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THE PRODUCTION AND CHARACTERISATION OF MONOCLONAL, POLYCLONAL AND PHAGE DISPLAY ANTIBODIES TO THE CYSTIC FIBROSIS TRANSMEMBRANE REGULATOR

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DECLARATION

The monoclonal antibodies described in Chapter 4 were raised by Judy Watson, Department of Biochemistry, University of Bristol. All other the work within this thesis is my own unless otherwise indicated. The views expressed within this study are my own and not necessarily those held by the University.

J. H. Walker

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ABBREVIATIONS

Ab	antibody
ABC	ATP-binding cassette transporter family
ABTS	2′,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) diammonium
ADP	adenosine-5'-diphosphate
AFLM	Association Franchise de Lutte contre la Muscovosidose
Amp	ampicillin
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indonyl-phosphate
blotto	blocking solution
bp	base pairs
BSA	bovine serum albumin fraction V
cAMP	cyclic adenosine monophosphate
Carb	carbenicillin
cDNA	copy deoxyribonucleic acid
CDR	complementarity determining region
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane regulator
C_H1	antibody constant domain one
C_H2	antibody constant domain two
CsTFA	ceasium trifluoroacetate
DAB	diaminobenzidine
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
dNTP	monodeoxyribonucleoside-5'-triphosphates
ddNTP	dideoxyribonucleoside-5'-triphosphates
DMEM	Dulbecco's modified Eagle's medium

DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenedinitro tetra-acetic acid disodium salt
ELISA	enzyme linked immunoabsorbant assay
EPICS	electronic pulse intergrated cell sorter
Fab	antibody light chain and variable domain and first constant domain of the heavy chain
Fc	antibody heavy chain constant domains two and three, responsible antibody effector functions
Fd	variable domain and first constant domain of the antibody heavy chain
FITC	fluorescein isothiocyanate
Fv	antibody variable domains
g	grammes
G418	Geneticin
g3p	bacteriophage major coat protein III
g8p	bacteriophage major coat protein IV
GTG	genetic technology grade
H	Heavy chain antibody component
HAT	hypoxanthine/methotrexate/thymidine
HEPES	N-2-hydroxyethylenepiperazine-N'-2-ethanesulfonic acid
IF	immunofluorescence
IgA	class A immunoglobulins
IgG	class G immunoglobulins
IgM	class M immunoglobulins
IPTG	isopropyl-1-thio-β-D-galactoside
Kan	kanamycin

kb	kilo bases
kDa	kilo Daltons
kV	kilo volts
L	light chain antibody component
λ	bacteriophage lambda
LB broth	Luria Bertani medium
LB amp	LB broth/agar containing 50 mg/ml ampicillinLMP low melting point agarose
M	molar
mA	milli amps
Mab	Monoclonal antibody
MDR	multiple drug resistance
Mega-10	decanoyl-N -methylglucamide
μF	micro Farads
μg	micro grammes
mg	milli grammes
MHC	major histocompatibility complex
ml	milli litres
μl	micro litres
mM	milli molar
MOPS	3-(N-Morpholino) propanesulphonic acid
mRNA	messenger ribonucleic acid
MSD	membrane spanning domain
NBD	nucleotide triphosphate binding domain
NBT	nitroblue tetrazolium
NH	hydrogen bonded to the amino group
nm	nano meters
OD	optical density
OPED	ortho-phenylenediamine
PAGE	polyacrylamide gel electrophoresis

PBS	phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4.7 H2O, 1.4 mM KH2PO4)
PCR	polymerase chain reaction
$pd(N)_6$	random hexadeoxynucleotides
PEG	polyethylene glycol
PFU	plaque forming units
PMSF	phenyl methyl sulphonyl fluoride
QBT	750 mM NaCl, 50 mM MOPS, 15% ethanol adjusted to pH 7.0 and then 0.15% Triton X-100 added
QC	1.0 M NaCl, 50 mM MOPS, 15% ethanol adjusted to pH 7.0
QF	1.25 M NaCl, 50 mM MOPS, 15% ethanol adjusted to pH 8.2
R	unique highly charged cytoplasmic domain (of CFTR)
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
SCID	severe combined immune deficiency (mice)
ScFv	single chain Fv antibody fragment
SDS	sodium dodecyl sulphate
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SERC	science and engineering research council
SSC	0.15 M NaCl, 0.015 M sodium citrate adjusted to pH 7.0 with HCl
TAE	0.2 M Tris base, 0.0005 M EDTA and 0.12% acetic acid adjusted to pH 7.0
Taq	*Thermus aquaticus*
Tet	Tetracycline
TBE	0.1 M Tris base, 0.1 M orthoboric acid 0.00024 M EDTA finally adjusted to pH 8.3

TCA	tricarboxylic acid
TE	10 mM Tris/HCl, pH 8.0, 1 mM EDTA, pH 8.0
TEMED	N', N', N', N'-Tetramethylenediamine
Tris	hydroxymethyl aminoethane
Triton X-100	octyl phenoxy polyethaxyethanol
Tween 20	polyoxyethylene sorbitan monolaurate
UV	ultra violet light
UWGCG	University of Winconsin genetics computer group
V	volts
V_L	antibody variable light chain
V_H	antibody variable heavy chain
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

1.1 CYSTIC FIBROSIS

Cystic fibrosis (CF) is the most common fatal genetic disease in Caucasian populations. The principal manifestations of CF include increased concentrations of chloride ions in exocrine gland secretions, pancreatic insufficiency, chronic lung disease, intestinal blockage, malabsorption of fat and male and female infertility [Boat et al., 1989]. CF is caused by mutations in the gene encoding the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), so named because of its postulated role in electrolyte (specifically chloride ion) transport [Rommens et al., 1989]. The gene spans 250Kb containing 27 exons, which are spliced to produce mRNA of approximately 6500 nucleotides [Zielinski et al., 1991].

A model of the structure of CFTR had been predicted based on conceptual translation of the cDNA sequence [Riordan et al., 1989] (fig. 1.1). The key features of the proposed structure include:

- Two hydrophobic membrane spanning domains (MSD1 and MSD 2) each composed of six transmembrane α-helical segments.
- Two nucleotide triphosphate binding domains (NBD1 and NBD2) which are predicted to interact with ATP.
- A unique highly charged cytoplasmic domain (R) which contains several consensus phosphorylation sequences;
- Two external potential glycosylation sites.

This model did not resemble previously identified ion channels, but shared homology with a family of membrane associated ATP-dependent transport proteins, the ATP-binding cassette (ABC) transporter family [Hyde et al. 1990; Higgins 1992]. In the light of the primary symptoms of CF, the predicted structure led to speculation that CFTR might actively transport a regulatory

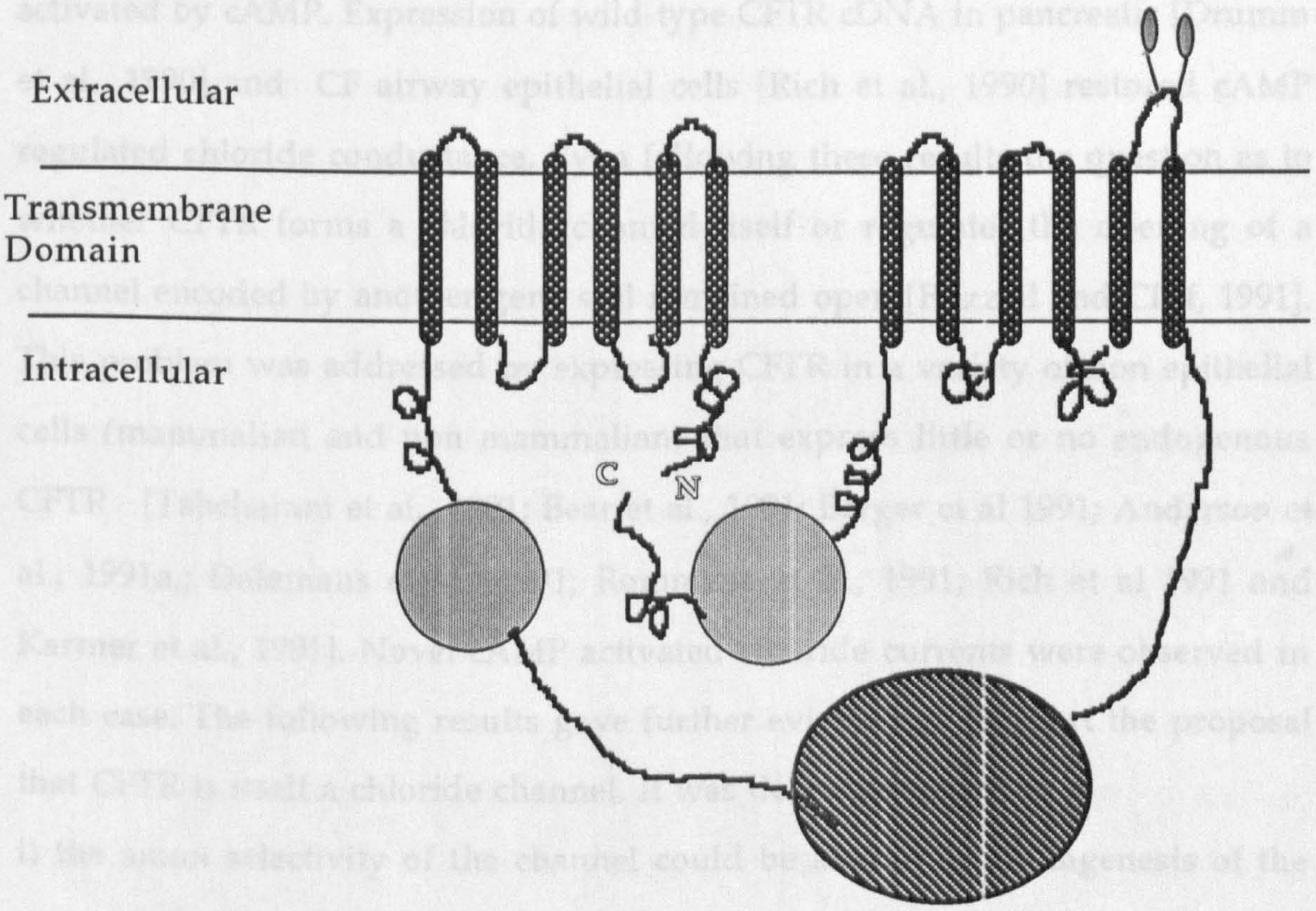


Fig.1. 1 Cartoon representing the predicted structure of CFTR.

● indicates R domain, ● indicates nucleotide binding domain,

▮ indicates the transmembrane domain, and ♀ indicates glycosylation site.

molecule rather than serve as a chloride channel itself [Ringe and Petsko, 1990].

1.1.1 The Cellular Functions of CFTR

Heterologous expression systems have been used to indicate that, as initially predicted, CFTR acts as a chloride ion channel which appears to be activated by cAMP. Expression of wild-type CFTR cDNA in pancreatic [Drumm et al., 1990] and CF airway epithelial cells [Rich et al., 1990] restored cAMP regulated chloride conductance. Even following these results the question as to whether CFTR forms a chloride channel itself or regulates the opening of a channel encoded by another gene still remained open [Frizzell and Cliff, 1991]. This problem was addressed by expressing CFTR in a variety of non epithelial cells (mammalian and non mammalian) that express little or no endogenous CFTR [Tabcharani et al., 1991; Bear et al., 1991; Berger et al 1991; Anderson et al., 1991a,; Dalemans et al., 1991; Rommens et al., 1991; Rich et al 1991 and Kartner et al., 1991]. Novel cAMP activated chloride currents were observed in each case. The following results gave further evidence to support the proposal that CFTR is itself a chloride channel. It was demonstrated that:

i) the anion selectivity of the channel could be altered by mutagenesis of the transmembrane regions of CFTR [Anderson et al., 1991b];

ii) functional channel activity could be restored in lipid bilayers containing reconstituted CFTR [Bear et al., 1992; Tilly et al., 1992];

iii) antisense oligonucleotides to the CF gene inhibit sweat duct chloride ion permeability in both a time and dose dependent manner [Sorcher et al., 1991].

The steps required in the activation of the CFTR encoded chloride channel appear to be phosphorylation of a number of sites on the R domain by protein kinase A [Cheng et al., 1991; Berger et al., 1991; Tabcharani et al., 1991] and hydrolysis of ATP on the NBF domains [Thomas et al., 1991; Anderson and Welsh, 1992]. Further studies will be needed to elucidate the exact mechanism of CFTR chloride channel activity.

The conclusion that CFTR is a chloride channel gives CFTR a unique function relative to other members of the ABC transporter family. However, it is possible that CFTR may have other transport functions, especially in the light of the observation that the human multiple drug resistance (MDR) gene (a member of the ABC transporter family closely related to CFTR) may function as a volume-regulated chloride channel [Valverde et al., 1992; Gill et al., 1992] and as an ATP channel [Abraham et al., 1993], as well as a unidirectional transporter of hydrophobic drugs [Gottesman and Pastan, 1988; Endicott and Ling, 1989; Juranka et al., 1989].

A variety of reports indicate that CFTR may indeed act as a multi-functional protein [Wine, 1993]:

• The protein may participate in cAMP dependent plasma membrane recycling [Bradbury et al., 1992] and in the acidification of intracellular organelles [Barasch et al., 1991]. However, there is still some debate over the actual involvement of CFTR in these roles [Dunn et al., 1994; Prince et al., 1994].

• ATP channel activity of CFTR has been identified recently [Reisin et al., 1994; Cantiello et al., 1994], which may serve to regulate activation of CFTR and perhaps other channels by selective delivery of nucleotides to the extracellular domain [Reisin et al., 1994.]. Involvement of CFTR has been implicated in the regulation of sodium ion transport [Jiang et al., 1993; Boucher et al., 1988].

• A recent report suggests that the amino-terminal portion of CFTR (MSD1, NBD1 and the R domain) alone is sufficient to form a regulated chloride ion channel [Sheppard et al., 1994]. Examination of this channel on a sucrose density gradient led to the conclusion that the functional protein was composed of dimerised amino-terminal portions of CFTR. If the amino-terminal portion alone is sufficient to form a functional chloride channel, the role of the apparently superfluous carboxyl-terminus of CFTR (NBD2 and MSD2) has yet to be determined.

From this it is clear that certain features of the activity of this protein still need to be elucidated.

1.1.2 Mutations in the CFTR Gene

By far the most common mutation identified in the gene is a three base pair deletion, resulting in the loss of a phenylalanine residue at position 508 of the protein sequence [Kerem et al., 1989]. The worldwide frequency of this mutation varies between 30% and 88%, but it is thought to be the predominant cause of the high incidence of CF in Caucasian populations [Cutting et al., 1992]. A CF Genetic Analysis Consortium has been established to coordinate efficient analysis of the numerous other CF producing mutations which occur at diverse sites within CFTR [Tsiu, 1992].

CFTR mutations have been classified into four groups based on the mechanisms by which they disrupt the function of the protein [Welsh and Smith, 1993]. These mechanisms are:

i) defective protein production [White et al., 1990; Hamosh et al., 1991],

ii) defective protein processing [Cheng et al., 1990; Denning et al., 1992b; 1992c; Armstrong, 1992; Lukacs et al., 1993; Yang et al., 1993],

iii) defective regulation of the CFTR channel [Anderson and Welsh, 1992], and

iv) defective conductance through the the CFTR chloride channel [Sheppard et al., 1993; Yang et al., 1993].

The varied nature and complex mechanisms of mutations responsible for CFTR makes the establishment of a universal therapy for the disease difficult. A possible way forward in treating CF, regardless of the mechanism of mutation in any particular case, would be to insert wild type CFTR into affected cells. This may be achieved by introducing the gene encoding CFTR or by delivery of the wild type protein itself to the appropriate cells. Much of the effort in CF research is now concentrated in these areas [Rosenfeld et al., 1991; 1992; Hyde et al., 1993; Porteous and Dorin, 1993; Zabner et al., 1993; Crystal et al. 1994; Marshall et al., 1994], especially with the recent availability of mouse models for CF [Snouwaert et al., 1992; Dorin et al., 1992; Colledge et al., 1992]. A full awareness of the expression of CFTR in different tissues is critical for the progress of gene and protein therapy strategies.

1.1.3 Location of CFTR

Northern blot analysis has identified CFTR mRNA in epithelial tissues affected by CF [Riordan et al., 1989]. These studies have been extended using *in situ* hybridisation in rat tissues which demonstrated CFTR mRNA in pancreas, salivary gland, lung, gastrointestinal tract, uterus and testis [Trezise and Buchwald, 1991]. Other researchers have followed a range of procedures to raise monoclonal and polyclonal antibodies to different epitopes within CFTR [e.g. Gregory et al., 1990; Crawford et al., 1991; Cohn et al., 1991; 1992; Marino et al., 1991; Denning et al., 1992a, 1992b; Kartner et al., 1991; Zeitlin et al., 1992]. Antibodies raised to CFTR have been used in a variety of experiments ranging from immunoprecipitation and immunoblot analyses to immunofluorescence assays and immuno-electron microscopy.

The main focus of antibody work has been in immunohistochemical tissue distribution studies of both developing and mature tissues. CFTR has primarily been located in the apical membranes of epithelial cells of organs in which the clinical manifestations of CF are apparent [see Tizzano and Buchwald, 1993; Welsh et al., 1992 and McCintosh and Cutting, 1992 for relevant recent reviews]. Somewhat surprisingly, the expression of CFTR in the lungs is not generally found in the respiratory epithelia, but appears to be concentrated in the submucosal glands of the bronchus [Engelhardt et al., 1991; Crawford et al., 1991], whereas expression is found in the epithelia of kidneys [Crawford et al., 1991] which are not usually associated with the pathogenesis of the disease. Immunohistological analysis of the location of CFTR is covered in more detail in chapter 4.

1.1.4 Topology of CFTR

Information about the topology of CFTR currently available agrees with predictions made from the primary sequence. Functional considerations place the R domain and probably the NBDs inside the cell (see section 1.1.1 above). Studies with antibodies in permeabilised and unpermeabilised cells have

confirmed the intracellular location of the R domain along with the carboxyl terminus and placed the loop between the first two membrane spanning domains on the extracellular surface [Denning et al., 1992b]. As there is no signal sequence the amino terminus is probably intracellular.

A more accurate model of CFTR would be of great benefit in terms of elucidating its exact biochemical functions (see section 1.1.2 above). The use of antibodies to different regions of CFTR would clearly help in the generation of such a model since they would provide information concerning both the topology of the protein in the membrane and the nature of those residues exposed on the surface of the folded protein. The success of using sequence specific antibodies to 'map' complex eukaryotic membrane proteins has been previously shown [Hughes and August, 1981; Georges et al., 1993; Banting et al., 1989; Wilde et al., 1992]. A panel of antibodies would also allow further detailed analysis of the cell and tissue distribution of CFTR to give a more complete understanding of the role it plays in the clinical manifestations of the disease, which in turn may influence approaches to therapy.

1.2 CHARACTERISATION AND PRODUCTION OF ANTIBODIES

The antibody molecule is composed of four polypeptide chains, two smaller (light) and two larger (heavy) chains. These chains fold into domains which have conserved three-dimentional conformations stabilised by disulphide bonds (Fig. 1.2). Each antibody domain consists of a sandwich of β-sheets which are stabilised by intra-chain disulphide bonds [see Amzel and Poljak, 1979 for a relevant review] . The domains associate to form structural regions, the functions of which have been characterised [see Davies and Metzger, 1983 for a relevant review].

The antigen binding domains are formed by the variable light (V_L) and the variable heavy (V_H) domains. The functional groups interacting with the

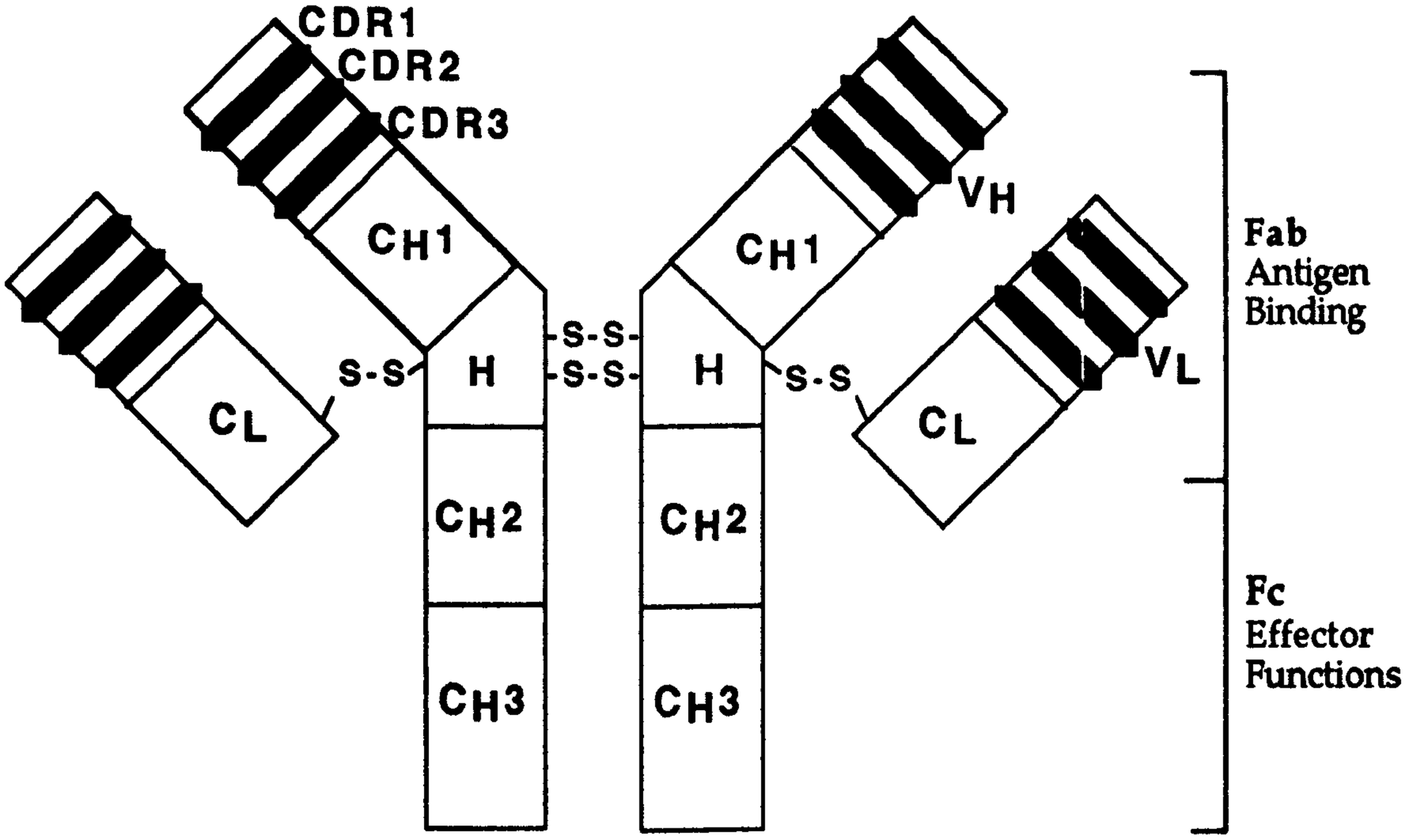


Fig. 1.2 The basic structure of IgG.
Modified from Burton and Barbas, 1993b.

antigen are formed by the amino acids in the six loops which connect the β-strands in the variable domains. These are hypervariable in sequence and length and are called the complementarity determining regions (CDRs) [Wu and Kabat, 1970].

The rest of the molecule is relatively constant in sequence and structure. The constant region domains are formed by the interaction of the remainder of the light chain with the first constant domain of the heavy chain and by the association of the remainder of the two heavy chains (see fig.1.2). The hinge region is a section of heavy chain between the first two constant region domains. The two 'arms' of the antibody are linked at this point by disulphide bonds and a degree of flexibility exists in this area allowing the two antigen binding domains to operate independently. The heavy chain constant regions are responsible for effecter functions, e.g. binding to receptors and complement fixation [see Davies and Metzger 1983, for a relevant review].

1.2.1 Antibody Fragments

The domain organisation of the antibody molecule means that it can easily be divided into a number of fragments. Several proteases are known to cleave the structure into component parts which have been very valuable in structural and functional studies of the antibody molecule [Roitt et al., 1993]. Some smaller units, like Fv fragments, cannot be easily prepared by protein cleavage and have therefore only really been amenable to characterisation since the advent of gene expression systems (see section 1.2.6.2 below) [Pluckthun, 1992].

1.2.1.1 The Fab Fragment

The Fab fragments can easily be prepared by proteolytic cleavage with papain [Porter, 1959] and have been thoroughly characterised. They are composed of the complete light chain and the heavy chain Fd region which

consists of the variable domain and first constant domain of the heavy chain. They have frequently been shown to bind antigens as well as the whole antibody [Nisonhoff et al., 1975]. Fab fragments are stable and associate well because the constant region contributes significant binding energy mainly from the formation of a disulphide bridge between the two chains.

1.2.1.2 The Fv Fragment

The Fv fragments consist of the heavy and light chain variable domains and as such are the smallest element containing the complete antigen binding site. Unlike Fab fragments they are not stabilised by interchain disulphide bonds. Initial reports indicated that they give good antigen binding activity [Skerra and Pluckthun, 1988]. These fragments are of particular importance in key areas of research, such as:

- Immunotoxin Therapy - genetic fusions of Fv fragments with potent toxins have demonstrated selective cytotoxicity [Chaudery et al., 1989; 1990].
- Tumour Medicine - ScFvs (discussed below) show a more even tumour penetration than larger, intact antibody counterparts [Yokota et al., 1992].
- Structural research - Fv fragments typically give better ordered crystals than Fab fragments [Bhat et al., 1990].

A major disadvantage, however, is that when diluted the Fv fragments have a tendency to dissociate into component chains. The dissociation constants will vary between Fv fragments because they are dependent on specific interactions between amino acids present in the variable loops [Chothia et al., 1985]. The consequence of this dissociation between V_H and V_L can be an apparent loss of binding activity. This problem can be alleviated by covalently linking the the two variable chains.

Three ways of covalently linking the V_H and V_L domains have been described. The first involves chemically cross linking the two domains with glutaraldehyde [Jaenicke and Rudolph, 1986]. The second uses a disulphide

bond cross link, thus imitating the disulphide bridge formed between C_{H1} and C_L in the Fab fragment [Reiter et al., 1994]. The third, and most commonly adopted means of cross linkage, connects the the two fragments by a peptide linker to produce a single-chain Fv fragment (ScFv) [Bird et al., 1988; Huston et al., 1988]. All three methods have been shown to give functional expression *in vivo* [Glockshuber et al., 1990]. The first two methods are advantageous because they provide considerably more stability to the antibody fragment than the linker approach, however they are not as universally applicable. A range of different linkers have been used, all of which tend to be hydrophilic and flexible. A sequence most frequently adopted for ScFv construction is $(Gly_4Ser)_3$. This 15-residue linker was designed to be unstructured and flexible to avoid strain on the native V_H and V_L domain interactions and its hydrophilic nature should reduce interference at the hydrophobic domain interface. The variable chains have been linked effectively in both orientations, V_L-linker-V_H and V_H-linker-V_L [Huston et al., 1993].

1.2.1.3 Single Domain and Smaller Antibody Entities

The next smallest units available for antigen binding are the V_H or V_L domains. Both V_L and V_H have a rather hydrophobic interface which is necessary for heterodimer formation [Chothia et al., 1985]. Different consequences result if both are not present. The V_L domains tend to dimerise [Azuma et al., 1974], thus blocking the antigen binding site. This homodimerisation phenomenon is not observed for the V_H domains which therefore have a tendency to be rather insoluble. A proportion of V_H fragments will remain soluble, but the exposed hydrophobic surface can lead to non specific interactions which may be wrongly interpreted as specific antigen binding. The formation of complexes between antigen and heavy chains has been demonstrated [Spielgelberg and Weigle, 1966., Ward et al., 1989], but the viability of using single V_H domains to obtain high affinity

antigen binding has to be questioned.

Attempts have been made to bind antigens with even smaller peptides derived from the CDR regions of antibodies [Williams et al., 1989]. The affinity of such binding is very low (10^3-10^6 mol^{-1}).

1.2.2 The Generation of Immunoglobulins

In the immune system, antibodies are created during the differentiation of hematopoietic stem cells into B lymphocytes. The sequence diversity for antibody binding sites is generated by the rearrangement of variable gene segments (V,D and J regions) to form variable genes [see Tonegawa 1983 for relevant review]. Because the potential diversity of the primary immune repertoire is far greater than the number of antibody displaying cells, each of these cells is likely to display an immunoglobulin of unique structure and antigen binding specificity.

Antibodies are displayed on the surface of the B lymphocytes. Antigen bound by a specific antibody is endocytosed, processed and presented to helper T cells in association with molecules of the major histocompatibility complex (class II). This induces the proliferation and differentiation of the relevant B lymphocyte to either plasma cells (which secrete considerable amounts of antibody into the bloodstream) or to memory cells which are involved in subsequent immune responses to the same antigen. The rearranged variable genes of the the memory cells undergo somatic mutations, resulting in the generation of altered antibodies, some of which have higher affinities than the initial antibody to the original antigen [Roitt et al., 1993].

1.2.3 Antibody - Antigen Interactions

Greater understanding of the nature of antibody-antigen interactions of immune complexes has been gained through X-ray crystallographic studies and molecular modelling [Poljak et al., 1973 and see Webster et al., 1994 and Rees et al., 1994 for relevant recent reviews]. The size, shape and charge

composition of the antigen binding site is defined by the length and configuration of amino acids in the six CDR loops. The number of hypervariable loops actually involved in antigen binding varies [Wilson and Stanfield, 1993]. Despite various proposals [Kelley and O'Connell, 1993; Tello et al., 1993; Ito et al., 1993], the thermodynamic basis of the antigen-antibody interactions is still under debate. However, evidence suggests that water molecules may play a key role in improving the fit between the interacting surfaces [Mariuzza and Poljak, 1993]. Several examples of such an 'induced fit' mechanism for antigen-antibody interactions have been identified [such as Bhat et al., 1990; Rini et al., 1992] and it seems likely that this is a common phenomenon [see Wilson and Stanfield, 1993 for a relevant recent review].

1.2.4 Polyclonal Antibodies

Polyclonal antisera derived from an immunised source are effective reagents in most immunochemical techniques [Harlow and Lane, 1988]. They are relatively easy to obtain, but their supply is dependent on the animal in which they are raised. Polyclonal antisera contain a heterogenous mixture of antibodies which will have a specificity biased towards the immunogen, but will also recognise many other antigens. The heterogeneity of the antisera is, however, reduced with repeated immunisations [Harlow and Lane, 1988]. Polyclonal antisera will usually contain antibodies that bind to a number of sites on an antigen, although one epitope is likely to be dominant. Multiple binding confers the advantage of higher avidity which makes them particularly useful tools for immunoprecipitations. Lack of specificity of polyclonal antisera can be a problem in certain immunochemical techniques. The preparation of homogeneous antibodies with a defined specificity was only possible after the development of hybridomas.

1.2.5 Monoclonal Antibodies

Since the mid 1970's, hybridoma technology [Kohler and Milstein, 1975] has led to many practical applications for monoclonal antibodies in research, industry and medicine. They are produced by the immortalisation of lymphocytes from an immunised animal by fusion with myeloma cells. Stable hybridomas secreting a monoclonal antibody (Mab) of the required specificity are identified by extensive screening. The technology has improved over the years. Manipulation of DNA sequences encoding specific Mabs has enabled the construction of a new generation of engineered antibodies [Winter and Milstein, 1991]. This includes a number of 'humanised' rodent antibodies [Winter and Harris, 1993] where rodent-derived CDR regions are grafted into a human-derived antibody. Humanised antibodies have a greatly reduced immunogenicity to humans, relative to rodent-derived Mabs, and are therefore very useful clinical tools [Hale et al., 1988; Isaacs et al., 1992].

The production and use of monoclonal antibodies does have limitations. Despite the painstaking and lengthy processes involved in obtaining hybridomas that produce Mabs, only a fraction of the B cells from an immunised source are successfully immortalised, at best a few hundred individual clones from a potential repertoire of tens of millions. In addition, the production of human monoclonal antibodies is restricted by the limitation of human immunisation protocols and the lack of a myeloma cell line that can routinely produce stable hybridomas when fused to human B cells [James and Bell, 1987; Thompson, 1988].

On commencement of this project, a selection of novel, monoclonal antibodies, raised to synthetic peptides corrisponding to specific regions of CFTR, were available. However these antibodies had not been fully characterised.

1.2.6 Combinatorial Antibody Libraries

More recently ,vector systems have been described that enable antibody fragments to be produced and isolated in prokaryotic systems (see section 1.2.6.2 below). The concept of the combinatorial approach to antibody production is based on the ability to directly clone diverse repertoires of genes coding for the antigen binding regions of the heavy and light chain immunoglobulin molecule in an appropriate expression vector. The two chains are cloned in a random combinatorial manner, such that any light chain could be joined with any heavy chain. The genes are then expressed to produce antibody fragments potentially capable of binding antigen. The antibody fragments are then exposed to antigen to directly select for binding combinations of heavy and light chains. The development of oligonucleotides capable of priming the amplification of heavy and light chain variable gene repertoires (see section 1.2.6.1 below) and the demonstration that functional antibody fragments can be expressed in *E.coli*. (see section 1.2.6.2 below) paved the way for the construction of combinatorial antibody libraries.

1.2.6.1 Amplification of Antibody Fragments by Polymerase Chain Reaction

To amplify a target sequence by polymerase chain reaction (PCR), oligonucleotide primer sequences need to be designed to anneal to either end of the target sequence [Saiki et al., 1985]. Amplification of antibody genes by PCR was initially demonstrated from hybridomas [Larrick et al., 1989a]. The first report of using 'universal' PCR primers, suitable for amplifying a range of heavy and light chain variable regions, was a key step towards making expression repertoires of rearranged V-regions [Orlandi et al., 1989; Larrick et al., 1989b]. The incorporation of restriction sites into the primers facilitates direct cloning into an appropriate expression vector. Nucleotide sequences of the variable region genes were extracted from the Kabat data base where all known antibody sequences are listed [Kabat et al., 1987]. Areas where nucleotide sequences were sufficiently conserved were used to design

degenerate PCR primers. The design of further sets of PCR primers has been prompted by the continuously increasing knowledge of antibody sequences. These include primer sets for the amplification of human, mouse and rabbit antibody repertoires, with back primers based in the V-gene region and forward primers based in the J-segment or the constant region [e.g. Sastry et al., 1989; Huse et al., 1989; Jones and Bendig, 1991; Kang et al., 1991a; Persson et al., 1991; Marks et al., 1991a; Ørum et al., 1993; Zhou et al., 1994; Sassano et al., 1994].

1.2.6.2 Cloning Antibodies in *E.coli.*

The development of genetic engineering has made it possible to express antibodies in *E.coli.* [Pluckthun and Skerra, 1989; Pluckthun, 1992]. Much is known about prokaryotic gene expression systems and they offer a number of important advantages, which include:

- The fast rate of *E.coli.* growth.
- The efficient transformation of *E.coli.* with DNA.
- The availability of selection markers.
- The ability to genetically manipulate cloned DNA using the immense range of molecular biological techniques available.
- The fact that larger scale fermentation is far less expensive and involves more straight forward conditions than are required for eukaryotic systems.

An essential requirement for a good antibody expression system is that the antibody fragment is expressed in a functional state so that the selection or purification procedures can make use of the antigen binding property.

Initial reports were of direct expression of antibody fragments without a signal sequence [Boss et al., 1984; Kenton et al., 1984]. Subsequently, the expression of antibody fragments in cytoplasmic fusion proteins [Baldwin et al., 1989] and in secreted fusion proteins [Pluckthun and Skerra, 1989] were described. A leading development was the employment of bacterial signal sequences to enable expression of secreted Fv [Skerra and Pluckthun, 1988] and

Fab [Better et al., 1988; Better and Horwitz, 1989] antibody fragments that were shown to bind antigen.

The success of signal peptide strategy was due to the ability to mimic in *E.coli.* the normal folding and assembly pathway in the eukaryotic cell. In antibody producing cells, heavy and light chains are targeted to the lumen of the endoplasmic reticulum where N-terminal signal sequences are cleaved off prior to the assembly of the antibody molecule [Wall and Kuehl, 1983]. The periplasm encapsulated by the inner and outer membranes of *E.coli.* provides an equivalent oxidative environment to that found in the endoplasmic reticulum [Wulfing and Pluckthun, 1994]. This provides conditions for the necessary steps of protein folding, disulphide bond formation and association of heavy and light chains into heterodimeric molecules to produce functional antibody molecules. A further advantage of periplasmic assembly is a reduction of the problem of protein degradation found in cytoplasmic expression systems. This reduction is due to the presence of fewer proteases in the periplasm, the protection achieved by disulphide bond formation and the folding to globular domains [Pluckthun, 1991]. Although correct folding of secreted heterologous proteins had been previously demonstrated [Briggs et al., 1986], antibody formation in the periplasm of *E.coli.* was the first demonstration of heteromeric assembly. The demonstration that antigen binding Fv and Fab fragments could be functionally expressed in *E.coli.* was a major advance leading to the development of antibody expression libraries.

As yet, there are no reports of the efficient expression of correctly folded whole antibodies (i.e. including the Fc region) in *E.coli.*. Not only is the large size of the entire antibody molecule a limiting factor, but also many of the functions of the whole antibody molecule depend on proper glycosylation of appropriate sites on the constant regions. Most antibodies are glycosylated in the C_H2 domain and others such as IgA and IgM can be glycosylated in the C_H1 and other domains [Nisonhoff et al., 1975]. Glycosylation of IgG is essential for the efficiency of the constant domain effector functions [Tao and Morrison,

1989]. It is also thought that glycosylation of the C_H2 domain may be an important requirement for the correct folding and the stability of the antibody structure, because at least in IgG1, only sugar residues are involved in the interaction of the two domains [Sutton and Phillips, 1983]. *E.coli.* cannot glycosylate proteins, therefore for Fc expression a eukaryotic host is preferred [see Morrison 1992 for relevant review]. Eukaryotic expression systems should be considered as complementary to prokaryotic systems and have been used in tandem to produce whole antibodies [Bender et al., 1993; Jespers et al., 1994]. DNA encoding for antigen binding antibody fragments that are selected from a phage library can be subcloned into a eukaryotic expression vector together with DNA encoding the rest of the complete antibody molecule. Thus fully glycosylated antibodies containing antigen selected variable sequences can be expressed in eukaryotic cells. However, glycosylation is not necessary for the functional expression of antigen binding antibody fragments because most antibody variable regions do not contain glycosylation sites and it has been shown that glycosylation of the whole molecule normally has no influence on the antigen-binding properties of the antibody [Wall and Kuehl, 1983].

1.2.6.3 Combinatorial Libraries in Lambda Phage

The coding sequences for numerous immunoglobulin heavy and light chain antigen binding sequences were inserted in a random combinatorial manner into modified bacteriophage λ vectors (called λ Zap vectors [Short et al., 1988]) and expressed in *E.coli.* to produce the first antibody combinatorial library [Huse et al., 1989]. The basic steps in the production of antibody fragments for cloning and expression in a bacteriophage λ library are outlined below and summarised in fig.1.3.

STEP 1 - mRNA is isolated from an appropriately immunised source.

STEP 2 - mRNA is converted to cDNA by reverse transcription.

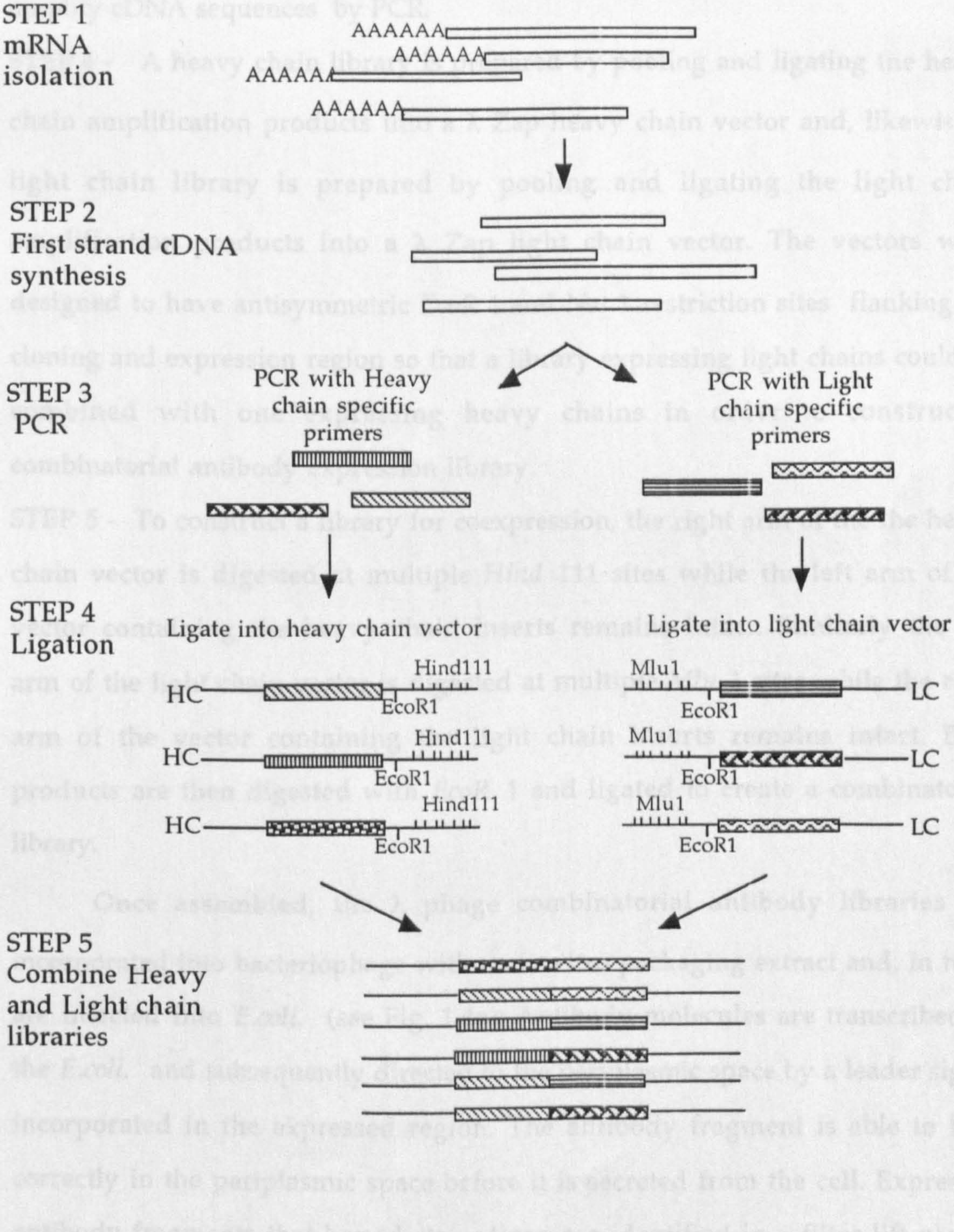


Fig. 1.3 Cartoon representing the basic steps in the preparation of antibody gene segments for cloning into a bacteriophage λ expression library.

STEP 3 - Heavy chain specific and light chain specific primers are used to amplify cDNA sequences by PCR.

STEP 4 - A heavy chain library is prepared by pooling and ligating the heavy chain amplification products into a λ Zap heavy chain vector and, likewise, a light chain library is prepared by pooling and ligating the light chain amplification products into a λ Zap light chain vector. The vectors were designed to have antisymmetric *EcoR* 1 and *Not* 1 restriction sites flanking the cloning and expression region so that a library expressing light chains could be combined with one expressing heavy chains in order to construct a combinatorial antibody expression library.

STEP 5 - To construct a library for coexpression, the right arm of the the heavy chain vector is digested at multiple *Hind* 111 sites while the left arm of the vector containing the heavy chain inserts remains intact. Similarly the left arm of the light chain vector is digested at multiple *Mlu* 1 sites while the right arm of the vector containing the light chain inserts remains intact. Both products are then digested with *EcoR* 1 and ligated to create a combinatorial library.

Once assembled, the λ phage combinatorial antibody libraries are incorporated into bacteriophage with an in vitro packaging extract and, in turn, are infected into *E.coli.* (see Fig. 1.4a). Antibody molecules are transcribed in the *E.coli.* and subsequently directed to the periplasmic space by a leader signal incorporated in the expressed region. The antibody fragment is able to fold correctly in the periplasmic space before it is secreted from the cell. Expressed antibody fragments that bound to antigen are identified in a filter lift plaque assay. The antibody fragments are 'lifted' from phage plaques grown on *E.coli.* lawns using nitrocellulose filters and subsequently incubated with labelled antigen. Detection of bound antigen at certain locations on the filter identifies the positions of phage expressing antibody fragments having an affinity for antigen.

Fig. 1.4a and b. Cartoons representing the pathways for the assembly of antibody fragments using **a.)** the λ phage expression system and **b.)** the filamentous phage expression system. In both situations the antibody fragments are directed to the periplasmic space by a leader sequence which is subsequently cleaved.The Heavy and Light chains assemble in the periplasm to form functional antibody fragments which are secreted from the cell either as **a.)** independent units or **b.)** attached to a coat protein of the filamentous phage.

This methodology was first used to express Fab fragments from an immunised mouse [Huse et al., 1989; Caton and Koprowski, 1990] and subsequently to generate a λ phage Fab library derived from immunised human peripheral blood lymphocytes [Mullinax et al., 1990; Persson et al., 1991]. Although the construction of the λ phage libraries was successful, the screening procedure had the disadvantages of:

i.) being very laborious because they were solid phase (i.e. based on membrane supports);

ii.) requiring a large amount of antigen;

iii.) potentially revealing false positives with certain antigens that bind to the membranes in the absence of antibodies.

Because of these intrinsic problems in library screening, this system is limited by the size of the combinatorial library that can be effectively examined (approximately 10^6 clones).

1.2.6.4 Combinatorial Libraries in Filamentous Phage

An alternative approach to screening larger libraries involves the expression of antibody fragments on the surface of filamentous phage. New expression systems have been developed, inspired by a method where diverse peptides are displayed on the surface of phage [Smith 1985; Palmer and Smith, 1988; Scott and Smith, 1990; Greenwood et al.; 1991 and see Cesareni, 1992 for relevant review]. In these systems, an antibody fragment is cloned into a phagemid vector (see section 1.2.6,4.2 below) next to a gene encoding a phage coat protein and subsequently expressed attached to the coat protein on the surface of the phage (see fig. 1.4b). Such surface expression allows the selection of specific antibodies based on their ability to bind to immobilised antigen. The phage particles displaying a particular antibody fragment contain the gene encoding that fragment, thus creating a 'genetic display package'. This mimics the immune system where the rearranged gene encoding an antibody is found

within the B lymphocyte on whose surface the antibody is displayed.

1.2.6.4.1 Filamentous Bacteriophage

Filamentous phage are long thin bacteriophage with a diameter of 6 to 10 nm and a native length of about 1-2μm, however the length can vary according to the length of DNA encapsulated by the phage coat [Model and Russel, 1988]. They consist of a single molecule of circular single stranded DNA contained within a protein coat [Beck et al., 1978; Newman and Swinney, 1977]. On completion of their life cycle, filamentous phage are secreted into the surrounding medium without damaging the host cell envelope (in contrast to virulent bacteriophage which lyse the infected bacterial cell) [see Rasched and Oberer, 1986 for a review].

The phage utilise the replication machinery of the host cell. The infecting single stranded circular DNA (the (+) strand) is converted to the double stranded replicative form (RF) by the combined action of the host RNA polymerase and the host DNA synthesis apparatus. The RF molecule serves as a template for transcription and protein synthesis. Subsequently the RF form undergoes rolling circle replication to generate single (+) strand DNA which either undergoes the same reaction as the incoming strand (i.e. becomes a new RF molecule) or is sequestered for packaging into new phage particles [Model and Russel, 1988].

While DNA synthesis goes on, there is concomitant synthesis of phage encoded proteins. The phage genome encodes 10 proteins of which 5 are virion structural proteins, 3 are necessary for phage DNA synthesis and 2 are required for phage assembly [Beck and Zink, 1981]. In addition, there is an 'intergenic region' which does not code for proteins, but contains signal sequences for the initiation of synthesis of both the (+) and (-) DNA strands, the initiation of capsid formation and the termination of RNA synthesis [Model and Russel, 1988].

The phage coat is made up of approximately 2710 copies of a 50 amino

acid helical protein, the product of gene VIII [Pratt et al., 1969; Newman and Swinney., 1977]. Both ends of the phage particle have structures formed by minor coat proteins, the products of gene VII and IX on one end and of VI and III on the other end. There are thought to be 3 to 5 copies of each of the minor coat proteins on each phage particle [Rasched and Oberer, 1986].

The genes encoding the major coat protein VIII (g8p) and the minor coat protein III (g3p) have been used to generate fusion proteins, facilitating the expression of antibody fragments on the surface of the phage particle (see section 1.2.6.4.3 below). These two coat proteins have a number of features in common. Both proteins have an acidic amino terminus which is exposed to the outside of the mature phage particle. They have a central hydrophobic core and carboxyl termini which are basic in nature and are an integral part of the phage coat [Armstrong et al., 1983].

The fate of the g3p and g8p coat proteins on bacteriophage infection of *E.coli.* and subsequent extrusion is represented in fig. 1.5. On infection, the phage coat is removed and the g8p and g3p coat proteins are deposited in the inner membrane of the host cell [Trenker et al., 1967]. During phage replication both g8p and g3p are initially synthesised as precursor proteins with amino terminal signal peptides [Sugimoto et al., 1977; Wickner et al., 1978]. These new coat proteins are also deposited in the inner membrane prior to phage assembly [Smilowitz et al., 1972]. In phage packaging the replicated viral DNA strands become associated with the inner membrane and encased by the coat proteins as they pass out of the host with the g3p proteins emerging last [Armstrong et al., 1983; Nambudripad et al., 1991].

Proteolytic cleavage of g3p with subtilisin yields two similarly sized domains [Armstrong et al. 1981] which have distinct functions. The exposed amino terminal domain is responsible for phage infection as it binds to the phage receptor on the F pilus of male bacteria [Nelson et al., 1981], whereas the membrane bound carboxyl terminal domain has a morphogenic role in the termination and stability of the virion assembly [Armstrong et al., 1981;

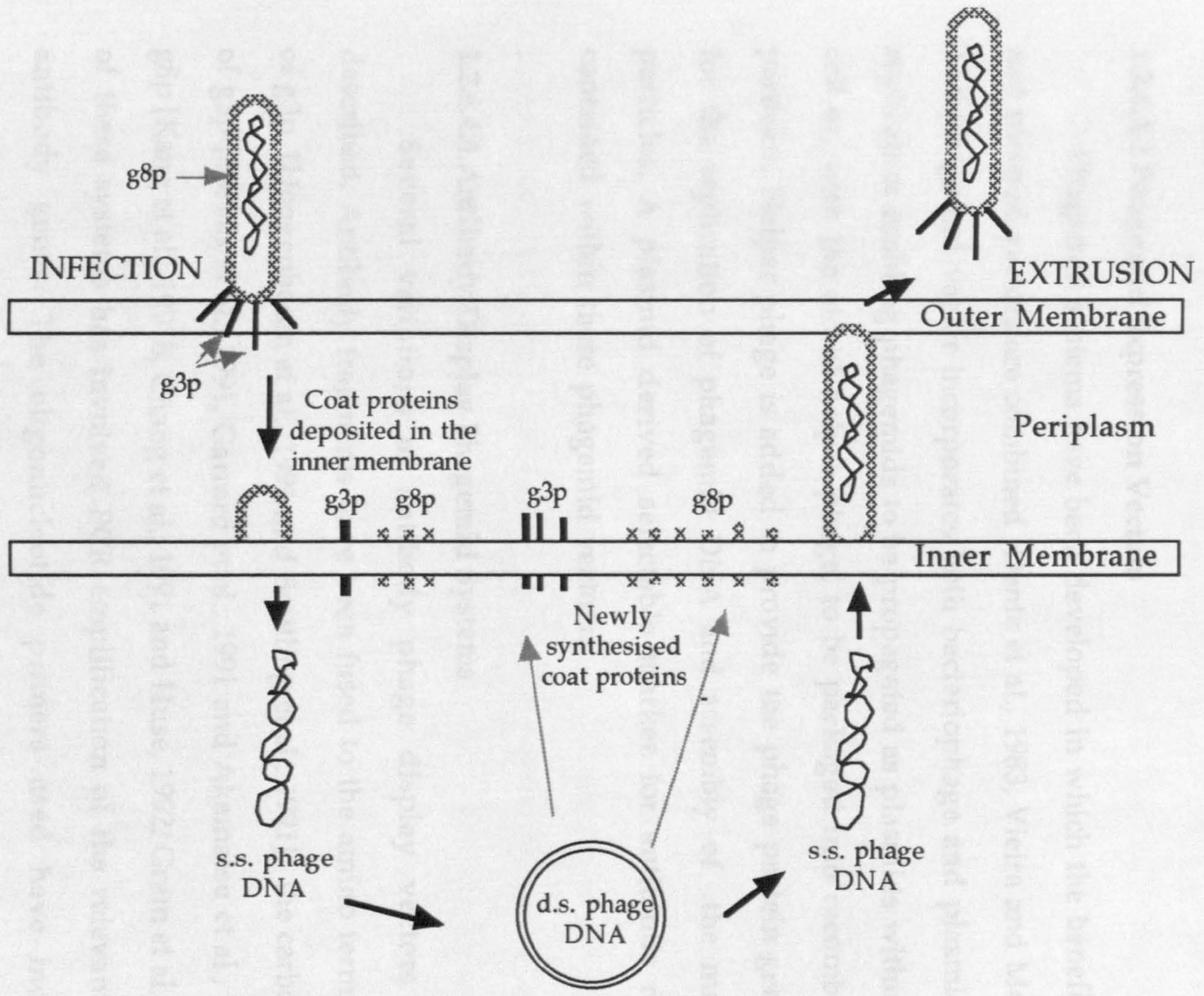


Fig. 1.5 Cartoon representing the fate of g3p and g8p coat proteins on bacteriophage infection of *E.coli,* and subsequent extrusion. The coat proteins that are stripped from the phage on cell infection and those that are newly synthesised accumulate in the inner membrane of the cell. As the assembling phage is extruded from the cell the single stranded DNA becomes encased in many copies of g8p and 'capped' by 3-5 copies of g3p.

Crissman and Smith, 1984]. After production of the g3p, the host bacterium becomes resistant to further bacteriophage infection [Boeke et al., 1982].

The filamentous phage are resistant to pH extremes and proteolytic (except with subtilisin (see above)) and nucleolytic digestion. They are, however, sensitive to detergents, organic solvents and mechanical shearing [Marvin and Hohn, 1969; Rasched and Oberer, 1986].

1.2.6.4.2 Phagemid Expression Vectors

Phagemid systems have been developed in which the benefits of phage and plasmid vectors are combined [Dente et al., 1983; Vieira and Messing,1987]. The phagemid vector incorporates both bacteriophage and plasmid origins of replication enabling phagemids to be propagated as plasmids within a bacterial cell or, with the aid of helper phage, to be packaged into recombinant phage particles. Helper phage is added to provide the phage protein genes required for the replication of phagemid DNA and assembly of the mature phage particles. A plasmid derived selectable marker for antibiotic resistance is contained within these phagemid vectors.

1.2.6.4.3 Antibody Display Phagemid Systems

Several variations of antibody phage display vectors have been described. Antibody fragments have been fused to the amino terminal domain of g3p [Hoogenboom et al., 1991 and Breitling et al., 1991], the carboxyl domain of g3p [Barbas et al., 1991, Garrard et al., 1991 and Akamatsu et al., 1993] and to g8p [Kang et al., 1991b, Chang et al., 1991 and Huse, 1992; Gram et al., 1992]. Each of these systems has involved PCR amplification of the relevant regions of antibody genes. The oligonucleotide primers used have included rare restriction sites in order to reduce the chances of digesting the imported immunoglobulin genes when restricting PCR products prior to cloning [Chaudary et al., 1990; Soderlind et al., 1992]. The two systems adopted within this study are outlined below. More extensive descriptions are given in the

introductions to the relevant chapters.

1.2.6.4.3.1 pCANTAB5

This phagemid vector was developed by Cambridge Antibody Technology and is commercially available from LKB-Pharmacia. In this system the antibody variable regions are constructed into a single gene (ScFv) using a DNA linker fragment and then cloned between the native leader sequence and the amino terminus of the g3p. The g3p leader sequence directs the fusion protein to the inner membrane of the *E.coli.* and the ScFv assembles in the periplasm.

