Characterization of the CD44 v6 Transcripts Expressed in Resting and Activated Human Peripheral Blood Mononuclear Cells

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by

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On page 2, the phrase "would healing and …" should read "wound healing and…".

On page 7, the phrase "the CD44 molecule contain distinct…." should read "the CD44 molecule contains distinct….".

On page 23, the phrase "Serum CD44 levels is significantly…." should read "Serum CD44 levels are significantly….".

On page 25, the phrase "however, has no peptide homology…" should read "however, it has no peptide homology…".

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ABSTRACT

CD44 is a type 1 transmembrane glycoprotein capable of generating a large number of isoforms by alternate splicing of the mRNA. A region of 10 variant exons (v1-v10) can be variably combined with one another and incorporated into the nascent RNA. The requirement of CD44 v6 exon containing isoforms for normal functioning of cells during allostimulation has been demonstrated in a rodent model.

This thesis examines the CD44 v6 transcripts in human peripheral blood mononuclear cells (PBMC) with specific aims to (i) define the CD44 v6 exon containing transcripts expressed in resting PBMC, (ii) characterize expression of these transcripts following *in vitro* and *in vivo* cellular activation by respective analysis of mitogenically stimulated PBMC and PBMC isolated from renal transplant patients and (iii) individually clone the CD44 v6 full length cDNA transcripts and ascertain their functional role during T cell activation.

Chapter 3 defines the variant exon composition of the CD44 v6 transcript by RT-PCR, demonstrating time-dependent changes in expression of these transcripts upon culture of PBMC onto plastic. Furthermore, the role of protein kinase C signalling pathway in the regulation of CD44 v6 isoform expression was observed by treatment of PBMC with phorbol ester.

Chapter 4 examines expression of the CD44 v6 transcripts during an *in vivo* alloimmune response by analysis of PBMC isolated from renal transplant patients.

The expression of a novel transcript, $CD44_{v6-7}$, was detected in 30% of this population with implications of maintained expression of this isoform throughout the life of the graft. Characterization of healthy individuals demonstrated expression of the $CD44_{v6-7}$ transcript in 6% of the population substantiating a 5-fold increase in the occurrence of this isoform in PBMC isolated from transplant patients. The conditions for the increased frequency in expression of the $CD44_{v6-7}$ transcript is unknown, however, may possibly be related to patient alloimmunity or the pharmacological actions of immunosuppressive drug therapy.

Chapter 5 describes the employment of a cloning strategy in generating full length CD44 v6 exon containing cDNA clones. Transfection into Jurkat cells demonstrated synthesis of mature peptide products as determined by Western analysis. This chapter also examined the function(s) of the CD44 v6 proteins, such as co-stimulation during CD3 activation, binding to hyaluronic acid and initiation of intracellular tyrosine phosphorylation, using the derived Jurkat transfectants.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any University or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Peter Laslo 31/8/98

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DEDICATION

I wish to dedicate this thesis to:

My parents for providing love, support and encouragement throughout the many years of my study. For believing in my abilities and allowing me to grow and achieve my personal best at all times. For this I am forever grateful and appreciative.

To a very close group of friends that can only be described as soul-brothers. At many times when things seemed no longer worth the effort and the strength to carry on was dwindling, these friends provided moral support and encouragement in the aid for me to continue. They go beyond simple friends and are more than family, a relationship I am only too proud to have with each of them. They are the following:

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ABBREVIATIONS

APC(s)	antigen presenting cell
bp	base pairs
ECM	extracellular matrix
ERM	ezrin-radixin-moesin
FACS	flow cytometric analysis
FCS	fetal calf serum
GAG	glycosaminoglycan(s)
HA	hyaluronic acid
hr	hour(s)
IL	interleukin
INF-γ	interferon-γ
mAb(s)	monoclonal antibody
min	minute(s)
NBT	4-nitro blue tetrazolium chloride
PBMC	peripheral blood mononuclear cell
PHA	phytohemagglutinin
PKC	protein kinase C
PMA	phorbol myristate acetate
TNF-α	tumour necrosis factor-α
UV	ultra-violet
v	variant exon
XPO_4	5-bromo-4-chloro-3-indoyl phosphate

Chapter One

Introduction

The most exciting phrase to hear in science, the one that heralds new discoveries, is not "Eureka! (I found it)" but "That's funny...."

Isaac Asimov

1.1 General Background

CD44 refers to a family of glycoproteins, which has been identified and independently studied by different laboratories. Several names have been consequently reported for the molecule including gp90HERMES (Jalkanen *et al.*, 1986b, Jalkanen *et al.*, 1986c, Jalkanen *et al.*, 1987, Jalkanen *et al.*, 1988), phagocytic glycoprotein Pgp-1 (Hughes *et al.*, 1981, Trowbridge *et al.*, 1985, Isacke *et al.*, 1986), extracellular matrix receptor III (Carter, 1982, Carter and Wayner, 1988), HUTCH-1 (Gallatin *et al.*, 1989, Idzerda *et al.*, 1989) and the hyaluronate receptor (Alho and Underhill, 1989).

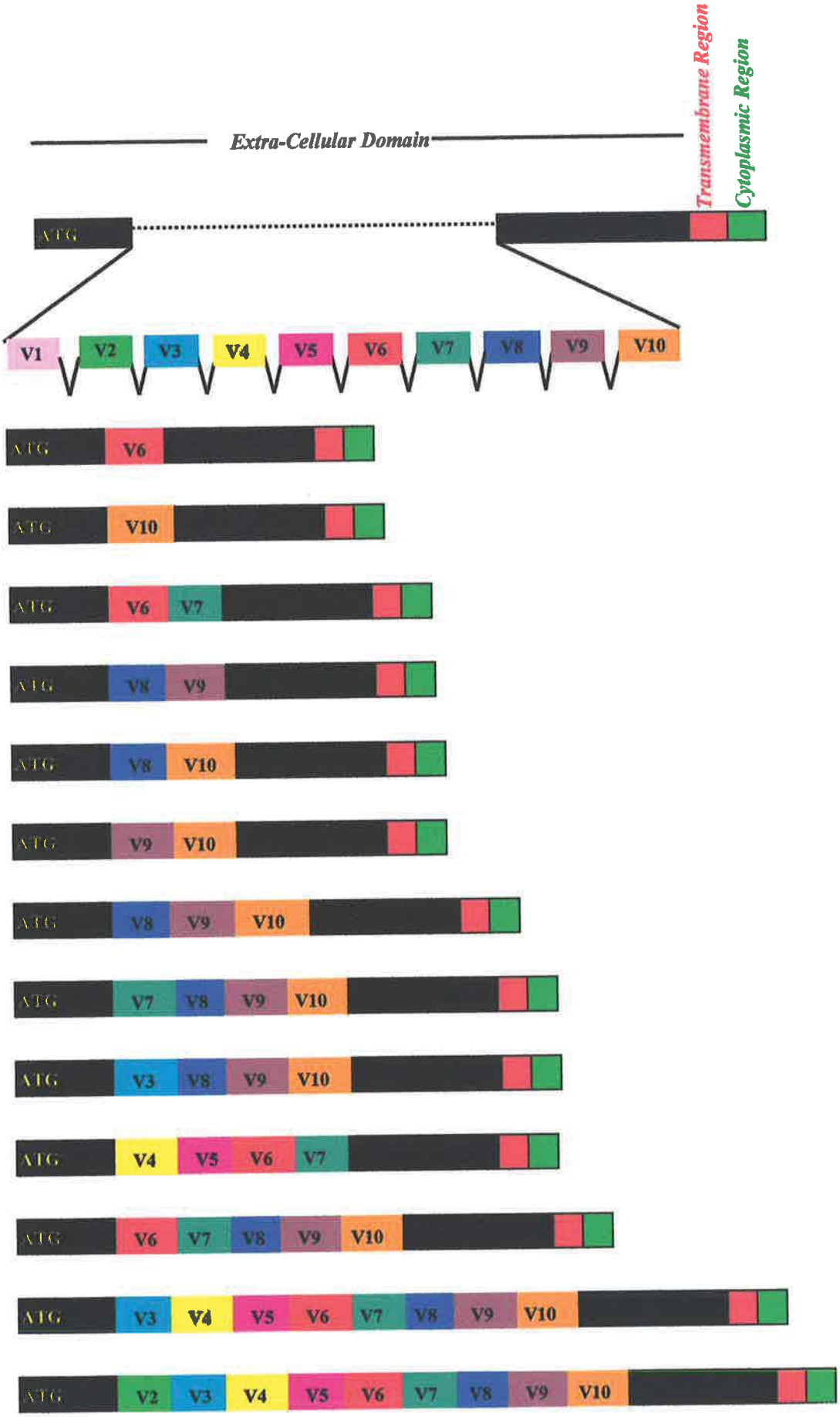
The CD44 protein has been characterized in a number of species including mouse (Nottenburg *et al.*, 1989), rat (Gunthert *et al.*, 1991), hamster (Aruffo *et al.*, 1990), dog (Milde *et al.*, 1994a), cow (Bosworth *et al.*, 1991), sheep (Krishnan *et al.*, 1995), horse (Tavernor *et al.*, 1993) and human (Screaton *et al.*, 1992). As to date no CD44 or CD44-like molecule has been described in organisms other than mammals.

It is widely accepted that CD44 is a cell adhesion molecule that is involved in cell-cell and cell-extracellular matrix adhesion (Lesley *et al.*, 1993a). CD44 belongs to a larger group of hyaluronic acid (HA) binding proteins, the hyaladherins (Toole, 1990), which include other protein members such as the link protein (Goetinck *et al.*, 1987), aggrecan (Doege *et al.*, 1991), versican (Zimmermann and Ruoslahti, 1989) and the arthritis-associated protein tumour necrosis factor-stimulated gene-6 (Lee *et al.*, 1992). Most of the functions described for the CD44 molecule can be attributed, at least in part, to the interaction with hyaluronic acid.

The adhesive interactions of CD44 are likely to play pivotal roles in lymphocyte activation (Huet *et al.*, 1989, Shimizu *et al.*, 1989, Haynes *et al.*, 1989, Denning *et al.*, 1990, Arch *et al.*, 1992, Thomas *et al.*, 1992, Naujokas *et al.*, 1993), cell-cell interaction (Shimizu *et al.*, 1989, St. John *et al.*, 1990, Koopman *et al.*, 1990), cell-extracellular matrix interaction (Aruffo *et al.*, 1990, Underhill, 1992, Thomas *et al.*, 1992, Lesley *et al.*, 1993a), tumour growth and metastasis (Sy *et al.*, 1991, Gunthert *et al.*, 1991, Herrlich *et al.*, 1993, Bartolazzi *et al.*, 1995), would healing and tissue remodelling (Kaya *et al.*, 1997), morphogenesis (Underhill, 1993, Underhill *et al.*, 1993, Fenderson *et al.*, 1993, Knudson and Knudson, 1993, Wheatley *et al.*, 1993, Edwards *et al.*, 1994, Campbell *et al.*, 1995), haematopoiesis (Lewinsohn *et al.*, 1990, Miyake *et al.*, 1990a, Delfino *et al.*, 1994) and lymphocyte recirculation (Jalkanen *et al.*, 1986c, Jalkanen *et al.*, 1987).

The diverse physiological functions of CD44 cannot be easily attributed to just one molecule and as such interest arose when different isoforms of CD44 were identified. Multiple mRNA isoforms of CD44 can be generated from alternate splicing of the nuclear RNA where additional coding information is integrated. A region consisting of 10 exons, termed variant exons (v1-v10), can be variably combined with one another and incorporated within the extracellular region of the CD44 molecule (Gunthert *et al.*, 1991, Screaton *et al.*, 1992, Tolg *et al.*, 1993, Herrlich *et al.*, 1993). In principle, the variant exons could be joined with each other in multiple combinations to result in a vast diversity in CD44 structure by the generation of potentially more than 100 different variant transcripts (Figure 1.1).

Figure 1.1 Schematic representation of the human CD44 cDNA and examples of alternately spliced variant transcripts.


Extra-Cellular Domain
Transmembrane Region
Cytoplasmic Region
ATG
V1
V2
V3
V4
V5
V6
V7
V8
V9
V10
ATG V6
ATG V10
ATG V6 V7
ATG V8 V9
ATG V8 V10
ATG V9 V10
ATG V8 V9 V10
ATG V7 V8 V9 V10
ATG V3 V8 V9 V10
ATG V4 V5 V6 V7
ATG V6 V7 V8 V9 V10
ATG V3 V4 V5 V6 V7 V8 V9 V10
ATG V2 V3 V4 V5 V6 V7 V8 V9 V10

Approximately 30 variant isoforms have been identified thus far and are involved variously in embryogenesis (Section 1.3), neoplastic alterations (Section 1.12.2) and lymphocyte activation (Section 1.13).

The CD44 variant proteins can be identified by monoclonal antibodies directed against specific exon products using flow cytometry or immunohistochemistry. However, this provides only partial information as the identification of the full length sequences cannot be determined as only the expression of a specific variant exon epitope is defined. For example, the v6 exon product can be identified by the anti-CD44 v6 monoclonal antibody (mAb). However, it is not known if the v6 exon is included with sequences from other variant exons or if it is expressed independently. The correlation between variant epitope expression and the identity of variant transcripts can be obtained by reverse transcription-PCR of the specimen thus defining the exon structure of the CD44 v6 containing isoforms.

As discussed throughout this review, CD44 is subject to further molecular diversity by post-translational modifications via glycosaminoglycan substitution and cell specific N- and O-linked glycosylation (Stamenkovic *et al.*, 1991, Jackson *et al.*, 1992, Bennett *et al.*, 1995b, Lesley *et al.*, 1995), shedding of cell surface CD44 (Bazil and Horejsi, 1992) and interaction of the CD44 cytoplasmic domain with cytoskeletal proteins (Kalomiris and Bourguignon, 1988, Bourguignon *et al.*, 1995). The generation of such diversity in the molecular structure of CD44 is likely to be responsible for the functional heterogeneity attributed to the protein. This review will focus on the complex organization of the CD44 molecule and the regulatory

mechanisms involved in controlling functional activity. In addition, the role of CD44 in T cell activation and the immune response will be discussed.

PART I REGULATION OF CD44 FUNCTION

1.2 Characterization of the CD44 gene

All CD44 isoforms are the product of a single gene located on the short arm of human chromosome 11p13. The CD44 gene spans approximately 60 kb of DNA and consists of 19 exons, exon 1- 19 (Goodfellow *et al.*, 1982, Screaton *et al.*, 1992, Lesley *et al.*, 1993a, Screaton *et al.*, 1993, Tolg *et al.*, 1993). Structural analysis of the CD44 promoter region has demonstrated an absence of TATA box and CCAAT consensus sequences, however, three copies of G/ C motifs, which typically bind the Sp-1 transcription factor, are located upstream the transcription initiation site (Sehgal *et al.*, 1988, Shtivelman and Bishop, 1991). The region located between +1 and –1000 bp of the CD44 promoter consists of approximately 65% G/ C residues and contains 3 Sp-1 binding sites and 2 immediate early gene (egr-1) motif regions (Shtivelman and Bishop, 1991, Maltzman *et al.*, 1996). Furthermore, the immediate 150 bp upstream sequence of the CD44 promoter can confer transcriptional activity in melanoma and neuroblastoma cells (Shtivelman and Bishop, 1991).

The exons of the CD44 gene can be classified into two groups, those that encode for the standard or variant nucleotide sequences of CD44. The CD44 isoform comprised of the first 5 exons as well as exons 16-20 is invariably expressed by all cells and the product is referred to as the standard form of CD44 ($CD44_{std}$). The human gene contains multiple poly A sites and generates mRNA species of 1.6, 2.2 and 4.8 kb in molecular size which exclusively express for the $CD44_{std}$ transcript.

Variant CD44 transcripts are generated by alternately splicing of exons 6 to 15 or more often called v1-v10 (Tolg *et al.*, 1993). Not only could the variant exons be joined together in multiple combinations, but also the standard exons 5 and 16 as well as exons 19 and 20 which encode for the cytoplasmic domain can be alternately spliced (Screaton *et al.*, 1992). In addition, two anomalous splice sites in the v3 and v10 exons can further enlarge the variability of the isoforms (Gunthert, 1996).

1.3 Expression of CD44 in various tissues

The tissue expression of CD44 and its variant isoforms is regulated and dependent upon cell specificity, stage of mammalian development and cellular activation.

The distribution of the CD44 protein in adult tissues has been characterized using $CD44_{std}$ and variant exon specific mAbs (Kennel *et al.*, 1993, Lesley *et al.*, 1993a, Wirth *et al.*, 1993, Fox *et al.*, 1994, Mackay *et al.*, 1994, Terpe *et al.*, 1994). Generally, the $CD44_{std}$ protein is expressed on the majority of cells and tissues whereas positive reactions against variant proteins containing the v4, v6 or v9 epitopes were only detected on some epithelial cells (Table 1.1). Furthermore, the analysis of CD44 mRNA expression in mouse has demonstrated expression of distinct alternately spliced transcripts in different tissues and cell types (Hirano *et al.*, 1994). These observations suggest that alternate splicing of mRNA regulates the tissue-specific restriction of CD44 variant protein expression.

Tissue-specific expression of the variant isoforms is also observed during mammalian embryogenesis. In contrast to the adult organism, the variant isoforms

Table 1.1 Distribution of CD44 standard and variant isoforms in human epithelial tissues.

Adapted from Terpe *et al.* (1994).

+++: strong staining of cells

++: strong staining in $^{2}/_{3}$ cells

+: strong staining in $^{1}/_{3}$ cells

(+): weak staining

Tissue	$CD44_{std}$	$CD44_{v9}$	$CD44_{v6}$	$CD44_{v4}$
SKIN:				
Epidermis				
Basal cell layer	+++	+++	+++	+++
Spinous cell layer	+++	+++	+++	+++
Other cell layer	+++	+++	+++	+++
Sweat gland				
Acinus cells	+++	+++	+++	
Ducts	++	++	+	
Sebaccous glands				
Acinus cells	+++[a]	+++[a]	+++[a]	
MAMMARY GLANDS:				
Ducts				
Epithelial cells	+++	+++	+	
Myoepithelial cells	+++	+++	++	
BRAIN:				
Ependymal cells				
Epithelium of choroid plexus				
Cells of arachnoid	+			
SALIVARY GLAND:				
Acinus cells	+++	+++		
Intercalated portion	+++	+++		
Ducts	+++[a]	+++[a]	+[a]	
THYROID GLAND:				
Follicular cells	+++	++		
LUNG:				
Respiratory epithelium	+++[a]	+++[a]	(+)	
Pneumocytes	+++	+++	(++)	
OESOPHAGUS:				
Squamous epithelium	+++[a]	+++[a]	+++[a]	+++[a]
Trachea	+++	+++[a]		
STOMACH:				
Epithelial cells	++[a]	+[a]		
Surface epithelium				
SMALL INTESTINE:				
Epithelial cells	++[a]	+[a]		
Surface epithelium				
LARGE INTESTINE:				
Epithelial cells	++[a]	+[a]		
Surface epithelium				
LIVER:				
Hepatocytes	+++	+++		
Bile ducts				
PANCREAS:				
Acinar cells				
Centoacinar cells	+++	+++		
Intercalated ducts	+++	+++		
Ducts	++	+		
Islets of Langerhans				
KIDNEY:				
Proximal tubules				
Distal tubules	(+)	(+)		
Collecting tubules				
URINARY BLADDER:				
Transitional epithelium	+++	+++	(+)	
UTERUS:				
Glands of endomerium	+++	++		
OVARY:				
Follicular epithelium	+++			
Germinal epithelium				
PROSTATE GLAND:				
Glands	+++	+++	+	
Ducts	+++	+++	(+)	
EPIDIDYMIS:				
Ductuli efferentes	+++[a]	+++[a]		

[a] Staining of the basal layer

are abundantly expressed during implantation, gastrulation and embryogenesis (Wirth *et al.*, 1993, Haegel *et al.*, 1994, Terpe *et al.*, 1994, Ruiz *et al.*, 1995, Weber *et al.*, 1996a, Zoller *et al.*, 1997). In addition, the $CD44_{std}$ and variant isoforms are expressed in a temporal manner in rat, mouse and human inferring a pivotal role for CD44 in cellular interactions that occur during early development, haematopoiesis and pattern formation (Fenderson *et al.*, 1993, Kennel *et al.*, 1993, Wirth *et al.*, 1993, Ruiz *et al.*, 1995, Weber *et al.*, 1996a).

However, only specific variant isoforms are associated with development. Unlike most CD44 variant epitopes which are transiently expressed during rat ontogeny on ectoderm- and endoderm-derived tissues and cells (Ruiz *et al.*, 1995, Weber *et al.*, 1996a), the CD44 v10 isoforms are expressed at late ontogeny (Rosel *et al.*, 1997). Thus it appears unlikely that CD44 v10 exon containing isoforms function in tissue formation or patterning during foetal formation.

The state of cellular activation can also regulate expression of the CD44 isoforms. CD44 is expressed at high levels on B-cell precursors yet down regulated with progression to immature B cells and subsequently upregulated again on mature B cells (Kansas *et al.*, 1990). Activation of both B and T cells and the subsequent differentiation into memory cells is associated with a significant, long term increase in the levels of CD44 expressed on the cell surface (Budd *et al.*, 1987, Camp *et al.*, 1991a, Hathcock *et al.*, 1993). Furthermore, upon activation of T cells with mitogens or cytokines, cell-surface expression of variant isoforms containing the v6 and v9 epitopes are up-regulated as determined by flow cytometry (Koopman *et al.*, 1993b, Mackay *et al.*, 1994, Galluzzo *et al.*, 1995).

This tight regulation in expression of the CD44 isoforms suggests a defined functional role of the variant isoforms within specific cells and tissues.

1.4 Protein structure of the CD44 molecule

1.4.1 Extracellular region of the CD44 molecule

The extracellular domain of the $CD44_{std}$ protein is composed of 268 amino acids encoded by exons 1-5 and 15-16 (Screaton *et al.*, 1992). Overall the sequence homology is strong among species (Figure 1.2) yet based on consensus similarity, two distinct regions can be identified. Approximately 190 amino acids of the NH_2 terminus has strong homology among the human, mouse, hamster, rat, equine and bovine sequences and suggests a conserved region of functionality. As discussed below, this region contains the HA binding region of the CD44 molecule. The membrane-proximal region (carboxy half of exon 5 and exons 15-16) has limited homology across species. Although strong homology exists between the rat, mouse and hamster sequences there is limited similarity between the human, bovine and equine sequences. This suggests an evolutionary divergence from the rodent sequences to that of the larger mammals. As to date no defined functional role has been associated with this membrane proximal region although peptide motifs for post-translational modifications are present.

As discussed below, the extracellular region of the CD44 molecule contains distinct peptide domains that allow protein binding to HA and also post-translational modification of the molecule by N- and O-linked glycosylation and glycosaminoglycan attachments.

Figure 1.2 Amino acid comparison of the CD44 extracellular domain of the human, mouse, hamster, rat, equine and bovine sequences.

Amino acid sequences obtained from:

Human: Screaton *et al.* (1992)

Murine: Nottenburg *et al.* (1989)

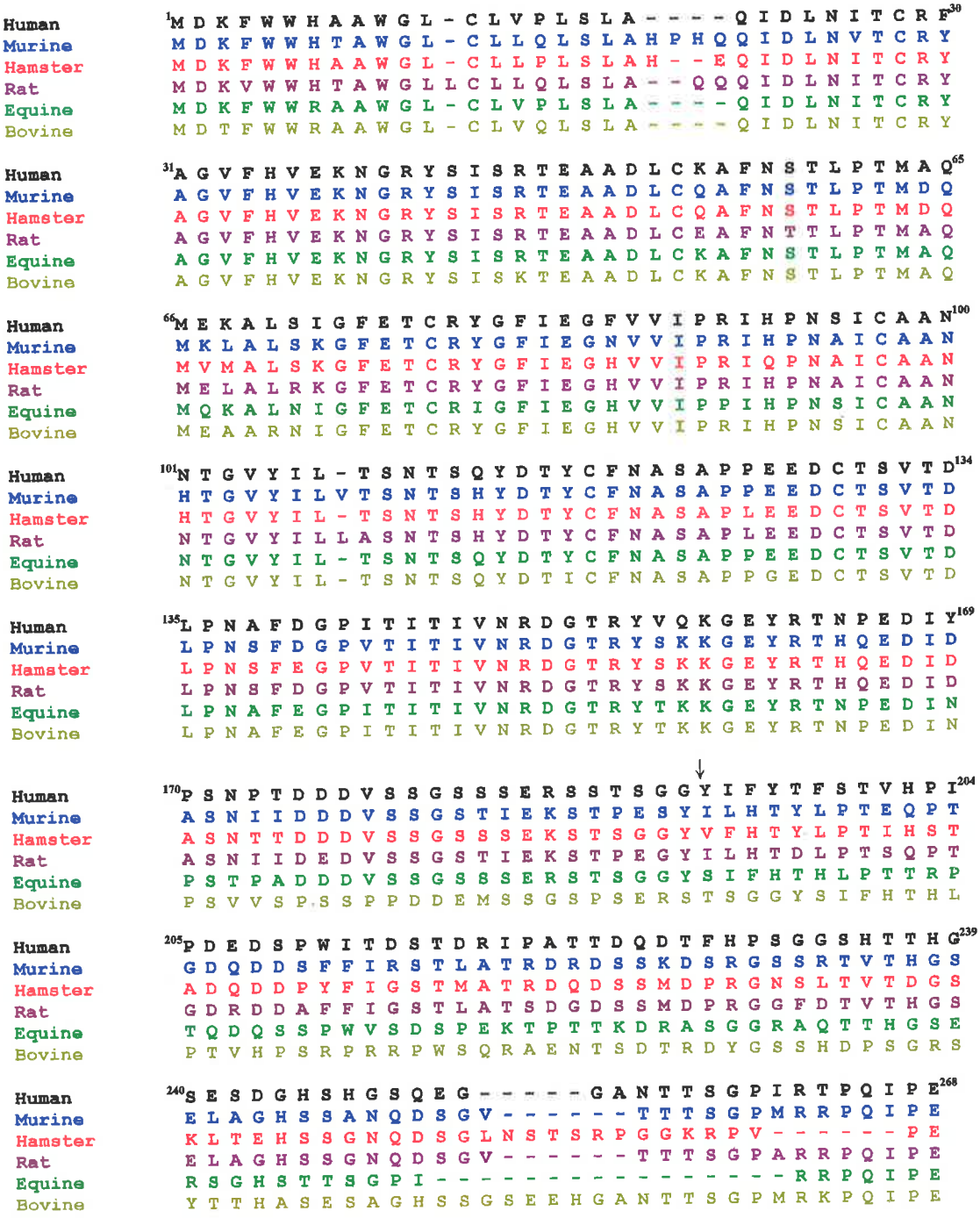
Hamster: Aruffo *et al.* (1990)

Rat: Gunthert *et al.* (1991)

Equine: Tavernor *et al.* (1993)

Bovine: Bosworth *et al.* (1991)

↓ delineation between the two conserved regions of the extracellular domain based on consensus homology of amino acid sequence.


Human 1MDKFWWHAAWGL-CLVPLSLA----QIDLNITCRF30
Murine MDKFWWHTAWGL-CLLQLSLAHPHQQIDLNVTCRY
Hamster MDKFWWHAAWGL-CLLPLSLAH--EQIDLNITCRY
Rat MDKVWWHTAWGLLCLLQLSLA--QQQIDLNITCRY
Equine MDKFWWRAAWGL-CLVPLSLA----QIDLNITCRY
Bovine MDTFWWRAAWGL-CLVQLSLA----QIDLNITCRY
Human 31AGVFHVEKNGRYSISRTEAADLCKAFNSTLPTMAQ65
Murine AGVFHVEKNGRYSISRTEAADLCQAFNSTLPTMDQ
Hamster AGVFHVEKNGRYSISRTEAADLCQAFNSTLPTMDQ
Rat AGVFHVEKNGRYSISRTEAADLCEAFNTTLPTMAQ
Equine AGVFHVEKNGRYSISRTEAADLCKAFNSTLPTMAQ
Bovine AGVFHVEKNGRYSISKTEAADLCKAFNSTLPTMAQ
Human 66MEKALSIGFETCRYGFIEGFVVIPRIHPNSICAAN100
Murine MKLALSKGFETCRYGFIEGNVVIPRIHPNAICAAN
Hamster MVMALSKGFETCRYGFIEGHVVIPRIQPNAICAAN
Rat MELALRKGFETCRYGFIEGHVVIPRIHPNAICAAN
Equine MQKALNIGFETCRIGFIEGHVVIPPIHPNSICAAN
Bovine MEAARNIGFETCRYGFIEGHVVIPRIHPNSICAAN
Human 101NTGVYIL-TSNTSQYDTYCFNASAPPEEDCTSVTD134
Murine HTGVYILVTSNTSHYDTYCFNASAPPEEDCTSVTD
Hamster HTGVYIL-TSNTSHYDTYCFNASAPLEEDCTSVTD
Rat NTGVYILLASNTSHYDTYCFNASAPLEEDCTSVTD
Equine NTGVYIL-TSNTSQYDTYCFNASAPPEEDCTSVTD
Bovine NTGVYIL-TSNTSQYDTICFNASAPPGEDCTSVTD
Human 135LPNAFDGPITITIVNRDGTRYVQKGEYRTNPEDIY169
Murine LPNSFDGPVTITIVNRDGTRYSKKGEYRTHQEDID
Hamster LPNSFEGPVTITIVNRDGTRYSKKGEYRTHQEDID
Rat LPNSFDGPVTITIVNRDGTRYSKKGEYRTHQEDID
Equine LPNAFEGPITITIVNRDGTRYTKKGEYRTNPEDIN
Bovine LPNAFEGPITITIVNRDGTRYTKKGEYRTNPEDIN
Human 170PSNPTDDDVSSGSSSERSSTSGGYIFYTFSTVHPI204
Murine ASNIIDDDVSSGSTIEKSTPESYILHTYLPTEQPT
Hamster ASNTTDDDVSSGSSSEKSTSGGYVFHTYLPTIHST
Rat ASNIIDEDVSSGSTIEKSTPEGYILHTDLPTSQPT
Equine PSTPADDDVSSGSSSERSTSGGYSIFHTHLPTTRP
Bovine PSVVSPSSPPDDEMSSGSPSERSTSGGYSIFHTHL
Human 205PDEDSPWITDSTDRIPATTDQDTFHPSGGSHTTHG239
Murine GDQDDSFFIRSTLATRDRDSSKDSRGSSRTVTHGS
Hamster ADQDDPYFIGSTMATRDQDSSMDPRGNSLTVTDGS
Rat GDRDDAFFIGSTLATSDGDSSMDPRGGFDTVTHGS
Equine TQDQSSPWVSDSPEKTPTTKDRASGGRAQTTHGSE
Bovine PTVHPSRPRRPWSQRAENTSDTRDYGSSHDPSGRS
Human 240SESDGHSHGSQEG------GANTTSGPIRTPQIPE268
Murine ELAGHSSANQDSGV------TTTSGPMRRPQIPE
Hamster KLTEHSSGNQDSGLNSTSRPGGKRPV------PE
Rat ELAGHSSGNQDSGV------TTTSGPARRPQIPE
Equine RSGHSTTSGPI----------RRPQIPE
Bovine YTTHASESAGHSSGSEEHGANTTSGPMRKPQIPE

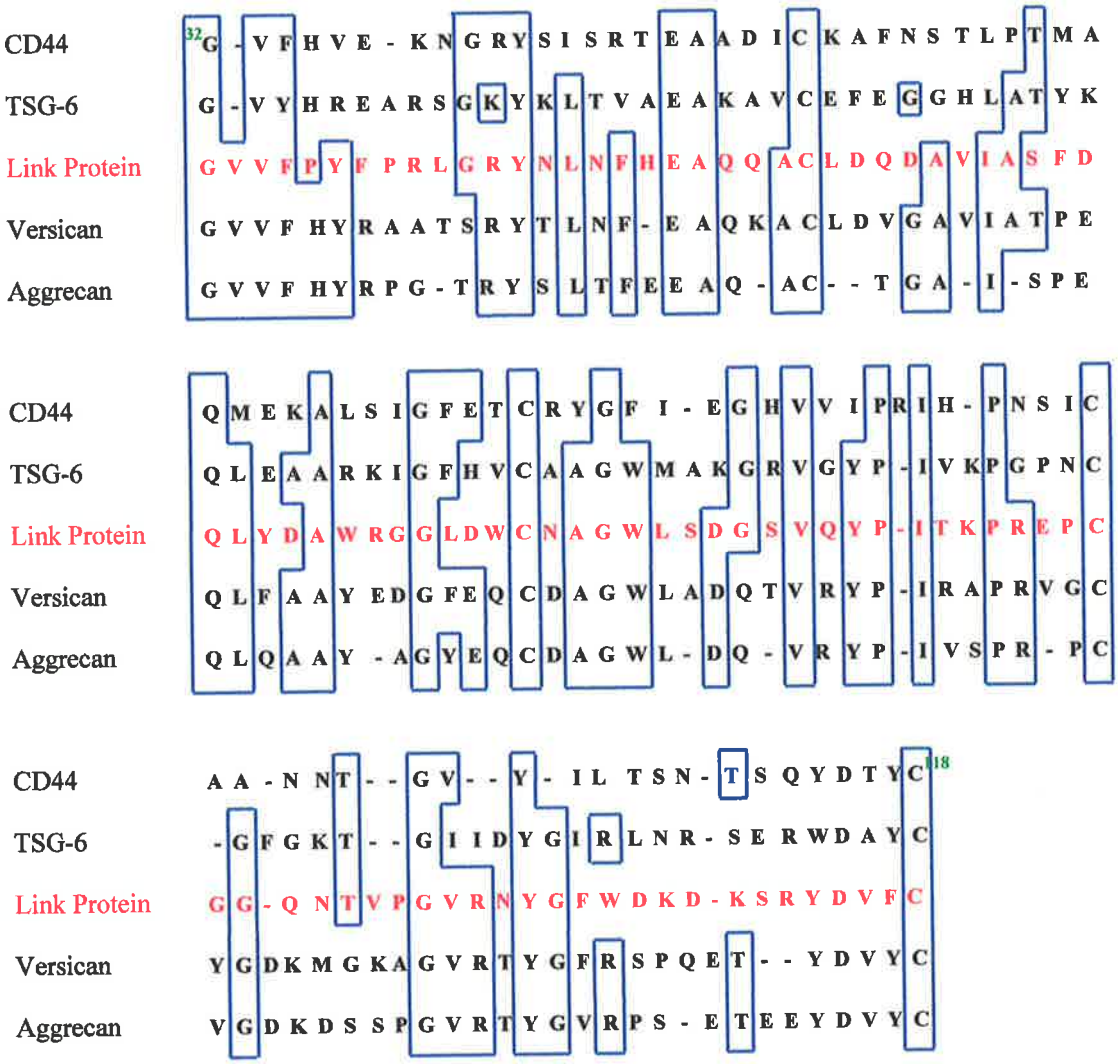
The CD44 molecule is a member of the hyaladherin family of proteins which share the ability to bind the extracellular matrix (ECM) component HA. Characterization of one protein member, the chicken cartilage link protein, has identified a discrete HA binding region of approximately 100 amino acids in length. This region is characterized by clusters of positively charged amino acids and the presence of cysteine residues that are capable of forming disulfide bonds (Goetinck *et al.*, 1987). Many proteins from the hyaladherin family contain a domain homologous to this HA binding domain and sequence similarity to this region is generally used to predict the HA binding property of other proteins (Goetinck *et al.*, 1987, Yang *et al.*, 1994). The NH_2-terminus of the CD44 molecule, encoded by exon 2 and 3, has approximately 30% sequence homology to the HA binding region of the link protein (Figure 1.3).

However, not all HA binding proteins have a homologous region to the primary amino acid sequence of the link protein HA binding domain. For example, the receptor for hyaluronate-mediated motility (RHAMM) binds HA with great affinity and specificity yet bears no sequence homology to the link protein (Hoare *et al.*, 1993). Characterization of the heparin binding sites of the human von Willebrand factor protein identified a consensus domain for heparin binding based on a positively charged motif rather than primary amino acid sequence (Ferran *et al.*, 1992, Sobel *et al.*, 1992).

Subsequently, a HA binding motif was defined within the RHAMM protein on the basis of a charged motif. This peptide region consists of 9 residues and contains two positively charged amino acids separated by 7 residues. The derived motif was

Figure 1.3 Amino acid comparison of the HA binding region of cartilage link protein to homologous regions in the CD44, TSG-6, versican and aggrecan proteins.

Adapted from Lee *et al.* (1992).


CD44
TSG-6
Link Protein
Versican
Aggrecan
32G - V F H V E - K N G R Y S I S R T E A A D I C K A F N S T L P T M A
G - V Y H R E A R S G K Y K L T V A E A K A V C E F E G G H L A T Y K
G V V F P Y F P R L G R Y N L N F H E A Q Q A C L D Q D A V I A S F D
G V V F H Y R A A T S R Y T L N F - E A Q K A C L D V G A V I A T P E
G V V F H Y R P G - T R Y S L T F E E A Q - A C - - T G A - I - S P E
CD44
TSG-6
Link Protein
Versican
Aggrecan
Q M E K A L S I G F E T C R Y G F I - E G H V V I P R I H - P N S I C
Q L E A A R K I G F H V C A A G W M A K G R V G Y P - I V K P G P N C
Q L Y D A W R G G L D W C N A G W L S D G S V Q Y P - I T K P R E P C
Q L F A A Y E D G F E Q C D A G W L A D Q T V R Y P - I R A P R V G C
Q L Q A A Y - A G Y E Q C D A G W L - D Q - V R Y P - I V S P R - P C
CD44
TSG-6
Link Protein
Versican
Aggrecan
A A - N N T - - G V - - Y - I L T S N - T S Q Y D T Y C118
- G F G K T - - G I I D Y G I R L N R - S E R W D A Y C
G G - Q N T V P G V R N Y G F W D K D - K S R Y D V F C
Y G D K M G K A G V R T Y G F R S P Q E T - - Y D V Y C
V G D K D S S P G V R T Y G V R P S - E T E E Y D V Y C

defined as $B(X_7)B$ where B is an arginine or lysine residue and X_7 contains at least one basic but no acidic residues. The importance of the arginine or lysine residues was demonstrated by site-directed mutagenesis which abolished HA binding (Yang *et al.*, 1993, Yang *et al.*, 1994). As to date, all known HA binding proteins contain a $B(X_7)B$ motif including the link protein (Yang *et al.*, 1994).

Two functional $B(X_7)B$ HA binding motifs have been defined within the CD44 molecule located at amino acids 38 to 46 and 144 to 167 (Figure 1.4). One motif lies within the homologous link protein region and contains an arginine at position 41, which is critical for HA recognition (Peach *et al.*, 1993). The second more proximal motif is centred on Arg^{154} (Figure 1.4) and is located within a domain that bears no significant homology with other hyaladherins (Peach *et al.*, 1993). Sequence analysis of this second motif has determined that Arg^{154} may be at either the N-terminus or the centre of a $B(X_7)B$ motif. Consequently, a third putative $B(X_7)B$ motif is proposed (Liao *et al.*, 1995).

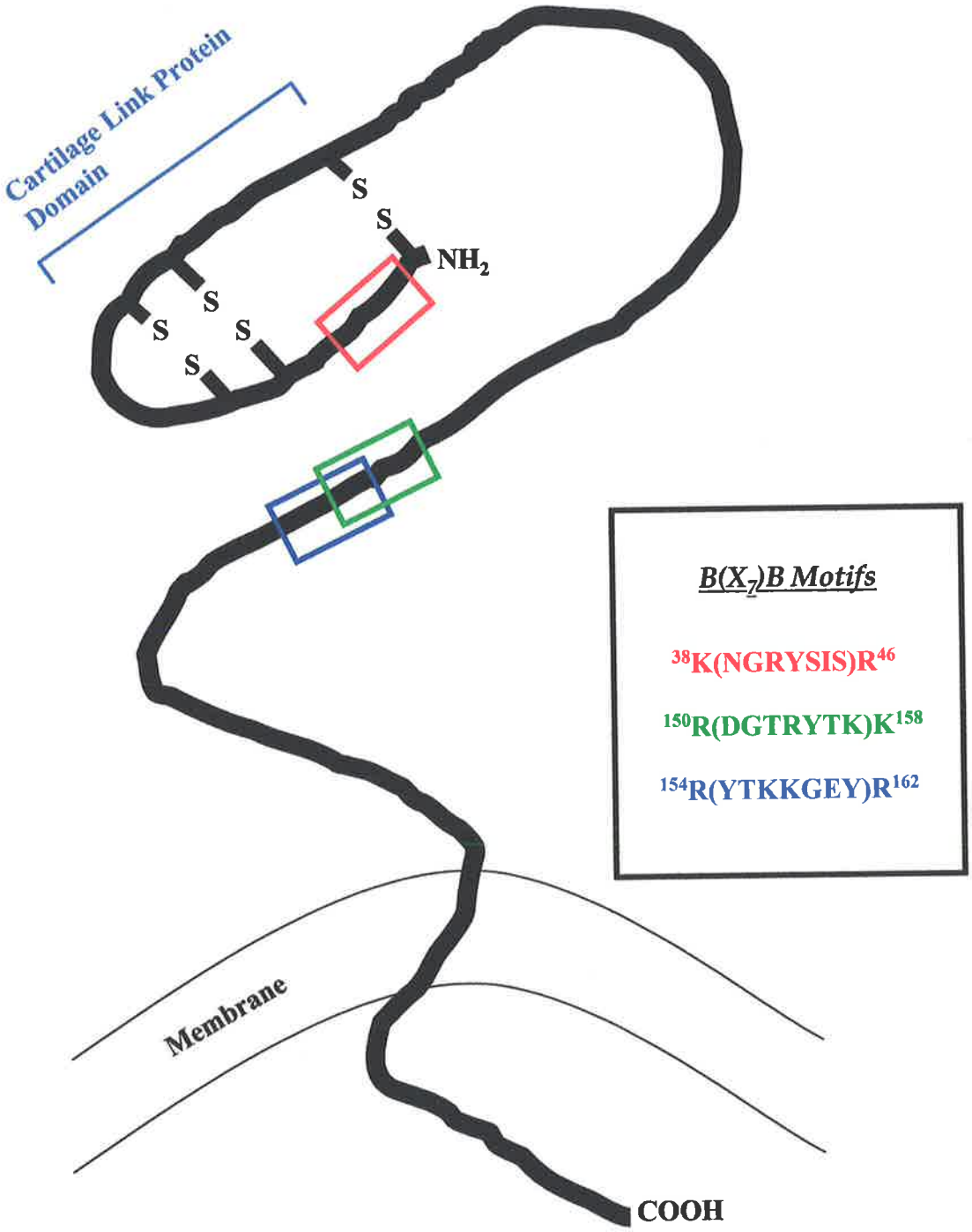
Although HA binding regions have been defined based on consensus motifs, other residues that potentially provide structural integrity towards the HA binding regions have also been identified. Using a model of the predicted tertiary structure of the NH_2-terminus HA-binding region, four residues (Arg^{41}, Tyr^{42}, Arg^{78} and Tyr^{79}) have been identified that form a tertiary cluster and are necessary for CD44-mediated HA binding (Bajorath *et al.*, 1998).

The Indian blood group system comprises of two antigens (In^a/ In^b) encoded by CD44 alleles (Telen *et al.*, 1996). The In^b antigen is of high frequency with low

Figure 1.4 Schematic representation of the B(X_7)B HA binding motifs of the CD44 protein.

Modified from Liao *et al.* (1995).

The three B(X_7)B motifs of the CD44 extracellular domain are represented by open boxes. Amino acid sequence of B(X_7)B motif is represented by single letter code with amino acid numbers corresponding to the sequence described by Screaton *et al.* (1992).


Cartilage Link Protein Domain
S
S
NH2
S
S
S
S
B(X7)B Motifs
38K(NGRYSIS)R46
150R(DGTRYTK)K158
154R(YTKKGEY)R162
Membrane
COOH

incidence of the In^a antigen, which is more common among populations of Middle East and Indian subcontinents. The molecular basis for the In antigens arises from a single amino acid substitution at Arg^{46} to a Pro of the CD44 molecule at both cDNA and genomic DNA sequence. Interestingly, Arg^{46} resides within the $B(X_7)B$ motif located in the homologous HA-binding link protein region. Although this polymorphism disrupts this motif it does not alter the HA binding capability of CD44 (Telen *et al.*, 1996).

The CD44 molecule can be presented on the cell surface as a proteoglycan protein by the attachment of one or more glycosaminoglycans (GAG) such as hyaluronan, chondroitin sulphate, dermatan sulphate, keratin sulphate or heparin/ heparan sulphate (Hardingham and Fosang, 1992). The Ser-Gly motif constitutes the minimal, although sub-optimal, consensus sequence for covalent modification with GAG (Bourdon *et al.*, 1985, Mann *et al.*, 1990). In total, four Ser-Gly motifs are located in the membrane proximal domain of CD44 (2 within exon 5 and one respectively in exons 16 and 17). As previously mentioned, the membrane proximal region of CD44 bears limited sequence homology to other species suggesting unique functional significance to the human CD44 protein. The GAG composition of the CD44 molecule can vary significantly between different cells indicating that the degree of GAG attachment is cell type dependent (Brown *et al.*, 1991, Stamenkovic *et al.*, 1991).

The CD44 extracellular domain can also be modified by N- and O-linked glycosylations. Serine and threonine residues, which serve as potential sites for O-linked glycosylation, constitute 21% of $CD44_{std}$ extracellular region. In addition, 5

potential N-linked glycosylation sites are located within the NH_2-terminus HA binding region (Bartolazzi *et al.*, 1996). As discussed later in Section 1.5, the cell-specific post-translational modifications have been shown to influence CD44 binding to the HA ligand.

1.4.2 Variant exon region of the CD44 molecule

The human CD44 variant exon region is comprised of 10 exons (v1-v10) and has strong sequence homology to variant exons of other species (Figure 1.5). The exons can be alternately spliced in various combinations with one another to generate in excess of 100 different isoforms (Screaton *et al.*, 1992, Tolg *et al.*, 1993, Gunthert, 1993, Herrlich *et al.*, 1993). Peptide products from the variant exons are inserted within the extracellular domain of the CD44 molecule (between residues 220 and 221) and can significantly alter protein structure and property. The molecular size of the $CD44_{std}$ core protein, which does not contain any variant exons, can be increased twofold upon incorporation of the entire variant exon region. It can therefore be conceived that this substantial modification to $CD44_{std}$ can confer a new or altered functional property to the molecule.

Peptide searches of the Genbank, PDB, SwissProt, Spupdate and PIR databases using BLASTP (Altschul *et al.*, 1990) did not reveal any homology of the variant exons in comparison with other mammalian protein sequences. While the precise function of the variant CD44 isoforms remain largely unknown tissue specific expression of the variant isoforms (Section 1.3) allude to functional specificity of these molecules.

Figure 1.5 Amino acid comparison of the CD44 variant exons of the human, rat, murine and canine sequences.

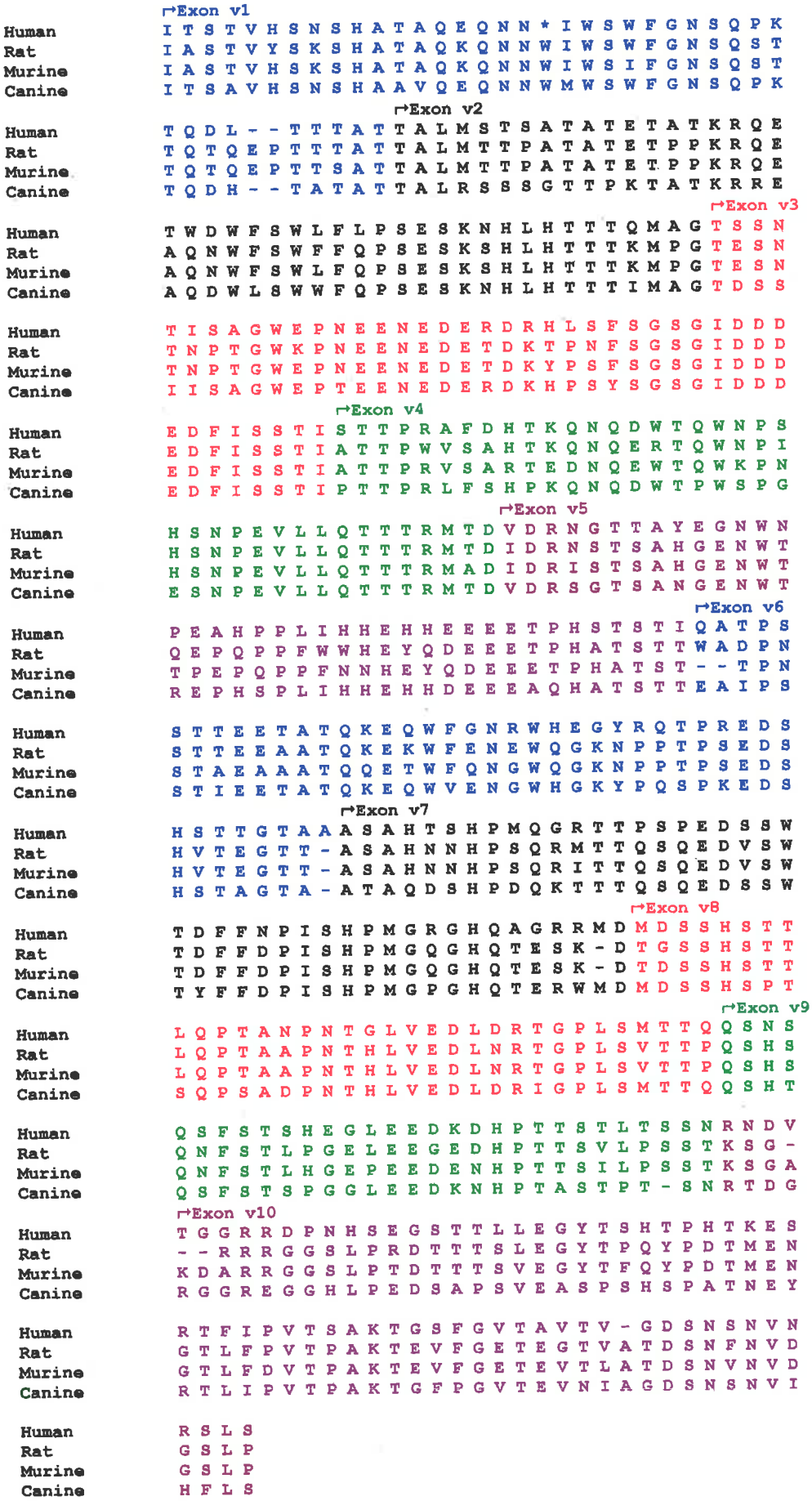
Amino acid sequences obtained from:

Human: Screaton *et al.* (1992)

Rat: Gunthert (1993)

Murine: Gunthert (1993)

Canine: Milde *et al.* (1994a)


↱Exon v1
Human I T S T V H S N S H A T A Q E Q N N * I W S W F G N S Q P K
Rat I A S T V Y S K S H A T A Q K Q N N W I W S W F G N S Q S T
Murine I A S T V H S K S H A T A Q K Q N N W I W S I F G N S Q S T
Canine I T S A V H S N S H A A V Q E Q N N W M W S W F G N S Q P K
↱Exon v2
Human T Q D L - - T T T A T T A L M S T S A T A T E T A T K R Q E
Rat T Q T Q E P T T T A T T A L M T T P A T A T E T P P K R Q E
Murine T Q T Q E P T T S A T T A L M T T P A T A T E T P P K R Q E
Canine T Q D H - - T A T A T T A L R S S S G T T P K T A T K R R E
↱Exon v3
Human T W D W F S W L F L P S E S K N H L H T T T Q M A G T S S N
Rat A Q N W F S W F F Q P S E S K S H L H T T T K M P G T E S N
Murine A Q N W F S W L F Q P S E S K S H L H T T T K M P G T E S N
Canine A Q D W L S W W F Q P S E S K N H L H T T T I M A G T D S S
Human T I S A G W E P N E E N E D E R D R H L S F S G S G I D D D
Rat T N P T G W K P N E E N E D E T D K T P N F S G S G I D D D
Murine T N P T G W E P N E E N E D E T D K Y P S F S G S G I D D D
Canine I I S A G W E P T E E N E D E R D K H P S Y S G S G I D D D
↱Exon v4
Human E D F I S S T I S T T P R A F D H T K Q N Q D W T Q W N P S
Rat E D F I S S T I A T T P W V S A H T K Q N Q E R T Q W N P I
Murine E D F I S S T I A T T P R V S A R T E D N Q E W T Q W K P N
Canine E D F I S S T I P T T P R L F S H P K Q N Q D W T P W S P G
↱Exon v5
Human H S N P E V L L Q T T T R M T D V D R N G T T A Y E G N W N
Rat H S N P E V L L Q T T T R M T D I D R N S T S A H G E N W T
Murine H S N P E V L L Q T T T R M A D I D R I S T S A H G E N W T
Canine E S N P E V L L Q T T T R M T D V D R S G T S A N G E N W T
↱Exon v6
Human P E A H P P L I H H E H H E E E E T P H S T S T I Q A T P S
Rat Q E P Q P P F W W H E Y Q D E E E T P H A T S T T W A D P N
Murine T P E P Q P P F N N H E Y Q D E E E T P H A T S T - - T P N
Canine R E P H S P L I H H E H H D E E E A Q H A T S T T E A I P S
Human S T T E E T A T Q K E Q W F G N R W H E G Y R Q T P R E D S
Rat S T T E E A A T Q K E K W F E N E W Q G K N P P T P S E D S
Murine S T A E A A A T Q Q E T W F Q N G W Q G K N P P T P S E D S
Canine S T I E E T A T Q K E Q W V E N G W H G K Y P Q S P K E D S
↱Exon v7
Human H S T T G T A A A S A H T S H P M Q G R T T P S P E D S S W
Rat H V T E G T T - A S A H N N H P S Q R M T T Q S Q E D V S W
Murine H V T E G T T - A S A H N N H P S Q R I T T Q S Q E D V S W
Canine H S T A G T A - A T A Q D S H P D Q K T T T Q S Q E D S S W
↱Exon v8
Human T D F F N P I S H P M G R G H Q A G R R M D M D S S H S T T
Rat T D F F D P I S H P M G Q G H Q T E S K - D T G S S H S T T
Murine T D F F D P I S H P M G Q G H Q T E S K - D T D S S H S T T
Canine T Y F F D P I S H P M G P G H Q T E R W M D M D S S H S P T
↱Exon v9
Human L Q P T A N P N T G L V E D L D R T G P L S M T T Q Q S N S
Rat L Q P T A A P N T H L V E D L N R T G P L S V T T P Q S H S
Murine L Q P T A A P N T H L V E D L N R T G P L S V T T P Q S H S
Canine S Q P S A D P N T H L V E D L D R I G P L S M T T Q Q S H T
Human Q S F S T S H E G L E E D K D H P T T S T L T S S N R N D V
Rat Q N F S T L P G E L E E G E D H P T T S V L P S S T K S G -
Murine Q N F S T L H G E P E E D E N H P T T S I L P S S T K S G A
Canine Q S F S T S P G G L E E D K N H P T A S T P T - S N R T D G
↱Exon v10
Human T G G R R D P N H S E G S T T L L E G Y T S H T P H T K E S
Rat - - R R R G G S L P R D T T T S L E G Y T P Q Y P D T M E N
Murine K D A R R G G S L P T D T T T S V E G Y T F Q Y P D T M E N
Canine R G G R E G G H L P E D S A P S V E A S P S H S P A T N E Y
Human R T F I P V T S A K T G S F G V T A V T V - G D S N S N V N
Rat G T L F P V T P A K T E V F G E T E G T V A T D S N F N V D
Murine G T L F D V T P A K T E V F G E T E V T L A T D S N V N V D
Canine R T L I P V T P A K T G F P G V T E V N I A G D S N S N V I
Human R S L S
Rat G S L P
Murine G S L P
Canine H F L S

The human v1 exon contains a stop codon at the 17th amino acid which results in the formation of a premature protein. Despite the strong sequence homology between different species this stop codon is not conserved in the rat, mouse or canine genes (Gunthert, 1993, Screaton *et al.*, 1993, Milde *et al.*, 1994b). The functional role of variant isoforms containing the v1 exon is unknown however its inclusion may yield soluble CD44 isoforms as detected in human serum (Bazil and Horejsi, 1992). Furthermore, differential splicing of the v1 exon may regulate variant CD44 production by either giving rise to a non-functional peptide product or decreasing transcript stability (Screaton *et al.*, 1993). Transcripts that contain a premature stop codon can result in a reduced mRNA half life as seen in the H-ras oncogene transcript (Cohen *et al.*, 1989).

Although the genomic organization of the CD44 gene has not been elucidated in all species, preliminary comparison has revealed strong homology of intron sequences between human and mouse genes. A strikingly high homology (65-80% identity) is noted specifically for the v2-v3, v4-v5 and v6-v7 intronic sequences (Screaton *et al.*, 1993). Usually introns are poorly conserved between species and the strong evolutionary preservation of introns within the CD44 variant exon region suggests the role of *cis*-acting sequences for the regulation of RNA splicing.

It is predicted that the variant exon region is capable of interacting with soluble ligands due to the presence of 60% hydrophilic residues in the resulting peptides (Gunthert, 1993). Furthermore, each variant exon (v2-v10) contains approximately 28% serine and threonine residues which serve as potential sites for O-linked

glycosylation and are generally conserved between different species (Screaton *et al.*, 1993). In contrast, the N-linked glycosylation sites of the variant exons are poorly conserved among species. However, interspecies variation both in the location and usage of N-linked glycosylation sites is a common feature among other prominent cell surface glycoproteins such as the CD2, CD4, CD5 and CD8 T cell markers (Gunthert, 1993, Screaton *et al.*, 1993).

Characterization of proteoglycan molecules from various sources has identified a consensus motif, SGXG (S represents serine, G glycine and X any amino acid), as an optimal site for addition of the GAG heparan- and chondroitin-sulphate (Bourdon *et al.*, 1987). The extracellular membrane proximal region of the $CD44_{std}$ protein contains four GAG attachment sites yet are of a sub-optimal Ser-Gly motif (Bourdon *et al.*, 1985, Jackson *et al.*, 1995). The identification and sequencing of the variant exons revealed that the v3 exon contains an optimal SGXG consensus motif in the context of 17 residues. This peptide region is fully conserved between mouse, human and rat (Jackson *et al.*, 1995). A second GAG assembly motif is located within the v10 exon, although in contrast with the v3 site, is of the sub-optimal Ser-Gly motif (Jackson *et al.*, 1992). Recent cloning of variant isoforms carrying either the v3 or v10 exons (Jackson *et al.*, 1995) demonstrated that the v3 containing isoforms ($CD44_{v3,8-10}$ and $CD44_{v3-10}$) were modified with GAG including both chondroitin and heparan sulphates. In contrast, little GAG were attached to the isoforms carrying the v10 exon ($CD44_{v10}$, $CD44_{v8-10}$, $CD44_{v7-10}$ and $CD44_{v6-10}$). In addition, rat CD44 variant isoforms containing sequences encoded by the v6 and v7 exons bind directly and avidly to GAG which included chondroitin-4-sulfate,

dermatin sulfate, chondroitin-6-sulfate, heparin, heparin sulfate and HA. In contrast the rat $CD44_{std}$ isoform only bound HA (Sleeman *et al.*, 1997).

In summary, the variant exons not only alter the physical structure of the $CD44_{std}$ protein by the incorporation of additional peptide sequences but can also provide additional post-translational modifications with the potential to bind novel ligands.

1.4.3 Transmembrane and Cytoplasmic regions of the CD44 molecule

The CD44 transmembrane region consists of 23 hydrophobic amino acids encoded entirely by exon 17 and is highly conserved among species (Figure 1.6). The final three residues constitute the beginning of the cytoplasmic region (Screaton *et al.*, 1992). The transmembrane domain and the following 18 residues of the long cytoplasmic domain (encoded by exon 19) are identical in mouse, baboon, horse, rat and human suggesting functional significance of this region (Isacke, 1994).

The clustering of human $CD44_{std}$ and variant isoforms into multimeric complexes is necessary for protein binding to HA (Lesley *et al.*, 1992, Liao *et al.*, 1993, Sleeman *et al.*, 1996a). PMA-induced binding of recombinant $CD44_{std}$ protein expressed in Jurkat cells to HA has been characterized to involve at least three distinct events: re-organization of the cytoskeletal proteins, clustering of CD44 and covalent dimerization of CD44 molecules (Liu and Sy, 1997). The Cys^{286} residue located within the transmembrane region is critical in the clustering and dimerization of the $CD44_{std}$ protein. Clustering of $CD44_{std}$ alone is insufficient to induce HA binding as PMA treatment could cluster $CD44_{std}$ mutants that lack the Cys^{286} residue

Figure 1.6 Amino acid comparison of the CD44 transmembrane and cytoplasmic regions of the human, murine, hamster, rat, equine and bovine sequences.

Amino acid sequences obtained from:

Human: Screaton *et al.* (1992)

Murine: Nottenburg *et al.* (1989)

Hamster: Aruffo *et al.* (1990)

Rat: Gunthert *et al.* (1991)

Equine: Tavernor *et al.* (1993)

Bovine: Bosworth *et al.* (1991)

TRANSMEMBRANE DOMAIN

Human	^{1}W	L	I	I	L	A	S	L	L	A	L	A	L	I	L	A	V	C	I	A	V	N	S^{23}
Murine	W	L	I	I	L	A	S	L	L	A	L	A	L	I	L	A	V	C	I	A	V	N	S
Hamster	W	L	I	V	L	A	S	L	L	A	L	A	L	I	L	A	V	C	I	A	V	N	S
Rat	W	L	I	I	L	A	S	L	L	A	L	A	L	I	L	A	V	C	I	A	V	N	S
Equine	W	L	I	I	L	A	S	L	L	A	L	A	L	I	L	A	V	C	I	A	V	N	S
Bovine	W	L	I	I	L	A	S	L	L	A	L	A	L	I	L	A	V	C	I	A	V	N	S

CYTOPLASMIC DOMAIN

Human	^{1}R	R	R	C	G	Q	K	K	K	L	V	I	N	S	G	N	G	A	V	E	D	R	K	P	S	G^{26}
Murine	R	R	R	C	G	Q	K	K	K	L	V	I	N	G	G	N	G	T	V	E	D	R	K	P	S	E
Hamster	R	R	R	C	G	Q	K	K	K	L	V	I	N	S	G	N	G	K	V	E	D	R	K	P	S	E
Rat	R	R	R	C	G	Q	K	K	K	L	V	I	N	S	G	N	G	T	V	E	D	R	K	P	S	E
Equine	R	R	R	C	G	Q	K	K	K	L	V	I	N	N	G	N	G	A	V	D	D	R	K	A	S	G
Bovine	R	R	R	C	G	Q	K	K	K	L	V	I	N	N	G	N	G	T	M	E	E	R	K	P	S	G

Human	^{27}L	N	G	E	A	S	K	S	Q	E	M	V	H	L	V	N	K	E	S	S	E	T	P	D	Q	F^{52}
Murine	L	N	G	E	A	S	K	S	Q	E	M	V	H	L	V	N	K	E	P	S	E	T	P	D	Q	C
Hamster	L	N	G	E	A	S	K	S	Q	E	M	V	H	L	V	N	K	E	P	S	E	T	P	D	Q	F
Rat	L	N	G	E	A	S	K	S	Q	E	M	V	H	L	V	N	K	E	P	T	E	T	P	D	Q	F
Equine	L	N	G	E	A	S	R	S	Q	E	M	V	H	L	V	N	K	E	S	S	E	T	Q	D	Q	F
Bovine	L	N	G	E	A	S	K	S	Q	E	M	V	H	L	V	N	K	G	S	S	E	T	P	D	Q	F

Human	^{53}M	T	A	D	E	T	R	N	L	Q	N	V	D	M	K	I	G	V^{70}								
Murine	M	T	A	D	E	T	R	N	L	Q	S	V	D	M	K	I	G	V								
Hamster	M	T	A	D	E	T	R	N	L	Q	N	V	D	M	K	I	G	V								
Rat	M	T	A	D	E	T	R	N	L	Q	S	V	D	M	K	I	G	V								
Equine	M	T	A	D	E	T	R	N	L	Q	N	V	D	M	K	I	G	V								
Bovine	M	T	A	D	E	T	R	N	L	Q	N	V	D	M	K	I	G	V								

yet were unable to bind HA. This observation demonstrates that the covalent dimerization of the CD44 protein at the transmembrane Cys^{286} residue is critical for HA recognition (Liu and Sy, 1996, Liu and Sy, 1997).

The CD44 cytoplasmic region is encoded by exons 18 or 19 which are alternately spliced. Exon 18 contains a premature stop codon resulting in a truncated cytoplasmic domain consisting of only 6 amino acids. In contrast, a long cytoplasmic domain consisting of 70 amino acids is encoded by exon 19. It is uncertain whether mature CD44 proteins containing exon 18 are expressed. However, CD44 transcripts encoding for this truncated $CD44_{std}$ isoform have been detected and approximated to consist of 0.5% of total CD44 expression (Goldstein *et al.*, 1990). Consequently, the majority of CD44 proteins expressed within any cell contain the long cytoplasmic region encoded by exon 19 (Goldstein *et al.*, 1990, Haynes *et al.*, 1991b, Koopman *et al.*, 1993a). Throughout the remainder of this review only the long tail cytoplasmic region will be discussed and as shown in Figure 1.6 this region is highly conserved among species.

A number of regulatory domains within the cytoplasmic region of the CD44 molecule have been defined. Madin-Darby canine kidney (MDCK) epithelial cells represent an *in vitro* model of polarized epithelia having two compositionally and morphologically distinct cell surface domains with disparate functions (Neame and Isacke, 1993). The two cellular domains consist of an upper apical surface which faces outwards into the body (e.g. towards lumen of kidney tubules) and is delineated from the basolateral surface by presence of tight junctions. CD44 is localized exclusively within the basolateral surface, however, upon truncation of the

cytoplasmic domain the tailless molecule is localized to the apical membrane (Neame and Isacke, 1993). This observation infers that the cytoplasmic region is critical in regulating the distribution of CD44 in polarized epithelial cells. Recently, 5 residues of the CD44 cytoplasmic region (His^{330}- Leu- Val- Asn- Lys^{334}) were identified as a localization motif as deletion of this region concentrated the CD44 protein to the apical membrane in MDCK cells. Further mutagenesis studies delineated the di-hydrophobic Leu^{331}-Val^{332} residues as critical for the correct plasma membrane localization of the CD44 protein in MDCK cells (Sheikh and Isacke, 1996).

As discussed in Section 1.7, the cytoplasmic domain can associate with protein kinases, lipids and cytoskeletal components which can regulate HA recognition of the CD44 protein (Kalomiris and Bourguignon, 1989, Bourguignon *et al.*, 1991, Guo *et al.*, 1994a, Tsukita *et al.*, 1994). Although the cytoplasmic domain includes consensus phosphorylation sites for protein kinase C and protein kinase A, as well cAMP- and cGMP-dependent protein kinases, there is no evidence that these sites are active *in vivo* (Wolffe *et al.*, 1990, Camp *et al.*, 1991b).

