaceutical to treat human disease. In order to overcome these obstacles antibody genes have been cloned and expressed in a wide range of host organisms, including mammalian cell lines(Riechmann *et al* 1988, Li *et al* 1995), transgenic plants(Owen *et al* 1992, yeast(Horwitz *et al* 1988) and bacteria, primarily *E.coli*. The cloning and expression of recombinant antibody gene sequences has several advantages over the use of hybridoma technology. Key among these is the fact that once cloned the antibody genes can be more easily manipulated to develop novel antibody based proteins, such as chimeric antibodies (Better *et al* 1988), murine variable regions grafted onto human constant regions, and humanised antibodies which contain murine CDR loops grafted onto human framework regions(Jones *et al* 1986, Winter 1991), immunotoxins, and bifunctional antibody conjugates for *in vivo* and *in vitro* diagnostic purposes. Recombinant antibody technology also allows for the development of human antibody fragments to specific antigens by the use of phage combinatorial libraries.

1.3.1 Cloning of antibody variable region genes

Antibody gene sequences can be cloned from antibody producing cell lines using a reverse transcriptase-PCR strategy. To create a cDNA antibody sequence mRNA from hybridomas, or any other antibody producing cell line, can be selected using oligo(dT) cellulose, the oligo (dT) being complementary to the poly A tail of mRNA. Subsequent treatment of the purified mRNA with reverse transcriptase produces a single strand cDNA/mRNA hybrid mixture.

The sequences of the V_l and V_H regions can be selected from the cDNA/mRNA library by PCR, using primers that are complementary to the conserved framework regions, FR1 which is 5' of the CDR1 and FR4 which is 3' of the CDR3, of both the heavy and light chain genes. As these sequences are highly conserved among antibodies of the same Ig class, consensus primers, that are complementary to these conserved framework regions, can be used to amplify the desired antibody sequence. The first clonings of antibody genes used a two stage cloning strategy(Huston *et al* 1988, Orlandi *et al* 1989, Pluckthun and Skerra 1988). The first stage PCR uses two separate pairs of consensus primers in two separate reactions. One primer pair being complementary for the V_l sequence and amplify the V_l sequence in one PCR reaction. The other primer pair being complementary for the V_H sequence and amplify the V_H sequence in another PCR reaction. The primers can also contain restriction enzyme sites, which allow for the forced cloning of the V_l and V_H sequences into plasmid vectors for expression as an Fv fragment. Both Fv and Fab fragments can be cloned by this methodology, the cloning of Fab fragments uses consensus primers that are complementary for C_{H1} and C_l regions as well as the variable region consensus primers.

Davis *et al*(1991) constructed an scFv of the antibody 4-4-20 using variable region DNA cloned from hybridomas, using the mRNA as template, with a PCRmethod that was an adaptation of splicing by overlap extension. Splicing by overlap extension, devised by Horton *et al*(Review 1993) is a method of fusing together two cDNA genes in a two stage process (*fig.* 1.3.1). The two desired genes are amplified independently by PCR using specially designed primers. The primers are designed with additional sequences which become incorporated into the amplified gene sequence. The resulting amplified fragments share complementary sequences, by the design of the primers, so that the two fragments will overlap.

The two fragments can be spliced together by bringing them together under PCR conditions, the two fragments denaturing then reannealing. As the additional sequences introduced by the primers are complementary the two fragments act as primers on each other forming a giant primer-dimer. The overlap is then extended using a DNA polymerase to give the recombinant product.

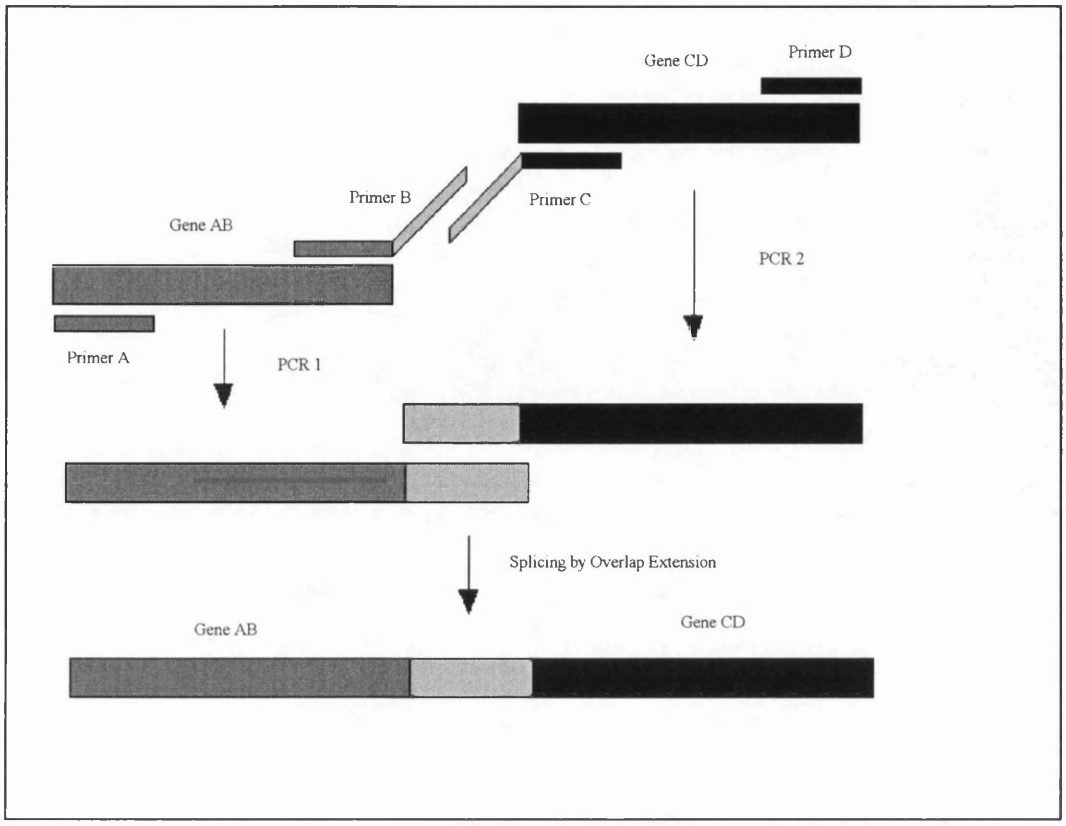

fig. 1.3.1 Splicing by overlap extension.

The scFv gene of the antibody 4-4-20 was constructed (Davis *et al* 1991), using primers that were complementary to the conserved framework regions of the V_l and V_H chains, $V_l$sense +$V_l$anti, $V_H$sense + $V_H$anti. The V_l and V_H were amplified by a first PCR step. In splicing by overlap extension one of the primers used for amplification of the V_l chain is complementary to one of the primers on the V_H chain in this case the complementary primers were $V_l$anti and $V_H$sense. The complementary primers also encoded for the polypeptide linker sequence required for the creation of the scFv. The PCR products of the first step were combined and a splicing by overlap extension step performed. The two PCR products from the first reaction were put together under PCR conditions, the two strands acting as primers on each other to form a single fused molecule. A second PCR reaction was performed with only the addition of the noncomplementary primers $V_l$sense and $V_H$anti to amplify the scFv gene.

Hybridomas contain antibody mRNA that encodes for not only for the functional antibodies produced by the hybridomas but also for non-functional antibody sequences(**1.1.2**). These sequences are derived from the myeloma cell line that was a fusion partner in the hybridoma. The myeloma fusion partners are selected as non Ig producing cell lines, the myelomas produce aberrantly rearranged

mRNA that is not translated to protein. The presence of these aberrantly rearranged mRNAs interferes with reverse transcriptase-PCR cloning strategies. These aberrant mRNA sequences are aberrantly rearranged at the V-J recombination site, but retain the conserved framework regions at their 5' and 3' ends, encoded by the 5' section of the V gene and the 3' section of the J gene, and so consensus primers are complementary for both genuine and aberrantly rearranged mRNA sequences. The high levels of aberrant mRNA present in hybridomas mean that any PCR procedure, using consensus primers that are complementary to the conserved framework regions of the Fv, may give products that may not encode for a functional protein sequence, as both functional and non functional mRNA is co-amplified (Carroll *et al* 1988, Nicholls *et al* 1993, Krebber *et al* 1997). In order to construct Fv or scFv genes it is necessary to compile a library of V_l and $V_H$genes, and screen them for the presence of aberrantly rearranged genes. Once the library is completed the correct functional V_l and V_H sequences can then be cloned into a suitable expression vector, or fused together to form an scFv.

Cloned murine immunoglobulin genes can be used to form chimeric or humanised antibodies to increase the therapeutic use of antibodies by decreasing their immunogenicity. A chimeric Fab utilises the variable regions from a murine antibody. the murine variable regions are spliced together with human constant regions, C_{H1} and C_l. The resulting hybrid protein retains the antigen binding properties of the murine antibody but because of the presence of the human constant regions is less immunogenic. A more extensive was described by Jones and Winter *et al*(1986), who devised a strategy of humanising rodent antibodies by loop grafting. The humanisation process involves the identification of the CDR residues of the desired antigen binding antibody and the *in vitro* grafting of those CDR residues onto human variable region framework regions. The resulting gene can be expressed and the subsequent humanised antibody possess the binding characteristics of the murine antibody but due to the extensive human origin of recombinant antibody its immunogenicity is reduced. However the reduction in immunogenicity may be at the cost of antigen binding. The design of the humanised antibody needs to take into account important residues in the framework regions that are either in contact with the antigen or are important in the orientation of the CDR loops.

1.3.2 Expression of antibody fragments in *E.coli*

Recombinant antibody gene sequences have been expressed in a number of host organisms, including tissue cultured mammalian cells, transgenic tobacco and *E.coli.* The *E.coli* based production systems would provide a more economical and potentially versatile technology than hybridoma-tissue culture technology. Once the antibody genes are cloned into a suitable vector they can be manipulated and fused to other gene sequences producing a wide range of antibody fragments and antibody fragment fusion proteins for a host of applications. There are however a number of problems with the use of bacterial expression systems for antibodies and antibody fragments, such as production of equivalent amounts of heavy and light chains, and incorrect folding of the chains. The first attempts to express cloned antibody fragments in *E.coli* often led to the recombinant antibodies forming insoluble inclusion bodies, which had to be solublised and correctly refolded (Bird *et al* 1988 and 1991).

The first research groups to have succeeded in producing active antibody fragments in *E.coli* were Better *et al*(1988) and Pluckthun and Skerra(1988). In order to produce active antibody fragments equimolar amounts of the heavy and light chains need to be synthesised and correctly folded. The two chain must also be correctly assembled, and the correct interchain disulphide bonds formed. Pluckthun and Skerra(1988) successfully expressed the active Fv of phosphorylcholine binding myeloma antibody McPC603. Pluckthun and Skerra tried to reproduce the same antibody folding pathway that exists in eukaryotic cells, in order to get active Fvs from bacterial systems. In the eukaryotic cell the two antibody chains are transported, with an NH_2 terminal signal sequence for transport which is then later cleaved, to the lumen of the endoplasmic reticulum. Working on the basis that protein transport to the periplasm in *E.coli* is the functional equivalent to transport of the protein to the endoplasmic reticulum in eukaryotic cells, Pluckthun and Skerra developed a system that expressed both chains of an Fv fragment in the same *E.coli* cell and then secreted both chains into the periplasm. Directing the expressed Fv to the periplasm was achieved using *E.coli* transport sequences, *ompA* and *phoA* fused to the V_l and V_H gene sequences. This system allowed for the synthesis of approximately equal amounts of each chain, transport to the periplasmic space and the subsequent folding and disulphide bridge formation to give active Fv fragments.

The plasmid used by Pluckthun and Skerra for Fv expression contained an origin of replication, ampicillin resistance gene for bacterial selection, and a *Lac* promoter-operator, for inducable protein production. The two chains were encoded as separate proteins on the same transcription unit downstream from the *Lac* promoter-operator. Each coding region was preceded by a ribosomal binding site, to form a regulatable dicistronic operon. The heavy chain was fused to the *ompA*, outer membrane protein A, signal sequence to direct the Fv to the periplasm, and the light chain to *phoA*, alkaline phosphatase signal sequence, also to direct the Fv to the periplasm. Protein production in the *E.coli* cultures was induced by the addition of IPTG, the *Lac* operator-promoter controlling the expression of both the V_H and V_l sequences. After induction the McPC603 Fv was produced in active from. The Fv fragment was purified from the periplasmic fraction of the cell lysate in a single step by using a phosphorylcholine affinity column.

The affinity constant of the McPC603 Fv produced in *E.coli*, for phosphorylcholine, was compared to the affinity constant of the whole antibody. Both affinity constants were determined by the same method, equilibrium dialysis, and found to be identical, within experimental error.

Better *et al* (1988) used a similar strategy to produce active Fab fragments in *E.coli* but used a *pelB*, pectate lyase from *Erwinia carotova*, leader sequence in place of the *ompA* signal used by Pluckthun and Skerra. The Better group produced the active Fab of L6 an antibody that binds specifically to the human carcinoma cell line C3347, in *E.coli*. The vector created by Better *et al* encoded the L6 Fab as a dicistronic operon. The recombinant L6 Fab was determined to possess the same binding characteristics as the Fab derived from proteolytic digestion of the whole antibody. The active expression of different antibody fragments, Fab and Fv, from different antibodies, McPC603 and L6, using different periplasmic signals, *ompA* and *pelB*, confirming the hypothesis that transport to the periplasm is the functional equivalent to transport to the lumen of the endoplasmic reticulum. These two groups established the technology of active antibody fragment expression in *E.coli* using periplasmic targeting signal sequences, as a general technique for the expression of recombinant antibody fragments.

Bird *et al* (1988) had created an scFv gene in which the two variable region domains were linked together by a short polypeptide sequence to form a single

molecule. The expressed protein formed insoluble inclusion bodies when the gene was expressed in *E.coli*, and was renatured to give an active antibody fragment. In an attempt to stabilise Fv fragments from disassociation Glockshuber and Pluckthun (Glockshuber *et al* 1990) produced a McPC603 scFv that was secreted as an active protein. The same expression system was used as for the McPC603 Fv, but the light chain gene was connected to the heavy chain gene by a sequence encoding for a $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_3$ linker sequence, formed by site directed mutagenesis. The *phoA* signal sequence fused to the V_l was deleted, and transport of the scFv to the periplasm relied on the *ompA* signal sequence fused to the V_H. The scFv was secreted as an active protein and as with McPC603 Fv purified in a single step affinity chromatography procedure, using a phosphorylcholine affinity column. The affinity constant of the scFv for phosphorylcholine was determined, by fluorescence titration, to be $1.3\times10^5M^{-1}$. The affinity constant for the Fv fragment was determined as $1.2\times10^5M^{-1}$, by equilibrium dialysis. The McPC603 scFv was also stabilised against irreversible thermal denaturation in comparison to the Fv fragment. As phosphorylcholine is a transition state analogue for the hydrolysis of choline carboxylate ester the McPC603 Fv fragment produced in *E.coli* was investigated for any catalytic activity and was found to catalyse the hydrolysis of choline carboxylate ester.

A comparison of the structures of the Fv and scFv fragment of McPC603 was performed using NMR, in order to determine if the linker sequence interfered with structure of the Fv (Freund *et al* 1993 & 1994). The N^{15} NMR experiments showed that the structures of the Fv and scFv fragments were identical and inferred that the linker sequence did not interfere with the folding of the scFv. These experiments also demonstrated that the $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_3$ linker sequence was the most flexible part of the molecule, and did not interfere in antigen binding.

Gibbs *et al.*(1991) used *E.coli* to express a scFv of the catalytic antibody NPN43C9, an esterase, called SCA protein 7A4-1/212. The SCA protein 7A4-1/212 gene sequence was constructed from the previously cloned sequence of NPN43C9. The V_l and V_H sequences were subcloned, and site directed mutagenesis used to generate useful restriction sites on the V_l and V_H sequences. The V_l and V_H sequences were fused to a linker sequence, 212, to form a scFv. The scFv gene sequence had the V_l sequence joined to the 5' end of the linker 212, and the V_H sequence joined to the 3' end. The 7A4-1/212 gene sequence

was then force cloned into expression plasmid pGX5526, the 7A4-1/212 gene sequence in pGX5526 was fused to *ompA*.

The expression plasmid pGX5526 was transformed into *E.coli*. host GX6712. The resulting expression vector/host strain combination provided temperature dependant transcription regulation. The cultures were grown at 30°C, then the temperature was raised to 42°C to induce protein expression. The 7A4-1/212 SCA protein was expressed as an insoluble protein. The crude protein was denatured, then refolded and subsequently purified using a two stage ion exchange chromatography procedure. The catalytic activity of the scFv and the whole catalytic antibody was compared and it was demonstrated that the renatured scFv retains the catalytic function of the whole antibody.

The work of Gibbs *et al* demonstrated that recombinant abzymes can be expressed in *E.coli*, correctly refolded *in vitro* and retain activity. Other groups however have been able to express abzyme fragments in *E.coli* in an active form by transport to the periplasm, using an inducible expression plasmid where the expression of the fragments is directed to the periplasm by a signal sequence (Kim *et al*, 1997, Ulirch *et al* 1995). these abzyme fragments, as Fv and scFv fragments, have shown comparable binding and catalysis characteristics to those abzyme from which they are derived.

1.3.3 Purification of recombinant antibody fragments

In order to aid the purification of antibody fragments some sequences have been fused to a purification handle. The use of affinity handles, or affinity tails was devised as a means of purifying recombinant proteins, the recombinant protein being produced as a fusion with the affinity handle. The affinity handle then acts as an affinity ligand which can be separated from cell debris by the use of an affinity column specific for the affinity handle. (Sassenfeld review 1990). Pluckthun (1991) developed the McPC603 expression plasmid to a more versatile system by introducing an affinity handle. Pluckthun modified the scFv gene by adding a tail of 5 histidine residues, to form an affinity tail. The subsequent protein could then be purified by an immobilised metal affinity chromatography (IMAC) procedure. The expressed Fv or scFv fragments could then be recovered by immobilised metal ion affinity chromatography, the hexa histidine tail chelating the immobilised metal ions. The use of an affinity tail simplifies purification procedures as it negates the need to create an antigen

column for purification of fragments and the same purification protocol can be used to purified any fragment carried by the vector regardless of antigen.

1.3.4 Phage antibody display libraries

The expression of antibody fragments in *E.coli* has lead to the creation of an *in vitro* alternative to hybridoma technology. Large libraries of antibody genes can be created from antibody mRNA, from hybridoma, immunised, and nonimmunised antibody producing cells, using the same reverse transcriptase-PCR strategy as for cloning V_l and V_H sequences from hybridoma mRNA, but instead of cloning a single antibody fragment cloning a large repertoire of antibody genes. The genes in the libraries can be combined *in vitro* and the resulting antibody variable regions can be displayed as a fusion to a phage coat protein and selected for antigen binding activity (Huse *et al* 1989, Winter *et al* 1993).

McCafferty *et al* (1990) and Clackson *et al* (1991) demonstrated that it was possible to clone antibody sequences into phage vectors, and for the antibody fragments to be expressed on the phage surface as a fusion to phage coat protein in a functional antigen binding form. Clackson *et al* (1991) cloned the rearranged V_H and V_l sequences from spleen mRNA of a mouse immunised against phOx. These sequences were randomly spliced together by PCR and then cloned into the vector fdDOG1 as a fusion with fd gene III. The resulting phage were panned against antigen and found a number of combinations of different V_H and V_l sequences, generating a range of scFv fragments with a variety of antigen affinities. The highest antigen affinity scFv's were found to be comparable to scFv's derived from hybridomas raised against phOx. Barbas *et al* (1991) demonstrated that antibody sequences cloned into phagemid vectors as fusions to carboxy terminal of phage major coat protein gVIII and to minor coat protein gIII would be expressed and packaged into the resulting phage particles, when the cells were infected with helper phage. The Fabs were displayed in an active form on the phage surface and the resulting phage particles could bind to immobilised antigen. A phagemid is a plasmid that contains a phage origin of replication. When cells containing phagemids are infected with a helper phage the phagemid, because of the phage origin, is incorporated into the phage. These *in vitro* phage display systems mimicked the *in vivo* antigen selection of B cells displaying the highest antigen affinity IgM. These libraries were constructed from the IgM

encoding mRNA from immunised cells and produced antibody fragments comparable to those generated by hybridoma technology.

Phage display of combinatorial antibody libraries can be utilised as a superior way of cloning antibody binding sites from hybridomas, Kreber *et al*(1997) used a phage display system to clone several monoclonal antibody sequences from hybridomas, which had previously proved difficult to clone. The phage library constructed from mRNA will contain both the functional antibody sequences but also the aberrant myeloma derived sequences, the phage display system allows for the isolation of the correct functional gene sequences by antigen binding, rather screening for functional genes by sequencing of the whole antibody cDNA produced from the hybridomas and selection of the correct sequences. Sastry *et al* (1991) constructed an abzyme Fab from a combinatorial library that had been generated from a hybridoma cell line. A hybridoma cell line had been raised against a transition state analogue for the hydrolysis of carboxyamide, and produced monoclonal antibody 43C9. The cDNA from this hybridoma cell line was cloned to generate libraries of heavy and light chain genes. These libraries were then phage displayed as Fab fragments, which was then screened for clones that had the highest hapten affinity, and the antigen selected Fab were demonstrated to catalyse the hydrolysis of carboxyamide.

The genes in a combinatorial library are spliced together in a number of ways, using similar methodologies as used in the manipulation of genes from hybridomas. Different methods are used if the library is to be an scFv library or a Fab library. The genes can be directly cloned using restriction sites introduced in the cloning primers. The primers can introduce a linker sequence containing a common restriction site which is utilised to randomly splice the V_H and V_l sequences together i*n vitro*. The resulting spliced sequence can be then inserted into an expression phagemid with a 5' periplasmic transport sequence and the phage gIII sequence 3'. Alternatively the V_H and V_l sequences can be sequentially cloned to construct a Fab library, by the use of a dicistronic construct with each sequence preceded by a transport sequence, and the second sequence fused to gIII. The first sequence is cloned into a phagemid and this library is used as an acceptor library into which the other sequence is cloned. The library is then displayed with the two antibody chains bonded together by the disulphide bond between the C_H-C_l domains of the Fab, with one of the chains fused to gIII. Splicing by overlap extension can also be used to construct the libraries with complementary overlapping primers. These primers are used to randomly

combine the V_H and V_l sequences into scFv fragments which can be cloned into expression phagemids.

As well as the production of the antibody fragments in *E.coli*, the antibody fragments from phage display can also be produced as full length immunoglobulins. Ames *et al* (1995) selected a Fab from a combinatorial library against complement component C5a, the V_H and V_l sequences were recovered using the same restriction sites as were used to clone them into the phagemid vector for phage display. The sequences were subcloned into separate vectors containing C_H and C_l domains which were cotransfected into cos cells, which subsequently secreted IgG with the same binding characteristics as the phage display Fab.

A variety of combinatorial libraries have been created from sources including hybridoma and immunised spleen cells. The use of phage displayed antibody combinatorial libraries has established an *in vitro* alternative to hybridomas for the generation of antibodies. Phage display libraries have also eliminated the need to immunise cells by the cloning and selection of antibody fragments from naive combinatorial libraries (Marks *et al* 1991, Winter *et al* 1993), and the need to immortalise the antibody producing cells by immortalising the antibody genes themselves. Such naive combinatorial libraries represent an advance for the production of therapeutic antibody fragment for use in humans as human antibodies can be developed for therapeutic uses without the need for immunising humans and creating human hybridoma cell lines (Little *et al* 1995, Vaughan *et al* 1996).

Marks *et al* (1991) created a naive combinatorial library using human peripheral blood lymphocytes, and multiple primers to designed to match each of the families of human V genes and J genes. This system allowed Marks *et al* to clone the expressed IgM and IgG present in the nonimmunised lymphocyte population, with V_H and V_l genes amplified separately and then splice the sequences together by PCR. The scFv sequences were cloned into a phagemid vector and subsequently displayed on phage.

Gram *et al* (1992) advanced antibody fragment combinatorial libraries a stage further by developing an *in vitro* equivalent to the *in vivo* immune response for the generation of the highest antigen affinity antibody from a naive library. A naive Fab library was created in λ phage from the bone marrow of nonimmunised

BALB/c mice. This naive Fab library was then displayed on the phage surface and the antigen binding Fabs selected, in this case the Fabs selected were for binding to a progesterone-bovine serum albumin conjugate. An error prone PCR process was used to perform random mutagenesis on the antigen selected Fabs, and the increased affinity Fabs, from the random mutagenesis were antigen selected. This work mimicked the *in vivo* immune system in that it antigen selected the highest affinity Fabs, produced from naive, germline, DNA library and then increased the antigen affinity of the Fabs by a random mutagenesis step that would be the equivalent of somatic hypermutation in the immune system.

While theses libraries have been shown to generate high affinity antibodies to a wide range of antigens from a single library, (Griffiths *et al* 1993, 1994, Nissim *et al* 1994) there are disadvantages. Key among these disadvantages is the unknown immune history of the donor, which influences the IgM and can influence any library derived from the IgM mRNA. Semi synthetic libraries have been developed where the V genes themselves rather than the IgM are cloned. The whole V_H and V_l regions are then constructed by the addition of synthetic D and J sequences to generate a semisynthetic antibody library, which is free of any bias introduced by the donors previous immune history (Hoogenboom *et al* 1997, Vaughan *et al* 1996).

An alternative to mutating phage displayed antibodies to increase antigen affinity is to increase the size of the antibody library, the larger the library the greater the probability of generating a high affinity antibody to any given antigen. Vaughan *et al* (1996) created a very large library of 1.4×10^{10} scFv's, from the functional V gene segments of 43 non-immunised human donors. This semi synthetic phage display librarywas used to produce various scFv's that bound to a range of antigens, including haptens proteins and "self" antigens. This very large scFv library has been shown to be a more versatile and powerful method of producing antibody fragment than production by hybridoma. The very large library produced scFv's of higher affinity than previous naive libraries and comparable to the affinities of antibodies produced by hybridoma The scFv's produced by the very large library, such as the scFv's that bound doxorubicin and the hormone oestradiol have no equivalent produced by hybridoma, as the very large library is an *in vitro* system and not subject to the same constraints as the immune system. The cancer drug doxorubicin is cytotoxic, and monoclonal antibodies raised against oestradiol have had good affinities but poor specificities, the oestradiol

binding antibodies produced by the very large library had equivalent affinities and improved specificity.

Essential for the construction of this large library was the use of the Cre/*lox* site specific recombinase system from bacteriophage P1(Waterhouse *et al* 1993). For very large libraries transformation efficiency becomes a limiting factor. Vaughan *et al* (1996) circumvented this limit by using combinatorial infection where the heavy chain repertoire is cloned into an ampicillin resistance carrying phagemid and transformed into *E.coli*, and the culture subsequently infected with phage carrying tetracycline resistance gene and the light chain repertoire, and a dummy heavy chain sequence. Both heavy chain sequences, the repertoire on the donor phagemid and the dummy on the phage where flanked by two *loxP* sites, one the wild type *loxP* and the other a mutant *loxP*11, the wild type *lox* being able to recombine with wild type but not mutant and vice versa. The resulting ampicillin and tetracycline resistant *E.coli* culture was then coinfected with a second phage carrying chloramphenicol resistance and the Cre protein, which catalyses the *lox* recombination. The light and heavy chain repertoires are then recombined by Cre in the *E.coli* cell, the heavy chain from the donor phagemid being incorporated into the acceptor phage in place of the dummy heavy chain, at the two *lox* sites. The use of the wild type and mutant *lox* sites means that the heavy chain sequences are not deleted in the presence of Cre and correct orientation of the heavy chains is maintained by having the incompatible wild type and mutant sites at the 5' and 3' ends of the heavy chain genes in the phage and the phagemid. The antigen binding phage are then selected from the resulting ampicillin/ tetracycline/ chloramphenicol resistant culture. As infection with phage is a more efficient process than transformation with plasmid or phagemid, the library of light chain heavy chain pairs is greater in size than that which can be generated by combination on the same phagemid and subsequent transformation. The limiting factor of transformation efficiency affects only the size of the heavy chain donor phagemid library not the whole library.

The very large naive combinatorial libraries is an *in vitro* copy of the immune system and large phage display libraries may now supersede hybridoma technology for the generation of antibody fragments. Phage libraries derived from non-immunised donors mean that human antibodies can be generated without, creating human hybridomas and exposing humans to hazardous antigens. The generation of antibodies from phage libraries is quicker, after initial construction

of the libraries, than by immunisation of animals and subsequent generation of hybridomas.

1.3.4 Applications of recombinant antibody fragment technology

Recombinant antibody technology holds great promise for a number of areas in research and therapy. The use of chimeric, humanised and human antibodies from phage libraries has meant that antibodies and antibody based therapeutics can be of use in the diagnosis and treatment of human disease. Many applications of antibodies have been described, both antibody fragments as binding agents and as antibody fusions as binding agents carrying an effector function, (Carter *et al* 1997).