1.2.6.4.3.2 pComb3H

This phagemid vector is a modified version of pComb3 [Barbas et al., 1991] and is designed for the cloning of Fab fragments. It has been derived from the phagemid pBluescript [Short et al., 1988]. The heavy and light chains are cloned sequentially into the phagemid vector. The heavy chain is fused to the carboxyl domain of the g3p. Appropriate signal sequences direct the fusion protein and the light chain to the periplasmic space of the *E.coli.*. The g3p domain with the heavy chain fused to it is anchored in the inner membrane. The Fab fragment is assembled as the light chain combines with the anchored heavy chain.

1.2.6.4.4 Strategies for Phage Display of Antibody Fragments

The detailed methods for the two systems used within this study are given in the appropriate chapters. However, the basic steps required for expressing antibody repertoires using phage display technology are outlined below and summarised in Fig.1.6.

BOX 1 - RNA is prepared from tissue rich in antibody producing cells (e.g. spleen, bone marrow or peripheral blood lymphocytes) from immunised or non-immunised animals or humans.

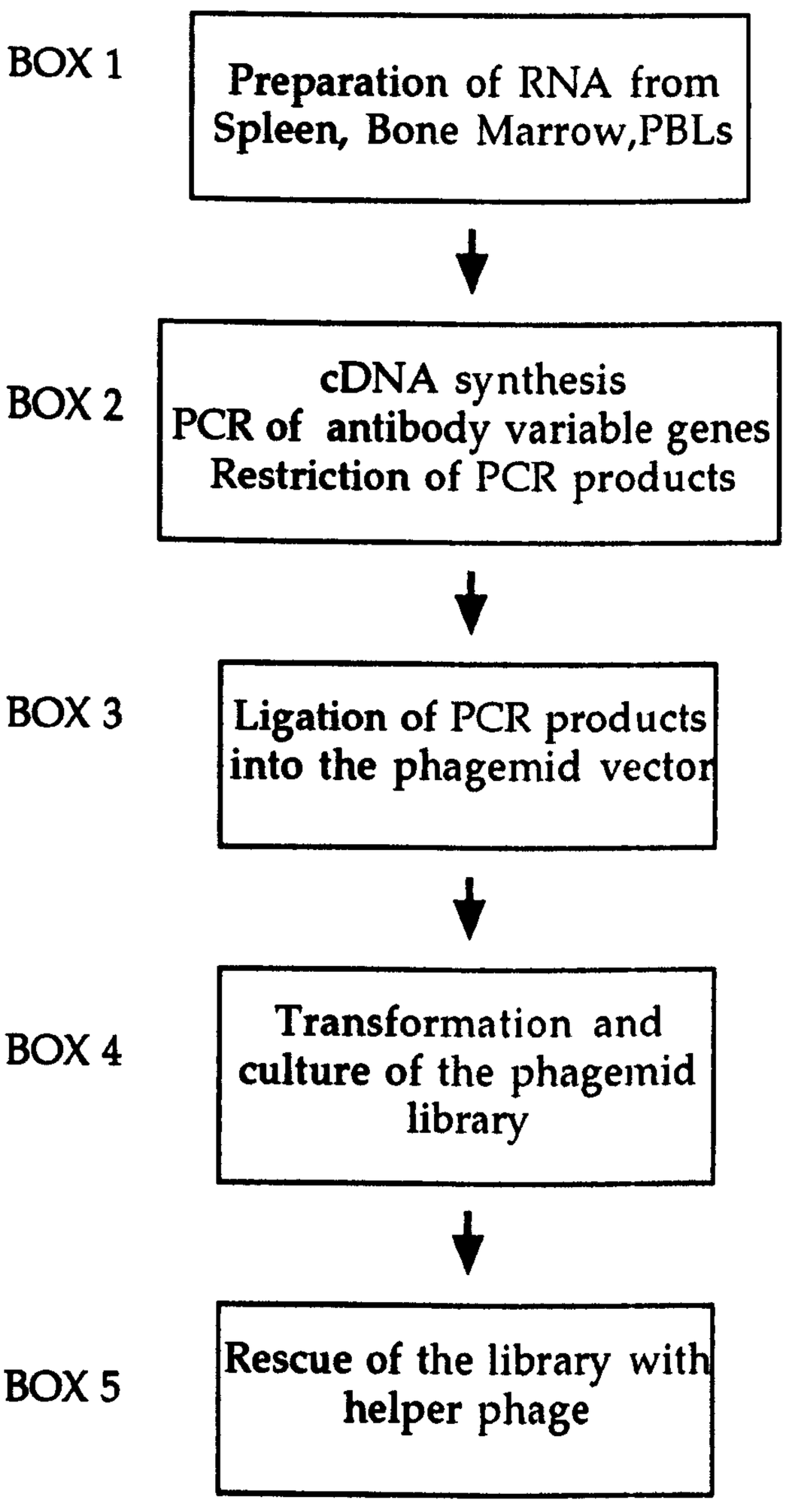


Fig. 1.6 Basic strategy for expressing combinatorial antibody **fragments** on the surface of bacteriophage.

BOX 2 - Reverse transcription of antibody heavy and light chain mRNA into first strand cDNA and amplification of the genes by polymerase chain reaction (see section 1.2.6.1 above). When ScFv fragments are being cloned, additional PCR assembly and further amplification steps are required. The prepared antibody fragments are digested with the appropriate restriction enzymes ready for ligation.

BOX 3 - The antibody fragments are cloned into the prepared vector adjacent to a leader peptide of a protein that is transported to the periplasm. (This signal sequence can be bacterial or phage in origin). As a result of cloning, some part of the antibody fragment is attached to the required domain of a bacteriophage coat protein (either to the amino or carboxyl domains of g3p or to g8p). For the construction of Fab fragments, the heavy and light chains are cloned sequentially into the phagemid vector.

BOX 4 - The cloned antibody libraries are transformed into a suitable *E.coli.* strain. The bacterial strains used allow high efficiency transformation and harbour an F factor that encodes the proteins forming the F pili, necessary for the absorption of the male specific phage. Bacterial cells carrying phagemid are selected by growth in medium containing the appropriate antibiotic dependent on the antibiotic resistance gene present on the phagemid (usually β-lactamase).

BOX 5 - Helper phage is added to the library culture to initiate phage replication and eventually yield recombinant phage particles that display antibody fragments. Since the helper phage contains a defective origin of replication, the phagemid DNA is more efficiently replicated and packaged than the helper phage genome [Vieira and Messing, 1987]. Helper phage with a selectable marker are used such that infected bacteria can be selected. The majority of phage produced therefore contain phagemid DNA encoding the antibody fragment expressed on their surface.

A combinatorial antibody library thus produced can be selected or

enriched for phage displayed antibody fragments capable of binding a specific antigen. This procedure is called 'panning'.

1.2.6.4.5 Selection of Antigen Binding Clones by Panning

Each phage particle in a combinatorial library contains the recombinant genome encoding the specific antibody displayed on its surface, allowing the antibody gene to be selected directly using the binding properties of the expressed protein.

The random combinatorial principles of the library construction result in a complete scrambling of association between protein sequences encoded by the heavy and light chain genes, i.e. any heavy chain can associate with any light chain. As a result, the original heavy and light chain pair are unlikely to be recovered [Winter and Milstein, 1991] and high affinity ($>10^8$ mol^{-1}) antigen binding heavy and light chain combinations are likely to be rare. Certain factors which improve the frequency of antigen binders in a library have been identified:

- Chain promiscuity - the ability of particular heavy chains to effectively bind antigen when combined with different light chain partners [Hudson et al., 1987; Caton and Kaprowski, 1990; Clackson et al., 1991; Kabat and Wu, 1991; Collet et al., 1992].
- Immunised sources - RNA from an immune source in library construction [Barbas et al., 1991; Clackson et al., 1991]. However, this is not always possible, for example tolerance mechanisms prevent a human immune response to self antigens. Methods are being established to circumvent this problem where necessary (see section 1.2.6.4.6 below).

Affinity purification of phage antibodies by 'panning' makes it possible to select even rare antigen binding antibody combinations from a library. Antibody fragments can be displayed to antigen in the following ways:

- antigen adsorbed to a plastic surface [Barbas et al., 1991; Marks et al., 1991b],
- columns of antigen linked to a matrix [McCafferty et al., 1990],

- biotinylated antigen in solution, subsequently captured on streptavidin coated magnetic beads [Hawkins et al., 1992],
- antigen expressed on the surface of an immobilised cell [Marks et al., 1993].

Non-binders can be removed by washing and the bound phage can then be eluted at low pH [Barbas et al., 1991], high pH [Marks et al., 1991b] or by addition of excess antigen [Clackson et al. 1991]. Successive rounds of selection can be achieved by infecting bacteria with the enriched phage and panning the phage prepared from the culture. The enrichment resulting from repeated rounds of selection should be sufficient to isolate specific phage occurring only singly in the initial library (i.e. about 1 in 10^7) [Burton and Barbas, 1993a].

Selected clones, either as phage [McCafferty et al. ,1990] or as expressed antibody fragment [Barbas et al.,1991; Hoogenboom et al., 1991], can be screened for reactivity with antigen, usually by conventional enzyme linked immunosorbant assay (ELISA).

1.2.6.4.6 Recent Advances in Antibody Phage Display Technology

Phage display technology has provided particular advantages in the production of antibodies against human antigens [Burton and Barbas, 1993b; Griffiths and Hoogenboom, 1993]. Several approaches have been adopted to circumvent the ethical and self-tolerence problems of human immunisation [French, 1992].

In one method, libraries have been constructed from mRNA derived from the bone marrow or peripheral blood lymphocytes of a sero-positive donor, being an individual shown to have antisera with high antibody titres against particular antigens [Burton et al., 1991]. Such humans have not been actively immunised, but have come in contact with antigen, usually in the form of an infectious disease. For donors who had not recently been exposed to antigen, the library could be constructed from RNA isolated from memory cells that had been selected with antigen coated magnetic beads [Hawkins and Winter, 1992]. Antibody fragments against several viral antigens have been

isolated from these human combinatorial libraries. Some of these anti-viral antibodies appear to be capable of inhibiting the infection of the particular virus, i.e. neutralising the virus by binding to its surface [Barbas et al., 1992b, 1992c; Williamson et al., 1993; Barbas et al., 1993]. Severe combined immune deficiency (SCID) mice [Bosma et al., 1983] can be used in conjunction with the combinatorial library approach [Duchosal et al., 1992]. Peripheral blood lymphocytes can be transferred from a sero-positive donor to SCID mice which are then stimulated with the appropriate antigen to boost a human antibody response outside the human body [Mosier et al., 1988]. RNA derived from antibody producing cells of these SCID mice can then be used to construct a combinatorial library from which antibody fragments binding the antigen can be selected.

An alternative approach which avoids human immunisation is to prepare naive libraries using RNA from an unimmunised source. However, the antibodies selected from such libraries tend to have only low or medium affinity (10^5-10^7 mol^{-1}) [Marks et al., 1991b; Gram et al., 1992]. Several strategies have been proposed for improving antibody affinities [Chiswell and McCafferty, 1992; Marks et al., 1992b]. These include chain shuffling (the process of pairing a specific heavy chain with several different light chains and vice versa) [Kang et al., 1991b; Marks et al., 1992a; Barbas et al.,1993d], or *in vitro* mutagenesis using, error-prone PCR [Lueng et al., 1989; Hawkins et al., 1992; Gram et al., 1992], spiked oligonucleotide primers [Derbyshire et al., 1986; Hermes et al., 1989] or growth of phage in mutator strains of *E.coli*. [Schaaper, 1988].

A process of 'guided selection' has been used to obtain antigen specific human antibodies from phage display repertoires using rodent derived monoclonal antibodies as a template [Johnson and Chiswell, 1993; Jespers et al., 1994; Figini et al., 1994]. In this approach, the rodent derived heavy chain is expressed paired with a phage library of human light chains. The phage are selected for binding to the specific antigen. The selected human light chains are

then paired with a repertoire of human heavy chains displayed on phage. The phage are selected again to obtain human Fab fragments that bind the same antigen as the original rodent derived monoclonal antibody.

Recently a novel approach of combinatorial infection has been used to increase a possible library size [Waterhouse et al., 1993]. This involves the infection of a library of light chains in a phagemid vector into *E.coli*. that have already been transformed with a library of heavy chains in a plasmid vector. *In vivo* recombination of the two libraries occurs to create a phage combinatorial library whose diversity is only limited by the efficiency of *E.coli*. infection ($>10^{12}$), rather than transformation ($\leq 10^{8}$). Theoretical studies have suggested that the larger and more diverse an antibody repertoire, the greater the chance of finding high affinity antibodies [Perelson and Oster, 1979]. High affinity human antibodies have been isolated from a large antibody repertoire [Griffiths et al., 1994].

As an alternative to the use of human genetic material, synthetic or semi-synthetic antibody libraries have been produced from cloned germline antibody genes or by randomising regions of single antibody clones [Barbas et al., 1992a; Hoogenboom and Winter, 1992 Lerner et al., 1992; Akamatsu et al., 1993; Barbas et al., 1993a; 1993b; Garrard and Henner, 1993]. In semi-synthetic libraries the antibody frameworks are derived from natural antibodies, but some or all of the CDRs are replaced by synthetic gene segments.

1.2.6.4.7 Future Prospects for Antibody Phage Display Technology

Antibody phage display systems have progressed rapidly and are becoming a powerful research tool. Immunised sources still seem to be the most appropriate for the construction of libraries with high affinity antibodies. However, it seems likely that with a greater appreciation of antigen-antibody interactions, the current methods for the production of phage display antibodies will be adapted to facilitate the isolation of high affinity antibodies to any antigen without the need for animal immunisation. The rapid progress

towards this goal has been promoted by the application to phage display technology of various known biological and molecular techniques, many of which are outlined above (section 1.2.6.4.6). Further advancement of the technology will no doubt be enhanced by the adoption of other innovative developments such as mutagenic DNA shuffling ('sexual PCR') [Stemmer, 1994a; 1994b; Smith, 1994] or transgenic mice that express human antibodies [Lonberg et al., 1994; Green et al., 1994].

In 'sexual PCR', recombination is promoted artificially by cleaving an initial population of DNA molecules into random fragments followed by a number of cycles of PCR in the absence of added primer. Fragments with complementary sequences (from the same or different original molecules) recombine during the annealing step to produce 'shuffled' molecules which are further amplified in a second conventional PCR with added primers. This process could be adapted to mimic the somatic mutation of the natural immune system.

Two groups have recently reported the insertion of elements of the human heavy and light chain locus into mice in which production of endogenous murine heavy and light chain was disrupted [Lonberg et al., 1994; Green et al., 1994]. These transgenic mice have been shown to express specific human antibody sequences in response to antigenic challenge. They may therefore prove to be an important immunisable source for the production of phage display libraries of human antibody sequences.

As antibody phage display technology continues to develop, it could become as widely used as the current monoclonal and polyclonal approaches. Antibody fragments isolated from phage display libraries will no doubt prove to be valuable tools in therapy and research.

1.3 PROJECT OBJECTIVES

The primary objective of this study was to raise a comprehensive range of antibodies to CFTR in order to facilitate a greater understanding of the topology and distribution of the protein. Such insights would in turn help elucidate the seemingly complex range of functions of CFTR and ultimately provide necessary information to direct therapeutic approaches to the disease.

In this study all three methods of antibody production described above (polyclonal, monoclonal, and phage display antibodies) have been adopted to raise a whole range of antibodies to CFTR. Polyclonal antibodies have been raised to CFTR fusion proteins and extensively characterised. Monoclonal antibodies raised to synthetic peptides corresponding to different regions of CFTR have been further characterised. The emphasis however is on the cloning and expression of antibody fragments (from sources immunised against CFTR) in combinatorial libraries using two different phagemid vector systems (pCANTAB5 and PComb3). The advantages and disadvantages of the two systems are compared and contrasted.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 General Chemicals and Materials.

The following were all purchased from Sigma Chemical Company Ltd., U.K.: Ethidium bromide, HEPES ($C_8H_{12}N_2O_4S$), octyl phenoxy poly ethaxyethanol (Triton X-100), polyoxyethylene sorbitan monolaurate (Tween 20), DNAse1, RNAseA, Lysosyme, 5-bromo-4-chloro-3-indole-phosphate (BCIP), nitroblue tetrazolium (NBT), N',N',N',N'-Tetramethylenediamine (TEMED), Ponceau S solution, phenyl methyl sulfonyl flouride (PMSF), Aprotonin, Benzamidine, Lysosyme, BCIP, ortho-phenylenediamine (OPED) and Coomassie Brilliant Blue R.

Acrylamide/bis-acrylamide stock solutions were obtained from Severn Biotech Ltd. and agarose from FMC Bioproducts. Bind Silane was obtained from LKB-Produkter AB.

Ethylenedinitro tetra-acetic acid disodium salt (EDTA) and Mowiol were purchased from CalBiochem Novabiochem (UK) Ltd., bovine serum albumin fraction V (BSA) from Park Scientific Ltd., and Marvel from Cadbury. The protease inhibitors, Antipain, Leupeptin and Pepstatin A were all supplied by Cambridge Research Biochemicals.

Conjugated antibodies were obtained from Dako Products. Streptavidin conjugated magnetic beads were purchased from Dynal, Oslo as was a (MPC-E) magnet.

Radiochemicals were obtained from Du Pont-NEN Ltd., UK. Radiolabelled gels were exposed to Amersham Hyperfilm. Polaroid 667 film was obtained from Polaroid (UK) Ltd..

Ampicillin (Penbritin) and Carbenicillin (Pyropen) were obtained from Beecham Research. Tetracycline (Achromycin) was obtained from Cyanamid of

Great Britain Ltd. and Kanamycin (Kannasyn) from Sanoji Winthop Ltd.. Penicillin, Streptomycin and Geneticin (G418) were purchased from Gibco BRL Ltd..

The following were obtained from LKB-Pharmacia: DNA molecular weight standard markers, Helper Phage M13KO7, nuclease-free BSA, RNA extraction kit (27-9270-01), mRNA purification kit (27-9258-01) and cDNA first strand synthesis kit (27-9661-01). Helper Phage VCSM13 was purchased from Strategene Ltd., Cambridge. Molecular weight standard markers for protein gels were obtained from Amersham International plc.

Preparative DNA columns were obtained from Qiagen Inc. and Promega Corporation (magic mini prep columns). A Promega TA cloning kit was also used. DNA purification columns were obtained from Clontech Laboratories Inc.. Centricon microconcentrators were supplied by Amicon Ltd.,UK.

Sequenase version 2.0 sequencing kit was obtained United States Biochemicals (USB), Amersham Life Sciences.

Restriction endonucleases and their appropriate reaction buffers were all obtained from Boehringer Mannheim UK Ltd., as were lithium salts of 2'-deoxy-nucleotides, *Thermus aquaticus* (*Taq*) Polymerase, pre-hybridisation solution, random prime DNA labelling kit and DNA Ligase. DNA Ligase was also obtained from Gibco BRL Life Technologies Ltd..

Unless stated otherwise, oligonucleotide primers and peptides (including biotinylated peptides) were synthesised within the SERC funded Molecular Recognition Centre, University of Bristol.

Nitrocellulose membranes for Western blotting were obtained from Schlescher and Schuell. Millipore 0.025μm membranes were used for microdialysis. Dialysis tubing obtained from Medicell International Ltd. was used for electroelution of DNA.

Bacto-tryptone and bacto-yeast extract were obtained from Difco, Detroit. Agar was obtained from Lab M, Bury, Lancs. Tissue culture media were obtained from Gibco,BRL Ltd.. Sterile pipettes for tissue culture work were

obtained from Sterilin Ltd., UK and tissue culture flasks and dishes from Costar Europe Ltd. , Corning Glass Works and Falcon, Becton Dickinson.

All other chemicals were purchased from BDH Chemicals Ltd., unless otherwise stated and were of AnalaR grade or the purest grade available.

2.1.2 Bacterial Strains and Growth Media.

Three different *E. coli*. strains were used. These were XL1-blue [Bullock et al., 1987], JM109 [Yanish-Perron et al., 1985] and TG2 (a recombination-deficient derivative of TG1) [Gibson 1984].

The following growth media were used:

LB broth : 10g/l bacto-tryptone, 5g/l bacto-yeast extract, 5g/l NaCl.

2TY: 16g/l bacto-tryptone, 10g/l bacto-yeast extract, 5g/l NaCl.

SOBAG : 20g/l bacto-tryptone, 5g/l bacto-yeast extract, 0.5g/l NaCl, 10mM $MgCl_2$, 0.1M glucose and 100μg/ml ampicillin.

SOC : 20g/l bacto-tryptone, 5g/l bacto-yeast extract, 0.5g/l NaCl, 2.5mM KCl, 10mM $MgCl_2$ and 20mM glucose.

SB: 30g/l bacto-tryptone, 20g/l bacto-yeast extract, 10g/l MOPS.

To propagate the *E.coli*. on solid media, agar was added to the appropriate broth to a final concentration of 1.6%.

2.1.3 Eukaryotic Cell Lines

A variety of different cell lines were used. They are listed below.

2wt - C127 cell line transformed with CL3AXBPVXT-NEO vector expressing CFTR [Marshall et al., 1994]

BPV - C127 cell line transformed with CL3AXBPVXT-NEO vector not expressing CFTR.

CaCo2 - human colon carcinoma cell line [Fogh et al., 1977]

Panc1 - human pancreatic carcinoma cell line expressing CFTR [Leiber et al., 1975]

C127 - mouse mammary tumour cell line [Lowry et al., 1978]

Heb7a - Hela derived cell line [Wallace et al., 1975]

HT29 - human colon carcinoma cell line [Fogh et al., 1975]

T84 - human colon carcinoma cell line [Murakami et al., 1980]

2.2 METHODS

2.2.1 Small Scale Preparation of Plasmid DNA by Alkaline Lysis

Method A -

This method is based on that described by Birnboim and Doly [1979]. A single colony of *E.coli.* cells was used to inoculate 5ml of LB amp broth containing 100μg/μl ampicillin and incubated at 37°C (or at 30°C when the pUEX 1 plasmid was involved [Bressan and Stanley 1987]) with vigorous shaking for a minimum of 16 hours. The cells were harvested from 3ml of culture by centrifugation in a microfuge for 2 minutes. The cell pellet was resuspended in 100μl of solution I (50mM glucose, 10mM EDTA, 25mM Tris-HCl pH8.0). 200μl of solution II (1%SDS, 0.2M NaOH) was added to lyse the cells while incubating on ice for 5 minutes. The solution was neutralised by the addition of 150μl of solution III (5M potassium acetate pH4.8). The solution was spun in a microfuge for 5 minutes, the pellet was discarded and an equal volume of phenol/chloroform/isoamyl alcohol (24:24:1) added to the supernatant. An emulsion was produced by vortexing and the two phases were separated by spinning in a microfuge for 2 minutes. 1ml of ethanol was added to the upper aqueous phase, the solution mixed and then kept at -20°C for 30 minutes. The precipitated DNA was collected by spinning in a microfuge for 15 minutes. The DNA pellet was washed with 400μl of 70% ethanol before vacuum drying. The pellet was resuspended in 50μl of 10mM Tris/HCl, pH8.0 and 1mM EDTA, pH8.0 (TE buffer) containing 20μg/ml RNaseA. The resultant solution of plasmid DNA was now suitable for analysis by restriction endonuclease digestion.

Method B-

This method is based on Promega Wizard Mini Prep system. A single colony of *E.coli.* cells were used to inoculate 5ml of LB amp broth containing 100μg/μl ampicillin and incubated at 37°C (or at 30°C where the pUEX 1 plasmid was involved [Bressan and Stanley 1987]) with vigorous shaking for a minimum of 16 hours. The cells were harvested from 3ml of culture by centrifugation in a microfuge for 2 minutes. The cell pellet was resuspended 200μl of cell resuspension solution (50mM Tris/HCl, 10mM EDTA pH7.5 containing 100μg/ml RNaseA). 200μl of cell lysis solution (0.2M NaOH, 1%SDS) was added and mixed by inverting the tube several times prior to addition of 200μl neutralisation solution (2.55M potassium acetate, pH4.8). Mixing was again achieved by inverting the tube before removing the cell debris by spinning in a microfuge for 5 minutes. The supernatant was removed to a fresh microcentrifuge tube containing 1ml of Wizard Mini Prep DNA purification resin and mixed by inverting the tube. The resin containing the bound DNA was decanted into the barrel of a 5ml syringe which was attached to the luer-lok extension of a Wizard Mini Prep column. The syringe plunger was inserted and the slurry gently pushed into the mini column. The mini column was washed with 2ml column wash solution (200mM NaCl, 20mM Tris/HCl, 5mM EDTA diluted with 50% (v/v) ethanol). The column was then dried by placing it in a microcentrifuge tube and spinning for 20 seconds. The mini column was then transferred to a fresh microcentrifuge tube and the DNA eluted by applying 50μl H_2O to the top of the mini-column and spinning for 20 seconds. The resultant solution of plasmid DNA was suitable for sequencing or analysis by restriction endonuclease digestion.

2.2.2 Medium Scale Preparation of Plasmid DNA

A single colony of *E.coli.* cells were used to inoculate 5ml of LB amp broth containing 100μg/ml ampicillin and incubated at 37°C with vigorous shaking for several hours until the broth was turbid. This culture was used to

inoculate 100ml LB amp broth, containing 100μg/ml ampicillin, which was incubated at 37°C with vigorous shaking for at least 16 hours. Cells were collected by centrifugation at 4500g for 20 minutes at 4°C, and resuspended in 4ml of 50mM Tris/HCl, 10mM EDTA containing 100mg/ml RNaseA. Cell lysis was achieved by adding 4ml of 200mM NaOH, 1%SDS, mixing gently and incubating at room temperature for 5 minutes. The solution was neutralised by adding 4ml of 2.55M potassium acetate pH4.8 and mixing gently. Cellular debris was pelleted by centrifugation at 30000g for 30 minutes at 4°C. The supernatant was applied to a Qiagen-tip 100 which had been pre-equilibrated with 4ml of buffer QBT (750mM NaCl, 50mM MOPS, 15% ethanol adjusted to pH7.0 and then 0.15% Triton X-100 added). The column was then washed with 2x10ml of buffer QC (1.0M NaCl, 50mM MOPS, 15% ethanol, pH7.0). The DNA was eluted from the column with 5ml buffer QF (1.25M NaCl, 50mM MOPS, 15% ethanol, pH8.2) and precipitated with 0.7 volumes of isopropanol. The DNA was recovered by centrifugation at 15000g at 4°C for 30 minutes. The DNA pellet was washed with 5ml 70% ethanol and air dried for 5 minutes before redissolving in a suitable volume of TE buffer or water.

2.2.3 Preparation of Total RNA

RNA was obtained using a LKB-Pharmacia RNA Extraction Kit (27-9270-01), following the manufacturer's protocol. Precautions were taken to minimise RNA digestion by ribonucleases present in the cells from which the RNA was being extracted. For RNA preparation from tissue culture cells the extraction buffer containing guanidinium thiocyanate was added directly to the cells in the tissue culture flask. For preparation of RNA from animal tissues the appropriate tissue (spleen from mice and bone marrow from rabbits) was removed aseptically and directly transferred to an aliquot of extraction buffer (in an Oakridge tube) [Chirgwin et al., 1979]. This was immediately homogenised with a polytron apparatus. RNA was separated from other cellular components by centrifugation through a caesium trifluoroacetate

(CsTFA) gradient [Okayama et al., 1987].

2.2.4 Preparation of mRNA from Total RNA

Total RNA was applied directly to oligo(dT)-cellulose spun columns for the affinity purification of polyadenylated RNA supplied in a LKB-Pharmacia mRNA Purification Kit (27-9258-01). mRNA was isolated following the manufacturer's protocol.

2.2.5 First Strand cDNA Synthesis

First strand cDNA was prepared from either total RNA or on mRNA using a LKB-Pharmacia First Strand cDNA Synthesis Kit (27-9261-01), following the manufacturer's protocol. The reaction was primed with primers supplied with the kit, using either 5μg of a *Not* I-d(T)18 bifunctional primer or 0.04μg pd(N)$_6$ in the reaction.

2.2.6 Digestion of DNA with Restriction Enzymes

Restriction endonuclease digests were carried out at 37°C (unless stated otherwise) using the stock concentrated buffers provided with the enzymes. The enzymes were generally diluted 1 in 10 in the final reaction solutions. The quantity of DNA digested, the amount of enzyme used and the incubation time varied according to requirements.

2.2.7 Polymerase Chain Reaction (PCR)

DNA sequences were amplified by PCR [Kawasaku 1990; Saiki 1990] in a Perkin Elmer-Cetus DNA thermal cycler, using *Taq* DNA polymerase (2.5 units) and the reaction buffer supplied by the manufacturer. Added to each reaction mix were: i) deoxyribonucleotides (dATP, dCTP, dGTP and dTTP) to a final concentration of 0.2mM, ii) 1μM of each the two specific primers required to recognise the DNA template and iii) an appropriate concentration

(between 10ng and 1μg depending on the source) of template DNA. The reaction components were overlaid with mineral oil. Unless otherwise stated, PCR reactions were incubated for 1 minute denaturation at 94°C, 2 minutes annealing at 52°C and 2 minutes extension at 72°C for 30 cycles followed by a 10 minute incubation at 72°C to allow complete extension of all the reactions.

2.2.8 Purification of DNA on Chromaspin Columns

Clontech chromaspin columns were used according to the manufacturer's protocol for rapid and efficient purification of DNA in the following situations: a) to selectively eliminate unextended PCR primers, b) to separate DNA away from enzymes and other proteins and c) to remove unincorporated nucleotides from DNA labelling reactions.

2.2.9 Agarose Gel Electrophoresis

All gel electrophoresis was carried out in TAE buffer (0.2M Tris base, 0.5mM EDTA and 0.12% acetic acid adjusted to pH7.8). Multi purpose agarose was used for analytical gels and genetic technology grade (GTG) agarose was used for preparative gels. The concentration of agarose in each gel used varied depending on the sizes of the DNA fragments to be separated but was chosen from the following table.

% agarose	size range (kilo base pairs)
0.8	0.5-10
1.5	0.2-4
4	<0.2-0.5

Each gel contained 0.2 mg/ml ethidium bromide and the DNA was visualised on a UVP UV transilluminator at wavelength of 302nm. Prior to loading samples onto the gel, 1/10th volume of sample buffer (0.4% xylene orange, 1mM EDTA pH8.0 and 50% glycerol) was added.

2.2.10 Purification of DNA from Agarose Gels

Method A - Phenol Freeze Extraction

Ethidium stained DNA was illuminated within the agarose gel by UV light (as described above) and a gel fragment cut from the gel containing the desired DNA band. The gel fragment was diced, transferred to a 1.5ml Eppendorf tube and an equal volume of TE buffered phenol added. The mixture was vigorously vortexed, snap frozen in liquid nitrogen and centrifuged in a microfuge for 15 minutes. The upper aqueous phase was removed and saved whilst the lower phenolic phase was back extracted with 200μl of TE buffer. The two aqueous phases were combined and the DNA precipitated by the addition of 1/10th volume of 5M ammonium acetate and two volumes of ethanol. The solution was mixed and incubated on dry ice for 20 minutes or at -70°C for 1 hour. The precipitated DNA was collected by centrifugation at 48000g for 30 minutes at 4°C. The DNA pellet was washed in 70% ethanol, air dried and resuspended in 10μl of TE buffer.

Method B - Electroelution of DNA

Ethidium stained DNA was illuminated within the agarose gel by UV light (as described above) and a gel fragment containing the appropriate band was cut from the gel. The gel chip was transferred to a length of pre-boiled dialysis tubing (size 5) with 400μl 0.1 x TAE buffer. All the air bubbles were removed before the tubing was clipped at both ends and allowed to rest just submerged, in an electrophoresis tank containing 0.2 x TAE. Electrophoresis was carried out at 200V for 30-60 minutes. The buffer containing DNA was illuminated over a UV transilluminator and removed to an Eppendorf tube. The solution was chilled on ice for 2 minutes, prior to spinning in a microfuge for 10 minutes, to remove any residual gel material. The supernatant was removed to a fresh tube, extracted first with an equal volume of phenol and then with an equal volume of phenol/chloroform/isoamyl alcohol (24:24:1). The DNA was precipitated out of the supernatant as described above.

2.2.11 Quantification of DNA

The concentration of DNA samples was ascertained on a 0.7% agarose plate containing 0.7μg/ml ethidium bromide. 1μl of the DNA solution to be assessed was dotted onto the plate along with 1μl of dilutions of plasmid DNA (of known concentration). The intensity of fluorescence of the dots was examined and compared by illumination with UV. The concentration of the DNA sample was estimated from the fluorescence it gave relative to fluorescence observed from the plasmid DNA dilutions of known concentration.

2.2.12 Subcloning Procedures

Unless otherwise stated, ligations were performed in a final reaction volume of 20μl using Boehringer Mannheim ligase and ligation buffer. Between 50 and 100ng of plasmid vector was used per reaction with a 3 fold molar excess of "insert" as recommended by Reevie et al., [1988]. Ligations were incubated at 16°C overnight. The ligation mixtures were microdialysed with water on 0.025μm membranes prior to electroporation into *E.coli*.

2.2.13 Preparation of Electrocompetent Cells

Method A -For preparation of TG2 cells [Gibson, 1987] and JM109 cells [Yannish-Perron,1985].

A single colony of *E. coli.* was picked from a fresh overnight agar plate into 5 ml of LB amp broth and incubated at 37°C with vigorous shaking until a swirl of cells could be seen (usually about 2 hours). The whole of this culture was transferred to 500 ml of LB broth (pre-warmed to 37°C) and shaken vigorously at 37°C until the optical density of the culture at 600 nm was between 0.5 and 0.7. The cells were then chilled on ice before being harvested by centrifugation at 4500 rpm and 4°C for 25 minutes in pre-chilled centrifugation bottles in a Sorvall RC3B. All further manipulations of the cells

were performed on ice and all further solutions used were pre-chilled on ice. The supernatant was removed and the cells resuspended in 500ml of 1mM HEPES, pH7.0 before being harvested by centrifugation as described above. A second wash with 200ml 1mM HEPES pH7.0 was performed as described above. The cells were resuspended in 50ml of sterile deionised water and harvested as described above. The cell pellet was then resuspended in 10ml of 10% glycerol in sterile deionised water and harvested as before. The final cell pellet was resuspended in 0.5ml of 10% glycerol in sterile deionised water to give a final volume of approximately 2ml. Aliquots (usually 40μl) of these cells could then be used for transformation by electroporation. Cells not to be used immediately could be stored at -70°C following the addition of an equal volume of 40% glycerol in sterile deionised water and snap freezing in liquid Nitrogen. Frozen cells were defrosted slowly on ice prior to their use in transformation.

Method B - For the Preparation of XL1-Blue Cells [Bullock et al., 1987] for transformation with Fab fragment antibody libraries.

A single colony was picked from a SB plate containing 10μg/ml tet into 10ml SB and incubated overnight at 37°C with vigorous shaking. 2.5ml of this overnight culture was inoculated into 0.5 litre of SB with 10ml 10% glucose and 5ml 1M $MgCl_2$. This was incubated with vigorous shaking at 37°C for approximately 4 hours until the optical density of the culture at 600nm was between 0.7 and 0.8. The culture was chilled on ice for 15 minutes before being harvested by centrifugation at 4500rpm and 0°C in bottles (pre-chilled to -70°C) in a Sorvall RC3B. All further manipulations were performed on ice using solutions pre-chilled on ice and pipettes chilled at -70°C. The supernatant was poured off and the cells were resuspended with gentle pipetting in 250ml 10% glycerol prior to recovering the cells by centrifugation as described above. A second wash with 250ml 10% glycerol was performed as above. The cellular pellet recovered after centrifugation following the second wash was resuspended in 25mls 10% glycerol and transferred to a 50ml conical tube (pre-

chilled to -70°C). The cells were harvested by centrifugation at 3500rpm for 15 minutes at 0°C in a Sorvall RC3B. The supernatant was decanted off from the cells until the loose cell pellet began to dislodge from the bottom of the tube. The cells were resuspended briefly, with a pipette, in the solution remaining in the tube and immediately aliquoted into 40μl or 200μl volumes into tubes placed in a ethanol/dry ice bath so that the cells froze instantaneously. These aliquots were stored at -70°C prior to their use in electrotransformation.

2.2.14 Transformation of Competent *E.coli* . by Electroporation

Electroporation was carried out as described by Dower et al. [1988], in 0.2cm gap cuvettes using a Bio Rad gene pulser set at 2.5kV, 25Ohms and 25μF. A frozen aliquot of cells was defrosted on ice. The DNA to be transformed was added to the cells and left on ice for 1 minute before transferring to a cuvette pre-chilled on ice. After pulsing the cuvette was immediately flushed with 1-3ml SOC medium at room temperature. The cells were usually allowed to recover by incubation at 37°C for 1 hour before an aliquot of this solution (between 10 and 100μl) was plated onto a LB agar plate containing 100μg/ml ampicillin. The plates were then incubated at 37°C (or 30°C in the case of cells electroporated with the pUEX series of plasmids) for at least 16 hours.

2.2.15 Double Stranded DNA Sequencing

This was performed using the Sequenase version 2.0 kit. 4μg of plasmid or phagemid DNA in 18μl H_2O was used for each sequencing reaction. To the DNA solution 2μl of 2M NaOH/2mM EDTA was added, mixed and incubated at room temperature for 5 minutes. The DNA was precipitated by the addition of 16μl of ice cold 5M ammonium acetate and 200μl of ethanol. After incubation at -20°C for 30 minutes, the DNA was collected by centrifugation at 48000g for 15 minutes at 4°C. The DNA pellet was washed in 70% ethanol, air dried and resuspended in 10μl of annealing buffer (40mM Tris/HCl, pH7.5,

20mM $MgCl_2$, 50mM NaCl) containing 10ng of sequencing primer. The annealing reaction was performed at 65°C for 5 minutes prior to cooling slowly to 35°C over 15-30 minutes and subsequent quenching on ice. The labelling reaction was set up by adding 5.5μl of labelling mixture (0.55μM each of dGTP, dCTP and dTTP, 18mM DTT, 5 units Sequenase enzyme and 5mCi α35S dATP [800 Ci/mmol]) and incubating at room temperature for 2 minutes. 3.5μl of this solution was added to 5μl of each of the termination mixes (each consisting of 80μM of each of the dNTPs and also 8μM of the same ddNTPs in 50mM NaCl) and incubated at 37°C for 5 minutes. The reaction was stopped by the addition of 4μl of stop solution (95% formamide, 20mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF) and incubation at 85°C for 4 minutes. The solution could be stored at -20°C or 2.5μl loaded immediately on a 6% acrylamide/urea gel.

2.2.16 Preparation and Running of Sequencing Gels

Sequencing reactions were electrophoresed on a 0.2 mm thick, 6% acrylamide gel preheated to 50°C. The gel mixture consisted of 1.8ml 25xTBE (2.5M Tris base, 2.5M orthoboric acid 0.06M EDTA finally adjusted to pH 8.3), 1.2ml deionised water, 27ml 6.7% acrylamide solution (60ml of 38% acrylamide/2% bis acrylamide, 192g urea made up to 360ml with deionised water), 130μl 10% ammonium persulphate and 17μl TEMED. This mixture was poured between 2 glass plates 0.2mm apart. Both glass plates were washed with ethanol. One plate was then coated with bind silane (0.3ml bind silane A-174, 100ml ethanol, 30μl acetic acid), which would cause the gel to stick to that plate. The other plate was coated with Repelcote, which would prevent the gel sticking to it.

The gel was assembled in a Touzart and Matignon electrophoresis apparatus with 0.8% TBE in the upper reservoir and 1.2% TBE in the lower reservoir. The gel was electrophoresed at 2500 V for approximately 1.5 hours

for a short run and 3-4 hours for a long run . The apparatus was disassembled and the gel and plate, to which the gel had stuck, was submerged in 1.5 litres of 10% acetic acid for 30 minutes to fix the gel. The gel was dried onto the glass plate in an 80°C oven. Once dry the gel, still attached to the plate, was cooled and exposed to X-ray film .

2.2.17 Eukaryotic Cell Culture

All manipulations were carried out in a vertical laminar flow cabinet. Cells were incubated at 37°C in a humidified atmosphere containing 5%(v/v) CO_2. All cells except hybridoma fusions were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal calf serum and 0.06mg/ml penicillin and 0.1mg/ml streptomycin. For the culture of BPV and 2wt cells 0.5mg/ml Geneticin (G418) was also added to the medium. Hybridoma fusions were cultured in RPMI 1640 media supplemented with 10% foetal calf serum, 0.06mg/ml penicillin and 0.1mg/ml streptomycin plus 100μM hypoxanthine, 16μM aminopterin and 10μM thymidine (HAT supplement) to select for fused myeloma-spleen cells [Littlefield, 1964].

2.2.18 Preparation of Membrane Proteins for SDS Page Electrophoresis

For protein extraction, HT29 cells were washed five times with cold PBS (137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4.7 H2O, 1.4 mM KH_2PO_4) and collected into a buffer containing 10mM Tris-HCl pH 8.0, 10mM $MgCl_2$ and the protease inhibitors antipain (50mg/ml), aprotonin (10mg/ml), benzamidine (300mg/ml), leupeptin (5mg/ml), pepstatin A (5mg/ml) and PMSF (phenylmethylsulphonyl fluoride) (175mg/ml). Cells were lysed by repeated passage through a 19 gauge needle. Cellular and nuclear debris were removed from the lysate by a 5 minute centrifugation at 300g and membranes pelleted by a 30 minute centrifugation at 100,000g. The membrane pellet was dissolved in 2.5% Triton X-100 in PBS or 10% Mega -10 in PBS.

2.2.19 Preparation of Decanoyl-N -methylglucamide (Mega - 10)

Mega-10 was prepared according to the method described by Hildreth, [1982]. This consisted of mixing 19.5g of N-methyl-D-glucamide with 150ml methanolin a 500ml round bottom side arm flask and refluxing it at 70°C until dissolved. Next 17.23g of decanoic acid was added to 10g of pyridine dissolved 100ml of ether on ice. After 10 minutes 11.5ml of ethyl chloroformate was rapidly added to the ether mix and allowed to stand for 20 minutes on ice. This mixture was then filtered through a sintered glass funnel into the side arm flask containing the methanol/N-methyl-D-glucamide mixture at 50°C. The funnel and the flask were rinsed with 50ml cold ether which was then added to the side arm flask. The resulting mixture was allowed to stand at room temperature for one hour before being stored at 4°C overnight. The solution was then filtered to remove any unreacted N-methyl-D-glucamide and the solvents removed by rotary evaporation at 30-50°C. The resulting oily liquid was poured into 300ml of ice cold ether, stirred and the solid product collected by pouring off the ether and allowing it to dry. The crude MEGA-10 was redissolved in the minimum volume of methanol/ether (1:9) at 50°C and allowed to crystallise out by cooling on ice. The powder was collected by filtration and allowed to air dry prior to use. Typically a yield in excess of 90% could be achieved using the above method.

2.2.20 Bradford's Analysis of Protein Concentration

Protein conncentrations were determined by the method described by Bradford et al., [1976]. Briefly this involves adding 1ml of Bradford reagent to a known volume of protein solution, leaving for 5 minutes for colour to develop and reading absorbance at 595nm.

The Bradford's reagent was made by dissolving 0.1g of Coomassie brilliant blue R in 50ml of 95% v/v ethanol, adding 100ml of 85% orthophosphoric acid and making the volume up to 1 liter prior to filterting

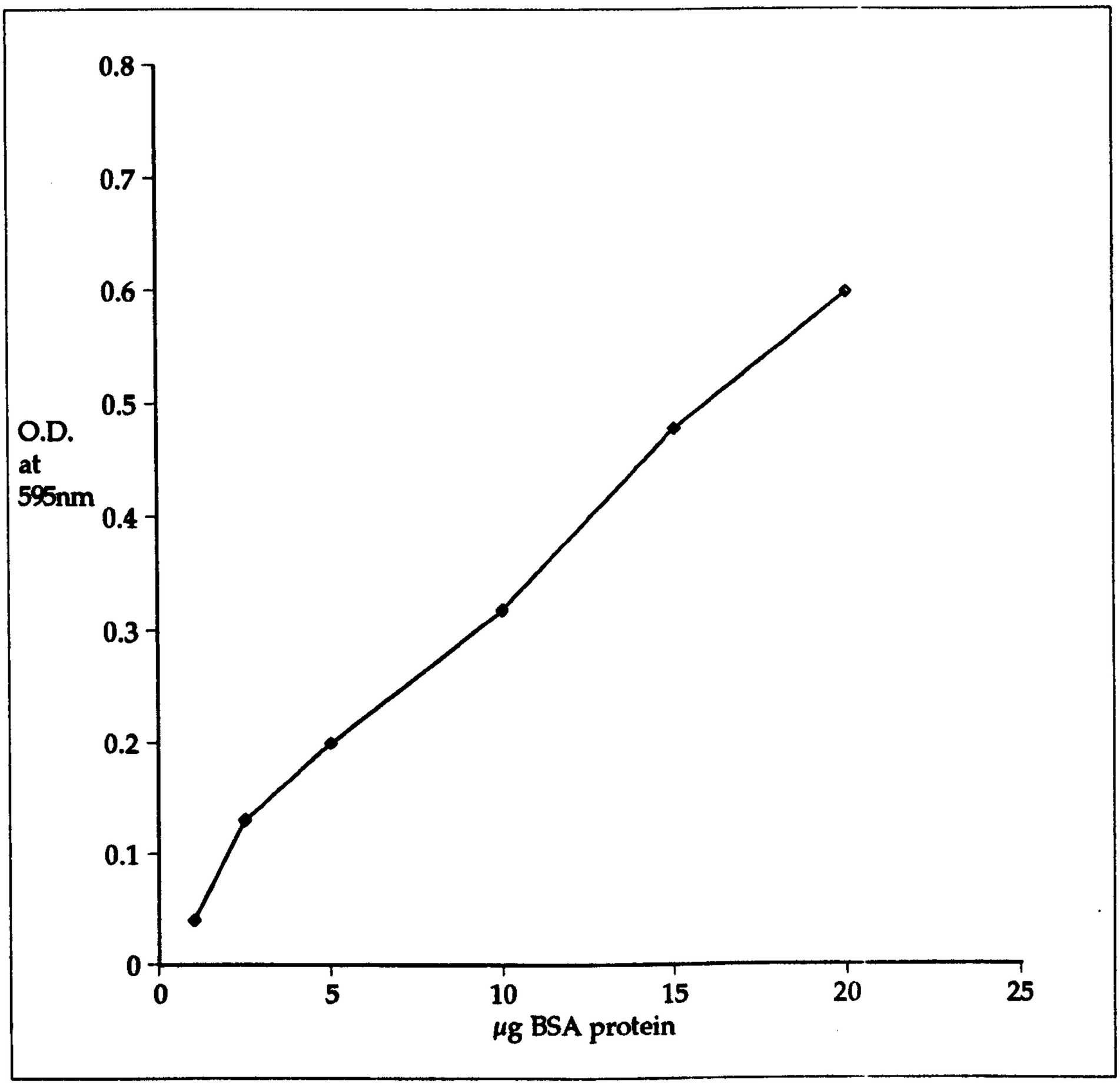


Fig. 2.1 Standard Bradford's Curve

the solution. Standard calibration curves were prepared for each batch of reagent using various volumes of 1mg/ml BSA solution, see fig.2.1 for a typical calibration curve.

2.2.21 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE)

This method of protein separation was carried out using the Bio Rad mini protean II SDS PAGE gel electrophoresis system and based on the method first described by Laemli, [1970]. The percentage of acrylamide varied according to the sizes of the proteins to be separated. The running gel contained acrylamide diluted from a stock acrylamide solution (consisting of a mixture of 30% w/v acrylamide and 0.8% w/v bis acrylamide) to the desired percentage, 0.4M Tris/HCl, pH 8.8, 0.1% (w/v) SDS. The gel solution was polymerised by the addition, to a final concentration of 0.1% w/v ammonium persulphate and 0.08% TEMED (N',N',N',N'-tetramethylenediamine). The stacking gel consisted of the stock acrylamide diluted to give a final acrylamide concentration of 5 % w/v, 0.13M Tris/HCl, pH6.8, 0.1% SDS and TEMED and ammonium persulphate added (as described above) to polymerise the gel. Samples were prepared by the addition of an equal volume of SDS gel sample buffer (200mM Tris/HCl, pH6.8, 25% glycerol, 5% SDS, 0.1% Bromophenol blue) and DTT to a final concentration of 50mM. The sample was either boiled for 5 minutes (if it was a fusion protein preparation) or heated at 37°C for 10 minutes (if it was a eukaryotic cell membrane preparation) before loading onto a gel. The sample was electrophoresed at 160V until the bromophenol dye front had reached the bottom of the gel. The proteins separated on the gel were either Western blotted directly onto nitrocellulose membrane or visualised by Coomassie brilliant blue staining.

To stain the proteins by Coomassie brilliant blue, the gels was soaked in stain solution (0.1% w/v Coomassie brilliant blue in 50% v/v tri-cholro-acetic acid) for 30 minutes. The background staining of the gel was removed by

soaking the gel in destain solution (30% v/v acetic acid and 10% v/v methanol) to reveal the stained protein bands.

2.2.22 Western Blotting

After a SDS PAGE gel had run to completion the gel was transferred to a piece of Whatman 3MM filter paper pre-soaked in Western blot transfer buffer (20mM Tris base, 150mM glycine). The gel was overlaid with a piece of nitrocellulose pre-soaked in transfer buffer, itself overlaid by another piece of Whatmann 3MM filter paper soaked in transfer buffer. This "sandwich" was placed between 2 buffer soaked sponges and then into the Bio Rad mini protean II Western blotting apparatus and immersed in chilled transfer buffer. The system was electro-blotted for a minimum of 45 minutes at 200mA. The proteins within the gel had now transferred to the nitrocellulose filter which could be probed with different antibodies as required.

2.2.23 Antibody Probing of Western Blotted Nitrocellulose Filters

On removal of the nitrocellulose filters, the proteins were visualised by incubation for 10 seconds in Ponceau S solution (0.1% Ponceau S w/v in 5% w/v acetic acid) and the molecular weight standard proteins marked in pencil. The Ponceau staining was washed off the filters whilst the filters were blocked. The nitrocellulose filters were incubated in the blocking solution 'blotto' (3% Marvel, 0.02 % Tween 20 in PBS) with gentle shaking for 1 hour. The filters were transferred to 10ml of 'blotto' containing a suitable dilution of primary antibody (antibody dilutions varied from neat to 1/1000 dilution depending on the antibody used) and incubated with gentle shaking for 1 hour. The filters were washed to remove the unbound antibody by 3 x 10 minute washes in 'blotto'. The filters were transferred to 10ml of 'blotto' containing a 1/1000 dilution of secondary antibody conjugated to alkaline phosphatase (obtained from DAKO) and incubated for 1 hour with gentle shaking. The filters were washed as described before and then developed. The filters were first washed

briefly in PBS. The filters were then equilibrated to an alkaline pH by soaking them in 100mM Tris/HCl, pH9.5, 100mM NaCl, 5mM $MgCl_2$. The colour reaction was started by the addition of 10ml of equilibration buffer containing 66μl of NBT solution (50mg/ml nitroblue tetrazolium dissolved in 70% dimethylformamide) and 66μl of BCIP solution (25mg/ml 5-bromo-4-chloro-3-indonyl-phosphate dissolved in dimethylformamide). The colour reaction was stopped by washing the filter in 10mM Tris/HCl, 1mM EDTA adjusted to pH8.0.

2.2.24 Immunofluorescence

Cells were cultured as described in Section 2.12 on glass microscope slide coverslips. When the cells reached approximately 80% confluency they were processed. The coverslips were transferred to a well of a 6 well plate and washed 3 times with 3ml of PBS. The cells were fixed by the addition of 3 ml of methanol (stored at minus 20°C) and incubated at -20°C for 5 minutes. The methanol was removed, replaced with 3ml of 0.2% BSA/PBS and incubated at room temperature for 20 minutes to 'block' the coverslips. The coverslips were probed with the primary antibody diluted accordingly. A 70μl aliquot of the diluted antibody was pipetted onto a piece of Lab film, on which the coverslip, cell side down, was placed. The coverslips were incubated with the primary antibody for 1 hour at room temperature. The coverslips were transferred back into the 6 well plate and washed twice with 3ml of PBS followed by two 3ml washes in 0.2% BSA/PBS. The coverslips were then probed with diluted secondary antibody (conjugated to either fluorescein of rhodamine and generally diluted 1 in 1000 in 0.2% BSA/PBS) in the same manner as described for the probing with the primary antibody. The coverslips were incubated with the secondary antibody for 30 minutes at room temperature. The coverslips were washed as described before and the mounted on microscope slides, cell side down on 20μl of Mowiol. The coverslips were viewed using a Zeiss microscope and lens. Photographs were taken of the coverslips using a Contax

167 Mt 35 mm camera,with Kodak TMAX 400 film.

2.2.25 Enzyme Linked Immunosorbant Assays (ELISAs)

Hybridoma supernatants and antisera were screened by ELISA against the relevant antigen. 96 well microtiter plates were seeded with 50-100µl antigen (10µg/ml or less) in PBS or 0.05M Na_2CO_3 (pH9.6) and left overnight at 4°C. All the subsequent steps were carried out at room temperature. The antigen was removed from the plates and 200µl 5% (w/v) BSA/ PBS was added to each well to block the non-specific sites. The plates were incubated for 30 minutes and then washed 4 times with 5% (w/v) BSA/PBS. 50µl of hybridoma supernatant or antisera diluted (usually 1/1000) in 5% (w/v) BSA/PBS and incubated for 1-2 hour. The wells were washed as before and incubated for 1 hour with peroxidase conjugated antibodies (rabbit anti-mouse antibody or swine anti-rabbit antibody) diluted 1/1000 in5% (w/v) BSA/PBS. The substrate solution containing 10ml citrate buffer (10.2g citric acid in 500ml H_2O), 10ml phosphate buffer (14.2g sodium phosphate in 500ml H_2O), 8mg OPED and 2µl H_2O_2 was prepared. The plates were washed as before and then developed with 100µl of substrate. The reactions were stopped after a suitable length of time by adding 50µl of 8M H_2SO_4 to the wells. Assay plates were read using a LabSystems Multiscanplus plate reader set at 450nm.

2.2.26 Animal Immunisations

All animal work was performed in the animal section of the University of Bristol by Mike Hill a suitably qualified animal technician .

CHAPTER 3

THE RAISING AND CHARACTERISATION OF POLYCLONAL ANTIBODIES TO FUSION PROTEINS PRODUCED BETWEEN CFTR FRAGMENTS AND BETA-GALACTOSIDASE.

3.1 INTRODUCTION

Topological studies of CFTR would be optimised if antibodies could be raised to recognise any part of the protein. Preferably the whole protein would be utilised for immunisation. Unfortunately, native CFTR protein is only expressed at low levels and has yet to be isolated and purified. This problem can be circumvented by using the identified gene to overexpress regions of CFTR as fusion proteins in *E.coli*. and isolating the products for immunisation.

A series of β-galactosidase/CFTR fusion constructs have been produced in pUEX 1 [Bressan and Stanely, 1987], a bacterial fusion protein expression vector (see fig. 3.1). Between them they cover almost the entire region of the expressed CFTR gene. In this chapter the generation of the fusion proteins and their use in the production of polyclonal antibodies to CFTR are described.

