Development of a cloning strategy to enable the isolation of novel membrane proteins

A thesis submitted for the degree
of a doctor of philosophy
at Glasgow university

Arvind.M.Hundal

Institute of Genetics
Glasgow university
Church Street
Glasgow

June 1991

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

The research reported in this thesis is my own original work except where otherwise stated and has not been submitted for any other degree.

Arvind.M.Hundal

To mum, dad and my brother Anand
this little black book is not much reward for
all your support and encouragement over the years
thankyou

CONTENTS

Page No.

SUMMARY..... 1

CHAPTER 1: INTRODUCTION

1.1 Background 4
1.2 The biological membrane..... 5
1.3 The evolution of eukaryotic organelles..... 6
1.4 Protein targeting..... 8
1.5 Mechanism of protein translocation across the ER membrane..... 10
1.6 The signal recognition particle 11
1.7 The signal recognition particle receptor..... 13
1.8 The signal sequence receptor..... 14
1.9 The signal peptidase 16
1.10 Import of proteins into sub-cellular organelles
(i) Lysosome..... 17
(ii) Mitochondria..... 20
(iii) Chloroplast..... 24
(iv) Peroxisomes (microbodies)..... 26
(v) Nucleus..... 27
1.11 Anchoring and topography of membrane proteins.... 28
(i) Monotopic membrane proteins..... 30
(ii) Bitopic membrane proteins..... 30
(iii) Membrane anchoring via GPI (Glycosyl Phosphotidyl inositol) anchor..... 32
(iv) Polytopic membrane proteins..... 33
(v) Positive inside rule..... 34
1.12 Cloning membrane proteins..... 37
1.13 Aim of the project..... 40
1.14 The reporter protein..... 41
1.15 Experimental strategy..... 43

CHAPTER 2: MATERIALS AND METHODS

2.1 (i) Bacterial strains 45
(ii) Plasmids 45
(iii) Microbial culture media 47
(iv) Indicators..... 47
(v) Antibiotics..... 47
(vi) Buffer solutions..... 47
(vii) Organic solvents 48
(viii)Other solvents..... 48
(ix) Sterilisation conditions 48
(x) Microbial growth conditions 49

2.2 Transformation of *Escherichia coli*
(i) Preparation of competent cells and transformation of *E. coli* using $CaCl_2$..... 49
(ii) Preparation of frozen competent cells for bacterial electroporation and transformation of *E. coli* using electroporation..... 50

2.3 General DNA purification procedures
(i) Extraction of proteins from DNA samples with organic solvents..... 51
(ii) Concentration of DNA samples by precipitation
(a) Ethanol 51
(b) Isopropanol 52

2.4 Preparation of plasmid DNA
(i) Mini-preparations of plasmid DNA
(a) For characterisation and subcloning 52
(b) For double stranded DNA sequencing 53
(ii) Large scale preparation of plasmid DNA
(a) Alkaline lysis method 54
(b) Purification of covalently closed circular plasmid DNA using CsCl gradients 54

2.5 DNA gel electrophoresis
(i) Agarose gel electrophoresis 55
(ii) Visualisation and photography of gels..... 56
2.6 Isolation of DNA fragments from gels
(i) Electroelution 57
(ii) Extraction of DNA from LMP agarose slices 58
(iii) Purification of DNA fragments using Gene Clean..... 58
2.7 DNA manipulations
(i) Digestion of DNA with restriction endonucleases
(a) Complete digestion of DNA
(i) single 60
or (ii) multiple 60
enzymatic digestions
(b) Partial digestion of DNA..... 60
(ii) Ligation of DNA fragments..... 61
(iii) Phosphorylation of synthetic linkers and oligonucleotides..... 63
(iv) Dephosphorylation of vector fragments..... 63
(v) Filling recessed 3' ends of double stranded DNA..... 64
2.8 Double stranded DNA sequencing
(i) Primer annealing 64
(ii) Sequencing reactions 65
(iii) Sequencing gels 66
(iv) Autoradiography 68
2.9 Isolation of RNA
(i) Preparation of RNase free materials
(a) Apparatus 68
(b) Solutions 69
(ii) Extraction of RNA from cultured cells..... 69
(iii) Analysis of RNA on non-denaturing agarose gels..... 71
2.10 Blotting of agarose gels
(i) Denaturing/neutralising DNA gels for Southern blots..... 71

(ii) Pouring and running RNA gels for Northern blots.....72
(iii) Setting up blots and fixing nucleic acids to nylon filters 73
2.11 Hybridisation analysis of nucleic acids immobilised on nylon
(i) Preparation of probes from LMP agarose..... 74
(ii) Radiolabelling of DNA probes by random primed synthesis 74
(iii) Hybridisation of radioactive probes to nucleic acids immobilised to nylon filters..... 75
(iv) Stringency of washing 76
(v) Autoradiography 77
2.12 Mammalian cell culture
(i) Cell lines 77
(ii) Cell culture media 77
(iii) Cell culture conditions 78
(iv) Transfection of mammalian cells using DEAE-dextran 79
2.13 Screening of transiently expressing cell lines with monoclonal antibody
(i) Panning (a) preparation of panning plates..... 81
(b) panning cells 81
(c) Hirt extraction..... 82
(ii) Cytospinning and immunofluorescent staining..... 83
2.14 Protein analysis
(i) SDS-polyacrylamide gel electrophoresis..... 85
(ii) Staining Coomassie blue 86
(iii) Electroblotting (Westerns) 86
(iv) Immuno-detection of proteins 87
(v) Development of blots
(a) 4-Chloronaphthol..... 87
(b) Enhanced chemiluminesence..... 88

(vi) *in vitro* labelling of cells using ^{35}S Met..... 88
(vi) Immunoprecipitation..... 88

CHAPTER 3: CONSTRUCTION OF A SERIES OF EXPRESSION VECTORS ENCODING NATIVE AND RECOMBINANT CD2 PROTEINS

3.1 Introduction 93
3.2 Transfer of the CD2 gene from CDM7 to CDM8..... 94
3.3 Cloning of the external domain of CD2 into CDM8 95
3.4 Construction of the CD2 fusion protein 96
3.5 Sub-cloning of the external domain of the CD2 gene containing the EcoRI linker into the vector pMTL-21..... 98
3.6 Isolation of the transmembrane sequence of the HLA-B7 gene..... 98
3.7 Partial digestion of the plasmid (pDP001) containing the HLA-B7 gene..... 99
3.8 Construction of the pCDFus-20 clone containing the external domain of CD2 and the Tm sequence of HLA-B7.....102
3.9 Sub-cloning the CDFus-20 construct into CDM8 103
3.10 The CD2 cloning vector 104
3.11 Ligation of the external domain of CD2 and the stuffer into pMTL-20 105
3.12 Sub-cloning of the CD2 external domain and stuffer sequence into CDM8.....106
3.13 Verification of frame by plasmid sequencing of the fusion construct 106

CHAPTER 4: EXPRESSION STUDIES OF THE pCD2, pCDFus-8 AND pCDStuff-8 CONSTRUCTS IN COS CELLS

4.1 Introduction 108
4.2 Optimisation of transfections 109
4.3 Estimation of transfection efficiency 110

4.4 Attempts to demonstrate that the external domain of CD2 is secreted.....114
4.5 Northern analysis of the recombinant CD2 mRNAs 118
4.6 Immunoprecipitation of the CD2 recombinant proteins.....119
4.7 Immuno-blotting of the CD2 recombinant proteins 121
4.8 Discussion.....123

CHAPTER 5: OPTIMISATION AND PRELIMINARY SCREEN OF THE NOVEL-MEMBRANE PROTEIN CLONING STRATEGY

5.1 Introduction 125
5.2 Assessment of the efficiency of panning 125
5.3 Estimation of the background number of clones arising from random DNA sequences generating membrane anchoring segments.....128
5.4 Subcloning cDNAs into pCDStuff-8.....133
5.5 Discussion 135

GENERAL DISCUSSION.....137

APPENDIX.....146

I Abbreviations 146
II Units 147
III Chemicals 148
IV Equipment 148

BIBLIOGRAPHY.....150

ACKNOWLEDGMENTS

I am indebted to more people than I can possibly mention. Firstly I have to thank Roger Sutcliffe for his support and advice during the completion of this thesis.

I'd like to thank the members of the first and third floors for their advice, and more importantly for the extensive use of their equipment. Thank you to the prep-room ladies who have provided me with an endless supply of media and sterile glassware. I am very grateful to Margaret and Linda for deciphering my scrawl and typing my reference section so promptly.

I have appreciated the assistance provided by members of my group both past and present, but two people require a special mention; Gertrudes, who kindly allowed me to use her computer to write this thesis, and to Martin, who drew my plasmids and helped me with the photography.

Thanks for the support from my friends, especially Joyce, Keith and David who helped me explore Glasgow when I first arrived, and to my fellow eating and drinking companions Leslie, Daniela, and Karen.

I can't complete these acknowledgements without mentioning the companionship of the late crew, Kevin for providing a floor for me to crash on, and to the innumerable people who have fed me and kept me supplied with chocolate over the years. Also to Rick and Andrew who, while proofreading, have tried to teach me (without much success) where to put commas and hyphens.

This work was supported by a grant from the Medical Research Council.

SUMMARY

The aim of the project was to develop a cloning strategy for the isolation of novel membrane proteins. The scheme evolved from the surface antigen screening system developed by Seed and Aruffo (1987). The system was adapted to enable the cloning of cDNAs coding for membrane proteins to which no antibodies exist. This was achieved by taking a previously cloned membrane protein gene (CD2), deleting its transmembrane (Tm) and cytosolic regions, and replacing them with the polylinker and stuffer sequences of CDM8. cDNAs were then sub-cloned into this expression vector to generate a fusion library. which was then screened for surface expression of the CD2 protein.

However, such an experimental strategy required a number of assumptions to be made:
a) The cloned membrane protein could not anchor to the cell surface without a Tm sequence.
b) Fusion of an in-frame foreign Tm sequence to the external domain of the membrane protein, would restore expression of the protein at the cell surface.
c) The polylinker/stuffer sequence and out of frame random cDNAs could not act as membrane anchoring sequences.

Assessing the validity of these assumptions, involved designing a series of constructs based on the CD2 gene in the shuttle vector CDM8. They all contained the coding region for the external domain of the CD2 protein either alone, or ligated to a foreign Tm sequence (HLA-B7 gene), or ligated to the polylinker/stuffer sequence sub-cloned into the CDM8 vector. Immunofluorescent staining and western blotting experiments, were carried out on transfected cells to determine the expression patterns of the products of the various constructs.

The only proteins expressed at the cell surface corresponded to native CD2 or the CD2-external domain fused to the HLA-B7 Tm sequence. Localising the Tm-less form of the CD2 protein, however, proved more difficult. Hirt extractions and Northern blot analysis confirmed that the plasmid had transfected and was being efficiently transcribed. Despite this, the results from surface and intra-cellular immuno-staining experiments were repeatedly negative, implying that the Tm-less CD2 protein either underwent rapid intra-cellular degradation, or was secreted into the medium. The latter conclusion seems more plausible, especially as the secretion of another transfected form of a CD2-Tm less protein has been demonstrated (Richardson *et al*;1988).

To determine whether random stretches of DNA could generate functional membrane anchoring segments, EcoRI digested genomic DNA from several sources was cloned into the CD2/stuffer construct. The results obtained when these genomic fusion libraries were screened using panning and immuno-staining procedures were consistently negative. As all of the assumptions central to this project were now valid, a preliminary attempt was made at constructing a CD2-cDNA fusion library. Unfortunately, a yield of only $3x10^4$ recombinants was obtained which may well account for the lack of any positive clones being detected. Verifying the utility of this cloning strategy for the isolation of novel membrane proteins, will have to await the construction of a more representative CD2-cDNA fusion library.

CHAPTER 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

1.1 Background

During pregnancy, the embryo becomes intimately associated with maternal tissues, and although genetically distinct from the mother (semi-allograft), the fetus is able to avoid immunological surveilence (Billington;1988). The first person to address this apparent paradox was Peter Medawar (1953). His observation that the fetal and maternal circulatory systems are separated at the placental interface, subsequently shown to consist of placentally derived trophoblast cells, lead to the concept of a trophoblastic barrier. It prevents the passage of maternal immuno-competent cells into the fetus, and limits the traffic of fetal lymphocytes in the opposite direction (Hunziker *et al*;1984). Consequently, placental trophoblast cells have become a major focus of attention in the study of maternal immunological tolerance.

Placental trophoblast cells can achieve direct contact with the maternal circulation. It would, therefore, seem necessary to arrest or neutralise, in some way the expression of the trophoblast antigens which could ultimately elicit a harmful immune response against the fetus. Obvious candidates for such trophoblast antigens which may undergo some form of modulation are the classical transplantation antigens: The Major Histocompatability Antigen (MHC).

Modification, inactivation or lack of placental MHC class I expression would be an elegant means to account for fetal tolerance, and though some sub-populations of placental tissue have been found to express MHC class I antigens on their cell surface, immunostaining experiments using monoclonal antibodies directed against monomorphic and

polymorphic determinants of HLA-A and HLA-B antigens have shown that the placental MHC class I antigens fail to bind HLA-A and HLA-B antibodies specific for the fetal phenotype (paternal antigens), As no antibodies for HLA-C loci antigens were available, it was concluded that the antigen expressed was either restricted to HLA-C or some other serologically, as yet, undefined class I antigen (Redman *et al*; 1984, Hsi *et al*;1984).

The identification and characterisation of placental proteins involved in maintaining the fetus during gestation will lead to a greater insight into the processes involved in immunological tolerance. This knowledge could also then be applied to the development of an anti-placental sera, for use as a contraceptive vaccine. Consequently, the aim of this project was to attempt to design a cloning strategy which would enable the isolation of cDNAs coding for such novel membrane proteins.

As aspects of membrane protein biochemistry are central to the strategy devised, a brief overview of the structure and function of biological membranes, as well as the mechanisms by which proteins target and anchor to membranes, will be described.

1.2 The biological membrane (Stryer;1981, Alberts *et al*;1989)

Biological membranes have similar basic structures, each sharing several common features:-

a) They are all sheet-like structures, 3nm thick, acting as closed boundaries between different compartments.

b) They are predominantely composed of lipids and proteins. The three main lipid constituents being cholesterol, glycolipids and phospholipids. These are amphipathic molecules, which in aqueous media spontaneously form bimolecular sheets which are able to block the flow of polar

molecules. Depending upon membrane function, their protein compositions vary from 20% to 80%, and these proteins mediate distinct membrane functions; acting as pumps, gates, receptors, energy transducers and enzymes.
c) They are fluid, asymmetric structures, which are held together by co-operative, non-covalent interactions; where both the main components, lipids and proteins, are able to diffuse freely in the plane of the membrane. However, they are restricted in their ability to move across the two sides of the bilayer (flip-flopping motion); to thus ensure that the asymmetry of the inner and outer faces of the membrane are maintained.

An integral membrane protein can be defined as a protein which requires the disruption of the lipid bilayer in order for it to be released from the membrane. Owing to their strong hydrophobic interactions with the lipid bilayer, integral membrane proteins are characteristically resistant to extraction with strong alkali (pH11). Consequently, this property is used as a criterion for defining an integral membrane protein.

1.3 Evolution of eukaryotic organelles

The plasma membrane which encloses a cell is not just a simple impervious barrier, but a highly selective filter and pump, which maintains the essential difference between cell contents and the environment. It regulates both the entry of nutrients and the exit of waste products, as well as functioning as a sensor to external signals, allowing the cell to respond to changes in the environment. At the simplest level, the bacterial cell is composed of a single compartment surrounded by a plasma membrane. Eukaryotic cells however, are 10-30 times larger and contain a number of membrane bound compartments enclosed by the plasma membrane.

These include:-

a) Endoplasmic Reticulum (ER), golgi, endosomes and lysosomes. These communicate with each other and with the exterior, via transport vesicles, by budding off one and fusing with another.

b) Mitochondria

c) Chloroplasts

d) Peroxisomes

e) Nucleus

Each of these organelles has a different function and contains a distinctive set of enzymes or other specialised molecules.

The precursor of the eukaryotic cell was thought to resemble a bacterium, composed of a single plasma membrane and lacking organelles; these possibly arose as a consequence of the bacterium increasing in size. This in effect decreased the cell surface area:volume ratio, rendering the cell incapable of sustaining vital membrane functions such as ATP synthesis. To compensate, internal membrane invaginations of the bacterial plasma membrane evolved. They are thought to have occurred in specialised regions of the membrane, where selected groups of membrane proteins aggregate (Gray and Doolittle;1988). Evidence to support this theory is based on physiological and structural comparisons of the bacterial plasma membrane with membranes of the various eukaryotic organelles.

For example:-

a) The membranes of the eukaryotic ER, golgi, lysosome and endosome are similar in structure to prokaryotic membranes.

b) The inner membrane and the lumen of both mitochondria and chloroplasts correspond in structure to the bacterial plasma membrane and cytoplasm respectively. Perhaps, they evolved from bacteria which were engulfed by other cells, and originally lived as symbiotes (Schwarz and Dayhoff;1982).

1.4 Protein targeting

The concept that proteins existed on or within a membrane was possibly first alluded to by the German scientist, Paul Ehrlich (1901). He depicted a receptor as a molecular antenna protruding from the cell surface, which had a unique chemical cluster that specifically bound to a certain type of extracellular molecule, or drug. Later, a model for cellular protein traffic and membrane flow was proposed by Stanley Bennett (1956). The mechanism was based on information obtained from electron micrographs prepared by George Palade. It described the passage of intracellular vesicles transporting material in two different directions, both from the ER to the cell surface (exocytosis), and from the cell surface to the interior of the cell (endocytosis). Bennett also suggested that extracellular material destined to be taken up by the cell had to first bind to a localised region of the cell surface, afterwhich, the patch of membrane invaginated, and pinched off to form an intraplasmic vesicle. It represented the first accurate description of the endocytic pathway.

In 1971, Bretscher described the first polypeptide chain (major human erythrocyte glycoprotein) that spanned the lipid bilayer of a biological membrane, and in 1972, Singer and Nicholson proposed the fluid mosaic membrane model. However, even before such reliable information on membrane structure was available, Redman and Sabatini (1966) had demonstrated that truncated polypeptide chains produced by the action of the protein synthesis inhibitor, puromycin, still segregated into the lumen of the RER. In addition, Sabatini and Blobel (1970) showed that microsomes could protect nascent chains from proteolytic degradation. Together, therefore, they generated the initial concept of protein transfer being tightly coupled to translation and occuring at the level of the RER. It was George Palade

(1975) however, who carried out the pioneering studies on intracellular routing of various non-cytoplasmic proteins. He established that:-

a) Proteins for export are synthesised on polysomes bound to the membrane of the rough endoplasmic reticulum (RER).

b) Proteins destined for export are never found in a mature form in the cytoplasm because they are immediately segregated into the lumen of the RER.

c) Secreted proteins are transported through the RER and the golgi apparatus to vesicles, where they remain stored until secreted.

As already discussed, membranes are composed of mainly lipids and proteins. With the exception of a few mitochondrial and chloroplast proteins, all protein synthesis occurs in the cytoplasm. These proteins, therefore, have to be targeted to their respective final destinations. This process of protein localisation, is consequently highly specific and very efficient. The first experimental evidence pointing to the location of export information in a protein was obtained by Milstein and co-workers (1972). Using a cell free translation system, they were able to demonstrate that the IgG light chain was initially synthesised as a larger precursor, extended at its N-terminus. This was illustrated more conclusively by Blobel and Doberstein (1975), using an *in vitro* assay for protein transport. mRNAs encoding for secretory proteins were translated in a cell free system containing ^{35}S-methionine and supplemented with canine pancreatic microsomes. Increasing quantities of microsomes added to the system increased the conversion of precursor protein into the mature secretory form. The secretory product was always translocated into the lumen of the microsomal membranes, concurrent with the loss of its N-terminal extension; the signal sequence. Translocation across the microsomal membrane protected the processed proteins from proteolytic degradation; a situation which could be reversed by disrupting the microsomal vesicles with detergent. Addition

of the microsomes, after protein synthesis had been completed, resulted in no translocation of the polypeptide chains being observed; a finding confirmed by Rothman and Lodish (1977) who reported that efficient translocation required the presence of microsomal membranes during the early stages of nascent polypeptide chain growth.

1.5 Mechanism of protein translocation across the ER membrane

Protein translocation across membranes, their integration into membranes and their subsequent cellular localisation, are elicited by topogenic sequences. These sequences are able to direct the translocation and sorting of numerous structurally and functionally diverse proteins.

Three classes of topogenic sequences have been identified (Blobel;1980):-

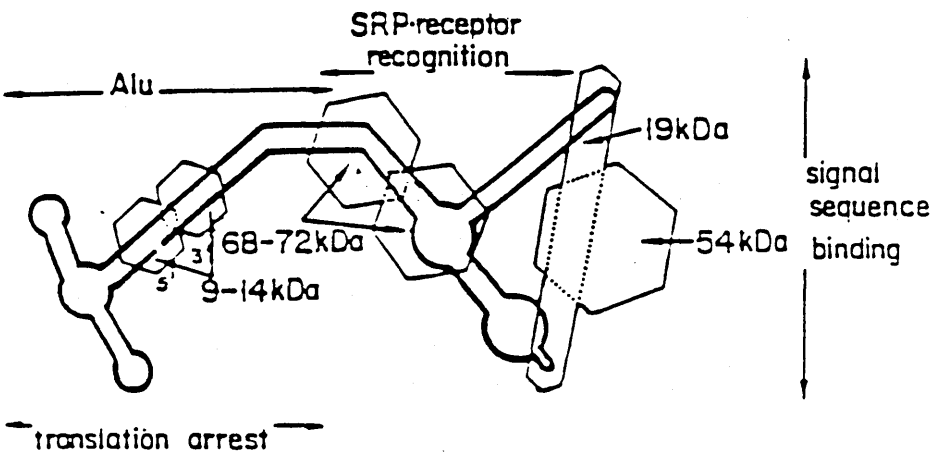
a) Signal sequences; which target proteins to specific cellular membranes and then effect translocation across them.

b) Stop-transfer sequences; which interrupt the translocation process initiated by a signal sequence, resulting in integration of the polypeptide chain into the bilayer.

c) Sorting sequences; which are involved in directing the traffic of proteins within the exocytotic and endocytotic pathways.

Topogenic sequences are decoded by membrane specific mechanisms. The signal sequence is recognised by its membrane receptor, and the signal recognition particle by its receptor and the signal peptidase complex; consequently, translocation is effected. As both the membrane specific signal sequence and the corresponding stop-transfer sequence are decoded by the same machinery, a polypeptide containing only a signal sequence will become completely translocated across a translocation competent membrane, whereas one

Figure:1.1



Model for mammalian SRP as determined by nuclaese accessability and footprinting and by reconstitution assays. Proteins are assembled on the 7S RNA core as shown. Only the 54kDa polypeptide is not in direct contact with the RNA (Pugsley;1989)

containing both a signal sequence and a stop-transfer sequence will be integrated into the membrane.

Translation is generally initiated on free ribosomes in the cytoplasm. The completed signal peptide, normally 15-30 amino acids in length, emerges from the ribosome after the synthesis of about 80 amino acids. It is responsible for specifying the destination of the polypeptide. When targeted to the ER membrane, the signal sequence is initially recognised by the signal recognition particle (SRP); a 250kDa, 11S ribonucleoprotein, made up of 6 non-identical polypeptides (9,14,19,54,68, and 72kDa molecular weight) and a single 7S RNA molecule (Walter and Lingappa;1986)(Fig:1.1). This SRP complex was the first soluble translocation specific recognition factor to be identified. It is 25x5nm in size, containing binding sites for the signal sequence, ribosome and ER membrane. The 9kDa and 14kDa polypeptides are concerned with ribosome binding, the 54kDa polypeptide with binding the signal sequence, and the 68-72kDa heterodimer is required for docking with the ER membrane (Siegel and Walter;1988).

1.6 The signal recognition particle

SRP interacts with the ribosome and the signal sequence in a co-operative manner; one interaction reinforcing the other. Effectively therefore, the SRP has a lower affinity for an unengaged ribosome than for one actively involved in the synthesis of a secreted protein (dissociation constants of 10^{-5} and 10^{-9} for unengaged and engaged ribosomes respectively, (Ceriotti and Colman;1988). The high affinity binding of SRP to the ribosome slows down or even stops translation, causing an "Elongation arrest". This enables the ribosome to engage the ER membrane prior to completing protein synthesis.

The SRP acts as an anti-folding device (Walter and Blobel;1981, Meyer *et al*;1982), segregating the bound signal sequence from the rest of the polypeptide chain. However, it is not capable of binding throughout protein synthesis, and its activity is restricted to a certain window defined by physical constraints. This prevents the interaction of SRP with the signal sequence, both before the signal sequence has completely emerged from the ribosome, and after that point at which it becomes buried in the folding polypeptide chain. SRP will only bind a certain proportion of the emerging nascent chains and this is dependent on its concentration and binding constant. It cycles between the cytosol and the ER membrane, so that once it has targeted an elongating peptide to the membrane, it is released to bind another emerging chain (Rapoport *et al*;1987, Siegel and Walter;1988). This ability to cycle means that a large quantity of SRP is not necessary to obtain a high overall targeting efficiency. The targeting efficiency however, not only depends on the binding constant between the signal sequence and SRP, but also on the size of the SRP window. Ultimately, therefore, it depends on the polypeptide sequence i.e. the intrinsic folding properties of the nascent chain. Signal sequence binding constants can range from 0.25-2.50nM (Rapoport *et al*;1987) thus allowing a high degree of discrimination in their recognition.

Photo-crosslinking studies have shown the 54kDa polypeptide of the SRP complex (SRP-54) to be the protein involved in binding to the signal peptide as it emerges from the ribosome (Krieg *et al*;1988, Kurzchalia *et al*;1986, Wiedmann *et al*;1987). Analysis of its amino acid sequence reveals the existence of two domains; an N-terminal domain containing sequence elements typical of a GTP-binding protein (G-domain), and a C-terminal methionine-rich (11%), M-domain. The M-domain has been implicated in signal sequence binding, based on secondary structure predictions which have shown all the methionine residues to lie on a single face of three amphipathic α-helices. It perhaps constitutes a flexible

binding groove on the surface of the protein, which can accomodate the hydrophobic core of the signal sequence (Bernstein *et al*;1989, Romisch *et al*;1989).

1.7 The signal recognition particle receptor

The SRP on complexing with the ribosome and the emerging polypeptide causes an elongation arrest, which is lifted when the SRP comes into contact with its receptor and binds with high affinity (Gilmore *et al*;1982). This results in a GTP-dependent displacement of both SRP and the ribosome (Connolly and Gilmore;1989, Rapoport;1990), thus allowing translation to resume. The SRP receptor (SRP-rec) is an integral membrane protein composed of two polypeptide chains; an alpha and a beta subunit of molecular weights 72kDa and 30kDa respectively (Tajima *et al*;1986). It is found almost exclusively on the cytosolic face of the RER (Meyer *et al*;1982, Romisch *et al*;1989), and was originally isolated by Gilmore and Connolly (1986). They demonstrated that salt washed microsomes lacking the SRP-receptor were unable to import secretory proteins. However, if the salt extract containing the receptor was added back, the import capabilities of the microsomes were restored.

Hann and co-workers (1989) have reported that the SRP-rec shares sequence homology with the SRP-54 protein in the G-domain. It has therefore been proposed that the SRP-rec also functions as a "kinetic proof reader", binding to the signal sequence to accomodate the degeneracy of the signal sequence. However, as yet, there is no experimental evidence to support this theory (Romisch *et al*;1989, Bernstein *et al*;1989).

1.8 The signal sequence receptor

After the SRP-translating ribosome complex has docked with the ER membrane, the signal sequence dissociates and is presented to another protein component in the membrane (Gilmore and Blobel;1985). This protein was identified using photocross-linking experiments: These were carried out by preparing short, signal sequence containing nascent polypeptide fragments of approximately 70 amino acids in length, and cross-linking them to proteins in the ER membrane. A 35kDa integral membrane protein was found coupled to the bound signal sequence, and was tentatively identified as the signal sequence receptor (SS-rec) (Wiedmann *et al*;1989). To confirm the results obtained, these experiments were repeated using longer nascent chains (with photoreactive groups), which still gave rise to cross-links with the same 35kDa protein (Krieg *et al*;1989, Wiedmann *et al*;1989).

The SS-rec is found almost exclusively on the RER. It is synthesised with a cleavable N-terminal signal sequence and is inserted into the ER membrane in an SRP-dependent manner. Hartmann and co-workers (1989) have purified the putative SS-rec from cannine pancreatic microsomes, where it constitutes around 1.5% of the total membrane protein, and Blobel (1989) has cloned and sequenced the SS-rec gene. This protein is composed of a single hydrophobic membrane-spanning domain, a 56 amino acid cytosolic region and a luminal portion containing two carbohydrate chains (Prehn;1990). The charge distribution in the vicinity of the N-terminus is highly negative with 23 of the 35 amino acids carrying a negative charge (about 66%), but the cytosolic tail carries a net positive charge (Gilmore and Blobel;1985).

The models for translocation put forward by Blobel and Doberstein (1975), and Rapoport (1985), have proposed that polypeptides are transported through a hydrophilic or amphiphilic tunnel assembled from Tm proteins. Although the

immunological data collected by Hartmann and co-workers (1989) have shown that the SS-rec is essential for protein translocation, both the SS-rec sequence, and its size, exclude the possibility of a single molecule of the 35kDa SS-rec forming such a tunnel.

Using bifunctional cross-linking reagents, Rapoport (1990) has recently demonstrated that other specific proteins are located in close proximity to the 35kDa SS-rec in the ER membrane. One of these is a 25kDa glycoprotein (Blobel;1989), which from sedimentation analysis appears to be tightly associated with the 35kDa SS-rec. Consequently, the two polypeptides have been called the α-35kDa and β-25kDa subunits of the SS-rec. The cross-linking experiments are also indicative of the close proximity of two adjacent alpha SS-rec molecules in the ER membrane, suggesting that the SS-rec may be composed of three heterodimers which associate to form a hexameric protein conducting channel. The SS-rec has been found in close proximity to nascent chains during membrane translocation, giving credence to it being a constituent of the putative channel (Wiedmann *et al*;1989, Krieg *et al*;1989). The cDNA of the 25kda α-subunit of this protein has also been cloned and sequenced. It is a bitopic integral membrane protein with a single Tm sequence (Blobel;1989).

The aqueous conducting channel has been proposed to consist of a combination of lipid and protein, analogous to those of ion conducting channels (Simon *et al*;1989) and contains both a cytosolic and a signal sequence binding domain. This channel remains accessible to aqueous perturbants, and can be extracted with either urea or a high pH (Gilmore and Blobel;1985) before or after the signal sequence has interacted with the membrane.

More concrete evidence for the presence of a protein conducting channel was obtained from electro-physiological experiments carried out by Simon and co-workers (1989).

Here, rough microsomes were fused to a planar lipid bilayer and maintained under voltage clamp conditions, in order to study the permeability of membrane channels. By measuring the conductivity, it was observed that the channels were open mainly at negative membrane potentials on the cytoplasmic face of the membrane, and closed at positive voltages. The results demonstrated that both sides of the membrane were involved in protein translocation, providing yet more evidence for the existence of a protein conducting channel.

Using the above technique, it was determined that although the rough microsomes they used were large enough to contain approximately 10-100 channels, occasionally, only a single channel was observed. This is apparently due to the blockage of conduction channels by nascent chains. This finding has been reinforced by repeat experiments utilising puromycin to produce shorter peptide chains. A dramatic increase in the number of conducting channels was observed, but only when puromycin was added to the cytoplasmic side of the fused rough microsomes (Blobel;1989).

Although, no actual protein conducting channel has been isolated to date, the use of voltage clamp technology in conjunction with the recently aquired ability to reconstitute translocation activity from detergent solubilised rough microsomes (Nicchitta and Blobel;1990), should enable the isolation of the protein conducting channel.

1.9 The signal peptidase

The hydrophobic signal peptide of soluble proteins acts as a start transfer signal, that remains anchored to the membrane throughout translocation. The rest of the protein is threaded continously through the membrane in the form of a large loop. Once the C-terminus has passed through the

membrane, only the signal peptide keeps the protein bound to the membrane. This peptide is then cleaved off by the signal peptidase, and the protein is released into the lumen of the ER. The signal peptidase has been isolated from canine pancreatic rough microsomes as a complex of five distinct polypeptide chains (Evans *et al*;1986). One of the five subunits is a glycoprotein containing a typical N-linked oligosaccharide chain (Evans *et al*;1986). The cDNAs of 3 of the 5 subunits have been sequenced (Blobel;1989), and two of the subunits have been found to be highly homologous to each other, with all three subunits containing at least one Tm sequence. It is not known why an endoprotease requires 5 subunits; possibly only one functions in cleavage, whereas the others may function as an exo-proteolytic signal peptide peptidase, a glycosyl transferase or another modifiying enzyme during translocation.

SRP and the SRP-rec occur in sub-stoichiometric amounts relative to the number of membrane bound microsomes, because they function only in the recognition and targeting step. The SS-rec and the signal sequence peptidase complex however, are present in approximately stoichiometric amounts relative to the number of membrane bound microsomes, implying that they are integral components of the translocation site in the membrane.

1.10 Import of proteins into the sub-cellular organelles

(i) Lysosome

Lysosomes are acidified cytoplasmic organelles that contain around 40 hydrolytic enzymes capable of degrading most biological molecules. These organelles carry out a number of vital functions including the killing of infectious agents taken into the cell by phagocytosis, degradation of

hormones and proteins which enter cells via receptor mediated endocytosis and the turnover of intra-cellular components of autophagy.

A proton pump maintains the pH of the lysosome at pH5.0, to provide the optimal working conditions for the acid hydrolases present. These enzymes are soluble glycoproteins with long half lives. It has been proposed that they are heavily glycosylated to protect them from degradation (Alberts *et al*;1989). The lysosomal membrane contains a number of transport proteins which allow the escape of breakdown products from digestion to be either recycled or excreted.

Both the lysosomal hydrolases and the lysosomal membrane proteins are sythesised in RER and transported through the golgi apparatus to the trans-golgi network. Here, transport vesicles bud off to deliver proteins via an endolysosome to the lysosome. In order to do this, lysosomal proteins have to be segregated from the other RER proteins, and targeted to the lysosome. The complete mechanism has not yet been determined, but substantial data exists on the manner in which lysosomal hydrolases reach their target.

The targeting system evolved in higher eukaryotes is elaborate and can be divided into a number of steps, each involving a recognition event (Kornfeld and Mellman;1989). Initially, secretory proteins, plasma membrane proteins, and lysosomal proteins are synthesised on the RER. They are co-translationally glycosylated (at Asparagine residues following cleavage of the signal sequence), and moved by vesicular transport to the lumen of the *cis* golgi (site of post-translational modification and segregation of proteins for targeting to their final destination).

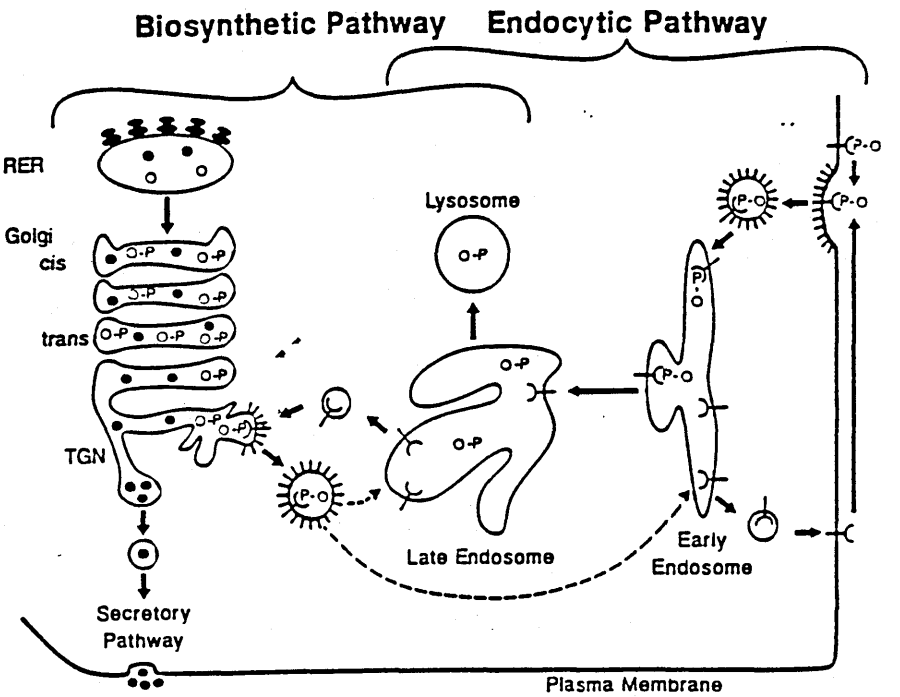
It can be concluded, therefore, that the information required to differentiate a lysosomal protein from other RER lumen proteins is already present in the peptide sequence.

Lysosomal hydrolases contain a unique marker, Mannose-6-phosphate, which is added exclusively to N-linked oligosaccharides. This is carried out by the action of two enzymes acting sequentially. N-acetyl-glucosamine phospho-transferase (Glc-N-Ac-P-transferase) first transfers the Glc-N-Ac-P portion of the sugar nucleotide UDP-Glc-N-Ac to selected Mannose residues of the lysosomal enzyme to give rise to a phosphodiester intermediate. Then, the second enzyme, a Phosphoglycosidase (α-N-acetyl-glucosaminidase) removes the N-acetyl glucosamine to generate a phospho-monoester.

The specificity of the reaction with lysosomal hydrolases is determined by the phosphotransferase, which recognises a protein determinant shared by lysosomal enzymes. The phosphomannosyl residues act as high affinity ligands which bind the Mannose-6-phosphate (M-6-P) receptors in the Golgi. By adding multiple M-6-P residues to lysosome hydrolases, (approximately a 10,000 fold amplification of the targeting signal can be achieved). The Phosphotransferase adds 1-2 Glc-N-Ac-phosphates and up to 5 Mannose residues per oligosaccharide chain.

The M-6-P receptors cluster in the trans-Golgi network. They are Tm proteins which bind lysosomal enzymes and separate them from the other proteins, concentrating them in clathrin coated transport vesicles. These vesicles exit the Golgi, lose their coats, fuse with acidic endolysosomes, thereby delivering their contents to the organelle. The fall in pH causes dissociation of the ligand from the receptor. Consequently, the enzyme is released into the lysosome, while the receptor recyles. It either returns to the Golgi, to repeat the process, or moves to the plasma membrane. Here, it functions to internalise exogenous lysosomal enzyme that has escaped sorting in the Golgi, and has thus been secreted. In fibroblasts, the secretion-recapture mechanism

Figure: 1.2



Model for lysosomal enzyme targeting to lysosomes. Lysosomal enzymes (◯) and secretory proteins (●) are synthesized in the RER and transported to the Golgi where the lysosomal enzymes acquire phosphomannosyl residues (◯-P). Most of the lysosomal enzymes bind to MPRs (Y) in the trans Golgi network (TGN) and are translocated to late and/or early endosomes where they are discharged due to the intraorganelle acidification. The enzymes are then packaged into lysosomes while the receptors cycle back to the Golgi or to the plasma membrane. In addition to the pathways shown, it is likely that MPRs also cycle to the plasma membrane from the Golgi (via leak into secretory vesicles) and from late endosomes. (Kornfeld, 1989)

functions as a minor salvage pathway, accounting for the delivery of only 5-10% of the total lysosomal enzyme content (Kornfeld;1989).

M-6-P is the major, and in some cells the only, sorting signal present for lysosomal enzymes. However, some lysosomal proteins exist which have no M-6-P residues e.g. in the human liver cell line which is devoid of N-acetyl glucosamine-1-phosphotransferase, or in lysosomal storage disease (I-cell disease). Therefore, despite the inability of these cells to phosphorylate Mannose residues, but they are still able to target their lysosomal enzymes, with only a very slight reduction in efficiency. This implies that these cells have a completely M-6-P independent system for sorting their lysosomal enzymes (Kornfeld;1986, Neufeld *et al*;1975) (Fig:1.2).

(ii) Mitochondria

Whereas translocation across the ER occurs co-translationally, translocation of proteins into mitochondria, chloroplasts and peroxisomes, has been shown to occur *in vitro* post-translationally; after synthesis of the protein has been completed and it is releasd into the cytosol. Import requires ATP hydrolysis but not ongoing protein synthesis. In the case of mitochondrial and chloroplast protein import, ATP hydrolysis is required to keep the protein unfolded as it passes through the membrane.

Although there is more information available on the import mechanisms in mitochondria than chloroplasts, it would seem that their mechanisms are virtually identical; despite the chloroplast having an additional membrane bound compartment, the thylakoid.

Mitochondria contain an outer and an inner membrane which enclose two separate compartments, the matrix and the inter-

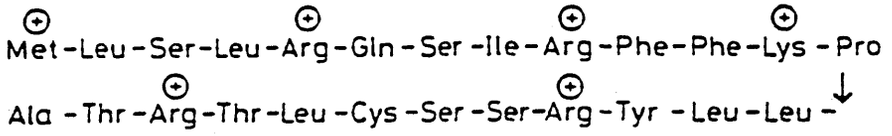
membrane space. Proteins to be imported into the mitochondrial matrix are usually taken up from the cytosol within 1-2min of their release from free polysomes. The proteins are synthesised with an N-terminal amphiphilic targeting sequence (Roise and Schatz;1988), generally 15-30 residues in length. It forms an amphipathic structure which is probably α-helical in nature, where the positively charged residues all line up on one side of the helix, while the uncharged hydrophobic residues line up on the opposite face (Fig:1.3). The precursor protein binds to the mitochondrial surface and is transported across the outer and the inner membrane at sites where the two membranes are in close proximity i.e. "contact sites". Passage across the membranes requires that the proteins are loosely folded.

Once the protein has reached its correct destination, its targeting sequence is cleaved and the mature polypeptide is allowed to fold into its native conformation (Attardi and Schatz;1988, Hartl and Neupert;1990). Premature folding in the cytoplasm is prevented by cytosolic "chaperones" (Ellis;1987) which selectively bind and stabilise partly folded proteins. The binding of the signal peptide to the mitochondrial membrane is carried out by specific receptors and transport across the membranes is catalysed by proteins forming transport pores or channels. The target sequence is then removed by a specific protease (similar to signal peptidase in SRP-mediated transport) and once released the folding of the matrix protein is facilitated by matrix-specific chaperones.

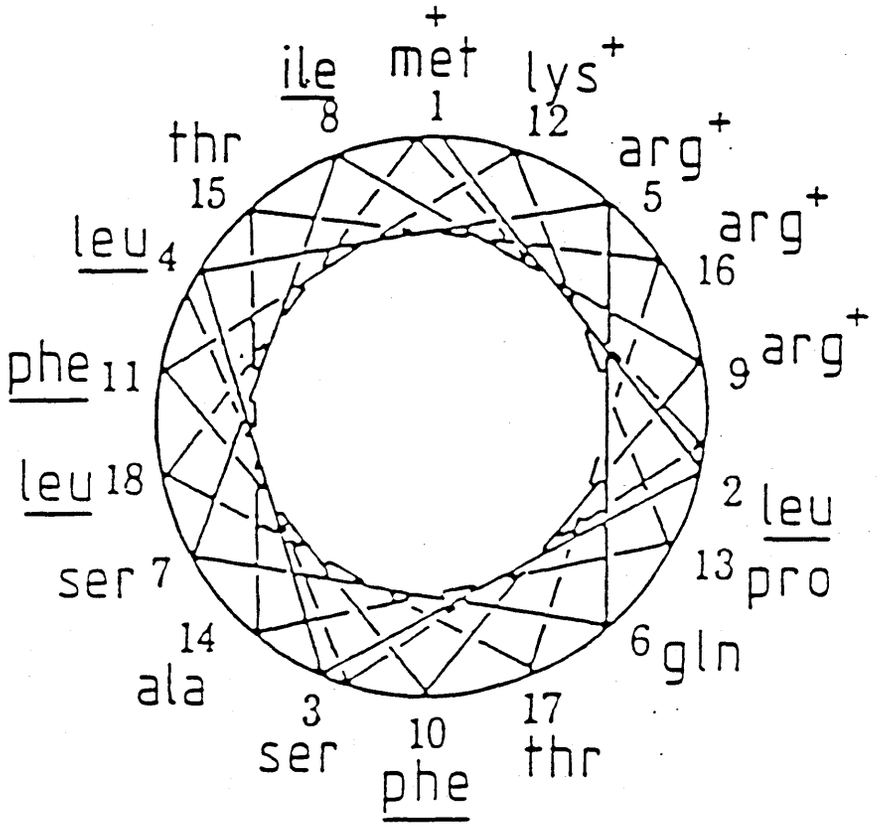
There appear to be five essential proteins involved in mitochondrial protein import. The unlinked nuclear genes *MAS1* and *MAS2* (Mitochondrial Assembly isolated from *S. cerevisiae*) encode the first two essential proteins which correspond to non-identical sub-units of the matrix-located protease (Pollock *et al*;1988, Witte *et al*;1988, Jensen *et al*;1988, Yang *et al*;1988). This protein is a water-soluble, metal-requiring heterodimer that removes the matrix

Figure: 1.3

a) PRESEQUENCE OF YEAST CYTOCHROME OXIDASE SUBUNIT IV



b)



a) The presequence of yeast ctochrome oxidase subunit IV in a linear representation

b) The same sequence presented as a helical wheel projection. Note the clustering of charged residues in the helical protjection, giving the helical surface a polar and an apolar face (Roise *et al*;1986)

targeting sequences from matrix precursor proteins (Baker and Schatz;1991). Removal of the amphiphilic matrix-targeting sequences from precursor proteins may be required to allow either proper assembly of imported proteins, or their dissociation from other components of the import machinery. Alternatively, the intra-cellular accumulation of proteins with amphiphilic targeting sequences may poison membrane function (Roise and Schatz;1988).

The third essential protein is the product of the *mif*4 gene (mitochondrial import function), encoding the mitochondrial heat shock protein hsp60 (Cheng;1989). It is a typical chaperone whose function closely resembles that of analogous proteins in both chloroplasts and bacteria (sharing strong sequence similarity with the *E. coli* chaperonin gene product, *gro*EL). It is a homo-oligomer composed of 14 subunits, which mediates the refolding of newly imported mitochondrial proteins. It binds to the proteins in the matrix and keeps them in a loosely folded conformation, releasing them in an ATP-dependent process requiring at least one other matrix protein.