The binding properties of antibodies hold out enormous potential as therapeutics, with their ability to bind cell surface antigens and other ligands such as growth factors and hormones and so act as high affinity inhibitors (Froyen *et al* 1993). Antibodies have been used to clear toxins from the blood stream, such as antidigioxin antibodies, (Huston *et al* 1988) and to block the effect of tumour necrosis factor to prevent sepsis. Anti viral scFv fragments have also been developed. Two strategies have been used to prevent infection. The first is to block key functions of the virus by raising antibodies against key proteins. Srikantan *et al* (1994) used this approach and developed a scFv which bound to the HIV-1 coat protein gp120 which plays a key role in HIV infection. The group subsequently used the scFv to neutralise cell free HIV-1. The second approach is to block cell surface receptors that virius will bind to. The low density lipoprotein receptor is the receptor for a group of human rhinoviruses, Hoditis *et al* (1995) raised an scFv against the LDLP receptor from a phage display library and demonstrated its ability to act as an inhibitor of rhinovirus infection.

One of the main thrusts of recombinant antibody research has been the use of antibody fragments to image tumours and to destroy them. Whole antibodies have found to have relatively poor tumour penetration characteristics, scFv's, because of their reduced size can penetrate deeper and more widely into tumours than the larger IgG (Yokota *et al* 1992). Although scFv are more rapidly cleared from the body than whole IgG, the specificity and tumour penetration characteristics of high affinity scFv make them ideal vehicles for the delivery of effector functions to tumour sites. Several groups have developed a number of

tumour associated antigen Fv based fusion proteins designed to destroy tumours by targeting toxins, drugs and the immune system to the tumour.

Wells *et al* (1992 & 1995) cloned and expressed an scFv derived from the monoclonal antibody FRP5. The FRP5 antibody binds to the erbB-2 receptor, which is over expressed in a high percentage of breast and ovarian tumours. The FRP5 scFv possesses the binding properties of the parent monoclonal antibody and so could be used to transport anti tumour agents to cancerous cells via the erbB-2 receptor affinity. Wels *et al.* then produced a scFv-exotoxin A fusion protein from a bacterial expression system. The scFv-ETA fusion protein was demonstrated to both bind to cells expressing the erbB-2 receptor, and be cytotoxic to erbB-2 receptor expressing cells. Other groups have also generated immunotoxins and demonstrated their *in vivo* efficacy, using a variety of carcinoma specific antigen targets and toxins. Yang *et al* (1995) have expressed a scFv-TNF fusion in *E.coli* and demonstrated that the fusion possessed the cytotoxicity of the tumour necrosis factor. The ability to target TNF to specific sites means that the efficacy of recombinant TNF is increased by decreasing the side effects of TNF use as a therapeutic, a decreased dose of targeted TNF being necessary to destroy a tumour.

Antibody directed enzyme prodrug therapy (ADEPT), (Deonain *et al* 1994 , Huennekens *et al* 1994, Melton *et al* 1996) utilises antibody binding sites as a delivery system for treatment of a specific cell type. Fv-enzyme fusions will bind to an antigen produced by the target cells, after the excess Fv-enzyme fusion is cleared from the circulatory system a relatively non-toxic prodrug is administered, which is converted to a cytotoxic drug by the Fv-enzyme fusion, at the site of the target cells. Rodrigues, Presta *et al* (1995) have developed an ADEPT antitumour therapeutic protein which is targeted to an antigen which is over expressed in some breast tumour cells, p185(HER2). A disulphide linked Fv was derived from the anti p185(HER2) antibody humAb4D5-8, this disulphide Fv was expressed as a fusion protein with β-lactamase at the carboxy terminus of the Fv. The Fv-βlactamase fusion protein efficiently activated a cephalothin doxorubicin prodrug. The Fv-βlactamase fusion protein was shown to target cell lines that over expressed p185(HER2) and activate the cephalothin doxorubicin prodrug, increasing its toxicity, cell lines that had normal p185(HER2) expression levels were unaffected. A further development of ADEPT is antibody directed abzyme prodrug therapy, ADAPT, the immunogenic prodrug activating enzyme is replaced with an abzyme that fullfills the same function. ADAPT

reduces the immunogenicicty of the therapeutic immunoconjugate by utilising an abzyme from a human phage display library, instead of a bacterially derived enzyme.

Antibody fragments have also been demonstrated as able to specifically target tumours for destruction by the immune system. George *et al* (1994) created a bifunctional scFv, the resulting molecule was capable of binding both to the specified tumour and to T cells simultaneously, and of causing T cell mediated lysis of the tumour cells. The bifunctional scFv molecule was constructed as a fusion of two scFv molecules. One specific for a tumour antigen and the other for the T cell receptor, and as a result the antibody fragment targets the anti tumour function of the T cell.

The versatility of antibody fragments means that many biotechnologies can utilises them. Molloy *et al*(1995) developed a powerful, highly specific purification system based on scFv fragments. The Molloy group utilised an scFv that bound to *Pseudomonas* bacteria, that was immobilised onto a column. The column was then demonstrated to be able to separate *Pseudomaonas* from mixed bacterial cultures, and to remove *Pseudomonas* from milk. Several researchers have utilised the binding properties to generate reagents for *in vitro* detection, in ELISA assays and western blotting, (Light *et al* 1992, Gandecha *et al* 1992 Lilley *et al* 1994). Some of these fragments are produced as reporter enzyme fusions and act as versatile research tools, for *in vitro* use in ELISA and western blots as an alternative to labelled antibodies derived from hybridomas. The Lilley group produced a scFv fusion to gp41 an epitope of HIV-1 , the scFv was raised against glycophorin A. The resulting bifunctional protein was used in a whole blood agglutination assay as was found to be able to discriminate between HIV-1 positive and negative sera, the anti HIV antibodies in the sera binding the gp41 epitope of the fusion.

The ability of antibody fragments to target effector functions to particular sites has been used to target viruses to specific cells. Somia *et al* (1995) created a fusion protein between an scFv and a viral envelope protein, which could target the resulting virus to a specific cell type for *in vivo* gene therapy. The group used the C7 hybridoma cell line which generated a monoclonal antibody against low density lipoprotein receptor (LDLR) and constructed an anti LDLR scFv envelope protein gene fusion. Recombinant virus particles were generated with this chimeric envelope protein fusion and were used to target LDLR

overexpressing HeLa cell line with β-lactamase as a model gene. This recombinant virus was demonstrated to be able to selectively introduce β-lactamase into LDLR expressing cell lines. The use of scFv envelope chimeric proteins is a useful and powerfully tool for the targeting of retroviral gene therapy vectors to human cells.

1.4 EXPRESSION PLASMID PPANG1C3SCFV

1.4.1 Construction of expression plasmid pPANG1.

The expression plasmid pPANG1 (*fig.* 1.4.1) was created by Gandecha *et al* (1994) Expression plasmid pPANG1 was based on the plasmid pBluescript SK II, (Stratagene cloning systems). A 200 bp fragment from another plasmid pTM2-2 (derived from pET-3a), containing a T7 ∅10 RNA promoter, the *E.coli ompA* signal peptide coding region, the Shine-Dalgarno sequence, and the multiple cloning site, was cloned into the *BamH* I site of the pBluescript plasmid to give the plasmid pANG1. A truncated *spA* sequence from plasmid pRIT5 was cloned by PCR using primers that incorporated into the sequence a *Xba* I site 5' and a *Nhe* I site 3' of the *spA* sequence. This cloned sequence was then digested with *Xba* I and *Nhe* I and then ligated into the *Nhe*1 site at the 3' end of the *ompA* signal coding region giving the plasmid pPANG1.

1.4.1.1 T7 RNA polymerase expression systems

Bacteriophage T7 RNA polymerase is used as the basis of an expression system for cloned genes in expression vectors, in *E.coli* (Studier *et al* 1990). T7 RNA polymerase expressed by the host strain will selectively transcribe only DNA under the control of a T7 promoter. T7 RNA polymerase is able to extend RNA sequences five times faster than *E.coli* RNA polymerase.

Bacteriophage DE3 carries a DNA fragment encoding for the *Lac* UV5 promoter and T7 RNA polymerase, this bacteriophage is used to construct *E.coli.* host cells for T7 based expression systems. Once the fragment from DE3 is inserted the *Lac* UV5 promoter can direct the transcription of T7 RNA polymerase, *Lac* UV5 being inducable by IPTG. The induced T7 RNA polymerase will then transcribe target DNA from expression vectors. T7 RNA polymerase is highly selective for specific promoters, such as T7 gene 10,(∅10), which rarely occur in DNA unrelated to T7.

In the (DE3) host expression systems there will be a low level of T7 RNA polymerase activity which will lead to some transcription of the target gene in uninduced cells. The basal activity of the T7 RNA polymerase can be reduced by T7 lysozyme, which is an inhibitor of T7 RNA polymerase. Host (DE3) strains have been created that carry plasmids, pLysS and pLysE, containing a clone of

the T7 lysozyme gene and that confer chloramphenicol resistance to the cells. The plasmid pLysE produces a higher level of T7 lysozyme than pLysS , pLysS has only a small effect on the expression of target genes causing a short lag in the production of protein, whereas the pLysE plasmid has a greater effect, the higher level of T7 lysozyme causing a greater lag in the production of protein and a reduction in the maximum level of expression relative to pLysS hosts. T7 lysozyme also cleaves a bond in the peptidoglycan cell wall, but T7 lysozyme produced from pLys plasmids is unable to pass through the inner membrane to reach the peptidoglycan layer. Once the inner membrane is disrupted the *E.coli* will rapidly lyse due to the presence of the T7 lysozyme.

The combination of DE3 infected host strain, carrying a pLys plasmid, and a T7 ∅10 promoter carrying expression vector produces a selective, tightly regulated and efficient expression system that can be induced by IPTG.

1.3.3.1 SpA affinity handles

Staphylococcal protein A is a 48KDa protein produced by *S.aureus*, which binds to most mammalian IgG(Nilsson *et al* 1990&1991). The wild type *spA* gene consists of three regions. The first region is the N terminus signal sequence, which is cleaved after translocation. The second region is the antibody binding region which contains five highly homologous binding domains. The third region acts as an anchor for the whole protein, by binding to the cell wall. The binding region of SpA binds IgG tightly, SpA domain B has an dissociation constant of 2 $\times 10^{-8}$ M for human IgG, and this affinity can be utilised for the purification of recombinant proteins.

A recombinant fusion protein including a SpA affinity handle can be easily purified using an IgG-sepharose column in an ligand affinity chromatography method. The spA fusion protein will bind to the IgG-sepharose while extraneous proteins flow through the column. The SpA fusion can be eluted off the column and then the SpA affinity handle can be cleaved from the target protein. The cleaved SpA can be removed by a second pass through the column which leaves the purified target protein.

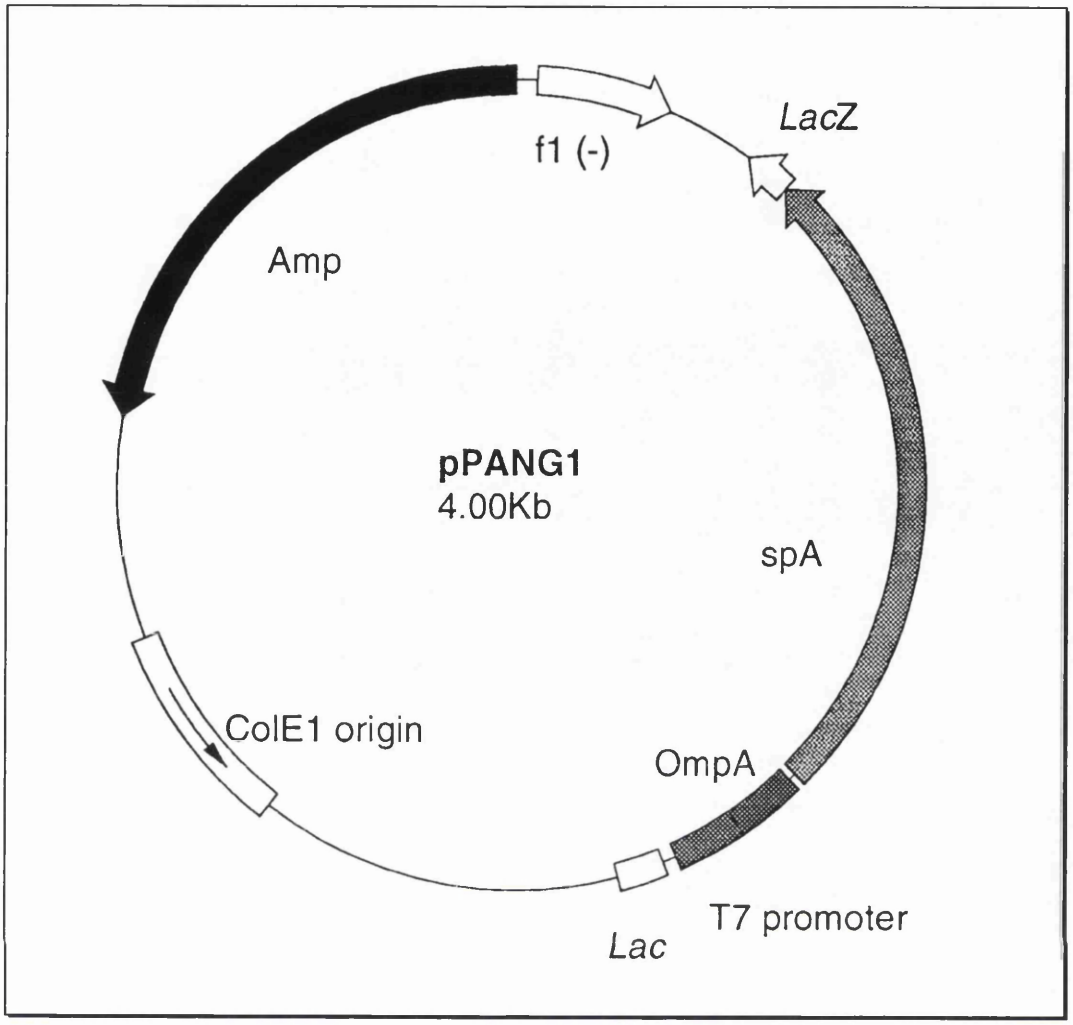

fig. 1.4.1 Diagram of expression plasmid pPANG1.

The expression plasmid pPANG1 was used by Gandecha *et al*(1994) to express an anti-phytochrome antibody, AS32, scFv-SpA fusion in *E.coli*. The expression of the AS32scFv being controlled by the T7 ∅10 promoter.

For expression of AS32scFv, pPANG1AS32scFv was transformed into *E.coli* strain JM109 (DE3). The *E.coli* cells were cultured at 30°C and production of AS32scFv-SpA induced by addition of IPTG to 1mM. The SpA sequence of the expressed AS32scFv-SpA fusion was utilised to purify the protein by affinity chromatography on human IgG-agarose columns, the SpA domain binding to the IgG constant regions of the IgG-agarose.

1.4.2 Construction of C3scFv, and expression vector pPANG1C3scFv.

The C3scFv was created from C3 hybridoma mRNA. The mRNA was treated with reverse transcriptase to give a single strand cDNA/mRNA hybrid. The V_l and V_H chains were amplified using PCR, with primers complementary for the conserved framework regions of the C3 V_l and V_H chains, V_l 5'RT+V_l 3' RT and V_H 5'RT+V_H 3'RT. The scFv was created using splicing by overlap extension, the complementary primers also encoding for a $(GlyGlyGlyGlySer)_3$ polypeptide linker, and the scFv amplified by PCR. The primer Vl5'RT contained *Xba* I and *Nco* I restriction site and the primer VH3'RT contained *BstE* II and *Kpn* I restriction sites. The C3scFv was cloned into the plasmid pGEM, and subsequently the expression plasmid pPANG1. Four pGEMC3scFv clones were created; pGEMC3scFv.2, pGEMC3scFv.4, pGEMC3scFv.7, pGEMC3scFv.9.

C3scFv.4 was cloned as a *Xba* I/*Spe* I fragment, from pGEMC3scFv.4, into the expression plasmid pPANG1 to give an *ompA-spA*-scFv fusion, pPANG1C3scFv.4 (fig. 1.4.2). The plasmid pPANG1 was digested with *Nhe* I and *Kpn* I and the *Xba* I / *Spe* I C3 scFv fragment ligated into the pPANG 1 vector. The *ompA* signal sequence would direct the translated protein to the periplasm of *E.coli*, so that the scFv would be secreted in an active form, production of the scFv being induced by IPTG. The SpA sequence could be used to purify the expressed scFv by affinity chromatography on IgG columns.

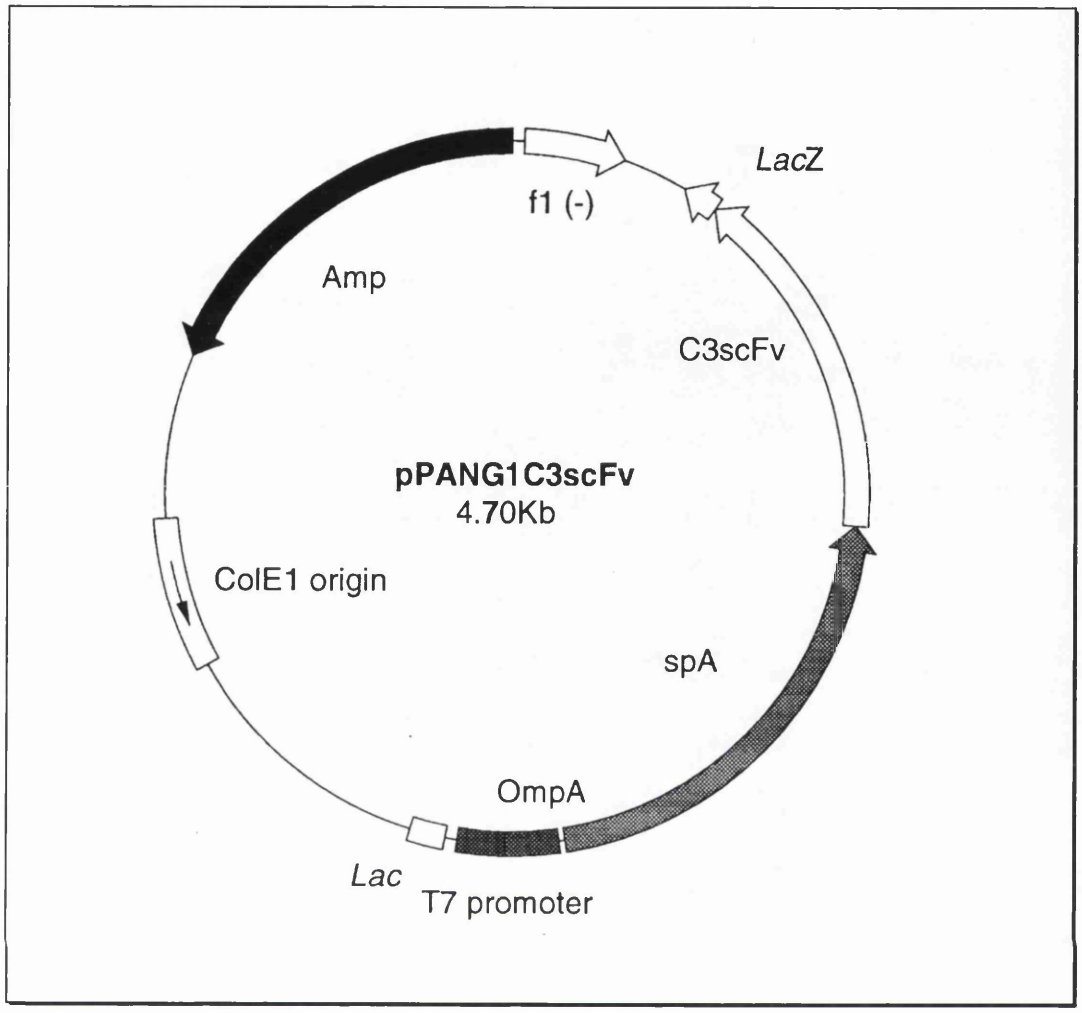

fig. 1.4.2 Expression plasmid pPANG1C3scFv

2 METHODS

2.1 TISSUE CULTURE

C3 hybridoma cells were cultured in RPMI 1640 medium (Gibco) supplemented with foetal calf serum, (FCS), as required, L-glutamine, 1% (v/v) of 200mM stock solutions stored at -20°C and 1% (v/v) of penicillin/streptomycin antibiotic mixture, 5000/5000μgml^{-1} stock solution stored at -20°C. RPMI 1640 medium was then stored at 4°C. Cells were cultured in either 25cm^3 or 75cm^3 tissue culture flasks (costar), presterilised by γ irradiation and incubated at 37°C. Cell cultures were handled under a sterile laminar flow hood, all surfaces and equipment were swabbed with 70% propan-2-ol. Contaminated cell cultures were sterilised by the addition of virkon, overnight, and the flasks were autoclaved before disposal.

2.1.1 Revival of C3 hybridoma cell line

Frozen cell suspension in an ampoule was thawed out, but the temperature of the cell suspension was not allowed to rise above 25°C. The cell suspension was washed with 20 ml of RPMI medium, centrifuged and the supernatant discarded. The cells were then resuspended in 5 ml of 20% FCS RPMI medium. The culture was fed with 20% FCS for one week then subsequently with 17% FCS RPMI medium, then 15% FCS RPMI medium for one week, then maintained with 10% FCS RPMI. Cell cultures were fed by centrifuging the cell cultures at 1000rpm for 5 minutes, removing the spent medium and resuspending the cell pellet in fresh medium.

2.1.2 Freezing of C3 hybridoma cell line

C3 culture was grown to late log phase and centrifuged. C3 cells were then resuspended in freezing medium (10%DMSO, 50%FCS, 1% L-glutamine 200mM stock solution ,1% penicillin/streptomycin 5000/5000μgml^{-1} stock solution) and transferred to labelled 2ml plastic freezing ampoules. The ampoules were sealed and placed in a polystyrene box, which was subsequently placed in a -70°C freezer overnight to slowly freeze the cells. The frozen cells were then stored in liquid N_2.

2.2 ELISA ASSAYS

The ELISA protocols all follow the same general methodology (fig. 2.2). Each protocol is a variation on this ELISA overview, and was a stage in the development of the final ELISA for the detection of IgG in C3 hybridoma culture supernatant.

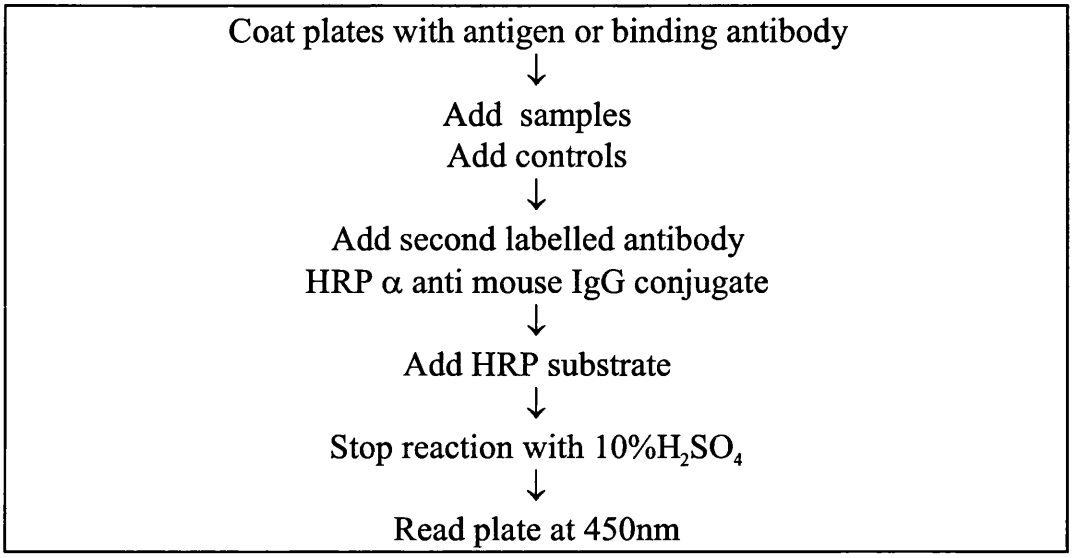

fig. 2.2 Overview of ELISA protocols for detection of C3 IgG

2.2.1 ELISA assays for detection of C3 IgG in C3 tissue culture

2.2.1.1 ELISA protocol 1, detection of C3 IgG by binding to immobilised hapten conjugate

- ELISA plates, 96 well Dynatech Immulon plates, were each coated with hapten, 4 - (2,3,4,5-tetrahydro-1-methyl-benzazepin-7-lyoxy) butanoic acid - transferrin conjugate, in coating buffer, 100µl per well. Two hapten transferrin conjugates were used as coating antigens in ELISA assay, KI/ipa/1, prepared 10/90 and used in the ELISA assay of previous C3 hybridoma cultures for IgG production, and SF/Htf/1 prepared as described.
- Plate wrapped in clingfilm and incubated over night at room temperature.
- Plate washed 4 times in wash buffer.
- Plate blocked with 10% NBCS blocking buffer, 250µl per well. Plate wrapped in clingfilm and incubated at 37°C for 1 hour.
- Plate washed 4 times in wash buffer.

- Spent medium from C3 RPMI 1640 + 2½%FCS tissue cultures loaded, in a predetermined order, onto plate and PBS as -ve control, 100µl per well. Plate wrapped in clingfilm and incubated at 37°C for 1 hour.
- Plate washed 4 times in wash buffer.
- Horse radish peroxidase α goat anti mouse IgG conjugate, 1:3000 in ¾PBS, ¼ sheep serum, loaded onto plate, 100µl per well. Plate wrapped in clingfilm and incubated at 37°C for 1 hour.
- Plate washed 4 times in wash buffer.
- TMB loaded onto plate, 100µl per well. Plate wrapped in clingfilm and incubated at room temperature for 50 minutes in a dark room, to develop.
- Reaction stopped by addition of 10% H_2SO_4, 50µl per well. Plate read at 450nm using Multiskan MCC/340 p version 2.2.

2.2.1.2 ELISA protocol 2, detection of Ig by direct binding to ELISA plate

- ELISA plates, 96 well Dynatech Immulon plates, were each coated with a purified antibody 3D11, in coating buffer, 100µl per well.
- Plate incubated at 37°C for 30 mins.
- Plate washed 4 times in wash buffer.
- Plate blocked with 1% casein blocking buffer, 250µl per well. Plate wrapped in clingfilm and incubated at 37°C for 5 mins.
- Plate washed 4 times in wash buffer.
- Horse radish peroxidase α goat anti mouse IgG conjugate, 1:3000 in ¾PBS, ¼ sheep serum, loaded onto plate, 100µl per well. Plate wrapped in clingfilm and incubated at 37°C for 30 mins.
- Plate washed 4 times in wash buffer.
- TMB loaded onto plate, 100µl per well. Plate wrapped in clingfilm and incubated at room temperature for 30 minutes in a dark room, to develop.
- Reaction stopped by addition of 10% H_2SO_4, 50µl per well. Plate read at 450nm using Multiskan MCC/340 p version 2.2.

2.2.1.3 ELISA protocol 3, detection of IgG by binding to immobilised sheep antimouse IgG

- ELISA plates, 96 well Dynatech Immulon plates, were each coated with $(NH_4)_2SO_4$ ppt sheep anti mouse IgG, 100µl per well. Plates wrapped in clingfilm and incubated overnight at room temperature.
- Plate washed 4 times in wash buffer.
- Plate blocked with 1% casein blocking buffer, 250µl per well. Plate wrapped in clingfilm and incubated at 37°C for 5 mins.
- Plate washed 4 times in wash buffer.
- Spent medium from C3 RPMI 1640 + 2½%FCS tissue cultures loaded, in a predetermined order, onto plate and PBS as -ve control, 100µl per well. Plate wrapped in clingfilm and incubated at 37°C for 30 mins.
- Plate washed 4 times in wash buffer.
- Horse radish peroxidase α goat anti mouse IgG conjugate, 1:250 in ¾PBS, ¼ sheep serum, loaded onto plate, 100µl per well. Plate wrapped in clingfilm and incubated at 37°C for 30 mins.
- Plate washed 4 times in wash buffer.
- TMB loaded onto plate, 100µl per well. Plate wrapped in clingfilm and incubated at room temperature for 30 minutes in a dark room, to develop.
- Reaction stopped by addition of 10% H_2SO_4, 50µl per well. Plate read at 450nm using Multiskan MCC/340 p version 2.2.