1.5 Post-Translational Modifications of the CD44 protein

The primary amino acid sequence of the $CD44_{std}$ protein structure estimates a mature peptide with a molecular mass of approximately 35 kDa. However, as a result of extensive post-translational modifications the molecular mass of the $CD44_{std}$ protein as expressed in all cell types exceeds 85 kDa. Variability in the protein structure of the CD44 isoforms can be generated by addition of carbohydrates via N- and O-linked glycosylation sites of the extracellular domain, GAG attachments and

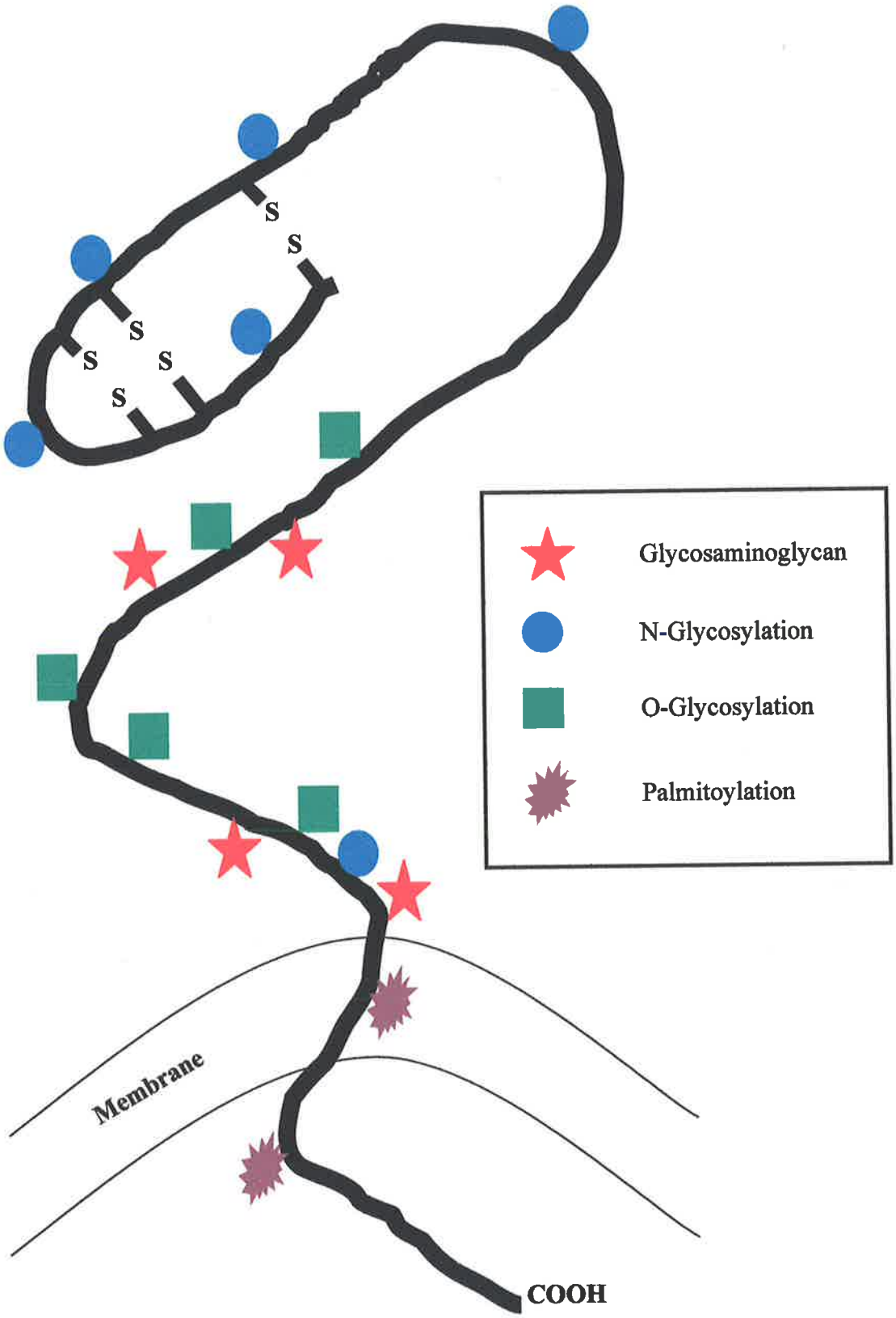
fatty acid modifications (Figure 1.7). These post-translational modifications are dependent on cell type and are capable of altering HA recognition and also the interaction with non-HA ligands (Stamenkovic *et al.*, 1989, Goldstein *et al.*, 1989, Camp *et al.*, 1991b, Hardingham and Fosang, 1992, Lesley *et al.*, 1993a, Guo *et al.*, 1994a, Jackson *et al.*, 1995).

1.5.1 Glycosylation of the CD44 molecule

Several studies have demonstrated that N- and O-linked glycosylation of CD44 can negatively or positively regulate the molecule's intrinsic affinity towards surface bound or soluble HA (Brown *et al.*, 1991, Lokeshwar and Bourguignon, 1991, Hathcock *et al.*, 1993, Katoh *et al.*, 1995, Lesley *et al.*, 1995, Bartolazzi *et al.*, 1996, Dasgupta *et al.*, 1996, Skelton *et al.*, 1998). Six potential N-glycosylation and five O-linked glycosylation sites are located within the extracellular domain (Nottenburg *et al.*, 1989, Zhou *et al.*, 1989, Wolffe *et al.*, 1990).

CD44-specific glycosylation can be regulated by local environment condition such as glucose concentration (Romaris *et al.*, 1995, Zheng *et al.*, 1997b). For example, a chinese hamster ovary (CHO) cell clone acquired the ability to bind HA when grown *in vivo* as solid tumour yet lost this ability when returned to *in vitro* culture conditions. However, when the glucose concentration of the culture media was reduced, HA recognition of the CHO clone was obtained. These observations suggest that HA-recognition of CHO-expressed CD44 is dependent on glucose concentrations (Zheng *et al.*, 1997b). Glucose deprivation in culture conditions has been demonstrated to diminish the post-translational modification of several proteins (Stark and Heath, 1979, Rearick *et al.*, 1981).

Figure 1.7 Schematic representation of post-translational modifications conferred upon the CD44 protein.


S
S
S
S
S
S
Glycosaminoglycan
N-Glycosylation
O-Glycosylation
Palmitoylation
Membrane
COOH

The precise mechanism by which glycosylation of CD44 can regulate HA binding is unknown. However, differential glycosylation may effect local CD44 receptor density, mobility of CD44 within the membrane or interactions of CD44 with other cell surface molecules (Katoh *et al.*, 1995, Lesley *et al.*, 1995).

Although the carbohydrate addition to CD44 can influence HA interaction, other carbohydrate-dependent functions have been ascertained. Oligomerization of specific CD44 variant isoforms is necessary for HA recognition by pancreatic carcinoma cells (Sleeman *et al.*, 1996a). This oligomerization was blocked by tunicamycin, an inhibitor of N-linked glycosylation, suggesting that glycosylation was required for formation of covalently dimerized CD44.

1.5.2 Glycosaminoglycan attachment to the CD44 molecule

The GAG moieties present on the CD44 molecule are capable of binding to growth factors and components of the ECM. For example, CD44 isolated from monocytes can bind to macrophage inflammatory protein-1β, via a GAG motif, and present this chemokine to T cells (Tanaka *et al.*, 1993). This demonstrates that the CD44 protein is capable of recruiting growth factors at the cell surface and subsequently presents them to specific receptors. Furthermore, a chondroitin-sulfated-modified CD44 protein from human lymphocytes is capable of binding both fibronectin and laminin components of the ECM (Jalkanen and Jalkanen, 1992). The addition of chondroitin sulphate chains may function in CD44-mediated cell motility and invasive properties (Faassen *et al.*, 1992, Faassen *et al.*, 1993, Knutson *et al.*, 1996) suggesting a potential role in neoplastic transformation or wound repair (Henke *et al.*, 1996).

As discussed in Section 1.4.2, the v3 and v10 exons contain motif sequences for GAG attachment. Characterization of the $CD44_{v8-10}$ isoform on epithelial cells demonstrated differences in the core protein structure and GAG composition in comparison to $CD44_{std}$ on haemopoietic cells. The majority of $CD44_{v8-10}$ was present as a high molecular weight proteoglycan form containing heparan sulfate and chondroitin sulfate chains presumably attached to the GAG motif located within the v10 exon (Brown *et al.*, 1991).

The precise function of the GAG modification of the v10 exon is unknown, however, a functional role for the heparan sulfate modifications of v3 containing isoforms was recently defined by the binding and presentation of heparin binding growth factors such as basic fibroblast growth factor and epidermal growth factor (Bennett *et al.*, 1995a, Jackson *et al.*, 1995).

From these observations the CD44 interaction with GAG may have three apparent functional consequences (Taipale and Keski Oja, 1997). First, they can promote the binding of CD44 to other cell surface or ECM components. Secondly, the interaction may initiate signal transduction via the CD44 molecule. Thirdly, the CD44 protein can bind chemokines and growth factors and present them to their receptors by facilitating high affinity interactions.

As discussed below, the incorporation of variant exons provides not only additional GAG modifications but can also enhance GAG addition of the entire molecule (Piepkorn *et al.*, 1997). The characterization of the $CD44_{v3-10}$ and $CD44_{v3,8-10}$ isoforms demonstrated that splicing of the v4-7 exon region was

associated with an increase glycanation by chondroitin-sulphate chains. However, the v4-7 peptide region does not contain any consensus motifs for GAG assembly. This suggests that the splicing of the v4-7 exons may allow greater utilization of distant Ser-Gly assembly sites by sulphotransferases possibly by providing an optimal protein structural configuration. Furthermore, destruction of the Ser-Gly-Ser-Gly GAG assembly motif located within the v3 exon correlated with an overall decreased chondroitin sulphate glycanation suggesting that alterations at the v3 locus has distal effects at other GAG assembly sites within the CD44 core protein. Taken together, these observations indicate that the variant exon splicing including those that do not contain potential GAG assembly motifs can exert distal effects on the overall carbohydrate substitution at other loci within the CD44 proteoglycan. Related conclusions have also been drawn for the other molecules such as the syndecan-1 protein (Zhang *et al.*, 1995).

1.5.3 Lipid modification of the CD44 molecule

Besides the addition of carbohydrates, CD44 can be covalently modified with long chain fatty acids (e.g. palmitic acid), a process known as protein palmitoylation (Bourguignon *et al.*, 1991, Guo *et al.*, 1994a).

Palmitoylation of proteins is an enzymatic process that covalently modifies proteins on internal cysteine residues (Towler *et al.*, 1988, Grand, 1989, Schmidt, 1989). Palmitoylated cysteines has been demonstrated in a number of membrane proteins including human HLA (Koch and Hammerling, 1986) and transferrin receptor (Jing and Trowbridge, 1987). Although the site for palmitic acid modification is not defined for the CD44 protein, analysis of the human cDNA

sequence indicates that either the transmembrane Cys^{286} or cytoplasmic Cys^{295} are possible targets.

Palmitic acid is incorporated into the $CD44_{std}$ protein *in vivo* with the quantity of incorporation increasing during CD44 cap formation (Bourguignon *et al.*, 1991). The precise function of CD44 palmitoylation is unknown, however, palmitoylation of murine CD44 is critical for its interaction with the ankyrin cytoskeletal protein (Bourguignon *et al.*, 1991). Furthermore, palmitoylation of human CD44 can interfere with anti-CD3 signalling pathway suggesting lipid modification of CD44 may play an active role in the interaction and signal transduction between these two receptors in normal T lymphocytes (Guo *et al.*, 1994a).

In summary, the post-translational modification of CD44 can (i) positively or negatively influence HA binding as dependent on cell specificity, (ii) bind growth factors thereby increasing functional diversity of CD44 and (iii) provide interaction with intracellular proteins through the cytoplasmic region of CD44.

1.6 Soluble CD44

Soluble CD44 protein has been detected in sera of a number of species including porcine, ovine, goat, equine, canine and human (Yang and Binns, 1993b). The shedding of CD44 from the cell surface is suggested to function as an important regulatory mechanism to control protein expression and function (Bazil and Horejsi, 1992). Protease cleavage of CD44 from the cell surface is suggested as a mechanism for shedding as mAbs directed towards the cytoplasmic region of CD44 fail to recognize soluble CD44. Furthermore, inhibitors of protease activity can reduce the

quantity of shed CD44 from the cell surface suggesting an endogenous proteolytic enzyme (Campanero *et al.*, 1991, Bazil and Horejsi, 1992). As yet, the natural proteases responsible for shedding of CD44 have not been identified.

Release of soluble CD44 from lymphocytes can be induced by anti-CD44 mAbs both *in vitro* and *in vivo* suggesting that ligation of CD44 might induce a signal for cleavage of the molecule (Bazil and Horejsi, 1992, Camp *et al.*, 1993). However, mAb-induced shedding appears to be cell-specific as CD44 on human fibroblast are unaffected by mAb treatment (Jacobson *et al.*, 1984).

The molecular weight of soluble CD44 can vary as a result of post-translational modifications of the extracellular region (Yang and Binns, 1993b, Guo *et al.*, 1994b, Katoh *et al.*, 1994, Ristamaki *et al.*, 1994). Soluble CD44 containing variant epitopes have also been detected in human serum by ELISA analysis with levels of soluble $CD44_{std}$, $CD44_{v5}$ and $CD44_{v6}$ in the range of 80 ng/ml, 35 ng/ml and 170 ng/ml respectively in healthy individuals (Ristamaki *et al.*, 1994, Kan *et al.*, 1995, Kittl *et al.*, 1997, Lein *et al.*, 1997).

The CD44 variant isoforms, $CD44_{v6\text{-}10}$, $CD44_{v7\text{-}10}$ and $CD44_{v8\text{-}10}$, demonstrated an enhanced sensitivity to spontaneous shedding of cell surface protein in comparison to $CD44_{std}$ in transfected Namalwa cells (Bartolazzi *et al.*, 1994, Bartolazzi *et al.*, 1995). However, variant isoforms containing the v3 exon, $CD44_{v3\text{-}10}$ and $CD44_{v3,8\text{-}10}$, displayed limited shedding suggesting that GAG associated with the v3 exon inhibit CD44 shedding by either conformational changes to, or masking, of the proteolytic cleavage site.

The concentration of soluble CD44 detected in the serum can vary dependant on a number of pathological and physiological factors. In pathological disorders, such as rheumatoid arthritis (Haynes *et al.*, 1991a), liver disease (Falleti *et al.*, 1997), lymphoma and B cell chronic leukaemia (De Rossi *et al.*, 1993, Ristamaki *et al.*, 1994) the concentration of soluble CD44 in the serum is greatly increased. Furthermore, in patients with large gastric or colon tumours, serum CD44 measured at time of diagnosis positively correlated with tumour burden and decreased after surgical resection of the tumour (Guo *et al.*, 1994b). The quantity of soluble CD44 containing v5 or v6 epitopes are also elevated in patients with breast cancer and healthy individuals that smoke cigarettes (Kittl *et al.*, 1997, Martin *et al.*, 1997).

The quantity of soluble CD44 can fluctuate in mice and is dependent on the rate of tumour growth and activity of the immune system (Katoh *et al.*, 1994). Serum CD44 levels is significantly reduced in immunodeficient mice and elevated in tumour bearing mice. In contrast, activation of the immune system by either induction of graft versus host reaction or in strains of mice with autoimmune diseases resulted in increased concentrations of soluble CD44.

The functional significance of soluble CD44 is as to date unknown. However, soluble $CD44_{std}$ can retain biological activity despite lacking the cytoplasmic domain. Soluble $CD44_{std}$ can adhere to ligands such as HA and fibronectin. $CD44_{std}$ expressing lymphocytes are inhibited to bind both HA and high endothelial venules in the presence of soluble CD44 (Ristamaki *et al.*, 1997). From these observations it is suggested that soluble CD44 may function as an anti-adhesive molecule by blocking binding sites on endothelial cell surfaces and ECM and thus prevent cell

attachment to these sites. Shedding of CD44 may also regulate the spread of tumours by enhancing the detachment of potential metastatic cells from the primary tumour mass (Bartolazzi *et al.*, 1995). Increased concentrations of soluble CD44 could also bind HA and block the subsequent binding to membrane-CD44 thus inhibiting any cell effector functions. Finally, soluble CD44 may itself be associated with chemokines and growth factors and function to deliver these signalling reagents to other cells (Tanaka *et al.*, 1993, Bennett *et al.*, 1995a).

1.7 Association of CD44 with intracellular proteins

Interactions between cytoskeletal proteins and the cytoplasmic domain of transmembrane proteins play an important role in several cellular activities including ligand-induced receptor patching and capping (Levine and Willard, 1983, Nelson *et al.*, 1983, Bourguignon and Bourguignon, 1984, Isenberg *et al.*, 1987), attachment of the cell surface to adhesion substrate (Geiger, 1983, Tamkun *et al.*, 1986), cell activation and proliferation (Geiger, 1983), cell motility and cell-cell interaction (Geiger, 1983). The interaction of CD44 with the cytoskeleton was initially suggested by the close correspondence between the distribution of CD44 on the cell surface and actin filaments beneath the plasma membrane (Jacobson *et al.*, 1984, Tarone *et al.*, 1984). Subsequent cell motility experiments have shown that CD44 is connected to the cytoskeleton (Isacke, 1994). This connection is dynamic and differs in various regions of a moving cell as CD44-cytoskeletal association is stronger at the leading edge of the cell membrane.

The interaction between the cytoplasmic domain of CD44 and intracellular proteins has been demonstrated to be critical in CD44-mediated effector function.

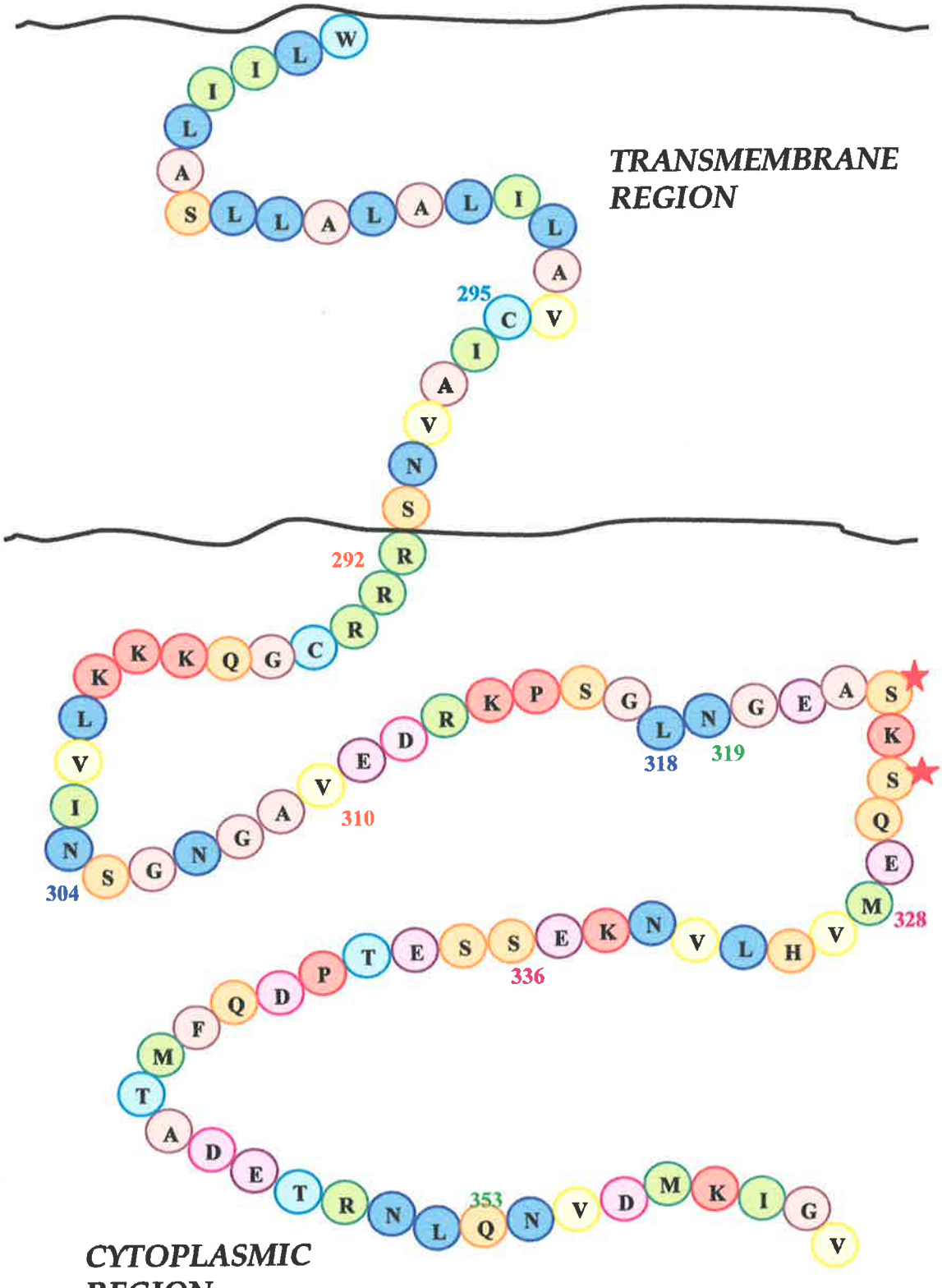
The disruption of cytoskeletal protein or inhibition of protein kinases can result in the complete abolishment of numerous CD44-mediated functions (Koopman *et al.*, 1990, Galandrini *et al.*, 1993, Funaro *et al.*, 1994, Galluzzo *et al.*, 1995, Galandrini *et al.*, 1996, Liu *et al.*, 1996). As to date the cytoplasmic region of the CD44 molecule has been associated with a number of intracellular proteins including ankyrin, ERM protein family, NF2 and protein kinases. As shown in Figure 1.8 the binding regions for some of these intracellular proteins within the CD44 cytoplasmic domain have been defined.

1.7.1 CD44 and ankyrin interaction

Ankyrin is a cytoskeletal protein that connects the submembranous part of the cell scaffolding with the actin filament meshwork (Bourguignon *et al.*, 1986, Kalomiris and Bourguignon, 1988). Several groups have demonstrated the association of CD44 with ankyrin (Lacy and Underhill, 1987, Carter and Wayner, 1988, Kalomiris and Bourguignon, 1988).

The ankyrin binding domain of the cytoplasmic region of CD44 has been mapped to residues Asn^{304} to Gln^{353} (Lokeshwar *et al.*, 1994). At least two subregions within this domain contribute to ankyrin binding: region I contains 15 amino acids between Asn^{304} and Leu^{318} with region II containing 35 amino acids between Asn^{319} and Gln^{353}. Region II is required for high-affinity ankyrin binding since its deletion results in a 2-fold decrease in the disassociation constant for ankyrin binding. This region is strongly conserved among species, however, has no peptide homology with other ankyrin binding proteins indicating a role as a CD44-specfic regulatory region for ankyrin binding in conjunction with region I.

Figure 1.8 Schematic representation of the CD44 transmembrane and cytoplasmic regions with demarcation of the binding domains required for interaction of CD44 with other intracellular proteins.


TRANSMEMBRANE REGION
295
292
304
310
318
319
328
336
353
CYTOPLASMIC REGION
292-310 ERM binding domain
328-336 GTP binding region
304-318 Ankyrin I region
319-353 Ankyrin II region
Phosphorylation

Deletion of region I and II of the ankyrin binding domain of the murine CD44 molecule abolishes HA binding suggesting that the intracellular and extracellular functions of CD44 are inter-connected (Lokeshwar *et al.*, 1994). Interaction between CD44 and ankyrin can be influenced by several post-translational modifications. Both phosphorylation and the attachment of lipid side-chains to purified CD44 enhances *in vitro* binding to ankyrin-derivatized beads (Kalomiris and Bourguignon, 1988, Bourguignon *et al.*, 1991).

Guanine nucleotide-binding proteins are capable of amplifying transmembrane signals, direct the synthesis and translocation of proteins, guide vesicular transport through cytoplasm, regulate cytoskeletal organization and control cell proliferation (Bourne *et al.*, 1991). It has been demonstrated that murine CD44 is a GTP-binding protein by displaying GTP binding and GTPase activity (Lokeshwar and Bourguignon, 1992). The binding of GTP to CD44 can enhance its interaction with ankyrin. It is suggested that ligand binding to CD44 can induce GTP binding which in turn enhances CD44-ankyrin interaction during lymphocyte activation (Lokeshwar and Bourguignon, 1992). However, it is not clear whether CD44 exists *in vivo* in a GTP-bound state.

1.7.2 CD44 and association with the ERM protein family

The ezrin-radixin-moesin (ERM) protein family are suggested to function as general cross-linkers between plasma membrane and actin filaments with the carboxy terminal half of the ERM proteins capable of binding directly with actin

filament (Bretscher, 1983, Pakkanen *et al.*, 1987, Lankes *et al.*, 1988, Tsukita *et al.*, 1989, Algrain *et al.*, 1993, Arpin *et al.*, 1994, Turunen *et al.*, 1994, Pestonjamasp *et al.*, 1995).

Murine $CD44_{std}$ and a variant isoform that contains at least the v9 and v10 exons can physically associate with ERM proteins thus creating a molecular peptide bridge between CD44 and actin filaments. This implies that variant isoforms, despite disruption of the extracellular conformation, can still associate with the ERM through its cytoplasmic region. In addition, the variant isoform had a greater affinity for the ERM proteins in comparison to the $CD44_{std}$ protein in baby hamster kidney cells (Tsukita *et al.*, 1994). This observation suggests that conformational structures induced by the variant exons can enhance the cytoplasmic interaction of CD44 with the ERM proteins.

Although CD44 is precisely localized with ERM proteins in cultured fibroblasts, the expression of CD44 varies among tissues and its distribution is not necessarily identical to those of ERM proteins in all cell types (von Andrian *et al.*, 1995, Berryman *et al.*, 1995, Nakamura and Ozawa, 1996). Recently, the ERM binding site of CD44 has been mapped to the Arg^{292}-Val^{310}amino acid region of the juxta-membrane cytoplasmic domain (Yonemura *et al.*, 1998). Within this ERM binding site, a number of positively charged amino acid clusters are present and a lysine cluster is critical for the association of CD44 with moesin (Isacke, 1994). This 19 amino acid region is also highly conserved among species, however, the precise functional role of the CD44-ERM complex is undefined.

1.7.3 CD44 and the Neurofibromatosis-2 protein

The neurofibromatosis-2 (NF2) gene has tumour suppressor properties as inactivation of this gene leads to a dominantly inherited disease manifesting as schwannomas, meningiomas and ependymomas (Thomas *et al.*, 1994, Louis *et al.*, 1995). The NF2 gene product has 49% amino acid identity to the ERM protein family suggesting possible interaction with the CD44 protein (Rouleau *et al.*, 1993, Trofatter *et al.*, 1993).

Recently, the interaction between the NF2 and CD44 proteins was demonstrated *in vitro* and its effect examined in Cos-1 cells (Sainio *et al.*, 1997). In untransfected cells, the CD44 protein was distributed diffusely across the cell surface. In contrast, upon expression of the NF2 protein, the distribution of CD44 was altered into the accumulation of clustered structures resembling thick microspikes and blebs at dorsal membrane. The NF2-dependent redistribution of CD44 and their *in vitro* interaction implies that the NF2 protein can regulate the adhesive function of CD44. However, the precise function of CD44 and NF2 interaction remains to be determined.

1.7.4 Intracellular phosphorylation of the CD44 molecule

Six serine residues are present within the human CD44 cytoplasmic domain of which four are conserved among rat, mouse, baboon, cow and hamster (Idzerda *et al.*, 1989, Stamenkovic *et al.*, 1989, Zhou *et al.*, 1989, Aruffo *et al.*, 1990, Bosworth *et al.*, 1991, Gunthert *et al.*, 1991). Phosphoamine acid analysis has revealed that phosphorylation of CD44 occurs primarily at serine and threonine residues suggesting that the serine residues are substrates for phosphorylation by protein

kinase C (PKC). Murine CD44 has been reported to be physically associated with PKC (Kalomiris and Bourguignon, 1988, Kalomiris and Bourguignon, 1989).

CD44 is constitutively phosphorylated on Ser^{325} and/ or Ser^{327} in mouse and Ser^{323} and Ser^{325} in humans (Neame and Isacke, 1992, Pure *et al.*, 1995). Recombinant CD44 mutated at either of the two residues in either mouse or human, were not phosphorylated when expressed in several different cell lineages (Pure *et al.*, 1995, Uff *et al.*, 1995). T cells expressing the non-phosphorylated murine CD44 mutants do not bind HA and are defective in CD44-HA-mediated adhesion to smooth muscle cells (Pure *et al.*, 1995). This observation infers that the cytoplasmic phosphorylation of murine CD44 is capable of regulating the extracellular interaction with HA. Furthermore, phosphorylation of the cytoplasmic region of CD44 can affect its association with ankyrin and possibly ERM (Kalomiris and Bourguignon, 1988, Bourguignon *et al.*, 1991.

The phosphorylation state of the cytoplasmic tail can therefore affect the function of the extracellular domain. The precise mechanism is unknown but may involve conformational changes in structure of the receptor, alterations in either the redistribution of the receptor or its association with other cellular proteins (Lazaar and Pure, 1995).

1.8 Ligands of the CD44 molecule

The elucidation of ligands bound by CD44 is of paramount importance in understanding its biological role. Although HA has been identified as the major ligand for CD44, other ligand binding activities must exist.

For example, CD44-mediated lymphocyte binding to mucosal high endothelial venules is independent of HA binding (Jalkanen *et al.*, 1987, Culty *et al.*, 1990). Furthermore, the administration of CD44 v6 mAb can block tumour growth, lymphocyte activation and limb bud outgrowth yet does not interfere with CD44-mediated HA binding (Arch *et al.*, 1992, Sleeman *et al.*, 1996b, Wainwright *et al.*, 1996).

A diverse range of ligands have been ascribed to the CD44 protein including mucosal addressin (Picker *et al.*, 1989), collagen type I (Carter and Wayner, 1988, Faassen *et al.*, 1992, Ishii *et al.*, 1993, Ehnis *et al.*, 1996), chondroitin sulfate form of the invariant chain of the MHC protein (Naujokas *et al.*, 1993), blood group antigens (Labarriere *et al.*, 1994), fibronectin (Jalkanen and Jalkanen, 1992, Verfaillie *et al.*, 1994, Cao *et al.*, 1996), macrophage inhibitory protein-1β (Tanaka *et al.*, 1993), serglycin (Toyama Sorimachi *et al.*, 1995), osteopontin (Weber *et al.*, 1996b) and sulfated proteoglycans (Underhill and Toole, 1979, Underhill *et al.*, 1983).

Thus the CD44 receptor is an example of a protein which can bind to multiple ligands and carry different functions. As to date, only the binding region of HA has been characterized on the CD44 molecule, yet it is conceivable that other ligand-specific binding domains must exist. The functional diversity of CD44-ligand interaction is exemplified below.

1.8.1 CD44 and hyaluronic acid

CD44 is the principal cell surface receptor for HA and also plays a key role in internalization and degradation of HA by acid hydrolases (Aruffo *et al.*, 1990,

Miyake and Kincade, 1990, Culty *et al.*, 1992). HA is a non-sulfated, unbranched extracellular and cell surface polysaccharide expressed in connective, neural and epithelial tissues (Ripellino *et al.*, 1985, Laurent and Fraser, 1992, Tammi *et al.*, 1994). As a major component of the ECM, HA has been suggested to play an important role as a provisional matrix for supporting cellular migration and adherence in a number of biological processes (Laurent and Fraser, 1992). These include wound healing (Oksala *et al.*, 1995, Kaya *et al.*, 1997), embryonic development (Underhill *et al.*, 1993), tumour growth (Sy *et al.*, 1991, Bartolazzi *et al.*, 1994), cell adhesion and aggregation (Pessac and Defendi, 1972, Green *et al.*, 1988), T cell activation (Haynes *et al.*, 1989, Galandrini *et al.*, 1994b), B cell maturation (Miyake *et al.*, 1990a), cell migration and cell proliferation (Underhill and Toole, 1981, West and Kumar, 1989, Turley *et al.*, 1991, Thomas *et al.*, 1993).

Cytosolic-free calcium, either entering through the plasma membrane or released from intracellular storage, is a regulator of a large number of cellular activities. The addition of HA to mouse T lymphoma cells and human T cells result in rapid elevation of intracellular calcium concentrations in a CD44-dependant manner (Bourguignon *et al.*, 1993, Galluzzo *et al.*, 1995). This suggests that HA can function as a cellular signalling molecule in addition to its cell scaffolding function.

Other examples of cellular signalling of HA are demonstrated by the induction of cytokine gene transcription in macrophages and fibroblasts. Small molecular weight HA fragments are capable of inducing gene expression of the cytokine and chemokine family including: macrophage inflammatory protein-1α, macrophage inflammatory protein-1β, cytokine responsive gene 2, IL-12, IL-1β, TNF-α and IL-8

(McKee *et al.*, 1996, Hodge Dufour *et al.*, 1997, Kobayashi and Terao, 1997). Therefore fragments of HA are capable of activating macrophages and fibroblasts to induce gene expression whose functions are relevant to chronic inflammation.

1.8.2 CD44 and serglycin

Serglycin is a secretory granule proteoglycan and is expressed in haematopoietic lineage cells including neutrophils, granulocytes, lymphocytes, spleen, thymus, lymph node and bone marrow (Stellrecht *et al.*, 1991, Toyama Sorimachi *et al.*, 1997). The serglycin transcript is regulated during tissue development and modulated by cellular activation (Stellrecht *et al.*, 1991, Birkenbach *et al.*, 1993). Although the function of serglycin remains to be fully defined it has been suggested that it is involved in myeloid cell differentiation as well as cell-mediated cytotoxicity (Stevens *et al.*, 1989, Masson *et al.*, 1990, Matsumoto *et al.*, 1995, Kolset *et al.*, 1996).

The CD44 molecule is capable of binding serglycin with subsequent enhancement of the CD3-dependent granzyme release from cytotoxic T cell clones. The addition of HA had no effect on granzyme release indicating that serglycin has a unique CD44-mediated function to that of HA in the cytotoxic T cell response (Toyama Sorimachi and Miyasaka, 1994, Toyama Sorimachi *et al.*, 1995). The binding of CD44 to serglycin is regulated and parallels that of the CD44-HA interaction, as peripheral lymphocytes cannot bind to serglycin unless treated with a stimulating CD44 mAb (Lesley *et al.*, 1993b, Toyama Sorimachi *et al.*, 1995).

1.8.3 CD44 and osteopontin

Osteopontin (Opn) is an extracellular phosphoprotein secreted by activated T cells, osteoblasts, macrophages and other cells (Patarca *et al.*, 1989, McKee *et al.*, 1992, Patarca *et al.*, 1993). Opn has a chemotactic ability and may function in cell attachment, regulation of inflammation, bone formation, angiogenesis and metastasis (Senger *et al.*, 1989, Reinholt *et al.*, 1990, Denhardt and Guo, 1993, Patarca *et al.*, 1993, Behrend *et al.*, 1994, Yue *et al.*, 1994).

Recently the murine $CD44_{v7\text{-}10}$ isoform was shown to interact with Opn (Weber *et al.*, 1996b). Transfected mouse embryonic A31 cells expressing the murine $CD44_{v7\text{-}10}$ protein migrated towards an Opn gradient in a manner reflecting chemotaxis. Antibodies to either Opn or CD44 inhibited cell migration indicating the necessary interaction between the two molecules for cell motility. In contrast no cell migration occurred within a HA gradient. As to date the precise binding site of Opn has yet to be defined.

HA can mediate homotypic aggregation of CD44-expressing haematopoietic cells and fibroblasts (Lesley *et al.*, 1990). While homotypic aggregation of the A31 $CD44_{v7\text{-}10}$ transfected cells was demonstrated to occur in the presence of HA, the addition of Opn, however, could not induce aggregation (Weber *et al.*, 1996b). This data indicates that Opn and not HA can induce CD44-dependent chemotaxis whereas HA and not Opn induces CD44-dependent cell aggregation. As suggested by Weber and colleagues, the interaction between Opn and CD44 may mediate cell migration out of the blood-stream and into sites of inflammation, where additional interactions between CD44 and HA may mobilize and activate these emigrant cells.

1.8.4 CD44 and macrophage inflammatory protein-1β

The macrophage inflammatory protein-1β (MIP-1β) can induce both chemotaxis and adhesion of T cells and is effective in augmenting cell adhesion of $CD8^+$ T cells to the vascular cell adhesion molecule-1 (VCAM-1). MIP-1β is produced in large quantities, both *in vitro* and *in vivo*, by activated monocytes, fibroblasts and lymphocytes (Sherry *et al.*, 1988, Zipfel *et al.*, 1989).

It has been proposed that cytokine interaction with cells is more effective when the cytokine is immobilized on luminal surface of the endothelium as constant blood flow would prevent the accumulation of effective cytokine concentrations (Rot, 1992). Chemokines like MIP-1β have GAG binding sites and can bind heparin (Butcher, 1991, Oppenheim *et al.*, 1991, Jackson *et al.*, 1991, Schall, 1991). Recently, MIP-1β was shown to be capable of binding to CD44 (Tanaka *et al.*, 1993). Furthermore, once retained on the CD44 molecule, MIP-1β could be presented to T cells resulting in an induced cell adhesion of the T cells to VCAM-1. Therefore, MIP-1β can bind to the CD44 proteoglycan molecule at the endothelial surface at inflammatory sites and be presented as an adhesion-inducing stimulus to passing lymphocytes.

1.9 Regulation of HA binding to CD44 protein

CD44 is expressed on a variety of cells and tissues and primarily interacts with the HA ligand. However, HA is expressed ubiquitously in the ECM and circulation and as such "promiscuous" binding of HA to CD44 may have an undesirable effect upon cellular function. Therefore, the interaction of CD44 with HA must be tightly

regulated and it is not surprising that the expression of the CD44 protein is required but not sufficient for HA recognition.

The differences in the ability of a single CD44 isoform to bind HA is dependent upon the activation state of the molecule and as such the binding property of CD44 towards HA can be inactive, inducible or constitutive (Lesley *et al.*, 1995). This is most clearly demonstrated in lymphocytes which express high levels of $CD44_{std}$ but do not bind soluble HA or adhere to HA coated substrates (Lesley *et al.*, 1992, Hathcock *et al.*, 1993, Murakami *et al.*, 1994, Lesley *et al.*, 1995). Upon lymphocyte activation with cytokines (Murakami *et al.*, 1990, Hathcock *et al.*, 1993, Katoh *et al.*, 1995, Legras *et al.*, 1997), ligation of CD3 receptor (Galandrini *et al.*, 1994b), specific anti-CD44 mAbs (Lesley *et al.*, 1993b, Liao *et al.*, 1993), lipopolysaccharide (Murakami *et al.*, 1990), concanavalin A (DeGrendele *et al.*, 1996), phorbol esters (Lesley *et al.*, 1990, Hyman *et al.*, 1991, Galandrini *et al.*, 1994b), association with the cytoskeleton (Kalomiris and Bourguignon, 1988, Lokeshwar and Bourguignon, 1992) or an *in vivo* allogeneic immune response (Lesley *et al.*, 1994) certain lymphocytes acquire CD44-mediated HA binding.

The ability of CD44 to bind HA is regulated in a cell specific manner as transfection of the same $CD44_{std}$ cDNA into different cell lines confers HA binding onto some cells but not others (Lesley *et al.*, 1993a). This suggests that in addition to the CD44 molecule, other cellular elements are required for HA-recognition. However, the precise factors controlling CD44-HA interaction is not fully understood. As discussed below, post-translational modification of CD44 and the interaction with intracellular proteins can regulate HA recognition.

1.9.1 Post-translational modifications of CD44 and its influence on HA interaction

The post-translational N- and O-linked glycosylation of CD44 can negatively or positively influence its binding to HA. For example, tunicamycin, an inhibitor of N-linked glycosylation, will inhibit HA binding to melanoma cells. Nevertheless, the same treatment of lymphoma, fibroblast, and ovarian cancer cells induces significant HA binding or has no effect (Katoh *et al.*, 1995, Lesley *et al.*, 1995, Bartolazzi *et al.*, 1996, Zheng *et al.*, 1997b). The precise mechanism by which glycosylation can regulate CD44-mediated HA binding is unknown, although the degree of CD44 glycosylation and the subsequent influence on HA recognition can be regulated by local environmental factors (Section 1.5.1).

1.9.2 CD44 variant proteins and HA binding

As to date the majority of studies characterizing the HA binding of human CD44 isoforms have concentrated on the $CD44_{std}$ isoform and as such little is known about the variant isoforms. The variant CD44 isoforms that have been cloned ($CD44_{v3\text{-}10}$, $CD44_{v3\text{-}8\text{-}10}$, $CD44_{v6\text{-}10}$, $CD44_{v7\text{-}10}$, and $CD44_{v8\text{-}10}$) and transfected into either Jurkat T cells or Namalwa B cells have a significantly diminished HA binding capacity in comparison to the corresponding transfectant expressing the $CD44_{std}$ isoform (Sy *et al.*, 1991, Liao *et al.*, 1993, Jackson *et al.*, 1995). The variant exons are inserted distally from the amino terminal HA binding region suggesting that conformational changes to the molecule can reduce HA recognition.

However, upon mAb modulation of the CD44 v6 or v9 proteins on *in vitro* activated human T cells, a marked reduction in HA-binding capability was

demonstrated suggesting that expression of the variant exons can enhance CD44-HA interaction (Galluzzo *et al.*, 1995). Furthermore, cloning of the murine $CD44_{v4-10}$, $CD44_{v6-10}$, $CD44_{v8-10}$ and $CD44_{v9-10}$ isoforms demonstrated HA binding to adhered stromal cells upon transfection into the AKR1 murine thymoma cell line (He *et al.*, 1992). These conflicting results can be reconciled with consideration that cell-specific post-translational modifications or cell-specific expression of intracellular proteins can influence the interaction of CD44 with HA.

1.9.3 Transmembrane and cytoplasmic regions of the CD44 molecule and its regulation of HA binding

As discussed earlier (Section 1.4.3) the Cys^{295} located in the transmembrane domain is critical for dimerization of CD44 receptors as a pre-requisite for binding of $CD44_{std}$ to HA in Jurkat cells (Liu and Sy, 1996, Liu and Sy, 1997).

The cytoplasmic portion of the CD44 molecule appears to influence HA-binding capacity as its truncation reduces binding efficiency (Lesley and Hyman, 1992, Thomas *et al.*, 1992, Liao *et al.*, 1993, He *et al.*, 1992, Uff *et al.*, 1995). The precise role of the cytoplasmic domain in the participation of HA binding is unknown, however, in murine cells the interaction between $CD44_{std}$ with ankyrin is essential for HA binding (Lokeshwar *et al.*, 1994). In addition, the ability of recombinant $CD44_{std}$ to bind HA can be inhibited by drugs which disrupt cytoskeletal protein function (Liu *et al.*, 1996).

However, cytoplasmic-truncated CD44 protein still retains the ability to be stimulated to bind HA by anti-CD44 mAbs (Lesley *et al.*, 1992). Furthermore,

soluble chimeric CD44 molecules lacking the cytoplasmic domain can bind HA *in vitro* (Peach *et al.*, 1993) and studies have demonstrated that binding of HA does not require interactions between CD44 and cytoskeletal proteins (Peach *et al.*, 1993, Murakami *et al.*, 1994). As such, whether the cytoplasmic domain of CD44 is important in binding of HA remains controversial.

In summary, these observations suggest that the interaction between CD44 and HA is a complex and dynamic process. Each domain of the CD44 protein (extracellular, transmembrane and cytoplasmic) can markedly influence HA binding and therefore understanding of CD44-HA interaction requires consideration of the entire molecule in association with other cellular proteins.

1.10 Signal transduction through the CD44 molecule

The specific binding of an extracellular molecule to either a cell adhesion protein or transmembrane receptor results in the elicitation of a cellular response (e.g. cell proliferation or gene transcription) by the initiation of a specific signal transduction pathway consisting of a cascade of intracellular events. Upon triggering of the CD44 receptor by either ligand or mAb, a number of downstream cellular effector functions, as discussed below, are initiated demonstrating the ability of CD44 to function as a transmembrane signalling molecule.

The accessory role of the $CD44_{std}$ protein in the co-stimulation of human T cells has been well documented. Binding of anti-CD44 mAbs, thought to mimic the CD44-ligand interaction, can augment the CD2 or CD3 activation of T cells (Shimizu *et al.*, 1989, Huet *et al.*, 1989, Denning *et al.*, 1990, Rothman *et al.*, 1991,

Conrad *et al.*, 1992). In addition, the ligation of variant isoforms containing the v6 or v9 epitopes can promote cytosolic free calcium mobilization and co-stimulate CD3 activated T cells (Galluzzo *et al.*, 1995).

However, some anti-CD44 mAbs can inhibit CD3-dependent cell proliferation yet continue to augment the corresponding CD2-dependent responses (Rothman *et al.*, 1991, Guo *et al.*, 1993, Lesley *et al.*, 1993a, Guo *et al.*, 1994a). The same anti-CD44 mAb that inhibits proliferation of CD3-activated T cells induces palmitoylation of CD44 suggesting that lipid modification of CD44 can interfere with signalling pathways (Guo *et al.*, 1994a). Furthermore, CD44 serves as a signalling molecule through which lymphocyte function-associated antigen-1 (LFA-1) can be triggered to an active conformation resulting in homotypic T cell aggregation (Denning *et al.*, 1990, Koopman *et al.*, 1990). These observations emphasize the significance of receptor cross-talk between CD44 and other cell surface molecules leading to either a positive or negative growth-transducing signal.

Another example of signal transduction through the CD44 molecule is demonstrated by induction of cytokine production from monocytes and macrophages upon ligation of the molecule by either mAb or HA. These cytokines include: IL-1β, TNF-α, IL-12, IL-8, insulin-like growth factor, MIP-1α, MIP-1β and cytokine responsive gene 2 (Denning *et al.*, 1990, Webb *et al.*, 1990, Gruber *et al.*, 1992, Noble *et al.*, 1993, McKee *et al.*, 1996, Hodge Dufour *et al.*, 1997, Kobayashi and Terao, 1997). In addition, the engagement of CD44 surface molecules can stimulate an increase in intracellular calcium (Bourguignon *et al.*, 1993, Galluzzo *et al.*, 1995) and protein phosphorylation (Galandrini *et al.*, 1996, Taher *et al.*, 1996) with

subsequent acquisition of effector functions such as HA binding (Lesley *et al.*, 1993b, Liao *et al.*, 1993, Zheng *et al.*, 1995), cell proliferation (Shimizu *et al.*, 1989, Huet *et al.*, 1989, Denning *et al.*, 1990, Rothman *et al.*, 1991, Conrad *et al.*, 1992) and cell cytotoxicitity (Galandrini *et al.*, 1993, Funaro *et al.*, 1994, Sconocchia *et al.*, 1994, Pericle *et al.*, 1996).

The association of CD44 signalling and cell proliferation is not restricted to haemopoietic cells as loss of CD44 in murine keratinocytes results in the inability of these cells to mount a proliferative response to phorbol ester both *in vivo* and *in vitro* (Kaya *et al.*, 1997). This suggests that the CD44 polypeptide is implicated in the regulation of phorbol ester-triggered keratinocyte stimulation.

As to date the signal transduction pathways bridging CD44 ligation to cellular effector function is undefined. However, in the presence of protein tyrosine kinase inhibitors a number of CD44-dependent functions are blocked suggesting the role of protein phosphorylation in CD44-mediated cell signalling (Galandrini *et al.*, 1996).

One of the earliest biochemical events to be initiated in the signal transduction of many cell surface molecules is the activation of intra-cellular protein tyrosine kinases, which in-turn, amplifies the signal response by the tyrosine phosphorylation of specific down-stream proteins (Berridge, 1997, Qian and Weiss, 1997). Ligation of CD44 on resting human peripheral T cells results in the rapid induction of tyrosine phosphorylation of several intracellular proteins (Taher *et al.*, 1996). By co-immunoprecipitation studies, the $CD44_{std}$ protein was demonstrated to be physically associated with the $p56^{lck}$ tyrosine kinase. Furthermore, ligation of CD44 results in

activation of $p56^{lck}$ kinase activity by the phosphorylation of ZAP-70 protein thus coupling the CD44 molecule to the T-cell receptor growth signal machinery. The functional association between the $CD44_{std}$ protein and the $p56^{lck}$ protein tyrosine kinase may, as suggested by Taher and colleagues (1996), provide a means by which the $CD44_{std}$ protein can augment the CD3 activation of T cells by the possible recruitment of $p56^{lck}$ to the T cell receptor/ CD3 complex. Whether the variant CD44 isoforms display differential association with $p56^{lck}$ and other components of the cellular signal transduction pathway remains to be determined.

1.11 Regulation of CD44 gene expression and alternate splicing

Regulation of CD44 promoter activity, like many other genes, is dependent upon the presence or absence of cell-specific transcription factors. Initial observation of CD44 gene regulation was demonstrated in human erythrocytes where expression of CD44 protein is under the genetic control of the dominant inhibitory gene *In(Lu)* which also downregulates expression of the Lutheran antigens (Telen *et al.*, 1983). Individuals with the dominant form of $Lu(a^-b^-)$ phenotype express reduced levels of CD44 on red blood cells and monocytes (Telen, 1995). As to date, neither the gene product of the *In(Lu)* gene is known nor how this gene can regulate expression of two genetically and structurally different proteins.

Characterization of the CD44 gene revealed a G/ C residue rich promoter with an absence of TATA and CCAAT consensus sequences (Shtivelman and Bishop, 1991). Transfection of reporter plasmid constructs containing various lengths of CD44 promoter sequence into a melanoma or neuroblastoma cell line demonstrated a downregulation of promoter activity in the neuroblastoma cells. This observation

implies that the presence of specific repressor transcription factors in neuroblastoma cells which upon binding to a corresponding *cis*-acting element can down-regulate CD44 gene transcription.

The expression of variant transcripts is restricted to certain tissues and cell types as well as different stages of cell activation (Section 1.3) suggesting that cell-type specific regulation of alternate splicing of CD44 must exist. Alternate splicing of mRNA can be stringently regulated by either positive or negative *trans*-acting factors (Konig *et al.*, 1996). The *trans*-acting factors would function similar to that of transcriptional regulators where a multitude of splice regulators with different pre-mRNA and variant exon binding specificities would allow differential expression of mRNA and protein isoforms from the CD44 gene (Konig *et al.*, 1996).

Negative regulators would inhibit the inclusion of variant exon sequences in CD44 mRNA thus generating expression of the $CD44_{std}$ transcript. Alternately, specific variant transcripts can be generated by the up-regulation of positive regulators that select the corresponding variant exon combination. With great diversity in the number of variant isoforms that can be generated by alternate splicing of the 10 variant exons it is suggested that specific positive regulators exists which are differentially activated by specific signal transduction pathways (Konig *et al.*, 1996). Evidence to support the existence of positive *trans*-acting factors was demonstrated by the use of somatic cell hybrids between the human keratinocyte HaCat cell line and rat embryonal fibroblasts. Human HaCat cellular proteins, which function as positive *trans*-acting factors, were capable of inducing the synthesis of

novel rat-specific alternately spliced transcripts within the fibroblasts (Konig *et al.*, 1996).

As discussed below, the activation of specific transduction pathways by either cellular activation or overexpression of downstream oncogenes can modulate CD44 gene transcription and alternate splicing of specific variant isoforms.

1.11.1 Cell activation and CD44 gene regulation

Resting human peripheral blood T lymphocytes weakly express CD44 v9 proteins and do not express CD44 v6 proteins (Koopman *et al.*, 1993b, Salmi *et al.*, 1993, Mackay *et al.*, 1994). However, CD44 v6 proteins can be transiently up-regulated upon lymphocyte activation by *in vivo* and *in vitro* antigenic stimulation, TNF-α, INF-γ, PHA, CD3 mAb and IL-1 or PMA (Arch *et al.*, 1992, Koopman *et al.*, 1993b, Mackay *et al.*, 1994, Galluzzo *et al.*, 1995). Furthermore, expression of CD44 v9 proteins are up-regulated by INF-γ, TNF-α or PHA treatment (Mackay *et al.*, 1994, Galluzzo *et al.*, 1995).

Factors present in serum have been shown to also modulate expression of CD44 variant isoforms. Variant proteins containing the v4, v6 and v9 epitopes are up-regulated on human peripheral blood monocytes cultured in the presence of human serum (Levesque and Haynes, 1996). Furthermore, expression of $CD44_{std}$ protein is upregulated in mouse fibroblasts by serum in a concentration-dependent manner and is associated with an induction of mRNA expression (Kogerman *et al.*, 1996a). This observation suggests that serum factors can modulate CD44 expression by a transcriptional mechanism. The precise serum factors that regulate the CD44

gene have not been identified, however, studies have implicated growth factors. Expression of $CD44_{std}$ protein can be up-regulated in mouse fibroblasts by addition of platelet derived growth factor (Kogerman *et al.*, 1996a). In addition, upon interaction with its receptor, epidermal growth factor can up-regulate cell surface expression of the $CD44_{std}$ protein and its mRNA (Zhang *et al.*, 1996). An epidermal growth factor responsive *cis*-acting element has been identified within the promoter of the mouse CD44 gene (Zhang *et al.*, 1997).

Stimulation of the human SK-N-SH neuronal cell line with PMA, insulin-like growth factor-1 or platelet derived-growth factor resulted in the transcriptional induction of the $CD44_{v6}$ mRNA (Fichter *et al.*, 1997). Furthermore, both the PKC and phosphoinositide 3-kinase pathways were shown to transduce the growth factor induced signals.

Antigenic stimulation of mature B lymphocytes initiates a number of effector functions including cell migration and adhesion, clonal proliferation and differentiation into memory and antibody-secreting plasma cells (Maltzman *et al.*, 1996). Following ligation of the B cell antigen receptor, expression of the immediate early gene, *egr*-1, is rapidly induced (Seyfert *et al.*, 1990). The *egr*-1 gene encodes for a zinc finger nuclear protein product that can target the CD44 gene inducing promoter activity. The *cis*-acting element for the *egr*-1 gene product was recently defined within the human CD44 promoter at 300 bp upstream the transcription initiation site (Maltzman *et al.*, 1996).

1.11.2 Oncogenes and CD44 gene expression

Oncogenes or cancer-producing genes were originally derived from viral DNA whose protein products produce cellular neoplasia by the constitutive activation of specific components of the signal transduction pathways. Analyses of more than 20 retroviral oncogenes have shown that they are homologous to normal human cellular genes. The products of oncogenes can be classed into different categories defined as: tyrosine kinases (e.g. *src* gene), growth factors (e.g. *sis* gene), growth factor receptors (e.g. *erb*B gene), guanyl nucleotide binding proteins (e.g. *ras* gene) or nuclear proteins (e.g. *myb* gene).

The *ras* oncogene belongs to a family of small molecular weight G proteins and is known to play a key role in regulation of cell proliferation and tumour metastasis (Barbacid, 1987). Overexpression of the *ras* oncogene can increase promoter activity of the mouse and rat CD44 gene (Jamal *et al.*, 1994, Kogerman *et al.*, 1996b). Site directed analysis of the rat CD44 promoter demonstrated the existence of a functional *cis*-acting element for the AP-1 transcription factor (Hofmann *et al.*, 1993). These observations suggest that *ras* induction of CD44 expression is mediated by the AP-1 transcription factor via the mitogen activated protein kinase cascade (Seger and Krebs, 1995). In addition, overexpression of *ras* can modulate the mRNA splicing machinery and generate CD44 v6 exon containing transcripts in transformed rat embryo fibroblasts (Hofmann *et al.*, 1993).

The $p185^{neu}$ oncogene is a transmembrane protein belonging to the epidermal growth factor receptor subgroup of receptor-linked tyrosine kinase superfamily (Stern *et al.*, 1986, Peles and Yarden, 1993). $p185^{neu}$ is often overexpressed in a

number of human tumours and is correlated with induction of tumour metastasis in nude mice and lymph node metastasis in breast cancer patients (Yu and Hung, 1991, Yu *et al.*, 1994, DiGiovanna and Stern, 1995). Overexpression of $p185^{neu}$ in mouse fibroblasts will result in the up-regulation of the $CD44_{std}$ protein and induce subsequent protein interaction with ankyrin (Zhu and Bourguignon, 1996).

In summary, initiation of signal transduction pathways by cytokines, mitogens, growth factors, antigenic interaction or overexpression of oncogenes can differentially activate nuclear transcription factors or RNA *trans*-acting splice proteins resulting in the regulation of CD44 promoter transcription and the alternate splicing of specific variant transcripts.

PART II FUNCTION OF THE CD44 PROTEIN

1.12 Involvement of CD44 in disease

The CD44 protein has been implicated in a number of physiological and pathological processes (Section 1.1) and the role of CD44 in inflammation and tumour metastasis are discussed below.

1.12.1 CD44 and its function in inflammation and rheumatoid arthritis

During chronic inflammation and tissue fibrosis the synthesis and degradation of ECM components, including HA, are increased with the additional accumulation of cytokines (Clark *et al.*, 1988). As previously discussed (Section 1.8.1) the interaction between CD44 and small molecular weight HA can induce the release of cytokines and chemokines from macrophages and monocytes subsequently activating

circulating lymphocytes. Furthermore, the cytokine-mediated activation of synovial endothelial cells can result in greater adhesiveness between lymphocytes and endothelial cells thus enhancing the recruitment of activated lymphocytes to the site of inflammation in arthritis.

Inflamed tissues are infiltrated by activated lymphocytes which possess increased expression of cell surface molecules including the CD44 variant protein isoforms (Arch *et al.*, 1992, Haegel *et al.*, 1993, Koopman *et al.*, 1993b, Mackay *et al.*, 1994, Galluzzo *et al.*, 1995). The precise role of $CD44_{std}$ and the variant isoforms in inflammation is unclear, however, its functional significance is exemplified by the pathogenesis of rheumatoid arthritis, an autoimmune inflammatory disease.

Rheumatoid arthritis patients display an increased expression of CD44 on cell surface of lymphocytes and macrophages infiltrating the inflamed joint (Fischer *et al.*, 1993, Kelleher *et al.*, 1995). In addition, the concentration of soluble CD44 in synovial fluid is increased in relation to the degree of inflammation (Haynes *et al.*, 1991a). This observation indicates that the induction of cell surface CD44 shedding upon onset of inflammation, however, the precise function of soluble CD44 remains to be understood (Section 1.6).

The expression of CD44 variant transcripts in synovial fibroblast has been detected and characterized as multiple alternately spliced isoforms containing the v3 and v6 exons (Croft *et al.*, 1997). CD44 variant mRNA expression was absent from synovial fibroblasts isolated from non-inflamed tissue. This observation suggests

that the induction of specific variant transcripts in fibroblasts occurs during inflammation, however, the precise function of the acquired CD44 splice variants is unclear.

Using *in vivo* rodent models of rheumatoid arthritis, similar implications for the functional role of the CD44 protein has been ascertained. The progression of inflammation in a model of adjuvant-induced arthritis in rats demonstrated upregulation of cell-surface CD44 protein expression on lymphocytes and macrophages (Halloran *et al.*, 1996). Mice immunized with collagen II and administered with anti-CD44 mAb demonstrated a delayed onset of arthritis (Verdrengh *et al.*, 1995). Furthermore, the treatment of a murine model of arthritis with anti-CD44 mAb rapidly reduced tissue swelling and lymphocyte infiltration (Mikecz *et al.*, 1995). The anti-CD44 mAb treatment induced cell surface shedding of CD44 suggesting that the anti-inflammatory properties of the mAb functions by the removal of CD44 from connective tissue and activated lymphocytes.

Although the function of CD44 in rheumatoid arthritis remains unclear, these observations signify an essential role for CD44 in lymphocyte recognition and migration towards sites of inflammation.

1.12.2 Relationship between CD44 and tumour metastasis

Metastatic spread of tumours requires a series of cellular interaction with components of ECM and/ or other cells. These interactions depend on cell-surface determinants such as cell surface receptors for ECM, surface bound proteolytic enzymes, growth factors and growth factor receptors (Gunthert *et al.*, 1991).

The participation of CD44 in the formation and progression of metastatic tumours was originally established by generation of the 1.1ASML mAb capable of antigenically discriminating between the spontaneous metastasizing rat BSp73ASML pancreatic adenocarcinoma and the non-metastasizing sub-clone (Gunthert *et al.*, 1991). The cloning of a full-length cDNA encoding for this epitope identified the metastasizing cell specific protein as a CD44 variant containing the v6 exon, later defined as the $CD44_{v4\text{-}7}$ transcript. Subsequent studies had delineated the epitope region recognized by the 1.1ASML mAb to be encoded by the v6 exon (Arch *et al.*, 1992).

The rat $CD44_{v4\text{-}7}$ protein is capable of conferring metastatic properties to the non-metastasizing BSp73ASML subclone as demonstrated by the formation of distant lung tumours upon injection into rats (Gunthert *et al.*, 1991). This transfer of metastatic function was confined to cellular properties of the variant peptide region of the $CD44_{v4\text{-}7}$ protein as transfection with $CD44_{std}$ did not confer metastasis. Furthermore, the v6 exon specific 1.1ASML mAb retarded the growth of lymph and lung metastasis of the BSp73ASML cell line and the $CD44_{v4\text{-}7}$ transfected subclone (Seiter *et al.*, 1993). These observations establish a causal involvement of the CD44 v4-7 peptide region in tumour metastasis.

The smallest variant isoform shown to confer metastatic potential is that of the $CD44_{v6\text{-}7}$ protein indicating that overexpression of rat v6 exon containing proteins is sufficient to confer metastatic potential to non-metastasizing cells (Rudy *et al.*, 1993). As to date, the metastatic properties of the rat $CD44_{v6}$ isoform has yet to be

reported and as such the capability of the v6 exon alone to confer metastatic properties is unknown.

The observation that rat-specific CD44 variant proteins are capable of initiating lymphatic spread of solid tumours has received great attention. In a variety of paired rat tumour subclones that grow locally or metastasize via the lymphatic system, expression of CD44 variant isoforms were exclusively detected in metastasizing subclones (Zoller, 1995).

The precise mechanisms whereby variant CD44 proteins enhance tumour growth and dissemination are unclear. Suggested possibilities include CD44-mediated signalling that promote cytokine and growth factor production, degradation of ECM components allowing for tumour invasion or the mimicking of tumour cells as activated lymphocytes thus exploiting homing receptors present on endothelial cells (Herrlich *et al.*, 1993, Lazaar and Pure, 1995).

Aberrant mRNA splicing of CD44 transcripts with relevance to human tumour diagnosis was initially reported in 1992, where carcinomas of the colon and breast were associated with the generation of novel splice variants (Matsumura and Tarin, 1992). This observation was followed by numerous investigations into the function of CD44 and the variant isoforms in epithelial and non-epithelial neoplasias (as reviewed in Fichtner *et al.*, 1997, Naor *et al.*, 1997, Rudzki and Jothy, 1997). However, expression of CD44 isoforms in human tumours is not as strongly restricted to metastasis formation as previously demonstrated for the rat (Jackson *et al.*, 1993, Zoller, 1995). For example, neuroblastoma tumours do not express variant

CD44 proteins (Gross *et al.*, 1994) and a reduced CD44 expression is implicated in cell metastasis of human endometrial carcinomas (Fujita *et al.*, 1994).

The analysis of tumour growth and metastasis of individual CD44 variant isoforms has been examined *in vivo* using mouse models. $CD44_{std}$ promoted tumour growth and metastatic organ colonization of transfected Namalwa B cells upon injection into nude mice (Sy *et al.*, 1991, Bartolazzi *et al.*, 1995). Transfection of Namalwa cells with variant isoforms, $CD44_{v8\text{-}10}$, $CD44_{v7\text{-}10}$ and $CD44_{v6\text{-}10}$ failed to promote tumour metastasis and demonstrated a reduced rate of haematogenous dissemination (Sy *et al.*, 1991, Bartolazzi *et al.*, 1994, Bartolazzi *et al.*, 1995). The delayed *in vivo* tumour spread of the variant isoform expressing cells was consistent with the reduced *in vitro* HA binding capabilities of the variant proteins (Section 1.9.2). These observations suggest that the degree of tumour metastasis of the CD44 transfectants may be dependant upon the *in vivo* interaction between CD44 and the HA ligand.

However, the interaction of variant isoforms with other ligands can also enhance tumour metastasis. Namalwa transfectants expressing CD44 v3 exon containing isoforms ($CD44_{v3\text{-}10}$ and $CD44_{v3,8\text{-}10}$) rapidly develop tumours in bone marrow despite their inability to bind HA (Bartolazzi *et al.*, 1995). Previous studies demonstrated the attachment of GAG to the v3 exon (Jackson *et al.*, 1995) suggesting that binding of novel ligands by the v3 exon bound GAG may facilitate cell migration to the bone marrow. Alternately, the bone marrow may provide an environment that preferentially stimulates the growth of CD44 v3 protein expressing

cells possibly by providing a reservoir of growth factors capable of interacting with GAG attached to the v3 exon.

The inhibition of cell metastasis by the v6-exon specific 1.1ASML mAb (Seiter *et al.*, 1993) strongly suggests that binding of a ligand to the variant peptide region is required for promotion of metastasis. In addition, characterization of the rat $CD44_{v4-7}$ protein demonstrated that binding to HA was not required for metastasis but rather dependent on the variant peptide endowed tumour cells with other molecular properties which promote tumour spread (Sleeman *et al.*, 1996b). These observations suggest that the binding of rat CD44 variant proteins to HA is not required for metastatic spread, yet the CD44 interaction with ligands other than HA may be rate limiting in metastasis. As to date the identity of the other ligands associated with cell metastasis is unknown, however, studies have demonstrated the capability of the rat $CD44_{v4-7}$ and $CD44_{v6-7}$ isoforms to bind to chondroitin-4-sulfate, dermatin sulfate, chondroitin-6-sulfate, heparin and heparin sulfate (Sleeman *et al.*, 1997).

In summary, these observations demonstrate that the relationship between CD44-HA interaction and tumour metastasis is not a simple correlation indicating that the process of CD44-mediated metastasis is complex and is likely to involve multiple cellular properties.

1.13 Function of CD44 in lymphocyte activation and the immune response

The involvement of CD44 in lymphocyte activation and the immune response is indicated by altered patterns of CD44 mRNA and protein expression in the

pathogenesis of many autoimmune diseases such as rheumatoid arthritis (Section 1.12.1), Grave's disease (Heufelder *et al.*, 1993), in MLR-lpr mice (Wang *et al.*, 1992), autoimmune uveitis (Foets *et al.*, 1992), experimental allergic encephalomyelitis (Zeine and Owens, 1992), multiple sclerosis (Girgrah *et al.*, 1991) and chronic graft versus host disease (Murakami *et al.*, 1991).

The precise function of CD44 and the variant isoforms in an immune response is unclear. However, as discussed below, ligation of the CD44 molecule can augment proliferation of T cells, activity of natural killer cells and cell-cell aggregation between T cells antigen presenting cells. Furthermore, by studying murine models, variant isoforms containing the v6, v7 or v10 epitopes have been demonstrated to be important in alloimmunity and delayed type hypersensitivity responses.

1.13.1 CD44 and cells of the immune system

The majority of haemopoietic cells express $CD44_{std}$ particularly T cells, B cells, natural killer, monocytes and dendritic cells (Shimizu *et al.*, 1989, Hathcock *et al.*, 1993, Sconocchia *et al.*, 1994, Levesque and Haynes, 1996, Weiss *et al.*, 1997). Cell surface expression of $CD44_{std}$ is upregulated during lymphocyte activation (Hamilton *et al.*, 1991, Lesley and Hyman, 1992, Hathcock *et al.*, 1993) and is accompanied by transient activation of HA binding capabilities (Hathcock *et al.*, 1993, Lesley *et al.*, 1994). Furthermore, induced expression of CD44 following lymphocyte activation appears to be permanent as memory cells express strong levels of CD44 (Budd *et al.*, 1987, Camp *et al.*, 1991a).

Like most cell adhesion molecules, CD44 can function as a co-stimulatory molecule by augmenting the CD3 or CD2 activation of T cells (Huet *et al.*, 1989, Shimizu *et al.*, 1989, Denning *et al.*, 1990, Conrad *et al.*, 1992). Ligation of the CD44 molecule by either HA or mAb can also result in a number of T cell effector functions including proliferation of human T cell clones (Denning *et al.*, 1990, Galandrini *et al.*, 1993, Galandrini *et al.*, 1994b) and the induction of IL-2 receptor expression and cytokine synthesis such as IL-2, IFN-γ, IL-4 and GM-CSF from peripheral blood mononuclear cells (Huet *et al.*, 1989, Denning *et al.*, 1990, Funaro *et al.*, 1994). Furthermore, activation of the lytic machinery of T cell clones with anti-CD44 mAbs leads to release of trypsin-like esterases with this process dependent upon protein tyrosine kinase activity (Galandrini *et al.*, 1993, Funaro *et al.*, 1994).

