IMMUNOLOGICAL AND MOLECULAR ~~BIOLOGICAL~~ APPROACHES TO CHARACTERISE ~~AN~~ ATION OF A NEW MEMBER OF THE EPIDERMAL GROWTH FACTOR ~~RELATED~~ GENE ~~PRODUCT~~ FAMILY

Guochin WU

University College and Middlesex
School of Medicine

Thesis submitted to the University of London
for the degree of Doctor of Philosophy,

1990

ABSTRACT

The aim of this project was to analyse the tissue distribution and function of a novel gene cloned by Jenny Dunne within ICRF. Sequence data revealed that the gene structure is homologous to the EGF-like protein family in the arrangement of cysteine and other conserved amino acids. The cloned cDNA was truncated at both the N- and C-termini. To study the function, tissue distribution and the relationship with other proteins of this gene product, an antibody reacting with the native form of the gene product was considered to be essential. Several strategies have been followed in attempting to achieve this aim.

Initially synthetic peptides derived from the gene sequence were used as immunogen, the reactivity of the polyclonal and monoclonal antibodies from immunised mice and rats did not correlate with the results of northern blot analysis and the antibodies did not react with native proteins. In a second series of experiments the truncated gene was linked in frame with a pseudo N-terminus and cloned into a vector which upon transfection can confer resistance to G418. The gene was stably integrated and transcribed. However, sera from mice immunised with the transfectants failed to stain living or fixed cells in a specific fashion. Surface iodination and internal labelling followed by one-D-gel analysis of cell associated or supernatant proteins did not show any difference between untransfected and transfected cells. This might

suggest that the products were not processed onto the cell surface as expected. A third approach was to make several TrpE fusion proteins as immunogens. Antisera from rats showed no specific reactivity with the fusion proteins. The difficulty of raising antibody to the gene product is discussed.

Northern blot analysis of various established lymphoid, myeloid, and epithelial cell lines, normal lymphoid tissues and activated lymphocytes showed that the gene is predominantly expressed in T-ALL cell lines. A striking feature of the northern analysis was the large number of different message sizes seen in different T cell lines. Cell surface phenotype analysis of these cells suggests that the pattern of transcripts is correlated with different stages of maturation. The implications of these findings for the function of this gene product and its possible role in malignant transformation are discussed.

INDEX

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AEV:	Avian erythroblastosis virus
APC:	Antigen presenting cell
APS:	Ammonium persulphate
ATL:	Adult T-cell leulemia
B-CLL:	B chronic lymphocytic leukemia
Brij 96:	Polyoxyethylene 10 oleyl ether
Brij 99:	Polyoxyethylene 20 oleyl ether
BSA:	Bovine serum albumin
CD:	Cluster of differentiation
cDNA:	Complementary DNA
CFA:	Complete freund's adjuvant
ConA:	Concanavalin A
cpm:	Counts per minute
CSF-1:	Colony stimulating factor-1
CTL:	Cytolytic T lymphocyte
DEPC:	Diethyl pyrocarbonate
DMSO:	Dimethyl sulphoxide
DTT:	Dithiothreitol
E^+:	Cells which form rosettes with sheep erythrocytes (T cells)
E^-:	C3N,N,N'N'-tetraacetic acid
ELISA:	Enzyme linked immunosorbent assay
ER:	Endoplasmic reticulum
FACS:	Fluorescence-activated cell sorter
FCS:	Foetal calf serum
FITC:	Fluorescein isothiocyanate

HEPES: 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid

HTLV-1: Human T-lymphotrophic virus-I

IAA: Iodoleacrylic acid

ICAM: Intercellular adhesion molecule

IFA: Incomplete freund's adjuvant

Ig: Immunoglobulin

IL: Interleukin

Influenza HA: Influenza haemagglutinin

I.P. Intraperitoneally

I.V. Intravenous

Kb(p): Kilobase (pairs)

Kd: Kilodalton

LDL: Low density lipoprotein

LFA: Lymphocyte function-associated antigen

LTR: Long terminal repeat

mAb: Monoclonal antibody

MEM: Minumum essential medium

MHC: Major histocompatibility complex

MOPS: 3- N-Morpholino propanesulfonic acid

mRNA: Messenger ribonucleic acid

NCAM: Neural cell adhesion molecule

NK cell: Natural killer cell

NP-40: Nonidet P-40

O.D.: Optical density

PBMC: Peripheral blood mononuclear cells

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PHA: Phytohaemagglutinin

PDGFR: Platelet-derived growth factor receptor

PMA: Phorbol 12-myristate 12-acetate

PMSF: Phenylmethylsulphonyl Fluoride

Pristane: 2,6,10,14-tetra-methylpentadecane

RaMIg: Rabbit anti-mouse Immunoglobulin

S.C.: Subcutaneous

SDS: Sodium dodecyl sulphate

SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SRBC: Sheep red blood cells

ssDNA: Salmon sperm DNA

SSV: Simian sarcoma virus

SV40: Simian virus 40

T-ALL: T-Acute lymphoblastic leukemia

TBS: Tris buffered saline

TCA: Trichloroacetic acid

TCR: T cell receptor

TdT: Terminal deoxynucleotidyl transferase

TEMED: N,N,N'N'-Tetramethylenediamine

TGF-1: Transforming growth factor type 1

TK: Thymidine kinase

TM: Transmembrane

TNF: Tumour necrosis factor

Trp: Tryptophan

TrpE: Anthranilate synthetase component I

Tween 20: Polyoxyethylene-sorbitan monolaurate

ACKNOWLEGMENTS

I am most grateful to my supervisor Professor P C L Beverley for his advice, help and constant encouragement during his supervision of this work. I would like to thank Dr M Owen and Ms J Dunne for their help and advice during the collaboration and for reviewing the manuscript. I am also indebted to the K C WONG Education Foundation Ltd in Hong Kong for financial support in the form of a loan.

I am particularly grateful to Dr Juliette Smith-Ravin for her friendship, help and constant encouragement during my study. I would also like to thank Dr I Goot and Dr P Jat in the Ludwig Institute for Cancer Research and Dr D Wallace in this laboratory for their help.

Finally, I would like to thank my husband Ming Zhou; his understanding, sacrifice, patience and encouragement during the three years of separation enabled me to finish this thesis.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Introduction

Lymphocytes express various molecules on their cell surfaces. These molecules have been found to play important roles in immune function. Antigen recognition, cell-cell interaction, cytokine-receptor interaction and signal transduction are all mediated via cell surface molecules. The advent of mAbs to lymphocyte specific cell surface antigens and the application of molecular biological techniques in the studies of surface molecules have contributed greatly to the classification and elucidation of the function of these molecules. The concept of clusters of differentiation (CD) has been widely accepted for the classification of different surface antigens. The elucidation of function of different CDs has been an important aspect of immunology for the past decade and these studies have permitted an explosive increase in our knowledge of their functions. This chapter reviews some of the important discoveries in this field and the history of an attempt to elucidate the function of an unknown molecule associated with T lymphocytes.

1.2 T Cell Ontogeny

The T lineage pathway of differentiation represents one of a few such pathways that are initiated during foetal development and persist throughout adult life. Bone marrow-derived prothymocytes go through several stages of differentiation in the thymus to give rise to immunocompetent T lymphocytes. The differentiation is

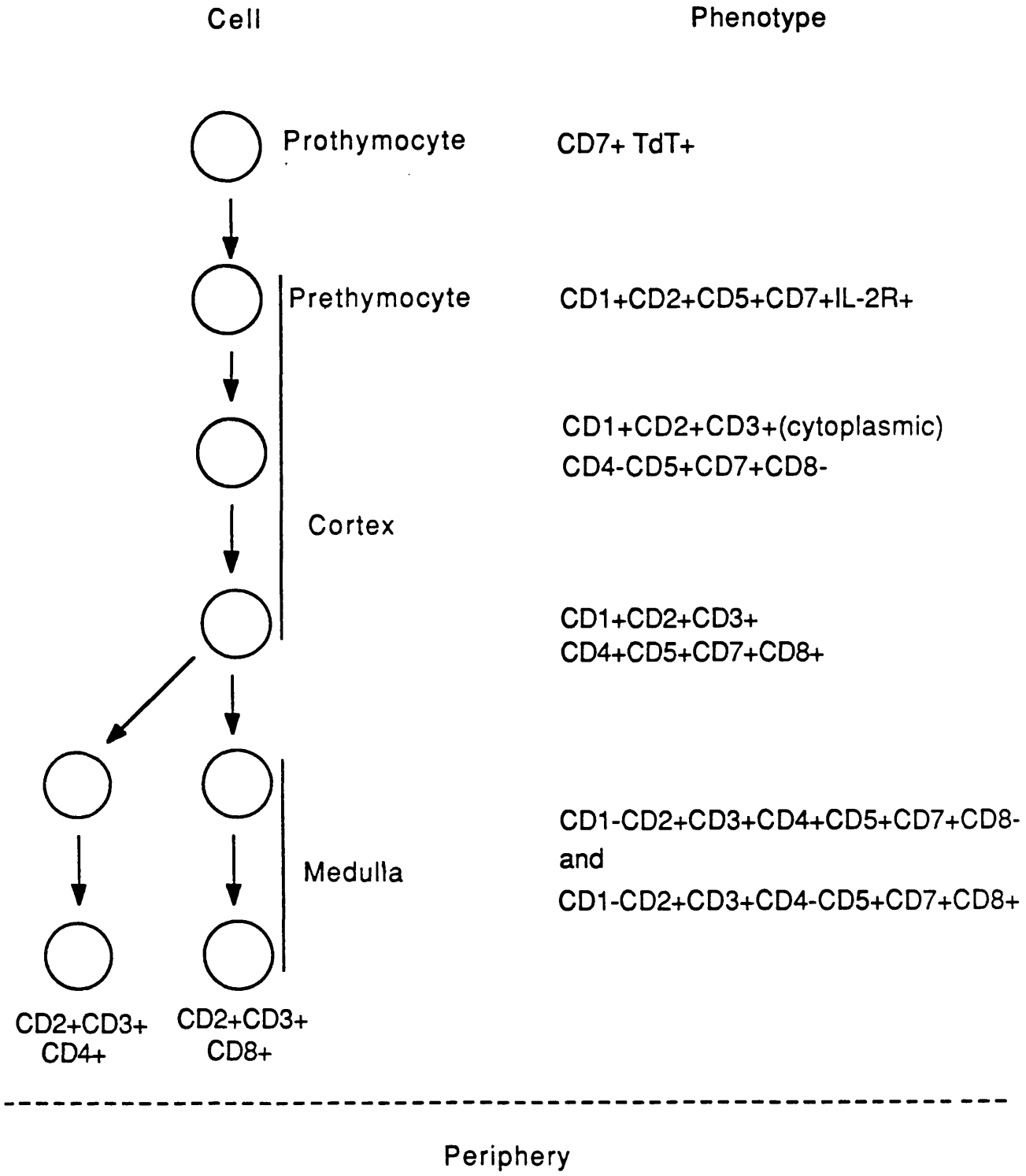
induced by the thymic microenvironment and is accompanied by changes in the expression of T cell specific cell surface antigens which serve as markers of the different stages of maturation. One possible scheme of T cell development is shown in Figure 1.1.

The thymus is anatomically divided into outer cortex, cortex and medulla. The earliest precursors of thymocytes are situated in the outer cortex of the thymus (reviewed in von Boehmer, 1988). Prothymocytes must enter the thymus with the use of a homing receptor. An early thymic marker that is candidate for the homing receptor is CD7 in man. Thymic development is actively driven by interactions with thymic epithelial cells and factors they produce. Early in murine thymic development, Thy-1, J11D, and the interleukin-2 receptor (IL-2R) appear in an unknown order. CD2 and CD1 are additional early thymocyte markers. Thus, in the mouse, Thy-1$^+$, J11D$^+$, IL-2R$^+$ thymocytes, and in man, CD7$^+$, CD2$^+$, CD1$^+$, and IL-2R$^+$ thymocytes, are early double negative (CD4$^-$CD8$^-$) precursors of more mature thymocytes (reviewed in Strominger, 1989). These cells also express components of CD3 intracytoplasmically, although they do not express surface CD3. Also during this early stage, CD5 begins to be expressed.

With differentiation, they begin to express CD8, then CD4, and become double positive. These double positive (CD4+, CD8+) cells reside mainly within the thymic cortex (Reinherz et al, 1980a). The majority of the cells will die before further maturation, only a small proportion eventually lose CD1 and differentiate into either CD4 or

Figure 1.1 Surface phenotype of T cells during thymic differentiation

(Adapted from Strominger, 1989)



CD8 subsets (single positive cells) and pass into the thymic medulla (McPhee et al, 1979). Immunological competence is acquired at this stage but is not fully developed until the thymocytes are exported into the peripheral compartment from the medulla.

One of the important features of immunocompetence is the specific recognition of antigen in association with the major histocompatibility complex (MHC) via T the cell receptor (TCR) on the surface of T lymphocyte. The T cell receptor consists of two disulphide-linked polymorphic glycoproteins ($\alpha\beta$, or $\gamma\delta$) (Oettgen et al, 1984; Ohashi et al, 1985) expressed in association with CD3 molecules as a TCR/CD3 complex (Oettgen et al., 1986; Allison and Lanier, 1987; Strominger, 1989; Clevers et al., 1988). Double negative thymocytes have intracellular CD3 proteins but the appearance of the TCR/CD3 complex on the surface of thymocytes requires the transcription of the genes of all of its members.

The TCR products will appear only after the DNA segments of the individual chains undergo gene rearragement. In mice the gene rearrangement follows the pattern of TCR-$\gamma\delta$ as the first genes to be rearranged, then TCR-β, and finally TCR-α. The gene rearrangement contributes to the polymorphism of the TCR repertoire. The order of gene rearrangement is reflected in the expression of TCR/CD3 complexes on the surface of thymocytes during T cell ontogeny. The presence of TCR-$\gamma\delta$/CD3 precedes that of TCR-$\alpha\beta$/CD3 during mouse thymic ontogeny. As T cells mature, TCR-$\alpha\beta$/CD3 become the dominant phenotype. The majority of mature T cells express

TCRαβ/CD3 while cells expressing TCRγδ/CD3 only constitute a small population of peripheral blood T cells. However TCRγδ/CD3 bearing cells comprise a much higher proportion of skin and intraepithelial T lymphocytes in the mouse.

1.3 Surface molecules of mature lymphocytes

Peripheral T lymphocytes express several pan-T cell markers which include CD2, CD3, CD5, CD7 etc. Apart from these pan-T markers, approximately two thirds of peripheral T lymphocytes express CD4 and one third express CD8 (Williams et al, 1977; Dialynas et al, 1983; Ledbetter and Herzenberg, 1979; Kisielow et al, 1975). This mutually exclusive expression divides the peripheral T cell population into CD4+ and CD8+ subsets. During the past several years, the functions of many surface molecules have been at least partially elucidated. The function of these surface molecules can be classified into general categories such as signal transduction, cell-cell adhesion, growth factor receptors or molecules with influence on the migration of cells (homing receptors) (see Table 1.1).

1.3.1 TCR/CD3 complex and T cell activation

T cells can be activated by mitogenic lectins such as concanavalin A (conA) and phytohaemagglutinin (PHA) in vitro. Under physiological conditions specific activation of the T cell induced by an antigen on the surface of an antigen presenting cell (APC) involves an interaction with the TCR/CD3 complex. This complex subserves two function in antigen-induced activation, (1)

Table 1.1 Major antigens expressed on T cells

CD Group	MW (kd)	Distribution	Comments
CD1	43-49	Thymocytes Dendritic cells Some B cells	
CD2	50	T cells NK cells	Alternative activation pathway Adhesion molecule
CD3	20-30	T cells	Complexed with the TCR and involved in signal transduction
CD4	60	T cell subset Some monocytes	Recognition of monomorphic determinants on Class II MHC Signal transduction
CD5	67	T cells Some B cells	
CD7	40	T cells NK cells	FcμR Candidate for the homing receptor
CD8	32	T cells subset Some NK cells	Recognition of monomorphic determinants on class I MHC Signal transduction

Adapted from Knapp et al., 1989. Molecular weights given are estimated from immunoprecipitation and SDS-PAGE analysis under reducing conditions.

Recognition function in which a specific antigen is recognised in the context of the appropriate MHC molecules, and (2) Effector function in which the recognition event is transmitted across the plasma membrane to the interior of the cell, with the resultant appearance of intracellular second messengers.

The structure of the TCR confers on the complex the first function: recognition. TCRs are disulphide-linked heterodimer glycoproteins of 80-90 kd which are composed of an acidic α chain of 43-54 kd and a more basic β chain of 38-44 Kd (Reinherz et al, 1983; Kappler et al, 1983a; 1983b). Initial peptide mapping and recent studies of the complementary DNA (cDNA) clones and genomic clones of these two chains (Yanagi et al, 1984; Sim et al, 1984) revealed that each chain contains both constant (C) and variable domains (V) for specific recognition which are homologuous to immunoglobulin (Ig) (see 1.4). They are both integral membrane proteins with only five amino acids as an intracytoplasmic tail. The relatively short cytoplasmic tail of these chains suggests that they are by themselves not responsible for transmembrane signalling events.

The prevailing opinion is that the signalling events are passed on by their associated cell surface molecule CD3, a complex of five distinct polypeptides, CD3 γ, δ, ε, ζ, and η (reviewed in Strominger, 1989; Clevers et al., 1988). CD3 polypeptide chains contain substantially longer intracellular domains than those of the TCR and are therefore likely to mediate signal transduction upon antigen recognition by the TCR heterodimer. The mechanism of T cell

activation via the TCR/CD3 complex is not completely understood. The early events of TCR/CD3 mediated activation include a rise in intracellular Ca^{2+}, and activation of protein kinase C of the T cells. Both of them serve as intracellular signals (Weiss et al, 1984; Truneh et al, 1985; Farrar and Rescetti, 1986).

Activated T cells undergo a number of morphological changes including an increase in cytoplasmic volume and nuclear size, expression of a number of novel activation antigens such as CD25 (which was lost when the thymocytes matured) and MHC class II antigen. Interaction of IL-2R with its ligand results in T cell proliferation. Activated T cells also produce a number of lymphokines which act on a variety of cell types (Table 1.2)

1.3.2 CD2 and LFA-3

The CD2 molecule is one of the earliest antigens of the T cell lineage and is a 50 kd glycoprotein on the surface of all T cells and thymocytes (Howard et al, 1981; Kamoun et al, 1981). This molecule was originally found as the sheep erythrocyte receptor. It is not polymorphic, therefore, it is not likely to play a major role in antigen binding. Although the physiologic function of this molecule is not clear, it has been reported that certain combinations of mAbs to the CD2 molecule induce T cell activation. The activation of T cells via the CD2 molecule has been termed the alternative pathway of human T cell activation to distinguish it from the antigen-

Table 1.2 Major lymphokines produced by T cells

(Adapted from Balkwill and Burke, 1989)

Factors	Other name/s	Target cells
IL-2	T cell growth factor	T cells, B cells
IL-3	Multi-colony-stimulating factor	multipotential stem cells
IL-4	B-cell growth factor 1 B-cell stimulating factor 1	B cells, T cells
IL-5	B-cell growth factor 2 Eosinophil differentiation factor	B cells, eosinophils
IL-6	IFN β_2 B-cell stimulating factor 2	B cells, fibroblast, macrophages
IFN-γ	Macrophage activating factor	B cells, macrophages
GM-CSF	Granulocyte/macrophage stimulating factor	myeloid precursor cells

dependent TCR/CD3 mediated pathway (Meuer et al, 1984; Siliciano et al, 1985).

Recent studies have demonstrated that lymphocyte function associated antigen-3 (LFA-3) represents the physiologic ligand of CD2 (Springer et al, 1987). This 55-70 kd heavily glycosylated protein is widely expressed on the surface of accessory cells and may provide assistance in cell-cell adhesion during T cell dependent function. CD2-LFA-3 interaction is important for cytolytic conjugate formation (Krensky et al, 1984; Shaw et al, 1986) and thymocyte-thymic epithelium adhesion (a reaction which could be important in prothymocyte homing). Sequencing of LFA-3 revealed that the ligand is homologous to its receptor CD2. Both are members of the Ig supergene family (section 1.4). CD2 is therefore an example of a molecule which has a dual function, binding to LFA-3 stabilises cell-cell contact but this also transmits an activating signal which may perhaps amplify specific signals transmitted via the TCR/CD3 complex.

1.3.3 CD4 and CD8

The expression of the CD4 or CD8 in the periphery divides mature T cells into two mutually exclusive subpopulations with distinct properties. CD4+ cells have been shown to provide helper/inducer function in T cell-T cell, T cell-B cell, T cell-accessory cell interactions, CD4 cells are required for an optimal CD8 cell mediated cytotoxic response to class I MHC antigens, and alone can provide

the signals necessary to induce autologous B cells to proliferate and differentiate into Ig secreting plasma cells. The CD8+ cells, which represent 20-30% of the resting T cells in the peripheral blood, in contrast, contain cells with suppressor or cytotoxic function, they are responsible for the elimination of virus infected MHC compatible cells (Cantor and Boyse, 1977; Reinherz et al., 1979a, b, 1980b). However, the establishment of specific cytotoxic CD4+ clones (Krensky et al., 1982a; 1982b) suggests that the expression of either CD4 or CD8 on the T cell surface does not necessarily commit a given T cell to one or another function.

The MHC restriction pattern of cells expressing CD8 or CD4 has been investigated. With few exceptions, CD4 cells recognise or are restricted by class II MHC proteins, while CD8 cells recognise or are restricted by class I MHC proteins (Swain, 1981, 1983; Swain et al., 1981; Engleman et al., 1981a, b; Meuer et al., 1982a, b; Krensky et al., 1982 a, b; Dialynas et al., 1983). The correlation between CD4 and CD8 expression and the class of MHC protein recognised led to the hypothesis that CD4 and CD8 might be receptors for nonpolymorphic regions on class I and class II MHC proteins, respectively. Recent data have provided extremely strong evidence in favour of this notion. In the case of CD8 a noncovalent association between CD8 and class I MHC proteins has been described on the surface of human T cell clones (Bushkin et al., 1988). Convincing evidence for binding between human CD4 and class II MHC proteins has been obtained in transfection experiments (Doyle and Strominger, 1987). The recently observed association of CD4 and CD8 with the T-cell specific tyrosine kinase $p56^{lck}$ which

phosphorylates the CD3 complex also suggests a direct role for CD4 and CD8 in signal transduction in response to antigen presentation (Rudd et al., 1988; Veillette et al., 1988; Barber et al., 1989).

The interaction of CD4 and CD8 molecules with MHC proteins increases the avidity of the interaction between T cells and the relevant target or accessory cells. It is clear that overall CD4 cells generally perform inducing functions while the CD8 subpopulation contains the bulk of cytotoxic activity. An important function of the CD4/MHC II interaction may be to act as a "guide" to stimulate appropriate cells to deal with extracellular parasites while CD8/MHC I interaction generates cytotoxic effectors able to combat intracellular microorganisms.

1.3.4 LFA-1 and ICAM-1

Antigen-specific cell contact in the immune system is strengthened by non-antigen-specific interactions mediated in part by lymphocyte function associated antigens (LFA) (Springer et al., 1987; Anderson and Springer, 1987). CD2 (LFA-2) and its ligand LFA-3 described in 1.3.2. are one such pair of interacting molecules. LFA-1, a member of the integrin supergene family of heterodimeric receptors, together with the Mac-1 and p150,95 heterodimers, which share a common β subunit with LFA-1, make up the leucocyte integrin subfamily (Kishimoto et al, 1987; Hynes, 1987). LFA-1 antigen is widely expressed on cells of haemopoietic origin. Recently, it has been shown that LFA-1 mediates intercellular

adhesion, at least in part, by binding to intercellular adhesion molecule-1 (ICAM-1) (Rothlein et al, 1986; Dustin et al., 1986; Marlin and Springer, 1987; Makgoba et al, 1988).

ICAM-1 is a single chain glycoprotein of 76-114 kd, its amino acid sequence contains Ig-like domains and is a ligand for LFA-1 (Marlin and Springer). It is weakly expressed on the surface of resting peripheral blood leucocytes. Its expression is increased by activation of T and B lymphocytes and monocytes (Clark et al., 1986; Dustin et al., 1986; Dougherty et al., 1988). The expression on non-haemopoietic cells is variable, but strongest on endothelium and at sites of inflammation (Dustin et al., 1986; Cotran et al., 1988). LFA-1 and ICAM-1 interaction mediates the leucocyte adhesion reactions underlying cytolytic conjugate formation, helper T-cell interactions, and antibody-dependent killing by natural killer cells and granulocytes. Cells freshly isolated from some Epstein-Barr virus (EBV) positive Burkitt's lymphoma tumours do not express ICAM-1 or the CD2 ligand, LFA-3, these cells are relatively resistant to EBV-specific cytolytic T lymphocytes (CTL). ICAM-1 and LFA-3 are expressed on some of the EBV-positive cell lines after in vitro culture and the expression is correlated with acquisition of susceptibility to EBV-specific CTL (Gregory et al., 1987).

1.3.5 Surface molecules as cytokine receptors

Interleukin 2 (IL-2) is an important mediator of immune responses released from activated T cells. This polypeptide possesses a

variety of biological activities and has the ability to stimulate: (1) the growth of thymocytes and mature T lymphocytes, (2) the differentiation of cytotoxic T cells, (3) the proliferation and activation of natural killer (NK) cells, and (4) the growth and differentiation of normal B lymphocytes (Smith, 1984; DeLa Hera et al., 1985; Ortaldo et al., 1984; Pike et al., 1984). The receptor for IL-2 was identified by studies with a mAb reactive with activated T lymphocytes (anti-Tac or CD25). Anti-Tac recognises a surface molecule that is expressed on activated T lymphocytes. It blocks IL-2 dependent T cell proliferation and inhibits the specific binding of radiolabeled IL-2 (Leonard et al., 1982). There are two classes of receptor, low affinity and high affinity (Depper et al., 1984; Robb et al., 1984). Both contain the Tac binding epitope. The majority of receptors are low affinity, but only high affinity sites appear to mediate biological acitvities of IL-2. Binding of IL-2 to its receptor leads to rapid internalisation of the receptor complex. Early studies showed that its major role is to mediate the progression of T cells from G_1 into S-phase (Smith, 1980).

The anti-Tac mAb precipitates a 55 kd glycoprotein. cDNA clones coding for the 55 kd Tac IL-2 receptor have revealed that unlike other growth factor receptors it has a very short cytoplasmic domain (13 amino acids) and lacks tyrosine-kinase activity. Recently, another chain of 75 kd containing a IL-2 binding site has been uncovered (Teshigawara et al., 1987; Tsudo et al., 1986). It has been named as IL-2R α chain while the one recognised by anti-Tac mAb has been designated as β chain. The α chain lacks the Tac epitope. The binding affinity of α chain is intermediate between

that of high and low affinity binding sites and it is able to transduce the growth signal (Smith, 1988).

IL-2R are not found on resting T cells but are induced by antigenic/mitogenic signals. The expression of these receptors is transient as removal of the immunostimulatory signals leads to a decline in receptor level (Cantrell and Smith, 1983). Leukemic cells from ATL patients and Human T-lymphotrophic Virus-I (HTLV-I) infected cells spontaneously (without stimulation) and continuously express IL-2R on their cell surfaces (Hattori et al., 1981; Depper et al., 1984; Waldmann et al., 1984; Uchiyama et al., 1985). It is suggested that abnormal IL-2R expression is associated with HTLV-I infection and may play a role in leukemogenesis by an autocrine mechanism (Yodoi et al., 1983; Uchiyama et al., 1985; Yodoi and Uchiyama, 1986).

1.3.6 CD5 antigen

The mouse CD5 antigen was defined initially with alloantisera in cytotoxic assays (Cantor and Boyse, 1977). Studies in human and mouse with mAbs revealed that normal mature T cells express CD5 regardless of functional subclass (Ledbetter et al., 1980; 1981), it is also expressed on fresh or established malignant T cells or T cell lines (Engleman et al., 1981c). It was believed at that time that CD5 was unique to T cells. Subsequent studies (Wang et al., 1980; Martin et al., 1980; 1981; Lanier et al., 1981), however, demonstrated that CD5 is also expressed on some B cell tumours, particularly, chronic

lymphocytic leukemia (B-CLL) and on a small proportion of apparently normal B cells (Calligaris-Cappio et al., 1982; Manohar et al., 1982).

Antibodies reacting with CD5 precipitate a 67 kd cell surface glycoprotein from radiolabeled lysates of thymocytes as well as malignant T cell lines. They do not show polymorphism among different individuals (Martin et al., 1980). The fact that anti-CD5 antibody precipitates the same 67 kd molecule from B cell tumours as from T cells might suggest that there is no difference between the CD5 on the T cells and that on B cells, though there is suggestion of a difference in glycosylation between them (Lanier et al., 1981). The function of the CD5 molecule is not clear. There are reports suggesting that it is involved in the proliferative response of activated T cells (Ledbetter et al., 1985; 1986) and in T cell helper function (Thomas et al., 1984; Hollander et al., 1981; Stanton et al., 1986; Lodgberg and Shevach, 1985). On the surface of B-CLL cells CD5 and CD21 (the receptor for the C3D component of complement) share an intimate spatial relationship and are involved in the redistribution of cytoskeletal proteins which may control the adhesive properties of these malignant B cells (Bergui et al., 1988).

1.3.6.1 CD5+ B Cells

Lymphocytes which express Ig either in the cytoplasm or on the surface or both belong to the B cell lineage. Studies both in humans and in mice demonstrated that a subpopulation of B cells expresses

the CD5 surface glycoprotein in conjunction with characteristic amounts of classical B cell surface molecules such as IgM, IgD, MHC class II antigens and other B cell markers. They do not express T cell markers except CD5 itself. The expression of CD5 on B cells is lower than that on T cells. Ontologically, CD5+ B cells are among the earliest B cells detectable in foetal spleen, however, their predominance declines as the conventional B cell population develops and fills the spleen. Thus, by 6 weeks of age, they represent less than 2% of total splenic B cells in mice, though there is variation of frequency in different strains (Hayakawa et al., 1983).

In adult mice, CD5 B cells mainly reside in the peritoneal cavity and comprise nearly half of the lymphocytes there. Studies in man revealed that the CD5+ B cell population are consistently present in the peripheral blood and spleens of healthy subjects and constitute about 17% of total B cells (Casali et al., 1987).

Functional studies in humans and mice readily distinguish CD5 B cells from conventional B cells. These cells produce many of the commonly studied IgM autoantibodies in autoimmune individuals (Hayakawa et al., 1984). Data also suggest that they may be responsible for producing much of the autoantibody (Hayakawa et al., 1984; Casali et al., 1987; Hardy et al., 1987) and anti-idiotype antibodies produced by normal individuals (Kearney and Vakil, 1986). In some autoimmune patients, the frequency of CD5 B cells is higher than that of controls (Hardy et al., 1987). CD5 negative B

cells, in contrast, tend to participate much less frequently in autoimmune responses and instead, appear to be concerned principally with producing antibodies to exogeneously introduced antigens (Hayakawa et al., 1984).

1.3.6.2 A differentiation marker or separate lineage?

There is some evidence that expression of CD5 may be associated with an early stage of B cell differentiation. CD5 B cells are present in large numbers in foetal spleen and lymph nodes (Bofill et al., 1984; Antin et al., 1986) and constitute the majority of graft-derived B lymphocytes that repopulate the host after bone marrow transplantation (Ault et al., 1985). In this context, chronic B lymphocytic leukemia cells, which consistently express CD5, may represent the neoplastic counterpart of the early stage of B cell differentiation. Further evidence for this notion is derived from the observation that CD5 negative malignant and normal B cells from patients and normal individuals have been induced to express CD5 antigen after in vitro exposure to PMA (Miller and Gralow, 1984). PMA is a tumour-promoting agent that has been shown to induce differentiation of human leukemia cells and normal lymphoid cells (Nagasawa and Mak, 1980; LeBien et al, 1982; Cossman et al., 1982). The induction of CD5 antigen by PMA suggests that the expression of this antigen may occur during the differentiation of B cells. Recent work by Werner-Favre et al (1989) suggests that CD5 is an activated B cell marker.

Studies in mice (Hayakawa et al, 1985; 1986), however, gave a different picture. By using bone marrow or peritoneal cells as a source of precursor B cells, it has been shown that CD5 B cells arise from a different precursor pool than CD5 negative B cells and may therefore represent a separate lineage. Palacios et al (1987) also demonstrated the establishment of CD5+ pro-B cell clones which can give rise to mature CD5+ B cells. In humans evidence of separate lineages is not available.

1.4 The Ig supergene family

Ig can be expressed either as cell surface-bound receptors (on B cells) or as secreted humoral antibodies. Ig is capable of recognising soluble or "free" antigen and plays a role in both the cell surface triggering of B cell differentiation and as the effector molecule of humoral immunity. The basic Ig structure is a tetramer constructed of two identical, disulphide-linked heterodimers, each composed of one light (L) and one heavy (H) chain. Both chains are divided into an N-terminal variable region (V) for antigen binding and a C-terminal constant region (C). Both V and C domains contain a characteristic intrachain disulphide bridge which stabilises a common fold, forming a sandwich of two β-sheets.

During the past decade, many molecules have been discovered and characterised within or outside the immune system. Some of them have been found to share Ig like structure and this led to the hypothesis that the Ig chains and other similar molecules may all

evolve from the same primordial gene (Cunningham et al., 1973; Strominger et al., 1980; Williams, 1984; Hood et al, 1985). Because Ig is the first member of this family to be sequenced, the group has become known as the Ig supergene family (Williams, 1987; 1982; Hood et al., 1985).

The criteria for inclusion of molecules in the Ig supergene family require that any members of the family show homology both in sequence alignment and predicted Ig domain-like structure which is stabilised by the conserved disulphide bond within the polypeptide chain. Members which meet these requirements are listed in Table 1.3. Most of the lymphocyte cell surface molecules described above are in this family. They include Ig, TCR, class I and class II MHC proteins, CD3, CD4, CD8, and CD2, LFA-3 and the ligand for LFA-1: ICAM-1. Some molecules of the family are found outside the immune system, such as neural adhesion molecule (NCAM), predominantly expressed in the nervous system (Cunningham et al, 1987; Barthels et al, 1987). N-CAM belongs to a limited number of cell adhesion molecule whose binding is involved in cell-cell interactions that coordinate patterns of migration, proliferation, and cellular differentiation during tissue development (Edelman, 1986). Some growth factor receptors such as platelet-derived growth factor receptor (PDGFR) (Lai et al, 1987; Yarden et al, 1986) and colony stimulating factor-1 (CSF-1) receptor also show Ig-like domains in their extracellular portions (Lai et al., 1987; Sherr et al., 1985).

Table 1.3 Molecules of the Ig supergene family (Adapted from Williams and Barclay, 1988)

Molecules	Tissue expression	Function	Recognition within the superfamily
Ig	B lymphocyte only	B lymphocyte antigen receptor	No, antibodies recognize antigen without involvement of other molecules
TCR	T lymphocytes, thymocytes	T lymphocyte antigen receptor	Yes, heterophilic; TCR binds MHC antigens plus peptides
CD3	T lymphocytes, thymocytes	Part of the TCR/CD3 complex, signal transduction?	CD3 associates with TCR but no known recognition of other molecules
MHC Class1 MHC Class2	Many cell types, induced by interferon	Present peptides from foreign antigen to the TCR	Yes, heterophilic; TCR interacts with Class 1 and Class 2 antigens
CD2	T lymphocytes, thymocytes	CD2 of T cells interacts with LFA-3 on other cell in adhesion reactions, anti-CD2 antibodies can trigger T cell activation	Yes, heterophilic, CD2 binds LFA-3
LFA-3	Widespread expression		

continued on next page

continued from previous page

Table 1.3. Molecules of the Ig supergene family

Molecules	Tissue expression	Function	Recognition within the superfamily
CD4	Thymocytes, T cell subset, macrophage	Interaction with Class II antigen	Yes, heterophilic, Class II antigen
CD8	Thymocytes, T cell subset, natural killer cells	Interaction with Class I antigen	Yes, heterophilic, Class I antigen
Thy-1	Neurons, fibroblasts, various lymphoid cells	Anti-Thy-1 antibody triggers mouse T cell division	No natural ligands known
ICAM-1	Widely expressed on cells of haematopoietic origin	Ligand for LFA-1	No, interacts with proteins in integrin family
NCAM	Neurons and glia, early embryonic tissue	Mediates adhesion of neural tissues	Yes, homophilic for NCAM via Ig-related parts.
PDGFR	Widespread on mesenchymal cells	Interacts with PDGF to trigger cell division	No.

As more surface proteins outside the immune system have been added to this family, the functional repertoire of these molecules has begun to emerge. In general, these molecules appear to mediate adhesion between cells via homophilic (protein A binds protein A) and heterophilic (protein A binds protein B) interaction on the opposed membrane surfaces (Table 1.3.). The importance of these interaction is broad, ranging from the modulation of embryonic development (Gallin et al, 1986) to the discrimination between self and non-self in the immune response (Williams, 1982; Hood et al, 1985).

1.5 The application of molecular biology in studies of surface antigens

The modern history of the study of cell surface molecules of lymphocytes can be traced back as far as the discovery of Thy-1 (Reif, 1963). In early days, most of the surface molecules were identified by alloantisera or heteroantisera. During that period, it was noticed that functionally different subpopulations of lymphocytes were correlated with distinct cell surface phenotypes (Raff, 1971). The advent of mAbs contributed greatly to the discovery and the systematic study of specific cell surface antigens. MAbs have been employed to separate subpopulations of lymphocytes for various functional assays, or have been added to in vitro culture to see if mAbs to a specific antigen would augment, inhibit or block certain cell functions. Immunoprecipitation with specific mAbs has revealed biochemical characteristics of antigens and the relationship between different surface molecules.

Recent advances in recombinant DNA technology and its application in the study of surface molecules have permitted an explosive increase in our knowledge of the structures of individual surface molecules, the gene regulation of proteins and the mechanisms of their functions. The application of molecular biology in these studies has benefited by the earlier studies with mAbs. The specificity of mAbs and the availability of large quantity of mAbs have made it possible to purify a specific antigen to homogeneity by immunoaffinity chromatography so that the amino acid sequence of the antigen can be determined and corresponding oligonucleotides can be synthesised as probes, or the antigen can be used as immunogen to make polyclonal antisera as protein probes. The selection of which probe to employ is dependent on what kind of cloning system is used. There are several reliable methods based on the exploitation of mAbs. They are described below.

1.5.1 λgt10

Phage libraries cloned in λgt10 are useful for screening with nucleic acid probes (Huynh, 1985). Depending on the purpose of investigation, probes may consist of any of the following: a previously cloned DNA fragment, genomic DNA, synthetic oligonucleotide specifying a particular amino acid sequence, RNA, or cDNA. The cDNA library is constructed in the phage λgt10 using poly(A)$^+$ RNA from cells which are positive for the antigen to be cloned. To prepare the probe, the first step usually begins with the

purification of the specific antigen to homogeneity by immunoaffinity chromatography. The N-terminal amino acid sequence of the molecule is determined by sequencing the purified protein. Usually several pools of corresponding oligonucleotides are synthesised and used as hybridisation probes to screen λgt10 cDNA library. Phages containing cDNA inserts complementary to the oligonucleotide probes are selected and the cDNA inserts can be employed for further screening or analysis.

1.5.2 λgt11

λgt11, is an expression vector capable of producing a polypeptide specified by a cloned DNA insert (Young and Davis, 1983a; b). Therefore, phage libraries cloned in λgt11 can be screened with antibody probes. In this system cDNAs are ligated into the λgt11 phage genome at an EcoR1 site within the lacZ gene encoding β-galactosidase. Under appropriate condition the lacZ gene can be expressed in bacterial cells. If its coding sequence is in frame with that of a cDNA insert then the result will be the synthesis of a fusion protein. Antibodies can be used to detect fusion proteins carrying antigenic determints encoded by the cDNA, and the cDNA sequences can be isolated from the corresponding cloned phage isolates.

Though mAbs can be produced in large quantity, the best probes may be polyclonal antisera raised against denatured purified protein. The advantage of polyclonal antisera recognising multiple

epitopes over mAbs would be the increased likelihood of detecting a fusion protein. A particular epitope recognised by a mAb might have an altered conformation in fusion proteins, might be dependent for its conformation on post-translational modifications which do not take place in bacterial cells, or might not be encoded by partial cDNAs. The advantage of using denatured protein as an immunogen would be that protein in this form would most likely mimic the conformation of the antigen amino acid sequences fused with β-galactosidase sequences and bound to nitrocellulose membrane (as would be the case when screening a λgt11 library). An antiserum which visualises the antigen strongly and specifically on western blots would probably be a good reagent for library screening.

1.5.3 Gene transfection and fluorescence activated cell sorting

L-cells lacking thymidine kinase (tk^-) are co-transfected with herpes simplex thymidine kinase (tk) gene and high molecular weight genomic DNA from antigen expressing or non-expressing cells. The transfectants are stained with fluorescein-tagged mAbs to the antigen to be cloned. The brightest 0.1-0.2% cells are viably separated using a fluorescence activated cell sorter (FACS). After growth of these selected cells, sorting and regrowth are repeated several times, resulting in a population which expresses the antigen and shows amplification of the gene coding the antigen. The technique is reliable and several cell surface antigens such as CD5, CD8, β_2m, HLA class I antigen etc, have been successfully

transfected into L cells (Kavathas and Herzenberg, 1983 a, b; Hsu et al., 1984).

1.5.4 Transient expression in COS cell

Another method has been developed in which cDNA is ligated to an expression vector πH3 which is transformed into *Escherichia coli* MC1061/p3. COS cells of 50% confluency are transfected with transformants by spheroplast fusion. Seventy-two hours after fusion, the cells are coated with mAbs, cells reacting with the antibodies are selected by sedimenting the cells in petri-dishes which had been coated with anti-mouse or rat Igs. The non-adherent cells are removed and plasmid DNA from adherent COS cells (containing the cDNA to be cloned) is extracted, transformed into *Escherichia coli* MC1061/p3, amplified and re-introduced into COS cells for further rounds of screening. After the third round of screening, the cDNA to be cloned has been enriched. DNA from individual bacterial colonies is then transfected into cells and assayed forantibody binding. The plasmid from positive cells is recovered and analysed. The best results are obtained by selecting positive cells with a panel of antibodies reacting with the same molecule at different epitopes. CD2, LFA-3 etc. have been successfully cloned by this method (Seed and Aruffo, 1987; Seed, 1987).

1.5.5 The application of cDNA clones

Once a cDNA is obtained, the sequence of the cDNA reveals the overall structure of a molecule. In comparison with other known molecules, it provides information on the relationship between different molecules. The existence of an Ig supergene family has been suggested for a set of proteins in and outside the immune system. This suggestion is based on the fact that a large number of proteins show sequence and predicted structural homology. Nearly all of them are engaged in protein recognition and adhesion. This suggests that structural similarity provides similar function. Therefore by grouping proteins according to their sequences, it is possible to predict the function of an unknown protein. In addition, by comparing the same molecules from different species, it is possible to obtain some clues to the evolution and function of the molecules. Conserved amino acids are usually important for retaining the function of a molecule. For example, human and murine CD4 molecules are homologous structures, with the greatest similarity occuring in the large intracytoplasmic domain, suggesting a conserved functional role for this region (Maddon et al, 1987).

However most significant has been the application of DNA mediated gene transfer to analyse structure-function relationship of gene products. An excellent example is the application of the method to study the control of MHC antigen gene expression, molecular assembly and the function of gene segments in immune recognition. Both class I and class II antigens have been studied in this way. The class II MHC category includes Ia molecules (I-A and I-E) in mouse and HLA-DP, DQ, and DR in man. The tissue distribution of

class II molecules is limited to certain cell types. Class II antigen is expressed primarily on B lymphocytes, monocytes, macrophages, dendritic cells, certain epithelial and skin-associated cells, and in species other than mouse, on activated T lymphocytes. Ia antigens are heterodimeric cell surface glycoproteins consisting of one heavy (33-34 kd), or α, and one light (28-29 kd), or β, chain. Both chains are subdivided into $\alpha 1$, $\alpha 2$ and $\beta 1$ and $\beta 2$ domains. The organisation of class II genes correlates well with the protein structure. Both α and β genes begin with a leader-encoding exon that also contains codons for 3-6 N-terminal residues of the mature protein. Exons 2 and 3 encode the α_1 or β_1 and α_2 or β_2 domains, respectively. There are additional exons encoding transmembrane and cytoplasmic domains.

Both Ia^+ cells (B lymphomas) and Ia^- cells (mouse L cells) express Ia on their cell surfaces when genes for α and β chains are transfected together. Single chain gene transfection (either α or β) results in accumulation of the protein chain in the cytoplasm of the cell, but not on the cell surface (Malissen et al., 1983; 1984; Gillies et al., 1984). This suggests that antigen assembly on the cell surface requires both chains to be expressed. Functional analysis of L cells transfected with MHC class II genes reveals that like normal APC, they are capable of presenting antigens to T-cell hybridomas (Malissen et al., 1984; Germain et al., 1983; Ben-Nun et al., 1984; 1985; Folsom et al., 1984; Norcross et al., 1984).

Experiments using "exon-shuffling" (regions of individual genes exchanged for the corresponding portion of an allelic form of the same gene, or variants created by combining pieces of unrelated genes) and transfection of the recombinant gene into recipient cells have given clues to the function of the individual portions of the genes. By exchanging the exons coding for the β_1 domain, it was demonstrated that the recombinant gene products behaved in a way identical to that of the donor of the β_1 domain (Folsom et al., 1985; Germain et al., 1985). This indicates the importance of this domain for MHC class II molecules.

Other studies have been performed by employing point mutagenesis to limit the mutation to a particular codon or codons resulting in a recombinant DNA with one or a few amino acids changed. The mutant DNA is subsequently transfected into recipient cells which are tested for their functions. One excellent example is the investigation of the binding site on CD4 for the human immunodeficiency virus (HIV). In man, CD4 is the target for attachment by HIV (Dalgleish et al., 1984; Klatzmann et al; 1984; Maddon et al., 1986), a process mediated by the viral envelop glycoprotein gp120 (McDougal et al., 1986). Through extensive analysis, the binding site for gp120 on CD4 has been localised within the first Ig-like domain on CD4 (V1) to a predicted loop structure of about 12 amino acids (Landau et al., 1988; Clayton et al., 1988; Mizukami et al., 1988; Arthos et al., 1989).

1.6 An attempt to Clone CD5 cDNA

In 1985 an attempt to clone the CD5 gene was stimulated by the important discoveries about the gene product described in 1.3.6. CD5 function was not clear in 1985, although the functional data described above suggest that the molecule might have a role in T cell activation and is implicated in autoimmune diseases. It was therefore decided to clone the gene in the hope that structural features would provide clues to the function. In addition the availability of the cloned gene would allow investigation of the function of the molecule by DNA transfection etc.

The strategy for cloning CD5 cDNA was based on the availability of a mAb to CD5 antigen, UCHT2 (produced by P C L Beverley). The mAb reacts with native CD5 molecule on cell surface, but does not recognise denatured CD5 antigen on western blot. It was however, decided to use λgt11 system to clone the gene. Therefore a cDNA library was constructed by Ms J Dunne and Dr M Owen using mRNA from a human T-leukemia cell line J6, which had been proved to be CD5 positive by antibody staining, and the cDNA was ligated into the expression vector λgt11 described in 1.5.2.

In the meanwhile the nonionic detergent (Nonidet P-40) soluble fraction of J6 microsome preparations was chosen for the purification of CD5 because of its ready availability in large amount. After J6 cells had been solubilised in the detergent, they were centrifuged and the nuclei were removed. The lysate was passed down a UCHT2-Sepharose 4B column, the bound CD5 molecules

were eluted and precipitated. The purity of the molecule was tested by applying a small aliquot of the elute to a SDS-PAGE gel which was silver stained. It was found to be pure enough to use as immunogen as the elute revealed a major band around 67 kd.

Rats were immunised with the eluted material to provide a polyclonal antiserum. The quality of the antiserum was tested by western blot and it reacted specifically with a 67 kd protein of the J6 lysate. The antiserum was then utilised to screen the J6 λgt11 cDNA library. After screening, a positive plaque was isolated and designated pT1A. This clone has an insert of about 500 base pairs. Later on a second clone of 2.4 kb, pT1AB was obtained from the same cDNA library using the insert of pT1A as a hybridisation probe. The sequencing data from these two clones show that pT1A is a part of pT1AB and has the same sequence as the 3' end of pT1AB. Although it was intended to clone the CD5 gene, the sequence of pT1AB is completely distinct from that of the CD5 gene (Jones et al.,1986) which was published at the time pT1AB was isolated.