Another stress protein hsp70, is the fourth essential mitochondrial protein required. It is encoded by the *SSC1* gene and is similar to the *E. coli dnaK* gene product, but it is synthesised with a 23 residue matrix-targeting signal which is cleaved (Scherer *et al*;1990, Craig *et al*;1989, Morishima *et al*;1990). It carries out one of the earliest essential functions by transiently associating with newly imported protein precursors. This association is disrupted by ATP, possibly as the bound proteins are transferred to mitochondrial hsp60 (Baker and Schatz;1991). The ATP mediated release may provide the driving force for the Tm movement of the polypeptide chain, and may also account for why protein import requires ATP in the matrix (Attardi and Schatz;1988, Hartl and Neupert;1990).

The fifth essential component of the protein import machinery *S.cerevisiae* is the 42kDa import site protein (ISP42) (Vestweber *et al*;1988). It is an integral outer membrane protein with no obvious Tm segment or N-terminal mitochondrial sequence (Baker *et al*;1990). It appears to be a sub-unit of a hetero-oligomeric transport channel for precursors across the outer membrane. Over production of ISP42 causes it to localise incorrectly and denature within the mitochondria. The *Neurospora* homologue of ISP42, *MOM38*, can be isolated as a complex with several other outer membrane proteins, including the two import receptors *MOM19*, and *MOM72* (Kiebler *et al*;1990). ISP42 is the only mitchondrial membrane protein known to be essential for cell viability.

Inactivation of the *Neurospora MOM72* gene impairs the binding and import of the ADP/ATP carrier protein (AAC), one of the most abundant mitochondrial proteins. Yet, 25-30% of the AAC protein is still imported. It has been proposed that this occurs because the AAC now gets imported via *MOM19*, which has some overlapping function with *MOM72* (Pffaner *et al*;1991), and thus accounts for the viability of *MOM72*-deficient cells. Both *MOM72* and *MAS70* (*S. cerevisiae* equivalent of *MOM72*) are found enriched at sites of contact between the two mitochondrial membranes. They may function to accelerate protein import, by binding to and delivering precursors to the import channel in the outer membrane.

Yet another mitochondrial import receptor has been identified in *S. cerevisiae*. It is a 32kDa protein (p32) that was detected with the aid of anti-idiotypic antibodies directed against a matrix targeting peptide (Pain and Blobel;1990, Murakami *et al*;1990). The deduced sequence of this protein resembles that of the phosphate transporter protein of a rat-liver, a 29kDa mitochondrial protein. Antibodies against it, only partially inhibit the import of several precursors into isolated mitochondria. Disruption of the *MIR1* gene (which encodes the p32 protein) is not lethal,

but renders the yeast respiration deficient. Using this anti-idiotypic technology, two further proteins have been identified (p65 and p63) by Blobel and co-workers (1990). Although no function has, as yet been assigned to them, but they too are found concentrated around the mitochondrial membrane contact sites.

Table:1.1

Components of mitochondrial protein import machinery in yeast and other organisms, listing the yeast genes and their homologues in *Neurospora*, Rat and Maize.

		Homologue	
Yeast gene	*Neurospora*	RAT	Maize
MAS1	*PEP*	*p52*	
MAS2	*MPP*	*p55*	
hsp60	hsp60		hsp60
MAS70	*MOM72*		
ISP42	*MOM38*		
MOM17?	*MOM19*		
MIR1 (p32)		29kDa protein protease II	

(iii) Chloroplast

The chloroplast genome, like the mitochondrial genome is too small to carry all the genetic information needed for the large number of different proteins that are functional in this organelle (Ellis;1981). Approximatlely 80% of the proteins are translated from nuclear encoded genes (Chua and Gilham;1977), and these are synthesised as precursors which are imported into chloroplasts in an energy dependent manner (Cashmore *et al*;1984). Associated with processing is the cleavage of an N-terminal signal peptide of approximately 50

amino acids (Schmidt *et al*;1979, Bedbrook *et al*;1980, Berry-Lowe *et al*;1982, Cashmore;1984). The protein once imported has to be targeted toward one of several differnt chloroplast compartments (outer membrane, intermembrane space, inner stromal membrane, thylakoid membrane or thylakoid lumen). This signal peptide has been shown to be able to direct bacterial proteins into the chloroplast (van den Broeck *et al*;1985, Schreier *et al*;1985).

Most of the research on protein import into chloroplasts has been carried out by studying the precursor of the Ribulose bis-phosphate carboxylase (RUBISCO) enzyme. This is a hetero-oligomeric protein composed of two different types of subunits, 8 large, plastid encoded and 8 small nuclear encoded polypeptides, the LSU and SSU polypeptides respectively. LSU is synthesised in the chloroplast on prokaryotic-type ribosomes and SSU is synthesised in the cytoplasm. It is then imported into the chloroplast stromal space, where it is assembled with LSU into the active holoenzyme.

The transport information deduced to date has been based on SSU, but appears to be valid for other stromal proteins. SSU is synthesised as larger precursor, with a signal peptide composed of a few acidic amino acids and a relatively high number Serine and Alanine residues. Shortly after transport of a precursor into the stroma, the signal peptide is cleaved by a chelator-dependent stromal peptidase (180kDa) which is highly specific for imported precursors (Robinson and Ellis;1984). Early import experiments with pre-SSU showed that hydrolysable ATP is the only energy source required for import. Therefore, unlike the mitochondrial system, import of chloroplast proteins does not require a proton motive force (Smeekens *et al*;1990).

Pretreatment of chloroplasts with trypsin inhibits protein import (Cline *et al*;1985), implying the existance of receptor/transport proteins on the chloroplast membrane.

Using anti-idiotypic antibodies, a 36kDa protein (p36) of the chloroplast envelope has been identified as the import receptor (p36) (Schnell *et al*;1990). The primary sequence of p36 is identical to the sequence of the phosphate translocator, an inner membrane protein of the chloroplast envelope (Pfanner *et al*;1991). It is located at the contact sites of the inner and outer chloroplast envelope membranes, and is reminiscent of the transport proteins involved in mitochondrial import (Hartl *et al*;1989). It is possible that the transport of proteins into chloroplasts shares some other critical features, such as dependence on hsp70 and hsp60, with the mitochondrial protein import system. The current information available on the transport apparatus of the chloroplast does not allow detailed conclusions to be made about the mechanisms involved.

(iv) Peroxisomes (microbodies)

Peroxisosmes or microbodies differ from chloroplasts and mitochondria, in that they are surrounded by a single membrane and do not contain either DNA or ribosomes. Therefore, all their proteins have to be supplied from the cytosol. Peroxisomes, thus, resemble the ER in being self replicating membrane bound organelles that exist with out their own genome. They are a concentrated source of 3 oxidative enzymes (in liver cells: D-amino acid oxidase, urate oxidase and catalse which account for 40% of the total peroxisomal protein content) and are the site of oxygen utilisation; a remnant of an ancient organelle that carried out all oxygen metabolism in the primitive eukaryotic cell).

Prediction about the import mechanism of proteins into microbodies/peroxisomes have not yet been elucidated; but the presence of signal sequences in the extreme carboxyl terminus of (Ser-Lys-Leu) of the firefly luciferase protein (Gould;1989) implies the existance of a distinct import mechanism.

(v) Nucleus

The nuclear pore forms a large channel connecting the nuclear and cytoplasmic environments. Transport through this pore is significantly different from protein transport into mitochondria or the ER. It occurs through a large aqueous channel and possibly more closely resembles the transport mechanism across a gap junction (Schwartzmann *et al*;1981). The channel is 90-100A in diameter, and therefore poses no barrier to either small molecules or proteins, but it does regulate the movement of large nuclear proteins and RNA molecules (Bonner;1978, Franke;1974, Dingwall and Laskey;1986, Newport and Forbes;1985).

Targeting of proteins to the nucleus, like the ER and mitochondria, is facilitated by a signal sequence. The signal sequence of large nuclear proteins appears to increase the diameter of the pore, to accomodate the protein and allow its entry into the nucleus. Nuclear proteins extracted from the nucleus retain the ability to re-enter the nucleus when microinjected into the cytoplasm of *Xenopus* oocytes (Gurdon;1970, Bonner;1975a, 1975b, De Robertis *et al*;1978). It was therefore concluded that the majority of nuclear proteins were targeted to the nucleus by an uncleaved signal sequence. Studies with nucleoplasmin, a pentameric 100kDa nuclear protein, enabled the location of the signal sequence to be determined (Khrone and Franke;1980, Dingwall *et al*;1982, Dingwall *et al*;1987, Buglin and De Robertis;1987).

Using molecular genetics, a number of nuclear transport signals have been identified (Lannford *et al*;1986, Richardson *et al*;1986, Burglin and De Robertis;1987), but the best characterised signal, is that of Simian virus 40 (SV40) large T-antigen (Lanford and Butel;1984, Kalderon *et al*;1984a, 1984b). Its signal sequence consists of seven

amino acids (extends from amino acids 126-132): Pro_{126}-Lys-Lys-Lys-Arg-Lys-Val_{132}. A point mutation in this sequence, where Lys_{128} is exchanged for either Thr or Asp, leads to a defect in nuclear transport of the T-antigen. It has also been demonstrated that synthetic peptides containing the wild-type SV40 signal sequence, when covalently coupled to non-nuclear proteins and injected into living cells, can direct these proteins into the nucleus (Goldfarb *et al*;1986, Lanford *et al*;1986). In contrast, these proteins when coupled to peptides containing point mutations at Lys_{128} are transport defective. These protein fusions have also been used to illustrate that transport into the *Xenopus* oocyte nucleus displays saturation kinetics, so that co-injection of large amounts of free signal-peptide inhibits the import of labeled signal-sequence-protein fusions, implying that transport into the nucleus is a receptor mediated process.

1.11 Anchoring and topography of membrane proteins

Membrane proteins integrate asymmetrically, with many different topologies in the lipid bilayer of a specific membrane compartment. Although, the mechanism has not been totally elucidated, the important elements involved in targeting membrane proteins have been identified (Sabatini *et al*;1982, Wickner *et al*;1984, Garoff;1985). Many of the membrane proteins require SRP or the bacterial Sec gene products for assembly, and use the same machinery for translocation as secretory proteins (Wiedmann *et al*;1987).

In addition to targeting, the signal sequence is also involved in protein topogenesis. It can be cleaved off, as previously described, by the signal peptidase complex in the lumen of the ER, or alternatively, serve as a membrane anchor. A second topogenic element, the stop transfer sequence, has also been identified. It is a hydrophobic segment which blocks the further translocation of a polypeptide chain. In the membrane integrated protein,

therefore, the stop transfer sequences as well as the uncleaved signal peptide can be embedded in the hydrophobic core of the bilayer to function as membrane spanning sequences.

The vast majority of integral membrane proteins have a similar and unique structural motif which was first identified by Henderson and Unwin (1975) using low resolution structural analysis of bacteriorhodopsin. These proteins are anchored in the bilayer by one or more principally hydrophobic transmembrane stretches of 15-30 amino acids (Sabatini *et al*;1982). These are thought to span the 3nm membrane as a single α-helix (Tanford;1978).

Signal sequences on the other hand, are composed of a hydrophobic core of only 8-14 non-polar residues (Adams and Rose;1985, Davis and Model;1985, Davis *et al*;1985, Cutler and Garoff;1986, Kaiser *et al*;1987, Spiess and Handschin;1987), and these usually lie within 5 amino acids of the N-terminus (Boyd and Beckwith;1990). However, it has been demonstrated that the signal sequence can also act as stop transfer sequence (Coleman *et al*;1985, Findori *et al*;1987) and that a stop-transfer sequence can functionally replace an internal signal sequence (Zerial *et al*;1987). Combinations of either a cleaved or uncleaved signal sequence with or without a following stop transfer sequence, can account for the number of different protein topologies observed with respect to the membrane.

Blobel (1980) classified integral membrane proteins on the basis of the number of times the polypeptide spanned the membrane.

1) Monotopic membrane proteins:- hydrophobically associate with the membrane but do not pass all the way across the bilayer.

2) Bitopic membrane proteins:- cross the membrane bilayer exactly once.
3) Polytopic membrane proteins:- cross the membrane more than once.

(i) Monotopic membrane proteins

Monotopic proteins are rare, an example of which may be the liver microsomal membrane protein, Cytochrome b5, (Anderson *et al*;1983), which associates with the bilayer by means of a hydrophobic hairpin loop. Although, this hairpin loop protein has been consistantly described as passing through only part of the membrane (Arinc *et al*;1987, Takagaki *et al*;1983), there is some evidence in the literature to suggest a bitopic conformation (Rose *et al*;1983).

(ii) Bitopic membrane proteins

Bitopic membrane proteins are fairly common, eg. the spoke proteins of enveloped viruses (Wiley and Skehel;1987, Rose *et al*;1980), Glycophorin (Marchesi *et al*;1976), HLA antigens of human cells (Lopez de Castro *et al*;1985), HLA-DR-associated invariant chain (Strubin *et al*;1984), asialoglycoprotein receptor (Speiss *et al*;1985), as well as a few secretory proteins such as ovalbumin (Lingappa *et al*;1979) and intestinal sucrase-isomaltase (Hunziker *et al*;1986). All of which have the majority of their mass on the extracellular face of the membrane. They are synthesised on the RER and are not cleaved during insertion into the lumen. This observation led to the the concept of an internal, uncleaved signal sequence (Blobel;1980, Sabatini *et al*;1982). It is defined, as a sequence which can be recognised by all the proteins of the translocation machinery (excepting the signal peptidase) and which can perform all the same functions as its cleaved counter part.

Studies where the uncleaved signal sequence of ovalbumin was substituted for a cleavable signal sequence, resulted in the targeting of these proteins, into microsomes. Both signal sequences are therefore functionally similar and can be interchanged (Sakaguchi *et al*;1984, Brown and Simoni;1984). Uncleaved signal peptides together with stop-transfer sequences and other internal membrane insertion domains, can generate the complex topologies observed with of different types of membrane proteins. For example, the bulk of influenza virus neuraminidase is extracytoplasmic and anchored to the membrane by a hydrophobic segment at its N-terminus. Neuraminidase does not undergo endoproteolytic cleavage during its biosynthesis, and the N-terminal hydrophobic domain acts as both a signal sequence and a membrane anchor (Bos *et al*;1984).

Other bitopic membrane proteins, such as the liver asialoglycoprotein receptor (Chiacchia and Drickamer;1984, Spiess and Lodish;1986) and the transferrin receptor (Zerial *et al*;1986) have major cytoplasmic domains. The epidermal growth factor receptor and the insulin receptor beta subunit are also very likely to be bitopic proteins, with major domains on both sides of the membrane. Bitopic membrane proteins can have long stretches of hydrophobic amino acids that bind strongly to the fatty acyl core of the membrane. As a result, the continued extrusion of the protein is blocked, implying the presence of a start-stop transfer sequence. The ribosome then continues to add amino acids to the nascent chain, and these remain on the cytoplasmic face of the membrane.

In the ER membrane of the eukaryote cell or the inner membrane of a prokaryote, the process of translocation or insertion is initiated in the same way. The signal sequence at the N-terminal of an unfolded chain intercalates into the translocator interface in an energy requiring process. As a

result, the N-terminal faces the cytoplasm and the C-terminal of the signal sequence faces the exterior (N-in and C-out), and subsequently, the signal sequence is cleaved.

Historically, Blobel's definitions have been the most commonly cited, however, it is now becoming more informative to classify integral membrane proteins according to their transmembrane topology and mode of insertion into the membrane. This is especially true for bitopic membrane proteins composed of a single membrane spanning segment which can be generated in one of three ways (von Heijne and Gavel;1988, Fig:1.4):

Type-I: with an amino-terminal cleavable signal peptide and an internal apolar stop-transfer sequence.
Type-II: with an amino-terminal or internal uncleaved signal peptide.
Type-III: with an amino-terminal start-stop transfer sequence.

(iii) Membrane anchoring via a GPI (Glycosyl phosphatidyl inositol) anchor

Recently, a mechanism for anchoring proteins to the plasma membrane has been described which involves the covalent attachment of a complex structure containing phosphatidyl inositol, carbohydrate and ethanolamine to the C-terminus of a protein. These proteins are glycoproteins composed of a single ectodomain, which is anchored to the membrane via covalent linkage to a glycophospholipid-glycosyl phosphatidyl inositol (GPI). The C-terminal amino acid of the extracellular protein moiety is linked via an ethanolamine residue to an oligosaccharide composed of Mannose, a variable number of galactose residues and (non N-acylated) glucosamine. This in turn is attached to the inositol portion of PI (phosphatidyl inositol). The diacylglycerol

Figure: 1.4

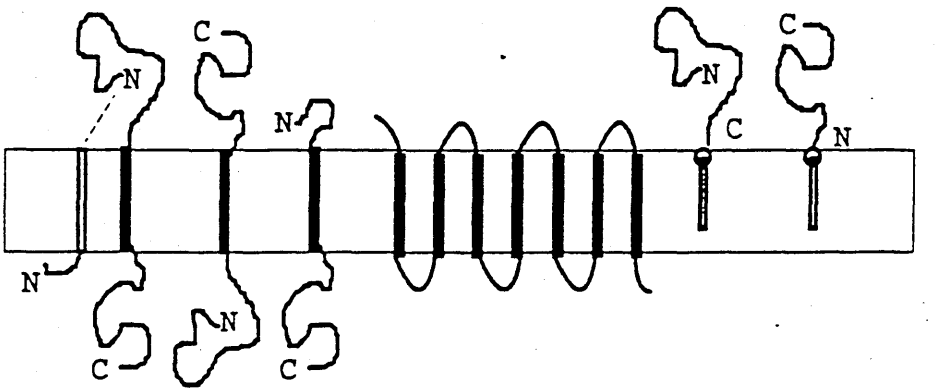


Diagram showing the different classes of membrane proteins. The horizontal shaded area represents the cell membrane; the black vertical rectangles represent transmembrane regions of proteins. Classes I, II, III, and IV are integral membrane proteins. GLYP represents those membrane proteins that are anchored by glypiation tails (see text). For class I integral membrane proteins, the N-terminal leader sequence (left open box) is shown cleaved (dotted line) from the N terminus of the mature protein. The number of transmembrane sequences of class IV proteins need not be seven, and the orientation of their N and C termini need not be as shown.

portion of PI provides the only site for membrane attachment in these proteins (Low and Saltiel;1988, Fergerson and Williams;1988).

GPI anchored proteins contain a conventional N-terminal signal sequence which serves to translocate the protein into lumen of the ER. In addition, there is a second cleavable hydrophobic sequence, at the C-terminal end of the protein, which serves as a signal for GPI attachment. This sequence is cleaved co-translationally in the lumen of the ER, and GPI is added as a block, by an as yet, uncharacterised set of enzymes (Caras *et al*;1987, Caras *et al*;1989). This sequence shares properties with the ER signal sequence, and has been shown to functionally replace the human growth hormone (hCG) signal sequence (Caras and Wedell;1989).

(iv) Polytopic membrane proteins

Polytopic proteins, where the polypeptide chain passes back and forth through the bilayer are thought to be achieved by internal signal sequences. These act as translocation initiating short transfer signals, with each translocation event proceeding to the next stop transfer peptide. The fundamental unit translocated is a loop of polypeptide between 2 hydrophobic segments; one start and one stop tranfer sequence, with one or both of these occuring as an α-helical membrane spanning domain in the mature protein.

The term multispanning polytopic protein was proposed by Blobel (1980). Examples include the protein opsin, which spans the membrane 7 times (Frielander and Blobel;1985), and the red cell anion exchanger protein Band III, which has more than 10 Tm domains (Kopito and Lodish;1985). The final topology achieved therefore, is due to the succession of alternating internal signal and stop transfer sequences (Frielander and Blobel;1985, Audigier *et al*;1987). Although, the first targeting/insertion event involves SRP and SRP-

rec, the insertion of subsequent domains is probably independent of these factors, and perhaps occurs spontaneously (Wickner and Lodish;1985). The postranslational insertion of the human glucose transporter *in vitro* (Mueckler and Lodish;1986) supports this model. Here, the hydrophobic side groups of the amino acids project outwards to interact with apolar fatty acyl core of the bilayer. Currently six categories, of polytopic membrane proteins have been assigned. Their definitions are based on the number of membrane spanning segments present, and the orientation of the two termini (according to the von Heijne and Gavel (1988) nomenclature they are classified as type-IV membrane proteins).

(v) Positive inside rule

Integral membrane proteins are characterised by long apolar segments that cross the lipid bilayer. Polar domains flanking these apolar segments have a more balanced amino acid composition typical of those found in soluble proteins. von Heijne and Gavel (1988) have shown that the apolar segments from three different kinds of membrane assembly signals do not differ significantly in amino acid composition. But, the inside/outside location of the polar domains, with respect to the interior of the cell, correlates strongly with their Arginyl and Lysyl residue content. This applies not only to bacterial inner membrane proteins, but also for bulk proteins from the ER, the plasma membrane, the inner mitochondrial membrane, chloroplast thylakoid membrane and to a lesser extent polytopic membrane proteins. A positive inside rule seems to apply to all the integral membrane proteins studied; apolar regions facilitating membrane integration and charged residues providing topological information.

Both prokaryote and eukaryote membrane proteins obey the positive inside rule, and approximately 2-4 times the number

of Arginine and Lysine residues are found in non-translocated segments when compared to translocated domains. Charge bias in complex membrane proteins is apparent only for polar segments less than 70-80 residues, suggesting that the mechanism for Tm translocation differs between short ($\leq$7-80 residues) and long ($\geq$100 residues) polar segments. All prokaryotic, and most of the eukaryotic signal sequences carry positively charged amino acids (Lys and Arg) at their N-terminus, even though these are not essential for signal sequence function. A signal sequence lacking basic amino acids, or even exhibiting a net negative charge is still capable of protein export, though at a somewhat slower rate (Vlasuk *et al*;1983, Puziss *et al*;1989).

Addition or removal of merely a single positively charged lysine residue, in one of two critical regions, can be sufficient to reverse the membrane topology of the molecule; from N-terminus out C-terminus out, to N-terminus in C-terminus in. Also inserting a positively charged amino acid before the signal sequence, results in proteins with a cleavable signal sequence and a Tm segment acquiring a stop-transfer sequence and an uncleaved signal sequence; i.e. from an N-terminal out C-terminal in to an N-terminal in C-terminal out conformation (Szczesna-Skorupa and Kemper;1989).

Negatively charged residues are far less potent, and only significantly affect the topology if present in high numbers. This implies that it is the number of positively charged residues, rather than the net charge of a particular region, which controls the localisation of a protein, relative to the membrane. Moreover, SRP-independent and SRP-dependent translocation mechanisms differ in their sensitivity to positively charged amino acids (Nilson and von Heijne;1990).

Recent results suggest that basic residues in the signal sequence may interact with acidic residues in components of

the secretory apparatus (Akital *et al*;1990). The further from the N-terminus the positive charge is located, the less the blocking effect on export; lysine having a much weaker effect when compared with Arginine. The presence of three Aspartatic acid residues have no effect, but six have a partial effect on blocking export. The orientation of the membrane spanning stretch is also influenced by the balance between the number of positively charged amino acids at the amino terminus and those in the hydrophilic domain.

A translocator protein mediates the passage of the polypeptide through the membrane. This mechanism must be able to discriminate between a signal sequence, a stop transfer sequence, and a non-hydrophobic sequence of similar length. As there is no absolute translocation sequence, 20 amino acids are engaged at a time (Singer and Yaffe;1990). To be able to translocate a mixed sequence vertically across the membrane, or a hydrophobic stretch horizontally into the membrane, the proposed translocator is thought to consist of a number of homologous subunits. These form a water filled Tm channel, down the central axis of the aggregate, similar in structure to the acetylcholine receptor. Its membrane spanning helices may be amphipathic with charged or polar residues confined to one face of the helix and the polar faces of several adjacent sequences could form a pore or channel through the membrane (Engelman and Tanford;1980, Neupert and Schatz;1983).

The translocation of the protein into, or across the membrane requires input of energy. This is either provided by Tm electrochemical potential, or by the folding of the protein during or after its translocation through the membrane. The loop model (Inouye and Halegoua;1980) requires the positively charged amino terminus of the signal peptide to interact with negative charges at the inner surface of the membrane. The hydrophobic section of the signal peptide is then inserted into the bilayer in the form a loop. The

loop is cleaved by the signal peptidase, leaving the N-terminus of the protein anchored to the cytoplasmic face of the membrane.

The "trigger hypothesis" emphasises the importance of the assembly information contained in the mature protein sequence, the importance of protein folding, and the direct hydrophobic interaction between the polypeptide chain and the hydrocarbon core of the bilayer (Wickner;1979). However, the importance of energetics in the transfer of polypeptide domains between water and hydrocarbon is only emphasised in the direct transfer model (von Heijne and Blomberg;1979) and the helical hairpin hypothesis (Englemen and Steitz;1981).

The helical hairpin hypothesis proposes that pairs of helices adopt a hairpin configeration which inserts spontaneously into the membrane, without the involvement of either SRP or the SRP-rec. Meanwhile, the nascent chain continues to grow in the cytoplasm. Domains with a hydrophobic surface are responsible for forming the helical hairpin, which by presenting only an apolar face to the fatty acyl chains of the phospholipids effectively shields its polar residues (Wickner and Lodish;1985).

1.12 Cloning membrane proteins

The significant advances made in the study of protein targeting and function are a testimony to the power and versitility of molecular biological and immunological techniques.

Several reliable methods for the construction and screening of cDNA libraries have been developed which, together with efficient expression systems, enable the production of virtually any recombinant protein. They utilise a series of vectors into which the gene of interest can be subcloned, and the protein gene product expressed in living cells.

These include the T7 and λ gt11 phage vectors propagated in the prokaryotic host, *E. coli*, and bacullo virus, vaccinia virus and COS cell based vectors propagated in a eukaryotic host.

Typically, therefore, an expression vector contains a DNA sequence that functions as a "promoter" to direct the synthesis of large amounts of mRNA, another sequence that facilitates the autonomous replication of the vector within the host organism, and in some cases, a sequence corresponding to a selectable marker which enables the identification of host cells carrying the vector.

All the available expression techniques have various advantages and disadvantages associated with them. For instance *E. coli* is cheap, it grows rapidly and, the fact that, it has been so well characterised enables manipulations to be performed out which can allow the expressed gene product to attain levels corresponding to 30% of the total protein synthesised. However, *E. coli* ~~are~~ is unable to carry out the correct post translational modifications of eukaryotic proteins, they are inefficient in producing recombinant secreted proteins, and the proteins expressed intra-cellularly, are often insoluble and form aggregates or inclusion bodies, thus, making extraction and purification difficult. In contrast mammalian expression systems are slower growing, more complex and expensive to maintain, and generally produced smaller yields of the recombinant protein. However, the proteins are invariably processed correctly and accurately targeted to their respective compartments.

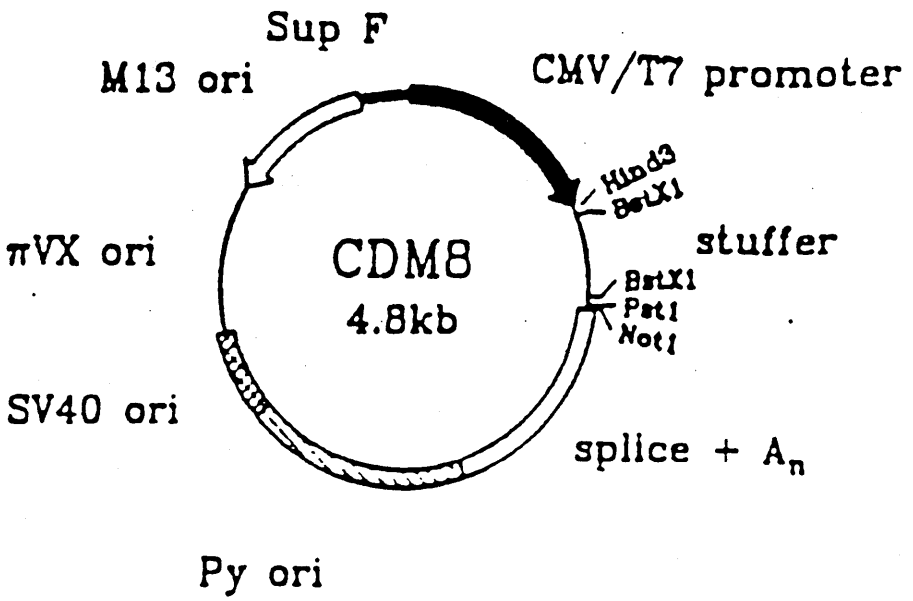
The effectiveness of using such a mammalian expression vector in conjunction with an immunological detection system, to screen a cDNA library is clearly illustrated in the work carried out by Seed and Aruffo (1987). Using this technology, they developed a monoclonal Ab based protocol for the enrichment of cDNAs encoding cell surface antigens.

Their strategy involved constructing a large size selected cDNA library in a high efficiency shuttle vector (CDM8, Fig:1.5). This vector contains a eukaryotic transcription unit which generates high levels of expression in transfected COS cells, when coding sequences are placed under its control. It also contains two identical BstXI sites (in inverted orientation), separated by a short replaceable fragment (the stuffer), to allow for the use of an efficient oligonucleotide-based strategy to promote cDNA insertion into the vector.

BstXI cleavage sites, CCAN'5NTGG, create a four base 3' extension. BstXI cleavage of the CDM7 vector, with its two identical BstXI sites, yields a linearised molecule which is incapable of self-ligating, but able to ligate to a fragment with the same ends as the stuffer region. Thus, synthetic oligonucleotides that generate termini complementary to the four base extension of the two BstXI sites, when attached to cDNA molecules, will generate cDNAs that cannot self-ligate, but are capable of ligating to the vector. Consequently, both the cDNA and the vector are used in the most efficient manner possible. The only disadvantage to this scheme, however, is that it does not allow for directional cloning of the cDNA.

The cDNA coding for the surface antigen of interest can be isolated from these libraries using an antibody enrichment method. This involves DEAE-dextran transfection, which introduces the library DNA into COS cells and allows for the replication and expression of the inserts. After a period of growth, the cells are harvested and incubated with mAbs specific for the surface antigen of interest. The surface antigen positive cells are separated from the rest by panning; pouring the cell suspension onto dishes coated with affinity purified anti-mouse IgGs. Cells expressing the surface antigen adhere while the rest are washed away. Hirt

Figure: 1.5



Shuttle vector CDM8 contains both SV40 and polyoma replication origins, a cytomegalovirus/T7 RNA polymerase promoter, and an M13 origin of replication (Seed; 1987)

(Not to scale)

extraction is used to re-isolate the cDNA from the adherent cells, and the DNA, amplified by electroporation into *E. coli*.

1.13 Aim of the project

This project evolved from the quest to isolate and characterise placental membrane specific proteins thought to be involved in conferring maternal acceptance of the fetus. Its goal was to develop and assess the feasibility of a cloning system that would enable the isolation of cDNAs coding for novel membrane proteins to which no antibodies exist. The strategy devised involved adapting the Seed and Aruffo surface antigen screening system, outlined above. Initially, therefore, a membrane protein expressed at the surface of eukaryotic cells must be selected, for which the gene has been cloned and monoclonal antibodies are available to detect the protein. The DNA coding for the external domain of this protein will be excised and cloned upstream from the stuffer region in the CDM7 vector for use as a "reporter molecule". After, appropriately, modifying the stuffer polylinker to facilitate cloning, cDNAs will be subcloned into this modified vector to generate a "membrane protein external domain-cDNA" or "reporter molecule-cDNA" fusion library. Those recombinants containing correctly orientated and in-frame membrane anchoring sequences, when transfected into COS cells, should synthesise fusion proteins which anchor in the membrane and express the reporter molecule at the cell surface. The panning technique will be used to screen this library, employing the mAb directed against the external domain reporter molecule encoded in the cloning vector. Following the initial screen, the positive clones obtained will be analysed in more detail.

The strategy descibed above, requires a number of assumptions to be made: Firstly it was essential that the

external domain used as the "reporter" to screen for membrane anchoring sequences, cannot anchor to the cell membrane in the absence of a Tm sequence. Secondly, the mAbs used to screen for the external domain should not cross react with any epitope normally found expressed on COS cells. Equally important are the assumptions that in-frame fusions with foreign Tm sequences will restore expression of the external domain at the cell surface, and that out of frame or random DNA sequences will be incapable of doing so.

1.14 The reporter protein

The chosen reporter was the CD2 (Cluster of differentiation molecule 2) protein, originally defined as the sheep red blood cell receptor (SRBC), because of its ability to form spontaneous aggregates (rosettes) with sheep erythrocytes (Brain *et al*;1970, Coombs *et al*;1970, Lay *etal*;1971). Expression of the CD2 antigen is initiated in the thymus, and it is found expressed on nearly all resting or activated human T-lymphocytes. It is involved in an antigen-independent adhesion process, as a ligand-binding molecule (Shaw *et al*;1986) facilitating the interaction between T-cells and antigen-presenting cells (Bierer *et al*;1988a, Bierer *et al*;1988b). It is also involved in the alternative T-cell activation pathway (Meuer *et al*;1988, Alcover *et al*;1987).

The natural ligand for CD2 has been identified as lymphocyte function associated antigen-3 (LFA-3) (Sayre *et al*;1987, Selvaraj *et al*;1987, Takai *et al*;1987), which is widely distributed on endothelial cells, thymic epithelial cells and most blood cells including erythrocytes (Krensky *et al*;1983, Seed;1987, Vollger *et al*;1987). Interaction of CD2 on thymocytes with LFA-3 on thymic stromal cells is important in the thymocyte activation pathway, and is regulated by a CD3-mediated signal in the later stages of maturation (Alcover *et al*;1986, Denning *et al*;1987, Ramarli

et al;1987). In addition, CD2-mediated adhesion and signaling in T-cells appears to be involved in natural killer (NK) cell-target cell interaction (Siliciano *et al*;1985, Seaman *et al*;1987, Anasetti *et al*;1987).

Certain CD2-specific Abs inhibit T-lymphocyte function, this inhibition is accompanied by a failure to express the interleukin-2 (IL-2) receptor. This IL-2 dependent, antigen-independent stimulation by CD2-specific Abs has been termed the alternative pathway of T-cell activation. This alternative pathway is thought to be involved in thymocyte differentiation. The thymocytes are thought to interact via their CD2 molecules with LFA-3 on thymic epithelial cells and thereby become activated. The CD2/LFA-3 complex also participates in Ag-driven T-lymphocyte stimulation, where CD2 on the T-lymphocyte interacts with LFA-3 on an antigen presenting cell (APC) to induce lymphokine secretion and simultaneously provide a proliferation signal for the T-lymphocyte (a signal that potentiates the resulting T-cell receptor-Ag and MHC interaction. These two pairs of adhesion molecules cooperate in mediating lymphocyte adherence) (Meuer *et al*;1984).

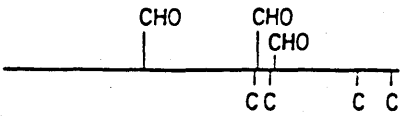
The CD2 gene is found on human chromosome 1 and murine chromosome 3 (Richardson *et al*:1988, Ruddle *et al*;1988). It is composed of five exons spanning 15kb of the genome (Lang *et al*;1988, Diamond *et al*;1988) (Fig:1.6). The CD2 cDNAs from a number of species have been cloned and sequenced; human (Sewell *et al*;1986, Seed and Aruffo;1987), rat (Williams *et al*; 1987) and mouse (Sewell *et al*;1987, Clayton *et al*;1987).

The CD2 protein is a non-polymorphic glycoprotein of approximately 45-55kDa. After the cleavage of a characteristic 19 amino acid N-terminal signal peptide, the mature CD2 polypeptide is predicted to be 327 amino acids in length. The unglycosylated protein has a calculated molecular weight of 36.9kDa (Sayre and Reinherz;1988), with

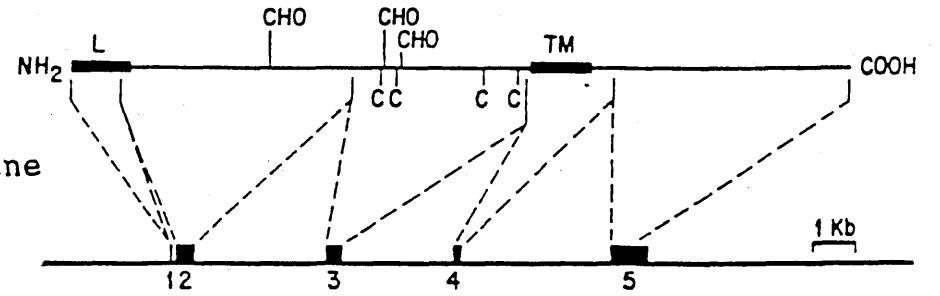
Figure: 1.6

C = Position of cysteine residues
CHO = Carbohydrate addition sites
Tm = Transmembrane region
L = Leader region

a) CD2Ex



b) CD2

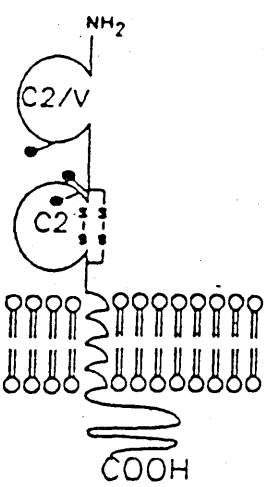


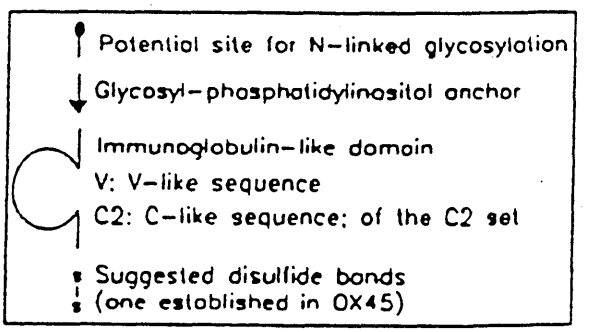
c) CD2 gene

a) and b) schematic structural diagrams of the external domain and native CD2 proteins

c) Diagram of the CD2 gene, exon 1 corresponds to CD2 amino acid residues -24 to -5, exon 2 to residues -4 to 103, exon 3 to residues 104 to 180, exon 4 to residues 181 to 221, and exon 5 to residues 222 to 327 (Sayre and Reinherz; 1988)

d)





CD2

d) structural model of the human CD2 protein in the membrane (Williams *et al*; 1987)

a single 25 amino acid internal hydrophobic membrane anchoring sequence which divides the protein into two regions: a 185 amino acid predicted external segment which consists of two immunoglobulin-like domains (Albertus *et al*;1988, Kileen *et al*;1988) containing three putative N-terminal glycosylation sites (Bierer *et al*;1989), and a cytoplasmic segment of 177 amino acids, 80 residues of which correspond to either Proline or basic amino acids (Lang *et al*;1988). This cytoplasmic domain has little resemblance to any other surface glycoprotein, but it is the most conserved region of CD2 protein (Sewell *et al*;1987, Clayton *et al*;1987, Williams *et al*;1987) (Fig:1.6). It has been postulated therefore, that this is the segment crucial for T-cell activation (Sewell *et al*;1989).

1.15 Experimental strategy

Initially, it would be necessary to establish that the assumptions made in section 1.12 are valid. In order to achieve this a series of control constructs will be designed. The reporter molecule selected, the CD2 protein, was chosen particularly because its cDNA was originally isolated using this surface antigen screening system. The constructs will be designed to express the following proteins:

(a) The external domain of the reporter membrane protein (CD2); to examine whether this domain can anchor to the membrane in the absence of its Tm region.
(b) The external domain of CD2 fused in-frame to a foreign Tm sequence; to determine whether the fusion protein is able to anchor in the membrane.

(c) The external domain of CD2 fused to the stuffer region; to determine whether random DNA sequences can function as membrane anchors.

The experiments necessary to analyse the expression and cellular location
of these proteins include:
(a) Immunofluorescent staining; to visualise the cell surface expression of the proteins.
(b) Immunoprecipitation; to determine whether a protein is secreted.
(c) Western blotting; to estimate protein sizes and study post-translational processing.

To evaluate the proportion of random DNA sequences capable of anchoring the CD2 external domain in the membrane; a fusion library, composed of fragments of genomic DNA ligated into the cloning vector, will be constructed. This genomic fusion library will be transfected into COS cells, and using the techniques described above (immuno-staining, panning and Hirt extractions), the number of potential background membrane anchoring fusion proteins will be calculated.

Finally, if all of the assumptions prove valid, cDNAs will be sub-cloned into the cloning vector to generate a CD2-cDNA fusion library which will be screened for membrane anchored fusion proteins.

CHAPTER 2

MATERIALS AND METHODS

2.1

(i) Bacterial strains

The bacterial strains used in these studies were derivatives of *Escherichia coli* K-12.

C1400 supE, supF, recA, hsd5, met$^-$, λ L512 (Amp^S Tet^S)

DS941 supE44, recF143, LacZ M15, lacIq derived from strain AB1157

DS946 Identical to DS941 except sup0 (as above)

MC1061 F$^-$, araD139, (ara-leu)7697, (lac)Y74, GalY, GalK, hsr$^-$, hsm$^+$, Str^r (Casadaban and Cohen;1980)

(ii) Plasmids

pBR322 (Sutcliffe;1978)

pAT153 (Twigg and Sherratt;1980)

pMTL20 (Chambers *et al*;1988)

pMTL21 (as above)

pCDM8 (Little *et al*;1983)

pRP1 (Jacob *et al*;1976, Windass *et al*;1980)

pDP001 (Sood *et al*;1985) cDNA clone of the HLA-B7 antigen (1410bp)

pCD2 (Seed and Aruffo;1987) cDNA clone of the T-lymphocyte cell surface protein CD2 (1504bp) cloned into the BstXI sites of the cloning vector pCDM7.

pMTL20 and pMTL21 code for the alpha-peptide of β - galactosidase (lacZ) which is able to complement the lacZ(D

M15) deletion in bacterial host strains and restore enzymatic activity. Colonies expressing β-galactosidase are blue on plates containing the chromogenic substrate X-gal. Sub-cloning into the multiple cloning site disrupts the alpha peptide sequence and prevents complementation resulting in colonies which are white. Analysis of transformants on X-gal plates therefore provides a ready means of screening for positive clones.

pCDM8 is derived from the cDNA expression vector pπH3 which in turn is derived from pπSV (Little *et al*;1983). It was constructed by inserting a synthetic transcription unit between the suppressor tRNA gene and the SV40 (Simian virus 40) origin. The transcription unit consists of a chimeric promoter, composed of the human cytomegalovirus AD169 immediate early enhancer sequence fused to the HIV (Human immunodeficiency virus) long terminal repeat (LTR) -67 to +80 sequence. Immediately downstream from the LTR +80 sequence it contains a polylinker with two BstXI sites separated by a 350bp "stuffer". The BstXI sites are flanked by XbaI and XhoI sites, which can also be used to excise the insert. The SV40 small tumour (t) antigen and the splice and early region polyadenylation signals derived from pSV2 are located downstream of the polylinker.

The strain MC1061 carries a single copy, stably maintained 57Kb plasmid called P3. It is derived from the "P" group of plasmids; the most well known being RP1 (Jacob *et al*;1978, Windass *et al*;1980). P3 specifies resistance to Kan and carries amber mutated Tet and Amp resistance genes. The shuttle vector pCDM8, has no selectable markers for growth in *E. coli*. It does however, carry a suppressor tRNA gene, supF. Therefore, when transformed into MC1061, the supF complements the amber mutations and confers Tet and Amp resistance to the cell.

(iii) Microbial culture media

L-broth	10g tryptone, 5g yeast extract, 5g NaCl, 1g glucose and 20mg thiamine, made up to 1l in distilled water and adjusted to pH 7.0 with NaOH.
L-agar	As L-broth, but containing 15g/l agar and no glucose.
Water agar	2% agar in distilled water.
SOC medium	20g/l tryptone, 5g/l yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$ and 2mM glucose.

(iv) Indicators

X-gal	2% (w/v) in dimethylformamide	} store -20C
IPTG	200mg/ml in dH_2O	

(v) Antibiotics

	Stock solution	Solvent	working concentration
Ampicillin	100mg/ml	Water	100ug/ml
Chloramphenicol	30mg/ml	Ethanol	30ug/ml
Kanamycin	25mg/ml	Water	50ug/ml
Rifampicin	5mg/ml	Methanol	50ug/ml
Streptomycin	30mg/ml	Water	30ug/ml
Tetracycline	12.5mg/ml	50% EtOH	12.5ug/ml

(vi) Buffer solutions

TE	10mM Tris.Cl, 1mM EDTA, pH 8.0
20x SSC	3M NaCl, 300mM sodium citrate, pH 7.0
20x SSPE	3M NaCl, 0.2M Na_2HPO_4, 20mM EDTA, pH 7.4

(vii) Organic solvents

Phenol — Phenol was redistilled at 160C and equilibrated to pH8.0 by washing with 1M Tris.Cl pH8.0, followed by 0.1M Tris.Cl pH8.0. 8-hydroxy quinoline (0.1%) was added as an anti-oxidant. Stock aliquots of phenol were stored under 0.1M Tris.Cl pH8.0, protected from light and at -20C.

Chloroform — Analytical grade chloroform was mixed with iso-amyl alcohol (24:1 v/v) and stored protected from light at RT.

Ethanol — Analytical grade ethanol and 70% ethanol (v/v) used for DNA precipitation, were stored at -20C

Formamide — Analytical grade formamide was deionised by the addition of a mixed bed ion exchange resin (amberlite) and mixing for 30min. Filtered, deionised formamide was stored protected from light at -20 C.

(viii) Other solvents

Methanol, acetic acid, ethyl acetate, formaldehyde, dimethyl formamide, DMSO and isopropanol were of analytical grade. They were stored at RT and used directly.

(ix) Sterilisation conditions

All growth media were sterilised by autoclaving at 120C for 15min. Supplements, buffer solutions and calcium chloride were autoclaved at 108C for 10min. Heat-labile reagents were sterilised by filtration through disposable 0.45um membrane filters. Plasticware was sterilised by autoclaving at 120C for 90min. Glassware underwent dry sterilisation.

(x) Microbial growth conditions

Liquid cultures of bacteria, for transformation and DNA isolation, were grown in L-broth at 37C with vigorous shaking. Plate cultures were incubated overnight at 37C. Antibiotics, X-gal and IPTG were added as required. Stocks of the bacterial strains were made by making the liquid cultures (1ml) 15% (v/v) with respect to glycerol and storing in a tghtly sealed vial at -70C.