Although the majority of functional CD44 mAbs have described the augmentation of T cell responses, some have been identified as inhibiting CD3-mediated T cell proliferation and IL-2 production (Rothman *et al.*, 1991, Guo *et al.*, 1993, Guo *et al.*, 1994a). These mAbs appear to inhibit T cell activation by either preventing increase in intracellular calcium by the rapid induction of cAMP synthesis or palmitoylation of the CD44 protein (Rothman *et al.*, 1993, Guo *et al.*, 1994a). The rapid rise in intracellular cAMP raises the possibility that the CD44 molecule is directly coupled to adenylate cyclase (Rothman *et al.*, 1993). Receptor-coupled G-proteins transmit signals from receptors to intracellular effectors such as adenylate cyclase (Birnbaumer, 1990, Simon *et al.*, 1991). The CD44 molecule possesses intrinsic GTPase activity (Lokeshwar and Bourguignon, 1992) suggesting G-protein-like activity of CD44 with direct interaction with adenylate cyclase.

Natural killer (NK) cells are a subpopulation of large granular lymphocytes endowed with the ability to mediate spontaneous as well as antibody dependant cellular cytotoxicity (Trinchieri, 1989). The ability of CD44 to augment pre-activated NK cell lysis has been demonstrated (Yang and Binns, 1993a, Galandrini *et al.*, 1994a, Sconocchia *et al.*, 1994, Tan *et al.*, 1995). Ligation of CD44 can increase the concentration of intracellular calcium in NK cells and enhance cell adhesion between NK and target cells thereby suggesting a possible mechanism for CD44-mediated up-regulation of cytotoxic activity. The acquisition of CD44-mediated effector functions requires *de novo* protein expression indicating that other proteins are required to link CD44 to the NK cell killing machinery (Sconocchia *et al.*, 1997). In addition, ligation of CD44 can stimulate TNF-α production and expression of the CD69 early activation antigen in human NK cells (Galandrini *et al.*, 1996). The CD44-mediated expression of TNF-α and CD69 can be blocked in the presence of a protein tyrosine kinase inhibitor (Galandrini *et al.*, 1993, Sconocchia *et al.*, 1997). This observation suggests that tyrosine kinases are required for CD44-mediated cytotoxic function in NK cells. A protein tyrosine kinase associated with CD44 in NK cells is yet to be identified, however, association with $p56^{lck}$ with CD44 on resting T cells has been reported (Section 1.10).

Expression of variant isoforms is limited in both adult tissues and in the absence of activating stimulus (Section 1.3). Activation of human T cells results in the induction of cell surface CD44 v6 and v9 containing proteins (Koopman *et al.*, 1990, Galluzzo *et al.*, 1995). In addition, activation of murine lymphocytes demonstrated an up-regulation of variant isoforms containing the v6 and/ or v7 epitopes (Wittig *et al.*, 1997). Furthermore, cytokines such as TNF-α and INF-γ can

specifically modulate CD44 v6 and v9 protein expression on monocytes with TNF-α preferentially upregulating CD44 v9 proteins and INF-γ that of CD44 v6 proteins (Mackay *et al.*, 1994). Activation dependant changes in CD44 protein expression is also characteristic of human langerhan and dendritic cells with the up-regulation of the v4, v5, v6 and v9 epitopes (Sallusto and Lanzavecchia, 1994, Weiss *et al.*, 1997).

The precise role of the variant isoforms on activated lymphocytes, monocytes and dendritic cells is unclear. However, ligation of CD44 v6 and v9 proteins can promote intracellular calcium mobilization and co-stimulate CD3-triggered T cell proliferation (Galluzzo *et al.*, 1995). The ability for the rat $CD44_{v4\text{-}7}$ protein to function as a co-stimulatory molecule in the activation of T lymphocytes has also been demonstrated (Moll *et al.*, 1994, Moll *et al.*, 1996). These observations demonstrate that the CD44 variant isoforms are capable of initiating cell signalling and effector functions of T lymphocytes.

As to date the corresponding ligands for the variant isoforms on activated lymphocytes is unknown. It is suggested that the ligands be of cellular origin rather than components of the ECM. These cellular ligands are likely to be expressed on antigen presenting cells (APC) and following interaction between the variant proteins and APC-expressing ligand, the cell-cell interaction and subsequent activation of T lymphocytes is further augmented. Evidence supporting this suggestion is provided by the demonstration that CD44 can function as a ligand for the chondroitin form of invariant chain (Ii). Ii is a non-polymorphic glycoprotein that associates with the major histocompatibility complex molecules (Jones *et al.*, 1979). The chondroitin sulfate form of Ii expressed on APC is required for efficient stimulation of primary

in vitro T cell responses to alloantigens (Naujokas *et al.*, 1993). The Ii protein can further function as a co-stimulatory molecule by binding to the CD44 molecule expressed by T cells thus facilitating T cell interaction with APC.

1.13.2 CD44 and cell-cell adhesion

Cell adhesion proteins can function as costimulatory molecules by either the initiation of signal transduction pathways or enhancing cell-cell adhesion between T cells and APC allowing the signal transmission of low avidity T cell receptor-antigen interactions (Springer, 1990, Hogg and Landis, 1993). The role of CD44-mediated signal transduction during T cell activation has been previously discussed (Section 1.10 and 1.13.1).

Initial observations of CD44-dependant cell aggregation was demonstrated in human melanoma cell suspension and several mouse cell lines where homotypic aggregation could be inhibited by the addition of anti-CD44 mAbs (Lesley *et al.*, 1990, Birch *et al.*, 1991). However, the addition of anti-CD44 mAbs could induce cell aggregation in cell suspension of human monocytes (Guo and Hildreth, 1993) and other haematopoietic cell lines (Murakami *et al.*, 1994, Cao *et al.*, 1995). This observation suggests the induction of homotypic aggregation by either linking the CD44 receptors of neighbouring cells or activation of other cell adhesion molecules.

Lymphocytes do not adhere spontaneously, however, upon cellular activation and ligation of the CD44 molecule, homotypic and heterotypic cell-cell aggregation can be induced (Belitsos *et al.*, 1990, Koopman *et al.*, 1990, Bruynzeel *et al.*, 1993, Toyama Sorimachi *et al.*, 1993). The mechanism of homotypic lymphocyte

aggregation is mediated by the CD44 activation of LFA-1/ ICAM interaction (Denning *et al.*, 1990, Koopman *et al.*, 1990, Funaro *et al.*, 1994, Vermot Desroches *et al.*, 1995). The CD44-mediated LFA-1-dependent cell aggregation is prevented by inhibitors of PKC and disruption of the cytoskeleton suggesting the requirement of functional kinase activity and cytoskeleton integrity (Koopman *et al.*, 1990). However, heterotypic cellular interactions may depend on alternate adhesion pathways to be activated. For example, the ligation of CD44 can increase cell adhesion between T cells and monocytes via CD2/ LFA-3 interaction (Haynes *et al.*, 1989, Denning *et al.*, 1990).

The role of CD44 in cell-cell adhesion is not limited to the $CD44_{std}$ protein as transfection of murine lymphoma TIL1 cells with the human $CD44_{v8\text{-}10}$ isoform can induce homotypic aggregation (Droll *et al.*, 1995). This aggregation was demonstrated to be dependent upon the v8-10 peptide region as transfection of $CD44_{std}$ protein did not produce cell aggregation. Cell mixing studies further demonstrated that homotypic aggregation of the $CD44_{v8\text{-}10}$ transfectants was mediated by adhesive interaction between the v8-10 variant peptide region and a common CD44 peptide region that is shared by both $CD44_{std}$ and $CD44_{v8\text{-}10}$ isoforms.

1.13.3 Role of CD44 variant isoforms in animal models of an immune response

Tumours in rats behave much like human tumours as both metastasize into the lymphatic system prior to dissemination in a blood-borne manner (Carr *et al.*, 1981, Neri *et al.*, 1982). This process resembles the trafficking of lymphocytes where upon contact with antigen, lymphocytes leave the periphery and enter the draining

lymphatic tissue and are selectively retained, activated and expanded before re-entry into circulation (Salmi and Jalkanen, 1997). Both tumour metastasis and lymphocyte trafficking involve highly mobile cells, cellular interactions with ECM and endothelium and cellular transport by blood and/ or lymph (Koopman *et al.*, 1990). Furthermore, cell adhesion receptors are believed to be critically important in both processes and the similarity between lymphogenic tumour spread and lymphocyte trafficking led to the postulation that the CD44 v6 protein involved in tumour cell metastasis (Section 1.12.2) may also have a physiological function in lymphocyte activation.

From these observations, the role of CD44 v6 proteins in an *in vivo* allogeneic response was studied (Arch *et al.*, 1992). Upon injection of allogeneic cells into rats, a transient up-regulation of the CD44 v6 epitope was noticed on lymphoid cells including T cells, B cells and macrophages. In addition, T cell-dependent and T cell-independent immune responses were suppressed upon administration of an anti-CD44 v6 mAb (1.1ASML mAb), which has been previously characterized to block tumour cell metastasis (Gunthert *et al.*, 1991, Arch *et al.*, 1992). The CD44 variant isoform expressed in T cells isolated from lymph nodes was identified as the $CD44_{v6}$ transcript. These observations demonstrate that cell surface expression of the CD44 v6 exon containing proteins is essential to the normal function of cells involved in the immune response.

The functional dichotomy of the CD44 v6 proteins in metastasis and the immune response was further ascertained by characterization of the metastatic-conferring rat $CD44_{v4\text{-}7}$ protein (Section 1.12.2). By the generation of transgenic

mice, murine lymphocytes expressing the rat $CD44_{v4-7}$ isoform were hyper-reactive *in vivo* and *in vitro* to T-cell dependent antigens and T cell mitogens demonstrating a co-stimulatory function of the $CD44_{v4-7}$ protein during T cell activation (Moll *et al.*, 1996). Furthermore, the accelerated cellular response was dependent on the expression of the transgene product as the addition of CD44 v6 specific mAb, 1.1ASML, reverted the response to that of control mice. These observations suggest that the inhibition of an *in vivo* allogeneic response by the 1.1ASML CD44 v6 mAb (Arch *et al.*, 1992) may not only retard cell migration of activated lymphocytes but also the co-stimulatory function of the CD44 v6 proteins in the activation of T cells.

T cells can be classified into two distinct subpopulations, Th_1 and Th_2 cells, each producing its own set of cytokines and mediating separate effector functions (Mosmann *et al.*, 1986). Th_1 cells produce IL-2 and INF-γ and mediate activation of macrophages and induction of delayed type hypersensitivity responses. Th_2 cells produce IL-4, IL-5, IL-10 and IL-13 which provide help for B cell function (Sayegh and Carpenter, 1997). Recently, the role of CD44 variant isoforms in a Th_1- or Th_2-mediated response was examined *in vivo* using murine models (Wittig *et al.*, 1997).

The trinitrobenzenesulphonic-induced colitis in mice has been characterized as a Th_1 cytokine mediated response (Kim and Berstad, 1992, Strober and Ehrhardt, 1993, Neurath *et al.*, 1995). Using such a murine model, the intrarectal application of CD44 v7 mAb completely abolished the signs of colitis yet the administration of mAbs directed against the v6, v10 or $CD44_{std}$ epitopes had no effect (Wittig *et al.*, 1997). Similarly, a dinitrofluorobenzene Th_1-dependent delayed type hypersensitivity (DTH) reaction of the skin (Gaspari and Katz, 1991, Meenan *et al.*,

1997) can be inhibited by administration of mAbs to either the v6 and v7 epitopes. However, the DTH response is further suppressed using an anti-CD44 v10 mAb suggesting different functional roles of the variant isoforms in a Th_1-dependent DTH reaction (Enk *et al.*, 1994, Rosel *et al.*, 1997).

In view of the distinct features of the anti-CD44 v6, v7 and v10 mAbs, subsequent examination of a Th_2-dependent DTH reaction by flouresceinisothycyanate (FITC)-induced allergic skin reaction ensued (Wittig *et al.*, 1997). The pattern of cytokine expression profile differed between anti-CD44 v6 and v7 mAb treated mice with those administered anti-CD44 v7 mAb having decreased number of IL-4 producing cells in comparison to anti-CD44 v6 mAb treated mice.

These observations demonstrate that both CD44 v6 and v7 epitopes are involved in Th_1-mediated activation but only the CD44 v7 mAb interfered with Th_2 activation. However, the specific variant v6 or v7 exon containing transcripts expressed in the T cells during the activation of the Th_1 and Th_2 responses are yet to be defined.

In summary, the functional importance of CD44 and the variant isoforms as a co-stimulatory molecule in T cell activation and in the normal functioning of cells involved in an immune response is without question. However, the precise role of the CD44 variant isoforms within different lymphoid cells remains to be further elucidated.

1.14 CD44-dependent migration of lymphoid cells

Mature lymphocytes continuously recirculate throughout the body, migrating from one lymphoid organ to another via blood and lymph (as reviewed in Jalkanen *et al.*, 1986a, Berg *et al.*, 1989, Salmi and Jalkanen, 1997). The migration of lymphocytes is critical in the physiology of the immune system and the initiation of an immune response. The continuous recirculation of lymphocytes allows the entire repertoire of clonal lymphocyte specificities to respond to antigens present at any site of the body.

Lymphocytes migrate from the blood to lymphoid organs via high endothelial venule (HEV) that serve as the entry into normal peripheral lymphoid tissues for recirculating lymphocytes and also as the entry for a variety of immune cell types extravasating at sites of tissue inflammation. Three different lymphocyte-HEV interactions have been described and mediate lymphocyte migration to peripheral lymph nodes, mucosa-associated lymphoid tissues (appendix, Peyer's patches) or inflamed joint (synovium) tissue (Butcher *et al.*, 1980, Butcher, 1986, Jalkanen *et al.*, 1986b). The majority of circulating lymphocytes are capable of interacting and migrating to all these different organ sites, however, some lymphocytes may express one form of HEV-specific receptor and subsequently only migrate to peripheral or mucosal lymphoid tissues or to sites of inflammation.

Tissue-specific migration of effector and memory lymphocytes are distinct and attributable, in part, to differential expression of adhesion receptors (Koster and McGregor, 1971, Issekutz, 1991, Mackay, 1991). Lymphocytes isolated from peripheral blood have a strong CD44 protein expression and constitute

predominantly of antigen stimulated memory lymphocytes circulating between blood and peripheral sites. In contrast, lymphocytes from the efferent lymph, which are mostly naive cells, express low levels of cell surface CD44 protein (Budd *et al.*, 1987, Camp *et al.*, 1991a, Lesley *et al.*, 1993a, Ayroldi *et al.*, 1996).

The CD44 molecule has also been termed as the lymphocyte homing receptor from the generation and characterization of the anti-CD44 Hermes series of monoclonal antibodies. Of the three mAbs (Hermes -1, -2 and -3) only Hermes-3 inhibited lymphocyte interaction with mucosal HEV yet did not inhibit lymphocyte binding to synovial or peripheral node HEV. However, polyclonal antisera against the CD44 molecule did inhibit lymphocyte interactions with HEV in peripheral lymph node, synovium and mucosal tissues (Jalkanen *et al.*, 1986b, Jalkanen *et al.*, 1987). These observations suggest that distinct regions of the CD44 molecule can differentially interact with the peripheral lymph node, synovium and mucosal HEV.

The adhesion interaction between CD44 molecules expressed on T cells and HA present on endothelial cells was recently suggested to be utilized by activated T cells for extravasation at sites of peritoneal inflammation (DeGrendele *et al.*, 1996, DeGrendele *et al.*, 1997). Using a parallel plate flow chamber designed to approximate postcapillary physiological flow, lymphoid cell rolling on cultured endothelial cells was demonstrated to be the CD44-dependent. The addition of anti-CD44 mAbs, soluble HA or hyaluronidase, blocked cell rolling thus indicating that lymphoid cell migration on endothelial cells is dependent on CD44-HA interaction. These observations suggest that in humans the CD44 molecule can confer tissue specificity to lymphocyte-HEV interactions. Due to the enormous variability in

protein structure different CD44 isoforms could endow the cell with a specific HEV-adhesion capabilities thus directing the migration of lymphocytes throughout the body. Potential mechanisms of CD44-mediated tissue-specific migration are differential post-translation modifications of CD44 or expression of variant CD44 isoforms.

Recently the importance of CD44 variant isoforms in the migration of dendritic cells was demonstrated. Epidermal langerhan cells (LC) belong to the dendritic cell family and are potent antigen presenting cells located in the suprabasal layers of the epidermis (Schuler *et al.*, 1993, Austyn, 1996). After antigen uptake, LC leave the epidermis and migrate via the afferent lymphatics into the regional lymph nodes to present antigen to T cells (Kripke *et al.*, 1990, Cumberbatch *et al.*, 1994).

Upon cellular activation, LC up-regulate expression of CD44 proteins containing the $CD44_{std}$, v5, v6 and v9 epitopes. Freshly isolated LC showed only weak binding to lymph node *in vitro* whereas activated LC adhered specifically to the paracortical T cell zone of lymph node (Weiss *et al.*, 1997). The pre-incubation with anti-CD44 v6 mAb significantly inhibited cell binding of activated LC to the T cell zone. Antibodies recognizing $CD44_{std}$ protein inhibited binding although less efficient, yet other mAbs directed to the CD44 v5 and v9 epitopes had no effect.

The *in vivo* role of LC in presenting antigen encountered within skin to T cells of the lymph node was assayed by the induction of an *in vivo* DTH response against an epicutaneously applied hapten. The DTH response is segregated into two stages described as the sensitization and challenge phase (Enk and Katz, 1995). The

sensitization phase occurs after the initial application of a hapten to skin where the hapten is taken up by LC which subsequently migrates into the regional lymph node to present the hapten to T cells. During the challenge phase, the same hapten is re-applied which induces an inflammatory response dependent the extravasation of lymphocytes into the application site. The challenge phase is independent of LC function (Becker and Knop, 1993, Grabbe *et al.*, 1995).

Antibodies to $CD44_{std}$ and the v6 epitope inhibited the challenge phase of DTH whereas only the anti-CD44 v4 and v6 but not $CD44_{std}$ mAbs inhibited the sensitization phase. These observations suggest that specific CD44 variant isoforms serve in different functions during the initiation of a DTH response by LC against epicutaneously applied haptens. It is suggested by Weiss and colleagues (1997) that during the sensitization phase CD44 variants containing the v4 and v6 epitopes play an essential role either during LC migration to lymph node and/ or their interaction with T cells. During the challenge phase $CD44_{std}$ and v6 epitopes appear to be required for lymphocyte extravasation into the site of hapten challenge.

1.15 Aims of the thesis

The literature in this review clearly demonstrates the role of CD44, and of particular interest the v6 exon containing isoforms, for normal function of the immune response. However, limited information is available pertaining to the precise identity of individual CD44 v6 transcripts expressed in human lymphoid cells and whether these transcripts are modulated upon cellular activation by either *in vitro* mitogens or *in vivo* alloimmunity.

The aims of this study was to:

(i) Define the variant exon composition of the CD44 v6 transcripts expressed in human peripheral blood mononuclear cells (PBMC).

(ii) Characterize expression of the CD44 v6 transcripts upon *in vitro* stimulation of PBMC with mitogens that mimic lymphocyte activation.

(iii) Characterize expression of the CD44 v6 transcripts from PBMC isolated from human renal transplant patients as representation of an *in vivo* immune response.

(iv) Design a cloning strategy to isolate full length CD44 v6 transcripts.

(v) Establish stable transfectants containing the full length CD44 v6 transcripts.

(vi) Functional characterization of the individual CD44 v6 proteins with objectives to provide further understanding of these isoforms during T cell activation.

Chapter Two

Materials and Methods

2.1 MATERIALS

2.1.1 Monoclonal antibodies

CD44 v6: murine IgG1 directed against an epitope expressed by the human CD44 v6 exon, purchased from Bender MedSystems (cat. # BMS116), Austria

CD44 v7: murine IgG1 directed against an epitope expressed by the human CD44 v7 exon, purchased from Bender MedSystems (cat. # BMS117), Austria

CD44 v9: murine IgG1 directed against an epitope expressed by the human CD44 v9 exon. The hybridoma (HB-258) was obtained from the American Type Culture Centre (ATCC), USA.

NIH-44: murine IgG1 directed against an epitope expressed by standard region of the human CD44 molecule. The hybridoma cell line was previously obtained within our laboratory.

Anti-CD3: murine IgG2a mAb (Orthoclone OKT®3) directed against an epitope expressed by the human CD3 receptor, purchased from Ortho Pharmaceutical Corporation, Immunobiology Division, New Jersey, USA

PY-20: murine IgG2b anti-phosphotyrosine mAb, purchased from Transduction Labs (cat. # P11120), Kentucky, USA

QE7.3E8: murine IgG1 directed against an epitope expressed by standard region of the human CD44 molecule. The hybridoma cell line was produced in the host lab (Kremmidiotis *et al.*, 1991).

X-63: murine IgG1 of unknown specificity however does not bind human tissue. The hybridoma cell line (P3X63Ag8) was obtained from the American Type Culture Centre (ATCC), USA.

2.1.2 Cell lines

Unless otherwise stated all cell lines were previously obtained within our laboratory and cultured in RPMI supplemented with 10% FCS.

HUT 78: human T cell line derived from peripheral blood of a 50 year old male with Sezary syndrome.

JM: human T cell line derived from peripheral blood of a patient with T-ALL.

Jurkat: human T cell line derived from peripheral blood of a male with leukaemia.

K562: human erythroleukemic cell line established from the pleural effusion of a 53 year old female with chronic myelogenous leukemia in terminal blast crisis.

Thp.1: human monocytic cell line derived from peripheral blood of 1 year male with acute monocytic leukemia. This cell line was cultured in RPMI supplemented with 10% FCS and 2 $x10^{-5}$ M β-mercaptoethanol.

U937: human cell line with monocytic characteristics established from malignant cells obtained from the pleural effusion of a 37 year old male with diffuse histiocytic lymphoma.

2.1.3 Enzymes

AmpliTaq® DNA Polymerase	Perkin Elmer, USA
M-MLV® Reverse Transcriptase	GIBCO BRL, USA
Restriction Endonucleases	
(Not I, Apa I, Eco RV)	Genesearch, Australia
RNasin® Ribonuclease Inhibitor	Promega, USA

2.1.4 Primer Sequences

Oligonucleotide primers were either designed *de novo* from published nucleotide sequences or taken from literature sources as indicated.

CD44 Primers

Primer A	5' CTT CCC AGT ATG ACA CAT ATT
Primer B*	5' CAG GCA ACT CCT AGT AGT AC
Primer C	5' CAG CTG TCC CTG TTG TCG AA
Primer D	5' CCC AAT CTT CAT GTC CAC ATT
Primer E*	5' CAG CCT CAG CTC ATA CCA GC
Primer F	5' TTG TCC TTC CTT GCA TTG GAT
Primer G	5' ATG GAC AAG TTT TGG TGG CAC GCA
Primer H	5' TTA CAC CCC AAT CTT CAT GTC CAC
V8* (forward)	5' TCC AGT CAT AGT ACA ACG CT
V9* (forward)	5' CAG AGC TTC TCT ACA TCA CA
V10*(forward)	5' GGT GGA AGA AGA GAC CCA AA

* Nucleotide sequences from van Weering *et al.*, 1993.

Other Primers

INF-γ (forward)	5' AGT TAT ATC TTG GCT TTT CA
INF-γ (reverse)	5' ACC GAA TAA TTA GTC AGC TT

Nucleotide sequence from Yamamura *et al.*, 1991.

β-actin (forward)	5' ATC ATG TTT GAG ACC TTC AA
β-actin (reverse)	5' CAT CTC TTG CTC GAA GTC CA

Nucleotide sequences from Fuqua *et al.*, 1990.

2.1.5 Reagents and supplements for culture media

Foetal Calf Serum (FCS)	CSL, Australia
Geneticin (G418)	Gibco BRL, Australia
HEPES	Sigma, USA
L-glutamine	Cytosystems, Australia
Penicillin/ Streptomycin	Cytosystems, Australia
RPMI 1640	Sigma, USA

2.1.6 Other chemicals and reagents

4-nitro blue tetrazolium chloride (NBT)	Boehringer Mannheim, Germany
5-Bromo-4-Chloro-3-Indoyl-β-D-Galactopyranoside (X-Gal)	Diagnostic Chemicals, Canada
5-bromo-4-chloro-3-indoyl phosphate (XPO_4)	Boehringer Mannheim, Germany
8-Hydroxyquinoline	Sigma, USA
Acrylamide (40%) 39:1 acrylamide: bis	Bio-Rad, USA
Agar bacteriological	Oxoid, UK
Agarose	Progen, Australia
Ammonium persulphate	Bio-Rad, USA
Ammonium sulphate	BDH, Australia
Ampicillin	Boehringer Mannheim, Germany
β-mercaptoethanol	Sigma, USA

Bacto-Tryptone	Oxoid, UK
Bacto-Yeast extract	Oxoid, UK
Blocking reagent	Boehringer Mannheim, Germany
Bovine serum albumin	Sigma, USA
Bromophenol blue	Bio-Rad, USA
Calcium chloride	Ajax, Australia
Chloroform	Ajax, Australia
Concanavalin A type IV (Con A)	Sigma, USA
Diethanolamine	Ajax, Australia
Diethylpyrocarbonate (DEPC)	Sigma, USA
Dimethyl formamide	BDH, Australia
Dimethyl sulphoxide (DMSO)	Ajax, Australia
DYNABEADS® M-450 goat anti-mouse IgG	DYNAL, Norway
Ethylenediamine tetraacetic acid (EDTA)	Sigma, USA
Ethanol	BDH, Australia
Ethidium bromide	Sigma, USA
Ethidium monoazide (EMA)	Molecular Probes, USA
FACS Lysing solution	Becton Dickinson, USA
Glacial acetic acid	BDH, Australia
Glucose	BDH, Australia
Glycerol	Ajax, Australia
Glycine	Sigma, USA
Guanidine isothiocyanate salt	Sigma, USA
Hyaluronan	Sigma, USA
Hybond N^+ membrane	Amersham, UK
Interleukin 2 (IL-2)	Genzyme Diagnostics, USA

Ionomycin	Sigma, USA
Isopropyl-β-D-thiolalactopyranoside (IPTG)	Boehringer Mannheim, Germany
Isoamyl alcohol	Sigma, USA
Isopropanol, anhydrous	Sigma, USA
Lymphoprep	Nycomed, Norway
Magnesium chloride	Sigma, USA
Maleic acid	Sigma, USA
Methanol	Ajax, Australia
Mineral oil	Sigma, USA
Mouse Immunoglobulin	Sigma, USA
Nitro-cellulose membrane	Bio-Rad, USA
N-lauroylsarcosine	Sigma, USA
Non-fat powdered milk	Dairy Vale, Australia
Pancreatic RNase A	Sigma, USA
Phenol	Progen, Australia
Phorbol myristate acetate (PMA)	Sigma, USA
Phosphate substrate tablets	Sigma, USA
Phytohemagglutinin (PHA)	Wellcome Diagnostics, UK
Potassium acetate	BDH, Australia
Pristane (2, 6, 10, 14 tetramethylpentadecane)	Aldrich Chemical Co, USA
Protein molecular weight marker	BioRad, USA
pUC 19 DNA marker	Bresatec, Australia
Rabbit anti-mouse immunoglobulin	DAKO, USA
Rose Bengal	Sigma, USA
Sheep anti-mouse immunoglobulin	Silenus Labs, Australia
Sheep anti-mouse alkaline phosphatase	Silenus Labs, Australia

Sheep anti-mouse FITC	Silenus Labs, Australia
Sodium acetate	Ajax, Australia
Sodium bicarbonate	Ajax, Australia
Sodium carbonate, anhydrous	Ajax, Australia
Sodium chloride (NaCl)	Ajax, Australia
Sodium citrate	BDH, Australia
Sodium di-hydrogen orthophosphate ($NaH_2PO_42H_2O$)	Ajax, Australia
Sodium dodecyl sulphate (SDS)	BDH, Australia
Sodium hydrogen carbonate	Ajax, Australia
Sodium hydrogen orthophosphate (Na_2HPO_4)	Ajax, Australia
Sodium hydroxide (NaOH)	Ajax, Australia
Sodium pyruvate	Ajax, Australia
SPP-1 DNA marker	Bresatec, Australia
Streptavidin-Alkaline phosphate conjugate	Boehringer Mannheim, Germany
TEMED	Bio-Rad, USA
Tritiated thymidine	Amersham, UK
Triton X-100	Bio-Rad, USA
Trizma base (Tris [hydroxymethyl])	Sigma, USA
Trizma HCl	Sigma, USA
Tween-20	Bio-Rad, USA
Water for irrigation (pyrogen free)	Baxter, Australia
Xylene cyanol	Sigma, USA

2.1.7 Molecular Biology Kits and Plasmid Vectors

5x first strand buffer	GIBCO BRL, USA
10x PCR Buffer	Perkin Elmer, USA
BRESA-CLEAN™ DNA purification kit	Bresatec, Australia
dNTPs mix	Promega, USA
JETstar plasmid purification kit	GENOMED, USA
pGEM T^+ PCR cloning kit	Promega, USA
pRC/ CMV expression vector	Medos, Australia
Protease inhibitor cocktail kit	ICN Biomedicals, Australia

2.1.8 Solutions for Molecular Biology

Sterilisation of solutions was by autoclaving for 20 min at 15 lb/sq inch. Filter sterilisation involved passing the solution through a 0.22 μm disposable filter.

2.1.8.1 Bacteriological Solutions

Alkaline lysis buffers for mini preparations of plasmid DNA

Solution A: 50 mM glucose, 25 mM Tris-HCl pH 8.0 and 10 mM EDTA pH 8.0 and was prepared as 100 ml batches, sterilised by autoclaving and stored at 4°C.

Solution B: 0.2 N NaOH and 1% SDS (v/v) and was prepared fresh prior to use.

Solution C: 3 M potassium acetate pH 4.8, stored at 4°C.

Ampicillin

A 100 mg/ml stock solution was prepared by dissolving 1 g of ampicillin in 10 ml distilled water. The solution was sterilised by filtration through a 0.22 μm disposable filter and stored as 1 ml aliquot at –20°C.

IPTG

A 200 mg/ml stock solution was prepared by dissolving 2 g of IPTG in 8 ml of distilled water. The volume was adjusted to 10 ml with distilled water and sterilised by filtration through a 0.22 μm disposable filter. The solution was stored as 1 ml aliquot at –20°C.

JETstar Solutions for Large Scale Plasmid Preparations

(as provided in kit)

Solution E1: 100 μg/ml RNase A, 50 mM Tris-HCl, 10 mM EDTA pH 8.0

Solution E2: 200 mM sodium hydroxide, 1% SDS (w/v)

Solution E3: 3.2 M potassium acetate pH 5.5

Solution E4: 600 mM NaCl, 100 nM sodium acetate, 0.15% Triton X-100 pH 5.0

Solution E5: 800 mM NaCl, 100 nM sodium acetate pH 5.0

Solution E6: 1.25 M NaCl, 100 mM Tris-HCl pH 8.5

Luria Bertani (LB) Medium

To 900 ml of distilled water, 10 g bacto-tryptone, 5 g bacto-yeast extract and 10 g NaCl were added. The solution was mixed thoroughly to completely dissolve all solutes and the pH adjusted to 7.5 by the addition of 5 N NaOH. The volume was adjusted to 1 L with distilled water and sterilised by autoclaving.

Preparation of LB plates with ampicillin/ IPTG/ X-Gal

To 1 L of LB broth, 10 g of bacteriological agar was added and solution boiled in a microwave oven. When the medium had cooled to approximately 50°C, ampicillin was added to a final concentration of 100 μg/ml, IPTG to a final concentration of 600 μg/ml and X-Gal to a final concentration of 50 μg/ml. In a biohazard hood approximately 35 ml of the LB-agar-ampicillin mixture was poured into 85 mm petri dishes. Air bubbles were flamed and cooled plates were stored at 4°C for up to 1 month.

RNase A

Pancreatic RNase A was dissolved to produce a final concentration of 10 mg/ml in 10 mM Tris-HCl pH 7.5/ 15 mM NaCl and heated to 100°C for 15 min. The sample was cooled to room temperature, aliquoted in 1 ml volume and stored at –20°C.

X-Gal

A 50 mg/ml stock solution was prepared by dissolving 1 g of X-Gal in 20 ml of dimethylformamide. The solution was wrapped in aluminium foil and stored as 1 ml aliquots at –20°C.

2.1.8.2 Agarose gel electrophoresis solutions

6x DNA gel loading buffer

The gel loading buffer contained 0.25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol and was stored at 4°C. This buffer was used when running DNA on agarose gels.

10x Tris-Acetate-EDTA (TAE) buffer

To 800 ml of distilled water, 48.4 g of Trizma base, 11.42 ml glacial acetic acid and 20 ml 0.5 M EDTA pH 8.0 were added and stirred until all solutes had dissolved. The volume was adjusted to 1 L with distilled water and stored at room temperature.

Ethidium bromide

A 10 mg/ml solution was made by adding 1 g of ethidium bromide to 100 ml of water and stirring on a magnetic stirrer overnight to ensure all solutes were dissolved. The bottle containing the ethidium bromide was wrapped in aluminium foil and stored at room temperature.

2.1.8.3 Southern hybridization solutions

20x SSC

To 500 ml distilled water, 175.3 g NaCl and 88.3 g sodium citrate were added and mixed thoroughly to completely dissolve all solutes. The pH adjusted to 7.0 by the addition of 1 M HCl and the volume was adjusted to 1 L with distilled water. The solution was stored at room temperature and used as a 1x reagent diluted in distilled water.

Alkali transfer buffer

To 500 ml distilled water, 26.6 ml 15 N NaOH and 120 ml 5 M NaCl were added and mixed thoroughly to completely dissolve all solutes. The volume was adjusted to 1 L with distilled water and stored at room temperature.

Blocking reagent

To 100 ml Buffer 1, one gram of blocking reagent was added and mixed thoroughly to completely dissolve all solutes. The solution was sterilised by autoclaving and stored at –20°C in 20 ml aliquots.

Buffer 1

To 1 L distilled water, 17.54 g NaCl and 23.22 g maleic acid were added. The solution was mixed thoroughly to completely dissolve all solutes and the pH adjusted to 7.5 by addition of 5 N NaOH. The volume was adjusted to 2 L with distilled water and stored at room temperature.

Buffer 3

To 415 ml distilled water, 50 ml 1 M Tris-HCl pH 9.5, 10 ml 5 M NaCl and 25 ml 0.5 M $MgCl_26H_2O$ were added. The solution was mixed thoroughly to completely mix all solutes and stored at room temperature.

Hybridization solution

To 50 ml distilled water, 25 ml 20x SSC, 1 g blocking reagent, 1 ml 10% n-laurylsarcosine and 200 µl 10% SDS were added. The solution was mixed thoroughly to completely dissolve all solutes. The volume was adjusted to 100 ml with distilled water and stored at -20°C in 20 ml aliquots.

NBT

A 75 mg/ml stock solution was prepared by dissolving 75 mg 4-nitro blue tetrazolium chloride (NBT) in 1 ml 70% dimethylformamide. The solution was mixed thoroughly to completely dissolve all solutes, wrapped in aluminium foil and stored at -70°C.

Southern hybridization substrate buffer

To 10 ml Buffer 3, 45 µl of NBT and 35 µl XPO_4 were added. The solution was mixed thoroughly to completely dissolve all solutes, wrapped in aluminium foil and used immediately.

XPO_4

A 50 mg/ml stock solution was prepared by dissolving 50 mg 5-bromo-4-chloro-3-indoyl phosphate (XPO_4) in 1 ml dimethylformamide. The solution was mixed thoroughly to completely dissolve all solutes, wrapped in aluminium foil and stored at -70°C.

2.1.8.4 Western blotting solutions

2x Laemmeli sample buffer

To 2 ml Milli Q water, 1.25 ml 0.5 M Tris-HCl pH 6.8, 2 ml 10% SDS, 2 ml 10% glycerol, 1 ml 10x protease inhibitor cocktail mix and 500 µl 0.05% bromophenol blue were added. Reduced lysis conditions was produced by addition of 100 µl β-mercaptoethanol. The solution was mixed thoroughly to dissolve all solutes. The volume was adjusted to 10 ml with Milli Q water and stored at –20°C in 200 µl aliquots.

2x PY-20 lysis buffer

To 4 ml Milli Q water, 2.5 ml 0.5 M Tris-HCl pH 6.8, 2 ml 10% SDS, 500 µl 100% glycerol and 100 µl β-mercaptoethanol were added. The solution was mixed thoroughly to dissolve all solutes. The volume was adjusted to 10 ml with Milli Q water and stored at –20°C in 200 µl aliquots.

10x SDS running buffer

To 1 L distilled water, 30 g Trizma base, 144 g glycine and 10 g SDS were added and mixed thoroughly to dissolve all solutes. The solution was stored at room temperature and used as a 1x reagent diluted in distilled water.

5% Non-fat powered milk solution

To 100 ml PBS, 5 g of skim milk powder was added and mixed thoroughly. The solution was made fresh prior to immediate use.

4% Stacking gel

To 6.34 ml Milli Q water, 2.5 ml 0.5 M Tris-HCl pH 6.8, 100 µl 10% SDS, 1 ml 40% acrylamide: bis (39:1), 50 µl 10% ammonium persulphate and 10 µl TEMED were added. The solution was inverted several times to mix all reagents and used immediately.

7.5% Resolving gel

To 2.97 ml Milli Q water, 5 ml 1.5 M Tris-HCl pH 8.8, 100 µl 10% SDS, 1.88 ml 40% acrylamide: bis (39:1), 50 µl 10% ammonium persulphate and 10 µl TEMED were added. The solution was inverted several times to mix all reagents and used immediately.

PY-20 Antibody buffer

To 100 ml PY-20 wash buffer, 5 g bovine serum albumin was added and solution mixed thoroughly until all solute dissolved and stored at 4°C for 1 hr prior to use.

PY-20 Wash buffer

To 600 ml distilled water, 5 ml 2 M Tris-HCl pH 7.5, 50 ml 2 M NaCl and 1 ml Tween-20 were added and mixed thoroughly. The volume was adjusted to 1 L by addition of distilled water and stored at room temperature.

TBS

To 1 L Milli-Q water, 2.42 g Tris-HCl and 29.22 g NaCl were added and mixed thoroughly to dissolve all solutes. The pH was adjusted to 7.5 by drop-wise addition of NaOH. The solution was sterilised by autoclaving and stored at room temperature.

Transfer buffer

To 1 L Milli-Q water, 6.06 g Trizma base, 28.8 g glycine and 400 ml methanol were added and mixed thoroughly to dissolve all solutes. The volume was adjusted to 2 L with Milli-Q water and stored at 4°C.

Western antibody buffer

To 45 ml PBS pH 7.2, 5 ml foetal calf serum and 25 µl Tween-20 were added and the solution mixed by inversion. The solution was stored on ice prior to use.

Western substrate buffer

To 100 ml distilled water, 10 ml 1 M Trizma pH 9.5, 10 ml 1 M NaCl and 10 ml 1 M $MgCl_2$ were added. The solution was mixed thoroughly to completely mix all solutes and stored at room temperature.

Western substrate solution

To 10 ml Western substrate buffer, 45 µl of NBT and 35 µl XPO_4 were added. The solution was mixed thoroughly to completely dissolve all solutes and wrapped in aluminium foil and used immediately.

2.1.8.5 General solutions and buffers

0.5 M EDTA pH 8.0

To 800 ml distilled water, 186.1 g of EDTA.$2H_2O$ sodium salt was added and stirred vigorously on a magnetic stirrer. The pH was adjusted to 8.0 by the gradual addition of NaOH dropwise. The volume was adjusted to 1 L with distilled water, sterilised by autoclaving and the solution stored at room temperature.

10% SDS

To 100 ml Milli Q water, 100 g SDS was added. The mixture was incubated at 65°C and mixed repeatedly until all solute dissolved. The solution was stored at room temperature.

Coating buffer

To 100 ml distilled water, 0.53 g sodium carbonate was added and mixed thoroughly to dissolve the solute. The pH was adjusted to 9.6 by the gradual dissolving of sodium bicarbonate, sterilised by autoclaving and used immediately.

Chloroform

All chloroform for RNA isolation was prepared as a 49:1 solution with isoamyl alcohol. All chloroform for DNA isolation was prepared as a 24:1 solution with isoamyl alcohol.

DEPC treated H_2O

To 100 ml of distilled H_2O, 100 µl of DEPC was added. The solution was shaken vigorously every 10 min for 1 hr prior to autoclaving.

Diethanolamine buffer

To 200 ml distilled water, 48.5 ml diethanolamine, 50 mg $MgCl_26H_2O$ and 0.1 g sodium azide were added and mixed thoroughly to dissolve all solutes. The volume was adjusted to 500 ml with distilled water, covered in aluminium foil and stored at 4°C for up to 3 months.

FACS washing buffer

To 1 L solution of PBS, 20 ml foetal calf serum and 0.1 g sodium azide were added. The solution was mixed thoroughly to dissolve all solutes and was stored at 4°C.

Phosphate buffered saline (PBS)

To 500 ml Milli Q water, 2.28 g Na_2HPO_4, 0.62 g $NaH_2PO_42H_2O$ and 7 g NaCl were added and mixed thoroughly to dissolve all solutes. The volume was adjusted to 1 L with Milli Q water and sterilised by autoclaving.

Phosphate substrate

To 10 ml diethanolamine buffer, two tablets of phosphate substrate were added, mixed thoroughly to dissolve all solutes and used immediately.

Phenol (water equilibrated) for RNA extraction

Hydroxyquinoline was added to colour the phenol yellow, prevent oxidation and inhibit the activity of RNases. The phenol was equilibrated by adding an equal volume of sterile distilled water and centrifuging at 850*g* for 5 min to separate the two phases. The aqueous phase was removed and the equilibration repeated. The second volume of aqueous layer was removed with a final replacement of fresh water.

RPMI media

RPMI 1640 was re-constituted in 1 L Milli Q water and supplemented with 50 U/ml penicillin/ streptomycin, 100 mM sodium pyruvate, 0.2% sodium bicarbonate, 10 mM HEPES and 2 mM L-glutamine. pH was adjusted to 7.3 by bubbling through CO_2.

Solution D

To 30 ml of distilled water, 25 g of guanidinium isothiocyanate, 1.76 ml 0.7 M sodium citrate pH 7.0, 2.64 ml 10% n-lauroylsarcosine and 360 µl β-mercaptoethanol were added and mixed thoroughly to dissolve all solutes. The volume was adjusted to 50 ml with distilled water and the solution wrapped in aluminium foil and stored at room temperature for up to 1 month.

2.2 METHODS

2.2.1 Isolation of mononuclear cells from human peripheral blood

Human blood was collected into heparinized tubes and centrifuged at 275*g* for 10 min followed by the removal of the platelet-rich plasma. The remaining blood was diluted 1:3 in PBS and distributed into v-bottomed 10 ml plastic tubes (Johns Biolab Scientific, Australia). The diluted blood was underlaid with 2 ml of Lymphoprep and centrifuged at 920*g* for 25 min.

The mononuclear cells at the Lymphoprep-plasma interface were removed by aspiration and washed twice in 3-fold excess PBS with centrifugation at 920*g* for 10 min. The cell pellet was resuspended in 10 ml volume PBS and the cell number determined with a Neubauer haemacytometer.

2.2.2 Stimulation of peripheral blood mononuclear cells

Mitogens

Peripheral blood mononuclear cells (PBMC) were cultured in RPMI 1640 supplemented with 10% fetal calf serum at a density 1 x10^6 cells/ml in 25 ml tissue

culture flasks (Corning, USA). PBMC cultures were maintained in a 5% CO_2 environment at 37°C and treated for 12 hr with PMA (10 ng/ml), IL-2 (100 U/ml), Con A (10 μg/ml), anti-CD3 mAb (5 μmg/ml), PHA (10 μg/ml) or Ionomycin (0.6 μg/ml).

Mixed lymphocyte reaction

PBMC from two donors were cultured, at a 1:1 ratio, in RPMI supplemented with 10% heat inactivated human AB sera at a density 1 $x10^6$ cells/ml in 96-well round bottomed plate. The plate was covered in cling wrap and incubated in a 5% CO_2 environment at 37°C for 12 hr.

2.2.3 Total RNA preparation by acid-guanidium thiocyanate phenol-chloroform extraction

Total RNA was prepared from PBMC and cell lines using a modified version of the method published by Chomczynski (1977).

Cells were counted and 5 $x10^6$ were lysed by the addition 500 μl of Solution D immediately followed by vigorous vortexing. RNA extraction was performed by the sequential addition of 50 μl sodium acetate pH 4.5, 500 μl DEPC-treated phenol and 100 μl chloroform: isoamyl alcohol (49:1). The cell suspension was vortexed thoroughly for 15 sec and incubated on ice for 10 min. The organic and aqueous phases were separated by centrifugation at 13,000*g* at 4°C for 10 min, with the aqueous phase removed and total RNA precipitated by the addition of equal volume isopropanol followed by incubation at –70°C for 20 min. Total RNA was pelleted by

centrifugation at 13,000*g* at 4°C for 30 min followed by two successive 70% ethanol washes of the pellet. The RNA pellet was air-dried and reconstituted in DEPC treated water.

2.2.4 Quantitation of DNA and RNA

To quantitate DNA and RNA, the sample was diluted $^{1}/_{50}$ in distilled water and the optical density at 260 and 280 nm was determined using by spectrophotometry. DNA and RNA were considered to be pure if the OD_{260}: OD_{280} was approximately 1.8 and 2.0 respectively. The concentration of DNA and RNA was based on one OD_{260} unit being equivalent to 50 μg of DNA per ml and 40 μg of RNA per ml of solution respectively.

2.2.5 Reverse transcription

First strand complimentary DNA (cDNA) was synthesized by reverse transcription. One microgram of total RNA was mixed with 4 μg oligo dT in a total volume of 20 μl of DEPC treated water. The RNA solution was denatured by heating to 65°C for 10 min followed by cooling on ice for 3 min. Twenty microlitres of a reaction mix containing 200 U M-MLV reverse transcriptase, 1x first strand buffer, 40 U RNasin® and 1 mM of each dNTP was added and reaction incubated at 37°C for 1 hr. Enzyme activity was destroyed by incubation of the reaction at 70°C for 10 min and snap chilling on ice. The reaction was diluted with 60 μl DEPC treated water and stored at –20°C.

2.2.6 Polymerase chain reaction

Unless otherwise stated all PCR reactions were carried out as follows. Target cDNA ($^1/_{20}$ of reverse transcription) was placed in a 0.5 ml reaction tube in a total volume of 50 µl containing 1 µM of each oligonucleotide primer, 200 µM of each dNTP, 1 U AmpliTaq® DNA polymerase in 1x PCR buffer. The reaction was overlayed with mineral oil to prevent evaporation. The DNA was denatured by heating to 94°C for 5 min before product amplification achieved by 23-50 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min followed by a final extension of 72°C for 7 min. To assess successful PCR amplification, a positive control containing known amplifiable cDNA was included. To assess whether contamination occurred, a negative control of no cDNA template was included. All PCR reactions were performed on a TC1 Perkin Elmer DNA thermal cycler machine (Perkin Elmer, USA).

2.2.7 Agarose gel electrophoresis

PCR products and restricted DNA fragments were routinely resolved by mixing with 6x DNA loading buffer and electrophoresis through an agarose 1x TAE horizontal slab gel. After electrophoresis, the gel was stained in ethidium bromide for 15 min and DNA bands visualised by subjecting the gel to UV (254 nm) light.

2.2.8 Southern transfer of DNA onto nylon membrane

A tray was filled with alkali transfer buffer and covered with a sheet of glass on which three sheets of Whatman 3MM filter paper were placed to form a wick. The gel was placed onto the wick and surrounded with cling wrap to prevent buffer

leakage. A sheet of Hybond N^+ membrane (cut to the size of the gel) was placed on top of the gel and 3 sheets of Whatman 3MM paper was placed on top of this. A stack of absorbent paper towels was placed upon the stack and a glass plate laid on the paper towels and weighted with a glass bottle to provide an even transfer of DNA onto the membrane.

2.2.9 Southern hybridization

Following an overnight transfer of DNA onto nylon membrane, the filter was pre-hybridized in blocking reagent for 4 hr at 42°C. The variant exon composition of the DNA fragments was determined by probing with 50 ng biotinylated exon-specific oligonucleotides in hybridization buffer. Hybridization was performed overnight at 42°C followed by stringency washes of 0.1x SSC/ 0.5% SDS. The oligonucleotide probes were labelled with $^1/_{5000}$ dilution streptavidin alkaline phosphatase conjugate in blocking reagent for 30 min at room temperature. The membrane was subsequently washed in buffer 1 for 30 min with the labelled oligonucleotides detected by addition of southern hybridization substrate buffer and placed in the dark until the enzyme reaction had completed to satisfaction. The reaction was stopped by washing the membrane in EDTA followed by washes in distilled water and left to air-dry overnight.

2.2.10 Cloning and subcloning of DNA fragments

2.2.10.1 Isolation of DNA fragments from TAE agarose gels

DNA fragments to be cloned or subcloned fell into two categories; (i) plasmid DNA which were digested with restriction endonucleases for the removal of DNA

fragments and its subsequent insertion into other plasmid vector and (ii) PCR products that were to be cloned directly. Both categories required isolation and purification of DNA fragments from contaminants prior to cloning.

DNA fragments (PCR products or digested plasmid DNA) were resolved through a TAE agarose gel for size separation of multiple DNA products. Samples were electrophoresed in duplicate lanes with one lane removed using a scalpel blade, stained with ethidium bromide and viewed under UV light. The DNA product of interest was marked above and below with a blade and the stained gel replaced along side its duplicate partner. Using the orientation markers, the DNA product from the unstained gel was excised.

DNA was extracted from the agarose gel slice using the BRESA-CLEAN™ DNA purification kit as per manufacturers instructions. Briefly, the gel slice was dissolved by the addition of 3 volumes of BRESA-SALT™ and incubated at 55°C for 10 min. BRESA-BIND™ was added (6 µl/µg DNA) and the solution mixed over 5 min at room temperature. The BRESA-BIND™/ DNA complex was pelleted by centrifugation at 13,000*g* for 10 sec, washed with BRESA-WASH™ with the pellet recovered by centrifugation. The pellet was resuspended in distilled water and DNA eluted by incubation at 55°C for 5 min followed by centrifugation for 2 min recovering the DNA in the supernatant.

2.2.10.2 *Ligations*

DNA ligations were set up in a 10 µl volume of distilled water containing 50 ng vector DNA, 2-3 fold molar excess insert fragment, 1x Ligase buffer and 0.5U T4 DNA Ligase. The reactions were incubated at 4°C overnight.

2.2.10.3 *Transformation of competent cells*

From an overnight culture of *E.coli* TG-1α, a $^{1}/_{500}$ dilution was performed in a total of 50 ml LB broth and culture grown in an orbital shaker until OD_{600} of 0.3-.06 was reached. The cells were collected by centrifugation at 3,000*g* for 5 min and resuspended in 2.5 ml solution containing 50 mM $CaCl_2$/ 20 mM $MgCl_2$. The cells were stored on ice for 1 hr at which time the cells were considered competent. A 5 µl aliquot of the ligation mixture was added to 200 µl aliquot of the competent cells and left on ice for a further 1 hr. Cells were then "heat shocked" by a 42°C incubation for 2 min followed immediately by incubation on ice for 2 min with a final incubation at room temperature for 2 min. A solution of LB broth containing 20% glucose was added and the cells were incubated in an orbital shaker at 37°C for 1 hr. The cells were pelleted by centrifugation at 13,000*g* for 10 sec and cell pellet resuspended in 100 µl LB broth. The cells were plated onto LB agar plates containing ampicillin/ IPTG/ X-Gal and incubated at 37°C overnight.

2.2.10.4 *Analysis of recombinant bacterial colonies*

Standard colour selection allowed white (recombinant) colonies to be differentiated from colonies containing the parent vector (blue). The colonies were picked from the plate using sterile toothpicks and used to inoculate 2 ml of LB broth

containing 100 µg/ml ampicillin. The inoculated cultures were incubated in an orbital shaker overnight at 37°C.

2.2.11 Small scale plasmid preparation: alkaline lysis

Small scale plasmid preparations were based on the method by Sambrook *et al.* (1989). The overnight bacterial cultures were pelleted by centrifugation at 13,000*g* for 5 min and the supernatant discarded. The bacterial pellet was resuspended in 100 µl of ice cold solution A and left at room temperature for 5 min. Two hundred microlitres of solution B was added and the sample mixed by inversion and left for 5 min on ice. Protein, chromosomal DNA and high molecular weight RNA were precipitated by the addition of 150 µl solution C and incubated on ice for a further 5 min prior centrifugation at 13,000*g* for 5 min. The supernatant was transferred to a fresh tube and bacterial RNA was degraded by the addition of RNase A at a final concentration of 20 µg/ml and incubated at 37°C for 30 min. Degraded RNA and RNase A was removed by extraction with an equal volume of phenol, the resulting aqueous phase was collected, extracted once with phenol: chloroform: isoamylalcohol (25:24:1) and further extracted with 500 µl of chloroform: isoamylalcohol (24:1). Plasmid DNA was precipitated with two volumes of absolute ethanol mixed by inversion and incubated at –70°C for 20 min. Plasmid DNA was pelleted by centrifuging at 13,000*g* for 30 min and washed in 70% ethanol, air dried, resuspended in distilled water and stored at –20°C.

2.2.12 Maxi preparation of plasmid DNA: JETstar method

Plasmid purification was performed on 500 ml bacterial culture using a JETstar maxi columns, according to the manufacturer's instructions. Briefly, the bacteria were collected by centrifugation at 15,000*g* at 4°C for 10 min. The bacterial pellet was resuspended in 10 ml of Solution E1. Following the addition of 10 ml Solution E2, the mixture was mixed by inversion and incubated at room temperature for 5 min. Solution E3 (10 ml) was added, the mixture immediately mixed by inversion and centrifuged at 15,000*g* at 20°C for 10 min. A JETstar maxi column was equilibrated with 30 ml of Solution E4 with the supernatant containing plasmid DNA applied to the tip allowing the lysate to run through by gravity. The column was washed with 60 ml of Solution E5 and the plasmid DNA eluted from the column with 15 ml of Solution E6 and precipitated with 0.7 volumes of isopropanol by centrifugation at 15,000*g* at 4°C for 30 min. The resultant DNA pellet was washed with 70% ethanol and air dried prior reconstitution in distilled water.

2.2.13 Electroporation of Jurkat 10.4 cell line

The Jurkat 10.4 cells were grown to a confluency of approximately 1 $x10^6$ cells/ml at which time the cells were collected by centrifugation at 3,000*g* for 10 min and resuspended to a density of 1.5 $x10^7$ cells/ml in RPMI supplemented with 10% FCS. To a 400 µl aliquot of cell suspension, 40 µl FCS and the required quantity of JETstar purified plasmid DNA were added and mixed by gentle pipette motion. The DNA/ cell mix was transferred into a 0.4 cm electrode gap Gene Pulser cuvette (Bio-Rad, USA) and placed into the chamber holder of the electroporator unit (Bio-Rad, USA). The appropriate voltage and capacitance was set on the unit and the pulse discharged with the time constant measured and recorded for future reference. The

cells were collected with 1 ml of RPMI supplemented with 10% FCS and cultured in a final 5 ml volume at 37°C with 5% CO_2.

2.2.14 Selection of recombinant Jurkat transfectants

Following a 48 hr recovery period after electroporation, Jurkat cells were seeded 1:5 in RPMI supplemented with 10% FCS and 2 mg/ml G418. The cells were continuously seeded 1:5 every 4-5 days for a period of approximately 8 weeks during which time non-recombinant cells were killed by G418. From this selection process, all surviving cells were considered stably transfected and subsequently cultured in RPMI supplemented with 10% FCS.

2.2.15 Dynabead® purification of CD44-expressing Jurkat cells

A total of 5 $x10^6$ Jurkat cells expressing recombinant cell surface CD44 protein was stimulated with 50 ng/ml PMA and 1 μg/ml PHA overnight at a density of 1 $x10^6$ cells/ml for induction of maximal protein expression. The cells were collected by centrifugation at 600*g* for 5 min and resuspended in 600 μl of PBS containing 2% FCS. The cells were pre-incubated with the murine anti-CD44 QE7.3E8 mAb by the addition of 400 μl overgrown tissue culture supernatant and incubated on a rotating platform at 4°C for 30 min. The cells were collected by centrifugation, washed twice in PBS containing 2% FCS and resuspended in a final 1 ml volume. A total of 2 $x10^7$ goat anti-mouse IgG Dynabeads® were added and incubated on a rotating platform at 4°C for 1 hr. The Dynabead®-rosetted CD44-expressing cells were collected by the application of a magnet to the outer wall of the tube. The supernatant containing non-expressing CD44 cells, which did not bind the QE7.3E8/

Dynabead® complex, were removed by aspiration. The pellet of Dynabeads® attached positive cells was washed four times with PBS supplemented with 2% FCS and CD44-expressing cells collected by application of a magnet to outer wall of the tube. The Dynabead® rosetted cells were finally resuspended in 5 ml RPMI supplemented with 10% FCS and allowed to expand in culture at 37°C with 5% CO_2.

2.2.16 Indirect immunofluorescence staining of cells for flow cytometric analysis

All cell samples were counted and approximately 0.5 $x10^6$ cells were used per antibody being tested. The cells were washed twice in cold FACS wash buffer by centrifugation at 200*g* for 5 min at 4°C. All subsequent washes in the procedure were performed in the same manner and the cells were resuspended in between by gentle flicking of the tube. All manipulations were performed on ice unless otherwise indicated.

After washing, the cell pellet was resuspended in cold FACS washing buffer. Aliquots of 100 µl were distributed into separate tubes and heat inactivated rabbit serum was added to a final concentration of 10% (v/v) to block non-specific antibody binding to Fc receptors. Ethidium monoazide (EMA) was also added to a final concentration of 10% (v/v) for discrimination between live and dead cells. Samples were then incubated in the dark for 30 min and kept in the dark as much as possible for all remaining manipulations. Following the incubation, primary antibody was added (50 µl of tissue culture supernatant or 10 µl of a $^1/_{500}$ dilution of commercially available mAb) and the samples placed under bright fluorescent light for 20 min to

photoactivate the EMA. All tubes were filled vigorously with FACS washing buffer and centrifuged at 200*g* for 5 min. The supernatant was removed from each tube and the cell pellet resuspended by flicking, prior to the addition of the FITC-conjugated sheep anti-mouse IgG secondary antibody at a final concentration of $^{1}/_{500}$ in FACS washing buffer. All samples were incubated in the dark for 20 min followed by 5 min incubation at room temperature. A 1 ml aliquot of freshly prepared 10% FACS lysing solution was added to each tube followed by a 20 min incubation at room temperature. The cells were washed twice by filling the tubes vigorously with FACS washing buffer and centrifuging at 200*g* for 5 min. Finally, the cells were resuspended in 200 µl of filtered saline and stored in the dark at 4°C until analysed on a Becton Dickinson FACScan flow cytometer.

2.2.17 Preparation of immune ascites

Balb/ c mice were injected twice at one week intervals with 0.5 ml Pristane. Three days after the second injection, 2 x10^6 washed hybridoma cells suspended in RPMI 1640 were intraperitoneally injected. The mice were inspected on a daily basis and upon marked abdominal distension (usually after 6-10 days) the mice were sacrificed and ascites aspirated by incising the peritoneal membrane. The sample was centrifuged at 3,000*g* for 10 min and ascites aspirated and stored at -70°C until required. In all procedures, the ascites were harvested using de-pyrogenated pasteur pipettes and collected in pyrogen-free sterile tubes to minimise introduction of endotoxins prior to antibody purification (Section 2.2.18).

2.2.18 Ammonian sulphate precipitation of monoclonal antibodies

Monoclonal antibodies were purified under stringent endotoxin-free conditions. This primarily involved the preparation of all reagents in pyrogen-free water (i.e. water for irrigation), using baked glassware and working under aseptic conditions. The technique is as described by Goding (1986).

Antibody was purified from ascites by the precipitation of immunoglobulin in a 50% solution of ammonium sulphate. An equal volume of cold (4°C) saturated solution of ammonium sulphate, adjusted to pH 7.2 with NaOH, was added dropwise to the supernatant preparation while being agitated with a vortex mixer. The mixture was continuously stirred for 30 min at 4°C. The resulting precipitate was washed three times with a 50% ammonium sulphate solution and resuspended in an appropriate volume of PBS. The final solution was dialyzed against PBS at 4°C with three changes of PBS prior to ELISA quantitation of immunoglobulin.

2.2.19 Monoclonal antibody concentration determination- ELISA

Monoclonal antibody concentration was determined using the method of Lems Van Kan *et al.* (1983).

The wells of a flat-bottomed 96 well microtitre plate (Corning, USA) were coated with 2 µg/ml sheep anti-mouse immunoglobulin in coating buffer by incubating at 37°C for 2 hr followed by overnight incubation at 4°C. The plate was washed 3 times with PBS/ 0.05% Tween-20. A standard curve was constructed by serially diluting at 1:3 mouse immunoglobulin (2 µg/ml in PBS). The mAb of

interest was serially diluted 1:3 commencing at $^1/_{10}$ or $^1/_{100}$ dilutions. The plate was wrapped in cling wrap and incubated for 2 hr at 37°C in a humid chamber. The wells were then washed 3 times with PBS/ 0.05% Tween-20 and 100 µl of a $^1/_{1000}$ dilution of sheep anti-mouse alkaline phosphatase conjugate added to each well. The plate was wrapped in cling wrap and incubated for 2 hr at 37°C in a humid chamber. Wells were then washed 3 times with PBS/ 0.05% Tween-20 and 100 µl of phosphate substrate was added. After a 15 min incubation at room temperature the OD_{405} was then obtained using a Titretek plate reader.

2.2.20 Protein detection by western blotting

2.2.20.1 Collection of samples

All cell samples to be analyzed were collected by centrifugation at 600*g* for 5 min. The cell pellet was resuspended in Laemmli sample buffer at 2 x10^6 cells/ 60 µl buffer. The cell suspension was vortexed thoroughly and boiled for 10 min. The sample was centrifuged at 13,000*g* for 5 min and a 35 µl aliquot was loaded onto a polyacrylamide gel for protein gel electrophoresis.

2.2.20.2 SDS polyacrylamide gel electrophoresis

Proteins were separated by electrophoresis using the mini Protean II gel apparatus (Bio-Rad, USA) using polyacrylamide microslab gels (8 cm x 7.3 cm) consisting of a 7.5% resolving gel and a 4% stacking gel as described by Matsudaira *et al.* (1978). The resolving gel was poured to allow for a 1 cm stacking gel and overlaid immediately with distilled water to prevent aeration and to ensure an even

level interface between the two gel types during polymerisation. After removal of the water, the stacking gel was poured with insertion of the gel comb (0.5 mm wide). Once the stacking gel had set, the comb was removed and wells were thoroughly washed to remove any residual acrylamide. Protein samples were loaded into the well with an appropriate protein molecular weight marker. The gel was run in 1x SDS running buffer at 30 mA for 60-90 min.

2.2.20.3 Transfer of protein onto nitro-cellulose membrane

Proteins from SDS-polyacrylamide gels were transferred onto nitrocellulose membrane using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, USA). The polyacrylamide gel was washed and equilibrated in transfer buffer for 30 min at room temperature with gentle agitation. The gel was sandwiched against a piece of nitrocellulose membrane between 2 stacks of 3 pieces of 3mm Whatman chromatography paper, each previously soaked in transfer buffer. The gel-membrane sandwich was then subjected to 900 mA for 30 min at 4°C. After transfer, the membrane was retrieved and washed for 1 min in PBS followed by 5 min in TBS solution.

2.2.20.4 Detection of proteins

Non-specific binding-sites on the nitrocellulose membrane was blocked by the incubation in 5% non-fat powdered milk (10 ml volume in hybridization plastic bag) for 2 hr at room temperature with gentle agitation. The membrane was transferred into a fresh hybridization bag containing the primary antibody (tissue culture supernatant diluted ½ in western antibody buffer) and incubated overnight at room temperature with gentle agitation. Unbound primary antibody was removed by

washing the membrane twice in TBS solution for 10 min with gentle agitation. The membrane was transferred into a hybridization bag containing sheep anti-mouse IgG alkaline phosphate conjugated antibody diluted $^{1}/_{5000}$ in western antibody buffer and incubated at room temperature for 1 hr. Unbound antibody was removed by washing the membrane twice in TBS solution and equilibrated in western substrate buffer for 5 min at room temperature. Bound antibodies were detected by placing the membrane in a hybridization bag containing western substrate solution and contained in the dark until the enzyme reaction had completed to satisfaction. The reaction was stopped by washing the membrane in EDTA followed by washes in distilled water and left to air-dry overnight.

2.2.21 Rose Bengal assay for insoluble HA binding

The Rose Bengal assay for detection of adhered cells was performed as essentially as described in Lyons and Ashman (1985). The wells of a 96 flat bottom plate (Corning, USA) were pre-coated at 4°C overnight with 10 mg/ml HA reconstituted in PBS or PBS alone. Excess HA was removed by thorough washing of the wells with PBS.

Jurkat cells were stimulated overnight with 50 ng/ml PMA and 1 μg/ml PHA, collected by centrifugation, washed three times in PBS and resuspended at a density of 1 x10^6 cells/ml. A total of 10^5 cells were added to a triplicate set on both HA-coated and PBS wells. The plate was incubated at room temperature for 1 hr with unbound cells removed by 3 washes with PBS. Bound cells were stained with 0.25% Rose Bengal stain for 10 min at room temperature with subsequent washes with RPMI supplemented with 5% FCS. Cells were lysed in 200 μl solution of 50%

ethanol in PBS and the quantity of cell-released Rose Bengal stain measured on a Titretek Multiskan analyser at 540 nm.

2.2.22 HA binding analysis using flow cytometry

The protocol for cell staining with FITC conjugated HA (HA-FITC; generous gift from Dr. Hyman, Salk Institute, California, USA) is essentially as described in Section 2.2.16 with the following modifications.

Jurkat cells were stimulated overnight with 50 ng/ml PMA and 1 μg/ml PHA at a density of 1 $x10^6$ cells/ml and cells collected by centrifugation at 200g for 5 min. The cells were washed three times in PBS prior to analysis. Following incubation of Jurkat cells with heat inactivated rabbit serum and EMA, the cells were immediately placed under bright fluorescent light for 20 min. The cells were then washed and HA-FITC was added to the cells at either $^1/_{400}$ or $^1/_{800}$ dilution as recommended by Dr. Hyman (personal communication). Following a 45 min incubation on ice and in the dark, the cells were fixed and analyzed as described in Section 2.2.16.

2.2.23 Monoclonal antibody ligation of cell surface receptor

A total of 2 $x10^6$ Jurkat cells were collected by centrifugation at 800*g* for 5 min and resuspended in 1 ml ice cold PBS. The cells were incubated with the addition of 10 μg/ml of anti-CD44 QE7.3E8 mAb, 10 μg/ml anti-CD3 mAb or PBS for 20 min on ice. Cells were collected by centrifugation, washed twice with ice cold PBS and resuspended in 200 μl ice cold PBS containing 5 μg/ml rabbit anti-mouse immunoglobulin. The cells were incubated at 37°C for either 2 or 5 min respectively

and the reaction stopped by the addition of 800 µl ice cold PBS with cells collected by centrifugation at 13,000*g* for 1 min and resuspended in 60 µl PY-20 lysis buffer.

The analysis of protein phosphorylation by western blotting using the PY-20 anti-tyrosine phosphorylation mAb is essentially as described in Section 2.2.20 with the following modifications. After transfer of protein onto nitrocellulose the membrane is incubated in PY-20 antibody buffer. The PY-20 mAb is diluted $^{1}/_{1000}$ in PY-20 antibody buffer and all subsequent membrane washes used PY-20 wash buffer.

2.2.24 DNA Nucleotide Sequencing

Plasmid DNA constructs were sequenced using the ABI Prism™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, USA) as per manufacturers instructions. Briefly, 250 ng purified plasmid DNA (Section 2.2.12) was placed in a 0.5 ml reaction tube in a final volume of 20 µl containing 3.2 pmole oligonucleotide primer in 1x Terminator Ready Reaction Mix (dye-labelled dNTPs, Tris-HCl pH 9, $MgCl_2$, thermal stable pyrophosphatase and AmpliTaq DNA polymerase). The reaction was over-layed with mineral oil to prevent evaporation. Cycle sequencing was achieved by 25 cycles of 96°C for 30 sec, 50°C for 15 sec and 60°C for 4 min. All PCR reactions were performed on a TC1 Perkin Elmer DNA thermal cycler (Perkin Elmer, USA).