1.7 pT1AB nucleotide sequence

The unpublished sequencing data reveal that the plasmid pT1AB contains a 2.4 kb cDNA insert. In an attempt to determine the protein-coding potential of the insert, the established primary sequence of the insert was examined for open reading frames. A diagram representing the entire nucleotide sequence is shown in

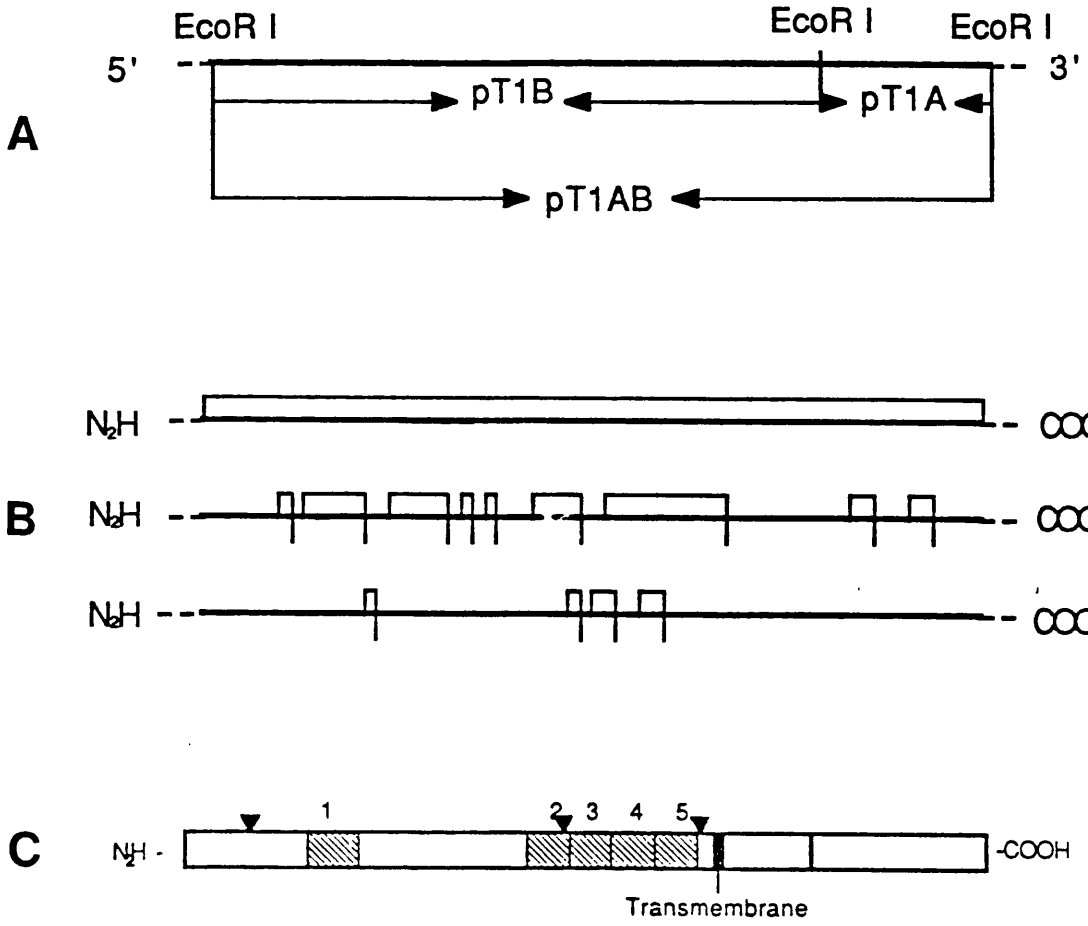


Figure 1.2. Primary structure of pT1AB and its predicted protein

The entire sequence corresponding to pT1AB is shown schematically in (A). The proposed translation initiation (AUG) and termination sites are missing. The positions of EcoR I sites which flank the pT1AB sequence and divide the sequence into pT1B and pT1A are shown. The open reading frames for the three possible forward translation frames are shown (B). The translational start (AUG) codons, indicated by lines extending above the sequence and stop codons (UAG, UGA, and UAA) by lines extending below the sequence. The presence of a single long open reading frame is apparent. The landmarks of the predicted amino acid structure of this long open reading frame are diagrammatically represented in (C). The location of a putative transmembrane region is indicated. The hatched boxes designate the location of the 5 cysteine-rich repeated units. The positions of potential glycosylation sites are indicated by small triangles.

Figure 1.2.A. The sequence is flanked by EcoR I sites at both 5' and 3' ends. An internal EcoR I site at 1,888 bp from the 5' end divides the whole sequence into pT1B and pT1A. The three possible translation frames of the insert sequence are depicted in Figure 1.2.B. Open reading frames are defined in this figure as segments that start with an AUG codon (lines extending above the sequence in the figure) and end with an UAG, UGA, or UAA codon (lines extending below the sequence). One of the three translation frames results in a long open reading frame with eight AUG codons but no stop codon. However no 'Kozak consensus sequence' for translation initiation (Kozak, 1986) is found surrounding these AUG codons, suggesting that pT1AB may contain a truncated cDNA.

The predicted sequence of the 806 amino acid polypeptide corresponding to this long open reading frame is shown in figure 1.3 under the nucleotide sequence. Several features of this sequence warrant comment. First, inspection of the sequence of pT1AB reveals an area of very high hydrophobicity as judged by the Kyte and Doolittle (1982) hydropathy scale which most likely represents a transmembrane region. The hydrophobic area consists of a 21 amino acid peptide (between amino acids 529 and 549) that is flanked by hydrophilic residues. Immediately adjacent to the membrane-spanning sequence there is, at the cytoplasmic interface, a 10 amino acid residue sequence that is highly enriched in basic amino acids (7 out of 10). Such an arrangement of hydrophobic and hydrophilic sequence is similar to that of the transmembrane domains of known membrane proteins or secreted proteins. This indicates that the cDNA insert encodes a membrane protein or a

DNA sequence 2400 b.p. GAATTCGTTGGT ... CCGTTACCGCCC linear

```
1   /   1                                   31   /   11
GAA TTC GTT GGT GAC TAC TGG CGC AAC TTC CAG CGA GCT TTA CGG AAC ATC CTG GGT GTG
glu phe val gly asp tyr trp arg asn phe gln arg ala leu arg asn ile leu gly val
61   /   21                                  91   /   31
AGG AGG AAC GAC ATA CAG ATT GTT AGT TTG CAG TCC TCT GAA CCT CAC CCA CAT CTG GAC
arg arg asn asp ile gln ile val ser leu gln ser ser glu pro his pro his leu asp
121   /   41                                 151   /   51
GTC TTA CTT TTT GTA GAG AAA CCA GGT AGT GCT CAG ATC TCA ACA AAA CAA CTT CTG CAC
val leu leu phe val glu lys pro gly ser ala gln ile ser thr lys gln leu leu his
181   /   61                                 211   /   71
AAG ATT AAC TCT TCC GTG ACT GAC ATT GAG GAA ATC ATT GGA GTT AGG ATA CTG AAT GTA
lys ile asn ser ser val thr asp ile glu glu ile ile gly val arg ile leu asn val
241   /   81                                 271   /   91
TTC CAG AAA CTC TGC GCG GGA CTG GAC TGC CCC TGG AAG TTC TGC GAT GAA AAG GTG TCT
phe gln lys leu cys ala gly leu asp cys pro trp lys phe cys asp glu lys val ser
301   /   101                                331   /   111
GTG GAT GAA AGT GTG ATG TCA ACA CAC AGC ACA GCC AGA CTG AGT TTT GTG ACT CCC CGC
val asp glu ser val met ser thr his ser thr ala arg leu ser phe val thr pro arg
361   /   121                                391   /   131
CAC CAC AGG GCA GCG GTG TGT CTC TGC AAA GAG GGA AGG TGC CCA CCT GTC CAC CAT GGC
his his arg ala ala val cys leu cys lys glu gly arg cys pro pro val his his gly
421   /   141                                451   /   151
                    1
TGT GAA GAT GAT CCG TGC CCT GAG GGA TCC GAA TGT GTG TCT GAT CCC TGG GAG GAG AAA
cys glu asp asp pro cys pro glu gly ser glu cys val ser asp pro trp glu glu lys
481   /   161                                511   /   171
CAC ACC TGT GTC TGT CCC AGC GGC AGG TTT GGT CAG TGC CCA GGG AGT TCA TCT ATG ACA
his thr cys val cys pro ser gly arg phe gly gln cys pro gly ser ser ser met thr
541   /   181                                571   /   191
CTG ACT GGA AAC AGC TAC GTG AAA TAC CGT CTG ACG GAA AAT GAA AAC AAA TTA GAG ATG
leu thr gly asn ser tyr val lys tyr arg leu thr glu asn glu asn lys leu glu met
601   /   201                                631   /   211
AAA CTG ACC ATG AGG CTC AGA ACA TAT TCC ACG CAT GCG GTT GTC ATG TAT GCT CGA GGA
lys leu thr met arg leu arg thr tyr ser thr his ala val val met tyr ala arg gly
661   /   221                                691   /   231
ACT GAC TAT AGC ATC TTG GAG ATT CAT CAT GGA AGG CTG CAG TAC AAG TTT GAC TGT GGA
thr asp tyr ser ile leu glu ile his his gly arg leu gln tyr lys phe asp cys gly
721   /   241                                751   /   251
AGT GGC CCT GGA ATT GTC TCT GTT CAG AGC ATT CAG GTC AAT GAT GGG CAG TGG CAC GCA
ser gly pro gly ile val ser val gln ser ile gln val asn asp gly gln trp his ala
781   /   261                                811   /   271
GTG GCC CTG GAA GTG AAT GGA AAC TAT GCT CGC TTG GTT CTA GAC CAA GTT CAT ACT GCA
val ala leu glu val asn gly asn tyr ala arg leu val leu asp gln val his thr ala
841   /   281                                871   /   291
TCG GGC ACA GCC CCA GGG ACT CTG AAA ACC CTG AAC CTG GAT AAC TAT GTG TTT TTT GGT
ser gly thr ala pro gly thr leu lys thr leu asn leu asp asn tyr val phe phe gly
901   /   301                                931   /   311
GGC CAC ATC CGT CAG CAG GGA ACA AGG CAT GGA AGA AGT CCT CAA GTT GGT AAT GGT TTC
gly his ile arg gln gln gly thr arg his gly arg ser pro gln val gly asn gly phe
961   /   321                                991   /   331
AGG GGT TGT ATG GAC TCC ATT TAT TTG AAT GGG CAG GAG CTC CCT TTA AAC AGC AAA CCC
arg gly cys met asp ser ile tyr leu asn gly gln glu leu pro leu asn ser lys pro
1021   /   341                              1051   /   351
AGA AGC TAT GCA CAC ATC GAA GAG TCG GTG GAT GTA TCT CCA GGC TGC TTC CTG ACG GCC
arg ser tyr ala his ile glu glu ser val asp val ser pro gly cys phe leu thr ala
1081   /   361                              1111   /   371                2
ACG GAA GAC TGC GCC AGC AAC CCT TGC CAG AAT GGA GGC GTT TGC AAT CCG TCA CCT GCT
thr glu asp cys ala ser asn pro cys gln asn gly gly val cys asn pro ser pro ala
1141   /   381                              1171   /   391
GGA GGT TAT TAC TGC AAA TGC AGT GCC TTG TAC ATA GGG ACC CAC TGT GAG ATA AGC GTC
gly gly tyr tyr cys lys cys ser ala leu tyr ile gly thr his cys glu ile ser val
1201   /   401                              1231   /   411                3
AAT CCG TGT TCC TCC AAC CCA TGC CTC TAT GGG GGC ACG TGT GTT GTC GAC AAC GGA GGC
asn pro cys ser ser asn pro cys leu tyr gly gly thr cys val val asp asn gly gly
1261   /   421                              1291   /   431
TTT GTT TGC CAG TGT AGA GGA TTA TAT ACT GGT CAG AGG TGT CAG CTT AGT CCA TAC TGC
phe val cys gln cys arg gly leu tyr thr gly gln arg cys gln leu ser pro tyr cys
1321   /   441                              1351   /   451   4
AAA GAT GAA CCC TGT AAG AAT GGC GGA ACA TGC TTT GAC AGT TTG GAT GGC GCC GTT TGT
lys asp glu pro cys lys asn gly gly thr cys phe asp ser leu asp gly ala val cys
1381   /   461                              1411   /   471
```

```
CAG TGT GAT TCG GGT TTT AGG GGA GAA AGG TGT CAG AGT GAT ATC GAC GAG TGC TCT GGA
gln cys asp ser gly phe arg gly glu arg cys gln ser asp ile asp glu cys ser gly
1441 /  481                             1471 /  491
AAC CCT TGC CTG CAC GGG GCC CTC TGT GAG AAC ACG CAC GGC TCC TAT CAC TGC AAC TGC
asn pro cys leu his gly ala leu cys glu asn thr his gly ser tyr his cys asn cys
1501 /  501                             1531 /  511
AGC CAC GAG TAC AGG GGA CGT CAC TGC GAG GAT GCT GCG CCC AAC CAG TAT GTG TCC ACG
ser his glu tyr arg gly arg his cys glu asp ala ala pro asn gln tyr val ser thr
1561 /  521                             1591 /  531
CCG TGG AAC ATT GGG TTG GCG GAA GGA ATT GGA ATC GTT GTG TTT GTT GCA GGG ATA TTT
pro trp asn ile gly leu ala glu gly ile gly ile val val phe val ala gly ile phe
1621 /  541                             1651 /  551
TTA CTG GTG GTG GTG TTT GTT CTC TGC CGT AAG ATG ATT AGT CGG AAA AAG AAG CAT CAG
leu leu val val val phe val leu cys arg lys met ile ser arg lys lys lys his gln
1681 /  561                             1711 /  571
GCT GAA CCT AAA GAC AAG CAC CTG GGA CCC GCT ACG GCT TTC TTG CAA AGA CCG TAT TTT
ala glu pro lys asp lys his leu gly pro ala thr ala phe leu gln arg pro tyr phe
1741 /  581                             1771 /  591
GAT TCC AAG CTA AAT AAG AAC ATT TAC TCA GAC ATA CCA CCC CAG GTG CCT GTC CGG CCT
asp ser lys leu asn lys asn ile tyr ser asp ile pro pro gln val pro val arg pro
1801 /  601                             1831 /  611
ATT TCC TAC ACC CCG AGT ATT CCA AGT GAC TCA AGA AAC AAT CTG GAC CGA AAT TCC TTC
ile ser tyr thr pro ser ile pro ser asp ser arg asn asn leu asp arg asn ser phe
1861 /  621                             1891 /  631
GAA GGA TCT GCT ATC CCA GAG CAT CCC GAA TTC AGC ACT TTT AAC CCC GAG TCT GTG CAC
glu gly ser ala ile pro glu his pro glu phe ser thr phe asn pro glu ser val his
1921 /  641                             1951 /  651
GGG CAC CGA AAA GCA GTG GCG GTC TGC AGC GTG GCG CCA AAC CTG CCT CCC CCA CCC CCT
gly his arg lys ala val ala val cys ser val ala pro asn leu pro pro pro pro pro
1981 /  661                             2011 /  671
TCA AAC TCC CCT TCT GAC AGC GAC TCC ATC CAG AAG CCT AGC TGG GAC TTT GAC TAT GAC
ser asn ser pro ser asp ser asp ser ile gln lys pro ser trp asp phe asp tyr asp
2041 /  681                             2071 /  691
ACA AAA GTG GTG GAT CTT GAT CCC TGT CTT TCC AAG AAG CCT CTA GAG GAA AAG CCT TCC
thr lys val val asp leu asp pro cys leu ser lys lys pro leu glu glu lys pro ser
2101 /  701                             2131 /  711
CAG CCA TAC AGT GCC CGG GAA AGC CTG TCT GAA GTG CAG TCC CTG AGC TCC TTC CAG TCC
gln pro tyr ser ala arg glu ser leu ser glu val gln ser leu ser ser phe gln ser
2161 /  721                             2191 /  731
GAA TCG TGC GAT GAC AAT GGG TAT CAC TGG GAT ACA TCA GAT TGG ATG CCA AGC GTT CCT
glu ser cys asp asp asn gly tyr his trp asp thr ser asp trp met pro ser val pro
2221 /  741                             2251 /  751
CTG CCG GAC ATA CAA GAG TTC CCC AAC TAT GAG GTG ATT GAT GAG CAG ACA CCC CTG TAC
leu pro asp ile gln glu phe pro asn tyr glu val ile asp glu gln thr pro leu tyr
2281 /  761                             2311 /  771
TCA GCA GAT CCA AAC GCC ATC GAT ACG GAC TAT TAC CCT GGA GGC TAC GAC ATC GAA AGT
ser ala asp pro asn ala ile asp thr asp tyr tyr pro gly gly tyr asp ile glu ser
2341 /  781                             2371 /  791
GAT TTT CCT CCA CCC CCA GAA GAC TTC CCC GCA GCT GAT GAG CTA CCA CCG TTA CCG CCC
asp phe pro pro pro pro glu asp phe pro ala ala asp glu leu pro pro leu pro pro
```

Figure 1.3 Combined pT1AB cDNA sequences and the encoded amino acids

The nucleotide sequence is a composite of two cDNA clones (pT1A and pT1B) as discussed in the text. The predicted amino acid sequence is shown below the cDNA sequence, beginning at nucleotide 1. The location of the repetitive elements are numbered and indicated by the heavy bars. Hydrophobic region is underlined with dashed lines. The asparagine residues as potential glycosylation sites are circled.

secreted protein. The transmembrane region divides the whole sequence into two parts, an extracellular domain of 528 amino acids and a cytoplasmic domain of 250 amino acids.

Secondly, a striking property of the protein is the presence of repetitive sequence elements in its extracellular domain illustrated in Figure 1.2.C and Figure 1.4. The predicted pT1AB amino acid repeat sequences are compared to each other. The segment of the sequence between amino acids 356 and 509, which has a cysteine composition of 15%, contains 4 tandem arrays of cysteine-rich repeats, approximately 40 amino acids long. Between amino acid 127 and 173 (46 amino acids), there is another similar segment consisting of one such repeat. The two segments of highly repeated elements are separated by a spacer of about 182 amino acids. The alignment shown in Figure 1.4 maximises the homology found between all five repeats and was derived by inspection of the sequences and insertion of spaces when these were needed to maintain the alignment of cysteines and other conserved amino acids.
In general, each of the 5 repeating units has six cysteines located at the same position. The spatial distribution of the cysteine residues follows a pattern consistent with the general formula: $CX_6CX_4CX_5CX_8CXCX_8$, where C is cysteine and X is any other amino acid. Some sequence gaps have to be introduced to achieve optimal alignment, but the high degree of similarity between the 5 sequence units is evident. In addition to the invariant cysteines, other residues such as glycine (G), phenylalanine (F), and tyrosine (Y) residues within each repeat appear to be also highly conserved. The consensus sequences are based on the presence of a specific amino acid at a given position within each repeat more than 50% of

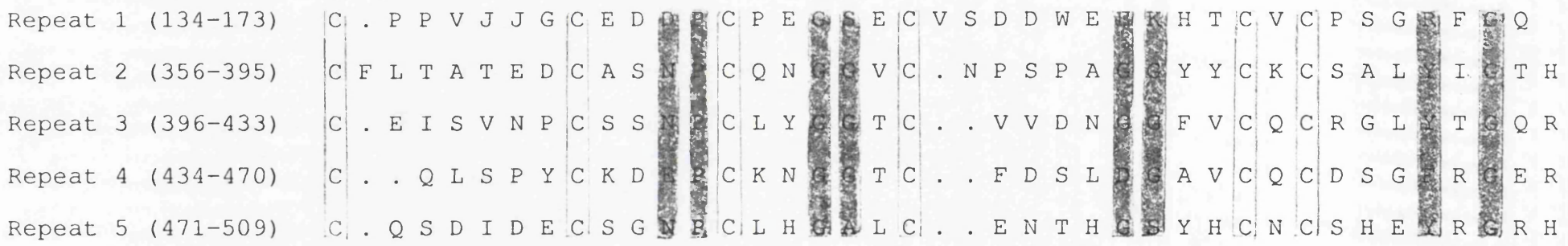


Figure 1.4. pT1AB EGF-like repeats

Alignment of 5 EGF-like cysteine-rich repeats in the predicted amino acid sequence of pT1AB. The conserved cysteines are boxed while the locations of other consensus sites are shaded. The consensus sequences are based on the presence of a specific amino acid at a given position within each repeat more than 50% of the time.

the time. Such repeats are homologous to the epidermal growth factor and other proteins containing EGF-like repeats (see 1.8.).

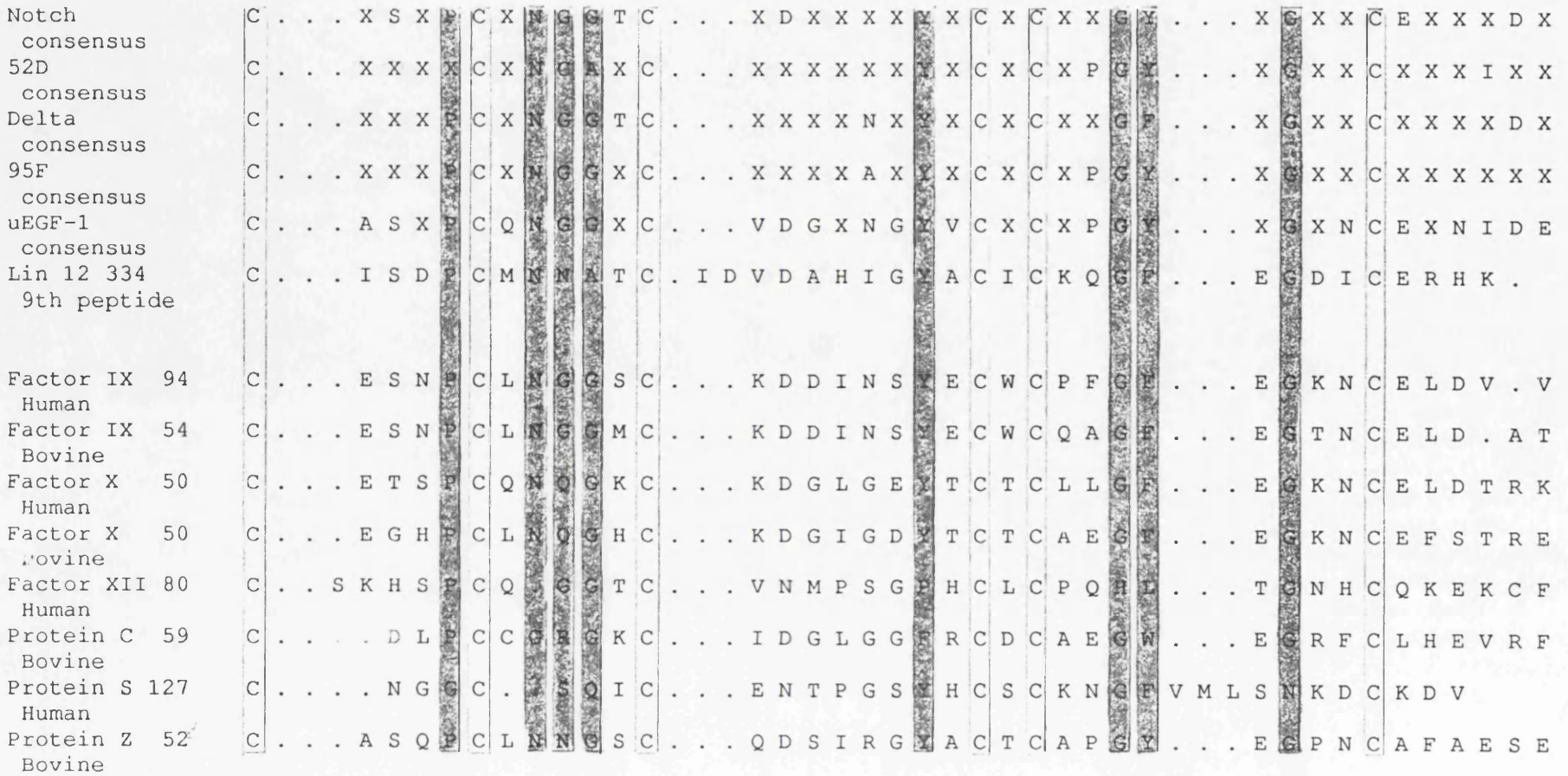
Thirdly, the amino acid sequence has also been examined for the occurence of potential N-linked glycosylation sites (tripeptides NXT and NXS), 3 of these tripeptides have been found and are indicated in figure 1.2.C and Figure 1.3. One such tripeptide is at amino acid 63, near the N-terminus. The other two are at amino acids 376 and 499, both are within the cysteine-rich repeats in the predicted extracellular domain. The nucleotide and protein sequences of pT1AB have been compared with the sequences in genbank and databases. There are no significant similarities with other known sequences. Preliminary northern blot analysis with pT1A among various cell lines including those of T cell and B cell origin, revealed that the gene expression is restricted to T cell lines with different message sizes (Ms J Dunne, personal communication).

1.8 The relationship between pT1AB and EGF-like proteins

The amino acid sequence of pT1AB reveals two segments of highly repeated cysteine-rich elements (see 1.7). They are found to be homologous to a set of proteins, of which the prototype is epidermal growth factor (EGF). The EGF-like protein family represents a rapidly expanding collection of proteins with a variety of functions (Table 1.4). The common feature of the family is that all members of the family exhibit one or several EGF-like repeats in their

Table 1.4 Molecules of the EGF-like proteins

Proteins	Amino acids No or MW (kd)	EGF-like repeat No.	Function
mEGF	53 a.a.	1	See Table 1.5
mEGF precursor	1,217 a.a.	9	Gives rise to EGF
EGF receptor	1186 a.a.	4 + 2 half repeats	Receptor for EGF and EGF-like growth factors
rTGF-1	50 a.a.	1	Transforming activity
VGF	140 a.a.	1	Transforming activity
LDL receptor	839 a.a.	8	Receptor for low density lipoprotein
Thrombomodulin	?	6	Receptor for thrombin
Factor IX	56 kd	2	Protease zymogen
Factor X	56 kd	2	Protease zymogen
Factor XII	80 kd	2	Protease zymogen
Notch	2703 a.a.	36	Neurogenesis in *Drosophila*
Delta	?	4	The same as above
Lin 12	?	11	Neurogenesis in *C. elegans*
MEL-14	372 a.a.	1	Lymphocyte homing receptor
Perforin	71 kd	2	Membrane-disrupting protein
C8 α	553 α.α.	2	Membrane-disrupting protein
C8 β	536 a.a.	2	Membrane-disrupting protein
C9	537 a.a.	2	Membrane-disrupting protein
pT1AB	More than 806 a.a.	5	Unknown


Notch consensus C . . . X S X P C X N G G T C . . . X D X X X X Y X C X C X X G Y . . . X G X X C E X X X D X
52D consensus C . . . X X X X C X N G A X C . . . X X X X X X Y X C X C X P G Y . . . X G X X C X X X I X X
Delta consensus C . . . X X X P C X N G G T C . . . X X X X N X Y X C X C X X G F . . . X G X X C X X X X D X
95F consensus C . . . X X X P C X N G G X C . . . X X X X A X Y X C X C X P G Y . . . X G X X C X X X X X X
uEGF-1 consensus C . . . A S X P C Q N G G X C . . . V D G X N G Y V C X C X P G Y . . . X G X N C E X N I D E
Lin 12 334 9th peptide C . . . I S D P C M N N A T C . I D V D A H I G Y A C I C K Q G F . . . E G D I C E R H K .
Factor IX 94 Human C . . . E S N P C L N G G S C . . . K D D I N S Y E C W C P F G F . . . E G K N C E L D V . V
Factor IX 54 Bovine C . . . E S N P C L N G G M C . . . K D D I N S Y E C W C Q A G F . . . E G T N C E L D . A T
Factor X 50 Human C . . . E T S P C Q N Q G K C . . . K D G L G E Y T C T C L L G F . . . E G K N C E L D T R K
Factor X 50 Bovine C . . . E G H P C L N Q G H C . . . K D G I G D Y T C T C A E G F . . . E G K N C E F S T R E
Factor XII 80 Human C . . S K H S P C Q . G G T C . . . V N M P S G P H C L C P Q H L . . . T G N H C Q K E K C F
Protein C 59 Bovine C . . . . D L P C C G R G K C . . . I D G L G G F R C D C A E G W . . . E G R F C L H E V R F
Protein S 127 Human C . . . . N G G C . . S Q I C . . . E N T P G S Y H C S C K N G F V M L S N K D C K D V
Protein Z 52 Bovine C . . . A S Q P C L N N G S C . . . Q D S I R G Y A C T C A P G Y . . . E G P N C A F A E S E

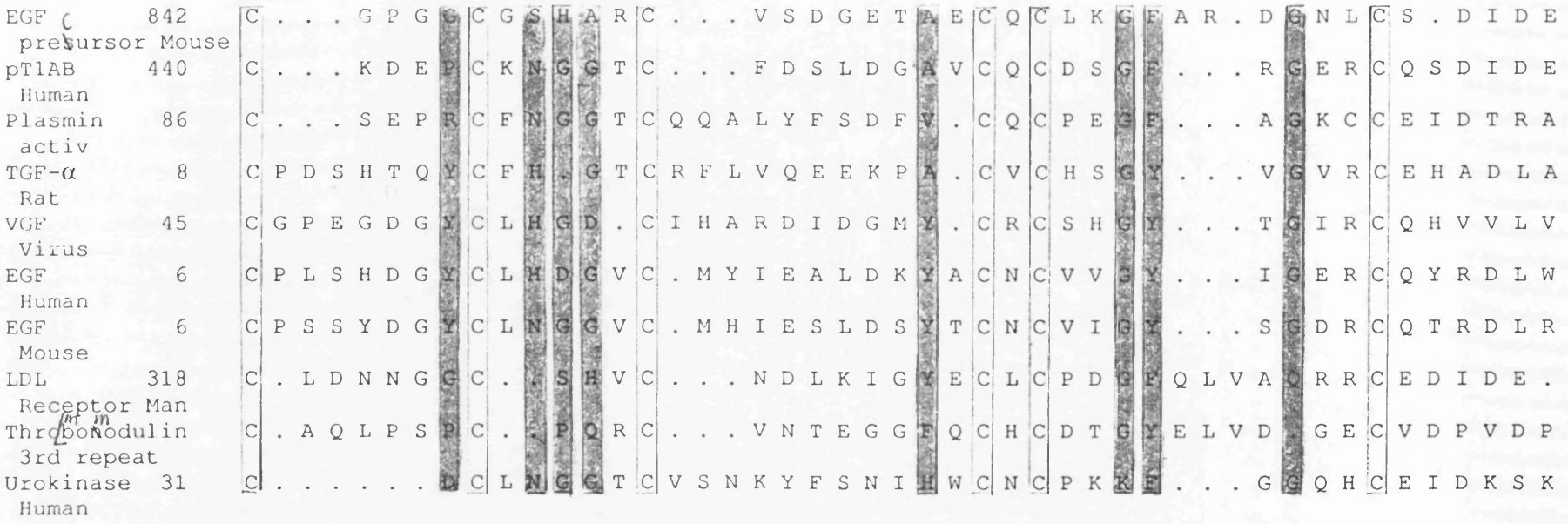


Figure 1.5 Protein sequences homologous to EGF- like repeats

Homologous sequences from invertebrates and vertebrates containing the characteristic EGF-like motif are compared. The number before each sequence refers to the position of the start of the EGF-like repeat within the total protein sequence. Where consensus repeat sequences are used they are based on the presence of a specific amino acid at a given position greater than or equal to 50% of the time. The conserved cysteine residues are boxed, while shading indicates a consensus among the protein sequences.

sequences. In addition to EGF itself, a number of receptors (receptors for low density lipoprotein and thrombomodulin (1.8.3); growth factors such as TGF-α, VGF (1.8.4); proteins involved in blood coagulation; neurogenic genes in invertebrates (1.8.2); as well as some molecules in the immune system have been shown to contain the EGF motif in their sequences (Table 1.4, Figure 1.5). The discovery of pT1AB cDNA containing EGF-like repeats with its restricted distribution in T cell lines prompts the suggestion that the gene product may play a role in supporting proliferation in these malignant cells. The elucidation of the function of the gene product will not only provide clues to the function of the gene product but also be helpful in understanding the EGF-like proteins in general. It should be noted that since the overall sequence homology of the EGF-like genes is low and the spacing between the cysteines in the repeats somewhat variable, inferences as to common functions of the gene products are speculative.

1.8.1 Epidermal growth factor and its receptor

The existence of an "epidermal growth factor (EGF)" was first detected about 30 years ago when injection of extracts of the submaxillary gland into newborn animals induced precocious eyelid opening and incisor eruption (Cohen, 1959; 1962). This was due to a direct stimulation of epidermal growth and keratinisation (Cohen and Elliott, 1962; Cohen, 1965). Mouse EGF (mEGF) is a single polypeptide chain of 53 amino acid residues. The primary amino acid sequence of mEGF includes three intramolecular disulphide bonds which are required for its biological activities (Figure 1.6) (Taylor et al, 1972; Savage et al., 1972). Functional studies with synthetic peptides from EGF revealed that amino acids 20-31 of EGF are important for EGF function (Komoriya et al., 1984).

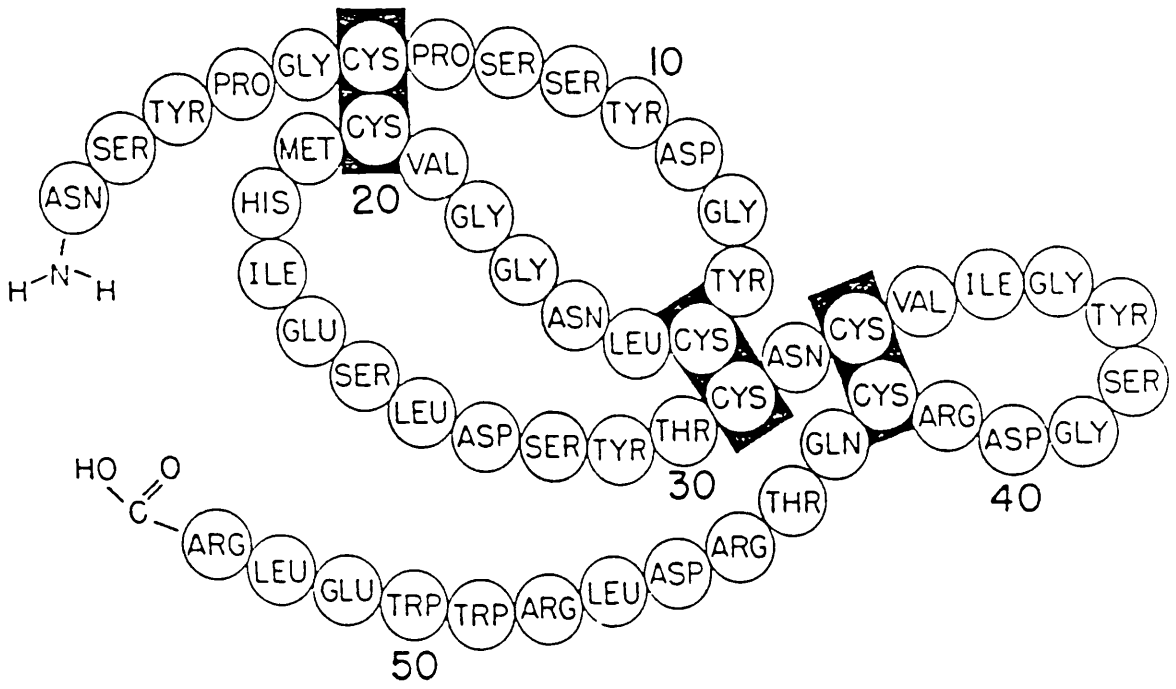


Figure 1.6 Amino acid sequence of mEGF with positions of disulphide bonds (Savage et al.,1973)

EGF is synthesised in the form of a large protein precursor of 1217 amino acids, which includes an internal stretch of 21 hydrophobic amino acids, flanked by polar residues, which could anchor the precursor in the membrane and would divide it into a 158 amino acid cytoplasmic domain and a 1,038-amino acid extracellular domain. The EGF precursor includes nine related peptide units, arranged in two groups separated by unrelated protein sequences (Scott et al., 1983; Gray et al., 1983); mature EGF is the ninth peptide in this arrangement and is liberated from the precursor by proteolytic cleavage at arginine residues. The precursor is more than 20 times larger than the mature growth factor. The significance of the phenomenon is not clear. There is a suggestion that the EGF precursor may be a membrane protein in addition to being the precursor of EGF (Pfeffer and Ullrich, 1985). EGF has been demonstrated to elicit significant biological responses in intact animals, organ cultures and in cell culture systems (Table 1.5). It has been shown to play an important role in many aspects of eukaryotic cell control, acting as signals for proliferation, growth inhibition and differentiation. Studies of human EGF have revealed that both mouse and human EGF share similar chemical characteristics and exhibit identical biological activities (Cohen and Carpenter, 1975). This indicates that these polypeptides have been retained throughout a long evolutionary process and probably have a function of general significance in nature.

The function of EGF is mediated via the EGF receptor, which, with the exception of haemopoietic cells, is detectable on a large variety

Table 1.5 Biological effects of EGF (adapted from Carpenter, 1979)

In vivo

Accelerated proliferation/differentiation
- of skin tissue
- of corneal epithelial tissue
- of lung and tracheal epithelia

Potentiation of methylcholanthrene carcinogenesis
Inhibition of gastric acid secretion
Increased activity of ornithine decarboxylase and accumulation of putrescine
Formation of fatty liver
Increase of disulphide group content in skin
Hepatic hypertrophy and hyperplasia
Potentiation of cleft palate

Organ cultures

Accelerated proliferation/differentiation
- of skin tissue
- of corneal epithelial tissue
- of mammary gland epithelial tissue

Induction of ornithine decarboxylase and accumulation of putrescine
Enhanced protein synthesis, RNA synthesis
Inhibition of palate fusion

Cell culture

Increased transport
- α-aminoisobutyrate
- deoxyglucose
- K^+

Activation of glycolysis
Stimulation of macromolecular synthesis
- hyaluronic acid
- RNA
- protein
- DNA

of cell types or tissues. The EGF receptor molecule is a single polypeptide chain of 1,186 amino acid residues that is divided into two domains by a hydrophobic membrane anchor sequence: an extracellular ligand binding site and a cytoplasmic growth factor-sensitive kinase domain having both the kinase activity and the autophosphorylation sites (Downward et al., 1984; Ullrich et al., 1984). The extracellular domain is of 621 amino acids which contain 51 cysteine residues, most of which are concentrated within two regions of about 170 amino acids located at positions 134-313 and 446-612. Alignment of these cysteine-rich sequences with one another reveals similarities in the spacing of the individual cysteine residues, possibly reflecting the involvement of these residues in the formation of two repeated structures and a common evolutionary origin by gene duplication for this part of the receptor. The pattern of cysteine spacing of the EGF receptor is different from that of the EGF precursor though they both contain cysteine-rich regions.

The 524 amino acid cytoplasmic domain of the EGF receptor contains a long core region of approximately 376 residues exhibiting extensive homology with v-*erb*-B oncogene (95% of the residues in this region are conserved). A 244 residue-long stretch within this region contains 60 residues (24.6%) which are conserved in other members of the tyrosine kinase family, of which the prototype is generally considered to be the *src* kinase of the Rous Sarcoma Virus.

Like several other growth factors, the binding of EGF to its cell surface receptor triggers a cascade of intracellular events, including induction of a tyrosine kinase activity intrinsic to the EGF receptor (Carpenter, 1987). Recently it has been demonstrated that the EGF receptor catalyses the phosphorylation of phospholipase C (PLC) (Meisenhelder et al., 1989). The phosphorylation stimulated by EGF is independent of receptor internalisation and extracellular calcium (Margolis et al, 1989; Wahl et al., 1989). PLC is a key enzyme responsible for reactions resulting in the production of intracellular second messenger molecules which transmit and amplify the extracellular signal initiated by the binding of EGF. This cascade ultimately stimulates cell growth (Ullrich et al., 1984; Berridge, 1987).

1.8.2 EGF-homologous proteins involved in neurogenesis in invertebrates

Neurogenesis, the process of segregation of neuroblasts from the ectodermal germ layer to form the primordium of the central nervous system, is mediated by neurogenic genes (Poulson, 1937; Wright, 1970; Lehmann et al., 1981; 1983; Campos-Ortega, 1985). The overall function of these gene products, revealed by studying function loss mutants in Drosophila, appears to be to provide a regulatory signal that leads to epidermal commitment. In these studies the loss mutations result in the development of all cells of the neurogenic ectoderm as neuroblasts, instead of the segregation between epidermal and neural tissues as in wild type individuals during ontogeny.

The similarity of phenotype deriving from mutations at the zygotic neurogenic loci, Notch (N) (Wharton et al., 1985), mastermind (mam), Delta (Dl) (Vassin et al., 1987; Knust et al., 1987), and slit (Rothberg et al., 1988) genes of *Drosophila melanogaster* and Lin-12 gene of *Caenorhabditis elegans* (Greenwald, 1985; Seydoux amd Greenwald, 1989) and a gene in sea urchin, uEGF (Hursh et al., 1987), prompts the suggestion that they act in concert to define the neural/epidermal dichotomy. The common features of these genes are that they all exhibit a striking structure consisting of a number of (36 fold for the Notch) tandemly repeated arrays of a cysteine-rich 40 amino acid sequence (EGF-like repeats). They show sequence homology with each other. Some of the members have been cloned by cross-hybridisation and washing with low stringency.

The biological role of the EGF-like peptides in the proteins encoded by these genes is still not understood. It is not known whether one or several of these repeated units are cleaved from a precursor protein and function as peptide hormones, in a way reminiscent of EGF itself. Genetic mosaics show that the gene products of Notch and Dl are unable to diffuse over long distances (Dietrich and Campos-Ortega, 1984; Hoppe and Greenspan, 1986), arguing against a hypothetical function of these gene products as peptide hormones. Alternatively, the entire membrane-bound protein (like Notch) may function as a receptor, or even interact directly with neighbouring cells.

1.8.3 EGF-homologous receptors and proteins involved in coagulation

The elucidation of the sequence of the cell-surface receptor for low density lipoprotein (LDL) has revealed an unexpected relationship between its stucture and that of the EGF-precursor (Russell et al., 1984; Yamamoto et al., 1984). Like the EGF precursor, the extracellular domain of the LDL receptor contains eight repeated, cysteine-rich units of 40 amino acids in length. The spatial distribution of the cysteine residues and other consensus features are conserved in each of the repeat units. In addition to non-EGF-like coding region, significant similarity (about 38%) was observed between EGF precursor amino acid residues 565-701 and residues 457-595 in the LDL receptor extracellular domain (Figure 1.7). Sequence homology decreases in regions surrounding this core, but remains statistically significantly (approximately 30%) for several hundred more amino acids. Located on the surfaces of body cells, the receptor binds the major cholesterol-carrying lipoprotein of human plasma, which is required for cell growth and transports it into the cell through the process of receptor-mediated endocytosis (Goldstein et al., 1985). The binding site of the receptor appears to be within the cysteine-rich domain and is stabilised by the disulphide bonds as long as there is no reducing agent present.

Recently, thrombomodulin, a specific endothelial cell receptor for thrombin (Jackman et al., 1986), as well as some soluble factors

involved in coagulation including the blood protein C light chain (Fernlund and Stenflo, 1982), factor VII, factor IX (Kurachi and Davie, 1982; Katayama et al., 1979), factor X (McMullen et al., 1983; Enfield et al., 1980), and factor XII have also been demonstrated to exhibit the EGF-like repeats in their sequences (reviewed in Furie and Furie, 1988). Thrombomodulin may be involved in the internalisation of thrombin. It has been noted that the internalisation and degradation of thrombin by an endothelial cell receptor-dependent mechanism could play a role in the growth of endothelial cells, since peptides derived from the enzyme are quite mitogenic (Bar-Shavit et al., 1985). Factor IX, XII, etc, contain two EGF-like repeats in their zymogen forms (see Table 1.5), however, the EGF-like repeats are not required for their protease activities.

1.8.4 EGF-homologous growth factors

Retrovirus-transformed cells and certain human tumour cells produce transforming growth factor type α (TGF-α) (Todaro et al., 1982). In contrast, control cells or cells infected with transformation-defective viral mutants do not produce and release detectable level of TGF-α. The complete amino acid sequence of rat TGF-α (rTGF-α) has been reported and its biological properties have been compared with those of mouse EGF (Marquardt et al., 1984). Structurally, rTGF-α belongs to the EGF family. Mature rTGF-α, sharing sequence homology with EGF, is a small polypeptide of 50 amino acids with three intramolecular disulphide bonds. Functional assays revealed that rTGF-α binds the EGF receptor and triggers

specific effects, including tyrosine phosphorylation of the EGF receptor, and produces the same biological activities as mEGF.

The cDNA of rTGF-α hybridises to a 4.5 kilobase (kb) messenger RNA that is 30 times larger than necessary to code for a 50 amino acid polypeptide. The mRNA is present not only in retrovirus-transformed rat cells but also at lower level in normal rat tissues. The nucleotide sequence of the cDNA predicts that rTGF-α is synthesised as a larger protein product and the larger form may exist as a transmembrane protein (Lee et al., 1985). Recently, it is shown that rTGF-1 mRNA increased during liver regeneration after partial hepatectomy in rats. The increase of rTGF-α mRNA coincides with an increase in the expression of EGF receptor mRNA during the first day after the operation. It is suggested that rTGF-α may function as a physiological inducer of hepatocyte DNA synthesis during liver regeneration by means of an autocrine mechanism (Mead and Fausto, 1989).

Brown et al reported in 1985 that a 19 kd polypeptide encoded by one of the early genes of vaccinia virus (VV) (Vankatesan et al., 1982) is closely related to EGF and rTGF-α. The sequence of residues 38-91 of vaccinia virus growth factor (VGF) appears to be related to those of rTGF-α and EGF to approximately the same degree that EGF and rTGF-α are related to each other. The three ligands are about 22% identical in amino acid sequence, but bind to the EGF receptor with nearly identical affinities and produce the same responses in target cells (Carpenter and Zendegui, 1986).

1.8.5 EGF-like proteins in the immune system

The EGF-like repeat structure has also been found in the immune system. A collection of membrane disrupting proteins including complement C8α (Rao et al., 1987), C8β (Howard et al., 1987), and C9 (Di Scipio et al., 1984; Shiver et al., 1986; Stanley et al., 1985) show 21-26% identity on alignment with each other. All three chains have a large internal domain almost free of cysteine residues, an N-terminal cysteine-rich domain homologous to LDL receptor non-EGF-like domain (see 1.8.3), and a C-terminal domain homologous to LDL receptor (EGF-like) sequences (Howard et al., 1987). Studies on perforin, the pore-forming protein present in the granules of cytotoxic T lymphocytes, show structural and functional similarity to the complement component C9 (Young et al., 1986). On the basis of similarities in their structure and function, it is suggested that these molecules share a close ancestral relationship and are members of a family of proteins that are capable of induced conformational changes leading to membrane interaction. It is unknown if the EGF-like repeats in these molecules are implicated in their function of membrane disruption.

The lymphocyte antigen identified by the MEL-14 mAb (Gallatin et al., 1983) has been found to be specifically involved in homing to peripheral lymph nodes (PN). The deduced MEL-14 antigen sequence reveals a number of interesting sequence homologies with other proteins. Apart from the lectin-like motif at the N-terminus,

there is a region (residues 160-193) showing a high degree of homology to the EGF-like protein family. The greatest degree of homology in this region is found with the twentieth repeat in the Drosophila neurogenic locus, notch (Lasky et al., 1989). It is suggested that this domain serves to strengthen the interaction between the lymphocyte and endothelium by binding to an EGF-receptor homolog on the endothelium surface.

1.8.6 The evolution of the EGF-like family

EGF-like proteins, as described above, consist of a collection of proteins with similar structures. The most characteristic feature among EGF and its homologous proteins are cysteine residues and other consensus amino acids found in a conserved arrangement. The arrangement is universally present ranging from Drosophila to mammals. Evolutionally these proteins may all originate from a single ancestral protein. The gene for this protein may have undergone duplication and expansion, and its descendents now perform widely divergent functions. It is also possible that the ancestor of all of these proteins was a gene for a small core protein that expanded and subsequently acquired additional domains. Alternatively, the ancestral gene may have encoded a large protein that encompassed all of its modern functions but lost domains selectively after expansion.

The distribution and the function of the EGF gene family is wider than the Ig supergene family, which is mainly concentrated within

the immune and nervous system. This suggests that the EGF-like protein family might have originated earlier during evolution than the Ig supergene family. Like the Ig supergene family, each member of the EGF-like family carries out its own distinct function/s, but one common factor is notable, it is that they are all implicated in extracelluar events and ligand-receptor interactions. A regular spacing of disulphide bonds is known to afford an ordered and energetically stable structure for EGF, which may aid ligand binding and/or receptor aggregation (Carpenter, 1979). The conserved arrangement of cysteines in EGF-like molecules must confer similar functions which have been indispensible during evolution.

