EXPRESSION OF THE ADHESION MOLECULE CD44 IN EPSTEIN-BARR VIRUS INFECTED B-CELLS

A dissertation submitted for the degree of Ph.D.

by

Joanne Gallagher B.Sc., M.Sc.

Under the supervision of Dr Dermot Walls

August 1999

School of Biotechnology, Dublin City University, Dublin 9, Ireland.

DECLARATION

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: Joanne Gallagher ID No. 95970851

Joanne Gallagher

Date: 20/9/1999

I dedicate this thesis with love to Mammy and Daddy
for all the love and support you have always given me.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr Dermot Walls, for giving me the opportunity to carry out my PhD in his lab group. I would like to thank him for all the help he has given me over the years but especially over the last two months with the preparation of my thesis.

Thanks to all the lab gang, in the early days to Carol Barbara and Susan for giving me a good start. To Olivia, Pam M, Brendan and Maria for being both good friends and colleagues and making working in DCU better on the days it was not so good. Melanie, thanks for the laughs, and for making me look at life from a different angle. To Pam P who has taken over Mels role as the lab "live wire". Una Mc, thanks for calling in and being always willing to listen. Thanks to Sinead in St James for the help and good advice.

Thanks to my house mates Olivia (again), Una, Sinead and Bernie, for all the glasses of wine, good conversation and laughs over the past four years.

To my life long friends Sharon, Kathy, Kara and Cepta for being there through the good and the bad, for the friendship we have shared for all the phone calls and words of encouragement you have all given me, even though secretly I know most of you wondered what I have really been doing all these years, thank you.

To all the Austen family for making me welcome in Wicklow, especially Louise and Stephen who treat me as part of the family and made Stilebawn cottage home from home.

To my Family who have been there through it all. To Teresa and Charlie my fairy godparents, for never leaving me out of the Christmas honours. Thanks to Uncle Charlie for all the "money for a disco" that have helped more than you can imagine over the years. To Anthony and Michelle and baby Christopher for giving me one of the most memorable "round" trips home I have had in a long time.

Thanks to my sister Mary for everything, to list all the kindness and good things you have done for me would take more time than I have right now. Thanks for all the support and the laughs and the love for all the "joint" pressies, for the trip to Paris and the credit free loans and the daily telephone calls keeping me up to speed with the rest of the world. For every thing thank you.

To Mammy and Daddy, for being the foundation of my belief in my self and the source of my strength and will power, for being always encouraging supportive in every way and above all for the unconditional love, thank you.

Finally thanks to Ben who has really seen the heart ache of the "one bad egg" over the past few years, who has listened tirelessly to my stories of cells and gels and so on, who has been there for me every day since we met. For the love we share I thank you.

ABSTRACT

The Epstein-Barr Virus is a human herpesvirus that is associated with African endemic Burkitt's Lymphoma (BL) and several other cancers of lymphoid/epithelial origin. The capacity of this virus to regulate the expression of cellular adhesion molecules, such as CD44, has important implications in the pathogenesis of EBV-associated malignant disease. CD44 is a multifunctional cell surface adhesion molecule involved in cell-cell and cell-matrix interactions. Many isoforms of CD44 exist, some of which have been implicated in metastasis. These are generated primarily as a result of complex alternate RNA splicing within the CD44 operon. In this thesis, CD44 expression was analysed in (I) EBV-positive BL-cell lines and (II) EBV negative derived cell lines that express viral latent proteins either after superinfection with virus, or after stable transfection of single viral genes. The results obtained illustrate that the expression of EBV latent proteins correlates with the induction of standard/variant isoforms of CD44 mRNA/protein in a BL cell background. When expressed as sole viral proteins, neither of the two principal viral effectors of cell transformation, the EBV latent membrane protein 1 (LMP1) nor the EB nuclear antigen 2 (EBNA2), were sufficient to induce CD44 expression. As part of this research a novel method for detecting CD44 mRNA splice variants was developed. Labelled antisense riboprobes derived from CD44 cDNA sequences were used in Ribonuclease Protection Assays (RPA) to analyse standard/variant exon usage in a range of cell lines including EBV-infected cells. The results obtained with this assay also indicated that the pattern of CD44 standard and variant exon usage is very complex but similar in type-III latency BL cell lines and lymphoblastoid cell lines.

The effect of EBNA2 and EBNA1 on cellular gene expression in an EBV negative BL background was also investigated by differential display reverse transcription polymerase chain reaction (DDRT-PCR). This was examined using EBV-negative BL cell lines in which EBNA2 or EBNA1 were expressed as sole viral proteins. A panel of six potentially differentially expressed gene were initially identified but subsequently northern blot analysis showed that none of these were in fact modulated by the relevant EBV protein.

ABBREVIATIONS

A	Adenosine
Abs	Absorbance
AIDS	Aquired Immune Deficiency Syndrome
amp	Ampicillin
AP	Alkaline phosphatase
APS	Ammonium Persulphate
ATP	Adenosine Tri-Phosphate
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BL	Burkitt's Lymphoma
BSA	Bovine serum albumin
C	Cystidine
CAT	Chloramphenicol acetyl transferase
CD44	Cluster of differentiation 44
CD44s	CD44 standard
CD44v	CD44 variant
cDNA	Complementary DNA
CIP	Calf Intestinal Phosphatase
CMV	Cytomegalovirus
CTP	Cystidine Tri-phosphate
DD	Differential display
DDRT-PCR	Differential Display Reverse Transcription Polymerase Chain Reaction
DEPC	Diethylpyrocarbonate
d H_2O	Distilled water
dATP	Deoxy Adenosine tri-phosphate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
EA-D	Early antigen-diffuse

EA-R	Early antigen restricted
EBER	Epstein-Barr virus Encoded RNA
EBNA	Epstein-Barr virus Nuclear Antigen
EBNALP	Epstein-Barr virus Nuclear Antigen Leader Protein
EBV	Epstein-Barr Virus
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FCS	Fetal calf serum
G	Guanosine
GTP	Guanosine Tri-Phosphate
HA	hyaluronic acid
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IL	Interleukin
IM	Infectious Mononucleosis
IPTG	Isopropyl β-D-Thiogalactopyranoside
LB	Luria-Bertrani broth
LCL	Lymphoblastoid cell line
LMP	Latent Membrane Protein
mAb	Monoclonal antibody
MCS	Multiple Cloning Site
MMLV-RT	Moloney murine leukaemia virus-reverse transcriptase
MOPS	3-[N-Morpholino]propanesulfonic acid
mRNA	Messenger RNA
NBT	Nitroblue tetrazolium
NPC	Nasopharengeal Carcinoma
NP-40	Nonylphenoxypolyethoxy ethanol - 40
OD	Optical density

OHL	Oral Hairy leukoplakia
ori	Origin of Replication
p	Plasmid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PKCγ2	Protein kinase C γ2
PMSF	Phenylmethylsulfonyl Flouride
POD	Peroxidase
PRB	Retinoblastoma protein
RBP-Jκ	Recombination Signal binding protein - Jκ
RNA	Ribonucleic acid
RNase	Ribonucleases
RPA	Ribonuclease protection assay
RT-PCR	Reverse transcription PCR
SA-PMPs	Streptavidin paramagnetic particles
SDS	Sodium dodecyl sulphate
SSC	Standard sodium citrate
T	Thymidine
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TBS	Tris buffer saline
TBST	Tris buffer saline plus tween 20
TE	Tris EDTA
TEMED	N,N,N',N'- Tetramethylethylenediamine
tRNA	Transfer RNA
upH_2O	Ultra pure water
U	Uracil
UTP	Uracil Tri-Phosphate
UV	Ultraviolet
v/v	Volume per volume

w/v	Weight per volume
X-Gal	5-Bromo-4-chloro-3indoyl-β-D-galactoside

UNITS

bp	Base pairs
Ci	Curies
Kb	Kilobases
KD	Kilodaltons
µg	Microgram
µl	Microliter
°C	Degrees Celsius
cm	Centimetre
g	g force
g	Grams
hr	Hour
Kg	Kilogram
L	Litre
M	Molar
mA	Milliamps
mg	Milligram
min	Minute
ml	Millilitre
mM	Milimolar
mol	Moles
ng	Nanogram
nm	Nanometre
pmole	Picomoles
s	Second
U	Enzyme units
V	Volts

FIGURES

TABLES

TABLE OF CONTENTS

CHAPTER 1
INTRODUCTION

1.0 EPSTEIN-BARR VIRUS

1.1 DISCOVERY AND CLASSIFICATION

In 1962 Dennis Burkitt described the clinical manifestation of a common childhood cancer in East Africa, Burkitt's lymphoma (BL), (Burkitt, 1962b). He suggested that a virus may be the cause of the lymphoma. In an attempt to confirm this hypothesis the Epstein-Barr virus (EBV) was discovered in cultured lymphoblasts from samples of African Burkitt's lymphomas by Epstein *et al.*, (1964). EBV is a ubiquitous human herpes virus, for which humans are the exclusive natural host, it infects between 90-95% of the population (Kieff, 1996). The herpesviruses are a family of DNA viruses found commonly in humans and animals. Herpes viruses are biologically classified into three subfamilies, alpha, beta and gamma, EBV belongs to the genera *Lymphocryptovirus* of the subfamily gammaherpesvirus. Viruses of this subfamily are characterised by their tropism for lymphoid cells and their capacity to induce cell proliferation *in vivo*, resulting in transient or chronic lymphoproliferative disorders and *in vitro* where many can immortalize the infected cell. Taxonomists have renamed EBV as human herpesvirus 4 (HHV4) but EBV is still its commonly used name (IARC Monographs, 1997). Like other herpesviruses, a mature EBV virion has a toroid-shaped protein core that is wrapped with double-stranded DNA this is surrounded by an icosahedral capsid with 162 capsomers (figure 1.1). The capsid is surrounded by an amorphous material, the tegument, composed of globular proteins. The envelope of herpesviruses have numerous glycoprotein spikes (Kieff, 1996).

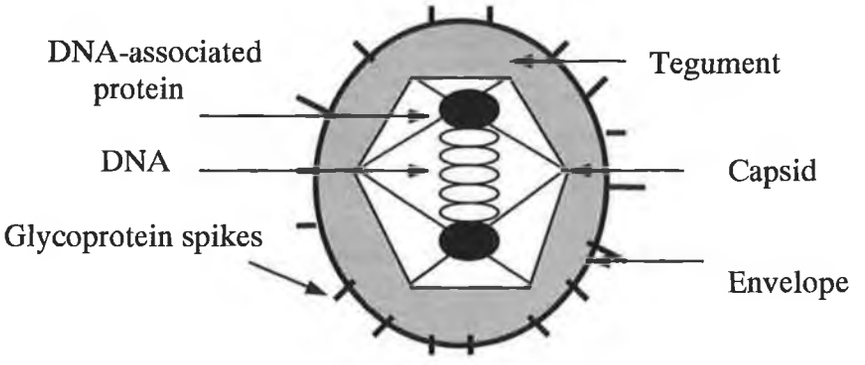


Fig. 1.1. **Schematic representation of herpes virus structure.**

1.2 EBV GENOME STRUCTURE

The EBV genome is a linear, double stranded 172 Kb DNA with a guanine/cytosine content of 60% (Kieff, 1996). The genome encodes an estimated 100 genes, but like many viruses there is complicated differential splicing of RNA transcripts and the number of proteins produced may be greater (Kieff, 1996). The EBV genome was completely sequenced from the EBV strain B95-8, initially cloned as a *Bam* H1 fragment library, therefore open reading frames (ORF), genes, sites for transcription or RNA processing are frequently referred to specific *Bam* H1 fragments (Baer *et al.*, 1984). For example, the BARF1 ORF is found in the *Bam* H1 A fragment (BA) and it is the first ORF (F1) extending in a rightward (R) direction. A simplified schematic representation of the EBV genome is outlined in figure 1.2.

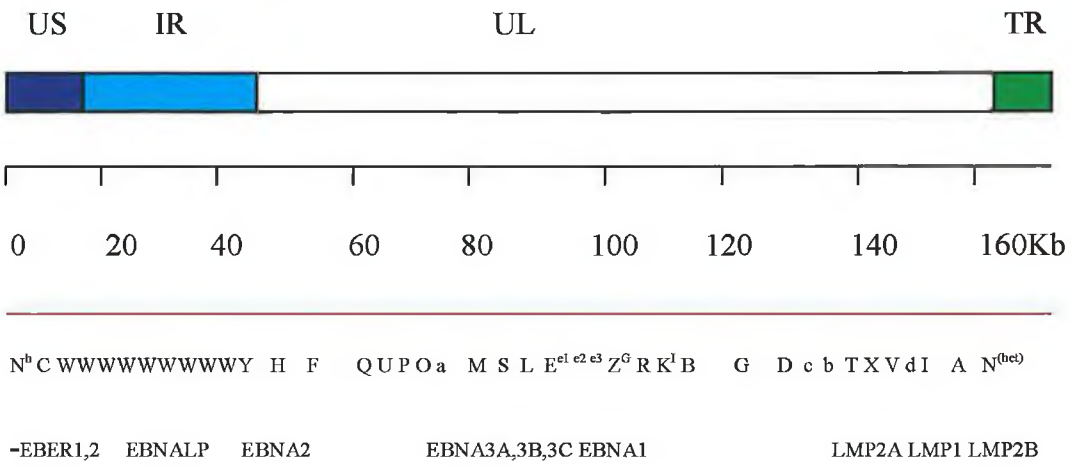


Fig. 1.2. A schematic representation of the EBV genome adapted from IARC Monographs (1997). The genome is divided up into repeat regions, 0.5 Kb terminal direct repeats (TR) and 3.0 Kb internal direct repeats (IR) that divides the genome into short and long largely unique sequence domains (US and UL). The *Bam* H1 fragments are represented by the letters below the red line. The *Bam* H1 fragment location of the EBV latent genes are indicated below the fragments. EBER, EBV encoded RNA; EBNA, EBV nuclear antigen; LMP, latent membrane protein; LP, leader protein.

The major DNA repeat elements serve as landmarks on the EBV genome map, however, serial passage of virus infected cells frequently results in differences in the number of tandem repeat reiterations (Dambaugh *et al.*, 1980; Heller *et al.*, 1981;

Brown *et al.,* 1986; Siaw *et al.,* 1986). Some of these repeats encode proteins and this can explain differences in protein sizes observed on immunoblots and can also serve as an important marker in identifying virus strains, or virus infected cells (Kieff, 1996).

It has been found that some EBV genes expressed during latent and lytic infection have no homology with other herpesvirus genes and may have arisen from cellular DNA. The EBV latent gene BCRF1 is the most striking example of an acquisition from the cell gene pool. BCRF1 is nearly identical to human interleukin 10 (IL10) in primary amino acid sequence (Moore *et al.,* 1993). Two EBV types circulate in most human populations (Gerber *et al.,* 1976; Young *et al.,* 1987; Rowe *et al.,* 1989). These genomes formerly known as type A and type B are now referred to as type-1 and type-2. The genomes are almost identical except for the genes that encode some of the Epstein-Barr nuclear antigens (EBNAs) such as EBNA2, EBNA3A, 3B, 3C and EBNALP, in latently infected cells (Nanoyama and Pagano, 1973; Bornkamm *et al.,* 1980). Apart from these genes the genomes appear to have little differences beyond those which characterise individual EBV strains. The differences in type-1 and type-2 EBV genomes are reflected in type-specific and type-common epitopes for antibodies (Young *et al.,* 1987) and T-cell recognition (Moss *et al.,* 1988). As type-1 EBV is more common in developed societies, most EBV immune human sera from these countries react preferentially or exclusively with type-1 EBNA2, EBNA3A, 3B, 3Cand EBNALP. African sera are almost evenly split in their serological reactivity, However, the recovery of type-2 virus from blood is unusual (Young *et al.,* 1987; Rowe *et al.,* 1989). In culture, EBV type-2 infected lymphocytes grow less efficiently *in vitro* than their type-1 infected counterparts (Rickinson *et al.,* 1987).

1.3 EBV STRATEGY OF INFECTION

Two forms of EBV-cellular infection are recognised, latent and replicative (or lytic). In latent infection, virus penetrates the cell and remains present either as circular episomal DNA (formed through fusion of the terminal repeats) or, less frequently as linear DNA integrated into the host genomic DNA. Episomes, present in low copy numbers in the host cell nucleus, are copied by host cell DNA replicating enzyme and pass to daughter

cells in mitosis (Joske and Knecht, 1993). EBV infection is largely restricted to primate B-lymphocytes *in vitro*. Most human peripheral blood B-lymphocytes are susceptible to EBV infection. The virus does not usually replicate in recently infected B-lymphocytes, instead they become stably latently infected. In latently infected B-lymphocytes, EBV expresses six different nuclear proteins or EBNAs, two integral membrane proteins or LMPs and two small nonpolyadenalated RNAs or EBERs. These viral gene products maintain the latent infection and cause the previously resting B-lymphocytes to continuously proliferate (Mark and Sugden, 1982). The EBV infected proliferating B-lymphocytes are similar to activated B-lymphocytes in their secretion of immunoglobulin and their adherence to each other (Klein, 1987; Zhang *et al.,* 1991). Approximately 1 in every 10^5-10^6 of the B-lymphocytes purified from the peripheral blood of previously infected people are latently infected with EBV. These latently infected B-lymphocytes may be cultured and will proliferate into long-term lymphoblastoid cell lines (LCL) (Sixbey and Pagano, 1985). LCL outgrowth is the simplest means for establishing immortal cell lines from individual humans for chemical, biological and genetic analysis.

Most non-EBV-infected continuous B-lymphocyte cell lines are derived from sporadically occurring EBV-negative Burkitt's lymphomas (BL) and many can be infected with EBV *in vitro* (Calender *et al.,* 1987; 1990). The growth of BL cells *in vitro* is attributed to constitutive *c-myc* expression (Dalla-Favara *et al.,* 1983) and to less characterised changes in chromosome 1 (Berger and Bernheim, 1985). BL cells grow as single cells and do not express activation markers and adhesion proteins associated with B-cell activation such as CD23, CD30, CD70 (Thorley-Lawson and Mann, 1985; Rowe *et al.,* 1985), or the cellular adhesion molecules LFA-1, LFA-3, ICAM-1 and CD44 (Gregory *et al.,* 1988). Usually EBNA1 is the only viral protein expressed (Marchini *et al.,* 1991; 1992a; 1992b). This form of latent infection (type-1 latency) is similar to that in many EBV infected BL cells *in vivo* (Rowe *et al.,* 1987; Sample *et al.,* 1991). In some EBV-positive BL cell lines the same EBV genes are expressed as in latently infected primary B-lymphocytes. The cells then express the same repertoire of B-lymphocyte activation markers and adhesion proteins as EBV-infected primary B-

Lymphocytes, they grow in clumps and to higher densities, this is known, as type-III latency (Calender *et al.,* 1987; 1990).

EBV infection of primary B-Lymphocytes *in vitro* involves binding of CD21 on the B-Lymphocyte plasma membrane. CD21 (also known as CR2) is the receptor for the C3d component of complement. After binding, aggregation of CD21 in the plasma membrane, the co-aggregation of surface immunoglobulins (sIg) and internalization of EBV into cytoplasmic vesicles occurs (Nemerow and Cooper, 1984; Carel *et al.,* 1990). The virus envelope then fuses with the vesicle membrane, releasing the nucleocapsid and tegument into the cytoplasm. Penetration is usually complete within 1-2h. Superinfection of established BL cell lines is somewhat different in that EBV binding does not result in as significant a patching of CD21 and sIg and the envelope fuses with the plasma membrane, releasing the nucleocapsid and tegument into the plasma membrane. The observed differences in mode of infection between primary B-lymphocytes and BL cells are likely to be due to the cytoskeletal abnormalities of the tumour cells (Kieff, 1996). The EBV outer envelope glycoprotein gp350 and gp220 are the CD21 ligand. The interaction of CD21, gp350 and gp220 mediates EBV absorption (Tanner *et al.,* 1987; Nemerow *et al.,* 1987; 1989). Another EBV glycoprotein gp85, has been implicated in the fusion of the EBV envelope with the vesicle membrane. Monoclonal antibodies to gp85 inhibit the fusion of the EBV envelope and the cell membrane (Miller and Fletcher, 1988). Little is known about EBV capsid dissolution, genome transport to the cell nucleus or DNA circularization. By comparing EBV to other DNA viruses that replicate in the nucleus it may be suggested that the cytoskeleton is likely to mediate EBV capsid transport to the nucleus (Dales and Chardonet, 1973). Cell transcription factors probably determine if latent or lytic infection ensues after the genome enters the nucleus and circularizes (Kieff, 1996).

1.4 EBV LATENT INFECTION

The usual outcome of B-lymphocyte infection with EBV is a persistent latent infection. Three forms of latent infection referred to as type-I-III have been demonstrated in EBV carrying B-cell lines and EBV carrying tumour biopsy samples. These distinct forms of

EBV latency which have been characterised are distinguished on the basis of expression of EBV latent genes and promoter usage (Sample *et al.,* 1986; 1991; Rowe *et al.,* 1986; 1987). Type-I latency is characterised by the expression of a single EBV protein EBNA1 (Rowe *et al.,* 1987), together with a high copy number of small non-polyadenylated RNA transcripts known as EBER1 and EBER2 (Rymo, 1979; Howe and Shue, 1989). The classic features of latency I are exhibited in endemic (BL) biopsies and in early passage cell lines derived from these tumours (Rowe *et al.*, 1987).

Cells in latency II resemble latency I cells as they express EBNA1 and the EBER RNAs. They also express LMP1, LMP2A and LMP2B. Two EBV-related clinical conditions exhibit the latency 2 program, nasopharyngeal carcinoma (NPC) and Hodgkin's disease (HD). Explanted BL cells grow continuously in culture and on serial passage some retain the phenotype of the original biopsy (type-I). However, during prolonged culture *in vitro* many BL cell lines show a dramatic phenotypic drift, with increased expression of B-cell activation antigens and adhesion molecules and the appearance in the culture of clumps of more lymphoblastoid-like cells (type-III). As the group-III phenotype cells dominate the culture, they frequently lose expression of CD10 and CD77 (which are BL-associated markers), while other LCL associated markers, such as CD40, intracellular adhesion molecules and Bcl-2 are up-regulated (Rooney *et al.,* 1986; Rowe *et al.,* 1987; Henderson *et al.,* 1991). Type-III cells express all the EBV latent genes and also the expression of cellular genes such as CD23 and a ligand for the EBV receptor CD21 is detected (Wang *et al.,* 1987). Two EBV-associated diseases best exemplify the latency III program, infectious mononucleosis and posttranplantation lymphoproliferative disorder (PTLD) which is a potentially fatal immunoblastic lymphoma in transplant patients.

Table 1.1. Pattern of EBV latent gene expression

Type of latency	Gene Product	Examples	Reference
I	EBERs, EBNA1	Burkitt's lymphoma	Rowe *et al.,* (1987)
		Gastric Carcinoma	Imai *et al.,* (1994)
II	EBERs, EBNA1,	Hodgkin's disease	Deacon *et al.,* (1993)
	LMP1, 2A, 2B,	Nasopharyngeal carcinoma	Hitt *et al.,* (1989)
	BARFO		Brooks *et al.,* (1992)
III	All EBV latent genes	PTLD, IM	Young *et al.,* (1989)
			Tierney *et al.*, (1994)
Other	EBERs, EBNA1, 2	Smooth muscle tumours	Lee *et al.,* (1995)

Table 1.1 was adapted from the IARC monograph (1997), PTLD, Post-transplant lymphoproliferative disorder, IM infectious mononucleosis.

1.5 EBV LATENT GENES

The binding of EBV to CD21 and the subsequent infection of the cell triggers a cascade of events leading ultimately to the immortalization of the cell. The immortalization of primary B cells requires the expression of several viral genes. The first viral proteins to be expressed in B cells upon EBV infection, namely EBNA2 and EBNALP are believed to play critical roles in the early stages of the immortalization process (Alladay *et al.,* 1989; Rooney *et al.,* 1989; Alfieri, *et al.,* 1991). Transcription of nuclear proteins is initiated at RNA polymerase II-dependent promoters in the *Bam* H1 C (Cp) and *Bam* H1 W (Wp) regions of the viral genome (Rogers *et al.,* 1992). EBNA2 and EBNALP are initially transcribed from the very strong promoter Wp, which is present in multiple copies in the major internal repeat. Once immortalization is established, Wp activity declines and transcription of the EBNA genes switches to using the Cp promoter (Woisetschlaeger *et al.,* 1990). All EBNA coding mRNAs are derived from the same transcriptional unit by alternative splicing and alternative polyadenylation (figure 1.3.). The coding exons for most of the EBNAs are towards the 3' end of the mRNAs and are

preceded by the highly spliced leader exons, which are encoded within the major internal repeat of the genome (Farrell, 1995).

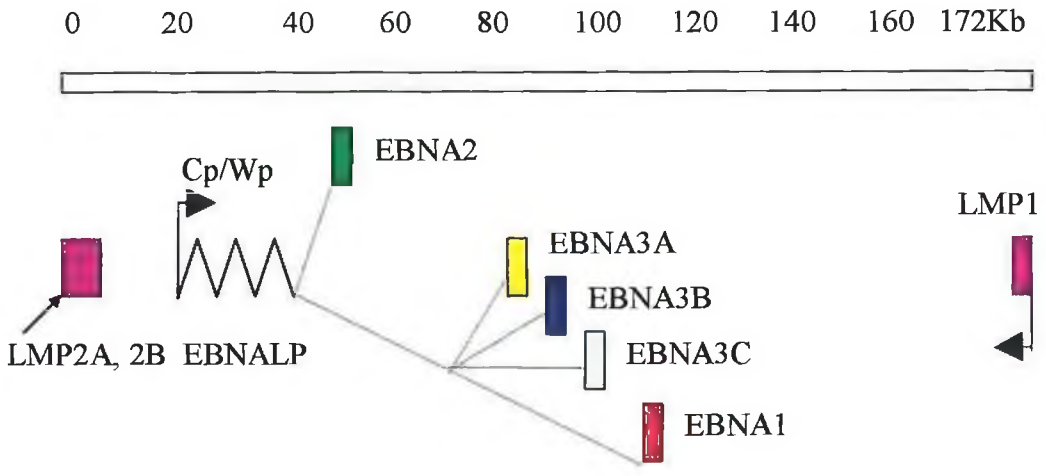


Fig. 1.3. A simplified outline of the splicing of the EBV nuclear antigen coding mRNAs. Transcription initiation is shown to arise from the Cp promoter. The EBNA gene mRNAs all derive from the same transcription unit by alternative splicing and alternative polyadenylation.

1.5.1 EBNA1

EBV nuclear antigen 1 is the only EBV latent gene which is detectable in all EBV infected cells. The 73 kDa protein consists of a short amino-terminal region a 20 kDa - 40 kDa, glycine alanine repetitive sequence flanked by arginine rich sequences and a highly charged acidic carboxy terminal sequence (Hennessy and Kieff, 1983). During latent infection of human host cells, EBV genomes are maintained as double-stranded DNA episomes that replicate once every cell cycle (Adams, 1987, Yates and Guan, 1991). The carboxy terminus of EBNA1 determines its nuclear localisation by interacting with a specific protein that is homogeneously distributed on chromosomes (Harris *et al.*, 1985; Petti *et al.*, 1990). This property is likely to be important for segregation of episomes into progeny nuclei during mitosis. Part of EBNA1 is also associated with the nuclear matrix. EBNA1 is the only EBNA that continues to be made during lytic infection (IARC Monograph, 1997).

EBNA1 binds to two components of the latent cycle origin of replication, *ori P* and it is the only virus encoded trans-acting factor required for episomal maintenance of the EBV genome (Ring, 1994). EBNA1 is a sequence specific DNA binding protein that

binds as a dimer to the sequence TGGATAGCATATGCTATCCA present in *ori P*. The replication origin is composed of 20 tandem repeats of the EBNA1 binding site, spaced about 1 Kb away from the 20 repeats are a further 4 copies of the binding site, 2 in dyad symmetry and two in tandem. The dyad symmetry component is stringently required for episome replication. The interaction of EBNA1 with the tandem repeats and dyad symmetry sites is co-operative and results in high-order structures that lead to bending of the DNA, distortion of the duplex and looping out of the intervening sequences (Frappier and O'Donnell, 1991; Orlowski and Miller, 1991; Frappier and O'Donnell, 1992). Regions of the protein important for DNA binding and transactivation of *ori P* are located in the carboxy-terminal third of the protein (Ambinder *et al.*, 1991). The functional domains of EBNA1 are outlined in fig. 1.4. Furthermore, *ori P* acts as an EBNA1 dependent enhancer and plays a crucial role in the regulation of viral transcription from both the C and the LMP1 promoter in growth-transformed cells (Sugden and Warren, 1989; Gahn and Sugden 1995).

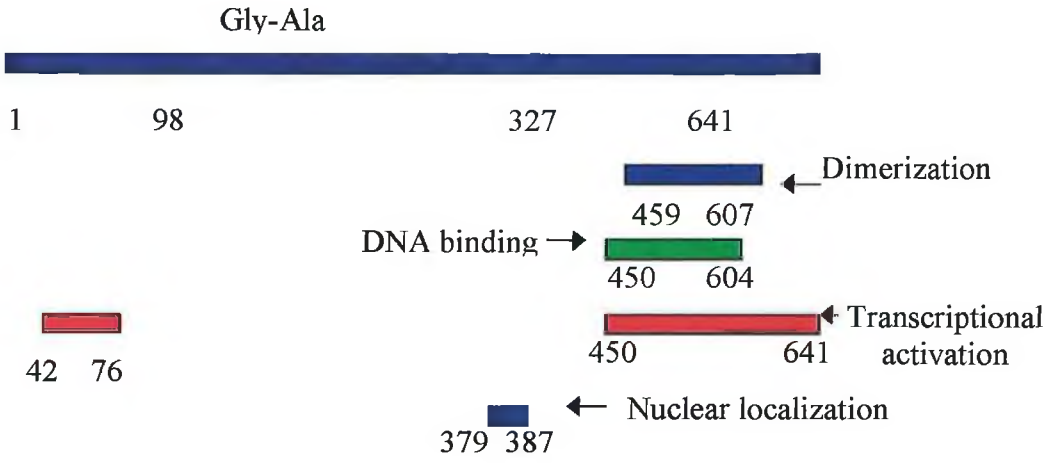


Fig 1.4. Functional domains of EBV nuclear antigen 1 (EBNA1). The Gly-Ala box is a repetitive region composed entirely of glycine and alanine, it varies in length between viral strains. Adapted from Farrell, 1995.

The promoter from which the EBNA1 gene is transcribed differs between cell types. In EBV transformed LCLs all the EBNA genes are derived from a highly spliced transcript that is generated by transcription from the C or W promoters located on the *Bam* H1 C and W fragments respectively (Middelton *et al.*, 1991). A recent study has indicated that RNA transcripts from latently infected early passage type-I BL cells, in the absence of a stimulus to induce virus replication, is initiated from a promoter distinct from Fp

located in the adjacent *Bam* H I-Q fragment (Nonkwelo *et al.,* 1995). This promoter is designated Qp. Qp does not contain a recognisable TATA box, which is consistent with multiple sites of transcription initiation from Qp (Nonkwelo *et al.,* 1996). Additionally, EBNA1 can negatively autoregulate expression within receptor plasmids containing both Fp and Qp through two binding sites downstream of the 3'-most Qp start site (Sample *et al.,* 1992; Snug *et al.,* 1994). Following the switch from latent to lytic infection EBNA1 transcription is controlled by the Fp upstream of Q (Lear *et al.,* 1992).

EBNA1 can bind RNA *in vitro* through arginine/glycine motifs (Snudden *et al.,* 1994). EBNA1 also activates expression of the lymphoid recombinase genes (RAGs) through an as yet unidentified mechanism (Srinivas and Sixbey, 1995). Activation of the RAGs could promote chromosomal rearrangements and translocations and possibly also facilitate viral integration. This may indicate that EBNA1 can activate expression of critical cellular genes and affect cellular growth control. Expression of EBNA1 in EBV negative cell lines has no obvious effect upon cellular growth characteristics. However, the expression of EBNA1 in the B cells of transgenic mice has been shown to be associated with the development of lymphocytic lymphoma and leukaemia suggesting that EBNA1 predisposes the mouse lymphocytes to oncogenic change (Wilson and Levine, 1992).

1.5.2 EBNA2

The EBV nuclear antigen 2 (EBNA2) is one of the first EBV encoded proteins expressed after primary infection of B lymphocytes. EBNA2 differs extensively between type-1 and type-2 EBV isolates (Aitken *et al.,* 1994) and is the primary determinant of the biological difference that enables the type-1 strains to transform B lymphocytes with greater efficiency (Rickinson *et al.,* 1987; Platt *et al.,* 1993). In nuclear fractions EBNA2 is associated with nucleoplasmic chromatin and nuclear matrix fraction (Platt *et al.,* 1993). EBNA2 is an 83 kDa protein containing a polyproline region, a glycine arginine repeat and a highly acidic carboxy terminus (Dambaugh *et al.,* 1984). Like EBNALP and EBNA1, EBNA2 is phosphorylated on serine and threonine residues. EBNA2 undergoes significant post-translational

modification other than phosphorlyation because the size of the nascent protein is smaller than that of the stable intranuclear EBNA2 (Kieff, 1996). EBNA2 is a *trans*-activator of viral and cellular gene expression. A set of activation molecules including the EBV receptor CD21 and the B-cell activation antigen CD23 which has been implicated as a growth and survival factor for B cells (Wang *et al.,* 1987, 1990a), are turned on by infecting EBV-negative BL cell lines with an immortalizing strain of the virus such as B95-8. When infected with a viral strain deleted for EBNA2 (P3HR-1) up-regulation of these genes was not observed. By stably transfecting EBNA2 into a P3HR-1 expressing BL, increased expression of CD21 and CD23 was observed, indicating a direct role for EBNA2 in the modulation of cellular genes implicated in B-cell proliferation (Wang *et al.,* 1987, 1990a). Other genes trans-activated by EBNA2 are the *c-fgr* oncogene (Knutson, 1990) and viral genes including LMP1 (Abbot *et al.,* 1990; Ghosh and Kieff, 1990; Wang *et al.,* 1990b; Tsang *et al.,* 1991; Fahraeus *et al.,* 1993) and LMP2 (Tsang *et al.,* 1991) also the *cis*-acting element upstream of the Cp promoter (Walls and Perricaudet, 1991; Sjoblom *et al.,* 1995).

Three regions have been located which appear to be stringently required for transformation and the *trans*-activating activity of EBNA2, between amino acid residues 95-110, 280-337 and 425-462. The role of the 95-110 region is unclear, the 425-462 region is essential due to its acidic *trans*-activating characteristic and the 280-337 region mediates the interaction with DNA sequence specific binding proteins that bring EBNA2 to its responsive elements as EBNA2 does not interact directly with DNA (IARC Monograph, 1997). All the promoters activated by EBNA2 share the core sequence GTGGGAA (Waltzer *et al.,* 1994). Through studies of its responsive elements a 28 amino acid polypeptide corresponding to residues 310-336 of EBNA2 (see figure 1.5) was used to purify a nuclear protein p63. The sequence of p63 revealed it to be the previously characterised recombination signal sequence binding protein RBP-Jκ, a widely expressed and highly conserved protein that probably acts as a key adapter for transcription regulatory factors of cellular genes. EBNA2 interacts with RBP-Jκ and binds DNA in this way (Grossman *et al.,* 1994; Henkel *et al.,* 1994).