PCR products were purified by ethanol precipitation (Section 2.2.11). Sequence analysis of each DNA sample was performed on the ABI 373A DNA Sequencer at the DNA Sequencing unit (IMVS Adelaide, South Australia).

Chapter Three

Identification and Characterization of the CD44 v6 Transcripts in Human Peripheral Blood Mononuclear Cells

3.1 INTRODUCTION

The focus of this chapter is based upon conclusions derived from a study that hallmarked the functional significance of the CD44 v6 protein isoforms in an alloimmune response (Arch *et al.*, 1992).

In an *in vivo* rat model of allogeneic stimulation, injection of rat lymphocytes to a recipient strain led to the observation of a transient increase in cell surface expression of CD44 v6 proteins in lymphatic tissues, bone marrow, spleen, lymph nodes and Peyers patches. It was subsequently identified by dual labelling experiments that the CD44 v6 protein expression was restricted to macrophages, T cells and B cells and that the relative proportions varied with the source of lymphoid tissue. For example, within the lymph nodes the T cells were mainly positive whereas in the peritoneal cavity CD44 v6 protein expression was limited to B cells and macrophages. Flow cytometric sorting of CD44 v6 positive cells from lymph node demonstrated that the predominant CD44 v6 transcript contained only the v6 exon. In addition, no other variant exons were associated with this product as detected by RT-PCR thus characterizing this isoform as the $CD44_{v6}$ transcript.

Furthermore, the administration of anti-CD44 v6 mAb impeded the activation of lymphocytes implicating a role for the CD44 v6 protein in the alloimmune response. Whilst the mechanism involved in the suppression of the alloimmune response is undefined, it is tempting to speculate that it might involve the blocking of receptor-ligand interaction and that the CD44 v6 protein mediates signal transduction that leads to cellular activation.

Although a significant contribution of the literature has analyzed the expression of the CD44 v6 proteins in man, the characterization of the CD44 v6 mRNA transcripts in human peripheral blood mononuclear cells (PBMC) is limited. Reports have demonstrated an altered mRNA splicing mechanism of the CD44 v6 transcripts to occur in malignant lymphoid disorders such as Non Hodgkins lymphoma (Stauder *et al.*, 1995) and large cell lymphomas (Salles *et al.*, 1993), however, the precise structural composition of these CD44 v6 transcripts was not characterized. Although not defined, the occurrence of multiple alternately spliced transcripts expressed in T cells has been observed (Koopman *et al.*, 1993b).

Given the lack of literature concerning the analysis of CD44 v6 transcripts in human PBMC, the aims of the experiments described in this chapter were to identify the variant exon composition of the alternately spliced CD44 v6 transcripts by RT-PCR. Furthermore, the expression of these transcripts were characterized *in vitro* upon mitogenic challenges that mimic lymphocyte activation.

3.2 RESULTS

3.2.1 Standardization of mRNA loading by the normalization of β-actin mRNA using RT-PCR

3.2.1.1 Reverse transcription of total RNA isolated from PBMC

A total of 5 $x10^6$ PBMC were cultured at a density of 1 $x10^6$/ml in RPMI containing 10% FCS at 37°C under 5% CO_2. At incubation periods of 0, 2, 4, 6, 12, 24 and 48 hr the cells were pelleted by centrifugation and RNA extracted (Section 2.2.3). Each RNA sample was quantitated by spectrophotometry and 1 μg of total RNA was reverse transcribed (Section 2.2.5).

All experiments in this chapter were conducted on individual PBMC preparations from three healthy volunteers (2 males, 1 female). The results presented in this chapter are from one representative individual.

3.2.1.2 Evaluation of mRNA loading as assessed by β-actin

The quality and quantity of all synthesized cDNA was initially evaluated by PCR amplification (Section 2.2.6) of β-actin mRNA. In preliminary experiments, PCR amplification cycle numbers were varied to ascertain optimal conditions for detection of β-actin cDNA. Based on these experiments it was established that PCR amplification at 23 cycles was non-saturating and therefore appropriate to compare mRNA loading between different samples. Subsequent PCR analysis of β-actin cDNA synthesized from 1 μg of total RNA (Section 3.2.1.1) demonstrated products

of uniform intensity when UV-visualized by ethidium bromide staining of agarose gel (Figure 3.1.A).

The human genome consists of 18 β-actin pseudogenes defined as double stranded DNA copies of the β-actin mRNA that has been randomly re-inserted into the genomic DNA (Taylor and Heasman, 1994). Consequently, β-actin primers can yield an identical molecular size product from either reverse transcribed cDNA or contaminating genomic DNA. To confirm that all amplified products are derived from β-actin cDNA rather than genomic DNA, 1μg of total RNA was PCR amplified under identical PCR conditions. No PCR products were observed implying that pseudogene contamination was too low for detection at 23 cycles of PCR amplification (Figure 3.1.B). However, amplification of the β-actin pseudogene was apparent when total RNA was subject to higher cycles of PCR amplification (>35 cycles) indicating genomic DNA carry over in the extracted RNA (Figure 3.1.C).

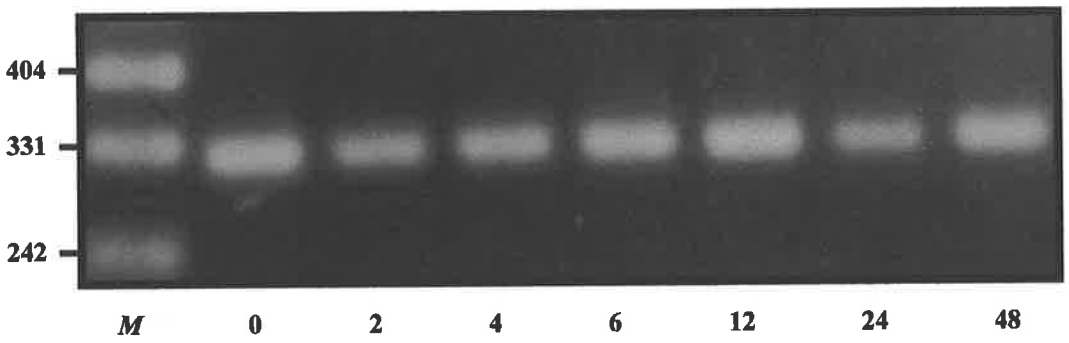
From these experiments, the mRNA loading of multiple cDNA templates with respect to expression of the β-actin mRNA was assessed at 23 cycles to avoid interference from β-actin pseudogene. Although the PCR fragments were not quantified, any cDNA samples that demonstrated unusually high or low β-actin expression were re-adjusted prior to the analysis of CD44 mRNA expression to achieve equal loading and allow an unbiased comparison of CD44 mRNA expression between different time points or treatments of PBMC *in vitro*.

Figure 3.1 Assessment of mRNA loading with respect to expression of β-actin mRNA as detected by PCR amplification.

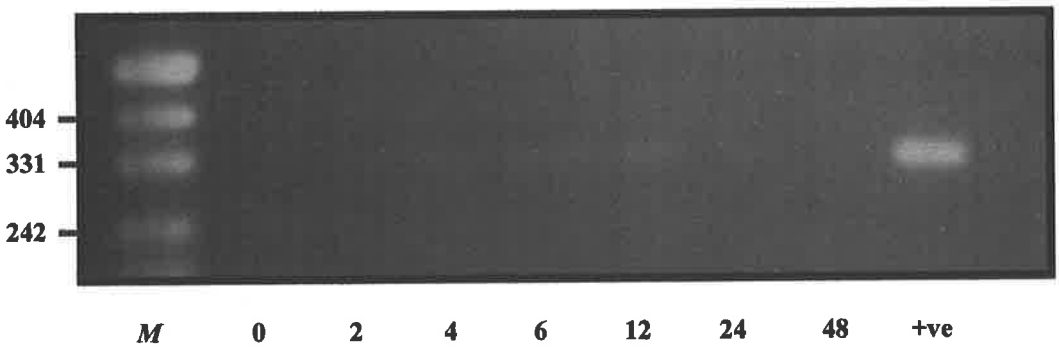
A total of 5 x10^6 PBMC were cultured for various time periods (0-48 hr) at a density of 1 x10^6/ml in T-25cm^2 flasks. Total RNA was extracted and 1 μg was reverse transcribed. β-actin cDNA was amplified at 23 cycles with PCR products (10 μl) electrophoresed in a 1.5% agarose gel and visualized under UV light after ethidium bromide staining (Panel A).

In addition, 1 μg of total RNA was PCR amplified at 23 (Panel B) or 35 cycles (Panel C). The validity of the PCR reaction was confirmed by inclusion of a positive cDNA template (+ve). Molecular weight marker (M) was pUC19 plasmid DNA digested with HpaII.

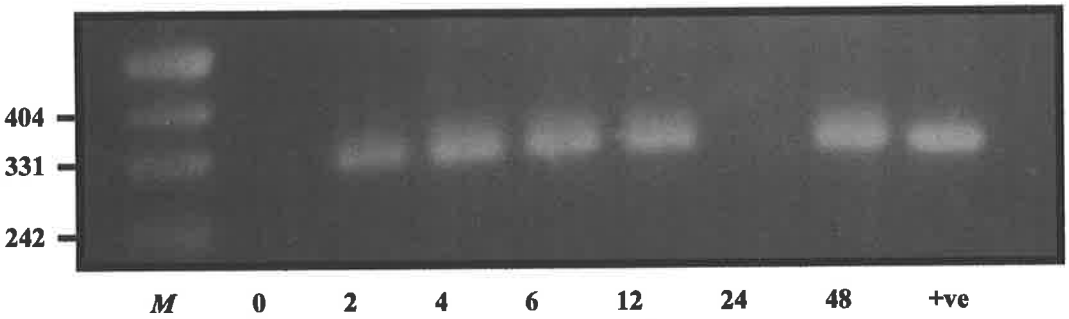
A.



B.



C.



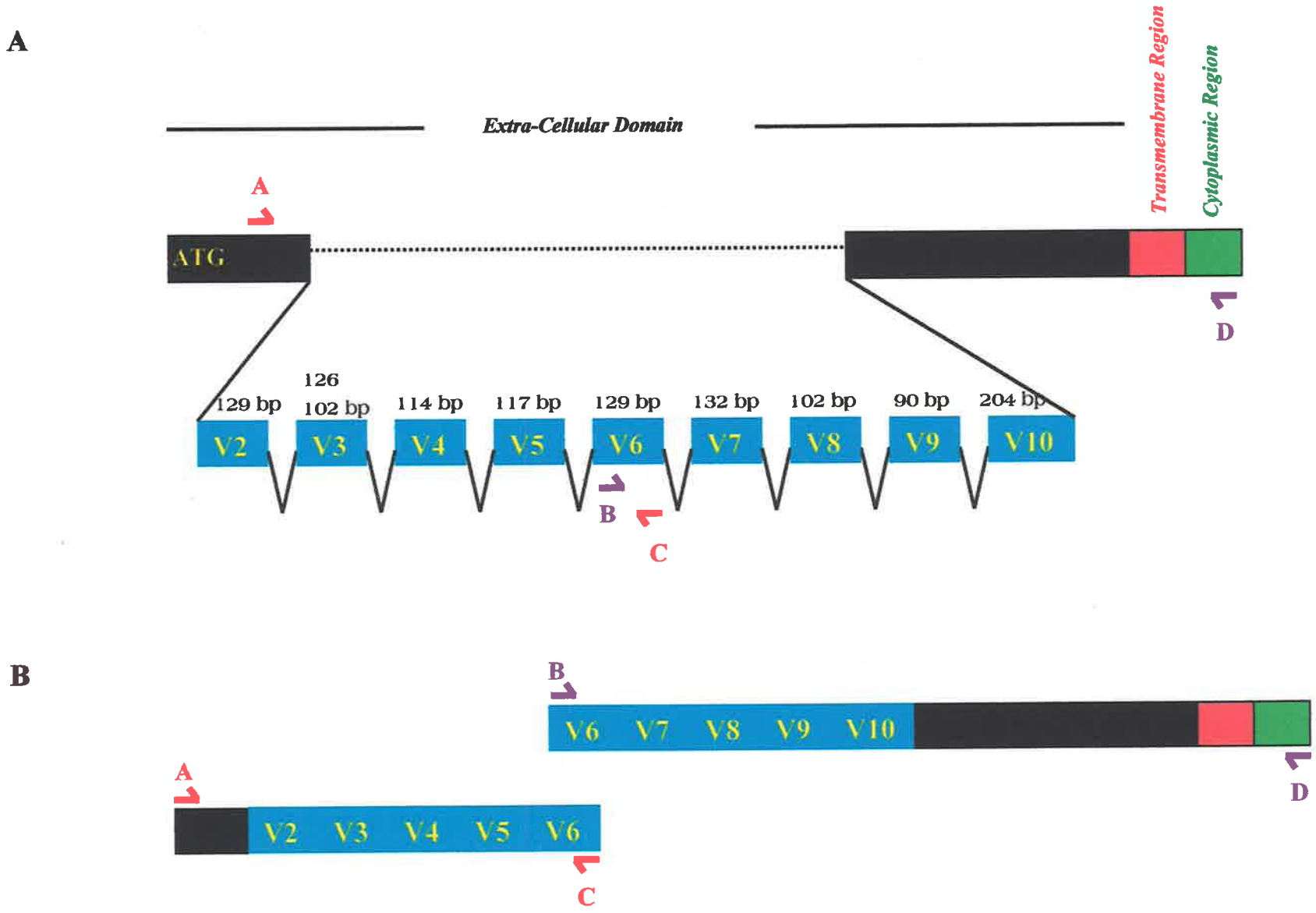
Time (hours)

3.2.2 Detection of CD44 v6 mRNA transcripts in resting human PBMC

3.2.2.1 Rationale for detecting CD44 v6 transcripts by the use of exon specific primers

PCR amplification using invariant primers that flank the CD44 variant exon region will often yield a dominant product representing the $CD44_{std}$ transcript (Matsumura *et al.*, 1994, Fichter *et al.*, 1997, Simon *et al.*, 1996). During the PCR amplification, the invariant primers can hybridize to common sequences present in both the $CD44_{std}$ and variant transcripts thus allowing amplification of both CD44 mRNA isoforms. However, because of the abundance of $CD44_{std}$ mRNA, the amplification of the variant transcripts is almost undetectable upon UV-visualization of ethidium bromide stained agarose gels.

To allow amplification of the CD44 v6 transcripts without competition from the $CD44_{std}$ transcript, v6 exon-specific primers were used in conjunction with invariant primers. The two pairs of primers were designed to characterize the variant exon region both up and down-stream of the v6 exon (Figure 3.2.A). A reverse v6 primer (primer C) in conjunction with a forward primer directed towards the invariant region (primer A) was used to amplify upstream of the v6 exon. Similarly, a forward v6 primer (primer B) was used in conjunction with a reverse primer directed towards the invariant region (primer D) to amplify downstream of the v6 exon (Figure 3.2.B).


A
Extra-Cellular Domain
Transmembrane Region
Cytoplasmic Region
A
ATG
D
126
129 bp
102 bp
114 bp
117 bp
129 bp
132 bp
102 bp
90 bp
204 bp
V2
V3
V4
V5
V6
V7
V8
V9
V10
B
C
B
V6
V7
V8
V9
V10
D
A
V2
V3
V4
V5
V6
C

3.2.2.2 PCR analysis of the composition of variant exons upstream of the v6 exon

Primers A and C were used to ascertain the variant exon composition upstream of the v6 exon. PCR amplification yielded a single product with a molecular size of approximately 460 bp (Figure 3.3.A). This fragment was present in all samples at a uniform intensity with no improved detection of other v6 containing transcripts despite the increase to 50 PCR cycles (Figure 3.3.B).

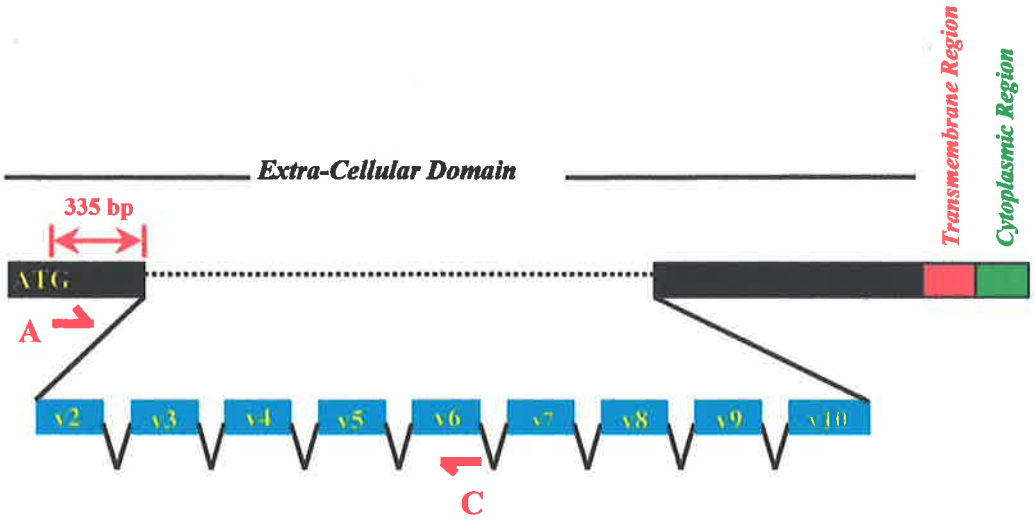
Based on the position of primers A and C (Figure 3.3.C) the 460 bp PCR product was deduced to consist of 335 bp invariant CD44 sequence and 126 bp v6 exon. In accordance with previous reports (Koopman *et al.*, 1990, Salles *et al.*, 1993) it was concluded that the CD44 v6 transcripts expressed in PBMC do not contain any combination of the v1-v5 exons.

3.2.2.3 PCR analysis of the composition of variant exons downstream of the v6 exon

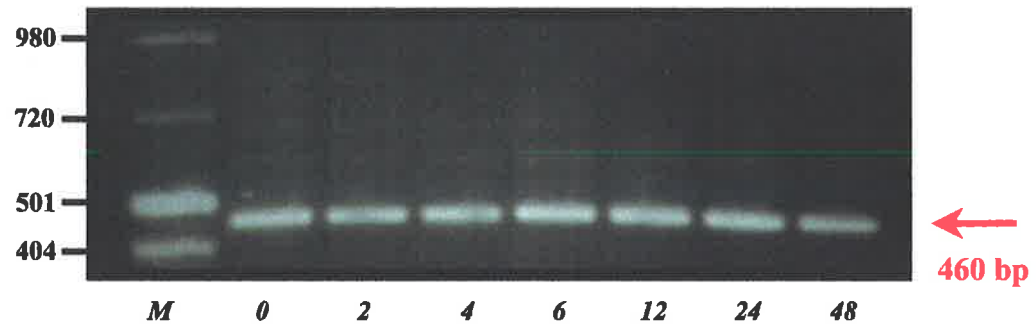
Primers B and D were used to ascertain the combination of variant exons downstream (v6-10) of the v6 exon. As shown in Figure 3.4, PCR amplification revealed the presence of four dominant products in freshly isolated PBMC with molecular sizes of 1100, 900, 740 and 530 bp respectively. From the location of primer D each amplified product contains 410 bp of invariant CD44 sequence. Subsequent calculations determined the molecular size of the variant exon inserts as 690, 490, 330 and 120 bp respectively.

Figure 3.3 Identification of the variant exon composition upstream of the v6 exon by PCR analysis.

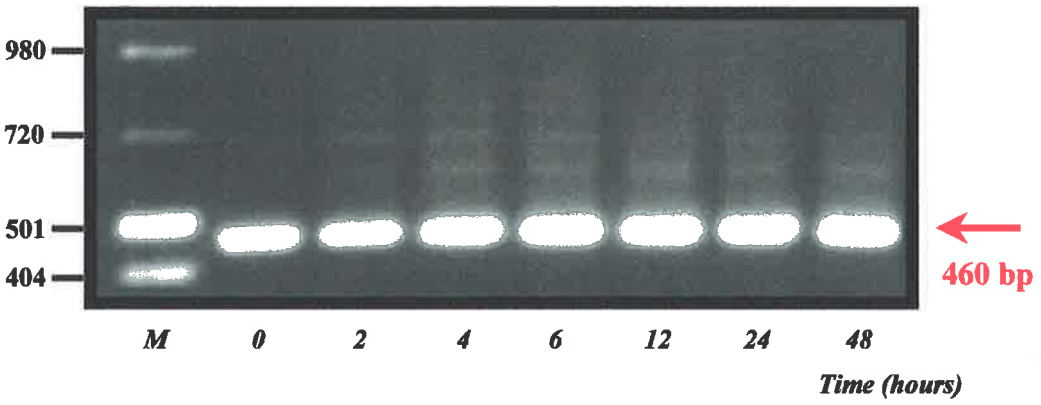
Total RNA was extracted from cultured PBMC and 1 μg was reverse transcribed. Using primers A and C, the composition of the variant exons upstream of the v6 exon was identified by PCR amplification at 35 cycles (Panel A) and 50 cycles (Panel B). The PCR products (10 μl) were electrophoresed in a 1.2% agarose gel and UV-visualized by ethidium bromide staining. The 460 bp product which was present in all samples was deduced to contain the invariant portion of the CD44 molecule immediately upstream of the v6 exon (Panel C). Molecular weight marker (M) was SPP-1 bacteriophage DNA digested with EcoRI and pUC19 plasmid DNA digested with HpaII.



A. *35 Cycle Amplification*



B. *50 Cycle Amplification*



C.

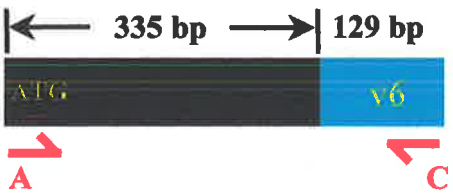
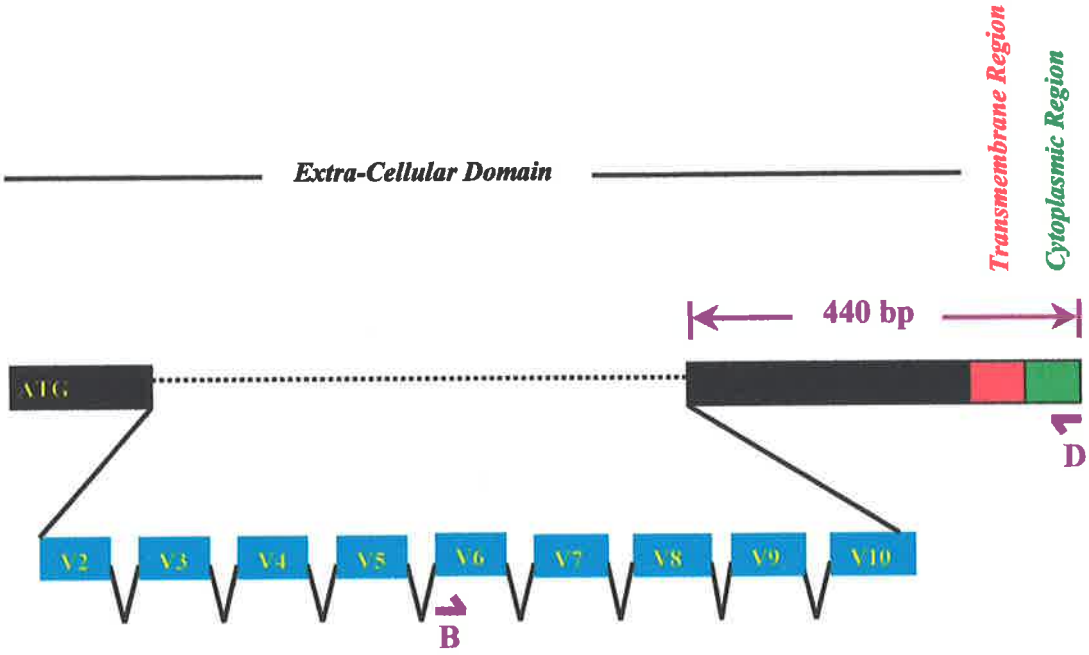
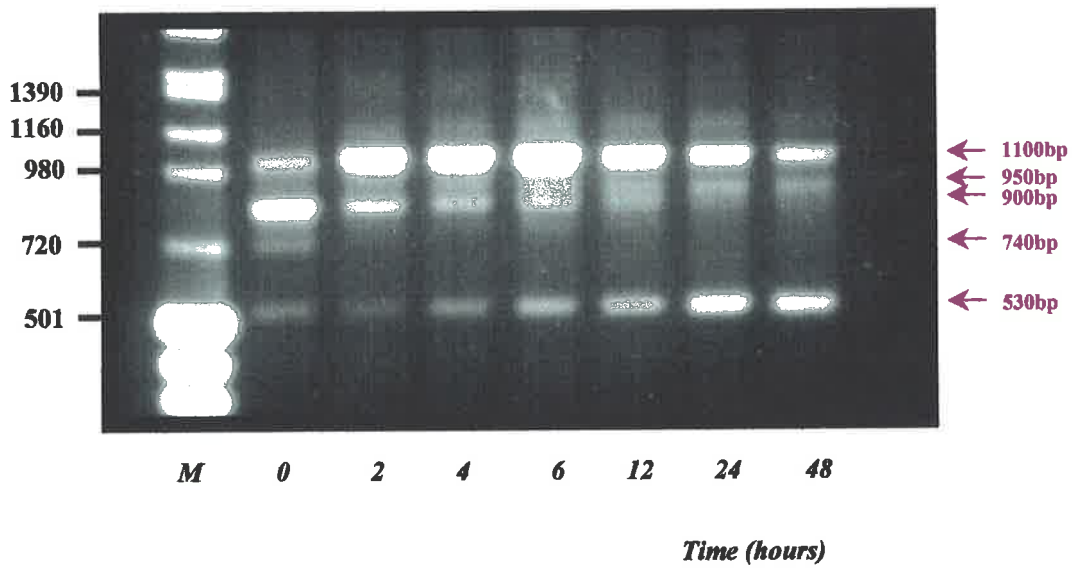


Figure 3.4 Characterization of alternately spliced transcripts detected by PCR amplification downstream of the v6 exon.

Total RNA was extracted from cultured PBMC and 1 μg was reverse transcribed. Using primers B and D, the variant exons downstream of the v6 exon was PCR amplified for 35 cycles. The PCR products (10 μl) were resolved in a 1.2% agarose gel and UV-visualized by ethidium bromide staining. Multiple alternately spliced CD44 v6 transcripts of molecular size 1100, 950, 900, 740 and 530 bp were detected. Molecular weight marker (M) was SPP-1 bacteriophage DNA digested with EcoRI and pUC19 plasmid DNA digested with HpaII.


Extra-Cellular Domain
Transmembrane Region
Cytoplasmic Region
440 bp
ATG
D
V2
V3
V4
V5
V6
V7
V8
V9
V10
B


1390
1160
980
720
501
1100bp
950bp
900bp
740bp
530bp
M
0
2
4
6
12
24
48
Time (hours)

Variant exon inserts of 690 and 120 bp correspond to products containing the maximal (v6-10) and minimal (v6) number of exons that can be incorporated from the v6-10 region. However, multiple exon usage of the v6-10 exons can generate variant exon inserts of 490 and 330 bp in molecular size. Consequently, the composition of the 490 and 330 bp variant exon inserts could not be deduced based on molecular size alone.

3.2.2.4 Changes in relative expression of the CD44 v6 transcripts upon culturing of PBMC

Culturing of PBMC onto plastic produced changes in the relative expression of the CD44 v6 transcripts. These changes transpired in a time dependant manner with expression of the 530 bp product increasing simultaneously with a decrease in the expression of the 1100 bp product. Furthermore, the expression of the 740 bp product is absent upon culturing of PBMC while expression of a 950 bp product appears initially at 12 hr of culture and is maintained with further periods of incubation.

3.2.3 Determination of variant exon composition of the CD44 v6 transcripts

3.2.3.1 Southern analysis of the CD44 v6 transcripts amplified using v6 exon specific primers

To define the variant exon composition of the four CD44 v6 transcripts (Section 3.2.2.3), the amplified products using primers B and D from the 0 and 6 hr time periods were blotted for Southern analysis. Replicates of PCR products from

the 0 and 6 hr samples were electrophoresed in a 1.2% agarose gel and transferred onto nylon membrane (Section 2.2.8). The nylon membrane was cut so that each strip contained PCR products from both 0 and 6 hr samples. Individual membrane strips were subsequently hybridized with biotinylated oligonucleotides specific for the v6, v7, v8, v9 or v10 exon (Section 2.2.9).

As shown in Figure 3.5, the PCR product with the molecular size of 1100 bp hybridized with all probes and was deduced to contain the v6-10 exons. This confirms the expected identity of this product as previously postulated (Section 3.2.2.3).

The 900 bp product hybridized with all but the v10 probe and thus confirmed that the transcript contained exons v6-9. It was previously determined that this product contains a variant exon region of approximately 490 bp (Section 3.2.2.3). From the nucleotide sequence of the CD44 gene the molecular size of the v6-9 exons is 453bp.

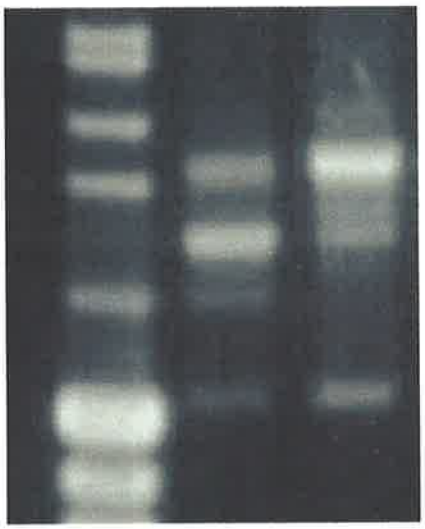
The 740 bp product hybridized with the v6, v8 and v9 probes identifying this product as containing the v6,v8-9 exons. The variant exon region of this product was previously concluded to be approximately 330 bp in molecular size (Section 3.2.2.3). Based on the nucleotide sequence of the CD44 gene the combined molecular size of the v6, v8-9 exons is 321 bp.

Figure 3.5 Identification of the variant exon composition downstream the v6 exon by Southern analysis.

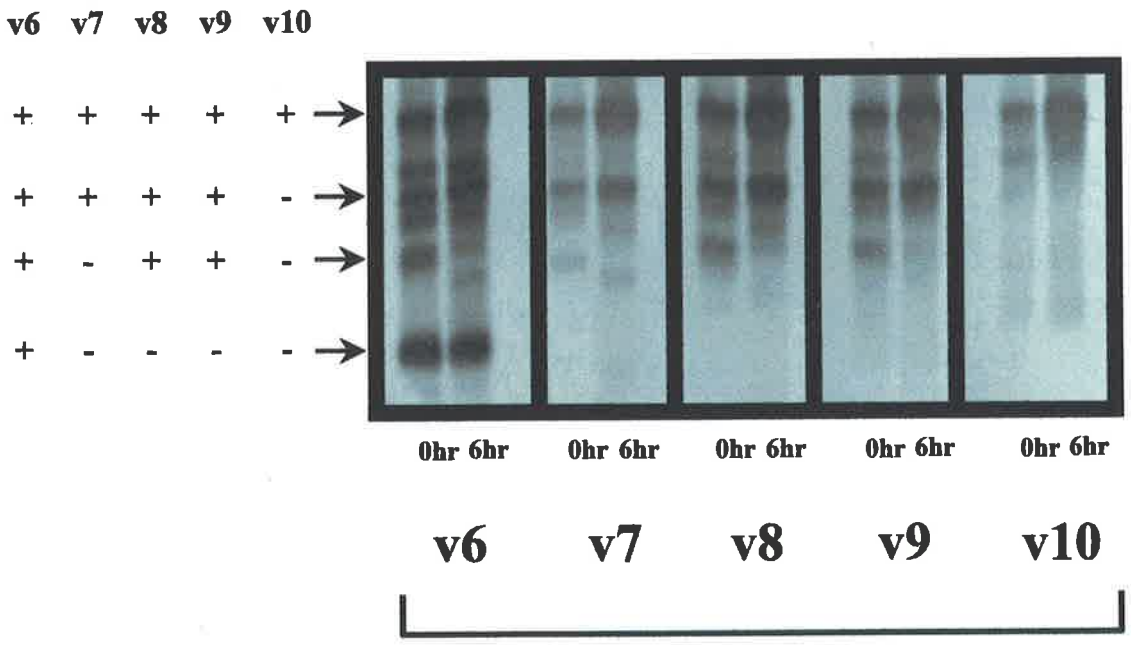
cDNA samples from freshly isolated (0 hr) and 6 hr cultured PBMC were PCR amplified using primers B and D for 35 cycles (Panel A). PCR products (10 µl) were electrophoresed in a 1.2% agarose gel in replicate pairs each containing the amplified fragments from the 0 and 6 hr time points. Following an overnight transfer onto nylon membrane, each replicate pair was separated and incubated in blocking solution for 4 hr at room temperature. The replicate pairs were individually probed overnight at 42°C with a biotinylated oligonucleotide directed towards the v6, v7, v8, v9 or v10 exon. After washing at 42°C in 0.1x SSC and 0.5% SDS, the hybridized biotinylated probes were detected with a streptavidin alkaline phosphatase conjugate and the Southern blot developed using the chromogenic substrates NBT/ XPO_4 (Panel B). Molecular weight marker (M) was SPP-1 bacteriophage DNA digested with EcoRI and pUC19 plasmid DNA digested with HpaII.

Each PCR product generated by amplification using primers B and D were ligated into the pGEM T^+ vector (Section 2.2.10.2) and transformed into competent *E.coli* TG1-α cells (Section 2.2.10.3). Positive recombinant colonies were selected for ampicillin resistance as well as blue/ white colour selection (Section 2.2.10.3). Plasmid DNA was prepared from recombinant colonies (Section 2.2.12) with subsequent determination of nucleotide composition of the insert by DNA sequencing (Section 2.2.24). Nucleotide sequence comparison (see Appendix) of each PCR product insert to that published by Screaton *et al* (1992) confirmed the identity of these isoforms as $CD44_{v6-10}$, $CD44_{v6-9}$, $CD44_{v6,8-9}$ and $CD44_{v6}$.

A.


1100 bp
900 bp
740 bp
530 bp
M
0 hr
6 hr

B.


v6 v7 v8 v9 v10
+ + + + +
+ + + + -
+ - + + -
+ - - - -
0hr 6hr 0hr 6hr 0hr 6hr 0hr 6hr 0hr 6hr
v6 v7 v8 v9 v10
Biotinylated oligonucleotide probes

The 530 bp product hybridized with only the v6 probe and was deduced to contain the v6 exon. This confirms the expected identity of this product as previously postulated (Section 3.2.2.3).

It is concluded on the basis of molecular size of the PCR products and Southern analysis that the full length v6 exon containing transcripts expressed in resting human PBMC are $CD44_{v6\text{-}10}$, $CD44_{v6\text{-}9}$, $CD44_{v6,v8\text{-}9}$ and $CD44_{v6}$ (Figure 3.6).

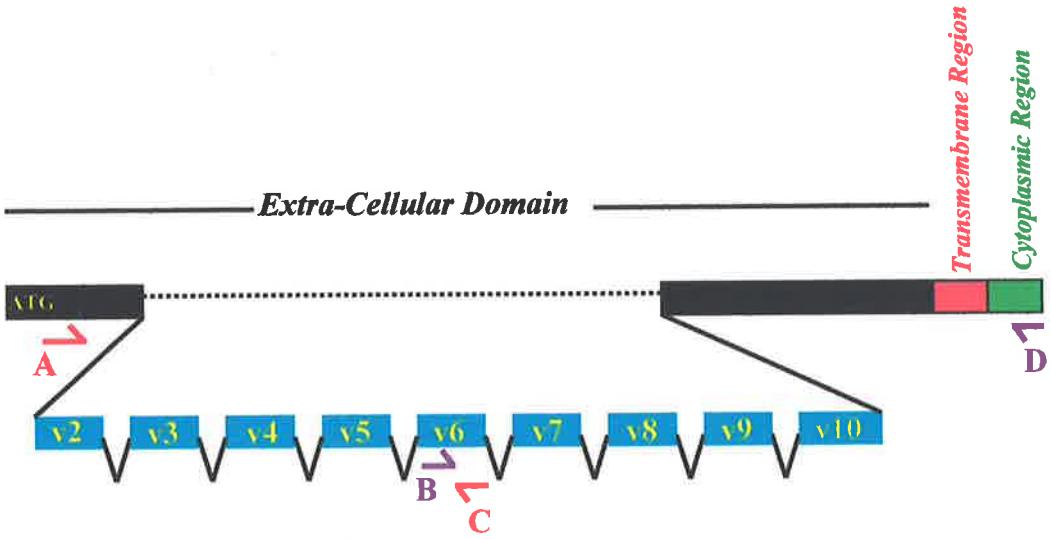
3.2.3.2 Confirmation of the CD44 v6 transcripts by PCR amplification using invariant primers

In several studies expression of CD44 variant transcripts was analyzed by RT-PCR using invariant primers that flank the variant exon region followed by Southern analysis using a labeled variant exon specific oligonucleotide (Salles *et al.*, 1993, Matsumura *et al.*, 1994, Stauder *et al.*, 1995, Woodman *et al.*, 1996, Croft *et al.*, 1997). This alternate method was therefore used to verify the identity of the CD44 v6 transcripts as previously determined (Section 3.2.3.1).

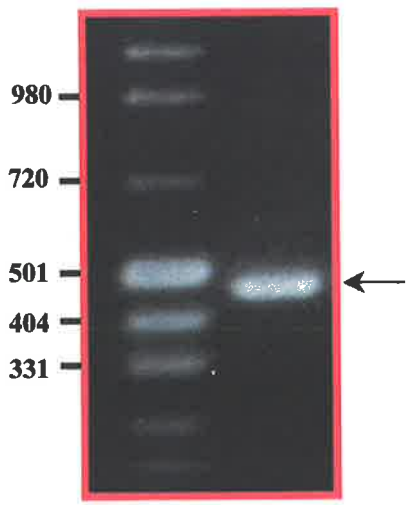
Essentially, cDNA samples from the 0, 6 and 48 hr time points were amplified for 50 cycles using the invariant primers A and D. The PCR products generated using primers B and D from the 6 hr time period (Figure 3.4) was included in the gel electrophoresis to serve as a positive control in the Southern hybridization and also as a transferable molecular size marker.

Figure 3.6 Characterization of the full length CD44 v6 transcripts expressed in human PBMC.

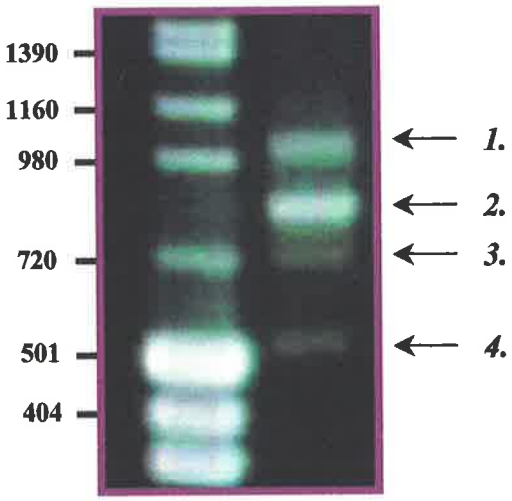
The CD44 v6 transcripts expressed in freshly isolated PBMC were identified by PCR analysis of the up- and down-stream regions flanking the v6 exon using specific primers directed towards the v6 exon in combination with invariant primers (Panel A). The combined PCR analysis of the v6 exon determined that the full length CD44 v6 transcripts expressed are $CD44_{v6\text{-}10}$, $CD44_{v6\text{-}9}$ $CD44_{v6,8\text{-}9}$ and $CD44_{v6}$ as schematically represented in Panel B.



A.

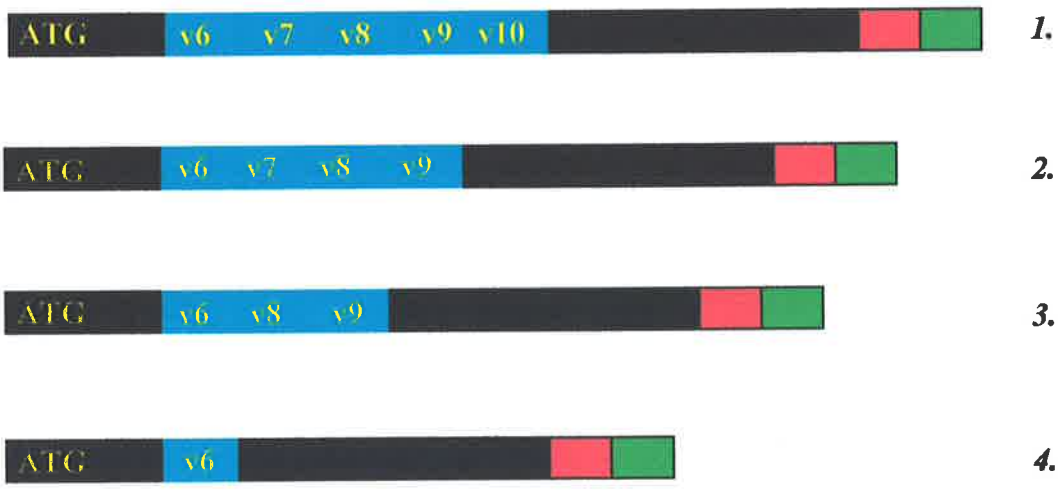


PCR analysis upstream the v6 exon using primers A and C



PCR analysis downstream the v6 exon using primers B and D

B.



A dominant product of 770 bp in molecular size and faint larger products were detected in all samples (Figure 3.7.A). Based on the position of the PCR primers it is concluded that the 770 bp product represents the $CD44_{std}$ transcript and the larger products those of variant transcripts.

For detection of the v6 containing transcripts, the amplified products were transferred onto nylon membrane and probed with a biotinylated v6 oligonucleotide (primer C) that hybridized to three distinct transcripts with estimated molecular sizes of 1400, 1200 and 860 bp respectively (Figure 3.7.B). By subtracting 770 bp of CD44 invariant sequence from the molecular sizes of the hybridized products, the variant exon regions were calculated to be 630, 430 and 90 bp, respectively.

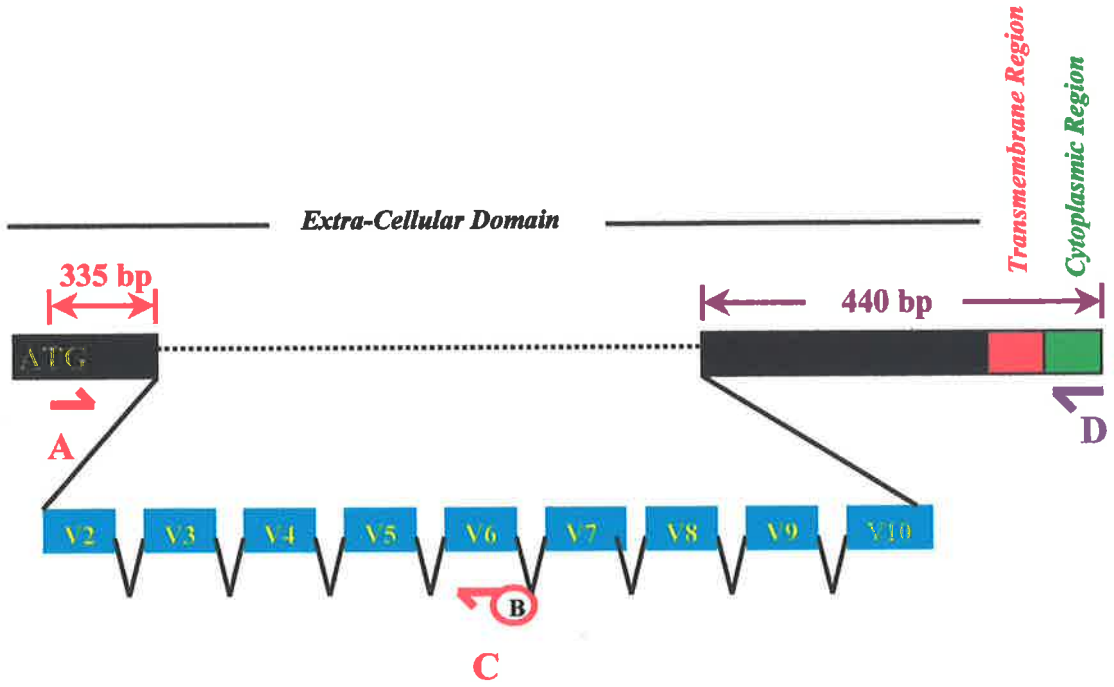
From the nucleotide sequence of the CD44 gene, the molecular size of the variant exon region of the $CD44_{v6\text{-}10}$, $CD44_{v6\text{-}9}$ and $CD44_{v6}$ transcripts are 657, 453 and 129 bp respectively. It is inferred from the molecular sizes of the estimated variant exon region and its correlation to the calculated nucleotide sequence that the 1400, 1200 and 860 bp hybridized products represent the $CD44_{v6\text{-}10}$, $CD44_{v6\text{-}9}$ and $CD44_{v6}$ transcripts respectively.

Comparing the two PCR-based approaches, the PCR amplification using invariant primers failed to amplify the $CD44_{v6,8\text{-}9}$ transcript and did not demonstrate temporal changes in the relative expression of the transcripts upon culturing of PBMC. From these observations it is concluded that PCR amplification using exon-specific primers is a more sensitive method for analyzing the expression of the CD44 variant transcripts.

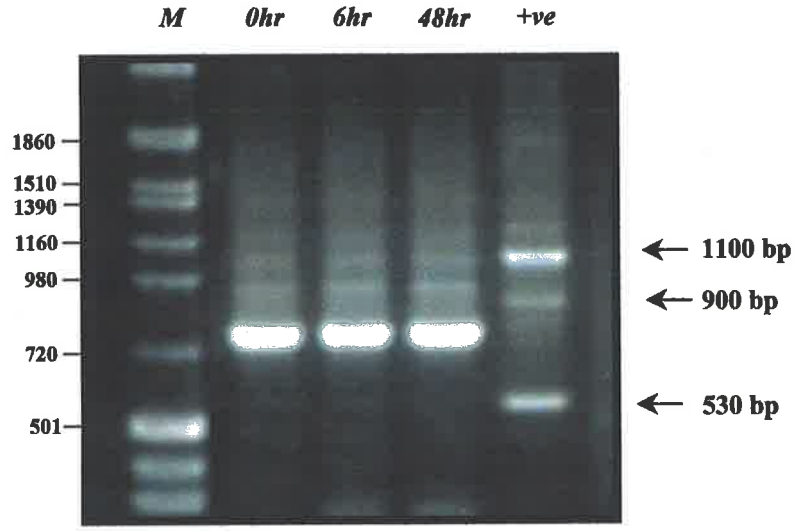
Figure 3.7 Verification of the variant exon composition of CD44 v6 transcripts by Southern analysis.

cDNA from PBMC samples (0, 6 and 48 hr) were PCR amplified using primers A and D at 50 cycles and products (10 μl) were electrophoresed in a 1.2% agarose gel and UV-visualized by ethidium bromide staining (Panel A). The amplified products generated using primers B and D (+ve) from 6 hr cultured PBMC (Figure 3.4) was included in the electrophoresis.

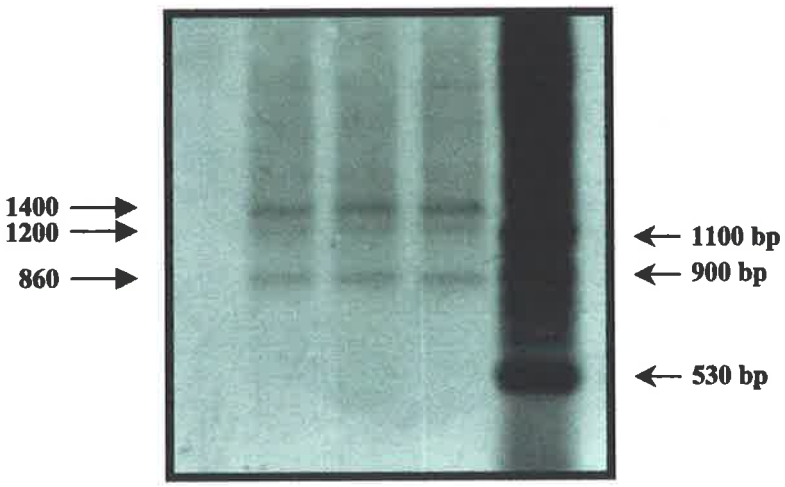
After overnight transfer onto nylon membrane and incubation in blocking solution for 4 hr at room temperature, the membrane was hybridized overnight at 42°C with a biotinylated oligonucleotide directed towards the v6 exon (primer C). After washing at 42°C in 0.1x SSC and 0.5% SDS, the hybridized biotinylated probes were detected with a streptavidin alkaline phosphatase conjugate and the Southern blot developed using the chromogenic substrates NBT/ XPO_4 (Panel B). Three CD44 v6 transcripts were detected in each sample with estimated molecular sizes of 1400, 1200 and 860 bp respectively. Molecular weight marker (M) was SPP-1 bacteriophage DNA digested with EcoRI and pUC19 plasmid DNA digested with HpaII.


Transmembrane Region
Cytoplasmic Region
Extra-Cellular Domain
335 bp
440 bp
ATG
A
D
V2
V3
V4
V5
V6
V7
V8
V9
V10
B
C

A.


M
0hr
6hr
48hr
+ve
1860
1510
1390
1160
980
720
501
1100 bp
900 bp
530 bp

B.


1400
1200
860
1100 bp
900 bp
530 bp

3.2.4 Effect of fetal calf serum on CD44 v6 mRNA expression

As previously demonstrated (Section 3.2.2.4), the relative expression of the CD44 v6 transcripts exhibit temporal changes upon *in vitro* culture of PBMC. It was therefore intended to investigate if fetal calf serum (FCS) could influence the expression of the CD44 v6 transcripts.

PBMC were cultured in the absence or presence of 10% FCS for 2 and 6 hr time periods. This experiment demonstrated no differences in the expression of CD44 v6 transcripts between the FCS concentrations (data not shown). From this observation it was concluded that FCS does not influence expression of the CD44 v6 transcripts.

3.2.5 Effect of cellular activation on the expression of the CD44 v6 transcripts

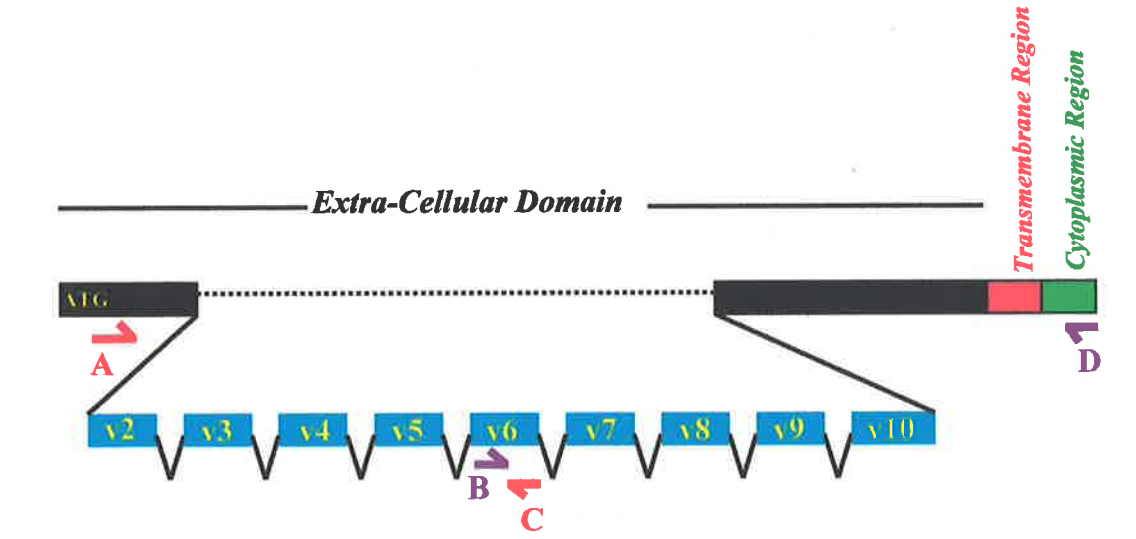
Having characterized the CD44 v6 transcripts in resting PBMC, it was of interest to ascertain if expression of these transcripts was altered by mitogenic stimulation (Section 2.2.2). PBMC were stimulated for 12 hr individually with either 5 μg/ml anti-CD3 mAb, 100 U/ml IL-2, 10 μg/ml PHA, 10 ng/ml PMA, 10 μg/ml Con A, 0.6 μg/ml Ionomycin or the mixed lymphocyte reaction.

PCR amplification upstream of the v6 exon using primers A and C yielded the previously characterized 460 bp product (Figure 3.8.A). This product was present in all samples at uniform intensity demonstrating that no changes were induced upstream of the v6 exon upon PBMC activation. However, PCR amplification

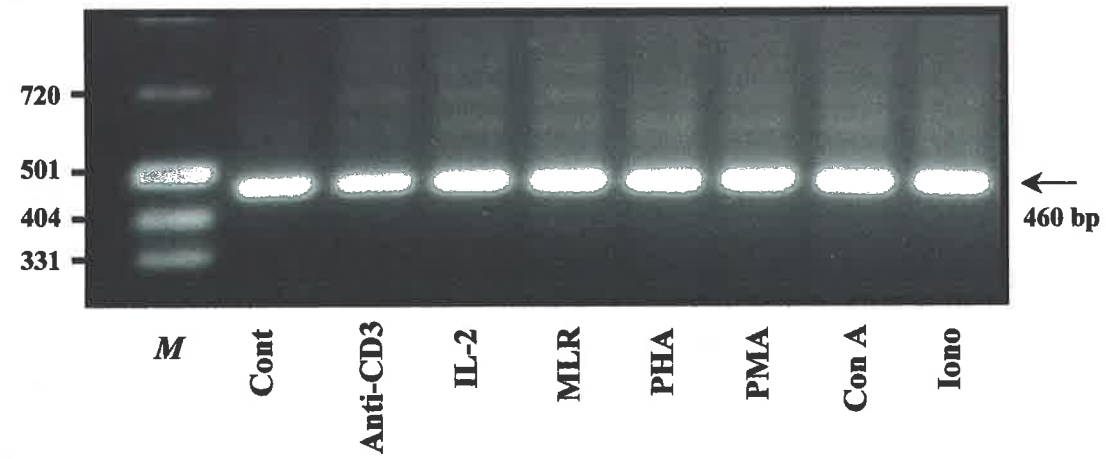
Figure 3.8 Expression of the CD44 v6 transcripts in mitogenically stimulated PBMC.

A total of 5 x10^6 human PBMC were subject to a variety of stimuli for 12 hr: 5 μg/ml anti-CD3 mAb, 100 U/ml IL-2, mixed lymphocyte reaction (MLR), 10 μg/ml PHA, 10 ng/ml PMA, 10 μg/ml Con A or 0.6 μg/ml Ionomycin. Total RNA was extracted from the cells and 1 μg was reverse transcribed. All PCR products (10 μl) were electrophoresed in a 1.2% agarose gel and UV-visualized by ethidium bromide staining.

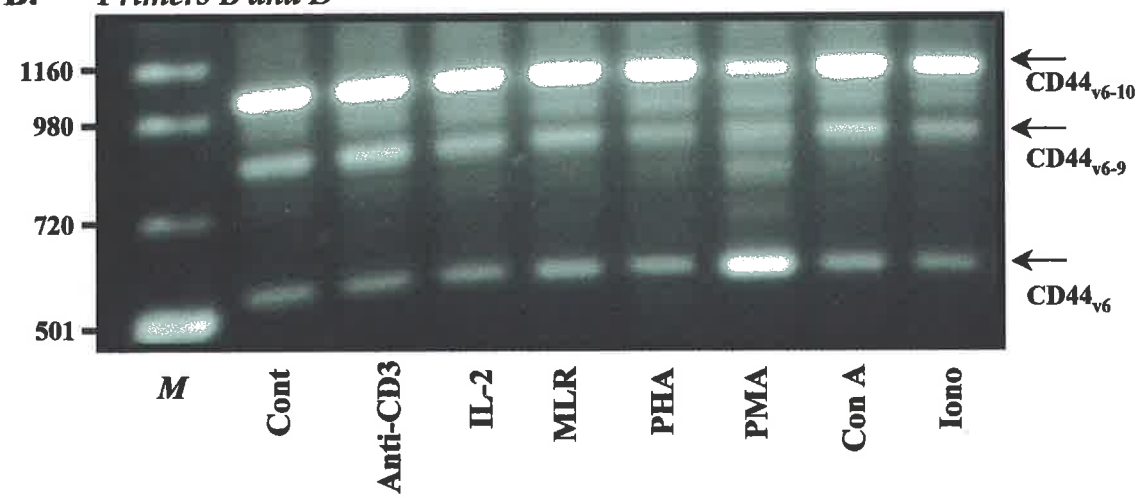
The upstream region flanking the v6 exon was analyzed by using primers A and C at 35 cycles of PCR amplification (Panel A). Subsequently, the downstream region flanking the v6 exon was analyzed using primers B and D at 35 cycles of PCR amplification (Panel B). Molecular weight marker (M) was SPP-1 bacteriophage DNA digested with EcoRI and pUC19 plasmid DNA digested with HpaII.



A. *Primers A and C*



B. *Primers B and D*



β-actin

downstream of the v6 exon demonstrated that PMA treatment alters expression of the v6 exon containing transcripts by increasing the expression of the $CD44_{v6}$ with a concomitant decrease in the $CD44_{v6\text{-}10}$ transcript (Figure 3.8.B).

Two novel products of approximately 800 and 600 bp in molecular size were detected in PMA treated cells. These products may represent newly synthesized CD44 v6 transcripts or heteroduplexes formed from the hybridization of complementary regions between the $CD44_{v6}$ and $CD44_{v6\text{-}10}$ or $CD44_{v6\text{-}9}$ transcripts.

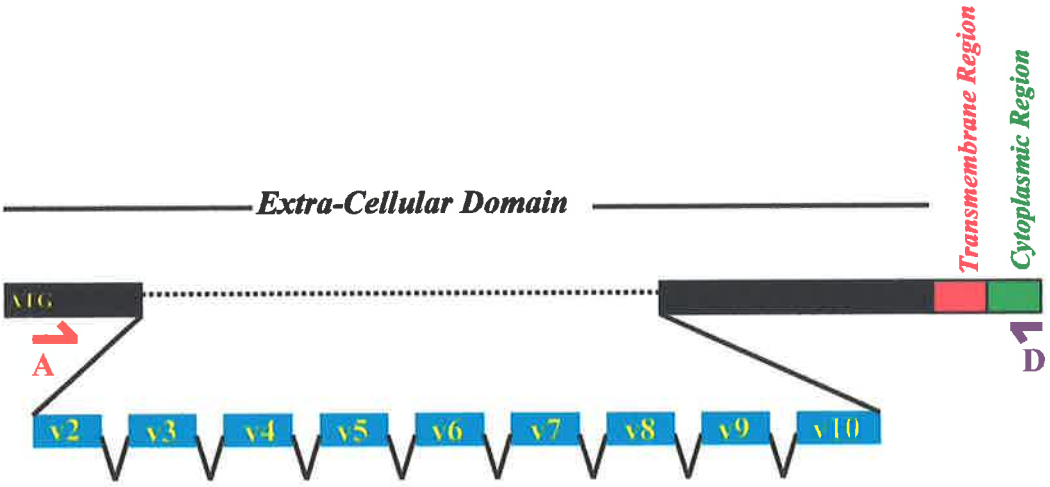
CD44 variant transcripts that do not contain the v6 exon may be induced upon cellular activation but are excluded from the PCR analysis based on the variant exon specificity of the primers used. Therefore all CD44 isoforms were amplified using primers A and D at 50 cycles of PCR amplification. As shown in Figure 3.9.A, the 770 bp band representing the $CD44_{std}$ transcript is predominant with no detection of any unique larger molecular sized products. Confirmation of cellular activation by mitogenic stimulation was demonstrated by the induction of the interferon-γ mRNA in treated cells by RT-PCR (Figure 3.9.B).

3.2.6 Further analysis of PMA stimulation of PBMC and expression of the CD44 v6 transcripts

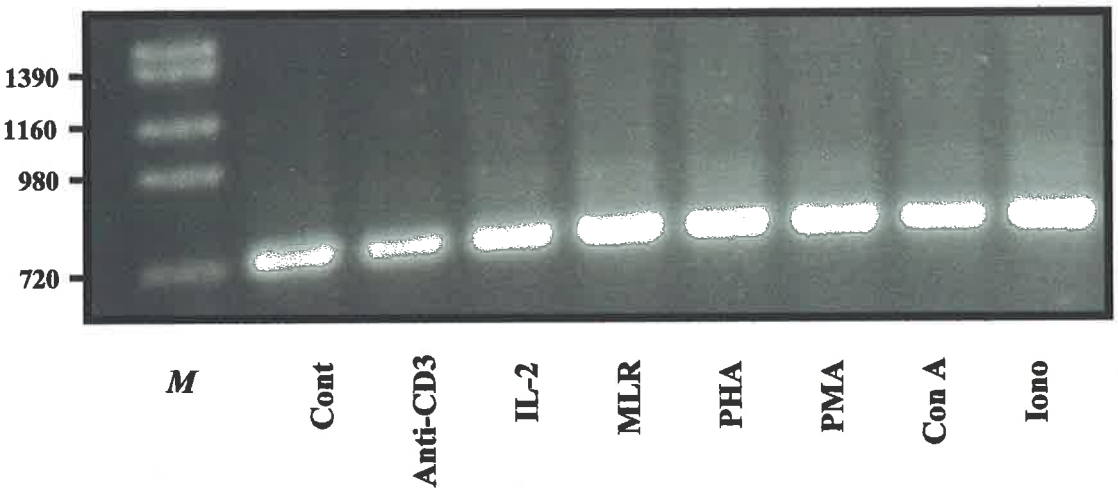
PMA is purchased as a dry chemical and is solubilized in dimethyl sulphoxide (DMSO). However, induction of the $CD44_{std}$ protein by 3% DMSO treatment has been demonstrated in the SW620 colon epithelial cell line (Omary *et al.*, 1992). This suggests that the changes in expression of the CD44 v6 transcripts induced by PMA treatment of PBMC may result from either the PMA solute or DMSO solvent.

Figure 3.9 Expression of total CD44 transcripts in mitogenically stimulated PBMC.

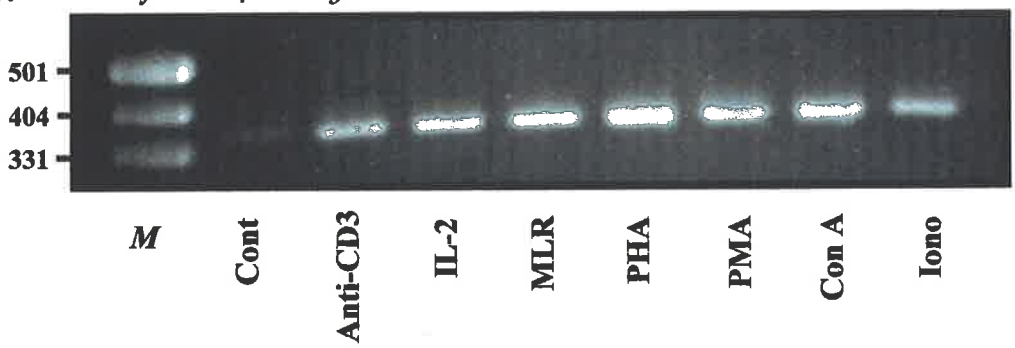
Total RNA was extracted from PBMC that were stimulated for 12 hr with a variety of stimuli and 1μg was reverse transcribed. Using the invariant primers A and D all isoforms of the CD44 molecule was amplified at 50 cycles. The PCR products (10 μl) were electrophoresed in a 1.2% agarose gel and UV-visualized by ethidium bromide staining (Panel A). Cellular activation of PBMC by the different stimuli was demonstrated by PCR amplification of the interferon-γ cDNA at 35 cycles (Panel B). Molecular weight marker (M) was SPP-1 bacteriophage DNA digested with EcoRI and pUC19 plasmid DNA digested with HpaII.



A. ***Primers A and D***



B. ***Interferon- γ Analysis***



PBMC were stimulated for 6 hr with 10 ng/ml PMA (in 0.1% DMSO) or 0.1% DMSO. Analysis downstream of the v6 exon demonstrated that DMSO treatment of PBMC did not change the expression of the CD44 v6 transcripts (Figure 3.10). However, PMA treatment had induced the previously mentioned changes in expression of the CD44 v6 transcripts (Section 3.2.5). It was therefore concluded that signal transduction pathways initiated by PMA will result in an altered expression of the CD44 v6 transcripts.

The kinetics of the PMA-induced changes in expression of the CD44 v6 transcripts was determined at the following time points: 2, 6, 12, 24 and 48 hr. Unstimulated time matched PBMC samples were included for comparison.

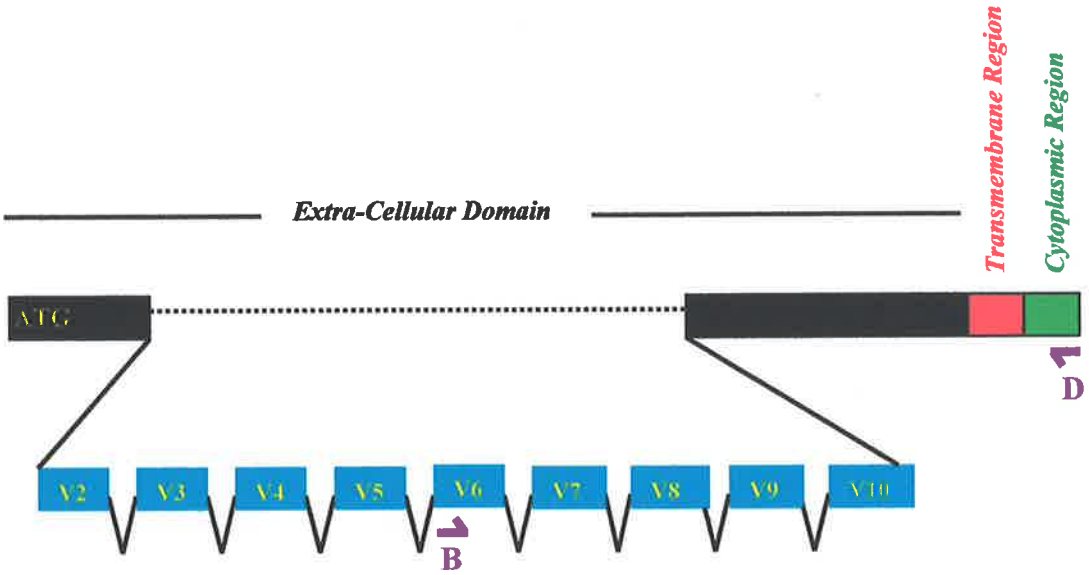
PCR amplification upstream of the v6 exon using primers A and C yielded the previously characterized 460 bp product (Figure 3.11.A). This product was present in all samples at uniform intensity. PCR amplification downstream of the v6 exon demonstrated an induced $CD44_{v6}$ expression concomitant with a decrease in the $CD44_{v6\text{-}10}$ transcript (Figure 3.11.B). These changes occurred as early as 2 hr stimulation and was consistent for up to 48 hr.

3.3 DISCUSSION

PCR amplification with exon-specific primers has been used in previous studies in the analysis of CD44 variant transcripts (Arch *et al.*, 1992, Koopman *et al.*, 1993b, Salles *et al.*, 1993, van Weering *et al.*, 1993, Konig *et al.*, 1996). Using this approach, the v6 exon containing transcripts expressed in resting human PBMC were

Figure 3.10 Effect of PMA and DMSO stimulation of PBMC on the expression of CD44 v6 transcripts.