1.9 The significance of pT1AB gene

The elucidation of the sequence of the pT1AB gene has revealed an unexpected relationship with the EGF-like protein family which is mainly implicated in receptor and ligand interaction. The structure of the pT1AB gene suggests a receptor-like protein. It resembles the structures of the EGF receptor, EGF precursor and the receptor for LDL, in terms of overall organisation and size. They all exhibit a large extracellular domain which contains several EGF-like repeats, a transmembrane region, followed by a cytoplasmic domain (Figure 1.7.). The incomplete sequence of pT1AB revealed an extracellular domain with 528 amino acids which contains five EGF-like repeats, cysteine-rich units of 40 amino acids in length. The cytoplasmic domain of pT1AB is of more than 250 amino acids (the C-terminus has yet to be cloned). The comparatively larger cytoplasmic domain

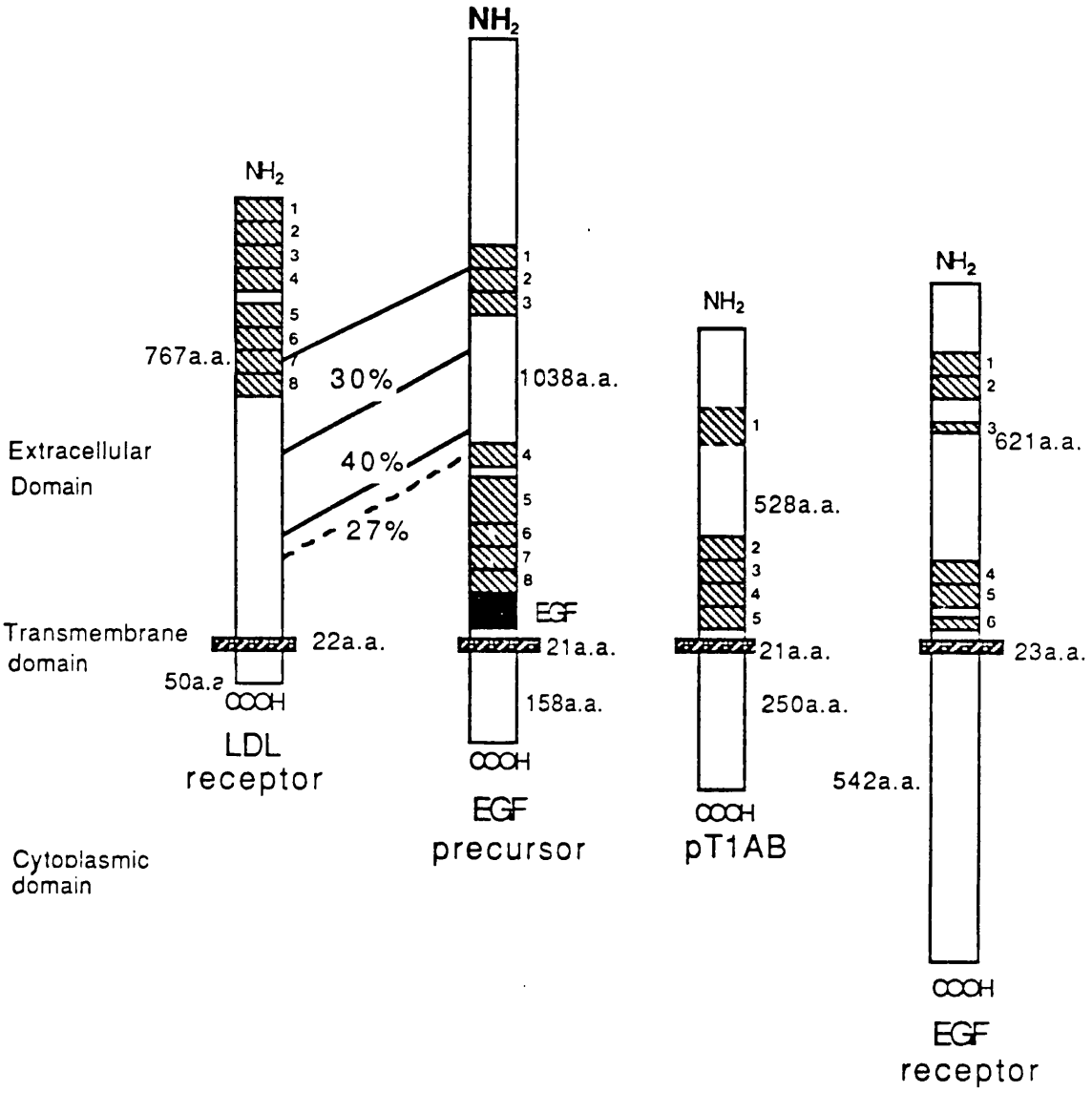


Figure 1.7. Presumed structure of pT1AB and its analogues

Presumed structure of pT1AB is compared with those of LDL receptor, EGF precursor and EGF receptor. Shaded bars represent the membrane bilayer, hatched, numbered boxes depict cysteine-rich repeat units. Sizes of structural domains are given in amino acids (aa). The extracellular domain of the mouse EGF precursor includes a putative signal sequence.

is characteristics of many receptors such as receptors for EGF, insulin, CSF-1, and PDGF. The large cytoplasmic domains of these receptors are mainly engaged in signal transduction initiated by the interaction with their ligands.

Initial northern blot analysis has revealed that the gene has a restricted distribution among malignant T cell lines. In an earlier section it has been noted that the aberrant expression of some surface molecule (CD25, the receptor for IL-2) may be associated with abnormal proliferation of certain malignant cells in which the cells are sustained in a proliferative phase via an autocrine mechanism. The restricted distribution of the gene product among malignant cells and the receptor-like structure of the gene may suggest a possible role of the gene product in supporting cell proliferation.

1.10 Aims

pT1AB is a partial cDNA clone which shows restricted distribution among T cell lines by northern blot analysis. Its sequence was found unexpectedly to share homology with the EGF-like protein family. The principal aim of this thesis was to identify the protein product(s) of the gene and study their distribution and function. An initial objective was to raise polyclonal and monoclonal antibodies in order to facilitate these studies.

CHAPTER TWO
MATERIALS AND GENERAL METHODS

2.1 Materials

2.1.1 Plastics

All plastics were of tissue culture grade polystyrene unless stated otherwise.

Sterile disposable containers included:	
30 ml universal container	Sterilin
7 ml bijou bottles	
15 ml V-bottomed tubes	
50 ml specimen container	
200 ml specimen container	
3.5 cm tissue culture dish	
8.5 cm tissue culture dish	
50 ml capped polystryrene tubes	Falcon
14 ml capped polystryrene tubes	
2 ml, 5 ml, 10 ml and 25 ml pippets	
25 cm^2, 75 cm^2 and 175 cm^2 tissue culture flasks	
96-well flat-bottomed multiwell plates	Flow labs
96-well round-bottomed multiwell plates	
both 250 µl capacity/well	
24-well flat-bottomed plates	Costar

2.5 ml capacity/well

Cryotube (1.8 ml capacity)	Nunc
0.2 µm cellulose acetate membrane filters	Gelman Sciences

2.1.2 Glassware

All glassware was soaked overnight in chloros, washed in clean 'N' (Gallay Ltd.) and given two successive rinses in distilled water. Dry glassware was sterilised by cutoclaving at 121^0C for 15 minutes.

2.1.3 Equipment

Zeta-probe membrane	Bio-Rad
Genescreen membrane	NEN
Intensifying film cassette	Du Pont
Hyperfilm	Amersham

2.1.4 Reagents and cell lines

All reagents used were of Analar grade unless stated otherwise.

1) Sera

a. Foetal calf serum (FCS): was obtained from Gibco Europe Ltd.

Batches were screened for their ability to stimulate optimal cell growth in *in vitro* liquid culture systems. FCS was heat-inactivated before use by incubation at 56^0C for 30 minutes.

b. Pooled human A+ serum, was obtained from Sigma.

c. Rabbit, rat and mouse sera were obtained from DAKO.

2) Media

a. RPMI-1640 supplemented with L-Glutamine and 20 mM Hepes was obtained from ICRF media department.

b. Minimum essential medium (MEM): ICRF media department.

c. Methionine free MEM: ICRF media department.

d. LB (Luria-Bertani) medium: ICRF media department.

e. LB with 1.5% Bacto-agar: ICRF media department.

f. M9 medium was prepared as described in Maniatis et al., 1982

g. Phosphate-buffered saline 'A' (PBS) was purchased from Oxoid and dissolved in ddH_2O as required.

3) Lectin

Phytohaemagglutinin from *Phaseolus vulgaris* in purified form was obtained from Wellcome Pharmaceuticals.

4) Antibiotics

Ampicillin and G418 (neomycin) were purchased from BRL.

5) Chemicals

Albumin bovine, Ammonium acetate, β-Mercaptoethanol, 3β-Indoleacrylic acid, Complete Freund's adjuvant (CFA), 4-Chloro-1-Naphthol, Citric acid, Diethyl pyrocarbonate (DEPC), Dithiothreitol (DTT), Diaminoethanetetra-acetic acid disodium salt (EDTA), Ethidium bromide, D-Glucose, Ficoll, Glycerol, 8-Hydroxyquinoline, Imcomplete Freund's adjuvant (IFA), Lithium chloride, L-Tryptophan, $MgCl_2$, 3-(N-Morpholino)propanesulfonic acid (MOPS), NaCl, Nonidet P 40 (NP-40), o-Phenylenediamine (o-PD), Phorbol 12-myristate 12 acetate (PMA), Phenylmethane sulphonyl floride (PMSF), Protein molecular weight standards, Sodium acetate, Sodium phosphate, Sucrose, Trichloroacetic acid (TCA), Tris base, xylene cyanol were purchased from Sigma.

Acrylamide, Agarose, Ammonium persulfate (APS), Cesium chloride, Guanidine isothiocyanate, Low melting agarose, Phenol, Sodium dodecyl sulfate (SDS), N,N,N'N'-Tetramethylethylenediamine (TEMED), Urea were obtained from BRL.

ATP, dATP, dCTP, dGTP, dTTP, Ficoll-Hypaque and CNBr-activated Separose 4B were from Pharmacia.

The following chemicals were purchased from Aldrich:
S-(2-Amino ethyl)-isothiouronium bromide hydrobromide (AET), 2,16,10,14-Tetramethylpentadecane (Pristane).

Acetic acid glacial, Calcium chloride, Chloroform, Dimethyl suphoxide (DMSO), Methanol, Sodium azide, Sodium hydroxide were from Fisons.

Bacto-agar, Casamino acids, tryptone, Yeast extract came from DIFCO.

6) Buffers

DNA loading buffer:

6x buffer
0.25% Bromophenol blue
0.25% Xylene cyanol
25% Ficoll (type 400) in H_2O

DNA denaturing buffer:

0.4 M NaOH
0.6 M NaCl

DNA neutralising buffer:

0.5 M Tris-HCl pH 7.5
1.5 M NaCl

DNA hybridisation buffer:

50% Formamide
1% NaCl
1% SDS
10% Dextran sulphate
100 µg/ml sonicated ssDNA

ELISA:

1.59 g Na_2CO_3
2.93 g $NaHCO_3$
0.2 g NaN_3
20.33 mg $MgCl_2$

Adjust to one liter (pH 9.5).

2x HBSP:

1.5 mM Na_2HPO_4
10 mM KCl
280 mM NaCl
12 mM Glucose
50 mM HEPES pH 7.0

Lysis buffer for SRBC (Gey's haemolytic solution)

Solution A: NH_4Cl, 35 g
KCl, 1.85 g
$Na_2HPO_4.2H_2O$, 1.5 g
KH_2PO_4 0.119 g
Glucose, 5 g
Gelatin (Difco), 25 g
1% Phenol red, 1.5 ml

Solution B: $MgCl_2.6H_2O$, 4.2 g
$MgSO_4.7H_2O$, 1.4 g
$CaCl_2$, 3.4 g

Solution C: $NaHCO_2$, 22.5 g

Each solution was made up to one liter with distilled water and autoclaved to sterilise. Immediately prior to use, a solution containing 2 ml A, 0.5 ml B, 0.5 ml C and 7 ml ddH_2O was prepared (Dresser, 1978).

Oligolabelling buffer (OLB):

OLB is made from the following components.

Solution O: 1.25 M Tris-HCl pH 8.0
0.125 M $MgCl_2$
Store at 4^0C.

Solution A: 1 ml Solution O
18 ul 2-Mercaptoethanol
5 ul dATP (0.1 M)
5 ul dTTP (0.1 M)
5 ul dGTP (0.1 M)
Store at -20^{0}C.

Solution B: 2M Hepes, titrated to pH 6.6 with 4 M NaOH.
Store at 4^{0}C.

Solution C: Hexadeoxyribonucleotides, evenly suspended in TE at 90 OD units/ml.
Store at -20^{0}C.

Mix solution A:B:C in a ratio of 100:250:150 to make OLB. Store at -20^{0}C.

Phosphate/citrate buffer pH 5.0

25.7 ml of 0.2 M Na_2HPO_4 was mixed immediately before use with 24.3 ml of 0.1 M Citric acid and the volume made up to 100 ml with double distilled H_2O.

RNA loading buffer:

6x buffer
50% Glycerol
1 mM EDTA
0.4% Bronophenol blue
0.4% Xylene cyanol

RNA hybridisation buffer:

50% Formamide
1% NaCl
1% SDS
10% Dextran sulphate
100 µg/ml sonicated ssDNA

RNA running buffer:

10x buffer
200 mM MOPS
50 mM NaAc
10 mM EDTA

SDS-PAGE

Running buffer:

0.025 M Tris base
0.192 M Glycine
0.1% SDS

Sample buffer:

2% SDS
10% Glycerol
0.125 M Tris-HCl pH 6.8
2 mM PMSF

Staining solution

50% Methanol
10% Acetic acid
0.125% Coomassie brilliant blue R-250

Destaining solution

50% Methanol
10% Acetic acid

SSC: 20x buffer

174 g NaCl
27.6 g NaH_2PO_4
7.4 g EDTA

Adjust pH to 7.4 with NaOH and adjust volume to one liter.

TAE:

0.04 M Tris-acetate
0.001 M EDTA

TBE:

0.089 M Tris-borate
0.089 M Boric acid
0.002 M EDTA

TBS:

0.02 M Tris-HCl pH 7.5
0.5 M NaCl

TE: 10 mM Tris-HCl
1 mM EDTA

Western blot transfer buffer:

0.192 M Glycine
0.025 M Tris pH 8.3

7) Antibodies

a. Antibodies used in this thesis are listed in Table 2.1

Table 2.1 Antibodies used in this thesis

mAb	Specificity	Isotype	Source	Reference
NA134	CD1	IgG2a	AJ McMichael	McMichael et al., 1979
OKT11	CD2	IgG2a	ATCC	Meuer et al., 1982a, 1982b
UCHT1	CD3	IgG1	PCL Beverley	Beverley and Callard, 1981a.
OKT4	CD4	IgG2b	ATCC	Kung et al., 1979
UCHT2	CD5	IgG1	PCL Beverley	Beverley and Callard, 1981b.
3A1	CD7	IgG1	ATCC	Haynes et al., 1979
UCHT4	CD8	IgG2a	PCL Beverley	Beverely, 1982

2D1	CD45	IgG1	PCL Beverley	Bradstock et al., 1980
L243	HLA-DR	IgG2a	ATCC	Lampson and Levy, 1980

b. Conjugated antibodies

Peroxidase conjugated rabbit anti-mouse Ig (RaMHRP)	DAKO
Peroxidase conjugated rabbit anti-rat Ig (RaRHRP)	DAKO
FITC conjugated rabbit anti-mouse Ig (RaMFITC)	DAKO

c. Production of ascites

BALB/c female mice were primed with pristane, 0.4 ml per mouse. After 10 days the primed mice were injected intraperitoneally (I.P.) with $1x10^7$ hybridoma cells washed free of medium and resuspended in RPMI1640. Swelling in the mice was apparent 6-10 days later at which time the ascitic fluid was harvested, separated from blood and pristane and stored at -20^0C.

d. Purification of antibody from ascites

Monoclonal antibodies were precipitated by 50% saturated ammonium sulphate and purified from a protein A-sepharose column with a stepwise pH gradient. Ascites was precipitated by adding an equal volume of cold saturated ammonium sulphate (in distilled water) dropwise and leaving the solution overnight at 4^0C. The precipitate was microfuged for 2 minutes, redissolved in 0.1 M

phosphate buffer (pH 8.0) and dialysed extensively against this buffer. A 2 ml protein A-Sepharose column was equilibrated with phosphate buffer. The dialysed protein was loaded into the column and left overnight at 4^0C. Unbound material was eluted with the phosphate buffer until the O.D. 280 nm of the eluate reached zero. A stepwise pH gradient was then applied to the column to elute Ig. IgG_1 antibody was eluted with 0.1 M citrate buffer pH 6.0, IgG_{2a} antibody was eluted with 0.1 M citrate buffer pH 4.5 and IgG_{2b} was finally eluted with 0.1 M citrate buffer pH 3.5. The fraction containing the antibody was dialysed against PBS and the quantity of Ig was determined by measuring an aliquot at 280 nm (an O.D. 280 of 1.4 was taken to correspond to 1 mg/ml of Ig).

8) Enzymes

The following enzymes were purchased from BRL:
Bam HI, Bgl II, EcoR I, Hind III, Sal I, Xho I, Large fragment of DNA polymerase I (Klenow fragment), T4 DNA ligase, T4 polynucleotide kinase.

Lysozyme, lactoperoxidase, D-Glucose oxidase were from Sigma.

RNase free DNase was from Pharmacia.
RNase (Sigma) that is free of DNase was prepared as described in Maniatis.

Thermalbase^{TM} Taq polymerase sequencing kit was purchased from Stratagene.

9) Radioisotopes

All the radioisotopes used were from Amersham International.
They include ^{125}I, ^{35}S-Methionine, ^{32}P-dATP and ^{32}P-dCTP.

10) Bacterial strains

The following strains of Escherichia coli were used:

JM101	(Amersham International)
C600	(Clontech Laboratories, Inc.)
DH5-α	(BRL)

11) DNA vectors and probes:

The following vectors were used:

pUC8	(BRL)
pIRV	(Dr JP Morgenstern, ICRF)
pMT2	(Dr I. Goot, Ludwig Institute for Cancer Research)
pATHn	(Spindler et al., 1984)
pT1-2	(CD5 probe from Professor Herzenberg, Stanford USA)
pT1A	Ms J. Dunne (ICRF)
pT1B	Ms J. Dunne (ICRF)

12) Human tissues

Human tonsils, spleens and intestinal lymphoid tissues were donated by hospitals in the Greater London area.

13) Cells and cell lines

Sheep red blood cells (SRBC) were obtained from Tissue Culture Services (Slough)

Cell lines were maintained in RPMI-1640 containing L-Glutamine, 20 mM Hepes, and 10% heat inactivated FCS. Table 2.2 lists all the cell lines used and their sources.

Table 2.2 Cell lines used in this thesis

Human cell line	Specificity and origin
CEM	T-ALL Foley et al., 1965
HPB-ALL	T-ALL Minowada et al, 1978
HSB-2015	T-ALL Adams et al., 1968
HUT78	T-ALL Gazdar et al., 1980
J6 and JM	Sublines of Jurkat (T-ALL) Gillis and Watson 1980
MOLT4	T-ALL Minowada et al., 1972
SUPT1	T-ALL Smith et al., 1986
PEER	from Lanier et al

GK	EBV-transformed B-lymphoblastoid line derived from patient with Lesch-Nyhan's syndrome (established by P. Beverley (ICRF HTIG).
Raji	B-lymphoblastoid line (EBV+) derived from a patient with Burkitt's lymphoma
LICR-LON-H-My2	B cell line derived from plasma leukaemia line ARH-77
HFB-1	B-myeloma line obtained from RJ Hartsman, Naval Medical Research Institute, Bethesda.
L6	Subline of U266 myeloma cell line, obtained from L. Olsson 1982.
WMPT	B cell line derived from patient with Waldenstrom's macroglobulinaemia, obtained from D. Crawford.
NALM6	ALL derived line.
Cole	B cell line derived from patient with Hodgkins disease
K562	Undifferentiated myeloid blast cell line from a patient with chronic myelogenous leukaemia (Lozzio and Lozzio, 1975).
U937	Monocytic cell line derived from a patient with histiocytic lymphoma. (Sundstrom and Nilson, 1976)
HL-60	Promyelocytic cell line (Collins et al., 1977)
PANT	Pancreatic tumour line from J. Hermon Taylor
L5174T	Colon cancer line from P Richman ICRF
CNE	Nasopharyngcal cancer line from Crawford

212	Neuroblastoma line from J. Kemshead
MDA157	Breast tumour line
MCF7	Breast tumour line
UCHNCu	Small cell lung cancer cell line started by F. Moss 1985
H-82	Small cell lung cancer cell line
T24	Bladder cancer line

Mouse cell lines

L cells	(P. Goodfellow ICRF)
NS-1/1Ag4.1	(Kohler et al., 1976)

Rat cell line

Y3-AG1.2.3	(Galfre et al., 1979)

2.2 General methods

2.2.1 Cryopreservation of cells

Cells for storage in liquid nitrogen were suspended in FCS and 10% DMSO on ice and rapidly aliquoted into 1.8 ml cryotubes and stored overnight in a closed polystyrene container at -70^{0}C. The tubes were then transferred to liquid nitrogen tanks for long-term storage.

2.2.2 AET-treated sheep red blood cells

Sheep red blood cells (SRBC) were washed 3 times with sterile PBS. 1 volume of packed SRBC was mixed with 4 volumes of AET at 40.2 mg/ml pH 9.0 and incubated for 15 minutes in a 37^0C water bath with frequent mixing. SRBC were then washed 5 times with PBS and stored as a 4% solution in RPMI-1640 supplemented with 10% FCS at 4^0C.

CHAPTER THREE
RAISING ANTIBODIES TO SYNTHETIC PEPTIDES

3.1 Introduction

The basis for the utilisation of synthetic peptide as immunogen was provided by early studies on the antigenicity of proteins. Macromolecular proteins usually express a large number of antigenic determinants. There are two types of epitopes: sequential and conformational (Sela, 1969). Whereas a sequential determinant is viewed as a sequence of several amino acid residues in its unfolded form, a conformational determinant is defined by a number of residues that are maintained in a particular conformation, to which long-range interaction at the levels of secondary, tertiary, and quaternary structure contribute.

A synthetic peptide usually contains a sequence of several continuous amino acids from a protein. If the sequence of residues happens to be exposed at the surface of the native protein it represents, then it is very likely that antibody specific for the peptide will also recognise the native protein. Successful examples of such antibody using peptides as immunogens have been reported.

Arnon et al in 1971 first showed that a synthetic peptide from egg-white enzyme elicited antibodies capable of recognising the native protein. Studies of the synthetic approach to vaccination for the influenza virus provided more data on this issue. After the antigenic determinants in the influenza hemagglutinin (HA) were identified by serological analysis, a peptide of 18 amino acid

residues corresponding to the sequence 91-108 of the HA molecule successfully elicited antibodies that recognise the native protein. Mice immunised with the peptide conjugated with tetanus toxoid were partially protected against further challenge infection with the virus (Muller et al. 1982).

Encouraged by these successful examples and the availability of the primary sequence of pT1A (the 500 bp insert) at the time, we decided to use synthetic peptides from the sequence as the first choice to produce antibodies to the gene product. Two peptides from pT1A (which were later on found to be within the cytoplasmic domain) at amino acids 33-43 and 58-69 with the sequences of NH_2-P S D S D S I Q K P S C-COOH and NH_2-C L S K K P L E E K P S-COOH respectively, predicted to be immunogenic by Dr Rothbard in ICRF, (Hancock and Evan, 1990) were synthesised and conjugated to either BSA or thyroglobulin* The immunisation schedules and characterisation of polyclonal and monoclonal antibodies from immunised mice and rats are presented in this chapter.

* using m-Maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) to cross-link the thiol group of cysteine on the peptide to an amino group on the carrier (Hancock and Evan, 1990).

3.2 Methods

3.2.1 Immunisation of mice and rats

1) Protocol I

Female or male BALB/c mice or Lou rats were used. The antigen injected, the amount, the route, the interval between the injections and the adjuvants used are shown in Table 3.1. Two adjuvants were

employed, complete or incomplete freund's (CFA or IFA). Mice or rats were sacrificed three to four days after the final injection and the serum and spleen harvested.

Table 3.1 Immunisation schedule

Peptide	ug/mouse	Adjuvant	Route	Day
33-43 or 58-69 conjugated with thyroglobulin	100 ug/mouse	CFA	S.C.	0
The same peptide conjugates	Same amount	IFA	S.C.	14
The same peptide conjugates	Same amount	IFA	S.C.	28
The same peptide conjugates	Same amount	No adjuvant	I.V.	42

Notes:

a. S.C. stands for subcutaneous injection; i.v. stands for intraveneous injection.

b. Three hundred micrograms of peptide-thyroglobulin were used for each rat.

c. The mice and rats were tail bled to test for specificic antibodies to the peptides after the third boost.

d. 3-4 days after the i.v. boost, the mice and rats were sacrificed for fusion

2) Protocol II (Intrasplenic immunisation)

Young adult mice (8-20 weeks) were anaesthetised by intraperitoneal injection of 4 mg/kg of "Hypnorm" (fluanisone) and 2 mg/kg of "Hypnoval" (diazepam). The spleen was exposed by laparotomy and injected with 25-50 μg of antigen in saline (50 ul) using a 26 gauge needle. The wound was closed by silk sutures to

the muscle layer and separate skin sutures. Five days later the mouse was sacrificed and the spleen removed for fusion.

3.2.2 Fusion procedure

The fusion procedure was based on the method of Kohler and Milstein (1975).

1) Fusion procedure for mouse mAbs

A mouse myeloma cell line NS-1 was used for fusion. The NS-1 cells were recovered from liquid nitrogen about 10 days before a fusion and maintained in log phase of growth in RPMI 1640 with 10% foetal calf serum. They were subcultured 1:5 every 3 days. The excised spleen was disrupted, the cell teased out into RPMI 1640, mixed with NS-1 cells ($2x10^7$ cells) and washed twice in RPMI 1640 to remove serum. The mixed cell pellet was carefully resuspended, over a period of 1 minute, in 1 ml of 50% polyethylene glycol (PEG) 1500 at 37^0C and then incubated for 1 minute at 37^0C. 20 ml of RPMI 1640 was slowly added, with gradual resuspension of the fused cells, over a period of 5 minutes.

The cells were washed and resuspended in complete medium supplemented with hypoxanthine, aminopterin and thymidine to give a final concentration of 10^{-4}M hypoxanthine, $4x10^{-7}$M aminopterin and $1.6x10^{-5}$M thymidine (HAT medium), and plated into four 96-well flat bottomed tissue culture plates. Half of the

medium in each well was replaced after 6 days and again at approximately 3 days intervals. Hybridomas were visible from the 10th day onward. Culture supernatant was harvested and tested for reactivity with peptide when the colonies occupied at least one-third of the well and at least two days after the medium was changed.

2) Fusion procedure for rat mAbs

The fusion procedure was similar to that performed for mouse monoclonal antibodies. The Y3 rat myeloma cell line, was used instead of NS-1. The splenic cells and Y3 cells were mixed at a ratio of 6 x 10^7 Y3 cells for every 10^8 spleen cells. The fused cells were plated in 24 well plates in complete medium at 1 ml/well. After 24 hours an equal volume of 2 x HAT was added together with 4 x 10^{-5} M β-mercaptoethanol. The subsequent medium changes and screening were similar to those used for mouse monoclonal antibody production.

3.2.3 Screening of hybridoma supernatants (ELISA)

The presence of mAbs specific for peptide was detected by its ability to bind the peptide BSA conjugates (Figure 3.1). The mice or rats were immunised with peptide thyroglobulin conjugates, therefore antibodies binding to peptide BSA conjugates were presumed to be specific for peptide. The screening assay is a modification of one used to detect reactivity with soluble proteins (Melero and Gonzales, 1984).

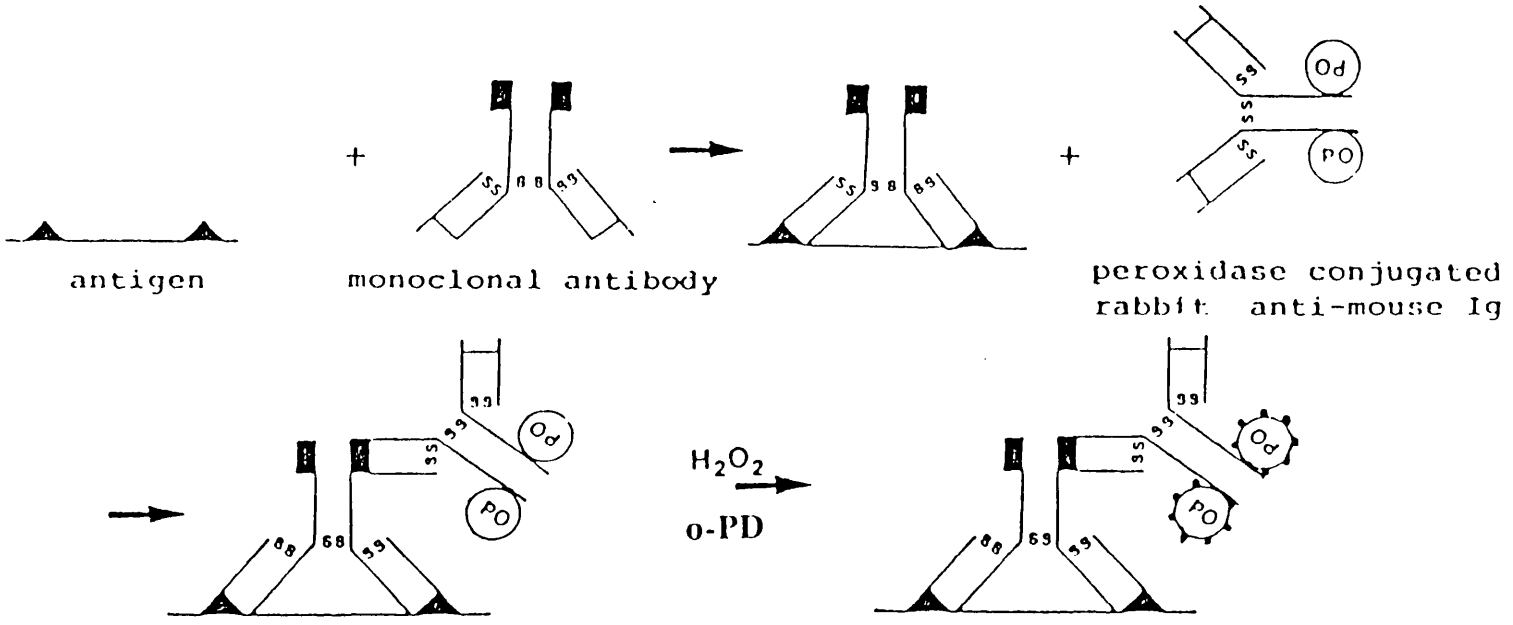


Figure 3.1 The indirect immunoperoxidase assay (ELISA)

Multiwell plates were coated with 100 ul of peptide BSA conjugate at 50 ug/ml in ELISA buffer for one hour at 37^0C or overnight at 4^0C. Each well was blocked with 3% BSA in PBS at 37^0C for 2 hours before 100 ul hybridoma supernatant was added and incubated for 1 hour at 37^0C. The supernatant was removed and the plates washed. 100 ul RaMHRP or RaRHRP at 1:1000 dilution was then added and incubated for a further hour at 37^0C.

The bound RaMHRP was visualised by adding 100 ul per well of the peroxidase substrate containing o-PD at 20 mg/ml in 0.1 M citrate phospate buffer, pH 5.0, with 0.05% Hydrogen peroxide. A brown colour gradually appeared within ten minutes in the dark at room temperature and the absorbance was read at 492 nM on a Titertek Multiscan.

3.2.4 Cell surface binding assay

The procedure is illustrated in Figure 3.2 and was similar to that described in 3.2.9. About $2x10^5$ cells were incubated in round-bottom microtiter plates at 4^0C for 30 minutes with 50 ul of 5% heat-inactivated rabbit serum in MEM containing 2 % FCS and 0.02% sodium azide. Cells were washed once in MEM and incubated with 50 ul of the appropriate antibody for 30 minutes. After 3 washes in MEM, 50 ul of RaMHRP at 1:500 dilution was added to the cells and incubated for another 30 minutes followed by 3 washes. It was finally washed once in PBS before the addition of 100 ul/well of

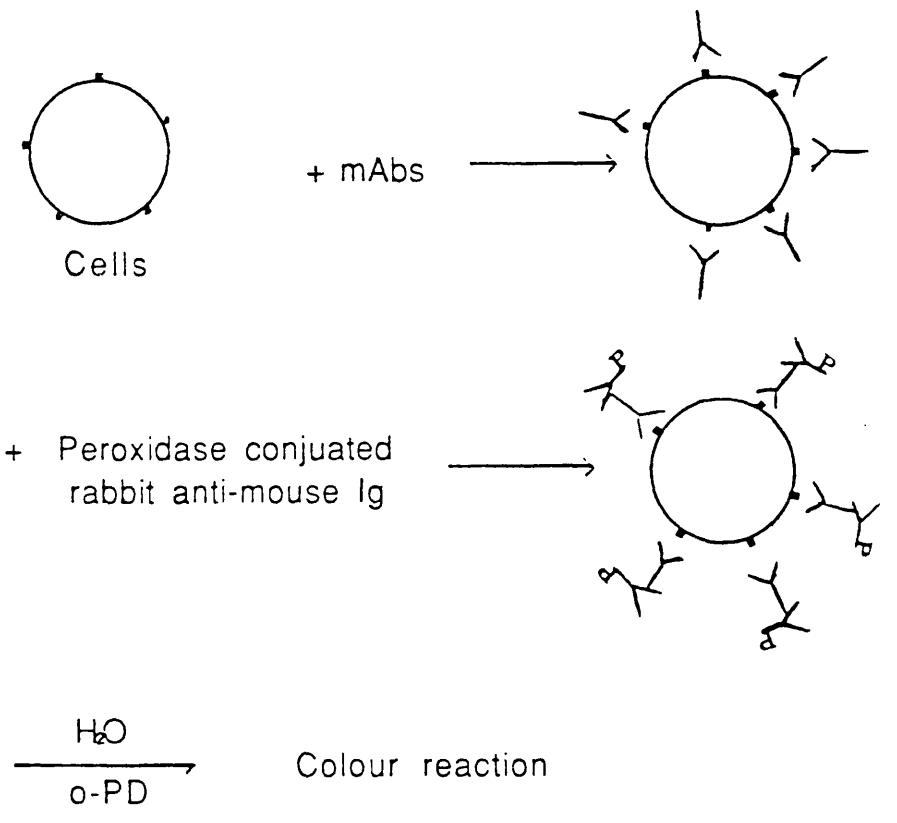


Figure 3.2 Cell surface binding assay

peroxidase substrate as for an ELISA assay. The result was read at 492 nM on a Titertek Multiscan.

3.2.5 Cloning by limiting dilution

When hybridoma cells were proliferating rapidly in 24-well plates or a small flask, they were diluted with warm medium and then plated out in 96-well plates at average concentrations of 0.5, 1 and 5 cells per well (The dilutions of the cells were carried out after counting by haemocytometer). Clones emerged in ten days. Flat bottom plates were used for cloning, which enabled single clones to be detected more easily. A mouse peritoneal macrophage feeder layer was always necessary for cloning.

3.2.6 Protein dot blot assay

a. Solubilisation of membrane antigen

Cells at 10^8 cells/ml were resuspended in 0.01 M Tris-HCl pH 7.3, 0.15 M NaCl, 0.02 % sodium azide at 4^0C. An equal volume of 10 % mass/volume of non-ionic detergent in the same buffer was then added. The detergent used was a 2:1 mixture of Brij 99 and Brij 96. To inhibit proteolysis, all the buffers contained 5 mM EDTA, 2.5 mM iodacetamide and 2 mM phenylmethylsuphonyl fluoride (PMSF) and were kept on ice at all times. The iodacetamide, dissolved in water, and the PMSF, dissolved in acetone were added at the time of use. The mixture was incubated at 4^0C for one hour with constant stirring and then centrifuged at 13,000 g for 30 minutes at 4^0C to

remove the nuclei. The supernatants were saved for analysis or stored at -70^{0}C until use.

b. Dot blot assay

SDS was added to the cell lysates prepared as above to a final concentration of 0.5%. 20 ul of cell lysates was loaded into each well of a preassembled 96 well dot blot microfiltration apparatus which contained zeta-probe membrane. The membrane was prewetted in TBS pH 7.5. The lysates were allowed to immobilised onto the membrane as they passed through the membrane. After washing 3 times with TBS containing 0.2 % Tween 20, 200 ul TBS containing 1 % BSA (blocking buffer) were added to each well and incubated at 37^{0}C to block the remaining binding sites.

Each well was then incubated with 100 ul of primary antibodies in triplicate for one hour at room temperature. After washing RaMHRP at 1:1000 in blocking buffer together with 1% normal human serum was added and incubated for one hour followed by 3 washes. The results were visualised by development in a substrate solution consisting of 60 mg 4-Chloro-1-Naphthol dissolved in 20 ml methanol, 100 ml of 0.1 M TBS (pH 7.5) and 60 ul H_2O_2. As soon as the coloured dots became apparent (about 10 minutes), the reaction was stopped by washing in tap water. The membrane was allowed to air dry and kept in the dark at room temperature.

3.2.7 Western blot

The method used was an adaptation of the protocol of Towbin et al (1979).

a. Polyacrylamide gel electrophoresis (SDS-PAGE)

A discontinuous gel system, based on that of Laemmli (1970), in which a running gel is overlaid with a stacking gel of different pH, was used throughout the studies. Generally, 10% acrylamide gels were used, prepared from a standard 30% acrylamide stock solution containing acrylamide and N,N'-methylene bisacrylamide. Gels were prepared containing 10 % acrylamide, 0.16 % bisacrylamide, 0.37 M Tris pH 8.8 and 0.1 % SDS. To 30 ml of acrylamide solution, 100 ul of 10 % ammonium persulphate solution (APS) in ddH_2O and 20 ul of N,N,N'N'-Tetramethylenediamine (TEMED) were added and poured between two glass plates with 1.5 mm thick spacers clamped together according to the instructions of the supplier of the gel apparatus.

Five milliliter ddH_2O was layered over the gel which was poured off after the gel had set. Stacking gel (20 ml) containing 10 % acrylamide , 0.16 % bisacrylamide, 0.125 M Tris-HCl (pH 6.8), 0.1 % SDS, 100 ul APS and 20 ul TEMED was poured on top of the running gel and a comb was placed in position. Samples were loaded immediately the stacking gel had set and were run at a constant 80 volts overnight.

b. Gel Transfer

After the electrophoresis, the SDS-PAGE gel was equilibrated in transfer buffer (Chapter 2, Buffers) for 30 minutes. The fibre pads, zeta-probe membrane and whatman filter paper of the same size as the gel were prewetted in transfer buffer. They were assembled into the gel holder in the order from cathode to anode of the gel holder: 1) fiber pad, 2) filter paper, 3) gel, 4) zeta-probe membrane, 5) filter paper, and 6) fiber pad. The transfer took place overnight at 280 mA with constant stirring of the transfer buffer.

c. Staining of the western blot

The transferred membrane was washed in TBS twice before blocking with TBS containing 1.5 % Blotto (nonfat milk) for 3 hours at 37^0C. They were then incubated with primary antibodies and secondary antibodies. The proteins identified by the antibodies were visualised in the same way as described for the protein dot blot assay (3.2.7).

3.2.8 Cell surface immunofluorescence staining

Two x 10^5cells were incubated in round-bottom microtiter plates at 4^0C for 30 minutes with 50 ul of 5% heat-inactivated rabbit serum in MEM containing 2% FCS and 0.02% sodium azide. Cells were washed once in washing buffer (MEM+2% FCS) and incubated with 50 ul of the appropriate antibody for 30 minutes. After 3 washes, 50 ul of RaMFITC at 1:200 dilution was added to the cells and

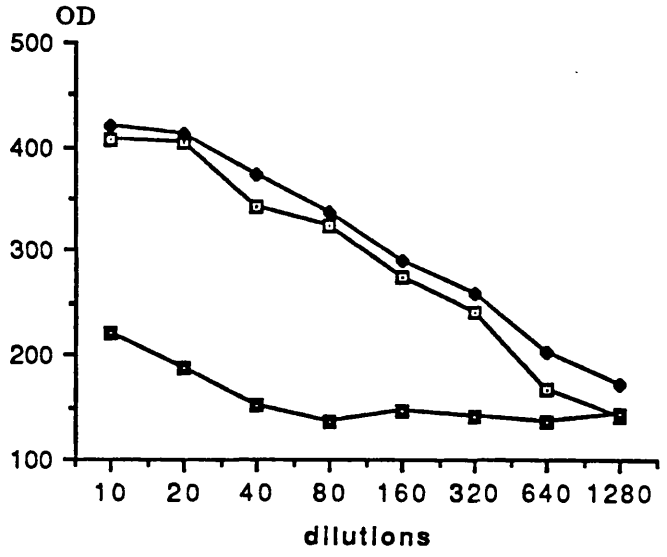
incubated for another 30 minutes followed by 3 washes. The samples were fixed in 1% formaldehyde and analysed within a week on a flow cytofluorometer (FACStar or FACScan).

3.3 Results

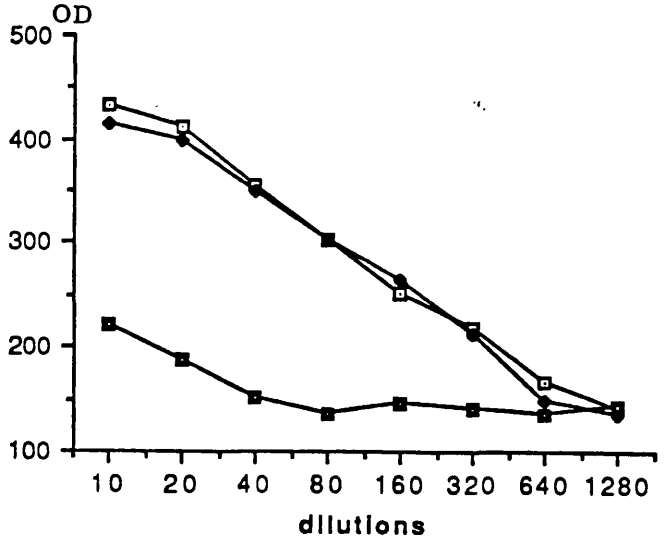
3.3.1 Mouse polyclonal antisera

Peptide-thyroglobulin was used as immunogen to produce mouse and rat mAbs. Two immunisation protocols were carried out during the study: Protocol I (The conventional immunisation protocol) and protocol II (the intrasplenic immunisation). Several mice and rats were immunised with either peptide to ensure that enough mice or rats producing antibodies to the peptides would be available for fusion. To improve the antigenicity of the peptides, multiple sites of injection were chosen and adjuvants were given (Table 3.1).

In the case of the protocol I immunisation, 10 days after the third boost, sera from immunised mice were pooled together and assayed for their specific reactivity for peptides by ELISA. The results are presented in Figure 3.3. Both antisera (mouse anti-peptide 33-43 and anti-peptide 58-69) showed higher titre than normal mouse serum (Figure 3.3.a and b). However, when the antisera were tested against irrelevant peptides (anti-33-43 sera were tested against 58-69 and vice versa), it was found that the antisera cross-reacted with both peptide conjugates with the same titration (Figure 3.3. a, b).



a. Titration of mouse anti 33-43-thyroglobulin serum against 33-43-BSA.
Titration of mouse anti 33-43-thyroglobulin serum against 58-69-BSA.
Titration of normal mouse serum against 33-43-BSA.



b. Titration of mouse anti 58-69-thyroglobulin serum against 58-69 BSA.
Titration of mouse anti 58-69-thyroglobulin serum against 33-43-BSA.
Titration of normal mouse serum against 58-69-BSA.

Figure 3.3 Titration of mouse polyclonal antisera

Since peptide 33-43 and peptide 58-69 shared the same three amino acids K P S near their C-terminal ends (see 3.1. Introduction), it was thought that the cross-reactivity of the polyclonal antisera might be due to immunogenicity of this common region and that the mAbs against this region might still be useful. Therefore fusions were made to obtain mouse mAbs.

3.3.2 Mouse mAbs

Three fusions were performed to obtain mouse mAbs: two for peptides 33-43 and 58-69 respectively by protocol I immunisation in mice, one for peptide 58-69 by intrasplenic immunisation (protocol II immunisation) in mice. The selection of the screening assay was dictated by the aims of the project which was to obtain antibodies recognising native proteins as well as the immunising peptide. Hybridoma supernatants were therefore screened both for binding to peptide in an ELISA assay (Figure 3.1) and for binding to native cell surface molecules in a cell surface binding assay (Figure 3.2).

In the first two fusions, spleen cells from mice immunised with 33-43-thyroglobulin and 58-69-thyroglobulin by protocol I (see 3.2.1), were fused with NS-1 myeloma cells. Supernatants from hybridoma growing wells were screened by ELISA against peptide-BSA as well as in a cell binding assay on J6 cells. Six hybridomas were positive in ELISA assay for peptide 33-43 and seven for peptide 58-69. The

mAbs were named after the numbers of the wells they came from in the original plates and were further charaterised in various tests.

In the cell surface immunofluorescence staining on J6 cells, no hybridomas were positive. Protein dot blot assay was then employed to decide if the antibodies would react with denatured proteins. The assay revealed that all the antibodies showed reactivity with the denatured J6 cell lysates. Two hybridomas, 2C7 and 4C4 from fusions for peptides 33-43 and 58-69 respectively were selected for further characterisation.

The two hybrids were cloned by limiting dilution. The antibodies were tested on various cell lysates to locate the tissue distribution of the antigen. Unfortunately, it was found that both antibodies reacted with all the cell lysates tested. In addition to T cell lines such as J6, CEM, and HUT78 which transcribe the pT1A gene, they reacted with lysates from HFB-1, a cell line of B cell origin which does not transcribe pT1A, as well as with lysates from fresh tonsillar E^+ and E^- cell fractions. On western blot the antibodies consistently failed to recognise any bands on the same lysates which were positive in protein dot blot assays.

Two aspects of these data were puzzling. The first was the discrepancy between binding of the mAbs to cell lysates and expression of pT1A message in the same cells and the second was the failure to detect binding of mAb in western blots when protein dot blots of the same lysates were positive.

In order to further explore the first question the mAbs were tested for binding to a variety of peptides including peptide 33-43, 58-69 and another irrelevant peptide derived from the human CD45 antigen with sequence of NH_2-Q S P T P S P T L E N L E P-COOH. The results are shown in Table 3.2.

Table 3.2 Cross-reactivity of mouse mAbs by ELISA

Peptide	Carrier protein	2C7	4C4
33-43	BSA	+	+
33-43	Thyroglobulin	+	+
58-69	BSA	+	+
58-69	Thyroglobulin	+	+
CD45	BSA	+	+
CD45	Thyroglobulin	+	+

The results clearly demonstrated that the mAbs cross-reacted with many peptide conjugates regardless of differences of peptide sequences and the carrier proteins. Because these mAbs were clearly not specific for either pT1A peptide, we did not further investigate the failure to detect bands on western blotting. It may be that there is a difference in sensitivity between protein dot blot and western blot or alternatively the higher concentration of SDS used (see method) in western blotting may have destroyed the epitopes detected by the mAbs.

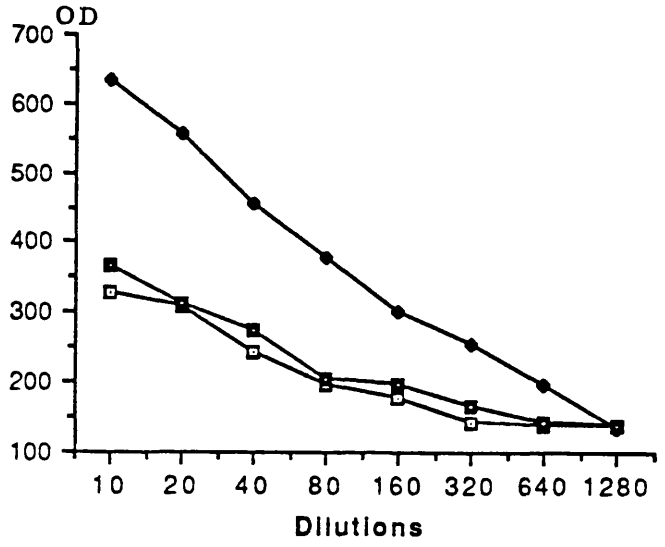
The third fusion for mouse mAbs was performed following protocol II immunisation. It was thought that a common conformation might exist among all the peptide conjugates which is dominant for the mouse immune system. In this case the repeated boosts of the conventional immunisation might select B cell clones for the dominant epitope at the expense of other specificities. To avoid this a single intrasplenic immunisation was performed by Dr Healey in this laboratory in the hope that antibodies of greater diversity would be generated and detected. The fusion was made 5 days later. The results from this fusion were similar to those obtained from traditional immunisation and fusion. All these data suggested that mouse mAbs produced by immunising with these peptides were prone to cross-reactitivity and that it would be difficult to raise desirable mAbs with this approach.

3.3.3 Rat polyclonal antisera

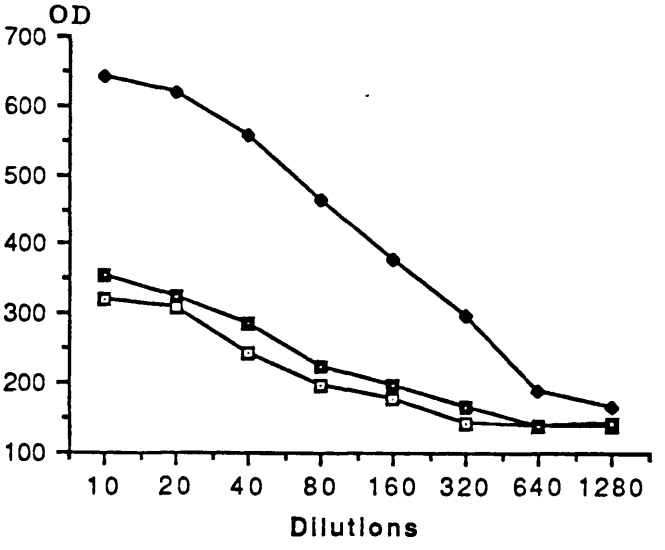
Pooled sera from the immunised rats were also tested for their specificity for the peptides. They were assayed in the same way as the sera from mice. The immune response from the rats was different from that of mice in that the polyclonal antisera from rats showed specificity for the immunising peptide (Figure 3.4).