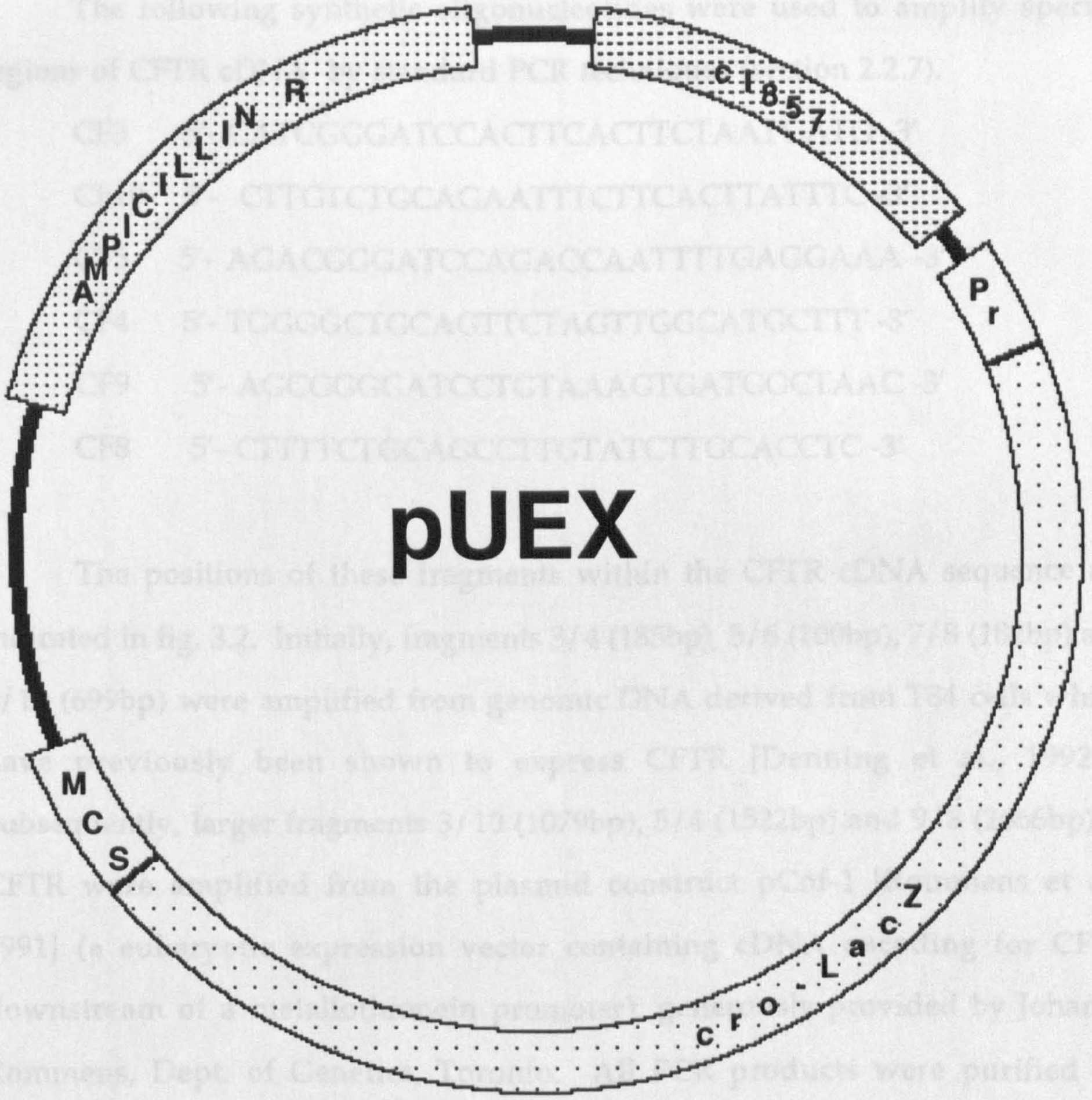


Fig. 3.1 Map of the bacterial expression vector pUEX

MCS	multiple cloning site
c1857	temperature sensitive repressor of the Pr promotor
Pr	promotor
cro-lac Z	fusion of the cro and lac Z genes

3.2 METHODS AND MATERIALS

3.2.1 Construction of pUEX Fusion Proteins

The following synthetic oligonucleotides were used to amplify specific regions of CFTR cDNA by standard PCR techniques (section 2.2.7).

CF3 5′- CATCGGGATCCACTTCACTTCTAATGATG -3′

CF10 5′- CTTGTCTGCAGAATTTCTTCACTTATTTC -3′

CF5 5′- AGACGGGATCCAGACCAATTTTGAGGAAA -3′

CF4 5′- TGGGGCTGCAGTTCTAGTTGGCATGCTTT -3′

CF9 5′- AGCGGGGATCCTGTAAAGTGATGGCTAAC -3′

CF8 5′- CTTTTCTGCAGCCTTGTATCTTGCACCTC -3′

The positions of these fragments within the CFTR cDNA sequence are indicated in fig. 3.2. Initially, fragments 3/4 (185bp), 5/6 (100bp), 7/8 (182bp) and 9/10 (699bp) were amplified from genomic DNA derived from T84 cells which have previously been shown to express CFTR [Denning et al., 1992a]. Subsequently, larger fragments 3/10 (1079bp), 5/4 (1522bp) and 9/8 (2666bp) of CFTR were amplified from the plasmid construct pCof-1 [Rommens et al., 1991] (a eukaryotic expression vector containing cDNA encoding for CFTR downstream of a metallothionein promoter), generously provided by Johanna Rommens, Dept. of Genetics, Toronto. All PCR products were purified on Chromaspin 100 columns prior to restriction enzyme digestion and ligation into the *BamH* I and *Pst* I sites of the multiple cloning site of pUEX1 (see fig. 3.1). The oligonucleotide primers were designed such that, using these restriction enzymes, the CFTR fragments were cloned in frame at the 3′ end of the β-galactosidase gene, thus generating DNA sequences encoding β-galactosidase/CFTR fusion proteins [Bressan and Stanley, 1987]. The constructs were transformed into *E.coli.* as described in section 2.2.14.

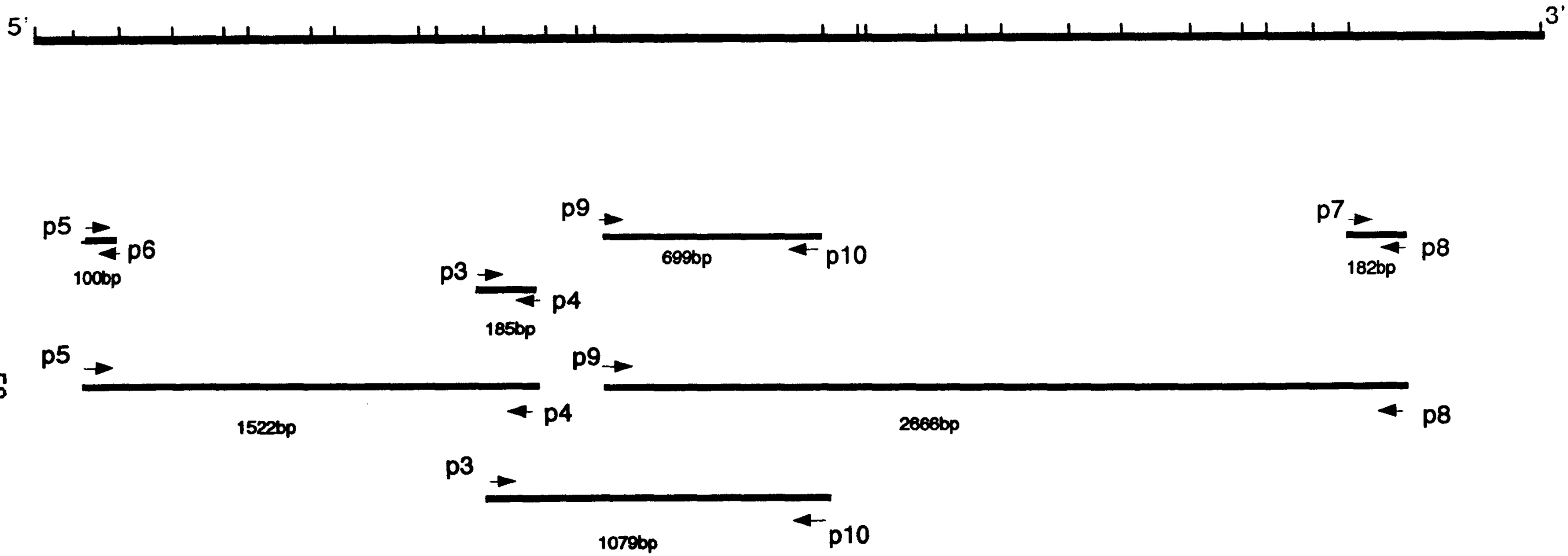


Fig. 3.2 Cartoon of CFTR cDNA illustrating areas coding for pcr p3-p10 primers used to generate fragments for cloning into the pUEX 1 fusion protein vector. The vertical dashes on the 5′ to 3′ cDNA represent exon boundaries.

3.2.2 Screening *E.coli* . Colonies with Oligonucleotide Probes

Transformed *E.coli.* colonies were streaked onto nitrocellulose filters, overlaid on LB amp agar plates and grown at 30°C for 16 hours. The filters were transferred onto 3MM Whatman paper presoaked in 5% SDS for 5 minutes and then transferred onto 3MM Whatman paper presoaked in denaturing solution (1.5M NaCl, 0.5M NaOH) for 5 minutes. The filters were blotted on 3MM Whatman paper to remove excess denaturing solution before being floated on neutralising buffer (0.5M Tris base, 1.5M NaCl, 37% HCl) for 5 minutes and then submerged in the same solution for a further 5 minutes. The nitrocellulose filters were pressed between 2 pieces of 3MM Whatman paper, rinsed in 6xSSC (20xSSC being 3M NaCl, 0.3M sodium citrate all adjusted to pH7.0 with HCl), air dried and then baked for 2 hours at 80°C.

The nitrocellulose filters were rehydrated by floating on 6xSSC and incubated in 50mM Tris/HCl, pH8.0, 1M NaCl, 1mM EDTA 0.1% SDS at 42°C in a Techne hybridiser HB1 hybridisation oven for 30 minutes. The nitrocellulose filters were blocked in 20ml of pre-hybridisation solution for 2 hours at 65°C. The pre-hybridisation solution was replaced by 20ml of fresh pre-hybridisation solution and 50μl of boiled cDNA probe. Hybridisation of the probe to the filters was allowed to proceed over a period of 16 hours at 65°C in hybridisation ovens.

To remove excess unbound cDNA probe, the nitrocellulose filters were washed for 20 minutes at room temperature in 2x SSC, 0.1% SDS, followed by 3x20 minutes washes at 65°C in 0.1x SSC, 0.1% SDS. Whilst still damp, the filters were wrapped in Saran wrap and exposed to X-ray film.

3.2.3 Radioactive Labelling of cDNA Probes

Appropriate oligonucleotide cDNA probes were synthesised using the Boehringer Mannheim random prime DNA labelling kit. The reaction requires 25ng of DNA in a final volume of 20μl. This solution was boiled for 10 minutes and snap chilled on ice. In the following order, 1 μl of the 0.5mM

dATP, dGTP and dTTP nucleotide stocks, 2μl of the 10x hexanucleotide reaction buffer, 5μl of a ^{32}P dCTP (10mCi/ml, 800mCi/mmol) and 1μl of 2units/μl Klenow were added to the denatured DNA solution. The reaction was allowed to proceed at 37°C for 30 minutes before being stopped by the addition of 2μl of 500mM EDTA and diluted to 50μl by the addition of sterile deionised water. This volume is suitable for application of the sample to a Chromaspin column. The labelled DNA was purified from unincorporated radionucleotides using a Chromaspin 100 spin column. The purified labelled DNA solution was boiled for five minutes and was then ready to be used to probe the desired filters.

3.2.4 Preparation of Bacterial Inclusion Bodies

This method was based on that described by Brake et al., [1990]. Two 5ml LB-amp broth aliquots containing 100mg/ml ampicillin were inoculated with *E. coli.* bearing a pUEX plasmid construct containing cDNA encoding CFTR regions of CFTR. The pUEX 1 vector allows the inducible expression of correctly inserted DNA sequences as β-galactosidase fusion proteins . Induction of fusion protein expression is driven by the Pr promoter of bacteriophage λ under the regulation of the temperature sensitive λ repressor *cI* 857. At 30°C the repressor is active and no fusion protein is produced, at 42°C the repressor is inactive and the strong Pr promoter leads to high level expression of the fusion protein. The inoculated medium was grown for 16 hours at 30°C with vigorous shaking. 8ml of the cultures were then transferred to 800ml of L-amp broth and grown, with shaking at 30°C, until the optical density of the culture at 600nm was 0.2. The entire culture was then transferred to a 2.5 litre flask, prewarmed to 42°C and then incubated for a further 2 hours with shaking. The bacterial cells were pelleted by centrifugation at 4500rpm for 20 minutes at 4°C in a Sorvall RC3B centrifuge. The bacterial cells were resuspended in 20ml of 100mM NaCl, 50mM Tris/HCl pH8.0 and transferred to a 50ml Falcon tube.

The cells were pelleted by centrifugation as before. The supernatent was discarded and the cells resuspended in 8ml of 15% sucrose, 50mM Tris/HCl pH8.0, 10mM EDTA. To this was added 2ml of a 10mg/ml Lysozyme solution. Then 2mg of DNAse I and 1 ml of 1M $MgCl_2$ were added, mixed and incubated on ice for a further 15 minutes. To lyse the cells, 6ml of detergent mix (10mM Tris/HCl, pH7.4, 100mM NaCl, 0.5% Deoxycholate, 1% Triton X-100) was added with complete lysis achieved after 5 minutes incubation on ice. The inclusion bodies were collected by centrifugation at 12000rpm for 10 minutes at 4°C in a Sorvall RC5C centrifuge. The pellet was resuspended in 10 ml of guanidinium wash solution (1.75M Guanidinium HCl, 1M NaCl, 1%Triton X-100) before centrifugation as before. These guanidinium washes were repeated twice. The pellet was resuspended in 10ml of 10mM Tris/HCl pH8.0 and centrifuged as before. The pellet was finally resuspended in 2ml of 10mM Tris/HCl pH8.0 before sonication to allow complete resuspension. The sample was aliquoted and mixed with an equal volume SDS sample buffer and DTT added to a final concentration of 100mM. The samples were then placed in a boiling water bath for 5 minutes and stored at -20°C.

3.2.3 Procedures for Rabbit Immunisations

Aliquots of the fusion protein preparations were defrosted, reboiled and run on 7.5% SDS polyacrylamide test mini gels prior to running larger preparative gels. Preparative gels were run at 10-20mA overnight until the loading buffer dye front had reached the bottom of the gel. The protein bands present in one inch width vertical strips that had been removed from the edge of each gel were visualised using Coomassie blue staining. The stained gel strips were aligned to the unstained gels and the overexpressed bands of fusion protein were cut from the unstained gels and shredded using scalpel blades. The gel was soaked in PBS at 4°C overnight and then sheared by repeated passage through needles attached to syringes. The resultant suspensions were injected into New Zealand white rabbits, both subcutaneously and

intramuscularly, on three occasions at monthly intervals. These were followed by intravenous injections at monthly intervals. For the intravenous injections the samples to be injected had had the acrylamide removed by centrifugation. Test bleeds were taken from the rabbits before the first injection (pre-bleeds) and 10 days after each intravenous injection. The blood was allowed to clot at 4°C for 16 hours and then centrifuged at 2000g for 10 minutes to separate serum from the clots and free red cells. The sera were removed, aliquoted and stored in the short term at 4°C and in the longer term at -20°C.

3.3 RESULTS

3.3.1 Production of Fusion Proteins

Specific regions of CFTR cDNA (fig.3.2) were amplified by PCR and cloned into an appropriate site in the plasmid based prokaryotic expression vector pUEX1 [Bressan and Stanley, 1987] as described above. This vector allowed the expression of a correctly inserted cDNA as the carboxy-terminal region of a β-galactosidase fusion protein. Individual clones of each construct were selected and sequenced to confirm that the insert was in the correct reading frame (data not shown). The correct sizes of the fusion proteins were established by testing aliquots of the expressed fusion proteins on mini SDS page gels (fig 3.3).

3.3.2 Production of Polyclonal Antibodies

Recombinant β-galactosidase fusion proteins were used to immunise rabbits. The antisera from immunised rabbits were designated CF3/4, CF5/6, CF7/8, CF9/10, CF3/10, CF5/4 and CF9/8 according to the construct used for immunisation.

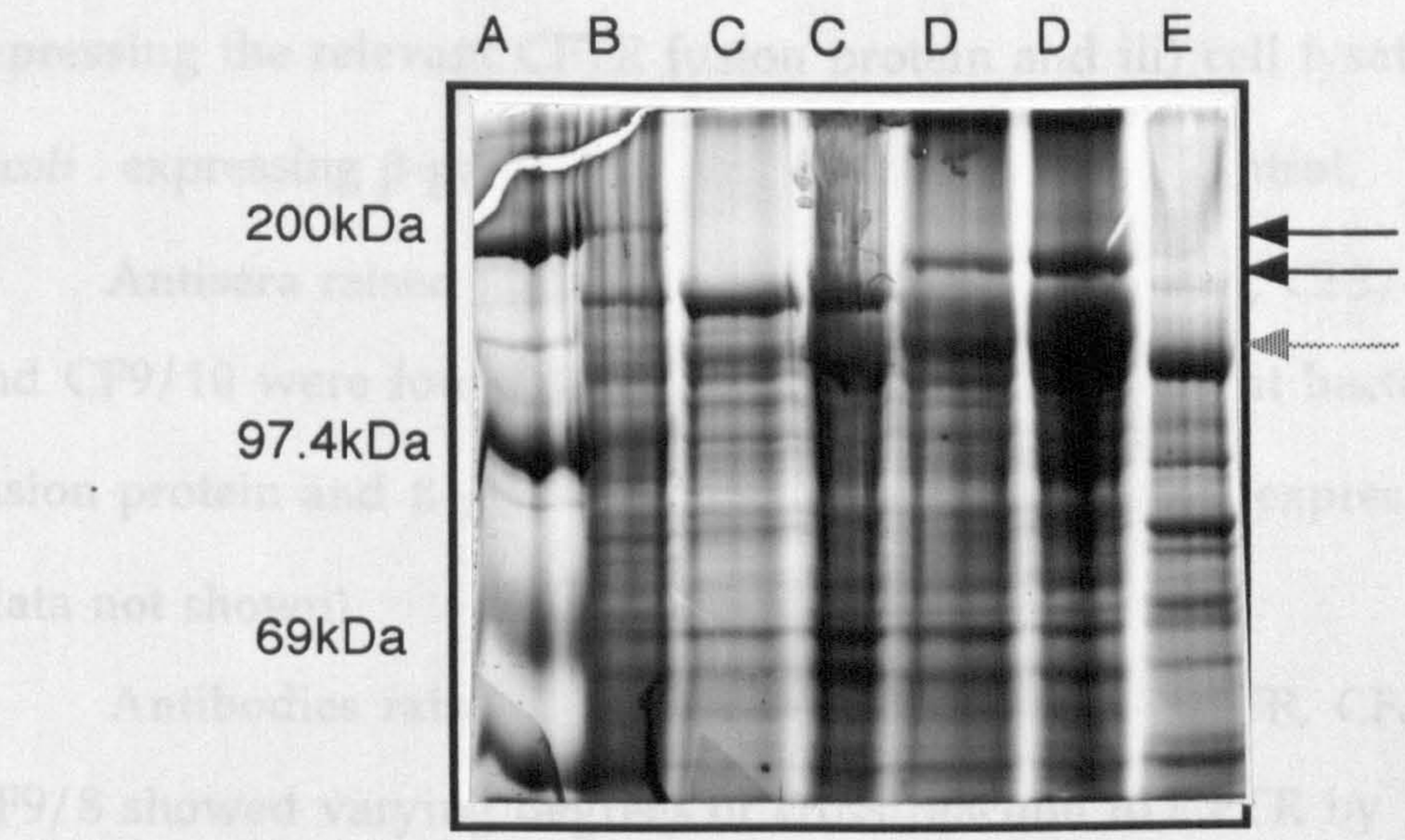


Fig. 3.3 8% SDS page gel of expressed proteins in pUEX.

A - Markers

B - CF 3/10 pcr product in pUEX - predicted size 156kDa

C - CF 5/4 pcr product in pUEX - predicted size 171kDa

D - CF 9/8 pcr product in pUEX - predicted size 213kDa

E - pUEX vector alone - predicted size 116kDa

⟵ indicates position of expressed β-galactosidase protein in pUEX alone

⟵ indicates position of expressed β-galactosidase protein in pUEX with insert from CFTR

3.3.3 Western Blot Analysis of Antibodies

HT29 cells had previously been shown to express CFTR [Denning et al., 1992a]. The polyclonal antisera were screened against Western blots of i) membrane lysate prepared from HT29 cells, ii) cell lysates prepared from *E.coli.* expressing the relevant CFTR fusion protein and iii) cell lysate prepared from *E.coli* . expressing β-galactosidase protein alone, as a control.

Antisera raised to the smaller fragments of CFTR, CF3/4, CF5/6, CF7/8, and CF9/10 were found to cross react with the relevant bacterially expressed fusion protein and β–galactosidase, but not with CFTR expressed in HT29 cells (data not shown).

Antibodies raised to the larger fragments of CFTR, CF3/10, CF5/4 and CF9/8 showed varying degrees of cross reaction to CFTR by Western blotting (fig 3.4). Rabbits immunised with 3/10 and 5/4 fusion proteins produced serum that recognised a ~170kDa HT29 protein on Western blots that was not recognised by the pre-bleed antisera taken from these animals. ~170kDa is the expected size of the glycosylated form of CFTR [Gregory et al.,1990]. The CF9/8 antisera failed to recognise any HT29 protein on Western blot. Antisera to all three larger fusion proteins were found to cross react with the relevant bacterially expressed fusion protein and with β–galactosidase on Western blots (data not shown).

3.3.4 Immunofluorescence Analysis of HT29 cells

HT29 cells were processed for immunofluorescence analysis (section 2.2.24) using the polyclonal antisera as the source of primary antibody. Each pre-bleed serum gave very weak background staining of HT29 cells when used in indirect immunofluorescence. There did not appear to be any significant increase in staining of the cells when polyclonal antisera CF3/4, CF5/6, CF7/8 and CF9/10 were used (data not shown). However a visible increase in staining of the cells was detected when they were screened with test bleed CF3/10, CF5/4

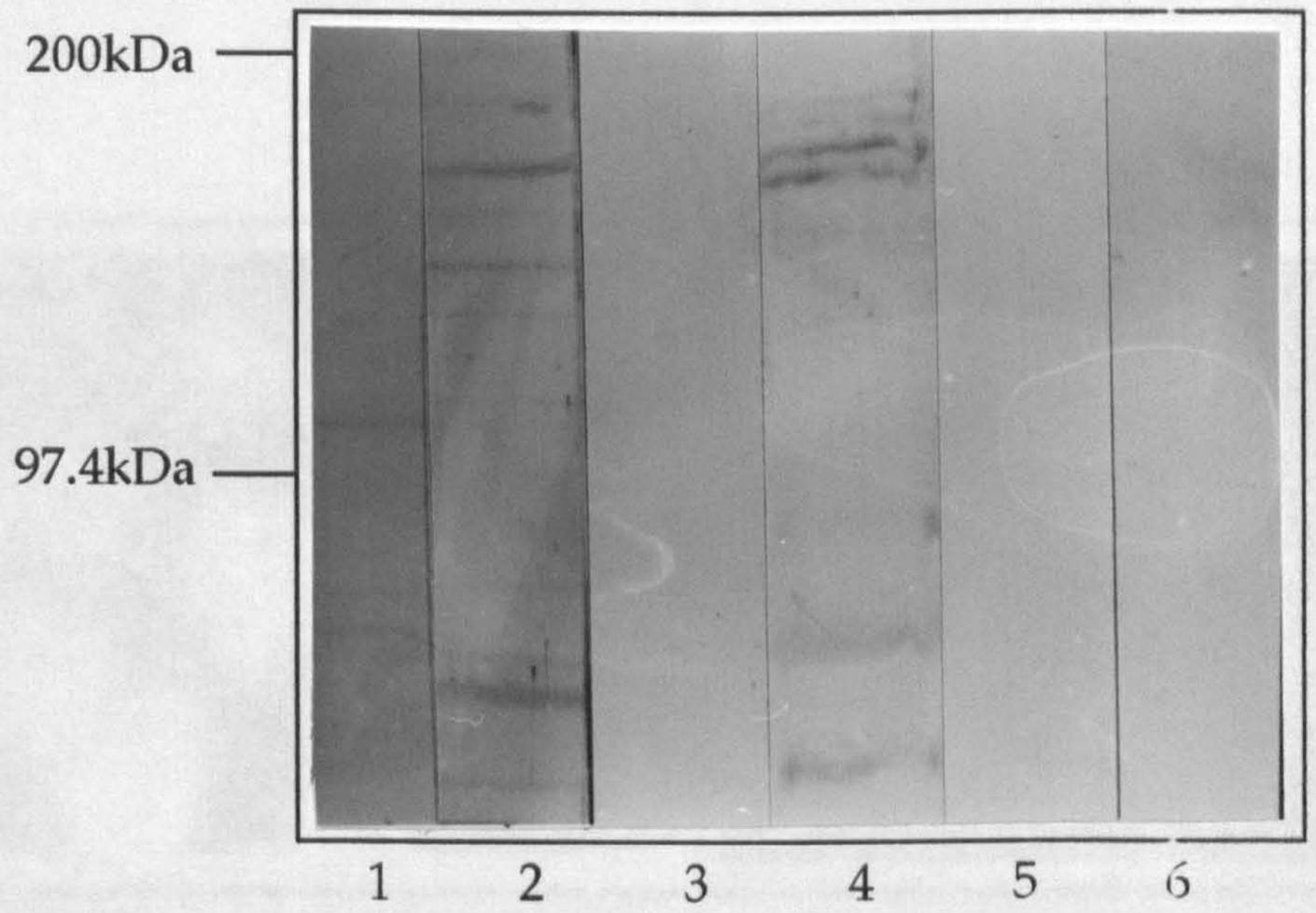


Fig.3.4 Immunoblots of HT29 membrane lysate probed with polyclonal CF3/10 (lane 2), polyclonal 5/4 (lane 4) or polyclonal 9/8 (lane 6) as primary antibody. Premmune sera from rabbits immunised with the CF3/10 (lane 1), CF5/4 (lane 3) or CF9/8 (lane 5) were used as controls. The relative mobilities of molecular weight standards, loaded in an adjacent lane, is indicated.

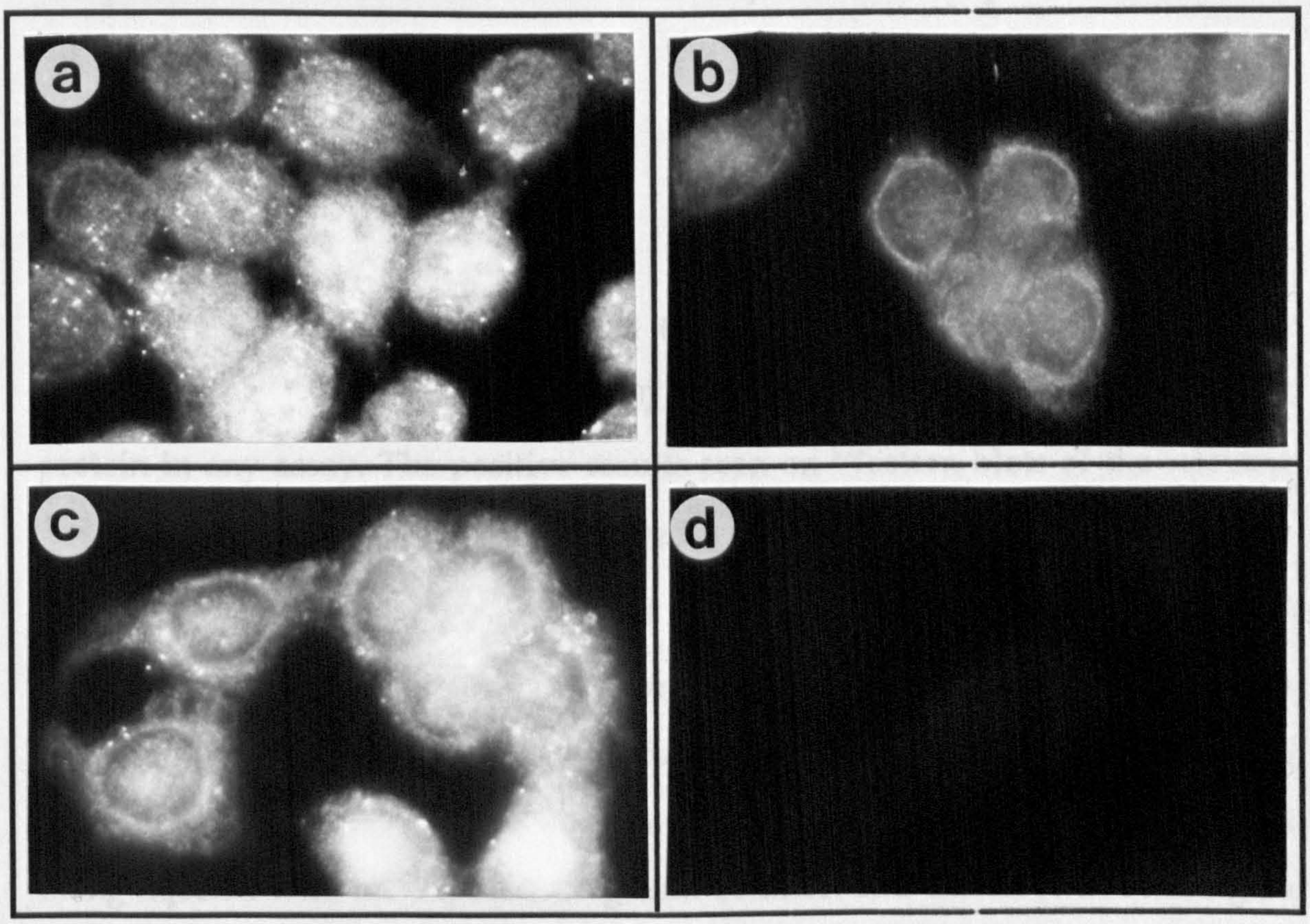


Fig. 3.5 Immunofluorescence analysis of methanol fixed HT29 cells with polyclonal antisera

a - CF3/10 test antiserum

b - CF5/4 test antiserum

c - CF9/8 test antiserum

d - CF3/10 pre-bleed antiserum

and CF9/8 antisera. Each gave a diffuse pattern of staining across the cell, with a concentration of fluorescence intensity around the periphery (fig 3.5). This pattern is consistent with a predominantly plasma membrane (or immediately sub-plasma membrane) localisation. CFTR has previously been localised to the plasma membrane and to vesicles adjacent to the plasma membrane [Crawford et al., 1991].

3.4 DISCUSSION

The polyclonal antisera raised to the smaller fragments of expressed CFTR (CF3/4, CF5/6, CF7/8, CF9/10) did not appear to recognise the CFTR protein in any assay. The positive results seen on Western blots of the relevant fusion proteins would most likely have been due to the presence of high titre anti β-galactosidase antibodies in the antisera. The small size of the CFTR components of the expressed fusion proteins made it difficult to determine a shift in the position of the expressed fusion protein band when run out on an SDS polyacrylamide gel. This problem may have resulted from the rabbits not being injected with the expected fragment of CFTR and therefore not raising an immune response to it.

The problem described above seemed to be overcome in the generation of polyclonal antisera to a second set of fusion proteins containing larger fragments of the CFTR protein (CF3/10, CF5/4 and CF9/8). The larger size of these fusion proteins meant that it was far easier to detect shifts in the size of fusion protein bands on SDS polyacrylamide gels. The immunoblot and immunofluorescence data were consistent with the assumption that these polyclonal antibodies recognise CFTR. However, it still had to be established conclusively that the ~170kDa protein recognised by these antibodies was CFTR and not another protein within the cell. Antibodies against bacterial fusion proteins can have a high level of specificity since the antigen is clonally pure

and contaminants in the antigen preparation are of bacterial origin and unlikely therefore to evoke antibodies that can cross react with mammalian cells [Tooze and Stanley, 1986]. However, further studies with these polyclonal antibodies raised doubts about their specificity and the possibility of them cross-reacting with proteins other than CFTR cannot not be ruled out. This problem is addressed in the next chapter.

Three of the β-galactosidase fusion proteins constructed contain the majority of the expressed CFTR sequence between them. All three have been used individually to raise an apparent antibody response to CFTR in rabbits. Therefore, it seemed realistic to immunise a single rabbit with all three fusion proteins and thereby elicit an immune response to virtually the entire expressed CFTR protein in one animal. This was done in preparation for the production of an anti-CFTR coliclonal antibody library from rabbit bone marrow, as described in chapter 8.

CHAPTER 4

CHARACTERISATION OF MONOCLONAL AND POLYCLONAL ANTIBODIES TO DIFFERENT REGIONS OF CFTR: DETECTION OF IMMUNOLOGICALLY RELATED PROTEINS

4.1 INTRODUCTION

The identification of the gene encoding CFTR and the its subsequent expression in heterologous systems has facilitated the development of a variety of polyclonal and monoclonal antibodies to the protein. These have been used in a range of immunocytochemical studies to investigate the patterns of expression and the biochemical functions of CFTR. Many of the published antibodies appear to recognise CFTR, however the specificity with which they do so has not always been exhaustively analysed. The lack of full characterisation of CFTR antibodies has been recognised as a particular problem by various groups in the field. This led to it being the subject of an international workshop sponsored by Association Franchise de Lutte contre la Mucovoscosidose (A.F.L.M.) in 1993.

The characterisation of a set of monoclonal and polyclonal antibodies raised to CFTR by immunoblot, immunoprecipitation, immunofluorescence and immunohistochemistry is reported in this chapter. The monoclonal antibodies were raised to thyroglobulin-peptide conjugates bearing peptides corresponding to different regions of human CFTR. The sequences of the peptides and their corresponding positions within the predicted structure of CFTR are indicated in fig.4.1. Antibodies produced by cloned hybridomas had been shown to recognise the corresponding BSA-peptide conjugates (by ELISA) and initial immunoblot and immunofluorescence results with these antibodies indicated that they may have been recognising CFTR. Supernatants from hybridoma clones producing antibodies to CF2, CF3, CF6, CF7 and CF8

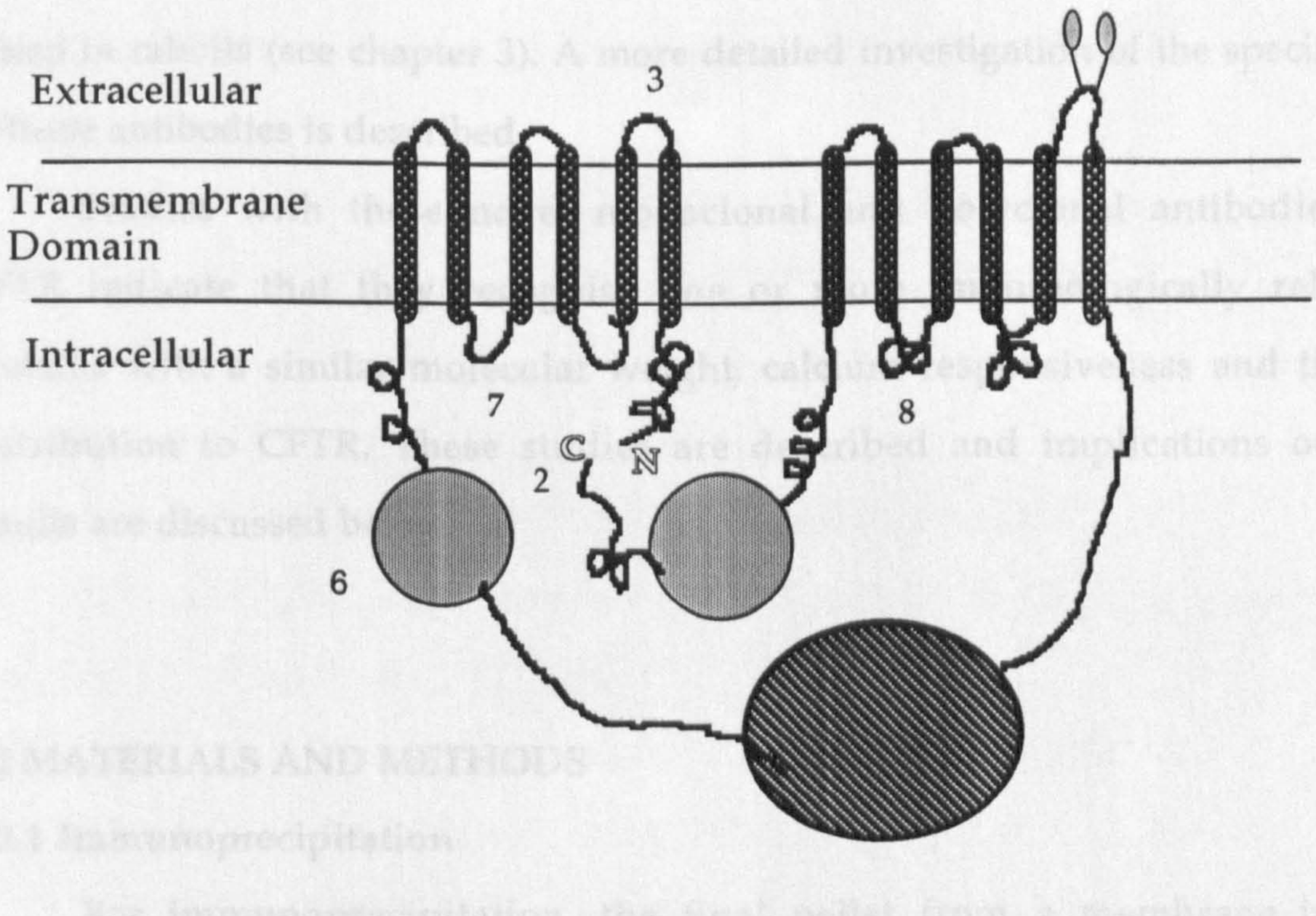


Fig. 4. 1

Cartoon indicating the positions of peptides within the sequence of CFTR.

● indicates R domain, ● indicates nucleotide binding domain,

▮ indicates the transmembrane domain, and ♀ indicates glycosylation site.

Peptide sequences are:- CF2: LKEETEEEVQDTRL,

CF3: GRIIASYDPDNKEER,

CF6: TIKENIIGVSYDEY,

CF7: RMMMKYRDQRAGKIS,

CF8: QTSQQLKQLESEGRSP.

were used in further immunocytochemical experiments as described below. The antibodies produced by these clones are referred to as MabCF2, MabCF3, MabCF6, MabCF7 and MabCF8 hereafter.

In addition to these monoclonal antibodies, polyclonal antibodies were raised in rabbits (see chapter 3). A more detailed investigation of the specificity of these antibodies is described.

Studies with these novel monoclonal and polyclonal antibodies to CFTR indicate that they recognise one or more immunologically related proteins with a similar molecular weight, calcium responsiveness and tissue distribution to CFTR. These studies are described and implications of the results are discussed below.

4.2 MATERIALS AND METHODS

4.2.1 Immunoprecipitation

For immunoprecipitation, the final pellet from a membrane lysate preparation (see section 2.2.18) from HT29 cells was dissolved in 5% Decanoyl-N-methylglucamide (Mega-10). A 1 in 25 dilution of polyclonal antiserum in membrane lysate solution was mixed for 1 hour at room temperature. Immune complexes were collected on Pepstatin A beads, which had been pre-washed in membrane lysis buffer, by agitating at 4°C for 1 hour. The beads were then washed four times before elution of the immune complexes by incubation in SDS-PAGE sample buffer and 50mM DTT at 37°C for 10 minutes. Eluted material was electrophoresed on an 8% SDS polyacrylamide gel, blotted onto nitrocellulose membrane and tested for reactivity with monoclonal antibodies to CFTR.

4.2.2 Reverse Transcription PCR

Total RNA was isolated and mRNA purified from tissue culture cells, then first strand cDNA synthesised using Pharmacia kits (27-9270-01, 27-9258-01 and 27-9261-01). Subsequent PCR reactions were carried out according to standard procedures (section 2.2.7) using the following pairs of oligonucleotide primers:

CF7 5′- AGACGGGATCCAAAGTGCGGCAGTACGAT -3′

CF8 5′- CTTTTCTGCAGCCTTGTATCTTGCACCTC -3′

CF7 and CF8 both prime within exon 24 of CFTR to generate a fragment of 192bp from CFTR genomic DNA or cDNA.

CFX 5′- AGACGGGATCCAGGATAGAAGCAATGCTG -3′

CF8 and CFX prime across exons 23 and 24 of CFTR to generate a fragment of 255bp from CFTR cDNA.

MIC2/11 5′- TACCAGAAAAAGAGATCTTGCTTCAAAG -3′

MIC2/13 5′- GATCCGCCTGGGCTGTTTCTGCCGACAAT -3′

MIC2/11 and MIC2/13 prime within exons 7 and 8 of MIC2 to generate a fragment of 144bp from MIC2 cDNA [Smith et al., 1990].

4.2.3 Immunohistochemistry (performed by Dr. C. Holmes, Department of Obsteterics and Gynaecology, University of Bristol.)

Adult human tissues were obtained either at post mortem or from surgical specimens and snap-frozen in liquid N_2-cooled isopentane (2-methylbutane). Acetone-fixed cryostat sections were prepared and stained by an indirect immunoperoxidase staining technique as described previously [Holmes et al., 1990]. Negative controls included sections incubated either in the absence of primary antibody or with an irrelevant primary antibody.

4.3 RESULTS

4.3.1 Immunoblot Analysis of HT29 Membrane Lysate

HT29 cells have previously been shown to express CFTR [Zeitlin et al., 1992]. It was decided to screen immunoblots of HT29 membrane lysate with selected monoclonal antibodies anticipating that each antibody would detect a protein of ~170kDa if it recognised fully glycosylated CFTR [Denning et al.,1992a]. The results are presented in fig. 4.2. Each of the antibodies tested detected a protein of the same apparent molecular weight, ~170kDa. It was shown in the preceding chapter that two of the three polyclonal antisera also recognise a ~170kDa band in the immunoblots of HT29 membrane lysate.

4.3.2 Immunofluorescence Analysis of HT29 Cells

HT29 cells were processed for immunofluorescence analysis using selected monoclonal antibodies as the primary antibody. As with the polyclonal antisera CF3/10, CF5/4 and CF9/8 (see section 3.3.4 and fig. 3.5). Each monoclonal antibody gave a diffuse pattern of staining across the cell with a concentration of fluorescence intensity around the periphery (fig. 4.3). This pattern is consistent with a predominantly plasma membrane (or immediately sub-plasma membrane localisation). HT29 cells have previously been shown to express CFTR [Denning et al., 1992a] and CFTR has previously been localised to the apical plasma membrane and to vesicles adjacent to the plasma membrane [Crawford et al., 1991].

4.3.3 Peptide Competition (performed by Judy Watson, Department of Biochemistry, University of Bristol.)

Peptide competition experiments were performed in order to check the specificity of the observed monoclonal antibody-antigen interactions. An immunoblot of HT29 membrane lysate was probed with either MabCF2, MabCF3 or MabCF8, alone or with one of these antibodies following their pre-incubation for 30 minutes with different BSA-peptide conjugates (fig. 4.4). In

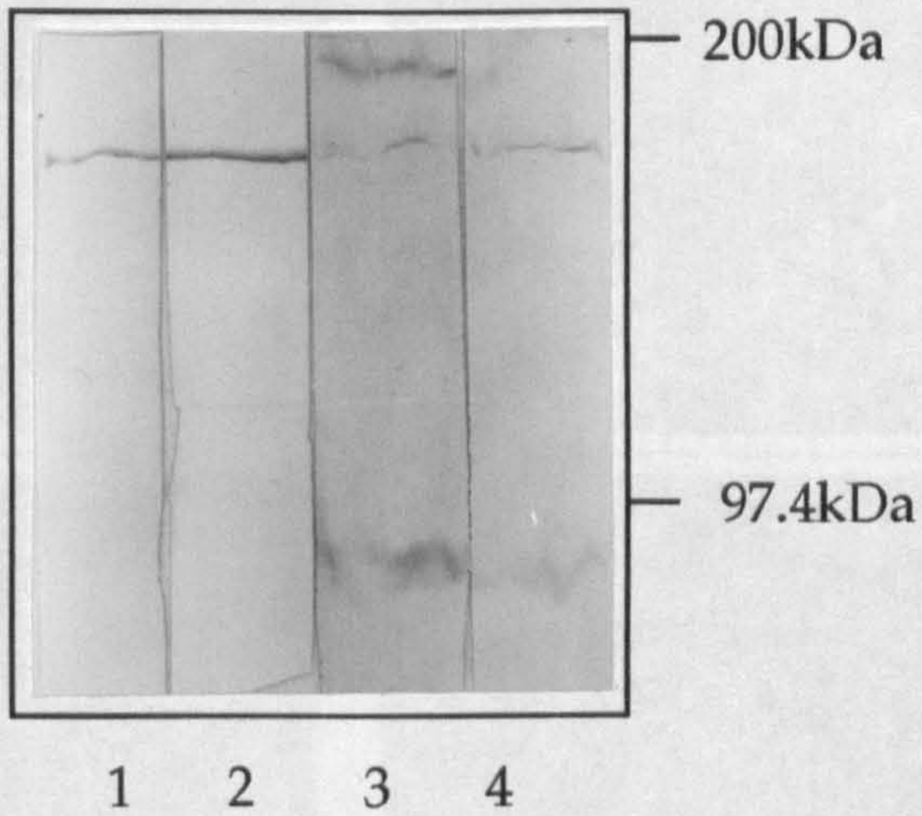


Fig.4.2 Immunoblots of HT29 membrane lysate probed with MabCF3 (lane 1), MabCF8 (lane 2), MabCF6 (lane 3) and MabCF7 (lane 4) The relative mobilities of molecular weight standards, loaded in an adjacent lane, are indicated.

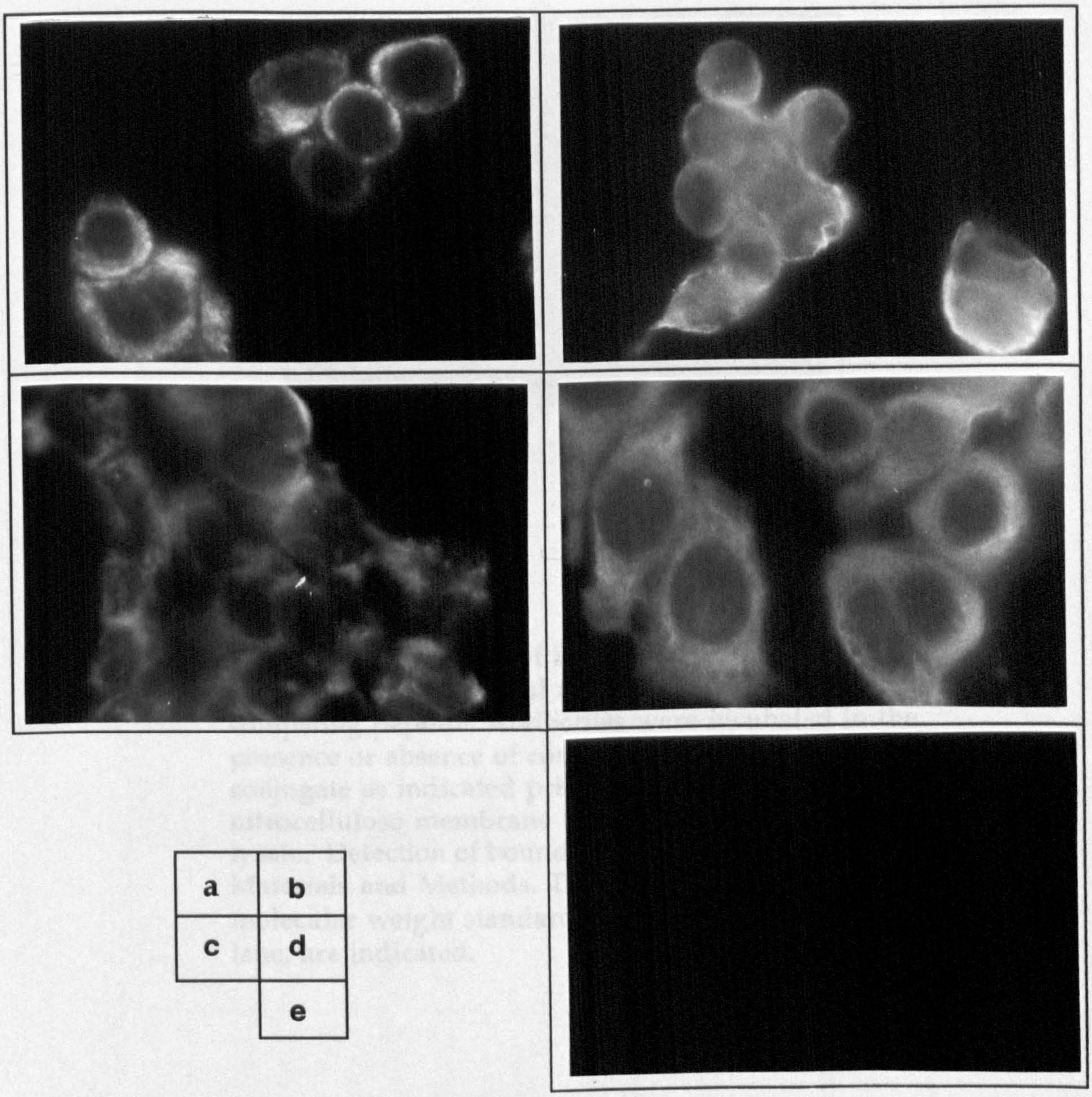


Fig. 4.3 Immunofluorescence analysis of methanol fixed HT29 cells using MabCF3 (panel **a**), MabCF6 (panel **b**), MabCF7 (panel **c**), MabCF8 (panel **d**) as first antibody. Panel **e** was incubated with no first antibody.

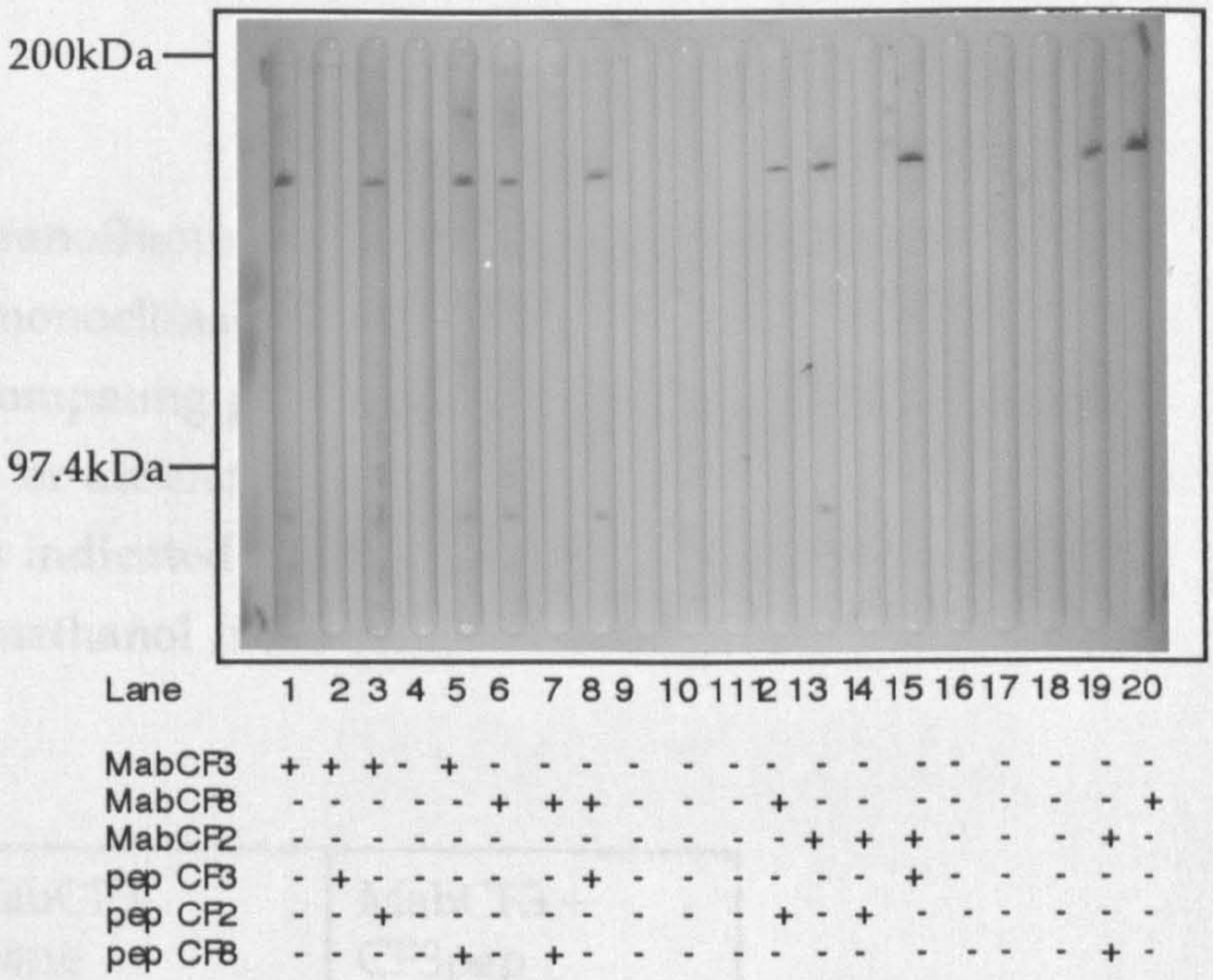


Fig. 4.4 Immunoblots of HT29 membrane lysate probed with monoclonal antibodies in the presence of competing peptide. Antibodies were incubated in the presence or absence of competing BSA-peptide conjugate as indicated prior to incubation with the nitrocellulose membrane bearing HT29 membrane lysate. Detection of bound antibody was as described in Materials and Methods. The relative mobilities of molecular weight standards, loaded in an adjacent lane, are indicated.

Fig. 4.5 Immunofluorescence analysis of methanol fixed T84 cells using monoclonal antibodies in the presence or absence of competing peptide. Antibodies were incubated in the presence or absence of competing BSA-peptide conjugate as indicated prior to use in immunofluorescence analysis of methanol fixed cells.

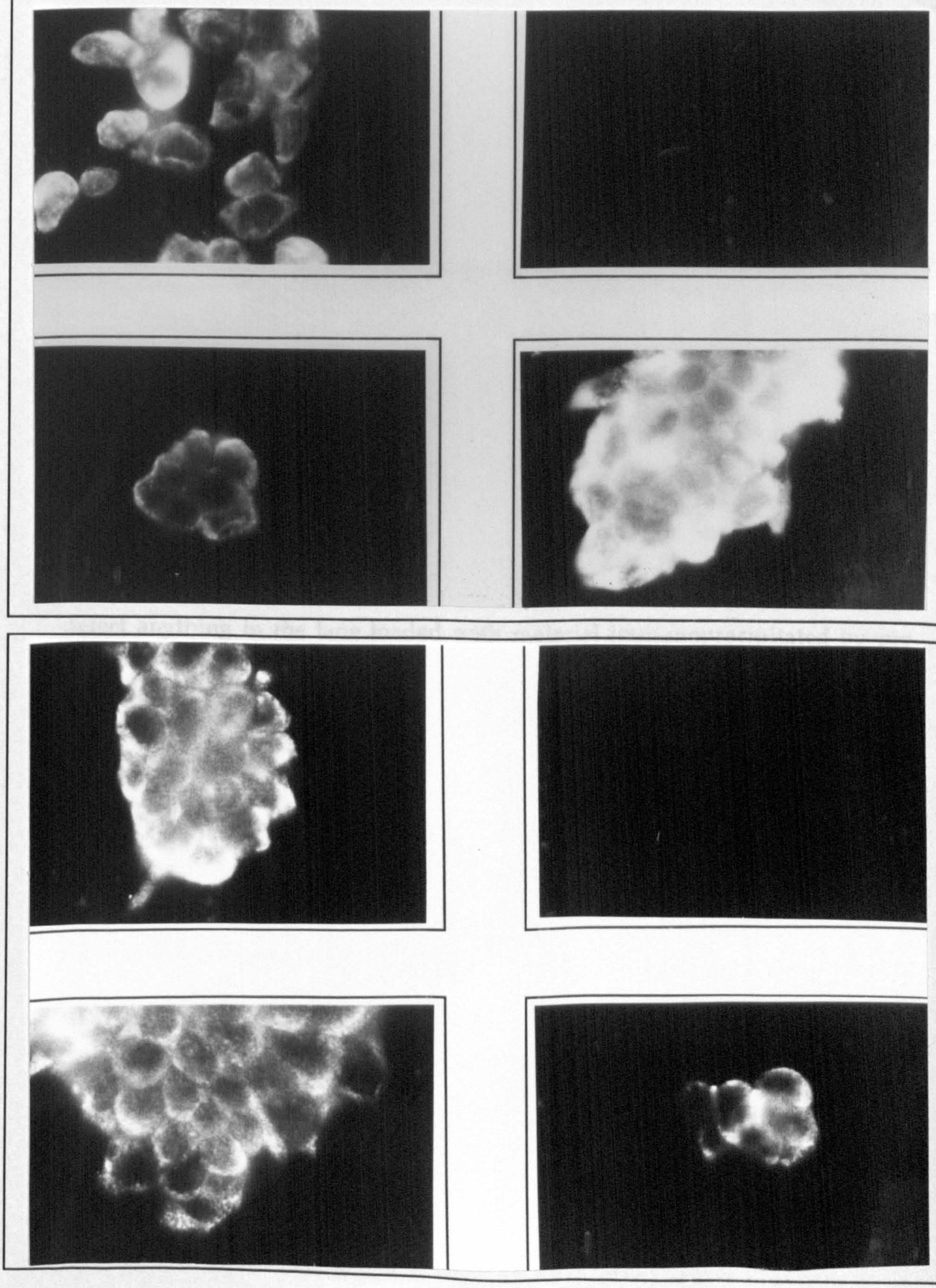
MabCF3 alone	MabCF3 + CF3pep
MabCF3+ CF2pep	MabCF3+ CF8pep

Upper Panel

MabCF8 alone	MabCF8 + CF8pep
MabCF8+ CF2pep	MabCF8+ CF3pep

Lower Panel

Fig. 4.5



all cases only the immunising peptide blocked binding of antibody to the ~170kDa protein. Similar results were obtained with MabCF6 (data not shown). T84 cells were also prepared for immunofluorescence as before and incubated with the same antibody/peptide cocktails used in the immunoblot analysis. Once again, in all cases, only the immunising peptide blocked binding of antibody (fig. 4.5 and additional data not shown).