2.2.1.4 ELISA buffers for C3 ELISA

	wash buffer	NBCS blocking buffer	casein blocking buffer
Tris-HCl	0.02M	0.02M	0.02M
Tween 20	0.05%(v/v)	0.05%(v/v)	
new born calf serum		10%(v/v)	
casein			1%(w/v)
pH	7.4	7.4	9.0

	substrate buffer
tetramethylbenzidine (6μgml^{-1} in DMSO)	250μl
acetate-citrate buffer (0.1M, pH 5.5)	25ml
30% H_2O_2	4μl

	HRP anti mouse IgG conjugate buffers (HRP)
PBS	9ml
sheep serum	3ml
horse radish peroxidase α goat anti mouse IgG conjugate, (sapu)	dilutions from 1:3000 to 1:250

2.2.2 ELISA for the detection of SpA in *E.coli* cultures

. ELISA plates, 96 well , were coated with 100μl of goat IgG(Sigma) at 100μgml^{-1} in 0.02M Tris pH9.0 buffer, plates were wrapped in cling film and incubated overnight at 4°C

. Plates washed 4 times in wash buffer

. Samples loaded at 100μl per well including +ve controls purified spA(Sigma), plate wrapped in clingfilm and incubated 1 hour at 37°C.

. Plates washed 4 times in wash buffer

. Mouse anti-SpA IgG added at 1:1500 dilution in PBS, 100μl per well, plate wrapped in clingfilm and incubated 1 hour at 37°C

. plate washed 4 times in wash buffer

. Goat antimouse IgG peroxidase conjugate added 1:1000 dilution in PBS, 100µl per well. plate wrapped in clingfilm and incubated 1 hour at 37°C.

. plate washed 4 times in wash buffer

. substrate OPD (Sigma), added 100µl per well plate incubated 30 mins at room temperature

. plate read at 450nm in MCC/340 Multiskan.

2.3.1 Chemical synthesis of C3 substrate, 4-nitrophenyl 5-(3-methoxyphenol)-pentanoate

In order to provide substrate for the C3 catalysis assays fresh , 4-nitrophenyl 5-(3-methoxyphenol)-pentanoate was synthesised using the same methodolgy as used by Khalaf et al (1992), (see section 1.2.3).

Redistilled(m-methoxyphenoyl)valeric acid (5.29g) was dissolved in thionyl chloride (10 ml). The reaction mixture was refluxed for 3 hours. The solvent, thionyl chloride was removed in vaccuo to give a yellow oil, (m-methoxyphenoyl)valeric acid chloride.

(m-methoxyphenoyl)valeric acid chloride (5g, 0.022 moles) was dissolved in dry dichloromethane (10ml) and added to a mixture of 4-nitrophenol (3.339g, 0.024 moles), 19ml and dry pyridine (1.898g,0.024 moles) in dry dichloromethane (10ml) at room temperature with stirring. The product was purified by column chromatography with silica gel and 1/5 ethyl acetate/ pet. ether. The purified mixture was recrystalized from ethyl acetate and analysed by NMR.

2.3.2 Conjugation of C3 hapten, 4-(2,3,4,5-tetrahydro-1-methyl-1 -benzazepin-7-yloxy)butanoic acid methiodate, to transferrin

The C3 hapten, 4-(2,3,4,5-tetrahydro-1-methyl-1 -benzazepin-7-yloxy)butanoic acid, transferrin conjugate was synthesised to provide a reagent for use in the C3 specific ELISA assays, (see section 3.1.1). The hapten conjugate was synthesised using the same methodolgy as used by Khalaf et al (1992), (see section 1.2.3).

4-(2,3,4,5-tetrahydro-1-methyl-1 -benzazepin-7-yloxy)butanoic acid methiodate (20mg in 200µl DMF), N-hydroxy succinimide, (10mg in 200µl DMF) and N (3-dimethylaminopropyl)-N-ethyl- carbodimide methodid 1- (3-dimethyl amino propyl)-3-ethyl-carbodimide methiodide, EDC, (12mg in 200µl DMF) were added together in an aluminium covered flask. The reaction mixture was left stirring overnight for 18 hours at room temperature.

The reaction mixture was then split into two 300µl aliquots. To one 300µl aliquot transferrin, (10mg in 2ml 0.9% NaCl(pH 6.5)) was added and the mixture stirred at 4°C for 4 hours.

The hapten-transferrin conjugate was then purified using a PD10 gel filtration column (Pharmacia Biotech), pre-equilibrated with PBS pH7.5 the hapten-transferrin conjugate reaction mixture (2.3ml)was loaded onto the column and the product was eluted with PBS, pH7.5. The absorbance of the eluted fractions at 280nm was measured. All fraction with an A_{280} greater than 1 were pooled. The concentration of the hapten-transferrin conjugate (3.3 $mgml^{-1}$) was determined by a Lowry based method, Pierce micro BCA protein assay kit, using BSA as a standard and measuring UV absorbance at 562nm. Hapten-transferrin conjugate, SF/Htf/1, (see section 3.1.1) was stored at –20°C.

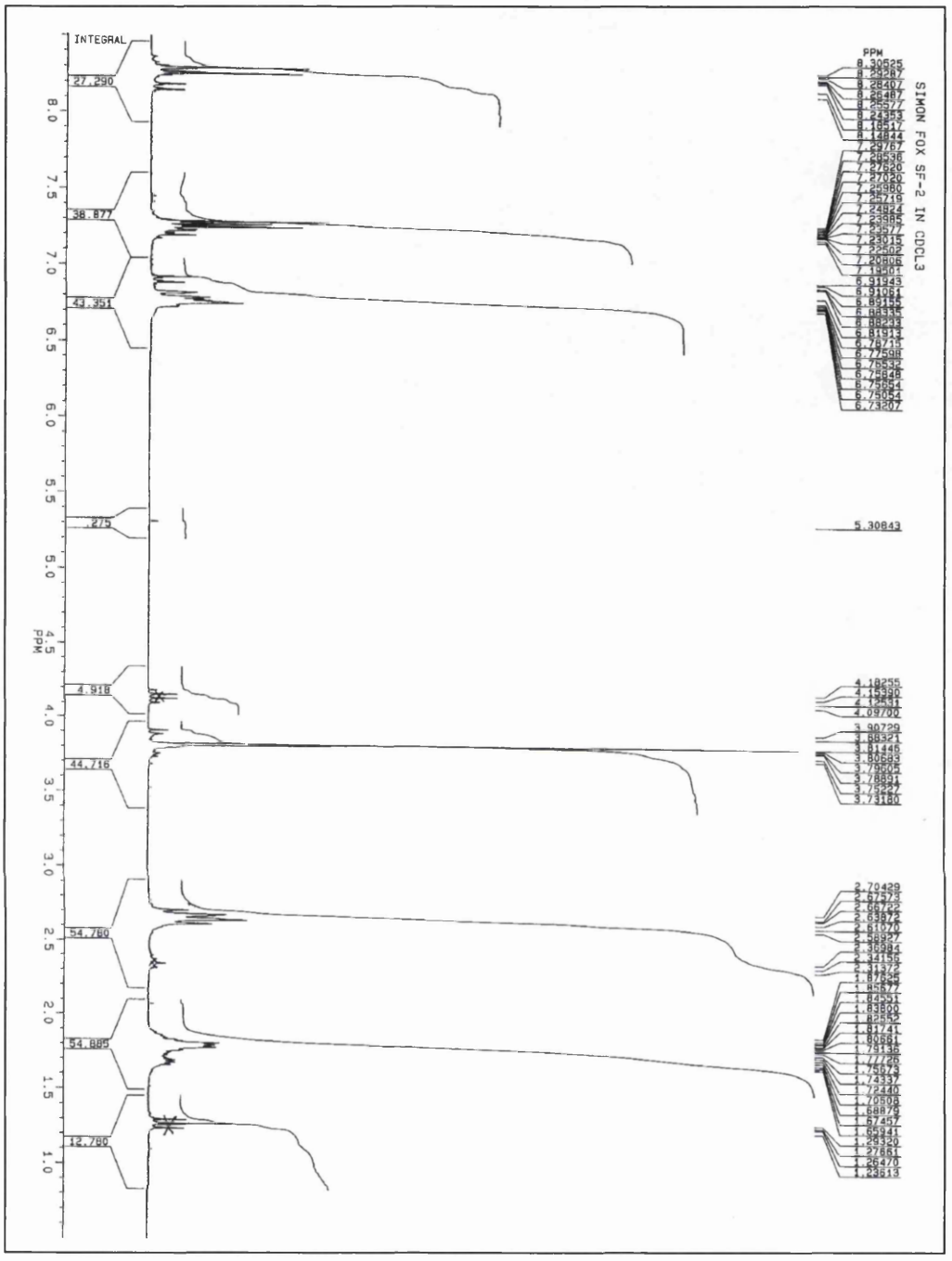

fig. 2.3.1 NMR spectra of C3 substrate 4-nitrophenyl 5-(3-methoxyphenol)-pentanoate

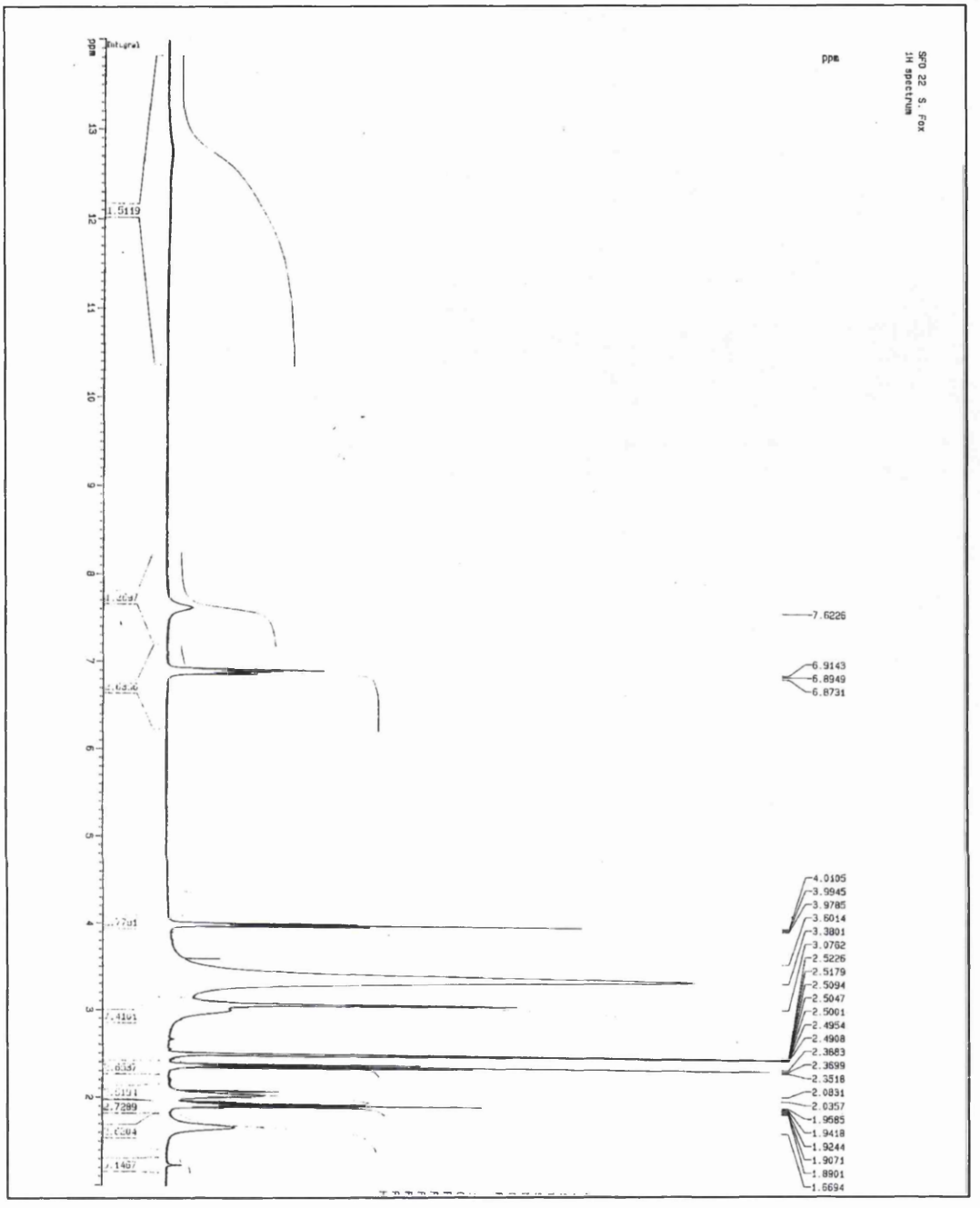

fig. 2.3.2.1 NMR spectra of C3 hapten 4-(2,3,4,5-tetrahydro-1-methy-1 - benzazepin-7-yloxy)butanoic acid methiodate

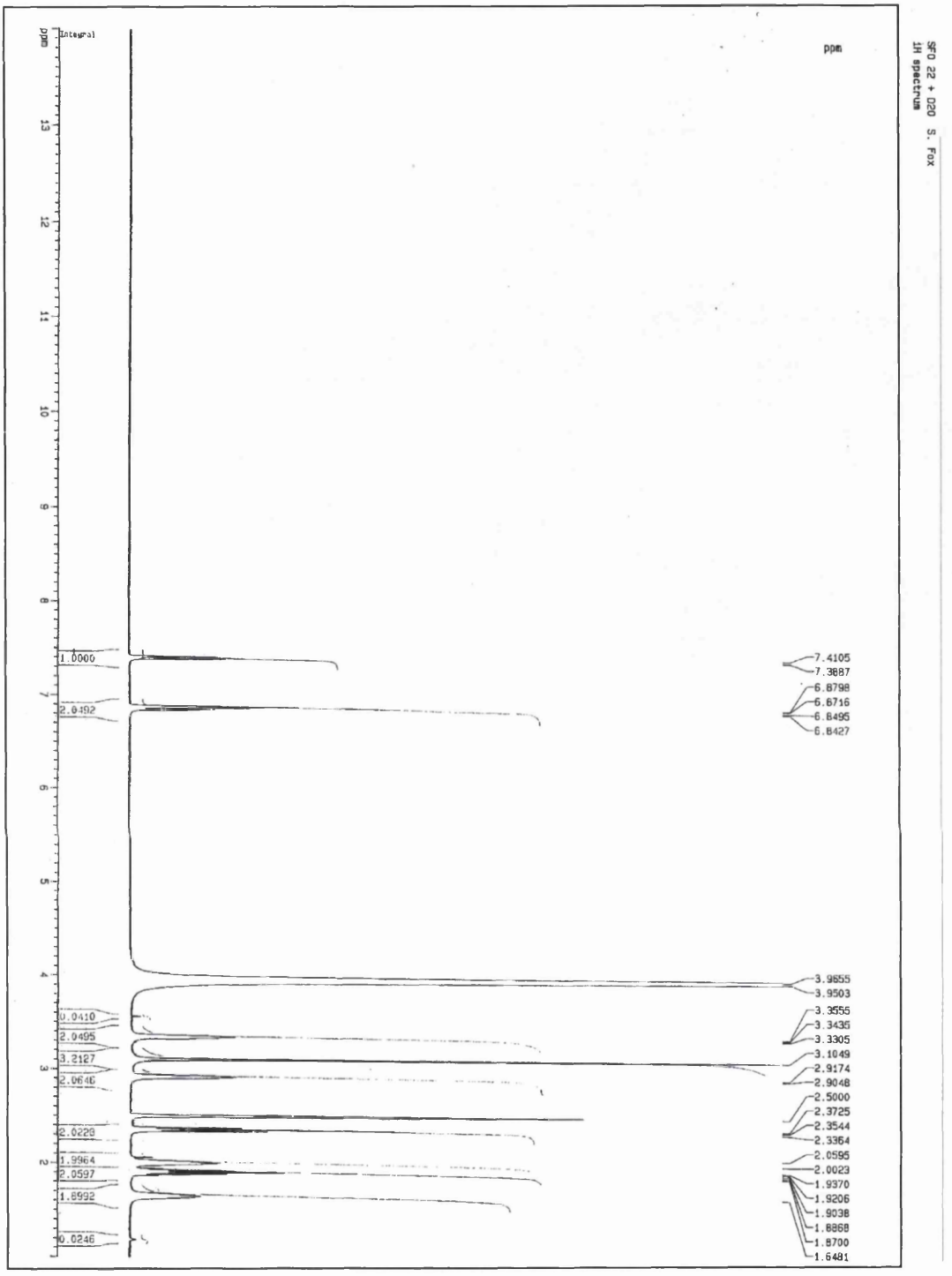

fig. 2.3.2.2 NMR spectra of C3 hapten 4-(2,3,4,5-tetrahydro-1-methy-1 -benzazepin-7-yloxy)butanoic acid methiodate + D_2O, deuterium exchange

2.4 BACTERIAL CULTURE METHODS

2.4.1 Revival and growth of *E.coli* strains

Culture of the various *E.coli* strains was performed using standard techniques as described in Sambook *et al* (Sambrook, Fritsch and Maniatis 1989)

Antibiotics were added as necessary, ampicillin used at a final concentration of $100\mu gml^{-1}$, and Chloramphenicol at a final concentration of $25\mu gml^{-1}$.

For small cultures used in plasmid preps, single colonies from freshly streaked selective plates, were picked and used to inoculate 10ml of broth containing appropriate antibiotics. Culture were grown overnight at 37°C in an incubator-shaker. Cells were harvested by centrifugation at 6000*g*.

For shake flask culture broth was made up and autoclaved in baffled shake flasks. When cooled selective antibiotics were added and the broth inoculated with a 1% inoculum from an overnight culture of the strain. Shake flasks were then incubated at selected temperature in an incubator-shaker. With inducible plasmids, pPANG1C3scFv and pQR627 expression was induced when the A_{600} of the culture reached 1 by addition of IPTG to a final concentration of 1mM.

2.4.2 Preparation of glycerol stocks of *E.coli* strains

From overnight 10ml cultures 1 ml of culture was transferred to a freezing capsule and 1ml of LB 60%/glycerol 40% mixture was added. The mixture was vortexed and the capsule frozen in liquid N_2, labelled with the *E.coli* strain and plasmid and subsequently stored in a -70°C freezer.

2.4.3 Preparation and transformation of competent *E.coli* cells and blue/white colour selection

E.coli strains were streaked onto fresh plates and incubated overnight at 37°C. Single colonies were picked from the plates and grown in NB for 2-3 hours at 37° C in an incubator-shaker . The cells were centrifuged (Beckman JA17 3500rpm for 10mins at 4°C, 4000*g*), the cell pellet was resuspended in 1ml of ice cold 0.1M$CaCl_2$. The cells were recentrifuged (Beckman JA17 3500rpm for 10mins

at 4°C, 4000*g*), and resuspended in 400µl of ice cold $CaCl_2$. The competent cells were either transformed or stored at -70°C for transformation at a latter date.

Competent *E.coli* cells were thawed on ice and the plasmid added to the cells, and incubated on ice for 45 mins. The *E.coli* cells were then incubated in a 37°C water bath for 10 mins . The transformed cells were transferred to 5ml of NB and incubated at 37°C for 1 hour. The transformed cells were then spread on agar plates, containing selective antibiotics as required, and incubated overnight at 37° C.

Plasmid	***E.coli* Host**	**Description**
pGEM-tC3scFv	XL-1 Blue	C3scFv cloning intermediate
pPANG1C3scFv	BL21 (DE3)	*ompA-spA*-C3scFv fusion expression vector and expression host
pQR627	JM 107	*ompA-spA*-$C3V_I$ expression vector created from pBluescript and PCR cloning from pPANG1C3scFv and host for blue/white colour selection of gene inserted pBluescript constructs
pQR627	BL21 (DE3)	*ompA-spA*-$C3V_I$ expression vector and expression strain
pEZZ18	BL21 (DE3)	SpA expressing vector (Pharmacia) and expression strain, for use as a +ve control in pQR627 expression experiments

Table 2.4.3 E.coli host/vector strains

For the selection of pBluescript constructs containing the pPANG1C3scFv expression cassette the ligation reaction mixture was transformed into *E.coli* strain JM107 and cells streaked onto X-gal plates (LB agar with 40µgml^{-1} X-gal, 20µgml^{-1} IPTG and 100µgml^{-1} Amp.) and incubated overnight at 37°C.

White colonies, containing pBluescript constructs with PCR fragment inserts, were picked from the X-gal plates and restreaked onto X-gal plates and incubated overnight at 37°C to isolate individual colonies for pBluescript constructs with PCR fragment inserts.

2.4.4 Fractionation by osmotic shock of *E.coli* BL21 (DE3)

2.4.4.1 protocol 1

E.coli cultures from the small scale expression experiments were harvested by centrifugation. Each cell pellet was then resuspended in 500 µl of 30mM Tris (pH8.0) 20% sucrose buffer solution. To 450 µl of the cell suspensions 2µl of 0.25M EDTA was added and incubated at 30°C for 15 mins. The cell suspension was then transferred to a cold eppendorf and centrifuged at top speed in a microcentrifuge (13000*g*) for 5 mins. The supernatant was removed and the cell pellet resuspended in 500µl ice cold deionised H_2O and whirlymixed for 30 seconds, then incubated on ice for 10 mins. The cell suspension was then centrifuged at top speed in a microcentrifuge (13000*g*) for 5 mins. The supernatant, the periplasmic fraction, was removed and stored at -20°C. The cell pellet was then resuspended in 500µl of ice cold deionised H_2O and centrifuged at top speed in a microcentrifuge (13000*g*) for 5 mins. The cell pellet was resuspended in 500µl of ice cold deionised H_2O and centrifuged at top speed in a microcentrifuge for 5 mins. The cell pellet, washed cell fraction, was then resuspended in 500µl of ice cold deionised H_2O and stored at -20°C

2.4.5.1 protocol 2 (French *et a*l 1996)

1ml of culture was removed and centrifuged at full speed in a microfuge for 5 mins. The supernatant was removed and stored. Cells were resuspended in 200µl of extraction buffer(20% sucrose, 1mM Na_2EDTA, 200mM TrisHCl, 500µgml^{-1}) and vortexed, then incubated at room temperature of 15 mins. Then 200µl of distilled H_2O added and mixed then incubated at room temperature for 15 mins. Samples then microfuged at top speed (13000*g*) for 10 mins. Supernatant, periplasmic fraction removed and stored, cell pellet resuspended in 500µl of 50mMTrisHCl and vortexed. Resuspended cells were then sonicated and microfuged at full speed (13000*g*) for 10 mins. Supernatant removed and stored as cell associated fraction.

2.4.5 Bacterial culture media

	LB per litre	LB agar per litre	TB per litre
tryptone	10g	10g	12g
yeast extract	5g	5g	24g
NaCl	10g	10g	
Agar		15g	
glycerol			4ml
TB salts, (0.17M KH_2PO_4, 0.72M K_2HPO_4)			100ml

2.5 MOLECULAR BIOLOGY METHODS

Standard molecular biology procedures were performed as described in Maniatis *et al.* Molecular biology kits were used in accordance with manufactures instructions.

2.5.1 Preparation of plasmid DNA

Cultures of *E.coli* strains containing plasmid DNA where grown overnight in appropriate medium, in an incubator-shaker. The cells were harvested by centrifugation and plasmid DNA was prepared by Promega Wizard or QIAgene plasmid prep kits.

The DNA samples from either miniprep system were concentrated, if necessary, by ethanol precipitation. Three volumes of ice cold ethanol was added to the DNA solution, which was stored on ice for 30 minutes. The DNA was recovered by centrifuging at top speed in a microcentrifuge (13000*g*) for 10 minutes and the supernatant removed. The microfuge tube was then half filled with 70% ethanol and recentrifuged at top speed in a microcentrifuge (13000*g*) for 2 minutes and the supernatant removed, twice.

The microfuge tube was then left open on a bench top at room temperature until the last traces of fluid have evaporated the DNA pellet was then resuspended in 20μl of water. The DNA samples were then stored at -20°C.

2.5.2 Restriction enzyme digestion of DNA

DNA samples were digested using the appropriate dilution of 10× restriction buffer and selected restriction enzymes, the restriction mixture being incubated in a 37°C water bath for at least 1 hour. The digestions were stopped by the addition of stop mix, and analysed by agarose gel electrophoresis on 1%, and 1.4% agarose TBE gels.

2.5.2.1 Restriction enzyme buffers

	10× restriction buffer	stop mix
Tris	500mM, pH 7.5	
$MgCl_2$	50mM	
Na_2EDTA		0.1 M
sucrose		40%(w/v)
bromophenol blue		$0.15mgml^{-1}$

2.5.3 PCR cloning of pPANG1C3scFv expression cassette protocols

PCR was performed on samples of pPANG1C3scFv plasmid DNA, prepared by QIAgen Maxiprep, using custom designed primers, *pfu* polymerase and a Hybaid omnigene thermal cycler.

The PCR was optimised to give the following PCR protocol. To a final reaction volume the following was added, 0.5µg template DNA, 20pM each of primers SFPCR1 and SFPCR2, (**5.2.1**), 50µM each dNTP, 5µl 10× *pfu* buffer and 1µl of *pfu* polymerase, made up to final volume, 50µl with TE buffer.

The reaction mixture was then placed in a preheated, 65°C, heating block connected to the Hybaid omnigene thermal cycler. Then 20 cycles of PCR was performed using the following temperature profile:

95°C for 1 minute
60°C for 1.5 minutes
75°C for 1.5 minutes

The presence of PCR product being determined by the analysis of a sample of the reaction mixture by agarose gel electrophoresis.

2.5.4 Sequencing of plasmid DNA

Sequencing of plasmid DNA was performed using a Pharmacia T7 sequencing kit, using a protocol designed for the sequencing of a double stranded template.

The template plasmid DNA was diluted in distilled water to give a final concentration of 1.5-2μg in a volume of 32μl. The template was denatured by the addition of 8μl of 2M NaOH. The mixture was vortexed and briefly centrifuged, then incubated at room temperature for 10 mins. After this incubation 7μl of 3M sodium acetate and 4μl of distilled water were added to the mixture. To precipitate the template 120μl of 100% ethanol was added and the mixture incubated on dry ice for 15 mins. The precipitated DNA was collected by centrifugation at top speed in a microcentrifuge for 15 mins, and the supernatant removed. The DNA pellet was then washed with 70% ethanol and recentrifuged for 10 mins, the supernatant was removed and the pellet dried in a 50°C oven for approximately 10 mins. The dried DNA pellet was then resuspended in 10μl of distilled water.

To the 10μl of template 2μl of annealing buffer and 2μl of primer were added and mixed by vortexing and centrifugation. The annealing mixture was then incubated at 65°C for 5 mins, then the annealing mixture was incubated at 37°C for 10 mins and then incubated at room temperature for 5 mins and briefly centrifuged.

Separate 2.5μl aliquots of 'A' mix short, 'C' mix short, 'G' mix short and 'T' mix short, were dispensed into labelled microfuge tubes. The stock T7 polymerase was diluted in enzyme dilution buffer, for two templates 1μl of T7 polymerase stock was diluted with 4μl of dilution buffer. To the annealed template-primer, 14μl, labelling mix A, 3μl, diluted T7 polymerase, 2μl, and labelled dNTP, [α^{-32}P]dATP, 1μl, was added to form the labelling reaction. The labelling reaction was then incubated at room temperature for 5 mins. The 'A'. 'C', 'G' and 'T' mix short aliquots were warmed at 37°C for at least 1 min.

The termination reaction was performed by the addition of 4.5μl of the labelling reaction to each of the pre-warmed mix short aliquots, and incubating at 37°C for 5 mins. Stop solution, 5μl, was added to each termination reaction which were then heated to 75-80°C, 3μl of the termination reaction were then loaded onto a sequencing gel and the remainder of the termination reaction stored at -20°C.

2.5.5 Purification of DNA from agarose electrophoresis gels

The PCR products were purified using a Bio 101 geneclean II kit. The total PCR reaction mixture was separated by agarose gel electrophoresis using 1% agarose

TBE gels and 1×TBE as running buffer. The required DNA bands were cut from the agarose gel using a scalpel, each gel band weighing approximately 0.3g, to the gels 1.35ml of NaI solution and 150 µl of TBE modifier were added and the mixture vortexed. The mixture was then incubated in a 55°C water bath until the agarose had dissolved completely, then 5µl of glassmilk was added and the mixtures vortexed and incubated on ice for 30 minutes, with occasional vortexing. The mixture were then centrifuged at top speed in a microfuge for 5 seconds and the supernatant removed, the pellet was recentrifuged at top speed in a microcentrifuge for 2 seconds and the supernatant removed. The pellet was then resuspended in 500µl of new wash, centrifuged at top speed in a microcentrifuge for 5 seconds and the supernatant removed. The wash stage was repeated three times. After the pellet was washed the pellet was centrifuged again and the supernatant removed, DNA was eluted from the pellet with TE. The cell pellet was resuspended in 5µl of TE buffer then centrifuged at top speed in a microcentrifuge for 30 seconds, the DNA containing supernatant was removed and the cell pellet resuspended in 5µl of TE and DNA eluted again by centrifugation. A sample of the eluent was then examined by agarose gel electrophoresis for the presence of DNA. Eluent containing DNA was then stored at -20°C.