3.3.4 Rat mAbs

A fusion was made to obtain rat mAbs specific for peptide 33-43. Eight out of 192 wells were positive when screened by ELISA. The



a. —◆— Titration of rat anti 33-43-thyroglobulin serum against 33-43-BSA.
—■— Titration of rat anti 33-43-thyroglobulin serum against 58-69-BSA.
—□— Titration of normal rat serum against 33-43-BSA.



b. —◆— Titration of rat anti 58-69-thyroglobulin serum against 58-69-BSA.
—■— Titration of rat anti 58-69-thyroglobulin serum against 33-43-BSA.
—□— Titration of normal rat serum against 58-69-BSA.

Figure 3.4 Titration of rat polyclonal antisera

supernatants were tested immediately against different peptides to ensure that the hybridomas selected was secreting antibodies specific for peptide 33-43 only. The results are shown in Table 3.3.

Table 3.3 The specificity of rat mAbs by ELISA

Peptide	Carrier protein	Reaction with rat mAbs
33-43	BSA	+
33-43	Thyroglobulin	+
58-69	BSA	-
58-69	Thyroglobulin	-
CD45	BSA	-
CD45	Thyroglobulin	-

The results indicated that the rat mAbs were specific for the 33-43 peptide amino acid sequence, not the carrier proteins. However on cell surface immunofluorescence staining, none of the clones reacted with J6 cells. Protein dot blot assays revealed similar results to those obtained with mouse mAbs. They reacted with lysates of various cell origin (see Figure 3.5) including T cells, B cells and epithelial cells.

As the rat mAbs showed specificity for the immunising peptide and in order to know if the results of protein dot blot assay were an artifact, the antibodies were tested by western blot assay. Cell lysates equivalent to $1x10^7$ cells from both tonsillar E^+ and E^- fraction (Both fraction were negative for pT1A messenger RNA, Chapter 6) were fractionated under reducing condition. In the E^+

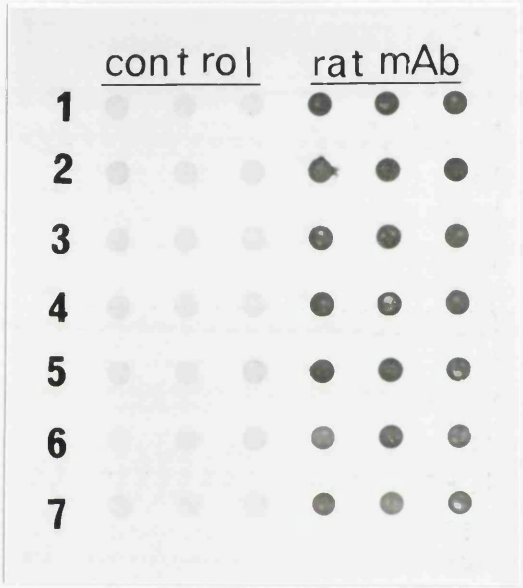


Figure 3.5 Protein dot blot analysis of rat mAb

Rat mAb was assayed in protein dot blot analysis on various cell lysates. Cell lysate sources were as follows: 1. J6 cells, 2. CEM cells, 3. HUT78 cells, 4. HFB-1 cells, 5. Tonsillar E^+ fraction, 6. Tonsillar E^- fraction and 7. T24 cells. The normal rat serum at 1:50 dilution was included as controls.

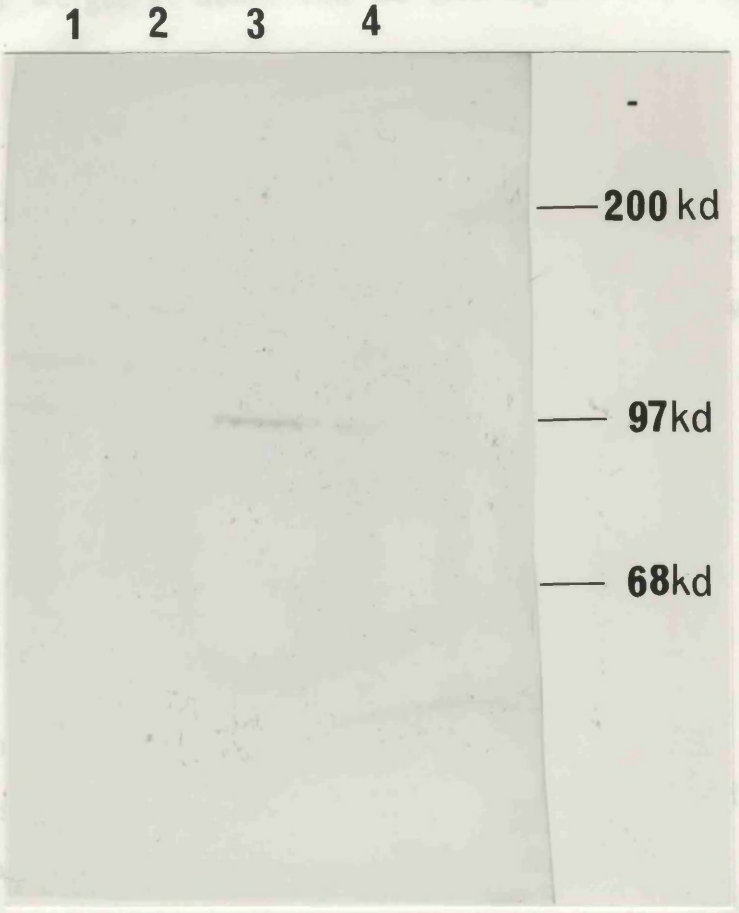


Figure 3.6 Western blot analysis by rat mAb

Cell sources were as follows: Lanes 1 and 2 Tonsillar E^- fraction under reduced condition. Lanes 3 and 4 are tonsillar E^+ fraction under reduced condition.

cell fraction the rat mAb recognised a 98 kd protein. In the E^- cell fraction the mAb revealed two bands, one was the same molecular weight as for E^+ cells and another band was of slightly higher molecular weight at about 120 kd (see figure 3.6).

The nature of the proteins recognised has not been investigated. It was clear that the rat mAbs did not meet the criteria for desirable mAbs because they were unable to recognise native proteins and had an unrestricted tissue distribution, which was not consistent with that of RNA expression (Chapter 6).

3.4 Discussion

The characterisation of the antibodies obtained from animals immunised with synthetic peptides indicated clearly that none of the mAbs derived met the criteria for desirable antibodies i.e. antibodies reacting with the peptide as well as native protein(s) with similar tissue distribution to the expressed gene (mRNA).

The mouse mAbs from both conventional and intrasplenic immunisation showed cross-reactivity with not only the two peptides derived from pT1A but also with other irrelevant peptide. In view of the cross-reactivity of the antibodies, it is hypothesised that a common conformation might exist in different peptide conjugates and that conformation is of dominant antigenicity for the mice. The view is strengthened by the fact that a single intrasplenic

immunisation elicited the same immune response as long term immunisation. The fact that mAbs specific for the peptide were selected from a rat fusion suggests that the screening system is capable of detecting mouse mAbs specific for the peptides.

The rat mAbs were specific for the peptide as tested by ELISA, but they failed to recognise native proteins. The failure to recognise native cell surface antigens by these antibodies is understandable because with more sequence data obtained it is clear that pT1A is within the cytoplasmic part of the molecule. Although they reacted with denatured proteins assayed by protein dot blotting and western blotting, the wide tissue distribution revealed by these assays is contradictory to the results from northern blot analysis which have shown that the gene has a restricted tissue distribution mainly among T-ALL cell lines.

It is therefore unlikely that the proteins recognised by the rat mAbs by the protein dot blot assay and western blot assay was the gene product. It is more likely that the peptide amino acid sequence recognised by the antibodies or the conformation of the peptide are frequently encountered, or the cytoplasmic part of the gene products might share some common sequence with other proteins which have a wide tissue distribution. This possibility awaits further investigation. However, the nature of this cross-reactivity was not investigated since the aim of the immunisation was to produce antibodies with specificity for the pT1A gene product.

With more sequence revealed, it would be possible to synthesise more peptides from the extracellular domain of the gene and try to obtain antibodies reacting with native protein. However rather than persevering with the synthetic peptide approach, which is time consuming, laborious and might still encounter the same problems, it was decided that the best route would be to use the gene products expressed in native form as immunogen. The next chapter describes an attempt to do so.

CHAPTER FOUR

RAISING ANTIBODIES TO TRANSFECTANTS

4.1 Introduction

The pT1AB clone, as described in 1.7, was a truncated gene with neither N nor C-terminus. Previous attempts to raise mAbs to synthetic peptides derived from the gene sequence were not successful. MAbs derived from that approach recognized proteins with a wide tissue distribution which was not consistent with that of northern blot analysis. Thus in order to raise antibodies recognizing native antigenic determinants of the gene product, it was considered essential to have the gene product expressed in a mammalian cell.

The sequence of the pT1AB gene showed a hydrophobic transmembrane region near its C-terminus, suggesting that the gene codes a membrane or secreted protein. Transfection of the DNA into L cells should therefore provide a means for expressing the gene product. If the gene is processed properly and expressed on the L cell surface, the transfectant would be an ideal immunogen to raise mouse antibodies, in the hope that the mouse immune system would recognise the gene product as a foreign antigen while other cell surface molecules on L cells would not be immunogenic. However, due to the truncated form of the cloned cDNA, the gene lacked essential elements which would allow it to be processed normally if introduced into mammalian cells without any modification. Neccessary modifications of the gene to be transfected

were based on the knowledge of normal processing of surface or secreted molecules. The process consists of several steps including transcription, translation and expression on the cell surface or secretion into the enviroment.

Transcription is the first step for a gene to be expressed. The resultant product is a messenger RNA which has a cap at the 5' end and a polyA tail at the 3' end. The general process of mRNA formation is outlined in Figure 4.1. In mammalian cells the synthesis and processing of mRNA is carried out by RNA polymerase II and a multitude of nuclear factors. Examination of the DNA sequences of a variety of different transcription units has revealed a short consensus sequence serving as a promotor for RNA synthesis. This highly conserved sequence has been named the TATA box, since it invariably contains TATA bases in this region.

Transcription starts about 30 bases downstream from the TATA box region. When the transcript is at about 20 to 30 nucleotides long, a methylated cap structure $m^7G_{ppp}N_1$ is added at the 5' end of each primary transcript. Transcription continues past the polyA addition site before terminating at about 15 to 30 nucleotides down-stream from the poly A site, a strongly conserved consensus sequence AAUAAA. This sequence and sequences downstream from the poly A site co-operate to signal the location of the poly A site, which results in cleavage of the primary transcript and addition of the polyA.

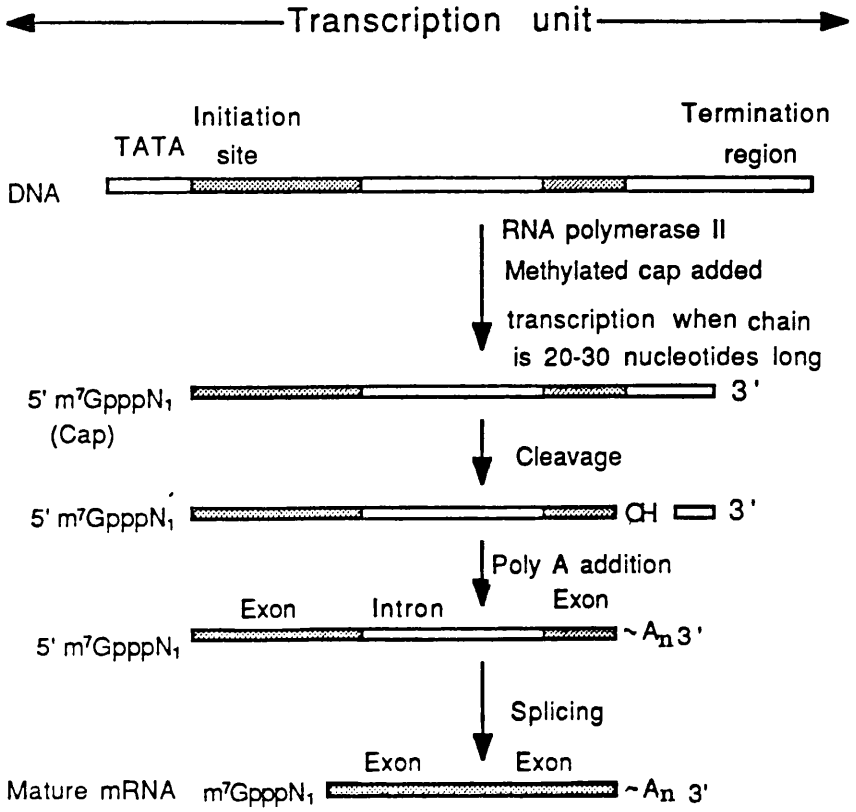


Figure 4.1 The general pathway of eukaryotic mRNA formation. Not all steps are necessary for all mRNAs. The initiation of RNA synthesis is influenced by several factors including the promoter, and enhancers which may exist near the template DNA or may be situated far away from the template DNA. Termination regions in the DNA are known, but the exact termination sites are not.

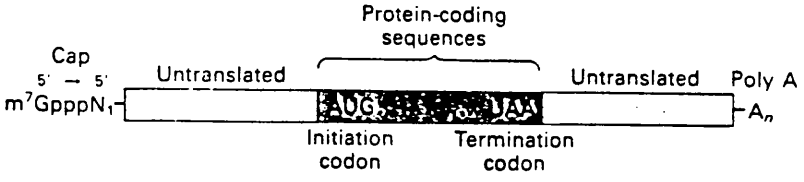


Figure 4.2 Regions of eukaryotic mRNAs, showing the terminal cap and poly A structures, the translation initiation and termination sites, and the untranslated regions at both the 5' and the 3' ends.

The primary transcript is spliced, if necessary, to complete the mRNA. The finished mRNA molecules have distinct features (Figure 4.2). Between the capped 5' end and the first AUG translation initiation codon, there are usually from 10 to as many as several hundred nucleotides that are not translated. Likewise, between the translation termination codon and the poly A at the 3' end of the mRNA molecule, there is another untranslated region that is usually at least 50 to as long as 2,000 nucleotides.

mRNA is the template for protein synthesis. The translation from mRNA to protein starts after the mRNA is transported into the cytoplasm of the cell. It starts at an AUG initiation codon and stops at UAA, UAG, or UGA termination codons. The translated products have to go through several processes before maturation. The process varies from protein to protein. Proteins expressed on the cell surface or secreted usually have a distinct biosynthetic pathway from that of other proteins.

Studies of mammalian cell surface and secreted proteins have revealed that such proteins are not merely a random cross-section of the total proteins of the cells, but rather are a unique subset which is segregated from those proteins that are to occupy intracellular locations. Despite their ultimate diversity of structure and function, all of the membrane proteins exhibit common features that cause them to be synthesized along biosynthetic pathways leading to the cell surface (Figure 4.3).

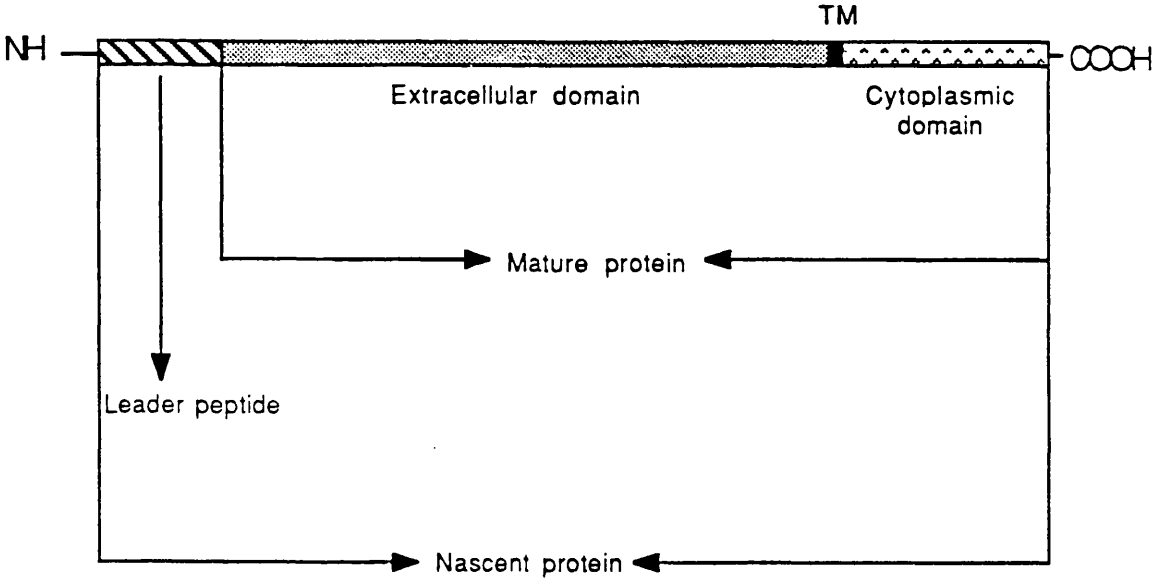


Figure 4.3 General features of transmenbrane proteins A standard transmembrane protein is composed of following elements: a transmembrane region (TM), an extracellular domain and a cytoplasmic domain. The nascent protein contains a hydrophobic leader peptide which is absent from the mature protein.

One important feature of these proteins is that they normally contain a hydrophobic region of about 24-30 amino acids as a transmembrane region (TM) that interacts with the lipid bilayer and causes them to be held securely in place in the plasma membrane. Another critical feature of these proteins is that almost always they carry at the N-terminus of the nascent polypeptide, a leader peptide, which is excluded in the final protein product. Leader peptides invariably have been found to be hydrophobic in nature and act as a signal to direct the growing peptide chain to sites in the membrane of the rough endoplasmic reticulum serving as portals to the biosynthetic pathway that leads to the cell surface (Reviewed in Blobel et al., 1979; Davis and Tai, 1980; Sabatini et al., 1982).

To ensure the proper processing of the pT1AB gene product after transfection into mammalian cells, the truncated pT1AB gene had to be modified to meet the requirements of the biosynthetic pathway of the membrane protein described above. pT1AB contains a hydrophobic transmembrane region near the C-terminus, but lacks an N-terminus which contains the leader peptide. A pseudo N-terminus containing a leader peptide had to be linked with the 5' end of the gene to ensure the proper processing of the gene product. This chapter describes an attempt to construct such a recombinant and the result.

4.2 Methods

Most methods described below were according to Maniatis et al (1982) unless otherwise indicated.

4.2.1 Restriction endonuclease analysis

Restriction enzymes were purchased from Bethesda Research Laboratories Inc, Boehringer and Biolabs. All digestions were performed at 37^0C in a standard buffer as recommended by the enzyme suppliers for one to two hours. The concentration of sodium chloride varied depending on the restrction enzyme used. Details are given on pages 100 and 101, in Molecular Cloning (Maniatis, 1982). DNA restriction fragments were analysed directly by agarose gel electrophoresis.

4.2.2 Agarose gel electrophoresis

Gels were formulated in the range 0.5-1% and were made by dissolving agarose powder in 1 x TAE buffer in a microwave oven. After cooling to about 50^0C, ethidium bromide was added to a final concentration of 0.5 ug/ml and the gel mixture poured into a horizontal plastic plate and with a comb in position. Once the gel had set, the plate was placed in a flat bed electrophoresis chamber and immersed in 1 x TAE. DNA samples were mixed with loading buffer and applied to the sample wells. After electrophoresis, the gel was viewed under UV illumination and photographed through an orange filter using Polaroid Type 57 Land film. The sizes of the

various restriction fragments obtained were estimated against the known sizes of fragments of λDNA derived from complete Hind III digestion.

4.2.3 DNA fragment preparation

The plasmid containing the fragment was cleaved by an appropriate enzyme and separated on a 0.7% agarose gel. The gel was stained with ethidium bromide at 0.5 ug/ml for 20 minutes in the dark and visualised by long wave UV illumination. The fragment of interest was excised and put into a dialysis tube containing 0.05 x TAE buffer. The DNA was eluted from the agarose gel at 250 V for 15 minutes in 0.05 x TAE. The fluid in the dialysis tube was recovered and passed through a siliconised pasteur pipet plugged with glass wool. It was extracted with an equal volume of phenol, then phenol : chloroform and chloroform. Then one tenth volume of 3 M NaAc pH 5.2 plus 2.5 volume of absolute ethanol were added and the DNA precipitated at -70^{0}C overnight. The mixture was contrifuged at 12,000 g at 4^{0}C for 30 minutes. The pellet was washed once with 70% ethanol and dissolved in TE pH 8.0. The concentration of the fragment was determined by running an aliquot of sample on a agarose gel together with serial dilution of λDNA at known concentration.

4.2.4 Kinase reaction

Five micrograms of DNA fragment was mixed with 5 ul 10 mM ATP, 5 ul 10 x kinase buffer which contained 500 mM Tris-HCl pH

7.6, 100 mM $MgCl_2$, 50 mM DTT, 1 mM spermidine and 1 mM EDTA. The final reaction volume was adjusted to 50 ul by adding ddH_2O. Two microliters (30 units) T4 kinase (BRL) were added and the mixture was incubated at 37^0C for one hour. The reaction was pass over a sephadex G-50 column. The kinased DNA fragment was eluted in TE pH 7.5 at known concentration.

4.2.5 Ligation

The ligation was carried out by mixing vector and insert at a ratio of 0.5 for sticky ends and 3 for blunt ends. The reaction was carried out in 1 x ligase buffer (BRL) together with 1 unit T4 DNA ligase (BRL) in a total volume of 10 ul. The ligation was allowed to take place overnight at 14^0C.

4.2.6 Klenow fragment filling reaction

One microgram of DNA was mixed with 5 ul 10x klenow fragment filling buffer which contained 500 mM Tris pH 7.5, 100 mM $MgCl_2$, 10 mM DTT and 500 ug/ml BSA, and 0.5 mM dNTP. The final reaction volume was adjusted to 50 ul. Two units of klenow fragment were added and the mixture was incubated at room temperature for 20 minutes.

4.2.7 Competent cell preparation

A fresh overnight culture of JM101 cells was diluted 40-fold into 1 liter of L broth (LB) medium and incubated at 37^0C with good aeration until an absorbance at 550 nm of 0.4 - 0.5 was reached. The culture was immediately chilled in an ice-water bath, centrifuged at 4^0C at 5,000 rpm for 10 minutes. The supernatant was decanted. The pellet was resuspended in 500 ml ice-cold 100 mM $CaCl_2$ and incubated on ice for 30 minutes. The cells were pelleted again at 5,000 rpm for 10 minutes at 4^0C. The cells were finally resuspended in 40 ml of ice-cold 100 mM $CaCl_2$, 15% glycerol and stored in 0.2 ml aliquots on ice for 12-24 hours. The tubes were frozen in ethanol-dry ice and placed immediately at -70^0C until use.

4.2.8 Transformation of competent cells

Frozen competent cells were thawed on ice. The plasmid DNA (from 0.1-10 ng/reaction) to be transformed was added and mixed with the cells gently. The mixture was incubated on ice for 40 minutes, heat-shocked at 42^0C for 90 seconds, then immediately quenched in ice for 60 seconds. Four volumes of LB were added and the tube was incubated at 37^0C for 1 hour. The culture was spread onto a LB agar plate containing 50 ug/ml ampicillin. After the solution had soaked in, the plate was inverted and incubated at 37^0C overnight.

4.2.9 Mini-preparation of plasmid

One and half milliliters of overnight culture from a single bacterial colony was poured into a microfuge tube and centrifuged for 1 minute. The supernatant was removed and the pellet resuspended by vortexing in 100 ul of an ice-cold solution containing 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0 (Solution I) and 5 mg/ml lysozyme. The lysis reaction was allowed to take place for 5 minutes at room temperature. Two hundred microliters of freshly prepared solution of 0.2 N NaOH, 1% SDS (Solution II) were added, mixed gently and incubated on ice for 5 minutes. Then 150 ul of 3 M KAc pH 4.8 (Solution III) was added and incubated on ice for another 5 minutes.

The tube containing the mixture was centrifuged and the supernatant was removed, extracted with an equal volume of phenol/chloroform and precipitated with 2 volumes of absolute ethanol for two minutes at room temperature. The DNA was pelleted by microfuging for 5 minutes. The pellet was washed once with 70% ethanol and resuspended in TE pH 8.0 containing 25 ug/ml RNase A. An aliquot of the sample was subjected to restriction enzyme digestion and analysed by agarose gel.

4.2.10 Large scale plasmid preparation

Once small-scale analysis of plasmid DNA had confirmed its identity, 500 ml cultures of bacteria were grown overnight in the presence of antibiotics. Bacteria were harvested and treated with Solution I with lysozyme, solution II and III as described for small-

scale plasmid DNA preparation. The cellular debris was removed by centrifugation at 10,000 rpm for 10 minutes, and following passage of the supernatant to a fresh tube, 0.6 volumes of propan-2-ol was added to precipitate nucleic acid. After 15 minutes at room temperature, it was centrfuged at 12,000 rpm for 30 minutes. After brief drying, the pellet was dissolved in TE buffer pH 8.0.

Solid caesium chloride was added to the nucleic acid solution at a concentration of 1 g/ml. 0.8 ml of ethidium bromide at 10 mg/ml was added to every 10 ml DNA mixture. The mixture was transferred to a 5 ml polycarbonate centrifuge tube, sealed and centrifuged at 58,000 rpm in VTi 65 (Beckman) overnight. The supercoiled plasmid DNA was visible in the middle of the gradient as a red band which was removed, extracted with Butan-2-ol and dialysed extensively against several changes of TE pH 8.0 at 4^0C. The plasmid DNA was quantitated by measuring the absorbance of a dilution of the sample at 260 nm in the spectrophotometer. It was precipitated with ethanol and redissolved in TE buffer at 1 mg/ml and stored at 4^0C.

4.2.11 DNA sequencing

The nucleotide sequence of recombinant DNA was determined by the dideoxynucleotide sequencing procedure (Sanger et al, 1977). It was carried out according to a protocol provided by Strategene (1988). Two micrograms of supercoil double-stranded DNA was denatured in 0.2 M NaOH, 0.2 mM EDTA in 20 ul for 5 minutes at

room temperature. Fifty nanograms of primer together with 2 ul of 2 M NH_4Ac pH 5.4 was added and mixed well. The DNA was precipitated with 55 ul cold absolute ethanol in a dry ice/ethanol for 5 minutes. The DNA was pelleted and washed once with 70% ethanol. The DNA pellet was resuspended in 1 x Taq sequencing buffer and then 2 ul of Taq sequencing extension mix in 1 to 5 dilution, 1 ul ^{32}p-dATP (>3000 Ci/mmol) and one unit of Taq polymerase were added. The mixture was incubated at 42^{0}C for 10 minutes.

Three and half microliters of the above reaction was aliquoted into each microfuge tube labeled as G, A, T, C, in which 2 ul of the appropriate ddNTP/dNTP had been added. The mixture was incubated at 70^{0}C for 5 minutes. The reaction was stopped by adding 5 ul of stop buffer. The resulting fragments were resolved electrophoretically on a 7 M urea, 6% acrylamide gel.

The sequencing gel was formulated by combining 63 g urea, 15 ml 10 x TBE, and 22.5 ml 40% acrylamide stock solution, the total volume was brought to 100 ml. The gel was poured by adding 1.3 ul 25% ammonium persulphate and 1 ul TEMED to every milliliter of gel solution. A 2.5 mm thick gel was used. Sequencing samples were boiled for 1 minutes and quenched immediately in ice for 5 minutes before loading onto the gel. The gel was kept at about 50^{0}C during the electrophoresis and stopped when the bromophenol blue had just migrated out of the gel. The gel was dried at 80^{0}C under vacuum for 45 minutes and autoradiographed at -70^{0}C.

4.2.12 DNA-mediated gene transfer (Transfection)

DNA was introduced into recipient eukaryotic cells in the following manner (Davis et al., 1986). L cells were seeded at 3 x 10^5 cells per 3.5 cm tissue culture dish the day before transfection, and re-fed with fresh complete medium 4 hours prior to transfection. In a sterile 20 ml universal, 436 ul dH_2O was added followed by 1 ug plasmid DNA, 42 ul 2 M $CaCl_2$. Five hundred microliters of 2 x HBSP which contained 1.5 mM Na_2HPO_4, 10 mM KCl, 280 mM NaCl, 12 mM Glucose and 50 mM HEPES pH 7.0, were slowly added to the bottom of the tube with a pippet aid. Five bubbles were expelled from the pipette to mix the DNA solution. A fine white co-precipitate of calcium phosphate and DNA was formed and allowed to stand at room temperature for 30 minutes.

The precipitate was added dropwise to the culture dishes at 1 ug DNA/dish and the cells were incubated for 4 hours at 37^0C in a 5% CO_2 incubator before glycerol shock. The glycerol shock involved incubation of the cells in 15% glycerol in 1 X HBSP at 37^0C for three minutes. This was followed by a wash with sterile PBS and addition of fresh complete medium. The medium was replaced by selection medium containing G418 after 48 hours. The transfectant colonies appeared in 2 weeks.

4.2.13 Preparation of high molecular weight cellular DNA

Cells to be used for genomic DNA extraction were cultured in complete medium. The cell monolayers or cell suspensions were drained of medium, washed twice with PBS, and then lysed in 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 5 mM NaCl 0.5% SDS and 100 ug/ml proteinase K. The mixture was incubated at 60^0C for one hour and then at 37^0C overnight. It was extracted sequentially with an equal volume of phenol, phenol/chloroform and chloroform. The supernatant was removed and dialysed against TE first at room temperature then at 4^0C. RNase free of DNase was added to a final concentration of 20 ug/ml and the mixture was incubated at 37^0C for one hour. It was again extracted with phenol and phenol/chloroform and chloroform. The DNA was ethanol precipitated and spooled out of solution around a sealed pasteur pipette and washed in 70% ethanol. The DNA was dissolved in TE buffer pH 7.5 and stored at 4^0C.

4.2.14 Southern blotting

The procedure followed the method originally described by Southern (1975). For southern blotting, gels were denatured for 30 minutes with gentle rocking in DNA denaturing solution, then soaked for 30 minutes in DNA neutralising solution (see Chapter 2, Buffers). DNA was tranferred to Genescreen plus membrane by capillary blotting in 20 x SSC. The gel was placed on a 3 MM paper wick which had been saturated with, and was dipping into, 10 x SSC. A genescreen membrane pre-soaked in 10 x SSC, was carefully laid over the gel, avoiding air bubbles, and the buffer was drawn up through the gel and membrane by placing dry paper tissues on

top of the filter with a 1 kg weight on the top. The transfer was allowed to take place overnight. The membrane was marked and rinsed in 2 x SSC briefly. After drying at room temperature, the membrane was baked at 80^0C for 2 hours and kept between sheets of 3 MM paper until use.

4.2.15 Preparation of cellular RNA

Cells from which total cellular RNA was to be extracted were washed with sterile PBS and lysed with a solution containing 4.0 M guanidine thiocyanate. The lysate was then overlaid on a 2.2 ml cushion of 5.7 M caesium chloride in 0.1 M EDTA and centrifuged at 33,000 rpm for 24 hours at 20^0C in a SW41 rotor. Under these conditions, the denatured proteins in the lysate were excluded from the caesium chloride, the DNA was sedimented to its buoyant density within the gradient, and the RNA was pelleted at the bottom. The supernatant and caesium chloride were removed by aspiration and the bottom containing the RNA pellet was cut from the tube. The pellet was rinsed with DEPC dH_2O and resuspended in 400 ul DEPC dH_2O. After extracting with an equal volume of phenol:chloroform, the RNA was precipitated with ethanol at -20^0C overnight. The RNA was recovered by centrifugation in microfuge for 5 minutes at 4^0C. The yield of RNA was estimated by measuring the spectrophotometric absorbance at 260 nm of a dilution of the nucleic acid, and the RNA was stored in ethanol at -20^0C until use.

4.2.16 Northern blotting

Flat bed agarose gels containing 1% agarose, 1 x MOPS and 2.2 M formaldehyde were prepared for electrophoresis of total RNA or polyA+ RNA. Twenty micrograms of total RNA or 5 ug of polyadenylated RNA, was denatured in RNA denaturing buffer at 60^0C for 5 minutes and electrophoresed overnight at 20 mA in 1 x MOPS buffer. The gel was soaked in 10 x SSC briefly and transferred onto the genescreen membrane as described for southern blotting.

4.2.17 Oligolabeling of the Probe

cDNA fragments, either purified or in blocks of low melting agarose, were labelled by the 'random hexamer' method of Feinberg and Vogelstein (1983), using ^{32}P-labeled dATP. The DNA fragment (between 50-100 ng) was boiled for 7 minutes and placed at 37^0C for 15 minutes before mixing with the following reagents: 1) 10 ul oligolabeling buffer, 2) 2 ul BSA at 10 mg/ml, 3) ddH_2O to total volume of 50 ul, 4) 5 ul ^{32}p-dATP at 10 uCi/ul (>3000 Ci/mmol), and 5) 2 units klenow fragment. The mixture was incubated at room temperature in a perspex container overnight. The reaction was terminated by addition of 2 ul 0.5 M EDTA. The probe was separated from unincorporated dNTPs by chromatography on a sephadex G-50 column. One microliter of the eluate was removed and counted for ^{32}p incorporation. Usually more than 1x 10^8 cpm/ug DNA was obtained.

4.2.18 Hybridisation

Prior to hybridisation, the filter was briefly washed in 2 x SSC to ensure complete wetting. It was sealed in a plastic bag containing 10 ml of prehybridisation buffer. The filter was incubated in this buffer with constant shaking at 42^0C for at least 3 hours as a prehybridisation step, after which the radioactive probe, prepared as described in 3.1.17. was added. After hybridisation overnight at 42^0C, the filter was washed twice in 500 ml of 2 x SSC containing 0.1% SDS for 5 minutes at room temperature. This was followed by two washes in about 500 ml of 0.1 x SSC, 0.1% SDS at 65^0C for 20 minutes. Following washing, the filter was blotted on tissue to remove excess solution, wrapped in cling film and exposed to Hyper film. Hybridisation conditions for northern blots were similar to those of southern blotting except that the northern blots were washed at 50^0C instead of 65^0C after hybridisation. Following hybridisation with pT1B or CD5 all filters were reprobed with a mouse actin probe to check that the quantity of RNA loaded was approximately equal in all tracks (see Figure 6.4).

4.2.19 Cell surface labelling with ^{125}I

To perform cell surfce iodination actively growing cells were removed from culture and washed three times with PBS. Cells (2 to $5x10^7$) wree suspended and labeled in a total volume of 0.5 ml PBS containing 2.5 mCi carrier-free sodium ^{125}I and 10 ug of lactoperoxidase in the presence of 0.1 ul glucose oxidase and 0.9 mg D-glucose. The reaction was carried out at room temperature for 10 minutes. The cells were washed extensively in cold PBS and lysed in 0.5 ml of lysing buffer. An aliquot was fractionated on an SDS-PAGE gel.

4.2.20 Biosynthetic labelling of cells

Typically, 2 x 10^7 cells were cultured for one hour in 10 ml medium without methionine and containing 5% FCS which had been dialysed against 0.9% (w/v) NaCl. The cells were pelleted and resuspended in 5 ml fresh medium and 0.5 mCi ^{35}S-methionine (>800 Ci/mmol) was added at the start of 4 hours of culture. After washing in ice cold PBS, the cells were resuspended in 5 ml lysis buffer and left on ice for 30 minutes. The cell lysate was cleared by centrifugation in a bench-top microfuge at 4^0C for 10 minutes. The lysates were analysed by SDS-PAGE or kept at -70^0C until use.

4.3. Results

4.3.1 pT1B insert

Inspection of the restriction map of pT1AB (Figure 4.4) revealed that the gene has three EcoR I sites, with one at either end and another one bewteen pT1A and pT1B. Because the cDNA was cloned at EcoR I sites , the gene was separated into pT1A and pT1B when it was generated from its original vector λgt11. When it was planned to construct a recombinant to transfect L cells, it was decided to use pT1B only. pT1B is about 1.9 kb in length. The transmembrane region located at 245 bp from the 3' end of pT1B divides the gene into two parts: an extracellular domain and a cytoplasmic domain. In order to retain as much extracellular domain as possible when the gene product is expressed on the cell surface, the unique

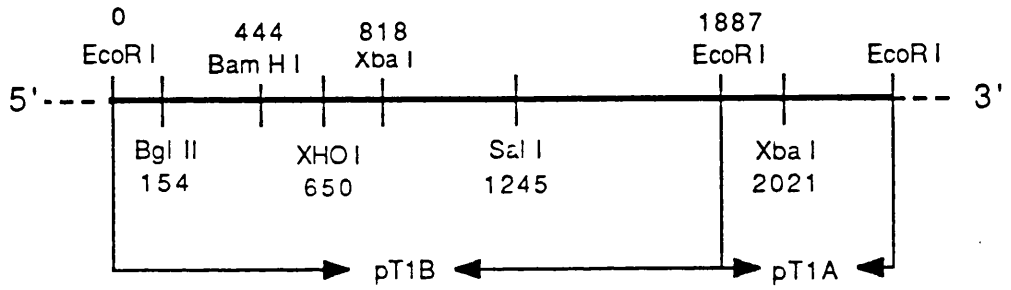


Figure 4.4 Restriction enzyme map of pT1AB. The restriction enzyme sites and their positions used throughout the thesis are illustrated. Those which have not been employed are omitted in the figure. pT1AB is divided into pT1A and pT1B by the EcoR I site at nucleotide 1887.

restriction site Bgl II (situated at 157 bp from the 5' end of pT1B) which is the nearest restriction site to the 5' end of pT1B was chosen as the site to join the pseudo N-terminus.

The strategy of cloning is diagramed in Figure 4.5. Plasmid containing pT1B was subjected to both Bgl II and EcoR I digestion. The fragment obtained was about 1.75 kb in length with a Bgl II site at the 5' end and an EcoR I site at the 3' end. The expected gene product would therefore contain 1.4 kb coding 466 amino acids as the extracellular domain (Figure 4.5) and 245 bp coding 82 amino acids as the cytoplamic domain.

4.3.2 Pseudo N-terminus

CD5 leader peptide was chosen as a pseudo N-terminus. Two complementary oligonucleotides of 110 bp corresponding to the amino acid sequence of the CD5 leader peptide were synthesised. The 3' end of the oligonucleotides was carefully decided by Ms J Dunne to make it Bgl II cohesive while the ligated product of the pseudo N-terminus and pT1B were kept in their original reading frames. The 5' end of the oligonucleotides was made Hind III compatible. Therefore the pseudo N-terminus contained a Hind III site at the 5' end to join the vector and Bgl II site at the 3' end to link the 5' end of the pT1B insert (Figure 4.5).

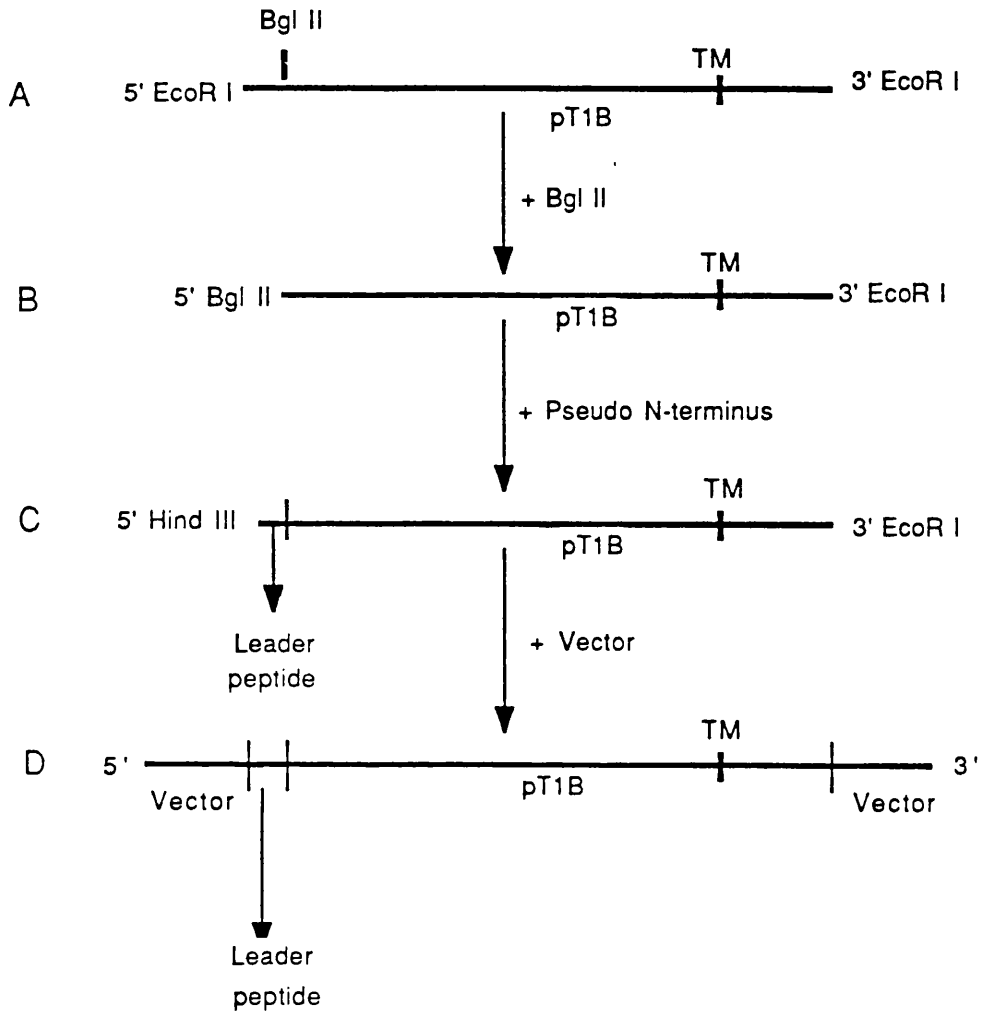


Figure 4.5 Outline of the cloning strategy.

The strategy of cloning pT1B and the pseudo N-terminus into the pIRV transfection vector is illustrated. a. pT1B cloned at EcoR I site in the original vector, b. the plasmid containing pT1B is cleaved with both EcoR I and Bgl II resulting in a fragment with Bgl II at 5' and EcoR I 3', c. pseudo N-terminus with Hind III at 5' and Bgl II at 3' is joined with the pT1B fragment, d. the recombinant of pT1B and the pseudo N-terminus is inserted in the right orientation into the pIRV vector which had been cut with both Hind III and EcoR I.

```
CD5 5'TACCCGGCCAGACACCCTCACCTGCGGTGCCCAGCTGAGGCAAGAGAAGGCCAGAAACC ATG
                                                                   Met

Pseudo N-terminus              5'AGCTTCTGAGGCAAGAGAAGGCCAGAAACC ATG
                                 HindIII                        Met


CCC ATG GGG TCT CTG CAA CCG CTG GCC ACC TTG TAC CTG CTG GGG ATG CTG
Pro Met Gly Ser Leu Gln Pro Leu Ala Thr Leu Tyr Leu Leu Gly Met Leu

CCC ATG GGG TCT CTG CAA CCG GTG GCC ACC TTG ATC CTG CTG GGG ATG CTG
Pro Met Gly Ser Leu Gln Pro Leu Ala Thr Leu Tyr Leu Leu Gly Met Leu


                        +1
GTC GCT TCC TGC CTC GGA CGG CTC AGC TGG TAT .....................
Val Ala Ser Cys Leu Gly Arg Leu Ser trp Tyr

GTC GCT TCC TGC CTC GGA CGG CTC CA 3'5'G ATC TCA ACA.............
Val Ala Ser Cys Leu Gly Arg Leu Gln      Ile Ser Thr  into pT1AB frame
```

Figure 4.6 The pseudo N-terminus and its relationship with the CD5 leader peptide.

The complete nucleotide sequence including the untranslated region and amino acid sequence of the CD5 leader peptide are shown. The first amino acid of mature CD5 protein (Arg) is designated as +1. The nucleotide and amino acid sequences of the pseudo N-terminus are aligned under the CD5 leader peptide. Segments different from the CD5 leader peptide are underlined including the synthetic Hind III site at the 5', Bgl II site at the 3' end and several amino acids coded by pT1B.

Combining the restriction sites of both pseudo N-terminus and pT1B insert, it is clear that the two fragments would join together only in one orientation at Bgl II sites. The recombinant would be 1.85 kb in length and contain a Hind III site at the 5' end and an EcoR I site at the 3' end, therefore the recombinant could be inserted into an expression vector which has both Hind III and EcoR I cloning sites in the right orientation.

4.3.3. Eukaryotic expression vector

Two eukaryotic expression vectors which mimic natural transcription units were employed. They consist of genomic sequences containing natural promoter elements and RNA processing signals. DNA inserted downstream of the promoter will be transcribed and the gene product will be expressed. The pIRV vector (Figure 4.7), constructed by Dr JP Morgenstern in ICRF is 6.5 kb in length and consists of the following parts: 1) the pST 1 fragment of PBR 322 (1980 bp) containing the origin of replication and β-lactamase gene which confers resistance to ampicillin; 2) The long terminal repeats (LTRs) at both 5' and 3' ends which serve as promoter and polyadenylation signal of the transcript respectively. Flanked between the LTRs are genes coding for gag, neomycin (neo), β-actin promoter and a polylinker where the DNA to be expressed is inserted.

Once inside the mammalian cell, the 5' LTR would drive the transcription of the neo gene which would render the recipient cell resistant to neomycin (G418). The polylinker, which is located downstream of the β-actin promoter, contains several cloning sites including Hind III, Bam H1, SnaB I, EcoR I, Sal I, and Cla I. Genes cloned in the polylinker would be transcribed under the control of the β-actin promoter in a mammalian cell (Figure 4.7).

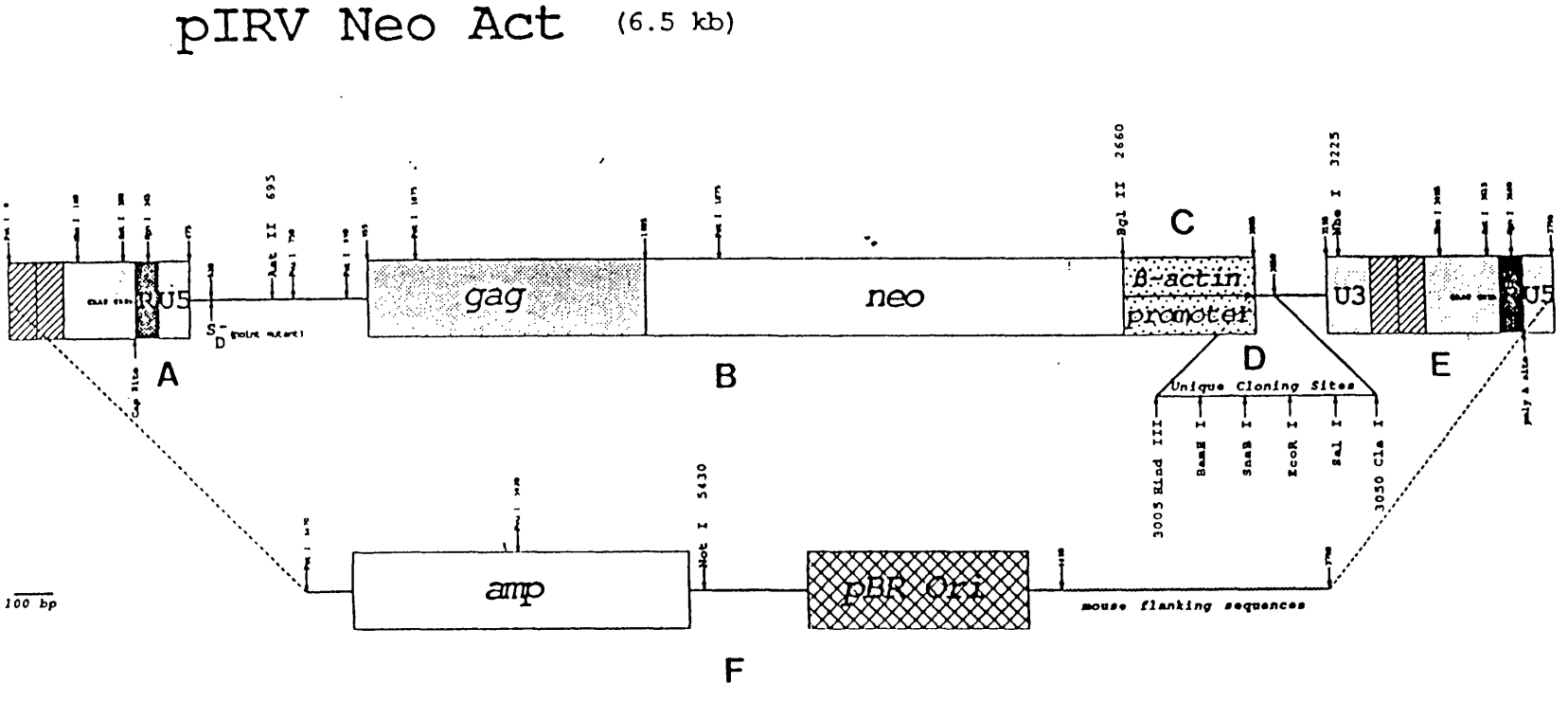


Figure 4.7 pIRV expression vector. A.E. are the long terminal repeats (LTRs) at the 5' and 3' ends respectively; B. The regions containing genes for gag and neo; C. β-actin promoter; D. polylinker for cloning and F. The region includes the bacterial origin and ampR gene from pBR322.