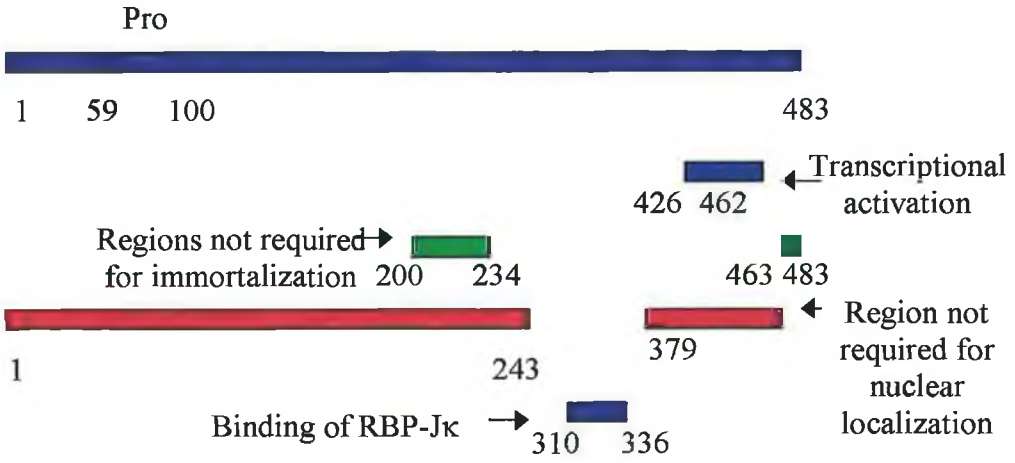


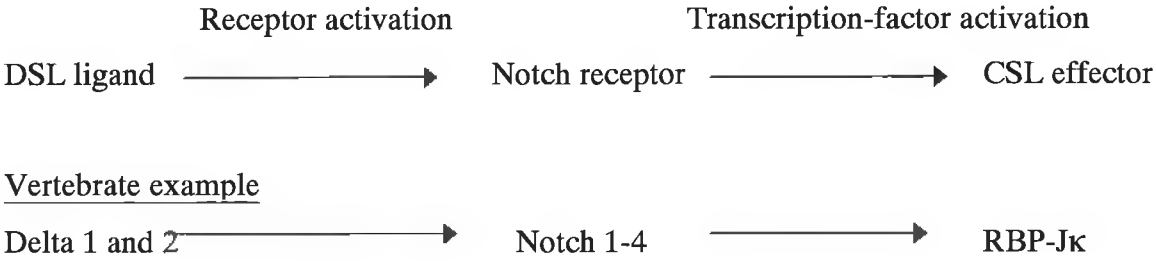
Fig 1.5. Functional domains of EBV nuclear antigen 2 (EBNA2). The pro box is a region composed entirely of proline, it varies in length between viral strains. Adapted from Farrell 1995.

The genetics of *Drosophila* provides a clue to the physiological role of RBP-Jκ. RBP-Jκ is a DNA binding protein and the down-stream target of a cellular receptor called Notch (Fortini and Artavanis-Tsakonas, 1994), which in mammals is part of a family of related proteins. The Notch gene of *Drosophila megalanogaster* encodes a 300 kDa transmembrane receptor with a large extracellular domain and 36 tandem epidermal growth factor (EGF)-like repeats as well as an intracellular domain with 6 tandem Ankyrin repeats and a PEST sequence (Artavanis-Tsakonas *et al.*, 1995). Notch participation in local intracellular communication was first appreciated in studies of embryonic neurogenesis in *Drosophila*. The Lin-12 and Glp-1 proteins of *Caenorhabditis elegans* are both structurally similar to Notch, although both possess fewer EGF-like repeats in their extracellular domains than Notch. These proteins have also been extensively studied in order to delineate the role of Notch (Artavanis-Tsakonas *et al.*, 1995). Within the past few years, several homologs of the *Drosophila* Notch proteins have been identified in vertebrates including humans. The vertebrate Notch genes are expressed throughout developing tissues at the embryonic stages and in the proliferative layer of mature tissue, the expression of vertebrate Notch is thought to play a role in the cell-fate specification in many developmental contexts (Artavanis-Tsakonas *et al.*, 1995). Antibodies to two of the four known human Notch proteins have been used to examine Notch protein levels in human tissue samples including

certain cancerous tissues (Zagouras *et al.,* 1995). In metaplastic cervical tissues as well as in certain cancerous lesions, Notch proteins are detected at elevated levels relative to the surrounding tissue (Zagouras *et al.,* 1995). This data supports the theory that Notch signalling activity is correlated with the differentiation state of these human tissues. Genetic and molecular studies have identified a family of structurally related ligands for the *Drosophila* Notch receptor and the *C. elegans* Lin-12 Gly-1 receptor proteins. These ligands encoded by the Delta and Serrate genes in *Drosophila* are all membrane-anchored extracellular proteins. The extracellular part of all putative ligands contain varying numbers of EGF-like repeats and a second cysteine-rich conserved motif referred to as the DSL region (Tax *et al.,* 1994). Further studies have identified further putative Notch ligands in humans. These vertebrate molecules have overall structures similar to the Delta and Serrate and all have extracellular regions with EGF-like repeats and cysteine rich DSL motifs. This DSL region is important in ligand function as shown by mutation analysis (Artavanis-Tsakonas *et al.,* 1995). A protein that interacts directly with the intracellular Ankyrin repeat region of Notch in *Drosophila* is the product of the Suppression of Hairless [Su(H)] locus, which has been strongly implicated in playing a central role in Notch signalling. The *Drosophila* Su(H) protein is highly related to RBP-Jκ (Fortini and Artavanis-Tsakonas, 1994).

Notch 1 is a human Notch first identified at the breakpoint of a recurrent chromosomal translocation associated with a subset of human T-cell acute lymphoblastic leukaemia/lymphomas (T-ALL). RNA transcripts from the normal Notch 1 gene are found in most cells but are present at highest levels in the developing thymus and brain (Aster *et al.,* 1997). A truncated form of Notch 1 has been identified in human T-ALLs and its oncogenicity has been confirmed. Notch has been shown to interact physically and functionally with RBP-Jκ and with components of the NF-κB signalling pathway and some Notch phenotypes in the fly appear to be independent of the Su(H). Studies conducted in T-ALLs indicate that the oncogenic capacity of truncated Notch 1 may be mediated at least in part by RBP-Jκ. As EBNA2, which is required for B-cell transformation, binds to and alters RBP-Jκ it may be that RBP-Jκ also has an important role in T-cell transformation (Aster *et al.,* 1997).

It has been proposed that Notch signalling is achieved by a DSL ligand activating the Notch receptor, which in turn, activates the CSL transcription factors (CSL = CBF1, Su(H), LAG-1). In addition feedback loops within the pathway reinforce and amplify the signalling.



A biochemical understanding of Notch signalling, however, is only just emerging and many details remain poorly understood (Kimble *et al.,* 1998).

Ligand binding activates Notch and subsequent processing and transport of Notch to the nucleus converts the repressor RBP-Jκ (Hsieh and Hayward, 1995; Waltzer *et al.,* 1995), into an activator and turns on target genes (Jarriault *et al.,* 1995; Hsieh *et al.,* 1996). EBNA2 and activated Notch interact with similar regions in the RBP-Jκ molecule (Hsieh *et al.,* 1996). Mutational analysis of an 80 bp EBNA2 responsive *cis*-element within the viral LMP promoter region identified two sequence elements involved in *trans* activation by EBNA2. Along with RBP-Jκ, a consensus binding site for Spi-1 (PU.1), a member of the Ets family of transcription factors, was identified which is also essential for transactivation of LMP1 by EBNA2 (Laux *et al.,* 1994; Johansenn *et al.,* 1995).

A consistent feature of BL cells is the transcriptional activation of the proto-oncogene c-*myc* by chromosomal translocation (Bornkamm *et al.,* 1988; Spencer and Groudine, 1991). The most frequent translocation t(8;14) fuses the c-*myc* gene locus on chromosome 8 to the constant region of the Ig heavy chain gene locus on chromosome 14. Since BL cells are thought to proliferate through activation of the c-*myc* gene the growth promoting function of EBNA2 may not be required in the setting of BL. A novel function of EBNA2 has been described using an oestrogen responsive system whereby the expression of EBNA2 is controlled by the presence or absence of oestrogen

(Jochner *et al.,* 1996). EBNA2 down-regulates surface IgM expression and transcription of the Ig-μ locus very efficiently. In BL cell lines with the t(8;14) translocation, down-regulation of Ig-μ is associated with concomitant transcriptional shut-off the c-*myc* gene, reflecting the fact that c-*myc* is under the control of Ig heavy chain locus in these cells. The function of EBNA2 as a negative regulator of Ig-μ provides an explanation for the growth inhibiting effect of EBNA2 in cells carrying a t(8;14) translocation (Jochner *et al.,* 1996). The down regulation of IgM expression by EBNA2 may also provide an explanation for the long standing observation that EBV negative BL cells in culture tend to have higher levels of Ig expression than their EBV positive counterparts (Benjamin *et al.,* 1982; Cohen *et al.,* 1987; Magrath *et al.,* 1990). Ig-μ and c-*myc* are down-regulated by EBNA2 at the transcriptional level and the transcription of Ig-μ and c-*myc* are affected by EBNA2 simultaneously. This suggests that EBNA2 is mediating its effect on expression through a common target, presumably a transcription factor (Jochner *et al.,* 1996).

A recent study using an LCL conditional for functional EBNA2 expression on the presence of oestrogen, demonstrated that the transcription of the proto-oncogene c-*myc* is activated by EBNA2. The precise mechanism of this transcription activation remains to be elucidated (Kaiser *et al.,* 1999). It was also concluded that in contrast to c-*myc* and LMP1, neither cyclin D2 nor cdk4 is a direct EBNA2 target. Cyclin D2 and cdk4 are both elements of the basic cell cycle machinery and drive cell cycle progression in early G_1. Since it has been shown that different B cell activation protocols can induce cyclin D2 and cdk4, it may be that the induction by proliferation of EBNA2 is a secondary event potentially driven by the primary viral and cellular EBNA2 targets (Kaiser *et al.,* 1999).

1.5.3 EBNALP

EBNA leader protein is so designated because it is encoded by the 5' leader sequence of bicistronic mRNAs specifying the other EBNAs. The translation initiation codon for EBNALP is created by a splicing event that occurs near the 5' end of the message (Sample *et al.,* 1986; Speck *et al.,* 1986; Rogers *et al.,* 1990). The EBNALP protein is

composed of amino-terminal repetitive segments followed by a unique carboxy-terminal sequence. EBNALP is frequently observed as a ladder of proteins in gel electrophoresis which differ in the number of amino-terminal repeats due to the fact that the repetitive part of the protein is derived from exons in the major internal repeat of the virus, which varies in copy number in any EBV population (Hammerschmidt and Sugden, 1989). The protein is strongly associated with the nuclear matrix, immunofluorescence microscopy illustrates its unusual location in the nucleus, a proportion is diffusely spread through the nucleus while the rest is concentrated in a few granules frequently distributed in curved linear arrays (Petti *et al.,* 1990; Jiang *et al.,* 1991). Association of EBNALP with pRb and p53 has been suggested based on *in vitro* biochemical interaction and colocalization of EBNALP with pRb as detected with one antibody and not another, the *in vivo* relevance of this is unknown (Jiang *et al.,* 1991; Szekely *et al.,* 1993). Transient transfection of EBNALP and EBNA2 into primary B-lymphocytes co-stimulated with gp350 indicated that the two proteins co-operate in the induction of G_0 to G_1 transition as marked by induction of cyclin D_2, however, the mechanism of action remains unclear (Sinclair *et al.,* 1994).

1.5.4 LMP1

Latent membrane protein 1 (LMP1) mRNA is the second most abundant viral transcript in latently infected cells (Fennewald *et al.,* 1984; Sample and Kieff, 1990). The product, encoded by three exons, is an integral membrane protein with at least 3 domains: a 20 amino acid hydrophilic amino terminus; a six amino acid hydrophobic domain and a 20 amino acid, alpha helicase transmembrane segments separated by five reverse turns, each five of 10 amino acids in length and a 200 amino acid carboxy terminus, rich in acidic residues (see figure. 1.6). LMP1 migrates on SDS-PAGE between 58 and 63 kDa depending on the EBV strain. Both the amino and the carboxy terminal sequences are on the cytoplasmic surface of the plasma membrane connected by the six transmembrane domains. This results in the exposure of three reverse turn loops on the external surface of the cell (Liebowitz *et al.,* 1986; Thorley-Lawson and Israensohn, 1987). LMP1 forms patches in the cell membrane, to which the intermediate filament protein vimentin colocalizes, however, patch formation does not

require vimentin or other EBV proteins (Liebowitz *et al.,* 1986; Moorthy and Thorley-Lawson, 1990, 1993a). LMP1 is the only known EBV gene which acting alone can transform rodent fibroblasts (Wang *et al.,* 1985). LMP1-expressing rodent fibroblasts, grown in low concentrations of serum, acquire anchorage-independent growth in soft agar and become tumorgenic (Wang *et al.,* 1985). LMP1 is highly toxic if expressed at high levels (Hammerschmidt and Sugden, 1989). At least half of the LMP1 protein expressed is associated with the cytoskeleton as defined by resistance to extraction with non-ionic detergents and co-localization with vimentin. Nascent, non-ionic detergent-soluble LMP1 has a half-life of less than two hours and is converted to an insoluble, cytoskeleton-associated form, with a half-life of 3-15h, that is phosphorlated on serine and threonine residues in the carboxy terminal domain (Moorthy and Thorley-Lawson, 1990, 1993a). LMP1 is transcribed during lytic infection and full size LMP1 is incorporated into virions, indicating that virion-associated products may affect the growth of newly infected cells (Mann *et al.,* 1985).

LMP1 also alters the growth of EBV-negative BL lymphoblasts when expressed stably or transiently at the appropriate level in such cells after gene transfer. In fact, LMP1 induces many of the changes usually associated with EBV infection of primary B-lymphocytes or with antigen activation of primary B-lymphocytes, including cell clumping, increased villous projections, increased vimentin expression, increased cell surface expression of CD23, CD39, CD40, CD44 and class II major histocompatability complex (MHC), decreased expression of CD10 and increased expression of the cell adhesion molecules LFA-1, ICAM-1 and LFA-3 (Wang *et al.,* 1988b; Birkenbach *et al.,* 1989; Wang *et al.,* 1990a; Liebowitz *et al.,* 1992; Peng and Lundgren, 1992; Zhang *et al.,* 1994a; Kieff, 1996). It has been suggested that LMP1 may contribute to malignant disease by mediating upregulation of the adhesion molecule CD44 which may be an important factor determining the progression and dissemination of EBV-associated tumours *in vivo* (Sy *et al.,* 1991; Walter *et al.,* 1995). LMP1 was shown to increase certain cytokines with B-cell promoting activity, such as IL-10 (Nakagomi *et al.,* 1994). It has also been shown to protect B-lymphocytes from apoptosis by inducing expression of the anti-apoptotic proteins Bcl-2 (Rowe *et al.,* 1994) and also that of A20 (Henderson *et al.,* 1991; Martin *et al.,* 1993; Fries *et al.,* 1996).

Transfection studies with LMP1-deletion mutants indicate that the cytoplasmic amino terminus is not responsible for the activating effects, the transmembrane domains are critical, probably due to their importance for LMP1 aggregation in the plasma membrane and the carboxy-terminal domain is essential (Wang *et al.,* 1988a,b; Martin and Sugden, 1991; Moorthy and Thorley-Lawson, 1993a,b). As expected EBV recombinants lacking LMP1 were unable to induce growth transformation of primary B cells (Kaye *et al.,* 1993). A recent study described the effect of LMP1 on EBV negative B cell growth using a tetracycline regulated cell line in which the expression of LMP1 is inducible. Results demonstrated that induced expression of LMP1 had a cytostatic effect upon B-cell lines due to an accumulation of cells at the G2/M phase of the cell cycle (Floettmann *et al.,* 1996).

The discovery that LMP1 efficiently transactivates the expression of the human immunodificiency virus type-1 long terminal repeat (HIV-1 LTR) by induction of NFκB activity, was evidence that at least part of LMP1 transcriptional activating effects are mediated by NFκB activation (Hammarskjold and Simurda, 1992). It was then shown that LMP1 engages signalling proteins such as tumour necrosis receptor associated factors (TRAFs) 1, 2 and 3. This provided further evidence for the role of TRAFs in signalling and linked LMP1-mediated transformation to signal transduction from the TNFR family (Devergne *et al.,* 1998). Constitutive LMP1 expression in EBV-transformed cells carrying a conditional EBNA-2 gene has provided evidence that LMP1 in the absence of functional EBNA2 promotes survival of the cells without maintaining proliferation, similar to the stimulation of the endogenous CD40 receptor, (CD40 is a member of the TNFR family), by CD40 ligand which is even more effective in promoting cell survival than LMP1 (Zimber-Stroll *et al.,* 1996). The functional similarity between LMP1 and CD40 is corroborated by the fact that the two proteins recruit TRAFs which mediate activation of NF-κB (Mosialos *et al.,* 1995). LMP1 mediates NFκB activation via two independent domains located in its carboxy terminal cytoplasmic tail, a TRAF interacting site that associates with TRAF 1, 2, 3 and 5 through a core motif PXQXT/S and a TNFR associated death domain (TRADD)-interacting site (see fig. 1.6), (Devergne *et al.,* 1998). Deletion of the TRAF interaction

domain within the LMP1 molecule abolishes B-cell transformation by EBV (Izami *et al.*, 1997). LMP1 aggregation at the plasma membrane is essential for signalling and for B-lymphocyte growth transformation (Floettmann and Rowe, 1997; Gires *et al.*, 1997). LMP1 constitutively signals because the six transmembrane domains enable ligand-independent continuous aggregation in the plasma membrane. As a consequence of LMP1 aggregation, TRAFs and TRADD constitutively associate with the LMP1 carboxy-terminal cytoplasm domain (CTD) (Devergne *et al.*, 1996; Izumi and Kieff, 1997). The anti-apoptotic gene A20 is upregulated by LMP1 and this up regulation of the A20 promoter is mediated through an NFκB-binding element upstream of the A20 promoter (Laherty *et al.*, 1992).

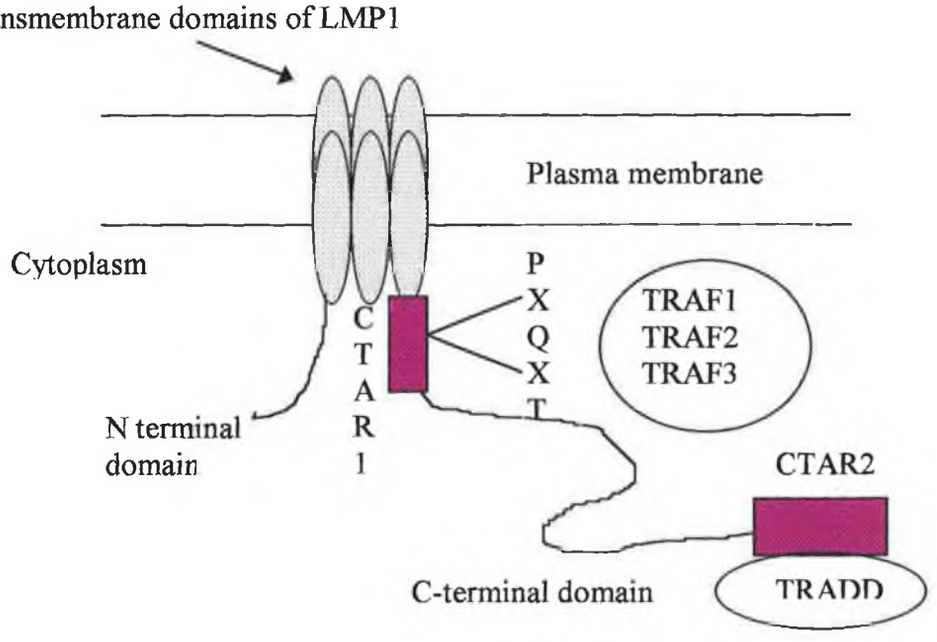


Fig. 1.6. Schematic representation of LMP1 adapted from Kieff, 1996. LMP1 consists of a N terminal cytoplasmic domain, six hydrophobic transmembrane domains separated by reverse turns and a 200 amino acid C-terminal domain (CTD). The two signalling domains in the CTD are represented by purple boxes.

While an important function of the amino terminal cytoplasmic amino acids (aa) appears to be in orientating the transmembrane domains (Izumi *et al.*, 1994), two regions of 200 aa CTD are critical for NF-κB activation and for B-lymphocyte growth

transformation. The region consisting of the membrane-proximal 45 residues of the cytoplasmic tail mediates less than 30 % of LMP1-induced NF-κB activation and has the functional designation CTAR1 (Carboxy-terminal activation region 1, see figure 1.6) (Mitchell and Sugden, 1995; Huen *et al.,* 1995). This domain is both necessary and sufficient for initial B-lymphocyte transformation. CTAR1 mediates the association of LMP1 with TRAFs (Devergne *et al.,* 1996). It has been suggested that TRAF2 or TRAF1/2 heterodimers mediate NFκB activation by CTAR1 while TRAF3 may act as a negative modulator by displacing TRAF1 and TRAF2 from LMP1 (Devergne *et al.,* 1996). The distal region of LMP1 CTD, encompassing aa 352-386 designated CTAR2, is the major NF-κB-inducing domain and mediates the association of LMP1 with TRADD (Izumi and Kieff, 1997; Devergne *et al.,* 1998;).

Studies have indicated that activation of the TNFR family member CD40 in conjunction with IL-4 stimulation can mimic EBV-induced immortalization of human lymphocytes in short-term culture (Saeland *et al.,* 1993; Galibert *et al.,* 1994). Expression of LMP1 in B-lymphocyte cells induces many of the phenotypic changes characteristic of EBV immortalization, suggesting parallel roles for the cellular CD40 protein and the viral LMP1 protein in signal transduction pathways. The expression of CD40 and LMP1 on malignant epithelial cells suggests that signals from these proteins may be involved in not only B-cell activation but also progression to malignancy in cells of epithelial origin such as NPC (Miller *et al.,* 1997). LMP1 has also been shown to induce expression of the epidermal growth factor receptor (EGFR) and A20 in human epithelial cells (Miller *et al.,* 1995). Upon stimulation with EGF, these cells demonstrated enhanced tyrosine phosphorylation of down stream targets of the EGFR and exhibit enhanced growth in serum free media. Induction of molecules such as EGFR and A20 in epithelial cells may be important from LMP1-mediated effects on cell growth and differentiation in established epithelial cell lines, as well as for EBV-infected epithelial malignancies such as NPC (Miller *et al.,* 1995).

It has been shown that overexpression of TRAF3 in a B-cell line expressing LMP1, displaces TRAF1 and TRAF2 from the membrane associated signalling complex, suggesting a negative regulator role for TRAF3 which is important in NF-κB activation.

LMP1 may modulate TRAF signal transduction events by binding negative regulators of the TRAF pathway such as TRAF3 (Devergne *et al.,* 1996). The relative levels of LMP1 and TRAF3 are apparently critical for the induction of the EGFR. These and other findings support the following model: In the absence of LMP1 or activated CD40, TRAF signalling molecules are present but are unable to activate signalling pathways resulting in A20 or EGFR expression. Expression of LMP1 in C33A epithelial cells then engages three distinct signalling pathways. The TRAF interaction domain in LMP1 activates the transcription factor NF-κB resulting in the induction of genes including the NF-κB regulated A20 gene. The TRAF interaction domain also induces expression of the EGFR through the activation of a pathway distinct from NF-κB activation alone. The distal LMP1 domain initiates the third signalling event. The distal domain which does not interact with the TRAF molecules, activates NF-κB, resulting in the induction of A20 expression, but has no effect on EGFR expression. The presence of two domains in LMP1 that can activate NF-κB through TRAF-dependent and TRAF-independent pathways suggests that NF-κB activation is carefully regulated during viral transformation (Miller *et al.,* 1997). The EGFR and other genes regulated by activation of the TRAF pathway are likely to mediate the oncogenic effects of LMP1 and CD40 expression in epithelial cells (Miller *et al.,* 1997).

1.5.5 LMP2A AND 2B

The genes encoding LMP2A and 2B have also been named terminal protein 1 and terminal protein 2 (TP1, TP2), because they are transcribed across the terminal repeat sequences of the linear viral genome of which are fused together upon infection to generate the intracellular episomal form of the viral genome (Laux *et al.,* 1988; Sample *et al.,* 1989). The two messages consist of different 5' exons and eight common exons and are predicted to encode nearly identical proteins differing only in the length of their hydrophilic amino termini (Ring, 1994). Transcription of the LMP2A starts 3Kb downstream of the LMP1 transcription start site (Laux *et al.,* 1988a; Sample *et al.,* 1989). The LMP2B and LMP1 promoters form bi-directional transcription units containing a common EBNA2 responsive element, while a separate EBNA2 response element regulates LMP2A transcription (Zimber-Strobl, *et al.,* 1993). LMP2A and 2B

are 54 and 40 kDa in size respectively. Both proteins are predicted to encode twelve highly hydrophobic membrane spanning domains and are localized to patches in the plasma membrane of infected cells, in close association with LMP1 (Longnecker and Kieff, 1990). LMP2A and 2B are not required for EBV mediated immortalization but they do improve efficiency of transformation (Longnecker *et al.,* 1992; Brielmeir *et al.,* 1996). The LMP2 proteins are phosphorylated on serine, threonine and tyrosine residues (Longnecker *et al.,* 1991) and have been shown to interact with *src*-family tyrosine kinases in EBV-infected B cells (Burkhardt *et al.,* 1992) They have also been associated with another stably phosphorylated tyrosine kinase *sky.* These interactions suggest that the LMP2s play a role in transmembrane signal transduction (Kieff, 1996). LMP2A has been shown to inhibit anti-immunoglobulin-mediated Ca^{2+} mobilization, PKCγ2 activation and anti-immunoglobulin-induced reactivation of the lytic cycle, which can be bypassed by TPA with Ca^{2+} ionophores (Miller *et al.,* 1994b, 1995a). These data are consistent with a model in which LMP2A sequesters the receptor associated tyrosine kinase, blocking its autophosphorylation and downstream signalling events (Miller *et al.,* 1995a).

1.5.6 EBNA3A, 3B, 3C.

EBNA3A, 3B and 3C are encoded by 3 genes located in tandem in the EBV genome (see fig. 1.3.). Each is encoded by a short and long exon (Hennessy *et al.,* 1985, 1986; Kallin *et al.,* 1986; Petti and Kieff, 1988; Ricksten *et al.,* 1988). The mRNAs that encode these proteins are the least abundant EBNA mRNAs, with only a few molecules occurring in each latently infected cell. The proteins are located in large nuclear clumps in the nuclear matrix, chromatin and nucleoplasmid fractions but not in the nucleolus (Petti *et al.,* 1990). Type-1 and 2 strains of EBV exhibit 84%, 80% and 72% amino acid identity in their EBNA3A, 3B and 3C sequences respectively (Sample *et al.,* 1990). There is increasing evidence that EBNA3C, like EBNA2, functions as a *trans*-activator of both cellular and viral genes. Transfection of an EBNA3C expression construct into an EBV negative BL cell line has been shown to result in the upregulation of the EBV receptor CD21 (Wang *et al.,* 1990). Furthermore, expression of EBNA3C in the Raji cell line (in which the EBV genome is deleted for most of the EBNA3C open reading

frame) induces an up-regulation of LMP1 and the cellular proteins CD23 and vimentin (Allday *et al.,* 1993; Ring, 1994). EBNA3A and 3C are required for transformation whereas EBNA3B is not (Tomkins and Kieff, 1992a; Tomkins *et al.,* 1993). EBNA3A, 3B and 3C proteins have been shown to inhibit the transcriptional activation of EBNA2 responsive promoters (Le Roux *et al.,* 1994) by destabilizing RBP-Jκ and EBNA2/RBP-Jκ complexes from binding to their cognate RBP-Jκ binding sites (see figure 1.7). EBNA3 proteins are thus believed to counter balance and finely tune the action of EBNA2 (Waltzer *et al.,* 1996; Robertson *et al.,* 1996; Zhao *et al.,* 1996).

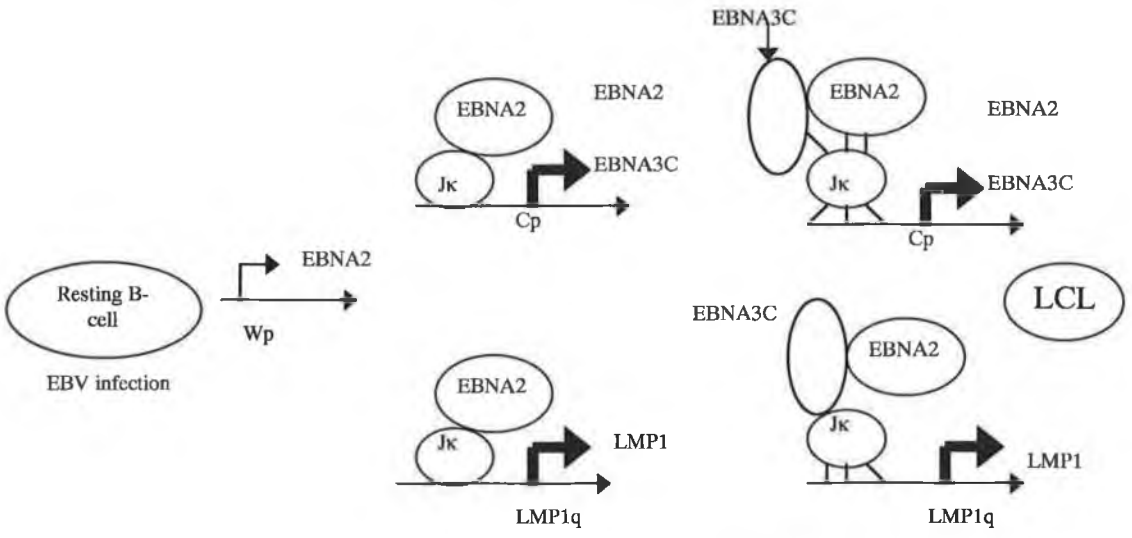


Fig 1.7. A schematic model of the mechanism by which EBNA3C counteracts EBNA2 mediated activation, by destabilizing the interaction of RBP-Jκ and RBP-Jκ/EBNA2 complexes binding DNA. Adapted from Roberston *et al.,* 1995.

EBNA2 can also be recruited to promoters through interaction with other factors such as the proteins from the PU.1 family thus inhibitors of EBNA2-mediated transcriptional activation by the EBNA3 proteins could thus be restricted to promoters activated through RBP-Jκ binding sites. This could be a way to differentially regulate certain viral or cellular genes (Waltzer *et al.,* 1996).

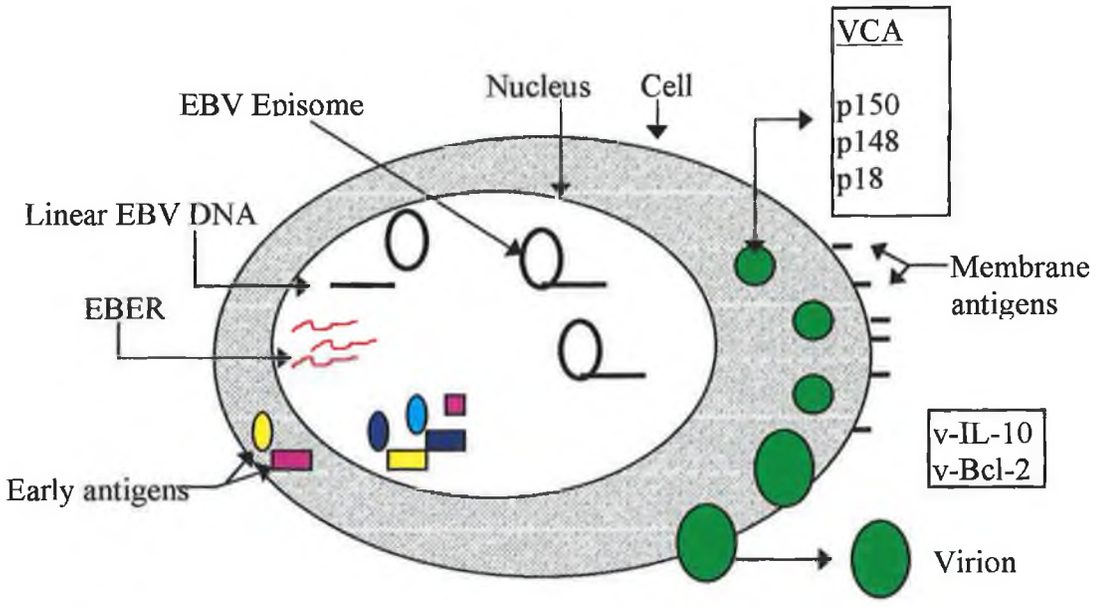
1.5.7 EBV ENCODED RNAS (EBERS)

The two EBV-encoded, small nonpolyadenylated RNAs, EBER1 and EBER2, are by far the most abundant EBV RNAs in latently infected cells, with an estimated abundance of 10^7 copies per cell. They are usually transcribed by RNA polymerase III although polymerase II may also be involved. Most EBERs are located in the nucleus and are associated at the 3' terminus with the cellular La antigen (Howe and Steiz, 1986; Howe and Shu; 1989). EBER 1 and 2 have extensive sequence similarity to adenovirus VA1 and VA2 and cell U6 small RNAs, both of which form similar secondary structures and complex with La protein (Rosa *et al.,* 1981; Glickman *et al.,* 1988). The role of the EBERs is unclear but based on the functions of VA and U6 RNAs two alternative roles have been proposed for the EBERs. In adenovirus infection VA1 RNA acts in the cytoplasm to directly inhibit activation of an interferon-induced protein kinase, which blocks transcription by phosphorylating the protein-synthesis initiator factor eIF-2α. EBER1 and 2 can partially complement the replication of an adenovirus with null mutations in VAI and VAII, but their effect on eIF-2α kinase activity is significantly less and they are not found in the cytoplasm (Kieff, 1996). Both of the proposed functions of EBERs are somewhat incompatible with the observation that their expression is delayed until after EBNA and LMP gene expression and initiation of DNA synthesis (Alfieri *et al.,* 1991). Nevertheless, the earlier events in primary B-cell infection are sensitive to interferon (IF) and EBERs may play a role in blocking eIF-2 kinase (Thorley-lawson, 1980; 1981).

1.6 GENES OF THE LYTIC VIRAL CYCLE

Only a small fraction of latently infected B-lymphocytes spontaneously enter the productive cycle, in these the viral DNA is amplified several hundred fold by a lytic origin of DNA replication, *ori Lyt* (Hammerschmidt and Sugden, 1988). Thus lytic infection is usually induced by chemicals (Luka *et al.,* 1979; Saemundsen *et al.,* 1980; Laux *et al.,* 1988b). Phorbol esters are among the most reproducible and most broadly applicable inducers, their effect is probably mediated by protein kinase C activation of

Jun-fos interactions with AP-1 upstream of the immediate early virus genes (Farell *et al.,* 1983; 1989; Farell 1992; Laux *et al.,* 1988). The Akata cell line which carries an LMP2A-deleted virus can be induced by cross-linking of surface immunoglobulins (sIg) to the extent that more than 50% of the cells enter the lytic cycle (Takada, 1984; Takada and Ono, 1989). A second approach to investigating viral replication is to induce the lytic cycle by superinfection of Raji cells with defective EBV from the P3HR-1 cell line (Mueller-Lantzsch *et al.,* 1980). Raji is an EBV-positive BL cell line with an unusually high EBV episome copy number, it is defective for DNA replication and late gene expression thus is tightly latent (Polack *et al.,* 1984a). Defective virions from P3HR-1 contain rearranged DNA molecules in which the intermediate early *trans*-activator of lytic cycle are expressed after superinfection (Cho *et al.,* 1984; Miller *et al.,* 1984). Studies with such cell lines has allowed the division of EBV replicative proteins into early antigens (EA), membrane antigens (MA) and virus capsid antigens (VCA). Early antigens are further subdivided into EA-D (diffuse) and EA-R (restricted) due to a different sensitivity to methanol fixation (Henle *et al.,* 1971a; 1971b). After induction, cells that have become permissive to viral replication undergo cytopathic changes characteristic of herpesviruses, including migration of nuclear chromatin, synthesis of viral DNA, assembly of nucleocapsids, envelopment of the virus by budding through the inner nuclear membrane and inhibition of host macromolecular synthesis (IARC Monograph, 1997) see figure 1.8.



EA	ORF
EBV DNA polymerase	**BALF5**
Thymidine Kinase	**BXLF1**
Ribonucleotide reductase	**BORF2 & BARF1**
Alkaline DNase	**BGLF5**
DNA binding protein p188	**BALF2**

MA	ORF
gp350/220	**BLLF1**
gp150	**BDLF3**
gp110	**BALF4**
gp85	**BXLF2**
gp78	**BILF2**
gp42	**BZLF2**
gp25	**BKRF2**

Fig. 1.8. A schematic representation of early and late EBV gene expression. The VCA the MA and the EA are illustrated, their open reading frames are written in bold.

Virus gene expression follows a temporal and sequential order (Farrell, 1992; Takada and Ono, 1989). Some virus genes are expressed independently of new protein synthesis, early after induction and are classified as immediate early genes. Early lytic virus genes are expressed slightly later and their expression is not affected by inhibition of viral DNA synthesis (Kieff, 1996).