4.3.4 Immunoprecipitation

The immunoblot and immunofluorescence data presented in figs. 4.2-4.5 are consistent with the assumption that the antibodies concerned recognised CFTR. However, it was considered that this point would be strengthened if protein immunoprecipitated from HT29 membrane lysate by any one of the polyclonal antisera was detected by the monoclonal antibodies in an immunoblot. The results of such an experiment are presented in fig. 4.6. Polyclonal antiserum 3/10 immunoprecipitated a protein of ~170kDa which was recognised in immunoblot analysis by MabCF8 (lane 2). MabCF8 failed to detect anything in the lane loaded with material immunoprecipitated by pre-immune serum derived from the rabbit which generated the 3/10 antiserum (lane 3). Another polyclonal antibody, 169, previously published as being a polyclonal antibody to CFTR [Crawford et al., 1991], was included in this experiment. MabCF8 detected a protein of ~170kDa in the material immunoprecipitated by 169 from HT29 membrane lysate (lane 1). MabCF3 was used in immunoblot analysis of the same material and gave results identical to those obtained with MabCF8 (data not shown). Similar experiments were performed with polyclonal antiserum CF9/8 as the immunoprecipitating antibody and MabCF3 and MabCF8 as the immuno-detecting antibodies. Both monoclonal antibodies recognised the ~170kDa protein immunoprecipitated by polyclonal antiserum CF9/8 (data not shown).

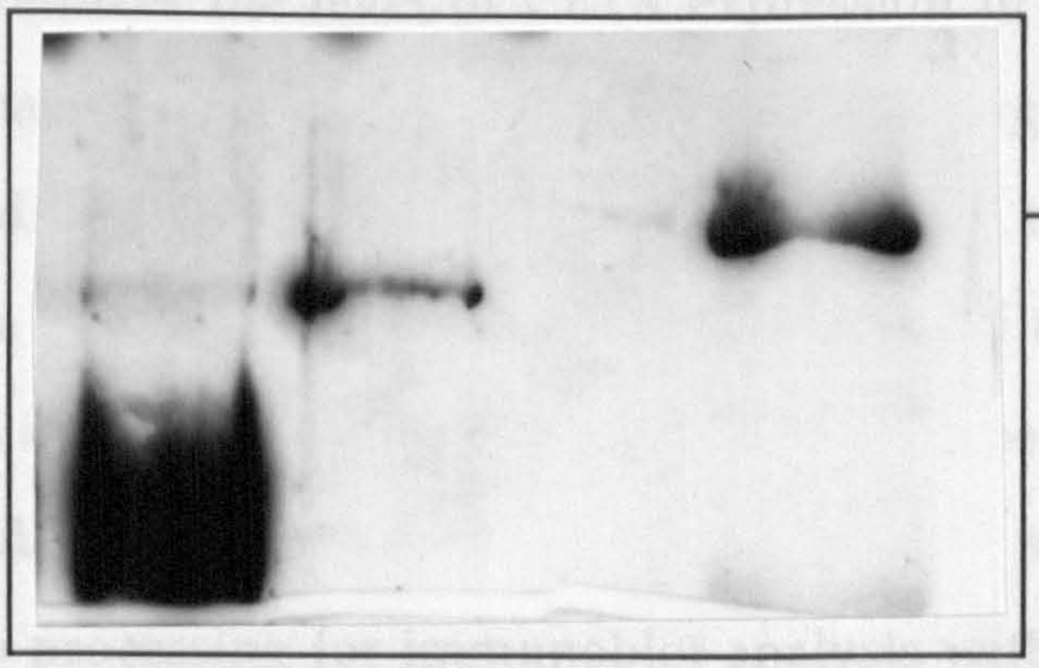


Fig. 4.6

Immunoblot analysis of material immunoprecipitated from HT29 membrane lysate. Polyclonal antisera 169 [Crawford et al., 1992] (lane 1) or 3/10 (lane 2), and preimmune sera from the rabbit which subsequently generated polyclonal 3/10 (lane 3) were used to immunoprecipitate material from HT29 membrane lysate. The immunoprecipitated material was subjected to SDS-PAGE, transferred to nitrocellulose and immunoblotted using MabCF8 as primary antibody. Pre-stained molecular weight standards were loaded in lane 4.

4.3.5 Expression of the Protein Recognised by the Monoclonal and Polyclonal Antibodies is Calcium Regulated

It has previously been shown that an increase in intracellular Ca^{2+} concentration, induced by treatment with the ionophores A23187 or ionomycin, reduces the level of CFTR expression in HT29, T84 and freshly isolated normal human bronchial epithelial cells [Bargon et al., 1992]. If these novel antibodies recognise CFTR, then ionomycin treatment of HT29 cells should induce a decrease in the level of expression of the ~170kDa protein that they detect in immunoblot analysis of HT29 membrane lysate. HT29 cells were therefore incubated both in the presence and absence of ionomycin for various times prior to processing for immunoblot analysis with MabCF3 as probe (fig. 4.7). The level of expression of the ~170kDa protein recognised by MabCF3 was reduced following ionomycin treatment (compare lanes 1, 2 and 3). Similar results were obtained with MabCF8 (data not shown). This work was performed by Aleksander Edelman, Inserm U323, Faculte de Medecine Necker, Enfants Malades, Paris.

4.3.6 Immunohistochemistry

Since the immunoblot, immunofluorescence and Ca^{2+} modulation data were consistent with the hypothesis that MabCF3 and MabCF8 recognised CFTR, it was decided to conduct preliminary immunolocalisation studies on cryostat sections of human tissues using MabCF3 or MabCF8 as the primary antibody. Some of the observed patterns of reactivity using MabCF8 are shown in fig. 4.8, similar results were obtained with MabCF3 (data not shown). In addition MabCF8 recognised epithelial cells lining ducts of the pancreas and showed weak reactivity in liver (where staining was more prominent on the bile ductular rather than the hepatocyte epithelium) and in kidney where some, but not all, the tubules were stained (data not shown). The antibody showed extensive reactivity in the colon where staining was present

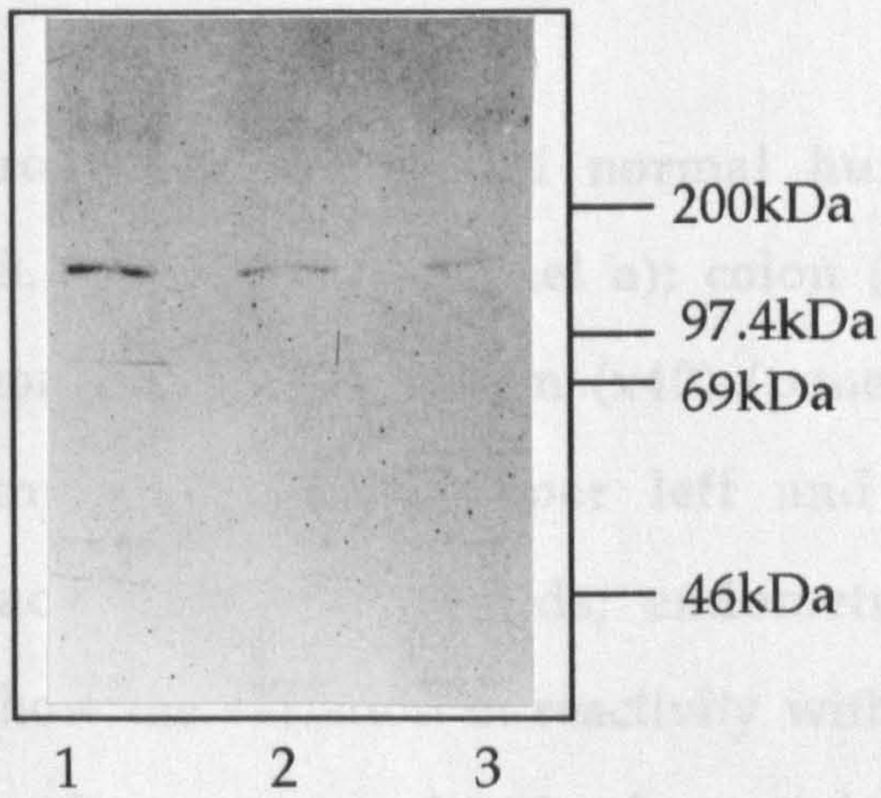
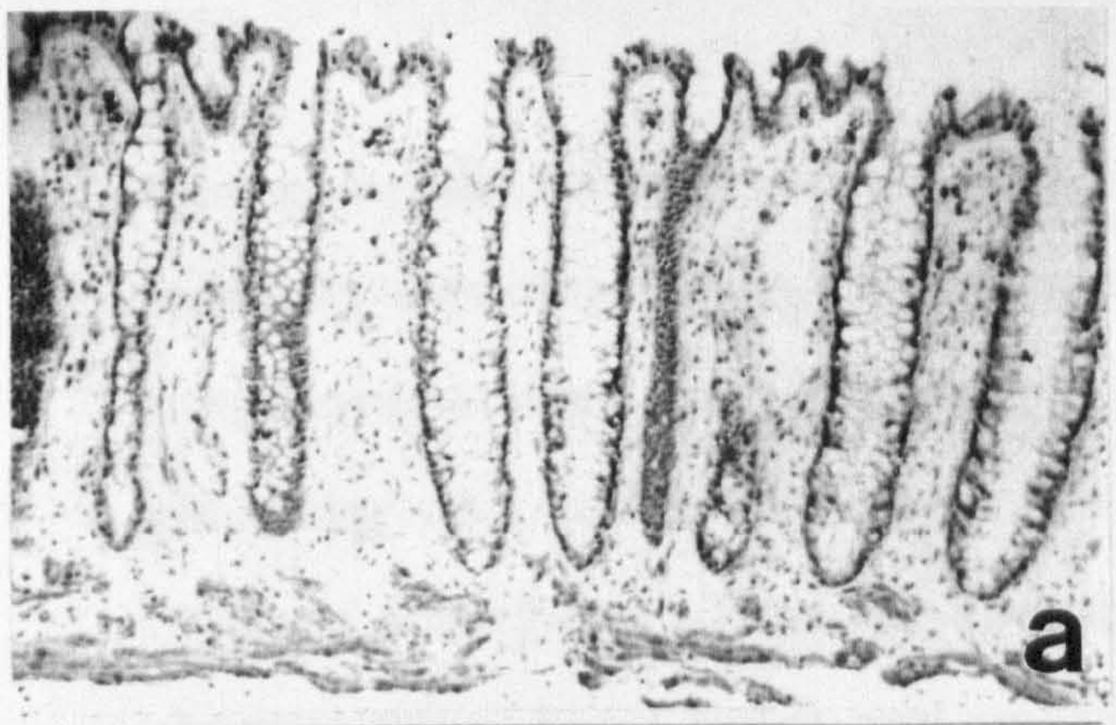


Fig. 4.7 Immunoblot analysis of lysate from ionomycin treated HT29 cells. MabCF3 was used as primary antibody in immunoblot analysis of HT29 membrane lysate prepared from cells which had been incubated in the presence (lanes 2 and 3) or absence (lane 1) of 2mg/ml ionomycin for 18 (lane 2) or 24 hours (lane 3). The relative mobilities of molecular weight standards, loaded in an adjacent lane, are indicated.

Fig. 4.8 Immunoperoxidase staining of normal human tissues with MabCF8. Colon (x40) (panel a); colon (x50) (panel b, negative control); endometrium (x40) (panel c), note the reactive myometrium at upper left and the heterogeneity in reactivity of the glands; endometrium (x160) (panel d), to show the variation in reactivity within a single gland and also the presence of stained stromal cells (arrow); tracheal epithelium (x100) (panel e); tracheal mucus gland (x100)(panel f); tracheal mucus gland, (x100) (panel g, negative control); prostrate (x40) (panel h); prostrate (x630) (panel i), to show reactivity on sub-glandular epithelial cells; testis, seminiferous tubule (x63) (panel j).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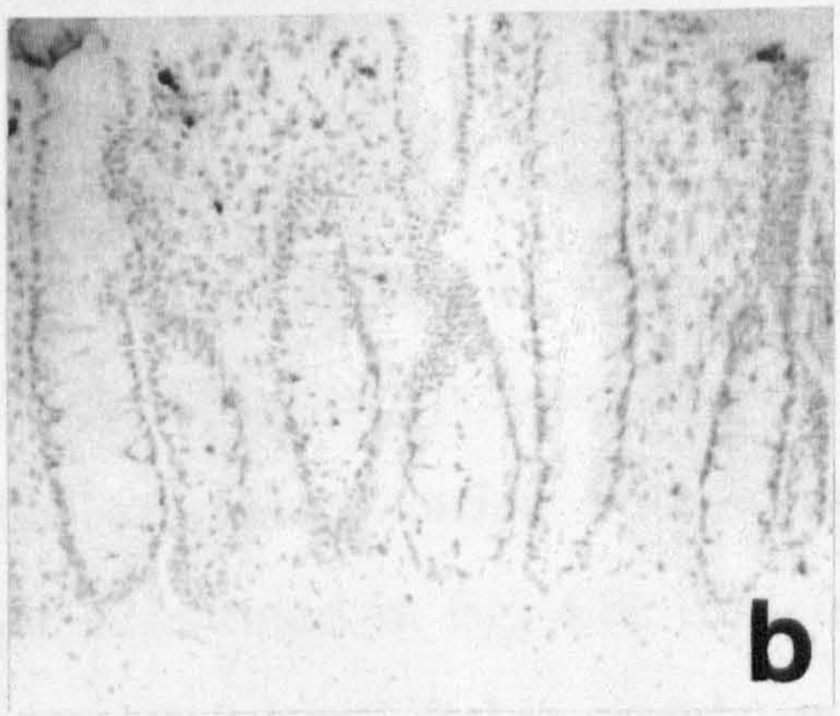
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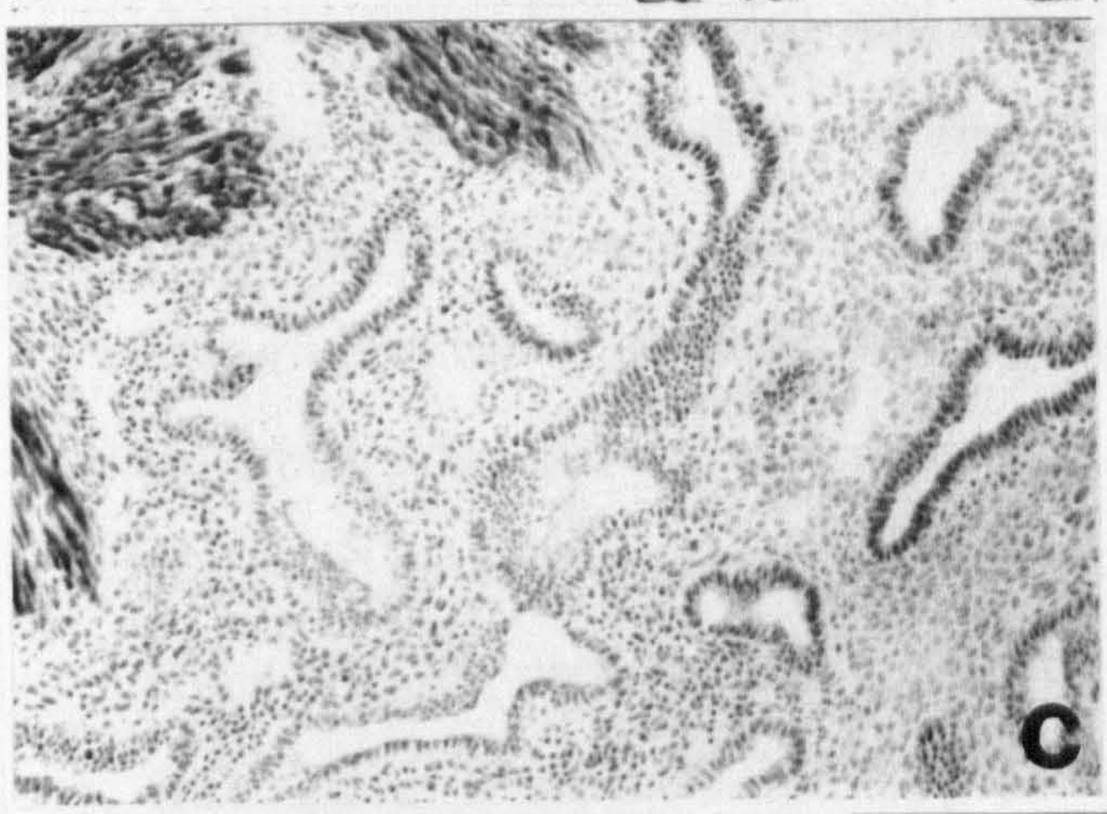
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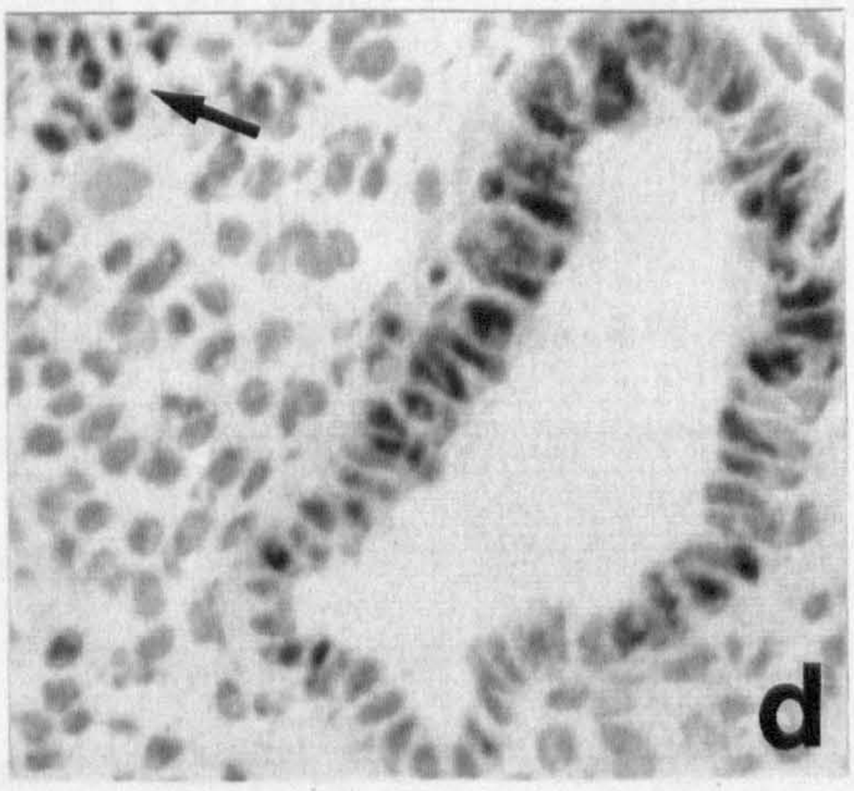
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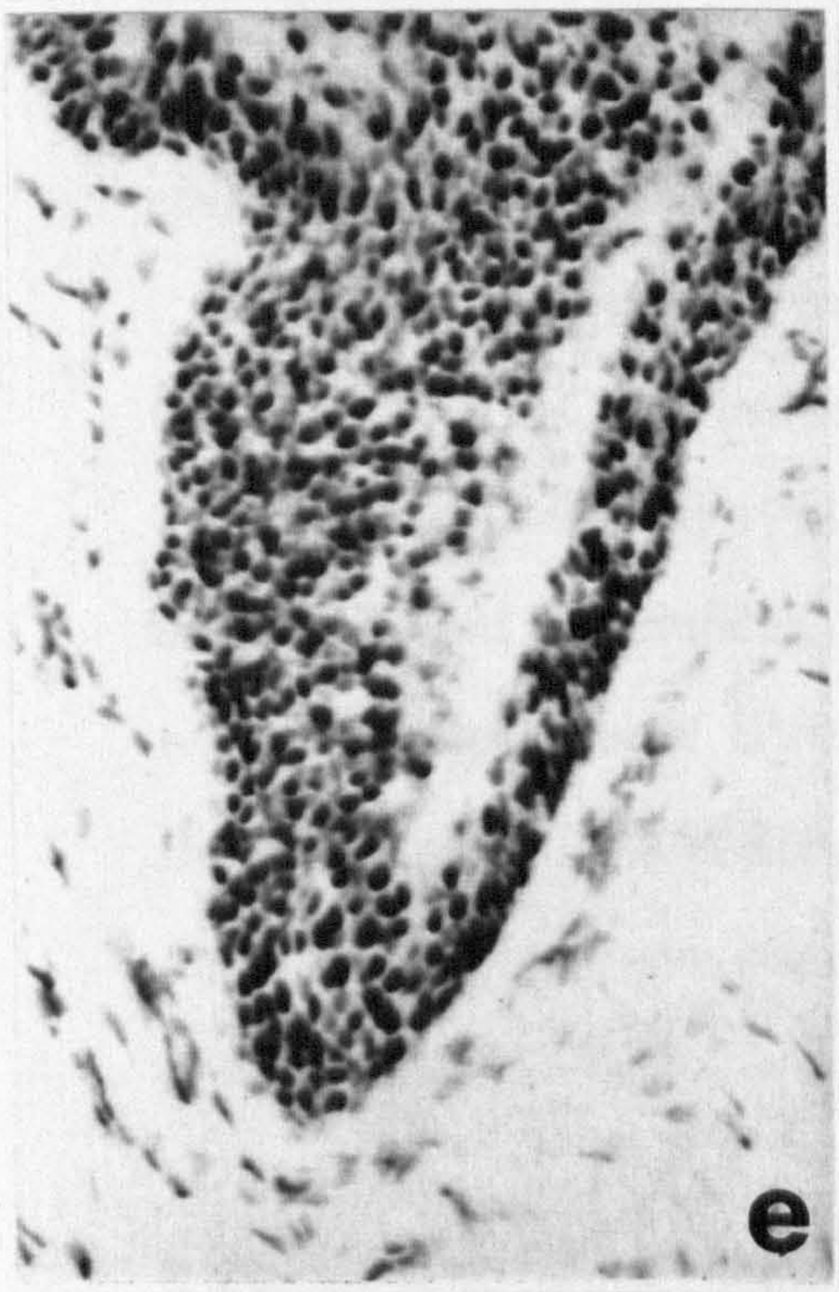
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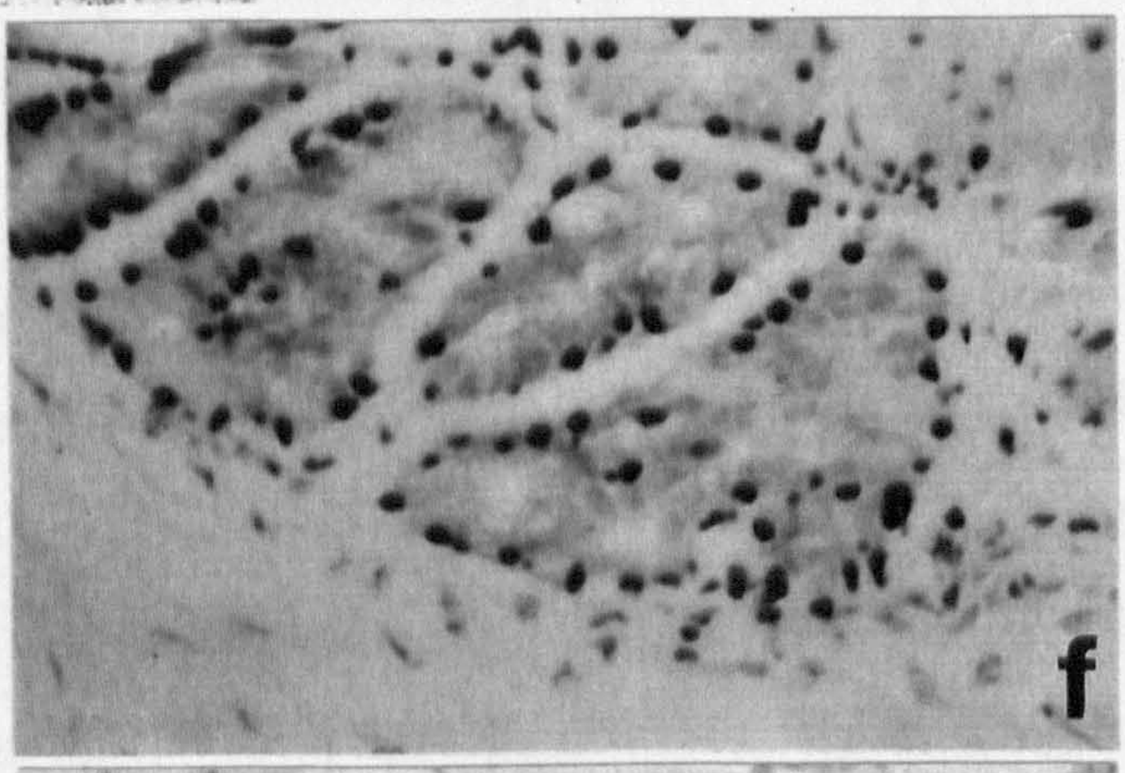
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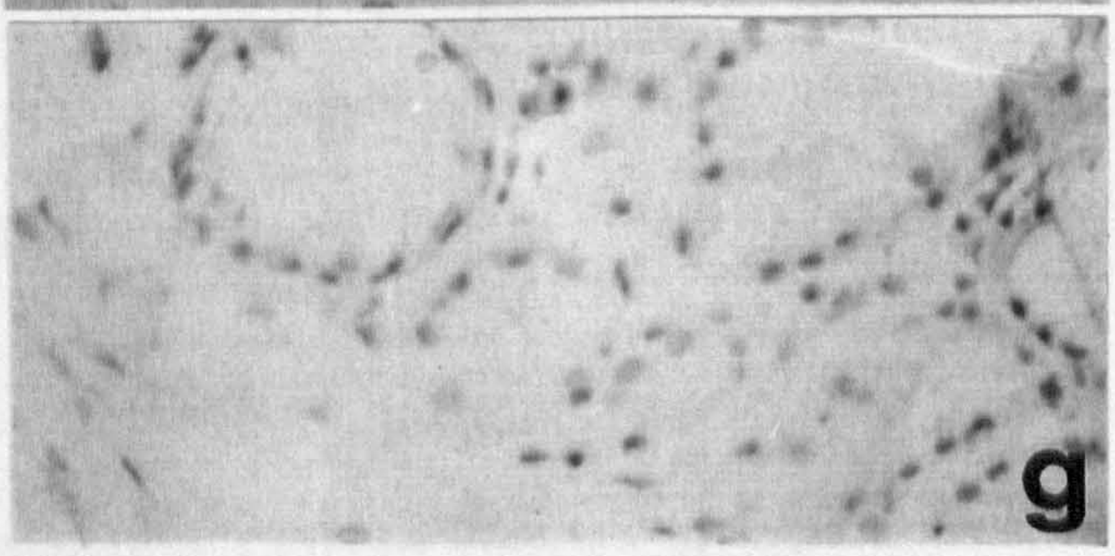
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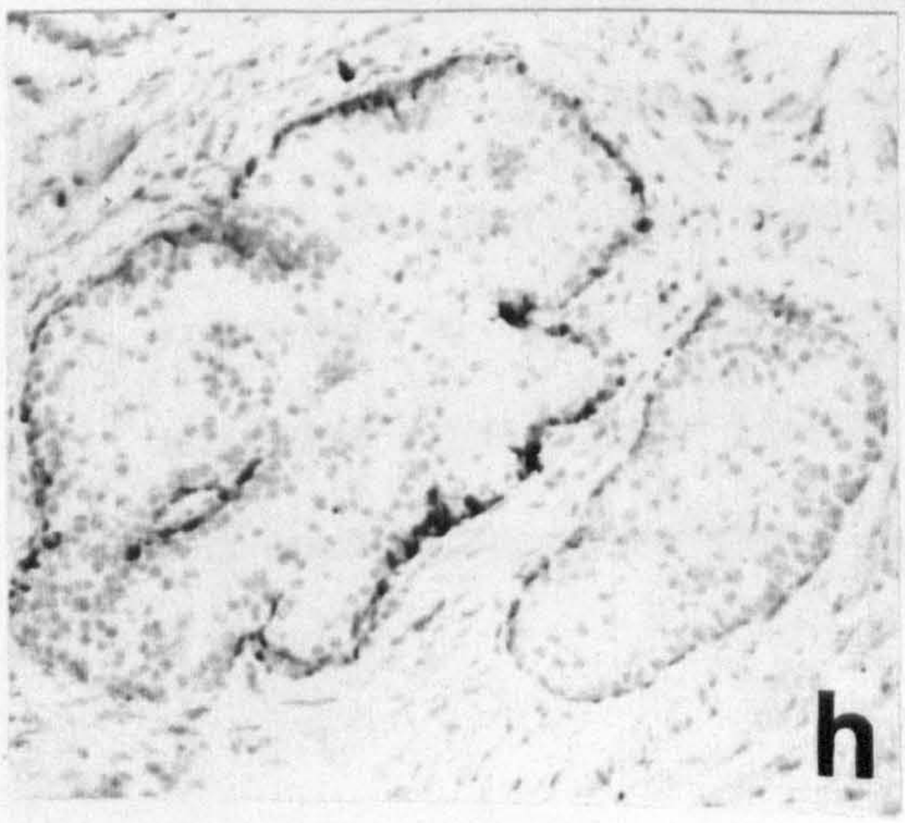
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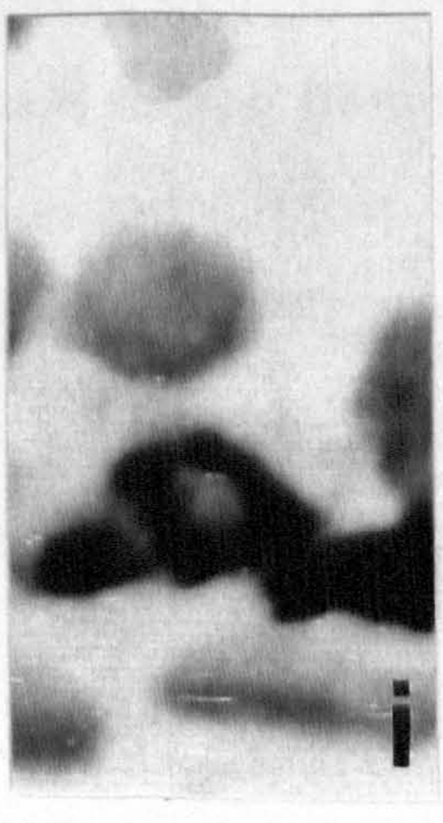
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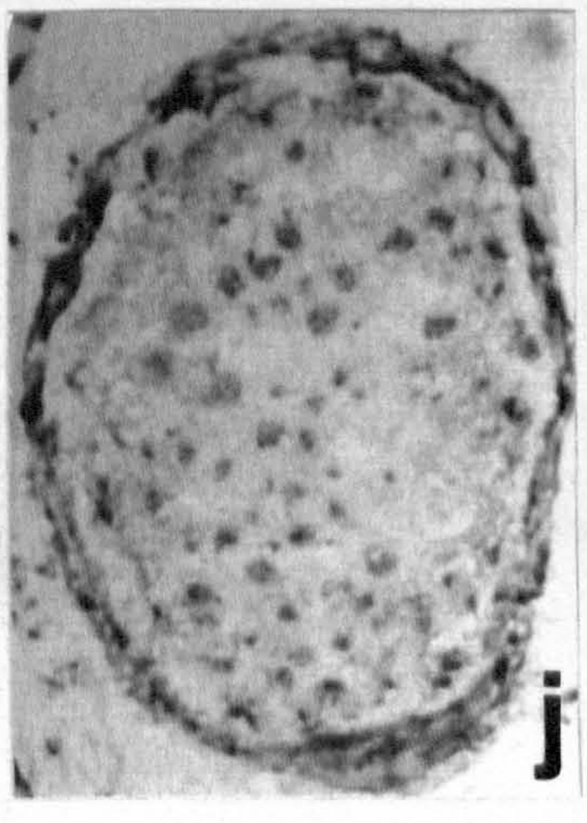
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throughout the colonic crypt epithelium (fig. 4.8a). A more complex pattern of reactivity was observed in the endometrium (fig. 4.8c and d); although MabCF8 consistently stained the myometrium (fig. 4.8c, upper left field), reactivity with glandular and stromal elements within the tissue varied both within and between specimens. Thus the specimen illustrated in fig. 4.8c contains both reactive (right-hand field) and unreactive (left-hand field) glands, whilst in fig.4.8d MabCF8 positive and negative cells are present, not only within an individual endometrial gland (upper and lower fields respectively), but also in the surrounding stroma (MabCF8-positive stromal cells are arrowed in fig.4.8d). Extensive reactivity was also observed with tracheal epithelium (fig.4.8e) with less pronounced staining in the underlying mucus glands (fig.4.8f). In prostrate, by contrast, the antibody showed intense reactivity throughout a discrete cell population underlying the unreactive glandular epithelium (fig. 4.8h and i). Weak reactivity throughout the germinal epithelium in seminiferous tubules of testis was also detected (fig. 4.8j).

These patterns of reactivity are similar to those previously described for CFTR [e.g. see Crawford et al., 1991; Cohn et al., 1991; Marino et al., 1991; Trezise and Buchwald 1991; Trezise et al., 1992; Tizzano et al., 1993], but there are significant differences, most notably in the fact that MabCF8 decorates the whole of the colonic crypt epithelium, an observation which conflicts with those of Trezise and colleagues who clearly show that the multidrug resistance and CFTR genes have complimentary patterns of epithelial expression [Trezise et al., 1993].

4.3.7 Recognition of CFTR Negative Cells in Tissue Culture

Since the pattern of tissue section immunoreactivity did not accurately reflect that previously described for CFTR it was decided to screen cell lines reported to lack CFTR expression (CFTR negative cell lines) using the antibodies produced. Heb7a cells were chosen as the potential CFTR negative cell line because they are a derivative of Hela cells which have been shown not

to express CFTR by mRNA analysis, functional studies [Anderson et al., 1991a] and by immunoprecipitation [Gregory et al., 1990]. Reverse transcription PCR (RTPCR) was used to confirm that Heb7a cells failed to produce any mRNA encoding CFTR (fig. 4.9). However, the novel monoclonal and polyclonal antibodies all recognised methanol fixed Heb7a cells in immunofluorescence analysis (fig. 4.10), giving a pattern of staining similar to that observed when HT29 cells had been used (fig. 4.3). The monoclonal and polyclonal antibodies also recognised a protein of ~170kDa in immunoblot analysis of Heb7a membrane lysate (fig. 4.11). This protein was indistinguishable in size from that detected in HT29 or CaCo-2 membrane lysates (compare lanes 1, 2 and 3). Similar results were obtained using another human cell line PANC-1, also shown by RTPCR not to express CFTR (data not shown).

The expression of the protein recognised by the monoclonal and polyclonal antibodies in CFTR negative cells also appeared to be calcium regulated. Heb7a cells were incubated in the presence or absence of ionomycin for various times prior to processing for immunoblot analysis with MabCF3 as probe. The level of expression of the ~170kDa protein recognised by MabCF3 was reduced following ionomycin treatment (data not shown). Similar results were obtained with MabCF8 (data not shown).

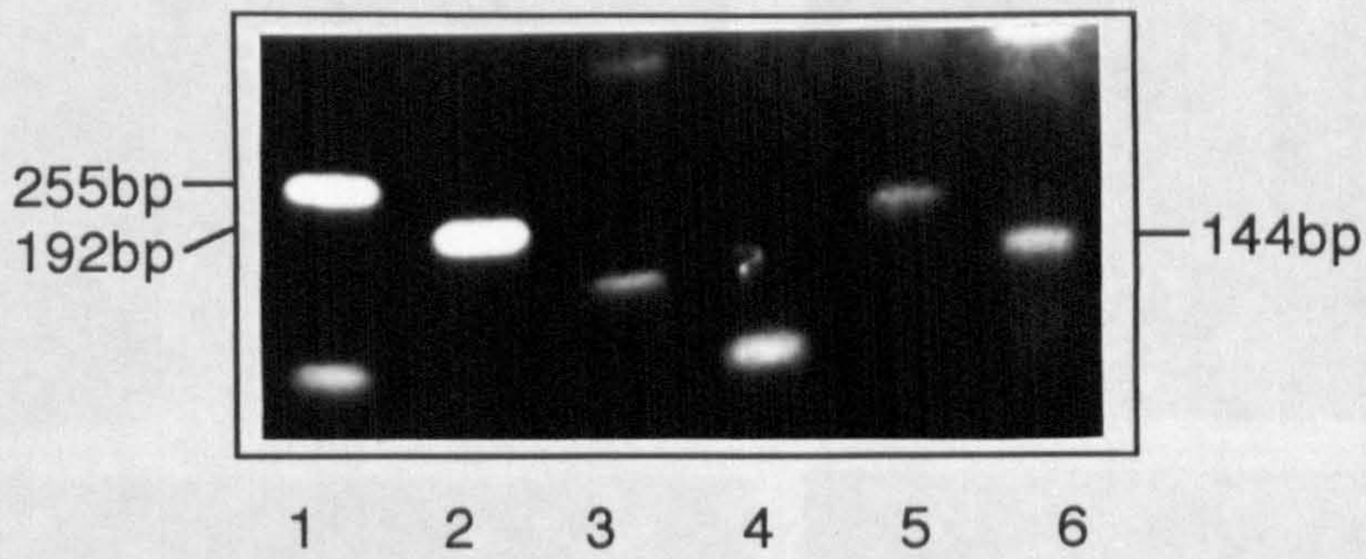
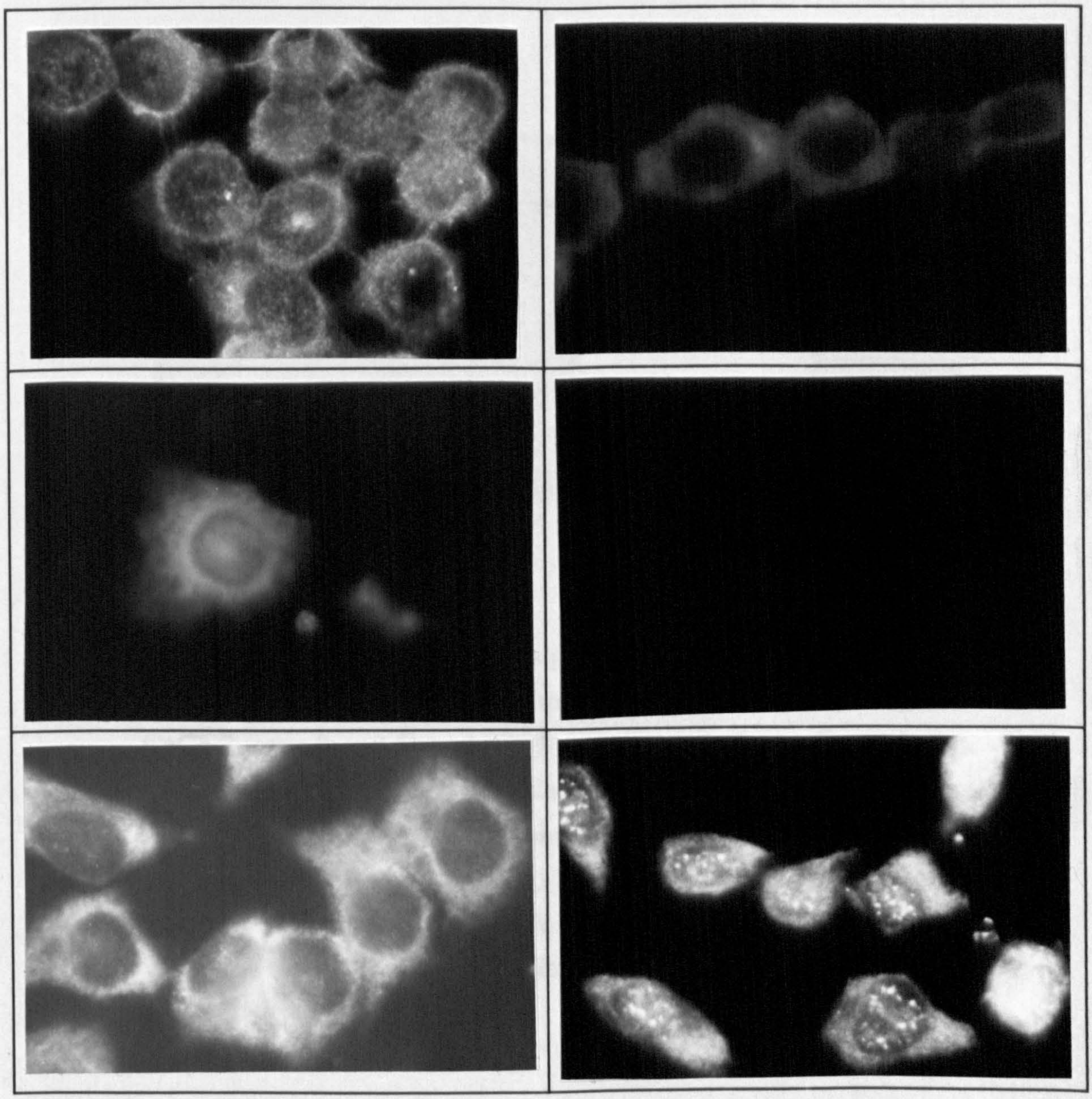


Fig. 4.9 Agarose gel analysis of PCR products from mRNA obtained from HT29 (lanes 1-3) and Heb7a (lanes 4-6) cell lines. Oligonucleotide primers designed to amplify a 255bp fragment spanning exons 23 and 24 of CFTR were used to amplify the material loaded in lanes 1 and 4. A band of this size is observed in lane 1 (HT29) but not lane 4 (Heb7a) indicating the presence of CFTR mRNA in HT29 but not Heb7a cells. Contamination of cDNA with genomic DNA was checked for using a pair of oligonucleotide primers designed to amplify a 192bp fragment within exon 24 of CFTR. The products of these reactions were loaded in lanes 2 and 5. A band of appropriate size is more intense in lane 2 (HT29) than lane 5 (Heb7a) but present in both lanes, indicating some contamination of the Heb7a cDNA with genomic DNA. The integrity of the template mRNA was tested using a pair of oligonucleotide primers designed to amplify a 144bp fragment spanning exons 7 and 8 of the MIC2 gene [Smith et al., 1993]. MIC2 encodes the E2 protein [Gelin et al., 1990] and is expressed in all human tissues [Banting et al., 1986]. A band of 144bp is present in lanes 3 (HT29) and 6 (Heb7a) indicating that intact mRNA was present in both original samples. DNA fragments of known molecular weight were loaded in an adjacent lane, their migration position is indicated.



a	b
c	d
e	f

Fig. 4.10 Immunofluorescence analysis of methanol fixed Heb7a cells using polyclonal antibody CF5/4 (panel a), polyclonal antibody 3/10 (panel b), polyclonal antibody 9/8 (panel c), MabCF3 (panel e) or MabCF8 (panel f). Pre-immune serum from the rabbits immunised with the CF5/4 construct was incubated with cells presented in panel d. Bound antibody was detected with a species specific FITC conjugated second antibody in each case.

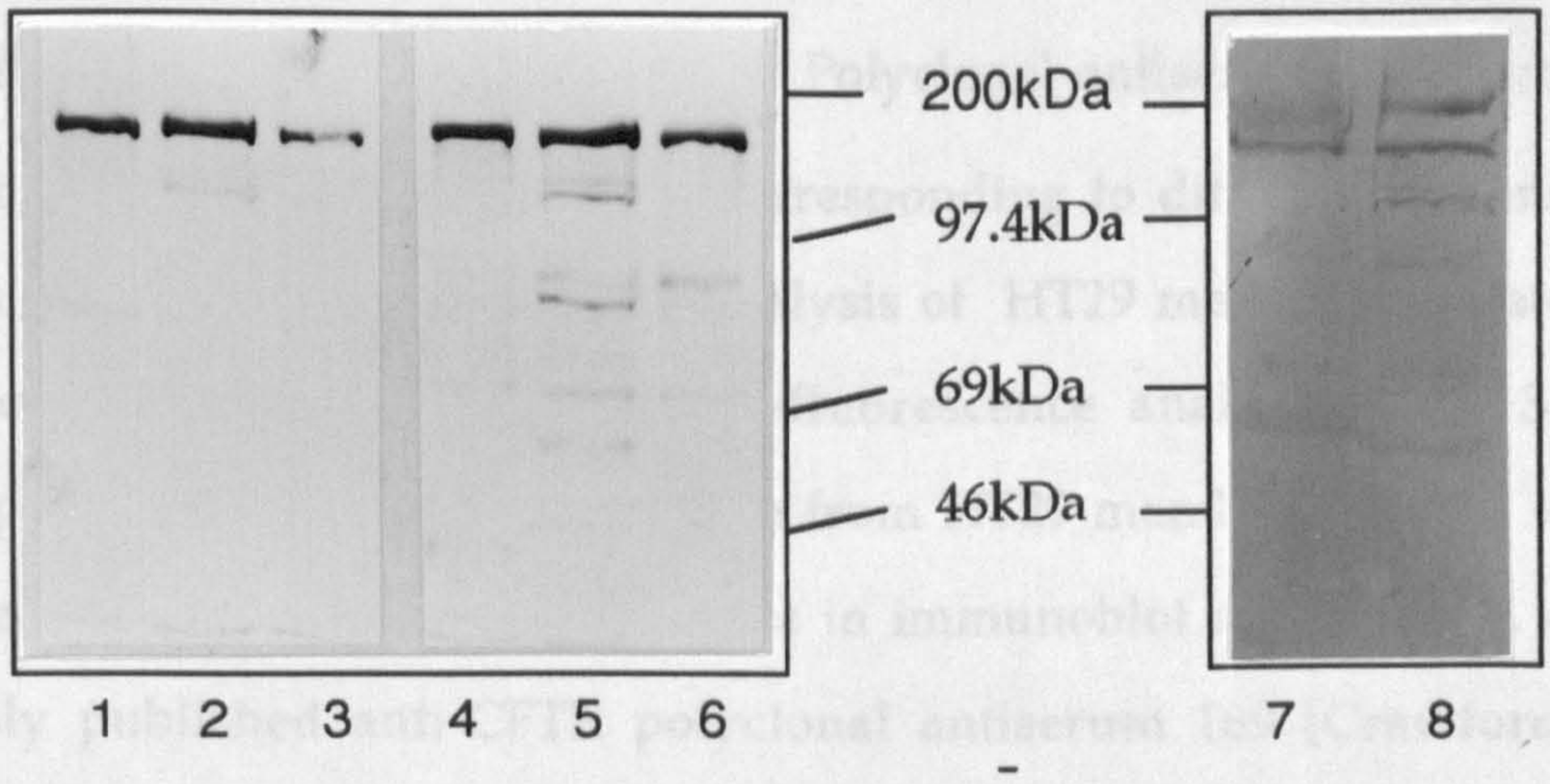


Fig. 4.11 Immunoblot analysis of membrane lysates using monoclonal or polyclonal antibodies. Heb7a (lane 1 and 4), CaCo-2 (lane 2 and 5) and HT29 (lane 3 and 6) membrane lysates were probed using MabCF3 (lanes 1-3) or MabCF8 (lanes 4-6) as primary antibody. Heb7a membrane lysates were also probed using polyclonal antibodies 3/10 (lane 7) or 5/4 (lane 8) as primary antibody. The relative mobilities of molecular weight standards, loaded in adjacent lanes, are indicated.

4.4 DISCUSSION

Monoclonal antibodies to synthetic peptides corresponding to five regions of CFTR (fig. 4.1) detect a protein of ~170kDa in immunoblot analysis of HT29 membrane lysates (fig. 4.2) and recognise HT29 cells in immunofluorescence analysis (fig. 4.3). Polyclonal antisera to β-galactosidase fusion proteins containing sequences corresponding to different regions detect a protein of ~170kDa in immunoblot analysis of HT29 membrane lysates (figs. 3.4), recognise HT29 cells in immunofluorescence analysis (figs. 3.5) and immunoprecipitate a protein of ~170kDa from HT29 membrane lysate which is recognised by the monoclonal antibodies in immunoblot analysis (fig. 4.6). A previously published anti-CFTR polyclonal antiserum 169 [Crawford et al., 1991] also immunoprecipitates a protein of ~170kDa which is recognised by the monoclonal antibodies in immunoblot analysis (fig. 4.6). The level of expression of the protein recognised by these antibodies in HT29 cells is modulated by intracellular Ca^{2+} levels (fig. 4.7), a phenomenon previously ascribed to CFTR [Bargon et al., 1992]. All of these data indicated that the novel antibodies raised recognise CFTR. Two of the monoclonal antibodies were therefore used in immunohistochemical screening of different human tissues (fig. 4.8). Both antibodies show a similar pattern of immunoreactivity, recognising epithelial cells in pancreas, gut, liver, kidney, placenta, prostrate, seminiferous tubules and trachea. The gross overall pattern of protein expression detected by these antibodies is similar to that previously described using either other anti-CFTR antibodies or in situ hybridisation techniques [Crawford et al., 1991; Cohn et al., 1991; Marino et al., 1991; Trezise and Buchwald 1991; Trapnell et al., 1991; Trezise et al., 1992; Tizzano et al., 1993). However, significant differences are noticeable:

- The entire length of the villi are stained in colonic epithelia (fig. 4.8a). This is in contrast to the data presented by Trezise et al., [1992] which show that, in rat, CFTR is expressed at the base of the villi and MDR at the top.

• There is clear expression of the protein recognised by MabCF8 in tracheal epithelia (Fig. 4.8e), whereas it has previously proved difficult to demonstrate CFTR expression in these cells by immunocytochemical means [Crawford et al., 1991] and assays of CFTR mRNA levels have suggested that there is low level expression of the protein in the respiratory tract [Trapnell et al., 1991].

• There appears to be expression of the protein recognised by MabCF8 in certain non-epithelial cells in some tissues (fig. 4.8d).

• Others have shown, by *in situ* hybridisation techniques, that in the rat uterus CFTR expression is limited to the epithelial lining of the endometrium with no expression in the underlying stroma or myometrium [Trezise and Buchwald 1991]. MabCF8 recognises the myometrium and some stromal cells in addition to epithelial cells of the endometrium (fig. 4.8c and d).

These differences posed the question of whether the antibodies cross-reacted with other proteins within cells.

CFTR is a member of the ABC transporter class of multiple membrane spanning proteins [Higgins and Hyde, 1991]. This family is large with almost 100 recognised prokaryotic and eukaryotic members [see Higgins 1992 for recent review]. A recently described yeast metal resistance protein (YCF1) has striking overall similarity to MDR and CFTR, including a region with strong sequence homology to the previously considered unique R domain in CFTR [Szczypka et al., 1994]. It seems likely that numerous other members of this family are still to be identified. It is not improbable that, since CFTR is an ABC transporter, anti-CFTR antibodies will recognise other members of this family. Indeed, it has recently been demonstrated that a monoclonal antibody raised against a synthetic peptide corresponding to part of the CFTR sequence recognises Hlyb, an *E. coli.* member of the ABC transporter family with limited linear sequence homology to CFTR (B. Holland pers. comm. [1]). It seems highly likely that some anti-CFTR antibodies will recognise other

1 Holland,B. 'A monoclonal antibody to the F508 region of CFTR recognises the equivalent ATPase domain of the haemolysin translocator HlyB' International Workshop on 'anti-CFTR antibodies' Paris, France 1993 sponsored by Association Franchise de Lutte contre la Mucovoscosidose (AFLM)

eukaryotic members of the ABC transporter family if immuno-cross-reactivity can occur between prokaryotic and eukaryotic members of that family.

Immunoblot (fig. 4.11) and immunofluorescence assays (fig. 4.12) clearly show that the novel antibodies described do recognise a protein in cells which fail to express CFTR (fig. 4.9). This protein (or possibly proteins) is of a similar size to CFTR (fig. 4.11) and is immunologically closely related to CFTR (as demonstrated by the fact that it is recognised by diverse monoclonal and polyclonal antibodies raised against different regions of CFTR). The data presented here indicate the importance of demonstrating the specificity of antibodies to CFTR.

The antibodies described here should provide a means of identifying other members of the ABC transporter family that are not only structurally, but may also be functionally, related to CFTR. The anticipated way forward in this work is to use these antibodies to immunoprecipitate proteins both from cell lines known to express CFTR and from those which have been shown not to express CFTR. Amino acid sequence of the ~170kDa proteins could then be ascertained by micro-sequence analysis. The antibodies could also be used to immunoscreen a cDNA expression library constructed using mRNA isolated from Hela cells. Immunopositive clones would be isolated and subjected to DNA sequence analysis. The identification and characterisation of novel sequences with strong homology to CFTR may prove to be highly significant in the understanding of the disease and have possible implications for therapy.

CHAPTER 5

PRODUCTION AND SCREENING OF ScFv ANTIBODIES

5.1 INTRODUCTION

Recombinant phage antibody technology has the power and versatility to mimic the features of immune diversity and selection. Two different systems for the production of phage display antibody fragments against CFTR have been adopted in this study, the first of which is described in this chapter.

The phagemid vector pCANTAB5 was developed by Cambridge Antibody Technology and is commercially available from LKB-Pharmacia. Most of the reagents needed for synthesis of an antibody library using this vector were supplied by Pharmacia prior to their commercial availability so that procedures could be tried, tested and modified. The pCANTAB5 vector is based on pUC119 [Vieira and Messing, 1988] and contains restriction sites *Sfi* 1 and *Not* 1 such that antibody fragments can be cloned between the leader sequence and the amino terminus of bacteriophage coat protein g3p (fig. 5.1). The expression of the antibody-g3p fusion protein is controlled by an inducible *lac* promoter. The phagemid is propagated in TG2 *E.coli.* which contains the *lac* Iq gene, encoding for the *lac* repressor required to block transcription of the *lac* promoter and prevent accumulation of excess antibody-g3p which can be toxic to the cell. Further repression of the relatively leaky *lac* promoter is achieved by incubating transformed *E.coli.* at 30°C (allowing slower growth than incubation at 37°C) and including glucose in the culture media which forces catabolite repression of cAMP stimulated transcription. When expression of the fusion protein is required (after the addition of helper phage) so that it can be accumulated into the assembling phage, the transformed *E.coli.* are grown at 37°C in the absence of glucose.