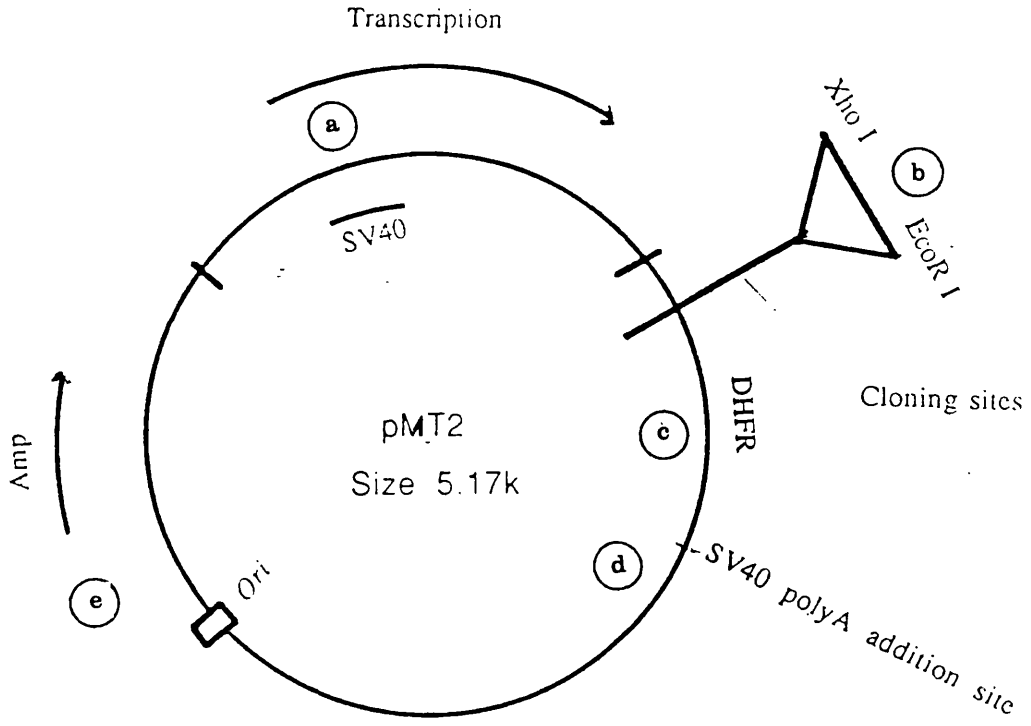


Figure 4.8 pMT2 expression vector. a. The SV40 early region promotor (340 bp); b. The position of the test gene cloned under the transcription control of the SV40 early region promotor; c. a region coding DHFR; d. Poly A addition site and e. The region includes the bacterial origin and ampR gene from pBR322.

Another vector pMT2 (Figure 4.8) contains the SV40 promoter with a polylinker situated downstream of the promoter containing cloning sites for Xho I and EcoR I. The vector does not contain neo gene, therefore, to select G418 resistant transfectants, L cells have to be co-transfected with a plasmid containing a neo gene, such as pIRV.

4.3.4 Cloning

1) Vector: pIRV was first digested with Hind III, then the reaction mixture was adjusted to a high salt concentration and further digested by EcoR1. Individual samples cut by only Hind III or EcoR1 were included as control and were tested by agarose gel electrophoresis to determine that all the plasmid had been linearised. The liberated small fragment from the cloning sites between Hind III and EcoR I, which otherwise would compete with the pseudo N-terminus and pT1B for the vector in a ligation reaction, was removed by precipitation and washing in 70% ethanol several times.

Before ligating the leader sequence and pT1B insert into the vector, the double cut vector was first tested in transformation assays. This involved transforming competent bacteria with the following preparations: (1) the unligated double-cut vector, (2) self-ligation of the double-cut vector, (3) self-ligation of the double-cut vector together with its original polylinker generated by the double cutting and (4) original vector. The unligated double-cut vectors did

not transform the bacteria, the self-ligated vector transformed poorly, while the ligation of vector together with its original polylinker and the uncut vector transformed the competent cells effectively. The results indicated that the double-cut vector had been digested completely with both Hind III and EcoR I. It was poor at self-ligation and was ready for ligation with the pseudo N-terminus and the insert.

2) Pseudo N-terminus: The pseudo N-terminus was synthesised by I. Goldsmith in the ICRF oligonucleotide synthesis laboratory and supplied as two complementary single strand oligonucleotides with hydroxyl groups at both 3' and 5' ends. To facility the ligation, the oligonucleotides were annealed and kinased as described in 4.2.4.

3) pT1B fragment: The plasmid containing pT1B was cleaved by both Bgl II and EcoR I and fractionated by electrophoresis. The fragment of interest was excised and purified as described in 4.2.3.

4) Ligation: The double-cut vector, the leader sequence and pT1B insert were quantitated by gel electrophoresis. The ligation was carried out by mixing 100 ng of vector, 100 ng leader sequence and 40 ng of insert as described in 4.2.5. Two microliters of ligation mixture were removed and analysed by gel electrophoresis together with a sample of vector ligated alone under the same conditions. The pattern of the gel electrophoresis of vector, leader

sequence and the ligated insert was different from self-ligated vector and un-ligated vector.

5) Transformation and screening by restriction enzyme mapping: The ligated mixture was diluted 1:5 in TE pH 8.0, and transformed into competent JM101 bacteria. The ligation of vector together with the pseudo N-terminus and pT1B insert generated significantly more colonies than that of self-ligated vector alone. Twelve arbitarily selected individual colonies were inoculated and grown up as overnight cultures. Mini-preparation of plasmid was made. The plasmids were subjected to restriction enzyme mapping.

4.3.5 Restriction enzyme map of the recombinants

The restriction sites of the right recombinant were deduced from the combination of the restriction maps of the double-cut vector, the pseudo N-terminus and pT1B insert. The recombinant would contain EcoR I, Bam H1 and Hind III as unique sites (see Table 4.1). The right recombinant subjected to any of these enzymes would generate a single linearised band about 8.3-8.4 kb while self-ligated vector would be around 6.4 kb.

There would be two Bgl II sites in the desired recombinant plasmid. An internal Bgl II site located at 335 bp upstream from the Hind III site in pIRV vector (Figure 4.7) and the Bgl II site at the joint of pT1B and the pseudo N-terminus. Bgl II digestion of the plasmids

would yield two fragments at about 450 bp and 7.9 kb respectively. The 450 bp fragment would consist of 335 bp between Hind III and Bgl II of the vector plus the 110 bp pseudo N-terminus. Digestion with EcoR I and Bgl II would generate three fragments. Apart from the 450 bp fragment, a fragment at 1.75 kb and another one at about 6.1 kb would be observed. The 1.75 kb fragment would be the pT1B fragment cloned. The 6.1 kb fragment was pIRV vector devoid of the Bgl II and Hind III fragment (Figure 4.7). Table 4.1 and Table 4.2 summarise the main restriction sites contributed by the vector, pseudo N-terminus and pT1B insert and the restriction map of the recombinant respectively.

Table 4.1 Restriction sites of the individual fragments

	pIRV	Pseudo N-terminus	pT1B	Number of sites after recombination
EcoR I	5'	-	3'	1
Hind III	3'	5'	-	1
Bam H I	-	-	1	1
Bgl II	1	3'	5'	2

Table 4.2 Restriction map of the desired recombinant

	Fragment Number	1st (kb)	2nd (kb)	3rd (kb)
EcoR I	1	8.3		
Hind III	1	8.3		
Bam H I	1	8.3		
Bgl II	2	7.9	0.45	
Bgl II + EcoR I	3	6.1	1.75	0.45

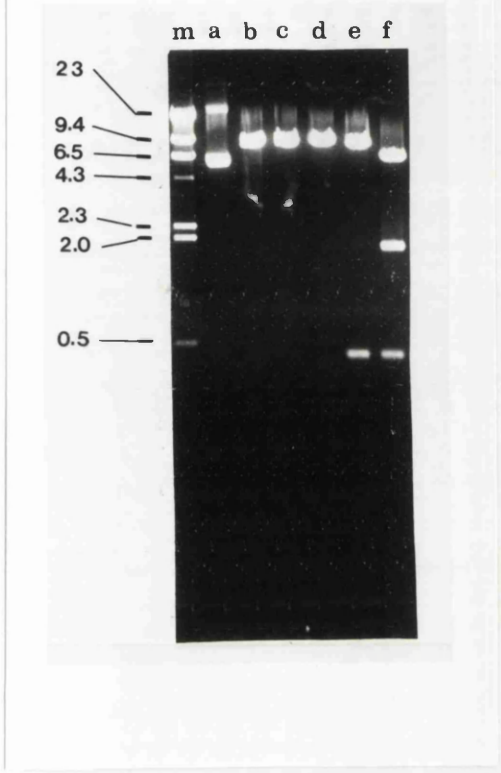


Figure 4.9 The restriction enzyme map of the selected recombinants. m. λDNA Hind III fragments serve as molecular weight marker; a. The intact recombinant without restriction enzyme treatment; b. EcoR I only; c. Hind III only; d. Bam HI; e. Bgl II only and f. EcoR I+Bgl II.

Initial screening was made by single EcoR I enzyme treatment. Among the 12 samples, two clones showed gel retardation with a single band at 8.3 - 8.4 kb while the remaining samples co-migrated with linearised pIRV at 6.4 kb. The two clones were designated as 7 and 11 respectively after their original number when the colonies were inoculated for mini-preparation analysis. Their identities were further confirmed by digestion with other enzymes.

The result of restriction enzyme mapping analysis is shown in Figure 4.9. The two plasmids were subjected to 1) EcoR I, 2) Hind III, 3) Bam HI, 4) Bgl II and 5) Bgl II plus EcoR I. Both plasmids gave identical restriction enzyme mapping pattern. EcoR I, Hind III and Bam HI treatment resulted in a single linearised band at 8.3 kb. Bgl II alone generated two fragments: 450 bp and 7.9 kb. The combination of Bgl II and EcoR I treatment gave rise to three fragments: 450 bp, 1.75 kb and 6.1 kb. The restriction enzyme map of the two plasmid selected were in agreement with that summarised in Table 4.2. It indicated that both plasmids selected met the criteria for a correct recombinant.

4.3.6 Sequence of the recombinants

The restriction enzyme mapping analysis of the selected plasmids suggested that the plasmid might be the right recombinant to transfect L cells. To ensure that the recombinant plasmid indeed contained the right reading frame for both the pseudo N-terminus

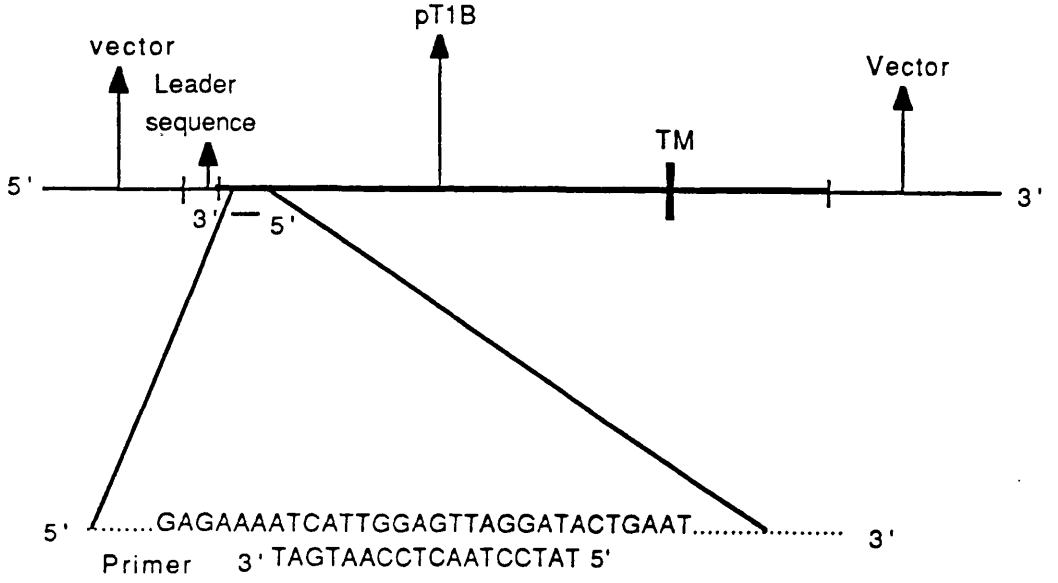


Figure 4.10 Sequencing strategy and the synthetic primer. The recombinant of pIRV, the pseudo N-terminus and pT1B joined in the right orientation is shown. A 18 mer synthetic oligonucleotide which is complementary to the sequence of pT1B near the joint with the pseudo N-terminus is underlined. The sequencing starts from the 3' end of the primer, goes through the leader sequence and then reaches the pIRV vector.

and pT1B insert, sequencing was performed. A 18 mer single strand oligonucleotide which is complementary to the sequence of pT1B positive strand near the joint was synthesised and used as a primer for the sequencing (Figure 4.10). The sequencing starts from the 3' end of the primer. The nucleotides incorporated were complementary to the template DNA. Therefore it was possible to read from the 3' end of the primer, through the Bgl II joint region, the pseudo N-terminus and finally into the vector.

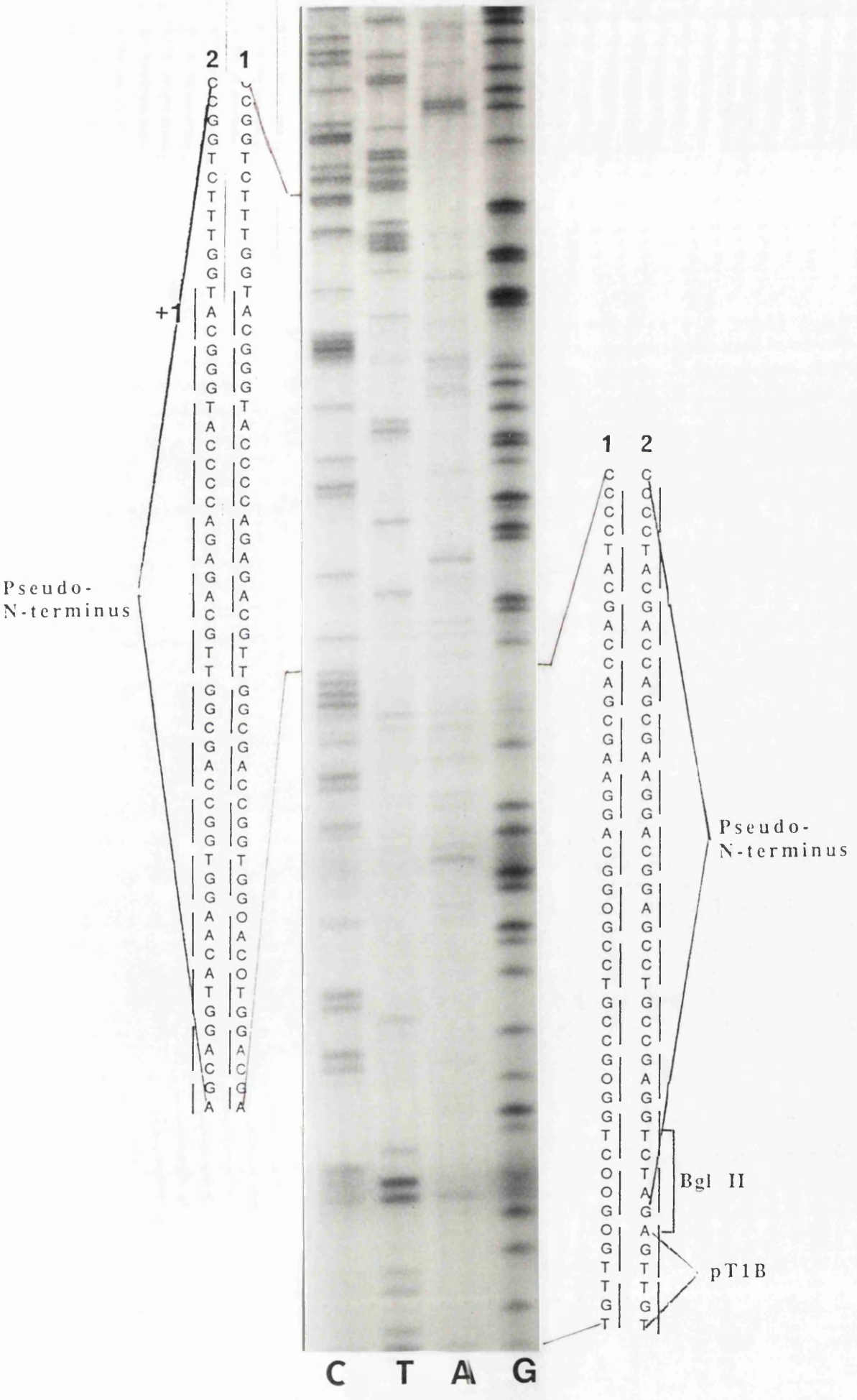
Double-stranded DNA sequencing was carried out by the method of Sanger et al (1977) in which the purified supercoiled recombinant plasmids (both plasmids were analysed as separate samles) were denatured and made single-stranded by alkaline treatment. Tthe primer was then added and annealed with the template DNA, following which dideoxy- and deoxynucleotides and Taq polymerase together with ^{32}P-dATP were added according to the standard protocol supplied by Strategene (1988).

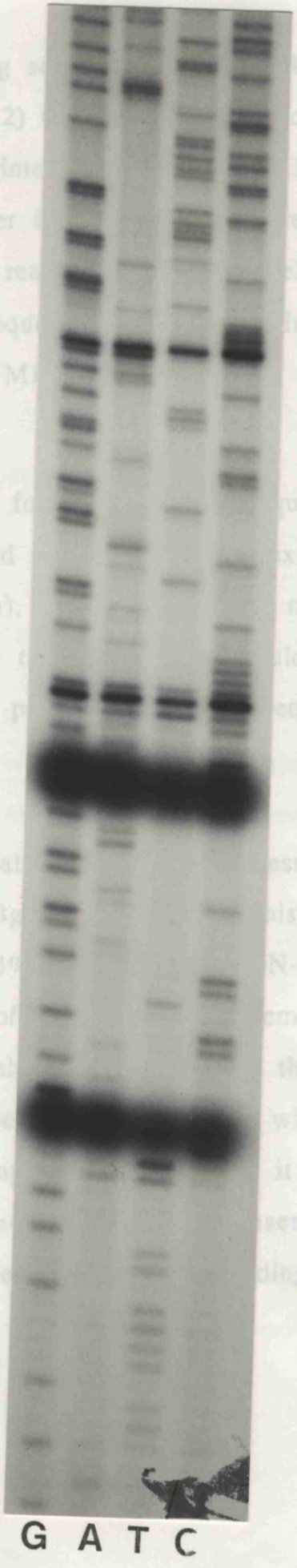
The sequencing was repeated several times due to various problems encountered. Both plasmids revealed identical patterns. A photograph of a sequencing gel is shown in Figure 4.11a. Unfortunately, bands in the A and T tracks are not of equal intensity to the bands in the G and C tracks. However, this is not an unusual phenomenon (Winter and Coulson, M13 cloning). The nucleotides which could be read with confidence are indicated beside the gel while those which are not clear enough are marked as circles. Difficulties can usually be overcome by following several

Figure 4.11 Sequence of the recombinant.

a. Shown is an autoradiograph of regions of gel analysing the sequence from pT1B to the end of the pseudo N-terminus. Sequence shown is the antisense strand (1). The corresponding initiation codon in the pseudo N-terminus is indicated as +1. The Bgl II site at the joint region is indicated. There were compressions in several regions including one at the Bgl II joint site. The sequence in the joint region was determined from the spaces between nucleotides as well as the fact that the Bgl II restriction site did exist in the region. Circles stand for spaces with unclear nucleotides. The expected sequence is displayed alongside the sequence obtained (2).

b. The sequence of the recombinant was determined by reading the nucleotides from several sequencing gels. Shown is a photograph containing regions which can be read with confidence.


2
1
+1
Pseudo-
N-terminus
1
2
Pseudo-
N-terminus
Bgl II
pT1B
C
T
A
G


G A T C

rules when interpreting sequencing data: 1) Upper C is always more intense than lower C; 2) Upper G is often more intense than lower G; Upper A is often less intense than lower A; Upper G is MORE intense than lower G whenever the double G is preceded by a T; 5) When reading a gel always read the spaces as well as the band to ensure that the proposed sequence fits exactly into the space available (Winter and Coulson, M13 cloning).

When these rules are followed and the sequence of the recombinant obtained is compared to that of the expected correctly joined plasmid (Figure 4.11a), it is clear that if most of the circles were replaced with A, then the recombinant would contain both the pT1B insert and the entire pseudo N-terminal sequence.

The sequencing gel also showed "compression" in several regions including one at the Bgl II joint region. This may be due to the high G:C composition (63%) of the pseudo-N-terminus, so that local secondary structure of the DNA may remain undenatured on the urea-polyacrymide gel. However, when the results from several repeat gels were combined, together with the results of the restriction enzyme mapping (Figure 4.9), it was concluded that the recombinants contained both pT1B insert and the pseudo N-terminus, and they were in the right reading frame.

4.3.7 Cloning DNA into pMT2 expression vector

The pseudo N-terminus and pT1B insert was also cloned into pMT2 vector. The sequence of pIRV-1B proved the recombinants selected contained the pseudo leader peptide and pT1B in the right reading frame. The Hind III and EcoR I fragment of pIRV-1B (plasmid 7 and 11) which contained both the pseudo leader peptide and pT1B insert was employed (Figure 4.5.). The cloning procedure and the results are simplified in Figure 4.12.

4.3.8 DNA transfection

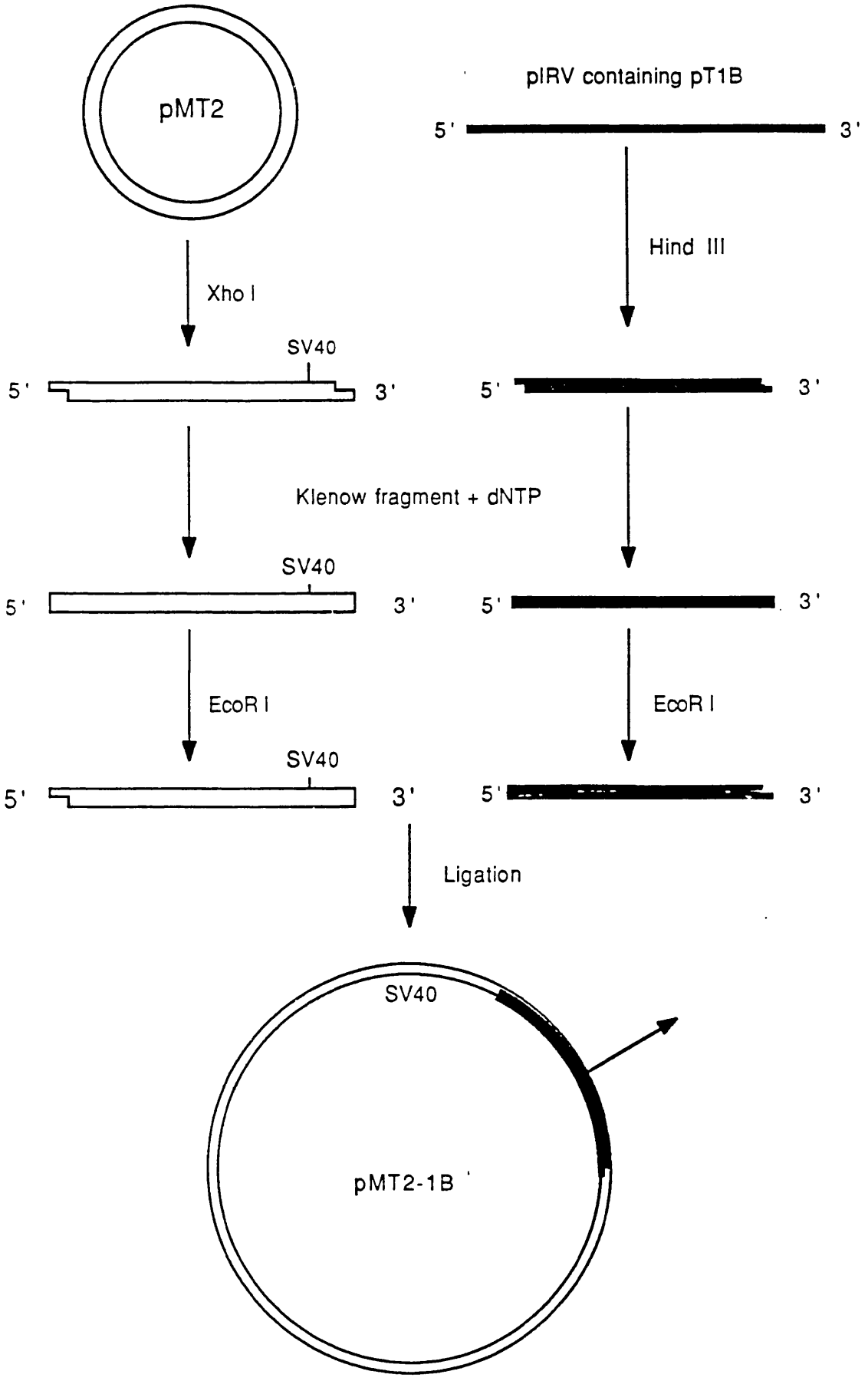
1) Titration of G418 on L cells: Prior to the commencement of DNA transfection, L cells were grown at different concentration of G418 ranging from 50, 100, 200, 400, 800 and 1000 ug/ml to decide optimal selection conditions. L cells grown at more than 200 ug/ml of G418 died in two weeks. Therefore G418 at 250 ug/ml was chosen for selection of G418 resistant transfectants.

2) Transfection: Transfection was carried out as described in 4.2.12. Plasmids 7 and 11 were kept as two individual samples. G418 resistant colonies appeared in about 2 weeks after the transfection. Usually around 10-15 colonies were observed from 3 x 10^5 seeded L cells. The colonies were pooled and kept in G418 all the time.

Figure 4.12 Cloning procedure for pMT2-1B.
Vector: pMT2 contains two cloning sites Xho I and EcoR I. The vector was digested to completion with Xho I. The protruding sticky ends were blunted by klenow fragment in the presence of four dNTPs. After phenol extraction to remove the klenow, the vector was digested with EcoR I. Therefore the vector had a EcoR I at 5' end and a 3' blunt end.

Fragment containg pseudo N-terminus and pT1B insert. pIRV-1B whose sequence had proved to containing the right insert was digested first with Hind III and blunted with klenow as for the vector. It was then digested with EcoR I so the fragment contained a 5' blunt end and a EcoR I site at the 3' end.

The vector and the fragment were ligated together in the right orientation. The pseudo N-terminus and pT1B insert was under the control of the SV40 promoter.


pMT2
pIRV containing pT1B
5'
3'
Hind III
Xho I
SV40
5'
3'
5'
3'
Klenow fragment + dNTP
SV40
5'
3'
5'
3'
EcoR I
EcoR I
SV40
5'
3'
5'
3'
Ligation
SV40
pMT2-1B

pMT2-1B did not contain the neo gene, L cells were co-transfected with pMT2-1B and pIRV at a molar ratio of 10:1 and selected by the same concentration of G418 pIRV-1B. Five to six G418 resistant colonies were observed for the transfection from every $3x10^5$ seeded L cells. Transfectants were pooled, expanded and subjected to southern and northern blot analysis.

4.3.9 pT1B gene is integrated in the L cell genome

High molecular weight DNA was extracted from J6 cells, L cells, and established transfectants 7 and 11. Ten micrograms of DNA from each sample were subjected to EcoR I digestion, fractionated on 0.8% agarose gel and transferred onto genescreen plus membrane. The membrane was probed with ^{32}P labelled Bgl II and EcoR I fragment of pT1B (1.75 kb). L cells transfected with plasmid 7 and 11 showed heavy bands around 23 kb (Figure 4.12 lane 3 and4), while J6 and L cells showed some faint bands at about 2.0 kb (Figure 4.12 lane 1 and 2). With long exposure these faint bands became much more evident (data not shown). The linearised plasmid 7 and 11 was about 8.3 to 8.4 kb, the detection of 23 kb from the transfectants on southern blot analysis therefore suggesting that the plasmid was integrated into L cell genomic DNA.

4.3.10 The transfectants transcribed pT1B message

RNA from J6 cells, L cells, L cells transfected with pIRV alone, L cells transfected with plasmid 7 and 11, L cells transfected with pMT2 alone and L cells transfected with pMT2-1B were extracted

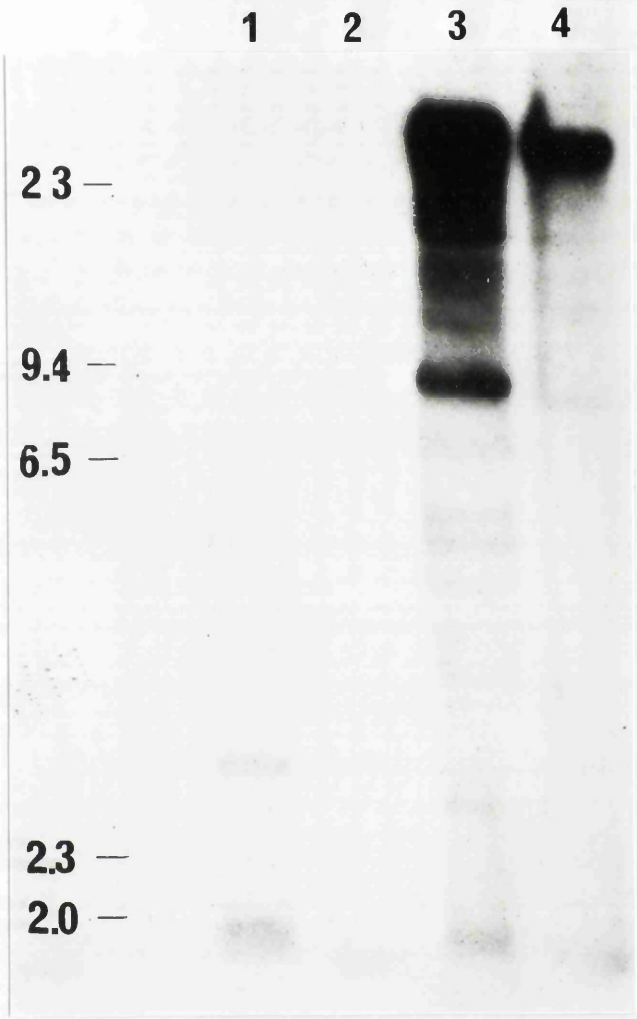


Figure 4.13 Southern blot analysis of transfectants.

DNA from J6 cells, L cells, L cells transfected with plasmids 7 and 11 was digested to completion with EcoR I, electrophoresed through a 0.8% agarose gel, blot transferred to a gene screen-plus membrane and hybridised with ^{32}P labelled pT1B fragment. The molecular weight reference is λDNA digested by Hind III. DNA sources are as follows: 1: J6 cells, 2: L cells, 3: L cells transfected with plasmid 7 and 4: L cells transfected with plasmid 11.

Figure 4.14 Northern blot analysis of pT1B-related transcripts. Twenty micrograms of total RNA was loaded on each slot of a 1% agarose gel, electrophoresed, blotted and hybridised with pT1B fragment. RNA souces were as follows: lane 1, J6; lane 2, L cells; lane 3, L cells transfected with pIRV vector only; lane 4, L cells transfected with plasmid 7; lane 5, L cells transfected with plasmid 11; lane 6, L cells transfected with pMT2 + pIRV; lane 7, L cell transfected with pMT2-1B plus pIRV. The reference RNA was an RNA ladder from BRL.

and fractionated as described in section 4.2.15 and 4.2.16. The northern blot was probed with, as for the southern blot analysis, ^{32}P labeled Bgl II and EcoR I fragment (1.75 kb). L cells, L cells transfected with pIRV alone and L cells transfected with pMT2 alone did not show any detective bands. Two transcripts were observed for L cells transfected with plasmids 7 and 11: 6.0 kb and 2.8 kb. As pIRV contains two RNA synthesis promoters: the two LTRs and the β-actin promoter, it is possible that the 6.0 kb transcript is mRNA synthesised under the control of the 5' LTR. It would contain gag (730 bp), neo gene (977 bp), β-actin promoter (345 bp) and the cloned insert (about 2.0 kb) (Figure 4.7), while the 2.8 kb transcript is synthesised under the control of the β-actin promoter upstream from the cloned pT1B insert.

L cells transfected with pMT2-1B showed one transcript of 3.2 kb, consistant with the fact that pMT2 only containing one promoter. Northern blot analysis of cells at different stages after transfection were performed, the results suggested that the transfectants transcribed pT1B-related messenger RNA stably throughout a period of several months (data not shown).

4.3.11 Immunisation of mice with transfectants

Mice were immunised with the established transfectants. Ten days after the third boosts, sera from immunised mice were pooled and analysed in several assays.

Antisera were first assayed by cell surface immunofluorescence staining. L cells, L cells transfected with pIRV, pMT2, and L cells transfected with pIRV-1B and pMT2-1B were stained with a series of dilutions of antisera. It was found that the antisera from immunised mice did not show significant specific staining of the transfectants. Table 4.3. summarises the surface staining results.

Table 4.3 Cell surface staining by mouse antisera

Antiserum dilutions	L cells	pIRV	7	11	pMT2	pMT2-1B
1:20	+	+	+	+	+	+
1:40	+	+	+	+	+	+
1:80	+	+	+	+	+	+
1:160	±	±	±	±	±	±
1:320	-	-	-	-	-	-
1:640	-	-	-	-	-	-
1:1280	-	-	-	-	-	-

Note: Two mice were immunised with each transfectant and the sera pooled. The results shown were obtained with antisera raised against transfectant 7. Results with the anti-transfectant 11 antiserum were identical to the resluts shown. Normal mouse serum gave a similar pattern of staining but the titre was one doubling dilution less.

The results suggested two possilities: 1) Though the gene product was presumed to be a surface molecule, it may not be expressed on the cell surface, especially, as the gene had been linked with a pseudo leader peptide; 2) The second possibility was that the antisera did not contain any antibody against the gene product. To test these possibilities the antisera were tested on fixed cytospins of the transfectants in an attempt to detect the gene product in the cytoplasm. The results obtained were similar to those from the surface staining assay (data not shown).

4.3.12 An attempt to locate the gene product by non-immunological means

The results from southern and northern blot analysis clearly demonstrated that pT1B was integrated into the genome of the transfectants and transcribed stably. The fact that the antisera from mice immunised with the transfectants showed poor and non-specific reactivity with the transfectants might suggest that the transcribed message was not processed properly in the transfectants. Alternatively the gene product may be non-immunogenic, so an attempt was made to locate the gene product by non-immunological means.

1) Cell surface: The search was first focused on the cell membrane as the gene was presumed to code for a membrane protein. According to the northern blot analysis, it is clear that when equal amount of RNA are loaded onto the gel, the transfectants synthesised far more pT1B-related RNA than the positive control J6 (Figure 4.14). Therefore it might be possible to detect the gene product by SDS-PAGE analysis of iodinated transfectants. L cells and L cells transfected with plasmids 7 and 11 in log phase growth were harvested and washed free of serum. They were catalysed with lactoperoxidase in the presence of ^{125}I and other agents as described in 4.2.19. The labelled cells were solubilised and the supernatant which contained membrane proteins were saved.

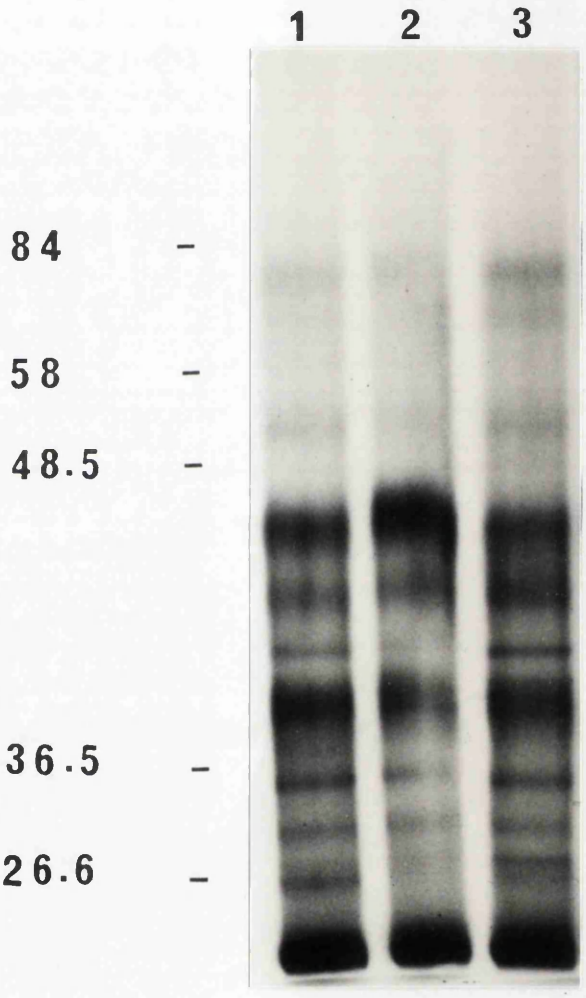


Figure 4.15 SDS-PAGE analysis of ^{125}I labelled transfectants.

Cells were iodinated by the lactoperoxidase method as described in 4.2.17. Cell lysates from $2x10^5$ cell equivalents were analysed by 10% SDS-PAGE. An autoradiograph of the gel is shown 1: L cells transfected with plasmid 11, 2: L cells transfected with plasmid 7, and 3: L cells.

Due to the lack of specific antibody to precipitate the gene product, the whole soluble fractions which contained membrane proteins from L cells, 7 and 11 were fractionated on a 10% SDS-PAGE gel. The autoradiograph of the gel is shown in Figure 4.15. It is apparent that there was no discernible difference in protein migration patterns between L cells and the transfectants.

2) Cytoplasm: L cells and transfectants were biosynthetically labelled with ^{35}S-methionine as described in 4.2.20. After solubilisation, the nuclei were removed by centrifugation. The soluble fraction was analysed on an SDS-PAGE gel. No difference was detected between L cells and the transfectants (Figure 4.16).

3) Supernatant: Since it was not sure if the mature pT1B gene product adopted a secreted form, the supernatants of L cells and the transfectants were also examined. L cells and transfectants washed free of serum were grown in methionine free RPMI 1640 medium supplemented with 0.5% FCS (dialysed extensively agaist PBS) together with ^{35}S-methionine for 12 hours. The supernatants were harvested and precipitated with Trichloroacetic acid (TCA). After contrifugation the precipitates were washed in acetone twice. They were then resuspended in SDS-PAGE sample buffer and subjected to SDS-PAGE analysis. No difference was observed between the supernatant from L cells and those of transfectants (data not shown).

Figure 4.16 SDS-PAGE analysis of biosynthetically labelled transfectants.

The nonionic detergent soluble fractions ($5x10^5$ cell equivalents) from biosynthetically labelled cells were analysed by SDS-PAGE. Lane 1: L cells transfected with plasmid 11, 2: L cells transfected with plasmid 7, and 3: L cells.

4.4 Discussion

It is clear that pT1B gene has been linked in frame with a pseudo N-terminus. The gene has been integrated into the genome of the transfectants and transcribed efficiently. Yet, the antisera from mice immunised with the transfectants did not show specific recognition in both immunofluoscence staining of cell surfaces and cytospin staining to detect a cytoplasmic gene product. Similarly the attempt to locate the gene product, on the cell surface, in the supernatant or in the cytoplasm by non-immunological means was not sucessful. Since a successful immune response depends on the immunogen used, the failure to detect the gene product might explain the poor immune response from the mice. Several problems may account for the failure of immunisation.

1) The gene was not processed properly as expected

The transfectants have been demonstrated to transcribe the pT1B-related mRNA but the fate of the mRNA cannot be determined due to the lack of sensitive probe (antibodies) for the gene product, it is difficult to conclude that the gene has or has not been translated. As indicated at the beginning of the introduction to this chapter, the truncated pT1AB has an internal EcoR I site within the cytoplasmic domain. When the gene is generated from the original cloning vector λgt11 to recombine with other DNA fragments, it is difficult to carry the whole pT1AB sequence to another vector. To simplify the procedure, only pT1B was used. Therefore the transcript

detected by northern blot analysis coded the pseudo N-terminus, part of the extracellular domain and an incomplete cytoplasmic domain of the pT1AB.

Such a construct was new. In theory, the transcript containing an initiation codon and the right reading frame should be translated and the gene product should be located somewhere within the cell or secreted. However in reality, it is difficult to know the ultimate destination of the mRNA. The leader peptide may work in its original context within the CD5 molecule, but may no longer provide the correct signal when it is linked with another protein. In particular, it is not yet clear whether cell surface proteins require features other than the signal peptide. In fact, it has been suggested that a sequence further "downstream" may also be recognised by the translocation machinery (Benson and Silhavy, 1983).

The incomplete cytoplasmic domain might also contribute to abnormal processing of the gene product. An example showing the importance of the cytoplasmic domain in directing the destination of a protein within the cell was provided by Nilsson et al., (1989). The adenoviral transmembrane E3/19K glycoprotein is a resident of the endoplasmic reticulum (ER). They demonstrated that the last six amino acid residues of the 15-membered cytoplasmic tail are necessary and sufficient for ER retention. Wild type CD4 and CD8 are cell surface molecules. When CD4 and CD8 were depleted of their original cytoplasmic tail and linked with the last six amino acid residues from E3/19, they were not found on the surface of the

transfectants even though they still retained the transmembrane region. The chimeric CD4 and CD8 were observed in the endoplasmic reticulum.

Their work was not aimed at studying the importance of the cytoplasmic domain in directing the protein to be expressed on the cell surface. However, it indeed provided some clues to the importance of the cytoplasmic domain in maintaining the full function of a protein.

2) The expression of pT1AB product may be tissue specific

The tissue distribution of pT1B revealed by northern blot analysis (Chapter 6) suggested that the gene has a restricted distribution, mainly among T-ALL cell lines. It is known that the expression of some surface molecules of lymphocytes requires the transcription and translation of the associated molecules. The CD3/TCR complex and MHC antigens are such examples. The expression of CD3 requires the transcription of the rearranged TCRα/β or TCRγ/δ (Chapter 1, section 1.2). The expression of MHC class II antigen in transfectants demands co-transfection of both α and β genes (Chapter 1, section 1.5.5).

Although many lymphocyte surface molecules have been successfully transfected into L cells, there is evidence that the

expression of some gene products is recipient cell type dependent. CEM is a class II negative T-ALL cell line, CEM cells transfected with class II genes are unable to express the gene products. (M. Moore personal communication). It is not known if the pT1B gene product is associated with some other protein. However it may need either physical association with other proteins or some specific processing signal provided by the recipient cell in order to be expressed.

3) The gene product is too conserved to elicit immune response

It is known that there is a collection of proteins which exhibit EGF-like structure. The EGF-like motif has been found to be widely distributed, ranging from drosophila to mammals. Since the EGF homologous domain structure is widely distributed among different animal species, it is likely that mouse or rat will have gene products with similarity to pT1AB which would share functionally important sequences with their human counterpart. And the sequence differences between them might be insufficient to stimulate a strong immune response. This argument is somehow strengthened by the finding that pT1B cross-hybridises with mouse DNA (section 4.3.9).

CHAPTER FIVE

RAISING ANTIBODIES TO FUSION PROTEINS

5.1 Introduction

The previous two chapters described attempts to raise antibodies to the pT1AB gene product. Initially synthetic peptides derived from the pT1A gene sequence were used as immunogens (Chapter 3), but the reactivities of the polyclonal sera and mAbs from immunised mice and rats detected by protein dot blots and western blots did not correlate with the results of northern blot analysis (Chapter 6), and the antibodies did not react with native proteins. In a second series of experiments, the truncated pT1B gene was linked in frame with a pseudo N-terminus and transfected into L cells. The gene was integrated into the genome of the transfectants and stably transcribed. The transfectants were used as immunogens, but sera from mice immunised with the transfectants failed to stain living or fixed cells in a specific fashion.

Because of the difficulty of obtaining the full length cDNA of pT1AB within the limited time available in order to do further transfections, a third strategy employing fusion proteins expressed from partial DNA sequences was followed. In this system, a DNA segment to be expressed is fused in frame to a portion of the TrpE protein of *Escherichia coli*.

TrpE fusion protein:

1) The pathway of tryptophan biosynthesis:

In bacteria the pathway of tryptophan biosynthesis is controlled by the tryptophan (Trp) operon containing a regulatory locus (operator) and four functionally related genes which convert chorismate to tryptophan (Yanofsky et al., 1981). The operator defined as controlling the transcription of the entire group of coordinately induced genes contains a binding site for tryptophan-activated repressor protein. The repressor and RNA polymerase binding are mutually exclusive (Squires et al., 1975), therefore the activation of the repressor can regulate transcription initiation (Bennett and Yanofsky, 1978; Gunsalus and Yanofsky, 1980). The presence of tryptophan in the culture medium of E. coli represses the formation of all the enzymes required to form tryptophan.

2) TrpE fusion protein vector:

The plasmid vector for production of TrpE fusion proteins contains DNA segments of the natural trp transcription promoter and trpE, thus the synthesis of TrpE protein will be regulated by tryptophan metabolism. In general, bacteria containing a recombinant plasmid are grown in a repressed state to prevent the synthesis of tryptophan. They are then induced with repressor inhibitor, 3β-indoleacrylic acid (IAA, a tryptophan-related compound which can bind competitively the repressor) to produce TrpE protein. If a DNA fragment containing the information for a segment of protein is fused in frame with the trpE protein, the end product will be a protein containing trpE as well as the foreign protein segment, a

fusion protein. The fusion protein can be purified and used as immunogen.

This chapter describes the construction of several fusion proteins from the pT1B gene and the antibodies derived by immunisation with these proteins.

5.2 Methods

5.2.1 Production of fusion protein

1) Induction of fusion protein: A single colony which contained a recombinant plasmid was inoculated in 1 ml M9 media containing casamino acids, ampicillin and tryptophan and grown at 37^0C overnight. 0.5 ml of the overnight culture was diluted into 5 ml M9 containing casamino acids, ampicillin and grown for one hour at 30^0C with great aeration. 25 ul of 1 mg/ml IAA in ethanol was added and the culture was further grown for 2 hours at 30^0C. Both whole cell lysate and the insoluble fraction of bacteria were tested for the production of the fusion proteins by SDS-PAGE.

2) Whole cell lysate: 1 ml of induced culture was pelleted by microfuging and resuspended in 50 ul cracking buffer containing 0.01 M Sodium phosphate pH 7.2, 1% β-Mercaptoethanol, 1% SDS and 6M urea. The lysate was incubated at 37^0C for 0.5-3 hours and 10 ul lysate was loaded onto a 12.5% SDS-PAGE gel.

3) Insoluble fraction: 1 ml induced culture was pelleted and resuspended in 0.1 ml TEN (50 mM Tris, pH 7.5, 0.5 mM EDTA, 0.3 M NaCl); 10 ul 10 mg/ml lysozyme was added and the mixture was left on ice for 15 minutes; 2 ul 10% NP-40 was then added and incubated for another 10 minutes on ice, finally 150 ul NaCl-Mg (1.5 M NaCl, 12 mM $MgCl_2$) together with 0.5 ul 1 mg/ml DNase were added and the mixture was incubated on ice for one more hour. The lysate was microfuged for 5 minutes, the pellet was washed twice with 100 ul TEN, and resuspended in 50 ul cracking buffer. It was incubated at 37^0C for 30 minutes before loading 10 ul onto a 12.5% SDS-PAGE.

4) Large scale fusion protein production: The procedure was a scaled up version of the method for the insoluble fraction according to Kleid et al., (1981).

5.2.2 Coupling protein to Sepharose CL 4B

Cyanogen bromide (CNBr)-activated Sepharose CL 4B 250 mg washed in 1 mM HCl, was mixed at room temperature for 2 hour with 1 mg fusion protein in 1 ml of coupling buffer (0.1 M NaHCO3 pH 8.3 containing 0.5 M NaCl). Uncoupled protein was washed off with coupling buffer and the sepharose beads were mixed at room temperature for 2 hour with 0.1 M Tris-HCl buffer pH 8.0. This was followed by 3 cycles of washes, each cycle consisting of a wash with 0.1 M Acetate buffer pH 4.0 containing 0.5 M NaCl followed by a

wash with 0.1 M Tris-HCl buffer pH 8.0 containing 0.5 NaCl. The coupled mixture was stored at 4^0C in PBSA.