2.5.6 Ligation of pPANG1C3scFv expression cassette into pBluescript.

The purified PCR product of the pPANG1C3scFv expression cassette and the plasmid vector pBluescript SK II were digested with the restriction enzyme *Sac* I in 10× restriction buffer and incubated in a 37°C water bath for 3 hours. A sample of the digested products from both the PCR fragment and pBluescript digestions was analysed by agarose gel electrophoresis to ensure that both had been restricted. A 1:10 dilution of the restricted pBluescript vector, in TE was made up and 2µl of this dilution added to 10µl of the restricted PCR fragment. To the ligation mixture 2µl 10× Ligation buffer and 4µl T4 DNA ligase were added. The ligation mixture was then incubated overnight at room temperature.

2.6 IgG AGAROSE PURIFICATION FOR SPA FUSIONS

E.coli cells from small scale expression experiments (4.1.2) were harvested by centrifugation and resuspended in 25× concentrate over broth sonication buffer. The *E.coli* cells were lysed by sonication and centrifuged to remove insoluble material, the supernatant was incubated with 0.75ml of human IgG agarose(Sigma A6284) which was pre-equilabrated with TBS, for 1.5 hours at room temperature. The slurry was transferred to a column (Pharmacia) and washed with TBS containing 0.1% (v/v) Tween 20. Bound protein was eluted off the column with 0.1M glycine-0.15M NaCl pH 2.4, (5 column volumes), into 0.25 volumes of 1M Tris pH 7.2.(Gandecha *et al* 1994)

2.6.1 IgG Agarose purification buffers

	Sonication buffer	**Ig column elution buffer**	**Ig column wash buffer**	**TBS**
Tris	50mM		50mM	50mM
NaCl		150mM	200mM	200mM
EDTA	1mM			
glycine		100mM		
Tween 20			0.1%	
glycerol	2%			
lysozyme	$10\mu gml^{-1}$			
pepstatin	$1\mu gml^{-1}$			
aprotinin	$2\mu gml^{-1}$			
leupeptin	$1\mu gml^{-1}$			
pH		2.4	7.4	7.4

2.7 GEL ELECTROPHORESIS

2.7.1 Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out on plasmid samples, obtained from Wizard and QIAgene minipreps, using 1% agarose(appligene)TAE + 0.5μgml^{-1} ethidium bromide, or 1% agarose TBE + 0.5μgml^{-1} ethidium bromide gels, with 1×TAE, diluted from 5×TAE stock solution as running buffer for TAE gels and 1 ×TBE diluted from 10×TBE as running buffer. Samples of 0.5-1μg of DNA sample, diluted with 5μl of water and 2μl of loading buffer, were used for gel electrophoresis. Powerpacks, plastic trays and electrophoresis tank from Pharmacia. Gels were removed from the electrophoresis apparatus and examined under UV light, then photographed, using a frame grabbing camera.

Agarose gel electrophoresis was carried out on the restriction digested samples of pPANG1C3scFv using 1.4% agarose TBE+ 0.5μgml^{-1} ethidium bromide gels, using 1×TBE, diluted from ×10 stock solution, as running buffer. Gels were removed from the electrophoresis apparatus and examined under U.V. light, then photographed, using a frame grabbing camera.

Agarose gel electrophoresis was carried out on the pPANG1C3scFv PCR products using 1.2% agarose TBE gels and using 1×TBE as running buffer. PCR reaction mixtures were diluted with 16μl of stop mix, and 20μl samples run on the gels.

2.7.1.1 Agarose electrophoresis gel buffers

	1×TAE	1×TBE
Tris-acetate	0.04M	
Tris base		0.09M
EDTA	0.001M	0.002M

	Gel loading buffer
Bromophenol blue	0.25%
xylene cyanol FF	0.25%
ficoll (type 400;Pharmacia)	15%

2.7.2 SDS-PAGE gel electrophoresis

SDS-PAGE gel electrophoresis was carried out on 12% acrylamide separating gels, with a 5% acrylamide stacking gel and using Tris/glycine running buffer.

SDS-PAGE apparatus assembled in accordance with individual kit instructions. Separating gel was first prepared and poured between the glass plates of the apparatus, then covered with water saturated butanol and left to set. The stacking gel was then poured on top of the separating gel and left to set.

1 volume of SDS-PAGE gel loading buffer was added to each sample. Samples were then immersed in boiling water for 3 mins, to denature, then loaded onto the gel(20-40µl per lane). The apparatus was assembled and running buffer tanks filled with 1× Tris glycine running buffer. A voltage of 80 volts was applied to the samples while in the stacking 5% gel and 120 volts in the separating 12%gel.

After electrophoresis gels were removed from the apparatus and placed in 5 volumes of gel staining buffer and placed on an orbital rotating platform for 2-3 hours. Gel staining buffer was then removed and gel destaining buffer added and the gel, returned to orbital rotating platform overnight. The gel destaining buffer was replaced and destaining carried out for a further 2-6 hours. The gel was then photographed, using a frame grabbing camera.

2.7.2.1 SDS-PAGE buffers

	Stacking gel 5% 10ml	**Separating gel 12% 50ml**
H_2O	6.8ml	16.5ml
30% acrylamide mix	1.7ml	20ml
1.5M Tris pH 8.8		12.5ml
1M Tris pH 6.8	1.25	
10%SDS	0.1ml	0.5ml
10% ammonium persulphate	0.1ml	0.5ml
TEMED	0.01ml	0.02ml

	SDS-gel loading buffer	Tris-glycine electrophoresis buffer
Tris	50mM	50mM
SDS	2%	0.1%
glycine		250mM
glycerol	10%	
bromophenol blue	0.1%	
dithiotheritol	100mM	

	Gel stain	Gel destain
methanol/H_2O (1:1)	90%	90%
acetic acid	10%	10%
Coomassie brilliant blue R250	0.25%(w/v)	

2.7.3 Western blotting

Samples from the periplasmic fraction of BL21 (DE3)pQR627 and pEZZ18 were run on a standard SDS-PAGE 12% gel. After electrophoresis was complete the gel was equilibrated in transfer buffer. Six sheets of whatman 3M filter paper, cut to the same dimensions as the gel, were preequillibrated in transfer buffer. Three of the filter paper sheets were stacked on top of each other, the gel was placed on the stack and then covered by a sheet of nitro-cellulose, cut to the dimension of the gel and preequillibrated in water. The remaining three filter sheets were then placed on the stack. The stack was then held in the transfer tank which was filled with transfer buffer. The proteins in the gel were transferred to the nitro-cellulose filter by the application of 30 mA current through the tank for 2 hours.

The nitro-cellulose filter was then blocked by incubating in PBS containing 0.5% milk powder overnight at room temperature. The nitro-cellulose filter was then washed three times in PBS containing 0.2% milk powder. The filter was then incubated in PBS containing 0.2% milk powder with 1:1000 mouse anti spA IgG for 2 hours at room temperature. The nitro-cellulose filter was then washed three times in PBS containing 0.2% milk powder. The filter was then incubated in PBS containing 0.2% milk powder with 1:1000 sheep anti mouse IgG peroxidase conjugate for 2 hours at room temperature. The nitro-cellulose filter was then washed three times in PBS containing 0.2% milk powder. The filter was

developed by incubating in OPD (Sigma) solution for 1 minute then transferring to distilled water. The filters were the photographed by frame grabbing camera.

2.7.3.1 Western blotting Buffers

	1×Transfer Buffer
Tris base	48mM
glycine	39mM
methanol	20%(v/v)
SDS	1.3mM

3 RESULTS

3.1 TISSUE CULTURE OF C3 HYBRIDOMAS.

C3 hybridomas were revived from liquid N_2 and cultured for the production of antibodies. For antibody production the FCS content of the medium was reduced stepwise to 2½% FCS. Spent medium was removed, labelled, and stored at -20° C. Spent medium from C3 tissue cultures was analysed by the ELISA protocol devised by Bence and Irvine (Khalaf *et al.* 1992) (2.2.1 ELISA protocol 1, detection of C3 IgG by binding to immobilised hapten conjugate) for detection of antibody production. This ELISA assay was based on the binding of C3 IgG3 to hapten, 4-(2,3,4,5-tetrahydro-1-methy-1-benzazepin-7-yloxy)butanoic acid, transferrin conjugate, KI/ipa 1, and failed to detect any antibody (plates 1-6 & 11).

Fresh hapten-transferrin conjugate was prepared, SF/Htf/1, ELISA assays using this hapten-transferrin conjugate and the same ELISA protocol (**2.2.1**) also failed to detect any antibody (plates 7-10). Crucially the positive controls used in these ELISA assays failed to work, so novel assay protocols were developed to detect expressed C3 IgG.

The first stage of assay development was to ascertain if the existing protocols could detect C3 IgG by allowing the antibody in the spent C3 hybridoma medium to bind directly to the ELISA plate and using a modified ELISA protocol 1 and ELISA protocol 2 (2.2.2). The ELISA assay (plate 12) failed to detect any Ig. The second stage of assay development was to ascertain if a known antibody, 3D11, bound to an ELISA plate would be detected using ELISA protocol 2 (plates 13 &14). The antibody 3D11 was a purified IgG produced by a research team at the University of Strathclyde, and bound to ELISA plates at known concentration. The ELISA protocol 2 (2.2.2) used in these plates was designed to assay for the total Ig bound to the plate. As a result of these experiments the concentration of the sheep anti mouse IgG-HRP conjugate was increased to 1:250 from 1:3000, to increase the sensitivity of the assay. This assay was able to detect immobilised 3D11 Ig.

The modified ELISA protocol 2 was then used to detect C3 IgG. This assay method attempted to bind C3 IgG in C3 supernatant directly to an ELISA plate. The ELISA assay was for the detection of total Ig bound to the plate and used the

3D11 antibody bound to the plate at a known concentration as a positive control. A second positive control was needed to determine if any Ig present in RPMI 1640 2½ FCS medium would bind directly to an ELISA plate. Supernatant from hybridoma cell line, 4E1, which produced aflatoxin binding IgG antibodies and was cultured under the same conditions as the C3 hybridomas was used to produce the necessary positive control. This ELISA assay, (plate 15) also failed to detect any Ig present in the spent C3 hybridoma medium. Neither C3 samples or Ig containing 4E1 samples gave a positive response in this assay system indicating that Ig in hybridoma supernatant will not bind to an ELISA plate, and that this assay could not detect antibody in spent hybridoma media.

To detect the production of C3 IgG, an ELISA assay method was devised that utilised the binding of sheep anti mouse IgG to mouse IgG (2.2.3 ELISA protocol 3, detection of IgG by binding to immobilised sheep anti mouse IgG). ELISA plates were coated with sheep anti mouse IgG that was used in an ELISA assay system to bind to any IgG in the C3 tissue supernatant, again 4E1 supernatant was used as a known Ig containing positive control for this assay system. The Ig containing 4E1 positive control was detected by this ELISA system (plate 16) validating the ELISA protocol 3 assay system for the detection of mouse IgG in RPMI 1640 2½ FCS hybridoma medium This sheep anti mouse IgG based ELISA also failed to detect any antibody production by the C3 hybridoma culture, (plates 17 & 18). The 4E1 positive control samples were detected in these assays proving that the ELISA assay was functioning properly. The C3 supernatant samples should have given a comparable result to the 4E1 controls if IgG was being produced by the C3 hybridoma culture. No antibody production was detected by this murine antibody assay, the C3 cell line was therefore not producing antibody.

To stimulate antibody production the concentration of FCS was increased to 10%, to stimulate growth then in subsequent feeds reduced to 5% then 2½% to induce antibody production in the hybridomas. Spent medium from the stimulated C3 hybridoma culture was then assayed using ELISA protocol 3, (plate 19), with 4E1 culture samples as positive controls. No antibody was detected by ELISA, the attempt to stimulate antibody production in the C3 hybridoma cultures was unsuccessful. No C3 IgG was produced by the hybridoma culture so no comparison of the catalytic activity of whole C3 IgG to C3scFv produced by *E.coli* was now possible.

3.1.1 ELISA assays for detection of C3 IgG by binding to immobilised hapten conjugate

Four ELISA plates were each coated with, KI/ipa 1 hapten transferrin conjugate, at 10 μgml^{-1} in 0.02M Tris, pH 7.0 (100µl per well). The spent C3 tissue culture medium was assayed using ELISA protocol 1(2.2.1.1)

An ELISA plate, plate 5, was coated with KI/ipa/1 at 30μgml^{-1}, in 0.02M Tris(pH 7.0). The spent C3 tissue culture medium was assayed using ELISA protocol 1. Rows A and B were loaded with C3 medium, same medium as loaded onto plate 4 and rows C-H with PBS.

	absorbance at 450nm				
	plate 1	plate 2	plate 3	plate 4	plate 5
C3	0.089±0.018	0.082±0.066	0.126±0.018	0.196±0.034	0.126±0.024
PBS	0.058±0.004	0.103±0.130	0.096±0.013	0.129±0.017	0.056±0.003

Table 3.1.1.1 Results for ELISA assays 1-5

The ELISA assays had failed to give a clear positive signal for the C3 media and so the assays were repeated with a positive control to determine if the assays were functioning correctly

One ELISA plate, plate 6, was coated with KI/ipa 1 at 10 μgml^{-1} in 0.02M Tris, pH 7.0. The spent C3 tissue culture medium was assayed using ELISA protocol 1. Rows A+B were loaded with spent medium from a previous C3 hybridoma culture, carried out by June Irvine, as a +ve control on the ELISA assay. This JI C3 sample had been previously assayed using the same ELISA protocol described here. The JI C3 sample had given a mean A_{450} of 0.344. Rows C+D were loaded with spent medium from current hybridoma culture, and rows E-H with PBS (table 3.1.1.1).

It was concluded that as the assay results of JI C3 did not agree with the previous recorded assay results, that the hapten conjugate had possibly decayed, (KI/ipa/1 hapten transferrin conjugate prepared Oct. 1990). New 4-(2,3,4,5-tetrahydro-1-methy-1-benzazepin-7-yloxy)butanoic acid transferrin conjugate was prepared as a coating antigen for ELISA assays, SF/Htf/1 (see section 2.3.2). Assays were then performed using this new hapten conjugate to detect C3 IgG.

An ELISA plate, plate 7, was coated with 30μgml^{-1} SF/Htf/1 in 0.02M Tris pH7.0. The spent C3 tissue culture medium was assayed using ELISA protocol 1. Row A was loaded with JI C3 as a +ve control for the assay, rows B-E were loaded with spent medium from current hybridoma culture and rows F-H were loaded with PBS. The assay was repeated with plate 8, 30μgml-1 SF/Htf/1, and plate 9, 120μgml-1 SF/Htf/1, using protocol 1. The increase in immobilised hapten conjugate concentration was an attempt to optimise the assay by increasing the signal produced by any bound C3 IgG.

	absorbance at 450nm			
	plate 6	plate 7	plate 8	plate 9
JI C3	0.075±0.008	0.057±0.004	0.105±0.029	0.064±0.005
C3	0.092±0.008	0.068±0.006	0.137±0.030	0.065±0.011
PBS	0.075±0.009	0.059±0.004	0.087±0.014	0.059±0.008

Table 3.1.1.2 Results from ELISA assays 6-9

The assays performed with the new hapten conjugate failed to detect any C3 IgG, and as with the assays using the old hapten conjugate the positive control, previously assayed hybridoma supernatant JI C3, also failed to produce a response.

An ELISA plate, plate 10, was coated with 30μgml^{-1} SF/Htf/1 **(2.3.2)** in 0.02M Tris pH9.0. The pH change in the coating buffer was designed to increase the amount of hapten binding to the plate and so optimize the assay for C3 IgG. Spent C3 tissue culture medium was assayed using ELISA protocol 1. Row A was loaded with C3 serum from the original immunised mice as a +ve control on the assay. The serum was loaded in doubling dilutions across the plate in PBS starting at 1:50. Rows C-E were loaded with spent medium from current C3 hybridoma culture and rows F-H were loaded with PBS.

An ELISA plate, plate 11, was coated with 30μgml^{-1} KI/ipa/1 in 0.02M Tris pH 9.0, Spent C3 tissue culture medium was assayed using ELISA protocol 1. Row A was loaded with C3 serum from the original immunised mice as a +ve control on the assay. The serum was loaded in doubling dilutions across the plate in PBS starting at 1:50. Rows B-E were loaded with spent medium from current hybridoma culture and rows F-H were loaded with PBS. In both ELISA plates 10 & 11 the original immunised mouse serum failed to give a positive result, and no C3 IgG was detected.

Neither assay system using KI/ipa/1 or SF/HTf/1 haptens in either pH7.0 or pH9.0 coating buffer was able to detect either positive controls, JI C3 or C3 mouse serum, or any C3 IgG in the spent hybridoma medium.

	absorbance at 450nm	
	plate 10	plate 11
C3	0.075±0.004	0.244±0.022
PBS	0.062±0.004	0.237±0.011

Table 3.1.1.3 Results for ELISA plates 10 & 11

3.1.2 ELISA assays for detection of C3 IgG by direct binding to ELISA plate

As no assay system using C3 hapten was demonstrated to function correctly, it was necessary to develop a new assay that could detect IgG produced in hybridoma culture. The first attempt at creating a new assay was to establish if antibody in spent media could be immobilised on an ELISA plate. An ELISA plate, plate 12, was not coated with antigen, this plate was loaded with spent C3scFv 2½% FCS medium so that antibody present in the medium would bind direct to the plate. Samples were loaded in the same arrangement as plate 11. Row A was loaded with C3 serum from the original immunised mice as a +ve control on the assay. The serum was loaded in doubling dilutions across the plate in PBS starting at 1:50. Rows B-E were loaded with spent medium from current hybridoma culture and rows F-H were loaded with PBS. The spent C3 tissue culture medium was assayed using ELISA protocol 1, stages 6-11.

In order to ascertain if this assay system would work, a purified antibody 3D11 that was unrelated to C3 was used as a positive control. An ELISA plate, plate 13, was coated with 10μgml^{-1} 3D11, a purified mouse antibody, in 0.01M Tris pH 9.0. The plate was coated with 3D11 in Tris buffer, in rows A-D and PBS in rows E-H. The 3D11 samples were then assayed using ELISA protocol 2.(**2.2.1.2**)

	absorbance at 450nm	
	plate 12	plate 13
C3	0.130±0.068	
3D11(purified IgG)		0.297±0.032
PBS	0.140±0.070	0.166±0.029

Table 3.1.2.1 Results for ELISA plates 12 & 13

An ELISA plate, plate 14, was coated with 10μgml^{-1} of 3D11. Rows B-D were coated with 3D11 in 0.02M Tris pH 9.0 and rows E-G with PBS, 100μl per well. The 3D11 samples were then assayed using ELISA protocol 2, stages 1-5. HRP anti mouse conjugate was then added (100μl per well), in increasing concentration across the plate; 1:3000 in column 2, 1:2500 in column 3, 1:2000 in column 4, 1:1500 in column 5, 1:1000 in column 6, 1:500 in column 7, the plate was covered with cling film and incubated for 30 mins at 37°C. ELISA protocol 2, stages 7-9 was then followed.

IgG-P	absorbance at 450nm					
conc	**1:3000**	**1:2500**	**1:2000**	**1:1500**	**1:1000**	**1:500**
3D11	0.380 ± 0.010	0.384 ± 0.008	0.483 ± 0.016	0.600 ± 0.016	0.297 ± 0.008	0.991 ± 0.016
PBS	0.055 ± 0.002	0.053 ± 0.0008	0.055 ± 0.006	0.064 ± 0.003	0.059 ± 0.005	0.067 ± 0.002

Table 3.1.2.2 Results for ELISA plate 14

This ELISA result showed that it was possible to directly bind an antibody to an ELISA plate and detect it using this assay system. The next stage in assay development would be to determine if the assay could detect C3 antibody in the culture medium.

An ELISA plate, plate 15, was not coated with antigen, this plate was loaded with spent C3scFv 2½% FCS medium so that antibody present in the medium would bind directly to the plate. The antibody 3D11 in 0.02M Tris pH 9.0 buffer was used as a +ve control for the assay. A second +ve control, spent 2½% FCS medium from the hybridoma culture producing the monoclonal antibody 4E1, was also used to ascertain whether IgG in 2½% FCS medium would bind directly to the ELISA plate. Row B was coated with 3D11 at 10μgml^{-1}, row C was loaded with 4E1 2½% FCS medium, rows D + E were coated with C3 2½% FCS

medium and rows F + G with PBS , 100µl per well. The plate was then wrapped in clingfilm and incubated at 37°C for 30 mins then washed four times in wash buffer. HRP antimouse IgG conjugate was then added, 100µl per well, in two concentrations, 1:250 in columns 2-6 and 1:500 in columns 7-11 The samples were then assayed using ELISA protocol 2, stages 7-9.

	absorbance at 450nm	
HRP	1:250	1:500
3D11	1.625 ±0.015	1.149 ±0.079
4E1	0.076 ±0.005	0.074 ± 0.010
C3	0.081 ± 0.005	0.074 ± 0.006
PBS	0.072 ± 0.008	0.058 ± 0.003

Table 3.1.2.3 Results for ELISA plate 15

These assay results proved that it was possible to directly immobilise antibody onto an ELISA plate and detect it with the optimised assay. This assay could not function as an assay for antibody produced in hybridoma culture as any antibody present will not bind directly to the plate, as shown by the lack of response of the 4E11 controls, which were known to contain antibody by a 4E11 specific ELISA. If it was possible for antibody in the culture supernatant to bind directly to anELISA plate then the 4E11 culture would have produced a positive signal in this assay.

3.1.3 ELISA assays for detection of C3 IgG by binding to immobilised sheep anti mouse IgG

Sheep anti mouse IgG, was used, to coat ELISA plates with, as a method of binding mouse antibody, produced by hybridomas, to an ELISA plate for detection. 5ml of saturated $(NH_4)_2SO_4$ was added dropwise to 5mls of sheep anti mouse IgG,(sapu), both stored on ice, over 30 mins. The precipitate was then recovered by centrifugation and the pellet resuspended in 5ml of PBS. This $(NH_4)_2SO_4$ precipitated sheep anti mouse IgG was then used as a stock solution, stored at -20°C, for the coating of ELISA plates.

An ELISA plate, plate 16, was coated with 100µl per well of three concentrations of $(NH_4)_2SO_4$ precipitated sheep anti mouse IgG. Columns 1-4 were coated with 1:500, columns 5-8 were coated at 1:1000, and columns 9-12 were coated at 1:2000. Row A was loaded with 10µgml-1 3D11 in 0.02M Tris pH 9.0, row B

with PBS, rows C + D with C3 2½% FCS medium, row F with 4E1 2½% FCS medium, and rows G + H with PBS. The samples were assayed using ELISA protocol 3.

	absorbance at 450nm		
3D11	0.325±0.178		
PBS	0.110±0.023		
anti mouse IgG	**1:500**	**1:1000**	**1:1500**
C3	0.231±0.020	0.239±0.012	0.225±0.007
PBS	0.197±0.007	0.227±0.019	0.192±0.027
4E1	0.763±0.022	0.715±0.017	0.713±0.009

Table 3.1.3.1 Results for ELISA plate 16

These results indicated that the assay was capable of detecting murine antibodies that were present in hybridoma culture media. The positive control 4E1 hybridoma media gave a positive signal in this assay, indicating that ELISA plates coated with the precipitated sheep anti mouse IgG were capable of binding the murine antibodies pesent in culture samples. However at this stage the C3 culture samples were not producing a signal comparable to that of the 4E1 culture.

Rows B-H of an ELISA plate, plate 17, were coated with a 1:500 dilution of $(NH_4)_2SO_4$ ppt sheep anti mouse IgG, 100µl per well. Row A was coated with 10 μgml^{-1} 3D11 in 0.01M Tris pH 9.0, rows B-E were then loaded with spent C3 2½% FCS medium, 100µl per well, row F with 4E1 2½% FCS medium, 100µl per well and rows G + H with PBS, 100µl per well. The samples were assayed using ELISA protocol 3.

Plate 17 was repeated as plate 18, as the 3D11, purified antibody, control had failed to produce the expected result(A_{450} approximately 1). Plate 18 was assayed using the same protocol and samples loaded onto the plate in the same arrangement, but with freshly prepared 3D11 control.

An ELISA plate, plate 19, was coated with a 1:500 dilution of $(NH_4)_2SO_4$ ppt sheep anti mouse IgG, 100µl per well. Row A was loaded with 4E1 2½% FCS medium, 100µl per well, rows B-F with spent C3 2½% FCS medium from C3 hybridoma cultures that had been shocked to stimulate IgG production. The FCS concentration in the tissue culture medium was increased to 10% from 2½%, then

decreased to 2½%, in subsequent feeds. Rows G-H were loaded with PBS as -ve controls. The samples were assayed using ELISA protocol 3.

	absorbance at 450nm			
	plate 17	plate 18	mean of plates 17 &18	plate 19
4E1	0.773±0.034	1.049±0.126	0.911±0.166	1.007±0.210
C3	0.250±0.027	0.402±0.046	0.326±0.111	0.371±0.155
PBS	0.178±0.018	0.459±0.112	0.313±0.155	0.211±0.024

Table 3.1.3.2 Results for ELISA plates 17 to 19

This ELISA assay system was proven to be able to detect murine antibody in hybridoma media, by producing positive results for the control 4E1. The working mouse antibody detecting ELISAs detected no antibody in the C3 medium, the attempt to stimulate the C3 hybridoma by increasing then decreasing the concentration of FCS also failed to produce any antibody that could be detected by this assay. The C3 hybridoma cell line was no longer producing any antibody. It would be impossible to continue with the project as originally intended, since no C3 IgG was produced and measurement of its catalytic properties with the newly prepared hapten would not be possible.

3.2 EXPRESSION OF C3SCFV IN *E.COLI*

3.2.1 Small scale expression of C3scFv in *E.coli*

Small scale expression experiments were carried out to evaluate the expression of *spA*-C3scFv fusion from pPANG1C3scFv, and to determine what consequences the aberrantly rearranged V_H sequence cloned into pPANG1 would have on the expressed product from pPANG1 C3scFv. In these experiments uninduced BL21 (DE3) pPANG1C3scFv cultures were to be used as negative controls, for comparison to induced BL21 (DE3) pPANG1C3scFv cultures. Induced expression of *spA*-C3scFv fusion from pPANG1C3scFv via the use of the T7 RNA polymerase system should produce a relative high titre of protein(Studier *et al* 1990, Anthony *et al* 1992), which can be detected by SDS-PAGE. The expression protocols for the production of recombinant protein from pPANG1C3scFv were based on those used by Gandecha *et al* (1994) to express the anti- phytochrome antibody scFv AS32 from the same vector pPANG1.

3.2.1.1 Comparison of periplasmic fractions from induced and uninduced BL21 (DE3) pPANG1C3scFv cultures

BL21 (DE3) pPANG1C3scFv bacterium were streaked from glycerol stocks onto LB agar plates and incubated overnight at 37°C, then stored at 4°C. Single colonies of BL21 (DE3) pPANG1C3scFv were picked of a LB agar plate and grown overnight in 10ml LB cultures, overnight at 28°C in an incubator-shaker. Four cultures were set up, two containing 1% glucose and two containing 0.1% glucose, from 10% glucose stock solution. When the A_{600} of the culture reached 1, IPTG was added, to a final concentration of 1mM, to one LB + 0.1% glucose culture and to one LB +1% glucose culture, to induce expression of the *spA*-C3scFv gene fusion in pPANG1C3scFv. After a further 16 hours incubation at 28 °C the cell cultures were centrifuged. The supernatants were removed and a sample taken for SDS-PAGE gel electrophoresis. Each cell pellet was then fractionated using cell fractionation by osmotic shock protocol 1 (2.4.5.1).