A total of 5 x 10^6 human PBMC were stimulated for 6 hr with 10 ng/ml PMA (in 0.1% DMSO) and 0.1% DMSO. Total RNA was extracted and 1 μg was reverse transcribed. PCR analysis downstream of the v6 exon using primers B and D was performed at 35 cycles and the products (10 μl) electrophoresed in a 1.2% agarose gel and UV-visualized by ethidium bromide staining. Molecular weight marker (M) was SPP-1 bacteriophage DNA digested with EcoRI and pUC19 plasmid DNA digested with HpaII.


Extra-Cellular Domain
Transmembrane Region
Cytoplasmic Region
ATG
V2
V3
V4
V5
V6
V7
V8
V9
V10
B
D

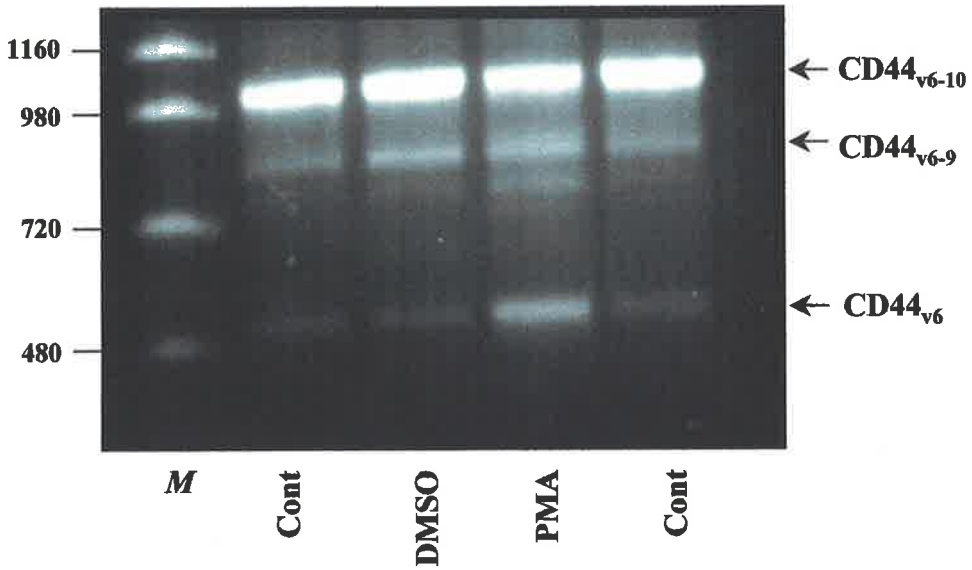
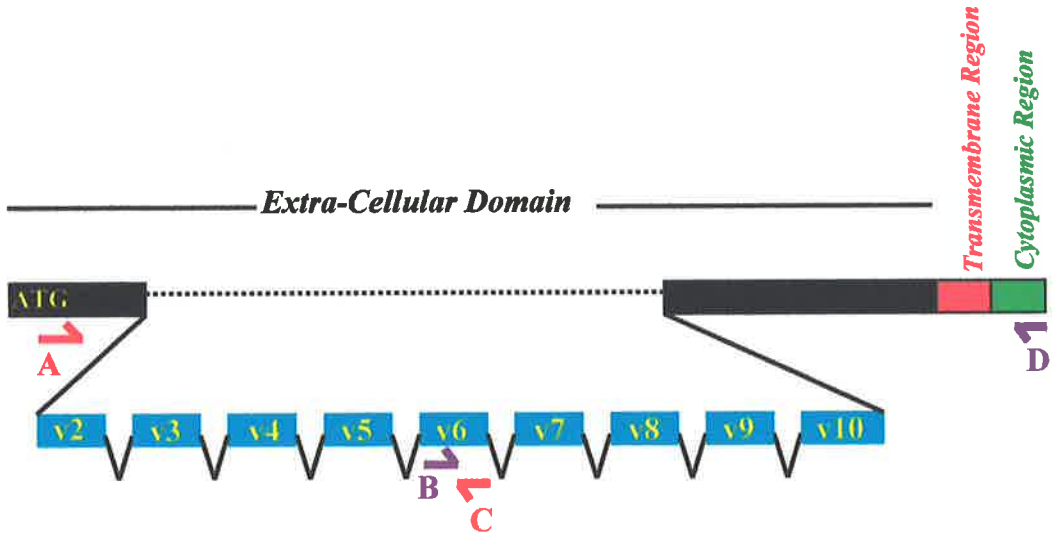
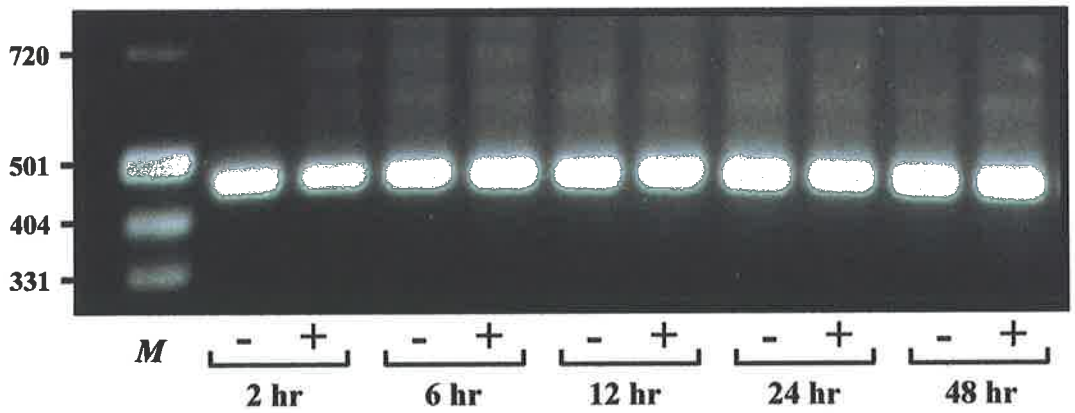

1160
980
720
480
CD44v6-10
CD44v6-9
CD44v6
M
Cont
DMSO
PMA
Cont

Figure 3.11 Kinetics of the PMA induced changes in the expression of the CD44 v6 transcripts in PBMC.

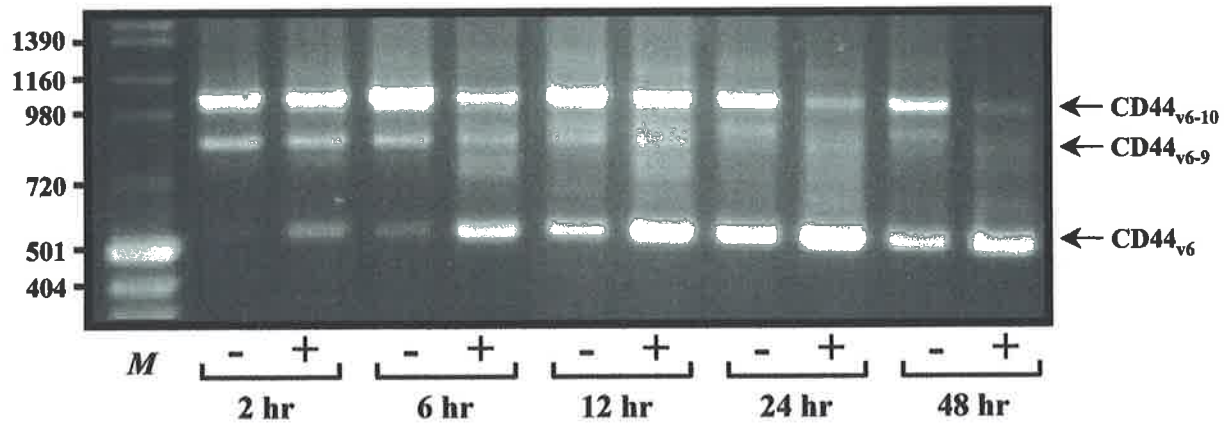
5 x10^6 PBMC were stimulated with 10ng/ml PMA for varying length of time and expression of the CD44 v6 transcripts was compared to time-matched untreated cells. Total RNA was extracted and 1 μg was reverse transcribed. All PCR products (10 μl) were electrophoresed in a 1.2% agarose gel and UV-visualized by ethidium bromide staining. The upstream region flanking the v6 exon was analyzed by using primers A and C at 35 cycles of PCR amplification (Panel A). Subsequently, the downstream region flanking the v6 exon was analyzed using primers B and D at 35 cycles of PCR amplification (Panel B). Molecular weight marker (M) was SPP-1 bacteriophage DNA digested with EcoRI and pUC19 plasmid DNA digested with HpaII.



A. ***Primers A and C***



B. ***Primers B and D***



- Unstimulated PBMNCs
+ PMA treated PBMNCs

identified as the $CD44_{v6-10}$, $CD44_{v6-9}$, $CD44_{v6,8-9}$, and $CD44_{v6}$ isoforms (Section 3.2.2). The rat homologue of the $CD44_{v6}$ transcript has been previously characterized and its significance to the alloimmune response is discussed earlier in this chapter (Arch *et al.*, 1992). The human $CD44_{v6-10}$ transcript has been cloned and encodes a 160kDa protein which shows poor HA binding capacity compared to the $CD44_{std}$ isoform (Jackson *et al.*, 1995). However, the occurrence of $CD44_{v6-9}$ and $CD44_{v6,8-9}$ transcripts has yet to be reported.

The identity of the CD44 v6 transcripts are the same as those previously reported in human T cells (Koopman *et al.*, 1993b). Although Koopman and colleagues (1993) observed three v6 exon containing transcripts only the identity of two were determined, on the basis of molecular size, as the $CD44_{v6-10}$ and $CD44_{v6}$ isoforms. The identity of the third transcript could not be defined since its molecular size could represent a variety of molecular species such as the $CD44_{v6-9}$, $CD44_{v6,9-10}$ or $CD44_{v6-7,10}$ isoforms. It is suggested, in accordance with data presented in this chapter, that the third transcript observed by Koopman *et al.* (1993) is the $CD44_{v6-9}$ transcript.

In contrast to the studies of Koopman *et al.* (1993), the data presented in this chapter identifies a fourth isoform, the $CD44_{v6,8-9}$ transcript. The T cells analyzed by Koopman and colleagues were cultured for a minimum of 24 hr and the absence of the $CD44_{v6,8-9}$ transcript could be due to the transient expression of the mRNA. The $CD44_{v6,8-9}$ transcript appears in freshly isolated PBMC and disappears upon culturing of these cells and does not reappear for 48 hr (Section 3.2.2.3). Furthermore, the $CD44_{v6,8-9}$ transcript may be a product of B cells or monocytes which are depleted in

the T cell preparation of Koopman *et al.* (1993) and yet are present in the mixed cell population of PBMC analyzed in this chapter.

It is interesting that although the CD44 v6 transcripts identified in PBMC do not carry variant exons from the v2-v5 region, alternatively spliced isoforms carrying the complete variant exon cassette have been identified in other tissues. The $CD44_{v2\text{-}v10}$ transcript is widely expressed in normal colonic crypt epithelium, (Gotley *et al.*, 1996) and the HPKII type variant ($CD44_{v3\text{-}v10}$) is expressed in skin (Hofmann *et al.*, 1991, Stauder *et al.*, 1995).

From these observations it is hypothesized that two distinct "groups" of CD44 v6 transcripts exist and are segregated by the presence or absence of alternately spliced exons from the v2-v5 region. CD44 variant transcripts containing the v2-v5 exons are expressed in epithelial tissues rather than haemopoietic cells suggesting tissue-specific regulation. It is therefore speculated that the expression of CD44 v6 transcripts that do not contain exons from the v2-v5 region ($CD44_{v6\text{-}10}$, $CD44_{v6\text{-}9}$, $CD44_{v6,8\text{-}9}$ and $CD44_{v6}$) predominate in haemopoietic cells.

To date the regulatory mechanism involved in the tissue specificity of alternate splicing of pre-mRNA remains unclear. However, two different mechanisms have been studied. The first suggests that cell-specific differences in the activity or concentration of general splicing factors can regulate alternate splicing (Krainer *et al.*, 1990a, Krainer *et al.*, 1990b). Secondly, selective splice sites can be negatively regulated by repressor proteins that bind specifically to pre-mRNA recognition sequences (Baker, 1989).

The culturing of PBMC resulted in four distinct changes to the relative expression of the CD44 v6 transcripts (Section 3.2.2.4). The four CD44 v6 transcripts are expressed in freshly isolated PBMC, and as a consequence to culturing on plastic the $CD44_{v6,v8\text{-}9}$ isoform is no longer detected for up to 48 hr. This sudden absence was demonstrated to occur within 30 minutes of the cells in culture (data not shown).

Expression of the $CD44_{v6\text{-}10}$ transcript dramatically increased within 2 hr of culture in comparison to freshly isolated cells. However, as the length of culture was extended the expression gradually decreased to similar concentration as that of freshly isolated cells.

The $CD44_{v6\text{-}9}$ transcript is predominantly expressed in freshly isolated cells yet dramatically decreases within 2 hr of culture. The expression of this transcript continues to decline until barely detected at 48 hr culture. Surprisingly, expression of a novel product of a slightly larger molecular size than the $CD44_{v6\text{-}9}$ transcript (approximately 950 bp in molecular size) was apparent. Expression of the 950 bp product was initially observed at 12 hr culture. Whether this 950 bp product represents a new alternately spliced transcript or a heteroduplex is yet to be defined.

Unlike the other three isoforms, the expression of the $CD44_{v6}$ transcript does not change within 2 hr of culture. However, as the cells are cultured beyond this time point, its expression is induced in comparison to freshly isolated cells.

Whether these changes to the expression of the CD44 v6 transcripts are inter-dependent or distinct from one another remains to be confirmed. Due to the competitive amplification of the PCR reaction, a reduction in the expression of one transcript can result in the relative increase of another suggesting two distinct changes have occurred. For example, freshly isolated cells predominantly express the $CD44_{v6-9}$ transcript yet within 2 hr of culture the expression decreases. Consequently the PCR amplification of the $CD44_{v6-10}$ transcript may increase because of the competitive decrease between these two transcripts for the availability of primer hybridization. Therefore, upon UV-visualization of the ethidium bromide stained products, the amplified increase in the $CD44_{v6-10}$ product may be misinterpreted as transcriptional induction rather than the decrease in the PCR competition.

The RNase protection technique is both quantitative and non-competitive and can be used to overcome the problems with competitive PCR amplification of multiple products. However, a disadvantage with the RNase protection assay is that large quantities of total RNA (>10 μg) may be required for adequate detection of the CD44 v6 transcripts. From the studies conducted in this chapter, approximately 25×10^6 cells would be required for the extraction of 10 μg total RNA, in comparison to 5×10^6 cells for a minimum of 2 μg total RNA for RT-PCR analysis.

As mentioned earlier, the amplification of multiple CD44 v6 transcripts may generate heteroduplexes that could be misinterpreted as novel transcripts. Heteroduplexes are formed by the hybridization between complementary DNA regions between two different strands of DNA. Electrophoresis of such products on

a non-denaturing gel allows the different sized strands of DNA to remain bound at their complementary regions. However, electrophoresis of PCR products on a denaturing gel does not permit the retention of the heteroduplex forms and therefore can be used in future studies to distinguish legitimate PCR products from heteroduplexes.

To understand the cellular mechanisms involved in the induction of the changes in expression of the v6 transcripts upon culture of PBMC, the influence of serum was investigated. Previous reports have demonstrated the regulatory effects of serum upon expression of the CD44 protein (Kogerman *et al.*, 1996b, Levesque and Haynes, 1996). However, it was concluded that the changes in expression of the CD44 v6 transcripts are independent of serum (Section 3.2.4) suggesting other factors are involved.

The *in vitro* culture of peripheral monocytes can induce cellular activation and differentiation (Stevenson *et al.*, 1981, Johnson *et al.*, 1977). Furthermore, monocytes can rapidly adhere to plastic within 30 minutes of incubation with only a minor increase to the number of adherent cells if the plating period is extended to 2 hr (Johnson *et al.*, 1977). These observations suggest a correlation with the culture-induced changes in expression of the CD44 v6 transcripts. Teflon coated vessels, which inhibit the adherence of monocytes, have been previously used for culturing of PBMC (Culty *et al.*, 1994, Levesque and Haynes, 1996). To investigate the correlation between adherence of monocytes and the changes in expression of the CD44 v6 transcripts, future experiments using these vessels can be conducted.

It is tempting to speculate that the changes induced upon expression of the CD44 v6 transcripts correlate with either:

(i) *in vitro* activation of monocytes consequent to cell adherence, or

(ii) activation of T and B cells subsequently stimulated by cytokines released from activated monocytes.

However, the experiments conducted in this chapter have analyzed a mixed population of mononuclear cells. As such, the precise expression of the CD44 v6 transcripts in each discrete sub-population (e.g. T cells, B cells and monocytes) is unknown. Future analysis of the CD44 v6 transcripts in each cell population is contemplated.

The finding that expression of the CD44 v6 transcripts in PBMC can be modulated is not unexpected as regulation of the CD44 gene by cellular signal transduction pathways has been previously reported (Section 1.11.1). It is therefore proposed that upon cell activation, the transmission of an external stimulus by intracellular signaling pathways can result in an alteration in the expression of the CD44 v6 transcripts. Furthermore, with the vast complexity and diversity of the signal transduction cascade (as reviewed in Berridge, 1997) the expression of the CD44 v6 transcripts can be differentially regulated and is determined by the specificity in the signaling pathways activated.

PBMC were stimulated with a variety of mitogens and it was surprising that only PMA stimulation altered the expression of the CD44 v6 transcripts (Section 3.2.6). From the importance of the rat $CD44_{v6}$ protein in the alloimmune response it was expected that the human mixed lymphocyte reaction, an *in vitro* model of an

allogeneic response, would influence expression of the CD44 v6 transcripts. Likewise, the PHA and anti-CD3 mAb stimulations, which induce the cell surface expression of CD44 v6 proteins on human T cells (Mackay *et al.*, 1994, Galluzzo *et al.*, 1995) were expected to modify the expression of the v6 exon containing transcripts.

From these observations it is concluded that the CD44 v6 transcripts expressed in human PBMC are regulated in a precise manner dependant upon the mode of cellular activation indicating a potential impact of the CD44 v6 transcripts upon cellular function.

PMA has been widely used to study the effects of protein kinase C (PKC) and has chemical properties of being both cell permeable and non-degradable thus resulting in the potent and prolonged activation of the PKC pathway (Nishizuka, 1984). The intracellular pathways by which PKC activation exerts its effect, although widely investigated remain largely unelucidated as the PKC pathway often activates and converges with other signaling pathways resulting in gene activation (Isakov and Altman, 1987, Nishizuka, 1995, Berridge, 1997).

Recently the direct association between initiation of the PKC pathway by PMA and induction of $CD44_{v6}$ transcript was demonstrated in neuroblastoma cells (Fichter *et al.*, 1997). The $CD44_{v6}$ transcript was strongly upregulated in the human SK-N-SH neuroblastoma cell line in response to PMA and the growth factors insulin like growth factor-1 (IGF-1) and platelet derived growth factor (PDGF). Using a specific inhibitor of the PKC signal transduction pathway, the PMA and IGF-1 up-regulation

of the $CD44_{v6}$ transcript was inhibited. Furthermore using a specific inhibitor of the phosphoinositide 3-kinase (PI 3-K) pathway, the up-regulation of the $CD44_{v6}$ transcript by IGF-1 and PDGF was partly reduced. These results indicate that the PKC and PI 3-K transduction pathways are capable of transducing intracellular signals resulting in an upregulation of the $CD44_{v6}$ transcript.

The PMA-induced changes in expression of the CD44 v6 transcripts are similar to those produced by culturing of PBMC where an increased expression of the $CD44_{v6}$ transcript with the concomitant decrease of $CD44_{v6\text{-}10}$ was demonstrated. As shown in Figure 3.11, PMA treatment of PBMC appears to accelerate the existing time dependent changes in the expression of the CD44 v6 transcripts such as to enhance the temporal onset of the induced $CD44_{v6}$ and decreased $CD44_{v6\text{-}10}$ expression. From this observation it is suggested that the PKC signal transduction pathway is utilized by PMA and the culturing of PBMC to induce changes in expression of the CD44 v6 transcripts.

Chapter Four

Identification and Characterization of a novel $CD44_{v6\text{-}7}$ mRNA Transcript: Expression in Normal Individuals, Renal Transplant Recipients and Cutaneous T Cell Lymphomas.

4.1 INTRODUCTION

The significance of the CD44 v6 proteins in an alloimmune response has been implicated by studies utilizing monoclonal antibodies towards these molecules (Arch *et al.*, 1992, Galluzzo *et al.*, 1995). For example, the proliferative response induced by monoclonal antibody-mediated ligation of the CD3 receptor on T cells can be further augmented by the additional ligation of CD44 v6 or v9 exon containing proteins. This observation demonstrates a convergence between the signal transduction pathways initiated by the CD44 variant proteins with that of the CD3 receptor. Furthermore, ligation of the CD44 variant proteins alone does not induce cell proliferation indicating the co-stimulatory function of these variant proteins in the CD3 activation of T cells. The corresponding ligands for the CD44 v6 and v9 proteins are unknown, however, it is postulated that these isoforms may interact with independent molecules dictated by the specific variant exon structure of the CD44 molecule.

However, these studies provide little information with regard to which specific CD44 v6 isoforms are involved in the cellular response. The CD44 v6 transcripts expressed in human PBMC have been previously defined (Chapter 3, Section 3.2.3). In addition, expression of these transcripts appears to be differentially regulated by the initiation of specific signal transduction pathways (Chapter 3, Section 3.2.5). From these observations it is suggested that an *in vivo* activation of the alloimmune response will modulate expression of the CD44 v6 transcripts in PBMC.

The aim of the studies in this chapter was to utilize a PCR based approach to analyze the expression of the CD44 v6 transcripts in PBMC isolated from human renal transplant patients, an *in vivo* model of alloimmunity, in comparison to healthy individuals.

4.2 RESULTS

4.2.1 PCR amplification of the CD44 v6 transcripts expressed in PBMC of renal transplant patients

All patients were admitted into the Renal Unit of the Queen Elizabeth Hospital (Adelaide, South Australia) for renal transplant surgery. After obtaining informed consent, 10 ml of peripheral blood was taken 24 hr post-transplant with two further samples collected at 48 hr intervals.

In preliminary experiments, peripheral blood specimens from 5 transplant patients were analyzed for expression of the CD44 v6 transcripts. The synthesis of cDNA from PBMC (Chapter 3, Section 3.2.1) and analysis of CD44 v6 transcript expression by PCR amplification (Chapter 3, Section 3.2.2) have been previously described.

In comparison to healthy individuals, the number of PBMC isolated from patient peripheral blood was low and as such the quantity of total RNA extracted was less than 1 μg based on spectrophotometric quantitation. To ensure successful amplification of the CD44 v6 transcripts from low concentrations of RNA, the entire quantity of RNA extracted was reverse transcribed.

PCR amplification of the CD44 v6 transcripts using primers B and D had identified the expression of a novel transcript in freshly isolated PBMC from one patient (data not shown). The visualization of this product by ethidium bromide staining was diffuse due to competitive PCR amplification of other v6 exon containing transcripts previously defined as $CD44_{v6\text{-}10}$, $CD44_{v6\text{-}9}$, $CD44_{v6,\ 8\text{-}9}$ and $CD44_{v6}$ (Chapter 3, Section 3.2.3). Previous studies had demonstrated that expression of these CD44 v6 transcripts can be influenced by further *in vitro* culturing of PBMC (Chapter 3, Section 3.2.2.3). Therefore, to ascertain if PCR amplification of the novel transcript could be enhanced, cells were cultured on plastic for 0, 2, 6 and 12 hr. Based on these experiments it was established that 6 hr culture of PBMC improved the PCR amplification and subsequent UV-visualization of the novel transcript (data not shown).

In summary, the experimental conditions for analysis of the CD44 v6 transcripts involved culturing of PBMC isolated from patients onto plastic for 6 hr, followed by reverse transcription of total RNA and PCR amplification at 50 cycles.

4.2.2 Identification and characterization of the novel CD44 v6 transcript

The novel transcript observed in PBMC isolated from a renal transplant patient was characterized by PCR amplification up- and down-stream of the v6 exon. Samples consisted of cDNA synthesized from PBMC isolated from both the patient expressing the novel transcript and a representative volunteer.

PCR amplification upstream of the v6 exon using primers A and C yielded a single product in both specimens (Figure 4.1). The product of 460 bp in molecular size has been previously identified to contain the invariant portion of the CD44 molecule immediately upstream of the v6 exon (Chapter 3, Section 3.2.2.2).

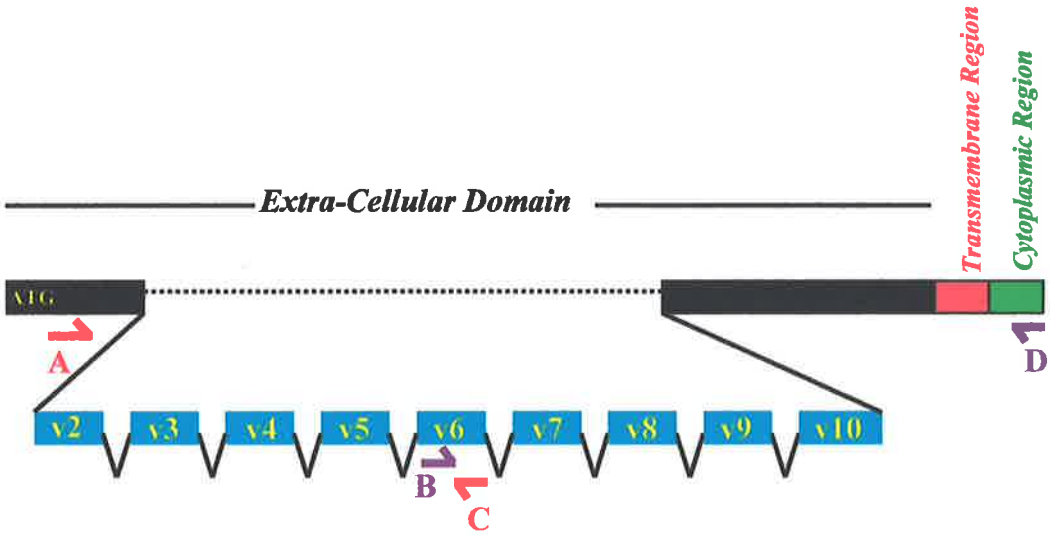
PCR analysis downstream of the v6 exon using primers B and D amplified the $CD44_{v6\text{-}10}$, $CD44_{v6\text{-}9}$ and $CD44_{v6}$ transcripts (Chapter 3, Section 3.2.2.3) in both samples. Amplification of a novel 650 bp product was detected in the patient sample and based on the position of primers B and D this fragment was deduced to consist of 410 bp invariant CD44 sequence, 126 bp of v6 exon and approximately 114 bp of variant exon region.

To define the variant exon composition of the novel transcript, the amplified products using primers B and D from the patient and volunteer samples were blotted for Southern analysis. Replicates of PCR products from both samples were electrophoresed through a 1.2% agarose gel (Figure 4.2.A) and transferred onto nylon membrane (Section 2.2.8). The membrane was cut so that each strip contained the PCR products from both patient and volunteer samples. Individual membrane strips were subsequently hybridized with biotinylated oligonucleotides specific for the v7, v8, v9 or v10 exon (Section 2.2.9).

The 650 bp product hybridized with only the v7 probe identifying this product as containing the v7 exon (Figure 4.2.B). It was concluded on the basis of molecular size of the PCR product and Southern analysis that the novel CD44 v6 transcript is $CD44_{v6\text{-}7}$ (Figure 4.2.C).

Figure 4.1 Characterization of the novel CD44 v6 transcript by PCR amplification using v6 exon specific primers.

cDNA samples synthesized from PBMC isolated from both a renal transplant patient expressing a novel CD44 v6 transcript and a healthy volunteer were analyzed by PCR amplification using v6 exon specific primers. The PCR products (10 μl) were resolved in a 1.2% agarose gel and visualized by ethidium bromide staining. The downstream region flanking the v6 exon was analyzed using primers B and D at 50 cycles of PCR amplification. Subsequently, the upstream region flanking the v6 exon was analyzed using primers A and C at 50 cycles of PCR amplification. Molecular weight marker (M) was SPP-1 bacteriophage DNA digested with EcoRI and pUC19 plasmid DNA digested with HpaII.


Extra-Cellular Domain
Transmembrane Region
Cytoplasmic Region
ATG
A
D
v2
v3
v4
v5
v6
v7
v8
v9
v10
B
C

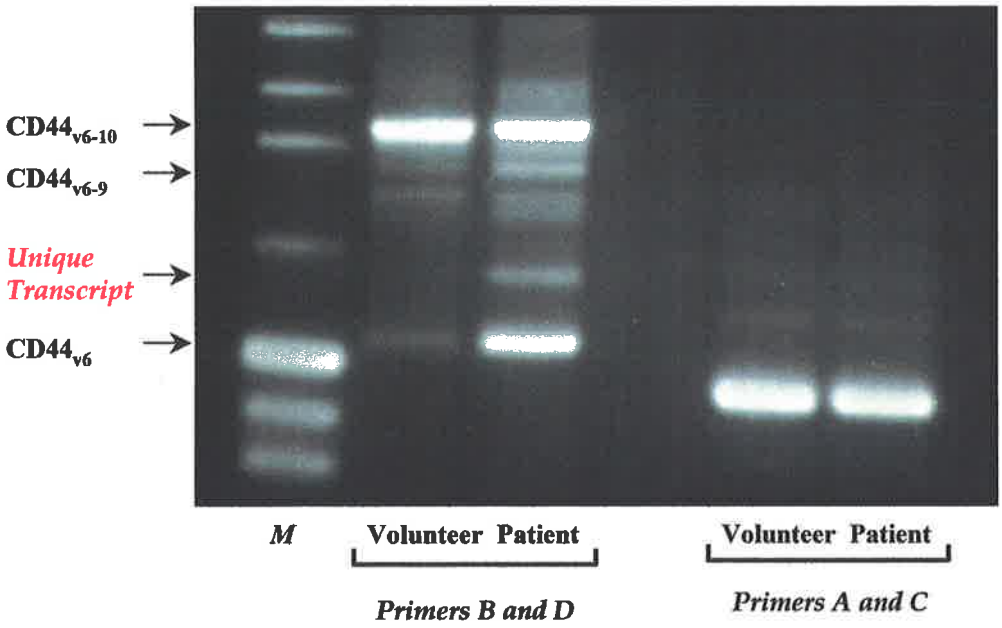
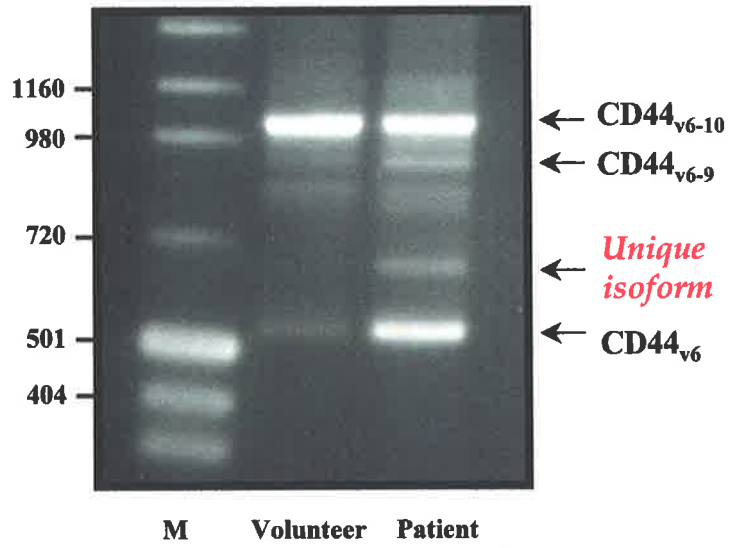

CD44v6-10
CD44v6-9
Unique Transcript
CD44v6
M
Volunteer Patient
Primers B and D
Volunteer Patient
Primers A and C

Figure 4.2 Identification of the variant exon composition of the novel CD44 v6 transcript.

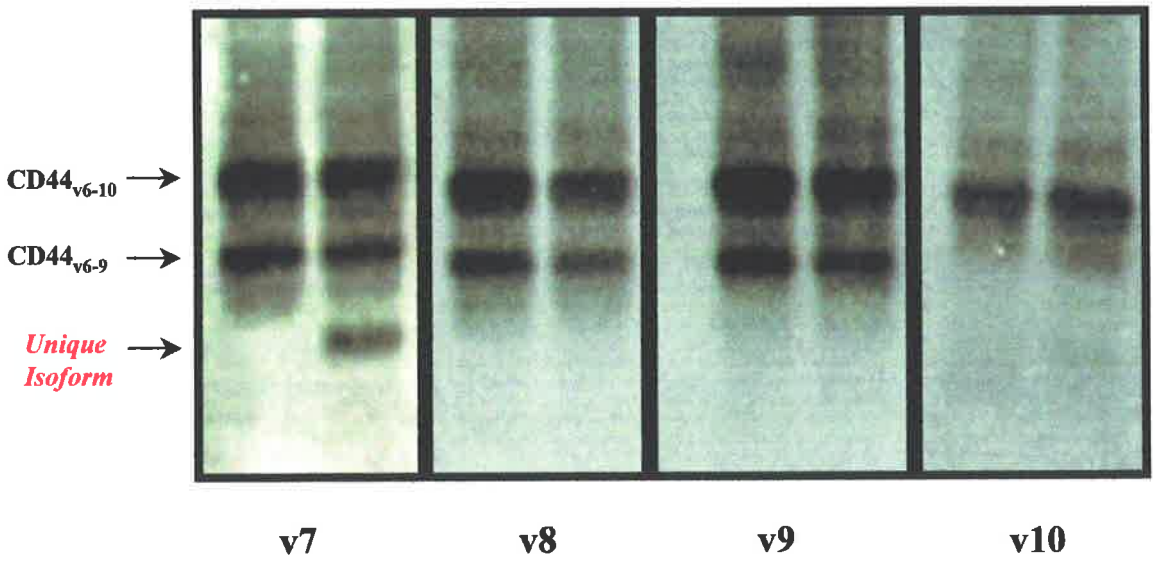
cDNA samples synthesized from PBMC isolated from both the renal transplant patient expressing a novel CD44 v6 transcript and a healthy volunteer were analyzed by using primers B and D at 50 cycles of PCR amplification. The resultant products (10 μl) were electrophoresed in a 1.2% agarose gel (Panel A) in 4 replicate sets. Following an overnight transfer onto nylon membrane, each pair was separated and incubated in blocking solution for 4 hr at room temperature. The pairs were individually probed overnight at 42°C with a biotinylated oligonucleotide directed towards the v7, v8, v9 or v10 exon. After washing at 42°C in 0.1x SSC and 0.5% SDS, the hybridized probes were detected with a streptavidin alkaline phosphatase conjugate and the Southern blot developed using the chromogenic substrates NBT/ XPO_4 (Panel B). Only the v7 probe hybridized to the novel 650 bp product defining this transcript as the $CD44_{v6\text{-}7}$ isoform (Panel C). Molecular weight marker (M) was SPP-1 bacteriophage DNA digested with EcoRI and pUC19 plasmid DNA digested with HpaII.

The up- and down-stream PCR products of the $CD44_{v6\text{-}7}$ isoform, as determined by Southern analysis, were utilized in the generation of full length CD44 transcript (see Chapter 5). Nucleotide sequence comparison of the full length clone to those published by Screaton *et al* (1992) confirmed the identity of this isoform (see Appendix).

A.


1160
980
720
501
404
CD44v6-10
CD44v6-9
Unique isoform
CD44v6
M
Volunteer
Patient

B.


CD44v6-10
CD44v6-9
Unique Isoform
v7
v8
v9
v10

C.


ATG
v6
v7

4.2.3 Detection of the CD44 v7 transcripts in PBMC of renal transplant patients

4.2.3.1 PCR amplification of the CD44 v7 transcripts by exon specific primers

To enhance PCR amplification of the $CD44_{v6-7}$ transcript, v7 exon specific primers were used in conjunction with invariant primers. The two pairs of primers were designed to characterize the variant exon region both up- and down-stream of the v7 exon (Figure 4.3.A). A reverse v7 primer (primer E) in conjunction with a forward primer directed towards the invariant region (primer A) were used to amplify upstream of the v7 exon. Similarly, a v7 forward primer (primer F) was used in conjunction with a reverse primer directed towards the invariant region (primer D) to amplify downstream of the v7 exon (Figure 4.3.B).

4.2.3.2 PCR analysis of the composition of variant exons upstream of the v7 exon

Primers A and F were used to ascertain the variant exon composition upstream of the v7 exon. Samples consisted of cDNA synthesized from PBMC isolated from both the patient expressing the $CD44_{v6-7}$ transcript and a representative volunteer. PCR amplification yielded a single product with molecular size of approximately 500 bp in both samples (Figure 4.4).

From the location of primers A and F, the 500 bp PCR product was deduced to consist of 335 bp invariant CD44 sequence, 40 bp of v7 exon and 125 bp variant exon insert. Based on the previous characterization of the CD44 v6 transcripts

Figure 4.3 Outline of the v7 exon specific PCR strategy used to characterize the CD44 v7 transcripts expressed in human PBMC.

Panel A depicts the human CD44 cDNA with the extracellular (■), transmembrane (■), cytoplasmic (■) and variant exon region (■) denoted. The relative positions of the primer pairs used for PCR analysis of the upstream (primers A and F) and downstream (primers E and D) regions flanking the v7 exon are shown. The corresponding regions of the CD44 cDNA amplified with these primer pairs are represented as an illustrated example in Panel B.

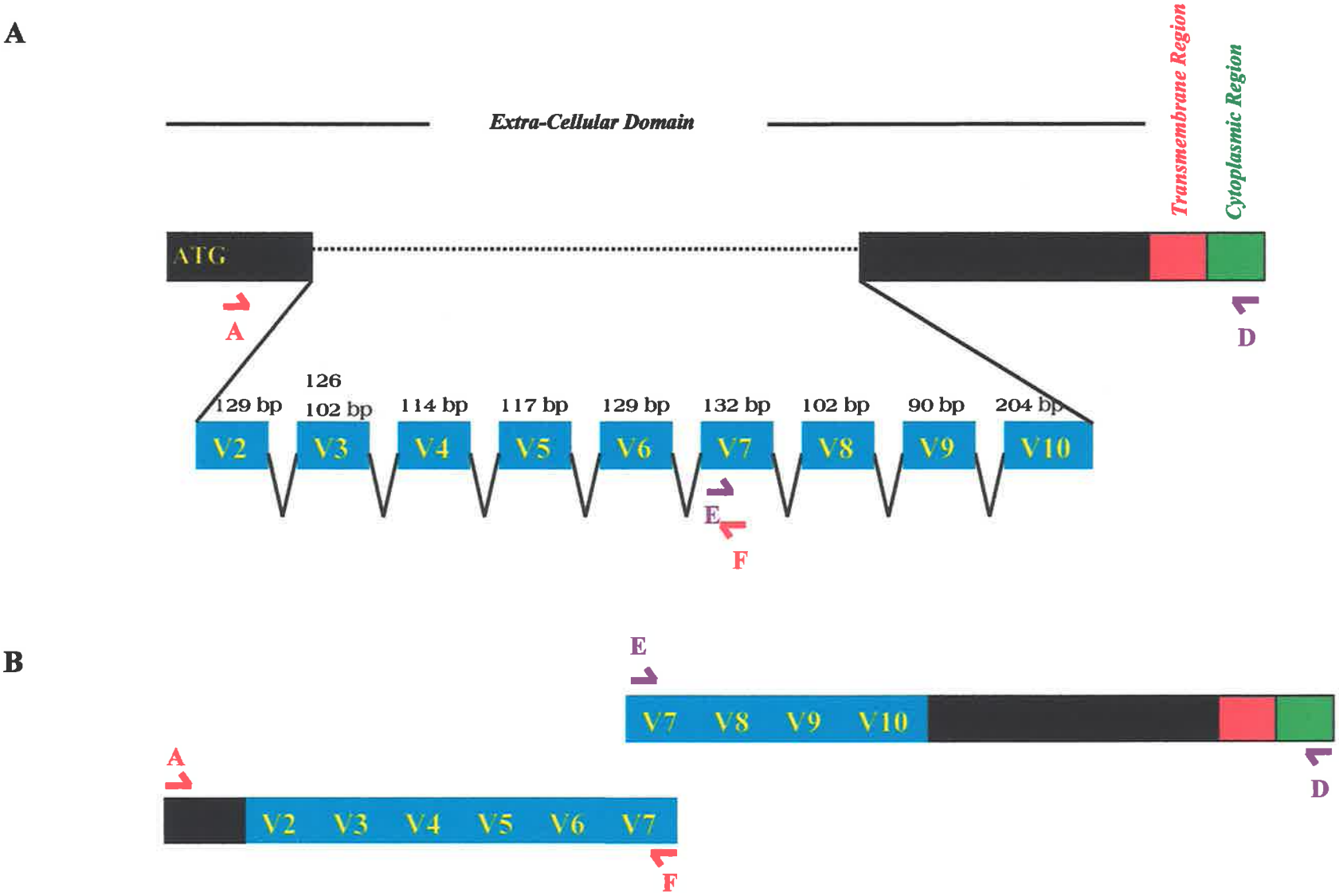
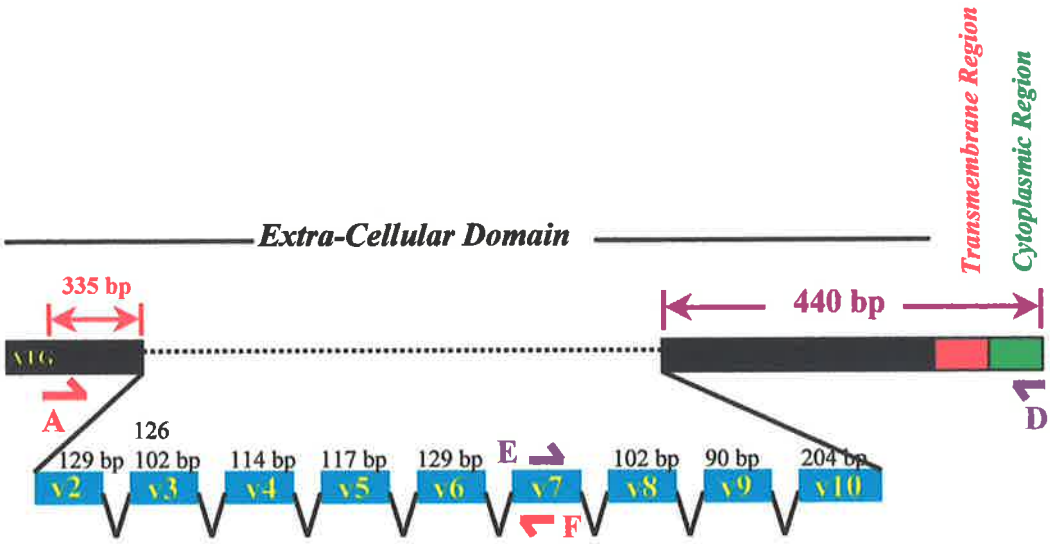
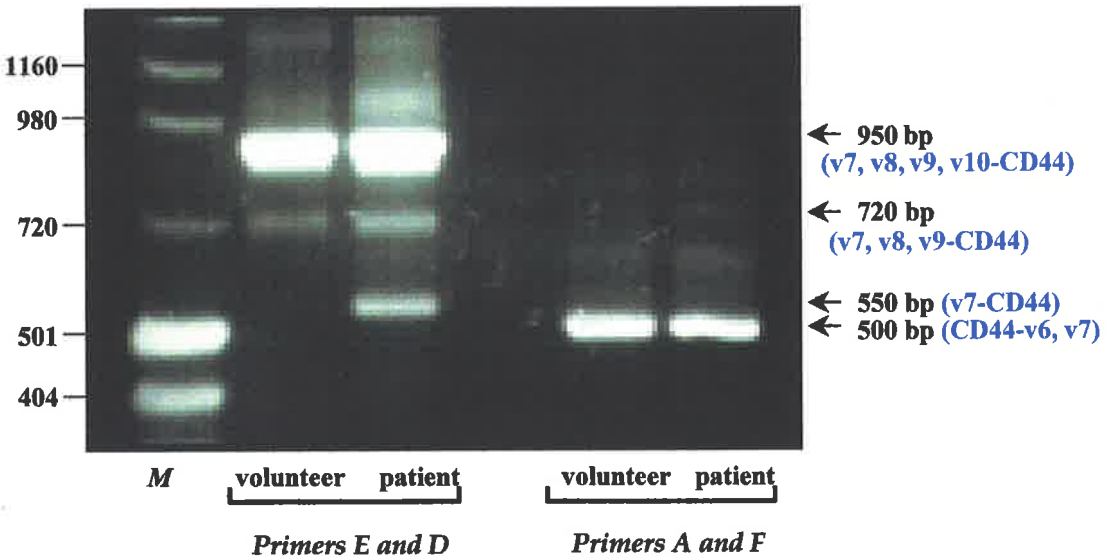

A
Extra-Cellular Domain
Transmembrane Region
Cytoplasmic Region
ATG
A
D
126
129 bp
102 bp
114 bp
117 bp
129 bp
132 bp
102 bp
90 bp
204 bp
V2
V3
V4
V5
V6
V7
V8
V9
V10
E
F
B
E
V7
V8
V9
V10
D
A
V2
V3
V4
V5
V6
V7
F

Figure 4.4 Characterization of the $CD44_{v6-7}$ transcript by PCR amplification using v7 exon specific primers.

cDNA samples synthesized from PBMC isolated from both a renal transplant patient expressing the $CD44_{v6-7}$ transcript and a healthy volunteer were analyzed by PCR amplification using v7 exon specific primers. The PCR products (10 μl) were resolved in a 1.2% agarose gel and visualized by ethidium bromide staining.

The downstream region flanking the v7 exon was analyzed using primers E and D at 50 cycles of PCR amplification. The upstream region flanking the v7 exon was analyzed using primers A and F at 50 cycles of PCR amplification. Molecular weight marker (M) was SPP-1 bacteriophage DNA digested with EcoRI and pUC19 plasmid DNA digested with HpaII.


Extra-Cellular Domain
Transmembrane Region
Cytoplasmic Region
335 bp
440 bp
A
D
126
129 bp
102 bp
114 bp
117 bp
129 bp
E
102 bp
90 bp
204 bp
v2
v3
v4
v5
v6
v7
v8
v9
v10
F


1160
980
720
501
404
950 bp
(v7, v8, v9, v10-CD44)
720 bp
(v7, v8, v9-CD44)
550 bp (v7-CD44)
500 bp (CD44-v6, v7)
M
volunteer
patient
volunteer
patient
Primers E and D
Primers A and F

(Section 4.2.2) it was concluded that the 125 bp variant exon insert represents the v6 exon.

4.2.3.3 PCR analysis of the composition of variant exons downstream of the v7 exon

Primers E and D were used to ascertain the combination of variant exons downstream of the v7 exon. Samples consisted of cDNA synthesized from PBMC isolated from both the patient expressing the $CD44_{v6-7}$ transcript and a representative volunteer. As shown in Figure 4.4, PCR amplification revealed the presence of three products with molecular sizes of 950, 720 and 550 bp respectively. The 550 bp product was amplified only in the patient sample.

From the previous characterization of the CD44 v6 transcripts (Chapter 3, Section 3.2.3) and in conjunction with the molecular size of the 950 and 720 bp products, it was concluded that they represent the $CD44_{v6-10}$ and $CD44_{v6-9}$ transcripts respectively. Based on the position of primers E and D, it was determined that the 550 bp product consists of 440 bp invariant CD44 sequence and 110 bp v7 exon. Subsequently it was deduced that the 550 bp product represents the $CD44_{v6-7}$ transcript.

4.2.4 Detection of the CD44 $_{v6-7}$ transcript in renal transplant patients

A total of 20 renal transplant patients were recruited into this study. The average age of this population was 44 years (range 18-67 years) and consisted of 12 males and 8 females. The pathological disorder predisposing these patients to a

transplant was diverse and 9 living related and 11 cadaveric donor transplant operations were performed (Table 4.1). Peripheral blood samples were collected between day 1-10 post-transplant and analyzed for the expression of the CD44 v6 transcripts by PCR amplification (Section 4.2.1 and 4.2.3). Five healthy volunteers consisting of 3 males and 2 females with an average age of 33 years (range 23-42 years) were used as the control population.

PCR amplification downstream of the v6 exon using primers B and D detected expression of the $CD44_{v6-7}$ transcript in 6 patients occurring at a 30% frequency within this population (Figure 4.5). The $CD44_{v6-7}$ transcript was not detected from PBMC isolated from healthy volunteers (data not shown). For all 20 patients and 5 volunteers, no discrepancies in detecting the $CD44_{v6-7}$ transcript were evident by PCR amplification downstream of the v7 exon using primers E and D (Figure 4.6).

Of the 6 positive patients, 2 were females and 6 were males. The $CD44_{v6-7}$ transcript was present in each post-transplant blood sample analyzed except patient T where the transcript was transiently expressed (Table 4.2). Upon re-examination of patient T, approximately 1 month after initial analysis, an absence in expression of the $CD44_{v6-7}$ transcript from seven blood samples was noted. Continued analysis could not be performed due to the death of this patient arising from infection.

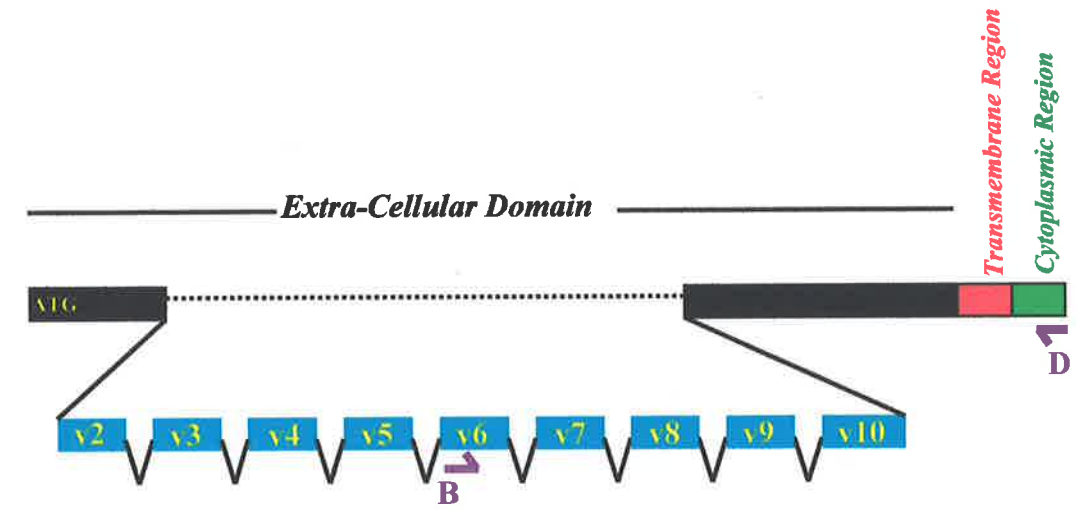
To assess if expression of the $CD44_{v6-7}$ transcript could correlate with an aggressive alloimmune response, subsequently resulting in graft rejection, the clinical data from each patient was examined at 2 months post-transplant (Table 4.2). A total of 8 patients exhibited episodes of graft rejection of whom 3 expressed the

Table 4.1 Clinical history of renal transplant patients.

Patient Identity	Sex	Age	Living/Cadaver	Reason for Transplant
A	M	34	Cadaveric	Diabetic Nephropathy
B	M	39	Cadaveric	Diabetic Nephropathy
C	F	59	Cadaveric	IgA Nephropathy
D	F	30	Cadaveric	Goodpasture
E	F	59	Cadaveric	IgA Nephropathy
F	M	50	Cadaveric	IgA Nephropathy
G	M	26	Living Related	Chronic Glomerulonephrits
H	M	50	Cadaveric	IgA Nephropathy
I	F	30	Cadaveric	IgA Nephropathy
J	F	28	Living Related	Haemolytic uraemic syndrome
K	M	46	Living Related	Polycystic Kidney
L	F	53	Cadaveric	Chronic Glomerulonephrits
M	F	65	Living Related	Analgesic Nephropathy
N	M	18	Living Related	Haemolytic uraemic syndrome
O	M	29	Living Related	Membranous Glomerulonephrits
P	M	38	Living Related	IgA Nephropathy
Q	M	54	Living Related	IgA Nephropathy
R	M	54	Cadaveric	IgA Nephropathy
S	F	67	Living Related	Crescentric Glomerulonephrits
T	M	59	Cadaveric	Interstitial Nephritis

Figure 4.5 Characterization of the CD44 v6 transcripts expressed in PBMC isolated from renal transplant patients.

PBMC were extracted from whole blood taken from renal transplant patients between days 1-10 post-transplant. The cells were cultured at a density of 1 $x10^6$/ml for 6 hr, collected by centrifugation and total RNA extracted and reverse transcribed. The downstream region flanking the v6 exon was analyzed using primers B and D at 50 cycles of PCR amplification. The PCR products (10 μl) from 6 representative patients and one healthy volunteer were resolved in a 1.2% agarose gel and visualized by ethidium bromide staining. Molecular weight marker (M) was SPP-1 bacteriophage DNA digested with EcoRI and pUC19 plasmid DNA digested with HpaII.


Extra-Cellular Domain
Transmembrane Region
Cytoplasmic Region
v1c
v2
v3
v4
v5
v6
v7
v8
v9
v10
B
D

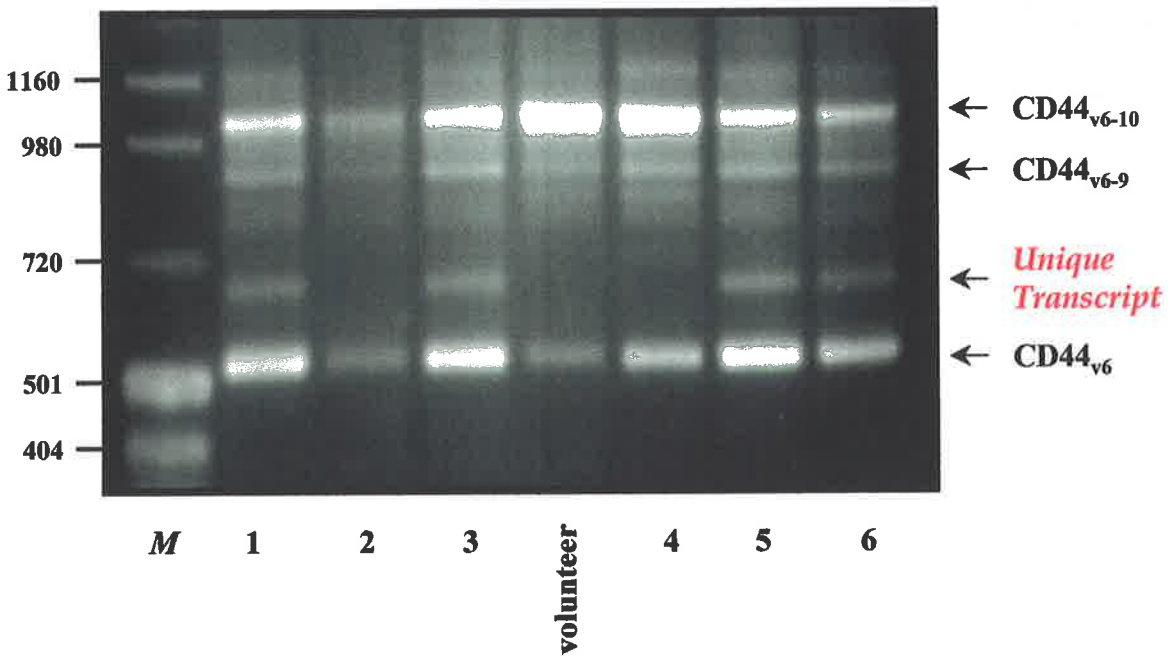
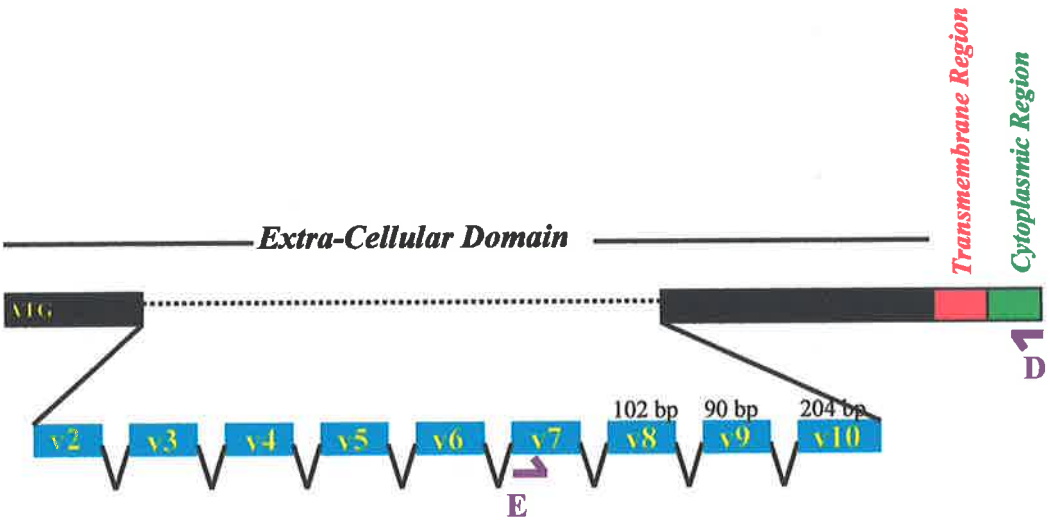

1160
980
720
501
404
CD44v6-10
CD44v6-9
Unique Transcript
CD44v6
M
1
2
3
volunteer
4
5
6

Figure 4.6 Detection of the $CD44_{v6-7}$ transcript in PBMC isolated from transplant patients by PCR amplification using v7 exon specific primers.

The cDNA samples used in this experiment were the same as described in Figure 4.5. Briefly, PBMC from renal transplant patients were cultured at a density of 1 $x10^6$/ml for 6 hr, collected by centrifugation and total RNA extracted and reverse transcribed. The downstream region flanking the v7 exon was analyzed using primers E and D at 50 cycles of PCR amplification. The PCR products (10 μl) from 6 representative patients and one healthy volunteer (as in Figure 4.5) were resolved in a 1.2% agarose gel and visualized by ethidium bromide staining. Molecular weight marker (M) was SPP-1 bacteriophage DNA digested with EcoRI and pUC19 plasmid DNA digested with HpaII.


Extra-Cellular Domain
Transmembrane Region
Cytoplasmic Region
ATG
D
v2
v3
v4
v5
v6
v7
v8
v9
v10
102 bp
90 bp
204 bp
E

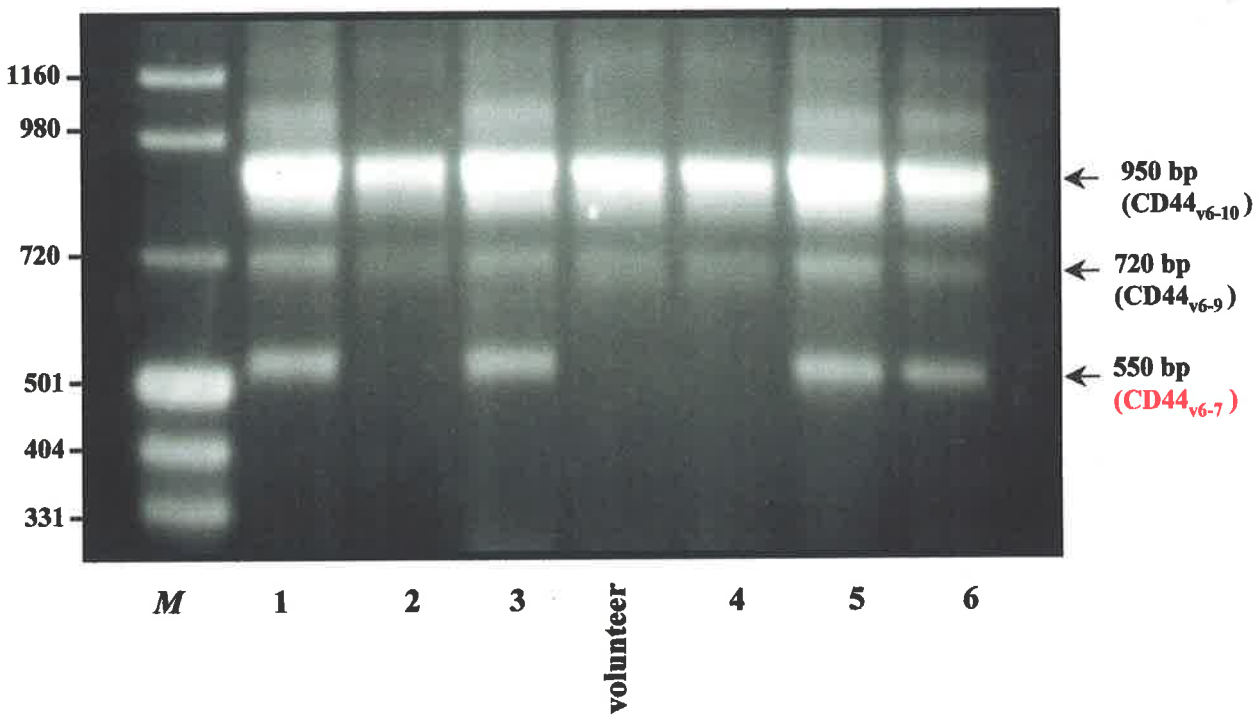

1160
980
720
501
404
331
950 bp
(CD44v6-10)
720 bp
(CD44v6-9)
550 bp
(CD44v6-7)
M
1
2
3
volunteer
4
5
6

Table 4.2 Clinical data and expression of the $CD44_{v6-7}$ transcript in renal transplant patients.

Normal functioning of the kidney was assessed by serum creatinine levels where levels less than 120 μmol/L was considered healthy.

Patient	Day of blood sample collection	CD44 $_{v6-7}$	2 month post-transplant
			Clinical Data
A	d1, 3, 6, 8, 10, 13, 15, 20		mild Rejection day 2,9,16
B	d1, 3, 6		mild Rejection day 15
C	d1, 3, 5		no Rejection
D	*d1, 3, 7*	*Positive*	*Rejection day 7. OKT3 therapy day 8-15*
E	d1, 3, 5		no Rejection. Cortical Necrosis day 5
F	d1, 3, 10, 12		no Rejection
G	d1, 3, 6		no Rejection
H	d1, 3, 6		no Rejection
I	d1, 3, 6		Rejection day 13, 29. OKT3 therapy day 3-6
J	d1, 4, 6		Cyclosporin toxicity
K	*d1, 3, 6, 10, 13*	*Positive*	*no Rejection. Infection day 9*
L	d1, 6, 16		no Rejection. Infection day 11
M	*d1, 3, 6*	*Positive*	*no Rejection*
N	d1, 3, 6		Possible Rejection day 6
O	*d1, 3, 6*	*Positive*	*Rejection day 15*
P	d1, 6, 8		Rejection day 8, 14
Q	*d1, 4, 6, 8*	*Positive*	*no Rejection. Infection day 3*
R	d1, 4, 6, 8		no Rejection
S	d1, 3, 5, 7		no Rejection
*T**	*d1, 3*	*Positive*	*Rejection day 6. OKT3 therapy day 1-15*
	d30, 33, 35, 37, 40, 42, 44		*Rejection day 35*

* Death related to lung infection arising from severe immunosuppression of the immune system

$CD44_{v6-7}$ transcript. Of the remaining 12 patients, 3 expressed the $CD44_{v6-7}$ transcript. With a small sample size, statistical analysis was not performed within this study. However, from this preliminary assessment it is unlikely that any correlation can be formed between the expression of the $CD44_{v6-7}$ transcript and clinical graft rejection.

4.2.5 PCR analysis of the $CD44_{v6-7}$ transcript in PBMC of stable renal transplant patients

The expression of the $CD44_{v6-7}$ transcript was analyzed in a group of stable renal transplant patients. These patients were defined as having received a kidney transplant a minimum of 12 months prior to this study and the donor graft still present within the patient. All stable renal transplant patients were consulted in the Renal Unit at the Queen Elizabeth Hospital. From each patient a single 10 ml blood sample was analyzed for expression of the $CD44_{v6-7}$ transcript by PCR amplification (Section 4.2.1 and Section 4.2.3).

The population of stable renal transplant patients comprised of 25 individuals (Table 4.3) consisting of 17 males and 8 females with the average life of the existing kidney graft of 9 years (range 2-24 years). The pathological disorders predisposing these patients to a transplant operation was diverse among the population and at the time of the study the majority had good kidney function as determined by serum creatinine. The expression of the $CD44_{v6-7}$ isoform was detected in a total of 7 patients, all males, occurring at a 28% frequency within this population.

Table 4.3 Clinical history and data of stable renal transplant patients.

Normal functioning of the kidney was assessed by serum creatinine levels where levels less than 120 μmol/L was considered healthy.

Patient	Sex	$CD44_{v6-7}$	Length of transplant*	Disease	Serum Creatinine**
st 1	M		7 Years	Medullary Cystic Disease	114
st 2	M		22 Years	FSGS	300
st 3	F		13 Years	Henochschonlein Purpura	115
st 4	*M*	*positive*	*21 Years*	*FSGS*	*95*
st 5	M		6 Years	IgA Nephropathy	100
st 6	*M*	*positive*	*2 Years*	*Uncertain*	*160*
st 7	F		7 Years	Polycystic Kidney	120
st 8	F		7 Years	Polycystic Kidney	89
st 9	*M*	*positive*	*11 Years*	*IgA Nephropathy*	*136*
st 10	M		3 Years	IgA Nephropathy	Dialysis
st 11	*M*	*positive*	*8 Years*	*unknown*	*110*
st 12	*M*	*positive*	*8 Years*	*Renal Hypoplasia*	*134*
st 13	F		13 Years	uncertain	77
st 14	M		11 Years	uncertain	80
st 15	*M*	*positive*	*10 Years*	*FSGS*	*130*
st 16	F		24 Years	GN	88
st 17	M		2 Years	FSGS	160
st 18	M		4 Years	Crescentric GN	120
st 19	M		3 Years	APKD	120
st 20	F		6 Years	APKD	120
st 21	M		6 Years	APKD	110
st 22	*M*	*positive*	*13 Years*	*FSGS*	*110*
st 23	M		13 Years	APKD	120
st 24	F		3 Years	Diabetes Type II	130
st 25	F		8 Years	FSGS	100

FSGS Focal and segmental glomerulosclerosis

* Duration of graft survival as of December 1997

** Serum Creatinine µmol/L as measured in December 1997

4.2.6 Re-examination of the $CD44_{v6-7}$ transcript expressed in the recent renal transplant patients

The $CD44_{v6-7}$ transcript was detected in approximately 30% of both the recent and stable transplant patient populations. This observation suggests that expression of this transcript is not transient as inferred by the previous analysis of patient T (Section 4.2.4).

To ascertain the kinetics in expression of the $CD44_{v6-7}$ transcript, the population of transplant patients (Section 4.2.4) was re-examined approximately 11 months after initial analysis. Acquiring blood samples from all 20 patients was not possible as the majority of patients were managed throughout other hospitals after transplant surgery. However, peripheral blood from 4 patients (patients I, M, N and S) were obtained. Previous PCR analysis had determined expression of the $CD44_{v6-7}$ transcript in patient M (Section 4.2.4). The expression of the $CD44_{v6-7}$ transcript was analyzed by PCR amplification (Section 4.2.1 and Section 4.2.3) and in accordance with earlier analysis, expression of the $CD44_{v6-7}$ transcript was detected only in patient M (data not shown).

4.2.7 Analysis of the $CD44_{v6-7}$ transcript in pre- and post-transplant PBMC

It was hypothesized that the $CD44_{v6-7}$ transcript is expressed upon activation of the alloimmune response. To examine this hypothesis, pre- and post-transplant blood samples were analyzed. Unfortunately obtaining pre-transplant blood samples was difficult due to the inconvenient time and urgency of the surgical procedure, nevertheless, 4 patients were analyzed.

A 10 ml peripheral blood sample was taken within the 24 hr period prior to transplant and day 1, 4 and 6 post-transplant. Expression of the $CD44_{v6-7}$ transcript was analyzed by PCR amplification (Section 4.2.1 and Section 4.2.3) and was detected in one male patient in both pre- and post-transplant blood samples (data not shown).