5.3 Results

5.3.1 Expression vectors

A series of fusion protein expression vectors, pATH1, pATH2 and pATH3 modified from Spindler et al., (1984) are shown in Figure 5.1. The construction of these vectors is virtually the same except for the reading frames of the polylinkers. They contain the E *coli* trp promoter as well as the entire trpE sequence which codes a 37 kd trpE protein. A DNA fragment to be expressed was inserted in the polylinker at the end of the trpE gene. Therefore the induced fusion protein would consist of the 37 kd trpE protein plus the amino acids coded by the pT1B gene fragment. To ensure that the foreign DNA is linked in frame with the trpE protein, the vector and the DNA to be used were carefully selected to fit the reading frames of both the vector and the DNA insert. Table 5.1 lists the cloning sites of pATH1, pATH2 and pATH3, and the reading frames of these restriction enzyme sites.

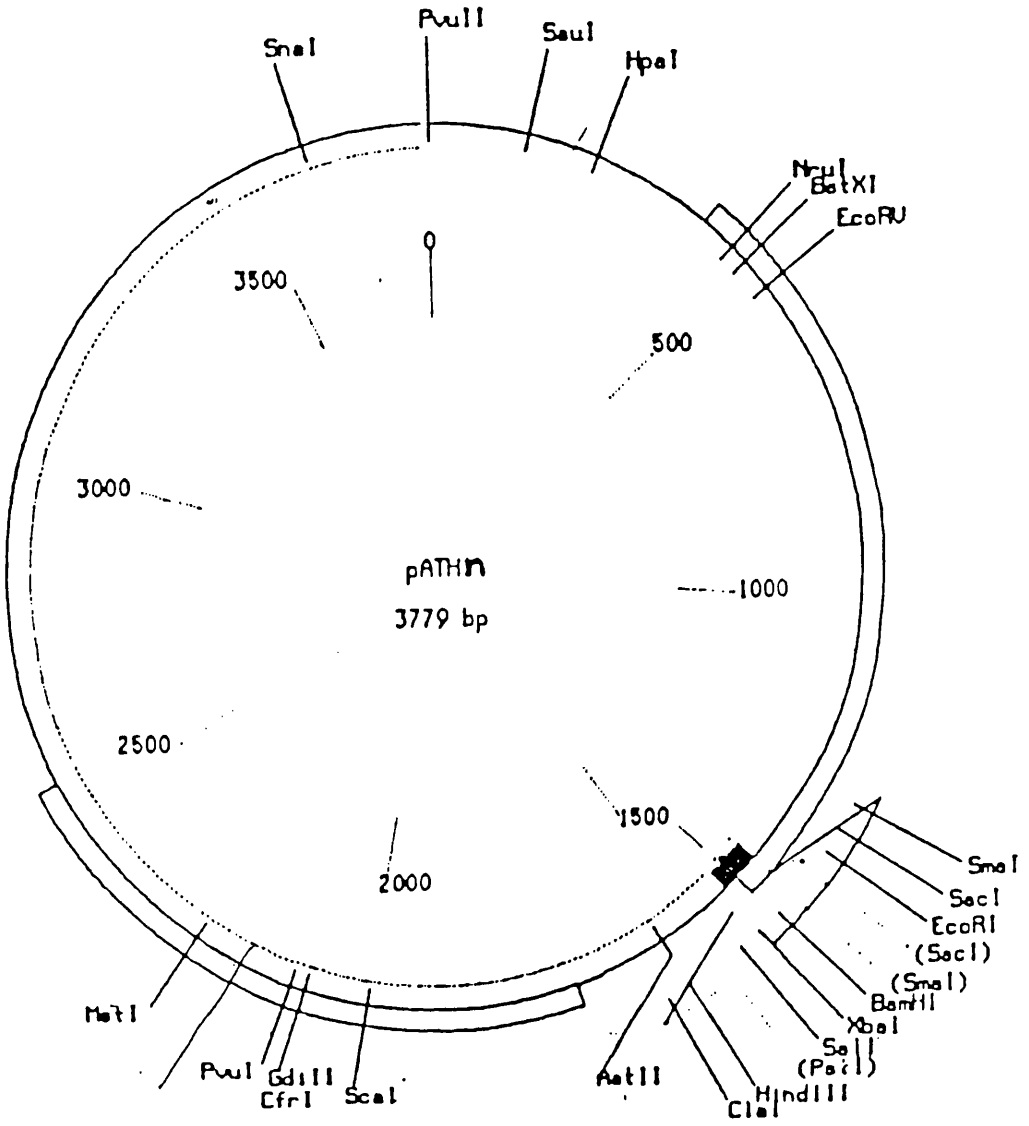


Figure 5.1 pATHn TrpE fusion protein vector

..... The region includes the bacterial origin and amp gene from pBR322.

TrpE coding region.

Synthetic polylinker

Table 5.1 The reading frames of cloning sites of pATHn

Enzymes	pATH1	pATH2	pATH3
Sma I	3m	3	-
Sac I	3m	-	-
EcoR I	2	-	3
Bam HI	2	3	1
Xba I	s	3	1
Sal I	s	3	1
Pst 1	m	m	m
Hind III	s	3	1
Cla I	s	1	2

Note:

1) This table lists where each enzyme cuts in the reading frame. e.g. "2" means the enzyme cuts after the second nucleotide,
Bam HI CCG GG'G ATC CTC.

2) s means that site is not usable as it occurs after a stop codon.

3) m indicates that there is more than one site in the vector.

5.3.2 Construction of inducible expression plasmids for pT1B

The construction of expression plasmids for pT1B was dependent on the availability and the location of the restriction enzyme sites of vector and pT1B. pT1B is 1.9 kb in length. The size of foreign DNA which can be fused with the trpE protein to produce stable fusion proteins is limited. A larger fusion protein may elicit an immune response more effectively than a smaller one but it is more prone to degradation during fusion protein production and purification. In

order to obtain modest-sized trpE-pT1B fusion proteins, several unique restriction sites within pT1B were chosen (Figure 5.2). The strategy of constructing the recombinant plasmids was summarised in Table 5.2.

Table 5.2 Construction of expression plasmids for pT1B

Fragments	Nucleotide sequence	a.a.	Restriction sites used	Reading frames F	V	Vector used
F2	444-819	125	Bam HI-Xba I	2	1	pATH3
F3	820-1246	142	Xba I-Sal I	3	3	pATH2

Note:

1) The enzymes employed divided pT1B gene into four fragments (Figure 5.2) only two fragments listed in the table (fragment 2 and fragment 3) were cloned into the plasmids to produce the fusion proteins.
2) F and V stand for fragment and vector respectively.

Unique restriction sites Bam HI and Xba I were used to generate a fragment (Fragment 2, F2) 375 nucleotides long from the pT1B cDNA clone. This 375 base pair fragment was then substituted into Bam HI and Xba I digested pATH3 vector. The recombinant plasmid contained the coding information for 125 amino acids including a partial EGF-like repeat in the predicted extracelluar domain (Figure 5.2).

For fragment 3 (F3), unique restriction sites Xba I and Sal I were used and the fragment was subcloned into Xba I and Sal I digested

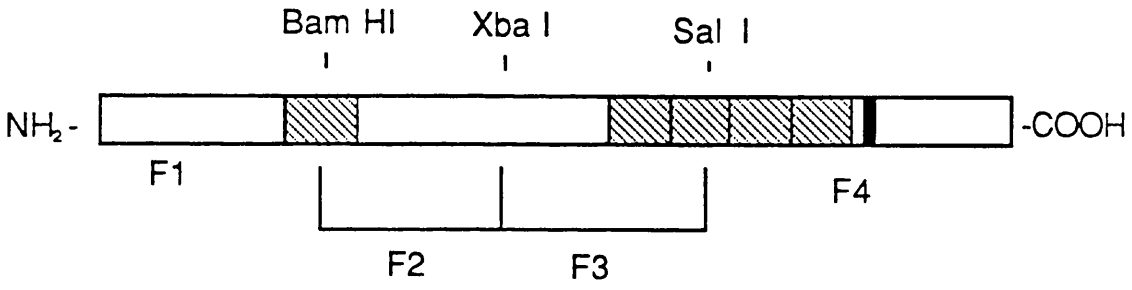


Figure 5.2 Fusion proteins and their relationship with pT1B.

The restriction enyme sites in pT1B used to construct fusion proteins are shown. These sites divide pT1B into four parts: Fragments 1, 2, 3 and 4 (F1, F2, F3 and F4). F2 and F3 were chosen to be expressed in fusion proteins. The relationship of the fragments and the EGF-like repeats in pT1B is also shown.

pATH2 vector. The protein coded by this fragment contained 142 amino acids including nearly one and half EGF-like repeats in the presumed extracellular domain of pT1B (Figure 5.2).

5.3.3 Production of fusion proteins

DH5-α was initially transformed with the recombinant plasmids; after screening, desired plasmids were used to transform C600.

Induction of trpE-pT1B fusion proteins

C600 strains containing recombinant plasmids for trpE, F2 or F3 respectively were tested for their ability to produce fusion proteins upon induction with indoleacrylic acid (IAA) (Doolittle and Yanofsky, 1968). The induction procedure was described as section 5.2.1. Cells which had been grown in a supressed condition were induced with 5 ug/ml IAA for two hours, and the whole cell lysates were collected and subjected to gel electrophoresis. Figure 5.3 shows the induced proteins from C600 E. coli containing pATH3, F2 and F3. In lane pATH3, there is a 37 kd polypeptide, the parent trpE protein. F2, the fused sequence containing 125 extra amino acids, would have an estimated molecular weight of 49 kd. The induced F2 fusion protein was at 47 kd. The estimated molecular weight of F3 was 51.5 kd, and an appropriate fusion protein was seen in induced bacteria.

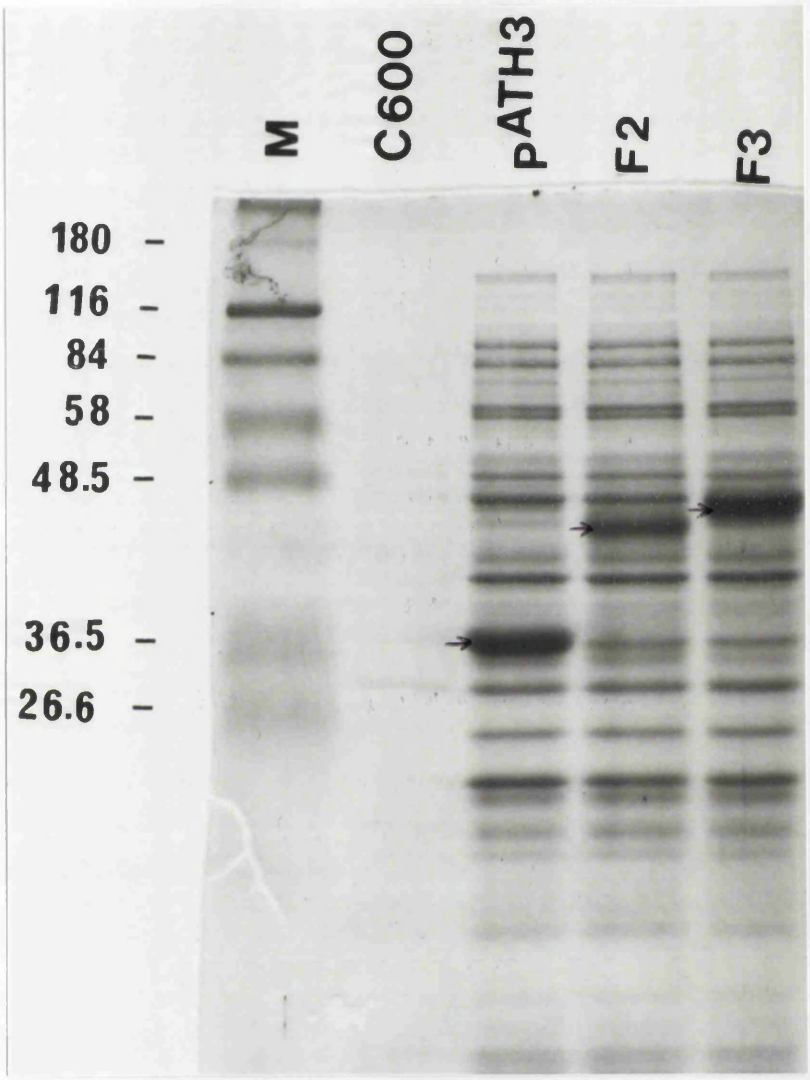


Figure 5.3 SDS-PAGE analysis of induced bacteria lysates.

Bacteria containing pATH3, F2 and F3 were induced to produce fusion proteins. Arrows indicate the relevant fusion proteins. Lysate from the parent bacteria C600 without plasmid was included as a control, however, the amount of lysate from C600 was less than expected.

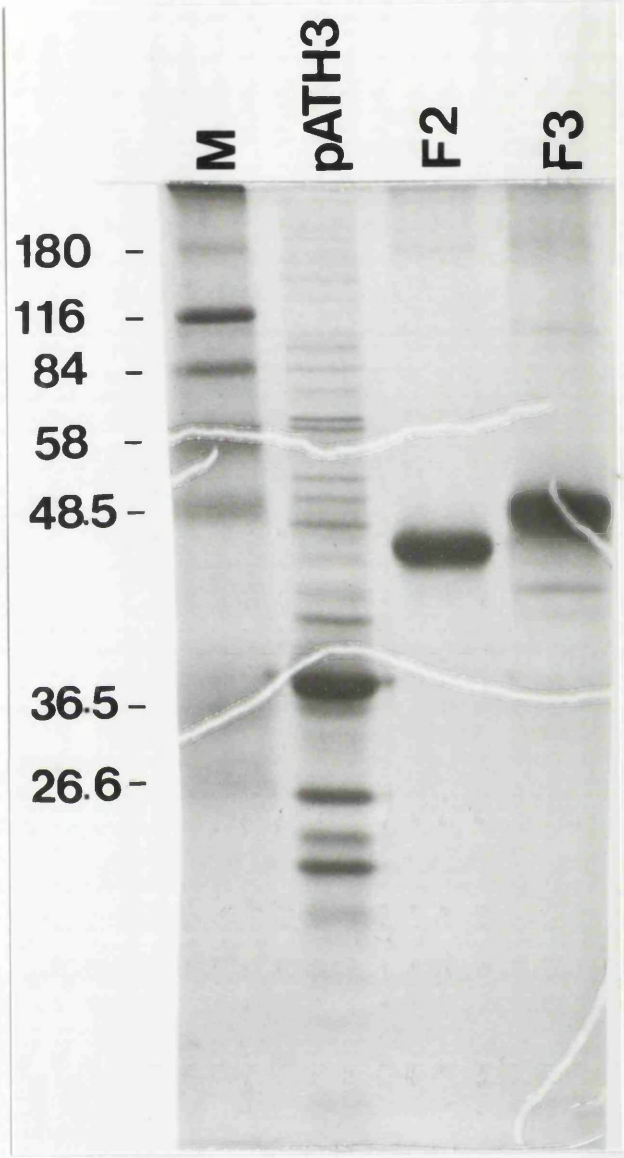


Figure 5.4 SDS-PAGE analysis of purified fusion proteins.

A 12.5% SDS-PAGE gel of purified proteins from bacteria containing pATH3, F2 and F3 is shown. The retardation observed for F2 and F3 compared with pATH3 indicated that the purified proteins contained the fused pT1B sequences.

Purification of fusion proteins

The fusion proteins were insoluble, relatively resistant to proteolytic degradation and accumulated inside the E. coli cell. The insoluble fractions were purified as described in section 5.2.1. Large amounts of protein (1 to 10 mg of fusion protein per liter of culture) were produced for tryE, F2 and F3. Figure 5.4 shows pATH3, F2 and F3 insoluble fractions after purification. It is clear that the insoluble fraction is greatly enriched for fusion proteins.

5.3.4 Antisera from rats immunised with fusion proteins

Fusion proteins were used to immunise rats as described in chapter 3 for the peptide immunisation. Ten days after the third boost, polyclonal antisera were obtained. Various assays were employed to analyse the antisera. They were first tested by protein dot blot assay. As shown in Figure 5.5, it was clear that in comparison with normal rat sera, the antisera reacted strongly with the fusion proteins. The reactivity was confirmed by western blot analysis in which pATH3, F2 and F3 were immobilised onto a nitrocelluse filter and probed with antisera to F2 and F3 respectively. The results from anti-F3 are shown in Figure 5.6. It was understandable that the antisera reacted with pATH3 TrpE protein as it was expected that the rat immune system would also recognise TrpE as foreign antigen.

In order to obtain antisera which were specific for the fused protein instead of trpE protein, the antisera were pre-absorbed

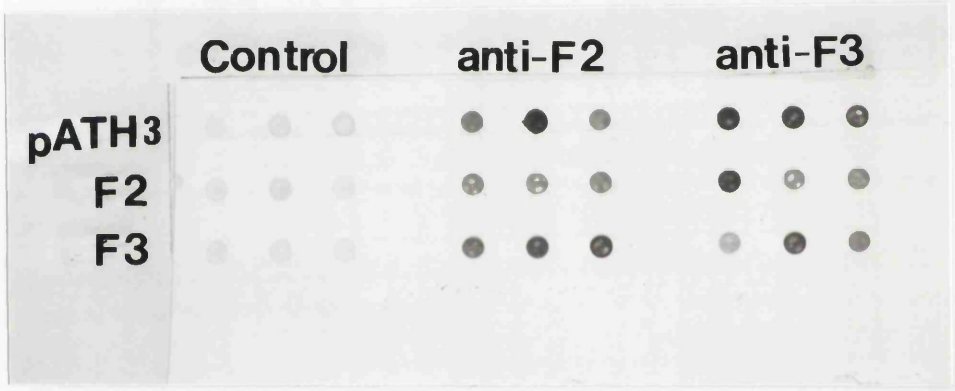


Figure 5.5 Protein dot blot analysis of polyclonal antisera to the fusion proteins.

Fusion proteins of pATH3, F2 and F3 were immobilised onto a nitrocellulose membrane which was probed with normal rat serum, anti-F2 serum and anti F3 serum respectively. Each serum was analysed in triplicates.

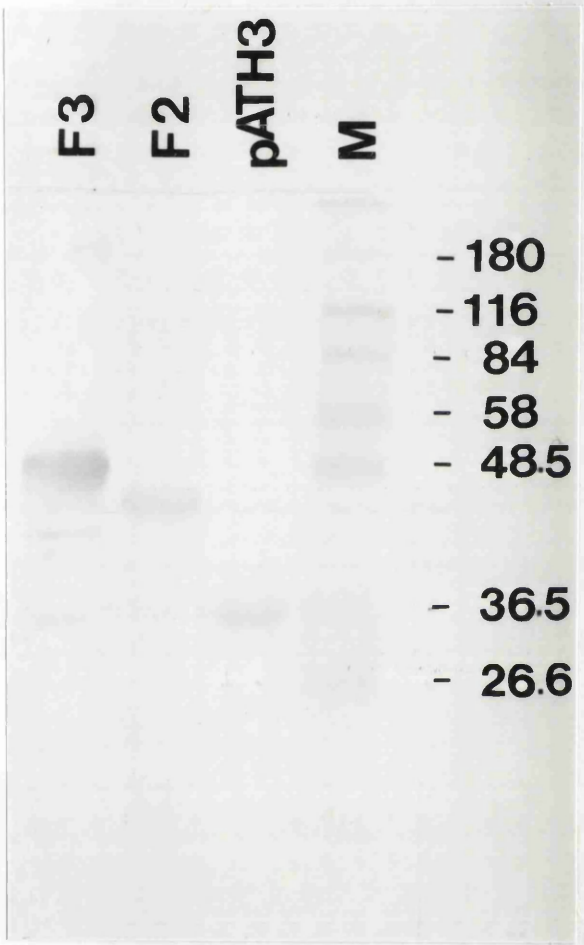


Figure 5.6 Western blot analysis of antiserum to the F3 fusion protein

Fusion proteins which had been fractionated on a 12.5% SDS-PAGE gel were immobilised onto the nitrocellulose membrane. The result obtained with anti-F3 polyclonal serum is shown. Similar results were obtained with anti-F2 polyclonal sera.

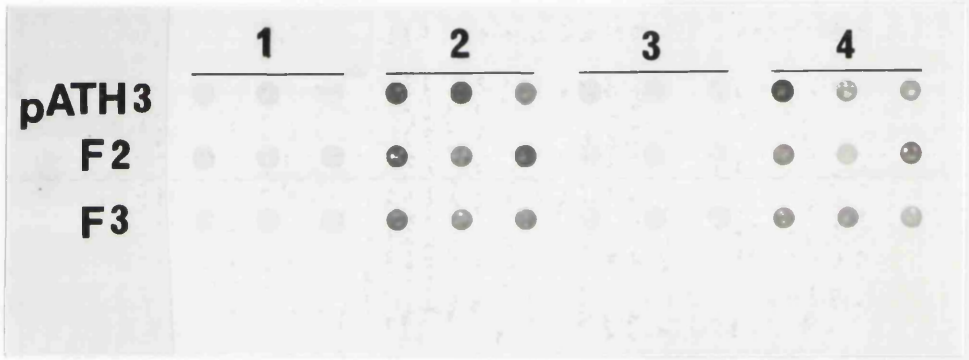


Figure 5.7 Reactivity of polyclonal antibodies after absorbed with TrpE.

Fusion proteins of pATH3, F2 and F3 were immobilised onto a nitrocellulose membrane. The membrane was probed with 1. Pre-absorbed anti-F2 serum, 2. Anti-F2 serum without absorption, 3. pre-absorbed anti-F3 serum, 4. Anti-F3 serum without absorption.

with trpE protein which had been coupled to CNBr-activated Separose Cl-4B in the hope that the specific antibodies would be left over. Unfortunately the reactivity to fusion proteins completely disappeared after absorption (Figure 5.7). It was clear the reactivity seen with unabsorbed antisera contained antibodies to trpE only or the reacticity for the fused proteins was too insignificant to be observed. Further analysis by surface immunofluorescence staining and cytospin staining did not reveal any specific reaction of the antisera with cellular components either (data not shown).

5.4 Discussion

It has been demonstrated that two fragments from pT1B sequences were linked in frame with TrpE in a fusion protein vector. The transformed bacteria were induced to produce large quantity of fused proteins which were isolated to reasonable purity. Polyclonal antisera derived from immunised rats reacted with the fusion protein when assayed in protein dot blot and western blot assays. However, on J6 cells, L cells and L cells transfected with pT1B gene (see chapter 4) in surface as well as cytospin staining, the polyclonal antisera showed no specific recognition. When the polyclonal antisera were pre-absorbed with TrpE, it was found that the antisera were no longer reactive with the fusion proteins. It is concluded that the activity seen with unabsorbed antisera for fusion proteins on dot and western blot was for the TrpE instead of the pT1B sequences fused.

In view of the various attempts to produce antibodies, it is apparent that it is not easy to produce antibodies specific for the gene product. The antibodies derived from the initial synthetic peptide approach reacted with proteins of wider tissue distribution than expected, polyclonal antisera from the transfectant and fusion protein approaches did not react with any cellular components.

It is known that a successful immunisation depends on the immunogen used. It is likely that in the case of fusion proteins, since TrpE protein is a protein of bacterial origin while the fused sequences are derived from human, the rat immune system might have already encoutered the trpE protein before and thus reacted with TrpE strongly at the expense of the pT1B fragments which are conserved over evalution. In this case repeated boosts would be likely to strengthen the immune dominance of the TrpE protein. In view of these possiblities, immunising germ-free animal or immunising with TrpE fusion protein and then boosting with β-galactosidase fusion protein or vice versa might overcome the problem.

CHAPTER SIX
TISSUE DISTRIBUTION OF PT1AB

6.1 Introduction

pT1AB was cloned accidently by screening a J6 λgt11 cDNA library with an anti-CD5 polyclonal antiserum. The nucleotide and the amino acid sequence of pT1AB share no homology with those of the CD5 molecule (Chapter 1). CD5 is expressed on normal and malignant T cells regardless of functional subclass. It is also expressed on a subpopulation of B cells (CD5+ B cell) and most B-CLL cells.

Initial northern blot analysis with pT1A (the 500 bp insert) by Ms Dunne suggested that pT1A expression is restricted to T-ALL cell lines. To further explore the tissue distribution of this novel gene and its relationship with CD5, analysis was extended to all the T cell lines available in this laboratory, as well as B cell lines, epithelial cell lines, normal and maligant lymphoid tissues. Since detection of proteins encoded by the pT1AB gene was impeded by the lack of a specific antibody, measurement of transcription was the only strategy by which to search for gene expression. This chapter describes the results obtained by this method.

6.2 Methods

6.2.1 Lymphocyte separation

Samples of peripheral blood from healthy volunteers were taken into preservative-free heparin (monoparin 2 u/ml) (Wedder Pharmaceuticals Ltd.) and diluted to twice the volume with RPMI-1640. This suspension was layered onto an approximately half volume of Ficoll-Hypaque according to the method of Boyum (1968), centrifuged at 1000 g for twenty minutes at room temperature and allowed to slow down with no braking force applied. Low density cells (peripheral blood mononuclear cells, PBMC) were collected from the interface between the medium and the Ficoll/Hypaque. The cells were washed twice (350 g, 10 minutes, room temperature) and resuspended in RPMI-1640 supplemented with 10% (v/v) FCS. An aliquot was counted.

The separation of lymphocytes from normal or malignant lymphoid tissues was essentially the same as described above except for the first step in which lymphocytes were teased out from the tissues into a single cell suspension. It was then loaded on a cushion of Ficoll/Hypaque to separate the lymphocytes from other cells.

6.2.2 Rosetting procedures

E+ cells (T cells) were prepared from PBMC with AET-treated sheep red blood cells using the method of Kaplan and Clark (1974). Briefly, suspensions of PBMC at 10^7/ml in RPMI-1640 were mixed with an equal volume of 4% AET-treated SRBC (section 2.2.2). The cells were then pelleted (350 g for 15 minutes) and kept on ice for

45-60 minutes. The pellet was then gently resuspended and layered onto Ficoll and centrifuged, (1000 g, 20 minutes, 4^0C). E^- cells were collected from the interface and the remainder of the sample except the pellet was discarded. E^+ cells (T-cells) were separated from the rosetted SRBC by hypotonic lysis with lysing reagent (see Chapter 2, Buffers). E^+ and E^- cells were washed three times in RPMI-1640 and were finally counted before use.

6.2.3 PHA or PMA activation of lymphocytes

E^+ or E^- fractions of lymphocytes at 1 x 10^6/ml were cultured with PHA (1ug/ml) or TPA (5 ng/ml) or both for 3 days at 37^0C. Cells were harvested and washed several times with sterile PBS before lysis for the RNA extraction.

6.2.4 Selection of polyadenylated messenger RNA

Polyadenylated ($polyA^+$) RNA was separated by chromatography on oligo (dT). For each milligram of total RNA, a 0.2-0.5 ml column of oligo (dT) was made in a sterile 2 ml column (Bio-Rad). The column was equilibrated in binding buffer (10 ml Tris-HCl, pH7.5; 500 mM NaCl; 0.1% SDS), and the RNA sample was loaded onto the column in the same buffer. The flowthrough was collected, re-applied to the column, and then kept as the nonpolyadenylated ($polyA^-$) fraction. The column was then washed several times, firstly with binding buffer, then with mid-wash buffer (10 mM Tris-HCl, 100 mM NaCl; 0.1% SDS) and finally with elution buffer (5 mM Tris-HCl, pH7.5; 1 mM EDTA, 0.1% SDS), to reduce the salt concentration and thus

release the polyadenylated RNA from the column. The polyA+ RNA fraction was ethanol precipitated and stored at -70^{0}C until use.

6.3 Results

6.3.1 pT1AB expression in T cell lines

To determine the size and tissue distribution of mRNA(s) encoding pT1AB gene product, northern blot analysis used mRNA isolated from various cell lines. Initial experiments were done using RNA from J6, CEM and HUT78 cell lines. The blot was probed with pT1B (the 1.9 kb cDNA insert) and different hybridisation patterns were obtained for these cell lines. For J6 cells, from which pT1AB was cloned, two transcripts of 2.8 and 3.3 kb were detected. In HUT78 cells pT1B hybridised with a transcript at 4.8 kb. For CEM cells, four transcripts of 3.3, 4.2, 4.8 and 5.3 kb were observed (Figure 6.1).

In most cases high molecular weight bands: 15kb for J6 cells and 8.4kb for CEM and HUT78 were also detected. However the intensity of these bands varied between different experiments. It is known that most known messages are less than 10kb with rare exception such as the gene for muscular dystrophy with a transcript of 14 kb which is spread over more than 2 megabases of the human X chromosome (Burmeister and Lehrach, 1986; van Ommen et a., 1986; kenwrick et al., 1987). The corresponding protein, dystrophin, has a relative molecular mass of 400,000

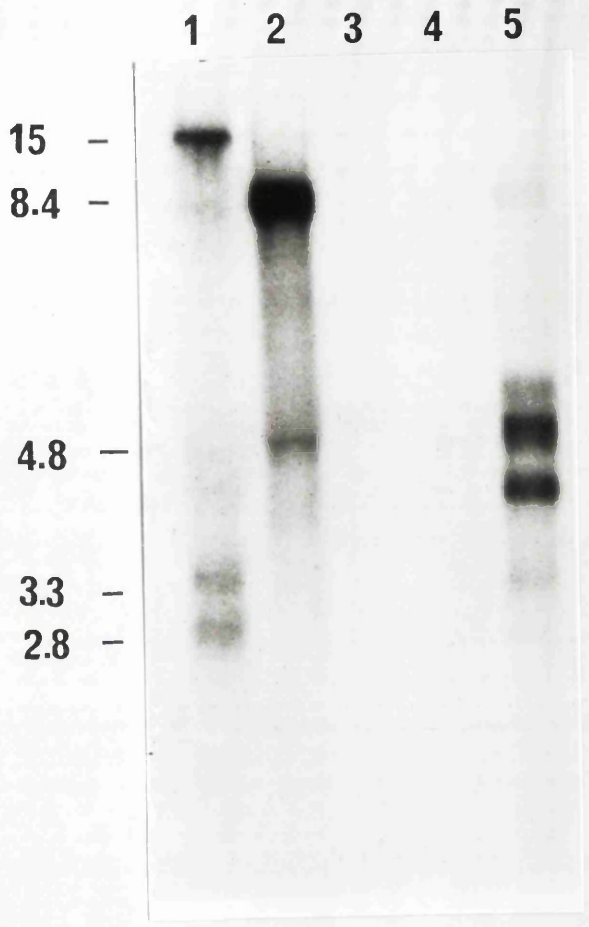


Figure 6.1 Northern blot analysis of J6, HUT78 and CEM cell lines with pT1B

^{32}P-labeled 1.9 kb cDNA (pT1B insert) was hybridised to RNA from: 1. poly(A)$^+$ from J6; 2. poly(A)$^+$ from HUT78; 3. non-poly(A)$^+$ from HUT78; 4. non-poly(A)$^+$ from CEM; and 5. poly(A)$^+$ from CEM. Poly(A)$^+$ RNA purified from 200 ug total RNA was used for all cell lines. The molecular weight of the transcripts was estimated by an RNA ladder (BRL) fractionated alongside the RNA samples.

(Hoffman et al., 1987). It is not yet known if the high molecular weight transcripts are precursors of the lower molecular transcripts.

The expression pattern of major transcripts (2.8, 3.3, 4.2, 4.8 and 5.3kb) for these cell lines was relatively stable, especially J6 and CEM cells. Repeated experiments revealed the same results (Figure 6.1).

These northern blot analysis data confirmed the results obtained by Ms J Dunne in which she has shown that pT1A (the 500 bp cDNA insert) hybridised with mRNAs of different sizes in these T cell lines (personal communication).

The analysis was then extended to other T cell lines available in this laboratory including JM, HPB-ALL, MOLT4, SUPT1, HSB2015, J.Jhan and PEER. Nearly all the T cell lines tested except for HSB2015, J. Jhan and PEER convincingly expressed some species of pT1AB-related transcripts (Figure 6.2). Most of these cell lines including JM, HPB-ALL, MOLT4 and SupT1 resemble the expression pattern seen with CEM cells (CEM-like) and express all the major transcripts. HSB2015, J.Jhan and PEER were either completely negative or at the most expressed extremely low levels of pT1AB transcripts compared to the other cell lines (see Figure 6.2 a. and b.).

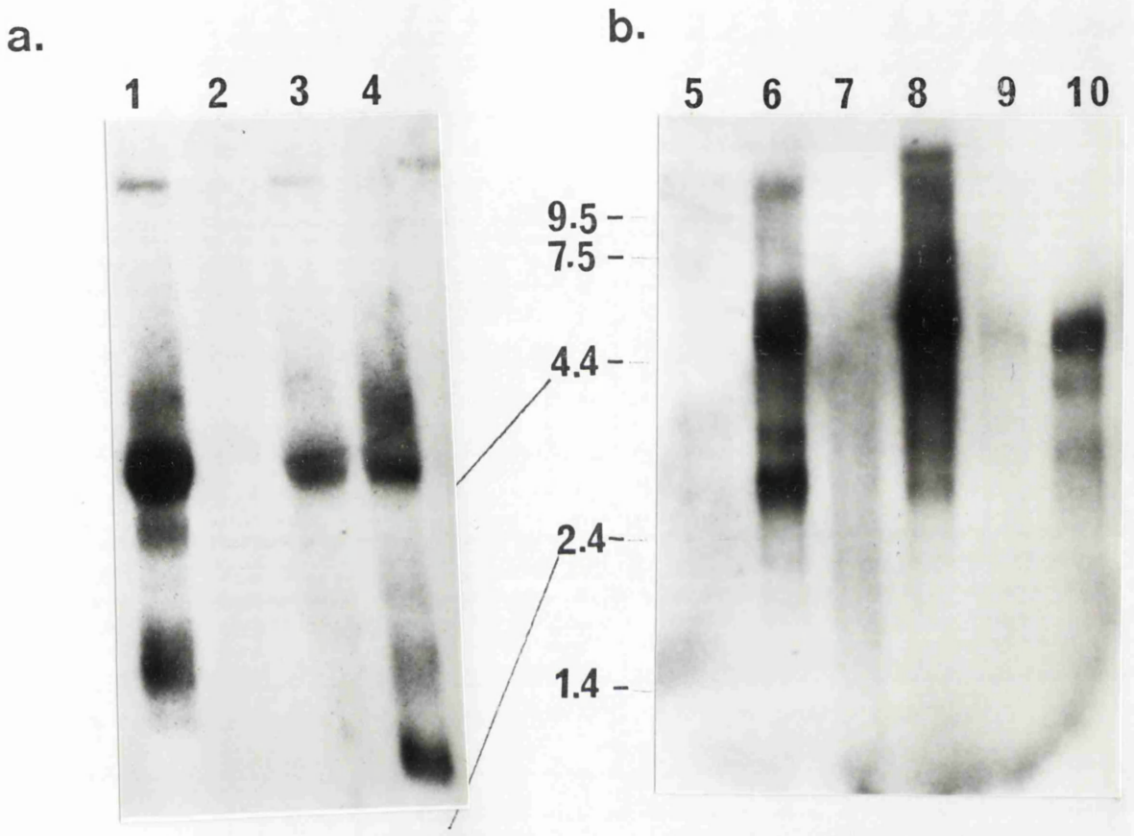


Figure 6.2 Nortern blot analysis of various T cell lines

Figure a. and b. represents two separate experiments. Lane 1. CEM, 2. HSB2015, 3. MOLT4, 4. HPB-ALL, 5. PEER, 6. SUPT1, 7. J.JHAN, 8. JM, 9. HSB2015, 10. MOLT. Numbers between the figures indicate sizes (in kb) of an RNA ladder (BRL).

6.3.2 pT1AB expression pattern and cell surface phenotype

The variation of pT1AB expression pattern seen in different T cell lines raised the speculation that pT1AB expression may correlate with different stages of T cell development. T cell lines are usually established from human T-ALL or T-cell lymphoma or by infection with HTLV-1 (Popovic et al., 1983). T-ALL is characterised by excessive accumulation of T-lymphoblasts and their progenitors. The leukemic lymphoblasts express the E-rosette receptor or other T-cell antigens corresponding to different stages of T-cell development. In T-ALL the phenotype of blast cells corresponds approximately to that of cortical thymocytes or their immediate precursors (Mills et al., 1975; Stein et al., 1976). T cell lines usually express relatively stable surface phenotype corresponding to their original leukemia or lymphomas (Baer et al., 1983) and can be divided into immature, intermediate and mature phenotypes (Roper et al., 1983).

In an attempt to examine if expression of the different transcripts detected by northern blot analysis is correlated with T cell differentiation, T cell lines were analysed by a panel of antibodies which distingush cells at different stages of maturation. They include antibodies to CD1, CD4, CD8, CD3 and several other pan T cell markers such as CD5 and CD7. An example of a FACScan profile is shown in Figure 6.3. The results of T cell phenotyping are summarised in Table 6.1.

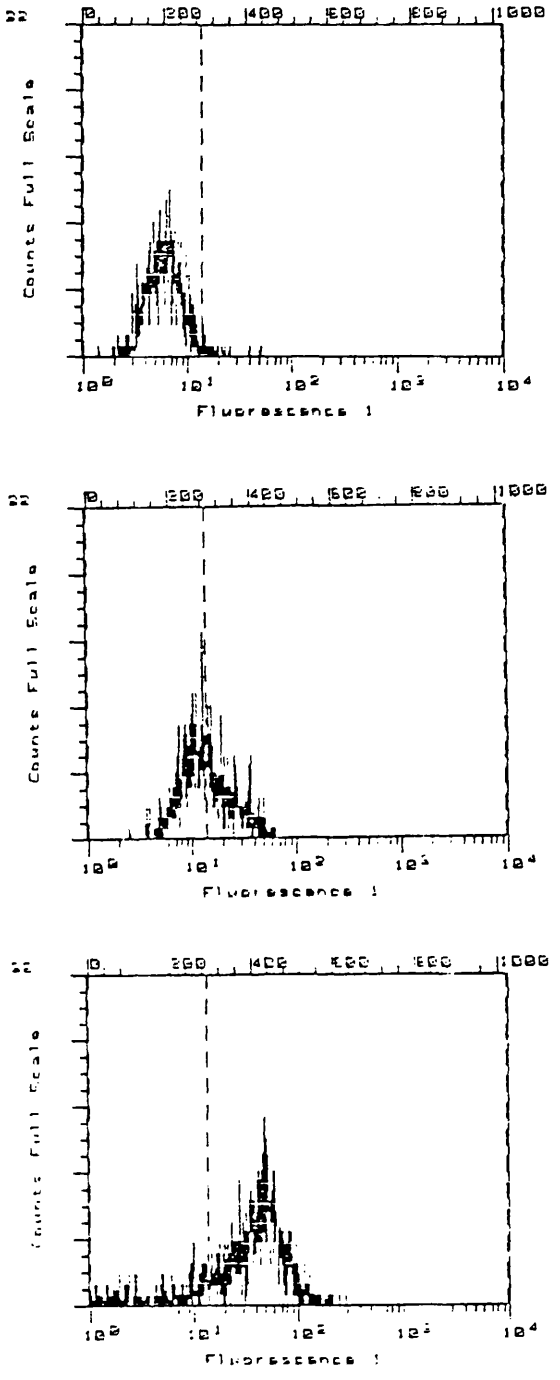


Figure 6.3 Cell surface staining and FACScan analysis

An example of FACScan analysis is shown. 1. Negative control, HUT78 cells stained with rabbit anti-mouse-FITC only, 2% stained cells; 2. HUT78 stained with UCHT1 (CD3) and rabbit anti-mouse-FITC, 45%; and 3. HUT78 stained with UCHT2 (CD5) and rabbit anti-mouse-FITC, 90%.

Inspection of both phenotype and mRNA expression of T cell lines reveals that the pT1AB mRNA expression pattern is correlated with the phenotype. The surface phenotype analysis divides T cell lines into different groups. The earliest phenotype is the $CD4^-$, $CD8^-$ (double negative) cells. The next group of cell lines are $CD4^+$ and $CD8^+$ (double positive). The last category includes those which express a more mature $CD4^+$ or $CD8^+$ (single positive) phenotype and CD3.

Among all the T cell lines studied, there were two cell lines HSB2015 and J6 which were negative for both CD4 and CD8 (immature phenotype). The two cell lines differ from each other in that J6 expresses CD1 while HSB2015 is negative. It is known that CD1 is expressed on the major cortical thymocyte population during T cell development but both the earliest and mature thymocytes lack it (section 1.2). It is likely therefore that J6 is slightly more mature than HSB2015. The transcripts displayed by these two cell lines are different. HSB2015 does not express pT1AB-related transcripts while J6 cells consistently showed the 2.8 and 3.3 kb transcripts.

JM, HPB-ALL, SUPT1, MOLT4 and CEM share some common features of the T cell antigen expression pattern. They were all double positive (intermediate phenotype) though there was variation for

Table 6.1 Phenotype of T-cell lines and pT1AB expression

Cell lines	CD1	CD2	CD3	CD4	CD5	CD7	CD8	4.2, 4.8 and/or 5.3 kb	3.3 and/or 2.8 kb
HSB2015	-	-	-	-	+	++	-	-	-
J6	++	-	-	-	+	++	-	-	+
JM	++	++	-	+ +	+ +	+ +	++	+	+
HPB-ALL	++	++	-	++	++	++	+	+	+
CEM	-	-	-	++	++	++	+	+	+
MOLT4	-	-	-	++	++	++	+	+	+
SUPT 1	+	-	-	+	+	+	+	+	+
HUT78	+	-	+	+	++	++	-	+	-
J.Jhan	+	-	+	+	++	++	+	-	-
PEER	-	+	+	+	++	++	-	-	-

Note: The results are from at least 3 repeated analyses. Cells were stained by indirect immunofluorescence and analysed on the FACScan.
- represents <10% staining,
+ represents 10-90 % and
++ represents >90%.

other markers (see Table 6.1). Most cell lines within the group displayed all the major messenger RNA species.

Among the single positive T cells (mature phenotype) HUT78 expresses only the 4.8 kb messenger RNA. While J.Jhan expressed no detectable transcripts, similarly the TCRγδ positive cell line PEER lacked pT1AB message though mouse actin probe (which cross-hybridises with human actin mRNA) analysis indicated the presence of mRNA in these samples (Figure 6.2). Though the number of samples analysed was not large enough to reach firm conclusion, the data suggest that the expression pattern might be associated with the stage of T cell maturation.

6.3.3 B lymphocytes and myeloid cell lines

To further determine if pT1AB-related transcripts were expressed in other haematopoietic cells, mRNA from GK, WMPT, Raji, LICR-LONH-My2, HFB-1, L6, NALM6, COLE, K562, U937 and HL-60 were analysed. Table 6.2 indicates the nature of these cell lines used. Messenger RNA from J6 cells was used as a positive control and the quantity of mRNA loaded was determined by hybridising with mouse actin probe. No transcripts were detected among all these non-T haematopoietic cell lines (data not shown).

Table 6.2 Cell lines used for northern blot analysis

Cell lines	Tumour type

CEM, HPB-ALL,MOLT4, HSB2015, HUT78, J6, JM, SUPT1, PEER and J.Jhan	T-ALL
GK and WMPT	EBV transformed B lymphoblastoid lines
Raji	Burkitt line
LICR-LONH-My2, HFB-1 and L6	Plasmacytoid lines
NALM6	C-ALL line
COLE	Hodgkins cell line
K562, U937 and HL-60	Myeloid cell line
212	Neuroblastoma line
PANT, LS174T, CNE, MDA157, MCF7, UCHNCu, H-82 and T24	Carcinoma lines

Note: For further details and references to these cell lines see chapter 2 Table 2.2

6.3.4 Epithelial cell lines

Cell lines established from small cell cancer of the lung (UCHNCu, H-82), breast cancer (MDA157, MCF7), pancreatic tumour (PANT), liver cancer (Chang), colon cancer (LS174T), bladder cancer (T24), neuroblastoma (212) and nasopharyncal cancer (CNE) (see Table 6.2) were also examined for pT1AB gene expression. When a long exposure was carried out, the characteristic pT1AB bands were seen in the control CEM track (Figure 6.4 track 11). However with

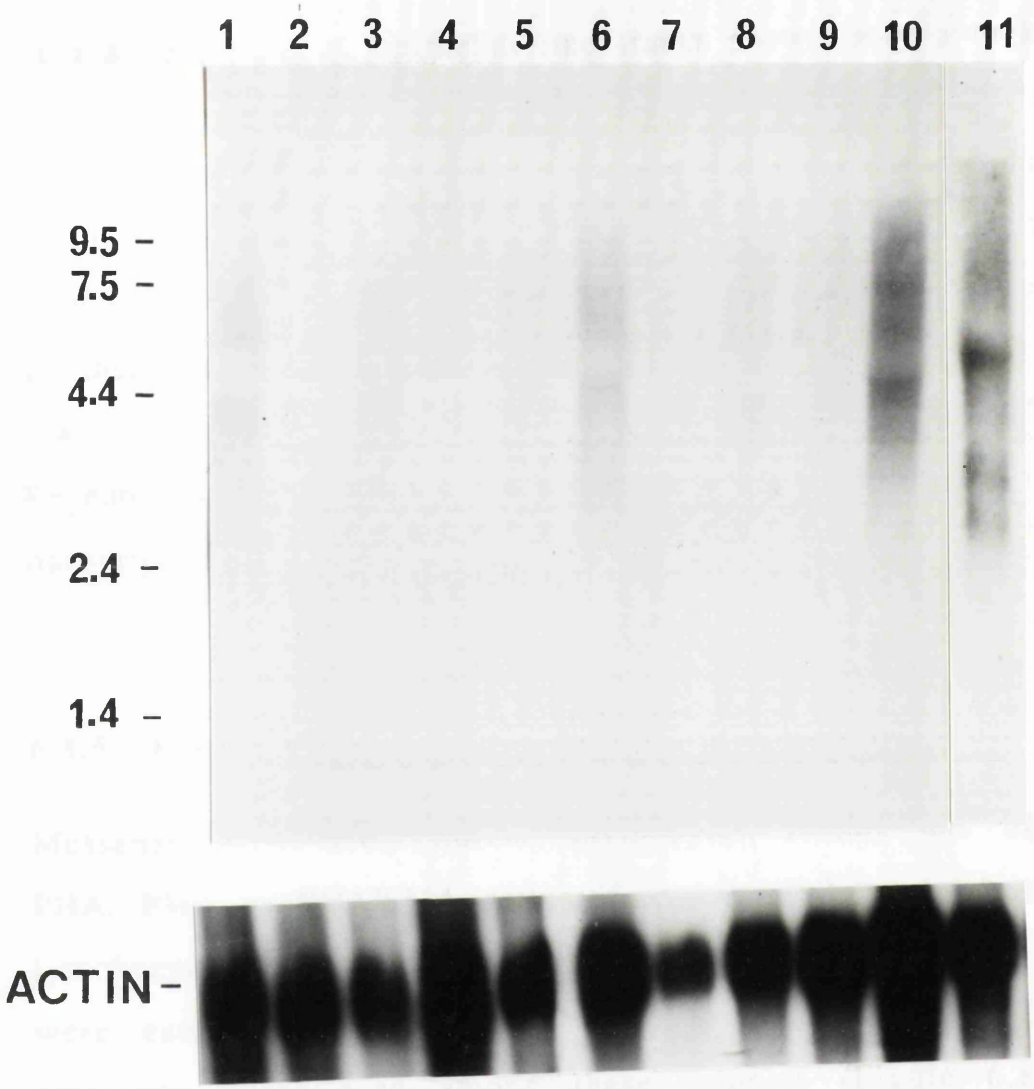


Figure 6.4 Northern blot analysis of mRNA from various epithelial cell lines

Lane 1. UCHNCu, 2. H-82, 3. MDA157, 4. MCF7, 5. PANT, 6. Chang, 7. L5174T, 8. T24, 9. 212, 10. CNE, and 11. CEM (an autoradiograph from a long exposure (day 7) of the same blot as the epithelial cell lines (day 2). Hybridisation with mouse actin probe is shown which was obtained with 4 hour exposure.

such an exposure the epithelial line tracks shown only a dark smear and no distinct bands could be seen. Figure 6.4 Tracks 1-10 therefore shows a shorter exposure in which only very faint bands can be seen. The molecular weight of these bands do not correspond to those of the pT1AB messages seen in T-ALL lines. We conclude 1), that if there is pT1AB message in epithelial cells it is at a very low level and 2), the message size differs from those in T cells. Repeated attempts to detect specific and more convincing pT1AB transcripts in epithelial lines failed to provide more clearcut data.

6.3.5 Fresh lymphoid tissues

Messenger RNA from normal thymocytes, resting and day three PHA, PMA or PHA/PMA activated (section 6.2.3) peripheral blood lymphocytes, tonsillar E^+ and E^- fractions, spleen E^+ and E^- fractions were examined for pT1AB gene expression. No pT1AB-related transcripts were seen among these samples (Figure 6.8). In a preliminary attempt to examine the gene expression in fresh T cell tumours, no convincing data were obtained due to either the small number of tumour cells available for each sample or the poor RNA recovery from frozen stocks of T cell tumours (data not shown).