A sample of the supernatant, whole cell, periplasmic fraction and washed cell fraction, of all four cultures was analysed by SDS-PAGE gel electrophoresis, (*fig.* 3.2.1.1)

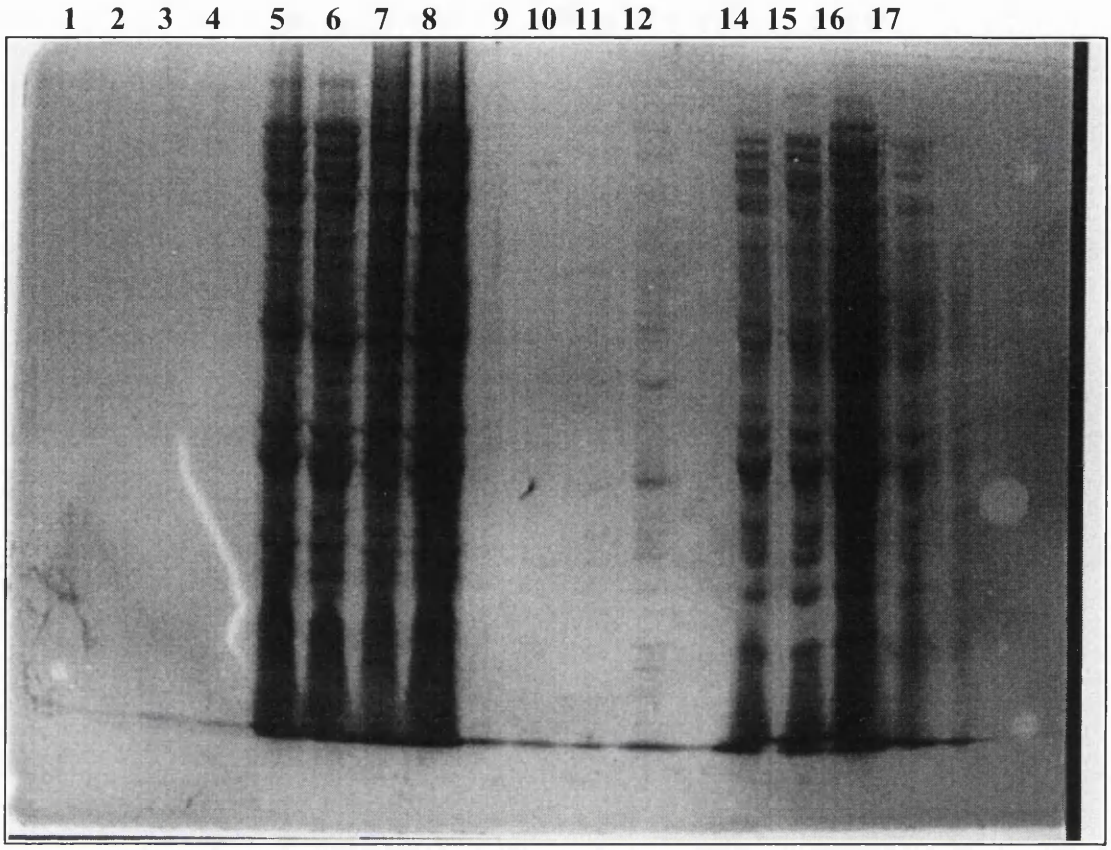

fig.*3.2.1.1 SDS-PAGE gel 1*

Lane	Culture conditions	Culture fraction
1	LB + 0.1% glucose, uninduced	supernatant
2	LB + 0.1% glucose, induced	supernatant
3	LB + 1% glucose, uninduced	supernatant
4	LB + 1% glucose, induced	supernatant
5	LB + 0.1% glucose, uninduced	whole cell fraction
6	LB + 0.1% glucose, induced	whole cell fraction
7	LB + 1% glucose, uninduced	whole cell fraction
8	LB + 1% glucose, induced	whole cell fraction
9	LB + 0.1% glucose, uninduced	periplasmic fraction
10	LB + 0.1% glucose, induced	periplasmic fraction
11	LB + 1% glucose, uninduced	periplasmic fraction
12	LB + 1% glucose, induced	periplasmic fraction
14	LB + 0.1% glucose, uninduced	washed cell fraction
15	LB + 0.1% glucose, induced	washed cell fraction
16	LB + 1% glucose, uninduced	washed cell fraction
17	LB + 1% glucose, induced	washed cell fraction

From SDS-PAGE gel 1, it was apparent that no induced protein product from the BL21 (DE3) pPANG1C3scFv had leaked into the supernatant. The *ompA* signal sequence should have directed the spA-C3scFv fusion sequence to the periplasm, the periplasmic fractions from the induced cultures should have shown a band on the SDS-PAGE gel corresponding to expressed SpA-C3scFv fusion which would have been lacking from the uninduced cultures. However the gel showed little detection of anything, possibly due to the proteins in the periplasmic fractions of the induced and uninduced cultures being at too low a concentration to be evaluated on an SDS-PAGE gel. The periplasmic fractions of all four cultures were concentrated by centrifugation with a centricon concentrator, and stored at -20°C.

3.2.1.2 Purification of spA fusion proteins from induced and uninduced BL21 (DE3) pPANG1C3scFv cultures

In order to determine if the pPANG1C3scFv construct was expressing a SpA-C3scFv protein a second set of expression experiments was performed. These expression experiments would be performed on a larger scale and utilise a spA-IgG affinity purification protocol to isolate any spA or spA fusion sequences produced by BL21 (DE3)C3scFv. BL21 (DE3)pPANG1C3scFv bacterium were spread from glycerol stocks on to LB agar plates and incubated overnight at 37° C, then stored at 4°C. Single colonies of BL21 (DE3)pPANG1C3scFv were picked off the plate and grown in four separate cultures, two 10ml LB and two 10ml TB. All four cultures were incubated at 28°C in an incubator-shaker, overnight.

Two 100ml LB and two 100ml TB cultures were set up in baffled flasks and inoculated with the overnight BL21 (DE3)pPANG1C3scFv LB and TB cultures. The four 100ml cultures were incubated at 28°C in an incubator-shaker, overnight. After the A_{600} of the cultures reached 1, IPTG was added to a final concentration of 1mM to one 100ml TB BL21 (DE3)pPANG1C3scFv culture and one 100ml LB BL21 (DE3)pPANG1C3scFv culture, to induce expression of the spA-C3scFv sequence in the plasmid pPANG1. Both induced and uninduced cultures were then incubated at 28°C in an incubator-shaker, overnight.

All four cell cultures were then centrifuged. The supernatant was removed and the cell pellet resuspended in sonication buffer, and then sonicated to lyse the *E.coli* and release any expressed spA-C3scFv fusion protein. The lysed *E.coli*

suspensions were then purified by IgG agarose purification for spA fusion proteins, protocol 1 (2.6).

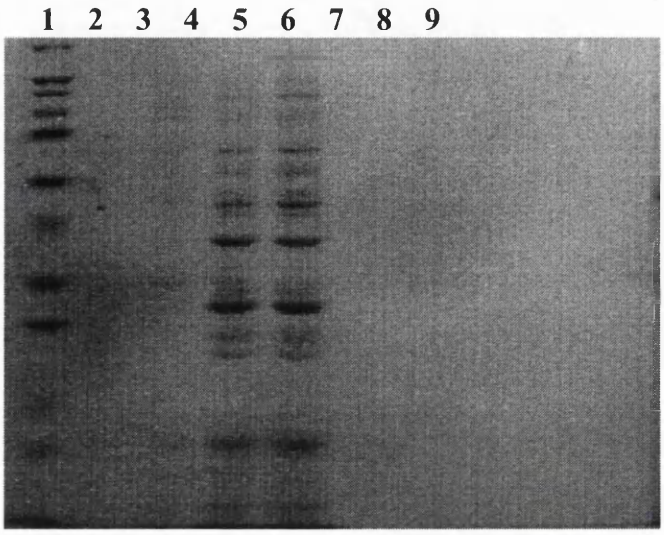

fig.3.2.1.2 SDS-PAGE gel 2

Lane	Sample from BL21 (DE3) pPANG1 expression
1	molecular weight marker, weights 205,116,97,84,66,55,45,36,29 KDa
2	concentrated periplasmic fraction of LB + 0.1% glucose, uninduced
3	concentrated periplasmic fraction of LB + 0.1% glucose, induced
4	concentrated periplasmic fraction of LB + 1% glucose, uninduced
5	concentrated periplasmic fraction of LB + 1% glucose, induced
6	eluted protein from IgG column for LB, uninduced
7	eluted protein from IgG column for LB, induced
8	eluted protein from IgG column for TB, uninduced
9	eluted protein from IgG column for TB, induced

The concentrated periplasmic fractions from the first set of expression experiments were analysed on SDS-PAGE gel 2, (*fig.* 4.1.2) along with the eluted fractions from the IgG agarose column for purification of spA fusions, from the second set of expression experiments.

The concentrated periplasmic fractions from the LB + 0.1% glucose cultures failed to show any protein. The concentrated periplasmic fractions from the LB + 1% glucose cultures did show protein, but there was now difference between the uninduced cultures, negative control, and the induced cultures. Expression of any kind of spA-C3scFv fusion would have given rise to an extra band in the periplasmic fraction, due to the OmpA mediated transport of the expressed SpA-

C3scFv fusion to the periplasm. No such protein band was detected suggesting that the *spA*-C3scFv gene was not expressed, (lanes 2-5). Neither gel showed a protein band of the expected size for the SpA-C3scFv fusion.

The second set of experiments examined all of the soluble products from the *E.coli* cells, the cells being resuspended and lysed by sonication. The detection of spA-C3scFv fusions relied on the binding of SpA domains to IgG, and would have detected the presence of SpA domains among any of the soluble products of the *E.coli.* cells. The IgG agarose purified samples from the second set of expression experiments contained no protein that could be detected on a SDS-PAGE gel, (lanes 6-9).

The results from both sets of expression experiments, both shown on SDS-PAGE gel 2, indicate that the BL21 (DE3) pPANG1C3scFv cells were not producing any inducable protein, or spA fusion protein, and the *spA*-C3scFv gene in the pPANG1 vector was not being expressed.

3.2.2 pGEM C3 scFv DNA sequence analysis

The C3scFv gene was sequenced by Julia Bartley at the University of Durham. Sequence data was obtained by using M13-20, M13 reverse primers and a primer designed by Julia Bartley for internal sequencing. The sequence of the C3 scFv gene from the sequencing data was determined to be,

TCGTCTAGACCCATGGACATCCAACTGACCCAGTCTCCA TCCAGTCTG
Ser Ser Arg Pro Met Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu
V_l5′RT
TCTGCATCCCTCGGAGACACAATTACCATCACTTGCCGTGCCAGTCAG
Ser Ala Ser Leu Gly Asp Thr Ile Thr Ile Thr Cys Arg Ala Ser Gln

CACATTAATATTTGGTTAAGCTGGTACCAGCAGAAACCAGGAAATATT
His Ile Asn Ile Trp Leu Ser Trp Tyr Gln Gln Lys Pro Gly Asn Ile

CCTAAACTGTTGATCTATAAGGCTTCCAACTTGCACATAGGCGTCCCA
Pro Lys Leu Leu Ile Tyr Lys Ala Ser Asn Leu His Ile Gly Val Pro

TCAAGGTTTCGTGGCAGTGGTCTCGGAACAGATTTCACATTAACCATC
Ser Arg Phe Arg Gly Ser Gly Leu Gly Thr Asp Phe Thr Leu Thr Ile

AGCAGTCTGCAGCCTGAAGACATTGCCACTTACTACTGTCTACAGGGT
Ser Ser Leu Gln Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Leu Gln Gly

CGAGATTATCCTCTGACGTTCGGTGGAGGGACCAAGCTGGAGATCAAA
Arg Asp Tyr Pro Leu Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys

CGTGTCAAC GGCGGTGGCGGATCTGGTGGTGGTGGCTCCGGCGGTGGC
Arg Val Asn Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly

linker
GGTTCT GGGGATGTGCAGCTGGTGGAGTCTGGACCTGGCCTGGTGGCG
Gly Ser Gly Asp Val Gln Leu Val Glu Ser Gly Pro Gly Leu Val Ala

CCCTCACAGAGCCTGTCCATCACATGCACCGTCTCAGGGTTCTCATTA
Pro Ser Gln Ser Leu Ser Ile Thr Cys Thr Val Ser Gly Phe Ser Leu

ACTAGCTATGGTGTACACTGGGTTCGCCAGCCTCCAGGAAAGGGTCTG
Thr Ser Tyr Gly Val His Trp Val Arg Gln Pro Pro Gly Lys Gly Leu

GAGTGGCTGGTAGTGATATGGAGTGATGGAAGCACAACCTATAATTCA
Glu Trp Leu Val Val Ile Trp Ser Asp Gly Ser Thr Thr Tyr Asn Ser

GCTCTCAAATCCAGACTGAGCATCAGCAAGGACAACTCCAAGAGCCAA
Ala Leu Lys Ser Arg Leu Ser Ile Ser Lys Asp Asn Ser Lys Ser Gln

GTTTTCTTAAAAATGAACAGTCTCCAAACTGATGACACAGCCATGTAC
Val Phe Leu Lys Met Asn Ser Leu Gln Thr Asp Asp Thr Ala Met Tyr

TACTGTGCCAGAGAGCCTCCCACGACGTACGTTTGCTTAC TGGGGCCA
Tyr Cys Ala Arg Glu Pro Pro Thr Thr Tyr Val Cys Leu Leu Gly Pro

AGGGACCACGGTCACCCTCTCCTCA**TAATAA**GGTACCGAG
Arg Asp His Gly His Pro Leu Leu Ile Ile Arg Tyr Arg
V_H3′RT

The sequence for the V_l and V_H sections of the scFv gene were compared with sequences at Daresbury gene sequence database, seqnet. The closest match for the V_l was found to be a murine κ light chain from a anti-dinitrophenyl antibody, clone AN04K as sequenced by Leahy *et al* (1988). The V_H sequence was found to be virtually identical to that of a cDNA of an RNA transcript of a aberrantly rearranged heavy chain sequence, (1.4). The C3 V_H sequence only varied from the aberrantly rearranged V_H sequence by 5 bases, all of which were in the VH3'RT primer sequence, suggesting that the changes were the result of PCR of the same mRNA transcript but with different primers, the differences being

introduced by the primers. The aberrantly rearranged V_H sequence was murine V_H Ag8 aberrantly rearranged heavy chain variable gene (x58634), (Yamanaka *et al* 1995) The primer V_H3'RT contained two stop codons TAATAA, however as the cloned sequence was an aberrantly rearranged variable region gene sequence the two stop codons were no longer in the correct reading frame. As a result the plasmid pPANG1C3scFv.4 contained no stop codons directly 3' from the C3scFv gene sequence, and the V_H sequence in pPANG1C3scFv did not encode for a functional protein. As a consequence of the stop codons being shifted out of frame the translated protein would be targeted for destruction by SscrA (Keiler *et al* 1996). The SsrA peptide tag serves *E.coli* as a mechanism for the removal of polypeptides and proteins that have been translated from damaged mRNA. When a ribosome stalls translating an mRNA, probably as the result of a missing stop codon, the translated protein is tagged with the SsrA polypeptide. SsrA tagged proteins are targeted for proteolytic destruction in order to restart the ribosome. From the sequence data showing the out of frame stop codon the most logical explanation for the lack of expression by pPANG1C3scFv of any recombinant protein is the out of frame stop codons and subsequent Ssc targeted proteolytic destruction of translated proteins.

In order to reconstruct a pPANG1C3scFv expression plasmid that contained genuine C3 V_l and V_H sequences three more clones of the C3scFv were obtained, from the Dept. of Botany at the University of Leicester **(1.4)**; C3scFv.2, C3scFv.7, C3scFv.9. These clones were constructed at the same time as the C3scFv.4 and all C3scFv clones were carried on the pGEM plasmid in *E.coli* XL1-Blue. A sample of each of The C3 scFv clones was prepared using Wizard miniprep system as described. The V_H sequence of these clones was determined, sequencing data from Julia Bartley. The clones all contained the same aberrantly rearranged V_H sequence, as C3scFv.4, and as a consequence the same out of frame stop codon. No plasmids containing only C3V_H sequences were created during the cloning of C3, as cloning intermediates.

The only way to recreate a genuine C3scFv sequence would be to clone the C3 V_l and V_H sequences from hybridoma mRNA and then splice the two sequences together by overlap extension. In order to do this new consensus primers would have to be designed, to avoid recloning the aberrant heavy chain sequence again. A library of cDNA produced from hybridoma antibody mRNA would have to be constructed and sequenced. The library would be screened for the presence of the

known C3 V_l sequence and to screen out any aberrantly rearranged sequences, to leave a genuine C3V_H sequence which could then be spliced to the V_l sequence.

The C3 hybridoma culture was however found to be producing no antibodies at all (**3.3**), by ELISA for total IgG in culture supernatant. As a result it was no longer possible to create a C3scFv sequence by cloning of hybridoma mRNA, as the hybridoma line was no longer producing antibody (**3.1.3**).

3.3 RECONSTRUCTION OF EXPRESSION VECTOR PPANG1C3SCFV

As the C3scFv sequence was not being expressed in *E.coli*, (**4.1**), and reconstruction of the C3scFv sequence was no longer possible,(**4.2 & Chapter 3**), the expression vector pPANG1C3scFv would be modified in order to express at least the genuine C3 V_l sequence in *E.coli*. Two possible strategies were available for the excision of the aberrant V_H sequence, removal by restriction and creation of a new vector by PCR lacking the aberrant V_H sequence.

To evaluate the merits and possibility of the use of either strategy the expression vector had to be restriction mapped. The creation of a restriction map of the vector pPANG1C3scFv would dictate subsequent work.

3.3.1 Restriction mapping of pPANG1C3scFv

The C3scFv sequence in pGEM C3 scFv, and the *spA* sequence that was cloned into pPANG1 was mapped for restriction sites, using seqnet at Daresbury. Using the restriction maps for C3scFv, *spA*, pBluescript SK II(-), and data from the construction of pPANG1 from pBluescript SK II(-) a map of unique and twin unique restriction sites for the plasmid pPANG1C3scFv was created, (*fig.* 3.3.1.1 & *fig.* 3.3.1.2).

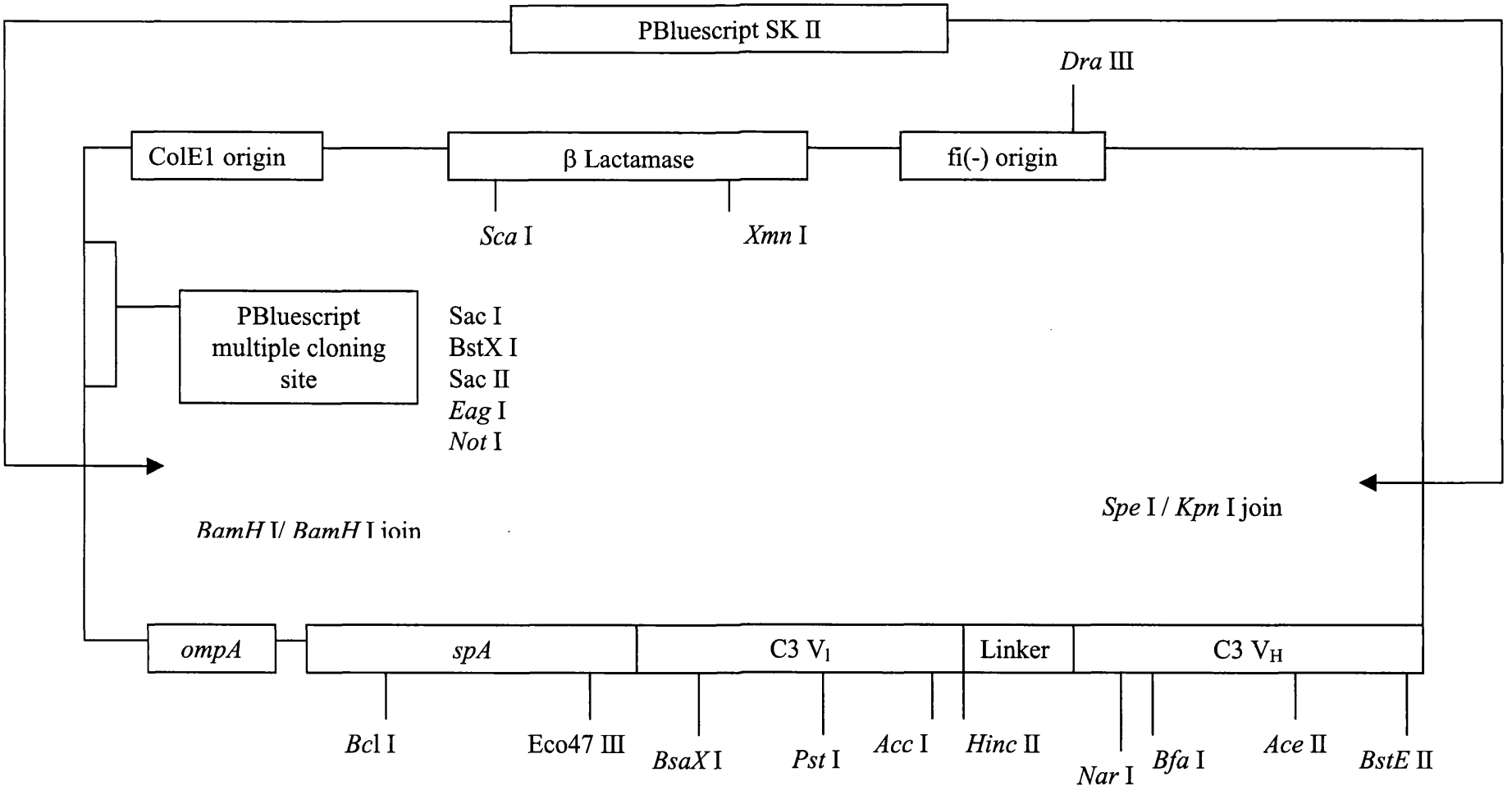

fig. 3.3.1.1 Unique restricition sites in pPANG1C3scFv

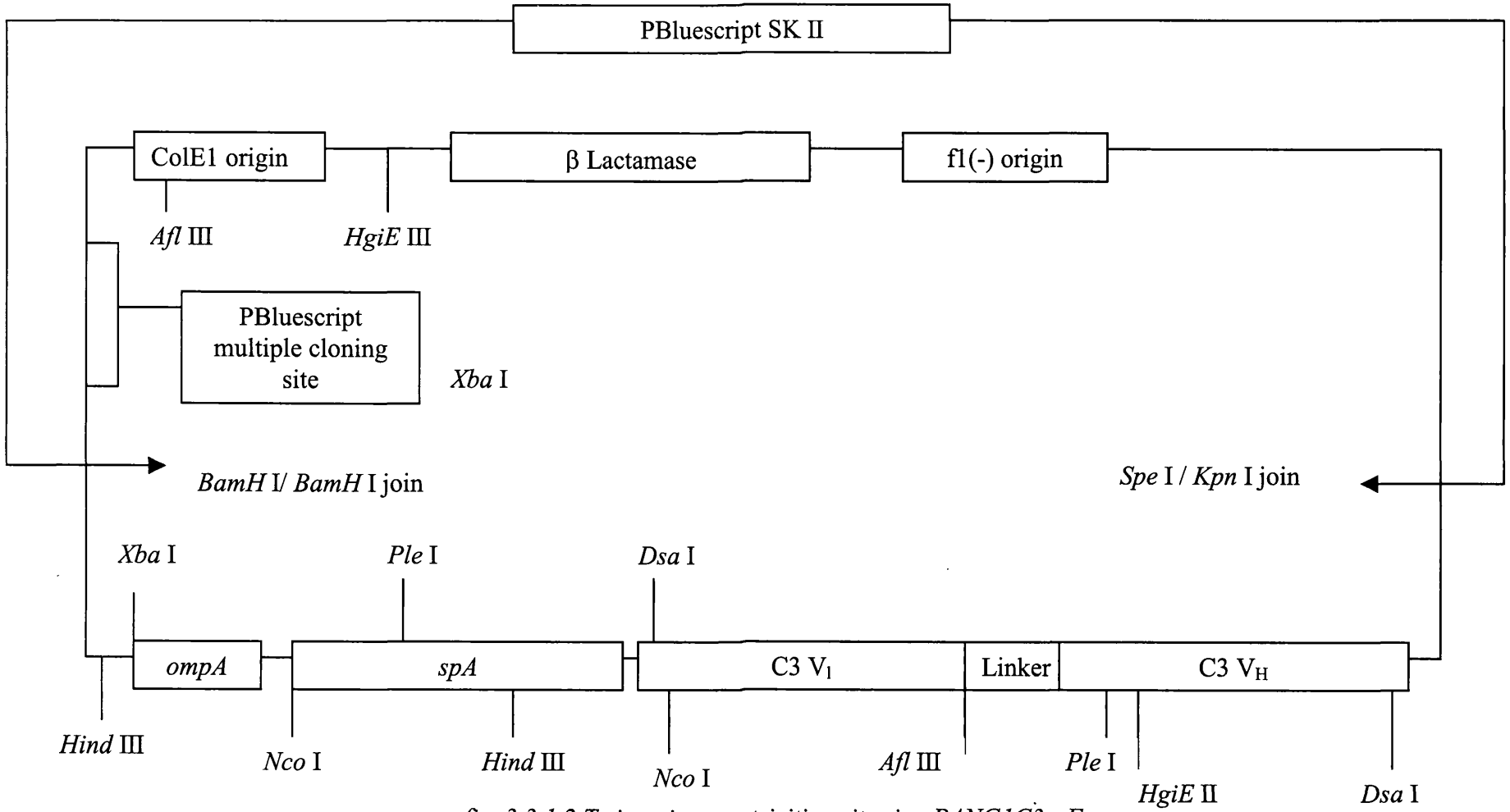

fig. 3.3.1.2 Twin unique restricition sites in pPANG1C3scFv

The aberrant V_H sequence could by removed, by restriction digestion of the vector pPANG1 C3scFv, in two ways. The first strategy would be to find a unique restriction site at the 3' end of the C3 V_l sequence, preferably in the linker sequence, the plasmid could be restricted at such a site and a short oligonucleotide, contain a stop codon, ligated into the plasmid. The second strategy would be to utilise twin unique restriction sites at the 3' and 5' ends of the aberrant V_H sequence and using these sites to remove the aberrant V_H sequence then ligating the plasmid after removing the aberrant V_H sequence. There was no twin unique sites in the pPANG1 C3scFv that could be used for removal of the aberrant V_H sequence, but a unique *Hinc* II site existed at the 3' end of the V_l sequence which could be used for the insertion of a stop codon.

In order to validate these unique and twin unique restriction site maps of pPANG1 C3scFv, diagnostic restriction digests were performed on the pPANG1 C3scFv plasmid DNA, for the validation of the pPANG1 C3scFv maps. The validation of the unique *Hinc* II site being the most important, as it could be a possible site for the insertion of a stop codon before the aberrantly rearranged V_H sequence in pPANG1 C3scFv.

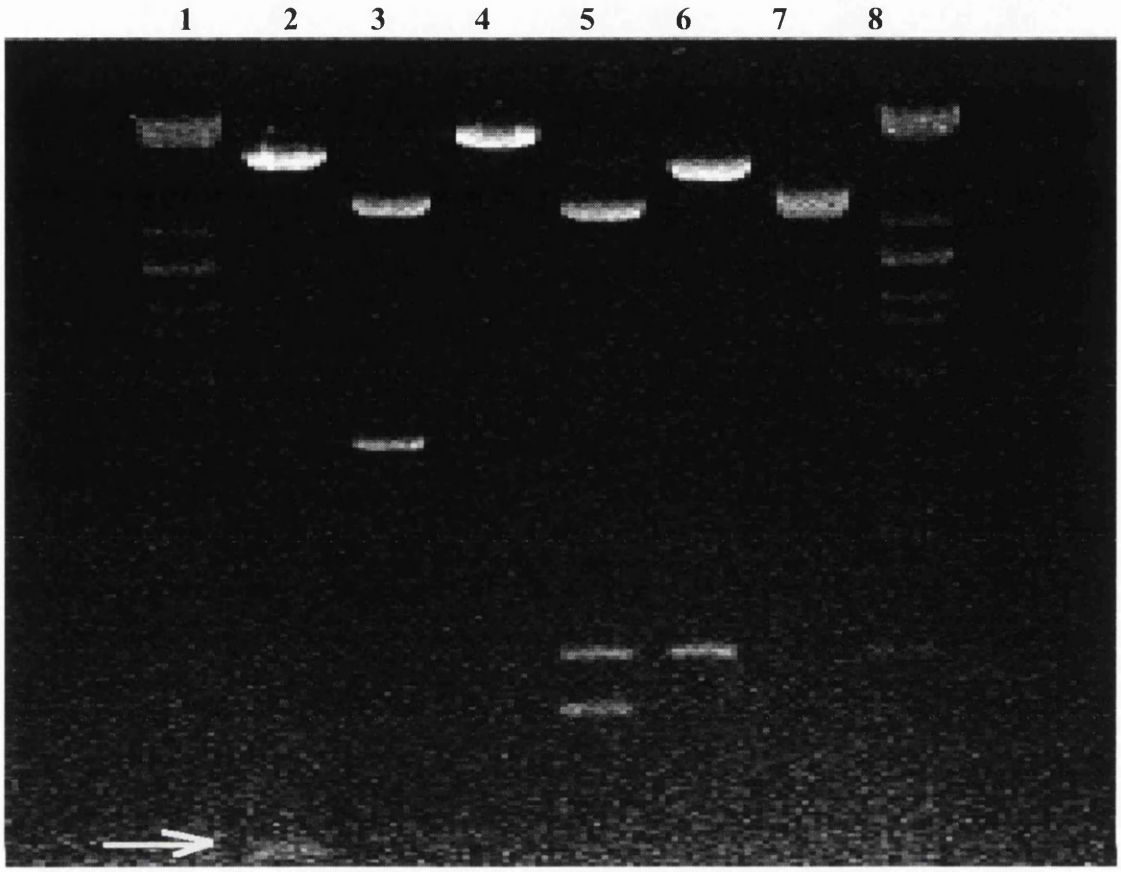

fig.3.3.1.3 Restriction digest gel 1

lane 1	lane 2	lane 3	lane 4	lane 5	lane 6	lane 7	lane 8
λPst marker	*Hinc* II	*Bsa* I	*BstE* II	*Sty* I	*Nco* I	undigested	λPst marker

The twin *Bsa* I, *Nco* I and the unique *BstE* II were all validated. The digest of pPANG1C3scFv plasmid DNA with *Hinc* II produced two fragments, the second fragment the weak DNA band highlighted with the white arrow, proving that the *Hinc* II site identified in the pGEMC3scFv sequence was not a unique site in the pPANG1C3scFv vector. The *BstE* II site, contained in the primer V_H3'RT was shown to be a unique site. To evaluate the utility of the *Hinc* II sites as part of a restriction strategy for reconstructing a vector to express a SpA-C3V_l fusion protein, the position of the second *Hinc* II site would need to be known. If the second *Hinc* II site was 3' from the known *Hinc* II site then a restriction strategy utilising *Hinc* II could still be a possibility for vector reconstruction, if the second *Hinc* II site was 5' from the known *Hinc* II site, possibly in the region of the *ompA*, T7 ∅10 sequences or at the *spA*-C3V_l junction, then a *Hinc* II based

restriction strategy would be invalidated, regardless of the exact location of the second *Hinc* II site.