2.2 Transfomation of *Escherichia coli*

(i) Preparation of competent cells and transformation of *E. coli* using $CaCl_2$

Two tubes containing 20ml L-broth were inoculated with 400ul of a fresh overnight broth culture and incubated with vigorous shaking for approximately 90min at 37C. After allowing growth to continue to mid log phase, the cells were transferred to two 50ml Falcon tubes and pelleted by centrifugation (MSE minor S bench top centrifuge 2.5Krpm 10min). The pellets were suspended in 10ml ice cold 50mM $CaCl_2$ and maintained on ice for 30min and the cells collected by centrifugation (MSE minor S bench top centrifuge 1.5Krpm, 10min). They were resuspended in 2ml of cold 50mM $CaCl_2$ and stored at 4C. Fresh competent cells, prepared as described above, were used for the majority of the transformation experiments.

Vector DNA (5-50ng in 10ul) was added to an aliquot of competent cells (100ul) in a 1.5ml microfuge tube, gently mixed and then maintained on ice for at least 15min. Following this, the cells were either heat shocked at 42C for 2min, or at 37C for 5min. L-Broth (1ml) was added to the mixture and incubation continued at 37C for 40min. The cells were then plated out on selective L-agar plates and

incubated overnight at 37C. A selection of transformants from these plates were picked for DNA isolation and analysis.

(ii) Preparation of frozen competent cells (MC1061/P3) for electroporation and transformation of *E. coli* using electroporation

A flask containing 1 litre of L-broth was inoculated with 10ml of a fresh overnight culture of MC1061/P3. The cells were grown with vigorous shaking at 37C to an OD600nm of 0.5-0.7 (OD600nm 1.0=$8x10^8$ cells/ml). The flask was stored on ice for 15 to 30min after which the cells were transferred to two 250ml plastic bottles (Beckman). The cells were pelleted by centrifugation in a pre-cooled Beckman JA-14 rotor (5krpm, 4C, 15min). The pellets were resuspended in a total volume of 1 litre of a cold low ionic strength wash medium (1mM HEPES, pH7.0) and collected by centrifugation (JA-14, 5krpm, 4C for 15min). They were resuspended in 0.5 litres of cold wash medium and centrifuged as before. The pellets were resuspended in a total volume of 20ml of 10% glycerol and the cell suspensions pooled. After collection by re-centrifugation, the cells were suspended in 20ml of 10% glycerol and aliquots (2.0ml) stored at -70C. These cells remained competent for approximately 6 months.

For transformation of *E.coli* using electroporation, an aliquot of frozen competent cells was allowed to thaw at room temperature and placed on ice. The cells were pelleted in a MSE micro-centaur microfuge (6.5krpm, 10min) and resuspended in 200ul of 10% glycerol. The cell suspension (40ul) was added to the DNA (1-10ng in 1-2ul water) maintained in a cold 1.5ml microfuge tube. After storage on ice for 1min, the contents were transferred to a cold 0.2cm bacterial electroporation cuvette (Biorad) and placed in the safety chamber. The cells were pulsed at 25uF, 2.5KV (Biorad

Gene Pulser electroporation apparatus) with the pulse controller set between 800-1000W to generate an optimal time constant of 15-20msec (field strength 12.5kV/cm). Immediately after pulsing, the cells were suspended in 1ml of SOC medium. After transfer to a 10x75mm glass tube, the cells were incubated with shaking at 37C for 1hr, plated out on selective L-agar plates and incubated overnight at 37C.

2.3 DNA purification and concentration procedures.

(i) Extraction of proteins from DNA samples with organic solvents.

Contaminating proteins were removed from DNA samples by sequential extraction with buffer saturated phenol, phenol/chloroform (1:1 v/v) and chloroform. The DNA samples were mixed with equal volumes of these solvents by vortexing for 1min. Phase separation was enhanced by centrifugation in a microfuge for 10min (unless otherwise stated all microfuge procedures were carried out in an MSE micro-centaur at 13.5krpm). Proteins partition into the organic phase while the DNA remains in the aqueous phase. Residual organic solvents dissolved in the aqueous phase were removed during the course of ethanol or isopropanol precipitation of the DNA.

(ii) Concentration of DNA samples by precipitation.

(a) Ethanol precipitation.

A 1/10th volume of 3M sodium acetate pH 5.2 was added to the DNA sample, followed by two volumes of cold ethanol. The mixture was either stored on ice for 10min, -20C for 30min or at -70C for 15min. The DNA was pelleted by centrifugation (15 to 30min, 4C) and excess salt removed by repeatedly

washing the pellet with cold 70% ethanol and re-centrifuging. The DNA pellets were vacuum dessicated and resuspended in water or the appropriate buffer.

(b) Isopropanol precipitation.
With this procedure, no salt is required to induce precipitation provided the DNA concentration exceeds 70ug/ml. Isopropanol (0.6vol) was added to the DNA sample. The mixture was vortexed and allowed to stand at RT for 10min. The sample was centrifuged for 15min and washed with 70% ethanol. This removes any remaining traces of isopropanol and facilitates the rapid dessication of the DNA pellet. When compared with ethanol, isopropanol has the added advantage of precipitating the DNA in a smaller final volume, although it also precipitates proteins with a higher efficiency.

2.4 Preparation of plasmid DNA.

(i) Minipreparations of plasmid DNA.

(a) For characterisation and subcloning.
Plasmid mini-preparations (STET-preps) were performed according to the boiling method of Holmes and Quigley (1974). Overnight cultures (1.5ml) of a plasmid-containing clone, grown in selective L-broth, were transferred to 1.5ml microfuge tubes. The cells were pelleted by centrifugation (20sec, RT) and resuspended in 350ul of STET buffer; to this was added 25ul of freshly prepared lysosyme solution (10mg/ml). The mixture was briefly vortexed and the tube placed in a boiling water bath for 45sec.

After centrifugation (10min, RT), the pellet was removed with a toothpick and 400ul of isopropanol added to the supernatant. The tube was briefly vortexed, incubated at RT for 10min and the plasmid DNA pelleted by centrifugation in the microfuge (15min, RT). The pellet was washed in 70%

ethanol and re-centrifuged for 5min. It was then dried and resuspended in 50ul of sterile water which usually approximates to a final DNA concentration of 20ug/ml. From these mini-preparations of DNA, 5ul was digested with restriction enzymes and analysed by gel electrophoresis so as to characterise the plasmids.

(b) For double stranded sequencing.

Minipreparations of plasmid DNA for double strand sequencing were executed by a modification of the Holmes and Quigley (1974) boiling preparation. This protocol remains identical to that described previously up to the point where the viscous genomic DNA pellet is removed. Thereafter, in this modified version, 5ul of a 50ug/ml solution of DNase-free RNase (50mg/ml) was added, the contents vortexed briefly and incubated at 37C for 10min. DNA was precipitated by the addition of 400ul of isopropanol and the resulting pellet washed with 1ml 70% ethanol and re-centrifuged. The pellet was dried under vacuum and resuspended in 200ul of sterile water. Proteinase K (25mg/ml) was added to a final concentration of 25ug/ml in order to degrade proteins bound to the DNA and the contents incubated at 37C for 30min. The sample was then extracted with phenol and the DNA precipitated with ethanol. The resulting pellet was collected by centrifugation in a microfuge (5min, RT), washed with 1ml of cold 70% ethanol, dried under vacuum and resuspended in the appropriate volume of sterile water for use in the sequencing reactions.

Starting with 1.5ml of a stationary culture containing a high copy number plasmid (200-300 copies/cell), this procedure yields approximately 2-5ug of clean plasmid DNA suitable for sequencing.

STET buffer

8% Sucrose

5% Triton X-100

50mM EDTA

50mM Tris.Cl, pH 8.0

(ii) Large scale preparation of plasmid DNA

(a) The alkaline lysis method of plasmid preparation. (A modification of that described by Birnboim and Doly (1979)).

An overnight culture (200ml) of a plasmid-containing clone in selective L-broth was transferred to a 250ml plastic centrifuge bottle. The cells were pelleted by centrifugation (JA-14 Beckman rotor, 5Krpm, 4C, 10min), resuspended in 8ml GET containing 10mg/ml lysozyme and stored at RT for at least 5min. Freshly made NaOH/SDS (16ml) was added, the contents mixed gently and stored on ice for 10min. Cold potassium acetate (12ml) was added, the mixture shaken gently and stored on ice for 10min. Chromosomal DNA and cell debris were precipitated by centrifugation (JA-14 rotor, 10Krpm, 4C, 20min) and the supernatant transferred into two 35ml plastic centrifuge tubes (Oakridge). Isopropanol (0.6vol), was added and the mixture stored at RT for 15min. Plasmid DNA was collected by centrifugation (JA-20 rotor, 18Krpm, RT, 20min). The pellet was washed with 70% ethanol (5ml), re-centrifuged and vacuum dessicated.

GET	NaOH/SDS	Potassium Acetate/l
25mM Tris.Cl, pH8.0	0.2M NaOH	60ml 5M Potassium acetate
50mM Glucose	1.0% SDS	11.5ml Glacial acetic acid
10mM EDTA, pH8.0		28.5ml Distilled water

(b) Purification of covalently closed circular (ccc) plasmid DNA using caesium chloride (CsCl) density gradients.

Solid CsCl (10g) was added to 10ml of a large scale plasmid preparation in TE containing 0.8mg/ml of ethidium bromide

(EtBr), so as to yield a final density of 1.6g/ml. The mixture was clarified by centrifugation (JA-20 rotor, 5Krpm, RT, 10min) and the supernatant sealed into a 12ml Ti70 ultra-centrifuge tube. Density gradients were formed by centrifugation (Ti70 fixed angle rotor, 49krpm, 25C, 18h) and viewed under a longwave UV source (366nm). The lower band corresponding to ccc plasmid DNA was removed using a syringe and a 21-guage needle, and the EtBr extracted by repeated (4x) partitioning into isopropanol. The ccc plasmid DNA was divided between two 10ml centrifuge tubes (Oakridge). Water (2vol) was added followed by 6vol of ethanol and the plasmid DNA precipitated by incubation for 30min at -20C. The DNA was recovered by centrifugation (JA-21 rotor, 18krpm, 4C, 20min) and the pellets washed in 70% ethanol. After re-centrifugation, the pellets were vacuum dessicated and dissolved in 500ul of sterile water. Plasmid DNA yield was estimated from the UV absorbance of the sample at 260nm and the relative purity determined from the ratio of the 260/280nm UV absorbances. Sterile water was added to the purified plasmid DNA to give a final concentration of 0.5mg/ml.

2.5 DNA gel electrophoresis.

(i) Agarose gel electrophoresis.

Agarose gel electrophoresis was used for the separation and analysis of DNA fragments greater than 200bp in size. Smaller fragments were analysed on polyacrylamide gels. Electrophoretic mobility is inversely proportional to $\log^{10}$ molecular weight. The molecular weights of isolated DNA fragments were estimated by comparison with DNA standards of known molecular weight, electrophoresed on the same gel. The most commonly used molecular weight standards are generated by digestion of wild type phage lambda (λ) DNA with either HindIII or a combination of HindIII and EcoRI.

During the course of this study, agarose gels of different sizes, concentrations and applied voltages were used. This depended on the size of the fragments being separated and the degree of resolution required.

Gel sizes	Baby gels 7.5cmx5.0cm 20ml
	Mega gels 20.0cmx16.5cm 250ml
Agarose concentrations	0.3% - 2.5%
Applied voltages	2.0V/cm - 10.0V/cm

The agarose was dissolved by boiling in 1x running buffer and EtBr (10mg/ml) added to a final concentration of 0.5ug/ml. The DNA samples were mixed with 1/10th vol of 10x loading buffer prior to loading and electrophoresis.

10x TBE		/l	
0.90M	Tris-borate	121.1g	Tris.base
		55.0g	Boric acid
0.20M	EDTA pH8.3	7.4g	EDTA

50x TAE		/l	
0.40M	Tris acetate	242.0g	Tris base
		57.1ml	Glacial acetic acid
0.05M	EDTA pH8.3	100ml	0.5M EDTA pH8.0

10x Loading dye/50ml

0.25%	Bromophenol blue
0.25%	Xylene cyanol
0.25%	Orange G
25%	Ficoll 400

(ii) Visualisation and photography of gels.

DNA fragments were visualised by UV-induced fluorescence of EtBr on short (254nm) or medium (302nm) or long (366nm)

wavelength transilluminators. Gels were photographed using a polaroid camera loaded with polaroid 545- or 667- type land film and fitted with a Kodak Wratten filter No.9.

2.6 Isolation of DNA fragments from agarose gels.

Purified DNA fragments are usually required for further enzymatic manipulations. It is important, therefore, to ensure that the DNA does not contain impurities which may interfere with these reactions. In order to minimise this, ultra pure agarose was used and all gels were formed and run in TAE gel buffer, as the presence of residual borate ions from gels run in TBE can inhibit the efficiency of subsequent enzymatic digestions and ligations.

(i) Electroelution.

This is a simple method for recovering DNA fragments from gel slices. However, the DNA is often recovered in a large volume and thus is dilute. Ethanol precipitation of DNA from large volumes is rather inefficient and therefore the gel slice to be electroeluted should contain a minimum of 0.5ug of DNA.

The DNA samples were run on gels containing 0.5ug/ml EtBr. Once adequate resolution of the DNA had been achieved, the band of interest was visualised and excised under long wavelength (366nm) UV light (to reduce photo-nicking of the DNA). The gel slice was placed in buffer-filled (200ul) dialysis tubing, clamped and subjected to electrophoresis. DNA migration out of the gel slice was followed using a hand-held long wavelength UV light and electrophoresis discontinued when the DNA was observed to have adhered to the dialysis tubing. After removal of the gel slice the tubing was resealed and placed back in the gel tank. Electrophoresis was carried out under reverse polarity

(30sec) so as to detach the DNA from the dialysis tubing. The DNA solution was decanted and the tubing rinsed with buffer to recover any residual DNA bound to the walls. After phenol extraction to remove any residual agarose, the DNA was ethanol precipitated. The pellet was washed with 70% ethanol, dried and finally resuspended in the appropriate volume of sterile water.

(ii) Extraction of DNA from low melting point agarose gels.

Following preparative gel electrophoesis using low melting point (lmp) agarose in TAE buffer, the band was excised from the gel, including as little excess agarose as possible. The slither of agarose was weighed, placed in a 1.5ml microfuge tube and to this 5 volumes of sterile water was added. The tube was heated at 65C for 10min to dissolve the agarose followed by the addition of an equal volume of buffered phenol (not phenol/chloroform). The mixture was vortexed vigorously and centrifuged (15min, RT). The aqueous phase was set aside and the phenol phase re-extracted with an equal volume of sterile water. The aqueous phases were combined and the DNA precipitated with ethanol. The pellet was washed with 70% ethanol, vacuum dried and resuspended in sterile water.

(iii) Purification of DNA fragments using "Gene Clean".

This is a very rapid method for isolating DNA fragments in a pure and concentrated form. Its major disadvantages are that the nature of the purification tends to shear large fragments of DNA (>10Kb), and also that small fragments of DNA (<250bp) are poorly recovered as they become irreversibly bound to the glass milk.

The DNA band was excised from an EtBr-stained agarose gel and the approximate volume of the gel slice was calculated

from its weight (1g is approximately equal to 1ml). The gel slice was transferred to a 1.5ml microfuge tube, to which 2.5 volumes of NaI stock solution was added. The tube was placed in a 55C water bath for 5min to dissolve the gel. Once dissolved, 5ul of the resuspended glass milk was added, the mixture was vortexed and the tube left on ice for 5-10min to allow the DNA to bind to the silica matrix. The DNA-glass milk complex was pelleted in a microfuge for 20sec, the supernatant decanted and 500ul of ice cold New wash buffer added. The pellet was vortexed until the glass milk was completely resuspended. The tube was spun for 20sec and the supernatant discarded. The "New wash" was repeated 3x and the residual liquid removed with a fine pipette. To elute the DNA from the glass milk, 15ul of sterile distilled water was added and the mixture was vortexed and heated to 65C. The glass milk was pelleted as before and the eluted DNA transferred to a fresh tube. The glass milk was re-eluted to maximise the DNA yield and the two elutions combined.

NaI stock solution

6M NaI
0.75g Na_2SO_3 was dissolved in 40ml dH2O, 45.62g of NaI was added 10g at a time until saturated. The mixture was filtered through a 0.45um filter and stored in the dark at 4C

New wash buffer

10mM Tris.Cl pH7.5 (at 40C)
125mM NaCl
0.5M EDTA
made up in 50% ethanol and stored at -20C

Glass milk

Glass powder suspension in 50% dH_2O or TE

DNA recovered from gel slices was quantified according to the following protocol. A microlitre of each of a set of DNA standards (400, 200, 100, 50, 25, 12.5, and 6.25ng/ul) was dotted onto an EtBr plate (1% agarose, 5ug/ml EtBr), together with 1ul of the DNA sample to be analysed. The

approximate concentration of the unknown sample was determined by comparison with the standards when visualised under medium wavelength UV light.

2.7 DNA manipulations

(i) Digestions of DNA with restriction endonucleases.

(a) Complete digestion of DNA

(i) Single enzymatic digestions.

All single digestions were carried out using 1-5ug DNA, 10U of enzyme and the BRL system of React buffers (See BRL catalogue), in a final volume of 20ul. The digestions were carried out at 37C for 1-3h, unless otherwise stated.

(ii) Multiple enzymatic digestion of DNA

Digestion of DNA with multiple enzymes was usually carried out in React 2 buffer, provided that each enzyme retained at least 50% of its activity (according to the table in the BRL catalogue). Alternatively, the "one for all" enzyme buffer system (KGB) was employed.

5x KGB (McClellend *et al*;1988)

500mM	Potassium glutamate
125mM	Magnesium acetate
250ug/ml	Bovine serum albumin (BSA)
2.5mM	2-Mercaptoethanol

The volume of restriction endonuclease added should be kept to less than 1/10th of the volume of the final reaction mixture. This is because the glycerol present in the enzyme storage buffer might have an inhibitory effect on the enzymatic digestion of DNA.

(b) Partial digestion of DNA.

It is often necessary to cleave DNA at only a subset of the reaction sites, particularly for subcloning segments of DNA in which the sites used for cloning are also present

internally within the segment. As with all enzymatic reactions, the rate can be influenced by time, temperature and concentration. Commonly, the extent of a restriction digest is modulated by adjusting the endonuclease concentration in conjunction with the reaction time.

A series of 3 digests was set up in duplicate, with the following dilutions of enzyme; 1/5, 1/10 and 1/50 (2.0, 1.0 and 0.2U/ul) in the appropriate restriction buffer. 1ul of each dilution was used to digest 3ug of DNA (final volume 20ul). The first set was digested for 15min and the second for 30min, and the reactions were stopped by the addition of 1ul of 10x loading dye. The digests were analysed by gel electrophoresis on an agarose gel and the conditions most suited to generating the required partial digest were determined. Scaled up reaction digests using the identical conditions were carried out to obtain sufficient quantities of the restriction fragment of interest.

(ii) Ligation of DNA fragments

T4 DNA ligase catalyses the formation of phosphodiester bonds between juxtaposed 5' phosphate and 3' hydroxyl termini in double stranded DNA (dsDNA). It is able to join restriction fragments with either blunt or compatible cohesive ends as well as repair nicks in duplex DNA.

The activity of T4 ligase is optimal at 30C. However, ligations between cohesive ends of DNA were carried out between 12-18C (based on their melting temperatures) to allow adequate annealing of the DNA. Ligation of flush-ended DNA was carried out at room temperature as no annealing is required, and it is considerably less efficient (up to 100 fold less) than cohesive end ligations. To optimise ligation efficiencies, the inhibitory effects of salt and ATP (co-factor) were minimised by maintaining their concentrations below 150mM and 10mM respectively.

Ligations were carried out at a total DNA concentration of 20ug/ml. Typically, 1U of T4 ligase was added to a reaction mix containing 1ul of each of the 10x ligation buffers (see below), and supplemented with 1ul of a 10mM stock of ATP, in a final volume of 10ul. To assess ligation efficiency, half the sample was analysed on a 20ml baby agarose gel and the remainder set aside for transformations (1-10ng DNA/transformation). Theoretically, maximal ligation efficiencies should result when the reaction contains equimolar concentrations of the ends. Practically, however, this is rarely the case.

The efficient ligation of an insert to a vector containing similar cohesive ends requires a molar excess of the insert (3:1) so as to prevent the vector from recircularising. Cloning into a vector with dissimilar ends (forced orientation cloning) or one that has been dephosphorylated lowers the optimal concentration of insert required.

For ligations between similar or dissimilar cohesive ends and for those between an insert and dephosphorylated vector, the ratio of ends (insert:vector) ranged from 2:1 to 5:1 and the reactions were carried out at 16C. Blunt end ligations, being less efficient, were carried out with a five-fold molar excess of insert. The ligations were carried out at the same total DNA concentrations (20ug/ml) but pre-incubated at RT for 3hr to enhance inter-molecular ligation events between the vector and insert. Thereafter, the sample was diluted two-fold in 1x ligation buffer (to increase the bias towards self-ligation), a further unit of enzyme added and the DNA ligated overnight at 16C. Ligation of small DNA fragments such as linkers are significantly more inefficient. For example, the ligation of a 12bp linker requires an linker to DNA ratio of approximately 10:1 whereas an 8bp linker requires a ratio of 100:1. Therefore, the molar ratios used were adjusted accordingly.

Ligation buffer I 10x	Ligation buffer II 10x
(50ul aliquots, -20C)	(50ul aliquots, -20C)
60mM Tris.Cl pH7.5	1mM ATP
60mM $MgCl_2$	20mM DTT (Dithiothreitol)
50mM NaCl	1mg/ml BSA
2.5mg/ml BSA	10mM Spermidine
70mM 2-Mercaptoethanol	

ATP	Ligase
10mM (10ul aliquots -20C)	1U/ul (-20C)

10ul Ligation reaction
6ul DNA/water (total approximately 200ng)
1ul Ligation buffer I 10x
1ul Ligation buffer II 10x
1ul ATP 10mM
1ul Ligase 1U/ul

(iii) Phosphorylation of synthetic linkers and oligonucleotides

Chemically synthesised oligonucleotides lack the 5' phosphate group essential for the formation of phosphodiester bonds in DNA. To facilitate the cloning of these molecules, addition of the phosphate group (phosphorylation) is carried out enzymatically. 1-10ug of oligonucleotide was phosphorylated in a reaction mix containing 3ul of 10x React 2 buffer, 3ul of 10mM ATP, 20U of T4 polynucleotide kinase in a final volume of 30ul. The mixture was incubated at 37C for 60min and the reaction terminated by the addition of 1ul 0.5M EDTA.

(iv) Dephosphorylation of vector fragments

The vector (1-5ug) was digested to completion with the restriction endonuclease of choice in a final volume of

20ul. The volume was increased to 50ul in 1x restriction buffer and 1ul CIP (Calf intestinal phosphatase 1U/ul, stock stored at 4C) was added. The tube was vortexed briefly and incubated at 37C for 20min. To terminate the reaction, the mixture was extracted once with phenol/chloroform, followed by chloroform extraction and then ethanol precipitated. The resulting pellet was washed with 70% ethanol, dried and resuspended in an appropriate volume of sterile water.

(v) Filling in recessed 3' ends of double stranded DNA

Filling of 3' recessed ends following digestion of DNA with restriction enzymes does not require either the inactivation of the enzyme or altering of the buffer. 1ul of 0.5mM mix dNTP (0.5mM, of each of the dNTPs) and 1-5U of Klenow polymerase was added to 20ul of a completed restriction digest (0.1-5ug DNA). The mixture was vortexed briefly and incubated at 30C for 30min. The reaction was terminated by the addition of 1ul 0.5M EDTA.

2.8 Double stranded DNA sequencing

This was carried out by the technique originally described by Chen and Seeburg (1985). Sequencing using double stranded templates requires purified RNA-free plasmid DNA. Small scale DNA preparations were carried out using the modified boiling preparation method, while large scale preparations used the alkaline lysis method followed by purification of the DNA on CsCl gradients; both of which have been described previously.

(i) Primer annealing

Plasmid DNA (2-5ug per sequencing reaction) was denatured in 0.2M NaOH, 0.2mM EDTA (30min at 37C) and neutralised by the

addition of 0.1vol 3M Na acetate (pH 5.0). The DNA was precipitated with 3 vol of ethanol (-70C, 15min) and centrifuged for 20min. The pellet was washed in 70% ethanol, dried and resuspended in 8ul of sterile water, 1ul 10x reaction buffer and 1ul (40ng) of primer (1:5 template:primer). The mixture was spun briefly to collect the contents and incubated at 37C for 15-30min.

10x Reaction buffer (BRL)	Primer
70mM Tris.Cl, pH7.5	15bp oligonucleotide
70mM MgCl2	stock solution 40ug/ml
500mM NaCl	(8pmoles/ul)

(ii) Sequencing reactions

The stock dNTPs and ddNTPs were stored at -20C at a concentration of 10mM (stable for 6 months). For use in the sequencing reactions the following series of dNTP and ddNTP mixes (A^o C^o G^o T^o)were prepared as follows.

1/20th dNTP stock	dNTP mixes (ul) A^o	C^o	G^o	T^o	ddNTP mix
0.5mM dCTP	20	1	20	20	1mM, ddATP (1/100th,10mM stock)
0.5mM dGTP	20	20	1	20	0.3mM,ddCTP (1/33th,10mM stock)
0.5mM dTTP	20	20	20	1	0.5mM,ddGTP (1/20th,10mM stock)
reaction buffer 10x	20	20	20	20	1.0mM,ddTTP (1/10th,10mM stock)

Owing to their lower concentration, these nucleotide mixes are less stable than the concentrated stocks, and may only be kept for 2 weeks at -20C.

During the course of the annealing reaction, four 0.5ml microfuge tubes were labelled A, C, G and T. To each of these, 1ul of each of the respective dNTP mixes and ddNTPs was added. Once the annealing reaction was complete, 3ul [α-^{35}S]-dATP, 1ul 100mM DTT and 1ul 2U/ul Klenow fragment were

added to the annealed template. The contents were mixed by pipetting up and down and centrifuged to collect the contents. A 3ul aliquot of the mix was dispensed into each of the four reaction tubes A, C, G and T, the contents mixed, centrifuged briefly and incubated at 30C for 20min. Following this a cold chase (1.5ul of a 0.125mM mix of all four dNTPs) was added to each of the four reaction tubes and these were incubated for a further 15min at 30C. The reactions were then terminated by the addition of 5ul of dideoxy stop buffer.

[∝-35S]dATP	aqueous solution 600Ci/mmole, 9uCi/ul $t_{1/2}$ = 87 days
DTT (dithiothreitol)	100mM
Klenow large fragment	2U/ul
Chase	0.125mM dNTP (all 4)

Dideoxy stop buffer	98% deionised formamide (w/w)
	10mM EDTA, pH 8.0
	0.2% Bromophenol blue (w/v)
	0.2% Xylene cyanol (w/v)

(iii) Sequencing gels

Analysis of the dideoxy sequencing reactions was carried out on polyacrylamide gels in the presence of high concentrations of urea (8.3M), to maintain the DNA fragments in the denatured (single stranded) state. An 8% polyacrylamide gel allows approximately 150-200bp of sequence to be read. Two tracking dyes contained in the stop buffer were used to monitor electrophoresis; and these, bromophenol blue and xylene cyanol, migrate on an 8% gel at positions equivalent to 15bp and 75bp respectively.

To 99ml of instagel, 1ml of freshly prepared APS and 10-20ul of TEMED were added to induce polymerisation. The gel was poured immediately and left for a minimum of 4h to allow

complete polymerisation. The gels were run in 1x TBE at high voltages (1500-1900V) so as to generate sufficient heat to maintain the DNA in the denatured state (the optimal surface temperature of the gel plate is 50C). In order to achieve a constant temperature, the gel was pre-electrophoresed at constant power (60W) for 15-20min prior to loading the samples.

Once pre-electrophoresed, the sample wells of the gel were rinsed out with a pasteur to remove any urea which had diffused into the wells. The DNA samples were heated to 100C for 5min and 3ul of each sample was loaded per well. The remainder was stored frozen at -20C (stable for 2 weeks). The gel was run until the bromophenol blue band reached the bottom of the gel. The plates were dismantled and the gel fixed in 5% methanol/5% acetic acid solution for 15-20min prior to drying under vacuum.

Acrylamide stock	40% (w/v) Acrylamide/Bisacrylamide (19:1) dissolved in water, filtered through Whatman 3MM and stored in a dark bottle at 4C (stable for 1 year).
Urea	Ultra pure, used at a concentration of 8.3M.
TBE (1x)	90-100mM Tris borate, pH8.3, 2mM Na_2EDTA.
TBE (10x stock)	1 mole Tris base titrated to pH8.3 with boric acid (generally 0.90-0.95 moles boric acid), 40ml 0.5M EDTA, pH8.0 made up to 1l with dH_2O.
Ammonium per - sulphate (APS)	10% (w/v) stable at 4C for 1 week.

Instagel 8%	40% (w/v) Acrylamide stock 20ml 50g Urea 10x TBE 10ml Water 30ml Stable for 1 month at 4C in a dark bottle.

(iv) Autoradiography

The dried gel was placed in a cassette adjacent to a sheet of Kodak Xar-5 film and exposed for 18-24h, at RT. Longer exposure times were used if the signal proved too weak (eg. 1 week).

2.9 Isolation of RNA

RNases are very stable RNA degrading enzymes, which generally require no cofactors to function. Therefore, even a small quantity of RNase contamination in an RNA preparation will result in degradation.

When undertaking any work with RNA, gloves were always worn. In addition, the following precautions were taken:

(i) Preparation of RNase free materials

(a) Apparatus.

The Glassware (flasks, beakers, pasteurs and pipettes) was baked at 200C for 4h (autoclaving is insufficient treatment to destroy all the RNases).

Sterile disposable plasticware (Falcon tubes, plastic pipettes, Sterilin tubes, plastic bijous and Beckman SW50.1 ultra centrifuge tubes) was assumed to be RF, provided the package was unopened and intact.

Reusable plasticware (beakers, tubing, measuring cylinders, tip racks and non sterile consumables eg. microfuge tubes

and tips), which could not be baked were soaked in a solution of 0.02% DEPC (diethylpyrocarbonate, a mild RNase inhibitor) for 4-8hrs, drained and autoclaved prior to use.

Equipment such as gel kits, formers and combs which can neither be baked or autoclaved were soaked in DEPC as before and then rinsed in sterile RF water. These were then handled only with gloves and when not in use were covered and kept aside.

(b) Solutions

Chemicals, reagents and solvents prior to opening were assumed to be RF and a set were kept aside exclusively for RNA work. All solutions (except those containing Tris, which reacts with DEPC to inactivate it) were treated with DEPC, at a final concentration of 0.02%. Vigorous shaking was required to dissolve the DEPC and the solutions were left for 4-8h before autoclaving. Solutions containing Tris were made up with RF chemical stocks dissolved in RF water and autoclaved.

(ii) Extraction of RNA from tissue culture cells

This procedure is modified from the method described by Chirgwin et al., (1979). The cells from 6-12 large tissue culture flasks (base area 150cm^2) were harvested and the cell suspension pelleted by centrifugation (500rpm MSE minor S benchtop centrifuge 10min) in a 50ml polypropylene Falcon tube. The pellet was made up to a total volume of 27ml with the Guanidinium denaturing solution and to this 194.5ul 2-Mercaptoethanol was added (=7.2ul/ml, i.e. a final concentration of 0.1M 2-Mercaptoethanol). The tube was vortexed vigorously (5-10min) to lyse the cells, 10.8g of CsCl was added (1.8g/2.5ml homogenate) and the tube vortexed again until the CsCl had completely dissolved. 1ml of a 5.7M CsCl solution (1/6 volume of the tube) was added to six Beckman polyallomer ultra centrifuge tubes (13x51mm) to form

a cushion, and the homogenate was then layered on carefully to within 2mm of the top. These were balanced (to 0.01g) and centrifuged (Beckman SW55 rotor, 35Krpm, 20C, 18h).

Denaturing solution.

4M Guanidiniumisothiocyanate	A 250g box of Guanidinmium isothiocyanate was dissolved in 293ml dH2O
25mM Sodium Citrate, pH7.0	17.6ml,0.75M Sodium citrate
0.5% Sarcosyl	26.4ml 10% Sarcosyl
1.0% 2-Mercaptoethanol	5.3ml 2-Mercaptoethanol

dissolved at 65C and stored at RT (stable for 6 months).

CsCl cushion /100ml

5.7M CsCl	95.8g CsCl
0.1M EDTA	1.26ml EDTA (0.5M)

pH7.5

After centrifugation the supernatant from each tube was carefully aspirated and the tubes inverted to drain away the residual liquid. The RNA pellets were dislodged from the bottom of the tube and each pellet was transferred to a separate 1.5ml microfuge tube. To ensure that all the RNA had been completely removed, the bottom of each ultra centrifuge tube was rinsed twice with 100ul of water. The RNA was ethanol precipitated with 3vol of ethanol, vortexed briefly and stored at -20C for 30min. No additional salt was added due to the high concentration of CsCl present. The RNA was pelleted at 13.5krpm for 15min, washed twice in 70% ethanol and dried. Each pellet was resuspended in 200ul of water, phenol extracted twice, chloroform extracted once and ethanol precipitated with 1/10th vol of 3M Na acetate (pH 5.0). The RNA was centrifuged for 15min, washed in 70% ethanol and vacuum dried. It was finally taken up in a total volume of 500ul water, pooled into one microfuge tube and stored at -20C.

(iii) Analysis of RNA on non-denaturing agarose gels

To obtain both an estimate of the quality and the quantity of the RNA preparation, 5ul (1/100th of the preparation) was run on a small non-denaturing agarose gel containing 0.5ug/ml EtBr. When visualised under UV light, an undegraded RNA sample will show 2 tight bands corresponding to 28S and 16S ribosomal RNA. Between these should lie the majority of the mRNA which is usually visible as a smear.

2.10 Blotting of agarose gels

(i) Denaturing/neutralising DNA gels for Southern blots

DNA agarose gels to be blotted were run normally in either 1x TAE or TBE, but certain precautions were taken. The wells were never overloaded (ie. no single band generated from a digest exceeded 1ug) and the gel was always run slowly (approximately 2.5V/cm) to eliminate the possibility of smearing. Although Southern blots are very sensitive (capable of detecting 10pg of DNA), the percentage of agarose was kept below 1.5% to ensure efficient transfer of the DNA. Once run, the gel was placed in a tray and the DNA denatured by the addition of 500ml of denaturing solution. The tray was rocked gently for 15min and the solution decanted. A further 500ml of denaturing solution was added and this procedure repeated. The denaturing solution was discarded and 500ml of neutralising solution added. The gel was left rocking for 30min, after which the neutralising solution was poured off and a second wash in neutralising solution carried out. At this stage the gel was ready to be blotted.

Denaturing solution	/l	Neutralising solution	/l
1.5M NaCl	87.5g NaCl	3.0M NaCl	175.5g NaCl
0.5M NaOH	20.22g NaOH	0.5M Tris	6.7g Tris.OH and 70.2g Tris.Cl

(ii) Pouring and running Northern blots

RNA is normally separated on denaturing agarose gels containing formaldehyde as the denaturant. The amount of RNA loaded varied from 5-20ug/lane depending on the abundance of the mRNA of interest. An appropriate quantity of RNA was ethanol precipitated, washed twice with 70% ethanol and vacuum dried. It was resuspended in approximately 5ul of water (RNA is highly soluble in water, up to 3ug/ul) and denatured in MMF (25-35ul) at 60C for 15min. Formaldehyde gel loading buffer (10-15ul) was added and the tube vortexed. The contents were collected by brief centrifugation and the RNA transferred to ice, ready for loading. The RNA was run on a 1.5% agarose gel which was prepared by boiling 1.5g agarose in 73ml H_2O (for every 100ml of gel). This was allowed to cool to 60C, before adding 10ml 10x MOPS and 16.2ml 37% formaldehyde. The components were mixed and the gel poured promptly (20x20cm agarose gel requires 300ml agarose). The combs used were 10mm wide and 1mm thick to form wells which could comfortably accomodate a volume of 50ul. The gel was run at 5V/cm for approximately 7h in a recirculating 1x MOPS buffer reservoir until the bromophenol blue had run 2/3 of the way down the gel.

MMF		10x MOPS/l
Formamide	500ul	500ul 0.2M MOPS (41.8g)
37% Formaldehyde	162ul	162ul 0.05M Na acetate (4.1g)
10x MOPS	100ul	100ul 0.01M EDTA (1.86g) pH 7.0
dH2O	283ul	133ul autoclave and store in a dark bottle at RT.

Formaldehyde gel loading buffer

50% Glycerol

1mM EDTA

0.4% Bromophenol blue

0.4% Xylene cyanol

(iii) Setting up blots and fixing nucleic acids to nylon filters (Southern;1975)

A neutralised DNA or a formaldehyde RNA gel (for Southern or northern blots respectively) was inverted and placed wells face down onto the Whatman wick of a blotting apparatus (for diagram see pg 2.9.3 in Ausubel *et al*;1989). Above this was placed a piece of nylon membrane (Pall Biodyne) which was cut 2mm smaller than the gel in both dimensions. The positions of the wells were marked on the membrane using a black biro. Following this, 3-5 pieces of Whatman 3MM paper were cut 5mm smaller in both dimensions than the nylon membrane. These were soaked in 20x SSC, drained and placed on top of the membrane. To ensure efficient transfer air bubbles trapped between the layers were displaced by rolling a 10ml pipette back and forth across the gel sandwich. A 3cm stack of paper towels was placed above this and covered with a glass plate onto which a 0.5Kg weight was placed to keep the sandwich compressed. Blotting occurs by capillary action and the rate at which this is achieved is dependent on the percentage of agarose and the thickness of the gel concerned. To ensure complete transfer of nucleic acids to the membrane, blotting of the gel was allowed to continue overnight. The gel sandwich was then taken apart, and the membrane lifted off the gel using blunt forceps. It was then sandwiched between two pieces of Whatman 3MM paper and baked at 80C for 1h to fix the nucleic acids to the membrane.

2.11 Hybridisation analysis of nucleic acids immobilised on nylon

(i) Preparation of probes from low melting point agarose

Plasmid or phage DNA was restricted to obtain the desired fragment. The sample was separated on a low melting point (lmp) agarose gel and the required fragment excised with the minimum amount of extraneous agarose. The DNA/agarose mix was diluted to a final concentration of 10ng/10ul; (1ug agarose =1ul H_2O). This was boiled for 10min and placed at 37C for a minimum of 10min. A 10ng (10ul) aliquot was taken for radioactive labelling and the remainder stored as a stock at 4C.

(ii) Radiolabelling of DNA probes by random primed synthesis

To a pre-boiled 10ul aliquot of a 1ng/ul mix of DNA/lmp agarose was added 24ul of sterile distilled water, 10ul of 5x Random prime mix, 2ul of BSA (ultra pure DNase-free, 2mg/ml), 1ul of Klenow (3U/ul) and 3ul of [α-^{32}P]dATP (10uCi/ul). The solutions were mixed by gentle pipetting and either incubated for 1h at 37C or overnight at RT. The labelled probe was separated from the unincorporated nucleotides by gel filtration on a Sephadex G50-150 column eluted with TE. 10ul of sample buffer containing dextran blue and phenol red were added to the mixture. The two dyes co-elute with the DNA and unincorporated nucleotide fractions respectively.

5x Random prime mix		Solution A	
200ul	Solution A	1ml	1.25M Tris/0.125M $MgCl_2$, pH8.0
200ul	Solution B	18ul	2-Mercaptoethanol
300ul	Solution C	5ul	dCTP (100mM dissolved in TE)
		5ul	dGTP (100mM dissolved in TE)
5ul	dTTP (100mM dissolved in TE)		

Solution B (store 4C)	Solution C
2M HEPES titrated to pH6.6 with 4M NaOH,does not completely	Random hexanucleotides (Hexa DNA Pharmacia) evenly suspended
10x Sample buffer	dissolve in TE at 90_{OD} unit/ml.
50% Glycerol	Supplied at 50U, resuspend in 556ul
0.25% Dextran blue	TE
0.15% Phenol red	

(iii) Hybridisation of radioactive probes to nucleic acids immobilised to nylon filters

Once baked, the blots were prehybridised in order to block any non-specific sites and thus prevent high backgrounds. This was achieved by placing the filter in a sealable plastic bag and adding the appropriate hybridisation buffer ($0.5ml/cm^2$). After displacing any air bubbles present, the bag was sealed and incubated in a shaking water bath for 2h (65C; Southern and 42C; northern). The corner of the bag was cut and the freshly denatured (by boiling for 5min) labelled probe was added. The air bubbles were displaced and the bag resealed. Hybridisation was carried out overnight in a shaking water bath for both Southern (65C) and Northern (42C) blots.

Hybridisation buffers

Southern hybridisation buffer

Stock	/100ml	Final concentration
20x SSPE	25ml	5x SSPE
10mg/ml hsDNA	1ml	100ug/ml
100x Denhardts	5ml	5x Denhardts
10% SDS	5ml	0.5% SDS
dH_2O	64ml	

Northern hybridisation buffer

Stock	/100ml	Final concentration
100% Formamide	50ml	50% Formamide
20X SSPE	25ml	5x SSPE
10% SDS	10ml	1% SDS
5% Na PPi	0.1ml	0.005% Na PPi
10mg/ml Poly A	0.15ml	15ug/ml Poly A
100x Denhardts	10ml	10x Denhardts
dH2O	3.75ml	

100x Denhardt$
2.0% Poly vinyl pyrollidone
2.0% Ficoll
2.0% BSA
dissolved in dH_2O

(iv) Stringency of washing

After hybridisation, the filter was removed from the bag and excess weakly hybridised probe (which had bound to a stretch of partially complementary sequence) and non-specifically bound probe was removed by a series of washes. These were carried out in steps of increasing stringency either by decreasing the salt concentration or increasing the temperature. After each wash, a Geiger counter was passed over the surface of the filter. When the number of counts per second (cps) over the majority of the filter had dropped to approximately that of background level, washing was discontinued and the filter sealed in a plastic bag in readiness for autoradiography.

Southern Blots

Salt concentration	Temp (C)	Time (min)	Frequency
1.00x SSPE 0.1% SDS	RT	15	x2
0.25x SSPE 0.1% SDS	RT	15	x1
0.25x SSPE 0.1% SDS	65	15	x2
0.10x SSPE 0.1% SDS	65	15	x2

Northern Blots

Salt concentration	Temp (C)	Time (min)	Frequency
5.0x SSPE 0.1% SDS	RT	15	x2
1.0x SSPE 0.1% SDS	RT	15	x2
0.5x SSPE 0.1% SDS	RT	15	x1
0.5x SSPE 0.1% SDS	37	15	x1

(v) Autoradiography

The filter was placed in a film cassette beneath a sheet of Xar-5 Kodak film and an intensifying screen. The film was exposed for 1-2 days at -70C prior to developing. Longer exposure times were used if the signal proved too weak (1-2 weeks).

2.12 Mammalian cell culture

(i) Cell lines

The cell line used in this study was the COS-7 cell line.

(ii) Cell culture media

For transfections and general passaging of COS cells GMEM (Glasgow Modified Eagles Medium) was used. However, for the cell labelling experiments using [$-^{35}S$] methionine, the richer media DMEM (Dulbecco's Modified Eagles Medium) lacking methionine was used.

GMEM

Made up from a 10x stock (stable for 2 weeks at 4C). For 200ml of 1x media, the following supplements were added:

150ml	ddH_2O
20ml	10x GMEM (BHK 21, Gibco) stored at 4C.
6.7ml	Sodium bicarbonate (7.5% w/v) stored at 4C.
20ml	FCS (Fetal Calf Serum) heat treated for 1h at 56C and stored at 4C.
2ml	Sodium pyruvate (10mM, 100x liquid 11.004g/l) stored at 4C.
2ml	Non-essential amino acids (100x liquid) stored at 4C (see below).
2ml	Pen/Strep (10,000 U/ml penicillin G sodium salt,10,000 U/ml streptomycin base dissolved in 0.85% saline and stored at 4C.
2ml	L-Glutamine (200mM, 100x liquid, 29.2 mg/ml in 0.85% saline pH4.7-5.6) stored at -20C.

Non-essential amino acids (100x liquid)

	g/l
L-Alanine	0.89
L-Asparagine	1.50
L-Glutamic acid	1.33
Glycine	0.75
L-Proline	1.15
L-Serine	1.05

When used for transfections, the FCS was additionally filter sterilised to remove large protein and lipid precipitates. DMEM was purchased as 1x complete media minus methionine and FCS. No supplements were required.

(iii) Cell culture conditions

The COS cell lines were grown as monolayers in small plastic tissue culture flasks (Bibby, base area $25cm^2$) under 10ml of GMEM at 37C in an atmosphere containing 5% carbon dioxide.

When confluence was reached, the cells were passaged as follows. The media was aspirated and the cells washed with 10ml of PBS (A) and detached by trypsinisation (1ml Trypsin-Versene (T/V) for 1min). The cells were dislodged by tapping the flask and dispersed by repeated pipetting in 9ml of freshly added media. Fresh cultures (4) were initiated with 1, 0.5, 0.2, and 0.1ml (1:10, 1:20, 1:50 and 1:100 dilutions respectively) of the cell suspension, made to 10ml with fresh media and incubated at 37C in 5% CO2.

PBS(A)	Trypsin-Versene (T/V)
5mM Na_2HPO_4	0.025% Trypsin (porcine)
7.5mM KH_2PO_4	500uM EDTA
2.75mM KCl	0.0015% Phenol Red
170mM NaCl	in PBS(A)

(iv) Transfection of mammalian cells using DEAE-dextran

To maximize efficiency, transfections were carried out on exponentially growing cells (30-50% confluent). In order to achieve this, the cells were seeded out in 10cm tissue culture dishes ($5x10^5$ cells /dish) and left to grow for one day prior to use (37C in 5% CO2). The plasmid DNA to be transfected (5-20ug) was ethanol precipitated and washed with 70% ethanol. The ethanol was aspirated in a culture hood using a finely drawn pasteur pipette and the pellets allowed to air dry. Once dry, the DNA was resuspended in 80ul of TBS and to this was added 160ul of DEAE-dextran solution and 4ul of chloroquine (final concentration-400ug/ml of DEAE-dextran).