6.3.6 pT1AB: relationship with CD5

Since the pT1AB gene was selected by screening a J6 λgt11 cDNA library with an anti-CD5 polyclonal antiserum, the question of the relationship between pT1AB and CD5 arises. It was possible that the two molecules might be associated on the T cell surface and had

been co-purified and used as immunogens to raise the anti-CD5 polyclonal antiserum. Confirmation of this speculation might account for the isolation of pT1AB. It therefore appeared worthwhile to compare the distribution and intensity of expression of the two genes.

a. Expression intensity

Experiments with CD5 and pT1AB probes on T cell lines revealed that pT1AB transcripts were expressed in low abundance. Figures shown above and assays for the non-T haematopoietic cells and epithelial cells were carried out with polyadenylated RNA of these cell lines. When a standard northern blot was performed in which 20 ug of total RNA was used, it was usually difficult to obtain clear signals with pT1B cDNA. In several experiments, pT1-2 (a 2.0 kb cDNA coding for CD5 antigen), which was nearly the same length as pT1B (1.9 kb), was included in the northern blot analysis of T cell lines. The results with these two probes are demonstrated in Figure 6.5. The pT1-2 probe readily detected CD5 transcripts (3.6 and 2.7 kb) in 20 ug/lane total RNA in all the T cell lines in a short exposure (Figure 6.5 a.), while the same exposure only revealed several faint bands with pT1B (Figure 6.5 b.) which became apparent only after long exposure (Figure 6.5 d.). CD5 is a pan-T cell marker and demonstrated comparable fluorescent intensity to other T cell markers such as CD2, CD3 etc when assayed in cell surface staining. The different intensity of transcripts detected by these two probes demonstrated that pT1AB gene expression was relatively low when compared with that of CD5.

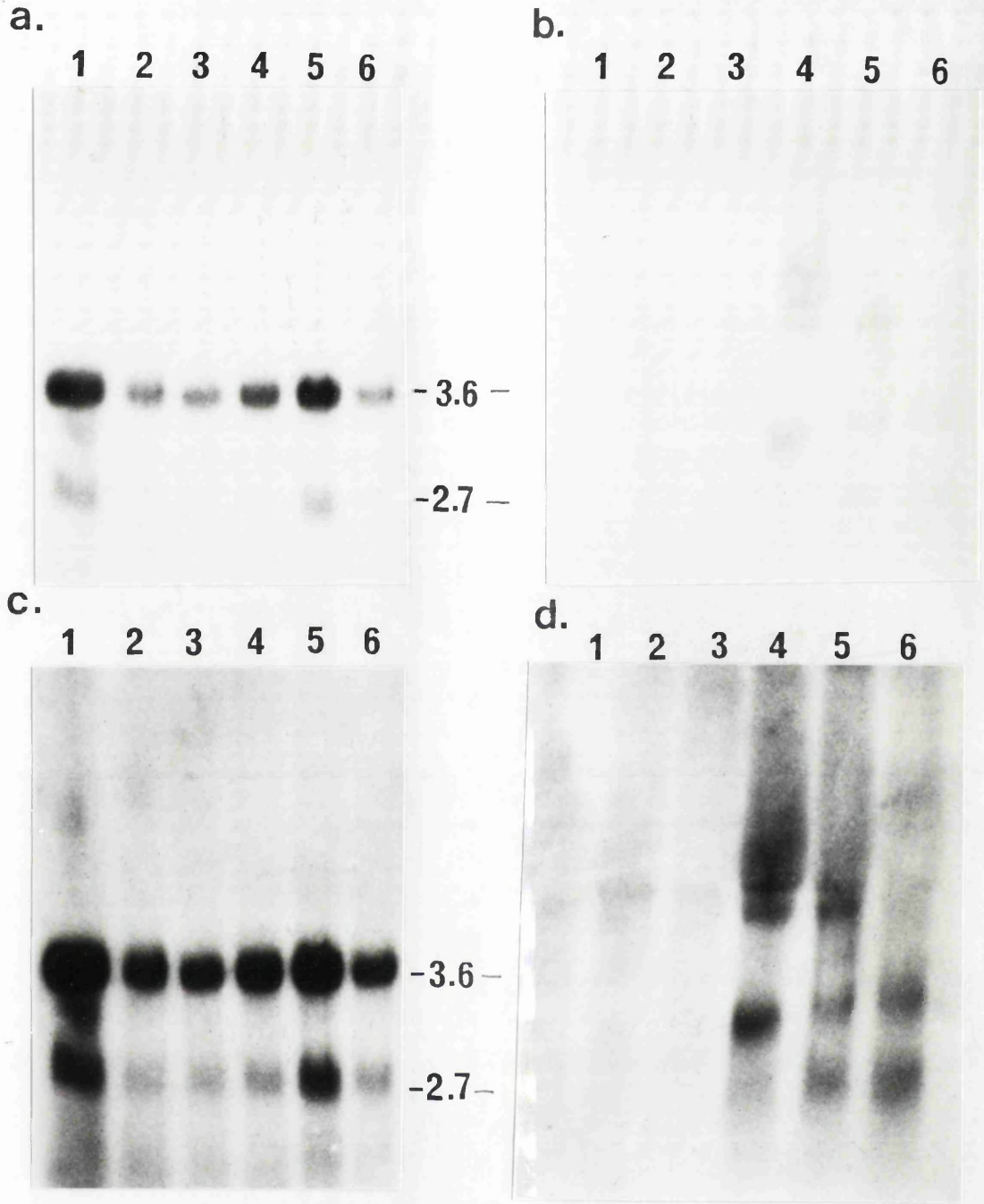


Figure 6.5 Comparison of expression intensity between CD5 and pT1AB

a. ^{32}P-labeled 2.0 kb cDNA (pT1-2 insert, a CD5 probe) was hybridized to RNA from 1. HUT78, 2. MOLT4, 3. HSB2015, 4. CEM, 5. JM, and 6. J6.

b. ^{32}P-labeled 1.9 kb cDNA (pT1B insert) was hybridized to RNA as described for the CD5 probe.

c. and d. are the autoradiographs of a. and b. after a long exposure respectively. Twenty micrograms of total RNA were used for all the cell lines.

b. Tissue distribution

A discrepancy between CD5 and pT1AB expression was observed in the analysis of normal lymphoid tissues. No pT1AB transcripts were seen in normal thymocytes although CD5 messages were easily detected (Figure 6.6). Experiments on peripheral blood, spleen and tonsil lymphocytes at first yielded inconclusive results. Figure 6.7 shows an experiment in which tonsil E^+ and E^-cells were activated in vitro and the message examined. CD5 transcripts are seen in resting and activated T cells (Figure 6.7 tracks 3 and 4), and in E^- cells activated with PMA (track 1). The results of probing with pT1B are less clearcut. Track 5 shows the positive control (J6) but the other tracks may have weak bands. Longer exposure did not make these more evident. The experiment was therefore repeated using polyA+ instead of total RNA. Under these conditions no pT1AB transcripts were detected (Figure 6.8).

Data from the tissue distribution and the expression intensity anlysis apparently indicate that the expression of pT1AB is not always associated with CD5, especially PMA induces the expression of CD5 transcripts in tonsillar E^- cells while the parellel studies with pT1B did not reveal any increase in pT1AB expression after the PMA treatment, though they are both expressed in malignant T cell lines.

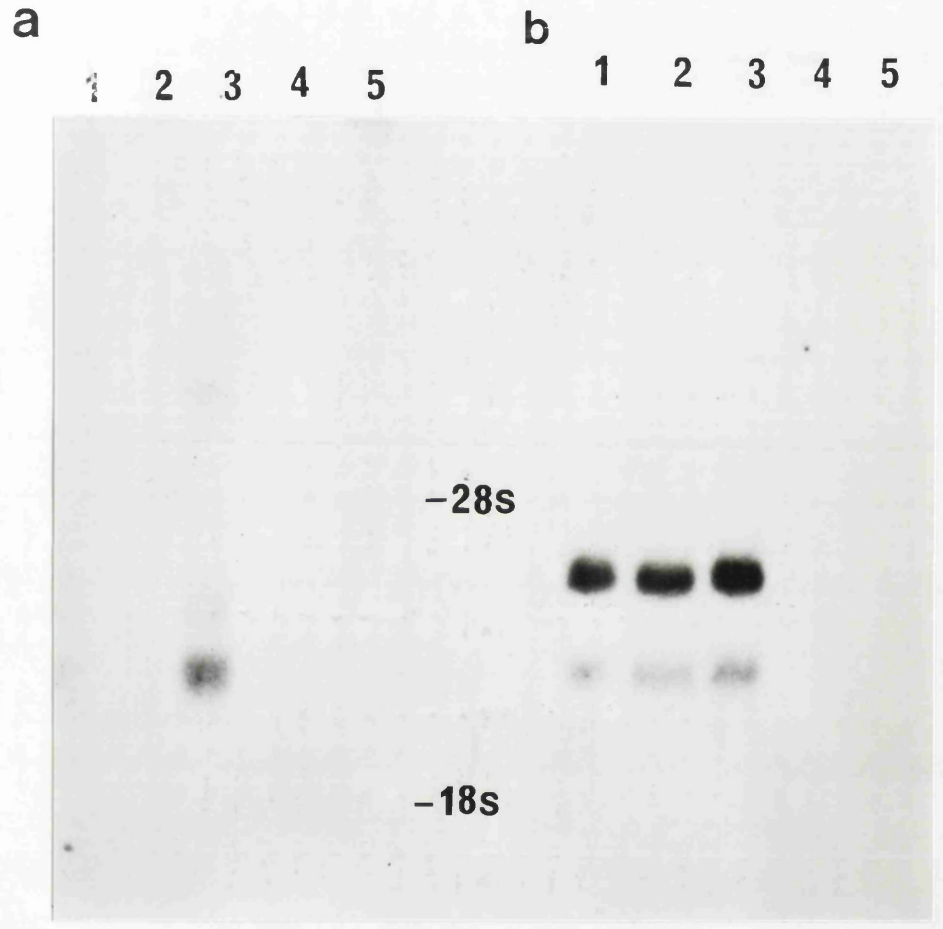


Figure 6.6 Northern blot analysis of mRNA from thymocytes

RNA sources were as follows: 1. J6, 2. Thymocytes, 3. HPB-ALL, 4. H-82 and 5 CNE. a. and b. are hybridisation pattern obtained with pT1B and pT1-2 (the CD5 probe) respectively.

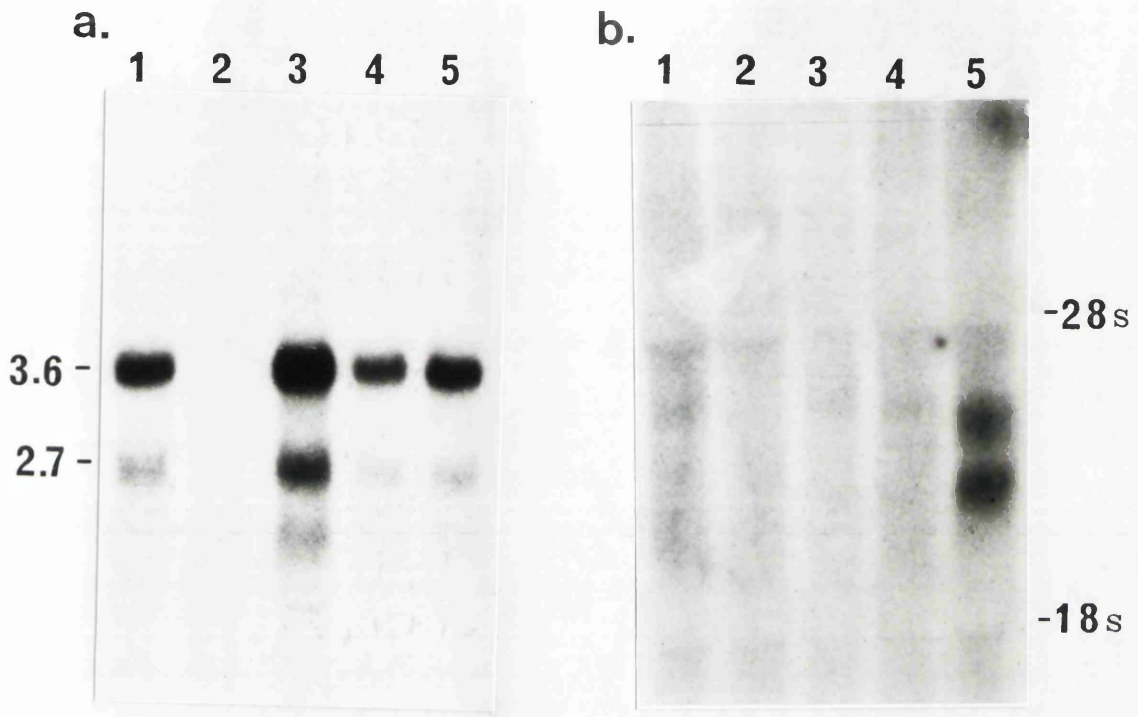


Figure 6.7 Comparison of tissue distribution between CD5 and pT1AB

RNA sources are as follows: 1. Tonsil E^- cells activated with PMA for 3 days , 2. Tonsil E^- fraction, 3. Tonsil E^+ fraction cells activated with PMA+PHA for 3 days, 4. Tonsil E^+ fraction, 5. J6. a. and b. represent the results with CD5 and pT1B probes respectively. Twenty micrograms of total RNA from cells indicated were used for all the lanes.

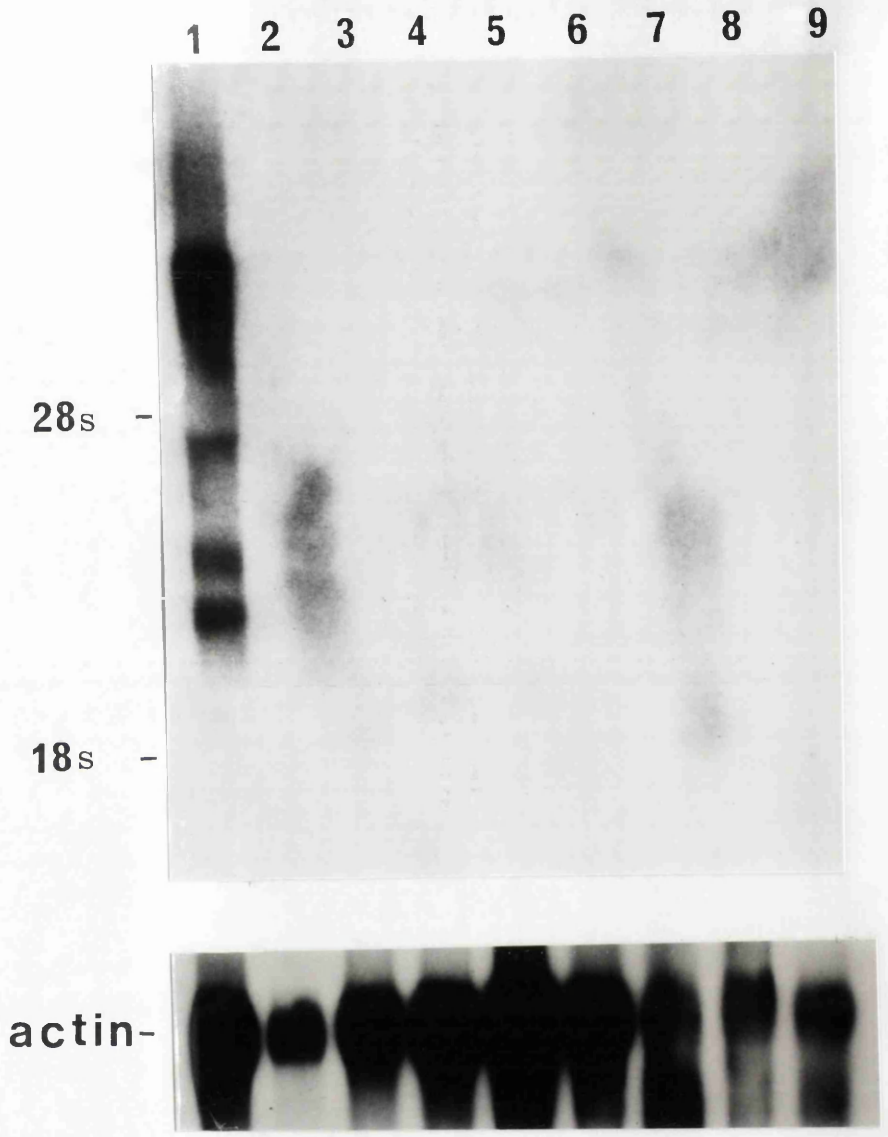


Figure 6.8 Northern blot analysis of mRNA from normal lymphoid tissues.

Messenger RNA from 1. J6, 2. Thymocyte, 3. PBM, 4. PBM activated with PHA/PMA, 5. Tonsillar E^+ fraction, 6. Tonsillar E^+ fraction activated with PHA/PMA, 7. Tonsillar E^- fraction, 8. Tonsillar E^- fraction activated with PMA, 9. Splenocytes, were analysed with pT1B probe.

6.4 Discussion

6.4.1 pT1AB tissue distribution: unanswered questions

It has been demonstrated that nearly all the T cell lines available in this laboratory displayed pT1AB-related transcripts while no transcript was observed among cell lines of other origin including B, myeloid and epithelial cells. The restricted tissue distribution of pT1AB suggests that gene expression is closely associated with T cells. It is surprising that normal thymocytes, and resting or activated lymphocytes from different lymphoid tissues do not exhibit discernible transcripts.

There are a number of possibilities which need to be borne in mind when interpreting such data. Firstly, this may be partly because the message is present only at certain restricted stages of T cell development. If this is true, the cells transcribing the gene might constitute only a small percentage of the total population. Secondly, even if the pT1AB gene is indeed expressed during T cell ontogeny, the message might be expressed only at low abundance. Comparison of the signals obtained with pT1AB and CD5 probes in northern blot analysis would support this notion (Figure 6.5). In situ hybridisation on tissue sections might reveal pT1AB in rare positive cells or transient expression and the use of polymerase chain reaction (PCR) should overcome the problem of low abundance. Finally, since the cell lines exhibiting pT1AB-related

transcripts are all established from malignant T cell leukemia or lymphomas, this raises the tantalising possibility that pT1AB gene expression is associated with malignant transformation.

6.4.2 Transcript pattern and cell phenotype

A striking feature of the northern blot analysis is the large number of different message sizes seen in different T cell lines. It is not known yet if the multiple transcripts detected are from a common transcript, which is spliced into various forms in different cells, or if these mRNA are transcribed from different genes which are closely related. Alternatively they might differ from each other only in the untranslated regions. Confirmation of these speculations must await the analysis of full-length cDNAs representing these transcripts. What is already known about the cells expressing different transcripts is that these cells differ from each other by their cell surface phenotype. The phenotypic analysis divides the T cell lines into three groups: early, intermediate and mature phenotype. Inspection of both the phenotypic and transcript pattern suggest that the pT1AB expression pattern is related to the stages of T cell maturation: the early cell line express the two low molecular weight transcripts (2.8kb and 3.3kb, J6 cells); the intermediate cell lines display all the major transcripts (CEM, JM etc.) and the mature phenotype cell line express only the high molecular weight transcript (HUT78 4.8kb) or no pT1AB-related transcripts (section 6.3.2).

6.4.3 pT1AB: functional implications

The study of pT1AB and its relationship to other molecules is hampered by the lack of a specific mAb to the gene product. The only available data are the amino acid sequence of the gene product and the tissue distribution obtained by northern blotting analysis. Approaches to protein structure and function predictions are not at present of a sufficiently sophisticated nature to allow assignments purely on the basis of amino acid sequences. However, a generally applicable principle has been that shared sequences tend to be reflected in some common properties of the proteins. Thus the high level of similarity of the pT1AB amino acid sequence with those of the EGF-like protein family members (Figure 1.5, Table 1.4) could provide useful pointers towards the design of informative experiments.

The physiological function of pT1AB is not known. The absence of pT1AB messenger RNA in normal and activated lymphocytes suggests that gene expression might correlate with the malignant transformation of these T-ALL cells. By analogy with what has been said in the General Introduction (Chapter 1) about EGF-like proteins, speculations might focus on a possible involvement of pT1AB as a growth factor and/or a receptor, or an oncogene or in membrane-membrane interaction in these T cell malignancies.

a. Growth factor and/or receptor: relationship with oncogenes

Investigations of the genetic alterations that cause normal cells to become malignant have focused on a small set of cellular genes. Acute transforming retroviruses have substituted viral genes necessary for replication with these discrete segments of host genetic information (Sharp, 1979). When incorporated within the retroviral genome, these transduced cellular sequences, termed onc genes, acquire the ability to induce neoplastic transformation. Proto-oncogenes can also be activated to become transforming genes by mechanisms independent of transduction by retroviruses. Genetic changes as small as point mutations (Tabin et al., 1982), as well as DNA rearrangements such as transpositions (Rechavi et al., 1982) and chromosomal translocations (Dalla-Favera et al., 1982), have been implicated in this process.

The profound cellular alterations induced by activated cellular transforming genes have some similarities to the growth promoting actions of hormones and growth factors. When expresssed with their cognate ligands, various growth factor receptors support autonomous cell growth by an autocrine mechanism. A prototypic example of transformation caused by autocrine activation is provided by the v-sis oncogene of simian sarcoma virus (SSV), which encodes a PDGF-like molecule (Doolittle et al., 1983; Waterfield et al., 1983; Robbins et al., 1983). The virally encoded growth factor is the homolog of the B chain of PDGF (Robbins et al., 1983), which itself is a potentially transforming cellular protein (Johnsson et al., 1984). The viral sis gene product induces mitogenesis by interaction with the PDGF receptor (Robbins et al.,

1983; Gazit et al., 1984; Leal et al., 1985). Another example is TGF-α (Chapter 1). Interaction of TGF-α with the EGF receptor leads to tyrosine kinase activation and induces mitogenesis.

A variety of oncogenes have been shown to be derived from growth factor receptors. Amino acid sequence homology between v-*erb*B oncogene product and human EGF receptor, v-*fms* and CSF-1 receptor provided the indication that the oncogenes had been generated by recombination of receptor sequences into a retroviral genome (Ullrich et al., 1984; Yamamoto et al., 1983; Downward et al., 1984). Comparison of the primary structures of these oncogenes with their normal cellular counterparts revealed the presence of a C-terminal sequence deletion in v-*erb*B, v-*fms* and deletion of most of the EGF receptor extracellular domain for v-*erb*B. It has been suggested that these structural alterations leads to constitutive kinase activity. V-*erb*B-2 is an analogue of v-*erb*B. The predicted amino acid sequence of the v-*erb*B-2 gene reveals the structural features of a growth factor receptor molecule with close similarity to the EGF receptor (Coussens et al., 1985; Yamamoto et al., 1986). Amplification of the oncogene v-*erb*B-2 has been detected in a variety of human tumours (Kraus et al., 1987; D'Emilia et al., 1989). These findings suggest that these proto-oncogenes mimic growth factor receptors and contribute to neoplastic growth in these tumour cells (King et al., 1985; Semba et al., 1985).

The proposal of pT1AB gene as a growth factor and/or receptor requires that pT1AB be an extracelluar and/or membrane protein,

for which the transmembrane region of pT1AB serves as the supportive evidence. The multiplicity of the EGF-like peptide motif of the gene suggests a parallel with EGF. EGF is synthesized in the form of a large protein precursor, which includes nine related peptide units, essentially arranged in two groups separated by unrelated protein sequences (Figure 1.7); mature EGF is the ninth peptide in this arrangement and is liberated from the precursor by proteolytic cleavage at arginine residues. Comparison of the protein sequences of EGF and the other eight peptide units, which have no known function, shows that they have diverged by both amino acid substitution and altered cysteine spacing, similar to that observed for the pT1AB peptides. TGF-α is also generated by proteolysis of a much larger precursor, with the cleavage occurring at an alanine-valine dipeptide (Derynck et al., 1984). One cannot predict protease cleavage sites for the pT1AB protein, but it is conceivable that a peptide or peptides could be released from a larger precursor by proteolysis.

Similarly a receptor function can be suggested on the basis of the predicted structure of the pT1AB gene product. The large cytoplasmic domain (Figure 1.7) is characteristic of many receptors such as receptors for EGF, insulin, CSF-1 and PDGF (reviewed in Carpenter, 1987). The cytoplasmic domains of these receptors are mainly engaged in signal transduction initiated by the interaction with their ligands.

b. Possible role in T cell malignancy

T-ALL is a malignant process characterised by abnormal growth and maturational arrest of lymphoid precursor cells. These abnormalities may be related to the escape by leukemic cells from normal genetic control mechanisms. Alternatively, T-ALL cells may become independent from the supply of exogenous growth factors, which are necessary for optimal proliferation and differentiation of their normal counterparts. This second mechanism could be due to the ability of T-ALL cells to synthesize and respond to growth factors. An autocrine secretion of growth factors may be operational in many malignancies (Sporn and Roberts, 1985). In the lymphoid system a family of growth factors that stimulate proliferation and differentiation of B and T lymphocytes have been identified (Dinarello and Mier, 1987). Examples include IL-1, IL-2, IL-3, IL-4, IL-5 and IL-6 (Ruscetti and Gallo, 1981; Clark and Kamen, 1987; Strober and James, 1988; Wong and Clark, 1988).

Another feature of T-ALL is the cytogenetic abnormalities observed in these tumours. These are principally chromosomal translocations, inversion or less commonly, deletions. The effects of chromosomal rearrangement are intimately linked to rearrangement and altered regulation of cellular oncogenes. These translocations typically juxtapose all or part of the relevant oncogene with a functionally acitve gene. As a consequence, the expression of the oncogene is changed. Altered levels of oncogene transcripts are reported in T-ALL. In addition to the myc gene, abnormalities in p53, myb, fos, fes, abl, hras, kras, fms, sis, and src mRNA have been observed

(reviewed in Champlin and Gale, 1989), though none of the oncogenes detected are specific for leukemia.

pT1AB has been shown to be predominantly expressed in various T cell lines (usually established from T-ALL cells) with a variety of different message sizes in different T cell lines. The significance of the confinement of pT1AB expression to T cell lines is not yet clear. It remains possible that the gene may be expressed only during early T cell development in a small population of developing thymocytes. In this case it would be difficult to detect by northern blotting analysis among a large population of non-expressing thymocytes. However, the fact that gene is not expressed on activated lymphocytes may suggest that the gene expression is associated with abnormal growth. Chromosome translocation, inversion or mutation in these T cells might turn on a previous silent gene. The expression of the pT1AB gene product might endow the cell growth advantage over normal cells therefore contributing to the malignant transformation of these cells.

CHAPTER SEVEN
CONCLUSION AND FUTURE PESPECTIVES

7.1 Conclusion

A novel gene was cloned by Ms J Dunne in a collaboration between this laboratory and that of Dr MJ Owen within ICRF. Sequence data revealed that the gene structure is homologous to the EGF-like protein family in the arrangement of cysteine and other conserved amino acids. The cloned cDNA was truncated at both the N- and C-termini. To study the function, tissue distribution and the relationship with other proteins of this gene product, an antibody reacting with the native form of the gene product was considered to be essential. Several strategies have been followed in attempting to achieve this aim.

Initially synthetic peptides derived from the gene sequence were used as immunogens, the reactivity by western blot analysis of the mAbs from immunised mice and rats did not correlate with the results of northern blot analysis and the antibodies did not react with native proteins. In a second series of experiments the truncated gene was linked in frame with a pseudo N-terminus and cloned into a vector which upon transfection can confer resistance to G418. The gene was stably integrated and transcribed in L cell transfectants. However, sera from mice immunised with the transfectants failed to stain living or fixed cells in a specific fashion. Surface iodination and internal labelling followed by one-D-gel analysis of cell associated or supernatant proteins did not show any difference between untransfected and transfected cells. This might

suggest that the products were not processed onto the cell surface as expected. A third approach was to make TrpE fusion proteins for use as immunogens. Antisera from rats showed no specific reactivity with the fusion proteins.

Northern blot analysis of various established lymphoid, myeloid, or epithelial cell lines, normal lymphoid tissues and activated lymphocytes showed that the gene is predominantly expressed in T-ALL cell lines. A striking feature of the northern analysis is the large number of different message sizes seen in different T cell lines. Cell surface phenotype analysis of these cells suggests that the pattern of transcripts is correlated with different stages of maturation.

7.2 Future perspectives

Preliminary data revealed that pT1AB gene is a member of the EGF-like protein family. It is mainly expressed in T cell lines with different message sizes. Nevertheless, little is known about the biochemistry and the function of the gene product. Further exploration of the properties of the gene product was greatly hampered due to the truncation of the cDNA clones and the lack of a specific antibody to the gene product. However, efforts can be made to overcome these shortcomings and some of the possibilities are discussed here.

7.2.1 Improving the strategies for producing antibodies

Previous attempts to produce mAbs to the gene product by synthetic peptides, transfectants and fusion proteins were not successful. The possible reasons for these failure have been discussed in previous chapters. It is known that a successful immune response depends on the immunogen used. In the first approach the synthetic peptides used were amino acid sequences within the cytoplasmic domain due to the limited nucleotide sequence (pT1A, the 500 bp cDNA insert) available at the time.

With more sequencing data obtained from the extended cDNA, it is possible to synthesize peptides from the predicted extracellular domain. EGF-like repeats may play an important role in the function of the gene product therefore antibodies to epitopes in these motifs would be useful to investigate the function of the gene product, but they may be too conserved to elicit an immune response. Since the EGF-like repeat is present in many proteins, antibodies to this structure might cross-react with other proteins. So with the aim of obtaining specific mAbs to the gene product, both EGF-like repeat and non-EGF-like sequences can be included as immunogens. The inclusion of EGF-like repeats as immunogens will be particularly important if the mature pT1AB gene product is cleaved to produce a EGF-like growth factor which is secreted.

Alternatively, transfectants can be made as immunogens when a full length cDNA is available. In case the expression of pT1AB gene

product is indeed recipient cell specific, both L cells and mouse T cell lymphoma lines such as BW5147 can be used in parallel.

Although it has been shown that CD5 expression is independent of pT1AB expression (CD5 has been transfected into L cells and the antigen is expressed properly), the expression of the pT1AB gene was found only in CD5+ cells (T cell lines which are CD5 positive). pT1AB was obtained by screening a J6 λgt11 cDNA library with an anti-CD5 polyclonal antiserum, which was raised against immunoaffinity purified denatured CD5 molecules. Silver staining of the purified CD5 revealed only a band of 67kd, however, this does not rule out the possibility that the expression of pT1AB and CD5 might be closely related and associated with each other on the cell surface. In view of these uncertainties, another approach would be to transfect a shuttle vector containing both pT1AB and the neomycin gene into a CD5 transfectant which had been established with HAT selection. Transfectants express both CD5 and pT1AB gene product will display resistance to both G418 and HAT.

Because of the difficulties so far encountered in raising antibodies to the pT1AB gene product, we suspect that it is not a very immunogenic molecule in mice and rats. Transfectants might only produce small amounts of the protein so that it may be sensible to employ combined immunisation strategies; for example prime with transfectant and boost with a fusion protein or peptide. Such a strategy would have the potential advantage that it might favour production of antibodies reacting with both native (transfectant)

and denatured (fusion) protein. Once an ideal antibody is obtained, it will be relatively easier to study the properties of the pT1AB gene product by various immunochemical and immunohistochemical methods.

7.2.2 Molecular biological studies

Exploitation of the partial cDNA: Before a full length cDNA is obtained, it would be possible to use the partial cDNA clones (pT1A and pT1B) to explore further the pT1AB gene. Partial cDNA clones can be used to:

1) screen cDNA libraries to select full length cDNA clones and screen genomic DNA libraries to obtain genomic clones;

2) perform gene mapping in which high molecular weight DNA is extracted from a panel of human-mouse somatic hybrids and subjected to the southern blot analysis. The hybrid containing the chromosome harbouring the pT1AB gene would give rise a positive signal. However, since pT1AB cross-hybridises with mouse DNA, therefore preliminary work has to be done to select the best restriction enzyme which distinguishes human and mouse DNA. Precise chromosomal location could be decided by *in situ* hybridisation. This might reveal if the chromosome abnormalities frequently seen in these T-ALL might paly a role in turning on pT1AB gene expression;

3) clone the mouse pT1AB analogue. Information on mouse pT1AB gene would provide not only information on functionally important

domains but also an animal model to conduct further analysis of the function of the gene product;

4) perform polymerase chain reaction (PCR). It is surprising that expression of pT1AB has not been detected in normal tissues. This may be partly because message is present only at low aboundance and comparison of the signals obtained with pT1AB and CD5 probes in northern blot analysis would support this (Figure 6.5). In addition pT1AB may be expressed only in cells at a particular stage of differentiation or cell cycle. Two approaches to resolving this problem are possible. In situ hybridisation on tissue sections might reveal pT1AB in rare positive cells and the use of PCR should overcome the problem of low abundance or transient expression.

The use of PCR would also facilitate analysis of tumour material. Even if pT1AB is expressed in some normal cells, it is certainly consistently expressed in T-ALL lines. It is tempting to speculate that this is more than a chance association. Confirmation of such a speculation will require analysis of more tumour material, particularly from fresh leukaemias rather that cell lines. Even if such an association is confirmed, the role of the pT1AB product in malignant transformation will need to be proved. However many growth factors and their receptors are now thought to be involved in a variety of tumours (reviewed in Yarden and Ullrich, 1988) and the EGF-like structure of pT1AB tends support to the possibility that pT1AB may be involved in transformation of T cells.

Full length cDNAs: Access to full length cDNAs coding for all the different transcripts would be ideal to carry out various studies:

1) They will provide information on the relationship between the different transcripts. It is not known yet if the multiple transcripts detected are from a common transcript, which is spliced into various forms in different cells, or if these mRNAs are transcribed from different genes which are closely related. Alternatively they might differ from each other only in the untranslated regions;

2) By comparing sequences of human pT1AB transcripts with their mouse counterparts, it may be possible to identify functionally important domains which might be conserved between different species;

3) The full length cDNA will facilitate transfection to obtain proper expression of the gene product;

4) It is also possible to use the full length cDNAs to perform in vitro transcription and in vitro translation to understand the biochemistry of the gene product.

7.2.3 Functional assays

It has been demonstrated that human malignant T lymphoblasts can be induced by PMA to differentiate and display mature phenotype (Greene et al., 1984). A comprehensive survey of pT1AB gene expression in T cells revealed that the expression pattern was relatively stable and was to a certain extent correlated with different stages of maturation. This correlation might be confirmed

if it can be demonstrated that the pT1AB-related transcript expression pattern changes following exposure to PMA.

It has been shown that fragment 1 and fragment 1-4 of laminin consist of cysteine-rich "EGF-like" repeats which stimulate thymidine incorporation in cultured cells possessing EGF receptors but have no effect on a cell line lacking this receptor (Panayotou et al., 1989). Synthetic peptides containing EGF-like repeats from the pT1AB gene or supernatant from pT1AB transfectants (which might contain the secreted pT1AB gene product) could be assayed for the effect on the growth of a number of established cell lines including fibroblastoid Swiss 3T3 (possessing EGF receptors) and a mutant cell line of Swiss 3T3 cells that lacks EGF receptors.

Another approach to investigate the function of the gene product would be to employ antisense oligomers of the different pT1AB transcripts. The antisense oligomers will block the translation of pT1AB transcripts, therefore the function of the gene product can be deduced from changes in cell phenotype and proliferation rate following the exposure to specific oligonucleotides (Gewirtz and Calabretta, 1989; Heikkila et al., 1987; Anfossi et al., 1989).

Finally, it has been pointed out in this chapter that much still has to be done concerning the pT1AB gene. The greatest challenge will be the elucidation of pT1AB's true physiological roles. It is hoped that tissue distribution and phenotype analysis presented here together

with the results of various approaches to production of antibodies, will provide useful information for selecting future strategies to study the gene product.

References

Adams, R.A., Flowers, A. and Davis, B.J. (1968). Direct implantation and serial transplantation of human acute lymphoblastic leukemia in hamsters, SB-2.
Cancer Res. **28** 1121

Allison, J.P. and Lanier, L.L. (1987). Structure, function and serology of the T-cell antigen receptor complex.
Ann. Rev. Immunol. **5** 503.

Anderson, D.C., and Springer, T.A. (1987). Leukocyte adhesion deficiency: an inherited defect in the Mac-1, LFA-1, and p150,95 glycoproteins.
Ann. Rev. Med. **38** 175

Anfossi, G., Gewirtz, A.M. and Calabretta, B. (1989). An oligomer complementary to c-myb-encoded mRNA inhibits proliferation of human myeloid leukemia cell lines.
Proc. Natl. Acad. Sci. USA. **86** 3379

Antin, J.H., Emerson, S.G., Martin, P., Gadol, N. and Ault, K.A. (1986). Leu-1$^+$ (CD5$^+$) B cells. a major lymphoid subpopulation in human fetal spleen: phenotypic and functional studies.
J. Immunol. **136** 505

Arnon, R., Maron., E., Sela, M. and Anfinsen, C.B. (1971). Antibodies reactive with native lysozyme elicited by a completely synthetic antigen.
Proc. Natl. Acad. Sci. USA **68** 1450

Arthos, J., Deen, C.K., Chaikin, M.A., Fornwald, J.A., Sattentau, Q.J., Clapman, P.R., Weiss, R.A., McDougal, J.S., Pictropaolo, C., Maddon, P.J., Trunch, A., Axel, R. and Sweet, R.W. (1989). Identification of the residues in human CD4 critical for the binding of HIV,
Cell **57** 469

Ault, K.A., Antin, J.H., Ginsburg, D., Orkin, S.H., Rappeport, J.M., Keohan, M.L., Martin, P. and Smith, B.R. (1985) Phenotype of recovering lymphoid cell populations after marrow transplantation.

Balkwill , F.R. and Burke, F. (1989). The cytokine network. Immunol. Today **10** 299

J. Exp. Med. **61** 1483

Baer, R., Forster, A. and Rabbitts, T.H. (1987). The mechanism of chromosome 14 inversion in a human T cell lymphoma.
Cell **50** 97

Barber, E.K., Dasgupta, J.D., Schlossman, S.F., Trevillyan, J.M. and Rudd, C.E. (1989). The CD4 and CD8 antigens are coupled to a protein-tyrosine kinase ($p56^{lck}$) that phosphorylates the CD3 complex.
Proc. Natl. Acad. Sci. USA **86**. 3277

Bar-Shavit, Z., Bing, D.H., Kahn, A.J. and Wilner, G.D. (1985) in Membrane Receptors and Cellular Recognition (Liss, New York).

Barthels, D., Santone, M.J., Wille, W., Ruppert, C., Chaix, J.C., Hirsch, M.R., Fontecilla-Camps, J.C., Goridis, C. (1987). Isolation and nucleotide sequence of mouse NCAM cDNA that codes for a Mr 79,000 polypeptide without a membrane-spanning region.
EMBO J. **6** 907

Bennett, G.N. and Yanofsky, C. (1978). Sequence analysis of operator constitutive mutants of the tryptophan operon of Escherichia coli.
J. Mol. Biol. **121** 179

Ben-Nun, A., Glimcher, L.H., Weiss, J., Seidman, J.G. (1984). Functional expression of a cloned I-A gene in B lymphoma cells.
Science **223** 825

Ben-Nun, A., Choi, E., McIntyre, K.R., Leeman, S.A., McKean, D.J., Seidman, J.G. and Glimcher, L.H. (1985). DNA-mediated transfer of major histocompatibility class II $I\text{-}A^{b}$ and $I\text{-}A^{bm12}$ genes into B lymphoma cells: molecular and functional analysis of introduced antigens.
I. Immunol. **135** 1456

Benson, S.A. and Silhavy (1983) Information within the mature LamB protein necessary for localisation to the outer membrane of E. coli K12.
Cell **32** 1325

Bergui, L., Teiso, L., Schena, M., Riva, M., Malavasi, F., Schulz, T., Marchisio, P.C. and Caligaris-Cappio, F. (1988). CD5 and CD21 molecules are a functional unit in the cell/substrate adhesion of B-chronic lymphocytic leukemia cells.
Eur. J. Immunol. **18** 89

Berridge, M. (1987). Inosital triphosphate and diacylglycerol: two interacting second messengers.
Ann. Rev. Biochem. **56** 159

Beverley, P.C.L. and Callard, R.E. (1981a). Distinctive functional charateristics of human T lymphocytes defined by E rosetting or a monoclonal anti-T cell antibody.
Eur. J. Immunol. **11** 329

Beverley, P.C.L. and Callard, R.E. (1981b). Re-definition of human T cells by monoclonal antibodies.
Protides Biol. Fluids **29** 653

Beverley, P.C.L. (1982). The application of monoclonal antibodies to the typing and isolation of lymphoreticular cells.
Proc. Roy. Soc. Edin. B **81** 221

Blobel, G., Walter, P., Chang, C.N., Goldman, B.M., Erickson, A.H. and Lingappa, V.R. (1979). Translocation of proteins across membranes: the signal hypothesis and beyond.
Symp. Soc. Exp. Biol. **33** 9

Bofill, M., Janossy, G., Janossa, M., Burford,G.D., Seymour, G.J., Wernet, P. and Kelemen, E. (1985). Human B cell development II. Subpopulations in the human fetus.
J. Immunol. **134** 1531

Boyum, A. (1968). Isolation of mononuclear cells and granulocytes from human blood.
Scand. J. Clin. Lab. Invest. **27** (suppl 97) 77

Bradstock, K.F., Janossy, G., Pizzolo, G., Hoffbrand, A.V., McMichael, A., Pilch, J.R., Milstein, C., Beverley, P.C.L. and Bollum, F.J. (1980). Subpopulations of normal and leukaemic human thymocytes: an analysis using monoclonal antibodies.
J. Natl. Cancer Inst. **65** 33

Brown, P.J., Twardzik, R.D., Marquardt, H. and Tadaro, J.G. (1985). Vaccinia virus encodes a polypeptide homologous to epidermal growth factor and transforming growth factor.
Nature **313** 491.

Burmeister, M. and Lehrach, H. (1986). Long-range restriction map around the Duchenne muscular dystrophy gene.
Nature **324** 582

Bushkin, Y., Demaria, S., Le, J. and Schwab, R. (1988).
Proc. Natl. Acad. Sci. USA **85** 3985

Calligaris-Cappio, F., Bobbi, M., Boffil, M. and Jonossy, G. (1982). Infrequent normal B lymphocytes express features of B-chronic lymphocytic leukemia.
J. Exp. Med. **155** 623

Campos-Ortega, J.A. (1985). Genetics of early neurogenesis in Drosophila melanogaster.
Trens Neurosci. **8** 245

Cantor, H. and Boyse, E.A. (1977). Lymphocytes as models for the study of mammalian cellular differentiation.
Immunol. Rev. **33** 105

Cantrell D.A. and Smith, K.A. (1983). Transient expression of interleukin 2 receptors: consequences for T cell growth.
J. Exp. Med. **158** 1895

Carpenter, G. (1979) Epidermal growth factor.
Ann. Rev. Biochem. **48** 193

Carpenter, G., Zendegui, J.G. (1986). Epidermal growth factor, its receptor, and related proteins.
Exp. Cell Res. **164** 1

Carpenter, G. (1987) Receptors for epidermal growth factor and other polypeptide mitogens.
Ann. Rev. Biochem. **56** 881

Casali, P.; Burastero, S.E.; Nakamura, M.; Inghirami, G.and Nothins, A.L.(1987). Human lymphocytes making rheumatoid factor and antibody to ssDNA belong to Leu-1+ B cell subset.
Science **236** 77

Clark, E.A., Ledbetter, J.A., Holly, R.C., Dinndorf, P.A. and Shu, G. (1986). Polypeptides on human B lymphocytes associated with cell activation.
Hum. Immunol. **16** 100

Clark, S.C. and Kamen, R. (1987). The human hematopoietic colony-stimulating factors.
Science **236** 1229

Clayton, L.K., Hussey, R.E., Steinbrich, R., Ramachandran, H., Hussain, Y., and Reinherz, E.L. (1988). Substitution of murine for human CD4 residues identifies amino acids critical for HIV-gp120 binding.
Nature **335** 363

Clevers, H., Alarcon, B., Wileman, T. and Terhorst, C. (1988). The T cell receptor/CD3 complex: a dynamic protein ensemble.
Ann. Rev. Immunol. **6** 629

Cohen, S. (1959). Purification and metabolic effects of a nerve growth-promoting protein from snake venom.
J. Biol. Chem. **234** 1129

Cohen, S. (1962). Isolation of a mouse submaxillary gland protein accelerating insisor eruption and eyelid opening in new-born animal.
J. Biol. Chem. **237** 1555

Cohen, S. Elliott, G.A. (1962). The stimulation of epidermal keratinization by a protein isolated from the submaxillary gland of the mouse. (Classical article).
J. Invest. Dermatol. **40** 1

Cohen, S. (1965). The stimulation of epidermal proliferation by a specific protein (EGF).
Dev. Biol. **12** 394

Cohen, S. and Carpenter, G. (1975). Human epidermal growth factor: isolation and chemical and biological properties.
Proc. Natl. Acad. Sci. USA **72** 1317

Collins, S.J., Gallo, R.C. and Gallagher, R.E. (1977) Continuous growth and differentation of human myeloid leukaemic cells in suspension culture.
Nature **270** 347

Cossman, J., Neckers, L.M., Arnold, A. and Korsmeyer, S.J. (1982). Induction of differentiation in a case of common acute lymphoblastic leukemea.
N. Engl. J Med. **307** 1251

Cotran, R.S., Pober, J.S., Gimbrone, Jr, M.A., Springer, T.A., Wiebke, E.A., Gaspari, A.A., Rosenberg, S.A. and Lotze, M.T. (1988). Endothelial activation during interleukin 2 immunotherapy A possible mechanism for vascular leak syndrome.
J. Immunol. **139** 1883

Coussens, L., Yang-Feng, T.L., Liao, Y.C., Chen, E., Gray, A., Mcgrath, J., Seeburg, P.H., Libermann, T.A., Schlessinger, J., Francke, U., Levinson, A. and Ullrich, A. (1985). Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene.
Science **230** 1132

Cunningham, B. A., Wang, J. L., Berggard, I., and Peterson, P. A. (1973). The complete amino-acid sequence of β_2-microglobulin.
Biochemistry **12** 4811

Cunningham, B. A., Hemperley, J.J., Murray, B.A., Prediger, E.A., Brackengury, R., Edelman, G.M. (1987). Neural cell adhesion molecules: structure, Ig-like domains, cell surface modulation and alternative RNA splicing.
Science **236** 799

Dalla-Favera, G. et al., (1982). Human c-myc onc gene is located on the region of chromosomal 8 that is translocated in Burkitts lymphoma cells.
Proc. Natl. Acad. Sci. USA **79** 7824

Dalgleish, A.G., Beverley, P.C.L., Clapham, P.R., Crawford, D.H., Greaves, M.F., Weiss, R.A. (1984). The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus.
Nature **312** 767

Davis, B.D. and Tai, P.C. (1980). The mechanism of protein secretion across membranes.
Nature **283** 433

Davis, G.L., Dibner, M.D. and Battey, F.J. (1986). Calcium phosphate transfection of nonadherent and adherent cells with purified plasmids.
In: Basic Methods in Molecular Biology p286

De La Hera, A., Tenibio, M.L., Marquez, C. and Martinez, C. (1985). Interleukin 2 promotes growth and cytolytic activity in human $T3^+4^-8^-$ thymocytes.
Proc. Natl. Acad. Sci. USA **82** 6268

D'Emilia, J., Bulovas, K., D'Ercole, K., Wolf, B., Steele Jr, G. and Summerhayes, I.C. (1989). Expression of the c-erbB-2 gene product (p185) at different stages of neoplastic progression in the colon.
Oncogene **4** 1233

Depper, J.M., Leonard, W.J., Kronke, M., Waldmann, T.A. and Greene, W.C. (1984). Augmented T cell growth factor receptor expression in HTLV-1-infected human leukemic T cells.
J. Immunol. **133** 1691

Derynck, R., Roberts, A.B., Winkler, M.E., Chen, E.Y. and Coeddel, D.V. (1984). Human transforming growth factor-alpha: precursor structure and expression in E. coli.
Cell **38** 287

Dialynas, D.P., Quan, Z.S., Wall, K..A., Pierres, A., Quintans, J., Loken, M.R., Pierres, M., Fitch, F.W. (1983). Characterization of the murine T cell surface molecule, designated L3T4 identified by monoclonal antibody GK1.5: Similarity of L3T4 to the human Leu3/T4 molecule.
J. Immunol. **131** 2445

Dietrich, U. and Campos-Ortega, J.A. (1984). The expression of neurogenic loci in imaginal epidermal cells of Drosophila melanogaster.
J. Neurogenet. **1** 315

Dinarello, C.A. and Mier, J.W. (1987). Lymphokines.
N. Engl. J. Med. **317** 940

Di Scipio, R.G., Gehring, M.R., Podack, E.R., kan, C.C., Hugli, T.E. and Fey, G.H. (1984). Nucleotide sequence of cDNA and derived amino acid sequence of human complement component C9.
Proc. Natl. Acad. Sci. USA **81** 7298

Doolittle, R.F., Hunkapiller, M.W., Hood, L.E., Devare, S.G. and Robbins, K.C. (1983). Simian sarcoma virus onc gene, v-sis, is derived from gene (or genes) encoding a platelet-derived growth factor.
Science **221** 275

Doolittle, W.F. and Yanofsky, C. (1986) Mutants of Escherichia coli with an altered tryptophanyl-transfer ribonucleic acid synthetase.
J. Bacteriol. **95** 1283

Dougherty, G.J., Murdoch, S. and Hogg, N. (1988). The function of human intercellular adhesion molecule-1 (ICAM-1) in the generation of an immune response.
Eur. J. Immunol. **18** 35

Downward., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J. and Waterfield, M.D. (1984). Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences.
Nature **307** 521

Doyle, C. and Strominger, J. L. (1987). Interaction between CD4 and Class II MHC molecules mediates cell adhesion.
Nature **330** 256

Dresser, D.W. (1978). Assays for immunoglobulin secreting cells.
In: Handbook of Experimental Immunology. D.M.Weir, ed., Blackwell Scientific Publications, Oxford. vol. **2** p22

Dustin, M.L., Rothlein, R., Bahn. A.K., Dinarello, C.A. and Springer, T.A. (1986). Induction by IL 1 and interferon-γ: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1).
J. Immunol. **137** 245

Edelman, G.M. (1986). Cell adhesion molecules in the regulation of animal form and tissue pattern.
Ann. Rev. Cell Biol. **2** 81

Edwards, P.A.W., Smith, C.M., Neville, A.M. and O'Hare, M.J. (1982). A human-human hybridoma system based on a fast growing mutant of the ARH-77 plasma cell leukemia-derived line.
Eur. J. Immunol. **12** 641

Enfield, D.L. et al. (1980). Amino acid sequence of the light chain of bovine factor X.
Biochemistry **19** 659

Engleman, E.G., Benike, C., Glickman, E., and Evans, R.L. (1981a). Antibodies to membrane structures that distinguish suppressor/cytotoxic and helper T lymphocyte subpopulations block the mixed leukocyte reaction in man.
J. Exp. Med. **154** 193

Engleman, E.G., Benike, C.J., Grumet, C. and Evans, R.L. (1981b). Activaiton of human T lymphocyte subsets: helper and suppressor/cytotoxic T cells recognize and respond to distinct histocompatibility antigens.
J. Immunol. **127** 2124

Engleman, E.F., Warnke, R., Fox, R.I., Dilley, J., Benike, C.J. and Levy, R. (1981c). Studies of a human T lymphocyte antigen recognized by a monoclonal antibody.
Proc. Natl. Acad. Sci. USA **78** 1791

Farrar, W.L., and Ruscetti, F. W. (1986). Association of protein kinase C activation with IL-2 receptor expression.
J. Immunol. **136** 1266

Feinberg, A.P. and Vogelstein, B (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity.
Anal. Biochem. **132** 6

Fernlund, P. and Stenflo, J. (1982). Amino acid sequence of the light chain of bovine protein C.
J. Biol. Chem. **257** 12170

Foley, G.E., Lazarus, H., Farbier, S., Uzman, B.G., Boone, B.A. and McCarthy, R.E. (1965) Continuous culture of human lymphoblasts from peripheral blood of a child with acute leukaemia.
Cancer **18** 522

Folsom, V., Gold, D.P., White, J., Marrack, P., Kappler, J. and Tonegawa, S. (1984). Functional and inducible expression of a transfected murine class II major histocompatibility complex gene.
Proc. Natl. Acad. Sci. USA **81** 2045

Folsom, V., Gay, D., Tonegawa, S. (1985) The b1 domain of the mouse Eb chain is important for restricted antigen presentation to helper T-cell hybridomas.
Proc. Natl. Acad. Sci. USA **82** 1678

Furie, B. and Furie, B.C. (1988) The molecular basis of blood coagulation.
Cell **53** 505

Galfre, G., Milstein, C. and Wright, B. (1979). Rat x rat hybrid myelomas and a monoclonal anti-Fd portion of mouse IgG.
Nature **277** 131

Gallatin, W.M., Weissman, I.L. and Butcher, E.C. (1983). Cell surface molecule involved in organ-specific homing of lymphocytes.
Nature **304** 30

Gallin, W.J., Chuong, C.M., Finkel, L.H. and Edelman, G.M. (1986). Antibodies to liver cell adhesion molecule perturb inductive interactions and alter feather pattern and structure.
Proc. Natl. Acad. Sci. USA **83** 8235

Gazder, A.F., Carney, D.N., Bunn, P.A., Russell, E.K., Jaffe, E.S., Schechter, G.P. and Guccion, J.G. (1980). Mitogenic requirements for the in vitro propagation of cutaneous T-cell lymphomas.
Blood **55** 409

Gazit, A., Igarashi, H., Chiu, I.M., Srinivasan, A., Yaniv, A., Tronick, S.R., Robbins, K.C. and Aaronso, S.A. (1984). Expression of the normal human sis/PDGF-2 coding sequence induces cellular transformation.
Cell **39** 89

Germain, R.N., Norcross, M.A., Margulies, D.H. (1983). Functional expression of a transfected murine class II MHC gene.
Nature **306** 190

Germain, R.N., Ashwell, J.D., Lechler, R.I., Margulies, D.H., Nickerson, K.M, Suzuki, G., Tou, J.Y.L. (1985) "Exon-shuffling" maps control of antibody and T-cell recognition sites to the NH_2-terminal domain of the class II major histocompatibility polypeptide Aβ.
Proc. Natl. Acad. Sci. USA **82** 2940

Gewirtz, A.M. and Calabretta, B. (1988). A c-myb antisense oligodeoxynucleotide inhibits normal human hematopoiesis in vitro.
Science **242** 1303

Gillies, S.D., Folsom, V. and Tonegawa, S. (1984). Cell type-specific enhancer element associated with a mouse MHC gene, Eb.
Nature **310** 594

Gillis, S. and Watson, J. (1980). Biochemical and biological characterisation of lymphocyte regulatory molecules V. Identification of an interleukin 2-producing human leukemia T cell line.
J. Exp. Med. **152** 1709

Goldstein, J.L., Brown, M.S., Anderson, T.G.W., Russell, D.W. and Schneider, W.J. (1985). Receptor-mediated endocytosis: concepts emerging from the LDL receptor system.
Ann. Rev. Cell Biol. **1** 1

Hancock, D.C. and Evan, G.I. (1990). Synthesis and use of synthetic peptides as immunogens.
Methods in Molecular Biology 8: Immunochemical Techniques. eds. M.M. Manson (series editor J.M. Walker).