Further restriction digests were performed on the plasmid pPANG1C3scFv, to locate the presence of the second *Hinc* II site. In order to determine if the second *Hinc* II site was 5' or 3' from the known *Hinc* II site.

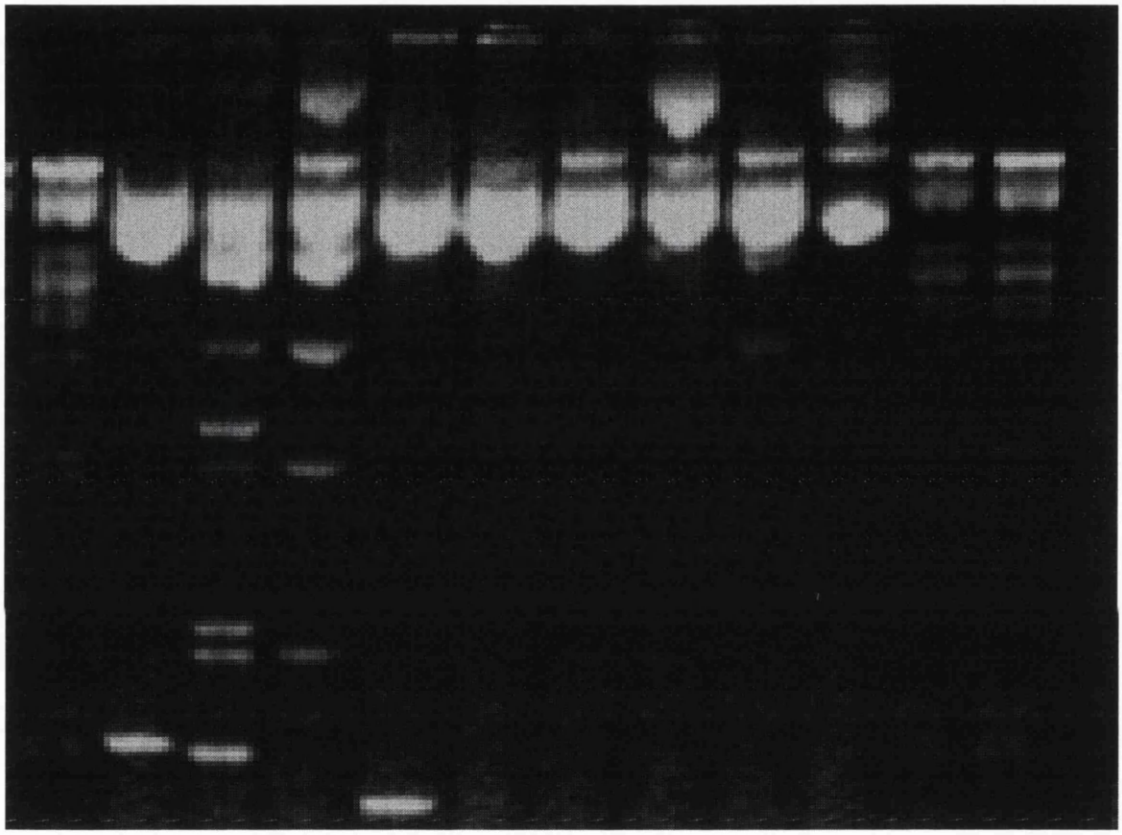

fig. 3.3.1.4 Restriction digest gel 2

lane 1	**lane 2**	**lane 3**	**lane 4**	**lane 5**
λPst marker	*Hinc* II	*Hinc* II+ *Hind* III	*Hind* III	*Hinc* II + *Acc* I

lane 6	**lane 7**	**lane 8**	**Lane9**	**Lane 10**
Acc I	*Sac* I	*Sac* II	Not I	Undige sted

3.3.2 PCR cloning of *ompA-spA*-C3V$_l$ expression cassette from pPANG1 C3scFv and creation of expression plasmid pQR627

A PCR based method of reconstruction was utilised to reconstruct an expression vector, from pPANG1C3scFv, that would be capable of expressing the *spA*-C3V$_l$ gene fusion present in pPANG1C3scFv. The PCR strategy would clone the expression cassette of pPANG1C3scFv, without the aberrant V_H sequence and clone it back into pBluescript SK II(-), to produce a vector that would be identical to pPANG1C3scFv but would lack the aberrant V_H sequence.

3.3.2.1 PCR cloning of ompA-spA-C3V$_l$ expression cassette from pPANG1C3scFv

The PCR strategy would insert suitable restriction sites 5' and 3' of the expression vector, by incorporating them into the primers for the cloning of the pPANG1C3scFv expression cassette. The 5' end primer would be designed from the known sequence of the pBluescript multiple cloning site, part of which is retained in pPANG1, pPANG1 being constructed from pBluescript, which would contain restriction sites that would be common to both pPANG1C3scFv and pBluescript. The ligation of the PCR produced expression cassette into one of the common pBluescript/pPANG1 restriction sites would produce a plasmid that would be a precise replica of the pPANG1C3scFv plasmid until the sequence introduced by the 3' primer used to clone the expression cassette. The 3' primer would be designed using the known sequence of the 3' end of the C3 V_l sequence, and incorporate a stop codon and a suitable restriction site for the ligation of the PCR cloned expression cassette into pBluescript.

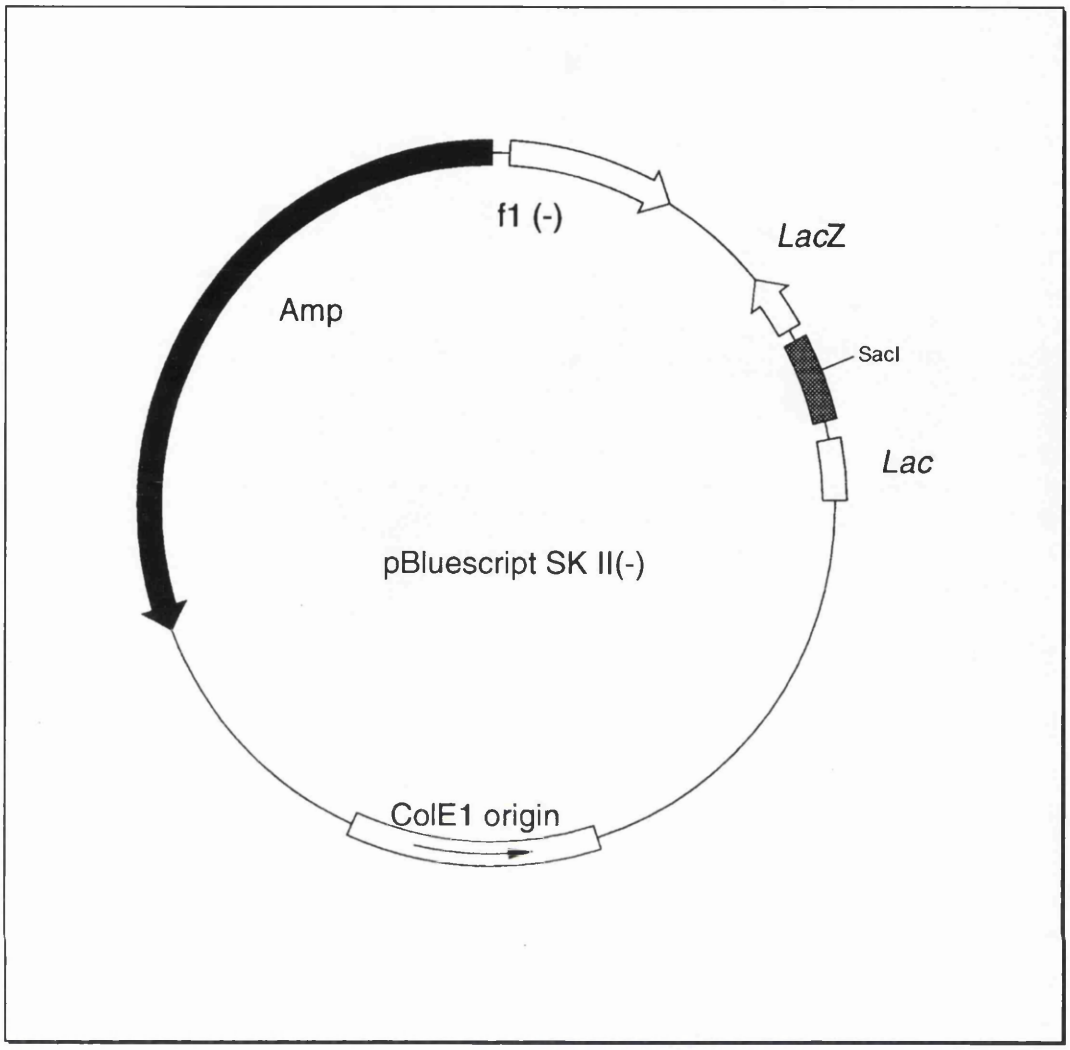

fig. 3.3.2.1 pBluescript SK II(-)

The selected restriction site for the forced cloning of the PCR produced expression cassette, from the common pBluescript/pPANG1 multiple cloning site sequence, was *Sac* I . The validity of the *Sac* I site having already been proven, and *Sac* I would produce sticky ends making the ligation of the PCR product into pBluescript more efficient. The use of *Sac* I for the ligation made for the simplest and most efficient strategy for force cloning the PCR produced expression cassette into pBluescript. The PCR protocols would be based on the enzyme *pfu* DNA polymerase, which possess proof-reading activity.

The primers for the PCR cloning of the expression cassette were produced by Pharmacia biotech;

primer 1 (based on known pBluescript sequence), ordered as SFPCR 1

5' 3'

GCGCGCAATTAACCCTCACTAAAGGG 26mer

primer 2, (end of C3 V_l + stop codon TAATAA+ *Sac* I restriction site), ordered as SFPCR 2

5' antisense strand 3'

GCGGGAGCTCTTATTAGCCGTTGACACG 28mer

5' sense strand 3'

CGTGTCAACGGC TAATAAGAGCTC CCGC

C3 V_l stop codons *Sac* I site

The primer SFPCR1 was designed to be complementary to a site 5' to where Gandecha *et al* (1994)had inserted their expression cassette to create pPANG1. The primer SFPCR2 was designed to be complementary to the 3' end of the C3V_l gene and to incorporate two stop codons and a *Sac* I site. The resulting PCR product would contain 2 *Sac* I sites, one incorporated from the *Sac* I site present at the multiple cloning site of pBluescript and retained in pPANG1C3scFv, the second was introduced by design in the SFPCR2 primer.

A PCR protocol for the cloning of the C3scFv expression cassette for the plasmid pPANG1C3scFv, using the described primers was optimised, (**2.5.3**) The PCR generated expression cassette was then purifed by geneclean and ligated into the vector pBluescript SK II(-), (Strategene). The pBluescript SK II plasmid was diluted 1:10 in distilled water and subsequently digested with *Sac* I, the genecleaned PCR expression cassette was also digested with *Sac* I and after heat inactivation the PCR product was ligated into the *Sac* I site of pBluescript SK II, (**2.5.6**)

3.3.2.2 Screening of pQR627 clones for orientation of inserted gene

The selected PCR and subsequent ligation strategy allowed for the gene to be inserted into the pBluescript SK II vector in either the same orientation as in pPANG1C3scFv, or in the counter orientation, (fig. 5.2.2). Theoretically the new construct would be able to produce the spA-C3V_l fusion protein regardless of the orientation of the expression cassette relative to the pBluescript vector, transcritption of the gene being initiated from the T7 promoter which was carried on the PCR cloned expression cassette. A host cell, such as BL21 (DE3), which could utilise the T7 expression system would produce protein irrespective of the expression cassettes orientation in the vector.

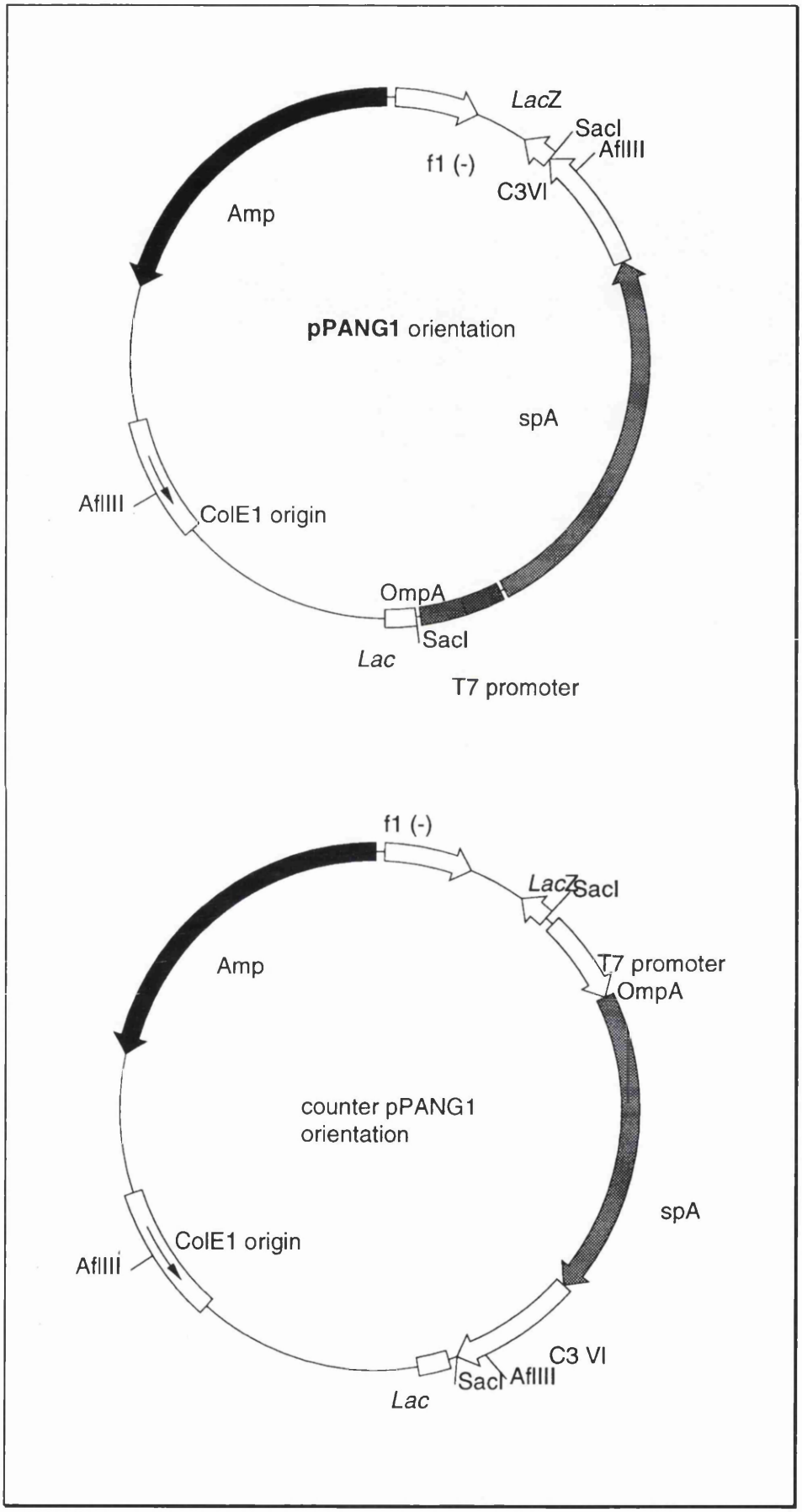

fig. 3.3.2.2 Orientations of gene inserts in pQR627 clones

The newly constructed plasmid DNA was transformed into competent JM107 *E.coli.* The *E.coli* strains containing pBluescript with the inserted expression cassette were selected by blue white colour selection on X-gal plates(**2.4.4**) White colonies of *E.coli* containing pBluescript vector with the gene insert were picked of the X-gal plate, then grown overnight in NB and the plasmid DNA from these colonies isolated by Wizard plasmid miniprep.

The plasmids from blue white selected colonies were then screened to determine the orientation of the inserted C3 V_l expression cassette relative to the pBluescript vector. This was achieved by miniprepping plasmid DNA from selected colonies and using a restriction digest screen, and then sequencing the pBluescript/PCR fragment junctions in the new construct pQR627.

Firstly the DNA from the minipreps was screened to ensure that the vectors carried the desired insert, this was achieved by the digestion of the plasmid DNA by *Spe* I and *Acc* I. These diagnostic digestions showed that the *ompA-spA*-C3V_l expression cassette was present in the new construct, digestions of both pQR627 clones and pPANG1C3scFv giving the same size fragment and therefore suggesting that both sets of restriction digestions produced the same fragment.

The plasmid DNA from the colour selected colonies was then screened for orientation using digestion with *Afl* III. The two orientations theoretically producing different sized fragments when digested with *Afl* III, (fig. 5.2.2). All of the blue white selected colonies produced plasmid DNA that carried the inserted gene in the counter orientation to that of the C3 expression cassette in pPANG1C3scFv. On digestion with *Afl* III all the pQR627 clones produced a smaller fragment than the pPANG1C3scFv digestion, the smaller sized fragment suggesting that the expression cassette had ligated into each of the clones in the counter pPANG1C3scFv orientation.

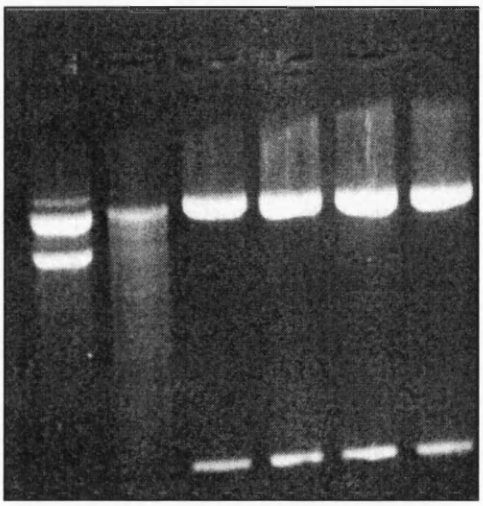

fig 3.3.2.3 Screening of pQR627 constructs by Afl III digestion

Lane 1	pPANG1C3scFv + *Afl* III
Lane2	PBluescript + *Afl* III
Lanes 3-6	PQR6227 constructs + *Afl* III

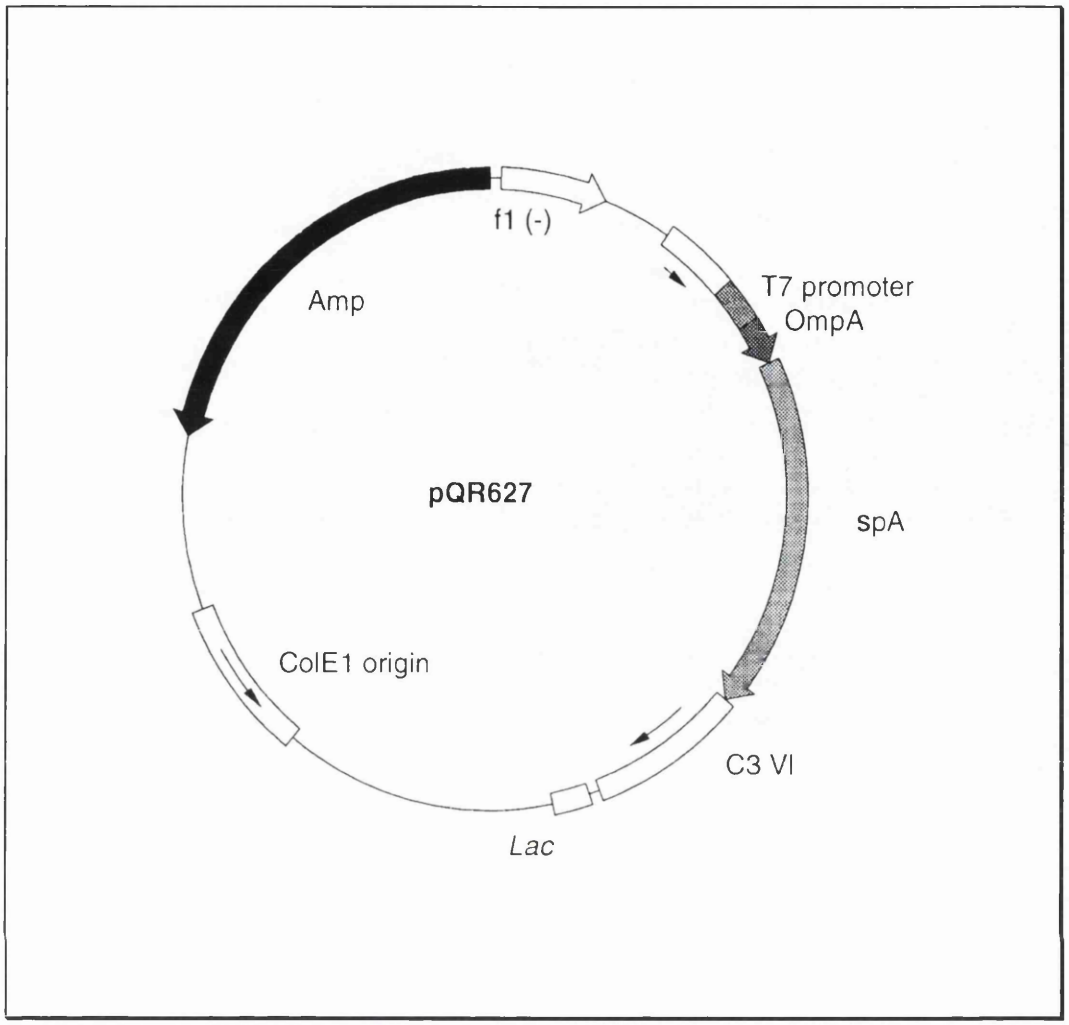

fig. 3.3.2.4 Expression vector pQR627

To completely determine both the presence of the inserted expression cassette and its orientation the pQR627 plasmid DNA was sequenced using primers sites being carried on the pBluescript vector, and which flanked either side of the inserted expression cassette. The sequencing confirmed the presence of the inserted expression cassette and the orientation of the inserted gene. Expression of protein from the construct was still theoretically possible as the *spA*-C3V_I gene was still in the correct orientation from the T7 promoter included in the PCR product from pPANG1C3scFv.

3.4 EXPRESSION OF SPA-C3V_L FUSION FROM PQR627

3.4.1 Detection of SpA fusion proteins in *E.coli* cultures

The SpA-C3V_l expression construct pQR627 was transformed into the *E.coli* expression strain BL21 (DE3)pLysS transformed cells being selected by streaking on double antibiotic selective plates, ampicillin and chloramphenicol, the *E.coli* strain BL21 (DE3)pLysS being chloramphenicol resistant, the pLysS plasmid carrying the chloramphenicol resistance gene and the gene for T7 lysozyme, a natural inhibitor of T7 RNA polymerase to regulate expression from the T7 promoter. To provide a negative control in small scale expression experiments with pQR627 untransformed BL21 (DE3)pLysS cultures were used. BL21 (DE3) cultures were grown overnight in NB containing chloramphenicol at 37°C or 28°C and 200rpm in an incubator-shaker. To provide a positive control for the subsequent expression experiments with the pQR627 vector a Staphylococcal protein A producing vector pEZZ18, (Pharmacia), was also transformed into the BL21 (DE3)pLysS strain. The pEZZ18 expression vector was developed as a non induceable expression vector for SpA fusion proteins, the SpA fusion being excreted into the supernatant. Previous experiments with a pEZZ18 derivative expressing a SpA-IGF-I fusion in *E.coli* strain HB101 gave yields of 75mgl^{-1} in a fermenter and 5mgl^{-1} in shake flasks. Cultures of BL21 (DE3)pEZZ18 were streaked from glycerol stocks onto double antibiotic selective plates, ampicillin and chloramphenicol and grown overnight in NB medium, containing ampicillin and chloramphenicol, at 37°C or 28°C and 200rpm in an incubator-shaker. The culture was centrifuged and the supernatant containing expressed SpA was retained along with the cells.

3.4.1.1 Detection of expressed proteins from vector pQR627 by SDS-PAGE

Small scale expression experiments were carried out with the BL21 (DE3)pQR627 *E.coli* strain in a similar manner as the small scale expression experiments with BL21 (DE3)pPANG1C3scFv *E.coli* strain. These experiments attempted to detected the induced SpA C3V_l protein by comparison of induced and uninduced cultures of pQR627 by SDS-PAGE. In these experiments BL21 (DE3) strain *E.coli* was used as a negative control, and *E.coli* strain BL 21 (DE3)pEZZ18 as a positive control strain.

Expression experiments with pQR627 were carried out at two separate temperatures 28°C and 37°C to ascertain if the culture temperature affected the expression of the SpA-C3V_1 protein. These expression experiments were comparable to those performed with pPANG1C3scFv. Cultures of BL21 (DE3)pQR627 were streaked from glycerol stocks onto double antibiotic, ampicillin and chloramphenicol, selective plates. Cultures were grown in NB containing ampicillin and chloramphenicol, in an incubator-shaker at 200rpm to an OD_{600} of 1, IPTG was then added to a final concentration of 1mM to the induced cultures, no IPTG was added to the uninduced cultures. After 3 hours cells were harvested by centrifugation, the supernatant was removed and stored. The cells were resuspended in ×25 concentrate over broth, resuspension buffer. The cell cultures were harvested three hours after induction as the T7 expression system utilised in the pQR627 and pPANG1C3scFv vectors, has been reported as producing detectable levels of recombinant protein three hours after induction (Studier *et al* 1990)

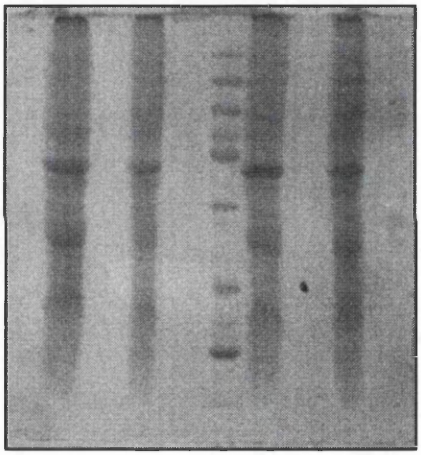

fig.3.4.1.1.1 SDS-PAGE gel of 37°C pQR627 expression

Lane	Sample
1	Uninduced BL21 (DE3)pQR627 cell fraction
2	Uninduced BL21 (DE3)pQR627 culture supernatant
3	Induced BL21 (DE3)pQR627 cell fraction
4	Induced BL21 (DE3)pQR627 culture supernatant
5	Marker
6	BL21 (DE3) pEZZ18 cell fraction
7	BL21 (DE3) pEZZ18 culture supernatant
8	BL 21 (DE3) cell fraction
9	BL21 (DE3) culture supernatant

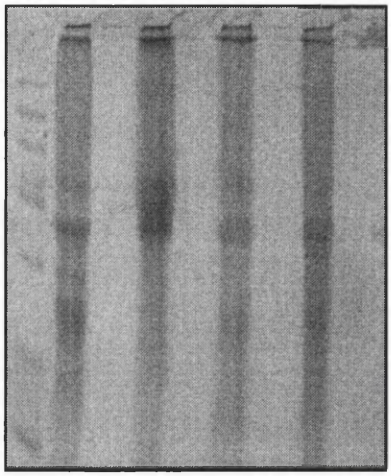

fig.3.4.1.1.2 SDS-PAGE gel of 28℃ pQR627 expression

Lane	Sample
1	Marker
2	Uninduced BL21 (DE3)pQR627 cell fraction
3	Uninduced BL21 (DE3)pQR627 culture supernatant
4	Induced BL21 (DE3)pQR627 cell fraction
5	Induced BL21 (DE3)pQR627 culture supernatant
6	BL21 (DE3) pEZZ18 cell fraction
7	BL21 (DE3) pEZZ18 culture supernatant
8	BL21 (DE3) cell fraction
9	BL21 (DE3) culture supernatant

As no induced protein was detected by SDS-PAGE a more sensitive assay specific for SpA had to be developed.