The media from the tissue culture dishes was aspirated (usually 3 at a time) and the cells washed twice with 5ml of PBS(A). To each dish, fresh media (4ml GMEM, 10% FCS) was added and the DNA/Dextran mix slowly pipetted dropwise evenly over the whole dish (minimum of 10 drops per dish). The dishes were swirled gently and incubated for 3.5hr at

37C in an atmosphere of 5% CO2. The progress of the reaction was monitored by observing the cells under a phase contrast microscope (Zeiss). The appearance of large vacuoles inside the cells was an indication that the DNA had been endocytosed.

Lopata and co-workers (1984) have demonstrated that DMSO when added to cells enhances DNA uptake. This "DMSO shock" was carried out by aspirating the media and then gently pipetting a solution of 10% DMSO/PBS (5ml) onto the cells. After incubation for between 1-2min, the solution was promptly aspirated and the cells washed once with PBS(A) followed by the addition of 10ml of fresh media. The culture dishes were placed overnight in a CO2 incubator (5% CO2, 37C).

The following day the media was aspirated from the dishes and the cells washed twice (5ml x 2) in PBS(A). They were then harvested with T/V (1ml/dish), left for 2min and the dishes tapped several times to promote lifting of the cells. Freshly added media (9ml) was pipetted up and down several times to facilitate cell dispersal, and the cell suspension transferred to a medium-sized tissue culture flask (base area 50cm^2). The volume was made up to 20ml with fresh media and the flasks incubated overnight at 37C in a 5% CO2 atmosphere. Following this, the cells were harvested for analysis.

TBS (Tris buffered saline)

25mM Tris.Cl, pH7.4
137mM NaCl
5mM KCl
0.5mM $MgCl_2$
0.6mM Na_2HPO_4
0.7mM $CaCl_2$

Filter sterilise, store at 4C.

DEAE-dextran

DEAE-dextran (10mg/ml) was made up in TBS. Filter sterilise, store at 4C. Mix well before use.

Chloroquine diphosphate	100mM, made up in TBS. Filter sterilise, store at 4C. Mix well before use.

Filter sterilisation was carried out by passing the solution through a 0.22uM filter.

2.13 Screening of transiently expressing cell lines with monoclonal antibodies

(i) Panning

The method for panning was based on the protocol described by Wysocki and Sato (1978).

(a) Preparation of panning plates

Panning plates were made by coating 10cm Falcon bacteriological plates with affinity purified Goat anti mouse IgG (Sera labs, 1mg/ml). 10ml of the diluted antibody (3ug/ml in 50mM Tris.Cl, pH9.5) was added to each plate and allowed to bind for 2h. Unbound antibody was removed by washing the plates with 5ml of 0.15M NaCl (3x). To reduce non-specific binding, the plates were incubated overnight at RT with 5ml of a 1mg/ml solution of BSA in PBS(A). This was then aspirated, and the plates were sealed in plastic bags and stored at -20C until required for use.

(b) Panning cells

The media was aspirated from the plates of transfected cells and 2ml of PEA (PBS/EDTA/Azide) added. The plates were left at RT for 5min and the sides of the dishes tapped to detach the cells. A short pasteur pipette was used to triturate the cells, the suspension transferred to a Falcon tube (15ml polypropylene) and pelleted (setting 3.5, 4min in an MSE minor S benchtop centrifuge). The cells were resuspended in 0.5-1.0ml PEA/5%FCS ($2x10^6$ cells/ml) and antibody added (1:10 to 1:100 dilution). The tube was mixed gently and the cells incubated on ice for 30min, periodically agitating the

tube to prevent the cells from settling. To remove unbound antibody, an equal volume of PEA was added and the cell suspension layered above 3ml of a PEA/2% ficoll solution, contained in a 15ml Falcon tube. The cells were centrifuged for 4min (setting 3.5, MSE minor S) and the supernatant containing the unbound antibody removed by aspiration. The cell pellet was taken up in 0.5ml of PEA and pipetted onto an antibody coated panning dish containing 5ml of PEA/5% FCS. The plate was swirled gently to obtain an even spread of cells and left at RT for 1-3hr. The unbound cells were removed by washing with PBS(A)/5% FCS (5ml, 3x).

PEA 0.5mM EDTA, 0.02% sodium azide, made up in PBS(A). Store at RT.

(c) Hirt Extraction (Hirt;1967)

A Hirt extraction allows for the efficient separation of plasmid or viral DNA from the cellular DNA and proteins of cultured cells. This procedure is based on the preferential precipitation of undegraded genomic DNA and protein in the presence of SDS and NaCl.

400ul of Hirt lysis buffer was added to a 10cm panned plate and the plate was tilted back and forth to obtain an even spread of the buffer and then left at RT for 20min. The viscous lysate was pipetted into a 1.5ml microfuge tube and 100ul of NaCl (5M) added. The tube was sealed and the contents gently mixed by inverting the tube three or four times. To increase the efficiency of precipitation, the sample was left overnight on ice and the pellet collected by centrifugation (4C, 4min). The supernatant was transferred to a fresh 1.5ml microfuge tube, phenol/chloroform extracted twice, chloroform extracted and ethanol precipitated with 1/10th of 3M NaAc (pH5.0), 2.5 volumes of ethanol and 10ug of linear polyacrylamide (which functions as a carrier and increases the efficiency of precipitating low concentrations of DNA). The tube was incubated at -20C for 30min and the precipitate collected by centrifugation (4C, 30min). The

pellet was washed with 70% ethanol, dried and resuspended in 100ul of sterile water (by heating to 65C for 10min) and then re-precipitated (10ul 3M NaOAc (pH5.0), 3vol ethanol, -20C for 30min). The pellet was collected by centrifugation (4C, 30min), washed with 70% ethanol, dried and resuspended in 50-100ul of sterile water.

Hirt buffer	Linear polyacrylamide
0.6% SDS	Acrylamide:Bis (9:1)
10mM EDTA	1-10ug/ml made up in sterile water.

(ii) Cytospinning and immunufluorescent staining

Prior to carrying out staining, the cells had to be harvested and fixed. Media from cultured cells was aspirated and the cells washed twice in 5ml of PBS(A) and then harvested using PEA (5ml, 5min, RT). The cell suspension was transferred to a Falcon tube (15ml) and pelleted by centrifugation (MSE minor S, setting 3, 5min, RT). The supernatant was decanted and the cell pellet resuspended in 2-4ml of 5% FCS/PBS (using a 1ml syringe fitted with a 21 gauge needle) to a final concentration of $3x10^4$ cells/ml (determined using a haemocytometer). The cytospin rotor (Shandon) was loaded with the 12 carriers, each containing a tightly clamped sandwich consisting of a slide, Shandon filter paper and the cell container. The cell suspension (100-300ul) was added to each container through an aperture in the lid and the cells centrifuged onto the slides (500rpm, 5min). The rotor was promptly unloaded, the filter discarded and the slides immediately immersed in an acetone bath (2min) to fix the cells. Following this, the slides were removed and air dried.

The fixed cells when observed under a phase contrast microscope have to be intact and well dispersed (they appear

refractile and shiny). Owing to the inherent variables in this technique, approximately 4-6 cytospins of a sample were required to generate a slide of acceptable quality.

The cytospun slides to be stained were labelled with an acetone resistant marker (Tex pen), placed in a humid slide box and wetted with TBS. The appropriate dilution of the respective primary antibody in TBS was made (1:5-1:500), the buffer on the slides drained and the area around the cells wiped dry. The diluted primary antibody was added (100ul/slide) and the cells incubated in the slide box for 15-30min at RT. Thereafter, the slides underwent 3 washes (5min each) in a circulating bath of TBS. The slides were removed from the bath, the area around the cells dried and the slides placed back in the humid slide box. Secondary antibody conjugated to FITC (Fluorescein isothiocyanate: 100ul of a 1:50 dilution) was added and the cells incubated at RT for 15-30min. The slides were washed and dried as previously described and propidium iodide (fluorescent nuclear stain)/glycerol (25ul) applied to each slide. A cover slip was placed over the cells prior to visualisation under the fluorescent microscope (Leitz).

PEA (for 100ml of 1x)	TBS/l
10ml PBS(A) 10x	9.5g NaCl
10ul 5mM EDTA	50ml 1M Tris.Cl pH7.5
0.2ml 10% Sodium azide	q.s. to 1l with water
89.7ml water	

Propidium iodide stock	1mg/20ml in 0.1% sodium citrate Store at 4C in the dark
Propidium iodide/Glycerol	10% propidium iodide stock, 50% glycerol (ultrapure), made up in TBS.

2.14 Protein analysis

(i) SDS-polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was performed on discontinuous Laemmli gels. The resolving gels (7.5-12.0%) were made up from a 30% stock of acrylamide (Acrylamide:Bis ratio is 30:0.8) diluted in resolving gel buffer and polymerised by the addition of APS and TEMED, both at a final concentration of 0.005%. Isobutanol was overlaid to prevent the entry of oxygen (an inhibitor of polymerisation) and the gel left for 90min at RT to complete polymerisation. Following this, the isobutanol was poured off and the surface of the gel was washed thoroughly with water and blotted dry with filter paper. The stacking gel (4% acrylamide) was prepared in stacking gel buffer and polymerised by the addition of APS and TEMED to a final concentration of 0.05% and 0.1% respectively. The gel was poured on top of the resolving gel, the comb inserted between the plates and the gel left at RT for 90min to polymerise. The samples were diluted 1:4 in SDS sample buffer, vortexed briefly and boiled (5min), prior to loading. The gels were run in Tris/Glycine buffer under constant current conditions for 4-5h (depending on length).

Acrylamide stock
30% Acrylamide (29.2g/100ml)
0.8% N,N' Methylenebisacrylamide (0.8g/100ml)
made up in distilled water. Filtered
through Whatman No.1 filter and stored in
a dark bottle at 4C.

4x Resolving gel buffer	4x Stacking gel buffer
1.5M Tris.Cl, pH8.3	0.5M Tris.Cl, pH6.8
0.4% SDS	0.4% SDS
store at 4C	store at 4C

APS 10% Ammonium persulphate

5x Tris/Glycine buffer /l	SDS sample buffer
15.0g Tris.base pH8.3	10% Glycerol
72.0g Glycine	5% 2-Mercaptoethanol
5.0g SDS	0.05% Bromophenol blue
	0.0625M Tris.Cl, pH 6.8

(ii) Staining SDS-page gels with Coomassie blue

After electrophoresis, the gels were stained in a solution of 0.1% Coomassie blue (30min) and destained by washing (30min, consisting of 5-6 washes, or until the background had cleared) in a solution of 40% Methanol/10% Acetic acid (Destain).

Coomassie blue stain	Destain
0.1% Coomassie blue R-250	40% Methanol
made up in Destain	10% Acetic acid

(iii) Electroblotting (Western blots)

After electrophoresis, the gel apparatus was dismantled, the stacking gel removed and the resolving gel placed in a tray of BEB (blotting electrode buffer). A piece of nitrocellulose membrane (Schleider and Schill, BA85, pore diameter 0.45um) was cut 5mm larger than the gel (taking care to handle the nitrocellulose with blunt forceps or gloved hands only). Four pieces of Whatman paper were cut 2mm larger in both dimensions than the nitrocellulose and soaked in BEB. The gel and nitrocellulose were sandwiched between the filter paper (two sheets on either side) and the air bubbles trapped in the layers removed by rolling a pipette back and forth over the surface of the stack. The sandwich was placed between 2 pieces of "Scotchbrite" pad clamped in a perforated perspex support and transferred to an electroblot tank (Nitrocellulose side facing the anode as

proteins from SDS reducing gels are negatively charged and migrate towards the anode). The tank was filled with BEB and electroblotting carried out for 16h at 30V, 0.1A followed by a further 1h at 100V, 0.3A. The sandwich was then disassembled, the nitrocellulose washed twice in TBS/Tween (5min) and the gel stained with Coomassie blue to determine the efficiency of transfer.

Blotting electrode buffer (BEB)
25mM Tris.Cl 3.0g/l
192mM Glycine 14.4g/l
20% (v/v) Methanol 200ml/l

(iv) Immunodetection of proteins

The non-specific protein binding sites on the nitrocellulose membrane (blot) were blocked by incubation in a solution of 5% dried milk in TBS/Tween (1h, RT with gentle agitation). The membrane was rinsed in 3 changes of TBS/Tween (initially for 15min and then twice for 5min) and transferred to a plastic bag. 10ml of diluted primary antibody was added (primary antibody diluted in TBS/Tween 1:10-1:100), and the bag was sealed and incubated with gentle shaking at RT for 1h. Following this, the membrane was washed as before, and then transferred to a fresh bag and 10ml of HRP-conjugated (horseradish peroxidase-conjugated) secondary antibody was added (1:50-1:1000 dilution of antibody). The bag was sealed and incubated on a rocking platform at RT for 1h. The membrane then underwent one 15min and four 5min washes in TBS/Tween.

(v) Development of blots
a) 4-Chloronapthol
The membrane was transferred to a tray and incubated in 94ml TBS containing 6ml of 4-chloronapthol(3mg/ml in methanol)

and 25ul of H_2O_2 (30%). The colour was allowed to develop for approximately 5min and the reaction terminated by washing the blot with several changes of distilled water.

b) Enhanced chemiluminescence (ECL) (Amersham Laminol)
Equal volumes of the two detection reagents (0.125ml/cm^2 of membrane) were combined and the membrane incubated for precisely 1min at RT in the mixture. The blots were drained, covered in Saran wrap, placed in a film cassette and initially exposed to Kodak Hyper film for 10sec. Depending on the signal obtained various exposure times were examined.

TBS	TBS/Tween
20mM Tris.base 2.42g/l	3% Tween-20
137mM NaCl 8.00g/l	made up in 1x TBS
3.8ml 1M HCl	

(vi) *In vitro* labelling of cultured cells with [α-^{35}S]Met following transfection

Cells transfected with DNA using the DEAE-dextran transfection method were passaged the following day from tissue culture dishes into culture flasks (75cm^2 base area) as previously described. Once attachment of the cells to the base of the flask was observed (5-8h), the media was aspirated and the cells washed twice in PBS. The GMEM media was then replaced with 5ml of DMEM-Met$^-$ media (Dulbecco's modified Eagles media without methionine) containing 10% dialysed FCS. Following a 30min incubation at 37C, 5% CO_2, * 5 125 uci [35S] - methionine was added.
the flasks were sealed and incubated at 37C for 12-18h.

(vii) Immunoprecipitation

Immunoprecipitations were carried out on transfected cells metabolically labelled with [α-35S]Met. The procedure was carried out in several stages as described below.

(i) Preparation

a) Samples (i) Media

or (ii) Cell lysate

b) Protein A agarose

c) Protein A agarose/antibody complex

(ii) Preclearing of the samples.

(iii) Immunoprecipitation

(iv) Enhancement and autoradiography

(i) Preparation

(a) Samples (i) Media

Immunoprecipitation of the media was carried out to demonstrate the presence of a secreted protein.

An aliquot of the media (1.5ml) was pipetted into a microfuge tube, PMSF (Phenyl Methyl Sulphonyl Fluoride), a mild protease inhibitor, added (10ul) and the tube inverted a few times to mix the solution. Cell debris and insoluble proteins were removed by centrifugation (4C, 30min) and the supernatant transferred to a fresh microfuge tube. A further 10ul of PMSF was added and the tube was vortexed briefly and placed on ice ready for preclearing.

(ii) Cell lysate

The media from the monolayer culture was poured off and the cells washed in PBS. Cell harvesting was carried out using PEA (10ml) and the resulting cell suspension transferred to a 15ml polypropylene screw cap tube (Falcon). The cells were pelleted by centrifugation (MSE minor S benchtop centrifuge, setting 3.5, 5min, RT) and the supernatant discarded. Lysis of the cell pellet was achieved by the addition of 1ml lysis buffer/PMSF and gently vortexing. The tube was then placed on a rock'n'roller (Gallenkamp) for 30min at 4C to increase the efficiency of cell lysis. Once complete, the lysate was transferred to a microfuge tube and the cell debris pelleted by centrifugation (10min, 4C). The supernatant was transferred to a fresh microfuge tube, to which 10ul of PMSF was added. The tube was vortexed briefly and centrifuged for

a further 30min at 13.5Krpm at 4C in an MSE Microcentaur microfuge to pellet insoluble proteins. The supernatant was pipetted into a fresh tube, 10ul of PMSF was added, and the tube was vortexed and placed on ice ready for preclearing.

b) Protein A agarose

Prior to use, the protein A bound non-specifically to the carrier agarose matrix had to be removed. This was achieved by repeated washing and centrifugation cycles (5min each, 3-5 times) in lysis buffer, discarding the supernatant between washes. Washed protein A agarose was finally resuspended at a concentration of 10%v/v in lysis buffer/PMSF and kept on ice.

c) Protein A agarose antibody complex (prot A complex)

Pre-washed protein A agarose (10% suspension v/v in lysis buffer/PMSF) was incubated with a 1:50 dilution of RAM (Rabbit anti-mouse IgG 20ug/ml) on a rock'n'roller (Gallenkamp) for 1h at 4C. The agarose beads were washed 3-5 times in lysis buffer to remove unbound antibody and the pellet resuspended as a 10% suspension v/v in lysis buffer/PMSF. MAb against the antigen to be immunoprecipitated was added (1:20 dilution (50ul/ml) anti-T11 mAb (Beckton Dickinson anti-CD2 mAb), or 1:50 dilution (20ul/ml) W6/32 mAb (Sera Lab anti-HLA.ABC)). The tube was vortexed briefly and placed on a rock'n'roller for 1h at 4C. Unbound antibody was removed by washing the matrix (3-5 times) in lysis buffer. The protein A complex was pelleted, resuspended in lysis buffer/PMSF (10%v/v), briefly vortexed and placed on ice.

(ii) Preclearing the sample

Prior to immunoprecipitation, the samples (both media and cell lysate) had to be precleared to remove non-specific binding proteins which might bind to either the protein A agarose or to the antibodies and hence increase the background. Therefore, NRS (Normal rabbit serum 50ul/ml) was added to each sample to adsorb proteins capable of non-

specifically binding to the antibodies. The tubes were vortexed briefly and incubated for 30min at 4C on a rock'n'roller (Gallenkamp). Pre-washed protein A agarose (50ul of 10%v/v suspension) was added to bind and remove the NRS. The tubes were vortexed briefly and incubated for 30min at 4C on a rock'n'roller. The protein A agarose was pelleted by centrifugation (2min, 4C) and the supernatant transferred to a fresh tube. A further 50ul of the protein A agarose suspension was added to remove both residual NRS and proteins that may bind non-specifically to protein A agarose. The mixture was incubated as before and the protein A agarose pelleted by brief centrifugation (2min, 4C). The supernatant was transferred to a fresh tube, PMSF (10ul) was added, and the tube was vortexed and placed on ice.

(iii) Immunoprecipitation

To the precleared samples 50ul of prot A complex (10% v/v suspension) was added. The tubes were briefly vortexed and placed on a rock'n'roller for 1h at RT to allow antigen binding (excessive incubation times increase the signal to noise ratio). The prot A complex was pelleted by centrifugation (2min, 4C) and the supernatant discarded. The pellet was washed twice in wash buffer I, once in wash buffer II and then recentrifuged to bring down the residual buffer which was removed with a finely drawn pasteur pipette. SDS page loading buffer (50ul) was added to the samples, which were then boiled for 10min and placed on ice. Insoluble material in the samples was pelleted (5min, RT) and aliquots of the supernatant loaded onto a 12.5% discontinuous SDS page gel.

(iv) Enhancement and autoradiography

Following electrophoresis the gel was stained with Coomassie blue and destained in acetic acid/methanol as described previously. The radioactive signal from the [^{35}S]Met labelled proteins was amplified by enhancement. This involved gently shaking the gel in 500ml of enhance solution (Enhance Dupont) for 30min at RT in a fume hood.

The gel was then washed with copious amounts of water, dried down and put up for autoradiography (Kodak Xar-5 film) at -70C for 18h.

PMSF
Stock made up in ethanol 100mM PMSF 0.174g/ml
Add 1ml of PMSF stock to 100ml lysis buffer (10ul/ml).

Lysis Buffer	/500ml
50mM Tris, pH7.5	50ml 0.5M Tris, pH7.5
5mM EDTA	5ml 0.5M EDTA
0.5% NP40	2.5ml 100% NP40
	442.5ml H_2O

TNEN Stock	/500 ml
20mM Tris, pH7.5	20ml 0.5M Tris, pH7.5
10mM EDTA	10ml 0.5M EDTA
0.1M NaCl	10ml 5.0M NaCl
0.5% NP40	2.5ml 100% NP40
	457.5ml H_2O

Wash Buffer I	Wash Buffer II
0.1% SDS	0.5M NaCl in 1/10th TNEN
1.0% BSA	
made up in TNEN	

SDS Loading Buffer
2% SDS
10% Glycerol
100mM DTT (Dithiothreitol)
60mM Tris, pH6.8
0.001% Bromophenol Blue

CHAPTER 3

CONSTRUCTION OF A SERIES OF EXPRESSION VECTORS ENCODING NATIVE AND RECOMBINANT CD2 PROTEINS

3.1 Introduction

The initial aim of the project was to generate the required series of constructs described in Chapter 1, so that control experiments could be carried out prior to attempting to clone putative Tm sequences.

a) The first of these was the CD2 cDNA, which was sub-cloned into the CDM8 shuttle vector for use as a positive control in transfection experiments. It would enable the transfection efficiency to be determined and demonstrate whether cells transfected with the CD2 cDNA could express the CD2 protein on their cell surface.

b) A truncated version of the CD2 clone, containing only the coding sequence for the external domains of the protein, was required to enable cell surface expression of the CD2 protein to be assessed in the absence of a Tm sequence (to ensure that it was secreted).

c) In addition, a construct containing an in-frame fusion of the external domain of CD2 to a foreign Tm sequence was essential for determining whether CD2 expression at the cell surface could be reproduced.

d) Lastly, a construct containing the coding sequence for the external domain of CD2 fused to the polylinker/stuffer region of CDM8 was necessary to provide an array of restriction sites into which cDNAs could be cloned. On transfection, these constructs, could potentially produce a

spectrum of fusion proteins which could be screened for cell surface expression of CD2, thus, allowing for the identification of Tm-like sequences, which can anchor the external CD2 domain on the membrane.

3.2 Transfer of the CD2 gene from CDM7 to CDM8

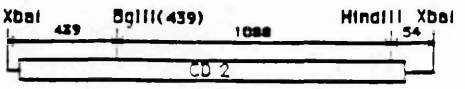
The donated CD2 clone was originally isolated from a plasmid library constructed in the shuttle vector CDM7. However, as all the gene fusions constructed utilised the vector CDM8, the CD2 gene was also sub-cloned into CDM8, in order to generate an appropriate control for the respective expression studies.

The CD2 vector was excised from CDM7 by digestion with XbaI. The resultant ~~the~~ 1581bp fragment was separated from the vector on an agarose gel and purified using Gene Clean. CDM8 was digested with XbaI and the vector DNA phosphatased using calf intestinal phophatase (CIP). The vector was separated from the 440bp stuffer DNA by agarose gel electrophoresis and purified by electro-elution. An estimate of the concentration was determined by comparison against a series of DNA standards dotted on an ethidium plate.

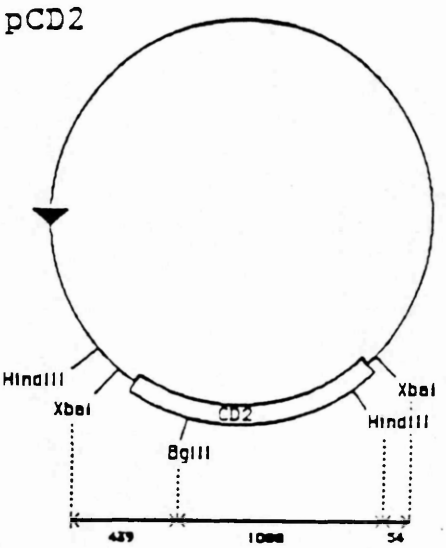
The $CD2_{\lambda}^{cDNA}$ fragment was then ligated into CDM8, and the DNA transformed into *E. coli* (MC1061/P3) by electroporation. Recombinants were selected on Tet and Amp plates and mini-preparations of plasmid DNA were isolated from a series of colonies. These were then digested with XbaI to verify the presence of the insert, and with BglII and HindIII to determine its orientation (Fig:3.1). The clone of CD2 in CDM8 was named pCD2.

Figure:3.1

a) Restriction map of CD2 excised from CDM7 using XbaI, and band purified using Gene Clean



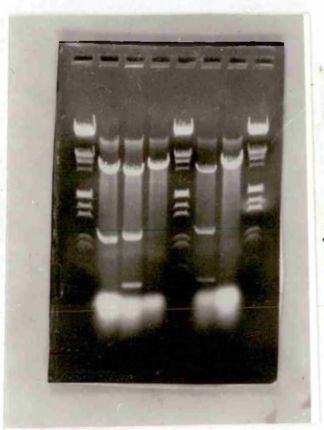
b) Restriction map of CD2 cloned into CDM8, the orientation of the fragment was determined by double digestion with HindIII and BglII



*

Boiling preparations of CD2 recombinants cloned into CDM8

1 2 3 4 5 6 7 8



-5005
-3501
-1088
-446

Lanes 1, 5, and 8 λ HindIII and EcoRI molecular weight markers

Lanes 4 and 7 CD2 subcloned into CDM8 digested with HindIII to linearise the plasmid 5005bp

Lanes 2, 3 and 6 CD2 subcloned into CDM8 digested with HindIII and BglII, to determine orientation of the recombinants

The Lanes 3 and 6 are in the correct orientation as the products of digestion include a fragment 1088bp in size. The 446bp fragment is obscured by the RNA present in the boiling preparation.

* Diagrams Not to scale

3.3 Cloning of the external domain of CD2 into CDM8

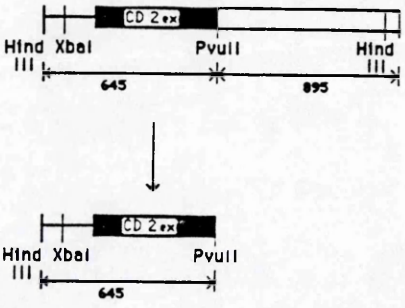
The CDM8 plasmid contains a very limited array of restriction sites in its polylinker and so, is unsuitable for carrying out numerous DNA manipulations. Therefore, the required manipulations were carried out in the vectors pMTL-20 and pMTL-21 (which differ in the orientation of the polylinker). These vectors carry the genes coding for ampicillin resistance and the α-peptide of the enzyme β-galactosidase, so colony colour screening using the chromogenic substrate X-gal is possible.

In order to construct the truncated version of the CD2 clone (pCDEx-21), pCD2 was digested with the restriction endonucleases HindIII and PvuII, producing two fragments; an 895bp fragment containing the transmembrane and cytoplasmic domain sequences and a 645bp fragment containing the external domain sequence (Fig:3.2a). This 645bp fragment was band purified and ligated into the HindIII and StuI sites of pMTL-21. The DNA was transformed into *E. coli* (DS941) using calcium chloride. The recombinants were selected on Amp and X-gal plates, and plasmid DNA from a number of white colonies was subjected to restriction analysis (Fig:3.2b).

The truncated CD2 fragment was cloned into CDM8 by digesting pCDEx-21 with XbaI and gel purifying the 650bp insert fragment. This fragment was then ligated to XbaI digested, phosphatased and band purified CDM8 vector, and the ligated material was transformed into *E. coli* (MC1061/P3) by electroporation. The recombinants were selected on Tet and Amp plates, and plasmid DNA from a number of positive clones was subjected to restriction analysis. XbaI digestions were carried out to confirm the presence of the insert, and fragment orientation was determined by double digestion with HindIII and SalI. The latter double digests were carried out in KGB restriction endonuclease buffer, though it later became apparent that the presence of glutamate in the buffer interfered with the migration of DNA in agarose gels. This

Figure:3.2a

a) Diagram showing the products of HindIII and PvuII digests of CD2. 2 fragments of DNA are generated the 645bp fragment coding for the external domain of CD2 and the 845bp fragment coding for the Tm and cytoplasmic domains. The 645bp fragment was band purified using Gene Clean and subcloned into HindIII and StuI sites of pMTL-21



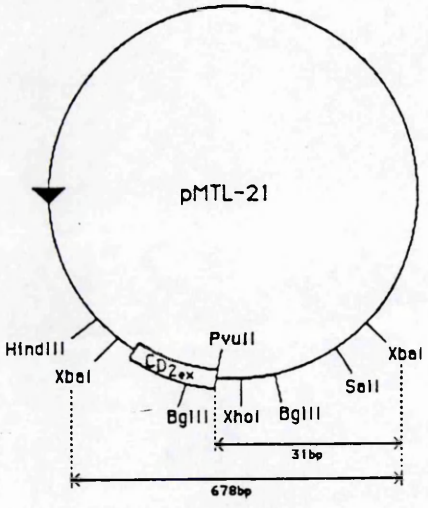
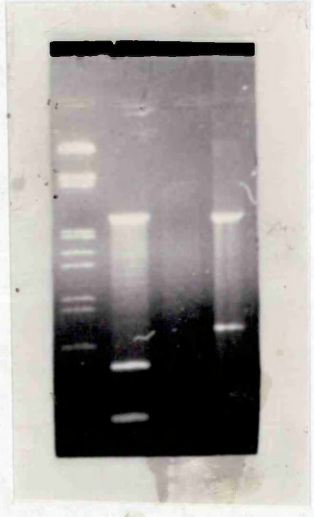


Diagram of CD2 external domain sub-cloned into pMTL-21

λ 1 2 3



-2476

-678

444-

208-

The gel shows the result of the diagnostic digests to confirm the subcloning of CD2Ex. An XbaI digest liberates the whole of the CD2Ex fragment, this was then subsequently subcloned into the XbaI sites of CDM8.

λ HindIII and EcoRI mwt markers

Lane 1, CD2 external domain sub-cloned into pMTL-21 HindIII and StuI sites. Plasmid pCDEx-21 digested with HindIII and BglII

Lane 2, -

Lane 3, CD2 external domain sub-cloned into HindIII and StuI site of pMTL-21 digested with XbaI

Figure: 3.2b

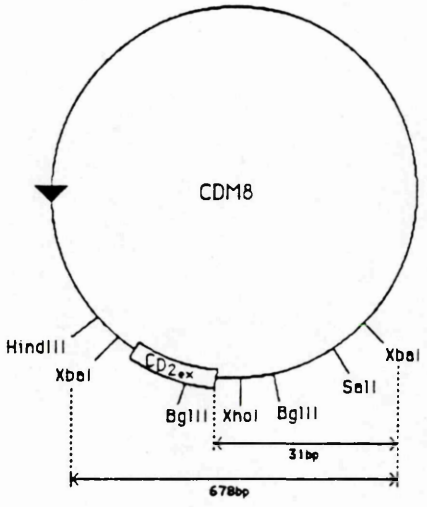
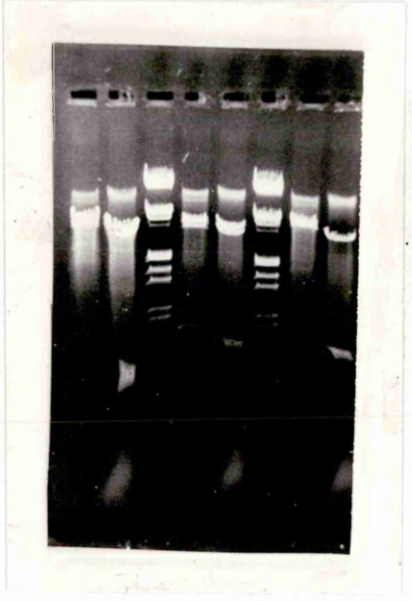


Diagram of the CD2 external domain subcloned into the XbaI sites of CDM8

1 2 3 4 5 6 7 8



To determine orientation of the insert a HindIII and SalI digest was carried out. The aberrant running of the gel is due to the presence of KGB buffer in the HindIII and SalI digests.

Lanes 1 an XbaI and Lane 2, HindIII and SalI digest of pCDEx-8 (CD2Ex subcloned into XbaI site of CDM8 (clone 1))

Lane 3, λ HindIII and EcoRI mwt markers

Lanes 4 and 5 as per 1 and 2 for clone 2

Lane 6, as per 3

Lanes 7 and 8 as per 1 and 2 for clone 3

Diagrams not scale.

is shown in Fig:3.2b where the fragment generated from the HindIII/SalI digest (lane 5) should have run the same distance as the 678bp fragment in lane 4. The lanes containing KGB buffer can be identified by the position of the dye front which is markedly retarded compared to those in the DNA marker lanes. The resulting plasmid was named pCDEx-8 (external domain of CD2 sub-cloned into the vector CDM8).

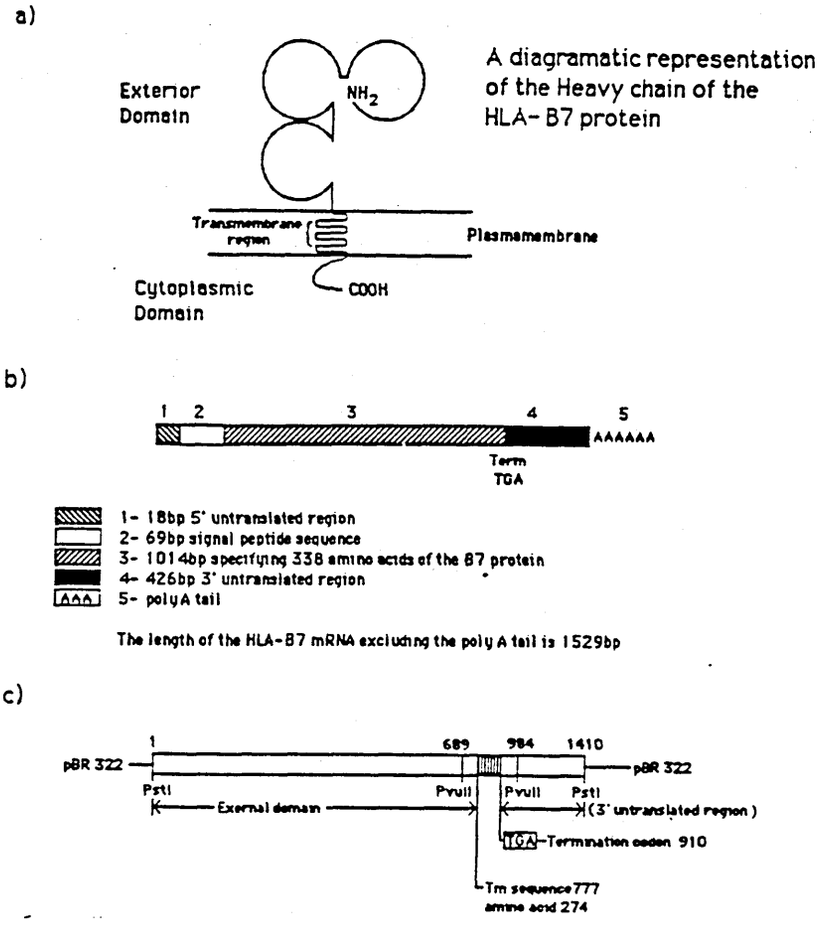
3.4 Construction of the CD2 fusion protein

To produce a fusion protein incorporating the external domain of CD2 fused to a Tm sequence, a cDNA coding for a membrane protein was required. As both the DNA sequence and a cDNA clone corresponding to the HLA-B7 gene were available, this was the membrane protein used.

The HLA-B7 protein is a class I antigen of the Major Histocompatibility Complex (MHC) (Sood *et al*;1985). It is a 44kDa glycoprotein expressed at the cell surface, and the cDNA clone used codes for its heavy chain (Fig:3.3). The extra-cellular portion consists of three domains, each approximately 90 amino acids in length, which are followed by a hydrophobic Tm domain and a relatively hydrophilic intra-cellular region (Fig:3.3a). The HLA-B7 mRNA is 1529bp in length (excluding the poly A tail) and contains a very short 18bp 5'-untranslated region and a 69bp signal peptide sequence. The open reading frame codes for 338 amino acids of the HLA-B7 protein and is followed by a TGA termination codon and a 3'-untranslated region (Fig:3.3b).

In the 1410bp HLA-B7 cDNA clone, pDP001 the transmembrane sequence starts at position 777 (amino acid 274), and is followed by the cytoplasmic domain and termination codon (position 910). Analysis of the DNA sequence shows that the first restriction site upstream from the Tm region is a PvuII site located at position 689. A second PvuII site is

Figure: 3.3



The pDP001 clone containing the 1410 bp HLA-B7 gene subcloned into the PstI site of pBR322.

a) A diagramatic representation of the HLA-B7 protein at the cell surface

b) mRNA of HLA-B7

c) cDNA of the HLA-B7 subcloned into the PstI site of pBR322 (pDP001). The two PvuII sites flanking the Tm and cytoplasmic domains are marked at positions 689 and 984bp

Diagram Not scale.

located 295bp downstream of the first and 14bp downstream of the termination codon (Fig:3.3c). As mentioned previously, the external domain of the CD2 gene can be isolated as a 645bp HindIII/PvuII fragment. The PvuII restriction endonuclease cuts at base 609 of the gene, between the second and the third nucleotides of a serine codon (see below).

```
                                        PvuII          >>
CD2 external domain 5' GAG CCT GTC AGC TGT CCA 3' Tm of CD2
                        Glu Pro Val Ser Cys Pro

CD2 external domain 5' GAG CCT GTC AG 3'
                        Glu Pro Val
```

The PvuII site (position 689) in the HLA-B7 clone lies between the first and second nucleotides of an alanine codon (see below).

```
                                  PvuII          >>
HLA-B7        5' AGG TGG GCA GCT GTG GTG 3' Tm of HLA-B7
                Lys Trp Ala Ala Val Val

                                  CT GTG GTG 3' Tm of HLA-B7
                                     Val Val
```

Although the ends of the fragment are compatible, direct ligation does not maintain the translational reading frames of the proteins. To overcome this an 8bp, double stranded EcoRI oligo-nucleotide linker was used as a bridge. Subsequent expression would then produce an in-frame fusion protein with some modifications at the junction. These included a substitution of a serine for an arginine, an alanine for a proline and the addition of a glutamic acid and a phenylalanine residue (see below).

<< EcoRI linker (8bp) >>

CD2 5' GAG CCT CTC AGG GAA TTC CCT GTG GTG 3' Tm of HLA-B7

Glu Pro Val Arg Glu Phe Pro Val Val

3.5 Sub-cloning of the external domain of the CD2 gene containing the EcoRI linker into the vector pMTL-21

pCD2 was digested with HindIII and PvuII, loaded on to an agarose gel, and the 645bp fragment coding for the external domain of the protein purified using Gene Clean as described previously. The EcoRI linker was ligated onto the flush PvuII end of the fragment, followed by an ethanol precipitation to remove the free linkers. The resuspended pellet was digested with EcoRI for a minimum of 3h to digest any polymeric chains of linker that may have been formed, and subsequently, the DNA was reprecipitated to remove the cut linkers and concentrate the DNA. The pellet was finally resuspended in sterile water and the concentration determined by comparison against a series of standards on an ethidium plate. The DNA was ligated into the HindIII and EcoRI sites of pMTL-21 and transformed into *E. coli* (DS941) using $CaCl_2$. White recombinants were selected on Amp/X-gal plates and the plasmid DNA isolated was subjected to restriction analysis (Fig:3.4). The plasmid generated was called pCDEx-R21.

3.6 Isolation of the DNA coding for the transmembrane sequence of the HLA-B7 gene

The sequence coding for the Tm region of the HLA-B7 clone can be isolated as a 295bp flush-ended fragment by digestion with the restriction endonuclease PvuII. However, to facilitate cloning, a larger fragment encompassing the whole of the 3'untranslated region was used. This involved partial digestion of the DNA with PvuII and complete digestion with PstI. The 721bp fragment generated contains the region from

Figure:3.4

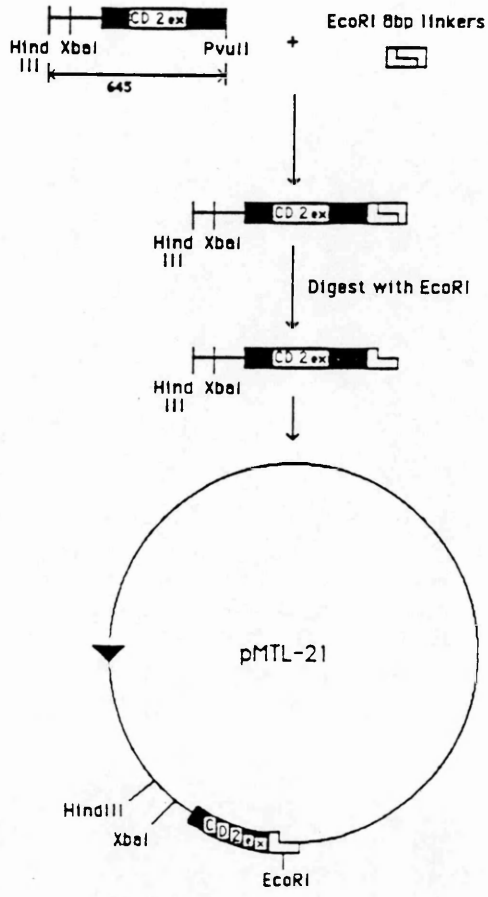
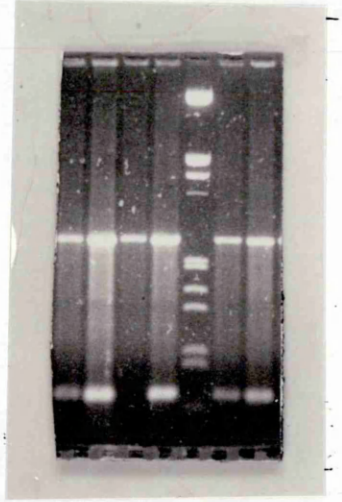


Diagram to show the stages of EcoRI linker addition to the external domain of CD2 and its subcloning into the HindIII and EcoRI sites of pMTL21 (pCDEx-R21)

1 2 3 4 5 6 7



Boiling preparations of putative pCDEx-R21 clones HindIII and EcoRI digests

Lanes 1, 2, 4, 6, and 7 positive clones

Lane 3, pMTL-21

Lane 5 λ HindIII and EcoRI mwt markers

Diagram Not to Scale.

the first PvuII site (position 692) to the end of the clone (position 1410) and spans the second PvuII site. The advantage of isolating this fragment over the 295bp PvuII fragment is that it has only one flush end, allowing linker ligation to proceed solely at the 5' end of the DNA. In addition, the different overhangs produced allow directional cloning of the fragment and simplifies the subsequent cloning of the fusion construct from the cloning vector pMTL-20 into the expression vector CDM8.

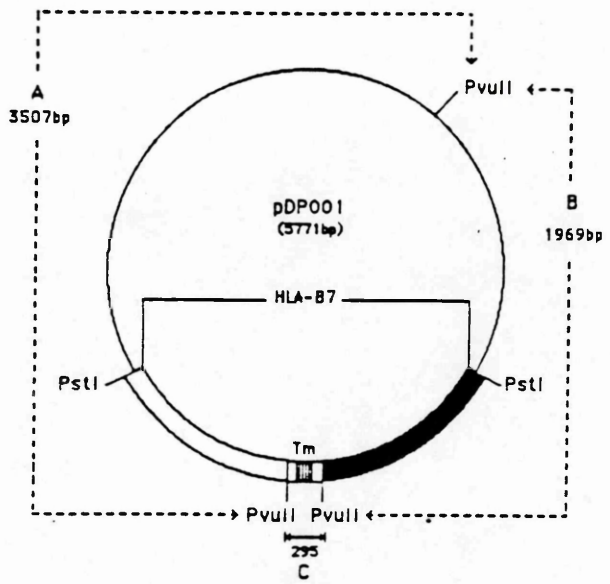
3.7 Partial digestion of the plasmid (pDP001) containing the HLA-B7 gene

The partial digestion of pDP001 plasmid DNA was complicated by the presence of a third PvuII restriction site, 1969bp downstream of the second. Furthermore, one of the products generated from a PstI/PvuII digest is a 689bp fragment which is only 32bp smaller than the 721bp segment of DNA containing the Tm region. Therefore, co-purification of these two fragments on an agarose gel was likely.

The three fragments generated following complete digestion of the plasmid pDP001 with PvuII, arbitrarily designated A, B, and C, are 3507bp, 1969bp, and 295bp in length respectively. Partial digestions, where two of the three possible restriction sites are cut, produce an additional three fragments; AB (5476bp), AC (3802bp), and BC (2264bp) (Fig:3.5a). Purification of BC, the 2264bp PvuII fragment which contains the Tm sequence, at this stage, would overcome the problem of trying to resolve the contaminating 689bp fragment from the 721bp fragment, following PvuII/PstI digestion.