1.6.1 IMMEDIATE EARLY GENES

After P3HR-1 superinfection of Raji or sIg cross-linking of Akata cells in the presence of protein synthesis inhibitors, three leftward mRNAs are transcribed. The BZLF1, BRLF1 and BILF4 encoded proteins are potent transactivators of early EBV lytic gene

expression (Takada and Ono, 1989; Marschall *et al.*, 1991; Kieff, 1996). The functional and physical interaction of BZLF1 with NFκB is an important mediator of LMP1 effects in EBV latent infection. BZLF1 can also downregulate the EBNA Cp promoter perhaps facilitating the transition from latent to lytic infection (Kenny *et al.,* 1989; Sinclair *et al.,* 1992).

1.6.2 EARLY GENES

The early genes are expressed when lytic cycle is induced in the presence of inhibitors of DNA synthesis. By this criterion at least 30 EBV mRNAs are early gene products (Hummel and Kieff, 1982a,b; Baer *et al.,* 1984). Two very abundant early proteins have been mapped to specific DNA sequences. The BALF2 protein is homologous to a HSV DNA binding protein and is important in DNA replication (Hummel and Kieff, 1982a; Kieff, 1996). The BHRF1 protein which is expressed in moderate abundance, has extensive collinear homology with *bcl-2* (Pearson *et al.,* 1983a; Austin *et al.,* 1988). BHRF1 can protect EBV negative BL cells from apoptosis (Mc Carthy *et al.,* 1996), however, EBV recombinants lacking the BHRF1 ORF are fully capable of initiating and maintain cell growth transformation and they can also enter the lytic cycle and produce virus (Lee and Yates, 1992; Marchini *et al.,* 1991). Several of the early genes are linked to DNA replication as indicated in figure 1. 8.

1.6.3 LATE GENES

The late genes code for structural glycoproteins or proteins that modify the infected cells in order to permit viral envelopment or egress (IARC Monograph, 1997). Among the non-glycoproteins the major nucleocapsid protein is encoded by BCLF1, BNRF1 encodes the major external nonglycoprotein of the virion and BXRF1 is likely to encode a basic core protein. The BFRF3 ORF encodes the tegument protein, (see figure 1.1) and VCA p18, which is strongly immunogenic in humans (Kieff, 1996). The genes encoding the EBV glycoproteins are illustrated in bold in figure 1.8. The late BCRF1 gene, which is located in the middle of the EBNA regulatory domain between *ori-P* and Cp, is a close homologue of the human IL-10 gene, with nearly 90% collinear identity

in amino-acid sequence (Moore *et al.,* 1990; Vieira *et al.,* 1991; Touitou *et al.,* 1996). BCRF1 has most of the activity of human IL-10, including negative regulation of macrophages and NK cell functions and inhibition of IF γ production. Thus, virally expressed IL-10 may have a local effect on these responses to reactivate infection (IARC Monograph, 1997).

1.7 EBV ASSOCIATED NON-MALIGNANT DISEASES

1.7.1 INFECTIOUS MONONUCLEOSIS

The classical clinical syndrome associated with primary EBV infection is infectious mononucleosis (IM), commonly known as glandular fever. Primary infection during early childhood appears to result in mild fever or no clinically detected symptoms (Henle and Henle, 1970). As is the case with some other viral illnesses the severity of primary infection increases with advancing age, infection during or after adolescence can give rise to IM in up to half of the infected individuals (Henle and Henle, 1979). Clinically apparent IM tends to be a disease of the socio-economic advanced countries where a greater number of people escape infection in childhood with a peak incidence occurring in people from 15-25 years of age (corresponding to the time at which they start dating) (Power and Walls, 1993). Hormonal changes and maturation of the immune response are thought to be possible reasons for this maturation related incidence of disease. After infection there is a 30-50 day incubation period, followed by a 3-5 day period where mild symptoms are experienced, these include headache, malaise and fatigue. In more than 80% of cases a sore throat will occur during the first week. Fever with temperatures reaching 39.5°C or higher lasts for about 10 days and then fall gradually over an additional 7-10 days. While IM is usually a benign, self-limiting disease, complications may ensue, including rupture of the spleen. Neurological complications, interstitial nephritis with renal failure, hepatitis interstitial pneumonia and anaemia have also been reported (Imoto *et al.,* 1995; Mayer *et al.,* 1996; Morgenlander, 1996; Sriskandan *et al.,* 1996). Fatal mononucleosis usually occurs in individuals with an underlying immune defect (Miller, 1990).

The acute phase of virus infection is characterised by a well-defined serological pattern. There is the absence of antibodies to EBNA and the presence of IgM antibodies to structural components of the virion, anti-VCA (viral capsid antigen) and anti-MA (anti-membrane or envelope antigen). Antibodies to early components of the viral replication cycle, early antigens (EA) are also readily detected (Henle and Henle, 1979). IgM antibodies to VCA evolve quickly with infection, persist for weeks to months and do not reappear. Their detection is presumptive evidence of recent primary infection. Antibodies to EA of the diffuse or restricted types develop in most primary infections and wane with time (Horwitz *et al.*, 1985). Transcriptional analysis suggests that a type-III EBV latency prevails in infectious mononucleosis, with expression of the full set of EBV latent genes, including Cp/Wp-driven EBNA1 (Falk *et al.,* 1990; Tierney *et al.,* 1994). A more detailed analysis of EBV gene expression at the level of the single cell reveals, however, a more heterogeneous picture. Only a subset of cells coexpress EBNA2 and LMP1, characteristic type-III latency. Most cells appear to be EBER positive but negative for EBNA2 and LMP1, suggesting a type-I latency and some large immunoblasts are seen which appear to express LMP1 in the absence of EBNA2 - type-II latency. There are also many small lymphocytes that express EBNA2 but no detectable LMP1. It is uncertain if this represents a new type of latency or a transitory phenomenon (Niedobitek *et al.,* 1997b).

1.7.2 X-LINKED LYMPHOPROLIFERATIVE SYNDROME

The X-linked Lymphoproliferative (XLP) syndrome is a hereditary immunodeficiency disorder characterised by a self-destructive immune response to primary EBV infection (Provisor *et al.,* 1975; Purtilo, 1976). Patients are usually asymptomatic until they encounter EBV, but may present symptoms of immuno deficiency prior to EBV infection. After primary EBV infection, the majority of patients develop IM with a fatal outcome. Patients who survive the primary infection are at high risk of developing malignant lymphoma, hypogammaglobulinaemia or aplastic anaemia. The XLP gene has been localised to Xq25, identification of the function of this gene is of prime

importance for better understanding the complex interaction between EBV and the host (IARC Monograph, 1997). The only curative treatment for X-linked lymphoproliferative syndrome is allogenic bone-marrow transplantation (Williams *et al.,* 1993).

1.7.3 ORAL HAIRY LEUKOPLAKIA

Oral hairy leukoplakia is an epithelial lesion of the tongue which was originally described in HIV-infected individuals but was subsequently found in immunosuppressed transplant patients. Oral hairy leukoplakia manifests itself as a raised white lesion, typically located at the lateral border of the tongue, but which may extend to other parts of the oral mucosa (IARC Monograph, 1997). In 1985, EBV DNA was detected in this lesion and the virus has been localised to the superficial epithelial cells. Linear virion DNA and the expression of viral lytic cycle antigens e.g. BZLF1 and VCA, have been shown, indicating that epithelial cells may support EBV replication (Greenspan *et al.,* 1985; Gilligan *et al.,* 1990a; Young *et al.,* 1991). Expression of BZLF1 and VCA, are restricted to the more differentiated upper epithelial cell layer (Greenspan *et al.,* 1985; Young *et al.,* 1991). In contrast to the abundance of the virus in the upper epithelial cells, viral genomes and EBV gene products associated with latent infection are absent from the basal or parabasal epithelial cells of oral hairy leukoplakia (Thomas *et al.,* 1991). Together with the absence of a detectable episomal population of EBV genomes, this indicates that oral hairy leukoplakia is an isolated focus of lytic EBV infection, with no detectable latent phase (IARC Monograph, 1997). Regression of oral hairy leukoplakia can be induced by treatment with acyclovir, indicating that this lesion is indeed caused by EBV (Resnick *et al.,* 1988).

1.8 EBV ASSOCIATED MALIGNANT DISEASES

1.8.1 BURKITT'S LYMPHOMA

Burkitt's lymphoma (BL) is the most common childhood cancer in certain parts of equatorial Africa and Papua New Guinea, with an annual incidence of more than 50 cases per million children below the age of sixteen. Burkitt's lymphoma now accounts for 30-70% of childhood cancers in equatorial Africa. The high incidence of BL in these locations is associated with geographic and climatic features, determined by altitude in East Africa and by rainfall in West Africa, coincident with holoendemic malaria (Haddow,1963; Burkitt, 1969; 1983; O'Connor, 1970). The fact that malaria infection might be a cofactor in the development of BL is supported by the observation that individuals with malaria have a reduced T-cell response to EBV-infected cells. In the United States the incidence of BL is 2-3 cases per million children per year. BL is a poorly differentiated malignant lymphoma in which the tumour cells show little variation in size or shape. The tumour cells are monoclonal B lymphocytes and they contain characteristic chromosomal translocations (Manolov and Manalova, 1972; Manalova *et al.*, 1979; Rowe and Gregory, 1989). The jaw is the most frequently involved site for tumours and the commonest presenting feature in patients with BL in equatorial Africa (Burkitt, 1958; 1970a) and Papua-New Guinea (Burkitt, 1967). Jaw tumours seem to be age dependent, occurring most frequently in young children, very young children often have orbital or maxillary tumours (Olurin and Williams, 1972). Abdominal involvement is found in a little more than half of equatorial Africa patients at presenting (Burkitt, 1970b) and as many as 80% of patients in other countries (Margrath, 1991; 1997). Burkitt's lymphoma is classified as a non-Hodgkin's lymphoma, invariably of B-cell origin, with B-cell markers such as CD19, CD20, CD22 and CD79a and surface immunoglobulin always detectable. The surface immunoglobulins are usually IgM (IARC Monograph, 1997). Other surface markers that are expressed in most BLs include CD10 and CD77 but CD23 and CD5 are absent (Harris *et al.*, 1994). BL cells express low levels of HLA class I adhesion and activation molecules such as CD54, CD11a/18 and CD58 (Massucci *et al.*, 1987; Billuad *et al.*, 1989; Anderson *et al.*, 1991).

In the areas of Africa where BL is endemic, more than 90% of the tumours contain EBV DNA and express EBNA1 (Geser *et al.*, 1983). However, in parts of the world where BL is sporadic (Western Europe and the Americas), only about 15-20% of BL tumours contain EBV DNA, this indicates that EBV is not essential for formation of the tumour. Therefore, EBV may not play a direct role in the pathogenesis of BL, but may simply increase the risk of development of BL by virtue of it's ability to immortalize B cells (including the cell population that gives rise to Burkitt's lymphoma) (Klein, 1979). This hypothesis is consistent with the lack of expression of EBV latent genes (e.g. EBNA2, EBNA3 and LMP) known to be necessary for the transformation of B cells (Alfeiri, *et al.*, 1991 ; Woisetschlaeger, *et al.*, 1991). The only latent gene invariably expressed in Burkitt's lymphoma, EBNA1, has never been shown to have transforming functions (Rowe *et al.*, 1988; Rowe *et al.*, 1987; 1992; Sample *et al.*, 1991; Magrath *et al.*, 1993).

The discovery of non-random chromosomal translocations associated with Burkitt's lymphoma (Bernheim *et al.,* 1981) paved the way to an understanding of the genetic derangements that are a central component of its pathogenesis. It has been observed that the chromosomal breakpoint on chromosome 8, band q24 is common to all three of the observed translocations in BL and that the breakpoints are located on chromosome 14, 2 and 22, at the heavy- and light-chain immunoglobulin loci (Croce *et al.,* 1979; Lenior *et al.,* 1982; Mc Bride *et al.,* 1982). The t(8;14) is the most frequent location of a breakpoint in African BL occurring in 75% of tumours and in 50% of Brazilian tumours (Gutierrez *et al.,* 1992). The net consequence of translocation appears to be that *c-myc* is regulated as if it were an immunoglobulin gene, i.e. it is constitutively expressed in these immunoglobulin-synthesising tumour cells.

1.8.2 NASOPHARYNGEAL CARCINOMA

Nasopharyngeal carcinoma (NPC) is a rare malignant tumour in most populations, however, it is highly prevalent in Southern China where it represents the most common tumour in males. More moderate rates are seen in the Inuit population, in other parts of

Southeast Asia and North Africa. (Ho, 1978). NPC is a disease with a remarkable racial and geographical distribution. It constitutes 75-95% of all malignant tumours occurring in the nasopharynx in low risk populations and virtually all of those in high risk populations (Ho, 1971; Levine and Connelly, 1985). The overall rates of NPC are high in China but the incidence in Southern China is 20% greater than that in Northern China (Parkin *et al.,* 1997). The rates of NPC are higher in men than in women in most populations studied, the number of both male and female cases increases steadily with age and the peak is at around 45-54 then declines at older ages (Parkin *et al.,* 1997).

NPC is derived from poorly differentiated epithelial cells and arises in the surface epithelium of the posterior pharynx (Parkin *et al.,* 1986). There is strong association between NPC and EBV as the presence of EBV is always detected in tumour cells. EBV infection is an essential step in the progression to malignancy. It has been shown that EBV DNA in NPC is clonal, arising from a single EBV infected cell (Raab-Traub and Flynn, 1986b). EBV is present in the cell at the time of carcinogenic transformation, thus suggesting that EBV contributes to the transformation event (IARC Monograph, 1997). NPC occurs primarily in adults and is unlikely to be the result of primary EBV infection as it is prevalent in areas where initial EBV infection occurs during childhood (Power and Walls, 1993).

Early studies have shown that NPC patients frequently possess elevated serum antibodies to two EBV lytic cycle antigens, viral capsid antigen (VCA) and early antigen (EA) (Henle and Henle, 1976; Ho *et al.*, 1976). Serum detection of these antibodies is a routine diagnostic test for NPC in South-east Asia. In about half the cases of NPC the presenting sign is a cervical mass resulting from spread to regional lymph nodes. Other symptoms may include nasal obstruction, postnasal discharge, possible impairment of hearing, tinnitus or otitis media. NPC may metastasise to the skeleton, the spine, the liver, lung and skin as well as to the peripheral lymph nodes (Miller, 1990).

Nearly all cases of NPC have detectable EBV DNA sequences, the detection of EBV DNA and EBERs has been useful in identifying carcinomas that have metastasised to lymph nodes when the primary tumour has not been identified (Ohshima *et al.,* 1991;

Chao *et al.,* 1996). Transcriptional expression of EBV latent genes in NPC cells has been studied by northern blotting/hybridization (Raab-Traub *et al.,* 1983; Gilligan *et al.,* 1990b, 1991; Karran *et al.,* 1992). BARFO, LMP2, EBER and EBNA1-coding transcripts are always expressed in NPC cells and LMP1 is detected in 50% of tumours (Fahraeus *et al.,* 1988; Brook *et al.,* 1992). Occasionally lytic cycle early genes are also detected in a few cells (Luka *et al.,* 1988; Cochet *et al.,* 1993).

The high incidence of NPC among the Cantonese population of China was first described by Ho in 1971, who also observed that salted fish is the principal source of supplemented food in the diet of these people (which consists mainly of rice). Further studies revealed that salted fish consumption was significantly related to the risk for developing NPC tumours and increasing frequency of intake was consistently associated with increased risk. The association with salted fish was stronger when exposure occurred during childhood as compared with adulthood (Huang *et al.,* 1981). Carcinogenic volatile nitrosamines have been detected in Chinese salted fish, however, their precise role in NPC has yet to be determined (IARC Monograph, 1997).

1.8.3 HODGKIN'S DISEASE

Following a report by Weiss and colleagues of EBV DNA in 50% of Hodgkin's disease (HD) tissues (Weiss *et al.,* 1987), the role of EBV in HD has been subjected to intense scrutiny (Joske and Knecht, 1993). Histologically HD is characterised by mononuclear Hodgkin cells (HC) and their multinucleated variants, the Reed-Sternberg cells (RS). The Rye classification distinguishes four major types of Hodgkin's disease: nodular lymphocytes predominant, nodular sclerosis, mixed cellularity and lymphocyte-depleted (Luka and Butler, 1966, Harris *et al.,* 1994). It is now accepted that lymphocyte-depleted HD represents a separate tumour entity and is considered separately from the other three classical forms of HD. Increasing evidence suggests that HD is not a single entity but a rather a heterogeneous group of diseases (Harris *et al.,* 1994). The clinical representation of HD varies with geographical location and in the western world HD usually arises as a unifocal lesion in cervical lymph nodes. Continuous spread of the tumour to adjacent lymph nodes gives rise to enlarged nodes. With spread of the

tumour through lymphatic channels, other organs are involved, the preferential sites of involvement including the spleen and distant Lymph nodes. Subsequently as the disease becomes more aggressive, other organs are involved, including the liver and the kidney, bone-marrow involvement in HD is indicative of extensive tumour infiltration (Kaplan, 1980).

In most western populations, very few cases occur among children, a rapid increase in incidence among teenagers is seen followed by a peak at about age 25, the incidence then plateaus with a second peak with increased age. There is an excess in males which is more pronounced at older ages (Mac Mahon, 1957). In poorer populations there is an initial peak in childhood only among boys with a relatively low abundance among young adults followed by a late peak in those of advanced age (Correa and O'Connor, 1971). There is evidence that the risk factor for HD in young adulthood through middle age is associated with higher education, higher social class, fewer siblings, less crowded housing and early birth rank. All of the above factors lead to increased susceptibility to later infections with the common childhood infections (IARC Monogragh, 1997).

Pallesen *et al.,* (1991a) and Herbst *et al.,* (1991a) reported that the EBV in HD has a restricted latent phenotype of EBNA1 and usually LMP1, LMP2A and LMP2B without detectable EBNA2 expression, as in NPC. These finding have been widely replicated. In multiple specimens of HD from case studies, molecular evidence of clonal EBV genomes with specifically restricted expression of latent viral proteins in the RS cells is found in 30-50% of cases. EBV genome status appears to be uniform in involved nodes within patients and over time in those patients studied longitudinally (Delsol *et al.,* 1992; Brousset *et al.,* 1994). The consistency of the finding of clonal EBV and the expression of LMP1 in about half of HD cases argues strongly against a passenger role for EBV in these cases. Seroepidemiology findings in multiple case studies show that patients with HD can be distinguished by an altered antibody profile to EBV. The evidence indicates that EBV is a causal factor in the etiology of HD.

The risk of HD after diagnosis of IM has been evaluated and this study revealed that overall there was a threefold increase in the risk of developing HD. Essentially all HIV-

1 infected patients with HD have a higher rate of EBV positivity, generally these patients present with advanced HD and have poor prognosis (Moran *et al.,* 1992; Tirelli *et al.,* 1995).

1.8.4 POST-TRANSPLANT LYMPHOPROLIFERATIVE DISORDERS

Post-transplant lymphoproliferative disorders (PTLD) are a major complication in allograft recipients, occurring in 1-20% of patients. The incidence tends to be lowest for renal transplant patients and highest for lung transplant patients which may reflect the amount of immunosuppressive therapy associated with the latter (Nalesnik and Starzl, 1994; Montone *et al.,* 1996). In PTLD the tumours proliferate unchecked due to the absence of adequate T-cell tumour suppression. The tumours can be polyclonal or monoclonal as determined by analysis of EBV terminal repeats or cellular gene rearrangement status (Joske and Knecht, 1993). It is believed that the pathogenesis of the condition starts with EBV driven polyclonal B-cell proliferation, eventually leading to fully developed malignant lymphoma. Typically B-cells in these lymphoproliferations express a broad spectrum of virus-encoded latent proteins, including EBNA1, EBNA2 and LMP1. This type-III form of latency is similar to that found in LCLs *in vitro,* accordingly these cells usually display LCL pattern of cellular gene expression, including lymphocyte activation and adhesion molecules (Young *et al.,* 1989; Thomas *et al.,* 1990). However, considerable variability has been found in EBV gene expression in and between lesions with type-I and type-II latency also observed (Delecluse *et al.,* 1995).

1.8.5 AIDS-RELATED LYMPHOMAS

In the US lymphomas are 60 times more frequent in AIDS patients than in the general population (Beral *et al.,* 1991). Non-Hodgkin's lymphomas are very common in HIV infected individuals, primarily at extranodal sites, particularly common are primary central nervous system lymphomas (Krogh-Jensen *et al.,* 1994). Morphologically, AIDS-related non-Hodgkin's lymphomas fall into two broad groups; diffuse large B-cell non-Hodgkin's lymphomas, which often show a prominent immunoblastic

component and Burkitt's lymphoma and Burkitt's-like lymphoma. Superficially the pathogenesis is the same as PTLD; EBV-immortalised B lymphocytes proliferate unchecked due to decimated T-cell numbers resulting in oligo- or monoclonal B-cell proliferations (Joske and Knecht, 1993). The two types of AIDS-related non-Hodgkin's lymphomas show striking differences in their relationship to EBV, suggesting different pathogenic mechanisms. Most diffuse large B-cell non-Hodgkin's lymphomas and all AIDS-related central nervous system lymphomas are EBV-positive (MacMahon *et al.,* 1991). Diffuse large B-cell lymphomas have been reported to occur relatively late in AIDS patients (Gaidano and Dalla-Favera, 1995) and more advanced depression of the immune system is a risk factor for their development (Pedersen *et al.,* 1991). Most AIDS-related non-Hodgkin's lymphomas appear to be monoclonal both with respect to their antigen receptor genes and to the EBV episomes, however, there may be rare polyclonal cases (Ballerini *et al.,* 1993; Delecluse *et al.,* 1993c).

HIV appears to contribute to the pathogenesis of some EBV-associated AIDS-related non-Hodgkin's lymphomas by inducing severe immunosuppression, leading to a loss of EBV-specific T-cell immunity (MacMahon *et al.,* 1991). As EBV-positive, AIDS-related B-cell lymphomas consistently lack the HIV genome, a direct contribution of HIV to tumorigenesis beyond suppression of the immune system is unlikely (Knowles, 1993). The relative risk for AIDS-related non-Hodgkin's lymphomas increases with duration of HIV infection and to a certain extent with immune suppression (Munoz *et al.,* 1993).

BL is up to one thousand fold more frequent in HIV-positive individuals than in the general US population (Beral *et al.,* 1991). The tumour is EBV positive in about 20% of cases (Beral *et al.,* 1991). AIDS related BL, both EBV-positive and EBV-negative, have been consistently shown to harbour the characteristic *c-myc* translocation. These translocations have been detected in a minority of diffuse large B-cell lymphomas and cases with morphological features between large B-cell lymphomas and BL (Ballerini *et al.,* 1993; Delecuse *et al.,* 1993c; Bhatia *et al.,* 1994). Other genetic changes implicated in the pathogenesis of AIDS-related non-Hodgkin's lymphoma whether EBV-associated or not, include *p53*, N-*ras* and K-*ras* point mutations and deletions in the long arm of

chromosome 6 (Gaidano and Dalla-Favera, 1995). HIV-positive individuals who develop HD are more likely to have advanced extra-nodal disease, not to respond to therapy and to die of opportunistic infections than those with HD alone (Ames, *et al.,* 1991).

The detection of EBV in T-cell lymphoma opposes the well established process of B-cell lymphotrophisim of the virus *in vitro.* The interpretation of the detection of EBV in T-cell non-Hodgkin's lymphomas and an assessment of the role of the virus in the pathogenesis of T-cell lymphoma are complicated by two factors. Firstly, if EBV infection of certain T-cells *in vitro* leads to predominantly lytic infection, EBV infection of T-cells may be accidental rather than part of the viral strategy to establish persistent infection. Such infection of cells not adapted to latent infection may contribute to the development of EBV-associated T-cell lymphomas. Secondly, in many cases the virus is detected in only a small proportion of tumour cells (Anagnostopoulos *et al.,* 1996). Although the virus may be present at the onset of the neoplastic process it may subsequently be lost from the tumour cell. While there is some evidence to suggest this may happen *in vitro* it has not yet been shown *in vivo.* The alternative scenario would be a secondary infection of established neoplastic T-cells with the virus, this would exclude the virus from an initial role in neoplasia but would be compatible with a role of the virus in contributing to the disease process. The frequent expression of LMP1 in T-cell lymphomas would seem to argue in favour of such a role (d'Amore *et al.,* 1992). However, the role of EBV in T-cell related malignancies remains uncertain to date.

1.9 CD44

1.9.1 Overview

CD44 is a family of ubiquitous multistructural and multifunctional cell surface adhesion molecules involved in cell-cell and cell-matrix interaction. Twenty exons are involved in the genomic organisation of this group of proteins. The first five and the last five exons are constant whereas the ten exons located between these regions are subjected to alternative splicing resulting in the generation of a variable region on the protein. Differential utilisation of the ten variable region exons, as well as variations of N-glycosylation, O-glycosylation and glycosaminoglycanation (by heparan sulphate or chondrotin sulphate) generates multiple isoforms of different molecular size (85-230 kDa). The smallest and most prolific being CD44 standard (CD44s) which lacks the entire variable region. CD44s is found in a wide variety of tissues including the central nervous system, lung, epidermis, liver and pancreas, whereas variant isoforms of CD44 (CD44v) appear to have a much more restricted distribution. CD44v is expressed in tissues during development including embryonic epithelia. Known functions of CD44 are cellular adhesion (aggregation and migration), hyaluronate degradation, lymphocyte activation, lymph node homing, myeleopoiesis, lymphopoiesis, angiogenesis and release of cytokines, presentation of chemiokines and growth factors to travelling cells and transmission of growth signals. The role of CD44 in neoplasia has been extensively investigated since it was uncovered that metastatic potential can be conferred on non-metastasising cell lines by transfection with a gene encoding a variant member of the CD44 family. High levels of CD44 are associated with several types of malignant tumour, the physiological function of CD44 indicates that it may be involved in the metastatic spread of tumours.

1.10 CD44 nomenclature

Prior to being given a standard name CD44 appeared in many guises probably reflecting its many functions. CD44 was first described as a molecule present on the surface of T-lymphocytes, granulocytes and coitical thymocytes (Dalchau *et al.,* 1980). It was

rediscovered as a phagocytic glycoprotein 1 (Pgp-1) and 80 kDa polymorphic surface glycoprotein expressed in large amounts on the surface of macrophages and granulocytes. The ability of CD44 to associate with the cellular cytoskeleton and bind the extracellular matrix components fibronectin, collagen type-I and IV and hyaluronan led to CD44 being described as the extracellular matrix receptor for type-III (ECM-III) and identified as the major receptor for hyaluronan (Lesley *et al.,* 1993). Furthermore, binding of specific antibodies to a protein that is known to be CD44 were shown to interfere with lymphocyte homing (Hermes 3 or $GP90^{Hermes}$) and with the binding of lymphocytes to high endothelial venules (HUTCH-1) (Lesley *et al.,* 1993). Other names attributed to CD44 were glcoprotein 85, Ly-24, hyaluronate receptor (H-CAM) and In (lu)-related p80 glycoprotein (Naor *et al.,* 1997). All of the above were included in the cluster of differentiation 44, or CD44 designation assigned by the Third International Workshop on Leukocyte Typing (Cobbold *et al.,* 1987).

1.11 CD44 STRUCTURE

Standard CD44 is a type-I transmembrane glycoprotein of 341 amino acids, forming a 248-amino acid extracellular domain and a 72-amino acid cytoplasmic tail (see figure 1.9) (Stamenkovic *et al.,* 1989). The N-terminal (membrane distal) region of CD44 folds into a globular domain through disulphide bonding of conserved cysteine residues. CD44 has the ability to bind hyaluronic acid (HA) in the extracellular domain. The amino acid sequence of CD44s predicts a polypeptide of approximately 37 kDa which contrasts with its apparent size SDS-PAGE of 80-90 kDa (Stamenkovic *et al.,* 1989). This difference is the result of extensive glycosylation and glycosaminoglycanation of the extracellular domain, which contains many sites for both N- and O-linked carbohydrates. Most of the potential N-linked sites are located in the membrane distal region while the membrane proximal domain has many O-linked sites (see figure 1.9). Chondroitin sulphate and heparan sulphate are examples of the glycosaminoglycans added during postranslational modification of CD44. These are large molecules with highly charged sulphate and carboxylate groups (Sneath and Mangham, 1998). The varying degree of glycosylation can affect some of the functions of the CD44 molecule. The negatively charged environment produced by these chains attracts positive ions and

so creates an osmotic potential (Sneath and Mangham, 1998). Differing CD44 isoforms appears to have different patterns of glycosylation, CD44s has a small amount of chondrotin sulphate whereas CD44v3-10 is glycosylated principally by heparan sulphate (Gallagher, 1992). There are four consensus sequences for attachment of chondroitin sulphate in the membrane proximal region (see figure 1.9) (Stamenkovic *et al.,* 1989; Goldstein *et al.,* 1989; Zhou *et al.,* 1989; Screaton *et al.,* 1992). There are isoforms of CD44 that occur in both the glycosylated and non-glycosylated forms, suggesting that some of the molecules functions are not dependant on the glycosaminoglycan side chains (Hardingham and Fosang, 1992). The CD44 glycoprotein is an acidic molecule, its charge largely due to sialic acid (Jalkanen *et al.,* 1988). The $t_{1/2}$ of CD44 turnover was found to be 8h (Lokeshwar and Bourguignon, 1991).

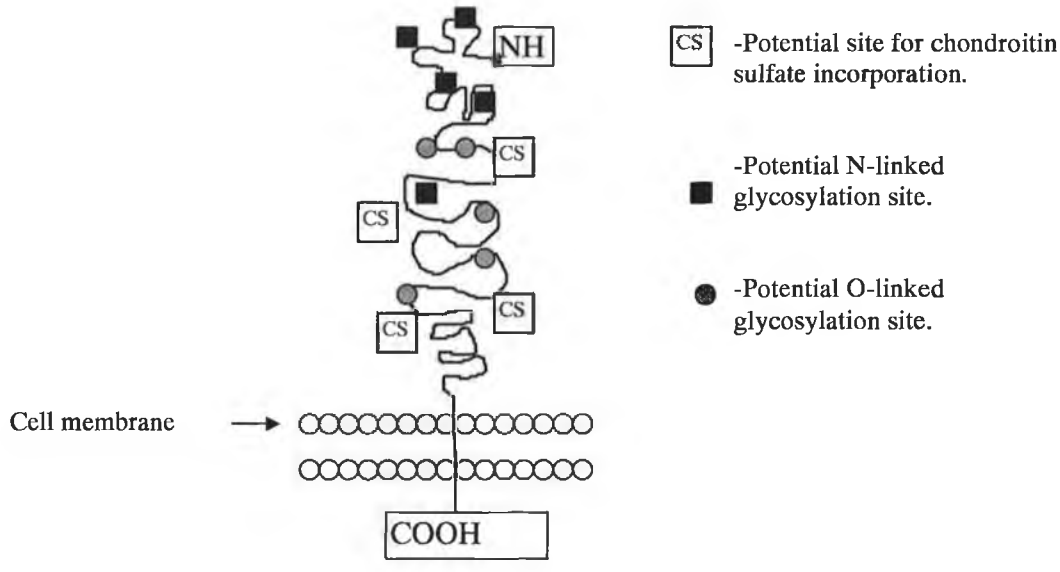


Fig 1.9. Schematic drawing of CD44 protein.

Polypeptide isoforms of CD44 are produced by alternative splicing of at least 10 of the 20 CD44 exons during mRNA processing. This introduces additional sequences at a single site between amino acids 202 and 203 in the membrane proximal extracellular domain of CD44s (Stamenkovic *et al.,* 1989; Dougherty *et al.,* 1991; Jackson *et al.,* 1992; Screaton *et al.,* 1992; Screaton *et al.,* 1993; Tolg *et al.,* 1993). Potentially any

combination of polypeptides encoded by these alternative exons could be inserted into the CD44 sequence, but no means all possible combinations seem to be expressed (Lesley *et al.,* 1993). To date approximately 30 different CD44 transcripts have been described. However, this is most likely not the final number, theoretically over 750 membrane bound CD44 isoforms can be generated by alternative use of the variant exons (van Weering *et al.,* 1993). The variant exons are called v1-v10 and the high molecular weight proteins that contain these sequences are identified by the specific exons used, for example: CD44v6 uses v6 of the variable region in conjunction with the 5' and 3' constant regions, CD44v8-10 uses v8, v9 and v10 of the variable region in conjunction with the 5' and 3' constant regions (Naor *et al.,* 1997). Note, however, that exon v1 is not expressed in humans as it contains a termination codon that is absent in the rodent CD44 gene (Screaton *et al.,* 1993). As well as increasing the size of the polypetide backbone CD44, insertion of variant exon sequences introduce extra potential glycosylation sites, particularly for O-linked oligosaccharides (Borland *et al.,* 1998).

1.12 CD44 GENOMIC ORGANIZATION

The CD44 gene is located on the short arm of human chromosome 11 and on mouse chromosome 2. The entire human CD44 gene covers between 50 and 60 kb of genomic DNA and contains at least 20 exons (figure 1.10). CD44 cDNA sequence alignments show remarkable conservation between species, such as the human and the rodent CD44, with overall similarities in excess of 70% (Naor *et al.,* 1997). The nomenclature used in this description is based on that of Screaton *et al.,* (1992), who described 19 of the 20 exons involved in the genomic structure of CD44, thus the exons are termed 1-19, with a missing exon, subsequently located between exons 5 and 6, designated 6a (Borland *et al.,* 1998). Exon 6a is also known in other studies as exon 5a or variant exon 1 (v1). CD44 proteins containing sequences encoded by v1 (exon 6a) are not observed in humans (Ponta *et al.,* 1998). The smallest CD44 transcript is the standard one, CD44s, which lacks the entire variable region with exon 5 of the 5' constant region being directly spliced to the 3' constant region (Idzerda *et al.,* 1989; Harn *et al.,* 1991;

He *et al.,* 1992). CD44 is expressed widely on hematopoietic cells thus CD44s is also designated CD44H.

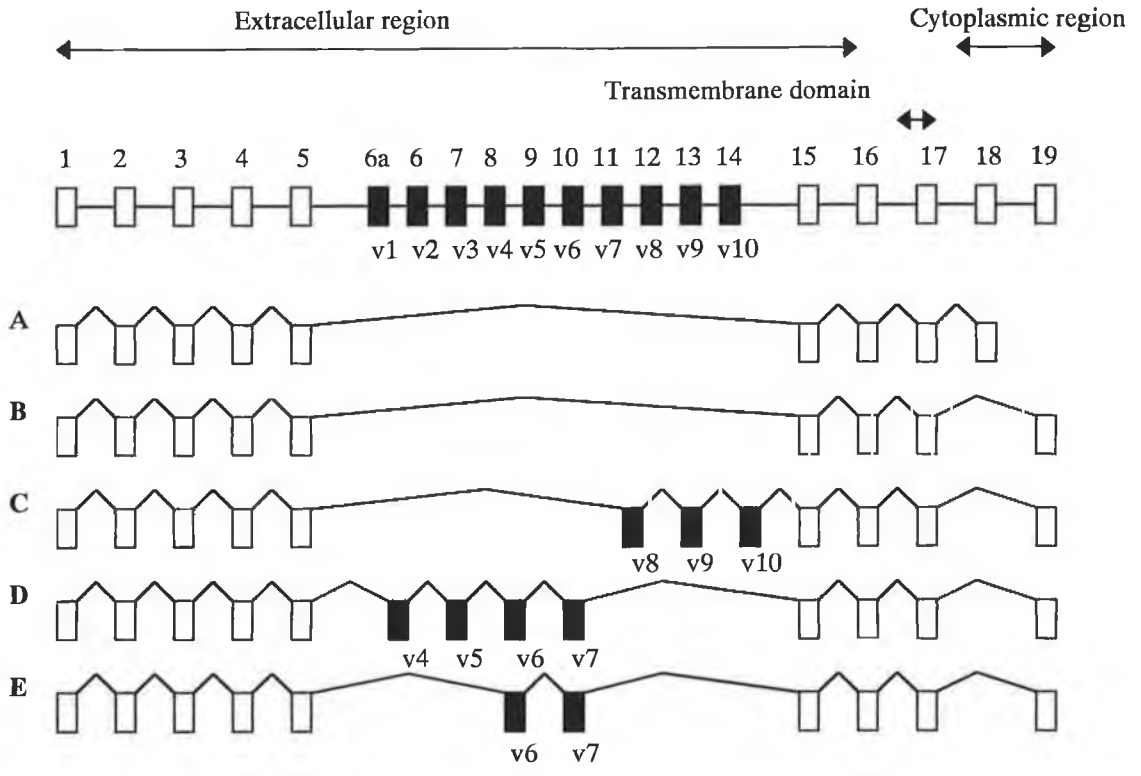


Fig 1.10. Schematic representation of the genomic structure of CD44 and multiple transcription products of CD44, adapted from Brooks *et al.,* (1995). A and B represent the standard forms of CD44. C represents the epithelial form (CD44E or CD44v8-10). D and E represents the metastatic forms pMeta-1 (CD44v4-7) and pMeta-2 (CD44v6-7) respectively.