This system is based upon the cloning of ScFv antibody fragments where heavy and light chain variable regions are joined together with the

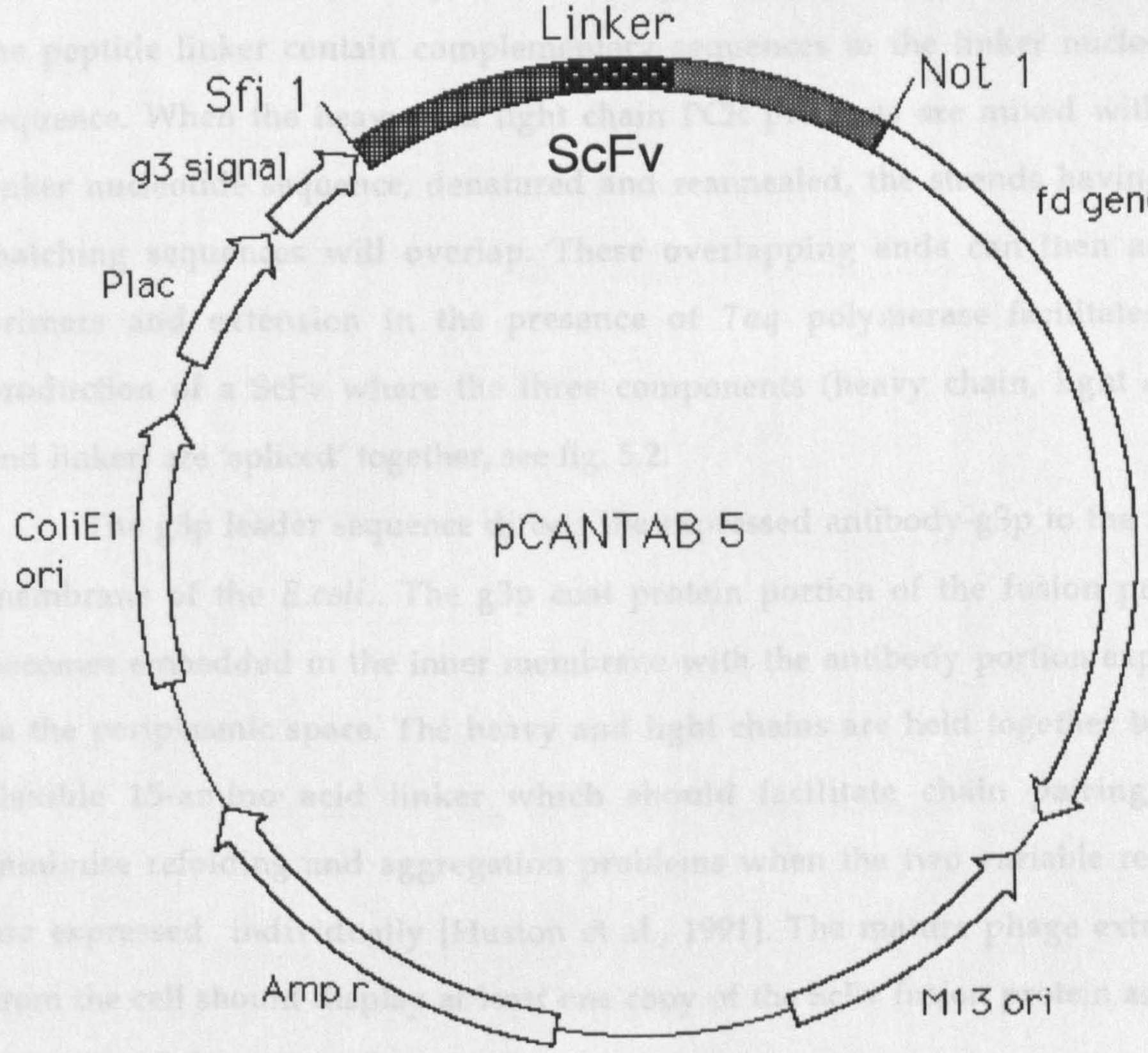


Fig. 5.1 pCANTAB 5 phagemid cloning vector, including ScFv inserted into *Sfi* 1 and *Not*1 restriction sites

P_{lac}	inducible *lac* promoter
Amp^r	β lactamase gene
ori	origin of replication

peptide linker ($(Gly_4\text{-}Ser)_3$ [Bird et al., 1988; Huston et al., 1988] by a process of 'splicing by overlap extension' [Horton et al., 1989]. The heavy and light chain variable regions are amplified separately by PCR. The primers are designed such that the ends of the amplified antibody sequences that are to be joined to the peptide linker contain complementary sequences to the linker nucleotide sequence. When the heavy and light chain PCR products are mixed with the linker nucleotide sequence, denatured and reannealed, the strands having the matching sequences will overlap. These overlapping ends can then act as primers and extension in the presence of *Taq* polymerase facilitates the production of a ScFv where the three components (heavy chain, light chain and linker) are 'spliced' together, see fig. 5.2.

The g3p leader sequence directs the expressed antibody-g3p to the inner membrane of the *E.coli*.. The g3p coat protein portion of the fusion protein becomes embedded in the inner membrane with the antibody portion exposed in the periplasmic space. The heavy and light chains are held together by the flexible 15-amino acid linker which should facilitate chain pairing and minimise refolding and aggregation problems when the two variable regions are expressed individually [Huston et al., 1991]. The mature phage extruded from the cell should display at least one copy of the ScFv fusion protein as well as copies of the native g3p protein.

In order to characterise the processes involved in the production of phagemid antibody expression libraries, the initial library was generated using mRNA from two different hybridoma cell lines shown by ELISA to produce monoclonal antibodies (MabCF2 and MabCF8) to peptides corresponding to regions of CFTR. Further characterisation of these monoclonal antibodies indicates that they recognise either CFTR or protein(s) with very similar characteristics to CFTR (see chapter 4). It was anticipated that using mRNA from these hybridoma cell lines would enable the production of a phagemid library that would be useful for testing and optimising the screening strategies that could subsequently be used against the phagemid library produced from

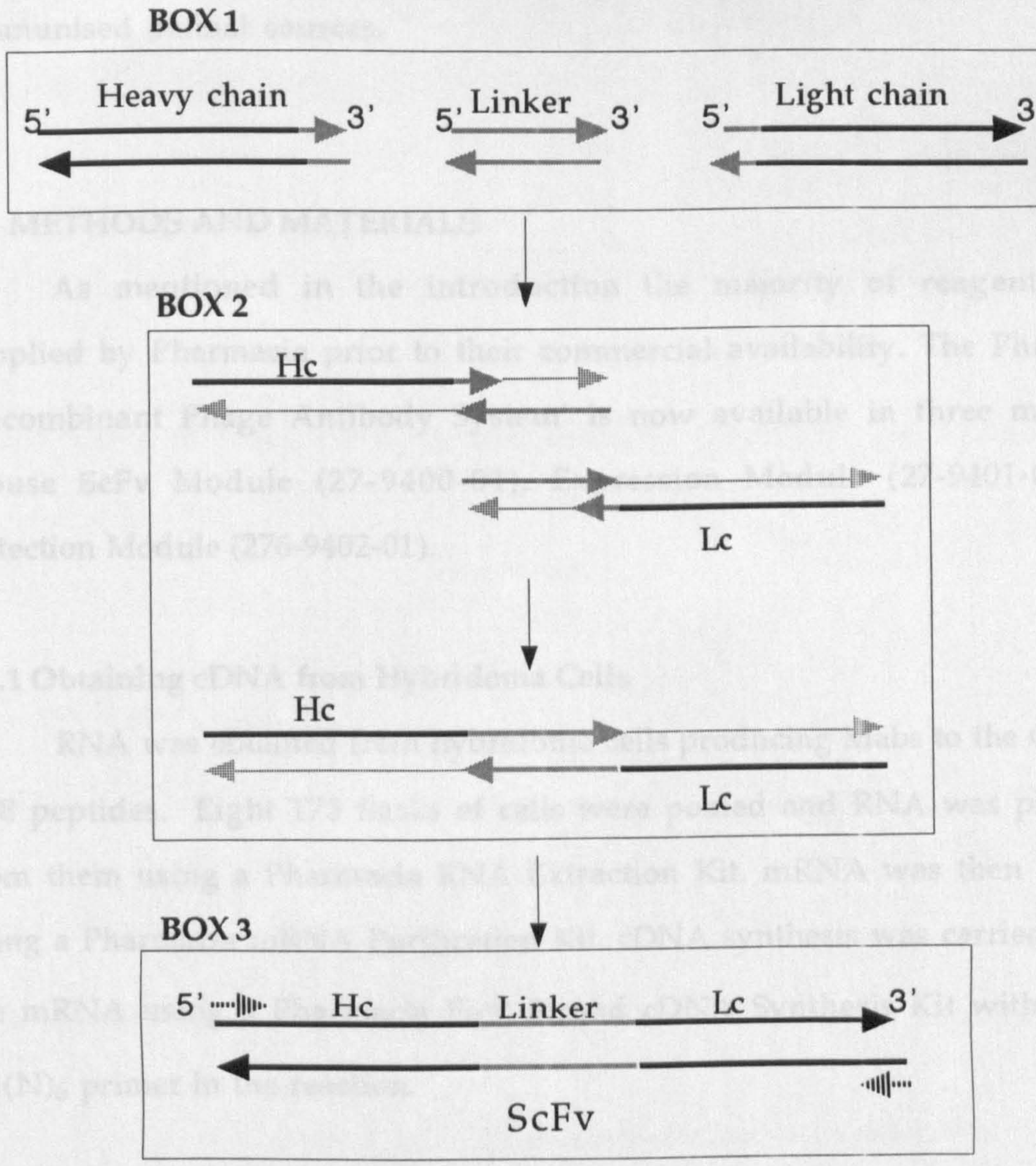


Fig. 5.2 Cartoon representing the construction of a ScFv by the mechanism of gene splicing by overlap extension.

BOX 1: Heavy (Hc) and light chain (Lc) antibody gene fragments which have been amplified independently by PCR are mixed with linker sequence, with all components in equal concentrations. Areas of the heavy and light chain sequences that are complementary to the linker sequence are depicted by ⟶.

BOX 2: The sequences are denatured and reannealed such that the overlapping regions can combine and act as primers for each other. Extension of the overlaps (depicted as ⟶) occurs in the presence of *Taq* polymerase enabling the component parts to be spliced together.

BOX 3: Addition of 5′ heavy chain and 3′ light chain primers (depicted by ⟶) and subsequent PCR gives amplification of the full length ScFv product.

immunised animal sources.

5.2 METHODS AND MATERIALS

As mentioned in the introduction the majority of reagents were supplied by Pharmacia prior to their commercial availability. The Pharmacia 'Recombinant Phage Antibody System' is now available in three modules: Mouse ScFv Module (27-9400-01), Expression Module (27-9401-01) and Detection Module (276-9402-01).

5.2.1 Obtaining cDNA from Hybridoma Cells

RNA was obtained from hybridoma cells producing Mabs to the CF2 and CF8 peptides. Eight T75 flasks of cells were pooled and RNA was prepared from them using a Pharmacia RNA Extraction Kit. mRNA was then isolated using a Pharmacia mRNA Purification Kit. cDNA synthesis was carried out on the mRNA using a Pharmacia First Strand cDNA Synthesis Kit with 0.02μg $pd(N)_6$ primer in the reaction.

5.2.2 Preparation and Cloning of ScFv Fragments

The procedures followed are summarised in fig. 5.3.

Primary PCR amplification was carried out on first strand cDNA to generate suitable quantities of antibody heavy (~340 b.p.) and light (~325 b.p.) chain DNA. The reactions were carried out using the primers provided by Pharmacia. The heavy chains were amplified using a single pair of primers whereas the light chains were amplified with a mixture of 10 primers (both 5′ and 3′). The individual oligonucleotide sequences of the primers were unspecified. Standard techniques (section 2.2.7) were followed with an annealing temperature of 55°C and 30 cycles. The amplified heavy and light chain antibody products were separated from other reaction components on a

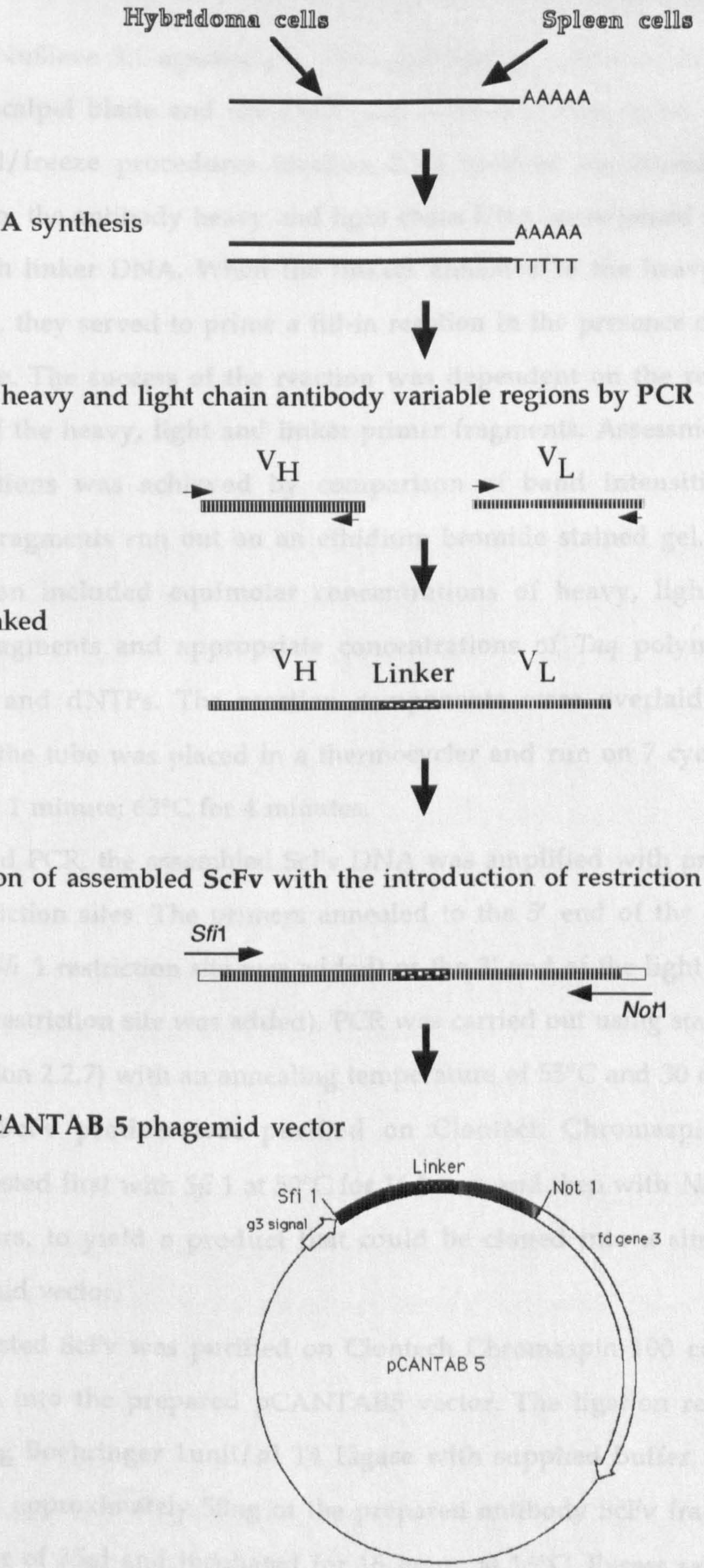


Fig. 5.3 Cartoon representing the preparation and cloning of ScFv fragments into pCANTAB5

preparative 1% NuSieve 3:1 agarose gel. The appropriate DNA bands were excised with a scalpel blade and the DNA was extracted from them using standard phenol/freeze procedures (section 2.2.9 method A). During the assembly reaction, the antibody heavy and light chain DNA were joined into a single chain with linker DNA. When the linkers annealed to the heavy and light chain DNA, they served to prime a fill-in reaction in the presence of *Taq* DNA polymerase. The success of the reaction was dependent on the relative concentrations of the heavy, light and linker-primer fragments. Assessment of these concentrations was achieved by comparison of band intensities of samples of the fragments run out on an ethidium bromide stained gel. The assembly reaction included equimolar concentrations of heavy, light and linker-primer fragments and appropriate concentrations of *Taq* polymerase enzyme, buffer and dNTPs. The reaction components were overlaid with mineral oil and the tube was placed in a thermocycler and run on 7 cycles as follows: 94°C for 1 minute; 63°C for 4 minutes.

In a second PCR, the assembled ScFv DNA was amplified with primers that added restriction sites. The primers annealed to the 5′ end of the heavy chain (where a *Sfi* 1 restriction site was added) or the 3′ end of the light chain (where a *Not* 1 restriction site was added). PCR was carried out using standard procedures (section 2.2.7) with an annealing temperature of 55°C and 30 cycles. The amplified ScFv product was purified on Clontech Chromaspin 100 column and digested first with *Sfi* 1 at 50°C for 16 hours and then with *Not* 1 at 37°C for 12 hours, to yield a product that could be cloned into a similarly digested phagemid vector.

The digested ScFv was purified on Clontech Chromaspin 100 column prior to ligation into the prepared pCANTAB5 vector. The ligation reaction was set up using Boehringer 1unit/μl T4 Ligase with supplied buffer, 100ng pCANTAB5 and approximately 50ng of the prepared antibody ScFv fragment in a total volume of 35μl and incubated for 16 hours at 16°C. Excess salt was removed from the ligation reaction by microdialysis prior to transformation

into *E.coli* . TG2 cells [Gibson, 1984].

5.2.3 Transformation and Rescue of the Recombinant Antibody Phage Library

40μl aliquots of freshly prepared competent TG2 cells (section 2.2.13 method A) were electroporated with 3μl of the ligation reaction as described in section 2.2.14. After incubation for one hour at 37°C the SOC media was supplemented with 100mg/ml ampicillin (to select for the phagemid) and 2% glucose, before further incubation at 37°C in a shaking incubator at 250rpm. After 3 hours, $2.5x10^9$ pfu of M13K07 helper phage was added to the cell suspension to 'rescue' the phagemid with its antibody ScFv gene insert from the transformed cells. M13K07 provided the necessary information for packaging the recombinant pCANTAB5 phagemid and for assembling the g3p-ScFv fusion protein on the tip of the assembled phage. The culture was incubated at 37°C for 30 minutes at 150rpm followed by 30 minutes at 250rpm, to facilitate helper phage infection. The sample was then centrifuged at 800g for 10 minutes, the supernatant was discarded and the pellet was resuspended in a glucose deficient medium containing 100mg/ml ampicillin (to select for cells with phagemid) and 50mg/ml kanamycin (to select for cells infected with M13K07). In the absence of glucose, the *lac* promoter present on the phagemid was no longer repressed and synthesis of ScFv-gene3 was facilitated. The cells were grown for 16 hours at 37°C in a shaking incubator with the M13K07 present to initiate phage replication and the production and release of complete phage particles. The cells were then pelleted by centrifugation at 1000g for 10 minutes and the supernatant containing the recombinant phage was recovered.

5.2.4 Enrichment of the ScFv Library

The phage-displayed antibodies capable of binding specific antigen in the form of the peptides CF2 and CF8 (to which the original Mabs were raised) were selected and enriched by two methods:

Method A- Panning against the antigen.

In the panning procedure, 5ml of recombinant phage culture was pre-incubated for one hour at room temperature with an equal volume of PBS containing 2% skimmed milk powder (blocking buffer). Panning was performed on a Petri dish that had been coated with antigen (2.5ml of each of the BSA linked CF2 and CF8 peptides diluted to 10mg/ml in PBS) by an overnight incubation at 4°C. Prior to panning, excess antigen solution was removed and the the panning plates were pre-incubated with blocking buffer as above. The blocking buffer on the panning plate was replaced with the pre-blocked phage solution. After 2 hours incubation at 37°C, the phage solution was removed and the dish was washed 20 times with PBS and 20 times with PBS containing 0.1% Tween 20. The bound phage was eluted by adding 1ml of 100mM triethylamine which was left for 10 minutes at room temperature before being removed and neutralised with 1M Tris-HCl, pH7.4. The neutralised recombinant phage solution was reinfected into log-phase TG2 cells by incubating with the cell culture for 30 minutes at 37°C with intermittent gentle shaking. The reinfected *E.coli.* were then cultured and the phage rescued as described previously.

Method B- Biotin-capture of phage on streptavidin coated magnetic beads.

Streptavidin-conjugated paramagnetic beads (M280 Dynal) were used to capture phage bound to biotinylated CF2 and CF8 peptides as described by Hawkins et al., [1992].

Prior to each round of selection, the titre of recovered phage supernatants was increased by PEG precipitation. 4% (w/v) PEG-8000 and 3% NaCl were added to phage supernatants, mixed and left on ice for 30 minutes. The precipitated phage was spun down at 9000 rpm in a Sorvall RC5C centrifuge for 20 minutes at 4°C. The supernatant was removed and the pellet allowed to drain before resuspension in 50-500µl TE buffer and transferred to

an Eppendorf tube. The resuspended phage solution was spun at full speed in a microfuge for 5 minute to pellet any residual cellular material. The supernatant was transferred to a fresh Eppendorf tube.

Precipitated phage was diluted 1:5 with blocking buffer (2% skimmed milk powder in PBS) and incubated for 1 hour at room temperature with continuous mixing. The streptavidin-conjugated paramagnetic beads were also pre-incubated in blocking buffer as above. 100nM of each of the biotinylated peptides CF2 and CF8 diluted in blocking buffer were added to the pre-incubated phage and incubated as above to allow the ScFvs on the phage to bind to the peptides. The pre-incubated magnetic beads were then pelleted on a Dynal magnet, the blocking buffer was removed and the beads were resuspended in the phage/peptide solution. After a 15 minute incubation at room temperature with continuous mixing, the magnet was used to isolate the beads along with biotinylated peptides attached to the streptavidin on the beads and any phage bound by ScFvs to the peptides. The supernatant containing any unbound peptide and phage was removed and the beads were washed 20 times with 0.1% Tween 20 in PBS followed by 20 times with PBS. Washes were performed by resuspending the beads in the wash solution, mixing well, magnetically isolating the beads and removing the supernatant. The washing steps should remove any residual unbound phage. The bound phage were eluted from the beads by incubation with log phase TG2 cells at 37°C for one hour. The beads were then isolated magnetically and the supernatant culture containing TG2 cells reinfected with selected phage were rescued as described previously.

Four rounds of enrichment were carried out with each of the methods described above. After the final round of enrichment the reinfected TG2 cells were plated out and grown overnight on nutrient agar plates containing ampicillin and 2% glucose. Individual colonies on these plates were then picked for microtiter plate rescue of the enriched clones and ELISA.

5.2.5 Microtiter Plate Rescue of Enriched Phage Clones

Single colonies from the quarternary enrichment step were picked and used to inoculate 900μl of 2TY containing 100μg/ml ampicillin and 2% glucose in wells of a 96 well microtiter plate (Falcon 3911). This master plate was incubated at 30°C overnight with shaking at 100-150rpm. 200μl aliquots of 2TY containing 100μg/ml ampicillin, 2% glucose and $1.05x10^{10}$ pfu of M13KO7 were added to each well of a second microtiter plate. 20μl of saturated culture from each well of the master plate was transferred to a corresponding well in the second plate which was then incubated at 37°C with shaking at 150rpm. At this point the master plate was sealed with parafilm and stored at 4°C. After 2 hours the cultures in the second plate began to appear turbid and the plate was spun at 400g for 10 minutes at 4°C in a MSE bench top centrifuge fitted with microtiter plate adapters. After removal of the supernatants the cell pellets were resuspended in 200μl of 2TY medium containing 100μg/ml ampicillin and 50μg/ml kanamycin. The plate was then incubated overnight at 37°C with shaking. After centrifugation (as described above) the supernatants were transferred to a fresh microtiter plate and incubated at room temperature for about 20 minutes with equal volumes of blocking buffer, ready for ELISA screening.

5.2.6 Selection of Individual Phage Clones Recognising Antigen by ELISA

The phage-displayed recombinant antibodies were detected in an enzyme-linked immunosorbant assay (ELISA) as described by McCafferty et al., [1990]. The ELISA plates were coated with either CF2 or CF8 peptide conjugated to BSA or biotin.

Diluted conjugated peptide (5mg/ml in PBS) was added to a 96 well flat bottom microtiter plate (Falcon) and incubated overnight at 4°C to coat the wells with antigen. The antigen solution was removed from each well and replaced with 200μl PBS containing 2% skimmed milk powder (blocking buffer) and incubated at room temperature for 1 hour. The blocking solution

was removed and replaced with the diluted recombinant phage supernatants from the microtiter plate rescue. After incubation 37°C for 1 hour the supernatants were removed from the wells and the wells washed 3 times in PBS with 0.05% Tween 20. A secondary antibody (raised to M13 phage and conjugated to horse radish peroxidase), at a dilution of 1 in 5000 in blocking buffer was then added to each well and incubated at 37°C for 1 hour. The solution was removed and the wells washed with PBS as described above. The ELISA was developed by the addition of 200μl of developing solution (ABTS (4.7mg 2',2'-Azino-Bis(3-Ethylbenzthiazoline-6-sulphonic Acid)diammonium) in 21ml 0.05M citric acid, pH4.0 and 37μl H_2O_2) to each well. The colour reaction was assessed after 40 minutes. (The intensity of the colour reaction was measured at 410 nm in a Labsystems Multiscanplus plate reader.)

5.2.7 Sequencing of ScFv Clones

Sequencing was carried out using the Sequenase version 2.0 kit (see section 2.2.15) with the following primers:

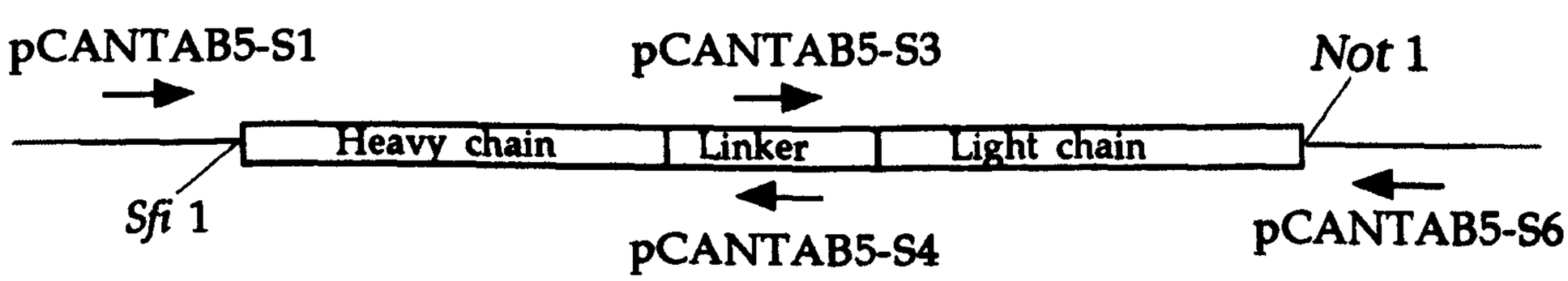
pCANTAB-S1 5'- CAACGTGAAAAAATTATTATTCGC -3'

pCANTAB-S3 5'- GGTTCAGGCGGAGGTGGCTCTGG -3'

pCANTAB-S4 5'- CCAGAGCCACCTCCGCCTGAACC -3'

pCANTAB-S6 5'- GTAAATGAATTTTCTGTATGAGG- 3'

The orientation of the primers in relation to the ScFv is represented below.



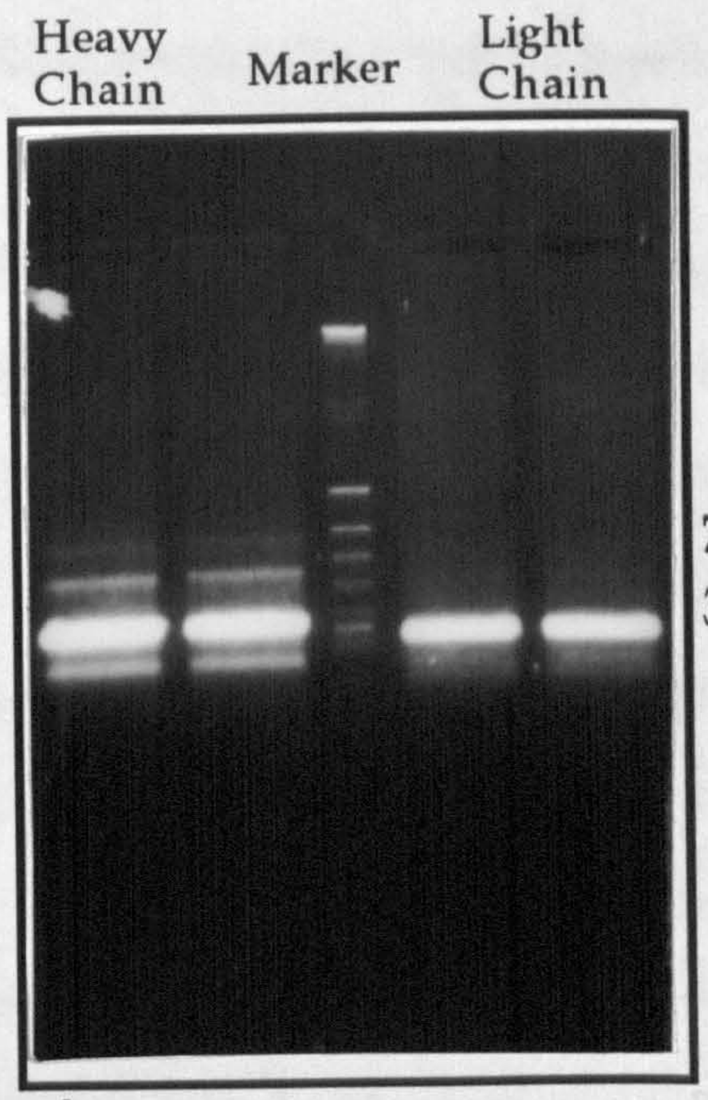
The oligonucleotide primers were synthesised within the SERC funded Molecular Recognition Centre, University of Bristol. However, they are now commercially available from Pharmacia as a pCANTAB5 sequencing primer

set (27-1585-01). Each sequencing reaction was run on short and long sequencing gels in order to maximise the length of readable sequence obtained.

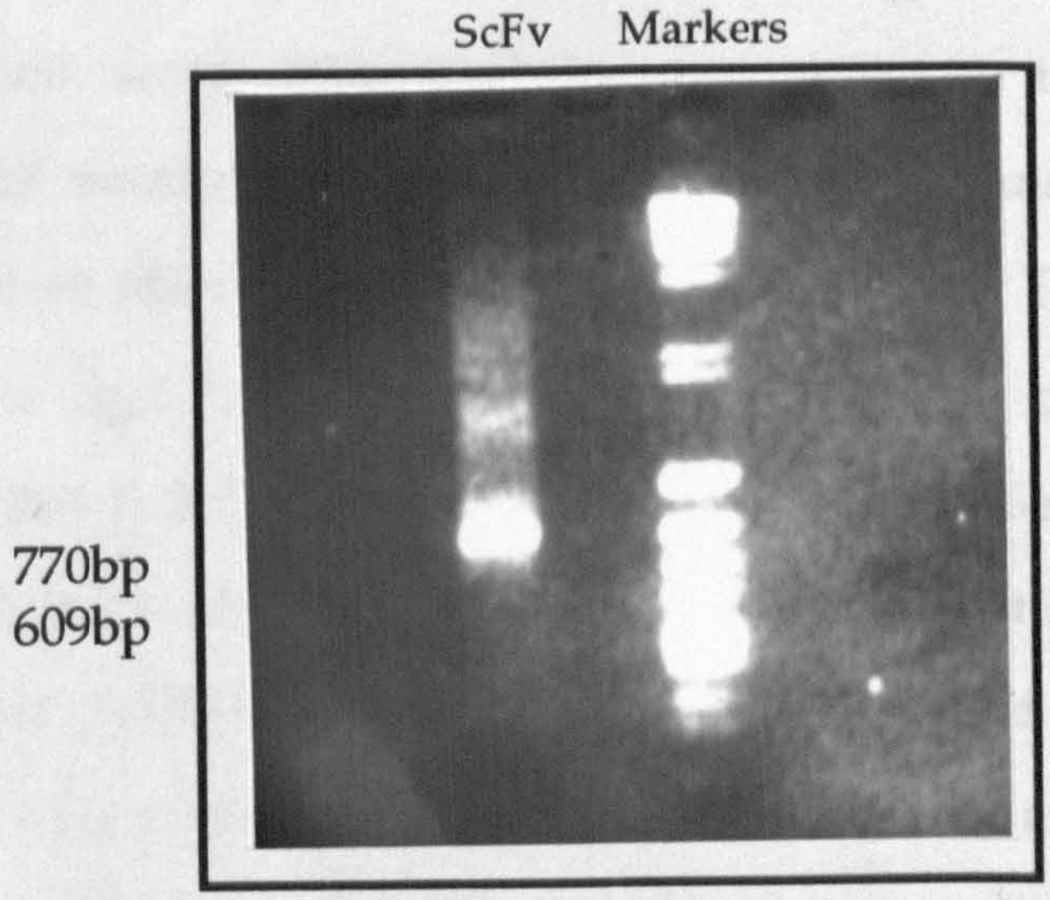
5.3 RESULTS

5.3.1 Construction of the ScFv Library

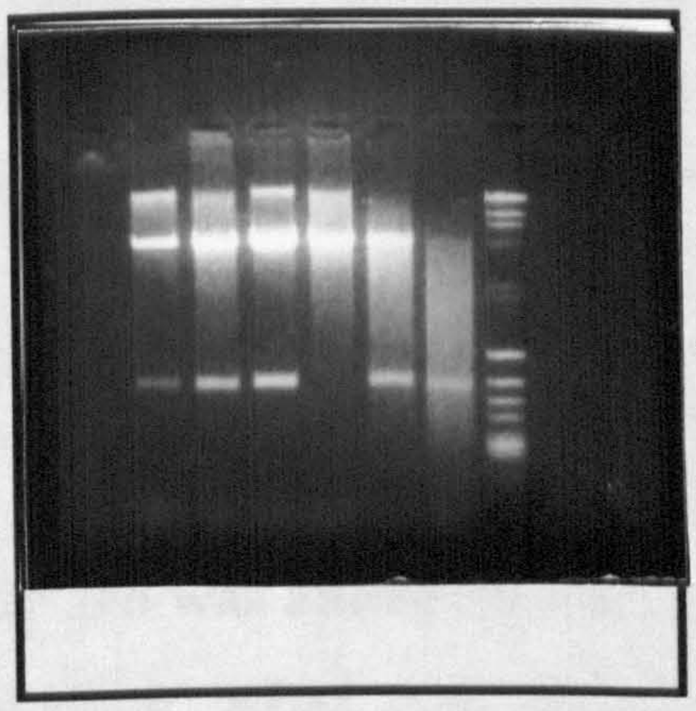
An ScFv antibody library was constructed from mRNA extracted and purified from the cells of two hybridoma cell lines which had previously been shown by ELISA to produce monoclonal antibodies to peptides found within the sequence of CFTR. Reverse transcription of the mRNA was followed by PCR to give amplification of heavy and light chain antibody sequences (see fig. 5.4a). Amplified PCR products were purified from an agarose gel and mixed with a suitable concentration of peptide linker nucleotide sequence. The heavy chain, linker and light chain sequences were then 'spliced' together by a process of overlap extension. In this process any light chain sequence could combine with any heavy chain sequence in a random manner. Once constructed, the ScFv sequences were further amplified by PCR with primers that contained *Sfi* 1 and *Not* 1 restriction sites to give products of approximately 750bp (see fig. 5.4b). The amplified ScFv sequences were digested with *Sfi* 1 and *Not* 1 restriction enzymes prior to ligation into the previously digested pCANTAB5 vector. Thus constructed, the ScFv antibody library was used to transform *E.coli.* by electroporation and 'rescued' with M13K07 helper phage. Phage particles extruded from the transformed bacteria were presumed to contain the single stranded phagemid DNA encoding for the specific ScFv antibody fragment that was expressed on its tip. Phagemid DNA prepared from six colonies of transformed *E.coli.* restricted with *Sfi* 1 and *Not* 1 and run on an agarose gel revealed that the majority of transformants contained a 750bp ScFv insert (see fig. 5.4c).



a)



b.)



c)

Fig. 5.4 Construction of the phagemid antibody library from hybridoma cells The standard markers were λ DNA*Hind* 111, ØX174 DNA *Hae* 111 digests

a) pcr products of heavy and light chain variable regions

b) pcr of assembled ScFv fragment

c) *Not* 1, *Sfi* 1 digests of plasmid DNA from TG2 cells trnsformed with pCANTAB5 showing ScFv inserts

5.3.2 Sequencing ScFv Clones

Phagemid DNA was prepared from six colonies of transformed *E.coli.* by Magic Mini Prep (see section 2.2.1 method B). The six DNA preparations were all sequenced with each of the four sequencing primers described above (section 5.2.7). The DNA sequences were then collated and analysed using the University of Wisconsin Genetics Computer Group (UWGCG) suite of programmes. The light chain sequences are given in fig.5.5. Of the six light chains analysed, five have almost identical sequences (ScFvLc2 - ScFvLc6). The sixth light chain sequence (ScFvLc1) only has limited homology to the other five. ScFvLc1 and ScFvLc6 were compared to sequences in the EMBL/Genbank data base release number 39 version 6, and the five most homologous sequences for each were identified and are given in fig. 5.6. The heavy chain sequences are given in fig.5.7. The six heavy chains analysed can be divided into two sets of three (Set 1: ScFvHc1, ScFvHc2 and ScFvHc3, Set2: ScFvHc4, ScFvHc5 and ScFvHc6) with each set containing almost identical sequences. One member of each set (ScFvHc1 and ScFvHc6) was compared to sequences in the EMBL/Genbank data base release, number 39 version 6, and the five most homologous sequences for each were identified and are given in fig.5.8.

5.3.3 Panning of the ScFv Library

Enrichment of the antibody library for ScFv which bound to peptide antigen was attempted by carrying out four consecutive rounds of selection by panning. After each round of panning, phage displaying antibodies were reinfected into *E.coli.* and rescued with helper phage. After the fourth round of panning, enriched clones were picked, cultured and rescued in a microtiter plate. ELISA analysis of supernatants from individual colonies did not indicate any positive clones expressing ScFvs that bound to either CF2 or CF8 BSA peptide conjugates.

```
ScFvLc1                    tcctccctgtctgcctctctgggagcagagtctccatcagt
ScFvLc2                  agtctccagccaccctgtctgtgactccaggagatagagtcagtcttt
ScFvLc3                 cagtctccagccaccctgtctgtgactccaggagatagagtcagtcttt
ScFvLc4                  agtctccagccaccctgtctgtgactccaggagatagagtcagtcttt
ScFvLc5                 cagtctccagccaccctgtctgtgactccaggagatagagtcagtcttt
ScFvLc6                  agtctccagccaccctgtctgtgactccaggagatagagtcagtcttt

1 tgcagggcaagtcaggacattagcaattatttaaactggtatcagcagaaaccagatgga
2 cctgcagggccagtcaaagtattagcaactacctacactggtatcgacaaaaatcacatg
3 cctgcagggccagtcaaagtattagcaactacccacactggtatcaacaaaaatcacatg
4 cctgcagggccagtcaaagtattagcaactacctacactggtatcaacaaaaatcacatg
5 cctgcagggccagtcaaagtattagcaactacctacactggtatcaacaaaaatcacatg
6 cctgcagggccagtcaaagtattagcaactacctacactggtatcaacaaaaatcacatg

1 actgttaaactcctgatctactacacatcaagattccactcaggagtcccatcaaggttc
2 agtctccaaggcttctcatcaagtatgcttcccagtccatctctgggatcccctccaggt
3 agtctccaaggcttctcatcaagtatgcttcccagtccatcactgggatcccctccaggt
4 agtctccaaggcttctcatcaagtatgcttcccagtccatctctgggatcccctccaggt
5 agtctccaaggcttctcatcaagtatgcttcccagtccatctctgggatcccctccaggt
6 agtctccaaggcttctcatcaagtatgcttcctagtccatctctgggatcccctccaggt

1 agtggcagtgggtctggaacagattattctctcaccattagtaacctggagcaagaagat
2 tcagtggcagtggatcagggacagatttcactctcagtatcaacagtgtggagactgaag
3 tcagtggcagtggatcagggacagatttcactctcagtatcaacagtgtggagactgaag
4 tcagtggcagtggatcagggacagatttcactctcagtatcaacagtgtggagactgaag
5 tcagtggcagtggatcagggacagatttcactctcagtatcaacagtgtggagactgaag
6 tcagtggcagtggatcagggacagatttcactctcagtatcaacagtgtggagactgaag

1 attgccacttacttttgccaacacggtaatacgcttccacgtggacattcggaggaggca
2 attttggaatgtatttctgtcaacagagtaacagctggcctctcacgttcggtgctggga
3 attttggaatgtatttctgtcaacagagtaacagctggcctctcacgttcggtgc.ggga
4 attttggaatgtatttctgtcaacagagtaacagctggcctctcacgttcggtgc.ggga
5 attttggaatgtatttctgtcaacagagtaacagctggcctctcacattcggtgc.ggga
6 attttggaatgtatttctgtcaacagagtaacagctggcctctcacgttcggtgctggga

1 ccaagatggaaa
2 caaagttggaaataaaa
3 caaagttggaaataaa
4 ccaagctggaaataaa
5 caaagttggaaataaaacgg
6 ccaagctggaaataaaacg
```

Fig. 5.5 Alignment of the light chain sequences of six ScFv clones. Sequences ScFvLc2 - ScFvLc6 are almost identical. Discrepencies between them are highlighted in **bold**. The ScFvLc1 sequence shown in *italics* has only limited homology to the other five sequences.

a)ScFvLc1

	Accession Number	Description	% Homology
i	M37021	Mouse Ig rearranged kappa-chain mRNA V-J region, partial cds.	96.9
ii	M32042	Mouse Ig kappa-chain mRNA V-J region, from hybridoma P6514-2, partial cds.	96.9
iii	M20281	Mouse Ig active kappa chain mRNA V-region VJk1, partial cds, clone 24F3.	96.9
iv	X05796	Mouse hybridoma 36-65 Ig L-chain rearranged V(k)-J(k)1 gene.	96.9
v	M20279	Mouse Ig active kappa chain mRNA V-region VJk1, partial cds, clone 4F8.	96.9

b)ScFv Lc6

	Accession Number	Description	% Homology
i	X02556	Mouse mRNA fragment for G1 kappa immunoglobulin A 20/44 light chain (V-J).	96.7
ii	X02555	Mouse mRNA fragment for G1 kappa immunoglobulin A 8/4 light chain (V-J).	96.7
iii	M35667	Mouse lysozyme-binding Ig kappa chain (HyHEL-10) V23-J2 region mRNA, partial cds.	95.4
iv	M93959	Mouse Ig active kappa-chain V-region partial cds.	95.3
v	M95945	Mouse mRNA sequence, partial cds.	95

Fig. 5.6 Tables presenting the 5 most homologous sequences identified for

a)ScFvLc1 and b)ScFvLc6 from the EMBL/Genbank data base, release number 39, version 6.

Further information and references for each of the sequences given can be obtained from the EMBL/Genbank data base via the accession numbers.

```
ScFvHc1   aggtccagctgcaggagtcaggacctgagctggtgaagcctgggg
ScFvHc2   aggtcaagctgcagcagtctggacctgagctggtgaagcctgggg
ScFvHc3   aggtgcagctgcaggagtcaggacctgagctggtgaagcctgggg
ScFvHc4   aggtgcaactgcagcagtccggggggaggcttagtgaagcctggag
ScFvHc5   aggtgcagctgcaggagttaggggggaggcttagtgaagcctggag
ScFvHc6   aggtgcaactgcagcagtcaggggggaggcttagtgaagcctggag

1 cttcagtgaagataccctgcaaggcttctggatacacattcactgactactacatgcact
2 cttcagtgaagatatcctgcaaggcttctggttactcattcactggctactacatgcact
3 cttcagtgaagatatcctgcaaggcttctggttactcattcactggctactacatgcact
4 ggtccctgaaactctcctgtgcagcctctggattcactttcagtagctatgccatgtctt
5 ggtccctgaaactctcctgtggagcctctggattcactttcagtagctatgccatgtctt
6 ggtccctgaaactctcctgtgcagcctctggattctctttcagtagctatgccatgtctt

1 gggtgaagcagagccatggaaagagccttgagtggattggaagtattaatcctaacaatg
2 gggtgaagcaaagccatgtaaagagccttgagtggattggacgtattaatccttacaatg
3 gggtgaagcaaagccatgtaaagagccttgagtggattggacgtattaatcctaacaatg
4 gggttcgccagactccagagaagaggctggagtgggtcgcatccattagtagtggtggta
5 gggttcgccagactccagagaagaggctggagtgggtcgcaaccattagtagtggtggta
6 gggttcgccagactccagagaagaggctggagtgggtcgcatccattagtagtggtggta

1 gtggtactatctacaaccagaatttcaagggcaaggccacattgactgtagataagtcct
2 gtggtactagctacaaccagaatttcaaggacaaggccagcttgactgtagataagtcct
3 gtgctactagctacaaccagaatttcaaggataaggccagcttgactgtagataagtcct
4 g...cacctactatccagacagtgtgaagggccgattcaccatctccagagataatgcca
5 gttacacctactatccagacagtgtgaagggccgattcaccatctccagagacaatgcca
6 g...cacctactatccagacagtgtgaagggccgattcaccatctccagagataatgcca

1 ccagcacagcctacatggagctccacagcctgacatctgaggactctgcagtctattact
2 ccagcacagcctacatggagctccacagcctgacatctgaggactctgcagtctattact
3 ccagcacagcctacatggagctccacagcctgacatctgaggactctgcagtctattact
4 ggaacatcctgtacctgcaaatgagcagtctgaggtctgaggacacggccatgtattact
5 ggaacatcctgtacctgcaaatgagcagtctgaagtctgaggacacggccatgtattact
6 ggaacatcctgtacctgcaaatgagcagtctgaggtctgaggacacggccatgtattact

1 gtgc
2 gtgcaaggccctatggtaactacccccccc
3 gtgcaaggccctatggtaactaccccccc
4 gtac
5 gtgcaagag
6 gtgcaagagtat
```

Fig. 5.7 Alignment of the heavy chain sequences of six ScFv clones. Sequences ScFvHc1 - ScFvHc3 given in *italics* are almost identical, discrepencies between them are highlighted in **bold**. Sequence ScFvHc4 - ScFvHc6 given in plain text are almost identical, discrepencies between them are highlighted in **bold**. Limited homology exists between the two sets of heavy chain sequences.

a)ScFvHc1

	Accession Number	Description	% Homology
i	L21020	Mus musculus immunoglobulin heavy chain (IgH) mRNA, VDJ region, partial cds.	94.4
i	M84429	Mouse rearranged heavy chain variable region gene sequence.	93.3
iii	D13201	Mouse gene for innunoglobulin heavy chain variable region.	93
iv	M36218	Mouse Ig heavy chain mRNA V region, partial cds, H220-23VH.	92.8
v	X02554	Mouse mRNA fragment for G2a kappa immunoglobulin A6/24 heavy chain (V-D-J).	92.8

b)ScFvHc6

	Accession Number	Description	% Homology
i	U04228	Mus musculus BALB/c 57-1M Ig germline heavy chain V region, Vh7183 family, partial cds.	99
ii	X59192	Mouse immunoglobulin variable region heavy chain (T5-625) mRNA.	98.6
iii	Z11163	M.musculus non-functional rearranged Ig heavy chain variable region V-BK.	95.6
iv	M92390	M.musculus Ig rearranged gamma chain mRNA, V-D-J region, partial cds.	94.3
v	X53400	M.musculus heavy chain immunoglobulin VH62H3.	94.3

Fig. 5.8 Tables presenting the 5 most homologous sequences identified for

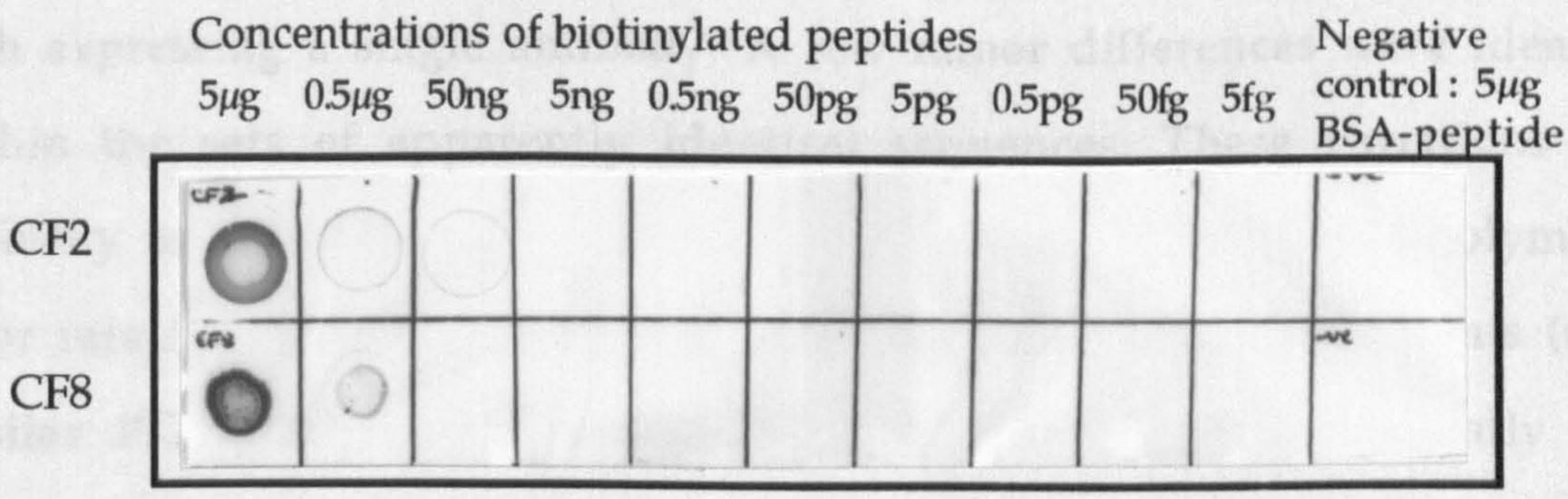
a)ScFvHc1 and b)ScFvHc6 from the EMBL/Genbank data base, release number 39, version 6.

Further information and references for each of the sequences given can be obtained from the EMBL/Genbank data base via the accession numbers.

5.3.4 Selection of ScFvs by Biotin-Capture

Prior to ScFv antibody selection with CF2 and CF8 biotinylated peptides, the ability of streptavidin to bind to serial dilutions of the biotinylated peptides was analysed on a dot blot (see fig.5.9) using standard techniques [Harlow and Lane 1988]. Streptavidin was found to bind to both CF2 and CF8 biotinylated peptides when 50ng or more peptide was present. However, streptavidin did not bind to non-biotinylated CF2 and CF8 peptides even when an excess of 5μg peptide was present. Selection for phage harbouring antibodies that bound to the biotinylated peptides was performed. Parallel control experiments were carried out where the phage were incubated with non-biotinylated peptides. Phage bound to peptide could only be recovered by capture of the biotinylated peptides on the streptavidin coated magnetic beads, i.e. phage bound to non-biotinylated beads could not be recovered. If selection was peptide specific, enrichment of the library could only be continued after incubation with biotinylated peptides. However, after four rounds of selection no significant phage enrichment was obtained with biotinylated peptides relative to the non-biotinylated peptides. ELISA analysis on supernatants from individual colonies, after four rounds of selection with biotinylated peptide, did not indicate any positive clones producing antibodies to CF2 or CF8. The possibility that binding the antigenic peptides to the wells on the ELISA might have masked the peptide epitope to which the ScFv fragment binds was addressed. The surface of the wells were pre-coated with streptavidin before incubating them with biotinylated peptide, thus enabling the peptide epitope to be more fully exposed for antibody binding. However ELISAs of the phage selected by biotin-capture, using such coated plates, were still negative for ScFv binding.

Fig. 5.9 Dot blot analysis of the binding of streptavidin to serial dilutions of biotinylated CF2 and CF8 peptides



5.4 DISCUSSION

Confirmation that the phagemid library derived hybridoma cells did contain ScFv fragments was established by sequencing a selection of library clones. The heavy and light chain sequences obtained all showed strong homology with previously identified murine antibody variable regions (see figs. 5.6 and 5.8). Two distinct sets of sequences were identified for each of these heavy and light chains. This result was anticipated because the library was produced from two clonal populations of hybridoma cells (CF2 and CF8), each expressing a single antibody. A few minor differences were identified within the sets of apparently identical sequences. These variations were probably introduced during the PCR amplification steps. The*Taq* polymerase error rate for PCR amplification of antibody variable gene segments (using similar PCR conditions to those used in this study) has recently been determined to be about 2.6×10^{-5}/bp per cycle [Ford et al., 1994]. As only two heavy and light chain sequences (disregarding minor differences) were obtained from the antibody library clones, it seemed reasonable to assume that approximately half of the heavy and light chain combinations in the constructed ScFvs would be those of the original pairings found within a clonal hybridoma cell.

ScFv antibody fragments have been found to successfully bind antigen by a number of different groups [Bird et al., 1988; Huston et al., 1988; Skerra and Pluckthun, 1988; Chaudhary et al., 1989; Glockshuber et al., 1990; Brietling et al., 1991]. They have also been used as the antigen binding unit in much of the pioneering phage display antibody library research done in Greg Winter's laboratory in Cambridge [McCafferty et al., 1991; Clackson et al., 1991; Griffiths et al., 1993; Marks et al., 1993; Nissim et al., 1994]. In the light of this, it seemed probable that ScFv fragments constructed from mRNA extracted from two clonal populations of hybridoma cells (CF2 and CF8) would bind to the peptides recognised by the monoclonal antibodies produced by the hybridoma cells (MabCF2 and MabCF8).

A ScFv phage expression 'library 'constructed from only two clonal cell lines should not require enrichment by rounds of selection (either by panning or biotin-capture) prior to determination of clones expressing ScFv fragments that recognise peptide antigen by ELISA. However, full enrichment selection was undertaken in order to test and optimise the selection strategies that could subsequently be used with diverse antibody libraries.

The apparent failure of the panning method to enrich for appropriate ScFv fragments could be explained by unsuccessful coating of the petri dish surface with peptide antigen. However, the conditions for coating the peptide onto the plastic surface were found to be satisfactory when the respective anti-peptide monoclonal antibodies were positively tested by ELISA. Another explanation may be that the phage displayed antibodies bound so strongly to the antigen that they were not eluted from it. Results did not improve when more stringent elution conditions were tested. It was predicted from positive ELISA results of the monoclonal antibodies against the peptides that the principal epitope for the ScFv fragments would be displayed and available for antibody binding on the panning dishes. Nevertheless, the epitope could have been more accessible if the antibodies were exposed to the peptides in solution. To facilitate this biotinylated peptides were prepared and the second method of antibody enrichment was undertaken. However, selection by biotin-capture and subsequent ELISA of enriched clones against biotinylated peptide also failed to isolate and identify ScFv fragments that bound to peptide antigen.

Sequence analysis of phagemid DNA prepared from transformed *E.coli.* indicated that the library did contain correctly assembled ScFv fragments. Non existent or only weak binding of the antibody fragments to antigen peptides would explain the apparent failure of enrichment and ELISA techniques. This lack of binding could be caused by either the ScFv sequences being different from those found in the original hybridoma cells or the ScFv structures (formed from correct sequences) not being capable of binding antigen in the same way as the original monoclonal antibodies.

Differences in the ScFv sequences relative to the variable chains found in the original hybridoma cells could be explained by the introduction of *Taq* polymerase errors during the PCR amplification steps. A single base pair change in a region of the sequence encoding for a significant part of the antigen binding recognition site could be sufficient to have detrimental influence on the antigen binding of the expressed ScFv [Ito et al., 1993]. If such a change occurred at the beginning of a set of PCR cycles it would be propagated through all subsequently amplified chains. This problem can be addressed by using DNA polymerase isolated from *Thermococcus litoralis* (Vent DNA polymerase available from New England Biolabs) to reduce the risk of *Taq* polymerase errors. Vent polymerase has 3'-> 5' proofreading activity which enhances the fidelity of replication by the enzyme [Mattila et al., 1991; Kong et al., 1993].

Assuming that at least some of the ScFv sequences are amplified correctly (i.e. without the introduction of *Taq* errors), then the possibility has to be considered that the inherent differences in the structure of ScFv fragments relative to the original monoclonal antibodies may have an adverse effect on antigen binding and possible reasons underlying this are considered in detail in chapter 9 of this study.

In the light of the difficulties encountered with the Pharmacia pCantab5 system in the screening of ScFv fragments derived from well characterised clonal hybridoma cells, it was decided not to pursue the use of the system in the production of antibody phagemid libraries from immunised animal sources. A second system which involved the expression of complete Fab fragments was adopted. This is described in chapters 6, 7 and 8.

CHAPTER 6

PRODUCTION AND SCREENING OF Fab ANTIBODIES FROM HYBRIDOMA CELLS PRODUCING Mabs TO THE CF8 PEPTIDE

6.1 INTRODUCTION

The most complex proteins to be heterologously expressed on the surface of filamentous phage are antibody Fab fragments [Barbas, 1993c]. The cloning of these fragments is the basis of the second system adopted for the production of phage display antibody fragments in this study.

The constant region domains present in Fab antibody fragments facilitate strong association between the heavy and light chains relative to the their smaller Fv counterparts [Nisohoff et al., 1975]. The binding between the heavy and light constant domains is both covalent (with the formation of a disulphide bridge) and non-covalent in nature. The resultant additional stability in the structure overcomes the need for an artificial covalent link between the chains such as the peptide linker in ScFv fragments.