The conditions for the partial PvuII digestions, therefore, had to be tuned so that the majority of the DNA was cut at least once. Results from a series of pilot experiments (section 2.7 chapter 2) suggested that the optimal reaction

Figure:3.5



Tm - Transmembrane region

Products of a complete digest of pDP001 with PvuII generates 3 fragments

A = 3507bp

B = 1969bp

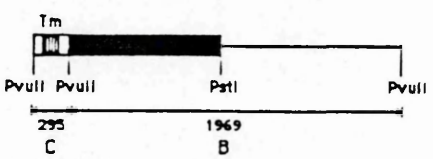
c = 295bp

partial products where 2 of the 3 restriction sites are cut

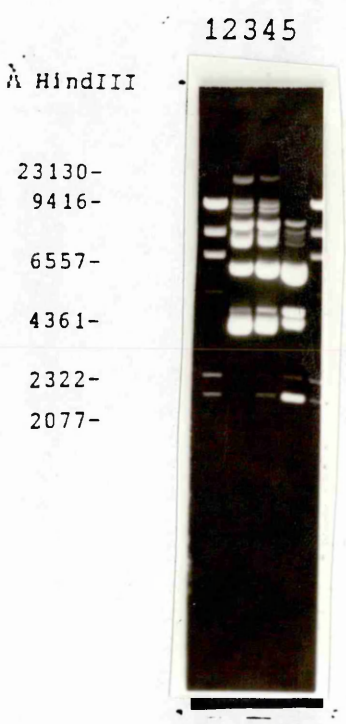
A + B = 5476bp

A + C = 3802bp

B + C = 2264bp Tm containing sequence



Single cut with PvuII linearises the plasmid = 5771bp



Partial products of a PvuII digest of pDP001

Lane 1, λ HindIII mwt markers

Lane 2, 0.5U PvuII, 3ug DNA, 20ul, digested 30min, 37C

Lane 3, 0.25U PvuII, 3ug DNA, 20ul, digested 30min, 37C

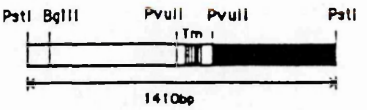
Lane 4, 0.05U PvuII, 3ug DNA, 20ul, digested 30min, 37C

Lane 5, λ HindIII mwt markers

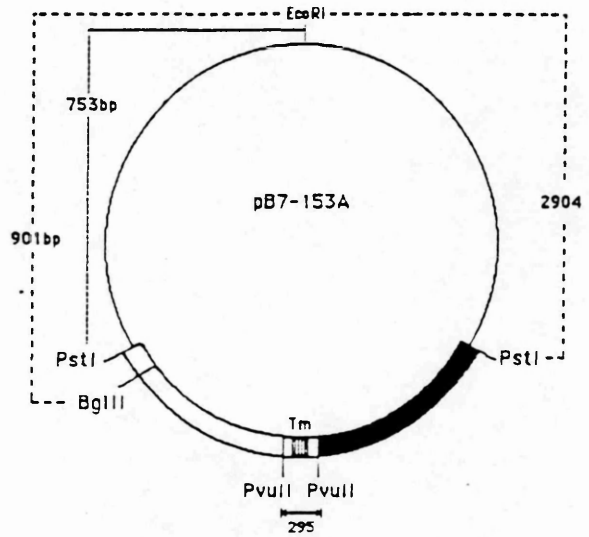
* = Fragment containing Tm sequence of HLA-B7

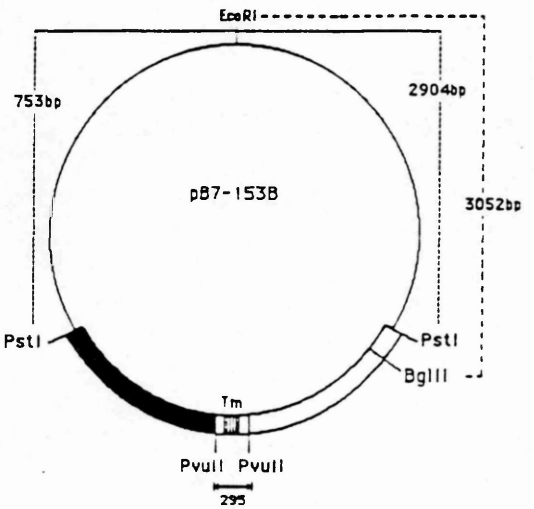
Diagram Not to Scale

Figure:3.6



HLA-B7



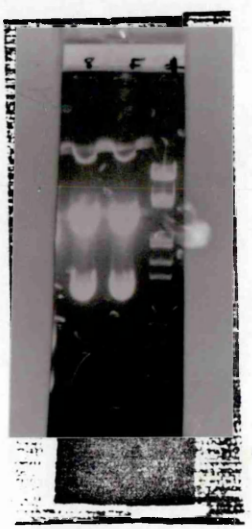


Tm - Transmembrane region

Tm - Transmembrane region

a) The HLA-B7 cDNA subcloned into pAT-153. The products of the diagnostic digests BglII and EcoRI are marked on the plasmid maps. These were used to determine the orientation of the insert

1 2 3



Lane 1, pB7-153A digested with PstI (2ug)

Lane 2, pB7-153B digested with PstI (2ug)

Lane 3, 1 HindIII and EcoRI mwt markers

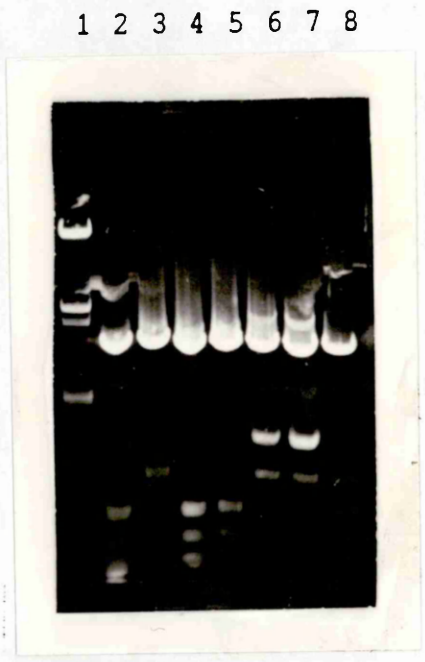
b) The gel shows a PstI digest of both pB7-153A and B Lanes 1 and 2, they are heavily over loaded

Diagram Not To Scale

conditions were to use 0.5U restriction endonuclease in 20ul of 1x reaction buffer, per 3ug of DNA, and to incubate the digestion at 37C for 30min (Fig:3.5b). However, the yield of the 2264bp product from the partial digestions was very low. Lane 2 of Fig:3.5b is loaded with a total of 3ug of partially digested pDP001 DNA. Of this, only about 100ng of the DNA appears to be in the 2264bp form. The yield for most DNA gel purification procedures is poor, with around 50% (or sometimes more) of the DNA being lost. So, essentially, this would leave 50ng of DNA which is insufficient to carry out the remaining manipulations.

Theoretically, scaling up the digests from 3ug to 30ug should generate approximately 1ug of the partial product. To attain adequate resolution, the digested DNA had to be electrophoresed in a single large well, approximately 7cm in length. The excised band, therefore, contained large quantities of agarose (relative to the amount of DNA) resulting in poor recovery of the DNA. Consequently, although ten times the amount of starting DNA was used, only a three fold increase in the yield of 2264bp fragment was obtained. It was necessary, therefore, to devise an alternative strategy. This involved sub-cloning the whole of the HLA-B7 gene as a PstI fragment, from the vector pBR322 to pAT153, a pBR322 based plasmid which contains no PvuII restriction sites. The band-purified PstI fragment of the HLA-B7 gene (1410bp) from pDP001 was ligated to phosphatased, PstI-digested pAT153. An aliquot of the ligation mix was transformed into the *E. coli* strain C1400, using the calcium chloride method, and recombinants were selected on Tet plates. Plasmid DNA from the putative positive clones was digested with PstI, to verify the presence of the insert, with BglII, to ensure that only one fragment had ligated into vector, and with both BglII and EcoRI, to determine the orientation of the insert (Fig:3.6). The plasmids were designated the names pB7-153A and pB7-153B depending on insert orientation.

Figure:3.7



Lane 1, λ HindIII and EcoRI mwt markers
Lane 2, 2ug DNA pB7-153A 0.5U PvuII, 60min
Lane 3, " " " " " " 30min
Lane 4, " " " " 1.0U PvuII, 60min
Lane 5, " " " " " " 30min
Lane 6, " " " " 0.2U PvuII, 60min
Lane 7, " " " " " " 30min
Lane 8, 400ng pB7-153A PstI and PvuII digested

Optimisation experiments for the partial digestions of pB7-153A following a complete digestion with PstI (See text for details)

Re-optimisation of the PvuII partial digestion had to be carried out for the pB7-153A and B clones, as now only one of the two restriction sites had to be cut. Hypothetically, therefore, a higher yield of the Tm containing fragment could be achieved.

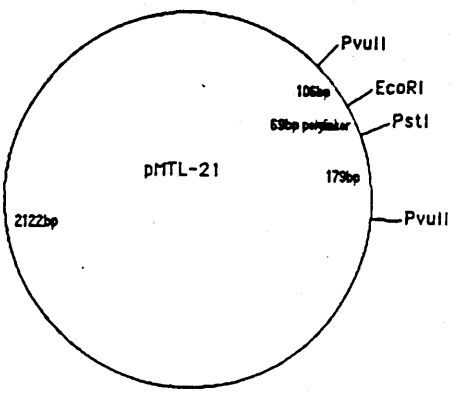
The optimisation experiments were set up by preparing an initial stock of DNA digested with PstI. PvuII digestions of 2ug aliquots of this DNA were carried out for either 30 or 60min at 37C (Fig:3.7), in a total volume of 25ul. After determining the optimal conditions (0.5U PvuII, 2ug DNA, in 25ul for 30min Lane 3 Fig:3.7), the procedure was repeated, but scaled up three fold. The DNA was electophoresed and the 689/721bp fragments were excised and purified using Gene Clean. EcoRI 8bp linkers were then ligated onto the fragment, and EcoRI was used to digest away any chains of linkers formed. Following reprecipitation, the DNA was digested again with EcoRI to ensure that all the linkers had been cut, and with BglII to cleave the 689bp fragment into two, generating a 148bp EcoRI/BglII fragment and a 541bp BglII/PstI fragment. The DNA was then phenol extracted and ethanol precipitated.

The vector pMTL-21 was digested with the restriction endonucleases EcoRI and PstI. It was then Gene Cleaned, in order to separate the vector from the 69bp polylinker fragment which irreversibly binds to the glass milk.

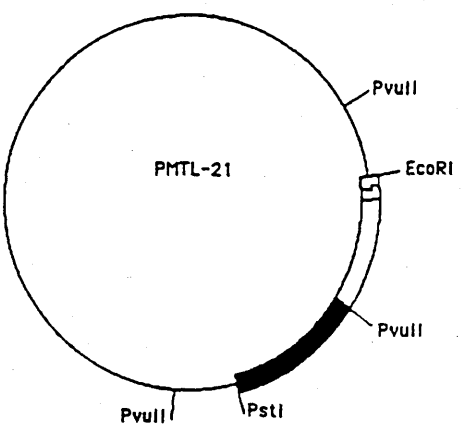
The ligation conditions were slightly modified to those described in chapter 2. This was due to the presence of the 148bp EcoRI/BglII and 541bp BglII/PstI fragments which are able to ligate to the above vector, but are unable to recircularise the plasmid. This in effect mops up the available vector DNA, so to compensate the molar ratio of ends employed was increased to 1:5 (insert:vector). An aliquot of the ligation mix was transformed into the *E. coli*

Figure:3.8

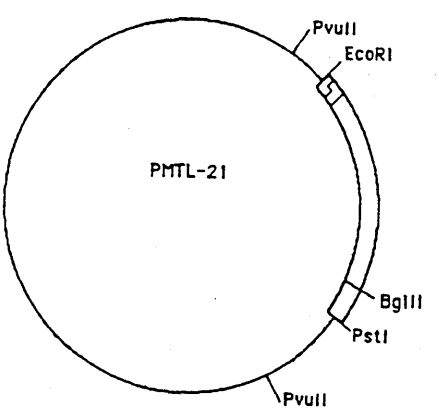
a) PvuII digested pMTL-21 generates 2 fragments 2122bp and 354bp in length respectively



b) HLA-B7 721bp Tm containing fagment plus 8bp EcoRI linker subcloned into PstI and EcoRI site of pMTL-21 digested with PvuII 3 fagments 2122bp, 605bp and 409bp in length



c) HLA-B7 689bp PstI and PvuII fragment plus an EcoRI 8bp linker subcloned into PstI and EcoRI sites of pMTL-21. PvuII digest generates 2 fragments 2122bp, and 974bp in length



BglII and EcoRI digest 2 fragments 2555bp and 549bp

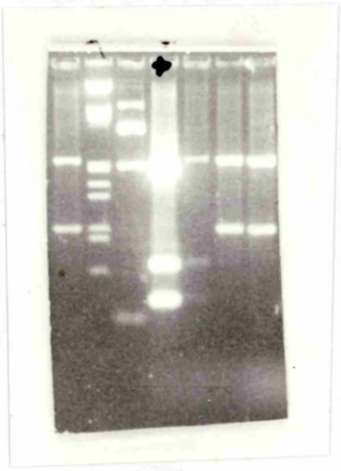
Diagram Not To Scale.

Figure:3.8

d)

(i) PvuII

1 2 3 4 5 6 7



-974

-605

-409

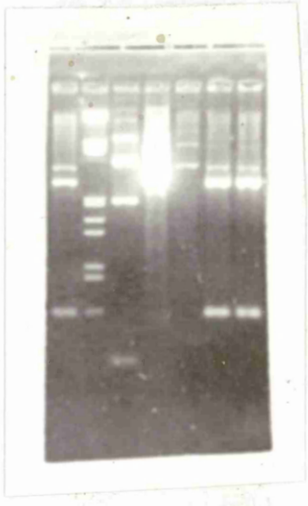
2555-

549-

354-

(ii) BglII and EcoRI

1 2 3 4 5 6 7



Lanes 1, 6, and 7, 689bp fragment subcloned into PstI and EcoRI sites of pMTL-21

Lanes 4, 5, 721bp fragment subcloned into PstI and EcoRI sites of pMTL-21

Lane 2, λ HindIII and EcoRI mwt markers

Figure:3.9

Mini-preparations of putative HLA-B7 Tm sequence plus EcoRI linker, sub-cloned as a PstI and EcoRI fragment into pMTL-21

Southern blot probed with the random primed 426bp PstI and PvuII digested fragment of pDP001 (black region in diagram below)

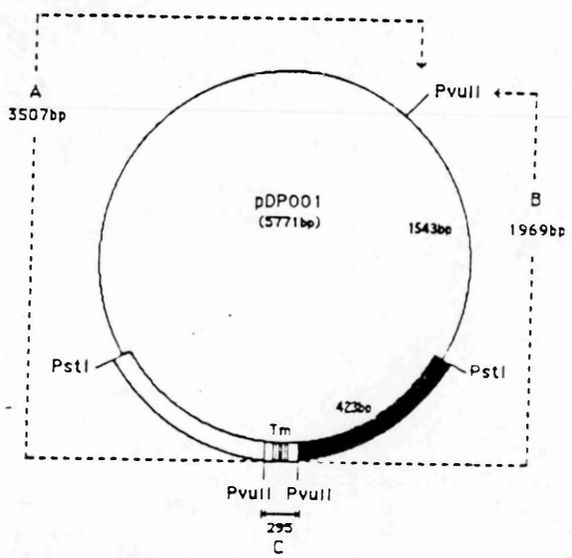
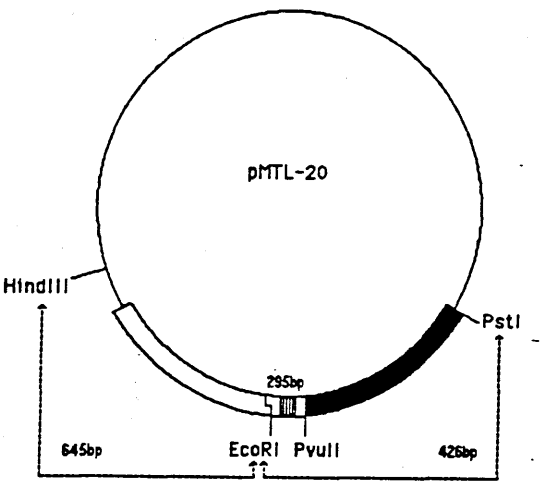


Diagram Not to Scale.

Figure:3.10



Simplified map of the fusion construct pCDFus-20 (refer to text for details on construction)

Diagram Not to Scale

strain C1400 and the positive recombinants selected on Amp plates. Mini-preparations of a number of these were digested with PvuII to verify the presence of the insert (Fig:3.8a).

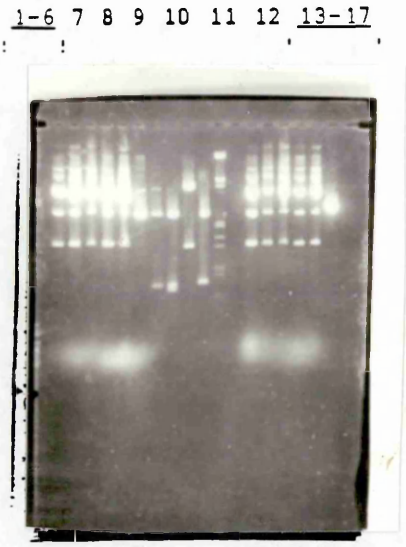
pMTL-21 contains two PvuII restriction sites positioned 179bp and 106bp on either side of the PstI and EcoRI sites respectively (Fig:3.8b). Digestion with PvuII produces two fragments of 2122bp and 354bp (Lane 3, gels (i) and (ii), Fig:3.8a), whereas, the positive clones containing the 721bp Tm fragment (Fig:3.8c) produce three fragments of 2122bp, 605bp and 409bp (Lanes 4 and 5, gel (i), Fig:3.8a). However, some PvuII digests of putative clones generated only two bands (2122bp and 974bp). These arose as a consequence of subcloning the 689bp PstI/PvuII fragment (Fig:3.8d) which co-purified with the 721bp fragment during band purification. This was confirmed by digesting the mini-preparation DNA with BglII and EcoRI (Lanes 1, 6,and 7, gel (ii), Fig:3.8a). The results indicated that the BglII digestion carried out prior to ligation had not gone to completion. The positive clones containing the Tm sequence of HLA-B7 were called pTm-R21.

To reconfirm the presence of the Tm containing insert in pMTL-21, a Southern blot of PvuII digested mini-preparation DNA was carried out. The membrane was probed with a random-primed, 426bp PstI/PvuII fragment of pDP001 (Fig:3.9).

3.8 Construction of the pCDFus-20 clone containing the external domain of CD2 and the Tm sequence of HLA-B7

To complete the CD2 fusion construct, the two components, the CD2 external domain and the Tm sequence had to be ligated into the HindIII/PstI sites of the vector pMTL-20, to produce pCDFus-20 (Fig:3.10). The CD2 external domain was isolated as a HindIII/EcoRI fragment from pCDEx-R21 and the Tm sequence of the HLA-B7 gene as an EcoRI/PstI fragment from pTm-R21. As double digestion of pMTL-20 with HindIII

Figure:3.11



The gel was run in duplicate and Southern blotted. One filter was probed with a random primed HindIII fragment of CD2 and the other with a random primed 426bp PstI and PvuII fragment of pDP001

Lanes 1-6, boiling preparations of putative pCDFus-20 clones digested with HindIII and PstI (fragments 3850bp and 1374bp)

Lane 7, pCDEx-21 digested with XbaI (fragments 2458bp and 678bp)

Lane 8, pCDEx-R21 digested with HindIII and EcoRI (fragments 2476 and 653bp)

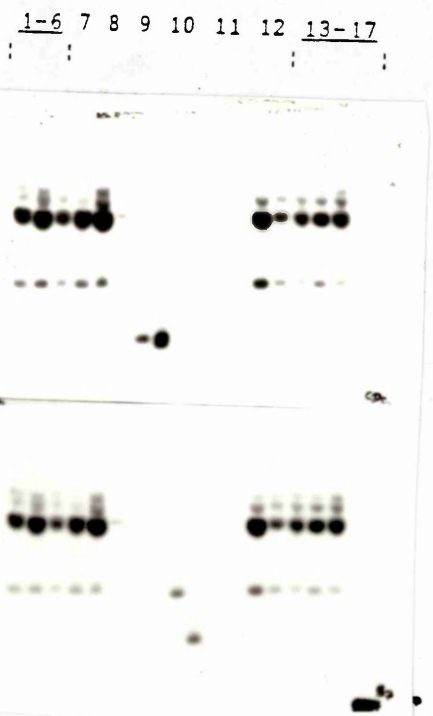
Lane 9, pDP001 digested with PstI

Lane 10, pTm-R21 digested with PstI and EcoRI

Lane 11, λ HindIII and EcoRI mwt markers

Lane 12, -

Lane 13-17, Boiling preparations of putative pCDFus-20 clones digested with HindIII and PstI (fragments 4363bp and 1410bp)



The blot was hybridised at 65C overnight washed 3x in 0.1x SSPE 65C (6h exposure of the autoradiograph at -70C with an intensifying screen)

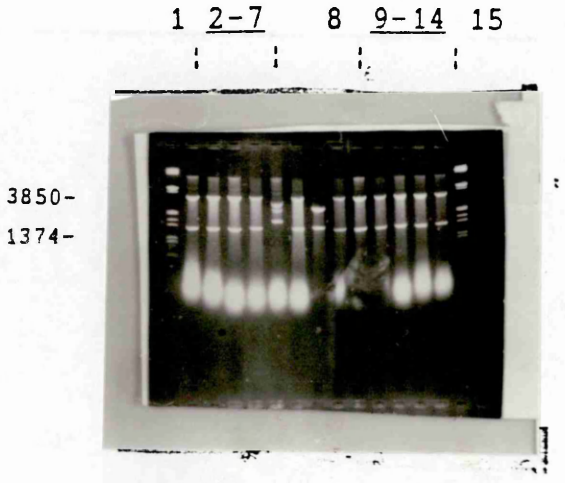
and PstI proved to be rather inefficient due to close proximity of these restriction sites (8bp), a derivative of pMTL-20, pStuff-20, was used. In this vector the HindIII/PstI sites are separated by a 440bp insert cloned between these two restriction sites. Thus double digestion of pStuff-20 with HindIII and PstI generated pMTL-20, which was then band purified.

The three components were ligated together and an aliquot of the ligation mix was transformed into the *E.coli* strain DS941 using the calcium chloride method. Positive recombinants were selected on Amp/X-gal plates and mini-preparation DNA from these colonies was subjected to restriction analysis (double digests with HindIII and PstI) to verify the presence of both inserts (Fig:3.11a). The gels were run in duplicate so that the identities of the fragments could be verified by Southern blotting and hybridisation. One of the blots was probed with a random-primed CD2, while the other was probed with random-primed, 426bp PstI/PvuII fragment of pDP001 (Fig:3.9b). DNA coding for the external domain of CD2 and the Tm sequence of HLA-B7 were included as hybridisation controls (Fig:3.11b).

3.9 Sub-cloning the CD2-fusion construct into CDM8

In order to express the fusion protein, the CD2-fusion construct pCDFus-20 had to be transferred from the cloning vector pMTL-20 to the expression vector CDM8. This involved digestion of pCDFus-20 with the restriction endonuclease HindIII and PstI. The 1374bp fragment (containing the CD2/B7 Tm fusion) generated, was Gene Cleaned and ligated into the band-purified, HindIII/PstI digested vector CDM8. The DNA was transformed into *E. coli* (MC1061/P3) using electroporation and the recombinant pCDFus-8 clones selected on Tet/Amp plates. DNA from a selection of these was digested with HindIII and PstI to verify the presence of the insert (Fig:3.12).

Figure:3.12



Lane 1, λ HindIII and EcoRI mwt markers

Lane 2-7, boiling preparation DNA of the pCDFus-8 constructs digested with HindIII and PstI

Lane 8, pCDFus-20 digested with HindIII and PstI

Lanes 9-14, boiling preparation DNA of the pCDFus-8 constructs digested with HindIII and PstI

Lane 15, λ HindIII and EcoRI mwt markers

Boiling preparations of putative pCDFus-8 clones (the fusion construct) subcloned from pMTL-20 into the HindIII and PstI sites of CDM8. Positve clones in all lanes except 6 which can not be determined because it did not cut.

3.10 The CD2 cloning vector

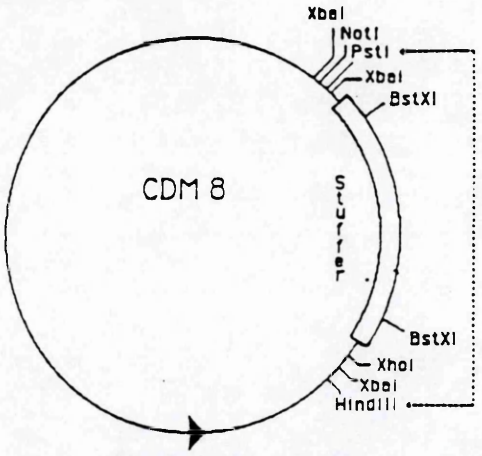
The CD2 cloning vector was constructed by ligating the external domain of CD2 to the stuffer sequence of CDM8. This was carried out in several stages. The stuffer region was excised from CDM8 as a 440bp HindIII/PstI fragment and was purified by phenol extracting the DNA from an LMP agarose gel, particularly because recovery using the Gene Clean procedure is poor for fragments less than 500bp in length.

Because of the close proximity of the HindIII and PstI sites in the pMTL-20 polylinker, double digestion with these enzymes is very inefficient. Separate digestions were therefore performed, using HindIII first, and then phenol extracting the DNA prior to digestion with PstI. Subsequently, the stuffer fragment was ligated into the HindIII/PstI sites of pMTL-20 and the DNA transformed into the *E. coli* strain DS941. Recombinants were isolated by Amp/X-gal selection, and mini-preparation DNA from these colonies digested with HindIII and PstI to verify the presence of the insert. The plasmid containing the stuffer sequence was named pStuff-20 (Fig:3.13).

To enable cDNA with either BstXI adaptors or EcoRI linkers to be sub-cloned into the CD2 cloning vector, the polylinker was modified to include an EcoRI site. Incorporation of the EcoRI restriction site was achieved by ligation of an EcoRI linker to the stuffer sequence in the pStuff-20 plasmid.

pStuff-20 contains a single StuI restriction site which lies immediately 5' of the PstI site in the polylinker. Digestion with StuI generated flush-ended plasmid DNA onto which EcoRI linkers (8bp ds oligonucleotides) were ligated. Following ligation, the DNA was ethanol precipitated, and the resuspended pellet was digested with EcoRI to remove linker multimers. After phenol extraction and precipitation, the

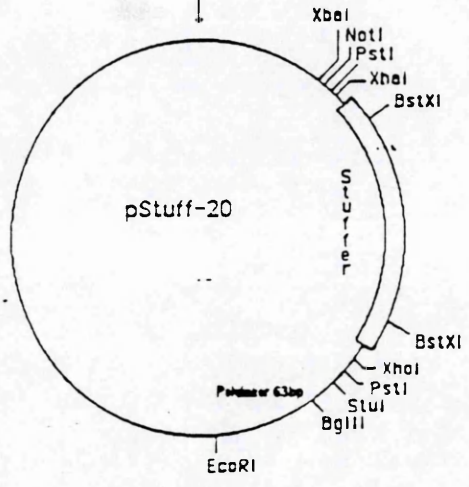
Figure:3.13



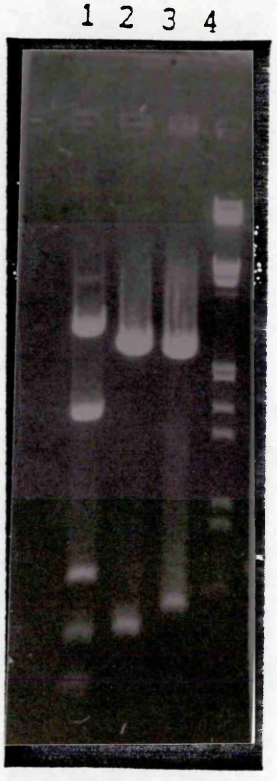
a) Map of CDM8 showing the stuffer region and the positions of the HindIII and PstI sites used to excise this fragment

Stuffer removed by HindIII/ PstI digest.

440bp stuffer fragment ligated into HindIII/ PstI sites of pMTL-20



b) map of the plasmid pStuff-20 containing the stuffer sequence subcloned into pMTL-20



c) Digestions of pStuff-20 with HindIII and StuI (fragment 443bp) and HindIII and EcoRI (fragment 503bp), to illustrate that the difference between the two fragments can be visualised on an agarose gel

Lane 1, pDP001 digested with PstI and PvuII

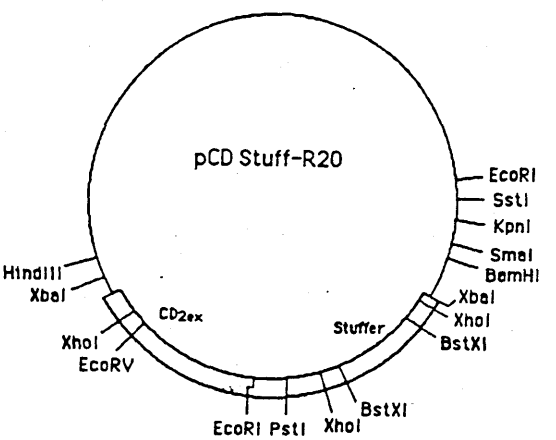
Lane 2, pStuff-20 digested with HindIII and StuI

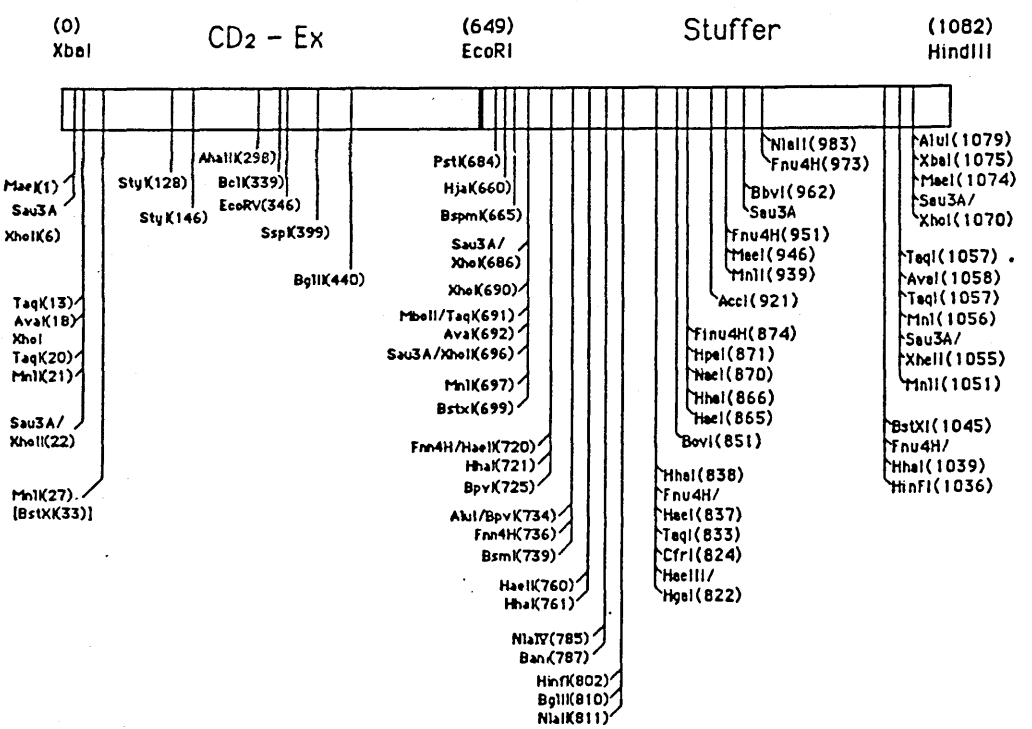
Lane 3, pStuff-20 digested with HindIII and EcoRI

Lane 4, λ HindIII and EcoRI mwt markers

Deagram Not to Scale.

b)



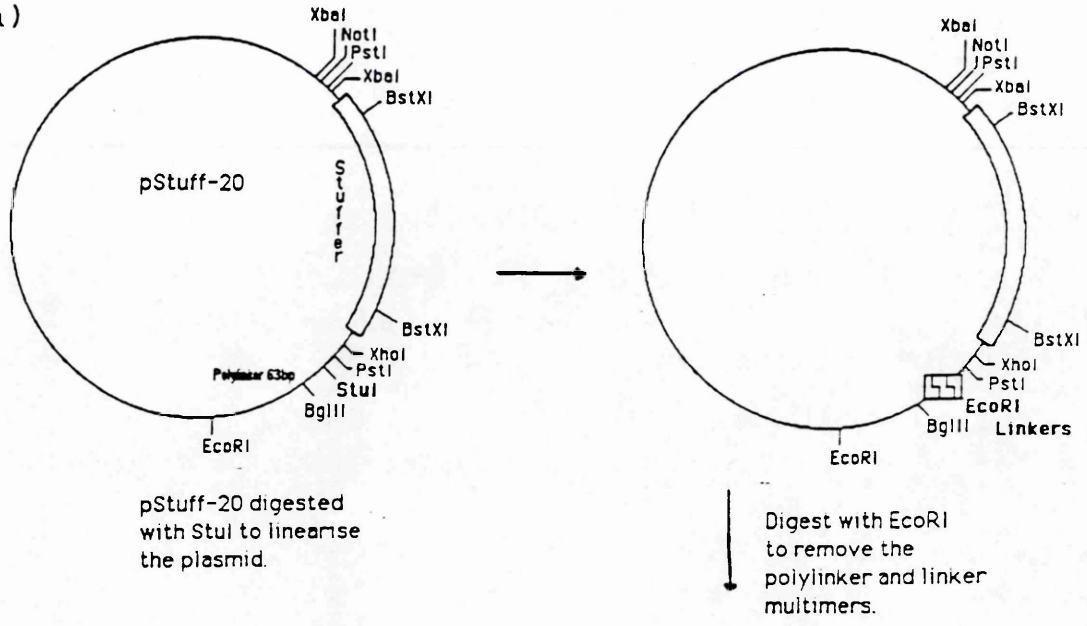


Restriction map of pCDStuff-20

Diagram Not to Scale.

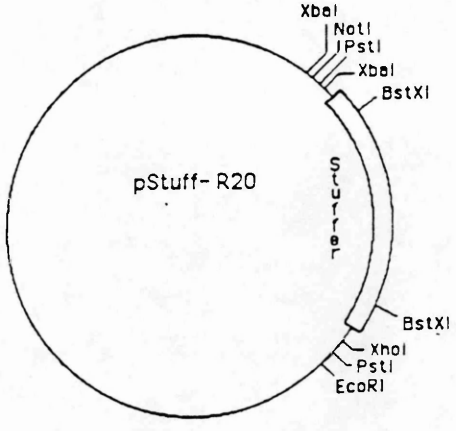
Figure:3.14

a)



a) A schematic diagram to show the addition of an EcoRI linker into the StuI site of pMTL-20, to form pStuff-R20

After religation.



b)

1 2 3 4

Lane 1, pStuff-R20 digested with HindIII and BglII to linearise the plasmid

Lane 2, λ HindIII and EcoRI mwt markers

Lane 3, PStuff-R20 digested with HindIII and EcoRI

Lane 4, pStuff-R20 digested with XhoI

Diagram Not to Scale

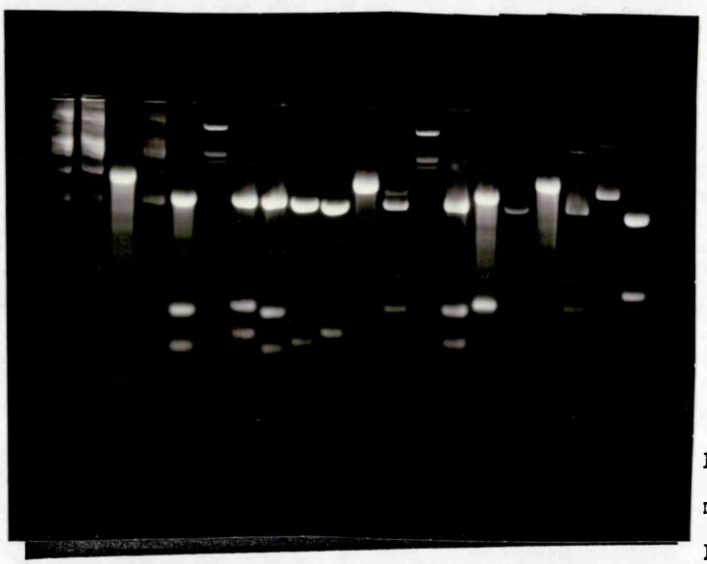
DNA was Gene Cleaned to remove both the excess linkers and the 63bp polylinker fragment, both of which bound irreversibly to the glass milk solution. The DNA was then religated and transformed into the *E.coli* strain C1400 using calcium chloride. Recombinants were selected on Amp plates and DNA mini-preparations of a selection of these were digested with both HindIII and EcoRI to liberate the stuffer fragment and consequently check for the presence of the linker. Double digestions with HindIII/BglII were also carried out to ensure that the remainder of the polylinker was deleted (correct clones linearised) (Fig:3.14). This plasmid was named pStuff-R20.

3.11 Ligation of the external domain of CD2 and the stuffer into pMTL-20

Following ligation of the EcoRI linker, the stuffer region was ligated to the external domain of CD2 via the EcoRI site and the combined fragment sub-cloned into pMTL-20. The stuffer DNA was isolated as an XbaI/EcoRI fragment from pStuff-R20 and the CD2 external domain was excised as a HindIII/EcoRI fragment from pCDEx-R21. The two portions were band purified, the stuffer sequence by phenol extracting the DNA from an LMP agarose gel, and the CD2 external domain sequence using Gene Clean. The two fragments were ligated together into the HindIII/XbaI sites of pMTL-20. An aliquot of the ligation mix was transformed into the *E. coli* strain DS941 using calcium chloride, the recombinants selected on Amp/X-gal plates, and DNA from a number of these digested with XbaI to confirm the presence of both inserts. A more detailed diagnostic restriction analysis of one of the positive clones was then carried out using a spectrum of restriction endonucleases (Fig:3.15). This clone was named pCDStuff-R20.

Figure:3.15

a)



a) digests of pCDStuff-20 unless otherwise stated

Lane 1, StuI
Lane 2,. BglII
Lane 3, BclI
lane 5, XhoI
Lane 6, λ HindIII and EcoRI mwt markers
Lane 7, XbaI and EcoRI
Lane 8, AvaI
Lane 9, HindIII and StuI
Lane 10, pStuff-20, digested with HindIII and EcoRI
Lane 11, BstXI
Lane 12, EcoRI, SmaI and XbaI
Lane 13, 1 HindIII and EcoRI mwt markers
Lane 14, XbaI and PstI
Lane 15, EcoRI
Lane 16, pCDEx-R21 digested with HindIII and EcoRI
Lane 17, HindIII and StuI
Lane 18, HindIII and EcoRI
Lane 19, HindIII and PstI
Lane 20, BglI

A series of diagnostic digests of the pCDStuff-20 plasmid

3.12 Sub-cloning of the CD2 external domain and stuffer sequences into CDM8

The pCDStuff-R20 plasmid was digested with XbaI to release the 1093bp CD2 external domain/stuffer fusion fragment and the CDM8 vector was digested with XbaI, and phosphatased. Both components were band purified using Gene Clean and then ligated together. The DNA was transformed by electroporation into the *E. coli* strain MC1061/P3 and recombinants selected on Tet/Amp plates. The plasmid DNA isolated from the putative positives was digested with XbaI, to confirm the presence of the insert and with HindIII/BglII, to determine its orientation. This plasmid was named pCDStuff-8 (FIg:3.16).

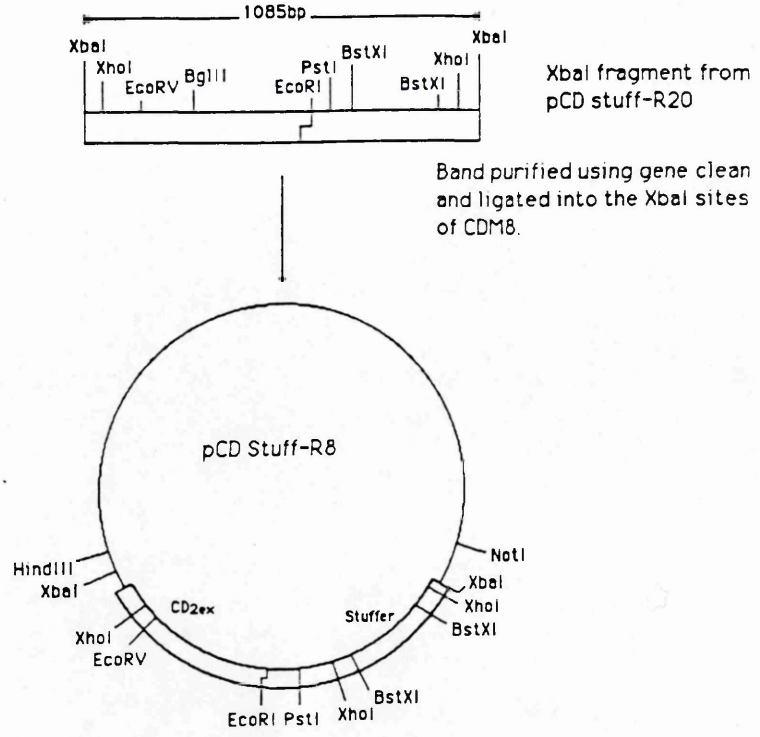
3.13 Verification of reading frame by plasmid sequencing of the fusion construct

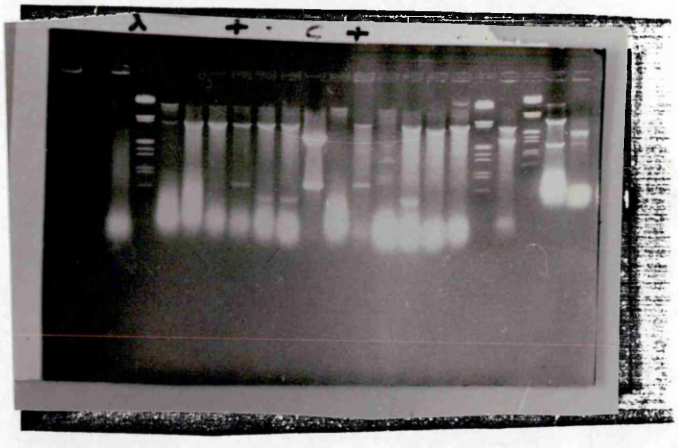
The limited number of restriction sites in the CDM8 poly-linker resulted in a large number of DNA manipulations being required to generate the various constructs. As this significantly increases the potential for error, it was essential to verify the frame of the various clones prior to embarking on the expression studies.

Restriction mapping alone was not sufficient proof that the reading frame had been maintained in the fusion constructs (pCDFus-20 or pCDFus-8). So plasmid sequencing was employed to verify the sequence across the junction of each clone. A 15bp oligonucleotide corresponding to nucleotides 546-561bp of the CD2 clone was synthesised as the primer, such that it annealed 50bp upstream of the region to be sequenced.

Plasmids pCDFus-20, pCDStuff-R20 and pCD2 were sequenced, the former two, to confirm the sequence at the junction and

Figure:3.16





HindIII and BglII digests of boiling preparation DNA of the pCDStuff-8 clones to determine orientaion of the insert

Lane 1 and 15, 1 HindIII
and EcoRI mwt markers

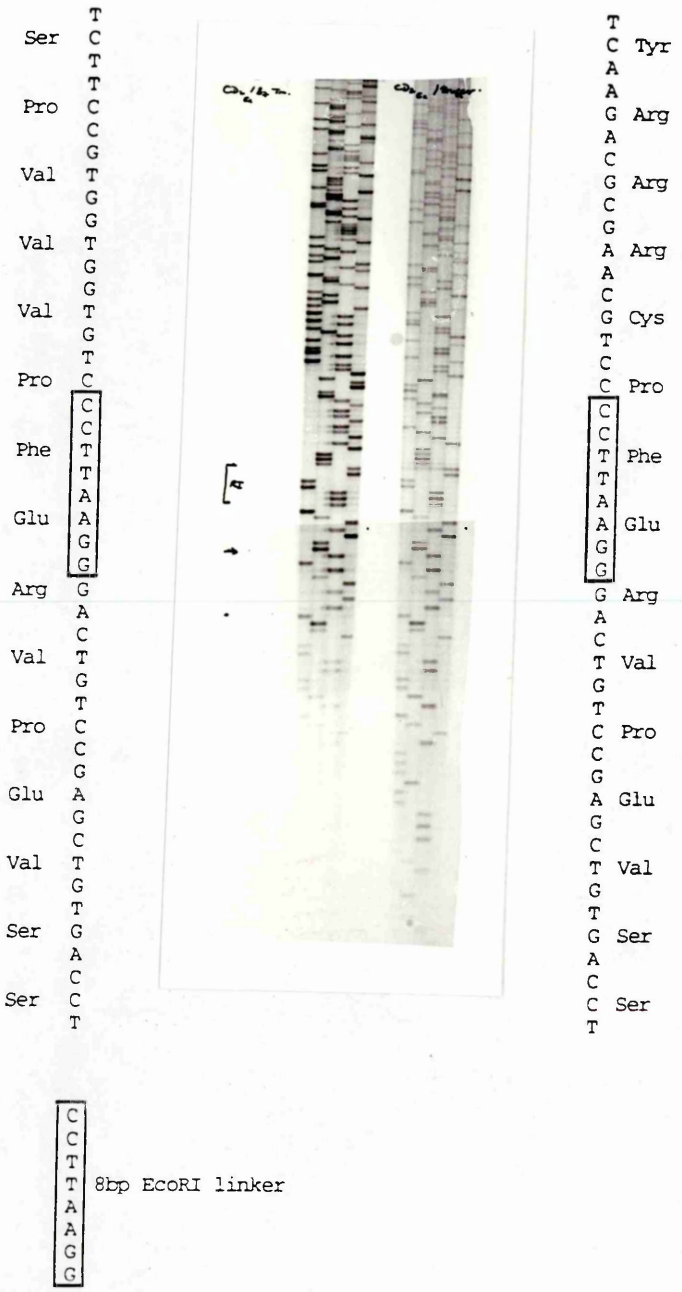
Lane 5 and 10, clones containing pCDFus-20 pCDStuff-20
the CDStuff fragment in the
correct orintation

Lane 9, pCDStuff-20 digested
with HindIII and BglII

Diagram Not to Scale.

Figure:3.17

a) Plasmid sequence of the recombinant CD2 constructs pCDFus-20 and pCDStuff-20.



b)

5' CCA CCA GCC TGA GTG 3'

15 base oligonucleotide primer hybridises to the CD2 50bp upstream of the PvuII site in the native CD2 clone (positions 546-561bp of CD2)

pCD2 as a control (Fig:3.17). The results obtained confirmed that the fusion construct was in frame and the junction of pCDStuff-20 was as expected.

The limited number of restriction sites in the CDM8 poly-linker resulted in a large number of DNA manipulations to generate the various constructs. Thus making it more critical to check the frame of the various clones prior to determining the targeting of the various expression products, in transfection experiments.

CHAPTER 4

EXPRESSION STUDIES OF THE pCD2, pCDFus-8 AND pCDStuff-8 CONSTRUCTS IN COS CELLS

4.1 Introduction

The predicted sizes of the proteins synthesised from the constructs pCDEx-8, pCDFus-8 and pCDStuff-8 (described in chapter 3) are 209, 298, and 230 amino acids respectively (Table:4.1). They all contain the coding sequence for the external domain of the CD2 protein (204 amino acids), which includes the 19 amino acid N-terminal signal peptide sequence and the three putative Asn-linked glycosylation sites (Fig:4.1). Theoretically therefore, these recombinant proteins should be glycosylated and completely processed through the ER and Golgi apparatus. However, as neither the pCDEx-8 nor the pCDStuff-8 constructs contain membrane anchoring sequences, it is likely that these recombinant proteins will be secreted. In contrast, the pCDFus-8 construct should express a protein, which is anchored to the membrane via the Tm sequence derived from the HLA-B7 surface antigen.