Exons 1-16 including exon 6a (v1) encodes the extracellular domain of the protein, exon 17 encodes a short transmembrane domain and exons 18 and 19 encode the cytoplasmic domain (Lesley *et al.,* 1993). Enormous heterogeneity in mRNA products transcribed from the gene is produced by alternative splicing of exons 6-14 in humans. These exons are known as variant exons v2-v10 and are capable of being individually spliced. Exons 18 and 19 which code for the intracellular portion of the CD44 protein are also subjected to alternative splicing generating two possible cytoplasmic tails (see figure 1.10). Short tailed isoforms of CD44 (those lacking exon 19), have been detected with PCR but in a 100-200 fold lower abundance than the long tailed isoforms and it is unclear whether the corresponding mature protein exists or if it has any functional relevance *in vivo* (Lesley *et al.,* 1993). The DNA sequence of exon 18 carries a long

A+T tract, possibly causing instability in the mRNA of the short version (Naor *et al.,* 1997). Consensus splice donor/acceptor sites are also found within exons 5 and 7, resulting in CD44 isoforms containing shortened versions of these exons. These isoforms have been found in some tumours, (Hoffmann *et al.,* 1991; Shtivelman and Bishop, 1991), but it is not known if they are used in normal tissue. An additional exon (called v9a) located between v9 and v10 and with a restricted expression pattern has been recently identified (Yu and Toole, 1996). This may enable cells to express a low molecular weight CD44 as a soluble isoform, which may turn out to have important functional properties in tissue remodelling in the foetus (Yu and Toole, 1996).

Northern blot analysis of RNA isolated from hematopoietic cell CD44 revealed three major transcripts in humans ~1.6, 2.2 and 4.8 kb (Goldstein *et al.,* 1989; Samenkovic *et al.,* 1989; Quackenbush *et al.,* 1990). Utilization of multiple polyadenylation signals may explain this heterogeneity (Harn *et al.,* 1991). A recently described alternative splicing assay has demonstrated a dramatic effect of intron length upon alternative splicing of the CD44 exons (Bell *et al.,* 1998). It was discovered that as intron length shortened exon inclusion increased, suggesting a kinetic proximity model for the splicing of alternative exons. Alternatively spliced exons are frequently included in contiguous blocks, a fact which may suggest that all of the variable exons may not have a separate function. Instead they may act as stuffers, extending the N-terminal hyaluronic acid binding domain from the cell surface (Bell *et al.,* 1998). This is in agreement with the observed number of variant isoforms of CD44 when compared to the possible number of variants through combinations of splice variants.

1.13 DISTRIBUTION OF CD44 ON NORMAL TISSUE

CD44 has been found on a wide range of tissues including the central nervous system, lung, epidermis, liver and pancreas. The distribution of CD44 standard and variant expression in normal human tissue is outlined in table 1.2.

Table 1.2. Differential expression of CD44 variants in normal human tissue. (Adapted from Sneath and Mangham, 1998).

TISSUE	CD44s	CD44v6	CD44v7	CD44v8	CD44v7-8	CD44v8-9	CD44v7-9
Skin	+++	+++	+++	+++	+++	+++	+++
Tongue	+++	+++	+++	+++	+++	+++	+++
Oesophagus	+++	+++	+++	+++	+++	+++	+++
Lung	+++	++	++	++	++	++	++
Stomach	+++	+	+	+	++	++	++
Colon	+++	+/++	+/++	+/++	++	++	++
Thyroid gland	+	+	+	+	++	+++	++
Pancreas	++	++	++	++	+++	+++	+++
Adrenal gland	-	-	-	-	+	+	+
Ovary	-	-	-	-	+	+	+
Cervix	+++	+++	+++	+++	+++	+++	+++
Kidney, cortex	+	-	+	+	+	+	+
Ureter	+	-	-	-	-	-	-
Bladder	+++	++	++	+++	+++	+++	+++
Spleen	++	++	++	++	++	++	++
Lymphocytes	+++	-	-	-	-	-	-

Expression: +++, Strong; ++, Moderate; +, Weak; -, Negative.

CD44 variant isoforms seem to have a much more restricted distribution than CD44s and their expression on a variety of epithelial cells seems to have a tissue specific pattern, suggesting that the process of alternative splicing is normally tightly regulated (Sneath and Mangham, 1998). This also suggests that these isoforms, with a specific exon sequence and restricted distribution have different additional functions to CD44s (Sleemann *et al.,* 1995). For example, numerous variants of CD44 have been found to be expressed strongly on tissues during stages of development, including expression on embryonic epithelia (Wirth *et al.,* 1993). CD44 was detected by indirect immunofluorescence in early preimplanted human embryos containing one to eight-cells. The intensity of expression was maximal at the eight cell stage and down-regulated at the morula, blastocyst and postimplantation stages (Campbell *et al.,* 1995).

Immunohistochemical studies with variant-specific mAb revealed the expression of CD44 variants in 10 week-old human embryos. CD44v9 was the predominant isoform found in many areas including the trachea, lung and thyroid gland, CD44v6 was also found in the epidermis and trachea (Terpe *et al.,* 1994b). CD44v isoforms have been detected in the rat apical ectodermal ridge (AER) (Wainwright *et al.,* 1996). The AER is a highly specialised ectodermal structure located at the leading end of the limb tip which is responsible for maintaining underlying mesenchymal cells in a constant state of proliferation. Presence of the AER is critical to limb out-growth as its removal abrogates further limb development. RT-PCR analysis of AER tissue demonstrated the expression of several large CD44v proteins containing v3 sequences, including one containing all of the 10 variant exons CD44v1-10 (Wainwright *et al.,* 1996). It was shown experimentally that upon treatment with mAb directed against an epitope encoded by the CD44 v6 exon, AER failed to support out growth of the limb bud (Wainwright *et al.,* 1996). Since a member of the fibroblast growth factor (FGF) family is able to replace the AER function (Niswnder *et al.,* 1993) and as the proteoglycan version of CD44 can present various growth factors it is tempting to speculate that the role of AER CD44v is to present FGF-like growth factors to growing limbs (Wainwright *et al.,* 1996). CD44 expression during foetal human thymus development was detected on the thymic epithelial cells and thymocytes begining at week 8 of foetal gestation. CD44 variants containing v4, v6 and v9 exon products emerge at 10 weeks of foetal gestation (Mackay *et al.,* 1994; Terpe *et al.,* 1994b; Patel *et al.,* 1994).

In humans the expression of CD44v6 and CD44v4 on cells of epithelial origin is widespread. They have been identified in epithelial cells of skin epidermis, hair follicles, oesophagus and tonsils (Naor *et al.,* 1997). CD44v6 but not v4 was also found on the epithelium of sweat glands, prostate gland, mammary glands and lung bronchi. CD44v9 has been detected on cells of the previously mentioned tissues as well as in the intestine, stomach, pancreatic duct, the tubular region of the kidney, hepatic bile ducts, thyroid gland, salivary gland and many more (MacKay *et al.,* 1994; Terpe *et al.,* 1994b; Stauder *et al.,* 1995). Contradictory findings regarding the presence of CD44v isoforms in other epithelial cells have been obtained. For example, thyroid and salivary glands were CD44v4 and v6 negative in one study by immunohistochemistry (MacKay *et al.,*

1994), but positive in another (Fox *et al.,* 1993). Similar conflicting results have been found on the endothelium. These discrepancies can be attributed to technical differences in methodology, varying degrees of staining sensitivity or the use of antibodies that recognise different epitopes (Naor *et al.,* 1997).

All types of hematopoietic cells, including erythrocytes, T and B lymphocytes, natural killer cells, macrophages, aveolar macrophages, kuffer cells, dendritic cells as well as granulocytes preferentially express CD44s (Naor *et al.,* 1997). Memory and activated T-cells have much higher levels of CD44s than naive T-cells, in addition they express CD44v9 (MacKay *et al.,* 1994). A substantial change in the CD44 repertoire has been noted after cell activation. Three to 14 days after *in vivo* antigenic stimulation of rat T-cells, B-cells and macrophages express (in addition to CD44s) CD44 variants containing the v6 exon (Arch *et al.,* 1992). Similarly activation of human T-cells transiently upregulates expression of CD44 variants containing v6 and v9 *in vitro.* CD44 transition has also been demonstrated during B-cell activation and differentiation in the germinal centre of human tonsils. Normal splenic human B-cells activated with anti-Ig antibody express increasing levels of CD44s, CD44v6 and a CD44v containing the v10 exon product (Salles *et al.,* 1993). Whereas resting human peripheral blood B cells express CD44s only, various CD44v isoforms such as, CD44v8-10, CD44v10, CD44 v6 and CD44v6-7 have been detected after stimulation with PMA, or IL-2 (Naor *et al.,* 1997). EBV-negative BL cells do not express CD44. In contrast CD44s, CD44v8-10 and CD44v10 were detected in EBV-positive BL cells and EBV-negative BL cells infected with EBV (Kryworuckho *et al.,* 1995).

In summary, it is clear that epidermal regions rich in proliferating cells express high levels of CD44v isoforms, especially exon v6. Similarly, activated leukocytes and epithelial cells upregulate v6 and v9 containing variants. The extensive locomotive and generative activities within the embryo are also accompanied by marked expression of CD44v exons, again the v6 variant is particularly conspicuous. Malignant cells, which share many properties with normal adult and foetal cells of generative tissues, bear similar CD44 isoforms (see section 1.16).

1.14 CD44 LIGANDS

1.14.1 CD44 AND HYALURONIC ACID

Perhaps the best studied aspect of CD44 is its ability to bind components of the extracellular matrix (ECM). The main ligand of CD44 is hyaluronic acid (HA), this glycosaminoglycan (GAG) is a major component of the ECM of many tissues. Also known as hyaluronan or hyaluronate, HA is a very high molecular weight GAG composed of repeating units of glucuronic acid-β1-3*N*-acetylglucosamine β1-4. Unlike other GAGs of the ECM, HA is not found covalently linked to ECM proteins, but associated non-covalently with a number of HA-binding proteins (such as aggrean in cartilage) through HA-binding domains (Borland *et al.,* 1998). The functions of HA are mediated through a family of HA-binding proteins called hyaladherins. The major leukocyte and epithelial hyaladherin is CD44 (Knudson and Knudson, 1990). Other members of this family include the receptor for hyaluronate mediated motility (RHAMM) (Turley *et al.,* 1991), cartilage link protein and the proteoglycan core protein (aggrecan) (Hardingham and Fosang, 1992).

HA is synthesised by fibroblasts (Teder *et al.,* 1995), chondrocytes (Mason *et al.,* 1989) and mesothelial cells (Honda *et al.,* 1991; Heldin *et al.,* 1992). The evidence for a receptor-ligand relationship between CD44 and HA has been established by several experimental approaches, resulting in a number of observations including; Anti-CD44 blocked hyaluronate binding of many CD44 positive cells and binding was sensitive to hylaluronidase: CD44 cDNA transfected into CD44 negative cells conferred HA binding and hyaluronidase-sensitive binding to high endothelial venules (HEV): Soluble CD44-IgGFc chimeric protein bound to purified hyaluronate and to lymph node HEV and binding was inhibited by hyaluronidase (Aruffo *et al.,* 1990; Stamenkovic *et al.,* 1991, Lesley *et al.,* 1992).

1.14.2 MECHANISM OF HYALURONATE BINDING

Early work on the fibroblast HA receptor found that the minimal HA fragment bound is a hexasaccharide. Like many of the Hyaladherins, CD44 contains a region (amino acid 31-120 at the N-terminus) homologous to the S loop of cartilage link proteins (CLP) (Stamenkovic *et al.,* 1989; Goldstein *et al.,* 1989). Conservation of this sequence between the proteins suggests that it fulfils an important function. Greater understanding of HA binding to CD44 and other hyaladherins came from the identification of a HA binding motif in the CLP domain (Aruffo *et al.,* 1990; Culty *et al.,* 1990; Yang *et al.,* 1994), which is found in all other hyaladherins. It consists of two basic amino acids separated by seven non-acidic amino acids (i.e. B(X7)B, where B is arginine or lysine, X is any non-acidic amino acid and X7 contains at least one basic aa), (Yang *et al.,* 1994). Three copies of this motif are found in CD44; one in the N-terminal CLP domain and there are two overlapping motifs in the central region of the extracellular domain (Yang *et al.,* 1994). CD44 has been shown to cluster on the cell surface and this clustering effect has been shown to be necessary for the binding of hyaluronate because it is a multivariant interaction (Underhill *et al.,* 1980). An intact cytoplasmic domain is also required for high affinity binding of HA (Lesley *et al.,* 1992). The binding affinity increases with molecular ionic strength because both molecules are negatively charged and binding is optimal at a neutral pH (Underhill *et al.,* 1992).

CD44 is heavily glycosylated on both N- and O-linked oligosaccarides. Nascent CD44 does not bind HA, but all its intermediate precursors along the glycosylation route possess the ability (Lokeshwar and Bourguigon, 1991). Although a certain level of cell surface glycosylation is probably required for CD44-dependent-HA binding, the complete glycosylation pattern may interfere with the CD44-HA interaction (Naor *et al.,* 1997). The ability of variant isoforms of CD44 to bind HA is variable (Stamenkovic *et al.,* 1991; Bennet *et al.,* 1995). It appears that although CD44v isoforms contain a HA-binding domain, the molecule's ability to bind HA is reduced by the presence of its variant exons (Bennet *et al.,* 1995). The inhibiting effect of the variant exons is additive as a result of the degree of their glycosylation. Binding studies

show that CD44 variants lacking O-linked glycosylation can bind HA as efficiently as CD44s. N-linked glycosylation has been shown to reduce the ability of CD44 to bind HA (Lesley and Hyman, 1992). This regulatory glycosylation can occur at the level of alternative splicing and also at the level of expression of glycosyltransferase (Bennet *et al.,* 1995).

Not all CD44-expressing cells are able to bind HA, although some acquire the property after activation or chemical modification. Thus the presence of CD44 is required but not sufficient for HA binding, this may suggest that the process is regulated by cellular factors (Naor *et al.,* 1997). Neither does HA binding correlate with the amount of CD44 expressed, as a cell with a large amount of CD44 may not bind HA, while another with a lower level of CD44 may be binding competent. It has been proposed that there may be a particular HA-binding form of CD44, although exactly what that is, is unclear (Borland *et al* 1998). Given the ubiquity of CD44 and HA, these observations are hardly unusual, as only tight binding regulation may prevent unnecessary or even harmful CD44-dependent cell adherence (Naor *et al* 1997). HA-binding is induced on some CD44-positive, non-HA-binding cell lines and normal haematopoietic cells when they are treated with an activating CD44 monoclonal antibody, or when the cells are activated or induced to differentiate by treatment with phorbol esters (Lesley *et al.,* 1990a, 1993a). The induction of HA-binding by activating antibodies could be due to a conformational change in CD44 or may occur as a consequence of clustering of CD44 on the cell surface (Borland *et al.*, 1998).

1.14.3 DEGRADATION OF HYALURONATE.

CD44 also plays an important role in the degradation of HA by pulmonary macrophages and other cells (Orkin *et al.,* 1982; Culty *et al.,* 1992). This degradation is a multi-step process in which HA is first bound to the cell surface, then internalized, brought into a lysosomal compartment and finally broken down by acid hydrolases. The first step of this process can be inhibited by the addition of lysomotrophic agents, which prevent the acidification of the lysosomal compartment necessary for the acid hydrolases (Culty *et al.,* 1992). The degradation of HA may be important in a number of situations. For

example, in the adult lung tissue excessive amounts of HA would interfere with the exchange of gas and blood. Here macrophages take up HA and remove it from the system. Another situation is the removal of HA from the lymphatic fluids. In this case a highly branched population of dendritic cells present in the lymph nodes appear to bind and degrade HA from the lymph (Fraser *et al.,* 1988; 1989). These cells also express relatively large amounts of CD44, suggesting that it plays a role in this situation as well.

1.14.4 NON-HYALURONATE LIGANDS OF CD44.

Although HA is the principal ligand of CD44 it is by no means the only one. CD44 can adhere to the ECM components collagen (Carter and Wayner, 1988; Lokeshwar and Bourguinon, 1991; Jalkanen and Jalkanen, 1992; Romaris *et al.,* 1995), laminin (Jalkanen and Jalkanen, 1992) and chondroitin sulphate (Aruffo *et al.,* 1990; Sy *et al.,* 1991), albeit, as indicated in some studies with lower affinity (Aruffo *et al.,* 1990; Peach *et al.,* 1993). The minor chondroitin-sulphated isoform of CD44 on human lymphocytes binds fibronectin, laminin and collagen type-I *in vitro* and binding depends on the chondroitin sulphate chains (Jalkanen and Jalkanen, 1992; Romaris *et al.,* 1995). Osteopontin (or Eta-1), a chemotactic factor secreted by a number of cell types including activated T-cells, osteoblasts and macrophages, is a CD44 ligand. This non-glycosylated phosphoprotein binds mouse fibroblasts transfected with CD44 v4-7 and also CD44s (Weber *et al.,* 1996). The serglycins are a heterogeneous family of proteoglycans that share a core protein composed primarily of Ser-Gly repeats and differ in the GAG attached. Serglycin/gp600 is a small chondroitin-sulphated proteoglycan stored in the intracellular secretary granules of lymphoid, myeloid and some tumour cells (Stevens *et al.,* 1989). The chondroitin 4-sulphate chains of serglycin /gp600 are essential to its interaction with CD44. The CD44-related binding of peripheral lymphocytes and CTLs to serglycin/gp600 allows CTL activated with anti-CD44 and anti-CD3 mAbs to release granzyme A, suggesting a physiological role for this proteoglycan (Toyama-Sorimachi *et al.,* 1995).

1.15 PHYSIOLOGICAL ROLES OF CD44.

The varied structure and distribution of CD44 suggests that the molecule has a variety of functions, some of which are discussed in the following section.

1.15.1 CELLULAR ADHESION

CD44 is used in many cell types to mediate HA-dependent adhesion. Aggregation of macrophages and lymphocytes is induced by exogenous HA (Pessac and Defendi, 1972; Green *et al.,* 1988). Cultured fibroblasts can aggregate spontaneously by using exogenous HA present on the cells (Underhill and Dorfman, 1978; Underhill and Toole, 1981). HA based adhesion is reduced in the presence of hyaluronidase, low levels of hyluronate, high concentrations of chondroitin, or monoclonal antibodies to the HA-receptor (Pessac and Defendi, 1972; Underhill *et al.,* 1987). CD44 binding to HA is relatively weak in comparison with other cell adhesion mechanisms, such as those involving cadherins or integrins. This has been suggested as an advantage where cells only need to be held close together for the exchange of chemical signals (Underhill 1992). Exon v10 has a serine glycine motif and functions as a chondroitin sulphate attachment and is used in preference to the CD44 chondroitin/HA binding site. This moiety is recognised by other CD44 molecules and is bound. Exon v10 can promote homotypic or heterotypic cell-cell adhesion *in vitro* (Cooper and Doughtery, 1995).

1.15.2 LYMPH NODE HOMING AND LYMPHOCYTE ACTIVATION

One of the main functions of CD44 is lymph node homing which was studied extensively in the late 1980's and the early 1990's. Depletion of CD44 positive cells from bone marrow using anti-CD44 monoclonal antibodies prevented their ability to reconstitute the thymus of irradiated mice. This illustrated that CD44 is a homing receptor for migrating thymus progenitor cells (O'Neill, 1989). In contrast migration of lymphocytes into the lymph nodes and other organs was normal, suggesting that other molecules are involved (Camp *et al.,* 1993). Lymph node homing is achieved by a specific interaction between the middle domain of CD44s on lymphocytes and a protein

present on the high endothelial cells of peyers patch and lymph nodes, called mucosal addressin (Huet *et al.,* 1989). The CD44v6 has been shown to have a crucial role in the movement and homing of antigen activated lymphocytes in lymph nodes (Arch *et al.,* 1992).

In addition, after antigenic and mitogenic stimulation, CD44 expression on activated B and T cells increased remarkably (Stauder and Gunthert, 1995). CD44v6 has been shown to be required for activation of B and T lymphocytes and maturation of stem cell progenitors (Arch *et al.,* 1992). The precise mechanism involved has yet to be elucidated though it is thought that activation and interaction of lymphocytes requires crosslinking of CD44 and additional cofactors and adhesion molecules. CD44v3 and CD44v6 have also been shown to be expressed transiently as an early event on the blast cells of lymph nodes and spleen after activation of lymphocytes (Arch *et al.,* 1992; Koopman *et al.,* 1993).

1.15.3 CYTOKINE AND GROWTH FACTOR PRESENTATION BY CD44

Cytokines, chemokines and growth factors may be best presented to travelling leucocytes if anchored to the substratum, thus preventing them from being swept away, as by the blood stream (Tanaka *et al.,* 1993). The role of macrophage inflamitory protein-1-β (MIP-1β) in T-cell adhesion has been reported by Tanaka *et al.,* (1995). Their results show that 1) MIP-1β is present on endothelium; 2) immobilized MIP-1β induces binding of T-cells to VCAM *in vitro*; and 3) MIP-1β is immobilized by binding to CD44. Circulating T-cells recognise adhesion molecules such as selectins expressed on endothelial cells and loosely attach to them. Subsequently, T-cells are activated through binding of MIP-1β immobilized by CD44 and then they adhere tightly and migrate to extravasate. Although, so far there is no evidence that tumour cells recognise cytokines presented by CD44, it can be speculated that similar events might happen in the interaction between tumour cells and endothelial cells.

CD44 is modified with heparin sulphate. Growth factors and cytokines which have affinity to heparin are potent binders of heparan sulphate. Heparin-binding growth

factors and cytokines comprise many factors including basic fibroblast growth factor (bFGF), heparin-binding epidermal growth factor (HB-EGF), hepatocyte growth factor (HGF), platelet derived growth factor PDGF and IL-8 in addition to MIP-1β. CD44 containing the alternatively spliced exon v3 in keratinocytes bind bFGF and HB-EGF via heparan sulphate (Bennet *et al.,* 1995). Since some of the heparin-binding growth factors stimulate tumour cell growth, motility and invasion, endothelial cell presenting growth factors on CD44 may offer favourable conditions for the survival, growth and extravasation of tumour cells. Conversely, binding of bFGF and IL-8 on CD44 heperan sulphate chains may stimulate enothelial cell angiogenesis (Hadmada *et al.,* 1998). CD44 has been implicated in many other physiological roles such as angiogenesis and wound healing and many more which are reviewed by Naor *et al* (1997).

1.16 THE ROLE OF CD44 IN NEOPLASIA

Tremendous interest in a possible role for CD44 in neoplasia was generated when Gunthert and colleagues conferred metastatic potential on a non-metastasising cell line by transfecting a variant of CD44 (Gunthert *et al.,* 1991). The initial work was to identify the membrane proteins on a metastasising rat pancreatic adenocarcinoma by the use of monoclonal antibodies. These antibodies were screened on a bacterial cDNA expression library and one of the cDNA clones was found to encode a variant isoform of CD44. This cDNA was transfected into a non-metastasising, rat pancreatic adenocarcinoma, which although expressing CD44s, did not express this particular variant. The new cell line was found to have gained metastatic properties when injected into rats (Gunthert *et al.,* 1991). CD44v6 gained further interest when it was discovered that T-cells activated in both mouse and humans are associated with transitional expression of v6-containing variants and that specific mAbs to CD44v6 inhibited various immunological function of the activated cells (Arch *et al.,* 1992; Moll *et al.,* 1996).

1.16.1 THE ROLE OF CD44 IN METASTASIS FORMATION

Metastatic spread requires a series of interactions between the tumour cell and the surrounding extracellular matrix and non-tumour cells. These interactions will depend on cell surface determinants such as extracellular receptors for matrix and lamina, surface bound proteolytic enzymes, cell adhesion molecules and growth factor receptors (Gunthert *et al.,* 1991). The metastasising tumour cell copies the same mechanism of normal cellular migration. CD44 can function as a cell surface determinant for many of the roles required for metastatic spread to occur (Sneath and Mangham, 1998). The function of CD44v6 in the metastatic process can be blocked by a mAb to CD44v6, however, this observation does not reveal in which step or steps CD44 is involved, although the mAb has to be given early on to stop metastatic process (Reber *et al.,* 1990; Seiter *et al.,* 1993).

The theoretical steps in the metastatic process are known as the metastatic cascade (Zetter, 1993; Jiang, 1994)and they consist of; 1) loss of contact with the surrounding tumour cells or neighbouring cells; 2) breakthrough of the basement membrane and penetration of the vessel walls; 3) survival of shearing forces in the blood/lymph stream; 4) adhesion and penetration through the vessel walls; 5) expansion into foreign tissue; 6) induction of vascularisation of the tumour. For a tumour cell to lose contact with its neighbouring cells, its adhesive properties must change. Changing the cellular CD44 profile could certainly achieve this. Increased expression of CD44 can enhance binding to HA and a pericellular matrix of HA may decrease the affinity of a cell for surrounding HA deficient cells by interfering with the adhesion processes, thus leading to detachment. This increased mobility is thought to be initiated by CD44, as it is linked to the cells cytoskeleton (Trochon *et al.,* 1996; Bourguignon *et al.*, 1993). A CD44 ligand complex may mediate the mechanical force and transmit intracellular locomotary signals via the cytoskeleton. This response may lead to the cells enhanced movement along a HA rich surface (Sneath and Mangham, 1998). As previously discussed, CD44 has the ability to uptake and degrade HA (see section 1.14.1-2), this property could allow tumour cells to escape entrapment within HA rich environments. The ability of CD44 to degrade HA could also be used for tumour cells to assist its path

through the basement membrane and vessel wall. Some cell adhesion molecules have an inhibitory role in the process of metastasis. Downregulation of adhesion molecules by tumour cells may free tumour cells from a tumour mass and allow migration and dissemination of the tumour cell. A tumour cell that can only bind but never free itself will never be able to metastasise. Therefore downregulation of CD44 may be beneficial for the growth or metastasis of some tumour cells (Sy *et al.,* 1997). Down regulation of CD44 on neuroblastoma is a marker for aggressiveness (Combaret *et al.,* 1995).

1.16.2 Role of CD44 in tumorigenesis

There are many possible theories about the possible role of CD44 in tumorigenesis. CD44 expression is associated with a high rate of cell division. The proliferation status of tumour cells increase when cultured on anti-v6 antibody coated plates. CD44v6 on the cell surface is though to cross link with other CD44v6 molecules, initiating signals of growth promoting activity (Zoller, 1995). Interaction between CD44 and its ligands might induce the tumour cells to produce autocrine growth factors which may be critical for tumour growth. The function of CD44 beyond cellular adhesion requires the transmission of intracellular signals. Some of these signals are thought to occur via the cytoskeleton and may enable CD44 to signal to both the locomotary and mitogenic machinery of the cell (Trochon *et al.,* 1996). Tumour cells expressing CD44 can adhere to the extra-cellular matrix through its ligand, including HA. This might allow tumour cells to colonise this environment more efficiently. The function of uptake and degradation of HA by CD44 could enable tumours cells to invade HA rich tissues (Sneath and Mangham, 1998).

1.16.3 CD44 in specific tumour types

Since the initial research illustrating the potentially crucial role of CD44 in tumorigensis and the metastatic cascade, there have been many studies investigating the pattern of CD44 in tumours. The following section gives a brief description of the role of CD44 standard and variant isoform expression in various cell types.

Lung carcinoma: CD44 has been detected in normal lung tissue and CD44 isoforms containing v6 or v9 were found on epithelial cells only. Some lung cancers retain the epithelial pattern of variant expression where as others show significantly reduced or absent, expression of CD44 standard or variants. No evidence correlating CD44v expression with tumour metastastic capacity has been observed (Givehchian *et al.,* 1996), suggesting that down regulation of CD44 standard and CD44 variants may contribute to metastatic spread, rather than enhanced expression of specific variant CD44 isoforms.

Melanoma: Whereas melanocytes express high levels of CD44s but not CD44v melanoma lesions express in addition to CD44s, CD44 variant containing v5 and v10, according to one study (Manter-Horst *et al.,* 1995) and CD44v7-8 according to another study (Korabiowska *et al.,* 1995). Tumour progression from common nevi through early and advanced primary melanoma to metastatic melanoma was followed by a gradual increase in the proportion of patients with v5 positive lesions (from 16 to 60%) (Manter-Host *et al.,* 1995).

Breast carcinoma: Studies investigating CD44 expression in breast carcinoma have indicated that expression could be regulated by hormones. Increase in the expression of oestrogen and progesterone receptors showed a positive correlation with CD44v6 (Friedrichs *et al.,* 1995). Several studies have evaluated CD44 expression in breast carcinomas with varying results. Sinn and colleagues found a correlation between CD44v3/v4 and CD44v6 and increased tumour grade (Sinn *et al.,* 1995), whereas Friedrichs and colleagues found a correlation between expression of CD44s and CD44v9 and tumour grade (Friedrichs *et al.,* 1995). Neither study found CD44 or any of its variants to be independent prognostic indicators, as was the case in a similar study by Kaufmann *et al.,* (1995). Differences between studies might result from varying techniques of recognition. Using immunohistochemistry, Sinn *et al.,* showed a higher expression of CD44 at the tumour-stroma interface and that all regional lymph node metastases were homogeneously positive for variants of CD44. A more recent study using immunohistochemistry on 218 primary breast carcinomas, found that CD44s and CD44v6 did not correlate with known predictors of poor prognosis (Stevens *et al.,* 1996).

Ovarian carcinoma: Immunostaining with polyclonal antibodies specific for CD44v3-10 revealed a detectable reaction with tumour cells in 75% of ovarian cancer specimens. When statistical analysis was confined to population with advanced disease, the overall survival of patients with tumours expressing CD44 variants were shorter than those of patients which did not express CD44 variants (Uhl-Steidl *et al.,* 1995). Sliutz and colleagues showed that ovarian tumour samples exhibit a more complex pattern of CD44 expression than normal ovarian tissue and proposed that this expression is reflected in its serum concentration (Sliutz *et al* 1995). However, the variants of CD44 detected in the serum did not correlate with any clinical parameter and it was suggested that the dominant serum CD44 variants were produced by natural sources masking the pathological output (Sliutz *et al.,* 1995).

Gastrointestinal carcinoma: The two main types of gastric cancer, intestinal and diffuse, differ in histological features and have different patterns of CD44 expression. The intestinal type of gastric cancer express mainly v6, whereas the diffuse type is mainly v6 negative (Dammrich *et al.,* 1995). It is interesting that the v6 positive intestinal type appeared to have a more infiltrating type of growth compared with the CD44v6 negative diffuse type. Mulder and colleagues found no correlation between CD44v6 expression in gastric carcinoma and prognosis (Mulder *et al.,* 1995). CD44v5 expression correlated with significantly shorter overall survival time. Some studies have found CD44v9 variants to be an independent prognostic indicator for gastrointestinal tumours. It was found that CD44v9 expression in primary gastric carcinoma had a statistical correlation with tumour recurrence and mortality (Mayer *et al.,* 1993). Yamaguchi and colleagues found that the rate of CD44v8-10 expression was significantly higher in tumours from patients with liver metastasis than those without, but no correlation was found between CD44v8-10 expression and prognosis (Yamaguchi *et al.* 1995). Lymph node metastasis is a critical prognostic factor for gastric cancer and a recent study illustrated that expression of CD44v6 increased the lymphatic potential of gastric cancer cells (Kurozumi *et al.,* 1998).

In normal colonic mucosa, CD44s or CD44v expression is mostly confined to the proliferating zone of the crypts, as indicated by immunohistochemistry (IHC). In other sites CD44s expression is practically absent and CD44v are not detected (Abbasi *et al.,* 1993; Wielenga *et al.,* 1993). Using IHC, RT-PCR and *in situ* hybridisation, some but not all investigators have demonstrated that CD44s and CD44v are expressed in the primary malignant colorectal tissue of at least 50% of patients and in lung, liver and lymph node metastases of all patients (Wielenga *et al.,* 1993; Suh *et al.,* 1995). Higashikawa and colleagues found that the colorectal cancers they examined over-expressed high molecular weight variants including the exon v6. CD44v6 was also found to correlate with tumour stage or metastatic status and was suggested to be a common phenotype of colorectal cancer. However, some studies have found the CD44v9 variant to be an independent prognostic indicator of gastrointestinal tumours. Another study found CD44v8-10 expression to be an independent prognostic indicator for colorectal cancer (Yamaguchi *et al.,* 1996). Takahashi group found that expression of CD44s by colon carcinoma cells enhances their HA binding and this enhancement seems to reduce both *in vitro* and *in vivo* growth of the carcinoma cells. They also reported that the HA binding activity of CD44 was less pronounced in highly metastatic cell lines compared with poorly metastatic cell lines. The difference in HA binding ability was suggested to be caused by post-translational modifications (Takahashi *et al.,* 1995). This lead to the conclusion that the functional consequences of the association of decreased CD44s expression, with colonic mucosa transformation, may be important as the expression of high molecular weight CD44 variants by tumours increases (Takahashi *et al.,* 1995). Furthermore, tumour tissues from 16-20 patients with colon cancer showed abnormal retention of intron 9 (located between exon v4 and v5) in CD44 transcripts. Such aberrant expression of intron 9 was also identified in the mRNA of oesophageal, colon and breast carcinoma cell lines (Yoshida *et al.,* 1995).

CD44 standard and variant expression has been examined in many other carcinomas such as thyroid, pancreatic leukaemia and prostrate for more comprehensive reviews see Gauntert *et al.,* 1995; Naor *et al.,* 1997; Sneath and Mangham, 1998. Although the data in the literature are often contradictory due to variable methodology and the misguided belief that all tumours must behave in an identical manner, the preliminary conclusion

that in many human cancers CD44 standard and variant expression are upregulated seems justified (Gunthert *et al.,* 1995; Naor *et al.,* 1997). The extensive assembly of CD44v and aberrant gene products in tumour cells may reflect the release from alternative splicing control, a phenomenon that is still ill-defined but that could eventually be used as a diagnostic tool. The contradictory findings do not at this stage allow the establishment of a conclusive concept regarding the role of CD44s or its variants in the development of cancer or the prognostic value of these molecules. On the other hand, the results are sufficiently interesting to encourage further research (Naor *et al.,* 1997). Thus, in conclusion it can be said that many but not all types of cancer cells are able so seek a selective advantage by the expression of CD44 proteins.

1.17 CD44 AND EBV

Due to the considerable body of evidence of a role for CD44 and CD44v isoform expression in neoplasia it is not surprising that the expression of CD44 in EBV infected cells and in EBV-related malignancies has received a lot of attention in recent years. A study aimed at characterising the pattern of CD44 isoform expression in nasopharyngeal carcinoma (NPC) employed both RT-PCR and monoclonal antibodies (mAb) directed against the variant exons of interest (Brooks *et al.,* 1995). The expression of CD44v6-10 and CD44v8-10 was detected by RT-PCR, however, the pattern of expression detected using mAbs differed. CD44s was widely distributed both on epithelial and on lymphoid cells, while the CD44v8-10 appeared restricted to the neoplastic epithelial cells and was not present on infiltrating stromal elements. Expression of v6 containing isoforms was detected on two thirds of the NPC biopsy samples, in such cases expression was usually strongest in isolated patches of tumour cells (Brooks *et al.,* 1995). Interestingly, a study on non-Hodgkin's lymphoma using mAbs revealed a similar weak focal pattern of staining on low grade malignancies and a strong upregulation of CD44v6 isoforms correlating with high grade malignancy (Terpe *et al.,* 1994). The expression of isoforms containing exon v6 has also been linked to advanced stages of colon cancer and to high grade human lymphomas (Koopman *et al.,* 1993; Terpe *et al.,* 1994). In view of the heterogeneous pattern of LMP1 expression in NPC and the fact that LMP1 has previously been shown to cause upregulation of CD44 when

transfected into the EBV-positive LMP1-negative BL cell line Daudi (Wang *et al.,* 1990), the pattern of CD44 isoform expression was also examined by Brooks and colleagues (1995) in LMP1 positive and LMP1 negative NPC tumours. A correlation between the LMP1 positive or negative status of NPC tumours and the expression of specific variant CD44 isoforms was not observed. However, only a small number of tumours, ten in total, were examined four of which were LMP1 positive, thus perhaps a larger study may reveal more conclusive results.