3.4.1.2 Detection of SpA fusion proteins from vector pQR627 by ELISA

An ELISA assay was developed to detect SpA utilising goat IgG immobilised onto a 96 well plate, and mouse anti SpA to detect SpA that had bound to the immobilised goat IgG. This assay was demonstrated to be able to detect purified SpA (Sigma). This purified SpA was used to calibrate the ELISA assays. As no equivalent SpA-V_I fusion was available as a positive control the purified SpA was used, as a substitute positive control. The purified SpA was not a perfect positive control as the SpA protein and the SpA-V_I fusion were of different molecular weights. Due to this difference in molecular weight the expression levels of recombinant SpA protein from pEZZ18 and pQR627 were calibrated in

units of equivalent SpA concentration, rather than in units of actual protein concentration.

The ELISA assays were also controlled using the same controls as for the SDS-PAGE gels, BL21 (DE3)pEZZ18 and untransformed BL21 (DE3). Cultures of BL21 (DE3) were grown in NB medium containing ampicillin and chloramphenicol in an incubator-shaker, at either 28°C or 37°C at 200rpm. When the cultures reached an A_{600} of one the were induced by the addition of IPTG to a final concentration of 1mM. After 3 hours the cells were harvested by centrifugation and resuspended in ×25 concentrate over broth resuspension buffer. The culture supernatant was also retained and assayed with the cells.

	37°C cell fraction	37°C culture supernatant	28°C cell fraction	28°C culture supernatant
BL21 (DE3)pQR627	1μgml^{-1}	0.4μgml^{-1}	0.9μgml^{-1}	0.7μgml^{-1}
BL21 (DE3)pEZZ18	4μgml^{-1}	3.3μgml^{-1}	4μgml^{-1}	3μgml^{-1}
BL21 (DE3)	-	-	-	-

Table 3.4.1.2 Expression of SpA fusion from pQR627

The initial shake flask experiments confirmed firstly that the ELISA assay could detect recombinant SpA expressed in *E.coli* as well as purified SpA. The major result from these experiments was that the pQR627 transformed cell lines were producing a recombinant SpA protein. The untransformed BL21 (DE3) control cultures produced a background noise level that was undetectable by ELISA, and confirmed that the ELISA assay was only detecting recombinant protein produced from the vectors pQR627 and pEZZ18. The 28°C and 37°C BL21 (DE3)pQR627 cultures both gave detectable levels of SpA at similar levels. The pEZZ18 control cultures were producing the recombinant SpA at higher levels, than the pQR627 cultures. The titre of SpA detected in the control pEZZ18 cultures was comparable to that previously reported with the vector. Theoretically the levels of SpA produced by the pQR627 cultures should have been greater than those for the pEZZ18 cultures, because the pQR627 vector utilises the T7 RNA polymerase expression system which should produce more protein than a *Lac* based expression system (Studier *et al* , Anthony *et al*). However direct comparison between expression from pEZZ18 and pQR627 is misleading, the two proteins being produced by different promoters, in the same strain but under different conditions. As the pEZZ18 culture were harvested after overnight

culture without induction, and the pQR627 cultures harvested three hours post induction the levels of *E.coli* present in each culture would be different. The use of the pEZZ18 acted to calibrate the pQR627 expression, giving a relative standard for recombinant SpA production from different vectors in the same *E.coli* strain, in spite of the differences in promoter and culture conditions.

3.4.2 Time course Expression from BL21 (DE3)pQR627

The expression of the SpA fusion protein from pQR627 cultures was further investigated using more sophisticated experiments. A time course induction of BL21 (DE3)pQR627 was performed. The purpose of these time course experiments was three fold. Firstly to determine accurately the levels of SpA fusion expressed by pQR627, the same SpA detection ELISA protocol was used as in previous expression experiments but the ELISA was recalibrated to be optimised over the range of SpA fusion levels suggested by the less accurate previous experiments. The second objective was to ascertain how the levels of SpA fusion altered over time after induction. The inclusion of an overnight culture sample in this time course would give a comparable sample to that of the pEZZ18 culture which was also cultured overnight. The analysis of SpA fusion produced over time would provide important data for the optimisation of the expression. The third objective was to determine the distribution of the expressed SpA fusion between the supernatant, periplasm and the cell associated fractions of the culture, and their variation over time. The SpA fusion should be most concentrated in the periplasm, as expression of the SpA fusion would be directed by the ompA leader sequence. The supernatant could also contain detectable levels of the fusion as *E.coli* cells containing plasmids encoding for SpA fusion proteins such as the pRIT series and the pEZZ18 plasmids (Nilsson *et al.* 1990 & 1991) secreted some of the recombinant protein into the culture supernatant.

The time course expressions were conducted at both 37°C and 28°C to establish if the growth temperature would affect the production of SpA fusion. Cultures of BL21 (DE3)pQR627 cells were grown in 100ml of NB containing ampicillin and chloramphenicol, in an incubator-shaker to an A_{600} of 1, then one culture was induced by the addition of IPTG to a final concentration of 1mM, the other was not induced to act as a negative control. Samples were taken from both cultures and fractionated as described in **2.4.6.2** (French *et al* 1996). Samples were taken at 30 minute intervals over a period of three hours after induction and a final overnight sample taken. These time points were chosen because of the reported

ability of the T7 promoter system to produce detectable protein three hours after induction (Studier *et al* 1990), The final overnight time point was included to enable some comparison of these pQR627 cultures with the pEZZ18 overnight cultures, and the pPANG1AS32, the same expression plasmid as had been intended for C3scFv expression and on which pQR627 was based, but containing a scFv that had been proven to be expressed in *E.coli.* cultures as reported by Gandecha *et al*(1994), who harvested the pPANG1AS32 after induction overnight.

3.4.2.1 Time course expression of BL21 (DE3)pQR627 at 37°C

The time course experiments were performed as described above with samples taken at 30 minute intervals and a final sample taken after the cultures had grown overnight. The concentrations of SpA as detected by ELISA in each of the culture fractions over the time course is shown below (table 3.4.2.1.1). The assays were performed four times on the samples.

time after induction(hours)	0.5	1.0	1.5	2.0	2.5	3.0	O/N
supernatant induced	400±130Ngml^{-1}	440±140Ngml^{-1}	400±90Ngml^{-1}	380±90Ngml^{-1}	470±210Ngml^{-1}	410±110Ngml^{-1}	620±140Ngml^{-1}
supernatant uniduced	300±80Ngml^{-1}	410±120Ngml^{-1}	390±100Ngml^{-1}	370±150Ngml^{-1}	350±90Ngml^{-1}	330±90Ngml^{-1}	400±130Ngml^{-1}
periplasm induced	400±90Ngml^{-1}	620±150Ngml^{-1}	600±150Ngml^{-1}	780±200Ngml^{-1}	760±90Ngml^{-1}	770±80Ngml^{-1}	400±60Ngml^{-1}
periplasm uninduced	320±200Ngml^{-1}	390±150Ngml^{-1}	510±200Ngml^{-1}	620±240Ngml^{-1}	610±200Ngml^{-1}	600±150Ngml^{-1}	200±90Ngml^{-1}
cell associated induced	560±170Ngml^{-1}	700±150Ngml^{-1}	620±170Ngml^{-1}	610±140Ngml^{-1}	660±180Ngml^{-1}	640±170Ngml^{-1}	100±90Ngml^{-1}
cell associated uniduced	300±50Ngml^{-1}	420±60Ngml^{-1}	500±60Ngml^{-1}	460±50Ngml^{-1}	420±120Ngml^{-1}	440±150Ngml^{-1}	90±70Ngml^{-1}

Table. 3.4.2.1 Levels of SpA, (Ngml-1) detected in pQR627 time course induction at 37 'C
(Assays repeated four times)

The most important feature of the 37°C time course expression is that the levels of SpA fusion in both induced and uninduced cultures are very similar. The level of total SpA fusion protein, in both induced and uninduced cultures, showing little fluctuation, over the observed time period, but with a slightly reduced concentration of SpA fusion in the overnight culture samples. In comparison the titre of recombinant SpA produced after overnight culture was less than that of the pEZZ18 culture.

The distribution of SpA fusion in the cultured cells did vary over time. The periplasmic fraction of both induced and uninduced cultures showed a slight increase in SpA fusion over 3 hours, overnight however the levels decreased to that comparable to the levels first observed. A similar pattern was observed in the cell associated fraction of the culture with a slight elevation in the levels of SpA-fusion over three hours followed by a decrease overnight. The decrease was more pronounced in the cell associated fraction, the levels of SpA fusion being markedly lower than the levels recorded 30 minutes after induction, and the overnight samples contained higher concentrations of SpA fusion in the periplasm than in the cell associated fraction.

The levels of SpA fusion in the culture supernatant remained fairly static over the first 3 hours, the induced cultures showing a slightly wider variation. Unlike the periplasmic and cell associated fractions the level of SpA fusion in the supernatant in both induced and uninduced cultures was increased overnight, and for the induced culture was detected at its higher concentration than for the first 3 hours.

The variation in the distribution of the SpA fusion of time suggests that the fusion protein was leaking out of the *E.coli* cells and into the culture supernatant as observed with previous SpA fusion proteins, (Nilsson *et al* 1991). Groups expressing antibody fragments in *E.coli* that have been shown to be cytotoxic to the host cells have reported a release of product into the supernatant due to cell lysis. The release of SpA fusion from pQR627 cultures could been taken as an indicator of toxicity, but as pEZZ18 cultures also secreted SpA into the supernatant it cannot be taken as a definitive indicator of the toxicity of the SpA-$C3V_1$ gene product on *E.coli.*

The level of expression of the SpA-$C3V_1$ gene was lower than expected for a T7 RNA polymerase controlled system (Deng *et al* 1990, Studier *et al* 1990,

Anthony *et al* 1992). These low levels of expression also suggest that the expressed fusion is cytotoxic to *E.coli* cells, supporting the evidence of the selection pressure shown by the β galactosidase clone selection when pQR627 was first created.(see section 3.3.2.2)

3.4.2.2 Time course expression of BL21 (DE3)pQR627 at 28°C

Time course experiments were also conducted at 28°C, using the same protocols as with the 37°C time course experiments. The assays were performed four times on the samples.

time after induction(hours)	0.5	1.0	1.5	2.0	2.5	3.0	O/N
supernatant induced	200±70Ngml^{-1}	220±70Ngml^{-1}	220±60Ngml^{-1}	200±70Ngml^{-1}	250±100Ngml^{-1}	260±90Ngml^{-1}	300±90Ngml
supernatant uniduced	200±80Ngml^{-1}	200±70Ngml^{-1}	220±70Ngml^{-1}	220±80Ngml^{-1}	230±80Ngml^{-1}	220±110Ngml^{-1}	290±110Ngm
periplasm induced	410±70Ngml^{-1}	520±180Ngml^{-1}	480±130Ngml^{-1}	610±190Ngml^{-1}	340±90Ngml^{-1}	450±140Ngml^{-1}	420±120Ngm
periplasm uninduced	430±120Ngml^{-1}	530±160Ngml^{-1}	560±150Ngml^{-1}	660±140Ngml^{-1}	630±180Ngml^{-1}	570±150Ngml^{-1}	460±180Ngm
cell associated induced	600±110Ngml^{-1}	570±110Ngml^{-1}	550±140Ngml^{-1}	560±160Ngml^{-1}	550±160Ngml^{-1}	590±180Ngml^{-1}	500±90Ngml
cell associated uniduced	550±150Ngml^{-1}	480±130Ngml^{-1}	440±140Ngml^{-1}	600±180Ngml^{-1}	520±160Ngml^{-1}	520±170Ngml^{-1}	390±90Ngml

Table 3.4.2.2 Levels of SpA, (Ngml-1) detected in pQR627 time course induction at 28 °C
(Assays repeated four times)

The time course induction of pQR627 at 28°C was comparably to the time course induction at 37°C. Although the total levels for SpA fusion production appear to be marginally lower for the 28°C cultures than for the 37°C cultures, the same features of SpA fusion expression are apparent in both cultures.

Both time courses showed a similar variation in SpA fusion distribution over time. The levels throughout the first 3 hours fluctuated for the levels of SpA fusion in both the periplasm and cell associated fractions but remained relatively static in the culture supernatant. However as with the 37°C cultures there was a marked increase in the concentration of SpA fusion in the supernatant in the samples taken after overnight growth, as compared to the first three hours. This increase was matched by a decrease in the periplasmic and cell associated concentrations of SpA, but the trend was less distinct than the 37°C culture trend. The variation in SpA fusion distribution over time again suggesting again that a certain amount of the recombinant protein was leaking out of the periplasm into the culture supernatant, although this was less pronounced in the 28°C cultures than in the 37°C cultures. In both cases the decrease in the concentration of SpA fusion in the periplasmic and cell associated fractions would probably be due to a combination of escape into the supernatant and proteolytic degradation by the *E.coli* proteases.

The concentration of SpA in the cell associated and periplasmic fractions of the 28°C cultures appear comparable, although marginally reduced, to those in the 37 °C culture, indicating that the temperature at which the pQR627 cells are cultured has little effect on the yield of recombinant protein. The levels of SpA in the periplasmic fraction of the uninduced pQR627 cultures as determined by ELISA are very marginally higher than those for the uninduced pQR627 cultures, indicating that the levels of expression in both cultures are only background levels even in the presence of IPTG. This suggests that the cytotoxic effect of the SpA fusion as observed by the selection pressure on the pQR627 clones when first created (see section 3.3.2.2) and in the 37°C time course expression experiments, is the overriding factor in the expression of the pQR627 protein.

The results of the time course experiments suggested that the large scale culture of pQR627 cells would not be feasible. Although further experimentation to optimise the expression system was feasible, such as culturing in different media and under different conditions, e.g. growth in a fermentor rather than simple

shake flask, the focus of the project was shifted away from expression of protein from pQR627 to its characterisation.

3.5 CHARACTERISATION OF SPA-C3V_L FUSION PRODUCED BY PQR627

The SpA fusion produced by pQR627 cultures was characterised by its relative molecular mass, its binding characteristics to the C3 hapten conjugate SF/HTf/1 and by its catalytic properties. The detection of any recombinant protein, in previous experiments, from pQR627 cultures has been dependant on the SpA domain of the fusion. No direct detection of the C3 V_l portion of the fusion has been attempted, and no direct evidence was available to suggest that the whole *spA*-C3V_l gene was being expressed, as only the SpA domain was detected. If the project could continue further it was necessary to determine if the whole fusion protein C3V_l was being expressed. If the whole *spA*-C3V_l gene was being expressed then the binding and catalytic properties of the C3V_l domain could be examined for comparison of those of the parent monoclonal antibody C3.

3.5.1 Western Blotting of pQR627 SpA fusion protein

The relative molecular mass of the SpA fusion was determined by western blotting. The relative molecular mass of the SpA fusion protein actually created in pQR627 cultures would indicate if the *spA*-C3V_l gene fusion was being expressed as a single protein, or if only a part of the fusion is present in the pQR627 cultures. The weight of the protein as predicted by the combined weight of the SpA binding domain and the weight of the C3V_l gene (approx 13.5KDa) which was predicted as 43-44KDa.

The western blotting of the periplasmic fraction of pQR627 cultures determined the relative molecular mass of the SpA fusion produced to be the same as the predicted relative molecular mass. This was the first evidence that the C3V_l gene was expressed with the *spA* gene, all previous evidence for production of recombinant protein in the pQR627 cultures relied only on the SpA domain. No direct evidence for the expression of the C3 V_l gene existed before the western blotting.

The SpA standard, fig 7.1 lane 6, is shown to consist of 4 distinct molecules, corresponding to sub units of SpA(Nilsson *et al* 1990 &1991). Both the pEZZ18 sample and pQR627 samples contained two separate bands that were detected by probing with anti-SpA IgG. The two pQR627 bands were of relative molecular masss 43KDa and 29KDa. The smaller 29KDa band was shown to be equivalent to the smallest band detected for the SpA standard, suggesting that the smaller

band was a single SpA binding domain and that the larger band, 43KDa was the SpA-C3V_I fusion, (29KDa +13.5KDa C3V_I). The presence of the two bands is evidence that there was some degree of cleavage of the SpA-C3V_I fusion produced in the BL21 (DE3)pQR627 cultures, the western blot detecting both fusion and cleaved SpA domain. The pEZZ18 samples also showed two bands, one 20.5KDa and one 31 KDa. The expected relative molecular mass of the pEZZ18 protein was 14KDa.

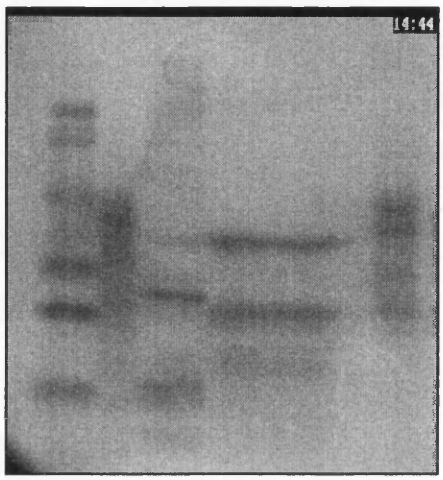

fig 3.5.1 Western blot of pEZZ18 and pQR627 samples probed with anti SpA IgG

Lane 1	Molecular weight markers,(KDa) 111, 77, 48.2, 33.8, 28.6, 20.8
Lane 2	purified SpA (sigma)
Lane 3	pEZZ18 culture samples
Lane 4	pQR627 periplasmic fraction sample
Lane 5	pQR627 periplasmic fraction sample
Lane 6	purified SpA (sigma)

The results of the western blot also had an effect on the ELISA assays used to measure expression levels from pQR627 and pEZZ18. These ELISA assays were detecting the combined level of SpA-C3V_I fusion and cleaved SpA from pQR627. The assays were calibrated using the SpA sample in lane 6, as a result the concentration of the control was not that of a single protein. The mass of protein weighed to make up the standards, was the mean mass of the mixture of 4 proteins. If both the SpA-C3V_I fusion and the purified SpA were a single protein as defined by the western it would be possible to convert the concentrations of SpA fusion produced by the ELISA, from units of SpA equivalent concentration which the ELISA was actually measuring, to units of actual protein

concentration, using the relative relative molecular masss determined by western. Due to the nature of the SpA standard as shown by the western it was now impossible to make this correction to the ELISA assays performed on the pQR627 expression experiments. The ability of the assays to determine protein concentration was marred by an unknown level of error introduced by the SpA standard not being a single protein.

3.5.2 Binding of pQR627 SpA fusion protein to C3 hapten conjugate SF/HTf/1

The binding of the SpA $C3V_1$ fusion to the original C3 hapten was assayed using an ELISA system. As no genuine positive samples from the original C3 abzyme programme existed it was necessary to develop a novel binding assay for the analysis of the binding properties of the SpA $C3V_1$ fusion produced by pQR627 cultures.

The ELISA system was based on the ELISA used to detect SpA in pQR627 cultures, the rationale behind the assay was that if the SpA portion of the pQR627 SpA $C3V_1$ could be detected in the periplasm of pQR627 cultures, then the $C3V_1$ portion could be detected by a similar system, if it retained C3 binding activity. The C3 binding activity ELISA utilised the bifunctional nature of the SpA $C3V_1$ fusion produced by pQR627. The transferin-hapten conjugate was immobilised onto the ELISA plate in place of the goat IgG, so that the $C3V_1$ portion of the pQR627 protein could bind, if it retained any of the original C3 binding activity. The same protocol as for SpA detection was used to detect the SpA portion of pQR627 protein that bound to the hapten conjugate. The ELISA was positively controlled by using the SpA detection ELISA protocol performed concurrently with the SF/HTf/1 binding ELISA on the same plate. The binding assays were negatively controlled by transferrin immobilised onto the plate. This negative control was necessary to determine if the SpA domain of the fusion bound to the transferrin portion of the hapten-transferrin conjugate, if the SpA domain did bind to transferrin it would cause an apparent increase the binding between the SpA-$C3V_1$ fusion and SF/HTf/1 and create a false positive for the $C3V_1$ binding assay. Khalaf *et al* (1992) had already established that C3 did not bind transferrin when screening the original C3 hybridoma fusion cultures. The samples used for the binding assays were the periplasmic fractions of pQR627 cultures, taken from the same culture, so that the sample being assayed for SpA activity was identical in every way to the sample being assayed for C3 binding activity.

immobilised protein	mean absorbance at 420nm
Goat IgG	0.740±0.164
SF/HTf/1	0.194±0.042
Transferrin	0.177±0.052

Table 3.5.2.1 Binding ELISA for assay of C3 binding activity in periplasmic samples of pQR627 SpA C3V$_l$ fusion

In an attempt to increase the signal from the SF/HTf/1-C3V$_l$ binding in the ELISA a sample of the periplasmic fraction was concentrated, using an amicon centrifugal concentrator. The binding assays were repeated with the concentrated periplasmic fraction sample.

immobilised protein	mean absorbance at 420nm
Goat IgG	1.127±0.35
SF/HTf/1	0.178±0.049
Transferrin	0.198±0.054

Table 3.5.2.2 Binding ELISA for assay of C3 binding activity in concentrated periplasmic samples of pQR627 SpA C3V$_l$ fusion

The results of the binding assay (tables 7.2.1 & 7.2.2) for the periplasmic and concentrated periplasmic samples of pQR627 both failed to show any binding activity for the hapten conjugate, SF/HTf/1.

The binding assays of the pQR627 SpA C3V$_l$ fusion protein were hampered by the lack of any original C3 hapten binding assays, or of any original C3 positive controls in order to validate the assays or to calibrate them. The assay system used for analysis of the C3V$_l$ binding activity was limited by the fact that unless the binding affinity of the C3V$_l$ to the C3 hapten was of the same order as that for the binding affinity of SpA for IgG, the calibration of the assay would mean that the binding activity of the C3V$_l$ would be below the lower limit of detection in the assay. However previous attempts to assay the binding activity of isolated V$_l$ domains of Fv's have produced data that suggests that V$_l$ domains do not retain the binding activity of the parent molecule. Pluckthun *et al* (1988) demonstrated that the V$_l$ of the antibody McPC603 possess none of the binding activity of the parent antibody. The results of the pQR627 SpA C3V$_l$ binding assays indicate that this is also the case for the C3 V$_l$. However because of the problem of calibration of the binding assays it was still possible that the C3V$_l$ was retaining a

fraction of the original activity, that could not be detected by the binding assay, even though the binding assay showed no evidence for activity.

3.5.3 Catalytic Properties of pQR627 protein

It was possible to attempt to use catalytic activity to determine if the $C3V_I$ domain retained any of the original IgG activity. The use of the C3 catalytic activity , to probe for C3 antibody fragment binding activity, was an original aim of the project. However as with the binding assays no original C3 material was available and so novel positive controls would have to be developed.

To perform catalysis assays pQR627 SpA $C3V_I$ protein was purified from periplasmic extracts of pQR627 cultures by affinity chromatography using Human IgG agarose (**2.7**). Eluted fractions were assayed for SpA activity and positive fractions pooled. Catalysis assays were performed on both purified SpA $C3V_I$ protein and SpA-$C3V_I$ containing periplasmic fraction from pQR627 culture.

Samples from the periplasmic fraction of BL21 (DE3)pQR627 and SpA purified fraction were pH adjusted to pH8.5. The samples were incubated with substrate in a 1ml cuvette. The progress of the reaction was monitored by the increase in absorbance at 410nm in a Uvikon922 spectrophotometer. The rate in the absence of SpA-$C3V_I$ was determined by incubation of the substrate in PBS pH8.5 and progress of the reaction monitored by the increase in absorbance at 410nm in a Uvikon922 spectrophotometer.

The periplasmic fraction of pQR627 cultures was used as a positive control for the catalysis assays. The theoretical reasoning behind this decision was the fact that the *E.coli* periplasm contains esterase that may exhibit activity against the C3 substrate. The genuine $C3V_I$ esterase activity would be determined by assay using the SpA-$C3V_I$ fusion purified by affinity chromatography with human IgG.

substrate concentration (mM)	periplasmic fraction (ΔA_{410})/min	SpA fraction (ΔA_{410})/min
0.05	0.0038±0.0003	0.0019±0.0001
0.10	0.0036±0.0003	0.0018±0.0005
0.15	0.0040±0.0005	0.0020±0.0002
0.20	0.0042±0.0002	0.0024±0.0002
0.25	0.0055±0.0002	0.0020±0.0002

Table 3.5.3 Catalytic activity of pQR627 periplasmic fraction and SpA purified fraction

The reaction was followed by change in absorbance at 410nm per unit time. As no obvious correlation between rate and substrate concentration was observed a mean rate from these experiments was used. Both unpurifed periplasmic extract and to a lesser extent the purified SpA fraction did appear to catalyse the hydrolysis of the substrate to produce 4-nitrophenol. The relative activity of the periplasmic fraction to the purified SpA fraction was determined in order to assess to what extent the observed activity was due to SpA-C3V_1 fusion and what was caused by the contents of the periplasm, the ratio of activities per unit SpA was determined. The purifed SpA fraction possessed 79% ± 26% of the periplasmic fraction activity, indicating that a large proportion of the observed activity was due to the presence of the SpA-C3 V_1 fusion.

Using this catalysis data it was also possible to compare the activity of the SpA-C3 V_1 fusion, to the catalytic properties of the whole C3 IgG as published by Khalaf *et al* (1992) and achieve a partial goal from the original concept of the project by comparing acivity of a whole antibody to that of a V_1 domain derived from it. The catalysis assays were performed with the same substrate, but produced at different times, but as the two batches of substrate were found to be identical by NMR a comparison between published data and the experimental data described here would be valid.

Using the experimentally derived mean rate for SpA-C3 V_1 fusion catalysis, the extinction coefficient for 4-nitrophenol, and the monomeric relative molecular mass of the SpA-C3 V_1 fusion as determined by western it is possible to determine a specific activity value for the SpA-C3 V_1 fusion.

Mean activity from purified SpA fraction = 0.0020 ΔA_{410} units per minute

moles product formed = $\Delta A_{410} \div$ extinction coefficient of 4-nitrophenolate

$= 0.002 \div 15 \times 10^{6} = 1.3 \times 10^{-10}$ moles 4-nitrophenolate formed per minute

moles SpA-C3V_l = mass SpA-C3$V_l \div$ Mw SpA-C3V_l

$= 0.65 \times 10^{-6}$g$\div 43000 = 1.5 \times 10^{-11}$ moles

specific activity of SpA-C3V_l = moles product per min per mole SpA-C3V_l

$= 1.3 \times 10^{-10} \div 1.5 \times 10^{-11} = 8.6$ moles product per min per mole SpA-C3V_l

SpA-C3V_l specific activity = 8.6 moles product per min per mole SpA-C3V_l

Khalaf *et al* (1992) published the whole C3 IgG k_{cat} as $2.4s^{-1}$. Converting this k_{cat} into specific activity in the same units as for SpA-C3 V_l fusion gives a value of

$2.4 \times 60 = 144$ moles product per min per mole C3 IgG

By comparison of experimental SpA-C3 V_l fusion specific activity and published whole C3 IgG specific activity the SpA-C3 V_l fusion as produced by expression in *E.coli* from the construct pQR627, possess 5.9 % of C3 IgG activity.

Due to the time limits on the project necessary control experiments were not performed. With the experimental data obtained the calculation of the K_M was not possible. The measurement of activity over a range of SpA-C3 V_l would be necessary to determine the enzyme concentration acivity relationship and thus K_M for the SpA-C3 V_l fusion. To determine if the SpA-C3 V_l fusion truly possessed catalytic activity inhibition experiments would have to be performed, using the hapten SF/HTf/1. A control SpA only catalysis experiment would also prove that the catalytic properties of the SpA-C3 V_l fusion were associated with the V_l domain or the whole fusion protein.

The unknown error introduced by the SpA used to calibrate the concentration of SpA-C3V_1 fusion used in the catalysis experiments, introduces the same unknown error into the specific activity calculations, the calculation of moles SpA-C3V_1 fusion being dependant on the results of an ELISA assay to determine protein concentration.

4 DISCUSSION

The cloning and expression of the C3 V_l gene provided evidence that the C3 V_l gene product was cytotoxic to *E.coli* cells. The first evidence of this toxicity was the selection pressure for counter pPANG1C3scFv orientation gene inserts in the pQR627 constructs, (**3.3.2**). Knappic and Pluckthun(1995), when describing the observed effects of the expression of recombinant antibody fragments in *E.coli* include the loss of clones from plasmid libraries as the severest of indicators of toxicity. The logical explanation for this selection pressure being the presence of the expressed *spA*-C3V_l gene product. This toxicity effect has been described by several other authors including Somerville *et al*(1994) and Forsberg *et al* (1997).