Table:4.1

Protein	No. amino acids	Glycosylation kDa	
		-	+
CD2 native protein	357	37	50
CD2/fusion protein	298	33	45
CD2/stuffer	230	25	38
CD2Ex	209	23	36

To verify the cellular expression and predicted targeting of these recombinant proteins, cultured cells were transfected

Figure:4.1

```
          1                                                  50
pCD2      MSFPCKFVAS FLLIFNVSSK GAVSKEITNA LETWGALGQD INLDIPSFQM
pCDFus-8  MSFPCKFVAS FLLIFNVSSK GAVSKEITNA LETWGALGQD INLDIPSFQM
pCDStuff-8 MSFPCKFVAS FLLIFNVSSK GAVSKEITNA LETWGALGQD INLDIPSFQM

          51                                                 100
pCD2      SDDIDDIKWE KTSDKKKIAQ FRKEKETFKE KDTYKLFKNGTLKIKHLKTD
pCDFus-8  SDDIDDIKWE KTSDKKKIAQ FRKEKETFKE KDTYKLFKNGTLKIKHLKTD
pCDStuff-8 SDDIDDIKWE KTSDKKKIAQ FRKEKETFKE KDTYKLFKNGTLKIKHLKTD

          101                                                150
pCD2      DQDIYKVSIY DTKGKNVLEK IFDLKIQERV SKPKISWTCINTTLTCEVMN
pCDFus-8  DQDIYKVSIY DTKGKNVLEK IFDLKIQERV SKPKISWTCINTTLTCEVMN
pCDStuff-8 DQDIYKVSIY DTKGKNVLEK IFDLKIQERV SKPKISWTCINTTLTCEVMN

          151                                                200
pCD2      GTDPELNLYQ DGKHLKLSQR VITHKWTTSL SAKFKCTAGNKVSKESSVEP
pCDFus-8  GTDPELNLYQ DGKHLKLSQR VITHKWTTSL SAKFKCTAGNKVSKESSVEP
pCDStuff-8 GTDPELNLYQ DGKHLKLSQR VITHKWTTSL SAKFKCTAGNKVSKESSVEP

          201       ¦--------------- Tm --------------¦     250
pCD2      VSCPEKGLDI YLIIGICGGG SLLMVFVALL VFYITKRKKQRSRRNDEELE
pCDFus-8  VREFPVVVPS GEEQRYTCHH VQHEGLPKPL TLGWEPSSQSTVPIVALVAR
pCDStuff-8 VREFPCRRRT GRYGRSLEIH CAGGRRAAPA *

          251                                                300
pCD2      TRAHRVATEE RGRKPQQIPA STPQNPATSQ HPPPPPGHRSQAPSHRPPPP
pCDFus-8  PPAVLAVVVI GPVVAVMCRR KSSGGKGGSY QAACTDSAQGSHVSLTA*

          301                                                350
pCD2      GHRVQHQPQK RPPAPSGTQV HQQKGPPLPR PRVQPKPPHGAAENSLSPSS

          351
pCD2      N*
```

NTT
NGT = Asn-X-Ser/Thr N-linked glycosylation sites

Tm = Transmembrane region

* = Stop codon

Amino acid sequence of the native and recombinant CD2 proteins

with the respective plasmid constructs, and the resulting proteins analysed using a variety of immunological techniques.

4.2 Optimisation of transfections

DEAE-dextran rather than $CaPO_4$ was used for the transfections particularly because, in transient expression experiments this method is not only more efficient (5-50% transfection efficiency), but also more reproducible. The mechanism by which transfection is achieved is not well understood, but it is thought that the DNA forms a complex with the DEAE-dextran and is consequently endocytosed by the cells; a process which is enhanced by either a glycerol or DMSO "shock". Transfection efficiency can be increased by using higher concentrations of DEAE-dextran or longer incubation times. However, DEAE-dextran also has the potential to be toxic to cells, with the degree of toxicity varying between cell lines. It was necessary, therefore, to optimise the transfection conditions for COS cells (Ausubel *et al*;1989).

Preliminary experiments were carried out to assess the toxicity of DEAE-dextran on COS cells. Nuserum (10%) added to the transfection mix has been described to increase cell tolerance to DEAE-dextran (Ausubel *et al*;1987), because it contains no lipoprotein (which tends to come out of solution from FCS over a prolonged incubation at 37C). However, due to its unavailability, other modifications were attempted.

Mock transfections (in the absence of DNA) were carried out using a DEAE-dextran concentration of 200ug/ml (the recommended range being 100-400ug/ml DEAE-dextran (Ausubel *et al*;1987):

a) in the absence of FCS

b) using 10% heat treated serum

c) using 10%, heat treated serum which had been passed through an 0.45um filter

The cells were observed at half hour intervals to monitor cell mortality. Depending on cell survival, a 1min DMSO shock was carried out after 3h (DMSO, although less efficient at enhancing DNA uptake than glycerol is less harmful to the cells).

Results

a) In the absence of FCS, initiation of cell death (cells lifted off the dish and began to swell) occured within 15min of adding the DEAE-dextran.

b) Cells incubated with DEAE-dextran in the presence of 10% heat-treated FCS appeared healthy. However, after 2h a lipoprotein precipitate had formed over the cells, with only a few cells lifting off after the 3h incubation with DEAE-dextran (implying that cell death was negligible to this point) but, after a 1min DMSO shock, extensive cell death (80%) was observed.

c) The cells incubated with a DEAE-dextran mixture containing filtered serum, produced the highest observed cell survival rate. No precipitate formed and the cells appeared to have suffered no adverse effects from either a 3h exposure to DEAE-dextran or a 1min DMSO shock.

A DMSO shock is not singularly responsible for high cell mortality, but in conjunction with the lipoprotein precipitate, which forms when using unfiltered serum, it is highly toxic. However, formation of the lipoprotein precipitate can be prevented if the serum is first passed through a 0.45um filter.

4.3 Estimation of transfection efficiency

Once the resistance of the cells, to 200ug/ml DEAE-dextran had been established, a series of experiments to assess the

optimum conditions for a high transfection efficiency were set up. Different concentrations and incubation times with DEAE-dextran were carried out on exponentially growing cells (between 30-50% confluent, at approximately $1x10^6$ cells/10cm dish). The DEAE-dextran mixture used contained 10% heat-treated filter sterilised serum and 5ug of plasmid DNA. Following incubation with DEAE-dextran the cells were subjected to a 2min DMSO shock.

Both the pCD2 and pCDFus-8 plasmids were tested at DEAE-dextran concentrations of 200ug/ml and 400ug/ml for either 1,2 or 3h. Also included, was a transfection of the pCDEx-8 plasmid (5ug) as well as a negative control of mock transfected cells (see 4.2). These were carried out for 2h using a DEAE-dextran concentration of 200ug/ml.

To assess the transfection efficiency, cytospins of the transfected cells were prepared for analysis using immuno-staining and fluorescence microscopy. Propidium iodide stained the nuclei red, while the membrane of the positive transfectants stained green using an anti-CD2 mAb (anti-T11) primary antibody followed by an FITC conjugated second antibody. The transfection efficiency was calculated by counting the mean number of fluorescent green cells in a random field of view (minimum sample size of n=5) and expressing this as a percentage of total cell number, determined by the number of fluorescent red nuclei.

$$\% \text{ Efficiency} = \frac{\text{Number of FITC stained cells (green)}}{\text{Total number of cells (number of red nuclei)}} \times 100$$

Results

Neither concentration of DEAE-dextran caused excessive cell death, either before or after the DMSO shock. On harvesting 48h after transfection, the cells appeared healthy and produced high quality cytospins (sick or aged cultures are more prone to lysis when cytospun). The percentage of cell lysis, following cytospinning, was determined by

immunostaining with an anti-HLA class-I mAb, W6/32, (which stained the surface of all the intact cells green) and was estimated to be approximately 5% (Fig:4.2).

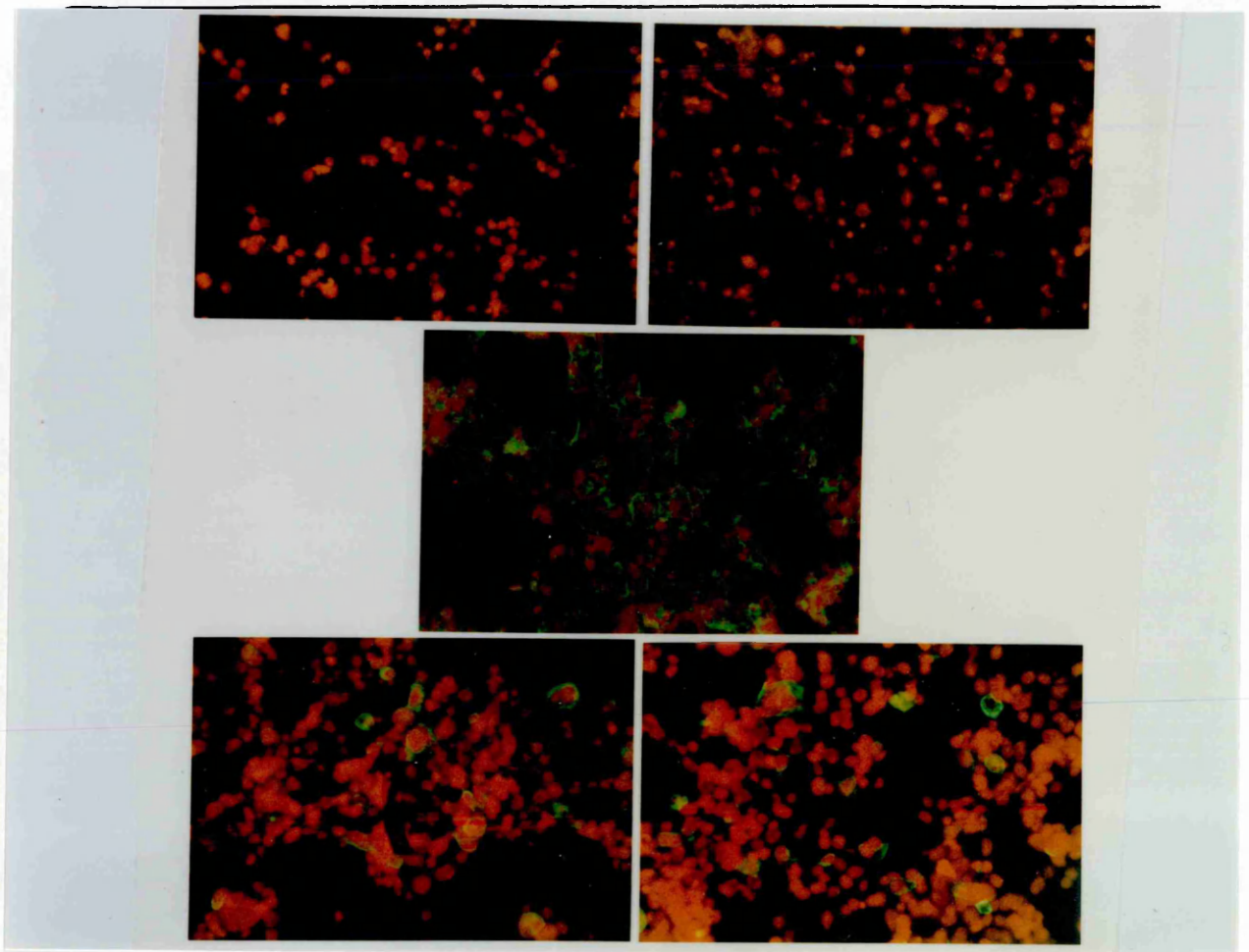
Cytospins of cells transfected with either pCD2 or the pCDFus-8 construct stained positive with the anti-T11 mAb (Fig:4.2). This demonstrated that both native CD2 and the CD2 fusion protein were expressed on the cell surface. It would seem, therefore, that the HLA-B7 Tm sequence acts as an adequate membrane anchor for the CD2 protein.

The transfection efficiencies for the plasmids at both DEAE-dextran concentrations and for each incubation time are listed in Table:4.2. As expected, the efficiency increased with both increasing concentrations of DEAE-dextran and longer incubation times. These ranged from 2% at 200ug/ml DEAE-dextran incubated for 1h; to a maximum value of 29% at 400ug/ml DEAE-dextran incubated for 3h. For each of the concentrations tested, both plasmids gave similar transfection efficiencies except for a single anomalous result obtained for the pCDFus-8 plasmid at 200ug/ml DEAE-dextran incubated for 2h. A DNA pipetting error during the transfection may account for this result.

Table:4.2

Cells $1.1x10^6$ were transfected with either 5ug of pCD2 or pCDFus-8. The cells were transfected for either 1,2, or 3h at 200 and 400ug/ml DEAE-Dextran, and shocked for 2min with DMSO 10%/PBS to enhance DNA uptake.

Figure:4.2



a) Mock transfected cells (200ug/ml DEAE-dextran, for 2h and a 2min DMSO shock) immunostained with anti-T11 (1/10 dilution), the negative control

b) COS cells transfected with pCDEx-8 (5ug), for 2h using 200ug/ml DEAE-dextran, 2min DMSO shock, immunostained with anti-T11 (1/10 dilution)

c) Positive control, COS cells immunostained with W6/32 (1/50 dilution)

d) COS cells transfected with pCD2 (5ug), for 2h using 400ug/ml DEAE-dextran, immunostained with anti-T11 (1/10 dilution)

e) COS cells transfected with pCDFus-8 (5ug), for 2h using 400ug/ml DEAE-dextran, immunostained with ant-T11 (1/10 dilution)

	pCD2			pCDFus-8		
DEAE-Dextran concentration	200ug/ml			200ug/ml		
Time	1h	2h	3h	1h	2h	3h
	(% positive cells)			(% positive cells)		
	0.6	7.3	14.3	5.6	25.4	30.3
	4.3	5.5	22.5	1.5	22.8	17.7
	3.9	6.3	22.2	6.8	21.0	19.7
	0.9	6.6	9.4	1.9	20.5	25.5
	1.7	13.2	16.0	3.7	23.7	23.1
mean x	2.3	7.8	16.9	3.9	23.7	23.1
n=5						

DEAE-Dextran concentration	400ug/ml			400ug/ml		
Time	1h	2h	3h			
	(% positive cells)			(% positive cells)		
	13.3	9.3	17.5	7.0	10.7	25.6
	15.2	10.2	44.6	7.3	8.6	25.7
	8.5	18.4	28.8	7.9	7.7	45.2
	8.6	17.0	29.6	9.4	14.4	17.2
	2.5	7.3	25.2	8.5	14.2	29.1
mean x	9.6	12.4	29.5	8.0	11.1	28.6
n=5						

The anti-T11 staining results for both the mock transfected cells and those transfected with pCDEx-8 were negative (Fig:4.2). Mock transfected cells were included as a negative control to demonstrate that non-specific binding of the anti-T11 mAb to the cell surface is negligible.

Asuming that the transfections were equally successful; the negative CD2-staining observed from cells transfected with the pCDEx-8 plasmid, implied that the external domain of the CD2 protein was unable to anchor to the membrane, and was thus secreted into the culture medium. Alternatively, a high protein turnover rate or the loss of the epitope due to a conformational change in the protein may also account for the negative staining observed. However, prior to further

speculation as to the fate of the truncated protein, it was necessary to demonstrate conclusively that experimental error, such as poor transfection efficiency, was not responsible for the negative result obtained.

4.4 Attempts to demonstrate that the external domain of CD2 is secreted

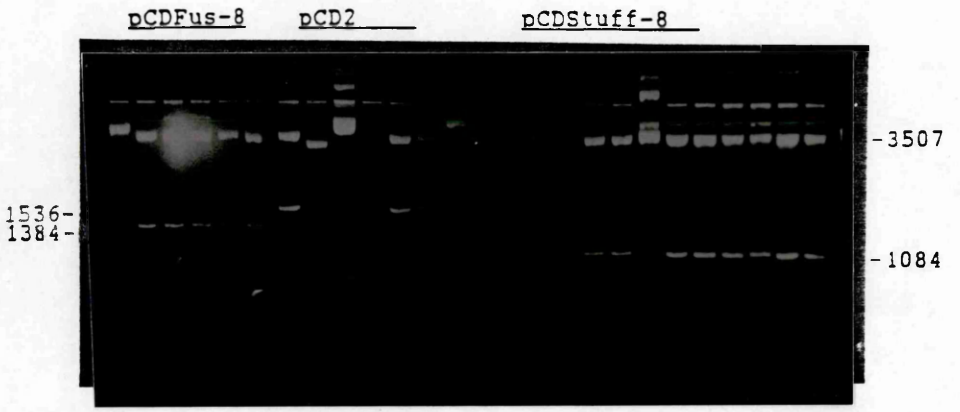
The plasmid pCDStuff-8, the cloning vector composed of the coding sequence for the CD2 external domain and the cloning cassette from CDM8, was transfected into COS cells. pCD2 and pCDFus-8 were included as positive controls and mock transfected cells as a negative control. The transfections were set up using 5ug of DNA under the optimal conditions described previously.

The cells were harvested 48h after transfection and dilutions of 1/20 and 1/100 of the cell suspension ($5x10^4$ and $1x10^4$ cells respectively) were taken for Hirt extractions. The remainder of the cells were used to prepare cytospins for immunofluorescent staining.

The purified DNA from the Hirt extractions (1/50) was electroporated into the *E. coli* strain, MC1061/P3, and the transformants plated out on Tet and Amp plates. The colonies were counted (Table:4.2) and a selection of these were grown up for boiling preparations. These were digested with XbaI and analysed by gel electrophoresis (Fig:4.3).

Slides of the cytospun cells were stained with W6/32 to determine the percentage of lysed cells, and with anti-T11 to determine the cells expressing the CD2 external domain on their surface. Propidium iodide was used as a nuclear stain to determine the total number of cells present (Table:4.4, Fig:4.4 slides were stained as described previously).

Figure:4.3



Boiling preparation DNA of a selection of the clones derived from electroporation of Hirt DNA from transfected cells. All samples were digested with XbaI

Lanes 1-6, pCDFus-8 (Lane 1 uncut)

Lanes 7-12, pCD2 (Lane 9 uncut, Lane 10 no sample)

Lanes 13-25, pCDStuff-8 (Lane 19 uncut)

Results

The plasmids pCD2, pCDFus-8 and pCDStuff-8 were successfully re-extracted from transfected COS cells. The transformation results from electroporation of 1/50 of the purified Hirt DNA from both $5x10^4$ and $1x10^4$ cells are listed in Table:4.3. Analysis of the number of colonies from each transformation showed a proportional relationship between the number of *E. coli* colonies obtained and transfection efficiency. It can be concluded therefore, that the transfection efficiency for the pCD2 ($5x10^6$ transformants) plasmid is 5 fold lower than that of either pCDFus-8 ($31x10^6$ transformants) or pCDStuff-8 ($25.5x10^6$) (Table:4.2).

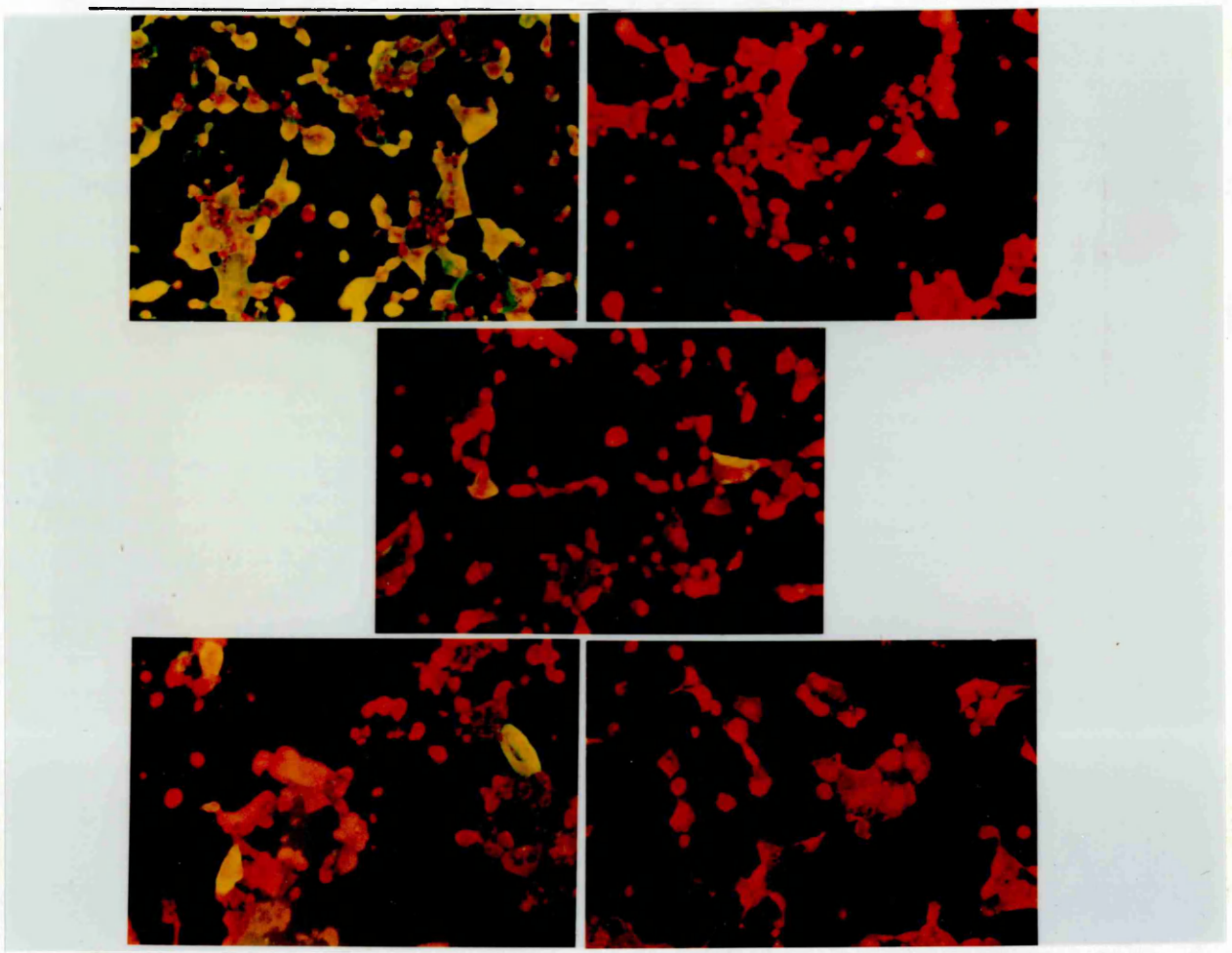
Table:4.3

The transformation efficiency for 10ng of CDM8 was $1x10^8$/ug using electroporation. The background number of colonies was assessed by mock transfection. 1/20 of the lysate was removed for Hirt extraction and a 1/50 of the extracted DNA was electroporated into *E. coli*. An average of 3 colonies/plate were obtained, which corresponded to a total of number of $3x10^4$ colonies arising as a result of background/transfection (background approximately 0.15%).

Plasmid	Dilution	No.colonies /plate	Total from Hirt mix	Total from transfection	mean $x10^6$
pCD2	1/20	514	$2.6x10^5$	$5.1x10^6$	5.5
	1/100	118	$5.9x10^4$	$5.9x10^6$	
pCDFus-8	1/20	3136	$1.6x10^6$	$31.4x10^6$	31.0
	1/100	614	$3.1x10^5$	$30.7x10^6$	
pCDStuff-8	1/20	2328	$1.2x10^6$	$23.3x10^6$	25.5
	1/100	556	$2.8x10^5$	$27.8x10^6$	

Immuno-staining of the cytospin preparations reproduced the results obtained previously. Cells transfected with pCD2 and pCDFus-8 stained positive with anti-T11 mAb, whereas those

Figure:4.4



a) COS cells immunostained with W6/32 (1/50 dilution) positive control

b) COS cells immunostained with anti-T11 (1/10 dilution) as a negative control

c) COS cells 48h following transfection with 10ug of pCD2 were harvested and immunostained with anti-T11 (1/10dilution). Cells expressing the CD2 protein ontheir cell surface can be clearly seen

d) COS cells 48h following transfection with 10ug of pCDFDus-8, were harvested and immunostained with anti-T11 (1/10 dilution). The CD2-fusion protein is expressed at the surface of the transfected cells with the same intensity as that seen for the native CD2 protein

e) COS cells 48h following transfection with 10ug of pCDStuff-8, were immunostained with anti-T11 (1/10 dilution) and were found to stain negative

transfected with pCDStuff-8 stained negative with anti-T11 mAb. Transfection efficiencies corresponding to the stained cells were determined as previously described. Values of 2.4% and 2.3% respectively, were obtained for pCD2 and pCDFus-8 (Table:4.4). Not only were these figures unexpectedly low (compare with Table:4.2), they also did not reflect the 5 fold difference in transfection efficiency observed between pCD2 and pCDFus-8 when Hirt DNA was transformed (Table:4.3).

Table:4.4

	Sample size						
pCD2	n=6						
No. of positive cells	3	2	4	3	2	4	3
Total/ field of view	114	54	70	78	45	163	143
	Percentage efficiency 2.4%						

	Sample size					
pCDFus-8	n=6					
No. of positive cells	2	3	4	5	2	3
Total/field of view	140	161	136	168	140	104
	Percentage efficiency 2.3%					

All intact cells stained positive with W6/32 (background cell lysis 7%). Both mock transfected cells and those transfected with pCDStuff-8 stained negative with anti-T11

Primary anitibody
anti-T11 1/10 dilution.
W6/32 1/50 dilution

Second antibody
FITC conjugated goat anti mouse 1/50 dilution

This discrepancy in transformation efficiencies between the two methods used, may be in part, due to the poor quality of the cytospins which contained a large number of lysed cells. This would effectively decrease the percentage of positively expressing cells; as percentage efficiency is calculated as

a fraction of total cell number and not a fraction of the number of intact cells. In addition, these cells may have an abnormally elevated surface protein content, due to high expression levels of the transfected protein. This may result in cells that are more fragile and susceptible to lysis. Consequently, this would introduce a negative bias in the calculation of the transfection efficiency.

Another consideration is that the mAb used, anti-T11, was raised against the native CD2 protein. A conformational change in the truncated CD2 protein, leading to the loss of the anti-T11 epitope, may also account for the negative staining observed with pCDStuff-8.

To clarify this, the transfections were repeated and the cells immunofluorescently stained with the RFT11 mAb. This mAb, unlike anti-T11, is able to bind both the native and denatured conformations of the CD2 protein external domain. Therefore, cells transfected with pCDStuff-8 should stain positive; irrespective of whether they express an abnormally folded form of the truncated CD2 on their cell surface, or accumulate the truncated protein intracellularly (the acetone fixing of the cytospin preparations permeablises the membrane, allowing access of the mAb to intracellular proteins).

However, the immunofluorescent staining results obtained using RFT11 were identical to those obtained with anti-T11; cells transfected with either pCD2 or pCDFus-8 stained positive and those with pCDStuff-8 stained negative. Thus, pCDStuff-8 derived expression of the external domain of CD2 was found to be both absent at the cell surface and undetectable within the cell.

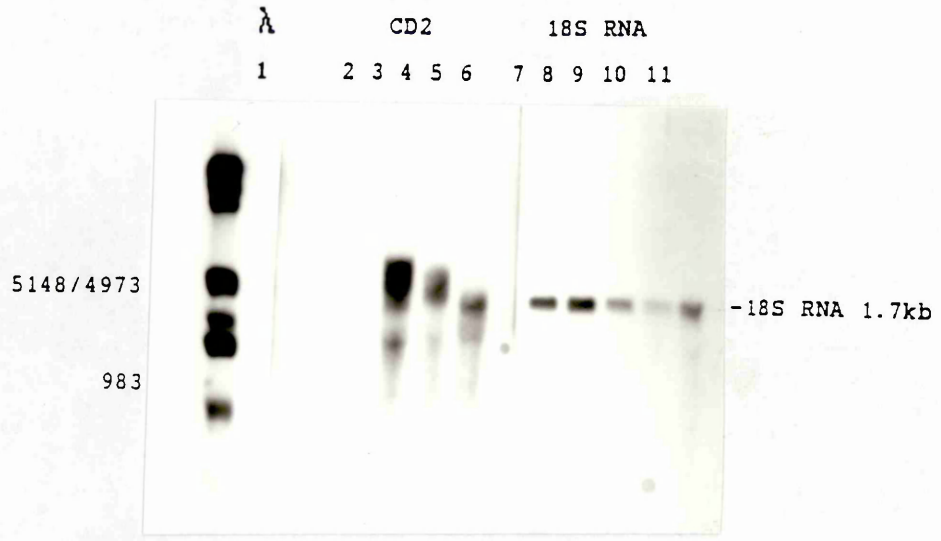
4.5 Northern analysis of the recombinant CD2 mRNAs

Regulation of protein expression can occur at both the translational and transcriptional levels, and may be reflected in the turnover rates for either the protein or the respective mRNA. To study this at the transcriptional level, $1x10^6$ cells were transfected with CDM8, pCD2, pCDFus-8, or pCDStuff-8. The cells were harvested 48h after transfection, 1/100 was removed for Hirt extraction (to confirm that the transfection was successful) and the total RNA extracted from the remainder of the cells. Aliquots of a 1/10 and 9/10 of the RNA extracted from each sample were run in parallel on a formaldehyde gel. After blotting, the membrane the 1/10 portion was probed with a random-primed 18S RNA probe and the 9/10 portion probed with a random-primed CD2 HindIII fragment (Fig:4.5).

The 18S RNA probe was used to determine the extent to which the RNA had degraded during purification. The yield of RNA from the cells transfected with CD2, pCDFus-8 and pCDStuff-8 was less than that for the negative controls, evidence of slight degradadation was apparent (assessed by the extent of smearing below 18S RNA band). The negative controls consisting of the mock transfected cells and those transfected with CDM8 were loaded with the most RNA, gave no signal when hybridised with random-primed CD2. However, three major bands 1.6-2.0kb, 1.7-1.9kb and 1.4-1.7kb in size were seen. These corresponded to the native CD2, the CD2-fusion and the CD2-stuffer mRNAs respectively. The bands were broad, probably because of overloading in the wells, but the length of the native CD2 mRNA agreed with the 1.7kb CD2 mRNA identified by Sayre and coworkers (1987).

In each instance, a second less intense band was visible below the first (ranging from 1.3-1.4kb in length) and can be accounted for in two ways. Firstly, examination of the CD2 cDNA sequence reveals the presence of an alternative 3'end polyadenylation signal AATAAA at position 1085 in the cDNA. This would produce a second 1.3kb native CD2 mRNA.

Figure:4.5



The total RNA was extracted from transfected COS cells (10ug DNA/transfection), the RNA was divided into 1/10 and 9/10. The 1/10 aliquot was probed with random primed 18S RNA, and the remainder probed with random primed CD2 DNA (Lanes 2-6 and 7-11 respectively). The northern blot was hybridised at 42C, in 50% formamide and washed to a maximum stringency of 1xSSPE at 42C. Autoradiographs exposed for 20h and 2h for RNA probed with CD2 and 18S RNA respectively

Lane 1, Denatured λ HindIII and EcoRI mwt markers
Lane 2, 9/10 RNA from mock transfected cells
Lane 3, 9/10 RNA from cells transfected with CDM8
Lane 4, " " " " " " pCD2
Lane 5, " " " " " " pCDFus-8
Lane 6, " " " " " " pCDStuff-8
Lane 7, 1/10 RNA from mock transfected cells
Lane 8, 1/10 RNA from cells transfected with CDM8
Lane 9, " " " " " " pCD2
Lane 10, " " " " " " pCDFus-8
Lane 11, " " " " " " pCDStuff-8

Sayre and co-workers (1987) have reported the isolation of two such CD2 mRNAs (1.3kb and 1.7kb) from resting T-lymphocytes. However, as this does not apply to the CD2-fusion and CD2-stuffer mRNAs, therefore, it is more likely that these secondary bands result from RNA degradation.

All three CD2 recombinant mRNAs could be detected in approximately equal amounts, implying that the steady state level of the CD2-stuffer fusion mRNA was on par with that of native CD2 and the CD2-fusion mRNAs. This eliminated the possibility that the truncated CD2 protein was not translated and supported the immunochemical evidence for its secretion. It seemed reasonable, therefore, to attempt to immunoprecipitate this protein from the culture medium, provided that its turnover rate was not abnormally high.

4.6 Immunoprecipitation of the CD2 recombinant proteins

In order to verify the hypothesis that the pCDStuff-8 derived, truncated form of the CD2 protein would be secreted, immunoprecipitations of culture media from [^{35}S]Met labelled transfected cells were carried out. The basic methodology for transfection, labelling with [^{35}S]Met and immunoprecipitation are outlined in chapter 2. The more specific details of the individual experiments are described below.

The preliminary transfections were set up with both pCD2 and pCDStuff-8 to immunoprecipitate both the membrane bound and putative secreted form of the CD2 protein. Approximately $2x10^6$ cells were transfected with 10ug of DNA. The cells were labelled for 8h with 125uCi [^{35}S]Met in 5ml of methionine minus media (5ml DMEM/culture flask, 75cm^2 base area) containing 10% dialysed FCS. The immunoprecipitations were carried out with 1.5ml of culture media or 200ul of cell lysate prepared from $5x10^6$ transfected COS cells. A 1/10 dilution of anti-T11, and 1/50 dilution of W6/32 were

used to precipitate CD2 and monkey class I-like antigens respectively. The negative controls included immunoprecipitations of transfected COS cell lysates and the respective culture media in the absence of a primary antibody. The samples were incubated for 1h at room temperature, with the aim of maximising antigen binding while keeping the level of non-specific binding to a minimum.

Prolonged exposure of the SDS-PAGE gel was required (two weeks at -70C with an intensifying screen) before any radiolabelled bands were apparent in the immunoprecipitate tracks (Fig:4.6). These corresponded to lanes 11, and 15, of the control W6/32 immunoprecipitations. They generated two bands; one at 45kDa, corresponding to HLA-I, and the other at 12kDa (β2-microglobulin molecule normally found associated with class I molecules). Despite the fact this protein complex is constitutively expressed on the surface of all COS cells, the bands obtained were still very faint. Extrapolating from this result, one may conclude that at this detection limit, a transfected protein of significantly lower abundance is unlikely to be detected.

Inefficient incorporation of radio-label into the proteins may account for the poor signal strength obtained. Therefore, the experiment was repeated using twice the amount of label, 250uCi, and increasing the incubation time from 8h to 12h. As a result, the amount of media covering the cells, during labelling, had to be increased (from 5ml to 9ml/culture flask 75cm^2 base area) to prevent the cells from drying out during the extended incubation. Although an increase in labelling efficiency was obtained (labelled protein bands of lysate and culture media could be visualised after 12h exposure of an autoradiograph, at -70C), it was accompanied by a substantial rise in background. In a bid to reduce background, the protein A complex was blocked with cold lysate prepared from untransfected COS cells. This

Figure:4.6

Lane 1, Control cells protein A sepharose, NRS and RAM
Lane 2, Cells RAM supernatant from protein A sepharose
Lane 3, Media, RAM and protein A sepharose
Lane 4, Supernatant from media, RAM and protein A sepharose
Lane 5, Cell lysate, cells transfected with pCDStuff-8
Lane 6, " " " " " pCD2
Lane 7, High molecular weight protein markers
Lane 8, Media from cells transfected with pCDStuff-8
Lane 9, " " " " " pCD2
Lane 10, IP of (pCDStuff-8) cells with anti-T11
Lane 11, " " " " " W6/32
Lane 12, " " " media " anti-T11
Lane 13, High molecular weight protein markers
Lane 14, IP of (pCD2) cells with anti-T11
Lane 15, " " " " " W6/32
Lane 16, " " " media " anti-T11
Lane 17, " " " " " W6/32
Lane 18, IP of (pCDStuff-8) media with W6/32

COS cells transfected with 10ug pCD2 or pCDStuff-8. 48h folowing transfection the cells were labelled with 125uCi α-^{35}Met for 8h, an aliquot of the media and the cell lysate were prepared for immunoprecipitation (IP). Normal rabbit serum (NRS) and rabbit anti mouse (RAM) were preincubated with the protein A sepharose to reduce the non-specific binding. Both the lysate and the media were precleared and incubated with 1/10 dilution of anti-T11 for IP of the CD2 protein and 1/50 dilution W6/32 for control IP of monkey MHC. The products of IP were run on a discontinous SDS-page gel 12.5%, the gel was Enhanced and the dried gel autoradiographed at -70C for 2 weeks with an intensifying screen

did not, however, eliminate the background sufficiently to permit identification of a band corresponding to the CD2 protein.

As, in the initial experiments, radio-labelled protein was also immunoprecipitated from the culture media, efforts were made to concentrate the media and thereby increase the signal strength. However, these efforts were limited by the the amount of serum present in the culture media (10%). To overcome this, transfected cells were labelled in the absence of serum enabling a five fold concentration of the media to be achieved. Unfortunately, this strategy failed to immunoprecipitate any specific proteins from the culture media.

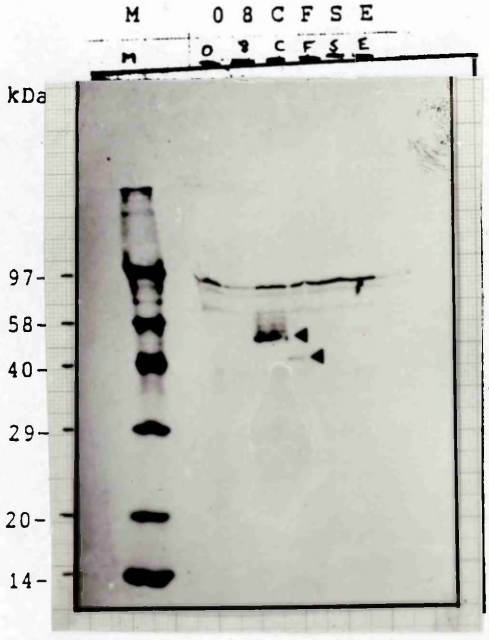
4.7 Immuno-blotting of the CD2 recombinant proteins

Immunoblotting, another versatile immunological technique, was applied towards visualising these recombinant proteins. Lysates, prepared from transfected cells, were run on 12% SDS-PAGE gels and electroblotted. The blot was hybridised for 1h with 1/10 dilution of RFT11 at room temperature and developed with 4-Chloronaphthol (Fig:4.7a). The cells transfected with pCD2 (lane 5) produced multiple bands ranging from 48kDa to 58kDa. This correlated well with the sizes quoted for CD2 in the literature (50, 53, and 55kDa, Sayre *et al*;1987). The protein possesses three putative N-terminal Asn-linked glycosylation sites. These may account for the various bands observed which correspond to differential glycosylation products. The pCDFus-8 derived fusion protein (lane 6) was present as a single 38kDa band. The predicted molecular weight from its amino acid sequence is 33kDa, which increases to 45kDa on glycosylation. The intermediate molecular weight obtained may imply that the protein is only partially glycosylated.

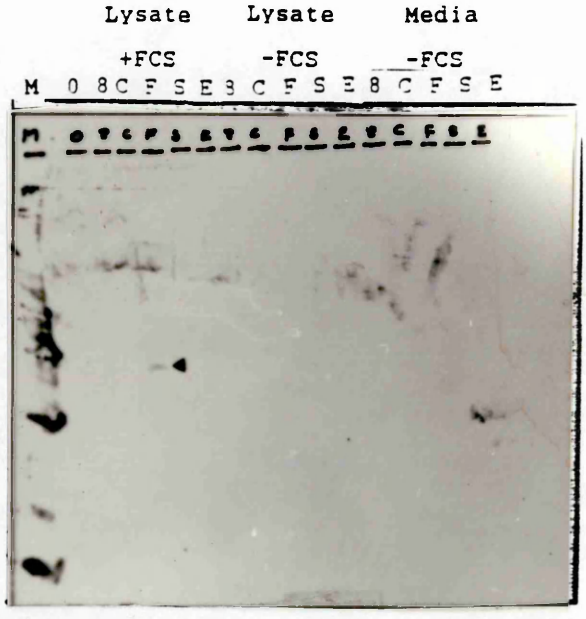
The experiment was repeated, but on this occasion the cells were divided into two aliquots 24h after transfection. One

Figure:4.7

a) 4-chloronapthol



b) ECL



0 = Mock transfected cells

8 = Cells transfected with 10ug CDM8

C = " " " 10ug pCD2

F = " " " 10ug pCDFus-8

S = " " " 10ug pCDStuff-8

E = " " " 10ug pCDEx-8

a) Cells transfected with 10ug of DNA, DEAE-dextran cells harvested 48h after transfection 25ul cell lysate and 25ul culture media run on 12.5% discontinous SDS-page gel and western blotted

Estimated molecular weights (log_{10} mwt vs distance migrated) CD2 = 48-58kDa CD2-fusion = 39kDa

b) A western blot probed with RFT11 mAb and developed using the ECL system.

Cell lysates were prepared from transfected COS cells which were divided into two (24h after transfection) and grown for 18h either in the presence or absence of serum. A negative result was obtained from both the lysate and the media from cells grown in the absence of serum. However, the CD2-fusion protein can be seen in the cells incubated with serum but the CD2 protein band was obscured by a bubble on the blot

aliquot was incubated in the absence of serum and the other in the presence of 10% serum. Samples of the lysate were taken for either a Hirt extraction, SDS-PAGE (12%) gel electrophoresis or immuno-blotting. Interestingly, in the absence of serum, no recombinant CD2 proteins were expressed (lanes 9-13) whereas, the original results were reproduced in the presence of serum (Fig:4.7a, Lanes 3-8). An air bubble on the blot, in the region of the native CD2 band (Lane 5), impeded protein transfer, allowing only the edge of the band to be visible when originally developed with 4-Chloronaphthol. This signal faded with time and efforts to redevelop the blot using ECL (enhanced chemiluminescence) failed to intensify this native CD2 protein band (Fig:4.7b)). It is adequate, however, to compare the expression of the CDFus-8 product, either in the presence or absence of serum (Lane 6 and Lane 11). It clearly illustrates that, in cells grown in serum deficient media, protein synthesis is significantly impaired.

The control mock transfected cells, and cells transfected with CDM8 gave the expected negative results. More importantly, however, the cells transfected with pCDStuff-8 also proved negative. In the light of Hirt extractions which confirmed that the transfections were successful, this data convincingly reinforced the immuno-staining results. It may be concluded, therefore, that the truncated CD2 protein does not accumulate within the cells to any appreciable extent.

In support of the idea that the external domain of the CD2 protein is secreted, Richardson and co-workers (1988) have used a high efficiency Baculo virus expression system to produce a membrane anchor minus form of the CD2 protein. The bacullo virus vector was used to infect *Spodoptera frugiperda* (SF9) cells, and metabolic radio-labelling studies carried out with [^{35}S]Met. The truncated CD2 protein was isolated from the media of litre cultures of SF9 cells (10^9 cells) by immuno-affinity chromatography. An affigel-10 column coupled to the anti-T11 mAb (3T48B5) was

used, and the protein visualised by SDS-PAGE. The purified truncated CD2 protein was estimated to be 28kDa in size, and its identity confirmed by N-terminal sequencing.

Their findings, together with the immuno staining and northern blot analysis data presented previously, provide overwhelming evidence to support the conclusion that the pCDStuff-8 product was secreted.

4.8 Discussion

In summing up the results obtained, both the pCD2 and pCDFus-8 products were found expressed on the cell surface of transfected COS cells. However, although the epitope for the anti-T11 mAb is present in the pCDFus-8 product, evidence from the immuno-blotting experiments suggest that this pCD2-fusion protein is not glycosylated as efficiently as the native molecule. This may be due to an alteration in the pattern of disulphide bonding. The CD2 external domain possesses four Cystine residues and thus forms 2 disulphide bonds. In the CD2-fusion protein (pCDFus-8) the fourth Cystine residue in the external domain is deleted, and the next Cystine residue occurs 15 amino acids further down stream in the stalk region of the HLA-B7 Tm sequence. This possibly renders the last CD2 Cystine residue either inaccessible or results in the formation of a new disulphide bridge, both of which result in an altered conformation of the protein which consequently modifies its glycosylation pattern (Fig:4.7).

Several studies were carried out to validate the assumption that the CD2 protein deleted of its Tm sequence would be secreted. Hirt DNA extractions were performed to demonstrate successful transfection and both immunoblotting and immunostaining methods verified that the protein was neither expressed at the cell surface nor accumulated within the cell. Efforts to isolate the protein from the culture medium, by immunoprecipitation, were either plagued by very

high backgrounds or affected by the detrimental effect on protein synthesis caused by the lack of FCS in the culture media. However, this data in conjunction with the work carried out by ~~Richard~~ Sayre and co-workers (1988), who isolated another form of CD2 from culture media, provides overwhelming evidence for the secretion of the pCDStuff-8 (CD2 minus Tm) protein product.

Although these results clearly illustrate the plausibility of this cloning strategy for isolating novel membrane proteins, two further components of this screening system require thorough study. The efficiency of the panning techniques as an effective library screening method, and the potential for random DNA sequences to generate functional membrane anchoring segments.

CHAPTER 5

OPTIMISATION AND PRELIMINARY SCREEN OF THE NOVEL-MEMBRANE PROTEIN CLONING STRATEGY

5.1 Introduction

Expression of the engineered CD2 protein product of pCDFus-8, and secretion of the external domain of the CD2 protein are good indications that potenially this cloning strategy devised is feasible. However, before a cDNA lbrary can be sub-cloned into the cloning vector pCDStuff-8, to carry out the preliminary screen for novel membrane proteins two additional factors have to be considered;

a) What is the sensitivity of the immunological screening system used.

b) What is the background number of positive clones that might arise from random cDNA sequences generating functional membrane anchors.

5.2 Assessment of the efficiency of panning

The efficiency of panning, (the immunological detection system employed in library screening) can be estimated by using either the pCD2 or pCDFus-8 plasmids in a series of transfection experiments. The proportion of positive cells bound to a panning plate can be calculated once the transfection efficiency has been determined. This can be achieved by counting the number of positively expressing cells bound to the panning plates, and expressing this as a fraction of the total. The transfection efficiency can then be determined (as per Chapter 4) either by calculating the

percentage of positive cells obtained in immuno-staining experiments, or by counting the number of colonies resulting from the transformation of an aliquot of Hirt DNA extracted from the transfected cells.