EBV has been identified in about 10% of gastric carcinomas (Leoncini *et al.,* 1993; Fukayama *et al.,* 1994). The EBV in EBV-associated gastric carcinoma (EBVaGC) is monoclonal and EBVaGC appears to have a better prognosis than EBV-negative gastric carcinoma (Chong *et al.,* 1994; 1997). Immunohistochemical analysis was used to detect CD44v expression in gastric carcinoma and to evaluate the role of EBV in CD44 expression in EBVaGC. Significant correlation was found between the expression of CD44v3-5 and v6 and EBVaGC. Several groups have reported that CD44v expression in gastric carcinoma is linked with poor prognosis, this does not appear to be the case in EBVaGC (Chong *et al.,* 1997). The profile of EBV encoded proteins expressed in gastric carcinoma has been shown to resemble type-I latency (Fukayama *et al.,* 1994). Therefore the expression of CD44vs might be differentially regulated from that of CD44s and independent of LMP1-expression in this EBV-associated epithelial malignancy. It is possible that EBV infection may influence CD44 expression by interacting with cytokine genes, such as those for TNFα, IFNγ and interleukin 10, which are known to modulate CD44 expression (Mackay *et al.,* 1994; Osada *et al.,* 1995).

Stable expression of CD44s but not the epithelial form of CD44 in the human BL cell line Namalwa, which is constitutively CD44 deficient, has been shown to accelerate local and disseminated tumour development in athymic mice (Sy *et al.,* 1991). The enhancing effect of CD44 expression on lymphoma growth is thought to be due in part to facilitation of cell interaction with host tissue stroma (Sy *et al.,* 1991; 1992). A study by Walter *et al.* (1995), illustrated that BL cells that formed local tumours after xenotransplantation into SCID mice disseminated to lymphoid tissue following

introduction of LMP1. The principal effect of LMP1 introduction was upregulation of CD44 expression and further experiments revealed that the introduction of CD44 into a BL cell line which was LMP1 and CD44 negative was observed to confer the disseminated growth pattern associated with LMP1. Together these results indicate that LMP1 may regulate expression of CD44 and play an important role in the behaviour of EBV-based lymphomas (Walter *et al.,* 1995).

Kryworuchko *et al.,* (1995) investigated the association between human B-cell activation and CD44v expression by analysing CD44 expression in resting and mitogenically stimulated B-cells. Results indicate that resting B cells express CD44s and that activation by PMA or surface immunoglobulin crosslinking alone or in the presence of IL-2 induces CD44E, CD44v10 and CD44v6-7 expression. B-cell activation by EBV infection induced the expression of CD44E and CD44v10 but not of CD44v6-7. These results indicate that CD44v6-7 expression depends on the mode of activation (Kryworuchko *et al.,* 1995). The role of EBV in inducing CD44 expression was further investigated using a panel of BL cell lines that differ in EBV status but share the same genetic background, i.e. BL30 (EBV negative BL cell line), BL30/P3HR1 (BL30 cells infected with an EBNA2 defective EBV strain EBV-P3HR1) and BL30/B95-8 (BL30 cells infected with a wild type EBV strain EBV-B958). Flow cytometry indicated that BL30 and BL30/P3HR1 were CD44 negative but BL30/B95-8 was positive for CD44s, CD44E and CD44v10. The fact that B95-8 strain and not P3HR1 induced CD44 expression in BL30 cells suggests that EBNA2 and or LMP1 may mediate the induction of these isoforms (Kryworuchko *et al.,* 1995).

A recent report by Kryworuchko *et al.,* (1999), investigated the effect of cytokines, especially those involved in B-cell activation and differentiation on CD44-HA interaction using a series of LCLs and EBV-positive BL cell lines. This study indicated that the ability of CD44 to recognise HA is dependent on the mode of activation and stage of transformation of human B-cells. Among the mitogens, PMA and among the cytokines, IL-4 alone induced strong HA recognition in the *in vitro* EBV-infected BL cells BL30/B95-8and *in vivo* infected B cell line IM. Attempts to delineate the molecular mechanism responsible for IL-4 and PMA-induced CD44-mediated HA

adhesion in BL30/B958 cells revealed that the increased HA adhesion correlated with enhanced expression of CD44s and isoforms containing v3, v6 and v9 and increased electrophoretic mobility that maybe due to differential glycosylation of the CD44s protein. Differential utilisation of v4 was also observed. In contrast, LCLs failed to recognise HA following PMA or IL-4. These results suggest that the signalling pathway that mediate CD44 expression and CD44-mediated HA binding are selectively inactivated in LCLs (Kryworuchko *et. al.,* 1999).

CD44 expression was examined using an LCL that was immortalized through a mutant EBV in which the expression of EBNA2 was regulated by the presence or absence of oestrogen. In this cell line, CD44 mRNA as well as CD44s protein levels were upregulated through the conditionally active EBNA2. Thus EBNA2 is implicated in the induction of CD44s in LCLs (Fichter *et al.,* 1997).

Two major subgroups of AIDS-related non Hodgkin's lymphomas (ARL) have been defined, Burkitt-type NHL (BL) and polymorphic centoblastic/immunoblast-rich large cell lymphomas (CB/IB LCL) (Kersten *et al.,* 1998). These subgroups differ in their association with EBV and thus possibly in their pathogenesis. It has been shown that patients with BL have significantly higher CD4-cell counts (greater immunity), 40% of these patients were EBV-positive and displayed type-1 latency. CD44 expression was not detected. In contrast the majority of CB/IB LCL had a low CD4-cell count and were EBV-positive, a high percentage of which displayed type-II or type-III phenotypes. CD44s expression was restricted to CB/IB LCL in which high expression of exon v6 containing isoform was also observed (Kersten *et al.,* 1998). The observed EBV-latency types and full expression of adhesion molecules suggested that defective EBV immunity is important in the pathogenesis of CB/IB LCL (Kersten *et al.,* 1998).

Thus the presence of EBV and the expression of EBV latent genes, particularly EBNA2 and LMP1 play an important role in the induction of both CD44 standard and variant isoform expression. However, this induction appears to be cell-type specific and stimulus specific, with LMP1 appearing to play an important role in CD44 expression in B-cell lines but not in epithelial cells (Wang *et al.,* 1990; Brooks *et al.,* 1995). Also

EBNA2 appears important for the expression of CD44 in an LCL context (Fichter *et al.,* 1997). The activation of B-cells by EBV infection also results in a specific pattern of CD44 isoform expression when compared with mitogen stimulated cells (Kryworuckho *et al.,* 1995). Overall the expression of CD44 in EBV infected cells appears to be related to the latent genes expressed and the cellular context in which they are expressed.

1.18 AIMS OF THIS THESIS.

The research presented in this thesis can be divided into two distinct sections. In the first section (chapters three and four), the expression of the adhesion molecule CD44 in Epstein-Barr infected B-cells is investigated. The effect of EBV-latent proteins on CD44 expression was examined in both type-I and type-III BL cell lines. As LMP1 and EBNA2 are the principal effectors of phenotypic change in EBV-infected B-cells their potential role in regulating CD44 expression was also addressed by expressing these proteins individually in an EBV-negative BL cell background. To this end flow cytometry, western blotting, northern blotting and RPA were employed.

The transcriptional expression pattern of CD44 splice variants was also investigated in EBV-positive BL and LCL cell lines in an attempt to define the splicing pattern of CD44 exons used. This was in part achieved by the development of a novel qualitative and quantitative CD44 exon-specific RPA. Identification of an EBV-positive BL splicing pattern may prove useful as a potential prognostic indicator for EBV-related BL. In addition the ability to accurately analyse CD44 mRNA splice patterns would be of use in helping to further our understanding of the factors that regulate the expression of this important adhesion molecule.

In the second section of this thesis (chapter 5), the effect of the EBV latent proteins EBNA1 and EBNA2 on cellular gene expression was examined by differential display polymerase chain reaction. As both EBNA1 and EBNA2 are required for B-cell immortalization, the identification of any novel gene which is differentially regulated by the expression of either of these latent viral proteins would be of interest.

CHAPTER 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 BIOLOGICAL MATERIALS

2.1.1 CELL LINES

Table 2.1. Cell lines used in this study

Cell Line	EBV Status	Cell Classification	Description
DG75	-	EBV negative BL	Lymphoid B cell line derived from an Israeli Burkitt-like lymphoma case (Ben-Bassat *et al.*, 1977).
DG75 EBNA1	-	Stable transfectant	EBV negative BL cell line stably expressing EBNA1 (Welinder *et al.*, 1987).
DG75 EBNA2	-	Stable transfectant	EBV negative BL cell line stably expressing EBNA2 (Welinder *et al.*, 1987).
DG75 tTA	-	Stable transfectant	EBV negative BL cell line stably transfected with the tetracycline-regulated transactivator only (Floettmann *et al.*, 1996).
DG75 tTA EBNA2	-	Stable transfectant	Tetracycline regulated system whereby the expression of EBNA2 can be induced by the removal of tetracycline from the growth media (Floettmann *et al.*, 1996).
DG75 tTA LMP1	-	Stable transfectant	Tetracycline regulated system whereby the expression of LMP1 can be induced by the removal of tetracycline from the growth media (Floettmann *et al.*, 1996).
Raji	+	Type III	EBV positive lymphoid B-cell line from an African Burkitt lymphoma (Pluvertaft, 1965).
Mutu 1	+	Type I	Early passage BL cell line expressing EBNA1 as the only viral gene (Gregory *et al.*, 1990).
Mutu 3 c95, c62, c148.	+	Type III	are stable clones of the early passage BL cell line Mutu 1 which have, upon serial passage in culture, "drifted" to express the full compliment of EBV latent genes (Gregory *et al.*, 1990).
Mutu 3 (LMP1-)	+	Type III	This is a partially characterised Mutu 3 clone which no longer expresses LMP1.

Kem BL, Rael BL.	+ +	Type I	Early passage BL cell lines expressing EBNA1 as the only viral gene (Patarroyo *et al.,* 1988).
BL41, BL41-B958, IARC 171.	+ + +	Type I Type III LCL	These cells are a matched set. BL41 is an early passage BL cell line expressing EBNA1 as the only viral protein, BL41 B958 is the cell line stably transformed with the EBV virus strain B958 expressing all the EBV latent genes, (Calender *et al.,* 1987) IARC 171 is a spontaneously transformed Lymphoblastoid cell line derived from the same patient (Andersson *et al.,* 1991).
BL72 III IARC 307	+ +	Type III LCL	These cells are a matched pair, BL72 is a group 3 BL cell line expressing all EBV latent genes. IARC 307 is a spontaneously transformed LCL from the same patient (Rowe *et al.,* 1990).
BL74 IARC 290B	+ +	Type I LCL	These cells are a matched pair, BL74 is a group 1 BL cell line expressing only EBNA1. IARC 290B is a spontaneously transformed LCL from the same patient.
Ag876	+	Type III	Type III BL cell line expressing all the EBV latent genes (Dambaugh *et al.,* 1984).
X50-7	+	LCL	Spontaneously transformed LCL (Miller *et al.,* 1984).
Hut 78	-	T cell	EBV negative T cell line.
Jurkat	-	T cell	Acute T-lymphocytic leukemic cell line (Brattsand, *et al.,* 1990).
HT29	-		A colon adenocarcinoma cell line (ATCC No. HTB-38).
C33A, C33A Neo, C33A LMP1.	- - -	Epithelial cell	These are cervical epithelial cell lines. C33A is the parental cell line, C33A Neo is stably transfected with an empty vector, C33A LMP1 is stably transfected with a vector constiutively expressing LMP1 (Miller *et al.,* 1995).

All BL cell lines and LCLs were obtained from Professor Martin Rowe, University of Cardiff, Wales. Hut 78 and HT29 cells were a gift from Dr Aideen Long, St James Hospital, Dublin Ireland. The epithelial cell lines C33A were a gift from Dr Nancy Rabb-Traub University of North Carolina, USA.

2.1.2 ANTIBODIES

BRIC 238, D2.1, L3D-1 and 44F10.2 antibodies to standard CD44 were a generous gift from Dr Sinead McGrath, St James Hospital, Dublin, Ireland. PE2 and CS1-4 (antibodies against EBNA2 and LMP1 respectively) were gifts from Professor Martin Rowe, University of Cardiff, Wales. The antibodies were supplied as cell culture supernatants and stored at 4°C or -20°C prior to dilution.

Monoclonal Antibody	**Supplier**
Anti-CD44 v3 and v6	R&D Systems
Anti CD44 v4, v4-5, v7 and v7-8	Bender Med
Anti-mouse-alkaline phosphatase (AP) conjugate	Promega
Rabbit anti Mouse IgG	Dako
Goat anti-rabbit HRP	Dako
Rabbit anti Mouse FITC	Dako

2.1.3 BACTERIAL STRAINS

E.coli DH5α, genotype: F-, *end* A1, hsdR17 (r_k-,m_k=), *sup*E44, *thi* -1,λ-, *rec* A1, *gyr* A96, *rel* A1,ϕ 80*lac* ZδM15.

E.coli NK5772, dam-, dcm-, MetB1, Gal T_{22},Lac Y1, Tax-78.

2.1.4 PLASMIDS

pGem 3Zf (+)	Cloning vector from Promega.
pGem 7Zf (+)	Cloning vector from Promega.
pAZ sCD44	CD44 standard cDNA cloned in to pAZ (pAZ is a reduced form of pSP65 vector from Promega) from Dr Ursula Gunthert, Basil, Switzerland.
pAZ vCD44	CD44 variant cDNA cloned into pAZ (pAZ is a reduced form of pSP65 vector from Promega) from Dr Ursula Gunthert, Basil, Switzerland.

pTAg Cloning vector from R&D Systems.

2.1.5 OLIGONUCLEOTIDES

Genosys Biotechnologies Europe Ltd.

β-actin

Forward primer

5' GAA ATC GTG CGT GAC ATT AAG GAG AAG CT 3'

Reverse primer

5' TCA GGA GCA ATG ATC TTG A 3'

CD44 v6-v7

Forward primer

5' CGC GGA TCCAGG CAA CTC CTA GTA GTA C 3'

Reverse primer

5' CGC GGA TCC AGC GTT GTA CTA TGA CTG GA 3'

2.1.6 COMMMERCIAL KITS AND RESTRICTION ENZYMES

All restriction enzymes were supplied by Boehringer Mannheim.

DD RTPCR	Gene Hunter Corporation
Enhanced Chemiluminesence substrate (ECL)	Amersham
InVitro Transcription kit	Promega
InVitro Transcription kit	Pharmingen
Protein assay dye reagent	BIORAD
Qiagen Tip -100	Qiagen
RPA kit	Pharmingen
Taq DNA polymerase	Perkin Elmer and Boehringer Mannheim.
The LigATor	R&D Systems
T7 Sequencing kit	Pharmacia

Wizard® PCR Preps DNA Purification System from Promega.

2.2 CHEMICAL MATERIALS

Protein prestained markers	NEB
^{35}S labeled dATP	Amersham
$\alpha^{32}P$ labeled UTP	Amersham
dNTPs	Pharmacia Biotech
RNase A	Pharmacia Biotech
Marvel	Premier Beverages
Chloroform	ROMIL
Isopropanol	ROMIL
Dimethyl formamide	Riedel-de-Haën

Sigma-Aldrick-Fluka Chemical Co.;

Urea, Dithiothreitol, Coomassie blue R, BCIP/NBT, Tetracycline, Liquid Phenol, Nitocellulose, Ampicillin, Potassium acetate, Tween-20, BSA, Sodium azide, Sigmacote, Ammonium phosphate, Mineral oil, α-Thiol-glycerol, PMSF, MOPS, BCS *E. coli* tRNA, Apoprotinin, Formaldehyde, Micophenolic acid, BCS, Xanthine.

Merck;

Boric acid, Ammonium persulphate, Sodium acetate, Magnesium chloride, Glucose, Sodium chloride, Potassium chloride, Sodium hydroxide, Sodium dodecylsulphate, Calcium chloride, Glycine, Methanol.

BDH;

TEMED, Bromophenol blue, Potassium dihydrogen phosphate, Potassium hydrogen phosphate, Sodium phosphate, Glycerol, Tris(hydroxymethyl)methylamine, EDTA, Magnesium sulphate, Ethidium bromide, Isoamyl alcohol, Hydrochloric acid, Acetic acid, Methanol, Isopropanol, Nondent P40, Sucrose, Paraformaldhyde.

Boehringer Mannheim;

Agarose, Low melt agarose, IPTG, Hygromycin B, Geneticin (G418), Leupeptin.

Oxoid;

Agar technical, Bacto-Tryptone, Yeast extract.

KODAK;

X-ray film, X-ray film developer, X-ray film fixer.

National diagnostics;

Acrylagel, Bis-acrylagel.

Gibco-BRL;

RPMI 1640, McCoys 5A, DMEMH, Trypsin EDTA, Fetal calf serum, Pencillin, Streptomycin, L-Glutamine, Hepes, Sodium Pyruvate, 1Kb DNA ladder, β-galactosidase (X-gal).

Promega;

100bp DNA ladder, RNA markers.

2.3 DNA MANIPULATION

Preparation of all solutions used in chapter two are outlined in appendix A.

2.3.1 STORAGE OF DNA SAMPLES

DNA samples were stored in TE buffer pH 8.0 at 4^{o}C. EDTA was used to chelate heavy metal ions that are needed for DNase activity while storage at pH 8.0 minimises deamidation. DNA was also stored in sterile distilled H_2O (dH_2O).

2.3.2 EQUILIBRATION OF PHENOL

Before use, phenol was equilibrated to pH 8.0 with TrisCl pH 8.0 as DNA partitions into the organic phase at <pH 7.8. Solid phenol was melted at 68^{o}C, hydroxyquinoline was added to a final concentration of 0.1% (w/v) (acts as an antioxidant, a chelator of metal ions, and an RNase inhibitor). An equal volume of buffer (0.5 M TrisCl pH 8) was added to the liquefied phenol and stirred for 15 min. The two phases were then allowed to equilibrate and as much as possible of the upper aqueous phase was removed. The extraction was repeated using equal volumes of 0.1 M TrisCl pH 8 until the pH of the phenol was > 7.8. An equal volume of TrisCl pH 8 and 0.2% (w/v) β-mercaptoethanol were added to the phenol, which was then stored at -20^{o}C in the dark.

2.3.3 PHENOL/CHLOROFORM EXTRACTION AND ETHANOL PRECIPITATION

Phenol/chloroform extraction and ethanol precipitation was carried out to concentrate nucleic acid samples or change the buffers in which a sample was dissolved. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the DNA solution, mixed by vortexing and centrifuged for 10 min at 13000 x g. The upper aqueous phase was removed, taking care not to take any material from the interphase, this was placed in a sterile microfuge tube. An equal volume of chloroform/isoamyl alcohol (24:1) was added to the aqueous phase, vortexed as before and centrifuged for 5 min at 13,000 x g. Again the upper aqueous phase was removed to a fresh tube. One

tenth volume of 3 M sodium acetate (pH 5.2) was added to the solution of DNA, mixed and then 2 volumes of 100% (v/v) ethanol. This mixture was vortexed and incubated at room temperature for 5 min. The DNA samples were then centrifuged for 30 min at 12,000 x g at 4°C, the supernatant was removed and pellets were washed with 1 ml 70% (v/v) ethanol to remove excess salts. The tube was centrifuged for 5 min at 10,000 x g, the supernatant was removed and pellets were air dried for approximately 10 min. Pellets were resuspended in an appropriate volume of sterile Tris-EDTA (TE) (pH 8.0) or dH_2O.

2.3.4 Restriction digestion of DNA

Restriction enzymes bind specifically to and cleave double-stranded DNA at specific sites within or adjacent to a particular sequence known as the recognition site. The restriction enzymes used were supplied with incubation buffers at a concentration of 10X (working concentration 1X). DNA was digested with restriction endonucleases for identification purposes or to linearise or cut fragments from a plasmid. DNA digests were performed by adding

200 ng - 1 μg of DNA (Final concentration of <300 ng/μl)
1 μl of enzyme/μg of DNA solution (~10 U).
10 X buffer to a final concentration of 1X
dH_2O to the final volume required

The reaction was gently mixed, centrifuged, then incubated for 2 hr at the optimum enzyme temperature (between 25°C and 50°C, usually 37°C).

2.3.5 Repair of DNA termini

The majority of restriction endonucleases digest DNA leaving either a 5' or a 3' overhang. When DNA is ligated together these ends must be compatible; if they are not the ends must be repaired and a blunt ended ligation carried out. This was achieved using the Klenow fragment of *Ecoli* DNA polymerase I. The DNA was resuspended at a concentration of 50 μg/ml in 1X Eco Pol buffer (supplied with the Klenow), dNTPs were added to a final concentration of 33 μM each, 1 μl of Klenow was added and the

reaction was placed at 25 °C for 15 min. The enzyme was inactivated by heating to 70°C for 10 min. This DNA was then purified by phenol/chloroform extraction and ethanol precipitation (2.3.3).

2.3.6 LIGATION OF DNA MOLECULES

Several strategies for ligation of DNA molecules were used depending on the nature of the termini. In the case of cohesive termini, ligations of equimolar amounts of vector and insert DNA (<1 μg) were carried out overnight at 16°C in a commercial ligation buffer (5 mM ATP) with 10 units of ligase/ml. After ligation, the samples were heated to 10 min at 70°C to inactivate the ligase (this appears to improve transformation efficiencies).

2.3.7 DEPHOSPHORYLATION OF LINEARISED PLASMID DNA

During ligation, T_4 DNA ligase will catalyse the formation of a phosphodiester bond between adjacent nucleotides only if one contains a 5' phosphate group and the other contains a 3' hydroxyl group. Recircularisation of plasmid DNA can therefore be minimized by removing the 5' phosphate groups after treatment with calf intestinal phosphatase enzyme (CIP). Digested DNAs (<100 ng/μl) were dephosporlylated using CIP in a 100 μl volume (CIP was added 1 unit/100 pmoles for cohesive termini and 1 unit/2 pmole for blunt termini). The solution was vortexed, centrifuged briefly and incubated for 30 min at 37°C. This was followed by an enzyme denaturation step achieved by heating to 75°C for 10 min. This DNA was purified by phenol/chloroform extraction and ethanol precipitation (2.3.3).

2.3.8 PREPARATION OF COMPETENT CELLS

The Calcium chloride ($CaCl_2$) method was employed to prepare competent cells. An *E.coli* strain was streaked from a glycerol stock on to an LB agar plate and incubated at 37°C overnight. An isolated colony was then picked using a sterile inoculating loop and used to inoculate 5 ml of SOB (appendix A) broth. This culture was incubated in a

shaking incubator at 200 rpm overnight at 37°C. The resulting culture (2 ml) was then used to inoculate 100 ml of sterile SOB and incubated at 37°C until the O.D. of the culture at 640 nm was between 0.4 and 0.8 (~2 hr 15 min). The cells were then transferred to two sterile 50 ml falcon tubes and incubated on ice for 10 min followed by centrifugation at 4,000 x g, 4 °C for 10 min. The resulting pellets were resuspended in 25 ml of 100 mM $CaCL_2$, mixed gently and incubated on ice for a further 20 min. Centrifugation was carried out as before (4,000 x g at 4°C for 10 min) followed by removal of the supernatant and the pellet was then resuspended in a 1% (w/v) volume of $CaCl_2$. The competent cells were stored on ice and used within 24 hr.

2.3.9 TRANSFORMATIONS

Two hundred microliters of competent cells were placed in a pre-chilled microcentifuge tube containing 10µl of DNA at a concentration of ~100 ng per 10 µl. The contents were mixed gently and incubated on ice for 30 min, during which time an aliquot of SOC (appendix A) was pre-heated at 42°C. After 30 min on ice the cells were heat-pulsed at 42°C for 90 s followed by incubation on ice for a future 2 min. One mililiter of preheated SOC was then added to the cells and incubated at 37°C in a shaking incubator for 1 hr 10 min. The cells were concentrated by centrifugation following which ~800 µl of supernatant was removed and discarded. The cells were resuspended in the remaining supernatant and plated out with the appropriate controls on LB plates containing ampicillin and incubated overnight at 37°C. If the cells are transformed they become ampicillin resistant thus only transformed cells will yield colonies. These were used to prepare broth cultures by inoculating 5 ml of LB containing ampicillin, incubated over night at 37°C and DNA minipreparations were carried out as described in section 2.3.10.

2.3.10 SMALL SCALE PREPARATION OF PLASMID DNA (MINIPREP)

This is a modification of the method of Birnboim and Doly, (1979) and Ish-Horowicz and Burke (1981). A single bacterial colony was used to inoculate 5 ml of LB medium (with appropriate antibiotic) and incubated overnight at 37°C. An aliquot (1.5 ml) of

this culture was added to a sterile microfuge tube and centrifuged for 30 s at room temperature, the remainder was stored at 4°C. The medium was removed from the tube, leaving the pellet as dry as possible. The pellet was resuspended thoroughly in 100 µl of solution I by vigorous vortexing. To this 200 µl of freshly prepared solution II was added, the tube contents were mixed by inverting the tube rapidly a number of times. Ice-cold solution III (150 µl) was added and the tubes were vortexed gently for 10 s.

The lysate was centrifuged for 5 min at 12,000 x g, the supernatant was transferred to a fresh tube, taking care not to carry over any of the white precipitate. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added, mixed by vortexing and centrifuged for 5 min at 12,000 x g. The upper aqueous phase was removed to a fresh tube, to which 2 volumes of 100% (v/v) ethanol were added, the solution was vortexed and centrifuged for 5 min at 12,000 x g. The supernatant was discarded, the pellet was washed with 1 ml 70% (v/v) ethanol, centrifuged as before and the supernatant was removed. The pellet was air-dried, then resuspended in 50 µl of TE (pH 8.0), 1 µl of DNase-free RNase A (20 µg/ml) was also added, vortexed, incubated at 37°C for 1 hr then stored at 4°C. Glycerol stocks of all bacterial cultures were prepared at this stage by the addition of 0.5 ml of a 50% (v/v) glycerol solution to 0.5 ml of the overnight bacterial culture of interest and storing at -80°C.

2.3.11 QIAGEN™ PLASMID DNA PURIFICATION PROTOCOL

Plasmid DNA was purified using the QIAGEN-tip 100 isolation system from Promega. All buffers used are described in appendix A. A glycerol stock of the bacteria of interest was streaked out on LB ampicillin agar and incubated overnight at 37°C, an isolated colony from this plate was used to inoculate a 5 ml LB ampicillin starter culture and incubated in a shaking incubator at (~300rpm) 37°C for 8 hr. One millilitre of the starter culture was used to inoculated 25 ml of LB ampicillin in a 250 ml sterile flask and incubated overnight in a shaking incubator at 37°C. The O.D. of the culture must read 1-1.5 at 600 nm. The following centrifugation steps were carried out using a JA-20 rotor in a Beckman centrifuge. The bacteria culture was transferred to a centrifuge tube and centrifuged by spinning at 6,000 x g for 15 min at 4°C. The supernatant was

removed and the pellet was dried by inverting the tube on tissue paper and allowing the supernatent to drain off. The bacterial pellet was resuspended completely in 4 ml of Buffer P1 containing RNase, 4 ml of freshly prepared Buffer P2 was added and incubated at room temperature for 5 min. Following incubation, 10 ml of prechilled Buffer P3 was added, immediately mixed gently by inverting the tube 5-6 times then incubated on ice for 20 min. The mixture was then centrifuged for 1 hr at 20,000 x g at 4°C.

The Qiagen-tip 100 was equilibrated by applying 4 ml of QBT buffer and allowing the column to empty by gravity. The column does not dry out at this stage as the flow of buffer will stop when the buffer reaches the upper filter. After the centifugation step the supernatant was removed immediately from the tube without disturbing the pelleted material and applied to the column by filtering through 1MM filter paper. The QIAGEN-tip was washed with 2 x 10 ml of Buffer QC. DNA was then eluted with 5 ml of Buffer QF. DNA was precipitated by adding 0.7 volumes of room-temperature isopropanol and centrifuged immediately at 15,000 x g for 30 min at 4°C and the supernatant was carefully removed. The resulting pellet was washed with 70% (v/v) ethanol, allowed to air dry for 5 min and re-dissolved in a suitable volume of TE or dH_2O. DNA was then quantified by spectrophotometric analysis as described in section 2.3.15.

2.3.12 Agarose Gel Electrophoresis of DNA

Electrophoresis through agarose gels is the standard method used to separate, identify and purify DNA fragments. The technique is simple, rapid to perform and can be used for the isolation of DNA fragments.

An appropriate quantity of agarose or low melt agarose was added to 100 ml 1X TBE /TAE buffer. The amount of agarose depends on the percentage agarose required. This was decided in relation to the size of the DNA fragments being separated. The mixture was boiled to dissolve, when cooled sufficiently (~60°C) the gel was cast into the Hybaid horizontal gel electrophoresis system, the comb was inserted. The gel was

allowed to set before filling the chamber with 1X TBE/TAE, the comb was then removed. To a 20 µl sample, 4 µl of DNA sample buffer was added and loaded into the wells made by the comb. DNA sample buffer was also added at 1X concentration to 500 ng of a 1Kb DNA ladder which was loaded as a size marker. The gel was run at constant voltage (5 V/cm) for 1-2 hr. When complete, the gel was stained in ethidium bromide (0.5 mg/ml) for 30 min, placed in distilled water to destain for 15 min and viewed under UV illumination.

2.3.13 Isolation of DNA from agarose gels

Low melting point agarose gels were prepared in 1X TAE buffer (gel isolation is not carried out in TBE buffer as borate ions are difficult to remove from the resultant DNA solution). Ethidium bromide was added to the samples before electrophoresis so as to minimise manipulations with the fragile low melting point agarose gels. After electrophoresis, the gels were viewed under 70% UV illumination. The time of exposure to UV light was kept to a minimum, as overexposure to UV would cause damage to the DNA. The DNA band of interest was excised from the gel using a clean scalpel, excess agarose was cut away to minimise the size of the gel slice which was then placed in a sterile microfuge tube.

2.3.14 Purification of DNA from low melt agarose

All DNA fragments of interest were purified from agarose using the Wizard® PCR Preps DNA Purification System from Promega as follows; the agarose gel slice (300 mg) was placed in a sterile microcentifuge tube at 70°C until the agarose had melted completely. One mililitre of the resin provided was added to the liquified solution and mixed gently but thoroughly for 20 s (not vortexed).

A 2 ml syringe (plunger removed) was attached to the wizard® minicolumn the DNA/agarose/resin mix was added to the syringe, the plunger was replaced and the mix was pushed gently through the column. The syringe was detached and the plunger removed again, then it was reattached and the column was washed with 2 ml of 80%

(v/v) isopropanol. The column was then spun at 10,000 x g for 2 min to dry the resin. The DNA was eluted by adding 50 µl of TE or dH_2O to the minicolumn and allowed to stand for 1 min at room temperature and centrifuged again at 10,000 x g for 20 s. The purified DNA was stored at 4 °C or at -20 °C.

2.3.15 SPECTROPHOTOMETRIC ANALYSIS OF NUCLEIC ACIDS

DNA and RNA concentration was determined by measuring the absorbance at 260 nm, which is the wavelength at which nucleic acids absorb maximally (λ_{max}). A 50 µg/ml preparation of pure DNA has an absorbance of 1 unit at 260 nm while 40 µg/ml of pure RNA also has an absorbance reading of 1 at this wavelength. The purity of an RNA or DNA preparation was determined by reading absorbance at 260 nm, the λ_{max} for nucleic acids and at 280 nm, the λ_{max} for proteins and obtaining the ratio for these absorbances. Pure DNA and RNA have A_{260}/A_{280} ratios of 1.8 and 2.0 respectively. Lower ratios indicate the presence of protein while higher ratios often indicate residues of organic reagents.

2.4 CELL CULTURE METHODS

All cell culture techniques were performed in a sterile environment using a Holten laminar air flow cabinet. Cells were visualised with an Olympus CK2 inverted phase contrast microscope.

2.4.1 CULTURE OF CELLS IN SUSPENSION

All media compositions and media supplements are given in appendix A. The cell lines DG75, DG75 EBNA 1, DG75 EBNA 2, DG75 tTA, DG75 tTA EBNA 2, DG75 tTA LMP1, HuT 78, HL60, Mutu 1, Mutu 3 c62, c148, c95, Mutu 3 LMP1-, X50-7, BL41, BL41-B958, IARC 171 Rael BL, Kem BL, IARC 307, IARC 290B, Ag876 III, BL72 III, BL74,and RAJI were maintained in supplemented RPMI 1640. Additional supplements were added to some culture media see section 2.4.3 Cultures were seeded at a density of 2 $x10^5$ to 5 x 10^5 cells per ml in 25 cm^2 flasks and expanded in 75 cm^2

flasks. Cells were sub-cultured two or three times per week by harvesting into a sterile centrifuge tube and centrifuging at 1000 x g for 5 min at room temperature. The cell pellet was resuspended gently in an appropriate volume of fresh media and replaced into the tissue culture flask. All cell lines were incubated in a humid 5% CO_2 atmosphere at 37°C in a Heraesus cell culture incubator.

2.4.2 CULTURE OF ADHERENT CELLS

HT29 cells were maintained in McCoy's 5A medium with L-glutamine supplemented with 10% (v/v) FCS, penicillin (100 units/ml) and streptomycin (1μg/ml). C33A, C33A Neo and C33A LMP1 were maintained in supplemented high glucose DMEM supplemented with 10% (v/v) FCS, penicillin (100 units/ml) and streptomycin (1μg/ml). Cultures were seeded into 25 cm^2 and 75 cm^2 tissue culture flasks. As the cells were strongly adherent trypsinisation was required for harvesting prior to sub-culturing. For trypinisation the medium was decanted and the cells were washed with 2 ml of sterile 1X PBS to remove any residual FCS which contains a trypsin-inhibitor activity (α_2-macroglobulin). Two mililitres of 0.25% (v/v) trypsin EDTA (HT29) or 1X trypsin (C33A C33A Neo and C33A LMP1) was then placed in each flask which was incubated at 37°C for 5-10 min or until all cells could be visualised as having detached from the flask surface. The cell suspension was then decanted into a sterile centrifuge tube containing 5 ml of sterile supplemented media (FCS inhibits trypsin) and centrifuged at 1000 x g for 5 min. Cells were resuspended in supplemented medium at 2 to 5 x 10^5 cells/ml, using 5 ml per 25 cm^2 flask and 15 ml per 75 cm^2 flask. Cells were then incubated as in section 2.4.1..

2.4.3 MEDIA SUPPLEMENTS

Supplements were added to the growth media of certain cell lines to (a) improve cellular proliferation or (b) to select cells containing transfected plasmids (all media supplements are outlined in appendix A). L-cysteine is required for the survival and proliferation of most group 1 BL cell lines. However L-cysteine is rapidly oxidated

under normal culture conditions. To improve proliferation of the group 1 Burkitt lymphoma cell line Mutu 1 α-thioglycerol was added to growth media as a stable substitute for L-cysteine. The α-thioglycerol was dissolved in bathocuproine disulfonic acid (BCS) which effectively prevents autoxidation of thiols in liquid solutions. Sodium pyruvate was also added to protect against H_2O_2 which may be generated. HEPES was added to maintain an alkaline pH of 7.4. The cell lines DG75 tTA EBNA2 and DG75 tTA LMP1 are tetracycline responsive cell lines in which the gene of interest is cloned downstream of a promoter containing a binding site for a hybrid tetracycline regulated transactivator (tTA) which is constiutively expressed from a second co-transfected plasmid. Tetracycline binds to the tTA and prevents it binding to the promoter which remains silent, but upon removal of tetracycline from the growth medium the tTA binds the promoter sequence and activates transcription. These cell lines were maintained in supplemented RPMI containing 1 μg/ml of tetracycline. Every three weeks the transfected cells were reselected by the addition of 500 μg/ml of hygromycin to DG75 tTA, 500 μg/ml of hygromycin and 1,000 μg/ml of geneticin (G418) to DG75 tTA EBNA 2 and 800 μg/ml of hygromycin and 2,000 μg/ml of geneticin (G418) to DG75 tTA LMP1.