The phagemid vector pComb3H-SS is a modified version of pComb3 which was derived from the phagemid pBluescript [Barbas et al., 1991; Short et al., 1988] (see fig. 6.1). In this system unique restriction sites are provided for the independent cloning of the heavy chain variable domain and first constant domain (Fd) sequences (*Xho* 1 and *Spe* 1) and light chain sequences (*Sac* 1 and *Xba* 1). The SS designation (see fig. 6.1) represents the presence of stuffer fragments in the antibody fragment cloning sites. These sites facilitate the juxtaposition of heavy and light chain sequences with the carboxyl termini of the protein leader peptides which are transported into the periplasm. The light chain is inserted next to the outer membrane *omp* A leader peptide of *E.coli.* [Ghrayeb et al., 1984] and the Fd chain is inserted between a *pel* B leader peptide of *Erwinia caratovora* [Lei et al., 1987] and the C-terminal domain of of the phage coat protein g3p. The g3p portion of the resultant expressed fusion

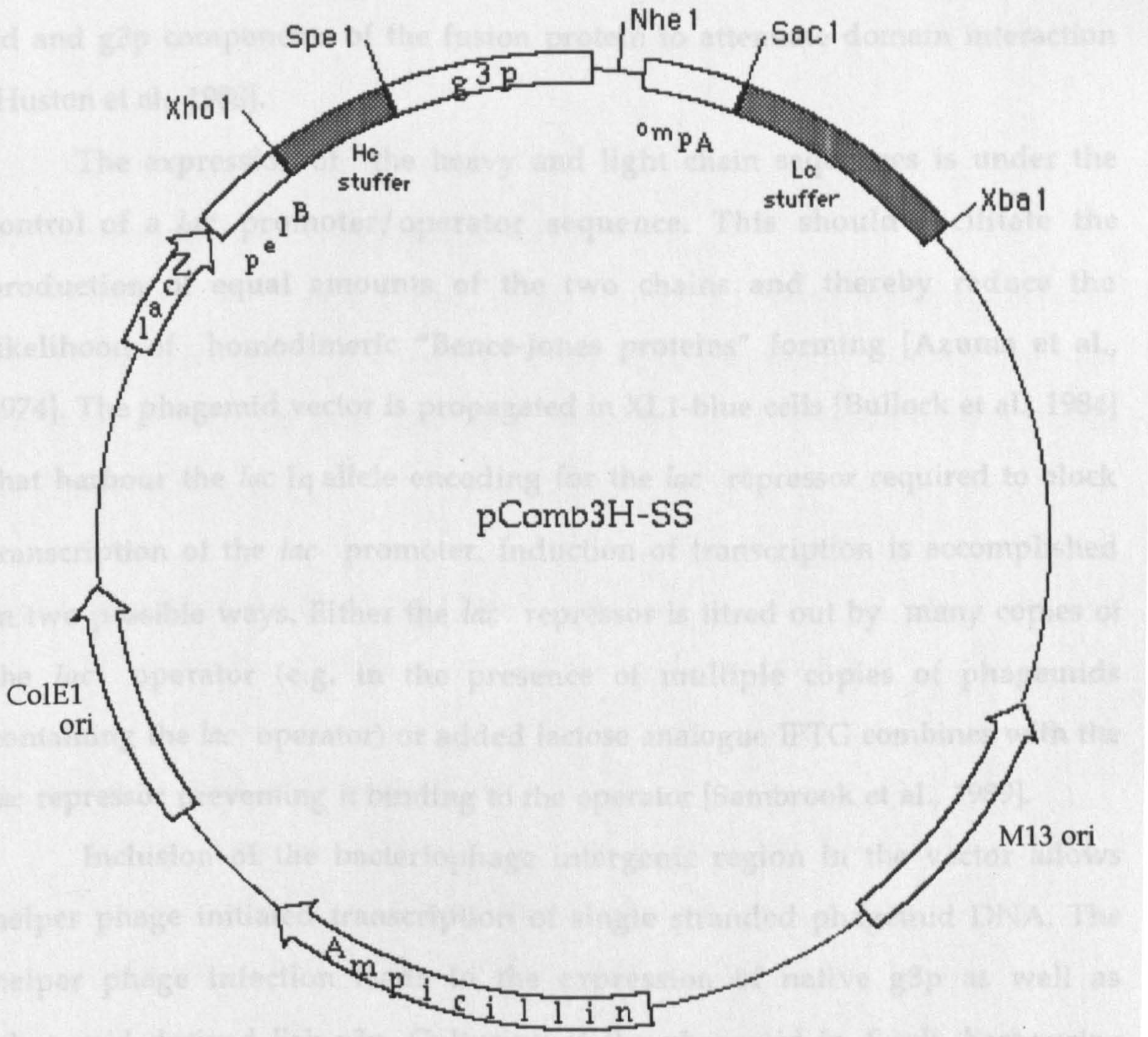


Fig. 6.1 pComb3H-SS phagemid cloning vector

protein accumulates in the inner membrane of the *E.coli.* with the Fd region in the periplasmic space where it can associate with a light chain to form a functional Fab fragment. A 5 amino acid flexible tether is located between the Fd and g3p components of the fusion protein to attenuate domain interaction [Huston et al., 1988].

The expression of the heavy and light chain sequences is under the control of a *lac* promoter/operator sequence. This should facilitate the production of equal amounts of the two chains and thereby reduce the likelihood of homodimeric "Bence-Jones proteins" forming [Azuma et al., 1974]. The phagemid vector is propagated in XL1-blue cells [Bullock et al., 1984] that harbour the *lac* Iq allele encoding for the *lac* repressor required to block transcription of the *lac* promoter. Induction of transcription is accomplished in two possible ways. Either the *lac* repressor is titred out by many copies of the *lac* operator (e.g. in the presence of multiple copies of phagemids containing the *lac* operator) or added lactose analogue IPTG combines with the *lac* repressor preventing it binding to the operator [Sambrook et al., 1989].

Inclusion of the bacteriophage intergenic region in the vector allows helper phage initiated transcription of single stranded phagemid DNA. The helper phage infection leads to the expression of native g3p as well as phagemid derived Fab-g3p. Culturing of the phagemid in *E.coli.* harbouring the *lac* Iq allele limits the fusion protein copy number [Bass et al., 1990]. The resultant packaged phage carries native g3p, which is necessary for infection, and the encoded Fab-g3p, which is displayed for selection. According to published reports , Fab fragments expressed with this system are monomeric in nature, thereby facilitating the isolation of specific, high affinity antibody clones [Bass et al., 1990; Burton et al., 1991; Barbas et al., 1991;1993c].

In this study a series of Fab antibody libraries have been developed. The heavy and light chain sequences for each of these was derived from sources immunised to CFTR protein sequence. The construction of a Fab 'library' from

cloned hybridoma cells producing monoclonal antibodies (MabCF8) to a peptide sequence corrisponding to a region of CFTR, is described in this chapter.

6.2 MATERIALS AND METHODS

6.2.1 Obtaining cDNA from Hybridoma Cells

RNA was obtained and mRNA purified from hybridoma cells producing Mabs to the CF8 peptide. Total RNA was isolated from eight T125 flasks of hybridoma cells and then first strand cDNA synthesised using Pharmacia kits.

6.2.2 Preparation and Cloning of Fab Fragments

The procedures followed are summarised in figure 6.2.

6.2.2.1 Preparation of Immunoglobulin DNA

The polymerase chain reaction was used to amplify heavy and light chain antibody fragments. Mouse immunoglobulin primers were synthesised based on the original published set [Kang et al., 1991b].

Heavy chain Fd 3′ primers:

IGg1 5′- AGGCTTACTAGTACAATCCCTGGGCACAAT - 3′

IGg2a 5′ - GTTCTGACTAGTGGGCACTCTGGGCTC - 3′

Heavy chain variable domain 5′ primers:

Hc1 5, - AGGTCCAGCTGCTCGAGTCTGG -3′

Hc2 5′ - AGGTCCAGCTGCTCGAGTCAGG -3′

Hc3 5′ - AGGTCCAGCTTCTCGAGTCTGG-3′

Hc4 5′ - AGGTCCAGCTTCTCGAGTCAGG-3′

Hc5 5′ - AGGTCCAACTGCTCGAGTCTGG -3′

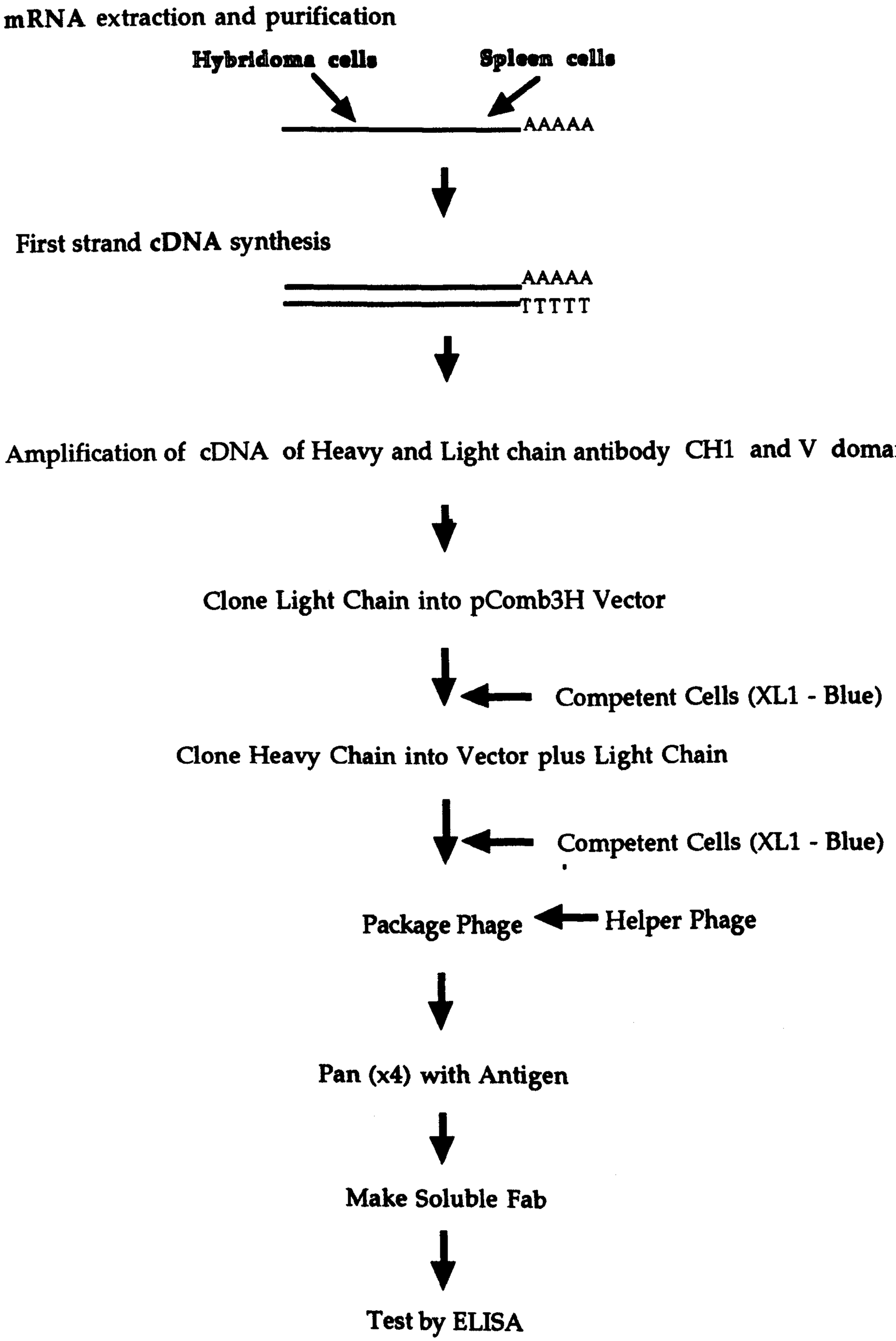


Fig.6.2 Cartoon representing the preparation and cloning of Fab fragments into pComb3H-SS

Hc6 5′ - AGGTCCAACTGCTCGAGTCAGG - 3′

Hc7 5′ - AGGTCCAACTTCTCGAGTCTGG - 3′

Hc8 5′ - AGGTCCAACTTCTCGAGTCAGG - 3′

Hc9 5′ - AGGTIIAICTICTCGAGTC(T/A)GG - 3′

Murine k light chain 3′ primer:

5′ - GCGCCGTCTAGAATTAACACTCATTCCTGTTGAA - 3′

Murine light chain variable domain 5′ primers:

Lc1 5′ - CCAGTTCCGAGCTCGTTGTGACTCAGGAATCT - 3′

Lc2 5′ - CCAGTTCCGAGCTCGTGTTGACGCAGCCGCCC - 3′

Lc3 5′ - CCAGTTCCGAGCTCGTGCTCACCCAGTCTCCA - 3′

Lc4 5′ - CCAGTTCCGAGCTCCAGATGACCCAGTCTCCA - 3′

Lc5 5′ - CCAGATGTGAGCTCGTGATGACCCAGACTCCA - 3′

Lc6 5′ - CCAGATGTGAGCTC TCATGACCCAGTCTCCA - 3′

Lc7 5′ - CCAGTTCCGAGCTCGTGATGACACAGTCTCCA - 3′

Standard techniques were followed using an annealing temperature of 52°C and 35 cycles. Each reaction was carried out with individual primer sets. An aliquot (7%) of each individual PCR reaction was run out on a 1% agarose gel to look for the presence of amplified antibody DNA. The mineral oil was carefully removed from the surface of the PCR reaction mixes and the PCR products with common 3′ primers were pooled, purified by phenol extraction and phenol:chloroform:isoamyl extraction before precipitating. The recovered DNA was run out on a preparative 0.8% Seakem GTG agarose gel. The appropriate (680bp) DNA bands were excised from the gel and extracted using standard electroelution techniques (section 2.2.10 method B). The DNA was quantified and digested with restriction enzymes for 3 hours at 37°C. The light chain was incubated with *Xba* 1 and *Sac* 1 and the heavy chain with *Xho* 1 and *Spe* 1. The number of enzyme units and buffers used are summarised in table

Table 6.1
Equivalent enzyme amounts and buffers for vector and PCR insert digestion (using enzymes and buffers obtained from Boehringer Mannheim)

Enzyme	Buffer	pComb3H-SS or pComb3H-H+L units/μg DNA	PCR inserts units/μg DNA
Spe1	H	3u	17u
Xho1	H	9u	70u
Sac1	A	5u	35u
Xba1	A	9u	70u

6.1. The restricted DNA was precipitated prior to purification for ligation by one of two methods. Either agarose gel electrophoresis followed by electroelution or by passage through a Chromaspin 100 column (section 2.2.8.) prior to ligation.

6.2.2.2 Preparation of Vector DNA

The phagemid vector pComb3H-SS [Barbas et al., 1991] was generously provided by Drs. Burton and Barbas, The Scripps Research Institute, La Jolla, California (see fig. 6.1).

Phagemid DNA was prepared using the Qiagen method (section 2.2.2). The vector DNA was digested with restriction enzymes for 3 hours at 37°C. Initially pComb3H-SS was incubated with *Xba* 1 and *Sac* 1 and, after ligation of the light chain into the vector, restriction with *Xho* 1 and *Spe* 1 was carried out. The number of enzyme units and the buffers used are summarised in table 6.1. The digested DNA was precipitated prior to being run out on a 0.8% Seakem GTG gel. The appropriate DNA band was excised from the gel and the DNA extracted using standard electroelution procedures (section 2.2.10 method B).

6.2.2.3 Ligation of DNA

The insert ligation efficiencies and the extent of background (vector alone) ligation were tested on a small scale prior to to library construction. For the library construction, 1400ng vector DNA was ligated with 450ng of the cut PCR product in the presence of 10μl BRL T4 Ligase and 40μl 5x buffer in a total reaction volume of 200μl. After overnight incubation at room temperature, the ligation reaction was stopped by heating at 65°C for 10 minutes, precipitated and resuspended in 15μl of water. This was microdialysed against water prior to transformation.

6.2.3 M13 Helper Phage Preparation

VCSM13 helper phage was prepared from an initial stock obtained from Stratagene. A series of dilutions of the phage stock were prepared in LB. 1μl of each dilution was added to 100μl cultures of XL1-blue cells O.D.A_{600}=1 which were then transferred to 3ml top agar at 42°C. The top agar was immediately poured onto LB plates which had been prewarmed to 37°C. The plates were incubated at 37°C overnight. The next day a single plaque was picked from a plate that had an appropriate distribution of phage and used to inoculate 10ml of a fresh XL1-blue cell SB culture. This was incubated at 37°C for 2 hours before being transferred to 500ml SB containing 10μg/ml tetracycline and 70μg/ml kanamycin and grown at 37°C overnight. The overnight culture was spun at 2500g for 15 minutes. The supernatant was transferred to a fresh tube, heated at 70°C for 20 minutes and recentrifuged as above. The supernatant was then stored at 4°C.

To calculate the titre of the helper phage preparation, serial dilutions were prepared in LB. 1μl of each dilution was transferred to 100μl of XL1-blue cell culture (O.D.A_{600}=1) which was in turn added to 3ml top agar at 42°C. The top agar was immediately poured onto LB plates prewarmed to 37°C, which were then incubated overnight at 37°C. The number of plaques on each plate were counted the following day and used to calculate the titre of the helper phage preparation.

6.2.4 Transformation of the Recombinant Antibody Libraries

The libraries were transformed into XL1-blue competent cells (section 2.2.13 method B) by electroporation (section 2.2.14). All 15μl of library DNA was transformed into 400μl of electrocompetent cells. Electroporation cuvettes were flushed out with a total volume of 5ml SOC which was then incubated, shaking at 37°C for 1 hour to allow the cells to recover.

6.2.5 Replication and Recovery of the libraries

10ml prewarmed (37°C) SB (20μg/ml carbenicillin (carb) and 10μg/ml tetracyline (tet)) was added to the transformed cells. 10μl, 1μl and 0.1μl of the culture were further diluted into 100μl SB and plated out onto LB (carb) plates. The plates were left to grow in a 37°C oven overnight. The cultures were plated in order to isolate individual library clones and to allow estimation of the size of the antibody library.

The 10ml culture was incubated for a further 1 hour at 37°C, shaking before the carbenicillin concentration was increased to 50μg/ml. After another 1 hour incubation shaking at 37°C, the 10ml culture was transferred to 100ml prewarmed (37°C) SB containing 50μg/ml carb and 100μg/ml tet and left at 37°C in a shaking incubator. For recovery of the light chain library this culture was left overnight. For recovery of the final Fab (heavy and light chain) library, 10^{12} pfu of VCSM13 helper phage was immediately added to the culture which was then incubated at 37°C shaking for 2 hours. 70μg/ml of kanamycin was added before leaving it to grow in a shaking incubator at 37°C overnight.

After construction of the both the light and heavy chain libraries, phagemid DNA was prepared from the overnight culture using the Qiagen method (section 2.2.2).

After construction of the Fab (light and heavy chain) library, the supernatant recovered after pelleting the cells was precipitated with PEG/NaCl on ice for 30 minutes. The precipitate was spun down at 9000rpm in an SS34 rotor at 4°C for 20 minute. The supernatant was discarded and the tubes containing the pellets were allowed to drain on paper towel for about 10 minutes to remove as much PEG solution as possible. The pellets were then resuspended in a total volume of 2ml PBS/1%BSA which was transferred to Eppendorf tubes and spun in a microcentrifuge for 10 minutes to pellet any residual cell debris. The supernatant was recovered to fresh tubes and stored at 4°C. Library phage preparations were always re-amplified prior to use in panning if they had been stored for more than 24 hours. Re-amplification was

achieved following the panning protocol (6.2.6) from the point marked *.

The phage suspension was titred by infecting 50μl volumes of XL1-blue cells (O.D.A_{600}=0.5) with 1μl of 10^{-3}, 10^{-6} and 10^{-8} dilutions of the phage suspension at room temperature for 15 minutes before plating out on LB carb plates and incubating overnight at 37°C.

6.2 6 Panning

Several rounds of panning were used to select for specific binding clones within the library.

Wells of an ELISA plate were coated overnight at 4°C with 1μg CF8-BSA peptide diluted in PBS. The following day the wells were emptied and washed three times with water before filling them with 3% BSA in PBS blocking buffer and incubating them at 37°C for 1 hour in a humidified incubator. The blocking buffer was then replaced with 100μl phage suspension and incubated for a further 2 hours 37°C in the humidified incubator. The phage was then removed and the wells were washed by filling them with PBS/0.5%Tween (PBST), pipetting vigorously up and down and leaving for 5 minutes before removal. In the first round of panning, the wells were washed once in this fashion, in the second round they were washed five times and in the third and subsequent rounds they were washed ten times. After washing the wells, the phage were eluted by adding 50μl of elution buffer (0.1M HCl (adjusted with glycine to pH2.2)/1mg/ml BSA) to each well. This was left at room temperature for 10 minutes prior to pipetting up and down vigorously and transferring to wells each containing 3μl 2M Tris. base neutralising buffer.

*The eluted phage was used to inoculate 2ml (per well) log phase (O.D.A_{600}=1) XL1-blue cells. The cells were then incubated at room temperature for 15 minutes to allow reinfection of the phage, before proceeding with replication as described for the original Fab library in section 6.2.5.

6.2.7 TA cloning of Heavy and Light Chain Sequences

Heavy and light chain variable region sequences were amplified by PCR from the prepared mRNA using the primers supplied by LKB-Pharmacia (as described in section 5.2.2). The PCR products were analysed and quantified by running 10% of each on a 1% agarose gel. The remainder of the PCR products were purified on a Chromaspin 100 column prior to direct ligation into the Promega pGEM-T TA cloning vector.

The linearised TA vector has 3′ terminal thymidine residues at both ends which greatly improves the efficiency of ligation of PCR products into the plasmid. The ligation reactions were set up in a final volume of 10μl with 50ng vector DNA, 10ng antibody insert DNA and T4 DNA ligase and reaction buffer supplied by Promega. The reaction was incubated at 15°C. After 3 hours the ligation mixtures were microdialysed with water (section 2.2.12) and 2μl of each of the heavy and light chain reactions were electroporated into *E.coli*.. (section 2.2.14).

6.2.8 Sequencing Antibody Clones

6.2.8.1 Sequencing pComb3H - Fab Clones

Sequencing was carried out using the Sequenase version 2.0 kit (see section 2.2.15) with the following primers:

Lc 5′- TGGCTGGTTTCGCTACCGTG -3′

Hc 5′- TGTTATTACTCGCTGCCCAA -3′

The primers were designed to sequence from the amino terminal of each (of the variable domains) to enable the comparison between variable domain sequences.

6.2.8.2 Sequencing TA Cloned Antibody Variable Regions Primers

Sequencing of TA cloned heavy and light chain variable regions was carried out with the following primers:

T7 5′ - TAATACGACTCACTATAGGGCGA - 3′ upstream of cloning site

SP6 5′ -ATTTAGGTGACACTATAG- 3′ downstream of cloning site

6.3 RESULTS

6.3.1 Construction of the Fab Library

A Fab antibody library was constructed from the mRNA of hybridoma cells that had been shown to produce monoclonal antibodies which recognised a peptide found within the CFTR sequence (see fig. 4.1). Following reverse transcription of the mRNA, the heavy Fd and light chain antibody sequences were amplified by PCR. Reactions with different pairs of 5′ and 3′ heavy or light chain primers were carried out separately to maximise the amplification of all appropriate mRNA sequences.

Initial test ligations performed prior to the construction of the light chain antibody library revealed that the background vector ligation was about 20% that of the test insert ligation, slightly higher than desired. The method for the purification of the restricted antibody chain sequences was changed from agarose gel electrophoresis and electroelution to passage through a Chromaspin column. This resulted in a reduction in the light chain background ligation to less than 1%, sufficiently low to proceed with the construction of the light chain library. The size of the light chain library was estimated to be 1.2×10^7 clones.

The restricted heavy chain sequences which had been purified on a Chromaspin column, were subsequently ligated into the prepared vector (containing the light chain sequences). Test ligations for the heavy chain insertion indicated a background of 8% and the estimated size of the heavy chain library was 1×10^6 clones. Phagemid DNA prepared from eight of the library clones, restricted with *Xba* 1 and *Sac* 1 and with *Xho* 1 and *Spe* 1 and run on an agarose gel, revealed that the majority of transformants contained both heavy and light chain sequences (see fig. 6.3).

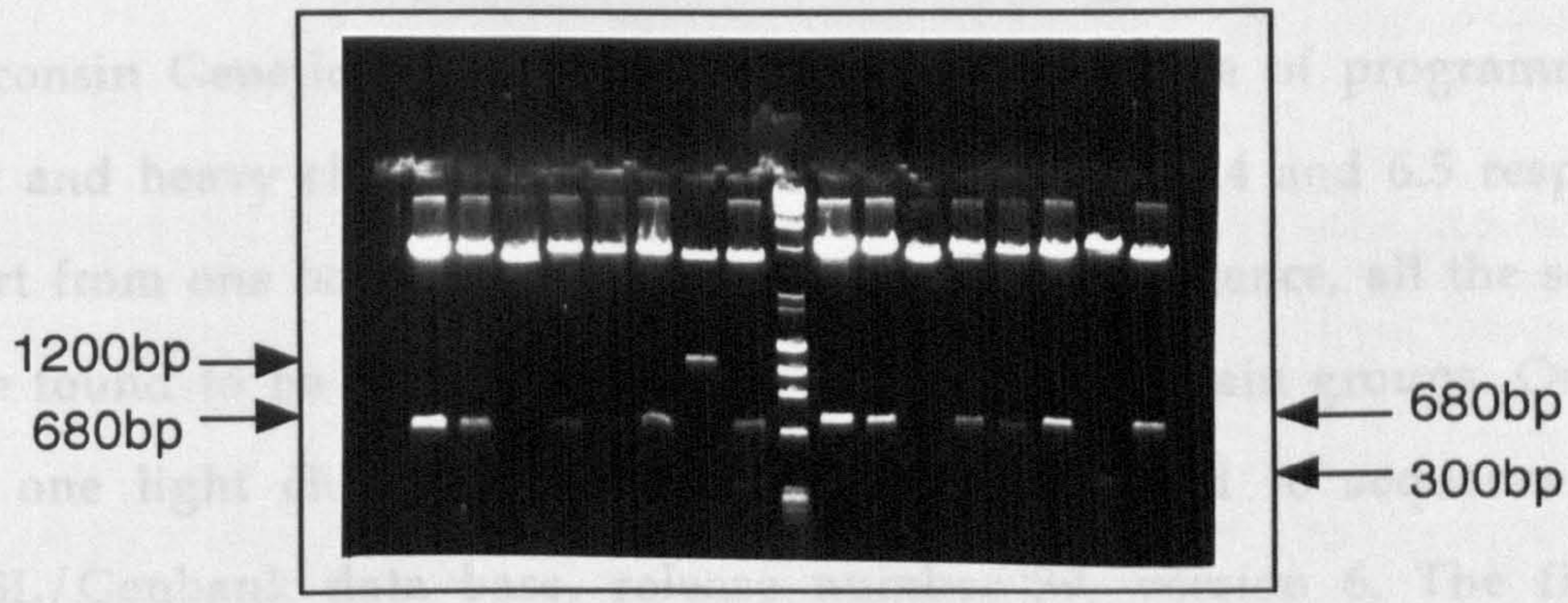


Fig. 6.3 Agarose gel analysis of restriction enzyme digests of mini prep DNA obtained from CF8 Fab library clones.

Lanes A - H: Clones 1-8 digested with *Xba* 1 and *Sac* 1 to cut out light chain inserts

Lane I: Lambda DNA-*Hind* III / Fx-174 DNA-*Hae* III digest markers

Lanes J - Q: Clones 1-8 digested with *Xho* 1 and *Spe* 1 to cut out heavy chain inserts

6.3.2 Sequencing Analysis of Antibody Fragments

6.3.2.1 Sequencing Fab Clones

Phagemid DNA was prepared from four library colonies by Wizard Mini Prep and sequenced using the 2 sequencing primers described above (section 6.2.8.1). The DNA sequences were collated and aligned using the University of Wisconsin Genetics Computer Group (UWGCG) suite of programmes. The light and heavy chain sequences are given in figs. 6.4 and 6.5 respectively. Apart from one base difference in a heavy chain sequence, all the sequences were found to be identical within their respective chain groups. One heavy and one light chain sequence were each compared to sequences in the EMBL/Genbank data base, release number 39, version 6. The five most homologous sequences were identified for each and are given in fig. 6.6a and b.

6.3.2.2 Comparison to ScFv Sequences

Cells from two different hybridoma lines producing monoclonal antibodies that recognise either CF8 and CF2 peptide sequences were the source of mRNA used in the construction of the ScFv antibody library described in the previous chapter. Hybridoma cells producing monoclonal antibodies that recognise CF8 peptide were the source of mRNA used in the construction of the Fab library described above. Both libraries contain the variable domains of heavy and light chain antibody sequences. It was therefore anticipated that the heavy and light chain sequences amplified with the PCR primers given in section 6.2.2.1 would have identical regions with sequences amplified with the PCR primers provided by Pharmacia (see section 5.2.2.). When the sequences were compared using the UWGCG suite of programmes this was not found to be the case. Two sets of sequences were identified for each of the heavy and light chain variable domains in the ScFv library (see section 5.3.3). Although the Fab heavy and light chain variable region sequences were not identical to any of the ScFv sequences they did show a far greater degree of homology to one of each of the respective heavy and light chain sequence sets (see figs. 6.7

```
Lc1          cccagtctccagccaccctgtctgtgactccaggagatagagtctctctt
Lc2                      ccaccctgtctgtgactccaggagatagagtctctctt
Lc3                      ccaccctgtctgtgactccaggagatagagtctctctt
Lc4          cccagtctccagccaccctgtctgtgactccaggagatagagtctctctt
```

```
1 tcctgcagggccagccagagtattagcgactacttacactggtatcaacaaaaatcacat
2 tcctgcagggccagccagagtattagcgactacttacactggtatcaacaaaaatcacat
3 tcctgcagggccagccagagtattagcgactacttacactggtatcaacaaaaatcacat
4 tcctgcagggccagccagagtattagcgactacttacactggtatcaacaaaaatcacat
```

```
1 gagtctccaaggcttctcatcaaatatgcttcccaatccatctctgggatcccctccagg
2 gagtctccaaggcttctcatcaaatatgcttcccaatccatctctgggatcccctccagg
3 gagtctccaaggcttctcatcaaatatgcttcccaatccatctctgggatcccctccagg
4 gagtctccaaggcttctcatcaaatatgcttcccaatccatctctgggatcccctccagg
```

```
1 ttcagtggcagtggatcagggtcagatttcactctcagtatcaacagtgtggaacctgaa
2 ttcagtggcagtggatcagggtcagatttcactctcagtatcaacagtgtggaacctgaa
3 ttcagtggcagtggatcagggtcagatttcactctcagtatcaacagtgtggaacctgaa
4 ttcagtggcagtggatcagggtcagatttcactctcagtatcaacagtgtggaacctgaa
```

```
1 gatgttggagtgtattactgtcaaaatggtcacagctttccgtacacgttcggagggg
2 gatgttggagtgtattactgtcaaaatggtcacagcttt
3 gatgttggagtgtattactgtcaaaatggtcacagctttccgtacacg
4 gatgttggagtgtattactgtcaaaatggtcacagctttccgtacacgttcggagggg
```

Fig.6.4 Alignment of the light chain sequences of four Fab clones.

No differences were identified between the sequences.

```
Hc1                                  gaagcctggggcttcagtgaagctgtcctgcaaggcttc
Hc2                                  gaagcctggggcttcagtgaagctgtcctgcaaggcttc
Hc3                gctgaactggtgaagcctggggcttcagtgaagctgtcctgcaaggcttc
Hc4                gctgaactggtgaagcctggggcttcagtcaagctgtcctgcaaggcttc
```

```
1 tggctacaccttcaccaactactggatgcactgggtgaagcagaggcctggacaaggctt
2 tggctacaccttcaccaactactggatgcactgggtgaagcagaggcctggacaaggctt
3 tggctacaccttcaccaactactggatgcactgggtgaagcagaggcctggacaaggctt
4 tggctacaccttcaccaactactggatgcactgggtgaagcagaggcctggacaaggctt
```

```
1 gagtggattggagagattaatcctagcaacggtcgtactaactacaatgagaagttcaag
2 gagtggattggagagattaatcctagcaacggtcgtattaactacaatgagaagttcaag
3 gagtggattggagagattaatcctagcaacggtcgtactaactacaatgagaagttcaag
4 gagtggattggagagattaatcctagcaacggtcgtactaactacaatgagaagttcaag
```

```
1 agcaaggccacactgactgtagacaaatcctccagcacagcctacat
2 agcaaggccacactgactgtagacaaatcctccagcacagcctacatgcaactcagcagc
3 agcaaggccacactgactgtagacaaatcctccagcacagcctacatgcaactcagcagc
4 agcaaggccacactgactgtagacaaatcctcca
```

```
1
2 ctgacatctgaggactc
3 ctgacatctgaggactctgcggtctattacgtgtcaagatccttctatgctta
4
```

Fig.6.5 Alignment of the heavy chain sequences of four Fab clones. Only one base difference was identified, this is highlighted in bold type.

a) CF8 Lc1 Light Chain Sequence Comparison

	Accession Number	Description	% Homology
i	M26003	Mouse Ig rearranged kappa-chain Vk 23.32 gene, partial cds, from Abelson virus transformed B cells	98.5
i	X65097	M.musculus mRNA for IG light chain VJ region (M-T408).	95.1
iii	M35667	Mouse lysozyme binding Ig kappa chain (HyHEL-10) V23-J2 region mRNA, partial cds.	91.3
iv	M19913	Mouse Ig rearranged kappa-chain mRNA, clone AN11k.	91
v	X59211	Mouse immunoglobulin variable region light chain (T9-1) mRNA.	90

b) CF8 Hc2 Heavy Chain Sequence Comparison

	Accession Number	Description	% Homology
i	M21814	Mouse/human Ig heavy chain constant region mRNA, segment 1.	95.4
i	S50856	Ig VH=chimeric anti-human neuroblastoma antibody (mice, Genomic, 706nt).	94.5
iii	U07204	Mus musculus clone 8 anti-C5a Ig heavy chain V region mRNA, partial cds.	93.2
iv	J00532	Mouse Ig germline H-chain, c57b1/6 b-np-related gene vh186-1, v-ii.	92.8
v	Z12781	M.musculus mRNA for VH-gene seq. of naturally occuring, somatically mutated memory B cell.	92.8

Fig 6.6 Tables presenting the 5 most homologous sequences identified for
a)Fab Lc1 clone and b)Fab Hc2 clone
from the EMBL/Genbank data base, release number 39, version 6.
Further information and references for each of the sequences given can be obtained from the EMBL/Genbank data base via the accession numbers.

and 6.8). The ScFv sequences with the greater homology to the Fab sequences were most probably those derived from MabCF8 producing cells rather than MabCF2 producing cells. Although a reasonable homology was found between the sequences, the differences between them were considered to be too great to be only due to *Taq* Polymerase, PCR errors. A likely reason for the variations was that the mRNA for the production of the two antibody libraries had been prepared from different clonal populations of MabCF8 producing hybridoma cells. This explanation was investigated.

6.3.2.3 TA Cloning of ScFv Heavy and Light Chain Sequences

The sequence differences described above could be explained if the mRNA for the production of the two antibody libraries had been prepared from different clonal populations of MabCF8 producing hybridoma cells. To test this theory, the cDNA used in the construction of the Fab library was amplified using the Pharmacia PCR amplification primers (section 5.2.2.). The PCR products were ligated directly into the Promega TA cloning vector (see section 6.2.7). DNA, derived from colonies of *E.coli.* that had been transformed with the TA vector, was sequenced using the primers described in section 6.2.8.2. When aligned to the respective Fab heavy or light chain sequences they were found to be identical (see figs. 6.7 and 6.8).

6.3.3 Panning of the Fab Library

Enrichment of the antibody library for Fab fragments which bound to peptide antigen was attempted by consecutive rounds of selection by panning. After each round of panning, eluted phage were reinfected into *E.coli.* and rescued with helper phage. Unfortunately, effective panning was prevented by the occurrence of considerable cell lysis in the reinfected and rescued phage cultures. This problem has been encountered by two groups responsible for pioneering research in phage antibody display technology (Andrew Griffiths (MRC, Cambridge) pers. comm. and Anthony Williamson (Scripps Institute,

```
1)Lc1       cccagtctccagccaccctgtctgtgactccaggagatagagtctctctttt
2)TA             tctccagccaccctgtctgtgactccaggagatagagtctctctttt
3)ScFvLc6     agtctccagccaccctgtctgtgactccaggagatagagtcagtctttt
4)ScFvLc1           tcctccctgtctgcctctctgggagcagagtcrccatcagt

1 cctgcagggccagccagagtattagcgactacttacactggtatcaacaaaaatcacatg
2 cctgcagggccagccagagtattagcgactagttacaccggtatcaacaaaaatcacatg
3 cctgcagggccagtcaaagtattagcaactacctacactggtatcaacaaaaatcacatg
4 tgcagggcaagtcaggacattagcaattatttaaactggtatcagcagaaaccagatgga

1 agtctccaaggcttctcatcaaatatgcttcccaatccatctctgggatcccctccaggt
2 agtctccaaggcttctcatcaaatatgcttcccaatccatctctgggatcccctccaggt
3 agtctccaaggcttctcatcaagtatgcttcccagtccatctctgggatcccctccaggt
4 actgttaaactcctgatctactacacatcaagattccactcaggagtcccatcaaggttc

1 tcagtggcagtggatcagggtcagatttcactctcagtatcaacagtgtggaacctgaag
2 tcagtggcagtggatcagggtcagatttcactctcagtatcaacagtgtggaacctgaag
3 tcagtggcagtggatcagggatagatttcactctcagtatcaacagtgtggagactgaag
4 agtggcagtgggtctggaacagattattctctcaccattagtaacctggagcaagaagat

1 atgttggagtgtattactgtcaaaatggtcacagctttccgtacacgttcggagggg
2 atattggagtgtattactgtcaaaatggtcacagctttccgtacacgttcggagggg
3 at.ttggaatgtatttctgtcaatagagtaacagctggcctctcacgttcggtcctggga
4 attgccacttacttttgccaacagggtaatacgcttccacgtggacgttcggaggaggca

1
2
3 caaagtggaaataaaacg
4 ccaagtatggaaa
```

Fig. 6.7 Alignment of light chain sequences of ScFv and Fab clones.

1) Fab fragment Lc1 sequence (see fig. 6.4)

2) TA cloned light chain variable domain sequence

3) ScFv light chain sequence 6 (see fig. 5.5)

4) ScFv light chain sequence 1 (see fig. 5.5)

Sequences 1 and 2 were derived from mRNA from one clonal hybridoma cell line producing monoclonal antibodies to the CF8 peptide. Sequences 3 and 4 were derived from mRNA from two clonal hybridoma cell lines producing monoclonal antibodies to the CF2 and CF8 peptides. 1, 2 and 3 are assumed to encode anti CF8 light chain sequences, variations between them are highlighted in bold type. The considerable differences in ScFvHc1 relative to FabLc1 and TA cloned sequences are probably due to different clonal derivation of the mRNA. Sequence 4 represented in *italic* is assumed to encode anti CF2 heavy chain.

```
1)Hc3                                              gctgaactggtgaagcctggggcttca
2)TA                 aggtgcaactgcaggagtctggggctgaactggtgaagcctggggcttca
3)ScFvH1             aggtccagctgcaggagtcaggacctgagctggtgaagcctggggcttca
4)ScFvH6             aggtgcaactgcagcagtcaggggaggcttagtgaagcctggagggtcc
```

```
1 gtgaagctgtcctgcaaggcttctggctacaccttcaccaactactggatgcactgggtga
2 gtcaagctgtcctgcaaggcttctggctacaccttcaccaactactggatgcactgggtga
3 gtgaagataccctgcaaggcttctggatacacattcactgactacaacatgcactgggtga
4 ctgaaactctcctgtgcagcctctggattctctttcagtagctatgccatgtcttgggttc
```

```
1 agcagaggcctggacaaggc.ttgagtggattggagagattaatcctagcaacggtcgtac
2 agcagaggcctggacaaggc.ttgagtggattggagagattaatcctagcaacggtcgtac
3 agcagagccatggaaagagccttgagtggattggagatattaatcctaacaatggtggtac
4 gccagactccagagaagaggctggagtgggtcgcatccattagtagtggtggtag...cac
```

```
1 taactacaatgagaagttcaagagcaaggccacactgactgtagacaaatcctccagctct
2 taactacaatgagaagttcaagagcaaggccacactgactgtagacaaatcctcc
3 tatctacaaccagaagttcaagggcaaggccacattgactgtagataagtcctccagcaca
4 ctactatccagacagtgtgaagggccgattcaccatctccagagataatgccaggaacatc
```

```
1 gcctacatgcaactcagcagcctgacatctgaggactctgcggtctattacgtgcaagatc

3 gcctacatggagctccacagcctgacatctgaggactctgcagtctattactgtgtgc
4 ctgtacctgcaaatgagcagtctgaggtctgaggacacggccatgtattactgtgcaagag
```

```
1 cttctatgctta

4 tat
```

Fig. 6.8 Alignment of heavy chain sequences of ScFv and Fab clones.

1) Fab fragment Hc3 sequence (see fig. 6.5)

2) TA cloned heavy chain variable domain sequence

3) ScFv heavy chain sequence 1 (see fig. 5.7)

4) ScFv heavy chain sequence 6 (see fig. 5.7)

Sequences 1 and 2 were derived from mRNA from one clonal hybridoma cell line producing monoclonal antibodies to the CF8 peptide. Sequences 3 and 4 were derived from mRNA from two clonal hybridoma cell lines producing monoclonal antibodies to the CF2 and CF8 peptides. 1, 2 and 3 are assumed to encode anti CF8 heavy chain sequences, variations between them are highlighted in bold type. The considerable differences in ScFvHc1 relative to FabHc3 and TA cloned sequences are probably due to different clonal derivation of the mRNA. Sequence 4 represented in *italic* is assumed to encode anti CF2 heavy chain.

La Jolla) pers. comm.) The phage used in the construction of antibody libraries do not routinely lyse the cells that they have infected. The extensive lysis of the cell culture was most likely to be due to either the presence of residual detergent on the glassware used or contamination of the culture with a lytic phage. Both these possibilities were addressed. Any glassware used in the culture of *E.coli.* harbouring the antibody phage library was thoroughly rinsed several times and filled with distilled water prior to sterilisation by autoclaving. The water should have assisted removal of any residual detergent during the autoclaving process. Attempts were made to avoid contamination of the cell cultures with a lytic phage. All solutions used in the panning process were freshly prepared and sterilised by autoclaving. Fresh competent cells were prepared for the initial transformation of the Fab antibody library and for reinfection of subsequently panned phage. Fresh helper phage were also prepared. Despite these preventative measures, the lysis of the cultures persisted.

6.3.4 ELISA Analysis of Phage Cultures

The population of phage recovered from *E.coli.* transformed with the Fab library was precipitated and then tested for binding to BSA conjugated CF8 and CF7 peptides by ELISA as described in the previous chapter (section 5.2.6). The CF7 peptide was included as a negative control to determine whether phage binding was specific to CF8 peptide. The colormetric readings of the developed ELISA plate did not indicate a significant difference in phage antigen binding to the two peptides.

6.4 DISCUSSION

Confirmation that the constructed Fab 'library' contained heavy and light chain antibody sequences was established by sequencing library clones. The sequences obtained showed strong homology to previously identified murine antibody sequences (see figs. 6.6a and b). Barring minor differences, the heavy chain sequences and the light chain sequences were found to be identical. This was anticipated because the 'library' had been constructed from mRNA derived from a clonal hybridoma cell line. Minor differences in sequence may be introduced as a result of *Taq* polymerase errors during the PCR amplification of the antibody sequences [Ford et al., 1994].

Comparisons were made between sequences found in ScFv and Fab antibody libraries, both of which had been prepared from MabCF8 producing hybridoma cells. Significant heavy and light chain sequence variations were found between the two libraries. This raised the possibility that the different primer sets used in the construction of the two libraries may preferentially amplify different sequences. This was unlikely to be the case because the number of heavy chain and light chain sequences available for amplification was limited to two for ScFv library construction and one for the Fab library construction, according to the number of clonal cell lines used. To clarify the situation, mRNA used in the construction of the Fab library was amplified with the PCR primers used for the construction of the ScFv library, cloned and sequenced. Sequence comparisons revealed that the hybridoma cells producing MabCF8 used in the construction of both the ScFv and Fab antibody libraries must have been derived from two different clonal populations (figs. 6.7 and 6.8).

Selection of antigen binding Fab fragments was attempted by panning. Unfortunately, progress in these enrichment steps was limited by the repeated lysis of reinfected phage cultures. Since the use of thoroughly rinsed, detergent-free glassware did not prevent cell lysis the most likely explanation for this problem was contamination of the culture with a lytic phage. This

contamination problem persisted despite the use of freshly prepared and sterilised materials. A way of overcoming this difficulty is addressed in chapter 8.

The Fab phage expression 'library' was constructed from a clonal cell line and from sequence analysis appeared to consist of only one heavy chain and one light chain sequence. Each Fab fragment should have been virtually identical and enrichment of such a library for antigen binding by panning a superfluous process. However, it was undertaken in order to assess and characterise the selection strategies that could be used in the analysis of a more diverse antibody library.

It is a well established phenomenom, that Fab antibody fragments bind antigen in a similar manner to the complete antibody molecule. It was anticipated that Fab fragments derived from a hybridoma cell line and expressed on the surface of phage would display the same specificity of antigen recognition as the monoclonal antibodies produced by the hybridoma cells. ELISA analysis had been used to establish that MabCF8 specifically bound to BSA conjugated CF8 peptide and not CF7 peptide. However the phage display Fab fragments did not appear to display the same discriminatory antigen binding. Three possible explanations for this lack of specificity are outlined below:

i) The cloned Fab sequence may have been slightly different to that found in the original hybridoma cells due to the introduction of *Taq* Polymerase errors during the PCR amplification step (see section 5.4).

ii) Fab dimers may be necessary to enable sufficient avidity for antigen binding [Crowthers and Metzger, 1972; Pack and Pluckthun, 1992; Pack et al., 1993]. The pComb3H vector is constructed in such a way as to permit on average only one Fab molecule per phage particle [Barbas et al., 1991] which may not exhibit sufficient antigen binding affinity to hold phage to the solid phase peptide [Clackson et al., 1991].

iii) The phage antibody specificity could have been masked by the presence

of Fab fragments ("stickies") that exhibit non specific binding to the solid phase antigen (Greg Winter (MRC, Cambridge) pers. comm.).

The first two of the problems described above may be overcome when more diverse Fab antibody libraries are panned and screened. When a specific antibody sequence needs to be maintained to ensure antigen binding, then the fidelity of the PCR amplification is likely to be critical. However, if a library is constructed from the random combination of a varied repertoire of heavy and light chain sequences, the introduction of *Taq* polymerase errors during PCR should have a less deleterious effect. Indeed, mutations introduced into the antibody sequences may enhance the antigen affinity of the assembled Fab fragments [Panka et al., 1988; Marks et al., 1991b; Barbas et al., 1992b].

The dimeric nature of antigen binding sites on antibodies increases the potential avidity with which the antibody can bind to antigen [Crothers and Metzger, 1972; Carter et al., 1992]. However, the presence of two antigen binding sites is not essential for antigen binding [Barbas, 1993c]. When selecting from a large diverse antibody library derived from an immunised source against the antigen to which the library has been raised, it is reasonable to anticipate that some monomeric antigen binding Fab fragments will be identified [Burton, 1991; Winter et al., 1994]. The following two chapters describe the construction of such libraries.

CHAPTER 7

PRODUCTION AND ANALYSIS OF Fab ANTIBODY LIBRARIES FROM MICE IMMUNISED WITH CELLS EXPRESSING CFTR

7.1 INTRODUCTION

CFTR has not yet been isolated and purified and is therefore not available for immunisation. One way of generating immunogen is to express regions of CFTR as fusion proteins (see Chapters 3 and 8). An alternative strategy, described in this chapter, is to use whole cells or membrane preparations from cells that express CFTR.

Cell lines BPV and 2wt were selected for use in the immunisation of mice. These cell lines were produced by transfection of C127 a (non-transformed) clonal cell line derived from the mammary tumour of an RIII mouse [Lowry et al., 1978]. 2wt cells were generated by the transfection of C127 cells with the eukaryotic expression vector CL3AXBPVXT-NEO [Reddy et al., 1987] into which the CFTR cDNA sequence had been cloned [Marshall et al., 1994]. BPV cells were derived from transfection of C127 cells with CL3AXBPVXT-NEO vector without a CFTR insert. Expression of sequences cloned into the CL3AXBPVXT-NEO plasmid is controlled by the mouse metallothionein I promoter. Other components of the vector include the bovine papilloma virus genome, which facilitates episomal maintenance of the plasmid, and the neomycin phosphotransferase gene, which confers resistance to the aminoglycoside antibiotic G418. The cell lines were generously supplied by Alan Smith (Genzyme).

CFTR is one of very many potential antigens expressed on the surface of the transfected cells. Strategies were pursued to maximise specific immunisation to CFTR, see fig. 7.1.

RIII mice were chosen for direct immunisation with the 2wt cell line which expresses CFTR. The cell line has been derived from the same mouse

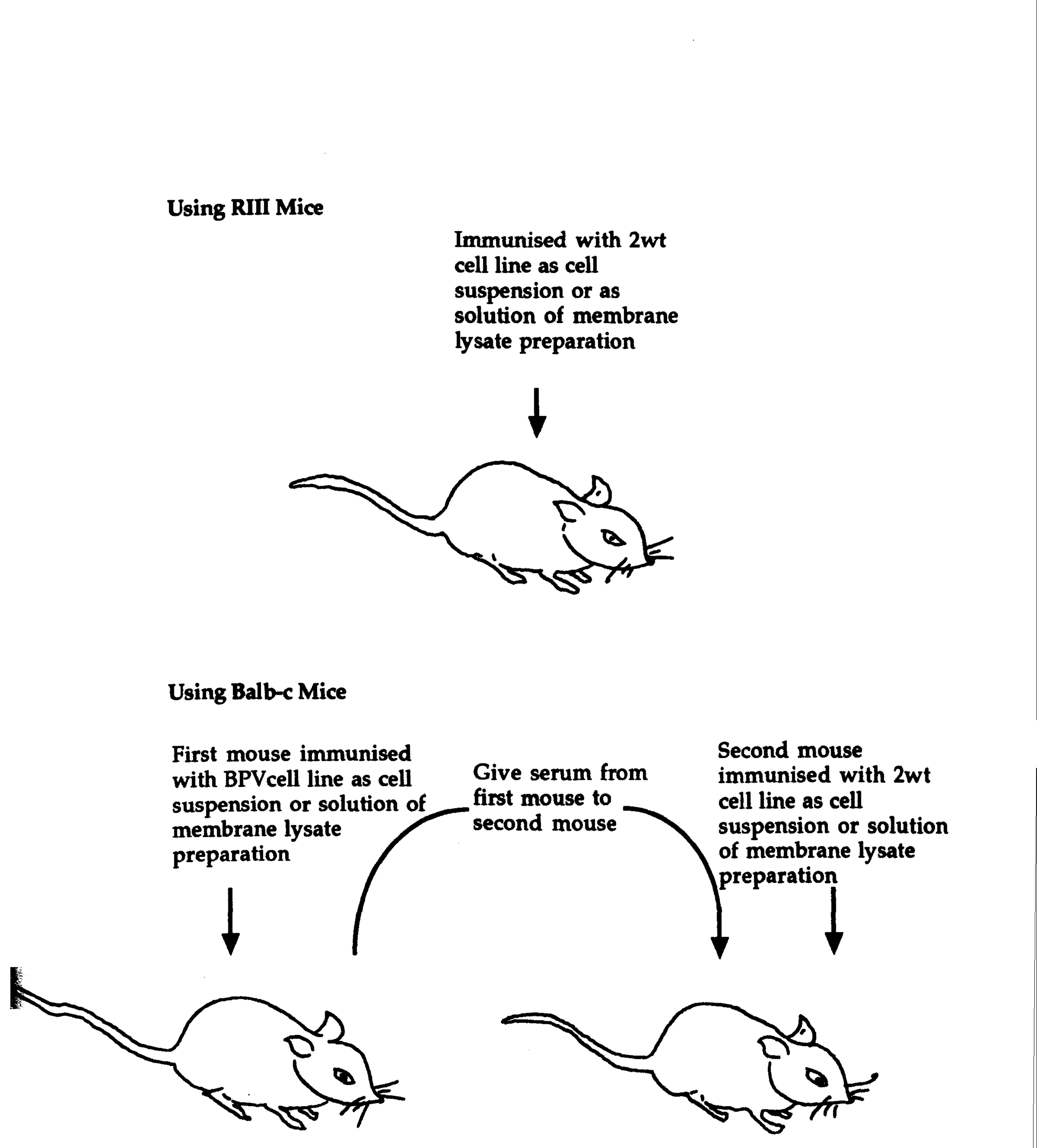


Fig. 7.1 Strategies for the immunisation of RIII and Balb-c mice.

strain, therefore its antigenicity should predominately be due to expressed products of the DNA with which it has been transfected, principally CFTR. A suspension of cells was given to one set of mice. However, because it was not known how long these cells would remain intact in the immunised mice, a second set of mice were given a solution of a membrane lysate preparation from the cells. This ensured that at least the second set of mice were exposed to all epitopes of CFTR that are internal and external to the cell membrane.

An alternative strategy was followed with Balb-c mice where the cell line was not syngeneic with the mouse strain and would therefore present many foreign epitopes to the murine immune system. To overcome this problem, a primary set of mice were immunised with cell suspension or a membrane lysate preparation from the BPV cell line. After three injections, serum from the primary set of mice was transferred to a secondary set of mice simultaneously with whole cells or a cell lysate preparation from the 2wt cell line which differs from the BPV cell line only in the fact that the CL3AXBPVXT-NEO vector used to tranfect the original C127 cells did contain a CFTR insert. Antibodies in the serum from the primary mice should mask all the antigenic epitopes in the 2wt cells except those presented by CFTR. Therefore the immune response raised in the secondary mice should be predominately to CFTR.

The pComb3H-SS vector system (see Chapter 6)was adopted for the construction of Fab fragment phage display libraries from mRNA derived from the spleens of the immunised mice.

7.2 MATERIALS AND METHODS

7.2.1 RTPCR

RTPCR was carried out on mRNA isolated from 2wt and BPV tissue culture cells, using the CF7, CF8, and CFX primers, as described in section 4.2.2. PCR was also carried out using the following pair of oligonucleotide primers:

Actin 31 5'- TGGAGAAGAGCTATGAGCTG -3'

Actin 32 5'- GGGTACATGGTGGTACCACC -3'

Actin 31 and 32 prime across exons of mouse beta-actin sequence to generate a fragment of 212bp from beta-actin cDNA [Alouso et al., 1986].

7.2.2 Immunisation Protocols

The mice were immunised with either suspensions of whole cells or solutions of membrane lysates prepared from the cells. Three mice were used for each immunisation scheme since, even in genetically identical animals, a single antigen preparation will elicit a different antibody response [Harlow and Lane, 1988] The immunisation strategies used in this study are summarised in fig. 7.1.

7.2.2.1 Immunisation with Whole Cell Suspensions

Transfected cells that had grown to confluency in T75 tissue culture flasks were removed from the flasks using Hank's Balanced Salt Solution (HBSS) containing 1mM EDTA and 5mM HEPES. The culture media was removed from the flask and replaced with 10ml supplemented HBSS. The flask was left for 30 minutes at room temperature on a mechanical rocker by which time the cells had come off the surface of the flask. The cells were recovered from the solution by centrifugation in a Sorvall RC3B at 1000rpm and 4°C for 5 minutes. The cells were washed several times in HBSS supplemented solution. For each wash, pelleted cells were gently resuspended in the solution and incubated at room temperature on a mechanical roller for 15 minutes prior to centrifugation as above. The final cell pellet was

resuspended in 0.4ml PBS and used for intra-peritoneal injection of the mice. One confluent T75 flask of cells was sufficient for the immunisation of 3 mice.

7.2.2.2 Immunisation with Membrane Lysate Preparations

Membrane lysate was prepared from confluent T75 flasks of transfected cells as described in section 2.2.18. The recovered membrane pellet was resuspended in 1% Mega-10 in PBS (200μl per T75 flask used). The solution was repeatedly passed through a 21 gauge needle attached to a 1ml syringe to ensure thorough resuspension of the pellet. The membrane lysate preparation was injected intra-venously into mice. Membrane lysate prepared from one confluent T75 flask of cells was sufficient for the immunisation of 2 mice.

7.2.2.3 Immunisation of RIII Mice

Three RIII mice were immunised with 2wt whole cell suspension and three with membrane lysate prepared from 2wt cells. Each animal was given four injections of the appropriate antigen at 2 weekly intervals. Ten days after the final injection, the mice were bled out and their spleens were removed for mRNA preparation. The antisera obtained from each set of mice were pooled and tested for 2wt cell specific binding by flow cytometry.