4.2.8 PCR analysis of the $CD44_{v6-7}$ transcript in PBMC of healthy individuals

Although expression of the $CD44_{v6-7}$ transcript was absent in PBMC isolated from five volunteers, a larger analysis was conducted by further recruitment of 28 individuals. A 10 ml peripheral blood sample was taken and expression of the $CD44_{v6-7}$ transcript was analyzed by PCR amplification (Section 4.2.1 and Section 4.2.3).

In total, 33 healthy volunteers comprising of 18 males and 15 females, with an average age of 33 years (range 21-55 years) were examined. The $CD44_{v6-7}$ transcript was detected in two individuals, both male, occurring at a 6% frequency in this population (Table 4.4). The expression of the $CD44_{v6-7}$ transcript in these two individuals was confirmed by the PCR analysis of a second blood sample.

4.2.9 Analysis of cell surface CD44 protein expression on PBMC isolated from transplant patients and healthy volunteers

Cell surface expression of the CD44 proteins was analyzed on PBMC from five patients and a representative volunteer. Monoclonal antibodies directed towards the

Table 4.4 Expression of the $CD44_{v6-7}$ transcript in healthy individuals.

Volunteer	Sex	Age	$CD44_{v6-7}$
1	M	31	
2	F	23	
3	M	34	
4	M	35	
5	F	24	
6	M	42	
7	F	28	
8	M	40	
9	M	25	
10	*M*	*40*	*Positive*
11	M	21	
12	M	42	
13	F	38	
14	F	37	
15	F	23	
16	M	25	
17	F	29	
18	F	26	
19	M	21	
20	M	24	
21	M	26	
22	M	27	
23	F	40	
24	M	26	
25	F	38	
26	F	25	
27	F	55	
28	M	23	
29	M	42	
30	F	55	
31	F	55	
32	F	42	
33	*M*	*35*	*Positive*

invariant region of CD44 (QE7.3E8) and the v6 and v7 exons were used. Freshly isolated PBMC from patient I, O, P, S and T were analyzed at day 7 post-transplant. Patients O and T express the $CD44_{v6-7}$ transcript as previously determined (Section 4.2.4).

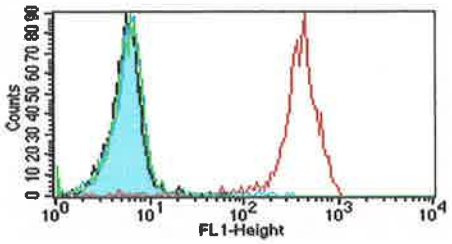
CD44 isoforms containing the v6 and v7 epitopes were not detected on PBMC isolated from either the patients or volunteer (Figure 4.7). Staining of volunteer PBMC with QE7.3E8 mAb demonstrated strong uniform expression of CD44 protein in >95% of the cells (Figure 4.7). However, the expression was distinctly bi-modal in patients P and S with patients I, O and T exhibiting a broad tri-modal expression of CD44 protein.

4.2.10 PCR analysis of the CD44 v6 transcripts expressed in haemopoietic cell lines

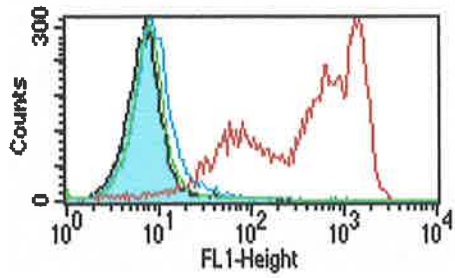
In experiments to ascertain the cellular phenotype(s) expressing the $CD44_{v6-7}$ transcript, the expression of the CD44 v6 transcripts was analyzed in human haemopoietic cell lines. The cell lines comprised of the erythroleukemic (K562), monocytic (U937 and Thp.1) and T cell (JM and HUT-78) lines. All cells were collected by centrifugation at logarithmic growth and lysed in Solution D with total RNA extracted and 1 μg reverse transcribed. The CD44 v6 transcripts were assessed by PCR amplification as previously described (Chapter 3, Section 3.2.2).

The upstream region flanking the v6 exon was analyzed by PCR amplification using primers A and C at 35 cycles. A single product approximately 460 bp in molecular size was amplified from all cell lines (Figure 4.8.A). This product has

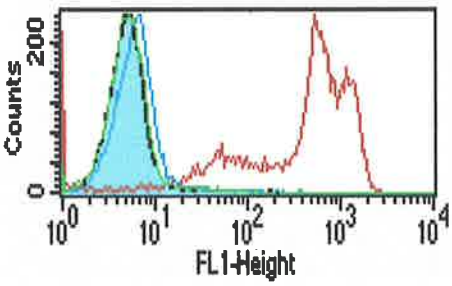
Figure 4.7 Characterization of cell surface CD44 protein expression on PBMC. PBMC isolated from transplant patients and a healthy volunteer were stained for cell surface protein expression by indirect immunofluorescence. Cells were stained with the negative control X-63 mAb (light blue histogram ■), the QE7.3E8 mAb (red overlay ■), the v6 mAb (dark blue overlay ■) and the v7 mAb (green overlay ■).



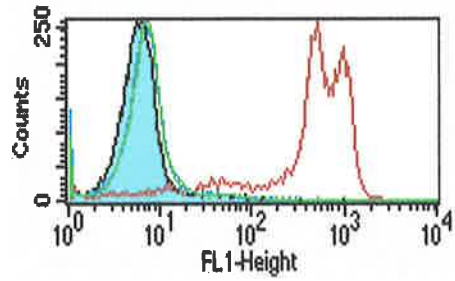
Volunteer



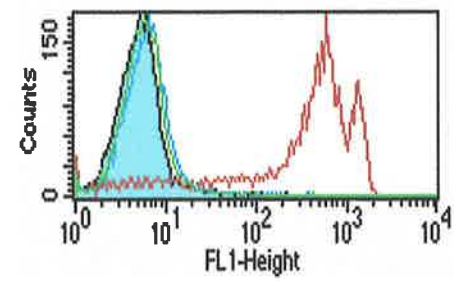
Patient I



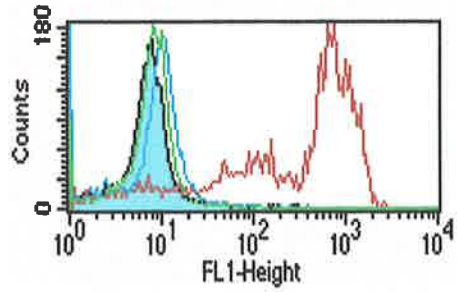
Patient O



Patient P



Patient S

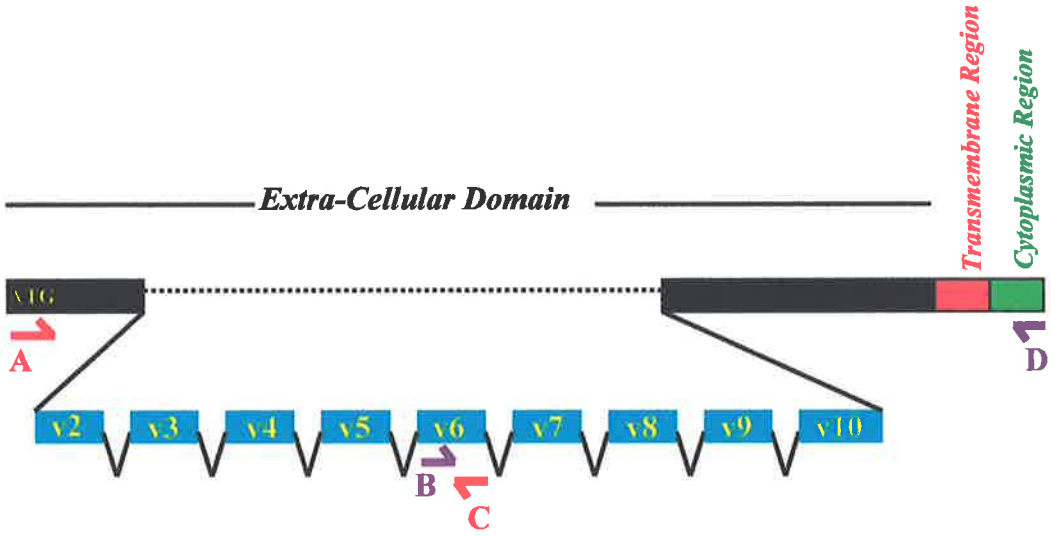


Patient T

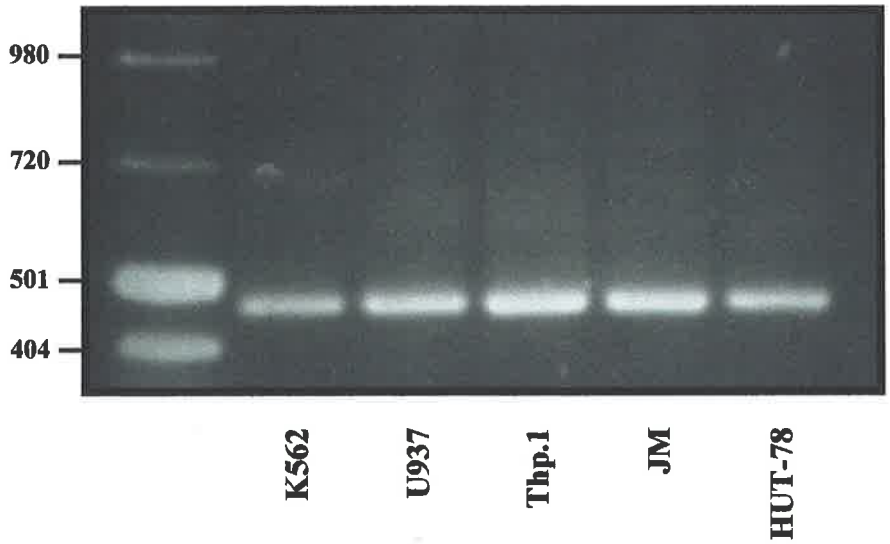
Figure 4.8 Characterization of the CD44 v6 transcripts expressed in haemopoietic cell lines.

The haemopoietic cell lines K562, U937, Thp.1, JM and HUT-78 were collected at logarithmic growth with total RNA extracted and 1 μg reverse transcribed. All PCR products (10 μl) were electrophoresed in a 1.2% agarose gel and visualized by ethidium bromide staining.

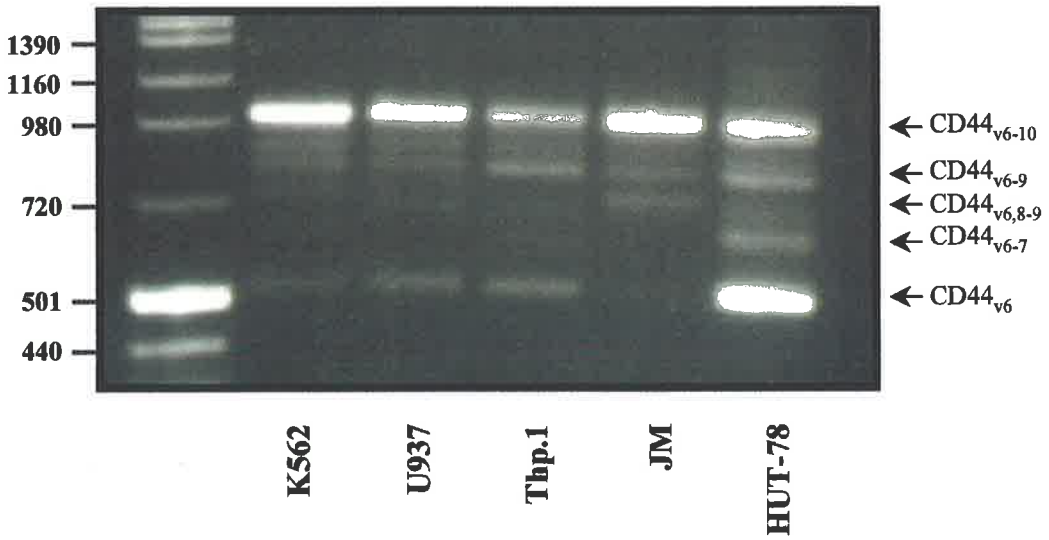
The upstream region flanking the v6 exon was analyzed using primers A and C at 35 cycles of PCR amplification (Panel A). The downstream region flanking the v6 exon was analyzed using primers B and D at 35 cycles of PCR amplification (Panel B). Molecular weight marker (M) was SPP-1 bacteriophage DNA digested with EcoRI and pUC19 plasmid DNA digested with HpaII



A. *Primers A and C*



B. *Primers B and D*



been previously identified to carry the invariant CD44 sequence immediately upstream the v6 exon (Chapter 3, Section 3.2.2.2).

The downstream region flanking the v6 exon was analyzed by PCR amplification using primers B and D at 35 cycles (Figure 4.8.B). The K562 cell line predominantly expressed the $CD44_{v6-10}$ transcript with $CD44_{v6}$ and $CD44_{v6-9}$ isoforms detected at low levels. Both the U937 and Thp.1 monocytic cell lines expressed a similar distribution of CD44 v6 transcripts as the K562 cells.

The expression of the CD44 v6 transcripts was different between the two T cell lines analyzed (Figure 4.8.B). The JM cell line predominantly expressed the $CD44_{v6-10}$ transcript with low levels of the $CD44_{v6-9}$ and the $CD44_{v6,8-9}$ transcripts. The HUT-78 cell line predominantly expressed the $CD44_{v6-10}$ and $CD44_{v6}$ transcripts with low level expression of the $CD44_{v6-9}$. Interestingly, the $CD44_{v6-7}$ transcript was amplified only in the HUT-78 cell line and verified by Southern analysis of the PCR products (data not shown).

4.2.11 PCR analysis of the CD44 v6 transcripts expressed in cutaneous T cell lymphoma

Mycosis Fungoides and Sezary Syndrome are neoplasias of malignant T lymphocytes that generally present with skin involvement (Dommann *et al.*, 1995, Toro *et al.*, 1997) and have been classified as cutaneous T cell lymphomas (CTCL). Sezary Syndrome is an advanced stage of Mycosis Fungoides with the HUT 78 cell line derived from peripheral blood of a male Sezary Syndrome patient (Gootenberg *et al.*, 1981).

Because of its expression in the HUT-78 cell line, the $CD44_{v6-7}$ transcript was studied in Sezary Syndrome and Mycosis Fungoides patients to investigate the significance of this transcript in the pathology of CTCL. Both disorders are rare with an incidence of 1-2/ 100, 000 persons per year (Dommann *et al.*, 1995). In collaboration with other institutes (IMVS, Adelaide, South Australia and Aarhus University, Aarhus, Denmark) peripheral blood, bone marrow and cell line specimens from both Sezary Syndrome and Mycosis Fungoides patients were collected (Table 4.5). The expression of the $CD44_{v6-7}$ transcript was analyzed by PCR amplification (Section 4.2.1 and Section 4.2.3).

As shown in Table 4.5, the $CD44_{v6-7}$ transcript was detected in only one of the two PBMC samples isolated from patients with Sezary Syndrome. Similarly, of the bone marrow specimens isolated from patients with Mycosis Fungoides, the $CD44_{v6-7}$ transcript was detected in one of the two. Both cell lines derived from PBMC of Sezary Syndrome patients expressed the $CD44_{v6-7}$ transcript whereas the cell line derived from PBMC of a Mycosis Fungoides patient was negative.

4.3 DISCUSSION

Although the CD44 v6 containing transcripts expressed in human PBMC have been previously defined (Chapter 3, Section 3.2.3), this chapter identifies the novel expression of the $CD44_{v6-7}$ isoform. The low frequency of the $CD44_{v6-7}$ transcript in healthy individuals (Section 4.2.8) suggests expression of an alternately spliced mRNA polymorphism. To verify such segregation, family studies could be conducted with characterization of the mRNA phenotype performed by PCR amplification as previously described (Section 4.2.1 and 4.2.3). Unfortunately these

Table 4.5 Expression of the $CD44_{v6-7}$ transcript in specimens obtained from Sezary syndrome and Mycosis fungoides patients.

Disease	Specimen type	$CD44_{v6-7}$	Sex of derived specimen
Sezary Syndrome *	*Peripheral Blood*	*Positive*	*Female*
Sezary Syndrome *	Peripheral Blood		Male
Mycosis Fungoides **	Bone Marrow		Unkown
Mycosis Fungoides **	*Bone Marrow*	*Positive*	*Unknown*
Mycosis Fungoides ***	Cell Line		Unknown
Sezary Syndrome ***	*Cell Line*	*Positive*	*Unknown*
Sezary Syndrome (Hut 78)	*Cell Line*	*Positive*	*Male*

* obtained from the Institute of Medical and Vetinary Science, Haematology Department , Adelaide, South Australia

** obtained from the Queen Elizabeth Hospital, Haematology Department, Adelaide, South Australia

*** kindly donated by Dr. Kaltoft, Department of Human Genetics, Aarhus University, Denmark

studies could not be performed due to difficulties in obtaining blood samples from family members of the two $CD44_{v6-7}$ positive individuals.

The cellular factors governing alternate splicing of the CD44 variant exons are unknown. However, it is suggested that retention of the v6-7 exons within the full length transcripts of certain individuals could arise by nucleotide changes in the exon/ intron boundary sequences. Possible nucleotide polymorphisms existing within the v5-v6, v6-v7 and v7-v8 introns can be investigated by either restriction fragment length polymorphism (RFLP) or single stranded conformational polymorphism (SSCP) analysis. In addition, expression of the $CD44_{v6-7}$ transcript in certain individuals may also arise from altered activity or concentrations of RNA splicing proteins. Possible candidates for such RNA splicing proteins are those of the SR family which, upon T cell activation, have been demonstrated to undergo complex changes in levels of mRNA expression concomitant with changes in alternate splicing of CD44 (Screaton *et al.*, 1995).

The specific function of the $CD44_{v6-7}$ protein is unknown, however, the rat homologue can confer metastatic behavior upon expression in non-metastasizing cells (Rudy *et al.*, 1993). In addition to conferring cell metastasis, some rat CD44 v6 containing proteins ($CD44_{v6}$ and $CD44_{v4-7}$) can also function as a co-stimulatory molecule in the activation of T cells, suggesting that the rat $CD44_{v6-7}$ isoform may possess a similar role (Gunthert *et al.*, 1991, Arch *et al.*, 1992, Rudy *et al.*, 1993, Moll *et al.*, 1996). Whether the human CD44 v6 proteins are capable of functioning as both a cell migratory and a co-stimulatory molecule is unknown. Studies by Galluzo *et al.* (1995) have however demonstrated the co-stimulatory function of the

CD44 v6 proteins. From these observations it is inferred that the human $CD44_{v6-7}$ isoform can function as a co-stimulatory molecule during activation of T cells.

The $CD44_{v6-7}$ transcript was detected in PBMC of recently transplanted patients at a 5-fold frequency to that of volunteers (Section 4.2.4). Earlier characterization of *in vitro* activated PBMC did not however detect the $CD44_{v6-7}$ transcript (Chapter 3, Section 3.2.5) suggesting a more complex cellular activation, such as those initiated during an *in vivo* alloimmune response, is required for induction of the $CD44_{v6-7}$ transcript. These observations suggest that, in certain individuals, expression of the $CD44_{v6-7}$ transcript may be induced upon activation of an *in vivo* alloimmune response.

Despite current advancements in medical technology, graft tolerance among transplant recipients is rarely achieved and consequently the immune system of the patient is perpetually activated throughout the life of the graft. From this observation it is expected that expression of the $CD44_{v6-7}$ transcript be maintained, in association with the patients activated immune system, throughout the life of the graft. Analysis of stable transplant patients (Section 4.2.5) demonstrated an induced frequency in expression of $CD44_{v6-7}$ with an occurrence (28% frequency) comparable to that previously concluded for recently transplanted recipients (30% frequency). Furthermore, re-examination of recently transplanted patients, at 11 months post-transplant, demonstrated continued expression of the $CD44_{v6-7}$ transcript in patient M (Section 4.2.6). These observations provide further support for the induced expression of the $CD44_{v6-7}$ transcript, in renal transplant recipients, upon activation and maintenance of an *in vivo* alloimmune response.

However, factors other than alloimmunity may influence the expression of the $CD44_{v6-7}$ transcript. Although the regulatory mechanisms involved are unclear, the predisposing renal disease and actions of immunosuppressive drugs were investigated as discussed below.

End stage renal disease can result from a variety of medical conditions. The pathological manifestations from these disorders may account for the increased frequency in expression of the $CD44_{v6-7}$ transcript in renal transplant patients. However, the predisposing disorder of each $CD44_{v6-7}$ expressing patient was different (Table 4.1 and 4.2). Therefore no correlation between expression of the $CD44_{v6-7}$ transcript and pathology of the predisposing disease could be formed.

With an activated immune system, all transplant recipients are dependent on immunosuppressive drugs throughout the life of the graft. The aim of immunosuppressive therapy is to interfere and ultimately interrupt the process of T cell activation. Various immunosuppressive agents act at distinct sites in the T cell activation cascade (as reviewed in Mandel, 1992, Russ, 1992, Suthanthiran, 1997) and can be classified on the basis of their primary site of action as inhibitors of cytokine gene transcription (Cyclosporin, FK506 and corticosteroids) or nucleotide synthesis and cell proliferation (Azathioprine and Myophenolate Mofetil). The large majority of patients in Australia currently receive initial treatment with two or more of Azathioprine, a corticosteroid and Cyclosporin. The doses of Cyclosporin and corticosteroids are reduced with time after transplantation, whereas the dose of Azathioprine is generally left at its initial level (Power and d'Apice, 1992).

The main action of immunosuppressive drugs such as Cyclosporin and FK506 are to inhibit induction of gene transcription. However, the consequences of these drug treatments can also be stimulatory to gene activity (as reviewed in Ruhlmann and Nordheim, 1997). Examples of genes whose transcription can be induced by Cyclosporin or FK506 treatment are cell adhesion molecules such as ICAM-1 (Frishberg *et al.*, 1996) and L-selectin (Kaldjian and Stoolman, 1995) and also the junB (Su *et al.*, 1996) and egr-1 (Metcalfe and Richards, 1990) transcription factors. The significance of the egr-1 transcription factor upon CD44 gene expression has been previously discussed (Section 1.11.1). From these observations, it can be inferred that expression of the $CD44_{v6-7}$ transcript may also be induced by actions of the immunosuppressive drugs.

Possible mechanisms for the proposed induction of the $CD44_{v6-7}$ transcript expression by immunosuppressive drugs are unclear, however, the involvement of RNA splicing proteins is speculated. Furthermore, data presented in this chapter (Section 4.2.4 and 4.2.5) suggests that only 30% of transplant patients are susceptible to the $CD44_{v6-7}$ inducing property of the immunosuppressive drugs. The influence of immunosuppressive reagents on expression of the CD44 v6 transcripts in PBMC can be analyzed by *in vitro* studies as described earlier (Chapter 3, Section 3.2.5). However, some immunosuppressive drugs are administered as prodrugs which must be enzymatically converted to the active reagent for functional activity (Russ, 1992). For example, Azathioprine is converted to 6-mercaptopurine by enzymes in the liver and other tissues.

Interestingly, the expression of the $CD44_{v6-7}$ transcript was transient in one patient (Section 4.2.4). Patient T was re-admitted to the hospital 1 month post-transplant suffering graft rejection at which time the dosage of the FK506 immunosuppressive drug increased 4 fold. Only at this increased dosage was expression of the $CD44_{v6-7}$ transcript absent. This observation suggests down-regulation in expression of the $CD44_{v6-7}$ transcript upon administration of an increased FK506 dosage to patient T. The precise mechanism for inhibition of the $CD44_{v6-7}$ mRNA expression is unclear, however, may be related to FK506-mediated severe depression of the immune system or by direct pharmacological actions of the higher dosage of FK506. Further analysis of patient T would be interesting, particularly once graft rejection subsides and dosage of FK506 is reduced. At such time, the possible re-appearance in $CD44_{v6-7}$ transcript expression can be determined. Unfortunately, patient T died from infection and subsequent studies could not be performed.

The action of the immunosuppressive drugs may also account for detection of the $CD44_{v6-7}$ transcript in pre-transplant PBMC from one patient (Section 4.2.7). All transplant recipients are treated with immunosuppressive drugs (Azathioprine and corticosteroids) for 48 hr prior to surgery. All pre-transplant blood samples were collected in the 24 hr period prior to transplantation. Therefore, it is not possible to exclude immunosuppressive drugs, during this 48 hr pre-transplantation period, as a contributing factor for expression of the $CD44_{v6-7}$ mRNA. Alternately, the $CD44_{v6-7}$ transcript expressed in the PBMC from the 2 healthy individuals (Section 4.2.8) may reflect constitutive mRNA expression and thus account for its detection in un-treated pre-transplant PBMC. Further studies investigating the expression of the $CD44_{v6-7}$

transcript in pre- and post-transplant PBMC specimens is contemplated with the recruitment of a greater number of patients. Future transplant candidates could be characterized for expression of the CD44 v6 transcripts in PBMC months prior to transplantation. The data obtained would determine if each patient constituitively express the $CD44_{v6-7}$ transcript. Consequently, the contributing factor for expression of the $CD44_{v6-7}$ transcript in any pre-transplant PBMC specimen can be defined as either constitutive or drug induced expression.

Following renal transplantation, both an active immune system and daily administration of immunosuppressive drugs are endured, throughout the life of the graft, by each patient. Therefore, it cannot be determined whether alloimmunity or immunosuppressive drugs are inducing transcriptional expression of the $CD44_{v6-7}$ mRNA within the renal transplant population. In summary, the data obtained from this study suggests that either an immunological or pharmacogenetic phenomenon is inducing expression of the $CD44_{v6-7}$ transcript in PBMC from renal transplant patients in comparison to healthy individuals.

Despite PCR amplification of distinct CD44 variant mRNA species containing the v6 and/ or v7 exons (Section 4.2.2 and 4.2.3), cell surface expression of corresponding epitopes were not detected on PBMC (Section 4.2.9). The poor correlation between CD44 variant mRNA and protein expression may result from limitations in detection of cell-surface bound antibodies, estimated to be in the order of 2000 antibody molecules per cell (Hulett *et al.*, 1973). A modified method capable of detecting fewer than 100 molecules of antibody bound per cell method has been reported (Zola *et al.*, 1990). It is suggested that future studies utilize the more

sensitive immunofluorescence method for detection of cell surface CD44 variant proteins.

Expression of total CD44 protein on PBMC from all transplant patients analyzed was bi-modal or tri-modal in comparison to the uniform expression detected from a representative volunteer (Section 4.2.9). This observation suggests an induced expression of CD44 protein in transplant recipients. In accordance with previous reports, it is suggested that $CD44_{std}$ protein is up regulated on T and B cells following *in vivo* cellular activation (Budd *et al.*, 1987, Camp *et al.*, 1991a, Hathcock *et al.*, 1993). The precise function of the $CD44_{std}$ protein in an alloimmune response is unknown, however, $CD44_{std}$ has been demonstrated to function in lymphocyte migration to extralymphoid sites of inflammation and a general role in cell adhesion, lymphocyte activation and signalling (Aruffo *et al.*, 1990, Denning *et al.*, 1990, Koopman *et al.*, 1990, Miyake *et al.*, 1990b, Camp *et al.*, 1993, Taher *et al.*, 1996).

As previously discussed, the delineation in expression of the CD44 v6 transcripts in sub-populations of mononuclear cells (e.g. T cell, B cell and monocyte) is unknown. In experiments to define the cellular origin of the $CD44_{v6-7}$ transcript a number of haemopoietic cell lines were characterized (Section 4.2.10). The $CD44_{v6-7}$ transcript was expressed in HUT-78 cells, a human T cell line derived from peripheral blood of a patient with Sezary syndrome (Gootenberg *et al.*, 1981).

Sezary syndrome is classified as a cutaneous T cell lymphoma (CTCL) with migration of circulating malignant T cells to the skin. Recently the expression of CD44 v6 proteins was examined in skin biopsies from CTCL patients with or

without systemic spread of the disease. Of the CTCL patients with systemic spread, $^{11}/_{12}$ samples showed a prevalent CD44 v6 protein(s) expression whereas no expression was detected from 18 CTCL patients with no systemic spread. Therefore expression of the CD44 v6 protein(s) on tumor T cells was strongly associated with an aggressive behavior of the cutaneous lymphoma (Dommann *et al.*, 1995). This study however, did not characterize the specific v6 exon containing isoforms as neither immunoprecipitation nor RT-PCR analysis was performed. The identity of the $CD44_{v6\text{-}7}$ mRNA in PBMC and T cell lines derived from Sezary patients (Section 4.2.11) suggests that this isoform may be associated with expression of the CD44 v6 protein(s) in skin biopsy specimens of CTCL patients.

Expression of the $CD44_{v6\text{-}7}$ transcript in Sezary cells (Section 4.2.11), in conjunction with the known metastatic properties of the rat $CD44_{v6\text{-}7}$ homologue, suggests the possible involvement of the human $CD44_{v6\text{-}7}$ isoform in the migration of malignant peripheral blood T cells to the skin. Although preliminary, the analysis of the $CD44_{v6\text{-}7}$ transcript in specimens and cell lines derived from CTCL patients suggests an increased frequency in expression of this transcript in comparison to volunteer population (Section 4.2.11). Future studies with the inclusion of greater numbers of patients should establish any relationship between expression of the $CD44_{v6\text{-}7}$ transcript and the pathogenesis of CTCL.

Chapter Five

Cloning and Functional Characterization of the $CD44_{v6\text{-}10}$, $CD44_{v6\text{-}9}$, $CD44_{v6\text{-}7}$, $CD44_{v6}$ and $CD44_{std}$ Proteins.

5.1 INTRODUCTION

An effective approach to study the function of CD44 variant proteins is to clone the full length cDNA transcript and to express the recombinant protein in a mammalian cell line that is devoid of CD44. Cell lines that have been previously used for expression and functional analysis of variant CD44 isoforms are the human B cell lymphoma Namalwa (Sy *et al.*, 1991, Bartolazzi *et al.*, 1995, Bennett *et al.*, 1995a, Jackson *et al.*, 1995) and acute T cell leukaemia Jurkat (Liao *et al.*, 1993, Liu and Sy, 1996, Telen *et al.*, 1996).

The aim of this chapter was to:

(i) develop a cloning strategy to isolate the full length cDNA clones of the previously identified CD44 v6 transcripts expressed in human PBMC,

(ii) establish stable transfectants containing the cloned cDNA in expression vectors using the Jurkat cell line and

(iii) characterize the cell surface expressed protein in functional assays. The objective of these experiments was to provide further understanding of the specific role of each CD44 v6 containing protein in the activation of human T cells.

5.2 RESULTS

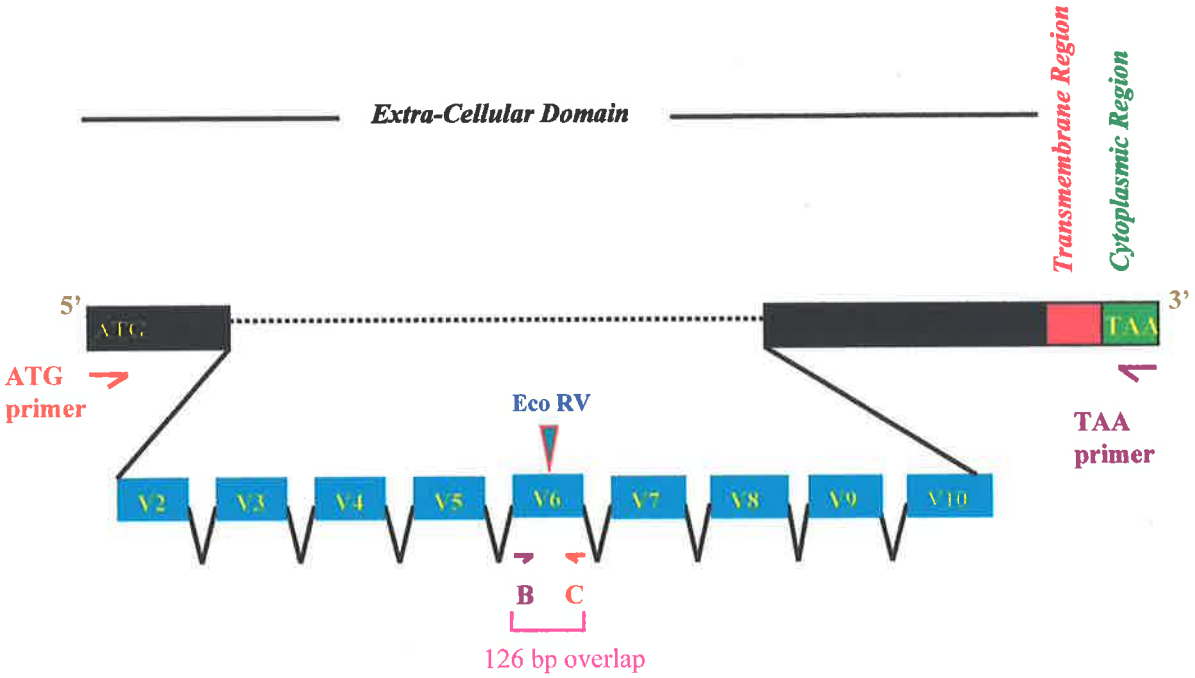
5.2.1 Strategy to clone the CD44 v6 transcripts expressed in human PBMC

The cloning of the full length CD44 v6 transcripts ($CD44_{v6\text{-}9}$, $CD44_{v6\text{-}7}$ and $CD44_{v6}$) was based on a strategy that utilized PCR amplification, restriction enzyme digestion and DNA ligation as outlined in Figure 5.1.

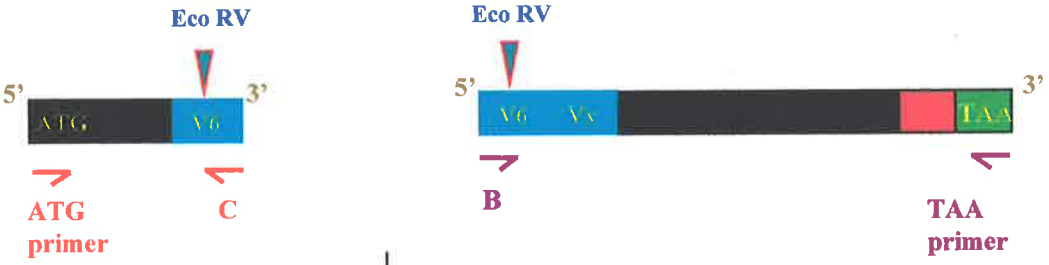
Figure 5.1 Strategy for the cloning of full length CD44 v6 transcripts.

Details of the cloning strategy to generate the CD44 v6 full length transcripts are outlined within the text.

vx represents 0, 1 or more variant exons.

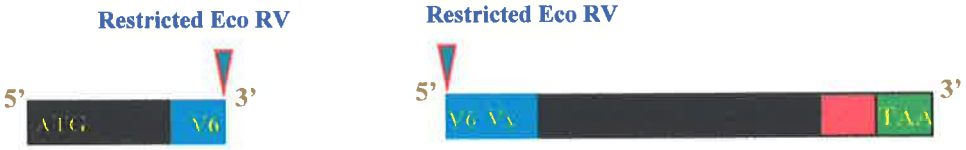


A.



Digest with Eco RV restriction enzyme

B.



Ligate the products together to re-create the Eco RV restriction enzyme site and generate a full length clone

C.

The up- and down- stream regions flanking the v6 exon are PCR amplified using v6 exon specific primers as previously discussed (Chapter 3, Section 3.2.2). From the position of primers B and C (see Figure 5.1) a 126 bp overlapping region of v6 DNA sequence is amplified in both PCR fragments. Contained within the v6 exon overlapping region is an Eco RV restriction enzyme site that is unique to the CD44 cDNA sequence and present in both up- and down- stream v6 exon PCR fragments (Figure 5.1.A). Following an Eco RV digestion of the PCR fragments (Figure 5.1.B), the restricted sites can be ligated together to re-create an in-frame enzyme site and generate a full length transcript (Figure 5.1.C). An alternative to this strategy is that each amplified fragment is cloned individually into the pGEM T^+ vector from which the full length transcripts are generated by Eco RV digestion and ligation as described.

5.2.2 Cloning of the full-length human CD44 v6 transcripts

5.2.2.1 PCR amplification of the up- and down-stream regions flanking the v6 exon

From the CD44 gene sequence (Screaton *et al.*, 1992) a forward primer was designed to incorporate the transcription ATG start codon (primer G). Similarly, a reverse primer was designed incorporating the termination TAA codon located in exon 19 (primer H). These primers were used in conjunction with corresponding v6 exon specific primers to amplify up- and down-stream of the v6 exon (Figure 5.2).

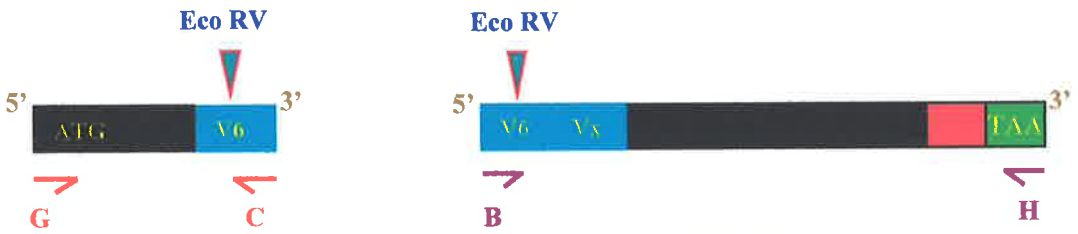
All PCR fragments were amplified from cDNA template previously synthesized using PBMC isolated from a healthy volunteer that expresses the $CD44_{v6-7}$

Figure 5.2 Cloning of the up- and down-stream PCR fragments flanking the v6 exon into the pGEM T^+ vector.

All PCR fragments were amplified using cDNA template synthesized from 6 hr cultured PBMC from a healthy individual expressing the $CD44_{v6-7}$ transcript.

The up- stream region flanking the v6 exon was PCR amplified using primers G and C at 35 cycles with the resultant (ATG~v6) fragment cloned into the pGEM T^+ vector. The down- stream region flanking the v6 exon was PCR amplified using primers B and H at 35 cycles with the resultant (v6~TAA), (v6-7~TAA) and (v6-9~TAA) fragments cloned into the pGEM T^+ vector. Recombinant plasmids resulting from the cloning were termed pGEM.ATG~v6, pGEM.v6-9~TAA, pGEM.v6-7~TAA and pGEM.v6~TAA respectively.

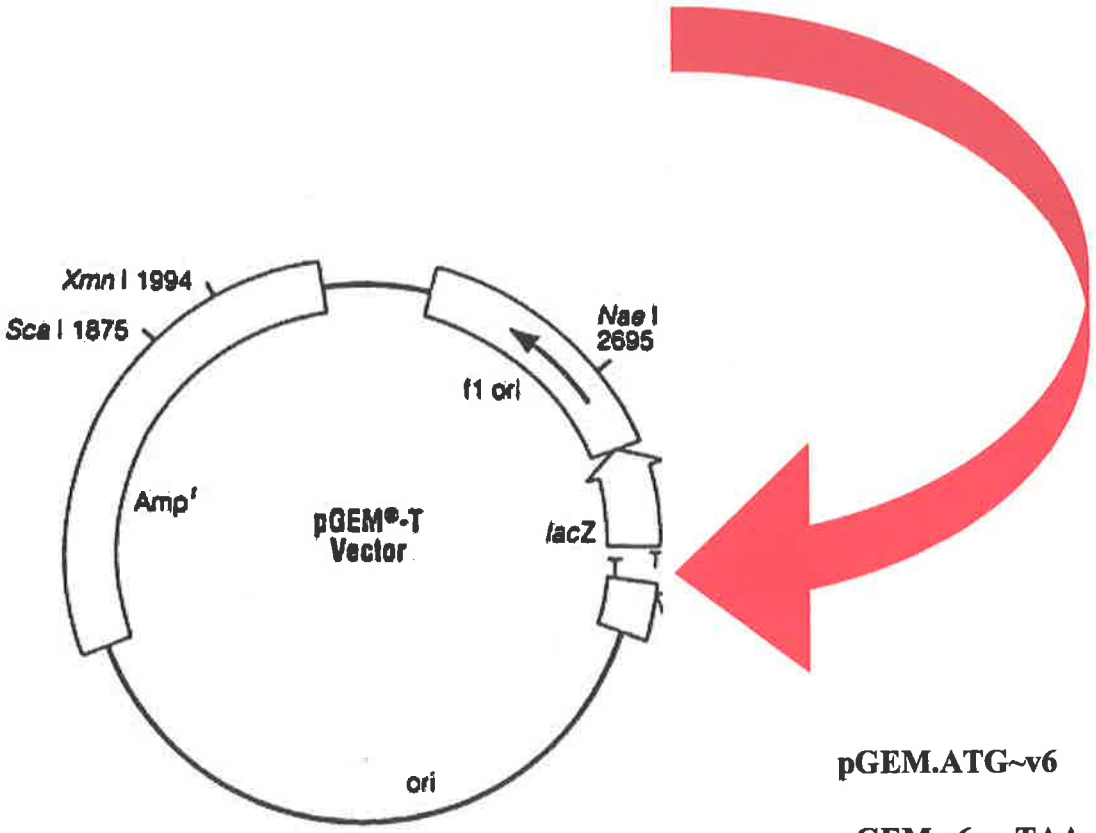
vx represents 0, 1 or more variant exons.



(ATG~v6) fragment

(v6~TAA)
(v6-7~TAA) fragments
(v6-9~TAA)

PCR Amplification of the up- and down-stream regions flanking the CD44 v6 exon and ligation into the pGEM T⁺ vector



pGEM.ATG~v6

pGEM.v6-x~TAA

transcript (Chapter 4, Section 4.2.8). PCR amplification at 35 cycles using primers G and C generated a single product previously defined (Chapter 3, Section 3.2.2.2) to contain the invariant region of the CD44 molecule immediately upstream of the v6 exon (data not shown). The PCR product was termed (ATG~v6) representing the presence of the start codon and the v6 exon within this fragment. Subsequent PCR amplification using primers B and H generated multiple products previously defined as the $CD44_{v6\text{-}10}$, $CD44_{v6\text{-}9}$, $CD44_{v6\text{-}7}$ and $CD44_{v6}$ transcripts (Chapter 4, Section 4.2.2) and termed (v6-10~TAA), (v6-9~TAA), (v6-7~TAA) and (v6~TAA) respectively.

5.2.2.2 *Cloning of the PCR fragments into the pGEM T$^+$ vector*

All PCR fragments were electrophoresed through a 1.2% agarose gel, purified (Section 2.2.10.1), ligated into the pGEM T$^+$ vector (Section 2.2.10.2) and transformed into competent *E.coli* TG-1α cells (Section 2.2.10.3). Positive recombinant colonies were detected by ampicillin resistance and isolated on the basis of blue/ white colour selection. Plasmid DNA was prepared from recombinant colonies (Section 2.2.11) and the presence of an insert verified by PCR amplification using appropriate pairs of invariant and v6 exon specific primers (data not shown).

The recombinant plasmids produced from this cloning were respectively termed pGEM.(ATG~v6), pGEM.(v6-9~TAA), pGEM.(v6-7~TAA) and pGEM.(v6~TAA) where in this nomenclature "pGEM" refers to the pGEM T$^+$ vector. For future reference the pGEM.(v6-9~TAA), pGEM.(v6-7~TAA) and pGEM.(v6~TAA) plasmids are collectively termed pGEM.(v6-vx~TAA), where "vx" represents 0, 1 or more variant exons.

A total of 4 recombinant plasmids were randomly chosen from the cloning of each PCR fragment and a single clone chosen on the basis of a specified criterion as outlined below. This criterion ensures the successful generation of full length CD44 v6 transcripts.

To verify that a single copy of each PCR product was cloned into the pGEM T^+ vector, plasmid DNA was digested with the Not I and Apa I enzymes. The restriction sites for these two enzymes flank the 3' thymidine insertion site of the pGEM T^+ vector. Neither enzyme site exists within the CD44 coding region and therefore under these digestion conditions the cloned PCR product is excised from the vector. Gel electrophoresis of Not I/ Apa I digested DNA revealed excised inserts of molecular sizes corresponding to that of the original PCR fragment (data not shown).

The presence of the unique Eco RV restriction enzyme site was confirmed in all recombinant plasmids by an Eco RV digestion. This restriction enzyme site is absent in the pGEM T^+ vector and therefore upon gel electrophoresis of restricted DNA a linearized fragment concluded the presence of a functional Eco RV restriction site (data not shown).

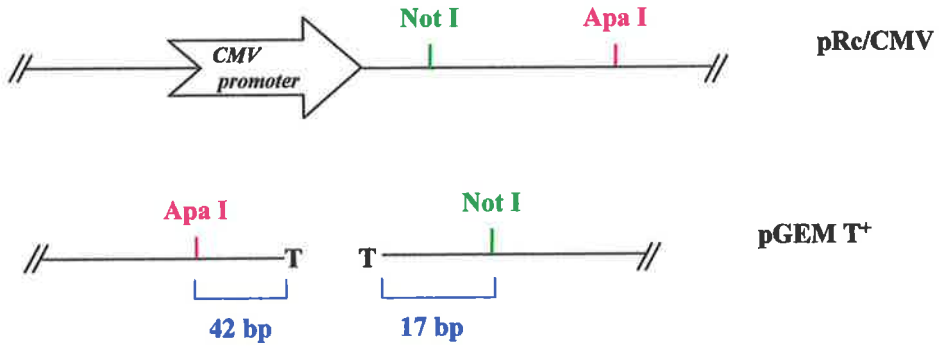
Analysis of the multiple cloning sites from the pGEM T^+ and pRc/CMV vectors reveal a common occurrence of both Not I and Apa I restriction enzyme sites (Figure 5.3.A). As previously mentioned, the restriction sites for these two enzymes are absent within the CD44 coding region and therefore can be utilized for subcloning full length transcripts from the pGEM T^+ into the pRc/CMV vector. The

Figure 5.3 Subcloning strategy for ligation of full length CD44 v6 transcripts from the pGEM T^+ vector into the expression pRc/CMV vector.

Details of the subcloning strategy of the CD44 v6 full length transcripts from the pGEM T^+ vector into the pRc/CMV vector is outlined within the text.

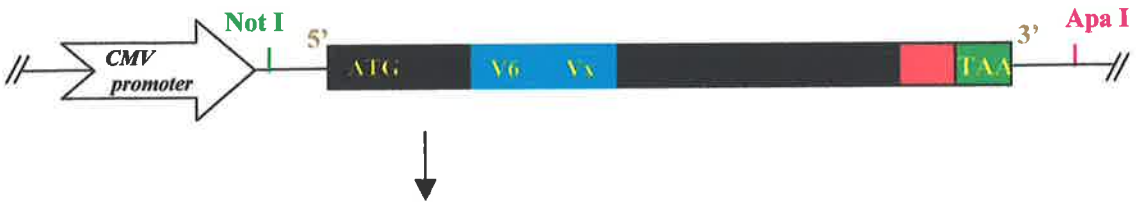
vx represents 0, 1 or more variant exons.

A. Analysis of Multiple cloning site of vectors



B. Orientation of transcription start and stop codons in relation to Not I and Apa I restriction enzyme sites

Full length CD44 transcript in pRc/CMV



Full length CD44 transcript in pGEM T+

Orientation of the up- and down-stream PCR products flanking the CD44 v6 exon in pGEM T+

pGEM.ATG~v6

pGEM.v6-x~TAA

final orientation of the full length transcripts within the pRc/CMV vector is determined by the location of the Not I and Apa I restriction sites in relation to the CMV promoter. The Not I site is adjacent to the CMV promoter. Consequently, subcloning of full length transcripts in a sense orientation into the pRc/CMV vector requires the ATG codon of the full length clones within the pGEM T^+ vector to be adjacent the Not I site (Figure 5.3.B).

The orientation of the (ATG~v6) fragment within the pGEM.(ATG~v6) plasmids were analyzed by Not I/ Eco RV digestion to isolate a clone that contained the ATG codon of the CD44 insert adjacent the Not I restriction site. Similarly, inserts within the pGEM.(v6-9~TAA), pGEM.(v6-7~TAA) and pGEM.(v6~TAA) plasmids were orientated by Apa I/ Eco RV digestion for clones that contain an insert with the TAA codon adjacent the Apa I restriction site (Figure 5.3.B). Based on the molecular size of the excised insert from these digestions, the orientation of the ATG and TAA sites within each recombinant plasmid was determined (Figure 5.4).

5.2.2.3 Generation of full length CD44 v6 transcripts

As mentioned previously the Not I enzyme site is located within the multiple cloning region of the pGEM T^+ vector and is absent from the CD44 cDNA sequence. Consequently, the digestion of the pGEM.ATG~v6 plasmid with Not I/ Eco RV (Figure 5.5.A) will excise the (ATG~v6) fragment containing an additional 17 bp of the multiple cloning site of the pGEM T^+ vector, yet missing 48 bp of the v6 exon (region pertaining from the Eco RV restriction site to primer C). Using identical enzyme digestion conditions, the pGEM.(v6-vx~TAA) plasmids are essentially linearized with the excision of an 87 bp fragment containing both pGEM T^+ vector

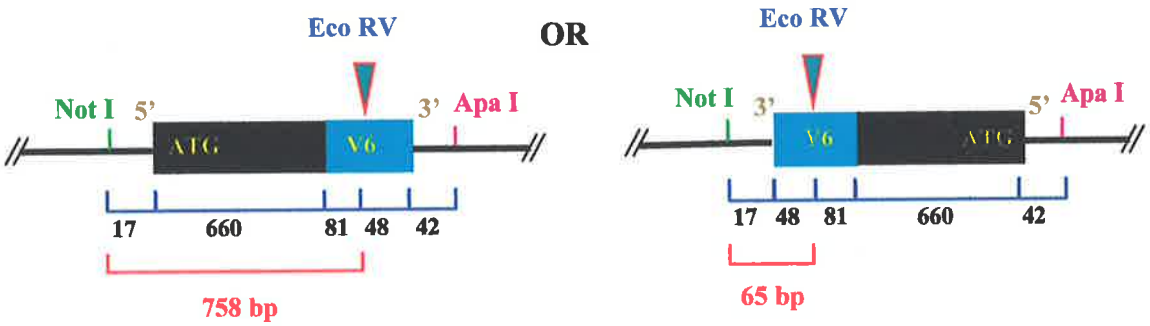
Figure 5.4 Restriction mapping of the pGEM.ATG~v6 and pGEM.v6-vx~TAA vectors.

From the physical restriction map of the pGEM.ATG~v6, pGEM.v6-9~TAA, pGEM.v6-7~TAA and pGEM.v6~TAA plasmids, the orientation of the PCR product inserts was determined by enzyme digestion. The pGEM.ATG~v6 plasmids were digested with the Not I and Eco RV enzymes and under these conditions, an insert of 758 bp is excised if the ATG~v6 PCR product ligates within the pGEM T^+ vector with the ATG codon adjacent the Not I restriction site. Conversely, if the ATG~v6 product lies in the opposite orientation a 65 bp fragment is removed from the vector. Under similar conditions the orientation of the v6-vx~TAA PCR products within the pGEM T^+ vector were determined using Apa I and Eco RV restriction digestion.

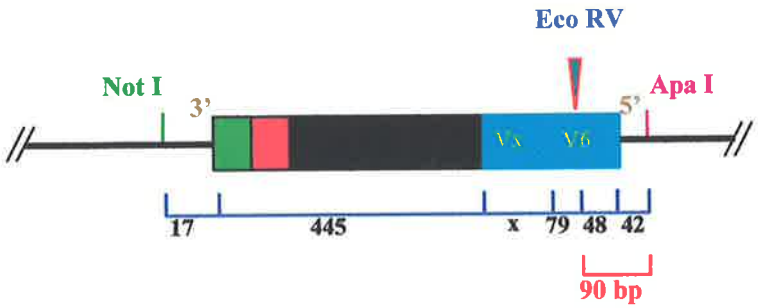
vx represents 0, 1 or more variant exons.

pGEM.ATG~v6 recombinant plasmids

(Not I/ Eco RV digests)



pGEM.v6-vx~TAA recombinant plasmids
(Apa I/ Eco RV digests)



OR

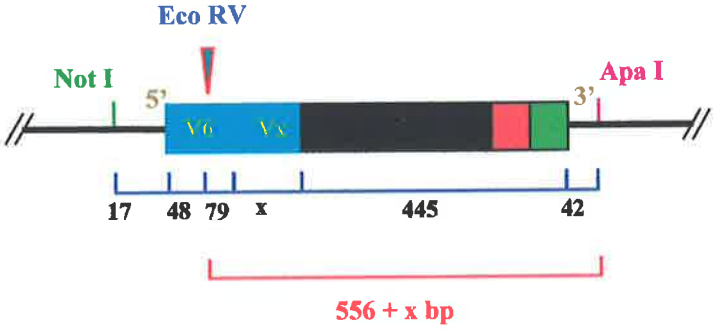
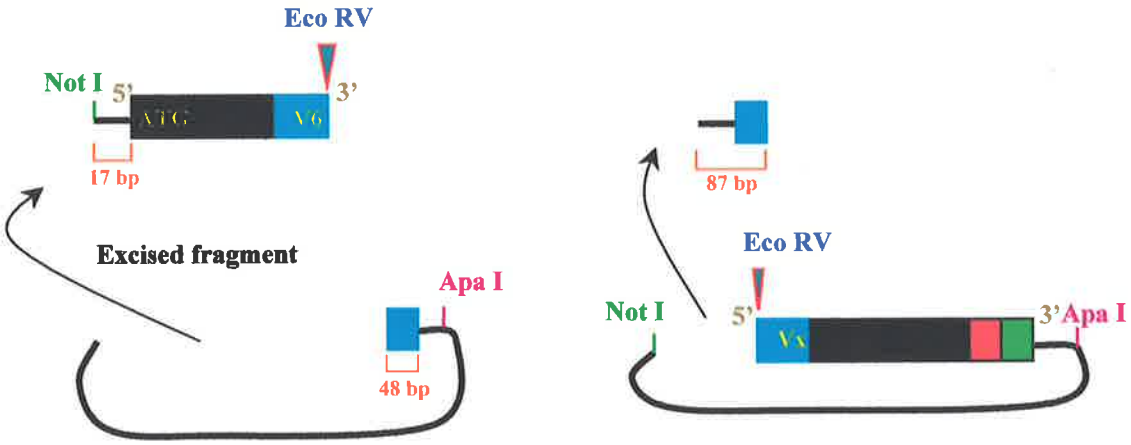


Figure 5.5 Generation of full length CD44 v6 transcripts.

Details of the cloning procedure used to synthesize the pGEM.$CD44_{v6}$, pGEM.$CD44_{v6\text{-}7}$ and pGEM.$CD44_{v6\text{-}9}$ plasmids are detailed in the text.

vx represents 0, 1 or more variant exons.

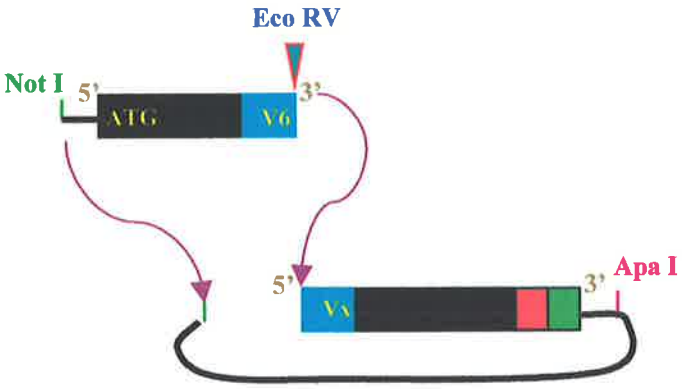
A. ***Digestion with Eco RV and Not I enzymes***



pGEM.ATG~v6

pGEM.v6-vx~TAA

B. ***Ligate the fragments to generate full length clone in pGEM T+***



pGEM.v6-vx~TAA

pGEM.CD44 $_{v6\text{-}vx}$

and v6 exon DNA sequence (Figure 5.5.A). As such the Not I/ Eco RV digestions prepares the pGEM.(v6-vx~TAA) plasmids as suitable vectors for insertion of the Not I/ Eco RV excised (ATG~v6) fragment.

All digested products were electrophoresed through an agarose gel and the products of interest were purified and ligated together at a 3:1 molar ratio of (ATG~v6) insert to pGEM.(v6-vx~TAA) vector DNA. Once transformed into the *E.coli* TG-1α bacteria, colonies containing plasmids with the full length CD44 v6 transcripts were identified by PCR analysis using primers G and H (data not shown).

Recombinant plasmid DNA was prepared and digested with the Eco RV enzyme where linearization of the plasmid demonstrated the presence of an in-frame restriction enzyme site (data not shown). Recombinant plasmids obtained from this ligation were termed pGEM.$CD44_{v6\text{-}9}$, pGEM.$CD44_{v6\text{-}7}$ and pGEM.$CD44_{v6}$ respectively (Figure 5.5.B).

The full length CD44 v6 transcripts were subsequently excised from the pGEM T^+ vector using the flanking Not I and Apa I restriction enzymes, ligated into Not I/ Apa I digested pRc/CMV vector and transformed into *E.coli* TG-1α bacteria. Bacterial colonies were randomly chosen and screened for full length transcript inserts by PCR analysis using primer G and H (data not shown).

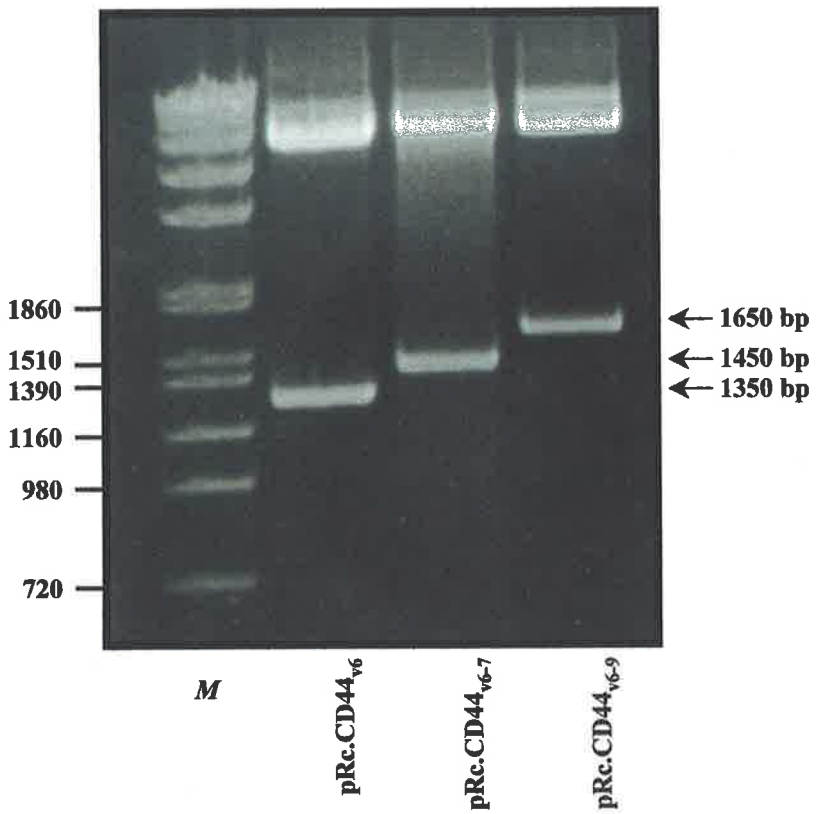
Plasmid DNA was prepared from recombinant colonies and presence of the full length transcripts confirmed by restriction enzyme digestion using the flanking Not I and Apa I restriction sites (Figure 5.6). The final recombinant plasmids obtained

Figure 5.6 Restriction enzyme analysis of the full length CD44 v6 transcripts within the pRc/CMV vector.

Presence of a full length CD44 insert within the pRc.CD44$_{v6}$, pRc.CD44$_{v6-7}$ and pRc.CD44$_{v6-9}$ plasmids was confirmed by the restriction digestion of plasmid DNA with the Apa I and Not I enzymes consequently excising the CD44 cDNA insert. The digested products were electrophoresed on a 1.2% agarose gel with CD44 cDNA inserts of approximately 1350 bp, 1450 bp and 1650 bp products excised from the pRc.CD44$_{v6}$, pRc.CD44$_{v6-7}$ and pRc.CD44$_{v6-9}$ plasmids respectively (Panel A). The molecular sizes of the inserts correspond to the calculated sizes of the respective full length transcripts as determined from the physical restriction map of each pRc.CD44$_{v6}$, pRc.CD44$_{v6-7}$ and pRc.CD44$_{v6-9}$ plasmid (Panel B). Molecular weight marker (M) was pUC19 plasmid DNA digested with HpaII.

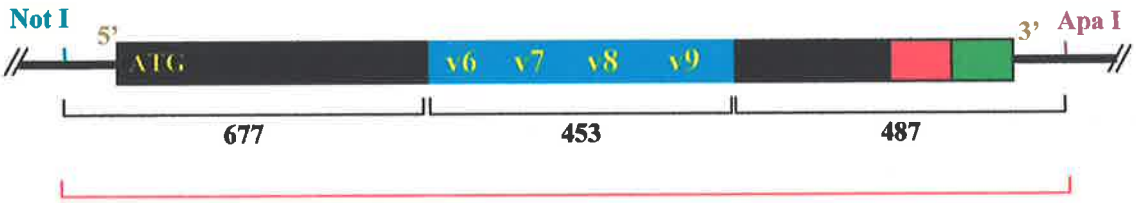
Nucleotide sequencing of the full length cDNA clones (pRc.CD44$_{v6-9}$, pRc.CD44$_{v6-7}$ and pRc.CD44$_{v6}$) demonstrated complete homology (see Appendix) to the sequence published by Screaton *et al* (1992).

A.



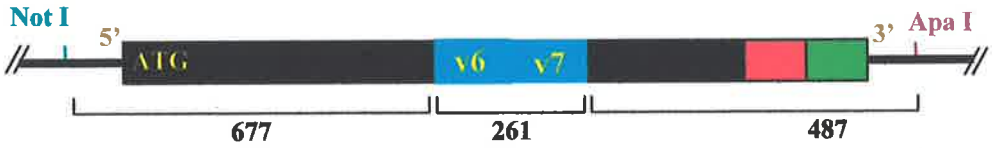
B.

pRc.CD44$_{v6-9}$



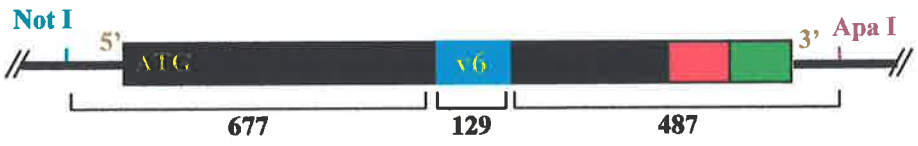
1617 bp

pRc.CD44$_{v6-7}$



1425 bp

pRc.CD44$_{v6}$



1293 bp

from this cloning were termed pRc.CD44$_{v6-9}$, pRc.CD44$_{v6-7}$ and pRc.CD44$_{v6}$ respectively, where in this nomenclature the "pRc" refers to the pRc/CMV vector.

5.2.3 Transfection of the Jurkat 10.4 cell line

5.2.3.1 Optimization of plasmid DNA electroporation into Jurkat 10.4 cells

A cloned derivative of the Jurkat cell line, Jurkat 10.4, was used for production of stable transfectants and was previously generated in the host laboratory. Jurkat 10.4 is characterized with strong cell surface protein expression of the CD2 and CD3 receptors yet no expression of CD44 protein or mRNA (data not shown).

The pRc.CD44$_{v6-10}$ and pRc.CD44$_{std}$ plasmid DNA clones (Jackson *et al.*, 1995) were kindly provided by Dr. Jackson (Oxford University, Oxford, England) and the pRc.CD44$_{v6-9}$, pRc.CD44$_{v6-7}$, pRc.CD44$_{v6}$ and pRc/CMV plasmids have been described earlier (Section 5.2.2). All plasmid DNA was extracted from bacterial culture using the Jetstar DNA affinity column (Section 2.2.12) and quantitated by spectrophotometry.

Conditions for the electroporation of Jurkat 10.4 cells (Section 2.2.13) was initially optimized using 20 μg of pRc.CD44$_{std}$ plasmid DNA. The Gene Pulser II electroporator was set at a constant 960 μF with voltage varied between 100 V and 500 V at 50 V increments. After a period of 48 hr, the transient cell surface expression of CD44$_{std}$ protein was measured by indirect staining using the anti-CD44 QE7.3E8 monoclonal antibody (Section 2.2.16). Maximal recombinant protein

expression was obtained with electroporation settings at 960 μF and 250 V (Figure 5.7).

With electroporation conditions set at 960 μF and 250 V, the Jurkat 10.4 cells were transfected with the varying quantities (10 μg, 20 μg, 40 μg or 60 μg) of pRc.CD44$_{std}$ plasmid DNA. Transient expression of recombinant CD44$_{std}$ protein was measured concluding that maximal protein expression was obtained with 40 μg plasmid DNA (data not shown).

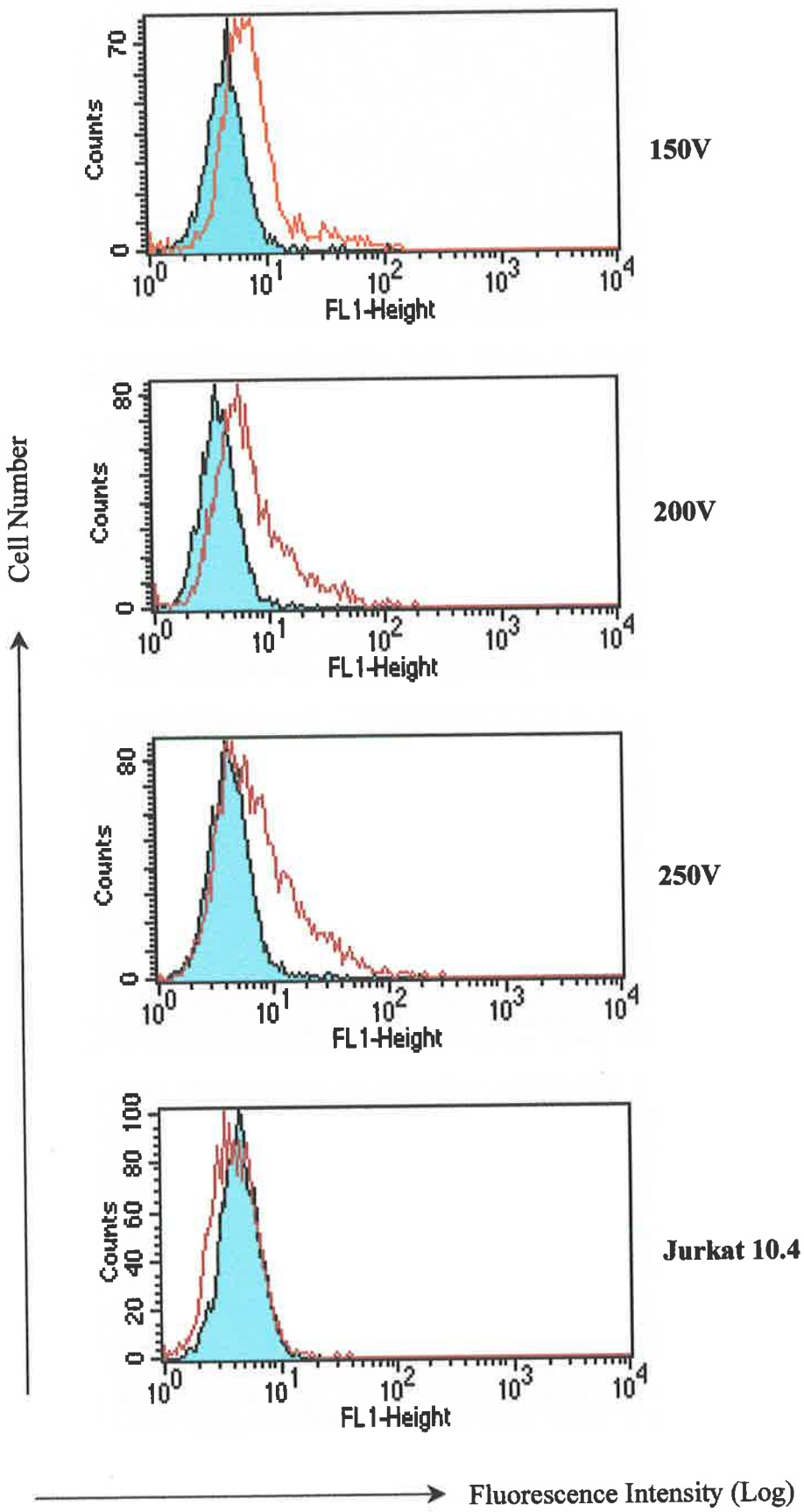
From these experiments the optimal conditions for transfection of the Jurkat 10.4 cell line was established with electroporation conditions at 960 μF and 250 V using 40 μg Jetstar purified plasmid DNA.