The stably transfected cell lines DG75 EBNA1 and DG75 EBNA2 were maintained in supplemented RPMI containing 0.5 μg/ml of microphenolic acid and 50 μg/ml of Xanthine. The stably transfected cell lines C33A Neo and C33A LMP1 were maintained in supplemented high-glucose DMEM containing 600 μg/ml of geniticin. The parental cell line C33A was maintained in supplemented high glucose DMEM.

2.4.4 Cell Counts

Cell counts were performed using an improved Neubaue haemocytometer slide. Trypan blue exclusion dye was routinely used to determine cell viability. Ten microlitres of trypan blue was added to 90 μl of a cell suspension and mixed. A sample of this mixture was added to the counting chamber of the haemocytometer and cells were visualised by light microscopy. Viable cells excluded the dye and remained clear while

dead cells stained blue. Cell numbers were acertained by multiplying the average cell count by the dilution factor and again by the volume of the haemocytometer.

2.4.5 CELL STORAGE AND RECOVERY

Cell stocks were prepared for long term storage as follows; Suspension cells: 1 x 10^7 cells in exponential phase were pelleted and resuspended in 800 µl of supplemented RPMI to which 100 µl of FCS was added, then placed on ice for 10 min. DMSO was added to a final concentration of 10% (v/v), mixed gently and transferred to a sterile cryotube. Adherent cells: one confluent 75 cm^2 flask of adherent cells was used per cell stock. Adherent cells were washed with 1X PBS followed by trypsinization and resuspension in 900 µl of FCS and 100 µl of DMSO. The cells were mixed gently and added to a sterile cryotube. The cryotubes were slowly lowered into the gas phase of liquid nitrogen and immersed in liquid nitrogen in a cryofreezer (Cooper Cryoservices Ltd). Cells were recovered from liquid nitrogen by thawing rapidly at 37°C and transferring to a sterile centrifuge tube containing 5 ml of prewarmed supplemented media. The cells were centrifuged at 1000 x g for 5 min, the pellet was resuspended in 5-10 ml of fresh supplemented medium, transferred to a culture flask and incubated at 37°C in 5% CO_2.

2.4.6 FLOW CYTOMETRY

All cells were passaged 24 hr before flow cytometry analysis; 2 x 10^5 cells were used in each experiment. The cells were pelleted at 1000 x g, the supernatant was removed and the pellet washed in 5 ml of PBS and recentirfuged. The supernatant was removed and the pellet stored on ice until required (up to 24 hr). Cell pellets were resuspended in 2 ml of PBS giving a final concentration of 2 x 10^5 cells. The quantity and dilution of the various antibodies used is outlined in table 2.2. All anti-CD44 variant antibodies were prefixed and all standard antibodies were not.

Table 2.2. Conditions for antibodies used in flow cytometry analysis.

Antibody	Name	Dilution	Quantity µl
Anti standard CD44	L3D-1	Neat	10
Anti standard CD44	44F10.2	Neat	5
Anti standard CD44	D2.1	Neat	10
Anti variant CD44	v3, v4-5, v6	1/100	10
Anti variant CD44	v4, v7, v7-8	Neat	5
Irrelevant Ab	IE	Neat	10
Rabbit anti-mouse FITC	FITC	1/50 Protect from light	5

Procedure: An FITC and an IE control were included with each cell line used, the FITC was a control for non-specific fluorescence and the IE was a non-specific murine antibody which controlled against non-specific antibody binding. After the pellets were resuspended, 200 µl of the cell suspension was aliquoted into each tube and the appropriate amount of Ab was added and incubated at room temperature for 10 min. The cells were then washed in 500 µl of washing buffer and microcentrifuged at 2,000 x g at 4°C for 2 min. This step was repeated and the cells were resuspended in 50µl of FITC Labelled monoclonal and incubated at room temperature for 10 min. The washing step was repeated twice as above. The cells were then resuspended in 500 µl of 2% (w/v) paraformaldehyde (fixative) and used in flow cytometry analysis. Alternatively the cells could be stored for up to 7 days when protected from light. When prefixing cells the initial 200 µl aliquot of cells were added to a tube containing 500 µl of 2% (w/v) paraformaldehyde and incubated at room temperature for 10 min. The cells were then washed twice as above and resuspended in 200 µl of 1X PBS. The procedure was carried out as above with the exception of the final step when the cells were resuspended in 500 µl of 0.5% (w/v) as opposed to 2% (w/v) paraformaldehyde.

2.4.7 Immunocytochemistry, Fixation and Staining of Tissue Culture Cells

Examination of the uniformity of EBNA 2 expression in the tetracycline inducible cell line DG75 tTA EBNA2 was examined by immunocytochemistry. Prior to starting the

procedure a bath of methanol and acetone were placed at -20°C for 2 hr. A cell suspension was prepared of 1 x 10^6 cells/ml in supplemented RPMI, 50 µl of this suspension was placed in a 1.5 ml microcentifuge tube which had a hole pierced in the bottom. This tube was then placed in a cytospin and centrifuged onto a clean microscope slide at 700 x g for 5 min. The slide was allowed to air dry for 5 min then placed in ice cold methanol for 5 min. The slide was then air-dried briefly and placed into an ice cold acetone bath for 1 min 45 s. This procedure fixed and permeated the cells. Slides were left to air dry overnight. Primary antibody (PE2 anti-EBNA2) was diluted 1/50 in PBS with 5% (v/v) FCS, 30 µl of the dilution was placed on the cell smear for 30 min in a humid chamber at 4°C. The slide was washed (3 x 3 min) with ~10 ml volumes of PBS, taking care to ensure that the slide did not dry out. Secondary Ab (Anti-mouse AP, Promega) was again diluted 1/50 in PBS containing 5% (v/v) FCS, 100µl was placed on the smear and incubated for 30 min in a humid chamber at 4°C. PBS washing was repeated as before and the slide was allowed to air dry. Finally, 100 µl of substrate (BCIP NBT) was added to the smear and colour was allowed to develop for a minimum of 30 min. When colour was apparent the slide was washed with water and viewed under microscope.

2.5 RNA ANALYSIS

2.5.1 RNASE FREE ENVIRONMENT

RNA is easily degraded by ubiquitous RNase enzymes and thus stringent measures were employed to avoid this potential hazard. All glassware and metal spatulas were baked prior to use at 180°C for 8 hr in order to inactivate any RNase. Sterile disposable plasticware is generally considered RNase free and thus did not require treatment. RNases are resistant to autoclaving but they can be deactivated by the chemical diethylpyro-carbonate (DEPC) when it is added to solutions at a final concentration of 0.1 % (v/v), incubated at room temperature for 18hr and autoclaved. Solutions which contain amines such as Tris cannot be DEPC-treated as the DEPC is inactivated by these chemicals. Solutions containing these chemicals were prepared using DEPC

treated H_2O followed by autoclaving. Hands are a major source of RNase contamination thus gloves were used at all times and changed frequently.

2.5.2 RNA EXTRACTION FROM CULTURED CELLS

Prior to RNA isolation the cells were examined by phase contrast microscopy to determine the condition of the cells. A cell count was performed as described in section 2.4.4. RNA was extracted from cultured cells using the commercial reagent RNA ISOLATOR™. Cells grown in suspension were pelleted and then lysed in RNA ISOLATOR™ by repetitive pipetting. One millilitre of RNA ISOLATOR™ was used per 1 x 10^7 cultured cells. Cells grown in monolayers were lysed directly in the cell culture flasks as trypsin can lead to the introduction of RNases, cells were removed from the flask by a sterile cell scraper and homogenised as above. The homogenised sample was incubated at room temperature for 5 min to allow complete dissociation of nuclear protein complexes, (the procedure may be stopped at this point by storing samples at -70°C). Phase separation was achieved by adding 0.2 ml of chloroform per 1 ml of RNA ISOLATOR. The samples were covered and shaken gently but thoroughly for 15 s or until completely emulsified. Samples were incubated at room temperature for 15 min. The resulting mixture was centrifuged at 12,000 x g for 15 min at 4°C. Following centrifugation the mixture separates into a lower red, phenol-chloroform phase, an interphase and a colourless upper aqueous phase. The aqueous phase, which contains the RNA, was removed to a fresh tube and RNA was precipitated by adding 0.5 ml of isopropanol per ml of RNA ISOLATOR used initially. The samples were stored for 10 min at room temperature, then centirfuged at 12,000 x g for 10 min at 4°C. The resulting RNA pellet was washed using 1 ml of 75% (v/v) ethanol by inverting the tube 5 times. The pellets were then recentrifuged at 10,000 x g for 5 min at 4°C and the 75% (v/v) ethanol was removed. Pellets were air dried and dissolved in DEPC treated upH_2O. The resulting RNA preparation was heated to 60°C and mixed gently to ensure a homogeneous solution prior to aliquoting. An aliquot was removed for spectrophotometric and gel electrophoretic analysis.

2.5.3 mRNA ISOLATION FROM TOTAL RNA

The PolyATtract mRNA isolation system from Promega was used to isolate mRNA from total RNA. This system employed a biotinylated oligo(dT) primer to hybridize at high efficiency in solution to the 3' poly(A) region present in most mature eukaryotic mRNA species. The hybrids were captured by streptavidin coupled to paramagnetic particles (SA-PMPs) and selected using a magnetic separation stand followed by washing at high stringency. The mRNA was eluted from the solid phase by the simple addition of ribonuclease free deionized water. This procedure yields an essentially pure fraction of mature mRNA after only a single round of magnetic separation. The procedure is outlined below.

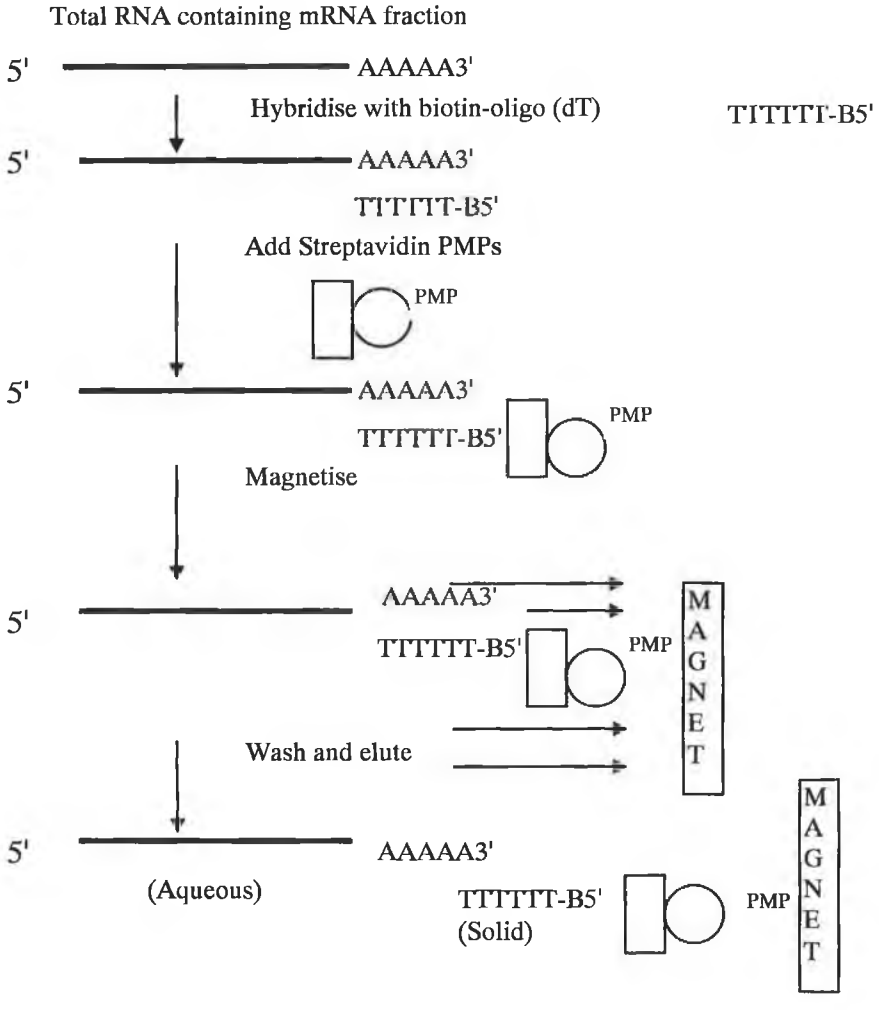


Fig 2.1. Schematic representation of PolyATtract mRNA isolation procedure.

2.5.3.1 ANNEALING OF PROBE

In a sterile RNase free tube 1 mg of total RNA was made up to 500 µl using RNase free water and placed in a 65°C water bath for 10 min. To the RNA 3 µl of biotinlyated oligo (dT) probe and 13 µl of 20X SSC was added, mixed gently and incubated at room temperature until completely cooled. This required approximately 10 min during which stock solutions of 0.5X and 0.1X SSC were prepared.

2.5.3.2 WASHING OF THE PARAMAGNETIC PARTICLES

The SA-PMPs were resuspended by gently flicking the bottom of the tube until they were completely dispersed and then captured using the magnetic stand provided. Thirty seconds were allowed for collection of all particles, the supernatant was removed and the particles were washed by resuspending three times in 0.3 ml of 0.5X SSC each time capturing them using the magnetic stand and carefully removing the supernatant. The washed particles were then resuspended in 0.1 ml of 0.5X SSC.

2.5.3.3 CAPTURE AND WASHING OF ANNEALED OLIGO (DT) - MRNA HYBRIDS

The entire contents of the annealing reaction were added to the tube containing the washed SA-PMPs and incubated at room temperature for 10 min. The SA-PMP were captured and the supernatant was gently removed and retained until after elution and quantification of the mRNA, when it was evident satisfactory binding had taken place. The particles were washed with 0.1X SSC by gently flicking the bottom of the tube ensuring that all particles were resuspended, capturing the particles and removing the supernatant with care. This was repeated four times.

2.5.3.4 ELUTION OF MRNA

In order to elute the mRNA, the particles were resuspended in 100 µl of RNase free water by a gentle flicking of the tube. The particles were magnetically captured and the eluted mRNA containing aqueous phase was removed to a sterile RNase free tube. The

particles were not discarded but the elution step was repeated using 150 µl of RNase free water. Particles which had been carried over were removed at this point by centrifugation at 10,000 x g for 10 min at 4°C and the mRNA was transferred to a fresh RNase free tube. The concentration of the mRNA was determined by spectrophotometric analysis as described in section 2.3.15.

2.5.4 RNA ANALYSIS BY GEL ELECTROPHORESIS

In order to check the integrity of RNA, isolated samples were run on 1% (w/v) agarose gels. These gels were prepared as outlined in section 2.3.12. The RNA samples (5 µl) were prepared for electrophoresis by adding 15 µl of RNA sample buffer and 3 µl of RNA loading buffer. The samples were heated to 65°C for 10 min prior to loading on the gel. The gel was run in 1X TAE as described in section 2.3.12. As ethidium bromide is included in the RNA loading buffer the gels did not require further staining and could be visualised directly on a UV transilluminator. The presence of two strongly staining bands represent the 28 S and the 18 S ribosomal RNAs, which indicated intact RNA. Degradation is observed by a smear running down the length of the gel.

2.5.5 REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION

2.5.5.1 REVERSE TRANSCRIPTION (RT)

This is the process whereby mRNA is transcribed into cDNA using a reverse transcriptase, in this case Moloney Murine Leukemia Virus reverse Transcriptase (MMLVRT). A typical reverse transcription reaction using a random hexamer is outlined below:

Total RNA	2 µg
Random hexamer (Promega)	100 ng
Magnesium Chloride	2.5 mM
BSA	100 µg/ml
rRNasin	40 units

RT MMLV	400 units
5X Buffer	1X
dH_2O	Final volume 40 µl

Program 20°C 1 min, 42°C 50 min, 99°C 6 min, 95°C 5 min.

2.5.5.2 Polymerase Chain Reaction Protocol (PCR)

The polymerase chain reaction (PCR) involves the amplification of specific DNA sequences using DNA primers which anneal to the DNA of interest. The primers are designed so that one anneals to the forward DNA strand and the other anneals to the reverse strand thus allowing polymerisation of both strands by the enzyme Taq DNA polymerase. This results in exponential amplification of the sequence of interest. PCR protocols varied with respect to the DNA amplified.

β-actin PCR

per 100µl reaction	µl
Sterile ultra pure water	71
10X enzyme buffer	10.0
25 mM $MgCl_2$	6.0
dNTP (1 mM each)	5.0
Forward primer 250 ng/µl	1.0
Reverse primer 250 ng/µl	1.0
Taq DNA polymerase 5U/µl	1.0
Template cDNA	5.0
Total volume	100

This reaction mix was overlaid with 50 µl sterile mineral oil before placing in the minicycler (Hrybid).

PCR program		
	94.5 °C 1 min	1 cycle
	94.5 °C 1 min	44 cycles
	34.0 °C 1 min	
	72.0 °C 1 min	
	72.0 °C 10 min	1 cycle

CD44 v6-v7

per 50µl reaction	µl
Sterile ultra pure water	36.5
10X enzyme buffer	5
25 mM $MgCl_2$	2.5
dNTP (5 mM each)	2
Forward primer 10 pM/µl	1
Reverse primer 10 pM/µl	1
Taq DNA polymerase	1
Template DNA 500 ng	1
Total volume	50

This reaction mix was overlaid with 50 µl sterile mineral oil before placing in the minicycler (Hybid).

PCR program	94.5 °C 5 min	1 cycle
	94.5°C 1 min	2 cycles
	53°C 1 min	
	72.0 °C 1 min	
	94.5°C 1 min	30 cycles
	68°C 1 min	
	72.0 °C 1 min	
	72.0 °C 10 min	1 cycle

All PCR products where visualised on agarose gels as outlined in section 2.3.12.

2.6 PREPARATION OF CELL PROTEIN

Prior to protein isolation cells were examined by phase contrast microscopy to determine the condition of the cell cultures. Cell counts were then performed as described in section 2.4.4. Two methods were employed to isolate protein as outlined below. All buffers required are outlined in appendix A.

2.6.1 PREPARATION OF TOTAL CELLULAR PROTEINS (METHOD A)

This method was employed to isolate total cellular protein including nuclear proteins from cultured mammalian cells. Approximately 6 x 10^7 cells from a cell line were used in each protein prep. Cells were pelleted at 1000 x g for 5 min and washed with 10 ml of ice-cold PBS. The cells were then spun at 3,000 x g and all the supernatant was removed. The volume of the pellet was estimated and the cells were dispersed in five volumes of ice-cold suspension buffer. This step was carried out rapidly to avoid proteolytic degradation. An equal volume of 2X SDS gel loading buffer was added, immediately after the suspension buffer, at which stage the sample becomes extremely viscous. Samples were then placed in a boiling water bath for 10 min. The DNA in each sample was sheared by sonication for 1 min on full power. The resulting lysates were transferred to an microcentifuge tube and centrifuged at 10,000 x g for 10 min at room temperature. Supernatants were aliquoted and stored at -20 °C. Samples were analysed by SDS PAGE, loading approximately 6 x 10^5 cells per lane as described in section 2.6.5.

2.6.2 PREPARATION OF CELLULAR PROTEINS (METHOD B)

Protein samples which excluded nuclear proteins were prepared as described by Kelleher and Long, (1992). Suspension cells were pelleted at 2,000 x g for 5 min and washed twice with 5 ml of PBS and the resulting pellet was drained. Adherent cells were washed twice with PBS and then lysed in the culture flasks. Cell lysis was preformed by adding the following per 1 x 10^7 cells: 0.5 ml PBS, 1 µl PMSF (0.1 mM), 3 µl leupeptin (10 mg/ml) and 0.5 ml 1% (v/v). The pellet was vortexed every 5 min for 30 min and then centrifuged at 400 x g for 5 min to deposit the cell debris. The samples are maintained on ice at all times. Supernatant were removed to ultracentrifuge tubes, overlaid with paraffin liquid and ultracentrifuged for 30 min at 100,000 x g. The supernatant was dispensed into aliquots and stored at -20 °C. Lysates were thawed out immediately before use. One 100 µl aliquot was stored on ice for immediate quantification using the Bradford assay as described in section 2.6.3.

2.6.3 ESTIMATION OF PROTEIN CONCENTRATION

In order to standardise the amount of protein sample applied to SDS-PAGE gels the protein concentration of each cell lysate isolated by method B was determined using the Bradford assay (Bradford, 1976). A standard curve was constructed using a stock solution of BSA. The standard solution contained 0, 25, 50, 75 μg or 1 mg of BSA protein per 1 ml of solution. The sample concentration was determined within this range and test solutions falling outside of this were diluted 1:2, 1:5, and 1:10. Standard and test solutions were prepared in a 1:1 solution of PBS and 1% (v/v) Np40. Standards were prepared as indicated in table 2.1.

Table 2.3 Preparation of standard BSA solutions for Bradford assay.

Concentration of standard (μg/100 μl)	**Volume of stock 100 μg/100 μl BSA (μl)**	**Volume of Diluent (μl)**	**Total Volume (μl)**
0	0	100	100
25	25	75	100
50	50	50	100
75	75	25	100
100	100	0	100

The Bradford reagent (Biorad) was diluted 1/10 and filtered through 1MM Whatman paper immediately before pipetting 5 ml into tubes containing 100 μl of sample. The tubes were vortexed briefly and colour development was allowed to proceed for 30 min. Absorbance was read at 595 nm against a reagent blank. A standard curve was plotted for BSA standards (concentration versus absorbance) which allowed estimation of the sample protein concentration.

2.6.4 ACETONE PRECIPITATION OF PROTEINS

Proteins isolated by method B have residual salts which may give rise to diffuse bands on SDS-PAGE, thus acetone precipitation was employed when preparing proteins isolated in this manner for SDS-PAGE. Having determined the protein concentration of

a sample, a sufficient volume was aliquoted to yield 50-100 μg of protein per lane. This volume was diluted 1:5 with ice cold acetone (stored at -20°C), vortexed and incubated at -20°C for a minimum of 30 min. The precipitated protein was briefly microcentrifuged (25,000 x g for 2 min) and the supernatant was removed. Sample buffer (1X) was added so that 20 μl contained the appropriate amount of protein required. The samples were incubated in a boiling water bath for 10 min after which they were subjected to SDS-PAGE or stored at -20°C.

2.6.5 PROTEIN ELECTROPHORESIS, PREPARATION OF SDS-PAGE GELS

A two phase SDS-PAGE system was used to analyse proteins with a 5% stacking gel and a 10% resolving gel as outlined below.

Resolving Gel

10 ml	10% resolving gel(ml)
acrylagel	3.33
bis-acrylagel	1.35
1.5 M Tris (pH 8.8)	2.5
ultrapure water	2.62
10% (v/v) SDS	0.10
10% (v/v) APS	0.10
TEMED	0.004

Stacking gel

2.5 ml	5% stacking gel(ml)
acrylamide	0.42
bis-acrylagel	0.168
1 M Tris (pH 6.8)	0.312
upH_2O	1.55
10% (v/v) SDS	0.025
10% (v/v) APS	0.025
TEMED	0.0025

2.6.6 POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

An ATTO protein gel electrophoresis system was used in this study. Glass plates were washed with detergent, rinsed first with tap water and then with dH_2O and finally wiped in one direction with tissue soaked with 100% (v/v) ethanol. The gasket was placed about the ridged plate, the plates were put together and secured with clamps. The depth of the resolving gel was marked on the outer plate. The resolving gel was then poured to within 2 cm of the top of the larger plate and overlaid with 100% (v/v) ethanol. When set, the ethanol was removed and the stacking gel was poured. A clean comb was inserted and the gel was allowed to polymerise for 45 min-1 hr. The electrophoresis tank was filled with 1X Tris glycine running buffer to the level of the horizontal rubber gasket. After polymerisation the gaskets clamp stands and comb were removed. Unpolymerised gel was removed by gently rinsing the wells with dH_2O, the wells were then straightened using a loading tip. The prepoured gels were lowered into the buffer at an angle to exclude air bubbles from the gel buffer interface. The gel plates were fixed firmly in place with the notched plate innermost. The chamber formed by the inner plates was filled with 1X running buffer, the samples were loaded and the electrodes were attached. The gels were run at 30 mAmps per gel for approximately 1 hr. When complete the plates were removed, separated and the gel was either placed in transfer buffer prior to western blotting or stained in Coomassie blue. Staining took place for 30 min, agitating constantly. The gel was then placed in destain (see appendix A) with constant agitation, until all background staining was removed. The destain was changed as it became saturated with stain.

2.6.7 WESTERN BLOT ANALYSIS

An SDS-PAGE gel was run as described, with pre-stained markers (New England Biolabs). Two pieces of 3MM filter paper were cut to the size of the gel as was the nitrocellulose membrane. The sponges from the transfer apparatus along with 4 pieces of 3 MM filter paper cut to size and the SDS gel were soaked in transfer buffer. Two sponges were placed on each side of the transfer apparatus and 2 pieces of filter paper in turn, on each of these. The gel was placed on one side of the filter paper. The

nitrocellulose membrane which had been pre-wet in distilled water and then soaked in transfer buffer for 5 min and placed on top of the gel, ensuring that no bubbles were trapped between any of the layers. The second stack of filter paper and sponges were placed on top of the membrane, the transfer apparatus was assembled and placed in the blotting apparatus with the gel on the side of the negative (black) electrode and the nitocellulose on the positive (red) side. The voltage was set at 80 volts for 2 hr. After transfer, the apparatus was disassembled and the membrane was washed briefly in TBS to remove any traces of gel, followed by blocking buffer for 1 hr. The membrane was then incubated with the appropriate antibody at the appropriate temperature overnight. Sodium azide was added to each antibody solution to a final concentration of 0.02% (w/v) as a preservative thus permitting reuse of the antibody.

Table 2.4. Incubation conditions for antibodies used in western blotting

Antibody	Name	Dilution	Incubation temperature
Anti-EBNA 2	PE2	1/50	4°C
Anti-LMP 1	CS14	1/50	4°C
Anti-Standard CD44	BRIC 238	1/100	room temperature
Anti-Standard CD44	D2.1	1/100	room temperature
Anti-Variant CD44	v4-v5, v7, v7-8	1/100	room temperature

After overnight incubation, the membrane was washed twice in TBST (0.1%(v/v) Tween-20) for 10 min and once in blocking buffer for 15 min. The filter was then incubated in the secondary antibody, a mouse anti-human alkaline phosphatase conjugated antibody (Promega) diluted 1/5000 in Blotto, for 1 hr at room temperature, followed by washing three times with TBST for 10 min each. All the above incubations were carried out with agitation. Membranes were then placed in a clean container and covered with BCIP/NBT substrate. The container was placed in the dark at room temperature without shaking for 30 min or longer if required. The filter was then rinsed in distilled water to stop the reaction, photographed then wrapped in cling film to store.

2.7 DIFFERENTIAL DISPLAY REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (DD-RTPCR).

DD-RTPCR was carried out using the RNAmap Differential Display system from the Gene Hunter corporation™.

2.7.1 DD-REVERSE TRANSCRIPTION OF MRNA.

Total RNA was used for reverse transcription. The integrity of all RNA used was verified by β-actin RT PCR (2.5.5.1) and visualised on a formaldehyde gel as described in section 2.7.8. RNA isolated from transformed rat embryo fibroblast was included in the kit as a control for reverse transcription dependent amplification of mRNA with any primer combinations. Four reverse transcription reactions were set up for each RNA, one for each of the anchored olig (dT) primers. The following were added for each RT reaction.

	µl
dH_2O	9.4
5X RT buffer	4.0
dNTP(250 µM)	1.6
Total RNA (DNA free)	2.0 (0.1 µg/µl, freshly diluted)
$T_{12}MN$(10 µM)	2.0
(M=A,C,G, N=A,C,T,G)	
Total	19.0

The mixture was placed in the thermocycler for 5 min at 65°C and 10 min at 37°C after which 1µl of MMLV reverse transcriptase (Promega) was added to each tube, mixed briefly, then incubated at 37°C for 50 min and 95°C for 5 min. After incubation tubes were centifuged briefly to collect condensation and stored on ice for immediate PCR or -20°C for long term storgage.

2.7.2 DD-POLYMERASE CHAIN REACTION.

PCR was carried out in a final volume of 20 µl. A core mix was prepared where possible to avoid pipetting errors, the RT mix and AP primer were aliquoted individually. AP primers used in this study were

AP-11 (2 µM) 5'-CAGACCGTTC-3'

AP-12 (2 µM) 5'-TGCTGACCTG-3'

AP-13 (2 µM) 5'-AGTTAGGCAC-3'

AP-14 (2 µM) 5'-AATGGGCTGA-3'

AP-15 (2 µM) 5'-AGGGCCTGTT-3'

The PCR reaction was set up at room temperature as follow:

	µl
dH_2O	9.2
10X PCR buffer	2.0
dNTP (25 µM)	1.6
AP-primer (2 µM)	2.0
$T_{12}MN$ (10 µM)	2.0
RT mix (containing the same $T_{12}MN$ for PCR)	2.0
α^{35}S-dATP (1200 Ci/mM)	1.0
AmpliTaq (Perkin-Elmer)	0.2
Total	20.0

The reaction was mixed well, overlaid with 20µl of mineral oil and placed in the thermocycler (MJ Research). PCR was carried out at 94°C, 30 s, 40°C, 2 min, 72°C, 30 s for 40 cycles, 72°C, 5 min, followed by electrophoretic analysis or storage at -20°C.

2.7.3 DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS

Differential display products were visualised by autoradiography after separation on a 6% (w/v) denaturing polyacrylamide gel using a sequencing apparatus. The constituents of the gel are out lined in appendix A.

2.7.4 PREPARATION OF THE SEQUENCING APPARATUS

Sequencing apparatus plates were washed in detergent to remove all traces of gel, rinsed thoroughly with tap water followed by upH_2O, wiped dry with clean dry tissue removing all tissue. One hundred percent ethanol was poured onto the plates and wiped in one direction along the entire length plate until dry. The large plate was siliconised using sigmacote, a few drops were applied at one end and wiped along the plate in one direction. Spacers and combs were wiped in 100% (v/v) ethanol and placed on one plate. The apparatus was then assembled and clamped together.

2.7.4.1 CASTING THE GEL

Starting with a clean dry casting tray a paper sealing strip was placed into the casting tray. To 20 ml of gel mix, 250 µl 10% (w/v) APS and 100 µl of TEMED was added. This gel solution was poured quickly onto the sealing strip and the assembled sequencing plates were placed on top and clamped into the tray. When set (tip to check), the main gel was pour at 45° angle using a 50 ml syringe slowly and constantly. The comb was inserted blunt end first and the gel was allow to set, allow 3 hr. Two litres of 1x TBE was prepared from the 10X stock with upH_2O. The comb was removed and inserted with the sharp tooth edge toward the gel. Four hundred millilitres of 1XTBE was poured in the bottom reservoir and the IPC chamber was filled, the wells were washed thoroughly, to remove bubbles or urea, with a syringe containing 1X TBE and a fine needle. The gel was pre-run for at least 45 min using sample loading dye in a few wells to ensure the samples would run correctly. After a gradual increase of power over a 10 min period the gel was run at 1700 volts for approximately 3 hr.

2.7.2 DRYING AND DEVELOPING

After electrophoresis the buffer was removed from the IPC chamber and the plates were separated. Two pieces of 3MM filter paper (cut to size) was placed on top of the gel, avoiding bubbles. The paper was lifted gently taking the gel with the paper. This was then covered with cling film and placed in the gel dryer, with the cling film facing up.

The gel was dried at 80 °C for 2 hr. Orientation markers were prepared by carefully streaking ^{35}S containing loading dye across a piece of filter paper, in the shape of an x and allowing it to dry. The markers were then wrapped in sellotape and stored until required. When the gel was dry the cling film was removed and the gel was placed in a cassette, two orientation markers were attached either side of the gel. In the dark room a sheet of X-ray film was placed on the gel and the cassette was sealed. The gel was exposed to the film for 24-96 hr. The film was removed from the cassette in the dark, placed in developer for 3 min, water for 1 min, fixer for 3 min and water again to rinse any residual developer or fixer (when developing the film was agitated continuously in each of the solutions). The film was then air-dried for 2 hr until fully dry.

2.7.5 Isolation of cDNA

After developing the autoradiogram bands of interest were identified and marked on the filter using a pencil. The autoradiogram was then placed on the dried filter and aligned using the orientation markers. The band of interest was removed by cutting through the autoradiogram and the filter paper using a clean razor blade. The gel slice including the filter paper was placed in an microcentifuge tube and soaked in 100 µl of upH_2O for 10 min, followed by boiling the tube with the lid tightly closed for 15 min. The mixture was then spun at 13,000 x g for 2 min to pellet the gel and the paper debris. The supernatant was transferred to a fresh tube to which 10 µl of 3M sodium acetate, 5 µl of glycogen (10 mg/ml) and 450 µl of 100 % (v/v) ethanol were added. The tube was allowed stand for 30 min at -80°C followed by spinning at 13,000 x g for 10 min at 4°C to pellet the DNA. The pellet was washed in 85% (v/v) ethanol, dried briefly and dissolved in 10 µl of dH_2O. Four microlitres were used for subsequent reamplification and the remainder was stored at -20°C.

2.7.6 Reamplification of cDNA probe

Reamplification was carried out using the same primer set and PCR conditions with the exception of the dNTP concentrations which was 20 µM. No radioactive isotopes was added.

	µl
dH_2O	20.4
10X PCR buffer	4.0
dNTP (250 µM)	3.2
AP-primer (2 µM)	4.0
$T_{12}MN$ (10 µM)	4.0
Isolated cDNA template	4.0
AmpliTaq (Perkin-Elmer)	0.4
Total	40.0

PCR was carried out at 94°C, 30 s, 40°C, 2 min, 72°C, 30 s for 40 cycles, followed by 72°C for 5 min. The PCR product (30 µl) was run out on a 1.5% (w/v) agarose gel and stained with Ethidium bromide (See section 2.3.12). In cases where the cDNA was not reamplified a 1:100 dilution of the first round PCR was made and 4µl of this dilution was used for a second round of PCR, using the same conditions as those described above. The size of the reamplified band was confirmed by agarose gel electrophoresis by comparison with a 100 bp DNA ladder (Promega). The PCR products were then cloned into the cloning vector pTAg from Invitrogen.

2.7.7 CLONING OF PCR PRODUCTS

The reamplified PCR products were cloned in to the pTAg cloning vector using the LigATor rapid cloning system from R&D systems. In order to reduce the risk of removing the A overhang by nuclease contamination unpurified PCR products were used for cloning. The use of freshly amplified PCR fragments yielded best results as storage of PCR products can lead to loss of the A overhang preventing ligation to the pTAg vector. Prior to ligation residual DNA polymerase activity was removed to avoid false positives by the addition of an equal volume of chloroform:isoamyl alcohol (24:1) to the PCR reaction and vortexing for 1 min. The tube was then microcentrifuged for 1 min at room temperature at 12,000 x g. The upper aqueous phase was transferred to a fresh tube, 2 µl of this aqueous phase was used in the ligation reaction. The maximum volume used was 2 µl as the salts may inhibit the ligation reaction.

2.7.7.1 LIGATION REACTION

The following reagents were combined in a 1.5 ml microcentifuge tube:

10X Ligase buffer	1 µl
100 mM DTT	0.5 µl
10 mM ATP	0.5 µl
50 ng/µl pTAg vector	1µl
Amplified fragment	2 µl
Nuclease free water	4.5 µl

The tube was vortexed briefly to mix and then microcentrifuged to collect the contents. T4 DNA ligase (0.5 µl) was added using a fresh tip and mixed gently without vortexing. The ligation reaction was incubated over night at 16°C, then placed on ice until required.