7.2.2.4 Immunisation of Balb-c Mice

Immunisation of Balb-c mice was a two stage process. In the first stage, three mice were immunised with BPV whole cell suspension and three mice with membrane lysate prepared from BPV cells. After three injections at fortnightly intervals, a test bleed was taken from each mouse and tested by flow cytometry. The second stage of immunisation protocol was initiated when a greater antibody response to BPV cells was detected relative to that found with pre-immunisation antiserum.

The Balb-c mice from the first stage of the immunisation protocol were bled out and the antisera obtained from each set of three mice was pooled. In

the second stage of the immunisation protocol, three mice were immunised with 2wt whole cell suspension and at the same time were injected intra-peritoneally with 0.15ml antisera derived from the mice immunised with BPV cells. Likewise, three other mice were immunised with membrane lysate prepared from 2wt cells and at the same time were given intra-peritoneal injections of 0.2ml antisera derived from the mice immunised with BPV membrane lysate. This dual injection procedure was repeated three more times at fortnightly intervals. Ten days after the fourth set of injections the mice were bled out and their spleens removed for mRNA preparation. The antisera from each set of mice were pooled and tested for 2wt cell specific binding by flow cytometry.

7.2.3 Flow Cytometry

Cell suspensions for flow cytometry were prepared in the same way as for the whole cell immunisation protocol described above (section 7.2.2). After several washes in EDTA and HEPES supplemented HBSS solution, they were pre-incubated for 30 minutes on ice in PBS/0.2%BSA/0.02% sodium azide followed by incubation for an hour with 1:50 dilution of mouse antisera. After three washes in PBS/BSA/azide the cells were incubated for 45 minutes on ice with FITC conjugated goat anti-mouse IgG diluted 1:500 in PBS/BSA/azide. Immediately prior to flow cytometry, cells were washed and resuspended in 0.2ml PBS/BSA/azide. Analysis was carried out at Bristol University Veterinary School on an EPICS CS fluorescence activated cell sorter (Coulter Electronics Ltd.) using an argon laser at 488nm collecting log green fluorescence and forward light scatter.

7.2.4 Preparation of Panning Wells for Fab Antibody Library Selection

The mouse derived Fab antibody libraries were to be panned against 2wt cell antigens. 2wt cells that had grown to confluency in a T25 tissue culture flask were washed twice with PBS before being scraped into 5ml ice cold PBS.

The cells were transferred to a conical tube and pelleted by centrifugation in a Sorvall RC3B at 1000rpm and 4°C for 5 minutes. The cells were lysed by resuspension of the pellet in 0.3ml 1% Mega-10 in 50mM Tris pH7. The solution was transferred to an Eppendorf tube and spun in a microfuge at full speed for 3 minutes to pellet any undissolved material. The protein concentration in the resultant supernatant was determined by Bradford's analysis (section 2.2.20). Wells of an ELISA plate were coated overnight at 4°C with 1μg of 2wt derived protein diluted in bicarbonate coating buffer pH9.5.

Panning was performed as described in section 6.2.6.

7.3 RESULTS

7.3.1 RTPCR

The immunisation protocols described above required the use of two cell lines, identical except for the fact that only one of them expressed CFTR. 2wt cells and that BPV cells were analysed for CFTR expression by reverse transcription PCR (RTPCR). Oligonucleotide primers designed to amplify a DNA fragment spanning two exons of CFTR gave a specific band of the appropriate size with cDNA derived from 2wt cells, but not with cDNA derived from BPV cells, see fig. 7.2.

7.3.2 Flow Cytometry

BPV and 2wt cells were processed for flow cytometry and tested with pre and post immunisation serum derived from RIII and Balb-c mice.

7.3.2.1 Flow Cytometric Analysis of RIII Antisera

As can be seen from from fig. 7.3, there was no significant difference between the intensity of fluorescence of 2wt cells relative to BPV cells when stained with antisera from mice immunised with 2wt cells or membrane lysate.

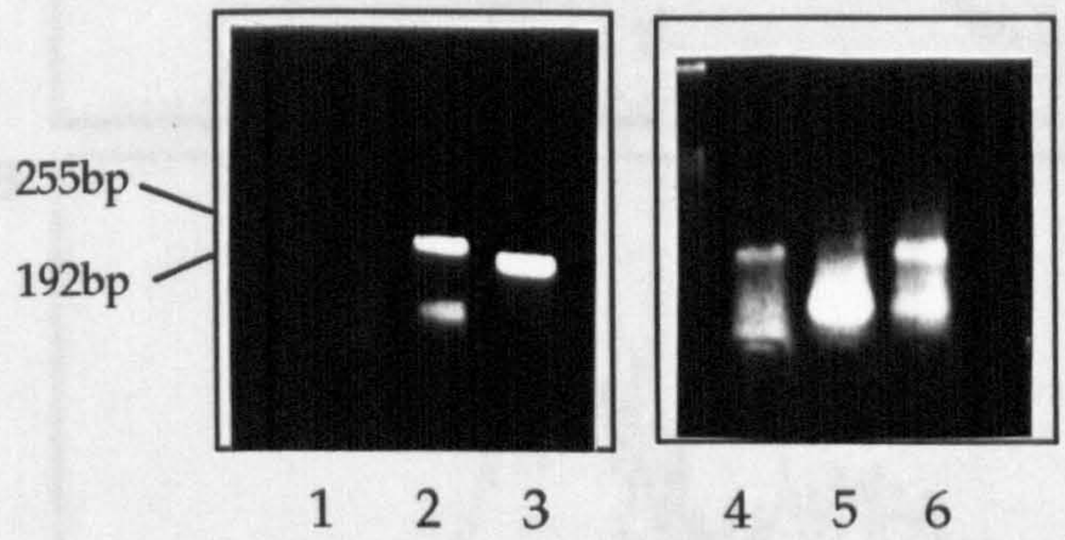


Fig. 7.2 Agarose gel analysis of PCR products from mRNA obtained from 2wt (lanes 1-3) and BPV (lanes 4-6) cell lines. Oligonucleotide primers designed to amplify a 255bp fragment spanning exons 23 and 24 of CFTR were used to amplify the material loaded in lanes 2 and 5. A band of this size is observed in lane 2 (2wt) but not lane 5 (BPV) indicating the presence of CFTR mRNA in 2wt but not BPV cells. Contamination of cDNA with genomic DNA was checked for using a pair of oligonucleotide primers designed to amplify a 192bp fragment within exon 24 of CFTR. The products of these reactions were loaded in lanes 3 and 6. A band of appropriate size is more intense in lane 3 (2wt) than lane 6 (BPV) but present in both lanes, indicating some contamination of the BPV cDNA with genomic DNA. The integrity of the template mRNA was tested using a pair of oligonucleotide primers designed to amplify a 212bp fragment spanning exons the mouse beta actin gene [Alousa et al., 1986]. A very faint band of 212bp is present in lanes 1 (2wt) and 4 (BPV) indicating that intact mRNA was present in both original samples. DNA fragments of known molecular weight were loaded in an adjacent lane, their migration position is indicated.

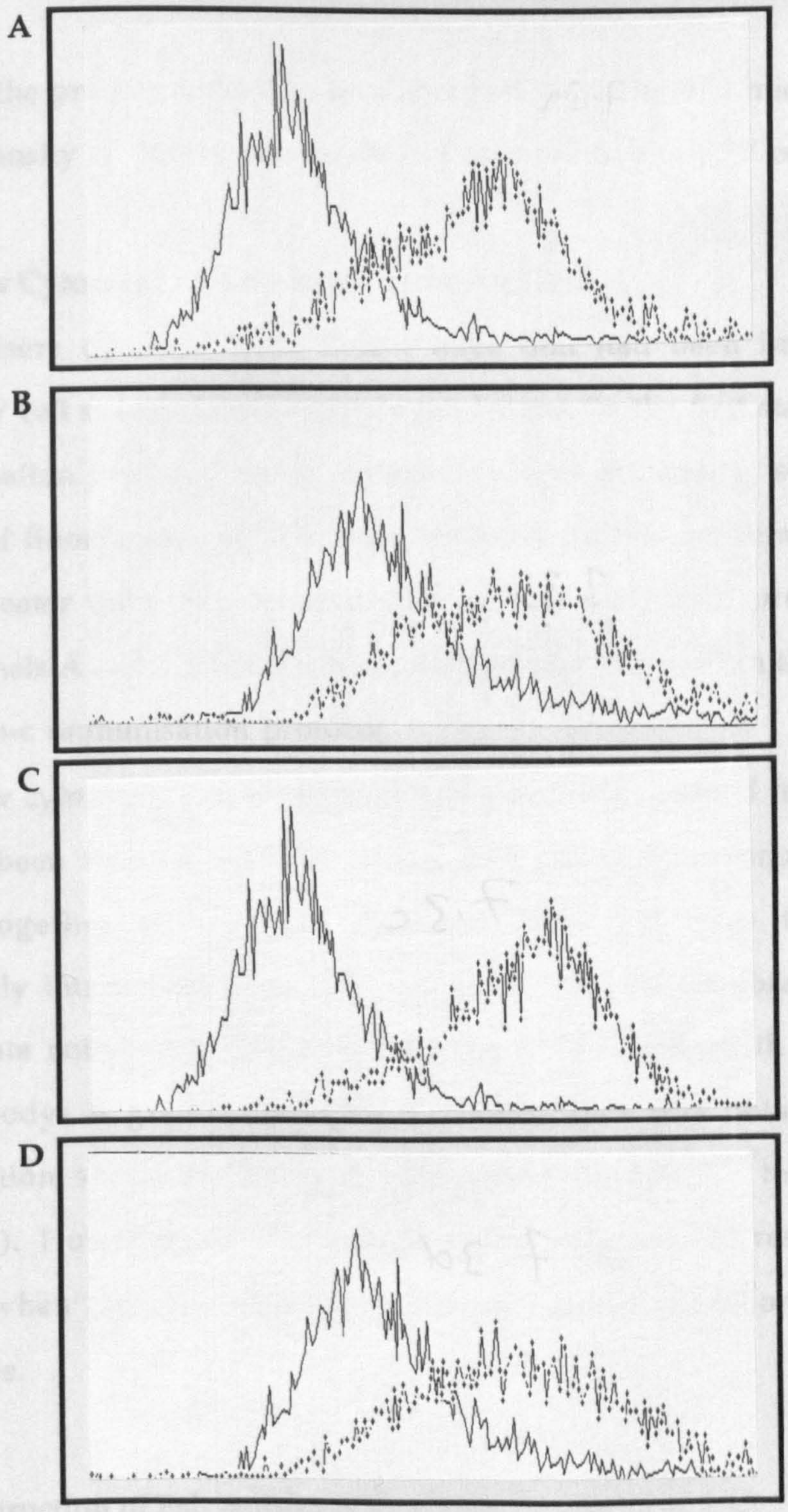


Fig. 7.3 Flow cytometric analysis of antisera obtained from 2wt immunised RIII mice. In each figure the uninterrupted line represents the staining of cells with pre-immunisation sera and the crossed line represents the staining of cells with post-immunisation sera.

A - BPV cells stained with sera from mice immunised with membrane lysate.

B - 2wt cells stained with sera from mice immunised with membrane lysate.

C - BPV cells stained with sera from mice immunised with cell suspension

D - 2wt cells stained with sera from mice immunised with cell suspension

Log green fluorescence is plotted on the x-axis versus number of cells on the y-axis. All graphs are plotted to the same scale.

However, the pre-immunisation sera obtained from the RIII mice did exhibit a greater intensity of fluorescence with 2wt cells relative to BPV cells.

7.3.2.2 Flow Cytometric Analysis of Balb-c Antisera

Antisera obtained from Balb-c mice that had been immunised with either BPV cell suspensions or membrane lysates (in the first stage of the Balb-c immunisation protocol) were analysed by flow cytometry, see fig. 7.4. The intensity of fluorescence of BPV cells stained with this antisera (panels B and D) was greater than that detected with cells stained with pre-immunisation serum (panels A and C). On this basis the antisera was used in the second stage of the Balb-c immunisation protocol.

Flow cytometry was performed using antisera obtained from Balb-c mice that had been immunised with either 2wt cell suspensions or membrane lysates, together with serum obtained from the mice that had been equivalently immunised with BPV cell suspension or membrane lysates, fig. 7.5 and data not shown. BPV and 2wt cells were stained with this antisera as first antibody. A greater intensity of fluorescence was obtained with post immunisation serum on the 2wt cells (panel B) relative to the BPV cells (panel A). However, this difference in intensity of fluorescence was not detected when the cells were stained with pre-immunisation sera from the Balb-c mice.

7.3.3 Construction of Fab Antibody Libraries

Random combinatorial Fab antibody libraries were constructed in the pComb3H-SS vector system from mRNA extracted and purified from the spleens of the immunised mice. The methods followed for the construction of these libraries are described in the previous chapter. Four sets of immunised mice were used, namely:

i) RIII mice immunised with 2wt cell suspensions,

ii) RIII mice immunised with membrane lysate prepared from 2wt cells,

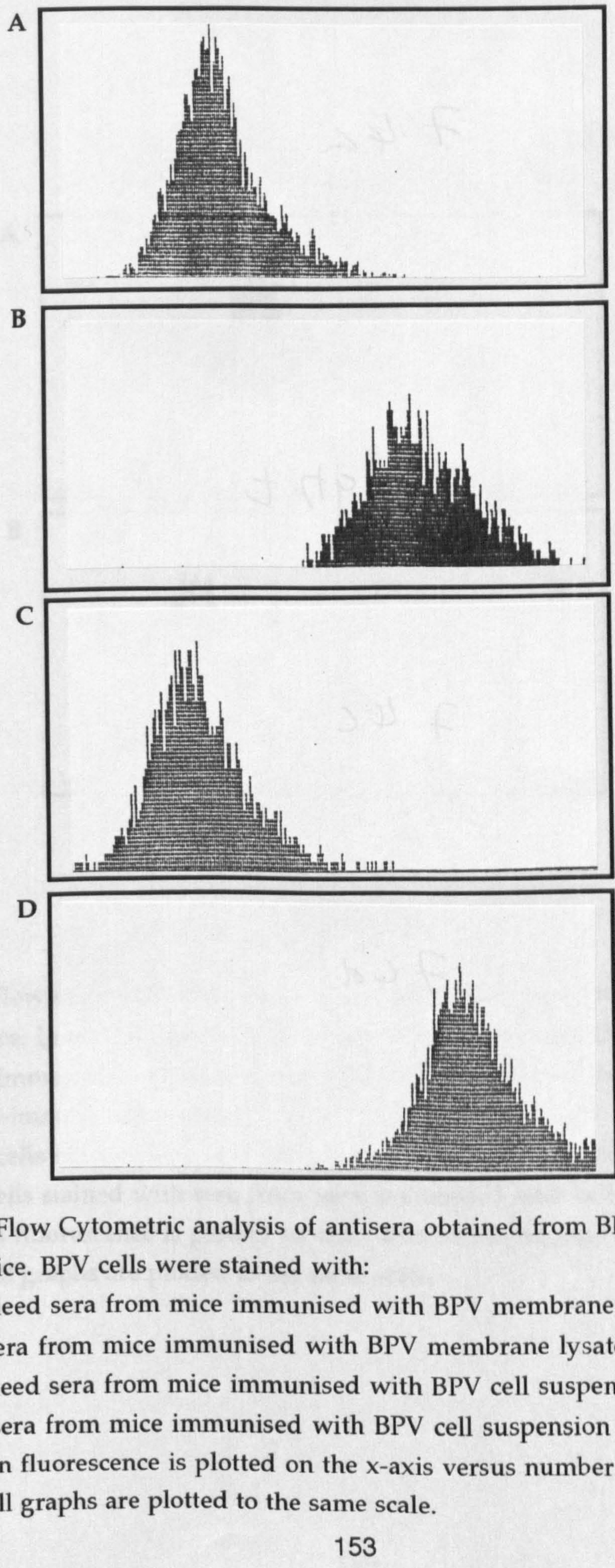


Fig. 7.4 Flow Cytometric analysis of antisera obtained from BPV immunised Balb-c mice. BPV cells were stained with:

A - prebleed sera from mice immunised with BPV membrane lysate.

B - antisera from mice immunised with BPV membrane lysate.

C - prebleed sera from mice immunised with BPV cell suspension

D - antisera from mice immunised with BPV cell suspension

Log green fluorescence is plotted on the x-axis versus number of cells on the y-axis. All graphs are plotted to the same scale.

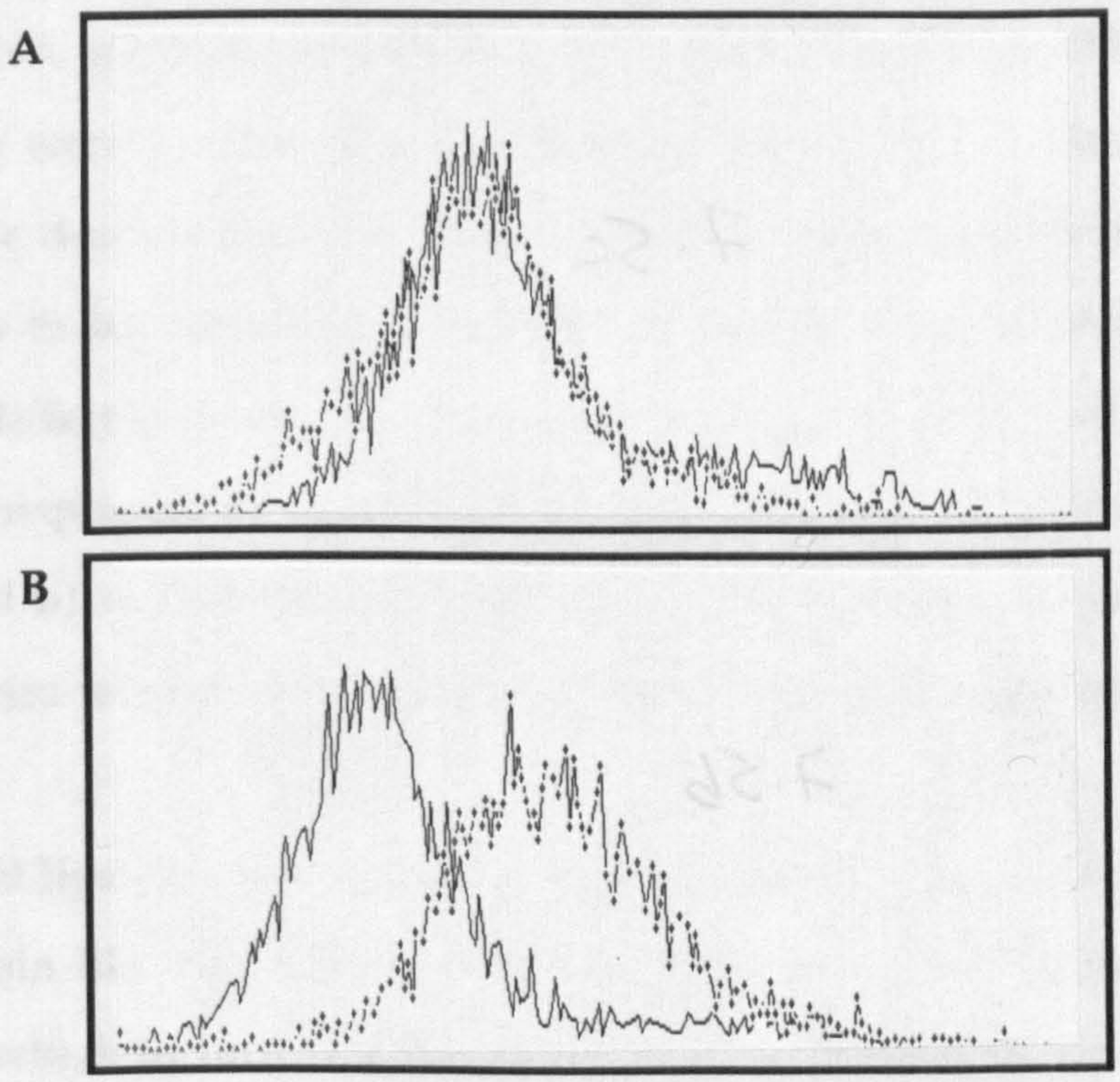


Fig. 7.5 Flow cytometric analysis of antisera obtained from 2wt immunised Balb-c mice. In each figure the uninterrupted line represents the staining of cells with pre-immunisation sera and the crossed line represents the staining of cells with post-immunisation sera.
A - BPV cells stained with sera from mice immunised with cell suspension
B - 2wt cells stained with sera from mice immunised with cell suspension.
Log green fluorescence is plotted on the x-axis versus number of cells on the y-axis. All graphs are plotted to the same scale.

iii) Balb-c mice injected with 2wt cell suspensions together with antisera from mice immunised with BPV cell suspensions, and

iv) Balb-c mice injected with membrane lysate prepared from 2wt cells together with antisera from mice immunised with membrane lysate of BPV cells.

cDNA synthesis and PCR amplification of antibody Fd and light chain sequences were performed independently on the mRNA obtained from each set of mice described above. This allowed for the maximum range of antibody sequences to be amplified from mRNA derived from mice immunised with the the different protocols. However after examination of the amplified antibody sequences by agarose gel electrophoresis the PCR products from mice sets i) and ii) were pooled as were those from mice sets iii) and iv). Thus two Fab libraries were constructed, one derived from RIII mice and one from Balb-c mice.

Test ligations were performed prior to the construction of the light and heavy chain libraries. This was to assess the extent of the background vector ligation, which in turn was indicative of how thoroughly the prepared vector DNA had been a) restricted and b) purified from uncut vector DNA. The restricted heavy and light chain sequences were purified on Chromaspin 100 columns prior to ligation into the prepared vector. For the RIII light chain, the background vector ligation was 9% that of the test insert ligation and for the Balb-c light chain it was 17%. These were considered to be low enough to continue with the construction of the light chain libraries. The size of the RIII light chain library was estimated to be 1.2×10^7 independent clones and that of the Balb-c library to be 8×10^6 independent clones.

For the heavy chain test ligations the background was found to be considerably higher, 30% for RIII heavy chains and 28% for the Balb-c heavy chains. These were considered to be too high to continue with the construction of the heavy chain libraries. The *Xho* 1 -*Spe* 1 stuffer fragment in the pComb3H-SS vector is only 300bp long, whereas the vector with inserted light

chain is 4Kb. When 20-30μg of vector DNA is restricted with *Xho* 1 and *Spe* 1, precipitated and run out on an 0.8% Seakem GTG gel (see section 6.2.2.2), it is difficult to distinguish linearised vector DNA with the heavy chain stuffer still present (≈4380bp) from vector DNA with the heavy chain stuffer fragment fully excise (≈4080bp). This might have resulted in the contamination of the *Xho* 1 and *Spe* 1 restricted and prepared vector with some vector still containing the heavy chain stuffer. One way around this problem was to improve the efficiency of *Xho* 1 and *Spe* 1 restriction of the vector DNA such that all the heavy chain stuffer was restricted out. In an attempt to accomplish this the vector preparation protocol described in section 6.2.2.2 and table 6.1 was modified in the following ways. The amount of *Xho* 1 enzyme used was increased from 9 units per μg DNA to be restricted to 12 u/μg and the amount of *Spe* 1 enzyme used was increased from 3 units per μg DNA to 5u/μg . The reaction times were also increased from 3 to 4 hours. Half of the total amount of enzyme was added at the beginning of the reaction time and half after 2 hours. Heavy chain test ligations were repeated using vector DNA that had been prepared in this manner. The background ligations were reduced to 1% for the RIII heavy chain and 5% for the Balb-c heavy chain. The heavy chain libraries were subsequently constructed. The estimated size of the RIII heavy chain library was $5x10^6$ independent clones and that of the Balb-c library was $4x10^6$ independent clones. Phagemid DNA prepared from library clones, restricted with *Sac* 1 and *Xba* 1 and with *Xho* 1 and *Spe* 1 and analysed on an agarose gel, revealed that 80% of transformants contained both heavy and light chain antibody sequences.

7.3.4 Sequencing Analysis of Antibody Fragments

Phagemid DNA was prepared by Wizard Mini Prep from 8 colonies of each of the two mouse derived Fab libraries. The DNA was sequenced with the heavy and light chain primers given in section 6.2.8.1. The DNA sequences

were all compared to sequences in the EMBL/Genbank data base, release number 39, version 6. The five most homologous sequences identified for each chain sequenced are given in fig. 7.6a and b and 7.7a and b.

7.3.5 Panning the Fab Libraries

Enrichment of the antibody libraries for Fab fragments that bound to antigens in 2wt membrane lysate was attempted by panning. After each round of panning eluted phage were reinfected into *E.coli.* and rescued with helper phage. However, effective panning was hindered by significant lysis of the reinfected and rescued cultures, as encountered in the panning of the hybridoma derived Fab library described in the previous chapter. Despite the considerable cell lysis the cultures did eventually grow sufficiently to continue with consecutive rounds of panning. Nevertheless, this was not considered to be valid enrichment because only those cells that survived cell lysis were selected, not necesssarily those that bound to the antigens.

Fig. 7.6a (pages 159 - 162). Light chain sequences 1 to 8, of Fab clones derived from the RIII mouse antibody library.

Fig. 7.6b (pages 163 - 166). Heavy chain sequences 1 to 8, of Fab clones derived from the RIII mouse antibody library.

Fig. 7.7a (pages 167 - 170). Light chain sequences 1 to 8, of Fab clones derived from the Balb-c mouse antibody library.

Fig. 7.7b (pages 171 - 174). Heavy chain sequences 1 to 8, of Fab clones derived from the Balb-c mouse antibody library.

The five most homologous sequences identified from the EMBL/Genbank data base, release number 39, version 6, for each light or heavy chain, are presented in tables below each sequence.

Further information and references for each of the data base sequences given, can be obtained from the EMBL/Genbank data base, via the appropriate accession number.

Fig. 7.6a R111 Light Chain 1. Length: 170

```
   1  gatatagtct ccagcactca tggctgcatc tccaggggag aaggtcacca
  51  tcacctgcag tgtcagccca agtataagtt ccagcaactt gcactggtac
 101  cagcagaagt cagaaacctc ccccaaacxc tggatttatt ggcacatcca
 151  acctggcttc tggagtccct
```

	Accession number	Description	% Homology
i	M36252	Mouse Ig light-chain mRNA V region, partial cds. H280-10.	98.2
ii	M36258	Mouse Ig light-chain mRNA V region, partial cds, from hybridoma H146-24B1VK.	98.2
iii	M36259	Mouse Ig light-chain mRNA V region, partial cds, from hybridoma H147-25H1VK.	98.2
iv	M36241	Mouse Ig light-chain mRNA V region, partial cds, from hybridoma H163-130H9.	98.2
v	M36240	Mouse Ig light-chain mRNA V region, partial cds, from hybridoma H163-130F2.	98.2

Fig. 7.6a R111 Light Chain 2. Length: 284

```
   1  atgtcccaga ctccactctc cctgcctgtc agtcttggag atcaagcctc
  51  catctcttgc agatctagtc agagccttgt acacagtaat ggaaacacct
 101  atttacattg gtacctgcag aagccaggcc agtctccaaa gctcctgatc
 151  tacaaagttt ccaaccgatt ttctggggtc ccagacaggt tcagtggcag
 201  tggatcaggg acagatttca cactcaagat cagcagagtg gaggctgagg
 251  atctgggagt ttatttctgc tctcaaagta cacatgttcc tgga
```

	Accession number	Description	% Homology
i	M28131	Mouse germline Ig kappa chain gene V-1A region.	99.3
ii	L22330	Mouse rearranged immunoglobulin kappa-chain mRNA V-J region, partial cds.	99.3
iii	D00080	Mouse kappa geremline V-region gene; subgroup VK-1 idiotype; anti-GAT.	99.3
iv	M15566	Mouse Ig germline V-kappa-1 chain gene (K5.1), exons 1and 2.	99.3
v	S54757	IgG3 VL=anti-DNA IgG3 light chain variable region {clone H161} (mice, mRNA, 324 nt).	99.3

Fig. 7.6a R111 Light Chain 3. Length: 236

```
  1  ctcacccagt ctccagcatc cctgtccatg gctataggag aaaaagtcac
 51  caacagatgc ataaccagca ctgatattga tgatgatatg aactggtacc
101  agcagaagcc aggggaacct cctaagctcc ttatttcaga aggcaatact
151  cttcgtcctg gagtcccatc ccgattctcc agcxgtggct atggtacaga
201  ttttxttttt acaattgaaa acatgctctc agaaga
```

	Accession number	Description	% Homology
i	M55318	Mouse IgK processed anti ds-DNA antibody chain V-region, from hybridoma C8.5.	98.3
ii	X16678	Mouse VK gene for kappa light chain variable region and J4 sequence.	96.2
iii	Z22104	M.domesticus IgK variable region.	94.9
iv	M34637	Mouse Ig rearranged kappa-chain (NC13-B12) mRNA Vk32-Jk5 region, partial cds.	94.5
v	M34631	Mouse Ig rearranged kappa-chain (NC9-B4) mRNA Vk32-Jk4 region, partial cds.	94

Fig. 7.6a R111 Light Chain 4. Length: 267

```
  1  gatgacccag actccactct ccctgcctgt cagtcttgga gatcaagcct
 51  ccatctctta cagatctagt cagagccttg tacacagtaa tggaaacacc
101  tatttacatt ggtacctgca gaagccaggc cagtctccaa agctcctgat
151  ctacaaagtt tccaaccgat tttctggggt cccagacagg ttcagtggca
201  gtggatcagg gacagatttc acactcaaga tcagcagagt ggaggctgag
251  gatctgggag tttattt
```

	Accession number	Description	% Homology
i	M28131	Mouse germline Ig kappa chain V-1A region.	99.3
ii	Z22114	M.domesticus IgK variable region	99.3
iii	Z22102	M.domesticus IgK variable region	99.3
iv	L22329	Mouse rearranged immunoglobulin kappa-chain mRNA V-J region, partial cds.	99.3
v	L22330	Mouse rearranged immunoglobulin kappa-chain mRNA V-J region, partial cds.	99.3

Fig. 7.6a R111 Light Chain 5. Length: 225

```
  1  gtgatgacac agtctccatc ctccctagct gtgtcagttg gagagaaggt
 51  tactatgagc tgcaagtcca gtcagagcct tttatatagt agcaatcaaa
101  agaactactt ggcctggtac cagcagaaac cagggcagtc tcctaaactg
151  ctgatttact gggcatccac tagggaatct ggggtccctg axcgttcaca
201  ggcagtggat ctgggacaga tttct
```

	Accession number	Description	% Homology
i	M12427	Mouse Ig aberrant recombined kappa-chain double recombinant product from plasmacytoma PC3609.	98.7
ii	M34634	Mouse Ig rearranged Kappa-chain (NC12-N5) mRNA Vk8-Jk1 region,partial cds.	98.7
iii	M62927	Mouse rearranged Ig kappa-chain gene V-region.	98.2
iv	Z29536	M.musculus (NZB X NZW)F1 mRNA for Immunoglobulin kappa light chain V-region	98.2
v	X65774	M.musculus DNA for IgE antibody light chain (VJ).	98.2

Fig. 7.6a R111 Light Chain 6. Length: 230

```
  1  ctcacccagt ctccaaccac catggctgca tctcccgggg agaagatcac
 51  tatcacctgc agtgccagct caagtataag ttccaattac ttgcattggt
101  atcagcagaa gccaggattc tcccctaaac tcttgattta taggacatcc
151  aatctggctt ctggagtccc agctcgcttc agtggcagtg ggtctggacc
201  tcttactctc tcacaattgg caccatggag
```

	Accession number	Description	% Homology
i	X59097	M.musculus mRNA (L14-3D10) for IgK light chain V-region.	99.6
ii	M34592	Mouse Ig kappa-chain mRNA V-J region partial cds.	99.6
iii	X59091	M.musculus mRNA (li-3B3) for IgK light chain V region.	99.6
iv	X14099	Mouse mRNA for kappa-immunoglobulin light chain variable region 38C13 variant 4 V-J4.	99.6
v	M57539	Mouse anti-idiotype antibody-resistant variant IgK (Vk-Ox1 gene family) mRNA, VJ4 region.	99.6

Fig. 7.6a R111 Light Chain 7. Length: 285

```
   1  gatgacacag tctccagcca ccctgtctgt gactccagga gatagcgtca
  51  gtctttcctg cagggccagc caaagtatta gcaacaacct acactggtat
 101  caacaaaaat cacatgagtc tccaaggctt ctcatcaagt atgcttccca
 151  gtccatctct gggatcccct ccaggttcag tggcagtgga tcagggacag
 201  atttcactct cagtatcaac agtgtggaga cagaagattt tggaatgtat
 251  ttctgtcaac agagtaacag ctggcctctc acgtt
```

	Accession number	Description	% Homology
i	M34528	Mouse Ig kappa-chain (anti-insulin Ab 126) mRNA V region, partial cds.	98.2
ii	X02556	Mouse mRNA fragment for G1 kappa immunoglobulin A 20/44 light chain (V-J).	97.5
iii	L08211	Mouse anti-Dna antibody kappa light chain variable region (Vk23 Vk family) mRNA.	97.5
iv	M16162	Mouse Ig kappa-chain VC-region (VJ5C) from hybridoma MAK33.	97.5
v	A07699	Synthetic pBT111 DNA sequence.	97.5

Fig. 7.6a R111 Light Chain 8. Length: 231

```
   1  ctccagatga cccagtctcc agcaatcatg tctgcatctc aggggagaa
  51  ggtcaccatg acctgcagtg ccagctcaag tgtaagttac atgtactggt
 151  aacctggctt ctggagtccc tgttcgcttc agtggcagtg ggtctgggac
 201  ctcttactct ctcacaatca gccgaatgga g
```

	Accession number	Description	% Homology
i	M97862	Mouse hybridoma Ig rearranged kappa-chain mRNA V-region, partial cds.	98.2
ii	M97866	Mouse hybridoma Ig rearranged kappa-chain mRNA V-region, partial cds.	98.2
iii	L06841	M.musculus Ig light chain (interleukin 2 receptor antibody HU-MIK-beta1) mRNA, V-region.	97.3
iv	M19906	Mouse Ig rearranged kappa-chain mRNA, clone AN02K.	97.3
v	K00746	Mouse Ig kappa active V-region mRNA from hybridoma nq2-6.1.	96.9

Fig. 7.6b R111 Heavy Chain 1. Length:216

```
   1  ggggctgaat tggtgaagcc tggggcttca gtgaagctgt cctgcaaggc
  51  ttctggctat accttcacca actattggat gcactgggtg aagcagaggc
 101  ctggacaagg ccttgagtgg attggagaga ttcatcctag tcacggtcgt
 151  gctagttata atgagaagtt caggagcaag gccacactga ctcgagacaa
 201  atcctccagc acagcc
```

	Accession number	Description	% Homology
i	L26880	Mus musculus (BALB-25) gene fragment.	93.1
ii	M21814	Mouse/human Ig heavy chain constant region mRNA, segment 1.	92.6
iii	M97865	Mouse hybridoma Ig rearranged H-chain mRNA V-region, partial cds.	92.6
iv	M19901	Mouse Ig rearranged gamma-chain mRNA, segment 1.	92.1
v	S50856	Ig VH=chimeric anti-human neuroblastoma antibody (mice, Genomic, 706nt).	91.7

Fig. 7.6b R111 Heavy Chain 2. length: 249.

```
   1  ggggctgagc tggtgaggcc tgggacttca gtaagctatc ctgcaaggct
  51  tctggctaca ccttcaccag ctactggatg aattgggtga agcagaggcc
 101  tggacaaggc cttgaatgga ttggtatgat tgatccttca gacagtgaaa
 151  tttactacaa tcaaatgttc aaggacaagg ccacattgac tgttgacaaa
 201  tcctccagca cagcctacat gcacctcaca gcctgacatc tgaggactc
```

	Accession number	Description	% Homology
i	M95940	Mouse mRNA sequence, partial cds.	95.2
ii	U01658	Mus musculus BALB/c anti-glycophorin A type M antibody A09 mRNA, complete cds.	94
iii	J00536	Mouse Ig germline H-chain, c57bl/6 b-np-related gene vh3, vhii.	93.6
iv	M34579	Mouse Ig heavy-chain mRNA V-D-J region, partial cds.	93.2
v	M32855	Mouse Ig rearranged H-chain mRNA V-D-J region, partial cds, from hybridoma CH-51.	93.2

.

Fig. 7.6b R111 Heavy Chain 3. Length: 220

```
  1  ggagctgggc tggtgaggcc tggggcttca gtgaagatat cctgcaaggc
 51  ttctggctac accttcactg cctactatct aaactgggtg aagcggaggc
101  ctggacaggg ccttgagtgg attggaaaga ttggtcctgg aagtggtagt
151  acttactaca acgagaagtt caagggcaag gcctcactga ctgcagacaa
201  atcctccagc acagcctaca
```

	Accession number	Description	% Homology
i	U07214	Mus musculus clone 103 Anti-C5a Ig heavy chain V region mRNA, partial cds.	95.9
ii	U07212	Mus musculus clone 102 Anti-C5a Ig heavy chain V region mRNA, partial cds.	94.1
iii	U07208	Mus musculus clone 100 Anti-C5a Ig heavy chain V region mRNA, partial cds.	94.1
iv	Z12760	M.musculus mRNA for VH-gene sequence of naturally occuring, somatically mutated mrmory B	91.8
v	X63047	M.musculus mRNA for IG heavy chain variable region (3F11).	91.4

Fig. 7.6b R111 Heavy Chain 4. Length: 314.

```
 51  ttctggctac atctttacta gctactggat gcactgggta aaacagaggc
101  ctggacaggg tctggaatgg attggataca ttaatccggx cagtggtcat
151  actaagttca atcagaagtt caaggacaag gccacattga ctgcagacaa
201  atcctccagc acagcctaca tgcaactgat cagcctgaca tctgaggact
251  ctgcagtctg ttactgtgca aggggggaac attactatgg tggtagccgt
301  actactttga ctac
```

	Accession number	Description	% Homology
i	Z22125	M.domesticus IgM variable region.	95.6
ii	M19898	Mouse Ig rearranged ganmma-chain mRNA, clone AN06g.	95.2
iii	X02458	Mouse germline immunoglobulin V(H)II gene H30.	94.5
iv	X02459	Mouse germline immunoglobulin V(H)II gene H13-3.	94.4
v	M28251	Mouse Ig rearranged gamma-chain (G-2a) mRNA V-region (V-J2), partial cds.	94.2

Fig. 7.6b R111 Heavy Chain 5. Length: 249.

```
   1  ggggctgaac tggcaaaacc tggggcctca gtgaagatgt cctgcaaggc
  51  ttctggctac acctttacta tgtactggat gcactggata aaacagagac
 101  ctggacaggg tctggaatgg attggataca ttaatctagt agtggttata
 151  ctgactacaa tcagaagttc aaagacaagg ccacattgac tgcagacatt
 201  tcctccagaa cagcctactt gcaactgacc agcctgacat ctgaggact
```

	Accession number	Description	% Homology
i	Z22125	M.domesticus IgM variable region.	94.4
ii	M36225	Mouse Ig heavy-chain V region, partial cds. H280-15VH.	94.4
iii	M28834	Gamma heavy chain of the monoclonal antibody 6A4.	93.2
iv	A13735	V-region of a monoclonal antibody that cross reacts with 19 known Pseudomonas aeruginosa	93.2
v	M19898	Mouse rearranged gamma-chsin mRNA, clone AN06.	93.2

Fig. 7.6b R111 Heavy Chain 6. Length: 239.

```
   1  ctgaactggc aaaacctggg acctcagtga agatgtcctg caaggcttct
  51  ggctacacct ttactagcta ctggatgatc tggatagcac agaggcctgg
 101  acagggtctg gaatggattg gggctattaa tcctttcaat ggttacattg
 151  aggacaatca gaagttcaag ggcaaggcca tattgactgc agacaaatcc
 201  tccaacacag cctacatgca actgagcagc ctgacatct
```

	Accession number	Description	% Homology
i	M36225	Mouse Ig heavy-chain mRNA V region, partial cds. H280-15VH.	92.1
ii	M28251	Mouse Ig rearranged gamma-chain (G-2a) mRNA V-region (V-J2), partial cds.	90.8
iii	M19898	Mouse Ig rearranged gamma-chain mRNA, clone AN06g.	90.4
iv	L22581	Mouse rearranged Ig heavy chain V-region sequence.	90.4
v	Z22125	M.domesticus IgM variable region.	90.4

.

Fig. 7.6b R111 Heavy Chain 7. Length: 253

```
  1  ggggqctaga gcttgtgaag cctggggctt cagtaaagct gtcctgcaag
 51  gcttctggct acaccttcac cagctactgg atgcactggg tgaagcagag
101  gcctggacaa ggcttgagtg gattggaagg attgatccta atagtggtga
151  tactaagtac aatgagaagt tcaagagcaa ggccacactg actgtagaca
201  aacccctccag cacagcctac atgcagctca gcagcctgac atctgaggac
251  tct
```

	Accession number	Description	% Homology
i	X67347	M.musculus immunoglobulin heavy chain V-D-J region (focus 1012 DNA #7).	98.4
ii	X67350	M.musculus immunoglobulin heavy chain V-D-J region (focus 1012 DNA #9).	98
iii	X67343	M.musculus immunoglobulin heavy chain V-D-J region (focus 1012 DNA #3).	98
iv	X67351	M.musculus immunoglobulin heavy chain V-D-J region (focus 1012 DNA #10).	98
v	J00529	Mouse Ig active H-chain, c57bl/6 b-np response from b1-8, vhii mRNA.	98

Fig. 7.6b R111 Heavy Chain 8. Length: 251

```
  1  ggggctgaac ttgtgaagcc tggggcttca gtggaaatgt cctgtaaggc
 51  ttctggctac accttcaccg actactggat aaactgggtg aagcagaggc
101  ctggacaagg cttgagtgga ttggagatat ttatcctggt agagggacca
151  ctaattacaa tgagaaattc aagggcaaga ccacactgac tgcagacaag
201  tcctccagca cagcctacat gcagctcagc agcctgacct ctgaggactc
```

	Accession number	Description	% Homology
i	K00603	Mouse Ig germline H-chain, BALB/c b-NP-equivalent VH3 pseudogene.	89.7
ii	J00535	Mouse Ig germline H-chain, c57bl/6 b-np-related gene vh6, vh-ii.	89.3
iii	M13788	Mouse Ig rearranged transcribed H-chain V-region VH558 mRNA, clone B4.	88.5
iv	M26808	Mouse Ig heavy chain mRNA B-region, partial cds.	88.5
v	Z12816	M.musculus mRNA for VH gene seq. of naturally occuring, somatically mutated memory B cell.	88.5

.

Fig. 7.7a Balb-c Light chain l. Length: 278

```
   1  ctccactcac tttgtcggtt accattggac aaccagcctc catctcttgc
  51  aagtcaagtc agagcctctt agatagtgat ggaaagacat atttgaattg
 101  gttgttacag aggccaggcc agtctccaaa gcgcctgatc tatctggtgt
 151  ataaactgga ctctggagtc cctgacaggt tcactggcag tggatcaggg
 201  acagatttca cactgaaaat cagcagagtg gaggctgagg atttgggagt
 251  ttattattgc tggcaaggta cacatttt
```

	Accession number	Description	% Homology
i	M20830	Mouse IgMk rearranged kappa light chain mRNA variable region(V-J-kappa) anti-DNA autoantibody	99.3
ii	M25996	Mouse Ig rearranged kappa-chain Vk 1.60 gene, partial cds, from B cell hybridoma 1D3-2.	99.3
iii	L22888	Mus musculus rearranged IgH mRNA, V-region, cell linr GB-1.	98.9
iv	M17722	Mouse Ig active kappa-chain VOX1-J2 region anti-dextran mRNA, hybridoma 42.48.12.2.	98.9
v	Z11917	M.musculus immunoglobulin kappa chain variable region.	98.9

Fig. 7.7a Balb-c Light Chain 2. Length: 228

```
   1  atgacccagt ctccatcctc cttatctgcc tctcgggaga aagagtcagt
  51  ctcacttgtc gggcaagtca ggacattggt agtagcttaa actggcttxa
 101  gcaggaacca gatggaacta ttaaacgcct gatctacgac acatccagtt
 151  tagattctgg tgtccccaaa aggttcagtg gcagtaggtc tgggtcagat
 201  tattctctca ccatcagctg tccttgag
```

	Accession number	Description	% Homology
i	X63811	M.musculus mRNA for IgM V(k)MRB11.	97.8
ii	J00565	Mouse Ig kappa active gene: vk41 v-j region.	97.8
iii	M36246	Mouse Ig light-chain mRNA V region, partial cds, from hybridoma H220-23.	97.7
iv	V00804	Murine kappa-immunoglobulin gene fragment including signal peptide and variable region.	97.4
v	M64168	Mouse Ig active kappa-chain mRNA V-region.	97.3

Fig. 7.7a Balb-c Light Chain 3. Length: 214

```
  1  tgctcaccca gtctccagtt atgacagctg catctctggg gcaaaaggtc
 51  accatcacct gcagtgccag ctcaagtgta agttacatac actggtacca
101  gcagaagtca ggcacctccc ccaaaccatg gatttatgaa atatccaaac
151  taacttctgg agtcccacct cgcttcagtg gcagtgggtc tgggacctct
201  tactctctca caat
```

	Accession number	Description	% Homology
i	X05554	Mouse mRNA for X24 kappa immunoglobulin light chain.	96.3
ii	X05555	Mouse germline gene for X24 kappa immunoglobulin light chain.	96.3
iii	X58204	M.musculus extrachromosomal DNA for V kappa and J kappa coding joint (clone pKDE 1).	95.8
iv	M15589	Mouse Ig kappa chain 5G11 mRNA, V-region (VJ5C) of phOx-specific hybridoma antibody.	90.2
v	M34586	Mouse Ig kappa-chain mRNA V-J region. partial cds.	90.2

Fig. 7.7a Balb-c Light Chain 4. Length: 198

```
  1  gacccagact ccactcactt tgtcggttac cattggacaa ccagcctcca
 51  tctcttgcaa gtcaagtcag agcctcttag atagtgatgg aaagacatat
101  ttgaattggt tgttacagag gccaggccag tctccaaagc gcctaatcta
151  tctggtgtct aaactggact ctggagtccc tgacaggttc actggcag
```

	Accession number	Description	% Homology
i	M25996	Mouse Ig rearranged kappa-chain Vk 1.60 gene,partial cds, from B cell hybridoma 1D3-2.	100
ii	M20830	Mouse IgMk rearranged kappa light-chain mRNA variable region(V-J-kappa) anti-DNA autoantibody	100
iii	L22888	Mus musculus rearranged IgH mRNA, V-region, cell line GB-1.	99.5
iv	M17722	Mouse Ig active kappa-chain VOX1-J2 region anti-dextran mRNA, hybridoma 42.48.12.2.	99.5
v	A07556	DNA sequence of monoclonal TSH antibody, kappa chain.	99.5

Fig. 7.7a Balb-c Light Chain 5. Length: 196

```
  1  cccagtctcc agcaataatg gctgcctctc tggggtagaa ggtcaccatg
 51  acctcgagtg ccagctcaag tgtaagttac atgcactggt accagcagaa
101  gtcaggcacc tcccccaaac catggattta tgaaatatcc aaactggctt
151  ctggagtccc tgctcgcttc agtggcagtg ggtctcggga cctctt
```

	Accession number	Description	% Homology
i	X59179	Mouse immunoglobulin variable region light chain (T2-150) mRNA.	93.8
ii	L31516	Mus musculus anticryptococcal Ig 386 L-chain mRNA V region, partial cds.	93.4
iii	M34527	Mouse Ig kappa-chain (anti-insulin Ab 123) mRNA V region, partial cds.	93.4
iv	Z22128	M.domesticus IgK variable region.	93.4
v	M34586	Mouse Ig kappa-chain mRNA V-J region, partial cds.	93.4

Fig. 7.7a Balb-c Light Chain 6. Length: 270

```
  1  ccatccagtc tgtctgcatc ccttggagac acaattacca tcacttgcca
 51  tgccagtcag aacattaatg tttggttaag ctggtaccag cagaaaccag
101  gaaatattcc taaactattg atctataagg cttccaactt gcacacaggc
151  gatcccatca aggtttagtg gcagtggatc tggaactggt ttcacattaa
201  ccatcagcag cctacagcct gaagacattg ccacttacta ctgtcaacag
251  ggtcaaagtt atccattcac
```

	Accession number	Description	% Homology
i	M19908	Mouse Ig rearranged kappa-chain mRNA, clone AN04K.	97.4
ii	L21021	Mus musculus immunoglobulin light chain (IgL) mRNA, VJ region, partial cds.	97.4
iii	M34636	Mouse Ig rearranged kappa-chain (NC6-C8) mRNA Vk10-Jk2 region, partial cds.	97.4
iv	L09033	Mus musculus Ig rearranged anti-Sm hybridoma mRNA V-region sequence.	96.7
v	Z22052	M.domesticus variable region.	95.9

Fig. 7.7a Balb-c Light Chain 7. Length: 223

```
  1  tctccatcct ccttatctgc ctctctggga gaagagtcag tctcacttgt
 51  cgggcaagtc aggaxattgg tggcttgctt aagctggctt xagcaxxaac
101  cagatggaac tattaaaacg cctgatctac gccgcatcca cgtttagatt
151  ctggtgtccc aaaaaggttc agtggcagta ggtctggtca gattattctc
201  tcaccatcag cagcctcgag tct
```

	Accession number	Description	% Homology
i	X02177	Mouse mRNA fragment for IgG light chain V-J region (Gloop1).	94.7
ii	X02178	Mouse mRNA fragment for IgG light chain V-J region (Gloop2).	93.8
iii	M36246	Mouse Ig light-chain mRNA V region, partial cds, for hybridoma H220-23.	93.3
iv	J00565	Mouse Ig kappa active gene: vk14 V-J region.	93.3
v	X63811	M.musculus mRNA for LgM V(k)MRB11.	93.3

Fig. 7.7a Balb-c Light Chain 8. Length: 279

```
  1  gactccactc actttgtcgg ttaccattgg acaaccagcc tccatctctt
 51  gcaagtcaag tcagagcctc ttagatagtg atggaaagac atatttgaat
101  tggttgttac agaggccagg ccagtctcca aagcgcctaa tctatctggt
151  gtctaaactg gactctggag tccctgacag gttcactggc agtggatcag
201  ggacagattt cacactgaaa atcagcagag tggaggctga ggatttggga
251  gtttattatt cgtggcaagt tacacatt
```

	Accession number	Description	% Homology
i	M20830	Mouse IgMk rearranged kappa light-chain mRNA variable region.	98.9
ii	M25996	Mouse Ig rearranged kappa light-chain Vk 1.60 gene, partial cds,for B cell hybidoma 1D3-2.	98.9
iii	M17722	Mouse Ig active kappa-chain VOX1-J2 region anti-dextran mRNA, hybridoma 42.48.12.2.	98.6
iv	L22888	Mus musculus rearranged IgH mRNA, V-region, cell line GB-1.	98.6
v	M20330	Mouse Ig rearranged kappa mRNA V-region, partial cds, from an anti-digoxin hybridoma 40-150.	98.2

Fig. 7.7b Balb-c Heavy Chain 1. Length: 149

```
  1  aagggctatt tccctgagcc agtgacagtg acctggaact ctggatccct
 51  gtccagcggt gtgcacacct tcccagctgt cctgcagtct gacctctaca
101  ctctgagcag ctcagtgact gtccccctcca gcacctggcc cagcgagac
```

	Accession number	Description	% Homology
i	U00927	Mus musculus antibody heavy chain FAB mRNA, partial cds.	100
ii	U00940	Mus musculus nucleic acid-binding antibody heavy chain Fab mRNA partial cds.	100
iii	S65761	anti-colorectal carcinoma heavy chain, glycoprotein CANAG-50 specific IgG1 kappa.	100
iv	M60430	Mouse Ig rearranged H-chain mRNA constant region.	100
v	M60432	Mouse Ig rearranged H-chain constant region.	100

Fig. 7.7b Balb-c Heavy Chain 2. Length: 251

```
  1  ggacctgagc tggtgaaacc tggggcctca gtgaaaatat cctgcaaggc
 51  ttctggatac acattcactg actacaacat acactgggtg aagcagagcc
101  atggaaagag ccttgaatgg attggatgtt ttcttcctta caatagtgat
151  actggctaca atcagaagtt caagagcaag gccacattga ctgtagacag
201  ttcctccagc tcagcctaca tggaactccg cagcctgaca tctgaggact
251  c
```

	Accession number	Description	% Homology
i	J00488	Mouse Ig germline H-chain V gene VH108A, subgroup VH-11.	94.4
ii	M83098	Mus musculus CD33 antigen mRNA, 5' end.	94.4
iii	M36218	Mouse Ig heavy-chain mRNA V region, partial cds. H220-23VH.	94.4
iv	X02461	Mouse germline immunoglobulin V(H)II gene H16.	94.4
v	L25332	Mus musculus immunoglobulin heavy chain variable region gene, exon.	92.8

Fig. 7.7b Balb-c Heavy Chain 3. Length: 313

```
   1  ggaggtggcc tggtgcagcc tggaggatcc ctgaaactct cctgtxcagc
  51  ctcaggattc gattttagtg gatactggat gagttgggtc cggcaggctc
 101  cagggaaagg gctagaatgg attggagaaa ttaatccaga tagcagtacg
 201  cgccaaaaat acgctgtacc tacaaatgag caaagtgaga tctgaggaca
 251  caggccttta ttactgtgca agaccccgct acggtagtgg ctacgactgg
 301  tacttcgatg tct
```

	Accession number	Description	% Homology
i	M15873	Mouse Ig active H-chain VD-J4-C mRNA (XRPC24 family), from myeloma W3129.	98.2
ii	V00774	Segment of a mouse germ line V-H gene (V-H 441) for a heavy chain variable region.	98.2
iii	J00541	Mouse germline IgH-chain gene, V441-region, subgroup VH-III, clone V-H-441.	98.2
iv	M38227	Mouse Ig heavy chain V-D region 10L126-7 mRNA, partial cds.	97.5
v	M74424	Mouse Ig rearranged heavy chain mRNA V-J1-region,partial cds, clone 402-10.	93.7

Fig. 7.7b Balb-c Heavy Chain 4. Length:281

```
   1  gggcctgagg tggtgaggcc tggggtctca gtgaagattt cctacaaggg
  51  ttccggctac acattcactg attatgctat gcactgggtg aagcagagtc
 101  atgcaaagag tctagagtgg attggaatta ttagtactta caatggtaat
 151  acaaaccaca accagaagtt taaggacaag gccacaatga ctgtagacaa
 201  tcctccagct ctgcctatat ggaacttgcc agaatgacat ctgaggattc
 251  tgccatttat tactgtgcaa gttcggggag g
```

	Accession number	Description	% Homology
i	S78361	AHT107 VH region=chimeric mouse/human Mab against the human p55 IL-2R H-chain V-region.	94.5
ii	J00494	Mouse Ig active H-chain V-region from bcl-1, subgroup VH-II.	93.8
iii	M25465	Mouse Ig rearranged H-chain gene V-region (V-D-J-sub-two), 5' end.	93.8
iv	M34523	Mouse Ig gamma-chain (anti-insulin Ab 123) mRNA V region, partial cds.	93.2
v	M20774	Mouse rearranged IgA-chain gene, V region, from 1.29 B cell lymphoma, clone L7.1	83.6

Fig. 7.7b Balb-c Heavy Chain 5. Length: 307

```
   1  ggggctgaac tggcttaaga ccttggggct cttcagtgaa gatgtcctgc
  51  aaggcttctg gctacacctt tactagctac acgatgcact gggtaaaaca
 101  gaggcctgga cagggtctgg aatggattgg atacattaat cctagcagtt
 151  attatactat ttacaatcag aagttcaagg acaaggccac attgactgca
 201  gacaaatcct ccagcacagc ctacatgcaa ctgagcagcc tgacatctga
 251  ggactctgca gtctattact gtgcaagatc agggtatggt aacggggact
 301  actgggg
```

	Accession number	Description	% Homology
i	X73076	M.musculus VH mRNA (VH5).	97.6
ii	K00606	Mouse Ig germline H-chain, BALB/c b-NP equivalent VH5 pseudogene, subgroup VH-II.	94.6
iii	X02458	Mouse germline immunoglobulin V(H)II gene H30.	94.2
iv	L24557	Mus musculus (SO3) monoclonal antibody; v-region.	93.2
v	M36225	Mouse Ig heavy-chain mRNA V region, partial cds. H280-15VH.	90

Fig. 7.7b Balb-c Heavy Chain 6. Length: 284

```
   1  ggagctgagg tgatgaagcc tggggcctca gtgaagatat ccctgcaagg
  51  cttctggcta cacattcagt acctactggg ttgagtggat aaagcagagg
 101  cctggacgtg gccttgagtg gattggagag attttacctg gaagaggtag
 151  tactaactac aatgagagtt tgaagggcaa ggccacattc actgcagata
 201  catcctccaa cacagcctac atgcaactca gcagcctgac atctgaggac
 251  tctgccgtct attacagagc aagttttaaa tcgg
```

	Accession number	Description	% Homology
i	M17723	Mouse Ig family J558 active mu-chain V-J3 region anti-dextran mRNA, hybridoma 19.1.2.	94.9
ii	M12392	Mouse Ig active alpha-chain V-JH3 region (J558 family) anti-dextran mRNA, hybridoma 14.6b.1.	93.8
iii	L17085	Horse immunoglobulin heavy chain mRNA.	93.7
iv	M12764	Mouse Ig active alpha-chain V-JH3 region (J558 family) anti-dextran mRNA, hybridoma 26.4.1.	93.8
v	M24320	Mouse active Ig mu-chain VJ3-region mRNA, partial cds, anti-alpha dextran hybridoma 27.7.2.	92.8

Fig. 7.7b Balb Heavy Chain 7. Length: 266

```
   1  tcaggacctg gcctcgtgaa accttctcag tctctgtctc tcacctgctc
  51  tgtcactggc tactccatca ccagtggtta ttattgaact ggatccggca
 101  gtttccagga aacaatctgg aatggatggg ctacataagc tacgacggta
 151  acaataacta caacccatct ctcaaaaatc gaatctccat cactcgtgac
 201  acatctaaga accagttttt cctgaagttg aattctgtga ctactgaggg
 251  cacagcttca tattac
```

	Accession number	Description	% Homology
i	M19895	Mouse Ig rearranged gamma-chain mRNA, clone AN03g.	98.1
ii	M64150	Mouse Ig active heavy-chain mRNA V-region.	98.1
iii	S51485	Influenza virus A/PR/8/34 hemagglutinin specific hybridoma antibody H chain variable region.	98.1
iv	X59105	M.musculus mRNA (L1-3B3) for IgH heavy chain V region.	98.1
v	M64148	Mouse Ig active heavy-chain mRNA V-region.	98

Fig. 7.7b Balb-c Heavy Chain 8. length: 206

```
   1  gaggcctgga gcttcagtga agctgtcctg caaggcttct ggctactcct
  51  tcaccaacta ctggatgaac tgggtgaaga agaggcctgg acaaggcctt
 101  gagtggattg gcatgattca tccttccggt agtgtaacta gattaaatca
 151  gaagttcaag gacacggcca cattgactgt agacagttcc tccagcacag
 201  cctaca
```

	Accession number	Description	% Homology
i	L26881	Mus musculus (BALB-26) gene fragment.	96.1
ii	Z25455	M.musculus immunoglobulin gamma heavy chain (DBA/1) gene, V region.	95.6
iii	X59200	Mouse immunoglobulin variable region heavy chain (T6-19) mRNA.	90.7
iv	X59113	M.musculus mRNA (L14-6CA) for IgH heavy chain V region.	88.8
v	X59186	Mouse immunoglobulin variable region heavy chain (T5-314) mRNA.	88.8

7.4 DISCUSSION

Analysis of antisera from immunised Balb-c mice by flow cytometry indicated a higher degree of binding to antigens on the surface of 2wt cells than to those on the surface of BPV cells. In addition, differences between pre- and post-immunisation sera were only apparent when tested on 2wt cells (fig. 7.4). Both cell lines were derived from the transfection of C127 cells with a CL3AXBPVXT-NEO vector. The only expected difference between the two cell lines was the insertion of the CFTR sequence into the vector with which the 2wt cells were transfected. RTPCR analysis of mRNA from both cell lines indicated that CFTR was expressed in 2wt cells, but not in BPV cells. On the basis of these results it seems reasonable to assume that injection of naive mice with antisera from BPV immunised Balb-c mice, together with 2wt cell suspension or membrane lysate, successfully reduced response to the major antigens present in 2wt cells, except for the novel antigen CFTR. Accordingly, the greater antisera recognition of 2wt cells relative to BPV cells was due to the presence of expressed CFTR on 2wt cell surface. RNA was extracted from the spleens of these mice for the construction of a Fab antibody library.