5.2.3.2 *Determination of G418 concentration for recombinant Jurkat 10.4 cells selection*

A total of 4 x10^6 Jurkat 10.4 cells was cultured at a density of 1 x10^6 cell/ml in RPMI supplemented with 10% FCS containing G418 at the following concentrations: 0 mg/ml, 0.5 mg/ml, 1 mg/ml, 1.5 mg/ml and 2 mg/ml. The cells were seeded every 4-5 days and cultured until cell death was apparent within the population. At the end of an 8 week period, the cells cultured in 1.5 mg/ml and 2 mg/ml G418 demonstrated 100% cell death. From these experiments it was determined that a G418 concentration of 2 mg/ml can be used for the selection of Jurkat 10.4 cells transfected with G418-resistant plasmid vector.

Figure 5.7 Flow cytometric analysis of transiently transfected Jurkat cells with the pRc.CD44$_{std}$ plasmid.

A total of 6 x10^6 Jurkat 10.4 cells were transfected with 20 μg pRc.CD44$_{std}$ plasmid DNA with electroporation conditions using a set capacitance of 960 μF with the voltage varying between 100 V to 500 V at 50 V increments. After a 48 hr recovery period, the cells were characterized for cell surface recombinant CD44$_{std}$ protein expression by indirect immunofluorescence with cells stained with the negative control X-63 mAb (light blue histogram ■) and the anti-CD44 QE7.3E8 mAb (red overlay ■). The flow cytometric analysis from the 150 V, 200 V and 250 V electroporations are shown with parental Jurkat cells also stained. The positive shift of fluorescence intensity of the QE7.3E8 histogram overlay away from the negative control X-63 mAb plot indicates detection of cell surface recombinant CD44$_{std}$ protein.


Cell Number
Counts
FL1-Height
150V
200V
250V
Jurkat 10.4
Fluorescence Intensity (Log)

5.2.3.3 Generation of stable Jurkat 10.4 transfectants

The pRc-CMV, pRc.CD44$_{std}$, pRc.CD44$_{v6-10}$, pRc.CD44$_{v6-9}$, pRc.CD44$_{v6-7}$ and pRc.CD44$_{v6}$ plasmids were transfected into the Jurkat 10.4 cell line (Section 5.2.3.1). After 8 weeks of culture selection with 2 mg/ml G418, viable cells were considered a stable transfected population. At such time the cells were removed from the G418 selection media and maintained in RPMI supplemented with 10% FCS. Stable transfectants were termed Jurkat-pRc/CMV, Jurkat-pRc.CD44$_{std}$, Jurkat-pRc.CD44$_{v6-10}$, Jurkat-pRc.CD44$_{v6-9}$, Jurkat-pRc.CD44$_{v6-7}$ and Jurkat-pRc.CD44$_{v6}$. In this nomenclature "Jurkat" refers to the Jurkat 10.4 clone.

Recombinant CD44 protein expression was measured by indirect immunofluorescent staining using the QE7.3E8 monoclonal antibody. Flow cytometric analysis demonstrated that although each transfectant expressed cell surface protein the overall transfection efficiency was low (Figure 5.8).

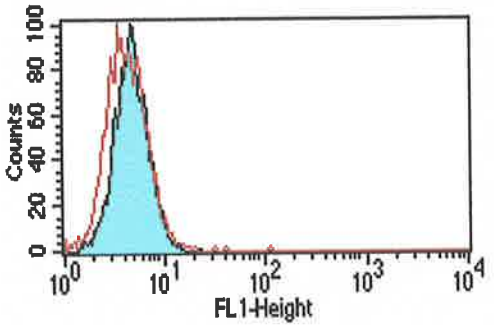
The promoter activity of CMV-derived expression vectors (e.g. pRc/CMV) can be enhanced within Jurkat cells by mitogenic treatment (Schifferli and Ciccarone, 1997). Therefore the Jurkat transfectants were stimulated overnight with 50 ng/ml PMA and 1 μg/ml PHA. Expression of cell surface recombinant protein was measured by immunofluorescent staining which demonstrated an induction of protein expression upon mitogenic stimulation in all transfectants. Figure 5.9 shows the representative analysis of stimulated Jurkat-pRc.CD44$_{v6}$ cells.

Approximately 70% of cells became adherent within 2 hr of mitogenic stimulation and 40% remained so after overnight treatment. This phenomenon was

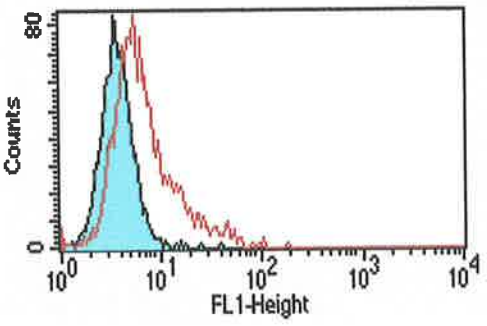
Figure 5.8 Flow cytometric analysis of CD44 cell surface protein expression from transfected Jurkat 10.4 cells.

A total of 6 x10^6 Jurkat 10.4 cells were transfected with 40 μg recombinant plasmid DNA (pRc/CMV, pRc.CD44$_{std}$, pRc.CD44$_{v6-10}$, pRc.CD44$_{v6-9}$, pRc.CD44$_{v6-7}$ and pRc.CD44$_{v6}$) with electroporation conditions set at 960 μF capacitance and a voltage of 200 V. After a 48 hr recovery period the cells were continuously cultured in RPMI supplemented with 10% FCS containing 2 mg/ml G418 for 8 weeks at which time the selection of recombinant transfectants was complete and the resultant cell culture was considered a stable transfectant population.

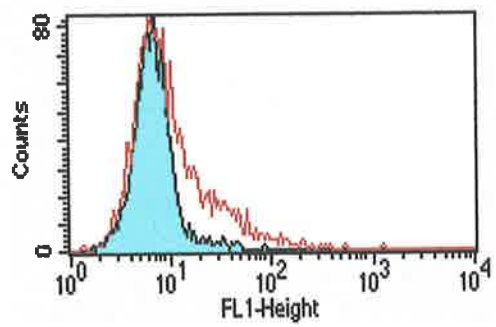
The stable transfectants were respectively termed Jurkat-pRc/CMV, Jurkat-pRc.CD44$_{std}$, Jurkat-pRc.CD44$_{v6-10}$, Jurkat-pRc.CD44$_{v6-9}$, Jurkat-pRc.CD44$_{v6-7}$ and Jurkat-pRc.CD44$_{v6}$. Expression of cell surface CD44 protein upon each transfectant was measured by indirect immunofluorescence with cells stained with the negative control X-63 mAb (light blue histogram ■) and the anti-CD44 QE7.3E8 mAb (red overlay ■).



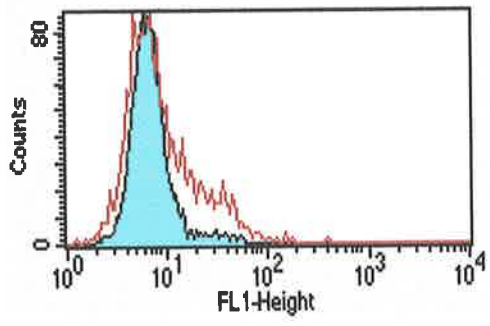
Jurkat-pRc/CMV



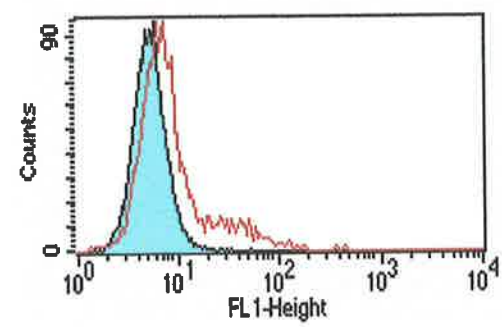
Jurkat-pRc.CD44$_{std}$



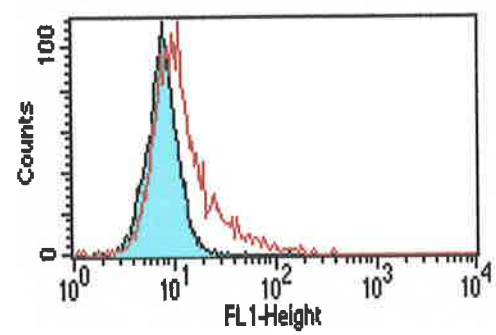
Jurkat-pRc.CD44$_{v6}$



Jurkat-pRc.CD44$_{v6-7}$



Jurkat-pRc.CD44$_{v6-9}$

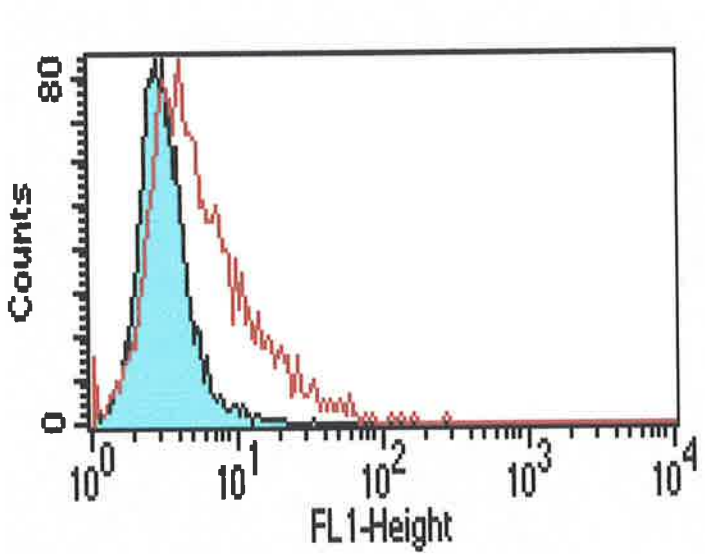


Jurkat-pRc.CD44$_{v6-10}$

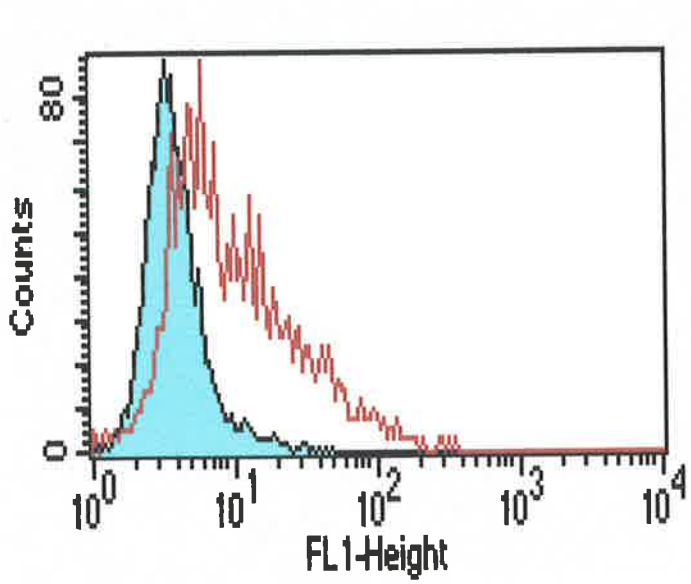
Figure 5.9 Flow cytometric characterization of the recombinant $CD44_{v6}$ protein expression from unstimulated and mitogenically stimulated Jurkat- pRc.$CD44_{v6}$ cells.

The Jurkat- pRc.$CD44_{v6}$ cell line was stimulated overnight with 50 ng/ml PMA and 1 μg/ml PHA at a final density of 1 $x10^6$ cells/ml. Expression of the $CD44_{v6}$ protein was analyzed on both the unstimulated (Panel A) and mitogenically treated cells (Panel B) by indirect immunofluorescence staining with the negative control X-63 mAb (light blue histogram ■) and the anti-CD44 QE7.3E8 mAb (red overlay ■).

A.



B.



Jurkat-pRc.CD44$_{v6}$

demonstrated to be CD44 independent as the PMA/ PHA treatment of Jurkat-pRc/CMV acquired the same adherent phenotype.

5.2.3.4 DYNAL selection of expressing transfectants

The population of CD44 expressing cells were enriched from each Jurkat transfectant by Dynabead® separation (Section 2.2.15). CD44 protein expression on Dynabead® purified cells was determined under unstimulated and PMA/ PHA stimulated conditions by indirect immunofluorescent staining using the QE7.3E8 mAb and flow cytometry (Figure 5.10). For all transfectants there was significant enrichment of CD44 bearing cells.

5.2.4 Characterization of the recombinant CD44 protein isoforms

5.2.4.1 FACS analysis of transfectants

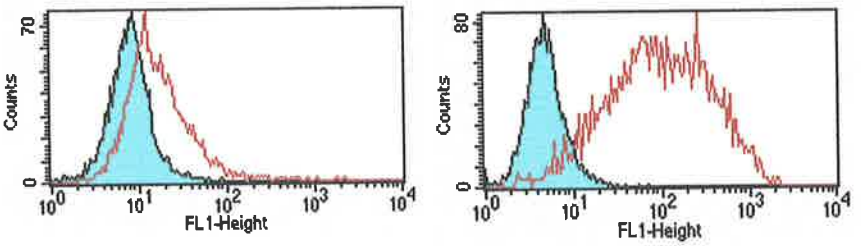
The CD44 protein isoforms were characterized by immunofluorescent staining with a variety of monoclonal antibodies recognizing different epitopes of the CD44 molecule. Monoclonal antibodies recognizing invariant CD44 epitope, QE7.3E8 (Kremmidiotis *et al.*, 1991) and NIH-44 (Shimizu *et al.*, 1989) and the variant exon-specific v6, v7 and v9 epitopes were used.

The Jurkat-pRc/CMV, Jurkat-pRc.CD44$_{std}$, Jurkat-pRc.CD44$_{v6-10}$, Jurkat-pRc.CD44$_{v6-9}$, Jurkat-pRc.CD44$_{v6-7}$ and Jurkat-pRc.CD44$_{v6}$ cells were mitogenically treated for maximal protein expression (Section 5.2.3.3). No antibody binding was detected upon staining of the Jurkat-pRc/CMV transfectant demonstrating the specificity of the antibodies towards the CD44 protein (data not shown).

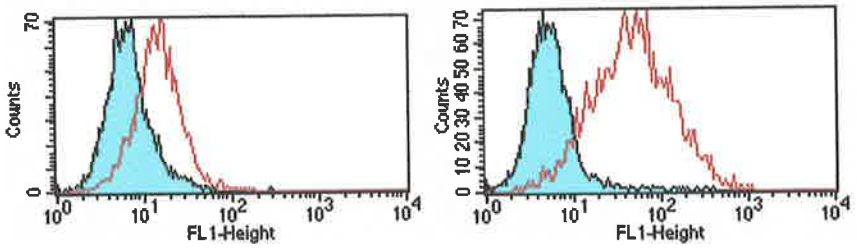
Figure 5.10 Characterization of Dynabead® purified CD44 expressing transfectants.

Jurkat transfectants were stimulated overnight with 50 ng/ml PMA and 1 μg/ml PHA at a final density of 1 $x10^6$ cells/ml. CD44 expressing cells were enriched by Dynabead® selection. Expression of the CD44 protein was analyzed under unstimulated (left panel) or PMA/ PHA treated (right panel) conditions by indirect immunofluorescence staining with the negative control X-63 mAb (light blue histogram ■) and the anti-CD44 QE7.3E8 mAb (red overlay ■).

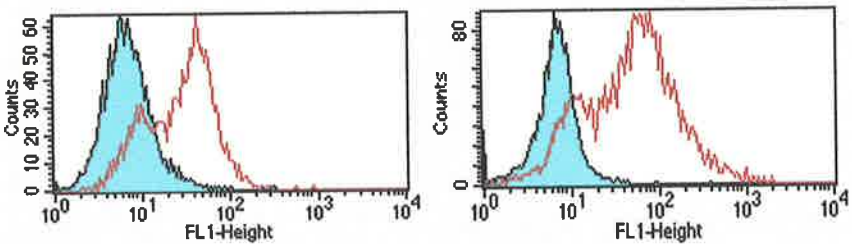
Jurkat-pRc.CD44$_{std}$



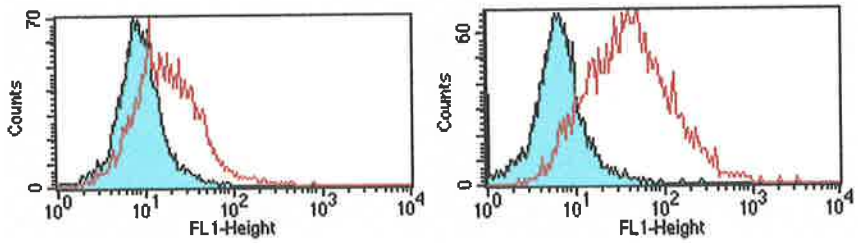
Jurkat-pRc.CD44$_{v6}$



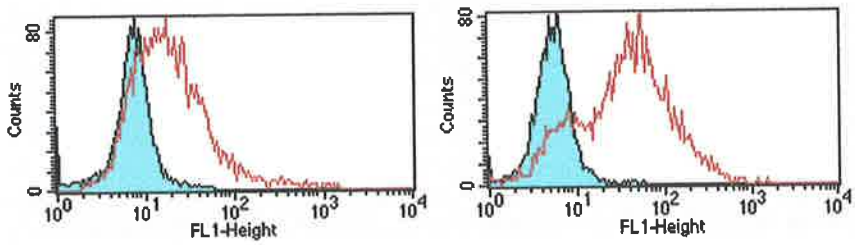
Jurkat-pRc.CD44$_{v6-7}$



Jurkat-pRc.CD44$_{v6-9}$



Jurkat-pRc.CD44$_{v6-10}$



Unstimulated **PMA/PHA Treated**

The QE7.3E8 and NIH-44 mAbs recognized and stained each CD44-expressing transfectant (Figure 5.11). The CD44 variant mAbs recognized only the transfectants expressing recombinant proteins carrying the corresponding variant exon. For example, the CD44 v6 mAb stained the Jurkat-pRc.$CD44_{v6-10}$, Jurkat-pRc.$CD44_{v6-9}$, Jurkat-pRc.$CD44_{v6-7}$ and Jurkat-pRc.$CD44_{v6}$ cells whereas the CD44 v9 mAb only stained the Jurkat-pRc.$CD44_{v6-10}$ and Jurkat-pRc.$CD44_{v6-9}$ cells (Figure 5.12).

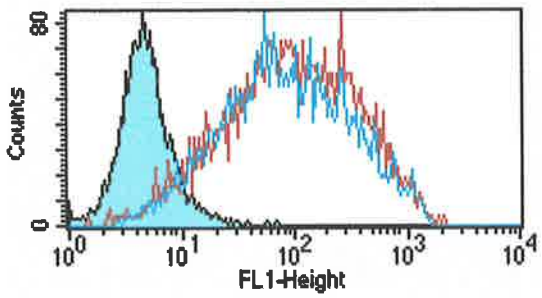
5.2.4.2 Western analysis of transfectants

The molecular size of the CD44 recombinant proteins was determined by western analysis of cell lysates. Briefly 5 $x10^6$ of unstimulated and overnight PMA/ PHA treated cells were collected and lysed in Laemmli loading buffer under reducing or non-reducing conditions. An equal volume of each protein lysate was electrophoresed through a 7.5% SDS-PAGE gel (Section 2.2.20.2), electro-blotted onto nitrocellulose membrane (Section 2.2.20.3) and probed overnight with the QE7.3E8 mAb. Bound QE7.3E8 antibody was detected by addition of sheep anti-mouse IgG alkaline phosphatase conjugate for 1 hr and the western blot developed using chromogenic substrate NBT/ XPO_4 (Section 2.2.20.4).

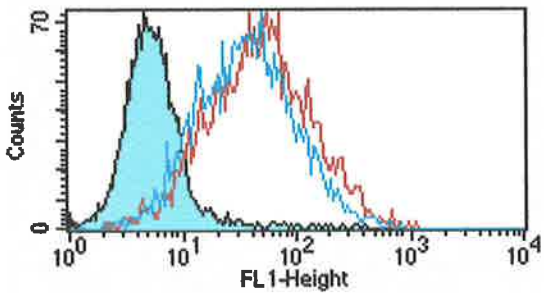
Recombinant CD44 protein of estimated molecular sizes of $CD44_{v6-10}$ (150 kDa), $CD44_{v6-9}$ (120 kDa), $CD44_{v6-7}$ (100 kDa), $CD44_{v6}$ (95 kDa) and $CD44_{std}$ (90 kDa) were detected from mitogenically stimulated transfectants using non-reduced Laemmli loading buffer (Figure 5.13). No recombinant CD44 protein was detected using a reduced Laemmli loading buffer suggesting destruction of protein structure resulting in the subsequent loss of epitope recognition by the QE7.3E8 mAb. In

Figure 5.11 Flow cytometric characterization of the Jurkat transfectants using CD44 monoclonal antibodies directed against the invariant region of the molecule.

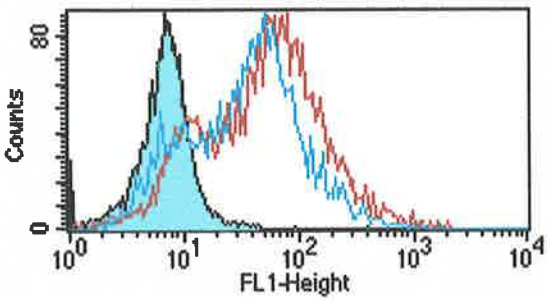
Jurkat transfectants were stimulated overnight 50 ng/ml PMA and 1 μg/ml PHA at a final density of 1 x10^6 cells/ml for maximal expression of the recombinant CD44 protein. Using two different anti-CD44 mAbs directed against the invariant region of the CD44 molecule, QE7.3E8 and NIH-44, each recombinant protein was characterized by indirect immunofluorescence staining with the negative control X-63 mAb (light blue histogram ■), the QE7.3E8 mAb (red overlay ■) and the NIH-44 mAb (dark blue overlay ■).



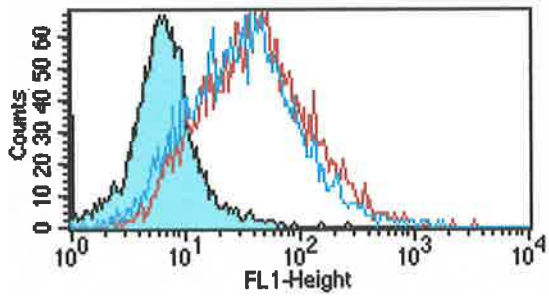
Jurkat-pRc.CD44$_{std}$



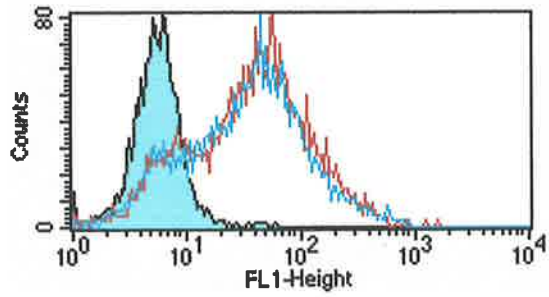
Jurkat-pRc.CD44$_{v6}$



Jurkat-pRc.CD44$_{v6-7}$



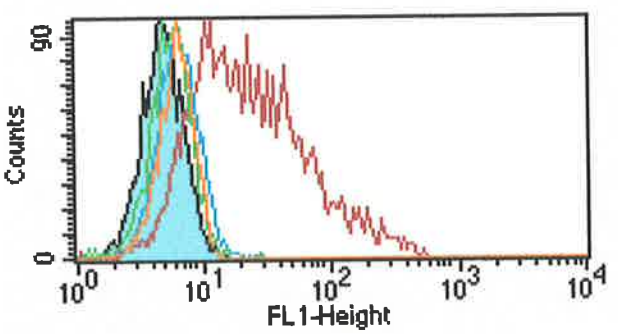
Jurkat-pRc.CD44$_{v6-9}$



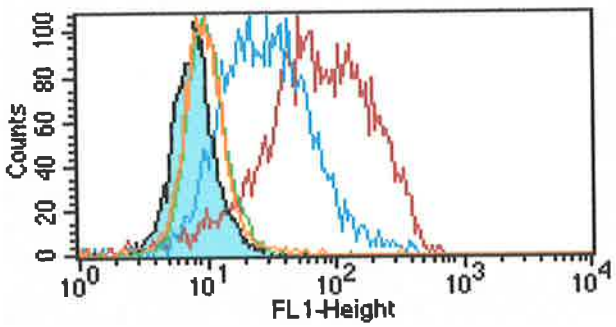
Jurkat-pRc.CD44$_{v6-10}$

Figure 5.12 Flow cytometric characterization of the Jurkat transfectants using CD44 monoclonal antibodies directed against the v6, v7 and v9 variant exons.

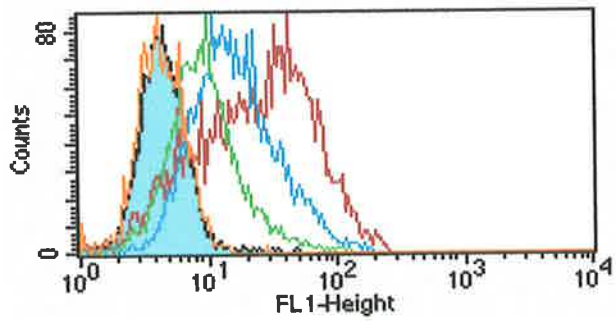
All Jurkat transfectants were stimulated overnight 50 ng/ml PMA and 1 μg/ml PHA at a final density of 1 x10^6 cells/ml for maximal expression of the recombinant CD44 protein. Using CD44 v6, v7 and v9 mAbs in comparison to the invariant CD44 QE7.3E8 mAb the recombinant proteins were characterized by indirect immunofluorescence staining with the negative control X-63 mAb (light blue histogram ■), the QE7.3E8 mAb (red overlay ■), the v6 mAb (dark blue overlay ■), the v7 mAb (green overlay ■) and the v9 mAb (orange overlay■).



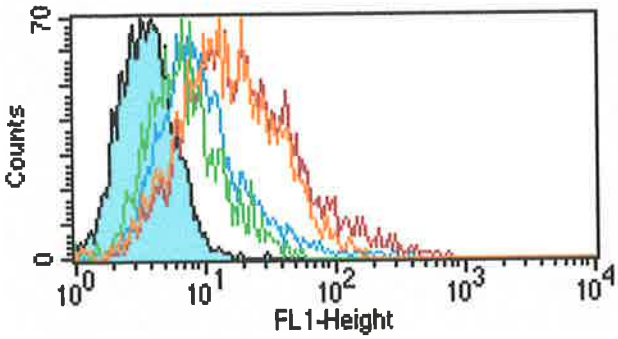
Jurkat-pRc.CD44$_{std}$



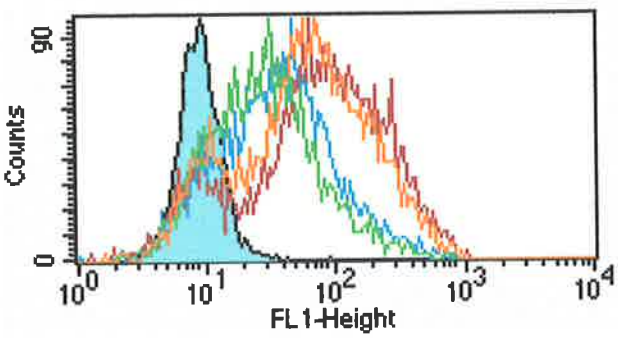
Jurkat-pRc.CD44$_{v6}$



Jurkat-pRc.CD44$_{v6\text{-}7}$



Jurkat-pRc.CD44$_{v6\text{-}9}$

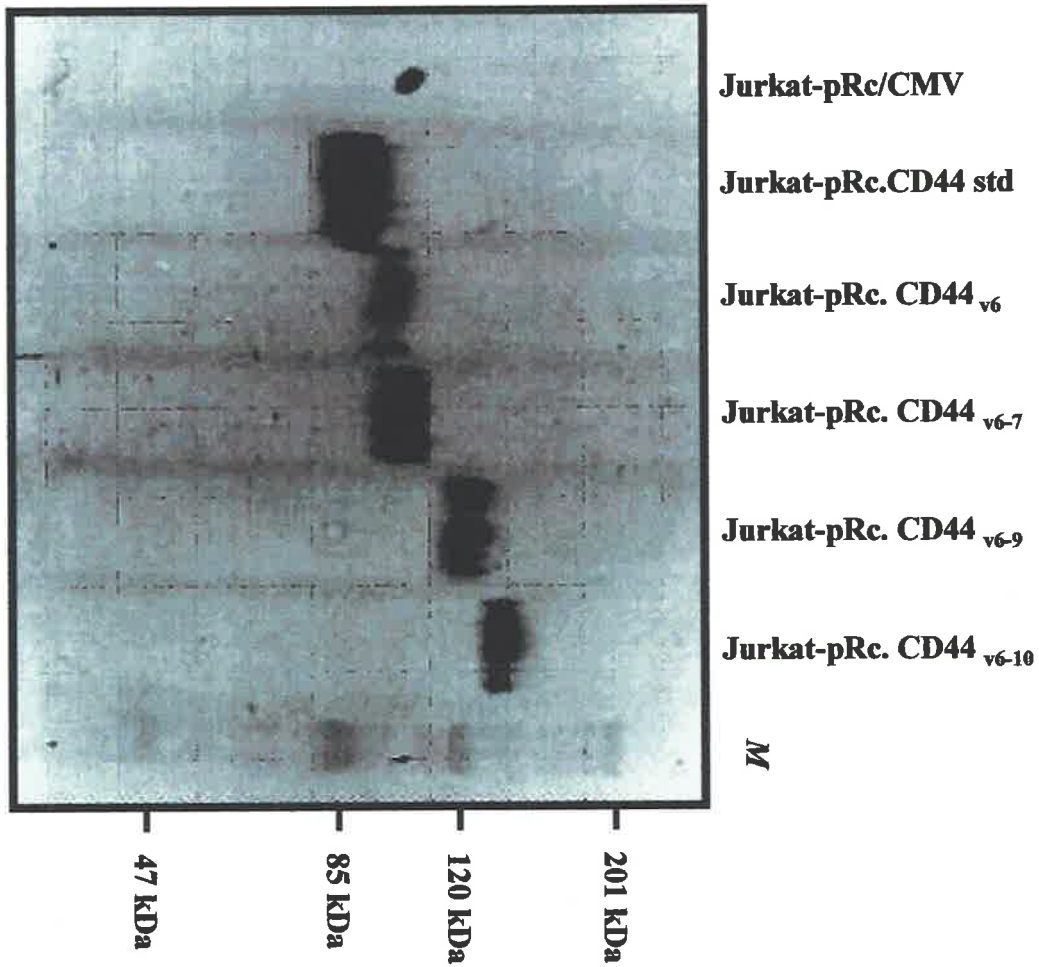


Jurkat-pRc.CD44$_{v6\text{-}10}$

Figure 5.13 Western analysis of the recombinant CD44 proteins.

Each Jurkat transfectant was stimulated overnight with 50 ng/ml PMA and 1 μg/ml PHA at a final density of 1 x10^6 cells/ml. The cells were pelleted by centrifugation and lysed in Laemmli sample buffer under non-reducing conditions with equal quantities of protein lysates electrophoresed through a 7.5% SDS-PAGE gel and electro-blotted onto nitrocellulose membrane. The membrane was incubated in 5% non-fat milk solution for 2 hr at room temperature and probed overnight by the addition of the primary anti-CD44 QE7.3E8 mAb. Unbound primary antibody was removed by two consecutive washes in TBS solution followed by addition of the secondary sheep anti-mouse IgG alkaline phosphate conjugated antibody for 1 hr at room temperature. The membrane was washed with the secondary conjugated antibody detected by chromogenic substrates NBT/ XPO_4.

Recombinant CD44 proteins of approximate molecular weight sizes of $CD44_{std}$ (90 kDa), $CD44_{v6}$ (95 kDa), $CD44_{v6\text{-}7}$ (100 kDa), $CD44_{v6\text{-}9}$ (120 kDa) and $CD44_{v6\text{-}10}$ (150 kDa) were detected.


Jurkat-pRc/CMV
Jurkat-pRc.CD44 std
Jurkat-pRc. CD44 v6
Jurkat-pRc. CD44 v6-7
Jurkat-pRc. CD44 v6-9
Jurkat-pRc. CD44 v6-10
M
47 kDa
85 kDa
120 kDa
201 kDa

addition, CD44 protein was not detected in unstimulated transfectants suggesting low level protein expression for western detection (data not shown).

5.2.5 Functional analysis of the recombinant CD44 proteins

5.2.5.1 Ligation of the CD44 protein and measure of cell proliferation

The capability of the CD44 v6 proteins to augment CD3 activation of T cells was investigated by ligation of the CD44 and CD3 receptors by monoclonal antibodies and magnitude of cellular activation measured by cell proliferation. In preliminary experiments, Jurkat-pRc/CMV cells were stimulated by ligation of the CD3 receptor using the anti-CD3 mAb. The degree of cellular activation was determined by analysis of cell proliferation as measured by tritium thymidine incorporation.

Briefly, a total of 2 x10^5 Jurkat-pRc/CMV cells were cultured in triplicate in 96 well round bottom plates in the presence of 2 μg/ml insoluble anti-CD3 mAb and cell proliferation measured at day 3 and day 5 culture. High counts of tritiated thymidine incorporation were obtained in the magnitude of 500,000 cpm at day 3 culture from both unstimulated and anti-CD3 mAb treated cells with no induction of cell growth by anti-CD3 mAb treatment (data not shown).

To reduce cell proliferation counts, both the cell number and culture time period were reduced. An additional positive control for induction of cell proliferation was included by stimulation of Jurkat cells with 50 ng/ml PMA. Although the average cell proliferation of the Jurkat-pRc/CMV cells was reduced, the mitogenic stimulation by anti-CD3 mAb could not be demonstrated by cell

proliferation analysis (Table 5.1). Stimulation of the cells with PMA produced an unexpected 20% reduction in cell proliferation at day 3 analysis. This reduced rate of cell growth may result from the adhesive property of the cells conferred by PMA stimulation (Section 5.2.3.3).

5.2.5.2 *Analysis of hyaluronic acid binding properties of the recombinant CD44 proteins*

5.2.5.2.1 Cell adhesion assay using insoluble hyaluronic acid

The ability of the recombinant CD44 proteins to bind HA was investigated by quantitation of cell adhesion to insoluble ligand (Section 2.2.21). The CD44 cell surface protein on each transfectant was adjusted to approximately equal quantities by overnight mitogenic treatment (Figure 5.10). Consequently, any differences in HA binding between the transfectants can be attributed to the intrinsic properties of the CD44 protein rather than variation in the concentration of cell surface protein.

The binding of the $CD44_{std}$ protein to the HA ligand has been unequivocally demonstrated (Culty *et al.*, 1990, Miyake *et al.*, 1990b, Aruffo *et al.*, 1990). As such the Jurkat-pRc.$CD44_{std}$ and Jurkat-pRc/CMV cell lines were used in preliminary experiments to demonstrate $CD44_{std}$-mediated HA binding.

No differences in the number of adhered cells to HA treated wells could be distinguished between the Jurkat-pRc.$CD44_{std}$ and Jurkat-pRc/CMV cells (Table 5.2). In addition, a greater number of Jurkat-pRc.$CD44_{std}$ and Jurkat-pRc/CMV cells adhered to non-treated wells in comparison to HA-coated wells suggesting non-specific cell adhesion.

Table 5.1 Cell proliferation analysis of the Jurkat-pRc.CMV transfectants under PMA and anti-CD3 mAb stimulation.

A total of 10^4 or 10^3 Jurkat-pRc.CMV cells were incubated in triplicate samples in a 96 well round bottomed plate and stimulated with either 50 ng/ml PMA or 2 μg/ml insoluble anti-CD3 mAb. The cells were cultured and cell proliferation determined by incorporation of tritium thymidine and measured at day 1, 2 and 3 respectively. The average and standard deviation from each triplicate sample are presented.

Density	Days in culture	Stimulation	Average cpm	Std Dev.
10^4 cells	1	unstim	**144,880**	**6,688**
		PMA	**158,420**	**8,541**
		anti-CD3	**143,386**	**7,485**
	2	unstim	**345,166**	**9,538**
		PMA	**314,366**	**7,548**
		anti-CD3	**315,666**	**53,978**
	3	unstim	**504,006**	**65,587**
		PMA	**406,093**	**25,796**
		anti-CD3	**523,213**	**40,653**
10^3 cells	1	unstim	**12,976**	**815**
		PMA	**11,784**	**1,960**
		anti-CD3	**11,058**	**685**
	2	unstim	**25,739**	**2,104**
		PMA	**21,112**	**770**
		anti-CD3	**25,282**	**1,289**
	3	unstim	**76,033**	**4,683**
		PMA	**43,526**	**3,064**
		anti-CD3	**63,003**	**8,290**

Table 5.2 Rose Bengal assay of the Jurkat-pRc/CMV and Jurkat-pRc.CD44$_{std}$ transfectants binding to insoluble HA.

Flat bottom 96 well plates were pre-coated with 10 mg/ml HA or PBS overnight at 4°C. A total of 10^5 pre-stimulated (50 ng/ml and 1μg/ml PHA) Jurkat-pRc/CMV and Jurkat-pRc.CD44$_{std}$ cells were incubated, in triplicate, to either HA or PBS treated wells for 1 hr at room temperature. Unbound cells were removed by gentle washing and adhered cells stained with 0.25% Rose Bengal for 10 min at room temperature. Cells were subsequently washed, lysed in 50% ethanol and quantity of released Rose Bengal stain measured at 540 nm.

Transfectant	Well Treatment	Average OD_{540}	Std Dev.
Jurkat-pRc/CMV	PBS	**0.19**	**0.066**
	HA	**0.071**	**0.006**
Jurkat-pRc.$CD44_{std}$	PBS	**0.156**	**0.047**
	HA	**0.061**	**0.013**

From these observations it is inferred that $CD44_{std}$-mediated binding of HA could not be demonstrated due to non-specific adhesiveness of the Jurkat cells conferred by PMA treatment (Section 5.3.3). The non-specific adhesion of the cells may be avoided by using unstimulated cells, however, previous studies have demonstrated the requirement for PMA treatment for induction of HA binding in CD44-expressing Jurkat transfectants (Liao *et al.*, 1993, Liu and Sy, 1997).

5.2.5.2.2 Soluble HA-FITC analysis by flow cytometry

The HA binding properties of the CD44 protein isoforms was further examined by immunofluorescent staining using soluble HA-FITC (Section 2.2.22). The Jurkat-pRc.$CD44_{std}$ and Jurkat-pRc/CMV cell lines were assayed under unstimulated and mitogenically treated conditions.

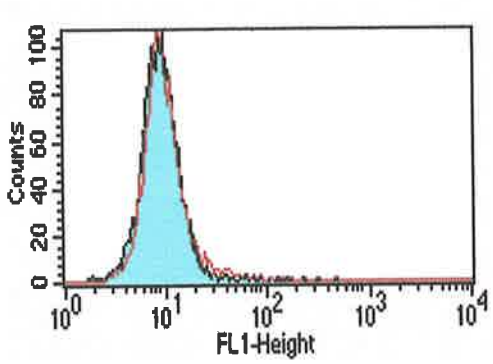
Initial experiments utilized HA-FITC at two different dilutions, 1:400 and 1:800 as recommended by Dr. Hyman, Salk Institute, California (personal communication). No HA-FITC binding to the unstimulated nor mitogen-treated Jurkat-pRc.$CD44_{std}$ cells was detected at either concentrations despite strong expression of $CD44_{std}$ protein (Figure 5.14).

The staining procedure was repeated using greater concentrations (1:10 dilution) of HA-FITC. Although HA binding was demonstrated this was determined to non-specific as demonstrated by positive staining of the Jurkat-pRc/CMV cells (Figure 5.14).

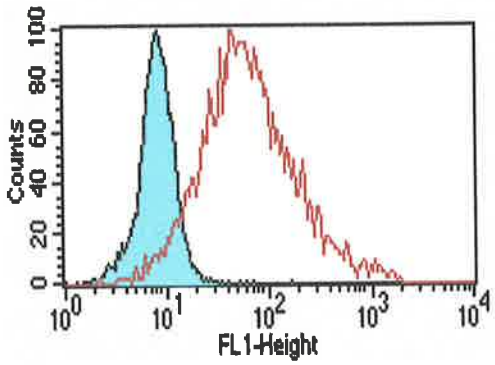
Figure 5.14 Flow cytometric analysis of soluble HA-FITC binding to mitogenically stimulated Jurkat-pRc/CMV and Jurkat-pRc.CD44$_{std}$ transfectants.

The Jurkat-pRc/CMV and Jurkat-pRc.CD44$_{std}$ transfectants were stimulated overnight 50 ng/ml PMA and 1 μg/ml PHA at a final density of 1 x10^6 cells/ml for maximal expression of the recombinant CD44 protein. The cells were pelleted by centrifugation and washed three times with PBS. Expression of the CD44 protein (Panel A) was analyzed by indirect immunofluorescent staining with the negative control X-63 mAb (light blue histogram ■) and the anti-CD44 QE7.3E8 mAb (red overlay ■). Analysis of soluble HA-FITC binding to the transfectants (Panel B) was measured at three different dilutions: $^1/_{800}$ (green overlay ■), $^1/_{400}$ (orange overlay ■) and $^1/_{10}$ (dark blue overlay ■).

A. CD44 Protein Expression

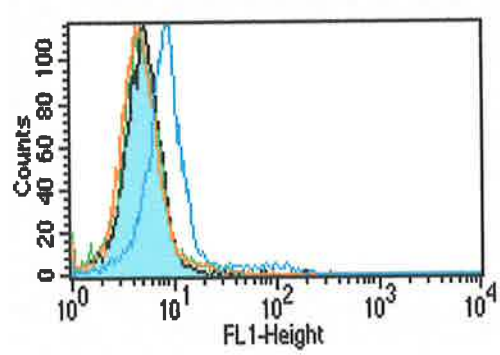


Jurkat-pRc/CMV

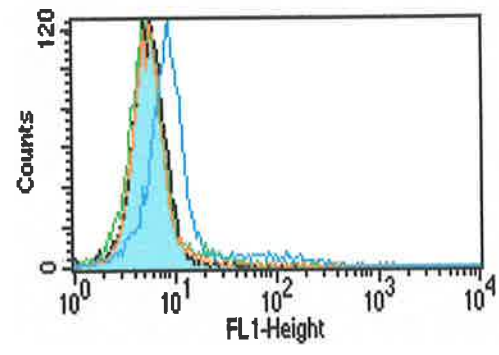


Jurkat-pRc.CD44$_{std}$

B. Soluble HA-FITC Binding



Jurkat-pRc/CMV



Jurkat-pRc.CD44$_{std}$

5.2.5.3 *Ligation of CD44 and analysis of protein tyrosine phosphorylation*

The capability of CD44 to transduce a signal pathway across the plasma membrane of resting human T cells has been demonstrated by mAb ligation of the molecule resulting in the rapid induction of tyrosine phosphorylated intracellular proteins (Taher *et al.*, 1996).

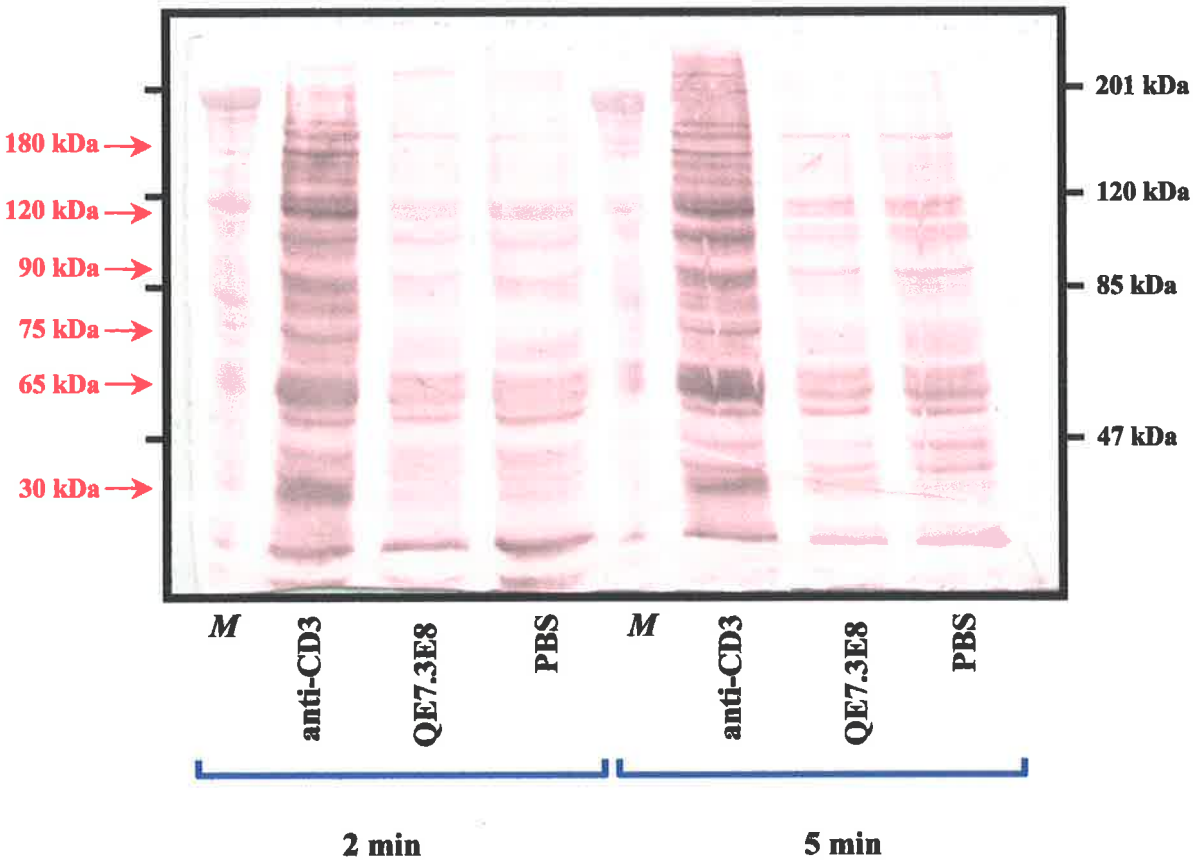
It is therefore postulated that different variant exon composition can alter the CD44-mediated signal transduction pathways thereby resulting in the tyrosine phosphorylation of novel intracellular proteins and the initiation of unique cellular effector functions. To investigate this, the CD44 proteins on the Jurkat transfectants were ligated with QE7.3E8 mAb and tyrosine phosphorylation of intra-cellular proteins examined by Western analysis. As a positive control the cross-linking of the CD3 receptor with the anti-CD3 mAb receptor was performed.

Briefly, 2 x10^6 cells were incubated with 10 μg/ml QE7.3E8 mAb, 10 μg/ml anti-CD3 mAb or PBS alone and stimulated with 50 μg/ml rabbit anti-mouse IgG for 2 and 5 min respectively. Cell lysates were prepared (Section 2.2.23) and electrophoresed through a 7.5% SDS-PAGE gel and electro-blotted onto nitro-cellulose membrane. Proteins phosphorylated on tyrosine residues were detected by western analysis using the anti-phosphotyrosine PY-20 mAb (Section 2.2.23).

Initial experiments assayed the Jurkat-pRc.CD44$_{std}$ cells with ligation of the CD3 receptor inducing phosphorylation of numerous proteins with molecular sizes of approximately 180 kDa, 120 kDa, 90 kDa, 75 kDa, 65 kDa and 30 kDa (Figure 5.15). In comparison to PBS treated cells, no induction of protein phosphorylation could be

Figure 5.15 Cross-linking of the CD3 and the $CD44_{std}$ protein on the Jurkat-pRc.$CD44_{std}$ transfectant and analysis of tyrosine phosphorylation of intracellular proteins.

A total of 2 $x10^6$ Jurkat-pRc.$CD44_{std}$ cells were incubated with 10 μg/ml of either anti-CD3 mAb, the anti-CD44 (QE7.3E8) mAb or PBS respectively at 37°C for 5 min. The primary antibodies were subsequently linked by the addition of 50 μg/ml rabbit anti-mouse IgG for 2 and 5 min respectively. Each reaction was stopped with equal protein lysates electrophoresed through a 7.5% SDS-PAGE gel and electro-blotted onto nitrocellulose membrane. The membrane was incubated in PY-20 antibody buffer for 2 hr at room temperature and subsequently probed overnight by the addition of the primary anti-phosphotyrosine (PY-20) mAb. Unbound primary antibody was removed by two consecutive washes in PY-20 wash buffer followed by addition of the secondary sheep anti-mouse IgG alkaline phosphate conjugated antibody for 1 hr at room temperature. The membrane was washed with the secondary conjugated antibody detected by chromogenic substrates NBT/ XPO_4.


180 kDa
120 kDa
90 kDa
75 kDa
65 kDa
30 kDa
201 kDa
120 kDa
85 kDa
47 kDa
M
anti-CD3
QE7.3E8
PBS
M
anti-CD3
QE7.3E8
PBS
2 min
5 min

detected with $CD44_{std}$ protein ligation. These experiments were repeated using the Jurkat-pRc.$CD44_{v6}$ cells, however, identical results were obtained (data not shown).

5.3 DISCUSSION

This chapter describes a PCR-based approach in cloning the full length CD44 v6 transcripts previously identified to be expressed in human PBMC. The PCR amplification of CD44 v6 transcripts from PBMC can, in effect, only be performed utilizing v6 exon specific primers (Chapter 3, Section 3.2). The terminal primers directed against the ATG and TAA codons (primers G and H) could be used for amplification of full length CD44 v6 transcripts, however, this is difficult as PCR amplification using invariant primer renders variant transcripts essentially un-amplifiable as previously discussed (Chapter 3, Section 3.2.2.1). Consequently, a cloning strategy was developed based on the PCR amplification of the up- and down-stream regions flanking the v6 exon and the successive ligation of these two fragments to generate full length $CD44_{v6-9}$, $CD44_{v6-7}$ and $CD44_{v6}$ clones (Section 5.2.1).

Due to time constraints nucleotide sequencing of the full length cDNA clones was not performed. However, western analysis of the recombinant CD44 proteins revealed an expected increase in the molecular sizes of each isoform concomitant with the introduction of new variant exons concluding the synthesis of mature CD44 peptides (Section 5.2.4.2).

Based on the difference in molecular size between the $CD44_{std}$ (90 kDa) and $CD44_{v6}$ (95 kDa) proteins it is determined that the v6 exon encodes for a peptide of

approximately 5 kDa. The expected molecular size of the v6 exon is 4.7 kDa based on calculations that the v6 exon is comprised of 43 amino acids with the molecular weight of each amino acid approximately 110 Da. Similarly, incorporation of the v7 exon peptide region (44 amino acid) within the $CD44_{v6}$ isoform should result in a 4.84 kDa increase in molecular size. This correlates with the 5 kDa molecular size difference between the $CD44_{v6}$ (95 kDa) and $CD44_{v6-7}$ (100 kDa) proteins as determined by western analysis.

The correlation between expected and determined molecular sizes of the peptide regions encoded by the v6 and v7 exons suggests a lack of post-translational modifications (glycosylation or glycosaminoglycan attachment) on either exons.

The incorporation of the v8 and v9 exons (64 amino acids) into the $CD44_{v6-7}$ peptide, thus generating the $CD44_{v6-9}$ isoform, is expected to increase the molecular size of the protein by 7 kDa. However, as determined by western analysis the determined molecular size difference between the $CD44_{v6-7}$ (100 kDa) and $CD44_{v6-9}$ (120 kDa) isoforms is approximately 20 kDa. These observations suggest that the v8 and v9 exons are substituted with post-translational modifications. No glycosaminoglycan attachment motifs have been identified within the v8 and v9 sequences implying that the additional molecular mass residing on the peptide region encoded by these exons are due to O- and N-linked glycosylations. The v8 and v9 exons are composed of 33% and 43% serine and threonine residues respectively which serve as potential sites for O-linked glycosylation. Whether the post-translational glycosylations occur mutually on both v8 and v9 exons or exclusively on only one exon is unknown.

The implication of post-translational glycosylation of the v8 and v9 exons are in accordance with characterization of other CD44 variant isoforms ($CD44_{v3,8-10}$, $CD44_{v6-10}$, $CD44_{v7-10}$, $CD44_{v8-10}$ and $CD44_{v10}$) which demonstrated that the variant peptide regions are heavily O-linked glycosylated (Jackson *et al.*, 1995).

The addition of the v10 exon (68 amino acids) into the $CD44_{v6-9}$ peptide, thus generating the $CD44_{v6-10}$ isoform, is expected to increase the molecular weight by 7.48 kDa. However, as determined by western analysis the determined molecular size difference between the $CD44_{v6-9}$ (120 kDa) and $CD44_{v6-10}$ (150 kDa) isoforms is approximately 30 kDa. These observations suggest that the incorporation of the v10 exon is substituted with post-translational modifications. The v10 exon carries motifs for glycosaminoglycan attachment (Chapter 1, Section 1.5.2) which may account for the anomaly between expected and determined molecular size of the peptide region encoded by the v10 exon. Furthermore, the v10 exon is comprised of 29% serine and threonine resides which serve as potential sites for O-linked glycosylations

Having generated full length CD44 v6 transcripts, stable transfectants using the Jurkat 10.4 cell line were produced (Section 5.2.3.3) and CD44-expressing cells enriched by Dynabead® selection (Section 5.2.3.4). Low expression of cell surface recombinant protein was obtained from Jurkat 10.4 transfectants which could be further induced upon stimulation of the cells (Section 5.2.3.3). From this observation it is suggested that transcription factors utilized by the CMV promoter are present at low levels within the Jurkat 10.4 cells and are upregulated upon cell activation thus increasing transcription and subsequent translation of recombinant CD44 protein.

However, strong expression of recombinant CD44 proteins has been reported in Jurkat cells transfected with CMV-promoter containing vectors (Liao *et al.*, 1993, Liu and Sy, 1996, Telen *et al.*, 1996). The discrepancy in CMV promoter activity between the data presented in this chapter and those of others may be accounted by clonal variation of the Jurkat cell line.

The introduction of additional variant exons into an existing CD44 molecule can significantly alter the tertiary structure thus hindering antibody-epitope recognition and binding. Using the Jurkat 10.4 transfectants, the CD44 proteins were characterized by flow cytometric analysis using mAbs that recognize CD44 invariant and variant-exon epitopes (Section 5.2.4.1). Each CD44 mAb was capable of recognizing its respective epitope in the CD44 proteins as expressed on the cell surface of Jurkat cells. No inhibition of CD44 mAb binding was apparent as demonstrated by the similar magnitude in positive fluorescence staining of each mAb to the respective CD44 protein. For example, the fluorescence intensity produced by staining of the Jurkat-pRc.CD44$_{v6}$, Jurkat-pRc.CD44$_{v6\text{-}7}$, Jurkat-pRc.CD44$_{v6\text{-}9}$ and Jurkat-pRc.CD44$_{v6\text{-}10}$ cells with the v6 mAb were of similar intensity. These observations suggest that the invariant and variant-exon (v6, v7 and v9) epitopes analyzed are structurally preserved among the alternately spliced variant isoforms.

Although multiple CD44 v6 transcripts are expressed in PBMC, whether each isoform is capable of co-stimulating the CD3 activation of T cells is unknown. To determine the capability of the CD44 v6 proteins to function as a co-stimulatory molecule, the role of the each isoform during CD3 activation of T cells was investigated (Section 5.2.5.1). However due to the rapid growth rate of the Jurkat 10.4 cell line, the proliferation assay was unable to demonstrate induction of cell

growth as a result of CD3 receptor ligation. Other determinants of cell activation such as the analysis of cytokine expression by semi-quantitative RT-PCR or ELISA measurements of soluble protein could be used to measure the co-stimulatory property of the CD44 v6 proteins. The ligation of CD44 expressed on human PBMC by either mAb or HA results in an increased synthesis of IL-2 protein indicating IL-2 as a candidate cytokine for future studies (Funaro *et al.*, 1994, Galandrini *et al.*, 1994b).

Hyaluronic acid is the principal ligand for the $CD44_{std}$ molecule yet information pertaining to the HA binding capabilities of the variant isoforms is limited. As to date the $CD44_{v3\text{-}10}$, $CD44_{v3,8\text{-}10}$, $CD44_{v6\text{-}10}$, $CD44_{v7\text{-}10}$ and $CD44_{v8\text{-}10}$ proteins have a significantly reduced HA binding capability in comparison to the $CD44_{std}$ isoform (Jackson *et al.*, 1995). The HA binding properties of the CD44 v6 isoforms was investigated, however, binding of Jurkat-pRc.$CD44_{std}$ cells to insoluble or soluble HA could not be demonstrated despite an abundance of cell surface $CD44_{std}$ expression (Section 5.2.5.2). As discussed earlier, the degree of glycosylation of the CD44 molecule (Chapter 1, Section 1.9.1) and the molecule's interaction with intra-cellular proteins such as ankyrin (Chapter 1, Section 1.9.3) can influence HA recognition. The Jurkat 10.4 clone may be defective in its ability to optimally modify CD44 or provide the necessary intra-cellular protein interaction thus resulting in an inactive HA-binding molecule. Consequently, the CD44 cDNA clones could be transfected into another previously characterized cell line (Namalwa cells or another Jurkat clone) for functionally active expression of recombinant CD44 protein.

Recently, the $CD44_{std}$ cDNA clone was transfected into the Namalwa cell line which demonstrated strong binding to soluble HA-FITC upon stimulation of the cells

(personal communication, Ms. A. Varelias, Transplant Immunology Lab, Queen Elizabeth Hospital, South Australia). This observation demonstrates that the inability of the Jurkat-pRc.CD44$_{std}$ cells to bind soluble HA is result of an intrinsic aberration within the Jurkat 10.4 clone.

The ability of the Jurkat-pRc.CD44$_{std}$ and Jurkat-pRc.CD44$_{v6}$ cells to transduce a signal across the plasma membrane through the CD3 receptor was demonstrated by the rapid tyrosine phosphorylation of intra-cellular proteins upon ligation with anti-CD3 mAb (Section 5.2.5.3). However, ligation of the CD44 protein did not result in protein phosphorylation despite previous reports of its signal transduction capabilities in human peripheral T cells (Taher *et al.*, 1996). The Jurkat 10.4 clone is suggested to render the recombinant CD44$_{std}$ protein inactive to HA binding inferring that the protein may also be functionally inactive to transduce a cell-surface signal. As such, the re-transfection of the CD44 cDNA clones into another Jurkat cell line is recommended with the signal transduction capabilities of the CD44 isoforms re-analyzed.

Chapter Six

Concluding Remarks

Without facts we have no science. Facts are to the scientist what words are to a poet. The scientist has a love for facts, even isolated facts, similar to a poets love of words. But a collection of facts is not science anymore than a dictionary is poetry. Around his facts the scientist weaves a logical pattern or theory which gives the facts meaning, order and significance.

Isoidor Issac Rabi

The functional characterization of the rat CD44 v6 proteins during an *in vivo* immune response was a significant factor in the decision to study the human homologues expressed in PBMC. Having strong peptide homology between rodent and human sequences, it is conceivable that the functions of the rodent CD44 v6 proteins, as determined by *in vivo* models (see Chapter 1), can also be attributed to the human isoforms.

This thesis has defined the variant exon composition of the CD44 v6 transcripts expressed in resting and activated human PBMC by RT-PCR using v6 exon specific primers to consist of the $CD44_{v6}$, $CD44_{v6,8-9}$, $CD44_{v6-9}$, and $CD44_{v6-10}$ isoforms (see Chapter 3). Previous studies have defined functional roles for the CD44 v6 isoforms, such as co-stimulation during T cell activation and HA binding (Galluzzo *et al.*, 1995), but the specific identity of the isoforms was not determined. Therefore, any isoform that contains the v6 exon may be associated with these cellular functions. It is now possible to attribute specific cellular function(s) to these individual CD44 v6 isoforms.

Furthermore, RT-PCR analysis of the CD44 v6 mRNAs indicates that these transcripts are expressed in low quantities in PBMC (see Chapter 3). The low expression of the CD44 v6 proteins has been confirmed previously by studies which demonstrate that the CD44 v6 proteins on *in vitro* activated T cells represent only 5% of total CD44 (Galluzzo *et al.*, 1995). Therefore, if the CD44 v6 proteins are expressed at low levels on the cell surface of lymphocytes then the biological relevance of these isoforms needs to be ascertained. Interestingly, mAb ligation of the CD44 v6 proteins expressed on T cells was shown to increase intracellular

mobilization of calcium ions and augment CD3-mediated cellular proliferation (Galluzzo *et al.*, 1995). It is therefore apparent from Galluzo's studies that the CD44 v6 isoforms can function as signalling proteins and consequently need only to be expressed in low quantities to affect cellular function. Despite the expression of several CD44 v6 transcripts, it is unknown which specific mRNA(s) is translated into protein and/ or distributed to the cell-surface and thus should be addressed in future studies.

The expression of the CD44 v6 isoforms in PBMC during an *in vivo* immune response was investigated using renal transplant patients. In these studies, a novel alternately spliced isoform, $CD44_{v6\text{-}7}$, was identified and shown to be expressed at an increased frequency in renal transplant recipients in comparison to a group of healthy individuals (see Chapter 4). Since the renal transplant patients were immunosuppressed, the pharmacological actions of the immunosuppressive drugs may explain its expression. However, an alternative hypothesis for the induced frequency of the $CD44_{v6\text{-}7}$ transcript in renal transplant recipients may be the result of an alloimmune response. With the expression of multiple CD44 v6 transcripts in PBMC it is possible to hypothesize that individual isoforms may participate in distinct steps in the alloimmune response. For example, certain isoforms may be associated with early phases of an immune response such as the co-stimulation of T cells upon antigen presentation whereas other isoforms may be utilized at a later stage such as the recruitment of activated T cells to inflammatory sites. Moreover, the association of CD44 during an immune or inflammatory response has been previously demonstrated by alterations in mRNA and protein expression in autoimmune disorders such as rheumatoid arthritis (Haynes *et al.*, 1991a, Fischer *et*

al., 1993, Kelleher *et al.*, 1995), Grave's disease (Heufelder *et al.*, 1993), autoimmune uveitis (Foets *et al.*, 1992), experimental allergic encephalomyelitis (Zeine and Owens, 1992), multiple sclerosis (Girgrah *et al.*, 1991) and chronic graft versus host disease (Murakami *et al.*, 1991). Future studies investigating the expression of the $CD44_{v6-7}$ transcript in such autoimmune disorders may establish a relationship between induction of this isoform and an *in vivo* immune response. Experiments in this thesis also showed the occurrence of the $CD44_{v6-7}$ transcript in the peripheral blood of CTCL patients. The presence of this transcript in these patients may have implications for the homing of tumour cells to skin and thus warrants further investigations to clarify this mechanism.

The identification of multiple CD44 v6 transcripts in resting and activated PBMC will permit the study of the specific function of each isoform. Therefore, a cloning strategy was developed to generate full length CD44 v6 exon containing cDNA clones. The transfection of the Jurkat 10.4 clone demonstrated that each of the CD44 v6 isoforms was indeed expressed on the cell-surface (see Chapter 5). In addition, the Jurkat cells transfected with $CD44_{std}$ were unable to bind HA despite stimulation with phorbol ester. Lesley *et al.* (1995) have reported similar results where a parental B cell lymphoma cell line which expressed constitutive CD44 demonstrated no HA binding activity. But, when HA-FITC stained cells were sorted by flow cytometry the investigators were able to select for subclones which demonstrated either inducible or constitutive HA-binding activity (Lesley *et al.*, 1995). They therefore suggested that the HA-binding conformation of the CD44 molecule is dependent on the genetic content of the cell and that clonal variation can result in the differential expression of these genes. That is, specific-gene products

can regulate CD44 function by directly or indirectly interacting with the molecule to produce an activated CD44 conformation. Such cell-specific regulation in CD44-mediated HA binding may be determined by either post-translational modification of CD44 (e.g. glycosaminoglycan attachments, N- and O-linked glycosylation or palmitoylation) or the interaction of CD44 with cytoskeletal or signal transduction proteins (e.g. ankyrin, protein kinase C). In experiments conducted in this thesis, the inability of the Jurkat-pRc.$CD44_{std}$ transfectant to bind HA suggests that the Jurkat 10.4 clone may lack the genetic content to convert CD44 from an inactive to an activated HA binding conformation. Indeed, other Jurkat clones used in transfection studies described by Liao *et al.* (1993) can be induced to bind HA upon cell stimulation with phorbol ester. It is conceivable that the specific identity of these regulatory genes may be revealed by the use of mRNA differential display or subtractive hybridization techniques (Wan and Erlander, 1997, Zheng *et al.*, 1997a) using different Jurkat clones that exhibit inactive, inducible or constitutive HA binding activity.

Others have demonstrated that the CD44 v6 isoforms expressed on activated T cells has been demonstrated to bind HA (Galluzzo *et al.*, 1995). However, the ability of the specific isoforms to bind HA has not been defined in this thesis. The incorporation of variant exons within the $CD44_{std}$ protein core may alter HA binding activity due to conformational and/ or further post-translational modifications imparted to the molecule. Subsequent determination of the HA binding property of the CD44 v6 isoforms will require the use of other cell lines or other Jurkat clones which have demonstrated HA binding upon transfection with the $CD44_{std}$ cDNA (Liao *et al.*, 1993, Liu and Sy, 1996, Telen *et al.*, 1996).

The occurrence of a specific ligand(s) capable of binding to the v6 exon has not been actively investigated. However, it is intriguing that a CD44 v6 mAb was able to block tumor growth (Sleeman *et al.*, 1996b), lymphocyte activation (Arch *et al.*, 1992) and limb bud outgrowth (Wainwright *et al.*, 1996) without interfering with CD44-mediated HA binding. Candidates for such ligands are those previously attributed to CD44 such as collagen type I (Carter and Wayner, 1988, Ishii *et al.*, 1993), glycosaminoglycans (Sleeman *et al.*, 1997), osteopontin (Weber *et al.*, 1996b), MIP-1β (Tanaka *et al.*, 1993), serglycin (Toyama Sorimachi *et al.*, 1995), mucosal addressin (Picker *et al.*, 1989) and fibronectin (Jalkanen and Jalkanen, 1992, Verfaillie *et al.*, 1994, Cao *et al.*, 1996). Henceforth, the identification and characterization of ligands of the CD44 v6 isoforms may lead to novel therapeutic strategies to target tumour metastasis, autoimmunity and transplant rejection.