Transfection of the plasmids pCD2, pCDFus-8, and CDM8 (negative control) were carried out, and 48h later the cells were harvested. A 1/20 of each cell suspension was removed for cytospinning and immuno-staining and the concentration of the remainder was determined using a hemocytometer (Table:5.1). The cell suspensions from pCD2 and pCDFus-8 were divided into five aliquots, and incubated with different dilutions of the anti-CD2 mAb (anti-T11) or W6/32 (1/100, 1/50, 1/10, 1/5 dilution of anti-T11, 1/50 W6/32). For cells transfected with CDM8, only incubations with W6/32 (1/50 dilution) and 1/10 dilution of anti-T11 were carried out. The cells were panned and the proportion of positive cells bound calculated. This was carried out by initially determining the area of the microscope's field of view as a fraction of the total area of the plate (Table:5.1). Then, the number of cells bound in five random fields of veiw (n=5) was counted, the mean value was corrected for total plate area, and as a percentage of the total number of cells panned (Table:5.2). The DNA from the bound cells was purified by Hirt extraction, a 1/50 electroporated into *E. coli* (MC1061/P3), and the number of colonies resulting from the transformed DNA calculated (Table:5.3). The transfection efficiency values were then compared to those obtained from the immuno-staining procedure (Table:5.4).

Results

Approximately 5 cells transfected with CDM8 bound per plate ($1x10^{-3}$%) when panned with anti-T11 (negative control), whereas 80% of the cells bound when panned with W6/32 (positive control). This correlated well with the immuno-staining results, where all the cells transfected with CDM8

stained negative with anti-T11, and positive with W6/32. The number of colonies obtained by transforming the Hirt DNA (1/50), extracted from the cells panned with W6/32, were pCD2 ($7.1x10^5$) and pCDFUs-8 ($0.8x10^5$). These values were 10 fold higher than any of the anti-T11 panning figures indicating that the panning plates were not entirely saturated with the W6/32 mAb.

Table:5.1

Total volume 5ml	pCD2	$7.6x10^5$ cells/ml
	pCDFus-8	$5.8x10^5$ cells/ml
	CDM8	$5.5x10^5$ cells/ml

Field of view under the microscope $2.52mm^2$
Area of panning plate with radius 45mm $6362mm^2$
Field of view is approximately 1/2500 of total plate

Table:5.2

Dilution of mAb anti-T11	pCD2 cells panned $7.6x10^5$		pCDFus-8 cells panned $5.8x10^5$	
	cells bound	% bound	cells bound	% bound
1/100	$1.26x10^4$	1.66	$5.05x10^3$	0.87
1/50	$7.32x10^4$	9.63	$2.02x10^4$	3.48
1/10	$1.54x10^5$	20.26	$6.06x10^4$	10.44
1/5	$1.36x10^5$	17.94	$6.82x10^4$	11.75

Table:5.3

Dilution of mAb	pCD2 Transformants (1/50) Hirt DNA	pCDFus-8 Transformants (1/50) Hirt DNA
1/100 Anti-T11	$6.7x10^4$	$1.5x10^3$
1/50 "	$8.8x10^4$	$3.0x10^3$
1/10 "	$1.1x10^5$	$1.0x10^4$
1/5 "	$9.4x10^5$	$1.5x10^4$
1/50 W6/32	$7.6x10^5$	$5.8x10^5$

Table:5.4

Dilution of mAb	pCD2 Immuno-staining % positive cells	pCDFus-8 Immuno-staining % positive cells
1/100 Anti-T11	0.98	1.88
1/50 "	2.39	3.78
1/10 "	15.49	12.62
1/5 "	13.24	18.88

Tables:5.2-5.4 illustrate that the optimum concentration of the anti-T11 mAb for panning is a dilution of less than a 1/10. More importantly, these figures demonstrate that although an estimate for transfection efficiency can be obtained, the techniques used are not precise enough to obtain an absolute value. Perhaps, an accurate method might be to use a FACS cell sorter to count the number of cells in suspension, before and after panning. The difference in cell number would account for the number of cells bound.

5.3 Estimation of the background number of clones arising from random DNA sequences generating membrane anchoring segments

The concept of experimentally determining the proportion of random stretches of DNA coding for membrane anchoring

sequences, was based on the work carried out by Kaiser and co-workers (1987). They were studying protein secretion, and more specifically, the role of the signal sequence. Their experiments involved replacing the normal signal sequence of the *Saccharomyces cerevisiae* invertase gene with essentially random peptide sequences, to functionally assess the specificity with which signal sequences are recognised. Approximately 20% of these sequences were able to function as export signals, implying that the specificity for recognition of a signal sequence is very low.

It has been shown that the basic common structural feature of over 200 signal sequences is a consecutive stretch of 7 hydrophobic amino acids (von Heijne;1985). Although, less rigid then the requirements specifying a membrane anchoring domain, the signal sequence shares structural similarity with it. Consequently, the potential for random cDNAs to generate functional membrane anchoring segments may be comparable. A background of such a magnitude, would virtually eliminate the prospect of using the library strategy as a viable scheme for isolating novel membrane proteins.

In order to quantitate the background arising from random cDNA fusions with the CD2-external domain, genomic DNA from yeast *Saccharomyces cerevisiae Drosophila melanogaster* (Canton S), Hela and V79 (Chinese hamster lung) cultured cells (kindly donated by J.Moore, A.Griffin and D.Donald) were digested with EcoRI and subcloned into EcoRI digested and phosphatased pCDStuff-8. 200 of the recombinants derived from sub-cloning fragments of Hela genomic DNA were grown up as separate 5ml cultures (to ensure that each clone was equally represented) and pooled into groups of fifty for maxi-plasmid preparations. The remainder of the Hela, and all the *D. melanogaster*, V79 and *S. cerevisiae* transformants were then grown up for maxi-plasmid preparations. Transfections using 10ug of each of the "plasmid-genomic fusion libraries" were carried out. The cells were panned

Figure:5.1

a) 0.3% agarose

Lane 1, uncut yeast genomic DNA
Lane 2, " Fly (Canton S) genomic DNA
Lane 3, " HELA genomic DNA
Lane 4, " V79 genomic DNA
Lane 5, " wild type λ DNA (50kb)

b) 0.8% agarose

Lane 1, Yeast genomic DNA digested with EcoRI
Lane 2, Fly " " " " "
Lane 3, HELA " " " " "
Lane 4, V79 " " " " "
Lane 5, λ HindIII molecular weight markers

a) Genomic DNA from various species, average size with minimal sheering should excede 50kb. The yeast preparation Lane 1, poor quality

b) Genomic DNA preparations digested with EcoRI. Lane 1, yeast DNA apppears with a large number of distinctive bands which result from the fact that the yeast genome is composed of highly repetitive DNA of ribosomal repeats and is thus characteristic of yeast DNA. The HELA DNA Lane 3, did not cut but a second preparation was digested successfully (not shown)

and immuno-stained with the mAb RFT11 (anti CD2 mAb). These experiments were repeated 3 times, in each case including pCDStuff-8 and pCD2 as negative and the positive controls respectively.

Results

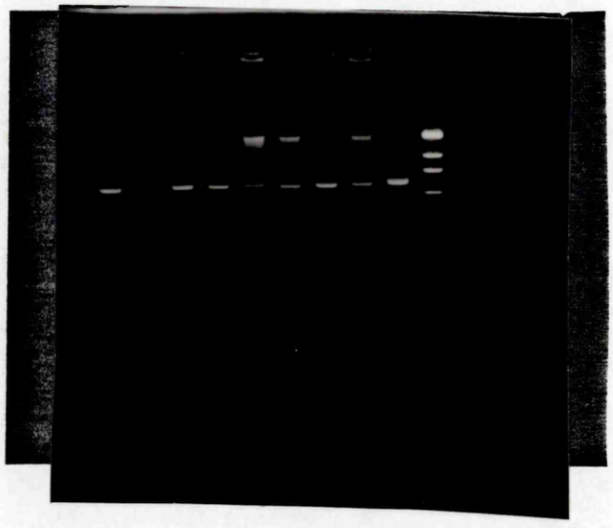
To assess genomic DNA integrty, aliquots were run on a 0.3% agarose gel. Uncut lambda DNA was included as a marker, to determine the extent of shearing that may have occurred during DNA purification (Fig:5.1a). Of the four genomic DNA preparations analysed, slight shearing was evident in only the yeast DNA (Lane 1). The DNA was then digested with EcoRI and an aliquot of each run on a 0.8% agarose gel (Fig:5.1b). The banding pattern seen in Lane 1 is characteristic of yeast DNA, and is caused by the high number of ribosomal repeats. The sample of Hela DNA (Lane 3) did not initially cut with EcoRI, but when repeated, a smear of fragments as per Lane 4, the V79 DNA was seen.

Aliquots of the ligation mixes (100ng) were run on an agarose gel (Fig:5.2). The controls included samples of self ligated PvuII digested pCD2 (Lane 2) and EcoRI digested and phosphatased pCDStuff-8 (Lane 3). The ligated material was electroporated into the *E. coli* strain MC1061/P3 and the number of transformants obtained are listed in Table:5.5.

Table:5.5

10ng pCD2 transformation efficiency		3.0×10^7
No. transformants	Yeast	1.4×10^4
	Fly	1.4×10^5
	Hela	2.2×10^5
	V79	1.5×10^5

Figure:5.2



Lane 1, pCDStuff-8 digested with XbaI

Lane 2, control ligation pCD2 digested with PvuII and ligated (110ng)

Lane 3, pCDStuff-8 digested with EcoRI phosphatased (cip) and ligated

Lane 4, PCDStuff-8 cut cip and ligated to Yeast genomic DNA

Lane 5, " " " " " " Fly " "

Lane 6, " " " " " " V79 " "

Lane 7, " " " " " " HELA 1 " "

Lane 8, " " " " " " HELA 2 " "

Lane 9, pCDM7 placental library uncut

Lane 10, λ HindIII mwt markers

The yeast genomic DNA ligations were very inefficient Lane 4. The first preparation of HELA genomic DNA when digested with EcoRI and ligated into pCDStuff-8 was also unsuccessful Lane 8

Boiling preparations (12 of each):

% with inserts		
	Yeast	8%
	Fly	60%
	Hela	86% *
	V79	75%

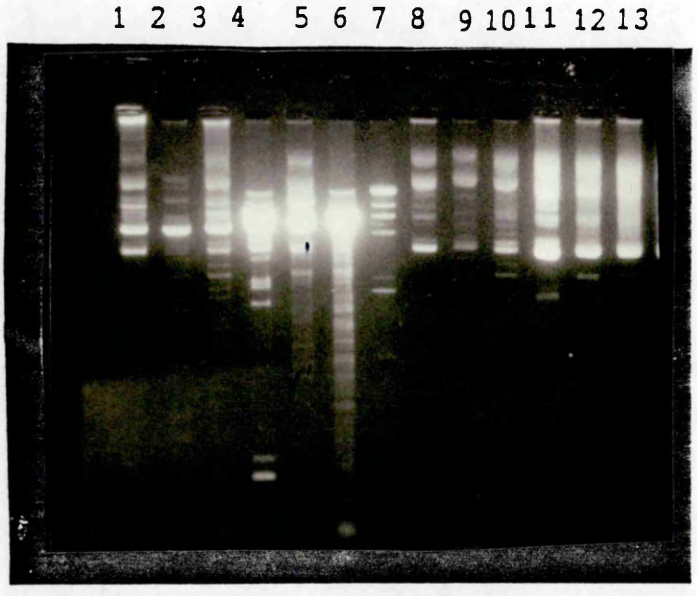
* = recombinants derived from Hela genomic DNA; 13.6% had no inserts, 18.2% had more than one insert with a size range of 0.9-9.0kb.

The 200 individual recombinants were selected from the Hela genomic fusion library because it had the highest number of transformants. On the other hand, the yeast genomic fusion library was considerably smaller. Only 8% of the yeast recombinants contained inserts discernable on the agarose gel, and the library was therefore discarded. Fig:5.3 shows the plasmid maxi-preparations of the various genomic fusion libraries, both uncut and digested with EcoRI. The four pools of 50 Hela recombinants were designated H1-4 respectively. The fourth, H4 (Lane 4) produced an unusual pattern of inserts and was not included in the transfections.

The transfections were carried out with 10ug DNA and the cells were harvested for analysis 48h later. It was observed from immuno-staining and panning experiments using, the anti CD2 mAb, RFT11 that cells transfected with pCDStuff-8 (negative control) stained negative and did not pan while those transfected with pCD2 (positive control) stained positive and transfected with an efficiency of 10.4%, as estimated from the number of cells panned (of the $1.2x10^6$ cells transfected and panned, a mean number of 50 cells bound to the panning plate/field of view (n=10)). Yet, none of the cells transfected with H1, H2 or H3 yielded positive results when panned or stained with the RFT11 mAb.

The negative staining obtained with RFT11 implies that none of the recombinant plasmids code for membrane anchoring

Figure:5.3



* Lane 1, H1 Preparations of the pools of 50 colonies
  Lane 2, H2 each grown up individually. Each pool was
* Lane 3, H3 digested with EcoRI to visualise the range
£ Lane 4, H4 of insert sizes
* Lane 5, 1ug of Fly genomic fusion library EcoRI digest
  Lane 6, " " V79 " " " " "
  Lane 7, 1 HindIII mwt markers
  Lane 8, H1 uncut
  Lane 9, H2 "
  Lane 10, H3 "
  Lane 11, H4 "
  Lane 12, Fly genomic fusion library uncut
  Lane 13, V79 " " " "

* = only partially digested
£ = Yeat genomic fusion library inserts with a strange banding pattern

The plasmid preparations of the genomic fusion libraries uncut and digested with EcoRI

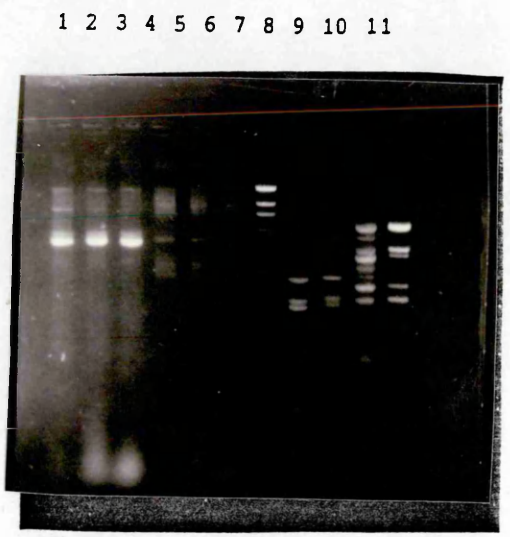
sequences or soluble fusion proteins which accumulate within the cell. Considering that $1.0x10^6$ cells were panned and that a transfection efficiency of 10% was obtained, approximately $1x10^5$ cells would contain transfected DNA. Consequently, even if only one fusion construct coded for a hydrophobic membrane anchoring sequence, the transfection would generate 2000 positive cells. Therefore, as the field of view of the microscope corresponds to approximately 1/2500 of the area of a panning plate, 80% of the areas scanned would contain a bound positive cell. A total of 150 recombinants were similarly screened and yielded negative results. Making allowances for the fact that only 86% of the recombinants contain inserts (i.e. effectively 130 recombinant clones screened), these negative results would still estimate the background at significantly less than 1%.

Negative results were also obtained when the genomic fusion libararies of fly, and V79 were screened. Perhaps, the requirements specifying a membrane anchoring sequence are more rigid than first envisaged and therefore, the background arising from random stretches of DNA coding for membrane anchoring sequences is actually very low, less than 0.0005% (background 5 cells bound/panning plate, $1x10^6$ cells panned with a transfection efficiency of 10%).

Another consideration in evaluating the background observed may be the size of the EcoRI fragments sub-cloned into pCDStuff-8. Large fragments when sub-cloned, may increase plasmid replication times and also decrease their stability. Therefore, in a mixed population, the smaller plasmids may compete out the larger ones and effectively exclude them from the experiment. Likewise, the synthesis of CD2 fusion-proteins with abnormally folded cytoplasmic domains may cause malfolding of the polypeptide chain; either increasing its rate of turnover directly or as a result of decreasing solubility, to the point of aggregation and thus, cause an increased breakdown of transfected fusion proteins. It is also possible that the low background observed is a

Figure:5.4

pCDM7 placental library digested with XbaI arrows indicate the smear excised, electro-eluted. Ends filled in with klenow, phenol extracted and precipitated.



Aliquots of ligation mix 1/20 (100ng)
Lane 1-3, CDM7 placental library XbaI smear 8, 10 and 12bp linkers
Lane 4-6, CDM7 placental library XhoI smear 8, 10 and 12bp linkers
Lane 7, λ HindIII markers
Lane 8, pCD2 digested PvuII
Lane 9, pCD2 digested PvuII and EcoRI linkers 8bps
Lanes 10, Control DNA EcoRI digested followed by self ligation
Lane 11, Control DNA digested with EcoRI

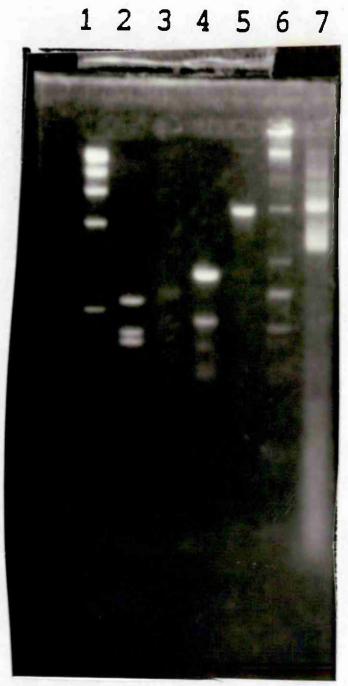
consequence of the CD2-fusion library coding for predominately secreted proteins. This, however, could not be confirmed due to the inability to sucessfully immunoprecipitating secreted CD2.

5.4 Subcloning cDNAs into pCDStuff-8

The background arising from random stretches of DNA coding for membrane anchoring sequences is less than 1%. Provided that this estimate is representative, the cloning strategy devised for screening novel membrane anchoring sequences would be entirely feasible. The sub-cloning of full-length cDNAs into the cloning vector pCDStuff-8 may generate fusions composed of two tandem external domains. Malfolding of the protein may result and thus prevent cell surface expression of the CD2-fusion proteins. Therefore, instead of constructing a cDNA library, a full length, size selected (inserts above 1kb in length) placental plasmid library (CDM7, donated by D.Simmons) was digested to liberate cDNA fragments ranging from 100-500bp in size and these were to be ligated into the pCDStuff-8 vector.

The main obstacle associated with this approach was the fact that the restriction sites which liberate the cDNAs from the CDM8 vector also released the CD2-stuffer segment in pCDStuff-8. The strategy employed, therefore, was to liberate the inserts from the CDM7 library using XbaI or XhoI, purify the smear by electro-elution (fragment sizes selected 0.5-2.5kb in length), fill-in the overhangs using Klenow and ligate EcoRI linkers (either 8, 10 or 12bp double stranded EcoRI, oligonucleotide linkers) onto the blunted cDNA fragments. Once digested with EcoRI the fragments were ligated into EcoRI digested and phosphatased pCDStuff-8, and the ligaced material electroporated into *E. coli* (MC1061/P3). The recombinants were grown up and the maxi-

Figure:5.5



Lane 1, 1 HindIII mwt markers
Lane 2, pCD2 digested with PvuII
Lane 3, pCD2 digested with PvuII and ligated
Lane 4, Control DNA digested with EcoRI
Lane 5, pCDStuff-8 digested with EcoRI and phosphatased
Lane 6, Cut control DNA ligated into phosphatased pCDStuff-8
Lane 7, CDM7 library ligated to phosphatased pCDStuff-8

Transformation efficiency $1x10^5$, grew up 10^4

plasmid preparation DNA isolated was transfected into COS cells. Panning and immuno-staining were carried out using RFT11 to identify positive clones.

Results

Fig:5.4a shows the DNA smear obtained when the placental library was digested with XhoI. The electroeluted smear DNA was divided into three aliquots, and 8, 10 and 12bp EcoRI linkers respectively, ligated to the fragments. The linkers were digested with EcoRI and following precipitation, the resuspended DNA was ligated into EcoRI digested and phosphatased pCDStuff-8 (Fig:5.4b smear of inserts derived from a XbaI (Lanes 1-3) and XhoI (Lanes 4-6) digested placental library. Both were blunt end EcoRI linker ligated to 8, 10 and 12bp linkers. Once cut, the EcoRI linkered fragments were ligated into pCDStuff-8). The experimental controls included;

(i) Trace labelling (^{32}P[α-dATP] 10uCi) of an aliquot of the electro-eluted smear to confirm Klenow activity in the fill-in reaction.

(ii) EcoRI linkers ligated to PvuII digested pCD2, EcoRI restricted and ligated (Fig:5.4b Lanes 8 and 9), and control DNA digested with EcoRI and then ligated (Fig:5.4b lanes 10 and 11): To determine ligation efficiency.

(iii) Control DNA and the CDM7 library, digested with EcoRI and ligated into phosphatased pCDStuff-8 (Fig:5.5 Lanes 5, 6 and 7), to ensure that the phosphatased EcoRI overhangs of pCDStuff-8 were not damaged.

Transformants ($3x10^4$) derived from the sub-cloning of the XbaI and XhoI fragments of the CDM7 libray were grown for maxi-plasmid preparation. Transfections using 10ug of the fusion library DNA, were carried out. Cells ($1.72x10^6$) were

panned using RFT11 and another aliquot used for immuno-staining. However, both analyses yielded negative results, despite the transfection efficiency, as estimated by panning, for the control plasmid (pCD2) being 8.4% (mean number of cells bound to panning plate/field of view =56 sample number n=10).

5.5 Discusion

Several factors may be responsible for a positive CD2 expressing cell not being detected. Each step involved in the construction of the library was prone to losses of DNA. Although, controls were included at each stage, in many instances their usefulness was limited. Controls, despite providing information as to whether a particular reaction is working, are inadequate indicators of whether the reaction has gone to completion. An example, would be the filling-in of the electro-eluted DNA using Klenow, ^{32}P[-dATP] was added to an aliquot of this reaction so that DNA incorporation of the label would correlate with the activity of the enzyme. But, whether, all the DNA ends had been completely filled-in could not be readily assessed, and incomplete filling-in, would obviously result in only partial linker ligation. Electrophoretic analysis showed that ligation had occurred, but not to completion, as uncut DNA was still observed in the control tracks (Fig:5.4b Lanes 10 and 11). Moreover, it was also possible that inefficient digestion and removal of the excess linkers may have contributed to the result.

The cumulative effect of these factors, in all probability accounts for the small number ($6x10^4$) of recombinants obtained, (total derived from separate linker ligations with DNA from XbaI and XhoI digestions of the CDM7 library). Only 46% of the recombinants contained inserts, possibly due to

the presence of residual EcoRI linkers ligating into pCDStuff-8. Effectively, therefore, only $2.76x10^4$ recombinants were screened in the panning experiments.

As this cloning strategy devised is capable of isolating any membrane anchoring sequence in its correct frame and orientation, a single positive CD2-fusion protein expressed at the cell surface, would adequately demonstrate its potential as a screening system. Consequently, it was decided that screening a fusion-library consisting only of $3x10^4$ recombinants would still be sufficient to isolate a few positive clones. In the light of the number of potential sources of DNA loss, it is evident that this was somewhat of an optimistic view.

In retrospect, the decission to transfer the cDNAs from the CDM7 library may be regarded as a poor choice. A more effective approach possibly, which circumvented the problem of common restriction sites in both vectors, would have been to sub-clone the cDNA from a lambda library. The cDNAs could have been excised with EcoRI, eliminating the need for either the fill-in step with Klenow or linker ligation.

GENERAL DISCUSSION

To gain a better understanding of mechanisms involved in the maternal acceptance of the fetus, many researchers have sought to identify and characterise some of the placental specific antigens thought to be involved in this process. However, this has proven to be a difficult task especially in instances where no protein specific antibodies are available. This project evolved in an attempt to overcome these problems by devising a cloning strategy which would facilitate the isolation of cDNAs coding for such novel membrane proteins. The cell surface antigen screening system developed by Seed and Aruffo (1987) was used as a basis for the strategy developed; the premise being that cell surface expression of a reporter molecule (the external domain of CD2) would occur as a result of an in-frame fusion to a cDNA sequence coding for a membrane anchoring domain. Theoretically, therefore, this cloning strategy would form a universal screen for membrane anchoring sequences, and consequently, for novel membrane proteins.

The constructs pCD2, pCDFus-8, and pCDStuff-8 were transfected into COS cells to determine whether the external domain of the recombinant CD2 proteins would be anchored at the cell surface. The immuno-staining results indicated that both the pCD2 and pCDFus-8 products were expressed on the cell surface of transfected COS cells. Alterations in conformation of the pCDFus-8 external domain are likely to be minimal, especially as the anti-T11 mAb raised against native CD2 was still able to recognise this CD2-fusion protein. The expression of CD2 and the CD2-fusion protein was equivalent at both the mRNA and protein levels. However, the fusion protein was not glycosylated to the same extent as the native CD2 protein, nor was there the repertoire of glycosylated forms observed with native CD2. This may have

resulted from altered disulphide bonding in the fusion protein, either reducing the efficiency of glycosylation or favouring one pattern of glycosylation over the others.

To demonstrate that the Tm-less CD2 protein was incapable of anchoring to the membrane was more complicated. Cells transfected with pCDStuff-8 stained negative with both the anti-T11 and RFT11 mAbs (anti-CD2 mAbs against native and denatured CD2 respectively), illustrating that the protein was neither expressed at the cell surface, nor produced and accumulated intracellularly. In order to rule out experimental error resulting from either a failed transfection or inadequate transcription, a Hirt extraction and Northern analysis was carried out. A CD2 mRNA transcript of the expected size was observed, implying that the truncated CD2 protein was either degraded rapidly within the cell or secreted into the culture medium. Unfortunately, attempts to immunoprecipitate this protein from the culture medium were not successful and failed to resolve the fate of this protein. However, in the light of the evidence for secretion of another Tm-less CD2 protein provided by Richardson and coworkers (1988), it is likely that this CD2 protein lacking a Tm sequence is also secreted.

The experimental scheme devised to calculate the number of spurious anchoring sequences in random stretches of DNA was carried out by screening three CD2-genomic fusion libraries (*D. melanogaster*; HeLa; and chinese hamster lung: V79). The estimated background derived from staining and panning experiments with transfected cells was less than 1%. The problem to be addressed, however, is how accurately do these experimental figures reflect the total number of potential random membrane anchoring sequences present?

In order to assess this, it is useful to attempt to calculate a theoretical value based on both a combination of lengths of open reading frame (ORFs) that would be expected to be found within random stretches of DNA, and the

probability of these containing a run of amino acids of sufficient length and hydrophobicity to anchor the CD2 external domain on the membrane.

In random DNA, assumed to have a 50% GC content, the distribution of open reading frames is expected to be a function of $(61/64)^n$, where n is the length of the ORF. Some values for the function are shown in Table 6.1. It can be seen that only in 6×10^{-6} segments of random DNA will there be a 250 codon stretch free of stop codons. At the simplest level, 64 codons encode 20 amino acids and three termination signals. The proportion of random stretches of DNA that encode an ORF of "n" or more amino acids without stop codons is therefore $(61/64)^n$ (Table:6.1). Thus, the frequency of synthesis of randomly encoded polypeptides greater than 140 amino acids in length is less than 0.1% of the total.

Table:6.1

n	$(61/64)^n$	$1-(61/64)^n$	n	$(61/64)^n$	$1-(61/64)^n$
1	0.953	0.047	40	0.147	0.853
5	0.787	0.213	50	0.091	0.909
10	0.619	0.381	60	0.056	0.944
15	0.487	0.513	70	0.035	0.965
17	0.442	0.558	80	0.021	0.979
20	0.383	0.617	90	0.013	0.987
25	0.301	0.699	100	8.22×10^{-3}	0.992
30	0.237	0.763	120	3.15×10^{-3}	0.997
			140	1.20×10^{-3}	0.999
			160	4.61×10^{-4}	0.9995
			200	6.76×10^{-5}	0.9999
			250	6.13×10^{-6}	0.99999
			300	5.56×10^{-7}	0.999999

n = length of amino acid sequence (ORF) encoded by a random stretch of DNA

$(61/64)^n$ = Frequency of generating a stretch at $\geq$ n amino acids

$1-(61/64)^n$ = Frequency of generating a stop codon at $\leq$ n amino acids (i.e. after 140 amino acids 99.9% of all ORS would have terminated)

It is then necessary to define the criteria for the occurrence of a membrane anchoring sequence both in terms of its length and composition. von Heijne (1981) concluded that amino acid sequences of Tm regions do not show homology except in gene families like HLA and andrenergic receptors. The most significant common feature of Tm segments is a high proportion of hydrophobic amino acids. In addition, certain charged residues are absent from Tm sequences. He calculated the hydrophobic character of each amino acid in terms of the energy associated with the transfer of residues from a helix in water, to a helix in a non-polar phase in the absence of hydrogen bonding capacity (-DG kJ/mole). The relationship between hydrophobicity and abundance can be seen when the frequency of each amino acid occurring in the Tm segments of single pass membrane proteins (von Heijne and Gavel;1988) are compared to their hyrophobicity (Table:6.2).

Table:6.2

Amino acid	-DG (kJ/mole)	Abundance (A)	Codons (C)
Phe (F)	-14.2	0.060	2
Met (M)	-11.3	0.018	1
Ile (I)	-10.5	0.139	3
Leu (L)	-10.1	0.283	6
Val (V)	-8.4	0.169	4
Trp (W)	-8.4	0.013	1
Cys (C)	-6.3	0.020	2
Ala (A)	-4.2	0.107	4
Gly (G)	0	0.085	4
Thr (T)	+3.8	0.042	4
Tyr (Y)	+4.6	0.013	2
Ser (S)	+6.3	0.036	6
Gln (Q)	+10.1	0	2
Asn (N)	+12.2	0.001	2
Pro (P)	+13.9	0.013	4
His (H)	+14.3	0.001	2
Lys (K)	+17.6	0	2
Glu (E)	+24.7	0	2
Asp (D)	+31.0	0	2
Arg (R)	+47.3	0	6
			61

Generally, hydrophobic residues occur at a higher frequency than polar ones in Tm segments, to the extent that certain amino acids, namely Gln, Lys, Glu, Asp, and Arg are never found, and His and Asn rarely occur (frequency 0.1%) in Tm sequences (Table:6.2). These "forbidden" amino acids account for 18 of the 64 codons. If it is now assumed that a Tm sequence spans the 3nm bilayer as an a-helix, approximately 17 amino acids would be required to cross the membrane. The frequency, therefore, of such a stretch of amino acids not containing any of these seven forbidden amino acids can be calculated as:

$$\left[\frac{(64-18)}{64}\right]^{17} = 3.6\times10^{-3}$$

The frequency of generating an ORF of 17 or more amino acids is 0.442 (44.2% of the total random peptides synthesised would be 17 or more amino acids in length, Table:6.1). Of this population, a frequency of 3.6×10^{-3} should be able to anchor the CD2 external domain on the membrane. Without accounting for the possibility that potentially any peptide greater than 17 amino acids in length could contain a hydrophobic region anywhere along its length, 0.16×10^{-3} (0.16%) of the recombinants from the genomic libraries should have anchored CD2 on the membrane ($0.442 \times 3.6\times10^{-3}$). However, it would seem to suggest that the minimum number of clones to be expected is well below the proportion Kaiser and coworkers (1987) obtained for the number of random DNA segments acting as signal sequences (20%). Fewer amino acids are necessary to form a signal sequence, but if this estimated value in any way approaches the actual value for the number of random anchoring sequences present the screening techniques used may not have been sensitive enough to detect them.

In terms of the number of recombinants screened, 9.0×10^{4} *Drosophila* (1.5×10^{5} transformants, 60% of which contained

inserts), and $1.1x10^5$ V79 recombinants ($1.5x10^5$ transformants, 75% of which contained inserts) from the genomic fusion libraries, as well as the $3x10^4$ recombinants from the transfer of cDNA fragments into pCDStuff-8; a total of $2.3x10^5$ potential fusion protein clones were obtained. This should have generated a mimimum of 368 clones (0.16% of $2.3x10^5$ clones) with the ability to anchor the CD2 external domain on the membrane. The significance of this calculated figure is difficult to determine especially in the light of the assumptions made. Presumably, the greater length required to specify a membrane anchoring domain and the strict requirements of hydrophobicity significantly decrease the probability of these occurring.

Apart from the possibility that the screening system was insensitive, the negative results obtained from the CD2-genomic fusion libraries may have been due to the production of malfolded CD2-fusion proteins, which are turned over at a high rate. Such an effect is suggested by the different glycosylation patterns observed between native and CD2-fusion proteins. In the latter the foreign transmembrane sequence may have slightly altered its conformation sufficiently to change its glycosylation, but not enough to increase its turnover rate. However, as both CD2 and HLA-B7 are related (they are both members of the IgG superfamily, Williams;1987) the fusion protein may not be recognised by the non-lysosomal degradation pathway, thus allowing its expression on the cell surface.

Alternatively, the majority of the fusion proteins synthesised may have been secreted. However, one must also contend with the fact that the low background obtained may be misleading, particularly if the size of the genomic EcoRI fragments sub-cloned into pCDStuff-8 were sufficiently large enough to decrease the stability of the plasmid (size range

of inserts 0.9-9.0kb) in COS cells. Also, the fact that large plasmids replicate at a slower rate than smaller plasmids may have precluded them from contributing to the background.

A CD2-cDNA fusion library, constructed by subcloning cDNA fragments from a placental CDM7 library, gave negative results when screened both by immuno-staining and panning. Factors which may have contributed to this result include the excessive number of manipulations involved in sub-cloning the cDNA into pCDStuff-8, and the limited number of recombinants screened. In retrospect the *de novo* synthesis of a cDNA library or isolating the cDNA from a lambda library would have overcome the necessity to Klenow fill-in the ends or add linkers to the fragments, possibly resulting in a more representative fusion library being screened.

Another consideration is that the ER is not a simple tube channeling proteins to their respective destinations. It contains a number of ER-resident proteins which aid in the folding, recognition and degradation of malfolded, incorrectly assembled proteins. An example of such a protein is the immunoglobulin heavy chain binding protein (BiP or GRP78), which associates tightly with newly synthesised proteins that are incorrectly glycosylated (Haas *et al*;1983, Bole *et al*;1986, Gething *et al*;1986). The pattern of expression for BiP suggests a very general role in protein assembly in the lumen of the ER. BiP is synthesised constitutively and accumulation of malfolded or mutant proteins in the lumen of the ER induces the synthesis of BiP in both yeast and animal cells (Kozutsumi *et al*;1988, Normington *et al*;1989, Rose *et al*;1989). BiP induction occurs regardless of whether the malfolding has resulted from errors in the primary amino acid sequence or as a consequence of incomplete glycosylation.

The majority of proteins which fail to fold correctly or assemble into complexes, are in most cases not released from

the ER (Lodish;1988, Hurtley and Helenius;1989, Rose and Doms;1988). They are retained and are eventually degraded, although the rate at which this occurs varies considerably. This is thought to be achieved by a non-lysosomal proteolytic degradation system, possibly in a pre-golgi compartment (Lippincott-Schwartz *et al*;1988). It would appear that this pathway is involved not only in the disposal of newly incorrectly folded or assembled proteins, but also in the regulation of metabolic pathways such as cholesterol biosynthesis (Klausner and Sitia;1990). Some of the membrane proteins degraded in the ER include the beta, delta, and gamma CD3 chains of the T-cell receptor complex (Bonifacino *et al*;1989, Chen *et al*;1988, Wileman *et al*; 1990), the asialoglycoprotein receptor (Amara *et al*;1989), IgG heavy chains and beta-hexoseaminidase (Lau and Neufeld;1989).

Experiments to determine the region of an ER protein which specifies that it should be rapidly degraded were carried out on the ζ-chain of the T-cell receptor (a protein with a short half life of 10-60min in the ER), where only its external domain, or its external domain fused to the transmembrane region of the IL-2 receptor (another rapidly degraded ER protein), were expressed in fibroblast cells. The turn over of the fusion protein was rapid but the half life of the truncated ζ-chain increased from 10-60min to over 8h (Bonifacino *et al*;1990). The results obtained strongly implicated the Tm region to carry the necessary information to potentiate rapid ER degradation of the protein.

In the light of these findings selecting CD2 as the reporter protein was probably not the ideal choice. A protein with a less complex tertiary structure, one without disulphide bonds or glycosylation sites, may be less likely to be affected by ER proteins monitoring changes in conformation as a result of the fusions.

Although the initial attempt at screening a library did not identify any membrane anchoring CD2-fusion proteins, several factors have been discussed which may have contributed to this result. Therefore, particularly in the light of the very favourable results obtained with the controls, screening a larger and more representive library needs to be carried out to adequately assess the viability of this cloning strategy.

APPENDIX

I ABBREVIATIONS

Ab	- Antibody
ADP	- Adenosine diphosphate
Ag	- Antigen
Amp	- Ampicllin
APS	- Ammonium per sulphate
ATP	- Adenosine triphosphate
Bis-acrylamide	- N,N'mathylene-bis acrylamide
BSA	- Bovine serum albumin
ccc DNA	- Covalently closed circular DNA
CD2	- Cluster of differentiation molecule 2
cDNA	- Copy DNA
CIP	- Calf intestinal phosphatase
ddNTPs	- Dideoxy nucleotides
ds	- Double stranded
dH_2O	- Distilled water
DMF	- Dimethyl formamide
DMSO	- Dimethyl sulphoxide
DNA	- Deoxyribo nucleic acid
DNase	- Deoxyribonuclease
dNTPs	- Deoxynucleotide triphosphates
DTT	- Dithiothreitol
EDTA	- Ethylenediaminetetra acetic acid
GAM	- Goat anti-mouse
HEPES	- N-2-Hydroxyethylpiperazine-N-2-ethane Sulphonic acid
HLA	- Human leukacyte antigen
IPTG	- Isopropylthio-B-D-Galactoside
λ	- Lamda
LFA	- Lymphocyte function associated
LMP	- Low melting point
LTR	- Long terminal repeat
mAb	- Monoclonal antibody
MHC	- Major histocompatibility complex
mRNA	- Messenger RNA
mwt	- Molecular weight
NRS	- Normal rabbit serum
PAGE	- Polyacrylamide gel electrophoesis
PBS	- Phosphate buffered saline
PEG	- Polyethelene glycol
PMSF	- Phenyl methyl Sulphonyl Fluoride
RAM	- Rabbit anti-mouse
RNA	- Ribonucleic acid
RNase	- Ribonulease
rRNA	- Ribosomal RNA
RT	- Room temperature
SDS	- Sodium dodecyl sulphate
SRBC	- Sheep red blood cell
SRP	- Signal recognition particle
ss	- Single stranded
SV40	- Simian virus 40

1 LSU - Large Sub unit
2 RER - Rough Endoplasmic Reticulum
3 RF - Ribose Free.
4 SSU - Small Sub-unit.

TBS	- Tris buffered saline
TEMED	- N,N,N',N'-tetramethylenediamine
Tet	- Tetracycline
Tm	- Transmembrane
Tris	- Tris (hydroxymethyl) aminoethane
tRNA	- Transfer RNA
T/V	- Trypsin/Versene
X-gal	- 5-Bromo-4-chloro-3- indolyl-α-β-galactoside

II UNITS

A	- Amps
mA	- milli amps
bp	- base pairs
kb	- kilo base pairs
C	- degrees centegrade
Ci	- Curie
mCi	- milli Curie
uCi	- micro Curie
CPM	- counts per minute
CPS	- counts per second
Da	- Daltons
h	- hours
kDa	- kilo daltons
g	- grammes
kg	- kilogrammes
mg	- milligrammes
ug	- microgrammes
ng	- nanogrammes
F	- Farrads
uF	- microfarrads
l	- litres
ml	- millilitres
ul	- microlitres
m	- metres
cm	- centimetres
min	- minutes
mm	- millimetres
M	- molar
mM	- millimolar
um	- micromolar
Ω	- Ohms
pH	- acidity ($-\log_{10}[H^+]$)
sec	- seconds
msec	- milliseconds
V	- volts
kV	- kilovolts
mV	- millivolts
rpm	- revolutions per minute
krpm	- kilorevolutions per minute

III CHEMICALS

General chemicals and solvents	BDH, Hopkins and Williams, Koch light laboratories, May and Baker
Media	Difco, Oxoid
Biochemicals	Sigma, Pharmacia, BRL
Agarose	BRL, IBI
Radiochemicals	NEN, Amersham
Antibiotics	Sigma
Restriction enzymes	BRL, Boehringer Mannheim, IBI, New England Biolabs
DNA-modifying enzymes	BRL, Boehringer Mannheim, Pharmacia
Scintillation fluid	National diagnostics
Antibodies	Ortho-immune, Becton Dickinson, Dako IgGs, Sera lab
Immunological reagents	Sigma, Dynal
Tissue culture media	Gibco, Northumbria biologicals

IV EQUIPMENT

Centrifuges	Eppendorf 5413, 54145 and 5415, MSE microcentrifuge, Beckman microfuge E, MSE minor S bench top centrifuge, Beckman L7-55 ultra centrifuge
Spectrophotometers	Beckman Du50, LKB ultraspec 4050

Scintillation counter	Beckman LS1801 liquid scintillation counter
Sequencing kit	BRL, Sequenase
mini gel kit	IBI BRL
Protein gel kit	Bio Rad
Electroblotter	Bio Rad
Electroporator	Bio Rad gene-pulser
Waterbaths	Grant
Microscopes	fluorescent - Leitz phase contrast - Zeiss
Cytospin	Shandon
Power supplies	Kikusui Electronic Corp., PAB DC power supply, LKB 2197 power supply
Balances	Sartorius
Membranes	nylon - PAL biodyne nitrocellulose - Amersham
Pipettmans	Gilson, Finnpipettes
X-ray film	Kodak hyper film

BIBLIOGRAPHY

Adams, G.A. and Rose, J.K. (1985). Structural requirements of a membrane-spanning domain for protein anchoring and cell surface transport. Mol. Cell. Biol. 5: (6) 1442-1448.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J.D. (1989). Molecular biology of the cell, 2nd edition. Gradand Publishing Inc.

Akita, M., Sasaki, S., Matsuyama, S. and Mizushima, S. (1990). Sec A interacts with secretory proteins by reorganising the positive charge at the amino terminus of the signal peptide in *Escherichia coli*. J. Biol. Chem. 265:8164-8169.

Albertus, Q.H., Beyers, D., Barclay, A.N. and Williams, A.F. (1988). A role in transmembrane signalling for the cytoplasmic domain of the CD2. T-lymphocyte surface antigen. Cell 54: 979-984.

Alcover, A., Alberini, C., Aluto, O., Clayton, L.K., Transy, C., Spagnoli, G.C., Moingeon, P., Lopez, P. and Reinherzz, E.L. (1988). Interdependence of CD3-Ti and CD2 activation pathways in human T lymphocytes. EMBO J. 71: 1973-1977.

Amara, J.F., Lederkremer, G. and Lodish, H.F. (1989). Intracellular degradation of unassembled asialoglycoprotein receptor subunits: in pre golgi non lysosomal endoproteolytic cleavage. J. Cell Biol. 109: 3315-3324.

Arine, E., Rzepecki, L.M. and Strittmatter, P. (1987). Topography of the C terminus of cytochrome b5 tightly bound to dimyristoyl phosphatidyl choline vesicles. J. Biol. Chem. 262: 15563-15567.

Attardi, G. and Schatz, G. (1988). The biogenesis of mitochondria. Ann. Rev. Cell Biol. 4: 289-315.

Audigigier, Y., Frielander, M. and Blobel, G. (1987). Multiple topogenic sequences in bovine opsin. Proc. Natl. Acad. Sci. (USA). 84: 5783-5787.

Ausubel, F.M., Brent, R., Kingston, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1990). Current Protocols in Molecular Biology.

Baker, K.P., Schanieli, A., Vestweber, D. and Schatz, G. (1990). A yeast mitochondrial outer membrane protein essential for protein import and cell viability. Nature 348: 605-609.

Baker, K.P. and Schatz, G. (1991). Mitochondrial proteins essential for viability mediate protein import into yeast mitochondria. Nature 349: 205-208.

Bedbrook, J.R., Smith, S.M. and Ellis, R.J. (1980). Molecular cloning and sequencing of cDNA encoding the precursor to the small subunit of the chloroplast enzyme ribulase-1,5-bisphosphate carboxylase. Nature **287**: 692-697.

Bennett, H.S. (1956). The concept of membrane flow and the concept of transport by membrane vesiculation in animal cells. J. Biophys. Biochem. Cytol. **2**: 99-103.

Bernstein, N.D., Poritz, M.A., Strub, K., Hoben, P.J., Brenner, S. and Walter, P. (1989). Model for signal sequence recognition from amino acid sequence of 54K subunit of signal recognition particle. Nature **340**: 482-486.

Berry-Lowe, S.L., McKnight, T.D., Shaw, D.M. and Meagher, R.B. (1982). The nucleotide sequence, expression and evolution of one member of a multigene family encoding the small sub-unit of ribulose-1,5-bisphosphate carboxylase in soy bean. J. Mol. Appl. Gen. **1**: 483-498.

Bierer, B.E., Barbosa, J., Herrmann, S. and Surkoff, S.J. (1988). Interaction of CD2 with its ligand LFA-3, in human T-cell proliferation. J. Immunol. **140**(10): 3358-3363.

Bierer, B.E., Peterson, A., Barbosa, J., Seed, B. and Burakoff, S.J. (1988). Expression of the T-cell surface molecule CD2 and an epitope-loss CD2 mutant to define the role of lymphocyte function-associated antigen-3 (LFA-3) in T-cell activation. Proc. Natl. Acad. Sci. (USA) **85**: 1194-1198.

Bierer, B.E., Golan, D.E., Brown, C.S., Herrmann, S.H. and Burakoff, S.J. (1989). A monoclonal antibody to LFA-3, the CD2 ligand, specifically immobilizes major histocompatibility complex proteins. Eur. J. Immunol. **19**: 661-665.

Bierer, B.E., Sleckman, B.P., Ratnofsky, S.E. and Burakoff, S.J. (1989). The biological roles of CD2, CD4 and CD8 in T-cell activation. Ann. Rev. Immunol. **7**: 579-599.

Billington, W.D. (1988). Maternal-fetal interaction in normal human pregnancy. Balleries Clin. Immun. and Allergy 2(3): 527-549.

Birnboim, H.C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acids. Res. **7**: 1513-1523.