The second part of the cytotoxicity evidence is the comparison between the levels of SpA fusion in pQR627 and pEZZ18 cultures, both constructs were expressing in the same *E.coli* strain but produced marked difference in the titres, particular the differences between the levels of SpA-C3V_l fusion detected in the overnight samples taken in the time course experiments from pQR627 and those detected in the pEZZ18 control cultures. The levels of expression from the pEZZ18 vector correspond to those expected suggesting that the BL21 (DE3) cell line will produce recombinant SpA protein, and that the expression of the recombinant SpA does not have a derogatory effect on the *E.coli*, and so any toxic effect of the SpA-C3V_l fusion was due to the V_l section of the fusion. As both pEZZ18 and pQR627 cultures had recombinant SpA in the culture supernatant, the presence of recombinant product in the supernatant cannot be taken as a definitive indicator of toxicity. Pluckthun *et al* (1995) had demonstrated the cytotoxicity of the expression of McPC603 Fv on *E.coli* by the measurement of product released into supernatant, product release being due to host cell lysis. The ability of recombinant SpA to be secreted , as described by Nillson *et al* , and demonstrated by the pEZZ18 expression described here, would effectively mask the release of SpA-C3V_l fusion into the supernatant by cell lysis.

The yield of SpA-C3V_l fusion as determined by ELISA was lower than would be expected for a vector utilising the T7 RNA polymerase/ promoter system that pQR627 utilised. By comparison other groups expressing Fv and scFv fragments in *E.coli* using a T7 RNA polymerase based system Burks *et al*(1995) describe a vector host system capable of producing 120mgL^{-1} of a digoxin binding scFv in shake flask culture . Anthony *et al*(1992), using a similar shake flask expression

system produced 14mgL^{-1} of active Fv using a periplasmic export system. The antibody fragment expressed by Anthony *et al* was the Fv of the antidigoxin antibody 26-10, the Fv gene being fused to *ompA* and under the control of the T7 promoter, Anthony *et al* noted that the use of a T7 expression system increased the levels of active Fv produced over a less powerful system such as *Lac* operon system. Direct comparisons between the expression of the antibody fragments by Anthony *et al*(1992) and Burks *et al*(1995) and the expression of C3V_l from pQR627 cannot be made due to the fact that the culture conditions between these expression experiments were not identical, however SpA-C3V_l is apparently produced at a level several orders of magnitude less than has been previously demonstrated for a similar system. The difference between the expression levels that a T7 system has been shown capable of producing, and that of pQR627 suggest that the presence of the V_l gene sequence in the *spA*-C3V_l gene fusion is having a derogatory effect on gene expression. Knappic and Pluckthun (1995) , Somerville *et al*(1994) and Forsberg *et al*(1997) all cite the toxicity of the antibody fragments they expressed in *E.coli* as a reason for low expression titres.

This antibody fragment sequence dependent expression level effect has been observed by many other researchers and could be interpreted, in the case of pQR627 in several ways. Firstly that the expressed sequence itself is a cytotoxic sequence, and as a consequence retards the expression levels of the spA-fusion. Second that the expressed protein is cytotoxic, and that cytotoxicity to *E.coli* is a function of the residual catalytic activity of the C3 V_l . Third that V_l only expression in *E.coli*, for C3 will only produce poor yields, and that the expression of undimerised V_l is responsible for the cytotoxic effect.

Many groups have expressed different antibody fragment in various *E.coli* expression systems, in shake flasks and fermentors. The yields from these experiments varied, from apparent high level expression to low level expression, but direct comparison of the results is not possible, due to the different expression systems. Several groups have tried to establish a sequence expression relationship by constructing and expressing mutant antibody fragment sequences, under identical conditions, to try and improve the expression of the fragment. These studies have also tried to establish sequence toxicity relationships as well.

Somerville *et al*(1994) expressed an scFv from a tumour binding antibody L6 in *E.coli*, and observed that the expressed protein had a cytotoxic effect on the host *E.coli*. The L6 scFv was expressed from two vectors, pIG101 as a fusion to *pelB*

and pIG373 without a *pelB* leader, in a range of hosts including HB101, JM101 and DH5-α . Induction of the *pelB*-L6scFv in any of the hosts lead to a loss of cell viability. Cells transformed with pIG101 were grown overnight and the presence of the plasmid determined, more than 99.99% of the cells from the overnight culture did not contain the pIG101 plasmid. The toxicity of L6scFv was determined by the cell viability and plasmid instability, it was also shown that the toxicity was associated with the transport of the antibody fragment to the periplasm. Comparisons between the *pelB* leader sequence plasmid, pIG101, and the plasmid without *pelB* pIG373, showed that the pIG373 transformed cells did not lose viability and did not undergo plasmid loss. Expression of L6 scFv from both plasmids was described as very low level, with total levels of L6 scFv from pIG101 as 59 $ngml^{-1}$.

Forsberg *et al*(1997) used an *E.coli* system to express a Fab molecule from the monoclonal antibody 5T4, the 5T4 Fab was expressed at a low level and was found to be toxic. Forsberg *et al* carried out chain swapping experiments to produce 5T4 Fab hybrids with another antibody Fab. The relative expression of these hybrid Fabs indicated that the 5T4 V_l domain was the expression limiting sequence. A number of mutants were constructed to probe its sequence toxicity relationship. The theoretical basis of the mutants was to test if increasing the hydrophillicity of the expressed protein also improved the production or decreased its toxicity. The mutants all carried a changes in the framework regions of the V_l. The cumulative effect of the seven point mutations on the yield was to increase it 15 fold. Even single residue changes had a beneficial effect on expression titre, a single Phe→Ser change at position 10 increased the expressed Fab from 39 to 92 $mgml^{-1}$. A model of 5T4 had been constructed which placed the position 10 Phe on the surface of the framework region and so was exposed rather than buried deep in the protein.

Kipriyanov, Moldenhauer, Martin *et al* (1997) reported that two single mutations could increase the yield of scFv in *E.coli.* The group had cloned the antihuman CD3 antibody OKT3 and expressed it as an scFv in *E.coli.* In order to increase the titre of scFv the group constructed a model in the same way that Forsberg *et al* did to identify residues that could exert an effect on expression. The OKT3 V_H sequence possessed two Cys at positions 92 and 100, in the functional form of the Fv the Cys at position 92 forms a disulphide with a Cys at position 22, the Kipryanov group reasoned that during folding the position 100 Cys may form a disulphide with the position 22 Cys leading to an incorrectly folded aggregating

product and that expression of the functional protein could be decreased because of this incorrect alternative disulphide. The position 100 Cys was replaced by Ser, the resultant mutant doubled the yield of expressed scFv, and the scFv retained its antigen binding characteristics. The second change had a more pronounced effect on the expression level than the Cys→Ser change. By comparison with the consensus sequences of the Kabat database the OKT3 V_H gene was determined to be mouse immunoglobulin subgroup IIb. The OKT3 V_H gene contained a glutamic acid at position 6, the consensus mouse immunoglobulin subgroup IIb position 6 was a glutamine. The position 6 residue was substituted for the consensus and the expression levels for scFv increased 30 fold and again did not change the antigen binding. The effect of both changes was cumulative the expression levels for the double mutant being 66 fold greater than for the original OKT3 sequence. However the Kipriyanov group could not give a mechanistic explanation for the position 6 change and could only speculate that position 6 was important in the correct folding of the V_H domain and that the change affected some folding intermediate.

Knappic and Pluckthun(1995) expressed different antibody sequences using the same system to indicate the effect of the antibody sequence itself on the yield of fragment. Two antibody sequences, McPC603, the murine myeloma antibody, and a humanised antibody hu4D5, were expressed under the same conditions, in the same vector host system, as Fv scFv and Fab fragments. The two sequences had a high degree of sequence homology between the respective framework regions the V_H framework regions being 78% identical, and the V_l 75%. All the hu4D5 fragments were expressed at a relatively much higher level than for the McPC603 fragments. A larger proportion of McPC603 was found in soluble exclusion bodies, than for hu4D5, the majority of expressed hu4D5 being correctly folded in the periplasm. The McPC603 fragment appeared to exert a toxic effect on the expressing bacteria as the growth profiles for McPC603 expressing cultures were retarded in comparison with the hu4D5 cultures. The leakiness of the bacteria in both cultures was compared by measurement of β-lactamase release into the culture supernatant, the McPC603 cultures were releasing greater amounts of β-lactamase into the media, than the hu4D5 cultures, showing that the McPC603 was causing membrane leakiness and eventual cell lysis. Knappic and Pluckthun(1995) constructed a series of back engineered McPC603 mutants designed to increase the expression of active fragments. A single mutation in McPC603 framework region significantly decreased the toxicity of the fragment, but did not reduce the levels of insoluble protein

aggregates. An unrelated double framework mutation significantly decreased the amounts of insoluble protein but did not decrease the toxic effects of McPC603. A construct containing all three changes increased the levels of expressed McPC603, but even the triple mutation McPC603 produced antibody at a lower level, with more insoluble protein and with reduced cell viability compared to the hu4D5. The group also grafted the McPC603 CDR's onto the hu4D5 frameworks, this McPC603/hu4D5 hybrid also showed reduced cell viability and greater aggregated product than for hu4D5.

This work demonstrated that the antibody sequence affected yield of active fragment in two ways. Knappic and Pluckthun had established that the yield was limited by folding problems of the fragments in the periplasm, and by the cytotoxic effects of the expressed fragment. The two effects were unrelated, the mutation that decreased the toxic effect did not improve the folding, and the converse was also true. This implies that it is not the aggregation of protein that causes the cytotoxicity, the different mutant constructs showed that the two effects were independent of each other. The constructs were expressed as Fv, scFv and Fab and each of the antibody fragments displayed the same characteristics, proving that the aggregation and toxicity effects were defined by the variable region sequence and not by the nature of the fragment itself. Knappic and Pluckthun(1995) concluded that the leakiness and cell lysis effects of the expressed protein was due to interaction with cellular components during the transport or folding of the sequence. The toxic effect of the antibody sequence was due to residues in both framework and the CDR's, framework mutations improved cell stability, but the McPC603/hu4D5 framework hybrid still displayed reduced viability in comparison with hu4D5, indicating that residues in the hypervariable loops were a component in the toxicity.

There is evidence that the expression of a single variable region in isolation will not match that observed for the whole Fv. Anthony *et al*(1992) not only expressed the whole 26-10 Fv, but also the V_H and V_l separately. The difference in the expressed levels of the two proteins from the same system was remarkable, the V_l expressed independently appeared to match the whole Fv level of expression. The isolated V_H however showed a marked decrease, against both Fv and V_l. Ulrich *et al*(1995) expressed a number of abzyme Fabs in *E.coli* and also expressed isolated light chain sequences, and described the expression levels of the light chain only constructs as strongly reduced compared to complete Fab fragments. Previous work had shown that expression yields of humanised Fabs,

in *E.coli*, was greater than those for the equivalent murine Fabs, with chimeric Fabs giving intermediate yields between humanised and murine Fabs (Carter *et al.* 1992). The aim of Ulrich *et al* work was to evaluate the utility of chimeric Fabs, as a general methodology for the production of Fabs. The use of chimeras increasing yield, but without the possible loss of antigen binding due to whole humanisation. The Ulrich group cloned a panel of 8 abzyme Fabs and expressed them in *E.coli* as chimeric Fabs. The expression yields varied with Fab sequence, but the group found no obvious correlation between the sequence and expression levels. The group subsequently compared the yields of the hybridoma derived Fabs and combinations of chains from one abzyme with the other seven abzymes to test the chimeric expression system for the production of antibody fragments where the light and heavy chains were not a pairing produced by an immune response, such as those combinations possible using a phage display library. In these studies the yield of the various pairings appeared to be limited by the poorer expressing partner, the levels of each of the different pairings being described as no worse than the lower yield partner's parent Fab.

Kim *et al* (1997) expressed a series of 4-nitrophenyl ester hydrolysis catalysing abzymes in *E.coli* as Fv and scFv. Their work centred on two abzymes D2.3 and D2.4 that catalysed the same reaction. The group expressed D2.3 and D2.4 scFv's and chain swapped hybrids, then measured the relative expression titres. They concluded that the D2.3 V_l decreased expression as the D2.3V_H/D2.4V_l showed an increase in expression levels over the D2.3 scFv and the D2.4V_H/D2.3V_l showed a decrease in expression levels from the D2.4 scFv. The same pattern of expression occurred for the Fv constructs the group created. Although the sequence of the D2.3 V_l appeared to have a derogatory effect on expression titres, it was not reported as having a toxic effect on the host *E.coli*. The fact that there was a difference in the levels of abzyme scFv produced by Kim *et al* and that these scFv catalysed the same reaction also suggest that it was the sequence not the catalytic activity that determined expression levels.

The argument that the cytotoxic effect is caused by the residual catalytic activity of the C3 V_l is the least supported. Stathopoulos *et al*(1996*)* described an OmpA-PhoA(bacterial alkaline phosphatase, a dimeric periplasmic enzyme) fusion expression system where the expression of the OmpA-PhoA fusion from a high copy number plasmid caused a cytotoxic effect. However both Ulrich *et al* and more relevantly Kim *et al* have expressed abzyme fragments in *E.coli* in an active form without reporting any toxic effects. The D2 series of abzyme fragments

expressed by Kim *et al* catalysed similar reactions to C3, the hydrolysis of 4-nitrophenyl esters. The expression titre of the recombinant abzymes was low, but no cytotoxic effects were observed. This would suggest that the production of the D2 abzymes was sequence dependant in the same way as the expression in *E.coli* of other antibody fragments is. The abzyme fragments were determined to retain the parental monoclonal catalytic activity and so infer that the presence of a heterologous esterase enzyme, such as D2, does not effect the viability of host cells.

The work of Anthony *et al* (1992) and Ulrich *et al* (1995) does however that sometimes an isolated variable domain will only express at a very low level compared to the whole Fv or Fab, and this may be the case with the $C3V_l$. The antidigoxin 26-10 Fv expressed by Anthony *et al* did not exert a toxic effect on the host *E.coli* cells, but expression of the isolated 26-10 V_H was drastically less than that of the whole Fv and V_l which expressed at equivalent levels and the V_H also did not possess a toxic effect. However the most likely explanation for both the cytotoxicity and low expression titre of C3 V_l would be that the sequence itself was the cause of the observed cytotoxicity.

The validity of these arguments could be tested by the creation of a number of hybrid scFv constructs based on the C3 V_l gene. If the C3 V_l gene was spliced into vectors carrying the V_H genes of other antibodies, a library of hybrid C3 V_l scFv sequences could be created, comparable to the hybrids created by Ulrich *et al.* Comparative expression of the hybrid scFv sequences, with the expression of $C3V_l$ and of the expression of the individual V_H sequences used to construct the hybrid scFv library, would determine if the presence or absence of a V_H sequence causes the cytotoxic effect. If the levels of antibody fragment increased, in scFv form, over those for the isolated $C3V_l$ then the cytotoxic effect would be established as being caused by the lack of heterodimer partner. The combination of the low yield V_l with a V_H partner may increase the yield of hybrid Fab, as described by Anthony *et al.* If the levels of scFv expression were decreased from that observed for isolated V_H then that would support the theory that the $C3V_l$ sequence itself was cytotoxic. The work of Ulrich *et al* and Kim *et al* suggested that for hybrid Fv or scFv sequences the expression titre will be determined by the lowest expression titre parent Fv. Any $C3V_l$ hybrids would therefore theoretically be produced with the same yield as for a whole genuine C3scFv which may be low due to the C3 sequence itself. The expression of C3 V_l in a hybrid scFv may not be the best strategy for increasing yield but would provide

evidence for the effect that isolated variable domain expression has in terms of protein yield and host cell toxicity.

The best way to establish a sequence toxicity and a sequence expression relationship for C3 V_l would be to create a series of mutants and compare the mutant and existing sequences for expression titre. Two strategies could be attempted to increase expression yield, the first would be that of the single point mutations as described above. No group generating single point mutants has yet been able to describe a unified strategy for increasing yield. Although Forsberg *et al*(1997)) operated on the assumption that increasing the hydrophillicity of the protein increased yield the Kipryanov (Kipryanov *et al* 1997) group assumed that their mutations helped stabilise a folding intermediate they could not define a mechanism for this. Knappic and Pluckthun(1995) could not give a definite mechanistic explanation of why the mutations increased yield, only that the mutants they made prevented the expressed protein from aggregating and that toxicity reducing mutations prevented possible folding intermediates interfering with the *E.coli* folding machinery. However by comparison of the mutants constructed by the above groups and the sequence of C3V_l two possible residues as candidates for reengineering have been identified. Two of the mutations made by the Forsberg group were V_l Thr77→Ser and Leu78→Val, and both were found to increase yield. Both these residues Thr77 and Leu78 are present in the C3 light chain sequence so based on the work of Forsberg *et al*(1997) the mutations Thr77→Ser and Leu78→Val would possibly increase the production levels of C3V_l in the same way as these mutations increase the 5T4 Fab yield.

All the sequence-expression relationship work has been based on the creation of mutants derived from a model of the antibody fragment. As no model for C3V_l has been created then any mutations appear to be pure speculation. The fact that both 4T5 and C3 possess the same residues at positions 77 and 78 may be sheer fluke as the surrounding residues show no homology. In the absence of a model and the C3V_l Thr77→Ser and Leu78→Val mutants, it is impossible to infer that the same increase in yield will occur, and that the same mechanism for an increase in 4T5 Fab and C3V_l expression levels exists.

The second strategy would be the strategy of loop grafting devised by Jung and Pluckthun(1997). The McPC603/4D5 CDR/framework hybrids, Knappic and Pluckthun(1995), demonstrated that both framework and CDR loop residues can influence the toxicity sequence relationship, they also demonstrated that the

antigen binding characteristics of a toxic, poorly expressed antibody fragment can be transferred to a higher expression titre fragment by grafting the hypervariable loops onto the superior expression frameworks. Jung and Pluckthun demonstrated the versatility of this approach, by extending the 4D5/McPC603 grafting work, but instead of using antibody sequences with a high degree of homology, as in the 4D5/McPC603 hybrids, grafting sequences from unrelated antibodies. The technique of loop grafting is one that is generally applicable to antibody engineering, the technique itself is developed from the humanisation techniques of Winter *et al* (review Winter and Milstein 1991). The CDR loops of the 4-4-20 antibody were grafted onto the frameworks of the 4D5 antibody. The strategy for grafting the hybrids was designed from a comparison of the two models for 4-4--20 and 4D5, derived from crystal structures. In addition to the grafted loops certain residues in the 4D5 frameworks were changed so that the orientation of the CDR's in the hybrid remained the same as in the parent 4-4-20 molecule, in all four changes were made to the framework regions to support the new CDR conformations. The 4-4-20/4D5 hybrid, 4D5Flu, was expressed in *E.coli* and purified at a yield of $2.5mgL^{-1}$ of culture at 24°C, under the same conditions no correctly folded 4-4-20 scFv was obtained. The grafting of the $C3V_I$ CDR's onto known higher expressing framework regions would circumvent the difficulties of low yield, however as stated above the CDR's themselves have an influence on the expression levels but loop grafting has been demonstrated as a facile and versatile way of increasing functional yield of antibody fragments.

The use of a loop grafting technique would probably increase the yield of the $C3V_I$. the sequence toxicity and sequence yield relationships having been shown to depend on both framework and CDR loop residues. It would also be a simpler way of increasing the yield than to try to improve expression characteristics by point mutations, as no comprehensive strategy to manipulating the sequence expression relationship has been proven. Loop grafting between antibodies with little sequence homology is feasible, the loop grafted hybrids retaining the antigen specificity of the parent antibody fragment. However as with humanisation and the 4D5/4-4-20 loop grafted constructs the role of framework residues in antigen binding is important. The construction of a molecular model for C3 V_I could identify which, if any framework residues are in contact with the antigen when bound, and which are crucial in maintaining the correct orientation of the hypervariable loops. In the absence of such a model a 'blind' loop graft onto a high yield framework such as 4D5 could increase yield but at the cost of antigen binding. With a greater amount of even 'blind' grafted material it would

be possible to conduct X-ray and NMR structure studies to generate a model for the 'blind' graft. This model could be used, by modelling antibody antigen or hapten interactions, to identify which residues, if any should be reverted to original C3 V_l residues in order to regain antigen binding. The re engineering of a high yield antigen active C3 V_l domain would be an achievable, but non trivial task.

The result of the C3 catalysis assay was unusual and unexpected. Most researchers that have expressed either isolated light or heavy chains have found that the antigen binding properties of the whole antibody have not been retained by the isolated variable domain. Some researches have demonstrated that some V_H domains do retain the parental IgG antigen binding characteristics. These observations are consistent with the theory that the V_H CDR3 plays the pivotal role in both antigen binding, and for abzymes catalysis. It would be expected that the C3 V_l would neither bind the hapten SF/HTf/1 or catalyse the hydrolysis of the C3 substrate, due to the lack of C3 V_H. The results of the binding assay indicated that C3 V_l did not bind the hapten, but the binding assays themselves are open to interpretation because of the lack of a C3 hapten binding positive control. The catalysis assays, although flawed are more interesting than the binding assays and did suggest that a fraction of the C3 activity (5.9%) was retained by the C3 V_l. This would be an unusual result due to the lack of the important V_H CDR3 at the active site. The catalysis experiments are flawed in two ways, firstly that they were not repeated due to time pressures, and second that adequate controls were not included. The lack of any inhibition data using the hapten SF/HTf/1, and catalysis data over a range of SpA-C3V_l fusion concentrations, means that the characterisation of the catalytic properties of the fusion is not complete. The comparison of specific activity for the SpA-C3V_l fusion and C3 IgG cannot be regarded as a definitive comparison because of the flaws in the catalysis data. The figure of 5.9% of activity retained by SpA-C3V_l cannot be regarded as an absolute proportion because of these gaps in the data, and the uncertainty introduced by the calibration of the ELISA assay used to determine the concentration of SpA-C3V_l fusion in the catalysis assay, but does give an indication that a fraction of the C3 activity is retained by the light chain, which is an unusual result considering the importance of the V_H CDR3 in antigen binding, and for abzymes catalysis.

However there has been a report of a catalytic light chain that retains activity, even without a heavy chain partner. Gao *et al*(1994) have cloned the light chain

from an antibody raised against vasoactive intestinal peptide (VIP). The light chain was found to catalyse the hydrolysis of VIP, and the hydrolysis of an unrelated tripeptide. The Gao group concluded that the both reactions are catalysed at a common catalytic site and that the light chain possessed a serine protease like catalytic site that displayed poor discrimination between substrates. The catalytic activity of this isolated light chain was compared to that of the non specific protease trypsin, the catalytic efficiency (k_{cat}/K_M) of the light chain was found to be 53 fold lower than that of trypsin. The work of Gao *et al* did demonstrate that it was possible for an isolated domain, such as C3 V_l, to be expressed in an active form in *E.coli* without a heavy chain partner. Although there was no comparison of whole antibody and light chain activity, this work also demonstrated that a recombinant isolated light chain can express a catalytic function, despite the lack of the important V_H CDR3 antigen binding loop. A molecular model of the light chain was constructed and used to locate a possible catalytic site that contained a catalytic triad found in serine proteases, Asp, Ser and His located in a hydrophobic pocket. The apparent binding and hydrolysis of C3 substrate by the SpA-C3V_l fusion is not therefore as surprising if no isolated light chain had been independently demonstrated. Due to these flaws the final result of the project, the comparison of the experimentally determined specific activity of the SpA-C3V_l produced from pQR627 and the specific activity of C3 IgG as published is not intended as a definitive comparison. The comparison is however an indication that at least a proportion of the original activity of the whole C3 IgG is retained. If the ratio between SpA-C3V_l and C3 IgG activity had been an order of magnitude or greater less than the estimate of activity generated here, then the inference that activity is retained by the V_l would be invalid.

The failure of both the C3 hybridoma culture and pPANG1C3scFv to produce the expected protein products has left this project with no definitive positive controls. The positive controls used in the work have been the closest reasonable alternatives that were available to the absolute positives, which would have been the C3 IgG itself and for the *E.coli* pQR627 expression work a second purified SpA-V_l fusion preferably produced from pPANG1. The design of the experiments has therefore been compromised and as a result almost all the experimental data included here includes a level of error that is incalculable. The positive controls used in ELISA assays for C3 IgG (**3.1**) failed to produce a positive response with either the existing hapten KI/HTf/1 or the freshly prepared hapten SF/HTf/1. The mouse anti IgG assay developed was demonstrated to be able to detect mouse IgG from hybridoma, and so prove that the C3 hybridoma

culture was not producing IgG. The lack of C3 IgG meant that the whole periplasmic fraction from pQR627 cultures had to be used as a positive control in the catalysis assays. Although the periplasmic fraction did have a greater activity than the purified SpA fraction, it was far from a perfect control for these catalysis assays. The ELISA assays that detected the expression of the SpA-C3V_l fusion from pEZZ18 and pQR627, also lacked a genuine positive control. The difference in relative molecular mass between the purified SpA used as a positive control, and the recombinant SpA produced in *E.coli* introduced an error in the expression titre data, which theoretically could have been removed when the relative molecular mass of each species was determined by western blotting, and the ratio between relative molecular masss used to correct the ELISA data. The multiple species in each sample, as detected by western blotting (see section 3.5.1), meant that this correction for different relative molecular masss was not possible. The expression titre of SpA-C3V_l could have been positively controlled by the expression of an unrelated V_l or scFv SpA fusion from the pPANG1 vector. Such a control would produce more accurate data on the expressed level of the SpA-C3V_l and would provide a comparison of absolute production between sequences from near identical vectors and so confirm the cytotoxicity of the C3 V_l sequence. The binding assays on SpA-C3V_l using SF/HTf/1 (see section 3.5.2) were also not positively controlled, again because of the lack of C3 IgG.

The loop grafting of C3V_l could also lead to the recreation of an abzyme scFv with similar properties to that of the original C3 antibody. A phage displayed framework grafted C3 V_l, which retained observed activity, could be used as an imprint template for the creation of a C3 like abzyme scFv. Jespers *et al* (1994) devised an alternative strategy to humanisation of murine antibodies, by using the phage displayed murine Fab as a template to select human Fab from a library with equivalent antigen binding. The murine anti human TNF antibody MAb32 was cloned and the heavy chain sequence inserted into a plasmid vector for expression in *E.coli*. The plasmid produced soluble MAb32 heavy chain. The MAb32 heavy chain producing *E.coli* was then infected with phage carrying a human light chain library. The subsequent phage displayed human light chain, which was fused to phage coat protein III, and which had dimerised with the murine heavy chain. The resulting murine human hybrid phage was selected for human TNF binding, the selected human light chains were then cloned from the phage into plasmid vectors and transformed into *E.coli*. This culture was then infected with a phage library of human heavy chains, and again the subsequent

phage displayed human heavy chain fused to pIII dimerised with the preselected light chain. This phage population was then screened for anti human TNF binding. This work generated a human Fab, the selection of which had been guided by the murine antibody that was used as a template, that bound to the same epitope of human TNF as the MAb32 with similar affinity.

Although the goal of this work was to generate a human antibody that bound to the same epitope as an existing antibody, rather than to generate a high affinity antibody against a given antigen, it does provide a theoretical basis for C3 binding site reconstruction. If it is possible to guide phage antibody selection to a particular epitope, then it is logical to assume that selection could be guided towards a transition state analogue. The resulting phage antibody could possess the same catalytic function as that displayed by a hybridoma raised against the same analogue, phage libraries having already been demonstrated as capable of generating abzyme fragments (Janda *et al* 1997). If a *E.coli* culture expressing soluble loop grafted $C3V_l$ light chain constructs was infected with a phage library of murine heavy chains the resulting phage displayed hybrids could be screened for SF/HTf/1 binding. The hapten binding positive clones could then be screened for C3 activity using the C3 substrate.

It is possible that the original heavy chain light chain pairing expressed by the C3 hybridoma could be reselected by the phage library. Hapten binding alone would not however guarantee catalysis, the C5 antibody bound hapten with similar properties to the C3 antibody but did not catalyse any reaction, but if the phage library is large enough it should produce a number of non catalytic and catalytic hapten binding clones. Huse, Sastry *et al*(1989) constructed a library from spleenic mRNA from a mouse immunised with a transition state analogue. The resulting Fab library was screened for transition state analogue binding and multiple clones identified as binding antigen. This library, because of its derivation from an immunised animal, would have been expected to produce such binders. A similar library derived from an immunised animal should also generate a C3 equivalent heavy chain. But generating a pseudo-C3 abzyme from an immunised animal would be repeating the experiments which produced the C3 hybridoma, but using a phage display system rather than hybridoma. Regeneration of the C3 active site from a naive library would be a further demonstration of the ability of phage display to duplicate the immune response for the generation of antibodies. As the original C3 heavy chain sequence is lost there is no way of determining if the reconstructed abzyme is identical to the

original C3 binding site, even if the original heavy chain sequence is not contained in the phage library it is still possible to duplicate the activity. The recovery of the original C3 heavy chain from a phage library would be inferred if the catalytic parameters of recovered C3scFv and C3 IgG were compatible.

As by Guo *et al* (1995) demonstrated that multiple abzymes can be generated in a single immune response to a transition analogue, with varying catalytic properties and mechanisms, the same would apply to a very large phage library. The recovery of C3 like abzyme would demonstrate if multiple abzymes and mechanisms could be generated against the same transition state analogue. A very large phage library that was capable of doing so would indeed be equal in versatility, power and application to the exploitation of the immune system by hybridoma technology.

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