Gray, A., Dull, J.T., Ullrich, A. (1983). Nucleotide sequence of epidermal growth factor cDNA predicts a 128,000-molecular weight protein precursor.
Nature **303** 722

Greene, W.C., Robb, R.J., Depper, J.M., Keonard, W.J., Drogula, C., Svetlik, P.B., Wong-Staal, F., Gallo, R.C. and Waldmann, T.A. (1984) Phorbol diester induces expression of Tac antigen on human acute T lymphocytic leukemic cells.
J. Immunol. **133** 1042

Greenwald, I. (1985). Lin-12, A nematode homeotic gene, is homologous to a set of mammalian proteins that includes epidermal growth factor.
Cell **43** 583

Gregory, C.D., Murray, R.J. Edwards, C.F. and Rickinson, A.B. (1988). Downregulation of cell adhesion molecules LFA-3 and ICAM-1 in Epstein-Barr virus-positive Burkitt's lymphoma underlies tumor cell escape from virus-specific T cell surveillance.
J. Exp. Med. **167** 1811

Gunsalus, R.G. and Yanofsky, C. (1980). Nucleotide sequence and expression of Escherichia coli trpR, the structural gene for the trp aporepressor.
Proc. Natl. Acad. Sci. USA **77** 7117

Hardy, R.R., hayakawa, K., Shimizu, M., Yamasaki, K. and Kishinoto, T. (1987). Rheumatoid factor secretion from human Leu-1+ B cells.
Science **237** 81

Hattori, T., Uchiyama, T., Toibana, T., Takatsuke, K. and Uchino, H. (1981). Surface phenotype of Japanese adult T-cell leukemia cells characterized by monoclonal antibodies.
Blood **58** 645

Hayakawa, K., Hardy, R.R., Parks, D.R. and Herzenberg, L.A. (1983). The "Ly-1 B" cell subpopulation in normal immunodefective, and autoimmune mice.
J. Exp. Med. **157** 202

Hayakawa, K., Hardy, R.R., Honda, M., Herzenberg, L.A., Steinberg, A.D. and Herzenberg, L.A. (1984). Ly-1 B cells: Functionally distinct lymphocytes that secrete IgM autoantibodies.
Proc. Natl. Acad. Sci. USA **81** 2494

Hayakawa, K., Hardy, R.R., Herzenberg, L.A. and Herzenberg, L.A. (1985). Progenitors for Ly-1 B cells are distinct from progenitors for other B cells.
J. Exp. Med. **161** 1554

Hayakawa, K., Hardy, R.R., Stall, A.M., Herzenberg, L.A. and Herzenberg, L.A. (1986). Immunoglobulin-bearing B cells reconstitute and maintain the murine Ly-1 B cell lineage.
Eur. J. Immunol. **16** 1313

Haynes, B.F., Eisenbarth, G.S. and Fauci, A.S. (1979). Human lymphocyte antigens: production of a monoclonal antibody that defines functional thymus-derived subsets.
Proc. Natl. Acad. Sci. USA **76** 5829

Heikkila, R., Schwab, G., Wickstrom, E., Loke, S.L., Pluznik, D.H., Watt, R. and Neckers, L.M. (1987). A c-myc antisense oligodeoxynucleotide inhibits entry into S phase but not progress from G_0 to G_1.
Nature **328** 445

Hoffman, E.P., Brown Jr, R.H. and Kunkel, L.M. (1987). Dystrophin: The protein product of the Duschenne muscular dystrophy locus.
Cell **51** 919

Hollander, N., Pillemer, E. and Weissman, I.L. (1981). Effects of lyt antibodies on T cell functions: Augmentation by anti-lyt-1 as opposed to inhibition.
Proc. Natl. Acad. Sci. USA **78** 1148

Hood, L., Kronengerg, M., and Hunkapiller, T. (1985). T cell antigen receptors and the immunoglobulin supergene family.
Cell **40** 225

Hoppe. P.E. and Greenspan, R. J. (1986). Local function of the Notch gene for embryonic ectodermal pathway choice in Drosophila.
Cell **46** 773

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

Howard, O.M.Z., Rao, A.G. and Sodetz, J.M. (1987) Complementary DNA and derived amino acid sequence of the β subunit of human

complement protein C8: identification of a close structural and ancestral relationship to the α subunit and C9.
Biochemistry **26** 3565

Hsu, C., Kavathas, P. and Herzenberg, L.A. (1984). Cell-surface antigens expressed on L-cells transfected with whole DNA from non-expressing and expressing cells.
Nature **312** 68

Hursh, A.D., Andrews, E.M. and Raff, A.R. (1987). A sea urchin gene encodes a polypeptide homologous to epidermal growth facotr.
Science **237** 1487

Huynh, T.V., Young, R.A. and Davis, R.W. (1985) in Glover, D.M. (ed) DNA cloning volume 1, IRL Press, Oxford, pp. 49-78

Hynes, R. O. (1987). Integrins: a family of cell surface receptors.
Cell **48** 549

Jackman, W.R., Beeler, L.D., VanDeWater, L. and Rosenberg, D.R. (1986). Characterization of a thrombomodulin cDNA reveals structural similarity to the low density lipoprotein receptor.
Proc. Natl. Acad. Sci. USA **83** 8834

Johnsson, A., Heldin, C.H., Wasteson, A., Westermark, B., Deuel, T.F., Huang, J.S., Seeburg, P.H., Gray, A., ullrich, A., Scrace, G., Stroobant, P. and Waterfield, M.D. (1984). The c-sis gene encodes a precursor of the B chain of platelet-derived growth factor.
EMBO J. **3** 921

Jones, N.H., Clabby, M.L., Dialynas, D.P., Huang, H.S., Herzenberg, L.A. and Strominger, J.L. (1986) Isolation of complementary DNA clones encoding the human lymphocyte glycoprotein T1/Leu-1.
Nature **323** 346

Kamoun, M., Martin, P. J., Hansen, J. A., Brown, M. A., Siadak, A. W., and Nowinski, T. C. (1981). Identification of a human T lymphocyte surface protein associated with the E-rosette receptor.
J. Exp. Med. **153** 207

Kaplan, M.E. and Clark, C. (1974). An improved rosetting assay for detection of human T lymphocytes.
J. Immunol. Methods **5** 131

Kappler, J., Kubo, R., Haskins, K., White, J., and Marrack, P. (1983a). The mouse T cell receptor: comparison of MHC-restricted receptors on two T cell hybridomas.
Cell **34** 727

Kappler, J., Kubo, R., Haskins, K., Hannum, C., Marrack, P., Pigeon, M., McIntyre, B., Allison, J., and Trowbridge, I. (1983b). The major histocompatibility complex-restricted antigen receptor on T cells in mouse and man: identification of constant and variable peptides.
Cell **35** 295

Katayama, K., et al (1979). Comparison of amino acid sequence of bovine coagulation factor IX (Christmas Factor) with that of other vitamin K-dependent plasma proteins.
Proc. Natl. Acad. Sci. USA **76** 4990

Kavathas, P. and Herzenberg, L.A. (1983a). Amplification of a gene coding for human T-cell differentiation antigen.
Nature **306** 386

Kavathas, P. and Herzenberg, L.A. (1983b). Stable transformation of mouse L cells for human membrane T-cell differentiation antigens, HLA and β_2-microglobulin: Selection by fluorescence-activated cell sorting.
Proc. Natl, Acad. Sci. USA **80** 524

Kearney, J. and Vakil, M. (1986). Functional idiotype networks during B cell ontogeny.
Ann. Immunol. (Inst. Pasteur) **137c** 77

Kenwrick, S., Patterson, M., Speer, A., Fischbeck, K. and Davies, K. (1987). Molecular analysis of the Duchenne muscular dystrophy region using pulsed field gel electrophoresis.
Cell **48** 351

King, C.R., Kraus, M.H. and Aaronson, S.A. (1985). Amplicaiton of a novel v-erbB-related gene in a human mammary carcinoma.
Science **229** 974

Kishimoto, T.K., O'Conner, K., Lee, A., Roberts, T.M. and Springer, T.A. (1987). Cloning of the β subunit of the leukocyte adhesion proteins: homology to an extracellular matrix receptor defines a novel supergene family.
Cell **48** 681

Knapp, W., Rieber, P., Dorken, B., Schmidt, R.E., Stein, H. and Borne, A.E.G.Kr.v.d. (1989). Towards a better definition of human leucocyte surface molecules.
Immunol. Today **10** 253

Kisielow, P., Hirst, J., Shiku, H., Beverley, P., Hoffmann, N.K., Boyse, E., Oettgen, H. (1975). Ly antigens as markers for functionally distinct subpopulations of thymus derived lymphocytes of the mouse.
Nature **253** 219

Klatzmann, D., Champagne, E., Chamaret, S., Gruest, J., Guctard, D., Hercend, T., Gluckman, J.C. and Mantagnicr, L. (1984). T lymphocyte T4 molecule behaves as the receptor for the human retrovirus. LAV.
Nature **312** 767

Kleid, D.G., Yansura, D., Small, B., Dowbenko, D., Moore, D.M., Grubman, M.J., McKercher, P.D., Morgan, D.O., Robertson, B.H. and Bachrach. H.L. (1981) Cloned viral protein vaccine for foot-and-mouth disease: responses in cattle and swine.
Science **214** 1125

Knust, K., Dietrich, U., Tepass, U., Bremer, A.K., Weigea, D., Vassin, H. and Campos-Ortega A.J. (1987). EGF homologous sequences encoded in the genome of Drosophila melanogaster, and their relation to neurogenic genes.
EMBO J. **6** 761

Kohler, G. and Milstein, C. (1975). continuous cultures of fused cells secreting antibody of pre-determined specificity.
Nature **256** 495

Kohler, G., Howe, S.C. and Milstein, C. (1976). Fusion between immunoglobulin secreting and nonsecreting myeloma cell lines.
Eur. J. Immunol. **6** 292

Komoriya, A., Hortsch, M., Meyers, C., Smith, M., Kanety, H. and Schlessinger, J. (1984) Biologically active synthetic fragments of epidermal growth factor: Localization of a major receptor-binding region.
Proc. Natl. Acad. Sci. USA **81** 1351

Kozak, M. (1986). Point mutation define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes.
Cell **44** 283

Kraus, M.H., Popescu, N.C., Amsbaugh, S.C. and King, C.R. (1987) Overexpression of the EGF receptor-related proto-oncogene erbB-2 in human mammary tumor cell lines by different molecular mechanisms.

EMBO J. **6** 605

Krensky, A.M., Clayberger, C., Reiss, C.S., Strominger, J.L. and Burakoff, S.J. (1982a). Specificity of OKT4+ cytotoxic T lymphocytes clones.
J. Immunol. **129** 2001

Krensky, A.M., Reiss, C.S., Mier, J.W., Strominger, J.L. and Burakoff, S.J. (1982b). Long term cytotoxic T cell lines allospecific for HLA-DR6 antigen are OKT4+.
Proc. Natl. Acad. Sci. USA **79** 2365

Krensky, A.M., Robbins, E., Springer, T.A. and Burakoff, S.J. (1984). LFA-1, LFA-2, and LFA-3 antigens are involved in CTL-target conjugation.
J. Immunol. **132** 2180

Kung, P.C., Goldstein, G., Reinherz, E. and Schlossman, S. (1979). Monoclonal antibodies defining distinctive human T cell surface antigen.
Science **206** 347

Kurachi, K. and Davie, E.W. (1982) Isolation and characterization of a cDNA coding for human factor IX.
Proc. Natl. Acad. Sci. USA. **79** 6461

Kyte, J. and Doolittle, R.F. (1982). A simple method for displaying the hydropathic character of a protein.
J. Mol. Biol. **157** 105

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4.
Nature **227** 680

Lai, C., Brow, M. A., Nave, K.A., Noronha, A.B., Quarles, R.H., Bloom, F.E., Milner, R.J., Sutcliffe, J.G. (1987). Two forms of IB235/myelin associated glycoprotein, a cell adhesion molecule for postnatal neural development, are produced by alternative splicing.
Proc. Natl. Acad. Sci. USA **84** 4337

Landau, N.R., Warton, M., Littman, D.R. (1988). The envelope glycoprotein on the human immunodeficiency virus binds to the immunoglobulin-like domain of CD4.
Nature **334** 159

Lanier,L.L., Warner,N.L., Ledbetter,J.A. and Herzenberg,L.A. (1981). Expression of Lyt-1 antigen on certain murine B cell lymphomas. J. Exp. Med. **153** 998

Lampson LA and Levy RH (1980) Two populations of Ia-like molecules on a human B cell line. J. Immunol. **125** 293

Lasky, L.A., Singer, M.S., Yednock, T.A., Dowbenko, D., Fennie, C., Rodriguez, H., Nguyen, T., Stachel, S. and Rosen, S.D. (1989). Cloning of a lymphocyte homing receptor reveals a lectin domain. Cell **56** 1045

Leal, F., Williams, L.T., Robbins, K.C.and Aaronson, S.A. (1985). Evidence that the v-sis gene product transforms by interaction with the receptor for platelet-derived growth factor. Science **230** 327

LeBien, T.W., Bollum, F.J., Yasmineh, W.G. and Kersey, J.H. (1982). Phorbol ester-induced differentiation of a non-T, non-B leukemic cell line: model for human lymphoid progenitor cell development. J. Immunol. **128** 1316

Ledbetter, J.A., Herzenberg, L.A. (1979). Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. Immunol. Rev. **47** 63

Ledbetter, J.A., Rouse, R.V., Micklem, H.S. and Herzenberg, L.A. (1980). T cell subsets defined by expression of Lyt-1,2,3 and Thy-1 antigens. Two parameter immunofluorescence and cytotoxicity analysis with monoclonal antibodies modifies current views. J. Exp. Med. **152** 280

Ledbetter, J.A., Evans, R.L., Lipinski, M., Cunningham-Rundles, C., Good, R.A. and Herzenberg, L.A. (1981). Evolutionary conservation of surface molecules that distinguish T lymphocyte helper/inducer and cytotoxic/suppressor subpopulations in mouse and man. J. Exp. Med. **153** 310

Ledbetter, J.A., Martin, P.S., Spooner, C.E., Wofsy, D., Tsu, T.T., Beatty, P.G. and Gladstone (1985). Antibodies to Tp67 and Tp44 augment and sustain proliferative responses of activated T cells. J. Immunol. **135** 2331

Ledbetter, J.A., Parsons, M., Martin, P.J., Hansen, J.A. Rabinovitch, P.S. and June, C.H. (1986). Antibody binding to CD5 (Tp67) and Tp44

T cell surface molecules: effects on cyclic nucleotides, cytoplasmic free calcium and cAMP mediated suppression.
J. Immunol. **137** pp3299

Lee, C.D., Rose, M.T., Webb, R.N. and Todaro, J.G. (1985). Cloning and sequence analysis of a cDNA for rat transforming growth factor-α.
Nature **313** 489

Lehmann, R., Dietrich, U., Jimenez, F. and Campos-ortega, J.A. (1981). Mutations of early neurogenesis in Drosophila.
Wilhelm Roux's Arch. Dev. Biol. **190** 226

Lehmann, R., Jimenez, F., Dietrich, U. and Campos-Ortega, J.A. (1983). On the phenotype and development of mutants of early neurogenesis in Drosophila-melanogaster.
Wilhelm Roux's Arch. Dev. Biol. **192** 62

Leonard, W.J., Depper, J.M., Uchiyama, T., Smith, K.A., Waldmann, T.A. and Greene, W.C. (1982). A monoclonal antibody that appears to recognize the receptor for human T-cell growth factor; partial characterization of the receptor.
Nature **300** 267

Lodgberg, L. and Shevach, E.M. (1985). Role of the Ly 1 antigen in interleukin 1-induced thymocyte activation.
Eur. J. Immunol. **15** 1007

Lozzio CB & Lozzio BB.(1975) Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome.
Blood **45** 321

Maddon, D.R., Dagleish, A.G., McDougal, J.S., Clapham, P.R., Weiss, R.A. and Axel, R. (1986). The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain.
Cell **47** 333

Maddon, P.J., Molineaux, S.M., Maddon, D.E. et al. (1987). Structure and expression of the human and mouse T4 genes.
Proc. Natl. Acad. Sci. USA **84** 9155

Makgoba, M.W., Sanders, M.E., Luce, G.E.G., Dustin, M.L., Springer, T.A., Clark, E.A., Mannoni, P. and Shaw, S. (1988). ICAM-1 a ligand for LFA-1-dependent adhesion of B, T and myeloid cells.
Nature **331** 86

Malissen, B., Steinmetz, M., McMillan, M., Pierres, M. and Hood, L. (1983). Expression of I-A^k molecules in mouse L cells after DNA-mediated gene transfer.
Nature **305** 440

Malissen, B., Peele-Price, M., Goverman, J.M., Mcmillan, M., White, J., Lappler, J., Marrack, P., Pierres, F., Pierres, M. and Hood, L. (1984). Gene transfer of H2 class II genes: Antigen presentation by mouse fibroblast and hamster B cell lines.
Cell **36** 319

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). Molecular cloning, a laboratory manual.

Manohar,V., Brown,E., Leiserson,W.M. and Chused,T.M. (1982). Expression of Lyt-1 by a subset of B lymphocytes.
J. Immunol. **129** 532

Margolis, B., Rhee, S.G., Felder, S., Mervic, M., Lyall, R., Levitzki, A. Ullrich, A., Ziberstein, A. and Schlessinger, J. (1989) EGF induces tyrosine phosphorylation of phospholipase C-II: A potential mechanism for EGF receptor signaling.
Cell **57** 1101

Marlin, S.D. and Springer, T.A. (1987). Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1).
Cell **51** 813

Marquardt, H. Hunkapiller, M.W., Hood, L.E., Todaro, G.J. (1984). Rat transforming growth factor type 1: structure and relation to epidermal growth factor.
Science **223** 1079

Martin, P.J., Hansen, J.A., Nownski, R.C. and Brown, M.A. (1980). A new human T-cell differentiation antigen: unexpected expression on chronic lymphocytic leukemic cells.
Immunogenetics **11** 429

Martin, P.J., Hansen, J.A., Siadak, A.W. and Nowinski, R.C. (1981). Monoclonal antibodies recognizing normal human T lymphocytes and malignant human B lymphocytes: a comparative study.
J. Immunol. **127** 1920

Matthias, H.K., Popescu, C.N., Amsbaugh, C.S. and King, C.R. (1987). Overexpression of the EGF receptor-related proto-oncogene erbB-2

in human mammary tumour cell lines by different molecular mechanisms.
EMBO J. **6** 605

McDougal, J.S., Dennedy, J.S., Seigh, J.M., Cort, S.P., Mawle, A. (1986). Binding of HTLV-III/LAV to T4 and T cells by a complex of the 110 k viral protein and the T4 molecule.
Sceince **231** 382

McMichael, A.J., Pilch, J.R., Galfre, G., Mason, D.Y., Fabre, J.W. and Milstein, C. (1979). A human thymocyte antigen defined by a hybrid myeloma monoclonal antibody.
Eur. J. Immunol. **9** 205

McMullen, B.A., Fujikawa, K., Kisiel, W., Sasagawa, T., Howald, W.N., Kwa, E.T. and Weinstein, B. (1983). Complete amino acid sequence of the light chain of human blood coagulation factor X.
Biochemistry **22** 2875

McPhee, D., Pye, T. and Shortmen, K. (1979). The differentiation of T lymphocytes. V. Evidence for intrathymic death of most thymocytes.
Thymus **1** 151

Mead, J.E. and Fausto, N. (1989). Transforming growth factor α may be a physiological regulator of liver regeneration by means of an autocrine mechanism.
Proc. Natl. Acad. Sci. USA **86** 1558

Meisenhelder, J., Suh, P.G., Rhee, S.G. and Hunter, T. (1989). Phospholipase C-γ is a substrate for the PDGF and EGF receptor protein-tyrosine kinases in vivo and in vitro.
Cell **57** 1109

Melero, J.A. and Gonzales-Rodriguez, J. (1984). Preparation of monoclonal antibodies against glycoprotein IIIa of human platelet: their effect on platelet aggregation.
Eur. J. Biochem. **141** 421

Meuer, S.C., Schlossman, S.F. and Reinherz, E.L. (1982a). Clonal analysis of human cytotoxic T lymphocytes: $T4^+$ and $T8^+$ effector T cells recognize products of different major histocompatibility complex regions.
Proc. Natl. Acad. Sci. USA **79** 4395

Meuer, S.C., Hussey, R.E., Hodgdon, J.C., Hercend, T., Schlossman, S.F. and Reinherz, E.L. (1982b). Surface structures involved in target recognition by human cytotoxic T lymphocytes.
Science **218** 471

Meuer, S.C., Hussey, R.E., Fabbi, M., Fox, D., Acuto, O., Fitzgerald, K.A., Hodgdon, J.C., Protentis, J.P., Schlossman, S.F., Reinherz, E.L. (1984). An alternative pathway of T-cell activation: A functional role for the 50 kd T11 sheep erythrocyte receptor protein.
Cell **36** 897

Miller,R.A. and Gralow,J. (1984) The induction of Leu-1 antigen expression in human malignant and normal B cells by phorbol myristic acetate (PMA).
J. Immunol. **133** 3408

Mills, B. Sen, L. and Borella, L. (1975). Reactivity of anti human thymocyte serum with acute leukemic blasts.
J. Immunol. **115** 1038

Minowada J, Ohnuma T and Moore GE. (1972) Rosette-forming human lymphoid cell lines. 1. Establishment and evidence for origin of thymus-derived lymphocytes.
J. Natl. Cancer Inst. **49** 891

Minowada, J., Janossy, G., Greaves, M.F., Tsubota, T., Srivastava, B.I., Morkawa, S. and Tatsumi, E. (1978). Expression of an antigen associated with acute lymphoblastic leukaemia in human leukaemia-lymphoma cell lines.
J. Natl. Cancer Inst. **60** 1269

Mizukami, T., Fuerst, T.R., Berger, E.A., Moss, B. (1988). Binding region for human immunodeficiency virus (HIV) and epitopes for HIV-blocking monoclonal antibodies of the CD4 molecule defined by site-directed mutagenesis.
Proc. Natl. Acad. Sci. USA **85** 9273

Muller, F.M., Shapira, M., Arnon, R. (1982). Anti-influenza response archieved by immunization with a synthetic conjugate.
Proc. Natl. Acad. Sci. USA **79** 569

Nagasawa, K., and Mak, T.W. (1980). Phorbol esters induce differentiation in human malignant T lymphoblasts.
Proc. Natl. Acad. Sci. USA 77 2964

Nilsson, T., Jackson, M. and Peterson, P.A. (1989) Short cytoplasmic sequences serve as retention signals for transmembrane proteins in the endoplasmic reticulum.
Cell **58** 707

Norcross, M.A., Bentley,D.M., Margulies, D.H. and Germain, R.N. (1984). Membrane Ia expression and antigen presenting accessory cell function of L cells transfected with class II major histocompatibility complex genes.
J. Exp.Med. **160** 1316

Oettgen, H.C., Kappler, J., Tax, W.J.M. and Terhorst, C. (1984). Characterisation of the two heavy chains of the T3 complex on the surface of human T lymphocytes.
J. Biol. Chem. **259** 12039

Oettgen, H., Pettey, C., Maloy, W., Terhorst, C. (1986). A T3-like protein complex associated with an antigen receptor on murine T cells.
Nature **320** 272

Ohashi, P.S., Mak, T.W., Van den Elsen, P., Yanagi, Y., Yoshikai, Y., Calman, A.F., Terhorst, C., Stobo, J.D. and Weiss, A. (1985). Reconstitution of an active T3/T-cell antigen receptor by DNA transfer.
Nature **316** 606

Ortaldo, J.R., Mason, A.T., Gerard, J.P., Henderson, L.E., farrar, W., Hopkins, R.F., Herberman, R.B. and Rabin, H. (1984). Effects of natural and recombinant IL 2 on regulation of IFNγ production and natural killer activity: lack of involvement of the Tac antigen for these immunoregulatory effects.
J. Immunol. **133** 779

Palacios,R.; Karasuyama, H. and Rolink, A. (1987) Ly1+ Pro-B lymphocyte clones: phenotype, growth requirements and differentiation in vitro and in vivo.
EMBO J. **6** 3687

Panayotou, G., End, P., Aumailley, M., Timpl, R. and Engel, J. (1989) Domains of laminin with growth-factor activity.
Cell **56** 93

Pfeffer, S. and Ullrich, A. (1985) Epidermal growth factor: is the precursor a receptor?
Nature **313** 184

Pike, B.L., Raubitschek, A. and Nossal, G.L. (1984). Human interleukin 2 can promote the growth and differentiation of single hapten-specific B cells in the presence of specific antigen.
Proc. Natl Acad. Sci. USA **81** 7917

Popovic, M., Sarin, P.S., Robert-Gurroff, M., Kalyanaraman, V.S., Mann, D. and Minowada, J. (1983) Isolation and transmission of human retrovirus (human T cell leukemia virus).
Science **219** 856

Poulson, D.F. (1937). Chromosomal deficiencies and the embryonic development of Drosophila melanogaster.
Proc. Natl. Acad. Sci. USA **23** 133

Raff, M.C. (1971). Surface antigenic markers for distinguishing T and B lymphocytes in mice.
Transplant. Rev. **6** 52

Rao, A.G., Howard, O.M.Z., Ng, S.C., Whitehead, A.S., Colten, H.R. and Sodetz, J.M. (1987). Complementary DNA and derived amino acid sequence of the α subunit of human complement protein C8: evidence of the existence of a separate α subunit.
Biochemitry **26** 3556

Rechavi, G., Givol, D. and Cannani, E. (1982). Activation of a cellular oncogene by DNA rearrangement: possible involvement of an IS-like element.
Nature **300** 607

Reif, A.E. and Allen, J.M.V. (1964) The AKR thymic antigen and its distribution in leukemias and nervous tissues.
J. Exp. Med. **120** 413

Reinherz, E.L., Kung, P.C., Goldstein, G. and Schlossman, S.F. (1979a). Separation of functional subsets of human T cells by a monoclonal antibody.
Proc. Natl. Acad. Sci. USA **76** 4061

Reinherz, E.L., Kung, P.C., Goldstein, G. and Schlossman, S.F. (1979b). Further characterization of the human inducer T cell subset defined by a monoclonal antibody.
J. Immunol. **123** 2894

Reinherz, E.L., Kung, P.C., Goldstein, G., Levey, R.H. and Schossman, S.F. (1980a). Discrete stages of human intrathymic differentiation:

analysis of normal thymocyte and leukemic lymphoblasts of T lineage.
Proc. Natl. Acad. Sci. USA **77** 1588

Reinherz, E.L., Kung, P.C., Goldstein, G. and Schlossman, S.F. (1980b). A monoclonal antibody reactive with the human cytotoxic/suppressor.
J. Immunol. **124** 1301

Reinherz, E.L., Meuer, S.C., Fitzgerald, K.A., Hussey, R.E.; Hodgdon, J.C., Acuto, O., and Schlossman,S.F. (1983). T cell subset previously defined by a heteroantiserum termed Th2.
Proc. Natl. Acad. Sci. USA **80** 4104

Robb, R.J., greene, W.C. and Rusk, C.M. (1984). Low and high affinity cellular receptors for interleukin 2. Implications for the level of Tac antigen.
J. Exp. Med. **160** 1126

Robbins, K.C., Antoniades, H.N., Devare, S.G., Hunkapiller, M.W. and Aaronson, S.A. (1983). Structural and immunological similarities between simian sarcoma virus gene product(s) and human platelet-derived growth factor.
Nature **305** 605

Roper, M., Crist, W.M., Metzgar, E., Ragab, A.H., Smith, S., Starling, K., Pullen, J., Leventhal, B., bartolucci, A.A. and Cooper, M.D. (1983). Monoclonal antibody characterization of surface antigens in childhood T-cell lymphoid malignancies.
Blood **61** 830

Rothberg, M.J., Hartley, A.D., Walther, Z. and Artavanis-Tsakonas, S. (1988). Slit: an EGF-homologous locus of D. melanogaster involved in the development of the embryonic central nervous system.
Cell **55** 1047

Rothlein, R., Dustin, M.L., Marlin, S.D. and Springer, T.A. (1986). A human intercellualr adhesion molecule (ICAM-1) distinct from LFA-1.
J. Immunol. **137** 1270

Rudd, C.E., Trevillyan, J.M., Dasgupta, J.D., Wong, L.L., and Schlossman, S.F. (1988). The CD4 receptor is complexed in detergent lysates to a protein tyrosine kinase (pp58) from human T lymphocytes.
Proc. Natl. Acad. Sci. USA **85** 5190

Ruscetti, F.W. and Gallo, R.C. (1981). Human T-lymphocyte growth factors: regulation of growth and function of T-lymphocytes.
Blood **57** 379

Russel, W.D., Scheider, J.W., Yamamoto, T., Luskey, L.K., Brown, S.M. and Goldstein, L.J. (1984). Domain map of the LDL receptor: sequence homology with the epidermal growth factor precursor.
Cell **37** 577

Sabatini, D.D., Kreibich, G. Morimoto, T. and Adesnik, M. (1982). Mechanisms for the incorporation of proteins in membranes and organelles.
J. Cell Biol. **92** 1

Sanger, F., Nicklen, S. and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors.
Proc. Natl. Acad. Sci. USA **74** 5463

Savage, C.R., Jr., Inagami, T., Colen, S. (1972). The primary structure of epidermal growth factor.
J. Biol. Chem. **247** 7612

Savage, C.R., Jr, Hash, J.H., Cohen, S. (1973). Epidermal growth factor. Location of disulfide bonds.
J. Biol. Chem. **248** 7669

Scott, J., Urden, M., Quiroga, M., Sanchez-Pescador, R., Fong, N., Selby, M., Rutter, J.W., Bell, I.G. (1983). Structure of a mouse submaxillary messenger RNA encoding epidermal growth factor and seven related proteins.
Science **236** 236

Seed, B. (1987). An LFA-3 cDNA encodes a phospholipid-linked membrane protein homologous to its receptor CD2.
Nature **329** 840

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

Sela M. (1969). Antigenicity: some molecular aspects.
Science **166** 1365

Semba, K., Kamata, N., Toyoshima, K and Yamamoto, T. (1985). A v-erbB-related protooncogene, c-erbB-2, is distinct from the c-erb-1/epidermal growth factor-receptor gene and is amplified in a human salivary gland adenocarcinoma.
Proc. Natl. Acad. Sci. USA **82** 6497

Seydoux, G. and Greenwald, I. (1989). Cell autonomy of lin-12 function in a cell fate decision in C. elegans.
Cell **57** 1237

Sharp, P.A. (1979). Molecular biology of viral oncogenes.
Cold Spring Harbor Symp. Quant. Biol. **44** 1305

Shaw, S., Luce, G.E.G., Quinones, R., gress, R.E. and Springer, T.A. (1986). Two antigen independent adhesion pathways used by human cytotoxic T-cell clones.
Nature **323** 262

Sherr, C.J., Rettenmier, C.W., Sacca, R., Roussel, M.F., Look, A.T. and Stanley, E.R. (1985). The c-fms proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor CSF-1.
Cell **41** 665

Shiver, J.W., Dankert, J.R., Danavan, J.J. and Esser, A.F. (1986). The ninth component of human complement (C9).
J. Bio. Chem. **261** 9629

Siliciano, R.F., Pratt, J.C., Schmidt, R.E., Ritz, J., Reinherz, E.L. (1985). Activation of cytolytic T lymphocyte and natural killer cell function through the T11 sheep erythrocyte binding protein.
Nature **317** 428

Sim, G.K., Yague, J., Nelson, Marrack, P., Palmer,E., Augustin, A., and Kappler, J. (1984). Primary structure of human T-cell receptor α-chain.
Nature **312**: 771

Smith, K.A. (1980). T-cell growth factor.
Immunol Rev. **51** 337

Smith, K.A. (1984). In "Fundamental Immunology" (W.E. Paul, ed.) pp. 559-576.
Raven, New York

Smith, K.A. (1988). The bimolecular structure of the interleukin 2 receptor.

Immunol. Today **9** 36

Smith, S.D., Morgan, R., Link, M.P., McFall, P. and Hecht, F. (1986). Cytogenetic and immunophenotypic analysis of cell lines established from patients with T cell leukemia/lymphoma.
Blood **67** 650

Southern, E. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis.
J. Mol. Biol. **98** 503

Spindler, K.R., Rosser, D.S.E. and Berk, A.J. (1984) Analysis of adenovirus transforming proteins from early regions 1A and 1B with antisera to inducible fusion antigens produced in *Escherichia coli.*
J. Virology **49** 132

Sporn, M.B. and Roberts, A.B. (1985). Autocrine growth factors and cancer.
Nature **313** 745

Springer, T. A., Dustin, M. L., Kishimoto, T. K., and Marlin, S.D. (1987). The lymphocyte function associated LFA-1, CD2, and LFA-3 molecules: cell adhesion receptors of the immune system.
Ann. Rev. Immunol. **5** 223

Squires, C.L., Lee, F. and Yanofsky, C. (1975). Interaction of the trp repressor and RNA polymerase with the trp operon.
J. Mol. Bio. **92** 93

Stanley, K.K., Kocher, P., Luzio, J.P., Jackson, P. and Tschopp, J. (1985). The sequence and topology of human complement component C9.
EMBO J. **4** 375

Stanton, T., Stevens, T.L., Ledbetter, J.A. and Wofsy, D.J. (1986). Anti-ly-1 antibody induces interleukin-2 release from T cells.
J. Immunol. **136** 1734

Stein, H. and Petersen, N. Gaedicke, G., Lennert, K. and Landbeck, G. (1976). Lymphoblastic lymphoma of convoluted or acid phosphatase type-a tumor of T precusor cells.
Int. J. Cancer **17** 292

Strober, W. and James, S.P. (1988). The interleukins.
Pediatr. Res. **24** 549

Strominger, J.L., Orr, H.T., Parham, P., Ploegh, H.L., Mann, D.L., Bilofsky, H., Saroff, H.A., Wu, T.T. and Kabat, E.A. (1980). An evaluation of the significance of amino-acid sequence homologies in human histocompatibility antigens HLA-A and HLA-B with immnoglobulins and other proteins using relatively short sequences.
Scand. J. Immunol. **11** 573

Strominger, J.L. (1989) Developmental biology of the T cell receptor.
Science **244** 943

Sundstrom C and Nilsson K. (1976) Establishment and charaterisation of a human histiocytic lymphoma cell line (U-937).
Int. J. Cancer **17** 565

Swain, S.L. (1981). Significance of Lyt phenotypes: Lyt2 antibodies block activities of T cells that recognize class I major histocompatibility complex antigens regardless of their function.
Proc. Natl. Acad. Sci. USA **78** 7101

Swain, S.L. (1983). T cell subsets and the recognition of MHC Class.
Immunol. Rev. **74** 129

Swain, S.L., Dennert, G., Wormsley, S. and Dutton, R.W. (1981). The Lyt phenotype of a long-term allospecific T cell line, both helper and killer activities to 1A are mediated by Ly-1 cells.
Eur. J. Immunol. **11** 175

Tabin, C.J., Bradley, S.M., Bargmann, C.I., and Weinberg, R.A. (1982). Mechanism of activation of a human oncogene.
Nature **300** 143

Taylor, J.M., Mitchell, W.M., Cohen, S. (1972). Epidermal growth factor, physics and chemical properties.
J. Biol. Chem. **247** 5928

Teshigawara, K., Wang, H-M. Kato, K. and Smith, K.A. (1987). Interleukin 2 high affinity receptor expression depends on two distinct binding proteins.
J. Exp. Med. **165** 223

Thomas, Y., Glickman, E., Demartino, J., Wang, J., Goldstein, G. and Chess, L. (1984). Biologic functions of the OKT1 T cell surface antigen I. The T1 molecule is involved in helper function.

J. Immunol. **133** 724

Todaro, G.J. et al in Tumor cell Heterogeneity: Origins and Implications, A. Owens, Ed. (Academic Press, New York, 1982), vol. **4** 205

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications.
proc. Natl. Acad. Sci. USA **76** 4350

Truneh, A., Albert, F., Golstein, P., and Schmitt-Verhust, A. (1985). Early steps of lymphocyte activation bypassed by synergy between calcium inoophores and phorbol easter.
Nature **313** 318

Tsudo, M., Kozak, R.W., Goldman, C.K. and Waldmann, T.A. (1986). Demonstration of a Tac peptide that binds interleukin 2. A potential participant in a multichain interleukin 2 receptor complex.
Proc. Natl. Acad. Sci. USA **83** 9604

Uchiyama, T., Hori, T., Tsudo, M., Wano, Y., Umadome, H., Tamori, S., Yodoi, J., Maeda, M., Sawami, H., and Uchino, H. (1985).
J. Clin. Invest. **76** 446

Ullrich, A., Coussens, L., Hayflick, J.S., Dull, T.J., Gray, A., Tam, A.W., Lee, J., Yarden, Y., Libermann, T.A., Schlessinger, J., Downward, J., Mayes, E.L.V., Whittle, N., Waterfield, M.D. and Seeburg, P.H. (1984). Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells.
Nature **309** 418

Vankatesan, S., Gershowitz, A. and Moss, B. (1982). Complete nucleotide sequences of two adjacent early vaccinia virus genes located within the inverted terminal repetition.
J. Virol. **44** 637

Vassin, H., Bremer, A.K., Knust, E. and Campos-ortega, A.J. (1987). The neurogenic gene Delta of Drosophila melanogaster is expressed in neurogenic teritories and encodes a putative transmembrane protein with EGF-like repeats.
EMBO J. **6** 3431

Veilette, A., Bookman, M.A., Horak, E.M. and Bolen, J.B. (1988). The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase $p56^{lck}$.
Cell **55** 301

Van Ommen, G.J.B., Verkerk, J.M.H., Hofker, M.H., Monaco, A.P., Kunkel, L.M., Ray, P., Worton, R., Wieringa, B., Bakker, E. and Pearson, P.L.C. (1986). A physical map of 4 million bp around the Duchenne muscular dystrophy gene on the human X-chromosome.
cell **47** 499

von Boehmer, H. (1988). The developmental biology of T lymphocytes.
Ann. Rev. Immunol. **6** 309

Wahl, M.I., Nishibe, S., Suh, P.G., Rhee, S.G. and Carpenter, G. (1989) Epidermal growth factor stimulates tyrosine phosphorylation of phospholipase C-II independently of receptor internalization and extracellular calcium.
Proc. Natl. Acad. Sci. USA **86** 1568

Waldmann, T.A., Greene, W.C., Sarins, P.S., Saxinger, C., Blayner, D.W., Blattner, W. A., Goldman, C.K., Bongiovanni, K., Sharrow, S., Depper, J.M., Leonard, W.J., Uchiyama, T. and Gallo, R.C. (1984). Functional and phenotypic comparison of human T cell leukemia/lymphoma virus positive adult T cell leukemia with human T cell leukemia/lymphoma virus negative sesary leukemia and their distinction using anti-Tac monoclonal antibody identifying the human receptor for T cell growth factor.
J. Clin. Invest. **73** 1711

Wang, C.Y., Good, R.A., Ammirati, P., Dymbort, G. and Evans, R.L. (1980). Identification of a p69,71 complex expressed on human T cells sharing determinants with B-type chronic lymphatic leukemic cells.
J. Exp. Med. **151** 1539

Waterfield, M.D., Scrace, G.T., Whittle, N., Stroobant, P. and Johnsson, A. (1983). Platelet-derived growth factor is structurally related to the putative transforming protein $p28^{sis}$ of simian sarcoma virus.
Nature **304** 35

Weiss, A., Imboden, J., Shoback, D., and Stobo, J. (1984). Role of T3 surface molecules in human T-cell activation: T3-dependent activation results in an increase in cytoplasmic free calcium.
Proc. Natl. Acad. Sci. USA **81** 4169

Werner-Favre, C., Vischer, T.L., Wohlwend, D. and Zubler, R.H. (1989) Cell surface antigen CD5 is a marker for activated human B cells.
Eur. J. Immunol **19** 1209

Wharton, A.K., Johansen, M.K., Xu, T., Artavanis-Tsakonas, S. (1985). Nucleotide sequence from the neurogenic locus Notch implies a gene product that shares homology with proteins containing EGF-like repeats.
Cell **43** 567

Williams, A.F., Galfre, F.G., Milstein, C. (1977). Analyses of cell surfaces by xenogeneic myeloma hybrid antibodies: Differentiation antigens of rat lymphocytes.
Cell **12** 663

Williams, A.F. (1982). Surface molecules and cell interactions.
J. Theor. Biol. **98** 221

Williams, A.F. (1984). The immunoglobulin superfamily takes shape.
Nature **308** 12

Williams, A.F. (1987). A year in the life of the immunoglobulin superfamily.
Immunol. Today **8** 298

Williams, A.F. and Barclay, A.N. (1988). The immunoglobulin superfamily-domains for cell surface recognition.
Ann. Rev. Immunol. **6** 381

Winter, G. and Coulson, A.R. (MRC Laboratory of Molecular biology). M13 Cloning.

Wong, G. and Clark, S.C. (1988). Multiple actions of interleukin 6 within a cytokine network.
Immunol. Today 137

Wright , T.R.E. (1970). The genetics of embryogenesis in Drosophila.
Adv. Genet. **15** 261

Yamamoto, T., Nichida, T., Miyajima, N., Kawai, S., Ooi, T. and Toyochima, K. (1983). The erbB gene of avian erythroblastosis virus is a member of the src gene family.
Cell **35** 78

Yamamoto, T., Davis, C.G., Brown, S.M., Scheider, J.W., Casey, M.L., Goldstein, L.J. and Russell, W.D. (1984). The human LDL receptor: a cysteine-rich protein with multiple alu sequences in its mRNA.
Cell **39** 27

Yamamoto, T., Ikawa, S., Akiyama, T., Semba, K., Nomura, N., Miyajima, N., Saito, T. and Toyoshima, K. (1986).
Nature **319** 230

Yanagi, Y., yoshikai, Y., Leggett, K., Clark, S.P., Aleksander, I., and Mak, T.W. (1984).
Nature **308** 145

Yanofsky, C., Platt, T., Crawford, I.P., Nichols, B.P., Christie, G.E., Horowitz, H., VanCleemput, M. and Wu, A.M. (1981) The complete nucleotide sequence of the tryptophan operon of Escherichia coli.
Nucleic Acids Research **9** 6647

Yarden, Y., Escobedo, J.A., Kuang, W.J., Yang-Feng, T.L., Daniel, T.O., Tremble, P.M., Chen, E.Y., Ando, M. E., Harkins, R.N., Francke, U., Fried, U.A., Ullrich, A., Williams, L.T. (1986). Structure of the receptor for platetet-derived growth factor helps define a family of closely related growth factor receptors.
Nature **323** 226

Yarden, Y. and Ullrich, A. (1988) Growth factors receptor tyrosine kinases.
Ann. Rev. Biochem. **57** 443

Yodoi, J., Uchiyama. T., K. and Maeda, M. (1983). T cell growth factor receptor in adult T-cell leukemia.
Blood **62** 509

Yodoi, J., and Uchiyama, T. (1986). IL-2 receptor dysfunction and adult T-cell leukemia.
Immunol. Rev. **92** 136

Young, J.D.E., Cohn, Z.A. and Podack, E.R. (1986) The ninth component of complement and the pore-forming protein (perforin 1) from cytotoxic T cells: structural, immunological, and functional similarities.
Science **233** 184

Young, R.A. and Davis, R.W. (1983a). Efficient isolation of genes by using antibody probes.

Proc. Natl. Acad. Sci. USA **80** 1194

Young, R.A. and Davis, R.W. (1983b). Yeast RNA polymerase II genes: isolation with antibody probes.
Science **222** 778