Contrary to expectations, flow cytometry analysis of pre-immunisation antisera from RIII mice indicated a higher degree of binding to antigens on the surface of 2wt cells than to those on the surface of BPV cells. This increased fluorescence was not seen with post-immunisation antisera (fig. 7.3). This result was indicative of the fact that the RIII mice had previously been exposed to antigens cross reacting with cell surface antigens present on 2wt cells and not on BPV cells. The principal protein that is present on the surface of 2wt cells and not on BPV cells is CFTR, which is a member of the ABC transporter class of multiple membrane spanning proteins [Higgins and Hyde, 1991]. The majority of recognised members of this group of proteins are prokaryotic [Higgins, 1992]. It is therefore highly conceivable that the RIII mice had been exposed to a prokaryotic pathogen that expressed a ABC transporter protein having significant homology to CFTR.

The quality of the antibody libraries was assessed by sequencing arbitrarily selected phagemid Fab clones. Important conclusions can be drawn from the analysis of these sequences, see figs. 7.6a and b and 7.7a and b. Firstly, each of the clones selected contain both heavy and light chain sequence. Each sequence had substantial homology (between 88%-100%) to at least 5 previously identified murine antibody sequences. Secondly, considerable diversity was found between either heavy or light chain sequences within each Fab library. The sequencing primers were designed to sequence from the amino termini of the variable domains of each of the antibody chains. The presence of a great variety of heavy and light chain variable domain sequences indicates that the libraries were representative of a diverse murine immune response.

The estimated size of the RIII Fab library was $5x10^6$ independent clones and that of the Balb-c library was $4x10^6$ independent clones. These figures are about half the estimated number of antibody producing cells in an average mouse, which is $1x10^6$-10^8 [Huse, 1992]. The libraries were produced from mice that had been immunised with antigen. Consequently, relatively high concentration antigen specific lymphocytes would have been present in the lymphoid tissue of the murine spleens from which the RNA was isolated, for the construction of the libraries [Harlow and Lane,1988]. Phage display libraries prepared from immunised sources have been used to produce high affinity antibodies ($> 10^7 mol^{-1}$) with regular success [Clackson et al., 1991; Burton et al., 1991; Zebedee et al., 1992]. However, it is unclear how often this random combinatorial approach leads to the recovery of the original pairs of heavy and light chains expressed in individual B lymphocytes [Winter and Milstein, 1991; Gheradi and Milstein, 1992]. Variable gene repertoires derived from immunised animals, such as the mice used in this study, should give antibody libraries that are enriched for heavy and light chains encoding part of an antigen combining site. It is anticipated that when these are recombined with

one another they should generate high affinity antigen binding fragments relatively frequently, even if the original *in vivo* heavy and light chain pairings are not represented [Burton, 1991].

Attempts were made to select for CFTR specific Fab fragments by panning the antibody libraries against prepared 2wt cell lysate. A different approach that could be undertaken would be to select for CFTR at the cell surface by panning against whole cells still attached to the surface of a tissue culture flask. The ability to isolate specific antibody fragments by selecting phage antibody libraries against specific cell surface antigens has been previously demonstrated [Marks et al., 1993]. The success of this approach depends on the presence of antigen specific Fab binders in the library that can be selected in preference to binders against other antigens present in and on cells. The immunisation protocols, prior to the construction of the Fab libraries described above, were designed to reduce the production of murine antibodies to 2wt cell antigens other than CFTR. If background binding of Fab fragments to antigens on the cells other than CFTR does prove to be a problem, it could be reduced by pre-absorbing the Fab libraries with BPV cells. These cells do not express CFTR, but should express all other potential antigens present in 2wt cells. Fab fragments that have not bound to BPV cell antigens could then be selected against 2wt cell antigens of which CFTR would be novel. So far the effective panning of the murine derived Fab libraries has been prohibited by the repeated lysis of the phage-antibody library cultures. This problem will have to be overcome before the potential of the Fab libraries as sources of antigen specific Fab fragments can be fully assessed. As discussed in the previous chapter, the lysis of the cultures is probably due to the presence of a contaminating lytic phage. A possible way of avoiding this lytic phage infection is discussed in the next chapter.

CHAPTER 8

PRODUCTION AND ANALYSIS OF Fab ANTIBODY LIBRARIES FROM RABBITS IMMUNISED WITH CFTR FUSION PROTEINS

8.1 INTRODUCTION

Tissue rich in antibody producing cells, such as spleen, lymph nodes, tonsils and bone marrow, is required for the construction of antibody phage display libraries, [Winter et al., 1994; Roitt, 1993]. Bone marrow has been identified as a major repository of differentiated B lymphocytes that produce antibodies to maintain circulating antibody titres [Lum et al., 1990]. Fab fragments have been successfully selected from antibody libraries produced from human bone marrow [Burton et al., 1991; Barbas et al., 1992b; 1992c; Williamson et al., 1993]. Rabbit bone marrow has also been used as the source of mRNA for the construction phage display antibody libraries (Carlos Barbas (Scripps Institute, La Jolla, California) pers. comm.).

Polyclonal antibodies have been raised in rabbits to three β-galactosidase/CFTR fusion proteins (see chapter 3). Characterisation of these antibodies indicates that they recognise CFTR and/or other protein(s) that have very similar properties to CFTR (see chapter 4). The three fusion proteins cover almost the entire CFTR sequence as represented in fig. 8.1. Two rabbits were immunised with all three fusion proteins. The pComb3H-SS vector system was then adopted for the construction of a Fab fragment phage display library derived from the bone marrow of immunised rabbits.

Selection of Fab libraries described in the previous two chapters has been inhibited by the deleterious infection of phage library cultures with lytic phage. The use of freshly prepared and sterilised materials failed to remove the lytic phage contamination. An alternative approach to overcoming this problem was to produce a cell line resistant to the contaminating lytic phage. The construction of the rabbit derived phage display antibody library and its culture

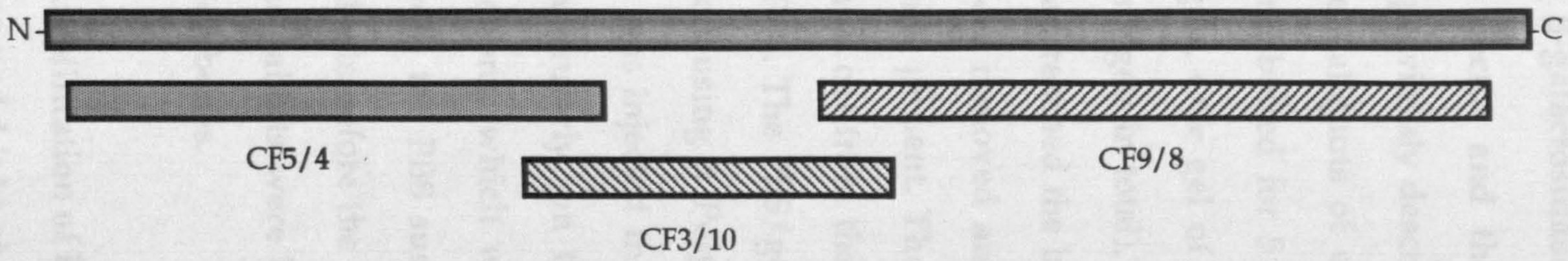


Fig. 8.1 Cartoon indicating the positions of the regions of CFTR cDNA amplified by PCR and sub-cloned into pUEX. The upper box represents the complete protein sequence of CFTR, the lower boxes represent the regions of that sequence which are encoded by the pUEX constructs designated CF5/4, CF3/10 and CF9/8.

in this cell line is described below.

8.2 MATERIALS AND METHODS

8.2.1 Rabbit Immunisation

The β-galactosidase/CFTR fusions were constructed in the pUEX1 expression vector and the fusion proteins were prepared from bacterial cultures as previously described in sections 3.2.1 - 3.2.4.

Frozen aliquots of each of the three fusion protein preparations were defrosted and boiled for 5 minutes prior to loading onto preparative 8% SDS page minigels. One gel of each of the fusion proteins was required for each rabbit (i.e. six gels in total). The gels were run at 120mA until the loading buffer dye front had reached the bottom of the gel. Proteins from strips off one edge of each gel were removed and stained in Coomassie blue solution to reveal the protein bands present. The overexpressed bands of the fusion proteins were identified and cut from the unstained gels, shredded using scalpel blades and soaked in PBS. The PBS/gel solutions from each of the gels were pooled and homogenised using a Polytron homogeniser with PT10 head. The resultant suspension was injected into two New Zealand white rabbits, subcutaneously and intramuscularly, on three occasions at monthly intervals. Prior to the fourth injections, which were given intravenously, the acrylamide gel was removed from the PBS suspension by centrifugation. Test bleeds were taken from the rabbits before the first injection (pre-bleeds). Ten days after the fourth injection the rabbits were bled out and the bone marrow was removed from their hind leg bones.

8.2.2 PCR Amplification of Rabbit Antibody Sequences

Heavy and Light chain antibody fragments were amplified by PCR as described in section 6.2.2.1. Rabbit immunoglobulin primers were synthesised based on sequences designated by Carlos Barbas III, Scripps Institute, La Jolla,

California.

Heavy chain Fd 3′ primers:

IgG 5′- TGGGCAACTAGTCTTGCTGCATGTCGAGGG -3′

IgM 5′- GCTCACACTAGTGGGCAGCTCTGAGTCCA -3′

Heavy chain variable domain 5′ primers:

RVH1 5′- CAGTCGBTGCTCGAGTCCGGGGGTCGCCT -3′

RVH2 5′- CAGTCGBTGCTCGAGTCCGGGGGAGGC -3′

RVH3 5′- CAGTCGBTGCTCGAGTCCGGGGGAGAC -3′

Where B= C, G or T

Light chain constant domain 3′ primers:

RCK1 5′- GCGCCGTCTAGATTAACAGTCACCCCTATTGAAGC -3′

RCK2 5′ GCGCCGTCTAGATTAACAGTCACCCCTATTGAAGC -3′

Light chain variable domain 5′ primers:

RVK1 5′- GCGCCGGAGCTCGTGATGACCCAGACTCCA -3′

RVK2 5′- GCGCCGGAGCTCGATATGACCCAGACTCCA -3′

RVK3 5′- GCGCCGGAGCTCGTGATGACCCAGACTGAA -3′

8.2.3 Preparation of a Cell Line Resistant to Lysis by Contaminating Phage

A cell line that was resistant to lysis by contaminating phage was prepared to enable several rounds of panning for selection of antigen specific Fab fragments from the antibody phage display library. Considerable cell lysis occurred in cell cultures that had been been infected with the Fab library and rescued. This lysis was apparent in the reduction in the turbidity of the cell culture solutions and by the presence of phage plaques on LB amp agar plates coated with a lawn of cells from the liquid cultures. These lytic phage containing plaques were used to prepare XL1-blue *E.coli*. that were resistant to

lysis by the contaminating phage.

A plaque was picked from the surface of a contaminated plate and transferred to 1ml Minimal media in an Eppendorf tube which was, in turn, vortexed. Serial hundred fold dilutions were made of the phage containing minimal media. 1μl of each dilution was transferred to 100μl log phase XL1-blue cells, then left at room temperature for 15 minutes before plating out onto LB plates. The plates were incubated overnight at 37°C. The following day, a plate was chosen where the dilution of the contaminating lytic phage was such that almost the entire lawn of *E.coli*. had been lysed. A few bacterial colonies were growing within the obvious phage plaque areas on the plate. These represented mutant *E.coli*. that were resistant to phage lysis. Five such colonies were picked and grown in 10ml aliquots of LB broth overnight in a shaking incubator at 37°C. 50μl of the cultures were plated out the next day on each of LB amp, LB tet, LB kan and Minimal media agar plates. Further 50μl aliquots of each culture were infected with 1μl of the undiluted lytic phage containing minimal media (described above) which, in turn, were plated out onto LB agar plates. All the plates were incubated overnight at 37°C. Growth of untransformed XL1-blue cells resistant to the contaminating lytic phage would have been anticipated on the LB tet, Minimal media and LB (post infection) agar plates, but not on the LB amp or LB kan agar plates. Cells thus produced could be cultured and prepared for DNA transformation by electroporation as previously described, section 2.2.13 method B.

8.2.4 Preparation of Panning Wells for Fab Antibody Library Selection

The three β-galactosidase/CFTR fusion protein antigens were prepared from mini SDS PAGE gels as described for the preparation of antigen for the final intravenous injection of the rabbits (see section 8.2.1 above). The supernatant recovered from the gel/PBS suspension was transferred to a Centricon 30 column for concentration by centrifugation in a Sorvall RC5C at

5000rpm and 4°C for about 40 minutes until the volume was reduced to about 150μl. The protein concentration of the concentrated solution was determined by Bradford's analysis (section 2.2.20) and wells of an ELISA plate were coated overnight at 4°C with 1μg of fusion protein antigen diluted in 0.1M bicarbonate coating buffer pH8.6.

8.3 RESULTS

8.3.1 Rabbit Immunisation

Two New Zealand white rabbits were each immunised with three β-galactosidase/CFTR fusion proteins that between them contain the majority of natively expressed CFTR sequence. The bone marrow was extracted from the hind legs of the rabbits and used as the source of mRNA in the construction of a phage display antibody library. Bleeds were taken from the rabbits pre- and post- immunisation. These sera were designated 38 and 49 according to rabbit code numbers.

8.3.2 ELISA Analysis of Rabbit Antisera

The rabbit antisera were diluted 1 in 400 and screened by ELISA against the fusion protein antigens as described in section 2.2.25. The post-immunisaton antisera 39 and 43 both gave colormetric readings that were about three times greater than those obtained using either pre-immunisaton antisera as the first antibody.

8.3.3 Western Blot Analysis of Rabbit Antisera

The rabbit antisera were screened against Western blots of membrane lysate derived from HT29 cells which have previously been shown to express CFTR [Denning et al., 1992]. The post-immunisation antisera from both rabbits recognised a 170kDa protein on Western blots that was not recognised by the

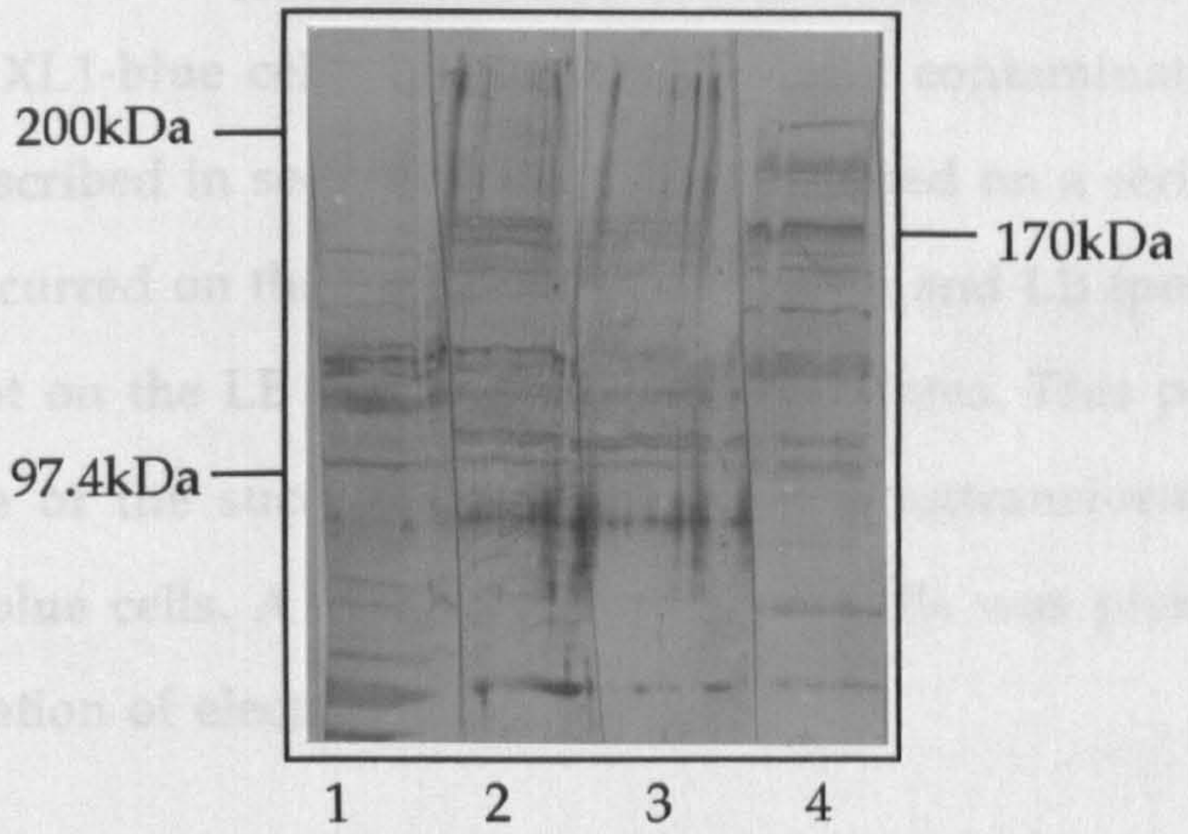


Fig. 8.2 Immunoblots of HT29 membrane lysate probed with polyclonal 39 (lane 2), or polyclonal 43 (lane 4) as primary antibody. Premmune sera from rabbit 39 (lane 1), or 43 (lane 3) were used as controls. The relative mobilities of molecular weight standards, loaded in an adjacent lane, are indicated.

pooled pre-bleed antisera see (fig.8.2). 170kDa is the expected size of the fully glycosylated form of CFTR [Gregory et al., 1990].

8.3.4 Cells Resistant to Lysis by Contaminating Phage

Mutant XL1-blue cells, resistant to lysis by contaminating phage, were cultured as described in section 8.2.3. When analysed on a series of agar plates growth had occurred on the LB tet, Minimal media and LB (post infection) agar plates, but not on the LB amp or LB kan agar plates. This pattern of growth was indicative of the successful preparation of untransformed, lytic phage resistant XL1-blue cells. A single clone of these cells was picked and cultured for the preparation of electro-competent cells.

8.3.5 Construction of a Rabbit derived Fab Antibody Library

A random combinatorial Fab antibody library was constructed in the pComb3H-SS vector from mRNA extracted and purified from bone marrow extracted from the immunised rabbits. The methods followed for the construction of this library are described in chapter 6. The mRNA derived from each of the two rabbits was independently reverse transcribed and amplified by PCR. PCR reactions with different 5′ and 3′ pairs of heavy and light chain primers were carried out separately to maximise the amplification of all appropriate mRNA sequences. However, after examination of the amplified sequences by agarose gel electrophoresis, all the light chain PCR products stemming from both rabbits were pooled, as were all the heavy chain PCR products, for the construction of a single rabbit derived antibody Fab library.

Test ligations were performed prior to the construction of the light and heavy chain libraries to assess the extent of the background vector ligation. The restricted heavy and light chain sequences were purified on Chromaspin 100 columns prior to ligation into the prepared vector. The light chain background vector ligation was 8% of that of the test insert ligation and the size of the

assembled light chain library was estimated to be $7x10^6$ independent clones. The pComb3H-S/light chain vector was prepared employing the modified restriction conditions described in section 7.3.3 of the previous chapter. The heavy chain background vector ligation was 2.5% that of the test insert ligation and the size of the assembled Fab library was estimated to be $8x10^6$ independent clones. Both the initial light and final Fab libraries were transferred by electroporation to freshly prepared lytic phage resistant XL1-blue cells.

8.3.6 Sequencing Analysis of Antibody Fragments

Phagemid DNA was prepared from eight Fab library colonies by Wizard Mini Preps and sequenced with the heavy and light chain primers given in section 6.2.8.1. The DNA sequences were all compared to sequences in the EMBL/Genbank data base, release number 39, version 6. The five most homologous sequences identified for each sequenced antibody chain are given in figs. 8.3a and b.

8.3.7 Panning the Fab Library

Enrichment of the Fab library for antibody fragments that bound to the CFTR fusion protein antigens was attempted by consecutive rounds of selection by panning. After each round of panning, the eluted phage were reinfected into mutant lysis resistant *E.coli.* and rescued with helper phage. Four rounds of panning were completed without the lysis of cell cultures that had been encountered on previous occasions (sections 6.3.3. and 7.3.5). Enrichment of phage was not detected during the panning process. The colony forming units (cfu) put in and recovered from each round of panning were:

ROUND 1	In - 2.7×10^{11}	Out - 4.8×10^7
ROUND 2	In - 6.3×10^{11}	Out - 2.8×10^6
ROUND 3	In - 2.1×10^{12}	Out - 1.6×10^5
ROUND 4	In - 1.4×10^{12}	Out - 1.6×10^4

Fig. 8.3a (pages 188 -191). **Light chain sequences 1 to 8, of Fab clones derived from the rabbit antibody library.**

Fig. 8.3b (pages 192 -195). **Heavy chain sequences 1 to 8, of Fab clones derived from the rabbit antibody library.**

The five most homologous sequences identified from the EMBL/Genbank data base, release number 39, version 6, for each light or heavy chain, are presented in tables below each sequence.
Further information and references for each of the data base sequences given, can be obtained from the EMBL/Genbank data base, via the appropriate accession number.

Fig. 8.3a Rabbit Light Chain l. Length: 143

```
   1  gtgtctgcag ctgtgggagg cacagtcacc atcaattgcc aggccagtga
  51  gaacatttat aacaatttag acctggtatc aacagaaacc agggcagcgt
 101  cccaagctcc tgatctacca ggcatccaaa ctggcatctg ggg
```

	Accession number	Description	% Homology
i	K01359	Rabbit Ig kappa (b9) complete mRNA.	90.2
ii	S50460	b41=immunoglobulin kappa L chain variable region (Oryctolagus cuniculus=rabbits).	87.4
iii	X14365	Rabbit vkB95r gene for immunoglobulin kappa light chain variable region.	87.4
iv	X00032	Rabbit mRNA for immunoglobulin kappa light chain b5 allotype.	86.7
v	K02131	Rabbit Ig germline kappa V-region V-kappa 20 and flanks.	85.2

Fig. 8.3a Rabbit Light Chain 2. Length: 163

```
   1  ctccagcctc cgtgtctgca gctgtgggag gcacagtcag catcagttgc
  51  cagtccagtg agagtgttta taagaacaac tacttatcct ggtatcagca
 101  gaaaccaggg cagcctccca agctcctgat ctattctgca tccactctgg
 151  catctggggt ccc
```

	Accession number	Description	% Homology
i	K02131	Rabbit Ig germline kappa V-region V-kappa-20 and flanks.	92.6
ii	X00977	Rabbit germ-line V kappa gene V18a.	89.6
iii	K02438	Rabbit germline gene V18b for immunoglobulin kappa light chain variable region.	89
iv	K02439	Rabbit germline gene V19a for immunoglobulin signal peptide; variable region	88.3
v	K01359	Rabbit Ig kappa (b9) complete mRNA.	88.3

Fig. 8.3a Rabbit Light Chain 3. Length: 220

```
   1  ctccatcctc cgtggaggca gttgtgggag gctctgtcac catcaagtxc
  51  caggccagtc agagcattag txcctactca tcctggtatc agcagaaacc
 101  agggcagcxt xccaagctcc tgatctacag ggccatccac tctggcatct
 151  ggggtctcat cgcggttcaa aggcagtgga tctgggacag agttcactct
 201  caccatcagc gacctggagt
```

	Accession number	Description	% Homology
i	X00032	Rabbit mRNA for immunoglobulin kappa light chain b5 allotype immunoglobilin.	87.3
ii	X14365	Rabbit vkB95r gene for immunoglobulin kappa light chain variable region.	86.8
iii	K01280	Rabbit Ig kappa light chain (bas) V-J-C mRNA.	86.3
iv	X02337	Rabbit germline gene V19a for immunoglobulin kappa light chain variable region.	85.9
v	K01359	Rabbit Ig kappa (b9) complete mRNA.	85.8

Fig. 8.3a Rabbit Light Chain 4. Length: 275

```
   1  ttccacgtct gcggctgtgg gaggcacggt caccatcaac tgccagtcca
  51  gtcagagtgt ttatggtaac accgcttagc ctggtatcag cagaaaccag
 101  gtcagcctcc caagctcctg atctacaggg catccaatct ggaatctggg
 151  gtcccatcgc ggttcaaagg cagtggatct gggacacagt tcactctcac
 201  catcagcgac ctggagtgtg acgatgctgc cacttactac tgtgcaggcg
 251  gtagtactgg tgagatttat ggttt
```

	Accession number	Description	% Homology
i	X00977	Rabbit germ-line V kappa gene V18a.	90.3
ii	K01358	Rabbit Ig kappa L-chain b4 allotype, mRNA.	86.7
iii	X02336	Rabbit germline gene V18b for immunoglobulin kappa light chain variable region.	86.5
iv	X14365	Rabbit vkB95r gene for immunoglobulin kappa light chain variable region.	84.3
v	X00032	Rabbit mRNA for immunoglobulin kappa light chain of b5 allotype immunoglobulin.	83.4

Fig. 8.3a Rabbit Light Chain 5. Length: 181

```
  1  ctgaatcgcc cgtgtctgcg gctgtgggag gcacagtcaa ggtattagta
 51  gtagctactt atcctggtat cacgagaaac cacgggaccg tcccaacgtc
101  ctgatctatg gtcgatcgaa actggcatct ggggtcccat cgcggttcaa
151  aggcggtgga tctgggacag agttcactct c
```

	Accession number	Description	% Homology
i	X00977	Rabbit germ-line V kappa gene V18a.	83.4
ii	X14365	Rabbit vkB95r gene for immunoglobulin kappa light chain variable region.	82.9
iii	K01358	Rabbit Ig kappa L-chain b4 allotype, mRNA.	82.9
iv	K01280	Rabbit Ig germline kappa V-region V-J-C mRNA.	82.3
v	X14364	Rabbit vkB95g gene for immunoglobulin kappa light chain variable region.	81.5

Fig. 8.3a Rabbit Light Chain 6. Length: 233

```
  1  ctccagcctc cgtgtctgca gctgtgggag gcacagtcac catcaactgc
 51  caggccagtc agagtgttta taataacaac ttcttatcct ggtatcagca
101  gaaaccaggg cagcctccca agctcctgat ctattatgca tccactctgg
151  catctggggt cccatcgcgg ttcaaaggca gtggatctgg gacacagttc
201  actctcacca tcagcgatgt ggtgtgtgac gat
```

	Accession number	Description	% Homology
i	K02131	Rabbit Ig germline kappa V-region V-kappa-20 and flanks.	91.8
ii	X02336	Rabbit germline gene V18b for immunoglobulin kappa light chain variable region.	90.6
iii	X00977	Rabbit germ-line V kappa gene V18a.	90.6
iv	X02337	Rabbit germline gene V19a for immunoglobulin kappa light chain variable region.	89.7
v	X14364	Rabbit vkB95g gene for immunoglobulin kappa light chain variable region.	89.3

Fig. 8.3a Rabbit Light Chain 7. Length: 138

```
  1  aactatggga ggcacagtca ccatcaagtg ccatgccagt gaaagcatta
 51  gcaactactt agcctggtat cagcagacac caggacagct ccaagtcctg
101  atctattatg catccactct ggcatctcgg ggtctcat
```

	Accession number	Description	% Homology
i	K02439	Rabbit germline gene V19a for immunoglobulin kappa light chain signal peptide variable region.	87.2
ii	J00667	Rabbit Ig kappa L-chain b4 allotype, mRNA.	86.5
iii	K01359	Rabbit Ig kappa (b9) complete mRNA.	85.8
iv	K01280	Rabbit Ig kappa light chain (bas) V-J-C mRNA.	85.1
v	X14365	Rabbit vkB95r gene for immunoglobulin kappa light chain variable region.	84.4

Fig. 8.3a Rabbit Light Chain 8. Length: 177

```
  1  ctccaccttc cgcgtctgaa cctgtgggag gcacagtcac catcaagtxc
 51  caggccagtg aggacattta cagctactta tcctggtatc agcagaaacc
101  aggxcagcct cccaagtctc atctatgata catccgatct ggcatctggg
151  gtcacatccc gcggttcaaa ggcagca
```

	Accession number	Description	% Homology
i	K01359	Rabbit Ig kappa (b9) complete mRNA.	87.2
ii	X00032	Rabbit mRNA for immunoglobulin kappa light chain of b5 allotype immunoglobulin.	86
iii	X14365	Rabbit vkB95r gene for immunoglobulin kappa light chain variable region.	84.2
iv	X14364	Rabbit vkB95g gene for immunoglobulin kappa light chain variable region.	83.1
v	K01280	Rabbit Ig kappa light chain (bas) V-J-C mRNA.	81.6

Fig. 8.3b Rabbit Heavy Chain 1. Length: 246

```
   1  gggggtcgcc tcttcaagcc agcggcctcc ctgacactca cctgcgcagt
  51  ctctggaatc gacctcagta ggaatccaat gagctgggtc cgccaggctc
 101  cagggaaggg cctgaatgga tcggaatcat ttatgataat ggtgacacat
 151  tctacgcgag ctggacgaaa ggccgattca ccatcaccag aaacaccaac
 201  gagaacacgg tgactctgaa aatgaccagt ctgacagccg cggaca
```

	Accession number	Description	% Homology
i	L03903	Rabbit immunoglobulin rearranged H-chain V-region, D-region and C-region complete cds.	86.6
ii	L03865	Rabbit immunoglobulin rearranged H-chain V-D-C region mRNA, 5' partail cds.	86.2
iii	M93172	Oryctolagus cuniculus Ig germline H-chain gene, V-region (VH1-a2), 5' end.	86.2
iv	M77079	O.cunniculus rearranged IgH heavy chain mRNA, VDJ region,allotype VHa2.	86.2
v	L03877	Rabbit immunoglobulin rearranged heavy chain V-D-C region gene, partial sequence.	86.2

Fig. 8.3b Rabbit Heavy Chain 2. Length: 301

```
   1  ggggagacc tggtcacgcc tgggacaccc ctgacactca cctgcaccgt
  51  ctctggattc tccctcagta gctactggat gaattgggtc cgccagcctc
 101  cagggacggg gctggagtgg atcggagtca ttaatagtgt accacaaggt
 151  acgcgaggtg ggcgaaaggc cgatttatta tctccaaaac ctcgtcgagt
 201  acggtggatc tgctgatgac cagtccgaca agcgaggaca cxxccaccta
 251  tttctgtgcc agagggctag atggtacaat actgcctggg ccagtatgggg
```

	Accession number	Description	% Homology
i	L27322	Oryctolagus cuniculus intervening DNA sequence.	86.2
ii	L03887	Rabbit immunoglobulin rearranged H-chain V,D,and C regions.	86.2
iii	L03877	Rabbit immunoglobulin rearranged H-chain V,D,and C regions.	86.2
iv	S63477	VH1-a1=immunoglobulin a1 VH region (rabbits, leukemic B cells, Genomic, 511nt).	85.6
v	M29947	Rabbit Ig rearranged H-chain (5.5) gene V-J-D region.	85.6

Fig. 8.3b Rabbit Heavy Chain 3. Length: 248

```
   1  gagtccgggg gtcgcctggt cacgcctggg acacccctga cactcacctg
  51  cacagtctcc ggaatcgacc tcagtagtta tagaatgggc tgggtccgcc
 101  aggctccagg gaaggggctg gaatggatcg gagtcattaa tactgatact
 151  agtagatatt acgcgagctg ggcgaaaggc cgattcacca tctccaaaac
 201  ctcgaccacg gtggatctga aaatcaccag tccgacaacc gaggacac
```

	Accession number	Description	% Homology
i	M93171	Oryctolagus cuniculus Ig germline H-chain gene, V-region (VH1-a1), 5' end.	91.5
ii	M29946	Rabbit Ig rearranged H-chain (5C3) gene V-D-J region.	91.5
iii	M29947	Rabbit Ig rearranged H-chain (5.5) gene V-D-J region.	91.5
iv	M29948	Rabbit Ig rearranged H-chain (4K7) gene V-D-J region.	91.5
v	X52450	O.cunigulus gene for VHa1.	91.5

Fig. 8.3b Rabbit Heavy Chain 4. Length: 291

```
   1  ccgggggagg cctaatcacg cctgggacac ccctgacact cacctgcaca
  51  gtctctggat tctccctcag tttctatgcc gtgacctggg tcgccaggct
 101  ccaggggagg ggctggaatg gatcggaacc attactacca ataataactc
 151  atactacgcg aactgggcga aaggccgatt caccatctcc aaaacctcga
 201  ccacggtgga tctgaaaatc accagtccga caaccgagga cacggccacc
 251  tatttctgtg ccaggatata tgctgcttat agtatgactg a
```

	Accession number	Description	% Homology
i	L27315	Oryctolagus cuniculus intervening DNA sequence.	87.7
ii	M21261	Rabbit Ig H-chain V-region (allotype VHa1)mRNA (V), clone RVH142.	86.4
iii	M60133	Rabbit (a2a2 haplotype) Ig H-chain V-region (allotype VHa1) gene, clone RVH799.	84.5
iv	M93177	Oryctolagus cuniculus Ig germline H-chain pseudo gene, V-region.	83.6
v	M21262	Rabbit Ig H-chain V-region (allotype VHa1) mRNA (V-D-J), clone RVH138.	83.4

Rabbit Heavy Chain 5. Length: 166

```
  51  gattctccct cagtaactat ggagtgagct gggtccgcca ggctccaggg
 101  aaggggctgg aatggatcgg aatcattagt agtagtggta ctacatacta
 151  cxcxacctgg gcgaaa
```

	Accession number	Description	% Homology
i	M29946	Rabbit Ig rearranged H-chain (5C3)) V-D-J region.	94
ii	M29948	Rabbit Ig rearranged H-chain (4K7) V-D-J region.	94
iii	M29947	Rabbit Ig rearranged H-chain (5.5) gene V-D-J region.	94
iv	X52450	O.cunigulus gene for VHa1.	94
v	M93171	Oryctolagus cuniculus Ig germline H-chain gene, V-region (VH1-a1), 5' end.	94

Rabbit Heavy Chain 6. Length: 207

```
   1  gagtccgggg gtcgcctggt cacgcctggg acacccctga cactcacctg
  51  cacagtctct ggaatcgacc tcagtagcta tgcagcgggc tgggtccgcc
 101  aggctccagg gaaggggctg gaatacatcg gaatcattga tcctagtggt
 151  cgcacatact agcgcgactg ggcgaaaggc cgatccacca tctccaaaac
 201  ctcgtcg
```

	Accession number	Description	% Homology
i	M93181	Oryctolagus cuniculus Ig germline H-chain gene, V-region (VH4-a1).	92.3
ii	M29948	Rabbit Ig rearranged H-chain (4K7) gene V-D-J region.	91.8
iii	M93171	Oryctolagus cuniculus Ig germline H-chain gene, V-region (VH1-a1), 5' end.	91.8
iv	M29946	Rabbit Ig rearranged H-chain (5C3) gene V-D-J region.	91.8
v	M29947	Rabbit Ig rearranged H-chain (5.5) gene V-D-J region.	91.8

Rabbit Heavy Chain 7. Length: 216

```
  1  gggggtcgcc tggtcacgcc tgggacagcc ctgacactca cctgcacagc
 51  ctctggattc tccctcacaa agtattggat gtactgggtc cgccagctcc
101  agggaagggg ctggaatgga tcggagtcat taacactggt ggtggcacat
151  ggtacgcgag ctgggcaaaa ggccgattca acatctccag aacctcgacc
201  acggtggatc tgaaaa
```

	Accession number	Description	% Homology
i	L03887	Rabbit immunoglobulin rearranged heavy chain V-D-C region.	89.5
ii	L27322	Oryctolagus cuniculus intervening DNA sequence.	89.5
iii	M60129	Rabbit (a2a2 haplotype) Ig H-chain V-region (allotype VHa1) gene, clone RVH732.	85.7
iv	M21261	Rabbit Ig H-chain V-region (allotype VHa1) mRNA (V), clone RVH142.	84.8
v	M12180	Rabbit Ig germline CL42.VH25 and CL.42VH34 genes, VHA3 allotype, 5' end.	84.3

Rabbit Heavy Chain 8. Length: 313

```
  1  ctcgagtccg ggggagacct ggtaacgcct ggaggaaccc tgacactcac
 51  ctgcacagtc tctagattct ccctcaatac ctatccaatc acctgggtcc
101  gccaggtccc agggaagggg ctggaatgga tcgggatcat ttatcctaat
151  actgatacat actacgcgaa cagggcgaaa ggccgattca ccatctccaa
201  aacctcgtcg accacggtgg atctgaaaat gaccagtctx xxaaccgagg
251  acacggcccc tatttctgtg ccagagggga ggctcatgtt ggtagtgatt
301  attcagacta ctt
```

	Accession number	Description	% Homology
i	M93181	Oryctolagus cuniculus Ig germline H-chain gene, V-region (VH4-a1).	86.5
ii	L03887	Rabbit immunoglobulin rearranged H-chain V-region, D-region and C-region.	84.9
iii	L27322	Oryctolagus cuniculus intervening DNA sequence.	84.9
iv	M12180	Rabbit Ig germline CL42.VH25 and CL.42Vh34 genes, VHA3 allotype, 5'end.	83.5
v	M29417	Rabbit Ig rearranged gamma-chain mRNA.	83.2

8.3.8 ELISA Analysis of Phage Cultures

The 'polyclonal' phage supernatants recovered from the initial transformed library cell culture and from the cell culture reinfected with phage eluted from the fourth round of panning were screened for binding to antigen by ELISA. This experiment was repeated in order to validate the results. The absorbancy readings at 410nm for the ELISA with the post fourth pan phage were 0.976 and 0.769 and the corresponding readings for the ELISA with unpanned phage were 0.591 and 0.563.

8.4 DISCUSSION

Positive binding of rabbit antisera 39 and 43 to the antigens to which the rabbits had been immunised was indicated by ELISA. Immunoblot analysis of these antisera revealed that they recognised a ~170 kDa protein which is the expected molecular size of fully glycosylated CFTR [Gregory et al., 1990]. Both of these results were consistent with the assumption that the rabbits had raised an immunogenic response to the CFTR components of the β-galactosidase/CFTR fusion proteins with which they were immunised. It was therefore anticipated that a phage display Fab library derived from these immunised rabbits would be biased towards the expression of antibody fragments that recognised regions of CFTR.

The successful construction of a phage display library expressing Fab fragments was confirmed by sequence analysis of 8 randomly selected library clones. Each sequence had considerable homology (>80%) to at least 5 previously identified rabbit antibody sequences. Some overlaps were seen between the top 5 homologous sequences found for the heavy and light chain sequences analysed. However, this does not necessarily mean that the diversity of this rabbit Fab library is limited. It is more likely a reflection of the fact that there are far fewer rearranged rabbit antibody gene sequences in the

EMBL/Genbank data, compared, for example, to murine counterparts.

XL1-blue cells that were resistant to contaminating lytic phage lysis were successfully prepared. The culture of the Fab library in these cells enabled consecutive rounds of phage-antibody reinfection and rescue which had been previously been prohibited (see chapters 6 and 7). The library was selected by panning for Fab fragments that bound to the fusion protein antigen. ELISA analysis indicated that the enriched phage bound antigen more strongly than the unpanned phage. This initial result was obtained with the polyclonal phage suspension. Further studies need to be carried out to ascertain whether individual Fab clones bind to the antigen.

The pComb3H-SS vector is designed such that free soluble Fab fragments can be expressed in *E.coli*. Removal of the g3p DNA from pComb3H/Fab vector DNA can be achieved by restriction with *Spe* 1 and*Nhe* 1 followed by direct religation of the compatible ends. The heavy and light antibody chains assemble in the periplasm of the *E.coli*. and soluble Fab fragments can be recovered from the culture supernatant or from bacterial lysate preparations [Burton et al., 1991]. Once individual positive Fab clones have been identified by ELISA, the specificity of their antigen binding will have to be analysed. Identification of which component of the fusion protein antigen mixture particular Fab fragments recognise, can be achieved by performing separate ELISA tests against each of the three β-galactosidase/CFTR fusion proteins and expressed β-galactosidase alone.

In conclusion, XL1-blue cells, apparently resistant to the lytic phage contamination that was disrupting previous Fab phage display antibody work, have been successfully cultured. This enabled antigen selection and binding studies to be performed with the rabbit derived, Fab antibody library. The results obtained so far augur well for the use of this library as a source of specific anti-CFTR antibodies. The mutated strain of XL1-blue cells will also facilitate further analysis of the murine derived Fab libraries described in

chapters 6 and 7.

CHAPTER 9

DISCUSSION

9.1 The production of CFTR Specific Antibody Libraries in *E.coli*..

The emphasis of this study has been on the construction of antibody expression libraries for the isolation of anti-CFTR antibody fragments. A variety of problems were overcome to enable the construction of five phage display libraries from sources immunised to CFTR. Each of these libraries appears to contain an appropriate diversity of heavy and light chain antibody sequences. Initial difficulties due to the contamination of the libraries with a lytic phage have been successfully addressed such that characterisation of the libraries by selection against specific antigens is now possible.

9.2 Progress in the Production of Phage Display Libraries

Antibody libraries based on phage expression were first described in 1990 [McCafferty et al., 1990] and rapid progress has followed leading to the construction of increasingly elaborate systems (see section 1.2.6.3.6). A number of problems associated with the methodologies have been surmounted. Other complications, which at present may inhibit a more widespread adoption of this technology, are gradually being overcome. Some of these complications are discussed below and, where appropriate, they are applied to the systems adopted in this study

9.2.1 PCR Amplification of Antibody Gene Fragments

The diversity of a combinatorial library is critically dependent on the efficacy of the oligonucleotide primers used to amplify the immunoglobulin gene fragments by PCR. The escalation of available information on immunoglobulin gene sequences has enabled the design of optimised PCR

primer sets that encompass essentially all heavy and light chain gene families [e.g. Marks et al., 1991a; Zhou et al., 1994]. However, certain complications are still intrinsically associated with the system.

It is likely that some heavy and light chain genes are primed relatively less efficiently. Coupled with the inherent amplification in PCR, this may lead to the under-representation of these chains in an antibody library. This problem can be reduced by performing separate PCR reactions with individual pairs of primers and pooling the products, rather than carrying out a single PCR reaction with a large mixture of primers [Sastry et al., 1989]. This method was adopted for heavy and light chain amplification prior to cloning into the pComb3H-SS phagemid vector (chapters 6, 7 and 8).

The introduction of *Taq* polymerase errors during PCR amplification may result in an altered antibody sequence that does not bind antigen as well as the original sequence found within an antibody producing B lymphocyte. This is most likely to be a problem in the amplification of sequences from clonal cell populations (see Chapters 5 and 6) which could be overcome with the use of Vent polymerase (sections 5.3 and 6.3) [Mattila et al., 1991]. However, mutations introduced during the PCR amplifications of heavy and light chain antibody sequences from a diverse repertoire of antibody producing cells may prove to be advantageous (section 6.4).

9.2.2 Reduction in the Toxic Effects on *E.coli.* of *in vivo* Antibody Production

In both the systems used in this study, the expression of the antibody fragments was under the transcriptional control of the *lac* promoter/operator. The *lac* promoter is repressible and has a relatively low leakage rate in the absence of induction, especially in the presence of glucose. This is particularly important in the production of antibody fragments to avoid toxic effects on *E.coli.* cells which, at least in part, are caused by incorrectly folded protein [Skerra et al., 1991; Knappick et al., 1993]. The stress caused to cells by the secretion of antibody fragments is an important consideration for the

maintenance of representative libraries. The problems associated with such instability of phage display antibody libraries have been recognised in the research community [Rapoport et al., in press]. It is quite conceivable that a library could be depleted of an important antigen recognising clone as a result of the poor growth of the *E.coli.* harbouring that clone. The molecular basis of the poor *in vivo* folding of antibodies has recently been elucidated and antibodies have been engineered with an altered framework that do not produce any insoluble protein and do not have a detrimental effect on cell growth [Knappik,A. and Pluckthun,A., submitted]. Antibody framework regions designed for improved stability and folding ability may form an appropriate basis for the production of semi-synthetic libraries in the future.

9.2.3 Efficiency of Antibody Selection from Phage Display Libraries

The efficiency of antibody selection depends on a number of factors, including the affinity of antigen binding, the number of antibody fragments on a single phage that can simultaneously bind antigen and the extent of non-specific binding to the antigen coated surface.

It has been demonstrated that the binding affinity of a single antibody fragment needs to be greater than 10^5 mol^{-1} to hold the phage fragment carrying it to a solid surface [Clackson et al., 1991]. A number of methods that have been adopted to increase the affinity of antibody binding were outlined in Chapter 1 (specifically section 1.2.6.4.6). One of these is based on theoretical studies that suggest that the larger an antibody repertoire, the greater the prospect of finding higher affinity antibodies to a given epitope [Perelson et al.,1979]. Given that the heterogeneous libraries produced in this study (Chapters 7 and 8) were from immunised animal sources and contained $4x10^6$ - $8x10^6$ clones, it is anticipated that they should contain high affinity antibodies to the appropriate immunogens (pers. comm. Carlos Barbas and Dennis Burton (Scripps Inst., La Jolla, California)).

The pCantab5 phagemid vector system could potentially facilitate the expression of more than one antibody fragment on the surface of an individual phage. This was not expected with the pComb3H-SS phagemid vector system [Barbas et al., 1991]. The advantage of multiple expression of antibody fragments is that low affinity antibodies may be selected on the basis of cumulative avidity [Griffiths et al.,1993]. If low antibody affinity is a problem, this can facilitate the selection of antibodies that can subsequently be manipulated to improve individual antigen binding affinities. Antibody fragments with specific higher binding avidities have been designed, for example "mini-antibodies" [Pack and Pluckthun., 1992] and "diabodies" [Hollinger et al.,1993]. However, a monomeric expression system (such as pComb3H-SS) is preferable for the isolation of high affinity antibody clones that are intrinsically present in library [Barbas, 1993].

Non-specific binding of antibody fragments has proved to be a significant problem in the isolation of rare antigen binding clones from phage display libraries [Rapoport et al., in press; Griffiths et al., 1994]. This phenomenom may introduce complications in the selection of the libraries described in this study. A novel approach to selection which links antigen recognition to phage replication has been published recently [Duenas and Borrebaeck, 1994]. This method is based upon the expression of antibody fragments on phage, rendered non-infectious by g3p deletions. Antibodies were selected with an antigen/g3p fusion protein. The antigen specific phage were thus rendered replication competent because the antigen/g3p fusion protein was capable of infecting the F-pili of the *E.coli*. Thus only the phage displaying antigen specific antibodies could be clonally amplified. This method of selection may be useful for the isolation of antigen specific clones from the rabbit Fab library described in Chapter 8 if non-specific antibody binding proves to be a problem. Fusion proteins could be created between the three CF PCR products (CF3/10, CF9/8 and CF5/4) and g3p to promote antigen specific selection.

9.3 The Relative Merits of Fab and ScFv Antibody Libraries

Both Fab and ScFv have been shown to bind antigen successfully [Winter et al., 1994]. As previously described in section 1.2.1.2, ScFv fragments have proved to be invaluable in circumstances where the small size of an antibody fragment is particularly important. Nonetheless, in general circumstances, Fab fragments are probably more functional and reliable research tools.

The Fab fragments are held together by a native disulphide bridge. The heavy and light chains in ScFvs are covalently joined by a short peptide. The rationale behind linking the heavy and light chain variable regions together into a ScFv is to promote the correct folding of the Fv region in the periplasm of *E.coli.* by bringing V_H and V_L domains sufficiently close together to interact and fold [Glockshuber et al., 1990; Huston et al., 1993]. The 15 residue $(Gly_4Ser)_3$ linker was designed to be long enough to avoid strain on the native Fv conformation while restricting steric interference with the antigen/antibody combining site from an excessively long peptide.

The additional extrinsic peptide sequence present in ScFv may interact with the antigen binding site and have a deleterious effect. In addition the variable domain order (either V_H-linker-V_L or V_L-linker-V_H) is normally predefined. It has been predicted from theoretical principles and experimental results that the conformation of a single chain binding site could sometimes be sensitive to the order and orientation of the variable domains [Huston et al., 1993; Anand et al.,1991].

NMR data indicate that the linker is a passive entity and as such does not influence the folding state reached by the interacting heavy and light chain variable regions [Freund et al., 1993]. However, it does not contribute to the thermal stability of the fragments. The linker may keep the heavy and light chains in the same vicinity in the periplasm of *E.coli.* , but it does not have a steric effect in keeping them folded together to form an antigen binding unit.

The weak interaction between heavy and light chain variable regions is not enhanced by the linker. Interactions between the heavy and light chain fragments are much weaker for ScFv than for Fabs because of the absence of interchain disulphide bonds.

Comparison of antigen affinities of a number of different ScFv fragments and their corresponding Fabs shows that in the majority of cases the ScFv fragments have a lower affinity [Bird and Walker, 1991; Huston et al.,1991; Ducancel et al., 1993].

In the light of the disadvantages of ScFv fragments mentioned above, most of the effort in the production of antibody libraries in this study was applied to the construction of anti-CFTR Fab antibody libraries. It seems likely that in the future most phage display antibody systems will be based on the expression of Fab fragments rather than ScFvs. The most recent significant phage display antibody library paper published by Greg Winter's group, who up until now have concentrated on the development of ScFv phage display systems, describes the production of a phage library based on the expression of a large synthetic repertoire of Fab fragments [Griffiths et al., 1994].

9.3.1 The LKB-Pharmacia Recombinant Phage Antibody System

The LKB-Pharmacia kit described in Chapter 5 of this study is based upon the phage expression of ScFv antibody fragments. As such it is prone to the disadvantages outlined above (section 9.1.3). Working with this system revealed a number of other innate limitations.

As described in section 9.1.1, the successful PCR amplification of a repertoire of antibody gene fragments is enhanced by carrying out separate reactions with individual pairs of primers. In the LKB-Pharmacia kit the primers are supplied as a mixed pool such that the amplification of all heavy or all light chains can only be carried out in a single PCR. Such reactions have previously been shown to not be very effective [Sastry et al., 1989].

It is recognised that the number of clones in a phage display library

directly affects the potential of that library to contain specific antigen binding antibody fragments [Winter et al., 1994]. In turn, library size is directly dependent on the amount of DNA used in the library construction, the efficiency of ligation and the competency of the *E.coli.* cells to be transformed. However, none of these details are emphasised as important in the methods provided with the LKB -Pharmacia kit.

It would be advantageous to be able to express soluble antibody fragments which are not attached to the g3p protein as is possible in the pComb3H-SS expression system (see section 8.3). This was not possible with the vector provided in the original LKB-Pharmacia system, although a new vector pCANTAB 5E is now available which facilitates soluble expression of ScFv. This is accompished by the addition of an amber stop codon between the cloned ScFv and the g3p sequence. In *E.coli.* strains lacking an amber suppressor (e.g. HB2151, suggested by Pharmacia), the ScFv is expressed as a soluble molecule.

9.4 The Production of Antibodies to CFTR

Monoclonal and Polyclonal antibodies raised to CFTR have been described in this study. Attempts to establish the specificity of these antibodies have led to the interesting speculation of the existence of an immunologically conserved family of proteins that is structurally and perhaps functionally related to CFTR [Walker et al., submitted]. Further studies with these antibodies, together with anti-CFTR antibodies isolated from the constructed Fab libraries, should lead to the identification of members of this family and the subsequent assessment of the significance of these findings to the cystic fibrosis field.

9.5 Epitope Mapping of CFTR

A precise confirmation of the predicted topology of CFTR is of key importance in the understanding of its exact functions. Until recently no experimental verification had been obtained. One of the primary objectives of this study was to produce a panel of antibodies that could be used to epitope map the protein. Such studies have been successfully carried out on mammalian MDR [Yoshimura et al., 1989].

The first systematic experimental evaluation of the original topological model of CFTR was reported this year [Chang et al.,1994]. Here, glycosylation site insertion studies were used to map the cytoplasmic and extracytoplasmic loops between the membrane spanning domains of the protein. The predicted cytoplasmic or extracellular locations of the loops between the transmembrane regions was verified.

In the light of the encouraging ELISA results obtained from panned polyclonal phage from the rabbit derived Fab library (section 8.3.8), it is anticipated that clonally pure anti-CFTR antibodies will be successfully isolated from the antibody display libraries described in this study. If the specificity of these Fab fragments is suitable, they should prove to be useful tools in the further analysis of CFTR topology, and the cell and tissue distribution of the protein.

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