Blobel, G. and Dobberstein, B. (1975). Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. J. Cell Biol. **67**: 835-851.

Blobel, G., and Dobberstein, B. (1975). Transfer of proteins across membranes. I. Reconstitution of functional rough microsomes from

heterologous components. J. Cell Biol. **67**: 852-862.

Blobel, G. (1980). Intracellular protein topogenesis. Proc. Natl. Acad. Sci. (USA). **77**: 1496-1500.

Bohnert, H., Herrera-Estrella, C., van Montagu, M. and Schel, J. (1985). Import of polypeptides into chloroplasts. Bio/Technology **3**: 803-808.

Bole, D.G., Hendershot, L.M. and Keeney, J.F. (1986). Postranslational association of immunoglobulin heavy chains in non secretory and secretory hybridomas. J. Cell Biol. **102**: 1558-1586.

Bonifacino, J.S., Suzuki, C.K., Lippincott-Schwartz, J., Weissman, A.M. and Klausner, R.D. (1989). Pre-golgi degradation of newly synthesised T-cell antigen receptor chains intrinsic sensitivity and the role of subunit assembly. J. Cell Biol. **109**: 73-83.

Bonifacino, J.S., Suzuki, C.K. and Klausner, R.D. (1990). A peptide sequence confers retention and rapid degradation in the endoplasmic reticulum. Science **247**: 79-82.

Bonner, W.M. (1975a). Protein migration into nuclei. I. Frog oocyte nuclei *in vivo* accumulate microinjected histones, allow entry to small proteins and exclude large proteins. J. Cell Biol. **64**: 421-430.

Bonner, W.M. (1975b). Protein migration into nuclei. II. Frog oocyte nuclei accumulate a class of microinjected oocyte nuclear proteins and exclude a class of microinjected oocyte cytoplasmic proteins. J. Cell Biol. **64**: 431-437.

Bonner, W.M. (1978). Protein migration and accumulation in nuclei in the cell nucleus (H. Bush, ed). **6**(C) N.Y. Acad. 97-148.

Boyd, D. and Beckwith, J. (1990). The role of charged amino acids in the localisation of secreted and membrane proteins. Cell **62**: 1031-1033.

Brain, P., Gordon, J. and Willetts, W.A. (1970). Rosette formation by peripheral lymphocytes. Clin. Exp. Immunol. **6**: 681-688.

Bretscher, M.S. (1971). Major human erythrocyte glycoprotein spans the cell membrane. Nature New Biol. **231**: 229-232.

Brown, M.H., Krissansen, G.W., Totty, N.F., Sewell, W.A. and Crumpton, M.J. (1987). Purification and N-terminal amino acid sequence of the human T-lymphocyte CD2 (T11) surface antigen. Eur. J. Immunol. **17**: 15-20.

Burgess, T.L. and Kelly, R.B. (1987). Constitutive and regulated

secretion of proteins. Ann. Rev. Cell Biol. 3: 243-293.

Burglin, T.R. and De Robertis, E.M. (1987). The nuclear migration signal of *Xenopus laevis* nucleoplasmin. EMBO J. 6: 2617-2625.

Caras, I.W., Davitz, M.A., Rhee, L., Weddell, G., Martin, D.W. Jr. and Mussenzweig, V. (1987). Cloning of decay accelerating factor suggests novel use of splicing to generate two proteins. Nature 325: 545-548.

Caras, I.W., Wedell, G.N. and Williams, S.R. (1989). Analysis of the signal for attachment of a glycophospholipid membrane anchor. J. Cell Biol. 108: 1387-1396.

Casadaban, M. and Cohen, S. (1980). Analysis of gene control signals by DNA fusion + cloning in *Escherichia coli*. J. Mol. Biol. 138: 179-207.

Cashmore, A.R. (1984). Structure and expression of a prenuclear gene encoding a chlorophyll a/b-binding polypeptide. Proc. Natl. Acad. Sci. (USA) 81: 2460-2464.

Ceroitti, A. and Coleman, A. (1987). Binding to membrane proteins within the endoplasmic reticulum cannot explain the retention of glucose regulated protein GRP78 in *Xenopus* oocytes. EMBO J. 9:(3) 633-638.

Chambers, S.P., Prior, S.E., Barston, D.A. and Minton, N.P. (1981) The pMTL-nic- cloning vectors. I. Improved pUC polylinker regions to facilitate the use of sonicated DNA for nucleotide sequencing. Gene 3: 9-13

Chang, H., Moingeon, P., Lopez, P., Krasnow, H., Stebbins, C. and Reinherz, E.L. (1989). Dissection of the human CD2 intracellular domain. Identification of a segment required for signal transduction and interleukin 2 production. J. Exp. Med. 169: 2073-2083.

Chen, E.Y. and Seeburg, P.H. (1985). Supercoil sequencing. A fast and simple method for sequencing plasmid DNA. DNA 4: 165-170.

Cheng, M.Y., Hartl, R.F.U., Martin, J., Pollock, R.A., Kaloussek, F., Neupert, W.J., Halberg, C.M., Halberg, R.L. and Horwich, A.L. (1989). Mitochondrial heat shock protein hsp60 is essential for assembly of proteins imported into yeast mitochondria. Nature 337: 620-625.

Chirgwin, J.J., Przbyla, A.E., Macdonald, R.J. and Rutter, W.J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18: 5294-5299. Chua, N-H and Gillham, N.W. (1977). The sites of synthesis of the principal thylakoid membrane polypeptides in *Chlamydamonas rheinhardtii*. J. Cell Biol. 741: 441-452.

Cline, K., Werner-Washburne, M., Lubben,T.W. and Keegstra, K. (1985). Precursors to two nuclear-encoded chloroplast proteins bind to the

outer envelope membrane before being imported into chloroplasts. J. Biol. Chem. **260**: 3691-3696.

Coleman, J., Inukai, M. and Inouye, M. (1985). Dual functions of the signal peptide in protein transfer across the membrane. Cell **43**: 351-360.

Connolly, T. and Gilmore, R. (1986). Formation of a functional ribosome-membrane junction during translocation requires the participation of a GTP binding protein. J. Cell Biol. **103**: 2253-2261.

Connolly, T. and Gilmore, R. (1989). The signal recognition particle receptor mediates the GTP-dependent displacement of SRP from the signal sequence of the nascent polypeptide. Cell **57**: 500-610.

Coombs, R.R.A., Gurner, B.W., Wilson, A.B., Holm, G. and Lindgren, B. (1970). Rosette-formation between human lymphocytes and sheep red blood cells not involving immunoglobulin receptors. Int. Arch. Allergy App. Immunol. **39**: 658-663.

Craig, E.A., Kramer, J. and Kosic-Smithers, J. (1987). SSC 1, an essential member of the yeast HSP70 multigene family, encodes a mitochondrial protein. Mol. Cell Biol **9**: 3000-3007.

Cutler, D.F., Melancon, P. and Garoff, H. (1986). Mutants of the membrane-binding region of Semliki Forest virus E2 protein. I Cell surface transport and fusogenic activity. J. Cell. Biol.. **102**: (3): 902-910.

Dalbey, R.E. and Wickner, W. (1987). Leader peptidase of *Escherichia coli*: critical role of a small domain in membrane assembly. Science **235**: 723-787.

Davis, N.G. and Model, P. (1985). An artificial anchor domain hydrophobicity suffices to stop transfer. Cell **41**: 607-614.

Davis, N.G., Boeke, J.D. and Model, P. (1985). Fine structure of a membrane anchor domain. J. Cell Biol. **181**: 111-121.

De Robertis, E.M., Longthorne, R.F. and Gurdon, J.B. (1978). Intracellular migration of nuclear protein in *Xenopus laevis* oocytes. Nature **272**: 254-256.

Diamond, D.J., Clayton, L.K., Sayre, P.H. and Reinherz, E.L. (1988). Exon-intron organization and sequence comparison of human and murine T11 (CD2) genes. Proc. Natl. Acad. Sci. (USA) **85**: 1615-1619.

Diesenhofer, J., Epp, O., Miki, K., Huber, R. and Michel, W. (1985). The structure of the protein subunits in the photosynthetic structure of the protein *sub* units in the photosynthetic reaction centre *Rhodopseudomonas viridis* at 3A resolution. Nature **318**: 618-624.

Dingwall, C. and Laskey, R.A. (1986). Protein import into the cell nucleus. Ann. Rev. Cell Biol. 2: 367-390.

Dingwall, C., Dilworth, S.M., Black, S.J., Kersey, S.E., Cox, L.S. and Laskey, R.A. (1987). Nucleoplasmin cDNA sequence reveals polyglutamic acid tracts and a cluster of sequences homologous to putative nuclear localisation signals. EMBO J. **6**: 69-74.

Dower, W.J., Miller, J.F. and Ragsdale, C.W. (1988). High efficiency transformation of *E. coli* by voltage electroporation. Nucl. Acids Res. **16**: 6127-6145.

Dustin, M.L., Selvaraj, P., Mattaliano, R.J. and Springer, T.A. (1987). Anchoring mechanisms for LFA-3 cell adhesion glycoprotein at membrane surface. Nature **329**: 846-848.

Ehrlich, P. (1901). Schlussbetrachtungen; Erkrankungen des Blutes; Nothnagel's Specielle Pathologie un Therapie **8**: 1817-1820.

Ellis, J. (1981). Chloroplast proteins: synthesis, transport and assembly. Ann. Rev. Plant Physiology **32**: 111-137.

Ellis, J. (1984). Proteins as molecular chaperones. Nature **328**: 378-380.

Emr, S.D., Hall, M.N. and Silhavy, J.D. (1980). A mechanism of protein location: The Signal Hypothesis and Bacteria. J. Cell Biol. **86**: 701-711.

Engelman, D.M. and Steitz, T.A. (1981). The spontaneous insertion of proteins into and across membranes: The helical hairpin hypothesis. Cell **23**: 411-422.

Evans, E., Gilmore, R. and Blobel, G. (1986). Purification of microsomal signal peptidase as a complex. Proc. Natl. Acad. Sci. (USA). **83**: 581-585.

Ferguson, M.A.J. and Williams, A.F. (1988). Cell surface anchoring of proteins via glycosyl-phosphotidyl inisorbitol structures. Ann. Rev. Biochem. **57**: 285-320.

Finidori, J., Rizzolo, L., Gonzales, A., Kreibich, G., Adesnik, M. and Sabatini, D.D. (1987). The influenza hemagglutinin insertion signal is not cleaved and does not halt translocation when presented to the endoplasmic reticulum membrane as part of a translocating polypeptide. J. Cell Biol. **104**: 1705-1714.

Franke, W.W. (1974). Structure biochemistry and functions of the nuclear envelope. Int. Rev. Cytol. **4**: 71-236.

Friedlander, M. and Blobel, G. (1985). Bovine opsin has more than one signal sequence. Nature **318**: 338-343.

Garoff, H. (1985). Using recombinant DNA techniques to study protein targetting in the eukaryotic cell. Ann. Rev. Cell Biol. **1**: 403-445.

Gething, M-J., McCammon, K. and Sambrook, J. (1986). Expression of

wild type and mutant forms of influenza hemagglutinin: the role of folding in intracellular transport. Cell **46**: 939-950.

Gilmore, R., Blobel, G. and Walter, P. (1982a). Protein translocation across the endoplasmic reticulum I. Detection in the microsomal membrane of a receptor for the signal recognition particle. J. Cell Biol. (1982a) **95**: 463-469.

Gilmore, R., Walter, P. and Blobel, G. (1982). Protein translocation across the endoplasmic reticulum. II Isolation and characterisation of the signal recognition particle receptor. J. Cell Biol. **95**: 470-477.

Gilmore, R. and Blobel, G. (1983). Transient involvement of signal recognition particle its receptor in the microsomal membrane prior to protein translocation. Cell **35**: 677-685.

Gilmore, R. and Blobel, G. (1985). Translocation of secretory proteins across the microsomal membrane occurs through an environment accessible to aqueous perturbants. Cell **42**: 497-502.

Goldfarb, D.S., Gariepy, J., Schoolnik, G. and Kornberg, R.D. (1986). Synthetic peptides as nuclear localisation signals. Nature **322**: 641-644.

Gordon, J.B. (1970). Nuclear transportation and the control of gene activity in animal development. Proc. Roy. Soc. (London) **176**: 303-314.

Gould, S.J., Keller, G-A., Hosken, N., Wilkinson, J. and Subramani, S. (1989). A conserved tri-peptide sorts proteins to peroxisomes. J. Cell Biol. **108**: 1657-1664.

Gray, M.W. and Doolittle, W.F. (1982). Has the endosymbiont hypothesis been proven? Microbiol. Rev. **46**: 1-42.

Greaves, D.R., Wilson, F.D., Lang, G. and Kioussis, D. (1989). Human CD2 3'-flanking sequences confer high-level, T-cell-specific, position independent gene expression in transgenic mice. Cell **56**: 979-986.

Gurdon, J.B. (1970). Nuclear transplantation and the control of gene activity in animal development. Proc. Roy. Soc. (London). B **176**: 303-314.

Haeuptle, M.T., Flint, N., Gough, N.M. and Dobertstein, B. (1989). A tripartite structure of the signals that determine protein insertion into the endoplasmic reticulum membrane. J. Cell Biol. **108**: 1227-1236.

Hann, B.C., Poritz, M.A. and Walter, P. (1989). *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* contain a homologue to the 54 kD subunit of the signal recognition particle that in *S. cerevisiae* is essential for growth. J. Cell Biol. **109**: 3223-3230.

Hartl, F-U and Neuport, W. (1990). Protein sorting to mitochondria: evolutionary conservations of folding and assembly. Science **247**: 930-938.

Hartman, E., Wiedmann, M. and Rapoport, T.A. (1989). A membrane component of the endoplasmic reticulum that may be essential for protein translocation. EMBO J. **8**: 2225-2229.

Haas, I.G. and Wabl, M. (1983). Immunoglobulin heavy chain binding protein. Nature **306**: 387-389.

Henderson, R. and Unwin, P.N.T. (1975). Three dimensional model of purple membrane obtained by electron microscopy. Nature **257**: 28-32.

= Von Heijne + Gavel. (1989)

Hirt, B. (1967). Selective extraction of polyoma DNA from infected maise cell cultures. J. Mol. Biol. **26**: 265-269.

Holmes, D.S. and Quigley, M. (1981). A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114**: 193-197.

Holland, E.C., and Drickamer, K. (1986). Signal recognition particle mediates insertion of a transmembrane protein which has a cytoplasmic NH2 terminus. J. Biol. Chem. **261**: 1286-1292.

Howard, F.D., Ledbetter, J.A., Wong, J., Bieber, C.P., Stinson, E.B. and Herzenberg, L.A. (1981). A human T-lymphocyte differentiation marker defined by monoclonal antibodies that block E rosette formation. J. Immunol. **126**: 2117-2122.

Hsi, B.L., Yeh, C.J.G. and Faulk, W.P. (1982). Human amniochorion: Tissue specific markers, transferrin receptors and histocompatibility antigens. Placenta **3**: 1-12.

Hunig, T., Tiefenthaler, G., Meyer, K.H., Buschefelde, Z. and Meuer, S.C. (1987). Alternative pathway activation of T-cells by binding of CD2 to its cell-surface ligand. Nature **326**: 298-301.

Hunziker, R.D., Gambel, P. and Wegmann, T.G. (1984). Placenta as a selective barrier to cellular traffic. J. Immunol. **133**: 667-671.

Hurtley, S.M. and Helenius, A. (1989). Protein oligomerisation in the endoplasmic reticulum. Ann. Rev. Cell Biol. **5**: 277-308.

Inouye, M. and Halegoua, S. (1980). Secretion and membrane localisation of proteins in *Escherichia coli*. CRC Crit. Rev. Biochem. **4**: 334-71.

Jacob, A.E., Cresswell, J.M., Hedges, R.W., Coetlee, J.N. and Beringer, J.E. (1976). Properties of plasmids constructed by *in vitro* insertion of DNA from *Rhizobium legunuminosrium* or *Proteus mirabilis* into RP4. Molec. Gen. Genet. **147**: 315-323.

Jensen, R.E. and Yaffe, M.P. (1988). Import of proteins into yeast mitochondria; the nuclear MAS 2 gene encodes a component of the processing protease that is homologous to the MAS1-encoded subunit.

EMBO J. 7: 3763-71.

Kaiser, C.A., Preuss, D., Grisafi, P. and Botstein, D. (1987). Many random sequences functionally replace the secretion signal sequence of yeast invertase. Science 235: 312 317 Kalderon, D.,

Richardson, W.D., Markham, A.F. and Smith A.E. (1984b). Sequence requirements for nuclear location of simian virus 40 large t-antigen. Nature 311: 499-509.

Kalderon, D., Roberts, B.L., Richardson, W.D. and Smith, A.E. (1989). A short amino acid sequence able to specify nuclear location. Cell 39: 499-509.

Kamoun, M., Martin, P.J., Hansen, J.A., Brown, M.A., Siadak, A.W. and Nowinski, R.C. (1981). Identification of a human T-lymphocyte surface protein associated with the E-rosette receptor. J. Exp. Med. 153: 207-212.

Kassenbrock, C.K., Garcia, P.D., Walter, P. and Kelly, R.B. (1988). Heavy chain binding protein recognizes aberrant polypeptides translocated *in vitro*. Nature 333: 90-93.

Krensky, A.M., Sanchez-Madrid, F., Robbins, E., Nagy, J.A., Springer, T.A. and Burakoff, S.J. (1983). The functional significance, distribution and structure of LFA-1, LFA-2 and LFA-3; cell surface antigens associated with CTL interactions. J. Immunol. 731: 611-614.

Kiebler, M., Pfaller, R., Sollner T., Griffiths, G., Horstmann, H., Pfanner, N. and Neupert, W. (1990). Identification of a mitochondrial receptor complex required for recognition and membrane insertion of precursor proteins. Nature 348: 610-616.

Killeen, N., Moessner, R., Arvieux, J., Willis, A. and Williams, A.F. (1988). The MRC OX-45 antigen of rat leukocytes and endothelium is in a subset of the immunoglobulin super family with CD2, LFA-3 and carcinoembryonic antigens. EMBO J. 7: 3087-3091.

Klausner, R.D. and Sitia, R. (1990). Protein degradation in the endoplasmic reticulum. Cell 62: 611-614.

Klein, P., Kanenisa, M. and De Lisi, C. (1985). The detection and classification of membrane spanning proteins. Biochim. Biophys. Acta 815: 468-476.

Kornfeld, S. (1986). Trafficking of lysosomal enzymes in normal and disease states. J. Clin. Investigation 77: 1-6.

Kornfeld, S. (1989). The cation-dependent mannose-6-phosphate receptor. Structural requirements for mannose-6-phosphate binding and oligomerisation. J. Biol. Chem. 264(19): 11458-11467.

Kornfeld, S. and Mellman, I. (1989). The biogenesis of lysosomes. Ann. Rev. Cell Biol. 5: 483-523

Kozutsuui, Y.m Normington, K., Press, E., Slaughter, C., Sambrook, J. and Gething, M.J. (1989). Identification of immunoglobulin heavy chain binding proteins as glucose-regulated protein 78 on the basis of amino acid sequence, immunological cross-reactivity and functional activity. J. Cell Sci. Suppl. 115-37.

Krieg, U.K., Walter, P. and Johnson, A. (1986). Photocross-linking of the signal sequence of nascent preprolactin to the 54 kilodalton polypeptide of the signal recognition particle. Proc. Natl. Acad. Sci. (USA) **83**: 8604-08.

Krohne, G. and Franke, W.W. (1970). Immunological identification and localisation of the predominant nuclear protein of the amphibian oocyte nucleus. Proc. Natl. Acad. Sci. (USA) **77**: 1034-1038.

Kurzchalia, T.V., Wiedmann, M., Girshovich, A.S., Bochkareva, E.S., Bielka, H. and Rapoport, T.A. (1986). The signal sequence of nascent preprolactin interacts with the 54K polypeptide of the signal recognition particle. Nature **320**: 634-636.

Kyewski, B.A., Jenkinson, E.J., Kingston, R., Actevogt, P., Owen, M.J. and Owen, J.J.T. (1989). The effects of anti-CD2 antibodies on the differentiation of maise thymocytes. Eur. J. Immunol. **19**: 951-954.

Kyte, J. and Doolittle, R.F. (1982). A simple method of displaying the hydropathic character of a protein. J. Mol. Biol. **157** 105-132.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**: 680-685.

Lanford, R.E. and Butel, J. (1984). Construction and characterisation of an SV40 mutant defective in nuclear transport of T antigen. Cell **37**: 801-813.

Lanford, R.F., Kanda, P. and Kennedy, R.C. (1986). Induction of nuclear transport with a synthetic peptide homologous to the SV40 T antigen transport signal. Cell **46**: 575-582.

Lang, G., Wotton, D., Owen, M.J., Sewell, W.A., Brown, M.H., Mason, D.Y., Crumpton, M.J. and Kioussis, D. (1988). The structure of the human CD2 gene and its expression in transgenic mice. EMBO J. 7(6): 1675-1682.

Lau, M.M. and Neufeld, E.F. (1989). A frameshift mutation in a patient with Tay-sacks disease causes premature termination and defective intracellular transport of the alpha-subunit of beba hexosaminidase. J. Biol. Chem. **264**: 21376-21380.

Lay, W.H., Mendes, N.F., Bianco, C. and Nussenzweig, V. (1971). Binding of sheep red blood cells to large population of human lymphocytes. Nature **230**: 531-533.

Lingappa, V.R., Lingappa, J.R. and Blobel, L. (1979). Chicken ovalbumin contains an internal signal sequence. Nature **281**: 117-121.

Lippincott-Schwartz, L., Bonifacino, J.S., Yuan, L.C. and Klausner, R.D. (1988). Degradation from the endoplasmic reticulum disposing of newly synthesised proteins. Cell **54**: 209-220.

Little, P.F.R., Treisman, R., Bierut, L., Seed, B. and Maniatis, T. (1983). Mol. Biol. Med. **1**: 473-488.

Lodish, H.F. (1988). Transport of secretory and membrane glycoproteins from the rough endoplasmic reticulum to the golgi. J. Biol. Chem. **263**: 2107-2110.

Lopata, M.A., Cleveland, D.W. and Sollnerwebb, B. (1984). High-level expression of a chloramphenicol acetyl transferase gene by DEAE-dextran mediated DNA transfection coupled with a dimethy sulphoxide or glycerol shock treatment. Nucl. Acids. Res. **12**: 5707

Lopez de Castro, J., Bragado, R., Strong, D.M. and Strominger, J.L. (1983). Primary structure of papain solubilised human histocompatibility antigen HLA-BHO (-BW60) an outline of alloantigenic determinants. Biochemistry **22**: 3961-3969.

Low, M.G. and Saltiel, A.R. (1988). Structural and functional roles of glycosyl-phosphatidyl inositol in membranes. Science **239**: 268-275.

McClelland, M., Hanish, J., Nelson, M. and Patel, Y. (1988). KGB: a single buffer for cell restriction and nucleus. Nucl. Acids Res. **16**: 364.

McGrath, J.P. and Varshavsky, A. (1989). The yeast STE 6 gene encodes a homologue of the mammalian multidrug resistance P-glycoprotein. Nature **340**: 400-404.

Marchesi, V.T., Furthmayr, H. and Tomita M. (1976). The red cell membrane. Ann. Rev/ Biochem. **45**: 667-698.

Medawar, P.B. (1953). Some immunological and endocrinological problems raised by the evolution of viviparity in vertebrates. Symp. Soc. for Exptal. Biol. **7**: 320-338.

Meuer, S.C., Hussey, R.E., Fabbi, M., Fox, D., Acuto, O., Fitzgerald, K.A., Hodgdon, J.C., Protentis, J.P., Schiossman, S.F. and Reinherz, E.L. (1984). An alternative pathway of T-cell activation, a functional role of the 50kd T11 sheep erythrocyte receptor protein. Cell **36**: 897-906.

Meyer, D.I., Krausse, E. and Dobberstein, B. (1982). Secretory protein translocating across membranes - the role of the 'docking protein'. Nature **247**: 647-650.

Milstein, C., Brownlee, G.G., Harrison, T.M. and Mathews, M.B. (1972). A possible precursor of immunoglobulin light chains. Nature

239: 117-120.

Morishima, N., Nakagana, K-I, Yomoto, E. and Shibata, J. (1990). A subunit of yeast site specific endonuclease 5 SceI is a mitochondrial version of the 70 kDa heat shock protein. J. Biol. chem. **265**: 15189-15197.

Murakami, H., Blobel, G. and Pain, D. (1990). Isolation and characterisation of the gene for a yeast mitochondrial import receptor. Nature **347**: 48-491.

2 — Murray (1981)

Neufeld, E.F., Lim, T.W. and Shapiro, L.J. (1975). Inherited disorders of lysomal metabolism. Ann. Rev. Biochem. **44**: 357-376.

Neuport, J. and Forbes, D.J. Fate of DNA injected into *Xenopus* eggs and in egg extracts: assembly into nuclei. Banbury report 20: Genetic manipulation of the early mammalian embryo. Cold Spring Harbor, New York: Cold Spring Harbor press pp. 243-250.

Neuport, W., Hartl, F-U, Craig, E.A. and Pfanner, N. (1990). How do polypeptides cross the mitochondrial membranes? Cell **63**: 447-450.

Newmeyer, D.D. and Forbes, D.J. (1988). Nuclear import can be separated into distinct steps *in vitro*: Nuclear pore banding and translocation. Cell **52**: 641-653.

Nilsson, I. and von Heijne, G. (1990). Fine-tuning the topology of a polytopic membrane protein. Role of positively and negatively charged amino acids. Cell **62**: 1135-1141

Normington, K., Kohno, K., Kozutsumi, Y., Gething, M.J. and Sambrook, J. (1989). *S. cerevisiae* encodes an essential protein homologous in sequence and function to mammalian BiP. Cell **57**: 1223-1236.

Palade, G. (1975). Intracellular aspects of the process of protein secretion. Science **189**: 347-358.

Paul, C. and Rosenbusch, J.P. (1985). Folding patterns of porin bacteriorhodopsin. EMBO J. **4**: 1593-1597.

Peterson, A. and Seed, B. (1987). Monoclonal antibody and ligand binding sites of the T-cell erythrocyte CD2. Nature **329**: 842-846.

Pfanner, N., Sollner, T. and Neuport, W. (1991). Mitochondrial import receptors for precursor proteins. TIBS **16**: 63-67.

Pfeffer, S.R. and Rothman, J.E. (1987). Biosynthetic protein transport and sorbing by the endoplasmic reticulum and golgi. Ann. Rev. Biochem. **56**: 829-852.

Plunkett, M.L., Sanders, M.E., Selvaraj, P., Dustin, M.L. and Springer, T.A. (1987). Rosetting of activating human T-lymphocytes with autologous erythrocytes. J. Exp. Med. **165**: 664-676.

Pollock, R.A., Hartl, F.U., Cheng, M.U., Ostermann, J., Horwich, A.

and Neupert, W. (1988). The processing peptidase of yeast mitochondria: the two cooperating components NPP and PEP are structurally related. EMBO J. 7: 3493-3500.

Prehn, S., Herz, J., Hartmann, E., Kurzchaha, T.V., Frank, R., Romisch, K., Dobberstein, B. and Rapoport, T.A. (1990). Structure and biosynthesis of the signal sequence receptor. Eur. J. Biochem. **188**: 439-445.

Pugsley, A.P. (1989). Protein targeting. Academic Press Inc.

Puziss, J.W., Fikes, J.D. and Bassford, P.J. (1989). Analysis of mutational alterations in the hydrophilic segment of the maltose-binding protein signal peptide. J. Bacteriol. **171**: 5536-5541.

Rapoport, T.A. (1985). Extensions of the signal hypothesis - Sequential insertion model versus amphipathic tunnel hypothesis. FEBS Lett. **187**: 1-10.

Rapoport, T.A., Heinrich, R., Walter, P. and Schulmeister, T. (1987). Mathematical modelling of the effects and the signal recognition particle on translation and translocation of proteins across the endoplasmic reticulum membrane. J. Mol. Biol. **195**: 621-636.

Rapoport, T.A. (1990). Protein transport across the ER membrane. T.I.B.S. **15**: 355-358.

Redman, O.M. and Sabatini, D.D. (1966). Vectorial discharge of peptides released by puromycin from attached ribosomes. Proc. Natl. Acad. Sci. (USA). **56**: 608-615.

Redman, C.W.G., McMichael, A.J., Stirrat, G.M., Sunderland, C.A. and Ting, A. (1984). Class I major histocompatibility complex antigens on human extra-villus trophoblast. Immunology **52**: 457-468.

Reinherz, E.L., Kung, P.C., Goldstein, G., Levey, R.W. and Schlossman, S.F. (1980). Discrete stages of human intrathymic differentiation: analysis of normal thymocytes and leukemic lymphoblasts of T-cell lineage. Proc. Natl. Acad.Sci. (USA) **77**: 1588-1592.

Richardson, W.D., Roberts, B.L. and Smith, A.E. (1986). Nuclear location signals in polyomer virus large-T. Cell **44**: 77-85.

Richardson, N.E., Hsdiu-Ching, L., Brown, N.R., Hussey, R.E., Sayre, P.H. and Reinherz, E.L. (1988). Adhesion domain of human T11 (CD2) is encoded by a single exon. Proc. Natl. Acad. Sci. (USA) **85**: 5176-5180.

Richardson, N.E., Chang, H., Brown, N.R., Gussey, R.E., Sayre, P.H. and Reinherz, E.L. (1988). Adhesion domain of human T11 (CD2) is encoded by a single exon. Proc. Natl. Acad. Sci. (USA) **85**: 5176-5180.

Robinson, A., Kaderbhai, M.A. and Austen, B.M. (1987). Identification of signal sequence binding proteins integrated into the rough endoplasmic reticulum membrane. Biochem. J. **242**: 767-772.

Robinson, C. and Ellis, R.J. (1984). Transport of proteins into chloroplasts: partial purification of a chloroplast protease involved in the processing of important precursor polypeptides. Eur. J. Biochem. **142**: 337-342.

Roise, D., Horvath, J.J., Tomich, J.M., Richards, J.H. and Schatz, G. (1986). A chemically synthesised pre sequence of an imported mitochondrial protein can form an amphiphilic helix and perturb national and artificial phospholipid bilayers. EMBO J. **5**: 1327-3332.

Rose, J.L., Welch, W.J., Sefton, B.M., Esca, F.S. and Ling, N.C. (1980). Vesicular stomatitis virus glycoproteins is anchored to the viral membrane by a hydrophobic domain near the cooH terminus. Proc. Natl. Acad. Sci. (USA) **77**: 3884-3888.

Rose, J.K. and Bergmann, J.E. (1983). Altered cytoplasmic domains affect intracellular transport of the vesicular stomatitis virus glycoprotein. Cell **34**: 513-524.

Rose, J.K. and Doms, R.W. (1988). Regulation of protein export from the endoplasmic reticulum. Ann. Rev. Cell Biol. **4**: 258-289.

Roise, D. and Schatz, G. (1988). Mitochondrial presequences. J. Biol. Chem. **263**: 4509-4511.

Romisch, K., Webb, J., Herz, J., Prehn, S., Frank, R., Vingrou, M. and Dobberstein, B. (1989). Homology of 54K protein of signal recognition particle, docking protein and two *E. coli* proteins with putative GTP-binding domains. Nature **340**: 478-482.

Rose, G.D., Geselowitz, A.R., Lesser, G.J., Lee, R.H. and Zeheus, M.H. (1985). Hydrophobicity of amino acid residues in globular proteins. Science **299**: 834-838.

Rose, M.D., Misra, L.M. and Vogel, J.P. (1989). KARZ, a karyogamy gene, in the yeast homology of the mammalian BiP/GRP78 gene. Cell **57**: 1211-1221.

Rothman, J.E. and Lodish, H.F. (1977). Synchronised transmembrane insertion and glycosylation of a nascent membrane protein. Nature **264**: 775-780.

Rothman, J.E. (1987). Protein sorting by selective retention in the endoplasmic reticulum and golgi stack. Cell **50**: 521-522.

Rothman, J.E. (1989). GTP and methionine bristles. (Signal-Peptide recognition). Nature **340**: 433-434.

Ruddle, F.H. and Reinherz, E.L. (1988). The gene for T11 (CD2) maps to chromosome 1 in humans and to chromosome 3 in mice. J. Immunology **140**: 3617-3621.

Sabatini, D.D. and Blobel, G. (1970). Controlled analysis of nascent polypeptides in rat liver cell fractions. II. Location of the polypeptides in rough microsomes. J. Cell Biol. **45**: 146-157.

Sabatini, D.D., Kreibach, G., Morimoto, T. and Adesnil, M. (1982). Mechanisms for the incorporation of proteins in membranes and organelles. J. Cell Biol. **92**: 1-22.
Sakaguchi, M., Mihara, K. and Sato E. (1984). Signal recognition particle is required for co-translational insertion of cytochrome P-450 into microsomal membranes. Proc. Natl. Acad. Sci. (USA). **81**: 3361-3364.

Sanger, D., Nicklen, S. and Coulson, A.R. (1977). DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. (USA) **77**: 5463-5466.

Sayre, P.H. and Reinherz, E.L. (1988). Structure and function of the erythrocyte receptor CD2 on human T-lymphocytes: a review. Scand. J. Rheumatol. Suppl. **76**: 131-144.

Scherer, P.E., Krieg, U.C., Hwang, S.T., Vestweber, D. and Schatz, G. (1990). A precursor protein partly translocated into yeast mitochondria is bound to a 70kd mitochondrial stress protein. EMBO J. **9**: (13) 4315-4322.

Schmidt, G.W., Devilliers-Thiery, A., Desruisseaux, H., Blobel, G. and Chua, N-H. (1979). NH_2-terminal amino acid sequences of precursors and mature forms of the ribolase-1,5-bisphosphate carboxylase small sub-unit from *Chlamydamonas rheinhardtii*. J. Cell. Biol. **83**: 615-622.

Schnell, D.J., Blobel, G. and Pain, D. (1990). The chloroplast import receptor is an integral membrane protein of chloroplast envelope contact sites. J. Cell Biol. **111**: (5) 1825-1838.

Schreier, P.H., Seftor, E.A., Schell, J. and Bohnert, H.J. (1985). The use of nuclear-encoded sequences to direct the light regulated synthesis and transport of a foreign protein into plant chloroplasts. EMBO J. **4**: 25-32.

Schwartz, R.M. and Dayhoff, M.O. (1978). Origins of prokaryotes, eukaryotes, mitochondria and chloroplasts. Science **199**: 395-403.

Schwartzman, G.O.H., Weigandt, H., Rose, B., Zimmerman, A., Ben-Haim, D. and Loewenstein, W.R. (1981). The diameter of the cell-to-cell junctional membrane channels, as probed with neutral molecules. Science **213**: 551-553.

Seczesna-Skoroupa, E. and Kemper, B. (1989). NH2-terminal substitutions of basic amino acids induce translocation across the microsomal membrane and glycosylation of rabbit cytochrome P450 C2. J. Cell Biol. **108**: 1237-1247.

Seed, B. (1987). An LFA-3 cDNA encodes a phospholipid linked

membrane protein homologous to its receptor CD2. Nature 329: 840-842.

Seed, B. and Aruffo, A. (1987). Molecular cloning of the CD2 antigen, the T-cell erythrocyte receptor, by a rapid immunoselection procedure. Proc. Natl. Acad. Sci. (USA) 84: 3365-3369.

Selden, R.F., Burke-Howie, K., Rowe, M.E., Goodman, H.M. and Moore, O.D. (1986). Human growth hormone as a reporter gene in regulation studies employing transient gene expression. Mol. Cell Biol. 6: 3173-3179.

Selvaraj, P., Plunkett, M.L., Dustin, M., Sanders, M.E., Shaw, S. and Springer, T.A. (1987). The T-lymphocyte glycoprotein CD2 binds the cell surface ligand LFA-3. Nature 326: 400-403.

Sen, J., Arceci, R.J., Jones, W. and Burakoff, J. (1989). Expression and ontogeny of murine CD2. Eur. J. Immunol. 91: 1297-1302.

Sen, J., Arlec, R.J., Jones, W. and Burakoff, S.J. (1989). Expression and ontogeny of murine CD2. Eur. J. Immunol. 19: 1297-1302.

Sewell, W.A., Brown, M.H., Dunne, J., Owen, M.J. and Crumpton, M.J. (1986). Molecular cloning of the T-lymphocyte surface CD2 (T11) antigen. Proc. Natl. Acad. Sci. (USA) 83: 8718-8722.

Sewell, W.A., Brown, M.H., Owen, M.J., Fink, P.J., Kozak, C.A. and Crumpton, M.J. (1987). The murine homologue of the T-lymphocyte CD2 antigen: molecular cloning, chromosome assignment and cell surface expression. Eur. J. Immunol. 17: 1015-1020.

Sewell, W.A., Brown, M.H., Clipstone, N.A. and Crumpton, M.J. (1989). The T-lymphocyte CD2 antigen - genetic and functional studies. Trans. Proc. 21(1): 41-43.

Shaw, S., Luce, G.E.G., Quinones, R., Gress, R.E., Springer, T.A. and Sanders, M.E. (1986). Two antigen-independent adhesion pathways used by human cytotoxic T-cell clones. Nature 323: 262-264.

3 — Shemet (1998)

Siegel, V. and Walter, P. (1988). Each of the activities of signal recognition particle (SRP) is contained within a distinct domain: Analysis of biochemical mutants of SRP. Cell 52: 39-49.

Simon, S., Blobel, G. and Zimmerberg, J. (1989). Large aqueous channels in membrane vesicles derived from the rough endoplasmic reticulum of canine pancreas or the plasma membrane of *Escherichia coli*. Proc. Natl. Acad. Sci. (USA). 86: 6176-6180.

Singer, S.J. and Nicolson, G.L. (1972). The fluid mosaic model of the structure of cell membranes. Science 175: 720-731.

Singer, S.J. and Yaffe, H.P. (1990). Embedded or not? Hydrophobic sequences and membranes. TIBS 15: 369-373.

Smeekens, S., Baurll, C., Haggman, J., Keegstra, K. and Weisbeck, P. (1986). The role of the transit peptide in the rating of precursors toward different chloroplast compartments. Cell **46**: 365-375.

Smeekens, S., Weisbeck, P. and Robinson, C. (1990). Protein transport into and with chloroplasts. TIBS **15**: 73-76.

Sood, A.K., Pan, J., Bird, P.A., Pereira, D., Srivastava, R., Reddy, V.B., Duceman, B.W. and Weissman, S.M. (1985). Structure and polymorphism of Class I MHC antigen mRNA. Immunogenetics **22**: 107-127.

Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**: 503-517.

Spagnoli, G., Clayton, L.K. and Reinherz, E.L. (1987). Molecular cloning and expression of T11 cDNAs reveal a receptor-like structure on human T lymphocytes. Proc. Natl. Acad. Sci. (USA) **84**: 2941-2945.

Spiess, M., Schwartz, A.L. and Lodish, H.F. (1985). Sequence of human asialoglycoprotein receptor cDNA: An internal signal sequence for membrane insertion. J. Biol. Chem. **260**: 1979-1982.

Spiess, M. and Lodish, H.F. (1986). An internal signal sequence: The asialoglycoprotein in receptor membrane anchor. Cell **44**: 177-185.

Spiess, M. and Handschin, C. (1987). Deletion analysis of the internal signal-anchor of the human asiologlycoprotein receptor H1. EMBO J. **6**: 2683-2691.

Stryer, L. (1981). Biochemistry, 2nd edition. W.H. Freeman & Co.

Sutcliffe, J.F. (1978). Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. Cold Spring Harb. Symp. Quant. Biol. **43**: 77-90.

Takagaki, Y., Radhaukrishnan, R., Wirtz, K.W.A and Khorawa, H.G. (1983). The membrane-embedded segment of cytochrome b5, as studied by cross-linking with photoactivatable phospholipids. II. The nontransferable form. J. Biol. Chem. **258**: 9136-9142.

Tajima, S., Lauffer, L., Rath, V.L. and Blobel, G. (1986). The signal recognition particle receptor is a complex that contains two distinct polypeptide chains. J. Cell Biol. **103**: 1167-1178.

Tanford, C. (1978). The hydrophobic effect and the organisation of living matter. Science **200**: 1012-1018.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from poly-acrylamide gels to nitrocellulose sheets. Procedure and some applications. Proc. Natl. Acad. (USA) **76**: 4350-4354.

Twigg, A.J. and Sherratt, D.J. (1985). Trans-complementable copy-

number mutants of plasmid ColE1. Nature **283**: 276-278.

Van den Broeck, G., Timko, M.P., Kausch, A.P.,Cashmore, A.R., van Montagu, M. and Herrera-Estrella, L. (1985). Targetting of foreign proteins to chloroplasts by fusion to the transit peptide from the small sub-unit of ribulase-1,5-bisphosphate carboxylase. Nature **313**: 358-363.

Verbi, W., Greaves, M.F., Scheider, C., Koubek, K., Janossy, G., Stein, H., Kung, P. and Goldstein, G. (1982). Monoclonal antibodies OKT11 and OKT11A have par-T reactivity and block sheep erythrocyte receptors. Eur. J. Immunol. **12**: 81-86.

Vestweber, D. and Schatz, G. (1988). A chimeric mitochondrial precursor protein with internal disulphide bridges blocks import of authentic precursors into mitochondria and allows quantitation of import sites. J. Cell Biol. **107**: 2037-2043.

Viera, J. and Messing, J. (1982). The pUC plasmids and the M13 mp7 derived system for insertion mutagenesis and sequencing with synthetic universal primes. Gene **19**: 259-268.

Vlasuk, G.P., Inouye, S., Ito, H., Itakura, K. and Inouye, M. (1983). Effects of the complete removal of basic amino acid residues from the signal peptide on secretion of lipoprotein in *Escherichia coli*. J. Biol. Chem. **258**: 7141-7148.

Vollger, L.W., Tuck, D.T., Springer, T.A., Haynes, B.F. and Singer, K.H. (1987). Thymocytes binding to human thymic epithelial cells is inhibited by monoclonal antibodies to CD2 and LFA-3 antigens. J. Immunol. **138**: 358-363.

von Heijne, G. (1986). The distribution of positively charged residues in bacterial inner membrane proteins correlates with the trans-membrane topology. EMBO J. **5**: 3021-3027.

von Heijne, G. and Gavel, Y. (1988). Topogenic signals in integral membrane proteins. Eur. J. Biochem. **171**: 671-678.

von Heijne G. (1989). Control of topology and mode of assembly of a polytopic membrane protein by positively charged residues. Nature **341**: 456-458.

Walter, P. and Blobel, G. (1980). Purification of a membrane-associated protein complex required for protein translocation across the endoplasmic reticulum. Proc. Natl. Acad. Sci. (USA). **77**: 7112-7116.

Walter, P. and Blobel, G. (1981). Translocation of proteins across the endoplasmic reticulum III. Signal recognition protein (SRP) causes signal sequence dependent and site specific arrest of chain elongation that is released by microsomal membranes. J. Cell Biol. **91**: 557-561.

Walter, P., Ibrahimi, I. and Blobel, G. (1981). Translocation of

proteins across the endoplasmic reticulum. I. Signal recognition protein (SRP) binds to *in vitro* assembled polysomes synthesising secretory protein. J. Cell Biol. **91**: 545-550.

Walter, P. and Blobel, G. (1982). 7S small cytoplasmic RNA is an integral component of the signal recognition particle. Nature **249**: 691-698.

Walter, P. and Lingappa, V.R. (1986). Mechanism of protein translation across the endoplasmid reticulum membrane. Ann. Rev. Cell Biol. **2**: 499-516.

Wessels, H.P. and Spiess, M. (1988). Insertion of a multispinning membrane protein occurs sequentially and requires only one signal sequence. Cell **55**: 61-70.

Wickner, W. (1976). Asymmetric orientation of phage M13 coat protein in *E. coli* cytoplasmic membranes and in synthetic lipid vesicles. Proc. Natl. Acad. Sci. (USA). **73**: 1159-1163.

Wickner, W.(1979). Assembly of proteins into membranes: The membrane trigger hypothesis. Ann. Rev. Biochem. **48**: 23-45.

Wiedmann, M., Kurchaia, T.V., Hartmann, E. and Rapoport, T.A. (1987). A signal sequence receptor in the endoplasmic reticulum membrane. Nature **328**: 830-833.

Wieland, F.T., Geason, M.L., Serafini, T.A. and Rothman, J.E. (1987). The rate of bulk flow from the endoplasmid reticulum to the cell surface. Cell **50**: 289-330.

Wiley, D.C. and Skehel, J.J. (1987). The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. Ann Rev. Biochem. **56**: 365-395.

Williams, A.F. (1987). A year in the life of the immunoglobulin superfamily: Review. Immunology Today **8**(10): 298-303.

Williams, A.F., Barclay, A.N., Clark, S.J., Patterson, D.J. and Willis, A.C. (1987). Similarities in sequence and cellular expression between rat CD2 and CD4 antigens. J. Exp. Med. **165**: 368-380.

Windass, J.D., Worsey, M.J., Pioli, E.M., Pioli, D., Barth, P.T., Atherton, K.T., Dart, E.C., Byrom, D., Powell, K. and Senior, P.J. (1980). Improved conversion of methanol to single-cell protein by *Methylophilis methylotrophus*. Nature **287**: 396-407.

Witte, L., Jensen, R.C., Yaffe, M.P. and Schatz, G. (1988). MAST, a gene essential for yeast mitochondrial assembly, encodes a subunit of the mitochondrial processing protease. EMBO J. **7**: 1439-1446.

Wysocki, L.J. and Sato, V.L. (1978). 'Panning' for lymphocytes: a method for selection. Proc. Natl. Acad. Sci. (USA) **75**: 2844-2848.

Yost, C.S., Hedgpeth, J.H. and Lingappa, V.R. (1983). A stop transfer sequence confers predictable transmembrane orientation for a previously secreted protein in cell-free systems. Cell **34**: 759-766.

Zerial, M., Melangon, P., Schneider, C. and Garoff, H. (1986). The transmembrane segment of the human transferrin receptor functions as a signal peptide. EMBO J. **5**: 1545-1550.

Zerial, M., Huylebroeck, D. and Garoff, H. (1987). Foreign transmembrane peptides replacing the internal signal sequence of transferrin receptor allow its translocation and membrane binding. Cell **48**: 147-155.

Zimmermann, R. and Meyer, D.I. (1986). 1986: A year of new insights into how proteins cross membranes. TIBS **11**: 512-515.