2.7.7.2 TRANSFORMATION REACTION

Competent cells used for the transformation reaction were provided with the LigATor Kit. One 1.5 ml tube contained 40 µl of cells which was sufficient for two transformation reactions. Cells were thawed on ice, 20 µl of which were gently pipetted into a prechilled sterile 1.5 ml microcentifuge tube. One microlitre of the ligation reaction was added to the cells and tapped gently to mix. The cells were then incubated on ice for 30 min. SOC media was also provided in the LigATor kit and it was thawed at room temperature. After 30 min on ice the cells were heat shocked at 42°C for exactly 30 s without shaking or mixing. The transformation reactions were then incubated on ice for 2 min, 80 µl of SOC media was added to each tube which were placed in a shaking incubator at 37°C for 1 hr. Prepared LB agar plates containing IPTG/X-Gal (see appendix A) were placed at 37°C for 30 min to equilibrate. Spread plates were prepared using 50 µl of the transformation reaction. The plates were left at room temperature to allow absorption of liquid and the incubated at 37°C overnight.

pTAg contains a *LacZα* peptide sequence which when functionally produced complements the N-terminal truncated *LacZ* peptide synthesised in the competent cells provided in the LigATor Kit. The resulting enzyme β-galactosidase, cleaves X-gal to give blue colonies. IPTG depresses the expression of the *LacZα* gene in cells containing pTAg. When an insert was cloned into the cut pTAg vector the *LacZα* peptide sequence is interrupted. This interfered with the function of the peptide and white colonies were produced on the plates. This formed the basis for the selection of colonies containing inserts. Transformed (white) colonies were inoculated into 5 ml of LB amp broth and incubated in a shaking incubator overnight at 37°C. DNA minipreparations were prepared from the resulting cultures as described in section 2.3.10. The presence of inserts and there orientation was determined by restriction digests (as described in section 2.3.4).

2.7.8 SEQUENCING OF CDNA PROBES

A T7 Sequencing ™ Kit from Pharmacia Biotech was used for sequencing, this kit is based on the dideoxy method of sequencing (Sanger *et al.,* 1977). The major steps involved in using T7 DNA polymerase to sequence DNA using a radioactive label were as follows; isolation of template DNA as described above, annealing of primer, labelling reaction, termination reaction, electrophoresis and autoradiography.

2.7.8.1 ANNEALING OF PRIMER TO DOUBLE STRANDED TEMPLATE

The concentration of DNA was adjusted to contain 1.5-2.0 μg of DNA in 32 μl of water (32 μl of miniprep DNA was used in each sequencing reaction). To denature the template DNA 8 μl of NaOH was added, the tube was vortexed and centrifuged briefly to collect drops and incubated at room temperature for 10 min. To precipitate the DNA 7 μl of 3 M sodium acetate (pH 4.8), 4 μl of dH_2O and 120 μl of 100% (v/v) ethanol were added to the denatured template, mixed gently and placed at -20 °C overnight. The precipitated DNA was collected by spinning at 13,000 x g for 15 min the resulting pellet was washed in 70% (v/v) ice cold ethanol the tube was then centrifuged for 10 min the supernatant was removed the pellet was air dried and resuspended in 10 μl of

dH_2O. Two microlitres of undiluted universal primer and 2 µl of annealing buffer was added to the template DNA vortexed and centrifuged briefly followed by incubation at 65°C for 5 min. The tube was then transferred to 37°C for 10 min followed by room temperature for 5 min and then used directly for labelling reactions.

2.7.8.2 LABELLING REACTION

The following were added to the annealed template/primer mix,

Labelling mix (dATP) 3 µl

Labelled dATP (S_{35}) 1 µl

Diluted T7 DNA polymerise 2 µl

These were mixed gently by pipetting and incubated at room temperature for 5 min.

Table 2.5. Dilutions of T7 DNA polymerase

no. of templates	polymerase	dilution buffer(µl)	total volume(µl)
2	1	4	5
3	1.5	6	7.5
4	2	8	10
5	2.5	10	12.5
6	3	12	15
7	3.5	14	17.5
8	4	16	20
9	4.5	18	22.5
10	5	20	25

2.7.8.3 TERMINATION REACTION

Four tubes were labelled A,C,G,T for each DNA template, 2.5 µl of each of the "read short mixes" were added to their corresponding tubes and incubated for 5 min at 37°C. To each of the 4 pre-warmed sequencing mixes, 4.5 µl of the labelling reaction was added, mixed by gentle pipetting, and incubated at 37°C for 5 min. Five microlitres of stop solution was added and mixed gently. Four microlitres of each reaction was added to a fresh tube, incubated at 75-80°C for 2 min and immediately loaded on the

sequencing gel. The remainder of the unheated reactions was stored at -20°C. Electrophoresis and autoradiography was carried out as described in section 2.7.4.

2.7.9 Riboprobe Synthesis

The cloned cDNAs were used to prepare probes for northern blots to confirm or refute the presence of differentially expressed cDNAs. Riboprobes (RNA Probes) were prepared using an *in vitro* transcription kit (Promega). Prior to labelling the plasmids containing the cDNA of interest were purified using the Qiagen purification protocol as outlined in section 2.3.11. Ten micrograms of purified plasmid DNA was linearized with the restriction enzyme *Hind* III, which did not cut the plasmid between the T7 promoter and the end of the cDNA fragment.

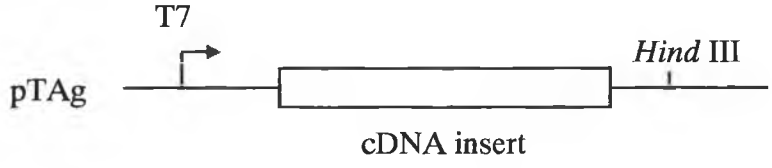


Fig. 2.2. Restriction of cloned DD cDNAs prior to labelling.

The linerised DNA was purified by phenol/chloroform extraction and ethanol precipitation as described in section 2.3.3 and resuspended in 10 µl of sterile DEPC treated H_2O.

2.7.9.1 Labelling reaction

The following components were added at room temperature in the order outlined below to a 1.5 ml microcentifuge tube.

	µl
Transcription optimisation buffer 5X	4
100 mM DTT	2
RNasin Ribonuclease inhibitor (20U)	0.5
ATP, GTP, UTP, 2.5 mM each	4 (mix)
10 µM CTP	2.4

Linearised DNA template (0.2-1.0 mg/ml)	1.2
[α-^{32}P] CTP (50 µCi/µl)	5
T7 RNA polymerase 15-20 U/µl	1
Final volume	20 µl

Reactions were incubated at 37°C for 60 min. Promega's RQ1 RNase-freeDNase (0.5 µl-1 U/µg of DNA) was added to the labelling reaction and incubated at 37°C for 15 min. The volume was adjusted to 50µl using DEPC treated H_2O and a phenol/chloroform and a chloroform extraction was carried out as described in section 2.3.3. The following were then added to the labelled probes, 20 µg of calf pancreatic tRNA, 0.5 volumes of 7.5 M ammonium acetate and 2.7 volumes of ethanol. Following overnight precipitation at -20°C the probe was pelleted at 13,000 x g for 30 min at 4°C. The pellet was washed in 70% (v/v) ethanol the supernatant was removed and the pellet was air dried until all residual ethanol had evaporated then resuspended in 20 µl of DEPC treated H_2O.

2.8 NORTHERN BLOTTING

Northern blotting was carried out according to Sambrook *et al.*, (1989). RNA of interest was first separated on a formaldehydye gel.

2.8.1 TREATMENT OF ELECTROPHORESIS APPARATUS

Prior to running an RNA gel the electrophoresis apparatus was treated to remove any RNase. The tank, gel tray, comb and lid were washed in detergent and rinsed well in DEPC-treated H_2O then air dried in 100% (v/v) ethanol. The tank, gel tray and comb were immersed in a 3% (v/v) solution of Hydrogen peroxide for 15 minutes. The apparatus was then rinsed thoroughly in DEPC treated upH_2O and allowed to dry.

2.8.2 ELECTROPHORESIS OF RNA/MRNA THROUGH GELS CONTAINING FORMALDEHYDE

As formaldehyde vapours are toxic these gels were prepared in a fume hood. The gel was prepared by melting the appropriate amount of agarose in water cooling to 60°C and adding 5X formaldehyde gel running buffer and formaldehyde to give a final concentration of 1X and 2.2M respectively (Appendix A). The gel was cast in a fume hood and allowed to set for at least 30 min. The samples were prepared by mixing the following in a microcentifuge tube:

RNA (up to 30 μg)	1.5μl
5X formaldehyde gel running buffer	2.0μl
Formaldehyde	3.5μl
Formamide	10μl

Ethidium bromide was added to samples (0.01 μg/sample) which were to be used for northern blots in order to examine the RNA briefly under UV (70% strength) for equal loading prior to blotting. The samples were incubated at 65°C for 15 min, chilled on ice then centrifuged briefly to collect the sample. Sterile DEPC treated gel loading buffer (2 μl) was added to each sample and applied to the gel immediately after prerunning the gel for five minutes at 5V/cm. Gels were run while submerged in 1X formaldehyde gel running buffer for approximately 3 hr or until the dye front had migrated two thirds of the way down the gel. The gel was removed from the buffer and viewed under UV light. Samples to be blotted were treated as described in the next section.

2.8.3 TRANSFER OF DENATURED RNA TO NITROCELLULOSE FILTERS

Gels which contained formaldehyde were first washed in several changes of DEPC treated H_2O. As the percentage agarose used to prepare the gel was greater than 1% (w/v), the gel was soaked in 0.05 M NaOH for 20 min this treatment partially hydrolyses the RNA and improves the efficiency of transfer. The gel was then rinsed in DEPC treated H_2O and soaked in 20X SSC for 45 min (Maniatis *et al*, 1989). Unused areas of the gel were trimmed away and the top left hand corner was cut for orientation

purposes in this and succeeding operations. Capillary transfer was used to transfer the RNA onto the filter, transfer was set up as illustrated in Fig 2.3.

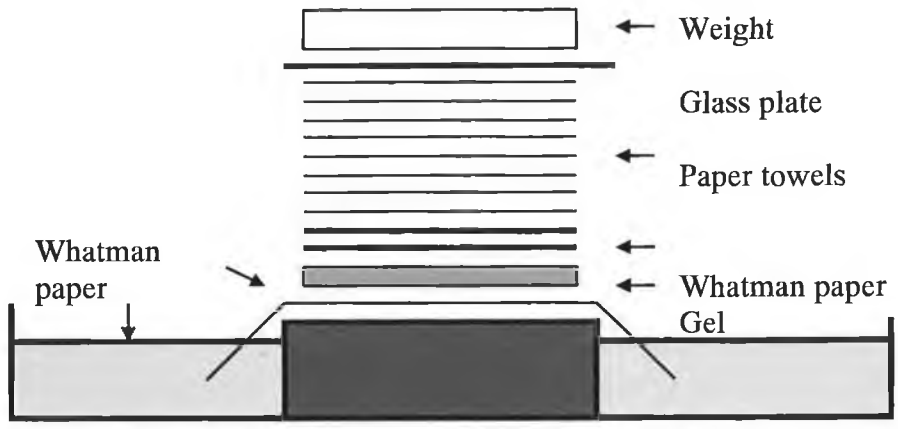


Fig 2.3. Capillary transfer of nucleic acids from agarose gels to solid supports. From Sambrook *et, al.,* 1989.

A solid support was placed in a bath of 20X SSC. A sheet of 3MM Whatman paper was cut to cover the support and dip down either side into the buffer. The washed gel is then placed (wells facing down) on to the whatman and covered by a piece of nitrocellulose membrane cut to the size of the gel. This membrane had been floated on a bath of deionized water and wetted completely from below then soaked in 20X SSC for at least 5 min prior to placing it on the gel. The buffer chamber was then covered with cling film. Two pieces of 3MM Whatman paper which had been soaked in 2X SSC were then placed on top of the filter paper, care was taken at all times to ensure that no bubbles were trapped when preparing the transfer. A stack of paper towels were then placed on top of the gel and held in place by a weight. Transfer was allowed to continue overnight. After transfer was completed the saturated paper towels were removed as was the Whatman paper. The gel and the filter paper were removed together and turned upside down on a clean piece of towel. The position of the wells were marked using a ball point pen, the gel was then discarded. The filter was washed briefly in 6X SSC to remove any remains of the gel then placed on a fresh sheet of Whatman paper and allowed to air dry for at least 30 min. The dried filter was then placed between two pieces of 3MM Whatman paper and baked in an oven at 80°C for 2

hr. The filter was then used directly for prehybridisation or stored at room temperature wrapped in aluminium foil.

2.8.4 PREHYBRIDIZATION AND HYBRIDIZATION PROTOCOL

The dried membrane was placed in a baked hybridization bottle, 10 ml of hybridization buffer A (Appendix A) was added and incubated in a Hrybid roller oven for 45 min to 1 hour at 55°C. Once the membrane was wet after baking it was not allowed to dry out again. To 10 ml of fresh hybridization buffer A which had been pre heated to 55°C, 5 µl of the labelled probe was added. The prehybridization buffer was removed and the preheated buffer containing the probe was promptly added. Hybridization was allowed to proceed overnight at 55°C.

2.8.5 MEMBRANE WASHING

Following the overnight incubation the hybridization buffer was removed and the membrane was washed as follows:

Table 2.6. Northern blot membrane washing.

Wash solution	Temperature	Time
1X SSC, 0.1% (w/v) SDS	room temperature	30 s
1X SSC, 0.1% (w/v) SDS	22°C	30 min
1X SSC, 0.1% (w/v) SDS	22°C	30 min
0.1X SSC, 0.1% (w/v) SDS	65°C	30 min
0.1X SSC, 0.1% (w/v) SDS	65°C	30 min

After washing the membrane was removed gently from the hybridization bottle and placed in a stomacher bag in a cassette. The blot was kept moist at all times with 0.1X SSC, 0.1% (w/v) SDS. The membrane was exposed to X-ray film for 24-96 hr at -70°C. Film was developed as described in section 2.7.4.2.

2.9 RNase protection assay

The ribonuclease protection assay (RPA) is a highly sensitive and specific method for the detection and quantitation of mRNA species. The RiboQuant® RNase protection assay system (PharMingen) was employed during this study. The procedure is outlined below.

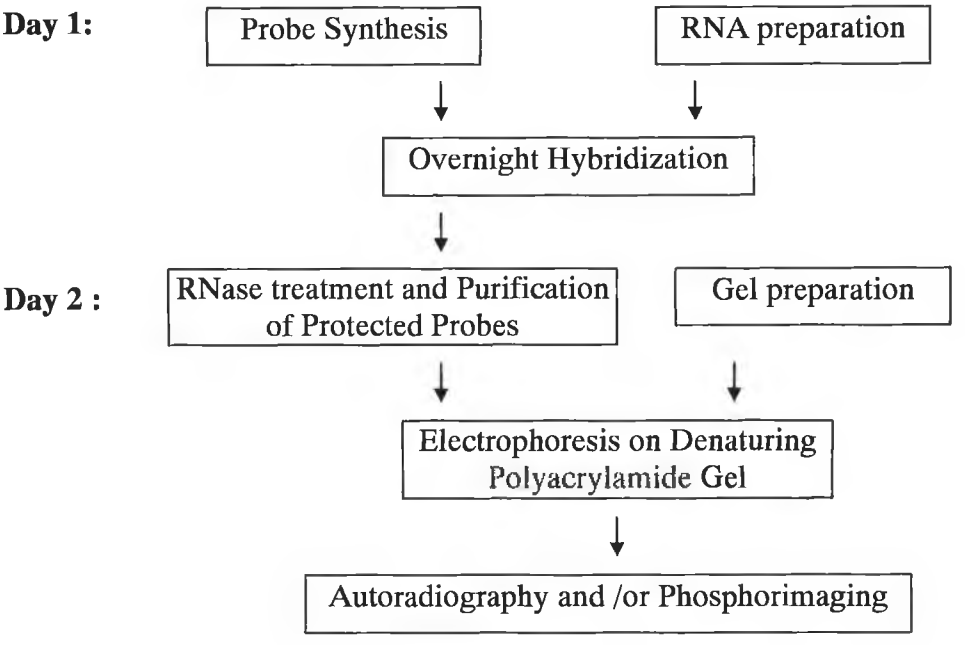


Fig. 2.4. Overview of the ribonuclease protection assay protocol.

2.9.1 Probe Synthesis

The [α-^{32}P]UTP, GACU nucleotide pool, DTT, 5X transcription buffer and the template DNA set was brought to room temperature prior to setting up the reactions. The following were added to a 1.5 ml microcentifuge tube for each probe synthesis:

RNasin	1 µl
GACU pool	1 µl
DTT	2 µl
5X transcription buffer	4µl
Template DNA	1 µl

[α-^{32}P]UTP	10 µl
T7 RNA polymerase	1 µl

In some cases cDNAs had been cloned in the SP6 orientation thus an SP6 enzyme (BohringerManheim) was employed which has a 10X transcription buffer the residual volume was made up with nuclease free water. The contents of the tube were mixed by gentle pipetting and centrifuged quickly followed by incubation at 37°C for 1 hour. The reaction was terminated by adding 2 µl of RNase free DNase mixing gently and incubating at 37°C for 30 min. The following reagents were then added to the reactions:

EDTA 20 mM	26 µl
Tris saturated phenol	26 µl
Chloroform:isoamyl alcohol (50:1)	25 µl
Yeast tRNA	2 µl

The contents were vortexed into an emulsion and centrifuged for 5 min at room temperature. The upper aqueous phase was transferred to a fresh tube containing 50 µl of chloroform:isoamyl alcohol (50:1), the tube was vortexed and microcentrifuged (top speed) for 2 min at room temperature. The upper aqueous phase was transferred to a sterile 1.5 ml tube to which 50 µl of 4M ammonium acetate and 250 µl of ice cold 100% (v/v) ethanol was added. The tube was inverted to mix and incubated at -70°C for 30 min followed by centrifugation at 4°C for 15 min. The supernatant was removed and the pellet was washed with 100 µl of ice cold 90% (v/v) ethanol after which the supernatant was removed and the pellet was air-dried for 5-10 min. The pellet was solubilized by the addition of 50 µl of hybridization buffer and gentle vortexing, contents were collected by a brief centrifugation. Duplicate 1 µl samples of the labelled probe were quantified in a scintillation counter. A maximum yield of ~3 x 10^6 Cherenkov counts/µl with an acceptable lower limit of ~3 x 10^5 Cherenkov counts/µl was expected. The probe was stored at -20°C until required. Generally probes can only be used for two overnight hybridizations when labeled with [α-^{32}P]UTP.

2.9.2 RNA PREPARATION AND HYBRIDIZATION

RNA was prepared using the RNA isolation method outlined in section 2.5.2, 20 μg of total RNA was used for each probe hybridization. Each RNA sample was made up to 50 μl with DEPC-treated upH_2O to which 50 μl of 4 M ammonium acetate and 250 μl of ice cold 100% (v/v) ethanol were added. The samples were mixed by inverting and stored at -70°C for one hr or -20°C overnight. The precipitated RNA was collected by centrifugation at 12,000 x g for 30 min at 4°C, the pellet was washed with 90% (v/v) ice cold ethanol. After removal of the supernatant and subsequent air-drying, the pellet was resuspended in 8 μl of hybridization buffer by gentle vortexing for 3-4 min followed by a brief centrifugation. Two microlitres of the probe was then added to each RNA sample and mixed by pipetting. A drop of mineral oil was added to each sample and the tubes were centrifuged briefly in the microfuge. Samples were then placed in a heating block for 3 min which had been preheated to 90°C, and then immediately turned down to 56°C, allowing the temperature to ramp down slowly, and incubated for 12-16 hr. The heating block was then turned down to 37°C prior to RNase treatment and again the temperature was allowed to ramp down slowly and then was maintained at 37°C for 15 min.

2.9.3 RNASE TREATMENTS

An RNase mixture was prepared by adding 2.5 ml of RNase buffer to 6 μl of RNase A + T1 mix, per 20 RNA samples (RNase A 80 ng/μl; RNase T1 250 U/μl). The RNA samples were removed from the heating block and 100 μl of the RNase cocktail was added underneath the oil into the aqueous layer (bubble). The tubes were microcentrifuged for 10 s and incubated for 45 min at 30°C. Before the RNase treatment was completed a Proteinase K mixture was prepared (per 20 samples),

Proteinase K buffer 1 X	390 μl
Proteinase K (10 mg/ml)	30 μl
Yeast tRNA (2 mg/ml)	30 μl

The mixture was mixed together and 18 µl aliquots were added to a sterile 1.5 ml microcentifuge tubes. The RNase digests were extracted from underneath the oil and transferred to the tube containing the proteinase K mixture (avoiding transfer of oil). The RNase/Proteinase K mixture was vortexed briefly, microfuged quickly and incubated for 15 min at 37°C. Tris saturated phenol (65 µl) and 65 µl of chloroform:isoamyl alcohol (50:1) were added to the samples, vortexed into an emulsion then centrifuged for 5 min at room temperature. The upper aqueous phase was extracted, avoiding the interphase, and transferred to a fresh tube to which 120 µl of 4M ammonium acetate and 650 µl of ice cold 100% (v/v) ethanol was then added. The tubes were mixed by inverting and were subsequently incubated at -70°C for 30 min. Samples were centrifuged for 5 min at 4°C, the pellet was then washed with ice cold 90% (v/v) ethanol, the supernatant was removed and the pellet was allowed to air-dry for 5-10 min. Pellets were resuspended in 5 µl of 1X loading buffer (provided in kit). Prior to loading onto the gel the samples were heated to 90°C for 3 min and placed immediately in an ice bath.

Five percent (w/v) gels were prepared according to the formula given in appendix A. The gel apparatus was prepared, cast, electrophoresed and dried as previously described in section 2.7.4. The dried gel was placed in a cassette which contained two intensifying screens, a sheet of X-ray film was placed on top of the gel and it was exposed overnight at -70°C. The film was developed as described in section 2.7.4.2.

CHAPTER 3

ANALYSIS OF CD44 EXPRESSION IN BURKITT'S LYMPHOMA CELL LINES

3.1 INTRODUCTION

Several reports indicate that the presence of EBV and the expression of EBV latent genes, particularly EBNA2 and LMP1, play an important role in the induction of both CD44 standard and variant isoform expression (see section 1.17). B-cell activation by EBV infection induces the expression of CD44v8-10 and CD44v10, further investigation illustrated that EBNA2 is required for the expression of these CD44 variant isoform, thus indicating that EBNA2 and/or LMP1 may mediate its induction (Kryworuchko *et al.,* 1995). EBNA2 has been shown to play a role in CD44 expression in lymphoblastoid cell lines (Fitchter *et al.,* 1997), also LMP1 has been implicated in the regulation of CD44 expression in EBV-based lymphomas (Walter *et al.,* 1995). In order to investigate further the effect of EBV latent genes on CD44 expression, we employed (1) the EBV-positive Burkitt's lymphoma (BL) lines Mutu I and Mutu III (exhibiting type-I and type-III latency programs respectively), (2) BL cell lines, DG75 tTA EBNA2 and DG75 tTA LMP1 in which EBNA2 and LMP1 expression is inducibly regulated by tetracycline.

3.2 ANALYSIS OF CD44 EXPRESSION

The expression of CD44 standard and variant isoforms was initially examined by flow cytometry, followed by western blotting as described in section 2.6.7. Flow cytometry may be used to analyse the expression of proteins at the cell surface and is an established method for the analysis of CD44 isoforms. These experiments were carried out by using a range of anti-standard and anti-variant CD44 antibodies. A flow cytometer is used to obtain quantitative information based on light scatter or fluorescence emission caused by individual cells in a population as they flow rapidly in a fluid stream in front of a light source. When a cell passes in front of the laser beam the light scatter or the fluorescence emitted from the cell is converted to an electronic signal that is proportional to a specific parameter for that cell (in this case fluorescence from a fluorescein (FITC)-labelled mAb bound to an anti-CD44 Ab bound to the cell surface). The information from a population of cells is displayed on a computer screen as a frequency histogram (Gilman-Sachs, 1994). Flow cytometry analysis was chosen as

it possesses many inherent advantages (a) it gives an immediate answer as to the expression of a molecule of interest on the cell surface (b) the number of cells required for analysis by each antibody is small (c) multiple samples can be prepared together and stored for up to 7 days (when protected from light) before analysis. As the panel of CD44 anti-standard and anti-variant antibodies used initially was quite large this was considered the best approach to establish the CD44 expression pattern of the cell lines investigated. The protocol used for flow cytometry is outlined in section 2.4.6. The antibodies used for analysis were as follows:

D2.1	Anti standard antibodies	v3	Anti-variant antibodies
L3D-1		v4, v4-5	
44F.10.2		v6	
		v7, v7-8	

A fluorescein (FITC)-labelled anti-mouse secondary antibody was used in all flow cytometry experiments. An FITC control was included for each cell line analysed, that is cells with out a primary antibody followed by the FITC label which resulted in a negative peak indicating that there was no non-specific interaction taking place between the cells and the secondary antibody. This control was included for each cell line and was negative in each case (results not shown). Also an irrelevant mouse antibody IE was used as a negative control, illustrating how non-fluorescing cells appear. This is represented by a dark peak on the left hand side of each of the histograms, fluorescence is measured against this negative peak. The positive peak, i.e. the antibody of interest detecting the protein of interest on the surface of a population of cells, is the clear peak on each of the histograms. When fluorescence is not detected this peak lies on top of the negative peak, indicating that the molecule of interest has not been detected. Positivity is measured along the horizontal X-axis of the histograms which is a log scale measuring fluorescence. The greater the number of cells expressing the molecule of interest then the further along the log scale the peak will appear. Two peaks represent a dual population in which some cells express the molecule of interest and some do not. The Y-axis is a measure of cell number.

In order to investigate the effect of EBV latent gene expression on CD44 standard and variant isoform expression, two EBV-positive BL cell lines, Mutu I and Mutu III were investigated. These cell lines were described by Gregory *et al.*, (1990), Mutu I is a type-I latency BL cell line expressing EBNA1 as the sole viral protein. These phenotypically small cells grow singly in culture and resemble very closely the morphology of cells from a BL tumour. Mutu III is a stable clone of the early passage BL cell line Mutu I which has, upon serial culture "drifted" to express the full compliment of EBV latent genes (type-III latency). This drift also involves a switch to the expression of B-cell activation antigens and other surface molecules including CD44 (Gregory *et al.,* 1990). Mutu III cells clump greatly in culture. The EBV-associated diseases that best exemplify the latency III program are infectious mononucleosis and postransplantation lymphoproliferative disorder (PTLD).

These cell lines provide a useful tool in examining the collective effect of the EBV latent genes on CD44 gene expression. The expression of the standard isoform of CD44 has previously been recorded in Mutu III but not Mutu I (Gregory *et al.,* 1990), however the pattern of CD44 variant expression has not been investigated in this type-III EBV positive cell lines.

The T-cell line HuT 78 was used initially as a positive control for the expression of CD44. Thus the pattern of CD44 expression in HuT 78 was determined by flow cytometry before examining cell lines of interest as illustrated in the following section. All cell lines were examined by flow cytometry twenty four hours after passage so that the culture conditions of the cells were consistent between experiments.

3.2.1 FLOW CYOMETRY ANALYSIS OF HUT 78

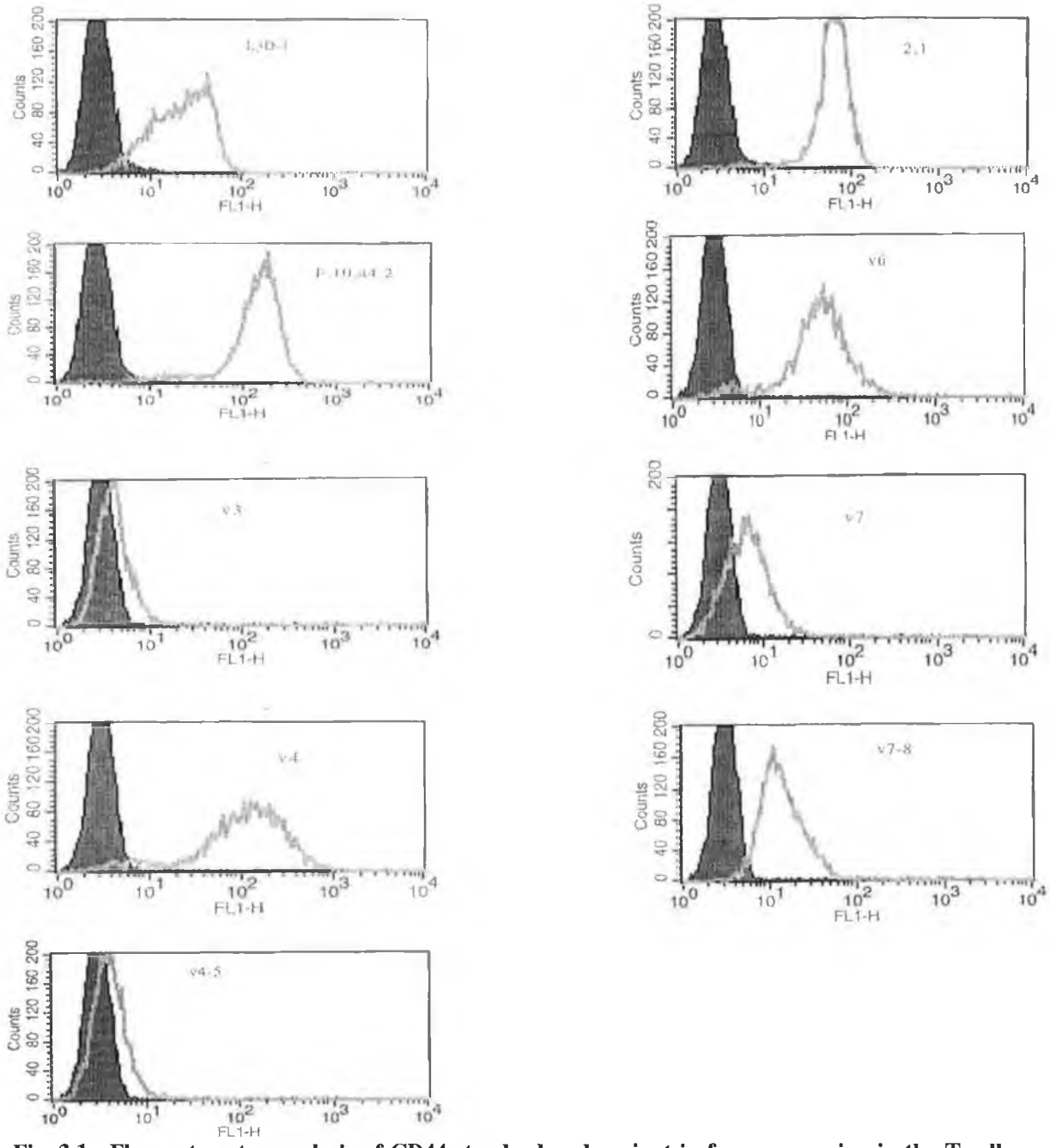


Fig. 3.1. Flow cytometry analysis of CD44 standard and variant isoform expression in the T-cell line HuT 78. Relative cell number is plotted on the Y axis and log fluorescence intensity on the X-axis. The antibodies used are indicated on each histogram (2.1=D2.1).

It can be seen that with the exception of anti-v3 and v4-5, all mAbs detected strong expression of CD44 in HuT 78.

3.3 ANALYSIS OF CD44 EXPRESSION IN MUTU I AND MUTU III

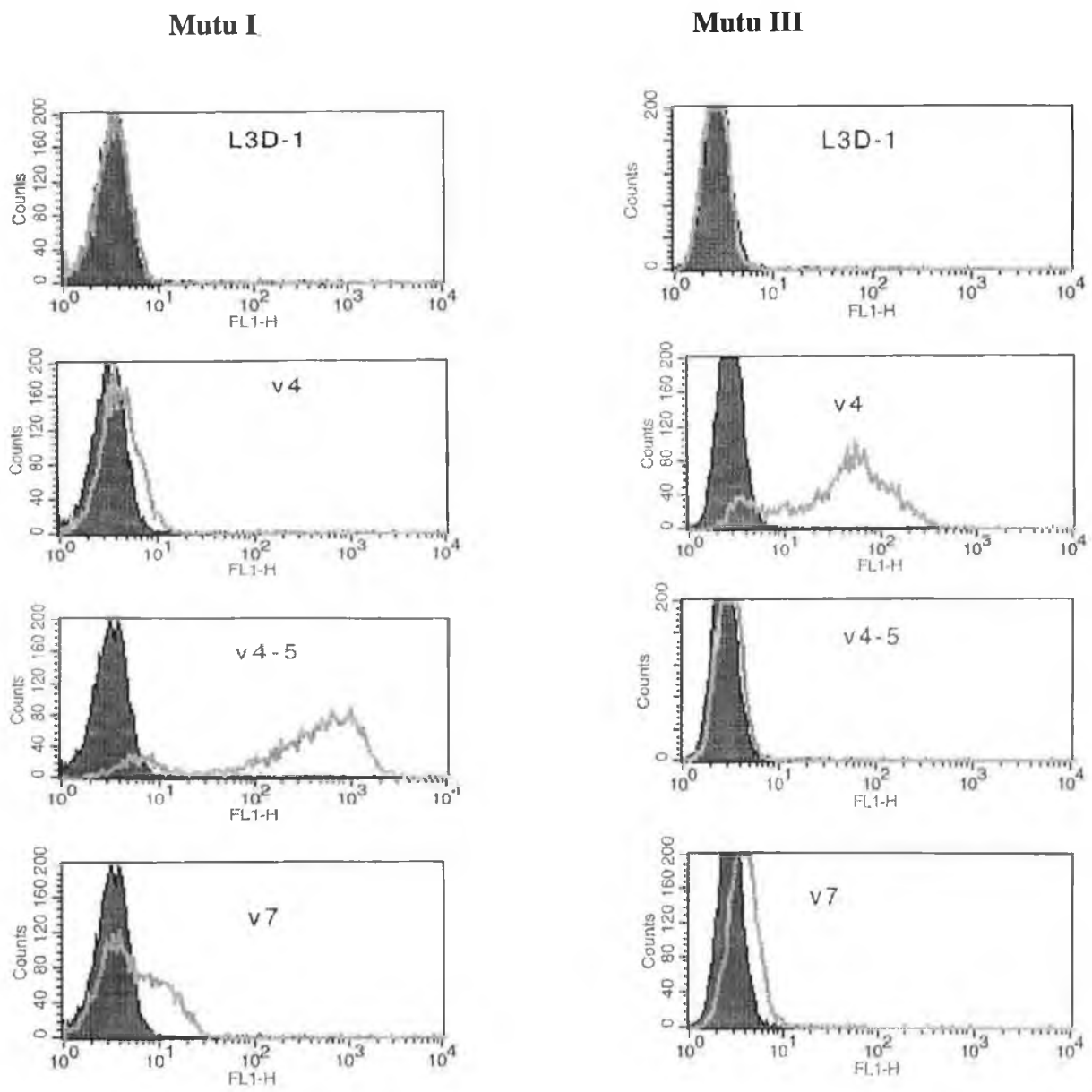


Fig. 3.2. Flow cytometry analysis of CD44 standard and variant expression in the BL cell lines Mutu I and Mutu III. Relative cell number is plotted on the Y-axis and log fluroescence intensity on the X-axis. The antibodies used are indicated on each histogram.

The results of the initial experiments indicate that both Mutu I and Mutu III were negative for CD44 standard expression however, considerable variant expression was detected in both cell lines. The fact that standard expression was not detected was possibly due to conformational changes in the CD44 molecule caused by the insertion

of variant epitopes, thus the anti-standard antibody L3D-1 was unable to detect CD44s. When comparing the variant expression pattern between both cell lines CD44v4 appears dramatically upregulated in Mutu III were as v4-5 appears down-regulated, a slight down regulation in expression of v7 was also observed in Mutu III. Both cell lines were strongly positive for v6 expression and negative for v3 expression, (results not shown).

Mutu I and Mutu III analysis was carried out using the anti-variant antibodies to variants which appeared differentially expressed in the initial experiments, v4, v4-5, and v7. Also included were the anti-standard antibodies D2.1 and 44 F.10.2.