$CD44_{v6,8\text{-}9}$ (v6~TAA PCR Product): Sequence using primer B

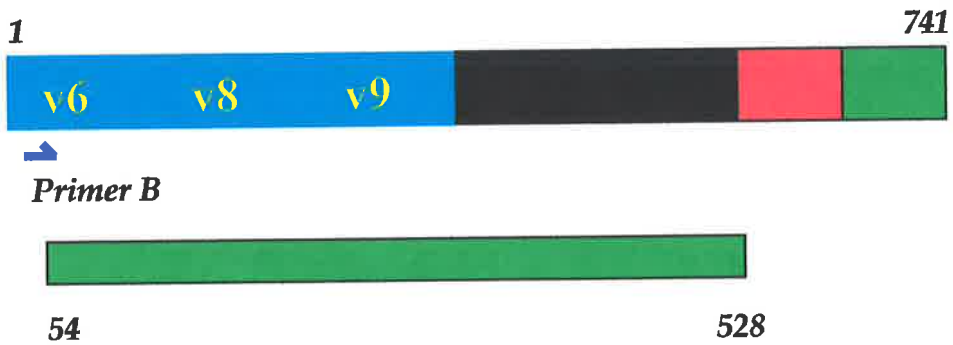
```
54
TGGTTTGGCAACAGATGGCATGAGGGATATCGCCAAACACCCAGAGAAGACTCCCATTCGACAACAGG
**********************************************************************
TGGTTTGGCAACAGATGGCATGAGGGATATCGCCAAACACCCAGAGAAGACTCCCATTCGACAACAGG
122
GACAGCTGATATGGACTCCAGTCATAGTACAACGCTTCAGCCTACTGCAAATCCAAACACAGGTTTGG
**********************************************************************
GACAGCTGATATGGACTCCAGTCATAGTACAACGCTTCAGCCTACTGCAAATCCAAACACAGGTTTGG
190
TGGAAGATTTGGACAGGACAGGACCTCTTTCAATGACAACGCAGCAGAGTAATTCTCAGAGCTTCTCT
**********************************************************************
TGGAAGATTTGGACAGGACAGGACCTCTTTCAATGACAACGCAGCAGAGTAATTCTCAGAGCTTCTCT
258                                285
ACATCACATGAAGGCTTGGAAGAAGATAAAGACCATCCAACAACTTCTACTCTGACATCAAGCAGAGA
**********************************************************************
ACATCACATGAAGGCTTGGAAGAAGATAAAGACCATCCAACAACTTCTACTCTGACATCAAGCAGAGA
326
CCAAGACACATTCCACCCCAGTGGGGGGTCCCATACCACTCATGGATCTGAATCAGATGGACACTCAC
**********************************************************************
CCAAGACACATTCCACCCCAGTGGGGGGTCCCATACCACTCATGGATCTGAATCAGATGGACACTCAC
394
ATGGGAGTCAAGAAGGTGGAGCAAACACAACCTCTGGTCCTATAAGGACACCCCAAATTCCAGAATGG
**********************************************************************
ATGGGAGTCAAGAAGGTGGAGCAAACACAACCTCTGGTCCTATAAGGACACCCCAAATTCCAGAATGG
462                                                                528
CTGATCATCTTGGCATCCCTCTTGGCCTTGGCTTTGATTCTTGCAGTTTGCATTGCAGTCAACAGTC
**********************************************************************
CTGATCATCTTGGCATCCCTCTTGGCCTTGGCTTTGATTCTTGCAGTTTGCATTGCAGTCAACAGTC
```

Nucleotide position 1 refers to the beginning sequence of the v6 exon

285: Beginning of DNA sequence that can be read from sequence using primer D

Shaded region: v6 exon
Shaded region: v8 exon
Shaded region: v9 exon

Sequence presented in <u>bold</u> is derived from published data from Screaton *et al* (1992)



(Sequence run: Primer B)

$CD44_{v6,8\text{-}9}$ (v6~TAA PCR Product): Sequence using primer D

285
AAAGACCATCCAACAACTTCTACTCTGACATCAAGCAGAGACCAAGACACATTCCACCCCAGTGGGGG
****
AAAGACCATCCAACAACTTCTACTCTGACATCAAGCAGAGACCAAGACACATTCCACCCCAGTGGGGG
353
GTCCCATACCACTCATGGATCTGAATCAGATGGACACTCACATGGGAGTCAAGAAGGTGGAGCAAACA
****
GTCCCATACCACTCATGGATCTGAATCAGATGGACACTCACATGGGAGTCAAGAAGGTGGAGCAAACA
421
CAACCTCTGGTCCTATAAGGACACCCCAAATTCCAGAATGGCTGATCATCTTGGCATCCCTCTTGGCC
****
CAACCTCTGGTCCTATAAGGACACCCCAAATTCCAGAATGGCTGATCATCTTGGCATCCCTCTTGGCC
489
TTGGCTTTGATTCTTGCAGTTTGCATTGCAGTCAACAGTCGAAGAAGGTGTGGGCAGAAGAAAAAGCT
****
TTGGCTTTGATTCTTGCAGTTTGCATTGCAGTCAACAGTCGAAGAAGGTGTGGGCAGAAGAAAAAGCT
557
AGTGATCAACAGTGGCAATGGAGCTGTGGAGGACAGAAAGCCAAGTGGACTCAACGGAGAGGCCAGCA
****
AGTGATCAACAGTGGCAATGGAGCTGTGGAGGACAGAAAGCCAAGTGGACTCAACGGAGAGGCCAGCA
625 *686*
AGTCTCAGGAAATGGTGCATTTGGTGAACAAGGAGTCGTCAGAAACTCCAGACCAGTTTATGACAGCT
****
AGTCTCAGGAAATGGTGCATTTGGTGAACAAGGAGTCGTCAGAAACTCCAGACCAGTTTATGAC
693 *739*
GATGAGACAAGGAAACCTGCAGAATGTGGACATGAAGATTGGGGTG*TAA*

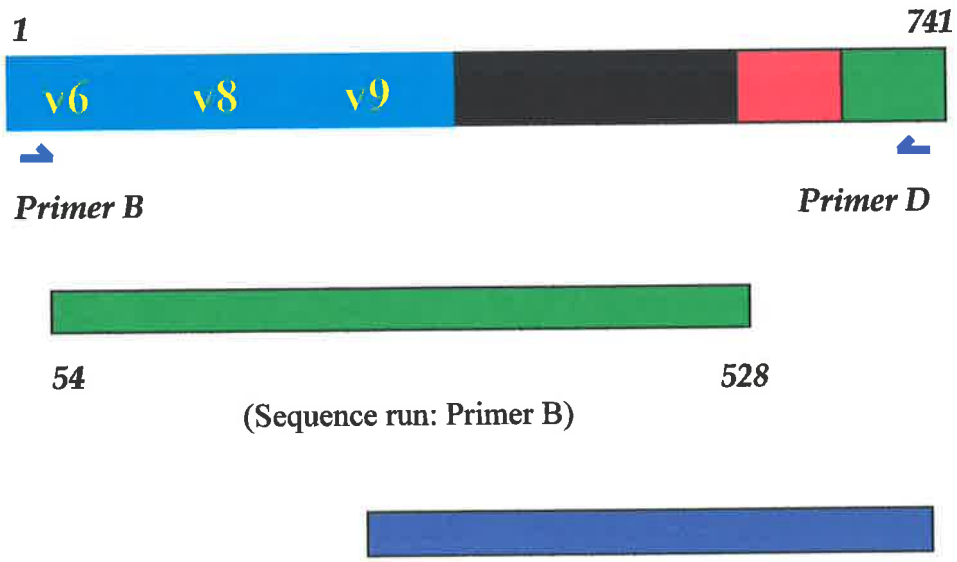
Note: The final 53 bp was not sequenced

Nucleotide position 1 refers to the beginning sequence of the v6 exon

Shaded region: v9 exon

739: TAA Stop codon

Sequence presented in bold is derived from published data from Screaton *et al* (1992)



$CD44_{v6\text{-}10}$ (v6~TAA PCR Product): Sequence using primer B

56
GTTTGGCAACAGATGGCATGAGGGATATCGCCAAACACCCAGAGAAGACTCCCATTCGACAACAGGGA
****
GTTTGGCAACAGATGGCATGAGGGATATCGCCAAACACCCAGAGAAGACTCCCATTCGACAACAGGGA
124 185
CAGCTGGCAGCCTCAGCTCATACCAGCCATCCAATGCAAGGAAGGACAACACCAAGCCCAGAGGACAG
****
CAGCTGGCAGCCTCAGCTCATACCAGCCATCCAATGCAAGGAAGGACAACACCAAGCCCAGAGGACAG
192
TTCCTGGACTGATTTCTTCAACCCAATCTCACACCCCATGGGACGAGGTCATCAAGCAGGAAGAAGGA
****
TTCCTGGACTGATTTCTTCAACCCAATCTCACACCCCATGGGACGAGGTCATCAAGCAGGAAGAAGGA
260
TGGATATGGACTCCAGTCATAGTACAACGCTTCAGCCTACTGCAAATCCAAACACAGGTTTGGTGGAA
****
TGGATATGGACTCCAGTCATAGTACAACGCTTCAGCCTACTGCAAATCCAAACACAGGTTTGGTGGAA
328 395
GATTTGGACAGGACAGGACCTCTTTCAATGACAACGCAGCAGAGTAATTCTCAGAGCTTCTCTACATC
****
GATTTGGACAGGACAGGACCTCTTTCAATGACAACGCAGCAGAGTAATTCTCAGAGCTTCTCTACATC

Nucleotide position 1 refers to the beginning sequence of the v6 exon

185: Beginning of DNA sequence that can be read from sequence using Primer E

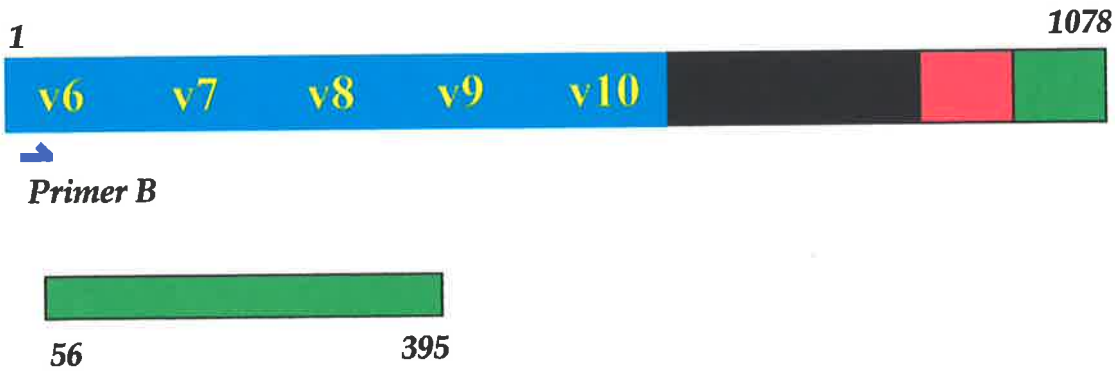
Shaded region: v6 exon

Shaded region: v7 exon

Shaded region: v8 exon

Shaded region: v9 exon

Sequence presented in <u>bold</u> is derived from published data from Screaton *et al* (1992)

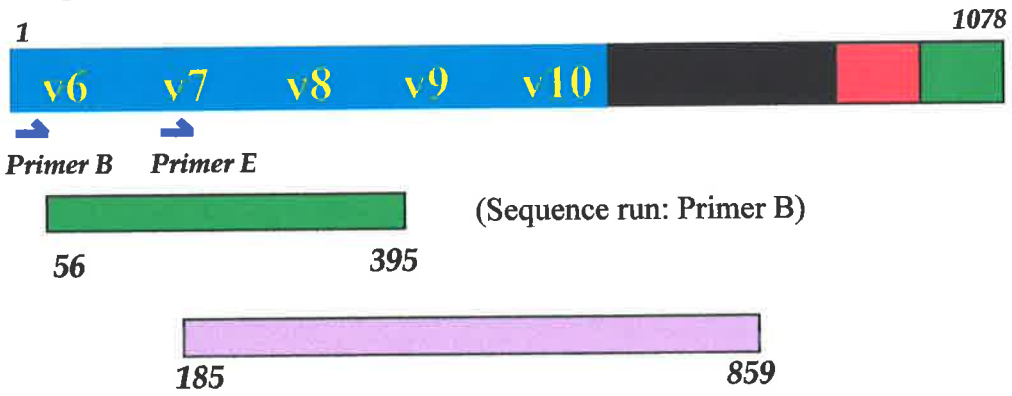


(Sequence run: Primer B)

$CD44_{v6\text{-}10}$ (v6~TAA PCR Product): Sequence using primer E

185
AGGACAGTTCCTGGACTGATTTCTTCAACCCAATCTCACACCCCATGGGACGAGGTCATCAAGCAGGA
****
AGGACAGTTCCTGGACTGATTTCTTCAACCCAATCTCACACCCCATGGGACGAGGTCATCAAGCAGGA
253
AGAAGGATGGATATGGACTCCAGTCATAGTACAACGCTTCAGCCTACTGCAAATCCAAACACAGGTTT
****
AGAAGGATGGATATGGACTCCAGTCATAGTACAACGCTTCAGCCTACTGCAAATCCAAACACAGGTTT
321
GGTGGAAGATTTGGACAGGACAGGACCTCTTTCAATGACAACGCAGCAGAGTAATTCTCAGAGCTTCT
****
GGTGGAAGATTTGGACAGGACAGGACCTCTTTCAATGACAACGCAGCAGAGTAATTCTCAGAGCTTCT
389
CTACATCACATGAAGGCTTGGAAGAAGATAAAGACCATCCAACAACTTCTACTCTGACATCAAGCAAT
****
CTACATCACATGAAGGCTTGGAAGAAGATAAAGACCATCCAACAACTTCTACTCTGACATCAAGCAAT
457 464
AGGAATGATGTCACAGGTGGAAGAAGAGACCCAAATCATTCTGAAGGCTCAAACTACTTTACTGGAAG
****
AGGAATGATGTCACAGGTGGAAGAAGAGACCCAAATCATTCTGAAGGCTCAAACTACTTTACTGGAAG
525
GTTATACCTCTCATTACCCACACACGAAGGAAAGCAGGACCTTCATCCCAGTGACCTCAGCTAAGACT
****
GTTATACCTCTCATTACCCACACACGAAGGAAAGCAGGACCTTCATCCCAGTGACCTCAGCTAAGACT
593
GGGTCCTTTGGAGTTACTGCAGTTACTGTTGGAGATTCCAACTCTAATGTCAATCGTTCCTTATCAGG
****
GGGTCCTTTGGAGTTACTGCAGTTACTGTTGGAGATTCCAACTCTAATGTCAATCGTTCCTTATCAGG
661
AGACCAAGACACATTCCACCCCAGTGGGGGGTCCCATACCACTCATGGATCTGAATCAGATGGACACT
****
AGACCAAGACACATTCCACCCCAGTGGGGGGTCCCATACCACTCATGGATCTGAATCAGATGGACACT
729
CACATGGGAGTCAAGAAGGTGGAGCAAACACAACCTCTGGTCCTATAAGGACACCCCAAATTCCAGAA
****
CACATGGGAGTCAAGAAGGTGGAGCAAACACAACCTCTGGTCCTATAAGGACACCCCAAATTCCAGAA
797 859
TGGCTGATCATCTTGGCATCCCTCTTGGCCTTGGCTTTGATTCTTGCAGTTTGCATTGCAGT
****
TGGCTGATCATCTTGGCATCCCTCTTGGCCTTGGCTTTGATTCTTGCAGTTTGCATTGCAGT

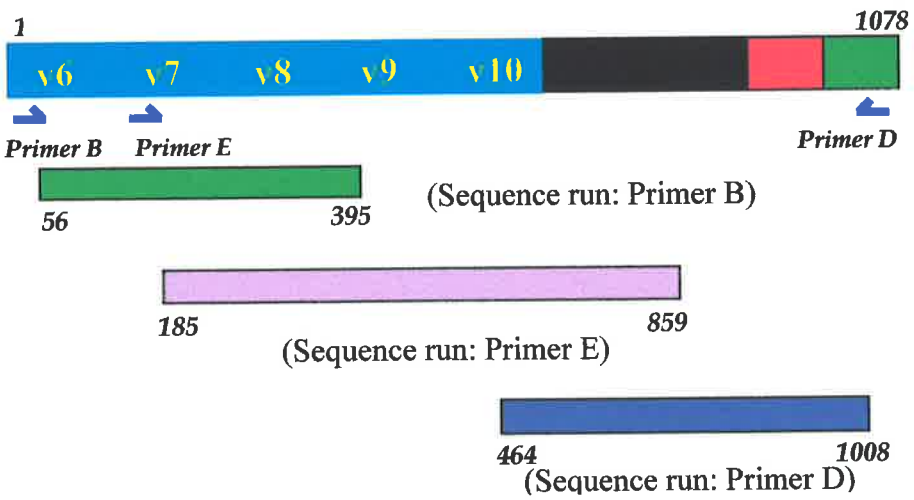
Nucleotide position 1 refers to the beginning sequence of the v6 exon
464: Beginning of DNA sequence that can be read from sequence using Primer D
Shaded region: v7 exon
Shaded region: v8 exon
Shaded region: v9 exon
Shaded region: v10 exon
Sequence presented in bold is derived from published data from Screaton *et al* (1992)



$CD44_{v6\text{-}10}$ (v6~TAA PCR Product): Sequence using primer D

464
ATGTCACAGGTGGAAGAAGAGACCCAAATCATTCTGAAGGCTCAAACTACTTTACTGGAAGGTTATAC
****
ATGTCACAGGTGGAAGAAGAGACCCAAATCATTCTGAAGGCTCAAACTACTTTACTGGAAGGTTATAC
532
CTCTCATTACCCACACACGAAGGAAAGCAGGACCTTCATCCCAGTGACCTCAGCTAAGACTGGGTCCT
****
CTCTCATTACCCACACACGAAGGAAAGCAGGACCTTCATCCCAGTGACCTCAGCTAAGACTGGGTCCT
600
TTGGAGTTACTGCAGTTACTGTTGGAGATTCCAACTCTAATGTCAATCGTTCCTTATCAGGAGACCAA
****
TTGGAGTTACTGCAGTTACTGTTGGAGATTCCAACTCTAATGTCAATCGTTCCTTATCAGGAGACCAA
668
ACACATTCCACCCCAGTGGGGGGTCCCATACCACTCATGGATCTGAATCAGATGGACACTCACATGGG
****
ACACATTCCACCCCAGTGGGGGGTCCCATACCACTCATGGATCTGAATCAGATGGACACTCACATGGG
736
AGTCAAGAAGGTGGAGCAAACACAACCTCTGGTCCTATAAGGACACCCCAAATTCCAGAATGGCTGAT
****
AGTCAAGAAGGTGGAGCAAACACAACCTCTGGTCCTATAAGGACACCCCAAATTCCAGAATGGCTGAT
804
CATCTTGGCATCCCTCTTGGCCTTGGCTTTGATTCTTGCAGTTTGCATTGCAGTCAACAGTCGAAGAA
****
CATCTTGGCATCCCTCTTGGCCTTGGCTTTGATTCTTGCAGTTTGCATTGCAGTCAACAGTCGAAGAA
872
GGTGTGGGCAGAAGAAAAAGCTAGTGATCAACAGTGGCAATGGAGCTGTGGAGGACAGAAAGCCAAGT
****
GGTGTGGGCAGAAGAAAAAGCTAGTGATCAACAGTGGCAATGGAGCTGTGGAGGACAGAAAGCCAAGT
940
GGACTCAACGGAGAGGCCAGCAAGTCTCAGGAAATGGTGCATTTGGTGAACAAGGAGTCGTCAGAAAC
****
GGACTCAACGGAGAGGCCAGCAAGTCTCAGGAAATGGTGCATTTGGTGAACAAGGAGTCGTCAGAAAC
1008
TCCAGACCAGTTTATGACAGCTGATGAGACAAGGAAACCTGCAGAATGTGGACATGAAGATTGGGGTG
****
TCCAGACC
1076
TAA

Note: The final 63 bp was not sequenced
Nucleotide position 1 refers to the beginning sequence of the v6 exon
Shaded region: v10 exon
1076: TAA Stop codon
Sequence presented in bold is derived from published data from Screaton *et al* (1992)



$CD44_{v6}$ clone: Sequence using primer G

30
GGGACTCTGCCTCGTGCCGCTGAGCCTGGCGCAGATCGATTTGAATATAACCTGCCGCTTTGCAGGTGTA
****
GGGACTCTGCCTCGTGCCGCTGAGCCTGGCGCAGATCGATTTGAATATAACCTGCCGCTTTGCAGGTGTA
100
TTCCACGTGGAGAAAAATGGTCGCTACAGCATCTCTCGGACGGAGGCCGCTGACCTCTGCAAGGCTTTCA
****
TTCCACGTGGAGAAAAATGGTCGCTACAGCATCTCTCGGACGGAGGCCGCTGACCTCTGCAAGGCTTTCA
170
ATAGCACCTTGCCCACAATGGCCCAGATGGAGAAAGCTCTGAGCATCGGATTTGAGACCTGCAGGTATGG
****
ATAGCACCTTGCCCACAATGGCCCAGATGGAGAAAGCTCTGAGCATCGGATTTGAGACCTGCAGGTATGG
240
GTTCATAGAAGGGCACGTGGTGATTCCCCGGATCCACCCCAACTCCATCTGTGCAGCAAACAACACAGGG
****
GTTCATAGAAGGGCACGTGGTGATTCCCCGGATCCACCCCAACTCCATCTGTGCAGCAAACAACACAGGG
310
GTGTACATCCTCACATCCAACACCTCCCAGTATGACACATATTGCTTCAATGCTTCAGCTCCACCTGAAG
****
GTGTACATCCTCACATCCAACACCTCCCAGTATGACACATATTGCTTCAATGCTTCAGCTCCACCTGAAG
380 *401*
AAGATTGTACATCAGTCACAGACCTGCCCAATGCCTTTGATGGACCAATTACCATAACTATTGTTAACCG
****
AAGATTGTACATCAGTCACAGACCTGCCCAATGCCTTTGATGGACCAATTACCATAACTATTGTTAACCG
450 *475*
TGATGGCACCCGCTATGTCCAGAAA
****
TGATGGCACCCGCTATGTCCAGAAA

401: Beginning of DNA sequence that can be read from sequence using primer A

Sequence presented in <u>bold</u> is derived from published data from Screaton *et al* (1992)

(Sequence run: Primer G)

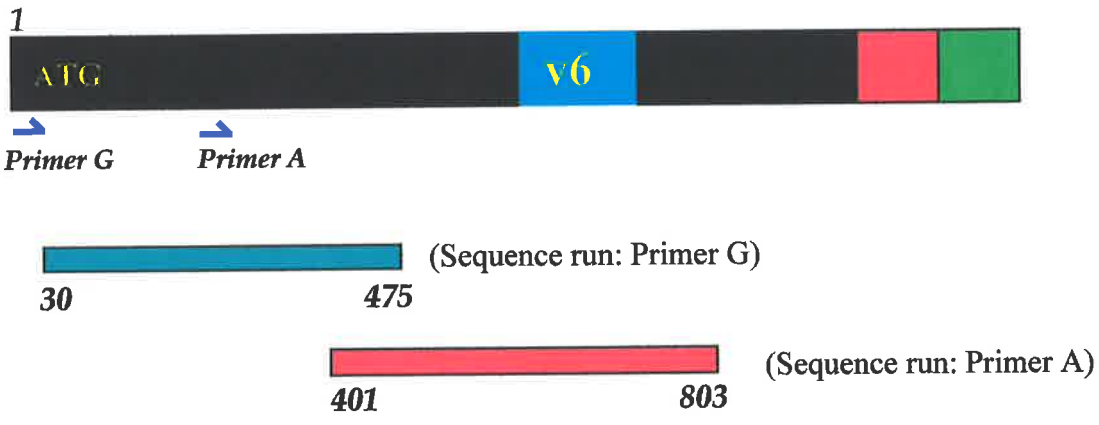
$CD44_{v6}$ clone: Sequence using primer A

401
ACCTGCCCAATGCCTTTGATGGACCAATTACCATAACTATTGTTAACCGTGATGGCACCCGCTATGTCCA
****
ACCTGCCCAATGCCTTTGATGGACCAATTACCATAACTATTGTTAACCGTGATGGCACCCGCTATGTCCA
471
GAAAGGAGAATACAGAACGAATCCTGAAGACATCTACCCCAGCAACCCTACTGATGATGACGTGAGCAGC
****
GAAAGGAGAATACAGAACGAATCCTGAAGACATCTACCCCAGCAACCCTACTGATGATGACGTGAGCAGC
541
GGCTCCTCCAGTGAAAGGAGCAGCACTTCAGGAGGTTACATCTTTTACACCTTTTCTACTGTACACCCCA
****
GGCTCCTCCAGTGAAAGGAGCAGCACTTCAGGAGGTTACATCTTTTACACCTTTTCTACTGTACACCCCA
611
TCCCAGACGAAGACAGTCCCTGGATCACCGACAGCACAGACAGAATCCCTGCTACCATCCAGGCAACTCC
****
TCCCAGACGAAGACAGTCCCTGGATCACCGACAGCACAGACAGAATCCCTGCTACCATCCAGGCAACTCC
681 715
TAGTAGTACAACGGAAGAAACAGCTACCCAGAAGGAACAGTGGTTTGGCAACAGATGGCATGAGGGATAT
****
TAGTAGTACAACGGAAGAAACAGCTACCCAGAAGGAACAGTGGTTTGGCAACAGATGGCATGAGGGATAT
751 803
CGCCAAACACCCAGAGAAGACTCCCATTCGACAACAGGGACAGCTGGAGACC
****
CGCCAAACACCCAGAGAAGACTCCCATTCGACAACAGGGACAGCTGGAGACC

715: Beginning of DNA sequence that can be read from sequence using primer B

Shaded region: v6 exon

Sequence presented in <u>bold</u> is derived from published data from Screaton *et al* (1992)



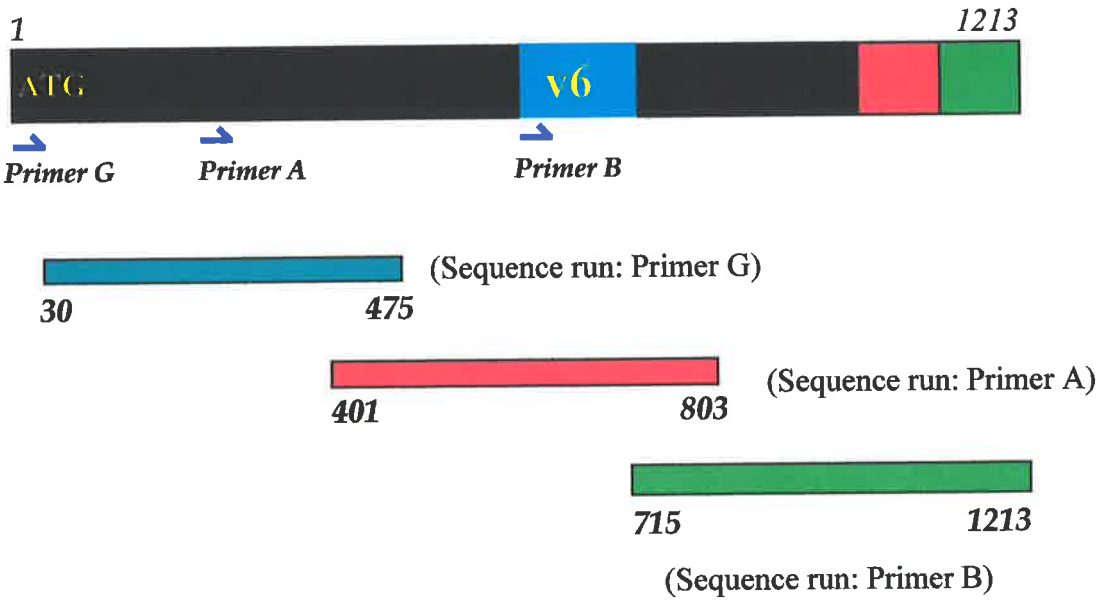
$CD44_{v6}$ clone: Sequence using primer B

715
CAGTGGTTTGGCAACAGATGGCATGAGGGATATCGCCAAACACCCAGAGAAGACTCCCATTCGACAACAG
****
CAGTGGTTTGGCAACAGATGGCATGAGGGATATCGCCAAACACCCAGAGAAGACTCCCATTCGACAACAG
785
GGACAGCTGGAGACCAAGACACATTCCACCCCAGTGGGGGGTCCCATACCACTCATGGATCTGAATCAGA
****
GGACAGCTGGAGACCAAGACACATTCCACCCCAGTGGGGGGTCCCATACCACTCATGGATCTGAATCAGA
855
TGGACACTCACATGGGAGTCAAGAAGGTGGAGCAAACACAACCTCTGGTCCTATAAGGACACCCCAAATT
****
TGGACACTCACATGGGAGTCAAGAAGGTGGAGCAAACACAACCTCTGGTCCTATAAGGACACCCCAAATT
925
CCAGAATGGCTGATCATCTTGGCATCCCTCTTGGCCTTGGCTTTGATTCTTGCAGTTTGCATTGCAGTCA
****
CCAGAATGGCTGATCATCTTGGCATCCCTCTTGGCCTTGGCTTTGATTCTTGCAGTTTGCATTGCAGTCA
995
ACAGTCGAAGAAGGTGTGGGCAGAAGAAAAAGCTAGTGATCAACAGTGGCAATGGAGCTGTGGAGGACAG
****
ACAGTCGAAGAAGGTGTGGGCAGAAGAAAAAGCTAGTGATCAACAGTGGCAATGGAGCTGTGGAGGACAG
1065
AAAGCCAAGTGGACTCAACGGAGAGGCCAGCAAGTCTCAGGAAATGGTGCATTTGGTGAACAAGGAGTCG
****
AAAGCCAAGTGGACTCAACGGAGAGGCCAGCAAGTCTCAGGAAATGGTGCATTTGGTGAACAAGGAGTCG
1135
TCAGAAACTCCAGACCAGTTTATGACAGCTGATGAGACAAGGAAACCTGCAGAATGTGGACATGAAGATT
****
TCAGAAACTCCAGACCAGTTTATGACAGCTGATGAGACAAGGAAACCTGCAGAATGTGGACATGAAGATT
1205 *1211*
GGGGTG<u>TAA</u>
****
GGGGTGTAA

Shaded region: v6 exon

1211: TAA stop codon

Sequence presented in <u>bold</u> is derived from published data from Screaton *et al* (1992)

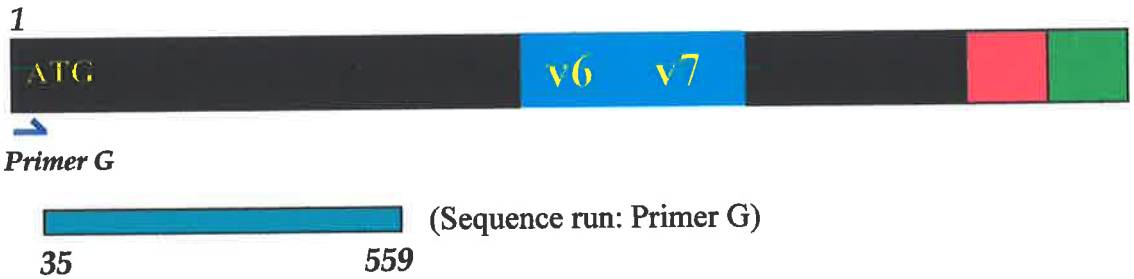


$CD44_{v6-7}$ clone: Sequence using primer G

```
                                                35
ATGGACAAGTTTTGGTGGCACGCAGCCTGGGGACTCTGCCTCGTGCCGCTGAGCCTGGCGCAGATCGA
                                                **********************************
                                                TCTGCCTCGTGCCGCTGAGCCTGGCGCAGATCGA
69
TTTGAATATAACCTGCCGCTTTGCAGGTGTATTCCACGTGGAGAAAAATGGTCGCTACAGCATCTCTC
************************************************************************
TTTGAATATAACCTGCCGCTTTGCAGGTGTATTCCACGTGGAGAAAAATGGTCGCTACAGCATCTCTC
137
GGACGGAGGCCGCTGACCTCTGCAAGGCTTTCAATAGCACCTTGCCCACAATGGCCCAGATGGAGAAA
************************************************************************
GGACGGAGGCCGCTGACCTCTGCAAGGCTTTCAATAGCACCTTGCCCACAATGGCCCAGATGGAGAAA
205
GCTCTGAGCATCGGATTTGAGACCTGCAGGTATGGGTTCATAGAAGGGCACGTGGTGATTCCCCGGAT
************************************************************************
GCTCTGAGCATCGGATTTGAGACCTGCAGGTATGGGTTCATAGAAGGGCACGTGGTGATTCCCCGGAT
273
CCACCCCAACTCCATCTGTGCAGCAAACAACACAGGGGTGTACATCCTCACATCCAACACCTCCCAGT
************************************************************************
CCACCCCAACTCCATCTGTGCAGCAAACAACACAGGGGTGTACATCCTCACATCCAACACCTCCCAGT
341                                                                  389
ATGACACATATTGCTTCAATGCTTCAGCTCCACCTGAAGAAGATTGTACATCAGTCACAGACCTGCCC
************************************************************************
ATGACACATATTGCTTCAATGCTTCAGCTCCACCTGAAGAAGATTGTACATCAGTCACAGACCTGCCC
409
AATGCCTTTGATGGACCAATTACCATAACTATTGTTAACCGTGATGGCACCCGCTATGTCCAGAAAGG
************************************************************************
AATGCCTTTGATGGACCAATTACCATAACTATTGTTAACCGTGATGGCACCCGCTATGTCCAGAAAGG
477
AGAATACAGAACGAATCCTGAAGACATCTACCCCAGCAACCCTACTGATGATGACGTGAGCAGCGGCT
************************************************************************
AGAATACAGAACGAATCCTGAAGACATCTACCCCAGCAACCCTACTGATGATGACGTGAGCAGCGGCT
545              559
CCTCCAGTGAAAGGA
***************
CCTCCAGTGAAAGGA
```

389: Beginning of DNA sequence that can be read from sequence using primer A

Sequence presented in <u>bold</u> is derived from published data from Screaton *et al* (1992)



$CD44_{v6-7}$ clone: Sequence using the primer A

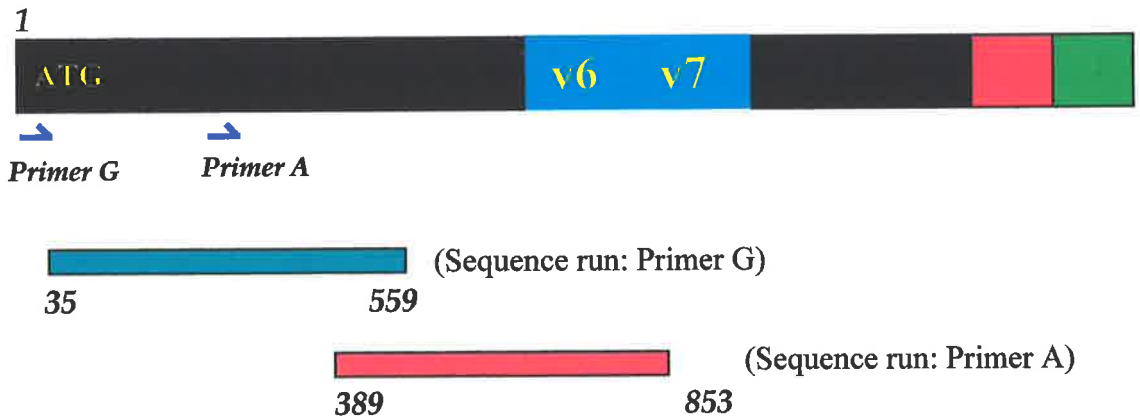
```
                                                                  389
ATGACACATATTGCTTCAATGCTTCAGCTCCACCTGAAGAAGATTGTACATCAGTCACAGACCTGCCC
                                                                  **********
                                                                  CATCAGTCACAGACCTGCCC
409
AATGCCTTTGATGGACCAATTACCATAACTATTGTTAACCGTGATGGCACCCGCTATGTCCAGAAAGG
************************************************************************
AATGCCTTTGATGGACCAATTACCATAACTATTGTTAACCGTGATGGCACCCGCTATGTCCAGAAAGG
477
AGAATACAGAACGAATCCTGAAGACATCTACCCCAGCAACCCTACTGATGATGACGTGAGCAGCGGCT
************************************************************************
AGAATACAGAACGAATCCTGAAGACATCTACCCCAGCAACCCTACTGATGATGACGTGAGCAGCGGCT
545
CCTCCAGTGAAAGGAGCAGCACTTCAGGAGGTTACATCTTTTACACCTTTTCTACTGTACACCCCATC
************************************************************************
CCTCCAGTGAAAGGAGCAGCACTTCAGGAGGTTACATCTTTTACACCTTTTCTACTGTACACCCCATC
613
CCAGACGAAGACAGTCCCTGGATCACCGACAGCACAGACAGAATCCCTGCTACCATCCAGGCAACTCC
************************************************************************
CCAGACGAAGACAGTCCCTGGATCACCGACAGCACAGACAGAATCCCTGCTACCATCCAGGCAACTCC
681                                                   714
TAGTAGTACAACGGAAGAAACAGCTACCCAGAAGGAACAGTGGTTTGGCAACAGATGGCATGAGGGAT
************************************************************************
TAGTAGTACAACGGAAGAAACAGCTACCCAGAAGGAACAGTGGTTTGGCAACAGATGGCATGAGGGAT
749
ATCGCCAAACACCCAGAGAAGACTCCCATTCGACAACAGGGACAGCTGCAGCCTCAGCTCATACCAGC
************************************************************************
ATCGCCAAACACCCAGAGAAGACTCCCATTCGACAACAGGGACAGCTGCAGCCTCAGCTCATACCAGC
817                                                   853
CATCCAATGCAAGGAAGGACAACACCAAGCCCAGA
**********************************
CATCCAATGCAAGGAAGGACAACACCAAGCCCAGA
```

714: Beginning of DNA sequence that can be read from sequence using primer B

Shaded region: v6 exon

Shaded region: v7 exon

Sequence presented in <u>bold</u> is derived from published data from Screaton *et al* (1992)



$CD44_{v6-7}$ clone: Sequence using primer B

714

TAGTAGTACAACGGAAGAAACAGCTACCCAGAAGGAACAGTGGTTTGGCAACAGATGGCATGAGGGAT

****

GGAACAGTGGTTTGGCAACAGATGGCATGAGGGAT

749

ATCGCCAAACACCCAGAGAAGACTCCCATTCGACAACAGGGACAGCTGCAGCCTCAGCTCATACCAGC

****

ATCGCCAAACACCCAGAGAAGACTCCCATTCGACAACAGGGACAGCTGCAGCCTCAGCTCATACCAGC

817 853

CATCCAATGCAAGGAAGGACAACACCAAGCCCAGAGGACAGTTCCTGGACTGATTTCTTCAACCCAAT

****

CATCCAATGCAAGGAAGGACAACACCAAGCCCAGAGGACAGTTCCTGGACTGATTTCTTCAACCCAAT

885

CTCACACCCCATGGGACGAGGTCATCAAGCAGGAAGAAGGATGGGAGACCAAGACACATTCCACCCCA

****

CTCACACCCCATGGGACGAGGTCATCAAGCAGGAAGAAGGATGGGAGACCAAGACACATTCCACCCCA

953

GTGGGGGGTCCCATACCACTCATGGATCTGAATCAGATGGACACTCACATGGGAGTCAAGAAGGTGGA

****

GTGGGGGGTCCCATACCACTCATGGATCTGAATCAGATGGACACTCACATGGGAGTCAAGAAGGTGGA

1021

GCAAACACAACCTCTGGTCCTATAAGGACACCCCAAATTCCAGAATGGCTGATCATCTTGGCATCCCT

****

GCAAACACAACCTCTGGTCCTATAAGGACACCCCAAATTCCAGAATGGCTGATCATCTTGGCATCCCT

1089

CTTGGCCTTGGCTTTGATTCTTGCAGTTTGCATTGCAGTCAACAGTCGAAGAAGGTGTGGGCAGAAGA

****

CTTGGCCTTGGCTTTGATTCTTGCAGTTTGCATTGCAGTCAACAGTCGAAGAAGGTGTGGGCAGAAGA

1157 1183

AAAAGCTAGTGATCAACAGTGGCAAT

****

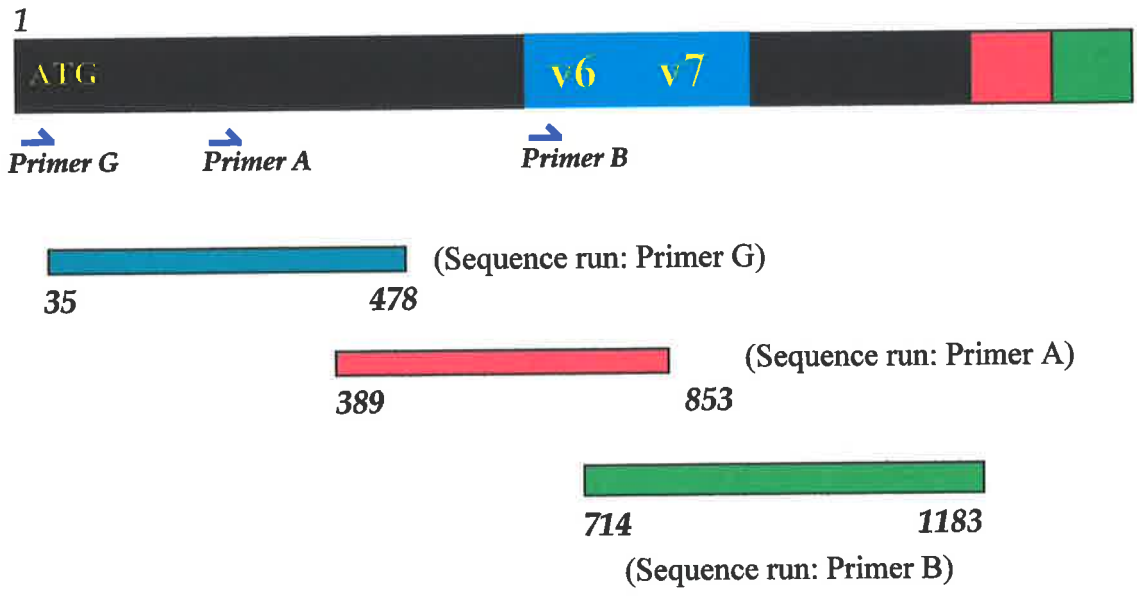
AAAAGCTAGTGATCAACAGTGGCAAT

853: Beginning of DNA sequence that can be read from sequence using primer E

Shaded region: v6 exon

Shaded region: v7 exon

Sequence presented in bold is derived from published data from Screaton *et al* (1992)



$CD44_{v6-7}$ clone: Sequence using primer E

853
CAGCCTCAGCTCATACCAGCCATCCAATGCAAGGAAGGACAACACCAAGCCCAGAGGACAGTTCCTGG
****
GACAGTTCCTGG

865
ACTGATTTCTTCAACCCAATCTCACACCCCATGGGACGAGGTCATCAAGCAGGAAGAAGGATGGGAGA
****
ACTGATTTCTTCAACCCAATCTCACACCCCATGGGACGAGGTCATCAAGCAGGAAGAAGGATGGGAGA

933
CCAAGACACATTCCACCCCAGTGGGGGGTCCCATACCACTCATGGATCTGAATCAGATGGACACTCAC
****
CCAAGACACATTCCACCCCAGTGGGGGGTCCCATACCACTCATGGATCTGAATCAGATGGACACTCAC

1001
ATGGGAGTCAAGAAGGTGGAGCAAACACAACCTCTGGTCCTATAAGGACACCCCAAATTCCAGAATGG
****
ATGGGAGTCAAGAAGGTGGAGCAAACACAACCTCTGGTCCTATAAGGACACCCCAAATTCCAGAATGG

1069
CTGATCATCTTGGCATCCCTCTTGGCCTTGGCTTTGATTCTTGCAGTTTGCATTGCAGTCAACAGTCG
****
CTGATCATCTTGGCATCCCTCTTGGCCTTGGCTTTGATTCTTGCAGTTTGCATTGCAGTCAACAGTCG

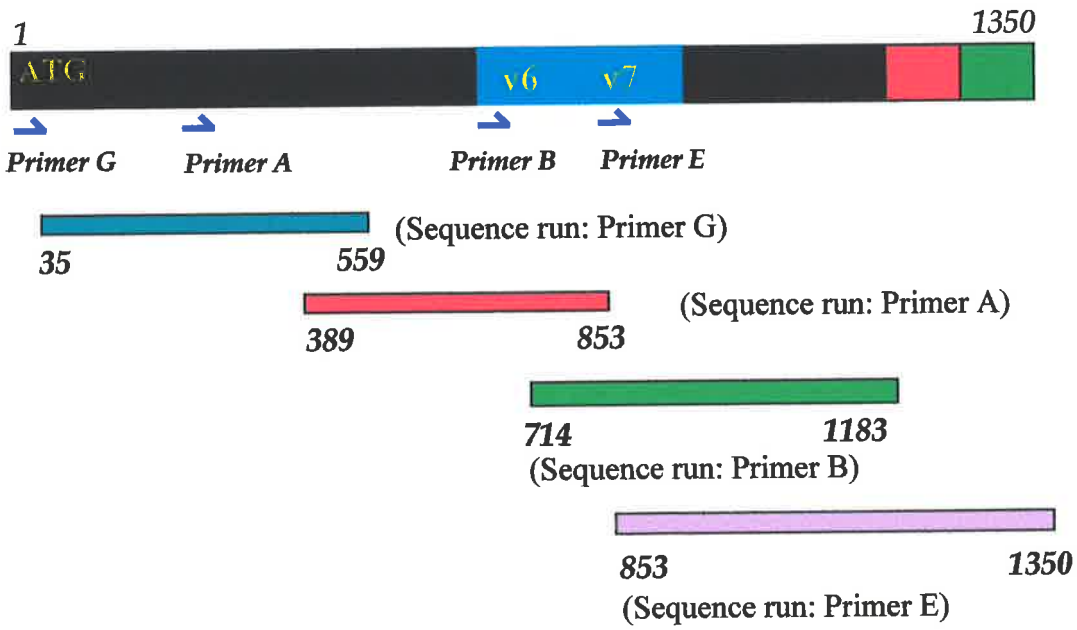
1137
AAGAAGGTGTGGGCAGAAGAAAAAGCTAGTGATCAACAGTGGCAATGGAGCTGTGGAGGACAGAAAGC
****
AAGAAGGTGTGGGCAGAAGAAAAAGCTAGTGATCAACAGTGGCAATGGAGCTGTGGAGGACAGAAAGC

1205
CAAGTGGACTCAACGGAGAGGCCAGCAAGTCTCAGGAAATGGTGCATTTGGTGAACAAGGAGTCGTCA
****
CAAGTGGACTCAACGGAGAGGCCAGCAAGTCTCAGGAAATGGTGCATTTGGTGAACAAGGAGTCGTCA

1273
GAAACTCCAGACCAGTTTATGACAGCTGATGAGACAAGGAAACCTGCAGAATGTGGACATGAAGATTG
****
GAAACTCCAGACCAGTTTATGACAGCTGATGAGACAAGGAAACCTGCAGAATGTGGACATGAAGATTG

1341 1347
GGGTGTAA
****
GGGTGTAA

Shaded region: v7 exon
1347: TAA Stop codon
Sequence presented in bold is derived from published data from Screaton *et al* (1992)



$CD44_{v6-9}$ clone: Sequence using primer G

35
ATGGACAAGTTTTGGTGGCACGCAGCCTGGGGACTCTGCCTCGTGCCGCTGAGCCTGGCGCAGATCGA
****
TCTGCCTCGTGCCGCTGAGCCTGGCGCAGATCGA
69
TTTGAATATAACCTGCCGCTTTGCAGGTGTATTCCACGTGGAGAAAAATGGTCGCTACAGCATCTCTC
****
TTTGAATATAACCTGCCGCTTTGCAGGTGTATTCCACGTGGAGAAAAATGGTCGCTACAGCATCTCTC
137
GGACGGAGGCCGCTGACCTCTGCAAGGCTTTCAATAGCACCTTGCCCACAATGGCCCAGATGGAGAAA
****
GGACGGAGGCCGCTGACCTCTGCAAGGCTTTCAATAGCACCTTGCCCACAATGGCCCAGATGGAGAAA
205
GCTCTGAGCATCGGATTTGAGACCTGCAGGTATGGGTTCATAGAAGGGCACGTGGTGATTCCCCGGAT
****
GCTCTGAGCATCGGATTTGAGACCTGCAGGTATGGGTTCATAAAAGGGCACGTGGTGATTCCCCGGAT
273
CCACCCCAACTCCATCTGTGCAGCAAACAACACAGGGGTGTACATCCTCACATCCAACACCTCCCAGT
****
CCACCCCAACTCCATCTGTGCAGCAAACAACACAGGGGTGTACATCCTCACATCCAACACCTCCCAGT
341 *401*
ATGACACATATTGCTTCAATGCTTCAGCTCCACCTGAAGAAGATTGTACATCAGTCACAGACCTGCCC
****
ATGACACATATTGCTTCAATGCTTCAGCTCCACCTGAAGAAGATTGTACATCAGTCACAGACCTGCCC
409
AATGCCTTTGATGGACCAATTACCATAACTATTGTTAACCGTGATGGCACCCGCTATGTCCAGAAAGG
****
AATGCCTTTGATGGACCAATTACCATAACTATTGTTAACCGTGATGGCACCCGCTATGTCCAGAAAGG
477
AGAATACAGAACGAATCCTGAAGACATCTACCCCAGCAACCCTACTGATGATGACGTGAGCAGCGGCT
****
AGAATACAGAACGAATCCTGAAGACATCTACCCCAGCAACCCTACTGATGATGACGTGAGCAGCGGCT
545 *577*
CCTCCAGTGAAAGGAGCAGCACTTCAGGA
****
CCTCCAGTGAAAGGAGCAGCACTTCAGGA

401: Beginning of DNA sequence that can be read from sequence using Primer A

Sequence presented in <u>bold</u> is derived from published data from Screaton *et al* (1992)


(Sequence run: Primer G)

35 *577*

$CD44_{v6-9}$ clone: Sequence using primer A

401
ATGACACATATTGCTTCAATGCTTCAGCTCCACCTGAAGAAGATTGTACATCAGTCACAGACCTGCCC
****
ACCTGCCC

409
AATGCCTTTGATGGACCAATTACCATAACTATTGTTAACCGTGATGGCACCCGCTATGTCCAGAAAGG
****
AATGCCTTTGATGGACCAATTACCATAACTATTGTTAACCGTGATGGCACCCGCTATGTCCAGAAAGG
477
AGAATACAGAACGAATCCTGAAGACATCTACCCCAGCAACCCTACTGATGATGACGTGAGCAGCGGCT
****
AGAATACAGAACGAATCCTGAAGACATCTACCCCAGCAACCCTACTGATGATGACGTGAGCAGCGGCT
545
CCTCCAGTGAAAGGAGCAGCACTTCAGGAGGTTACATCTTTTACACCTTTTCTACTGTACACCCCATC
****
CCTCCAGTGAAAGGAGCAGCACTTCAGGAGGTTACATCTTTTACACCTTTTCTACTGTACACCCCATC
613
CCAGACGAAGACAGTCCCTGGATCACCGACAGCACAGACAGAATCCCTGCTACCATCCAGGCAACTCC
****
CCAGACGAAGACAGTCCCTGGATCACCGACAGCACAGACAGAATCCCTGCTACCATCCAGGCAACTCC
681 720
TAGTAGTACAACGGAAGAAACAGCTACCCAGAAGGAACAGTGGTTTGGCAACAGATGGCATGAGGGAT
****
TAGTAGTACAACGGAAGAAACAGCTACCCAGAAGGAACAGTGGTTTGGCAACAGATGGCATGAGGGAT
749
ATCGCCAAACACCCAGAGAAGACTCCCATTCGACAACAGGGACAGCTGCAGCCTCAGCTCATACCAGC
****
ATCGCCAAACACCCAGAGAAGACTCCCATTCGACAACAGGGACAGCTGCAGCCTCAGCTCATACCAGC
817
CATCCAATGCAAGGAAGGACAACACCAAGCCCAGAGGACAGTTCCTGGACTGATTTCTTCAACCCAAT
****
CATCCAATGCAAGGAAGGACAACACCAAGCCCAGAGGACAGTTCCTGGACTGATTTCTTCAACCCAAT
885
CTCACACCCCATGGGACGAGGTCATCAAGCAGGAAGAAGGATGGATATGGACTCCAGTCATAGTACAA
****
CTCACACCCCATGGGACGAGGTCATCAAGCAGGAAGAAGGATGGATATGGACTCCAGTCATAGTACAA
953 1020
CGCTTCAGCCTACTGCAAATCCAAACACAGGTTTGGTGGAAGATTTGGACAGGACAGGACCTCTTTC
****
CGCTTCAGCCTACTGCAAATCCAAACACAGGTTTGGTGGAAGATTTGGACAGGACAGGACCTCTTTC

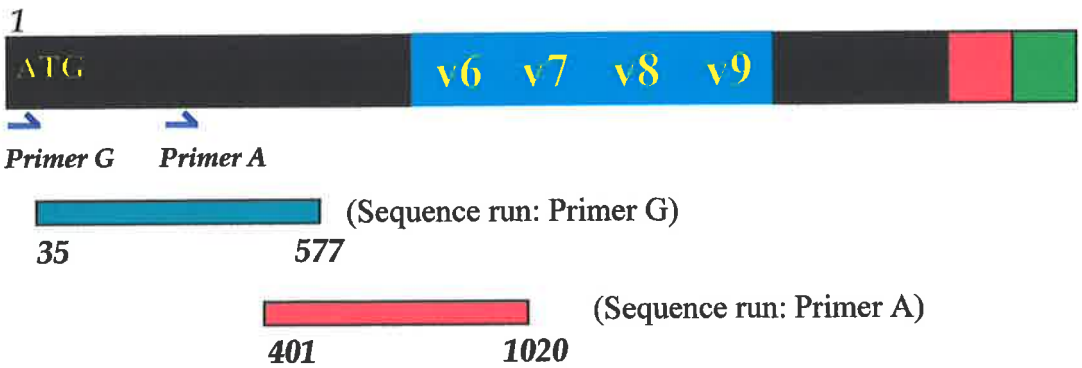
Shaded region: v6 exon

Shaded region: v7 exon

Shaded region: v8 exon

720: Beginning of DNA sequence that can be read from sequence using primer B

Sequence presented in <u>bold</u> is derived from published data from Screaton *et al* (1992)



$CD44_{v6-9}$ clone: Sequence using primer B

720
TAGTAGTACAACGGAAGAAACAGCTACCCAGAAGGAACAGTGGTTTGGCAACAGATGGCATGAGGGAT
****
GTGGTTTGGCAACAGATGGCATGAGGGAT

749
ATCGCCAAACACCCAGAGAAGACTCCCATTCGACAACAGGGACAGCTGCAGCCTCAGCTCATACCAGC
****
ATCGCCAAACACCCAGAGAAGACTCCCATTCGACAACAGGGACAGCTGCAGCCTCAGCTCATACCAGC

817 853
CATCCAATGCAAGGAAGGACAACACCAAGCCCAGAGGACAGTTCCTGGACTGATTTCTTCAACCCAAT
****
CATCCAATGCAAGGAAGGACAACACCAAGCCCAGAGGACAGTTCCTGGACTGATTTCTTCAACCCAAT

885
CTCACACCCCATGGGACGAGGTCATCAAGCAGGAAGAAGGATGGATATGGACTCCAGTCATAGTACAA
****
CTCACACCCCATGGGACGAGGTCATCAAGCAGGAAGAAGGATGGATATGGACTCCAGTCATAGTACAA

953
CGCTTCAGCCTACTGCAAATCCAAACACAGGTTTGGTGGAAGATTTGGACAGGACAGGACCTCTTTCA
****
CGCTTCAGCCTACTGCAAATCCAAACACAGGTTTGGTGGAAGATTTGGACAGGACAGGACCTCTTTCA

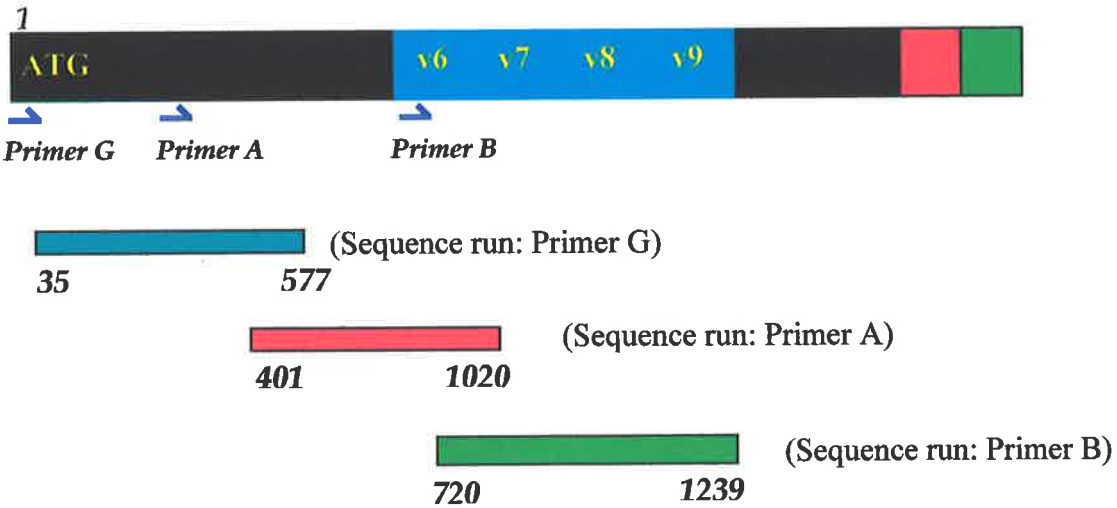
1021
ATGACAACGCAGCAGAGTAATTCTCAGAGCTTCTCTACATCACATGAAGGCTTGGAAGAAGATAAAGA
****
ATGACAACGCAGCAGAGTAATTCTCAGAGCTTCTCTACATCACATGAAGGCTTGGAAGAAGATAAAGA

1089
CCATCCAACAACTTCTACTCTGACATCAAGCAGAGACCAAGACACATTCCACCCCAGTGGGGGGTCCC
****
CCATCCAACAACTTCTACTCTGACATCAAGCAGAGACCAAGACACATTCCACCCCAGTGGGGGGTCCC

1157
ATACCACTCATGGATCTGAATCAGATGGACACTCACATGGGAGTCAAGAAGGTGGAGCAAACACAACC
****
ATACCACTCATGGATCTGAATCAGATGGACACTCACATGGGAGTCAAGAAGGTGGAGCAAACACAACC

1225 1239
TCTGGTCCTATAAGG
****
TCTGGTCCTATAAGG

853: Beginning of DNA sequence that can be read from sequence using Primer E
Shaded region: v6 exon
Shaded region: v7 exon
Shaded region: v8 exon
Shaded region: v9 exon
Sequence presented in bold is derived from published data from Screaton *et al* (1992)



$CD44_{v6\text{-}9}$ clone: Sequence using primer E

853
CAGCCTCAGCTCATACCAGCCATCCAATGCAAGGAAGGACAACACCAAGCCCAGAGGACAGTTCCTGG
****
GACAGTTCCTGG

865
ACTGATTTCTTCAACCCAATCTCACACCCCATGGGACGAGGTCATCAAGCAGGAAGAAGGATGGATAT
****
ACTGATTTCTTCAACCCAATCTCACACCCCATGGGACGAGGTCATCAAGCAGGAAGAAGGATGGATAT

933
GGACTCCAGTCATAGTACAACGCTTCAGCCTACTGCAAATCCAAACACAGGTTTGGTGGAAGATTTGG
**********+**********
GGACTCCAGTCATAGTATAACGCTTCAGCCTACTGCAAATCCAAACACAGGTTTGGTGGAAGATTTGG

1001
ACAGGACAGGACCTCTTTCAATGACAACGCAGCAGAGTAATTCTCAGAGCTTCTCTACATCACATGAA
****
ACAGGACAGGACCTCTTTCAATGACAACGCAGCAGAGTAATTCTCAGAGCTTCTCTACATCACATGAA

1069 1127
GGCTTGGAAGAAGATAAAGACCATCCAACAACTTCTACTCTGACATCAAGCAGAGACCAAGACACATT
****
GGCTTGGAAGAAGATAAAGACCATCCAACAACTTCTACTCTGACATCAAGCAGAGACCAAGACACATT

1137
CCACCCCAGTGGGGGGTCCCATACCACTCATGGATCTGAATCAGATGGACACTCACATGGGAGTCAAG
****
CCACCCCAGTGGGGGGTCCCATACCACTCATGGATCTGAATCAGATGGACACTCACATGGGAGTCAAG

1205
AAGGTGGAGCAAACACAACCTCTGGTCCTATAAGGACACCCCAAATTCCAGAATGGCTGATCATCTTG
**********+**
AAGGTGGAGCAAACACAACCTCTGGTCCTATAAGGACACCCCAAATTCCAGAATGGCTGATCATCCTG

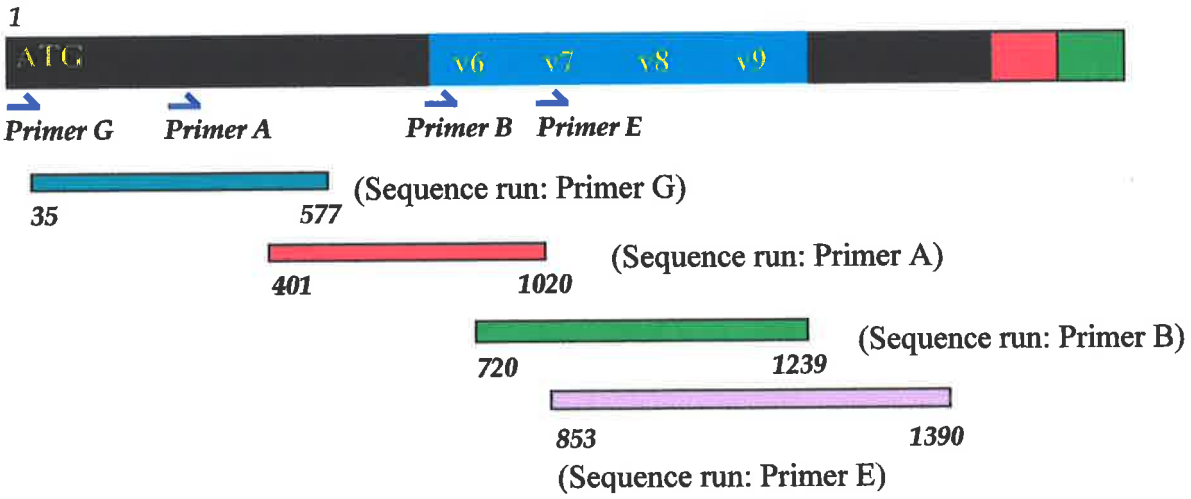
1273
GCATCCCTCTTGGCCTTGGCTTTGATTCTTGCAGTTTGCATTGCAGTCAACAGTCGAAGAAGGTGTGG
****
GCATCCCTCTTGGCCTTGGCTTTGATTCTTGCAGTTTGCATTGCAGTCAACAGTCGAAGAAGGTGTGG

1341 1390
GCAGAAGAAAAAGCTAGTGATCAACAGTGGCAATGGAGCTGTGGAGGAC
****
GCAGAAGAAAAAGCTAGTGATCAACAGTGGCAATGGAGCTGTGGAAGAC

1127: Beginning of DNA sequence that can be read from sequence using primer D
Shaded region: v7 exon

Shaded region: v8 exon

Shaded region: v9 exon
Sequence presented in <u>bold</u> is derived from published data from Screaton *et al* (1992)



$CD44_{v6-9}$ clone: Sequence using primer D

1127

CCATCCAACAACTTCTACTCTGACATCAAGCAGAGACCAAGACACATTCCACCCCAGTGGGGGGTCCC

****

AAGACACATTCCACCCCAGTGGGGGGTCCC

1157

ATACCACTCATGGATCTGAATCAGATGGACACTCACATGGGAGTCAAGAAGGTGGAGCAAACACAACC

****

ATACCACTCATGGATCTGAATCAGATGGACACTCACATGGGAGTCAAGAAGGTGGAGCAAACACAACC

1225

TCTGGTCCTATAAGGACACCCCAAATTCCAGAATGGCTGATCATCTTGGCATCCCTCTTGGCCTTGGC

****

TCTGGTCCTATAAGGACACCCCAAATTCCAGAATGGCTGATCATCTTGGCATCCCTCTTGGCCTTGGC

1293

TTTGATTCTTGCAGTTTGCATTGCAGTCAACAGTCGAAGAAGGTGTGGGCAGAAGAAAAAGCTAGTGA

****

TTTGATTCTTGCAGTTTGCATTGCAGTCAACAGTCGAAGAAGGTGTGGGCAGAAGAAAAAGCTAGTGA

1361

TCAACAGTGGCAATGGAGCTGTGGAGGACAGAAAGCCAAGTGGACTCAACGGAGAGGCCAGCAAGTCT

****

TCAACAGTGGCAATGGAGCTGTGGAGGACAGAAAGCCAAGTGGACTCAACGGAGAGGCCAGCAAGTCT

1429 1499

CAGGAAATGGTGCATTTGGTGAACAAGGAGTCGTCAGAAACTCCAGACCAGTTTATGACAGCTGATGA

****

CAGGAAATGGTGCATTTGGTGAACAAGGAGTCGTCAGAAACTCCAGACCAGTTTATGACAG

1547

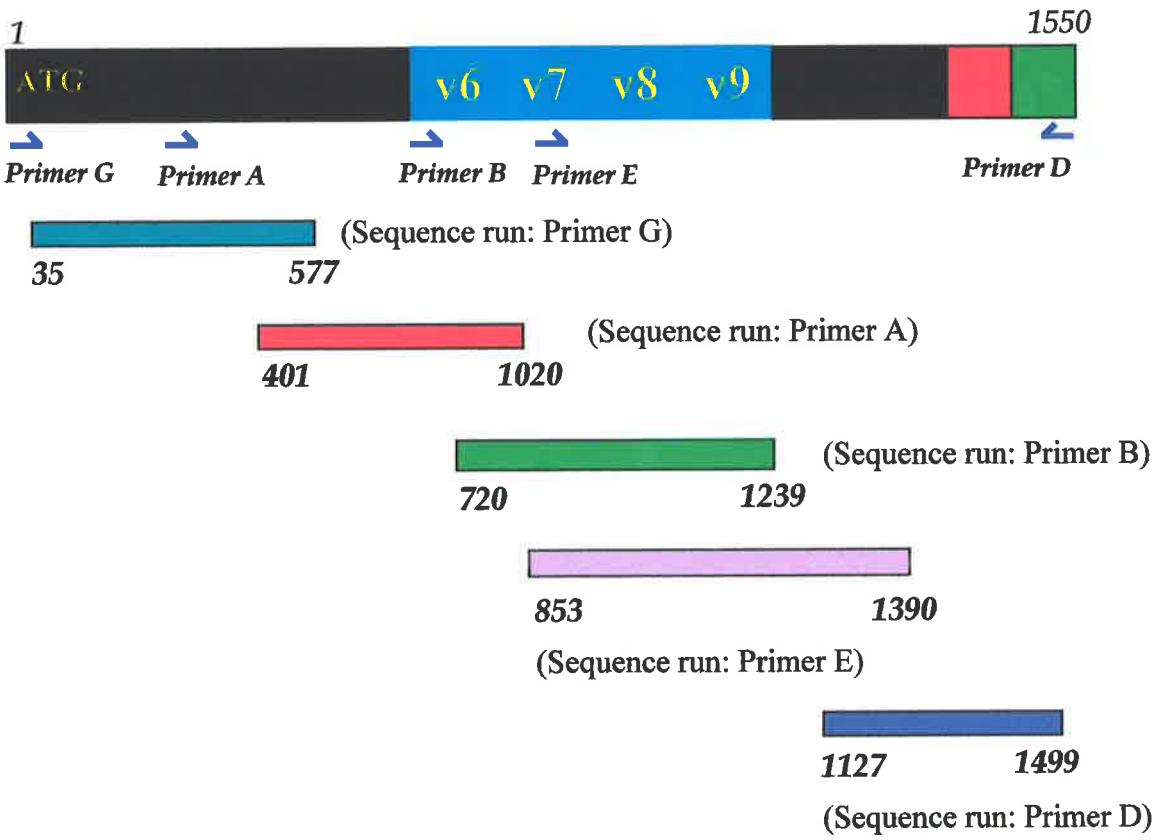
GACAAGGAAACCTGCAGAATGTGGACATGAAGATTGGGGTG*TAA*

Note: The final 50 bp of the $CD44_{v6-9}$ clone was not sequenced

Shaded region: v9 exon

1547: TAA Stop codon

Sequence presented in bold is derived from published data from Screaton *et al* (1992)



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