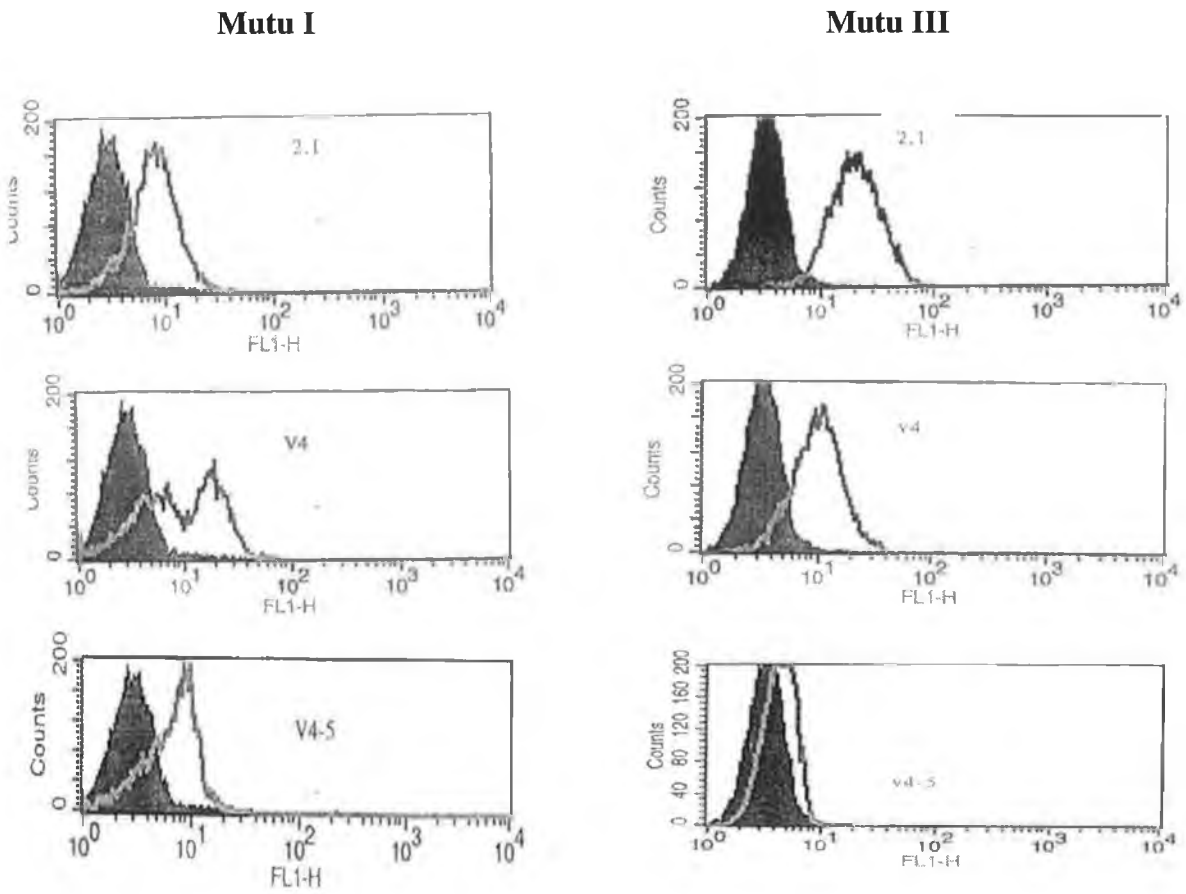


Fig. 3.3. Measurement of CD44 standard and variant isoform expression in the BL cell lines Mutu I and Mutu III by flow cytometry analysis. Relative cell number is plotted on the Y-axis and log fluroescence intensity on the X-axis. The antibodies used are indicated on each histogram (2.1=D2.1).

Using the anti-standard antibody 44F.10.2, CD44s was not detected in Mutu I or Mutu III (not shown), however, CD44s was repeatably detected using the anti-standard

antibody D2.1. Considerably more standard CD44 expression was detected in Mutu III than Mutu I. Although in previous reports, CD44s was detected in Mutu III but not in Mutu I, the results obtained here using flow cytometry with the anti-standard antibody D2.1 indicated that Mutu I did express CD44. The pattern of CD44 variant isoforms was examined, v4-5 and v7 (not shown) again appeared to be downregulated in Mutu III however, the pattern of v4 expression was not consistent as expression of these isoforms was detected in both cell lines. The differences in CD44 expression may have been caused by masking of epitopes due to conformational change caused by glycoslyation or possibly related to variations in isoform expression caused by the cell cycle.

Western blot analysis was carried out on protein isolated from Mutu I and Mutu III as described in section 2.6.7, using two anti-standard antibodies (1) BRIC 238 which recognises the standard form of CD44 at ~83 kDa and higher variant form appear as an upward smear from the 83 kDa band, and (2) D2.1 which recognises the CD44s 83 kDa band and a smaller band p45. P45 has previously only been detected in HuT 78 but is proposed to be a CD44 related protein as it recognises the HA binding site (Dr. Sinead McGrath, *pers comm*).

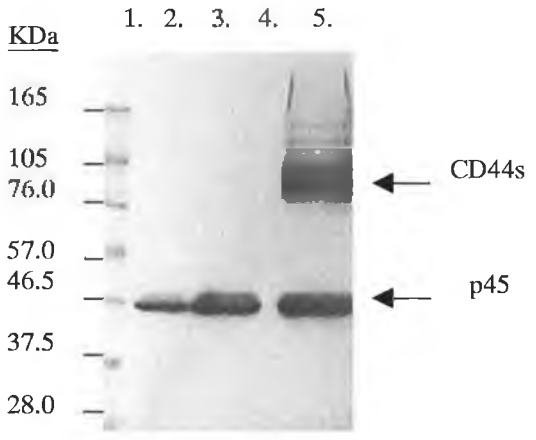


Fig. 3.4. Detection of CD44s expression by western blotting. CD44 standard expression was analysed using the anti-standard antibody D2.1 and 100 μg of protein per lane. Lane No. 1. Marker, 2. Mutu I, 3. Mutu III, 4. Blank, 5. HuT 78.

From figure 3.4 it is clear that CD44s was only detected in the positive control HuT 78, however p45 was detected in all cell lines and appeared to be several times more abundant in Mutu III. The apparent detection of CD44s by flow cytometry was

probably due to the interaction of D2.1 with p45, thus explaining why CD44s was not detected with the other anti-standard antibodies L3D-1 and 44F.10.2. The absence of CD44s in Mutu III is contrary to a previous report by Gregory *et al.,* (1990), who reported the detection of CD44s in Mutu III by flow cytometry. Thus the presence of CD44s in Mutu III was further investigated using another anti-standard antibody BRIC 238 and the results are shown in figure 3.5.

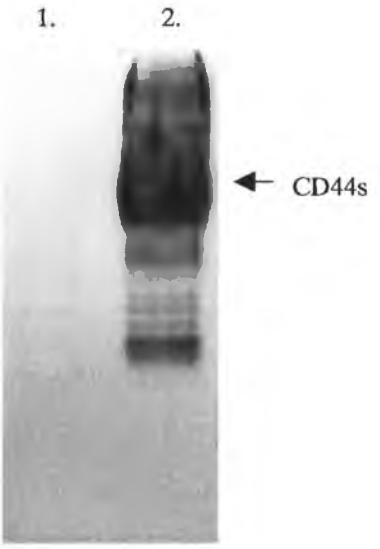


Fig. 3.5. Detection of CD44s expression by western blotting. The anti-CD44 antibody BRIC 238, was used to analyse the cell lines Mutu III and HuT 78 for the presence of standard CD44, 100 μg of protein was loaded per lane. Lane No. 1. Mutu III, 2. HuT 78.

It can be seen from figure 3.5 that CD44s was not detected in Mutu III (clone no. c148D). A partial characterisation of EBV latent gene expression in the cell clone Mutu III (c148D) was made using available antibodies as indicated in figure 3.6. The cell line B95-8 was used as a positive control as it expresses all EBV latent proteins, also included was a second clone of Mutu III clone c95.

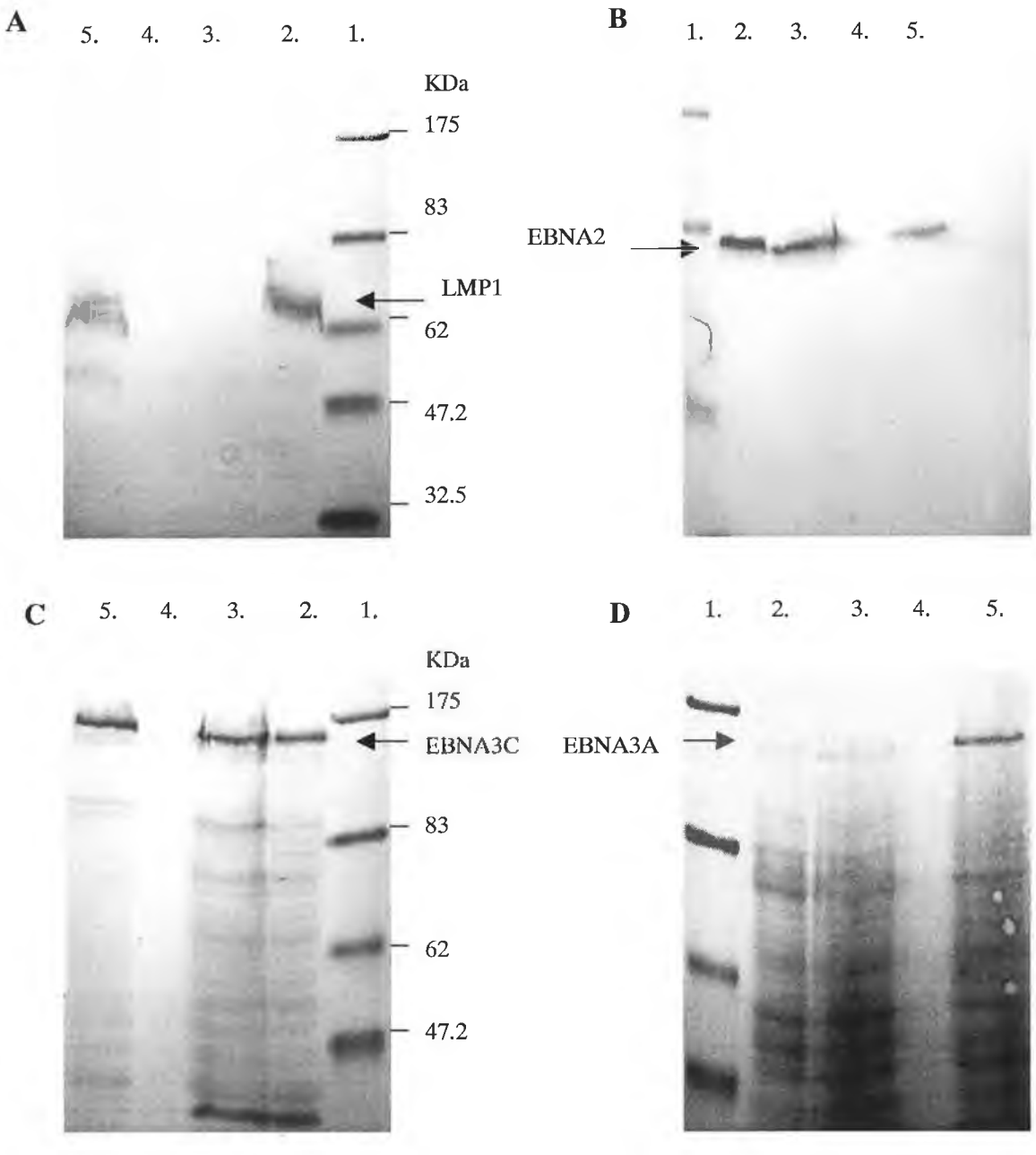


Fig. 3.6. Western blot analysis of the EBV latent proteins expressed in the cell line Mutu III c148. **A.** Analysis of LMP1 (~63 kDa) expression using the anti-LMP1 antibody CS1-4. **B.** Analysis of EBNA2 (~83 kDa) expression using the anti-EBNA2 antibody PE2. **C.** Analysis of EBNA3C (146 kDa) expression using the mAb A10. **D.** Analysis of EBNA3A (149 kDa) using the mAb T2.78. Lane No. 1. Protein marker, 2. Mutu III c95, 3. Mutu III c148D, 4. Blank, 5. B95-8.

It can be seen that both Mutu III clones express EBNA2, EBNA3A and EBNA3C, whereas, it is quite clear from figure 3.6 A., that Mutu III c148D does not express LMP1. Mutu III c95 does express LMP1 indicating that the absence of LMP1 in Mutu

III c148 is likely to be specific to this clone (Mutu III clone c148 is henceforth referred to as Mutu III LMP1-). A western blot was performed to establish if Mutu III c95 (now referred to as Mutu III LMP1+) expressed CD44s.

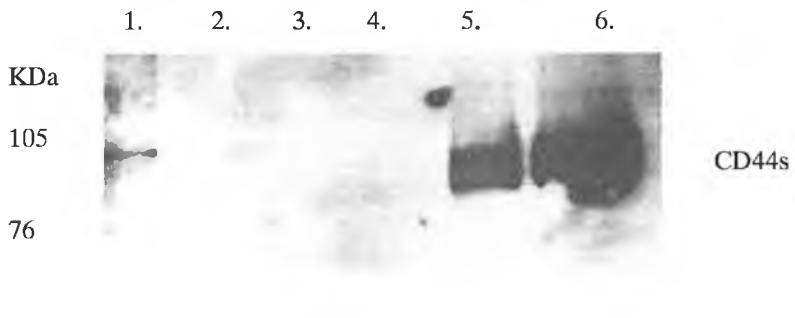


Fig. 3.7. Detection of CD44s expression by western blotting. The anti-CD44 antibody BRIC 238, was used to analyse 100 μg of protein for the presence of CD44s. Lane No. 1. Marker, 2. Mutu I, 3. Mutu III LMP1-, 4. Blank, 5. Mutu III LMP1+, 6. HuT 78.

CD44s was detected in Mutu III c95 (now referred to as LMP1+), thus it is clear that the presence of LMP1 in the type III latency BL cell line Mutu III, correlates with the expression of the standard isoform of CD44. This blot also confirmed that CD44s was not expressed in the type-I BL cell line Mutu I.

3.4 INVESTIGATION OF CD44 EXPRESSION AT THE RNA LEVEL

It was clear that the presence of LMP1 in the type-III latency cell line Mutu III correlates with CD44s expression. Transcription of the CD44 gene was analysed by northern blotting using mRNA from various cell lines and probed with a CD44s riboprobe. CD44 cDNAs, one of which corresponded to the standard CD44 exons only, and one which encodes the standard and the variant exons were used to generate riboprobes for northern blot and RNase protection assay (RPA). Both cDNAs (a gift from Dr Ursula Gunthert, Switzerland) were first subcloned as *Eco* RI fragments into pGEM 3Zf which has both SP6 and T7 promoter sequences flanking the multiple cloning site. The CD44s cDNA is 1300 bp and the CD44v cDNA is 2400 bp, see figure 3.8.

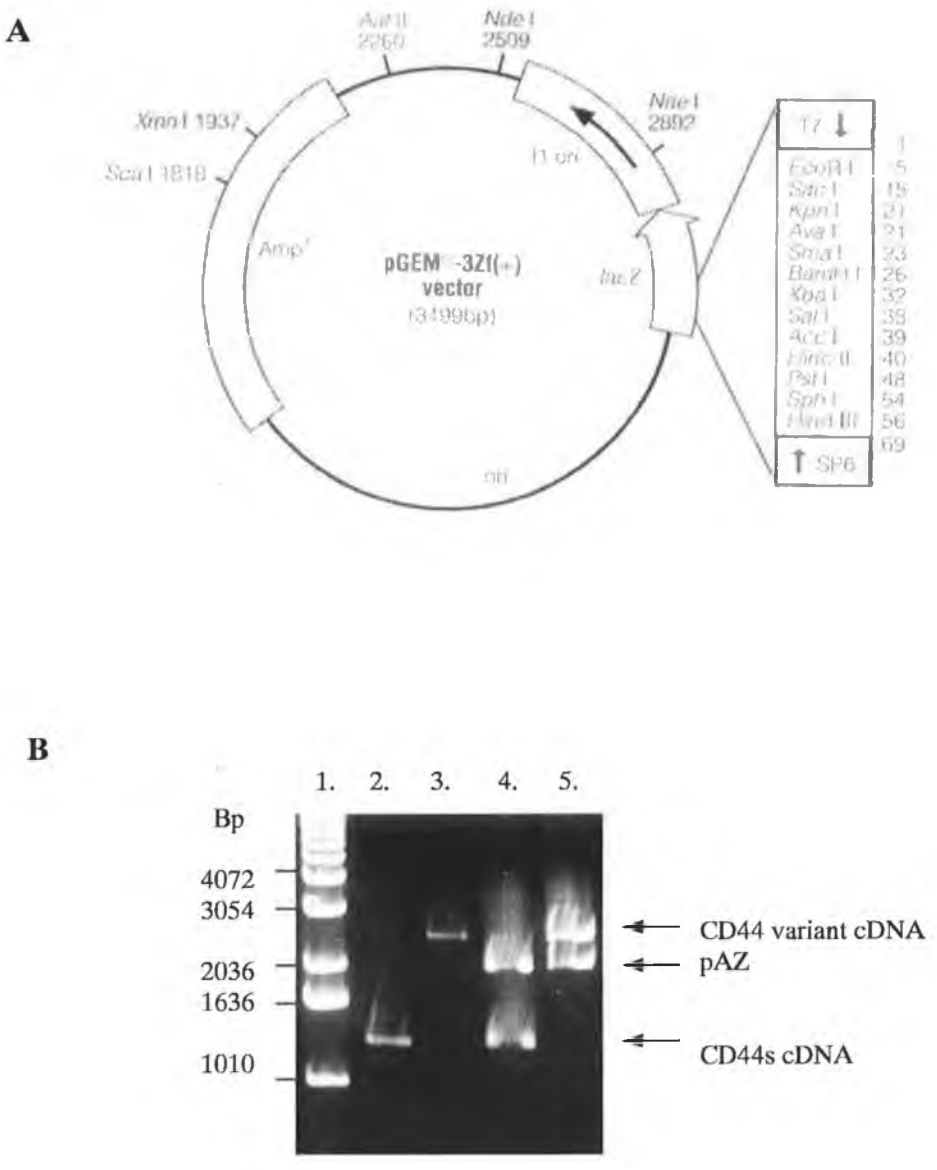


Fig. 3.8. A. Vector map of pGEM 3Zf (+). B. Restriction digestion of pAZ CD44s and pAZ CD44v with *Eco* RI and purification of cDNA fragments.. Lane no. 1. IKb DNA ladder, 2. CD44s purified cDNA fragment, 3. CD44v purified cDNA fragment, 4. pAZ CD44s, 5.pAZ C44v.

Recombinant pGEM-3Zf CD44s and pGEM-3Zf CD44v clones were analysed by DNA minipreps and digested with *Eco* RI and *Nco* I, to ensure the inserts were of the correct size as illustrated in figure 3.9.A.

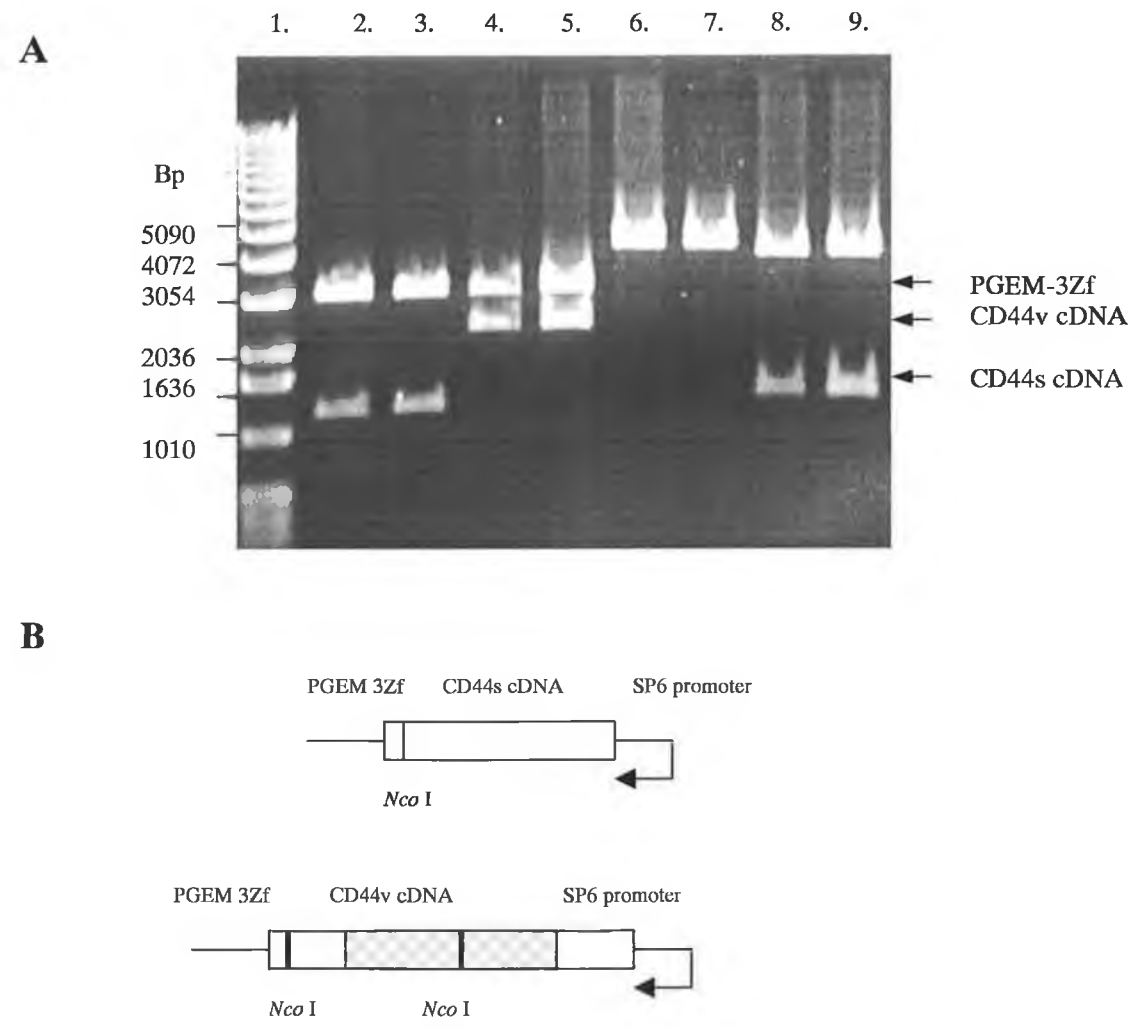


Fig. 3.9. A. Restriction analysis of pGEM-3Zf CD44s and pGEM-3Zf clones. DNA maxipreps of two pGEM-3Zf CD44s clones and two pGEM-3Zf CD44v clones were digested with *Eco* RI and *Nco* I. Lane no. 1. IKb DNA ladder, 2-3, pGEM-3Zf CD44s *Eco* RI, 4-5, pGEM-3Zf CD44v *Eco* RI, 6-7, pGEM-3Zf CD44s *Nco* I, 8-9, pGEM-3Zf CD44v *Nco* I. **B. Schematic representation of riboprobes.** CD44s cDNA sequences are shown above as open boxes and CD44v cDNA is shown as a shaded box in the sense orientation (left to right).

Eco RI digestion of pGEM-3Zf CD44s and pGEM-3Zf CD44v resulted in two correctly sized fragments in each case of 1300 bp and 3199 bp or 2400 bp and 3199 bp

respectively (figure 3.9. A). Orientation digests were carried out using *Bam* HI which illustrated clearly that the inserts were in the orientation whereby SP6 could be used for the generation of labelled antisense riboprobes, (results not shown). In order to construct a riboprobe from pGEM-3Zf CD44s the plasmid was cut (figure 3.9. B) using an enzyme which does not cut within the area of interest, in this case *Nco* I was chosen as it cuts near the 5' end of the cDNA encoding CD44 standard, leaving a 5' overhang. *Nco* I digestion of pGEM-3Zf CD44v resulted in two fragments as *Nco* I cuts twice within the CD44v cDNA, figure 3.9. B.

A purified DNA preparation of pGEM 3Zf CD44s was then labelled by *in vitro* transcription producing a ^{32}P-labelled riboprobe. Analysis of the riboprobe on denaturing PAGE revealed an undegraded fragment of the expected size (not shown). This riboprobe was then used to probe mRNA which had been blotted onto a nitrocellulose membrane as described in section 2.8. The results are shown in figure 3.10.

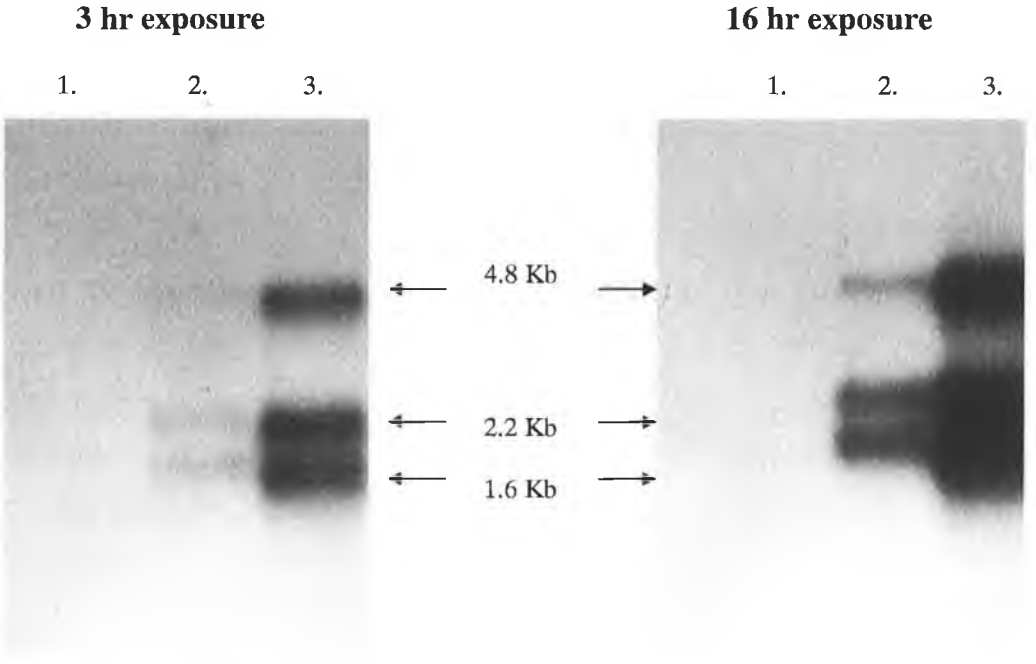


Fig. 3.10. Northern blot analysis of mRNA using a CD44s riboprobe. A CD44s ^{32}P-labelled riboprobe was used to probe 3 μg of mRNA revealing the three CD44 bands, these bands were

approximately sized by comparison to the ribosomal bands of total RNA which was electrophoresed along side the mRNA. Lane no. 1. Mutu I mRNA, 2. Mutu III mRNA, 3. HuT 78 mRNA.

It can be seen that CD44s transcript is present in the Mutu III LMP1+ clone and that CD44s transcript was absent in Mutu I. To determine if equal quantities of mRNA were applied to each lane a ^{32}P-labelled GAPDH probe was used to reprobe the same blot

Fig. 3.11. Northern blot analysis of mRNA using a GAPDH riboprobe. A ^{32}P-labelled riboprobe was used to probe the northern blot in figure 3.17. to demonstrate equal loading. Lane no. 1. Mutu I mRNA, 2. Mutu III mRNA, 3. HuT 78 mRNA.

Figure 3.11 illustrated that intact mRNA was present in each lane, HuT 78 appears overloaded but this is of no consequence as it only served as a positive control.

The ensemble of these results indicate that (1) CD44 expression was upregulated at the RNA level and (2) lack of CD44 expression in the type-III cell line Mutu III coincides with lack of LMP1 expression. As LMP1 and EBNA2 are the principal effectors of phenotypic change in BL cell lines it was decided to investigate the role of each of these viral proteins on CD44 expression when expressed as sole viral proteins in an EBV-negative BL background. The tetracycline regulated cell lines DG75 tTA LMP1 and DG75 tTA EBNA2 were used to this end.

3.5 Tetracycline Regulated Gene Expression System.

The tetracycline regulated system used by Floettmann *et al.*, (1996) is based on that developed by Gossen and Bujard (1992), in which the gene of interest is cloned downstream of a promoter containing binding sites for the hybrid tetracycline-regulated

transactivator (tTA). The plasmid tTA encodes a fusion protein of the sequence-specific DNA binding tetracycline repressor (TetR) and the C-terminal domain of the herpes simplex virus VP16 transactivator. A second plasmid contains the gene of interest cloned downstream of seven copies of the *Escherichia coli* Tn10 tetracycline operator *(tetO)* contiguous with a CMV-IE minimal promoter. When tetracycline is present, it binds to the tTA preventing it binding to the promoter. Upon removal of tetracycline the hybrid TetR binds to the *tetO* site positioning the VP16 domain so that it can transactivate the CMV-IE promoter (Gossen and Bujard, 1992).

Stable cell lines containing plasmids in which EBNA2 and LMP1 expression was regulated by tetracycline were generated by Floettmann *et al.*, (1996) as follows: The tTA expressing plasmid pUHD15-1 was modified by the addition of a hygromycin resistance gene under the control of an SV40 promoter creating the drug-selectable tTA-expressing vector pJEF-3. A neomycin resistance gene under the control of an SV40 promoter was cloned upstream of the tTA responsive promoter of pUHD10-3 to create the responsive vector pJEF-4. The EBNA 2 coding with a 5' rabbit β-globin intron was cloned into pJEF-4 to produce pJEF-31 (fig. 3.12). The LMP1 cDNA was inserted into pJEF-4 to produce pJEF-6, (fig. 3.12). pJEF-3 was then transfected into DG75 to give the stable cell line DG75 tTA, which was then transfected with pJEF-31 producing the cell line DG75 tTA EBNA2 or pJEF-6 giving the cell line DG75 tTA LMP1 (Floettmann *et al.*, 1996). This system allowed examination of either EBNA 2 or LMP1 in the same cell background before and after induction of the EBV protein thus eliminating clonal variations which may occur between cell lines.

TETRACYCLINE REGULATED CELL LINES DG75 tTA EBNA2/DG75 tTA LMP1

The host cell line DG75 is stably transfected with two plasmids pJEF-3 and pJEF-31 or pJEF-6 which are selected during cell culture using the drugs Hygromycin and G418 respectively.

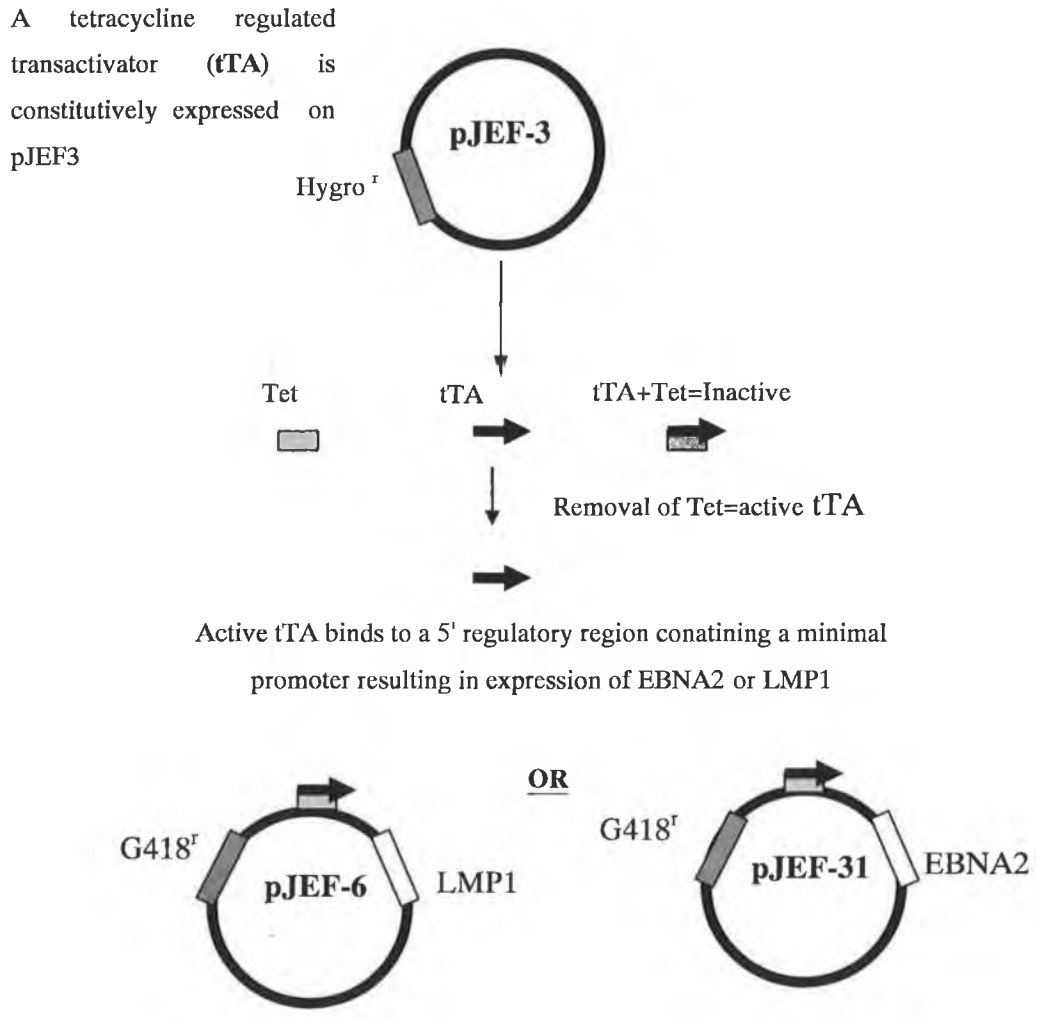


Fig. 3.12. A schematic representation of the tetracycline regulated system used to express of DG75 tTA EBNA2 and LMP1

Prior to commencing flow cytometric analysis of the cell lines DG75 tTA LMP1 and DG75 tTA EBNA2 for CD44 expression, the induction of the EBV latent proteins LMP1 and EBNA2 respectively were confirmed by western blotting. Proteins were isolated from cultured cells 24 hours after the removal of tetracycline, and from cells which were maintained in medium containing tetracycline.

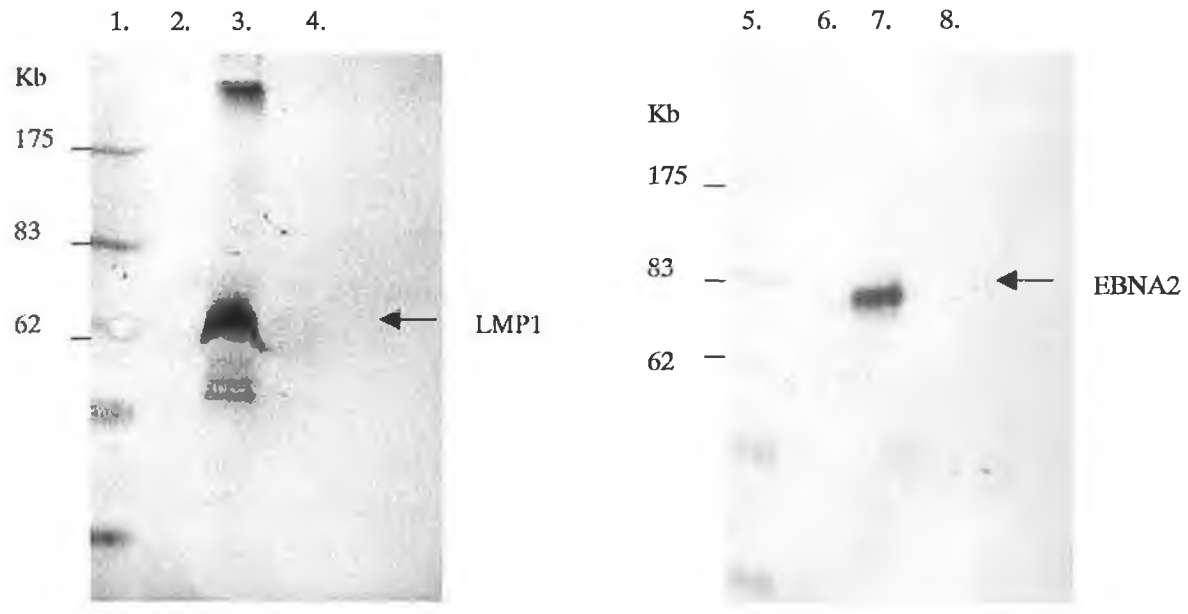


Fig. 3.13. Western blot analysis of the induction of LMP1 and EBNA2 in the tetracycline regulated cell lines DG75 tTA LMP1 (left) and DG75 tTA EBNA2 (right). Western blots were carried out using the anti-LMP1 antibody CS1-4 and the anti-EBNA2 antibody PE2. Lane no. 1, 5, Protein marker, 2. DG75 tTA LMP1 uninduced, 3. DG75 tTA LMP1 induced, 4. Blank, 6. DG75 tTA EBNA2 uninduced, 7. DG75 tTA EBNA2 induced, 8. Blank.

It is clear from figure 3.13 that 24 hr after the removal of tetracycline significant levels of LMP1 and EBNA2 are detected. Neither LMP1 nor EBNA2 are detectable in the protein extracts from cells before the removal of tetracycline from the growth medium.

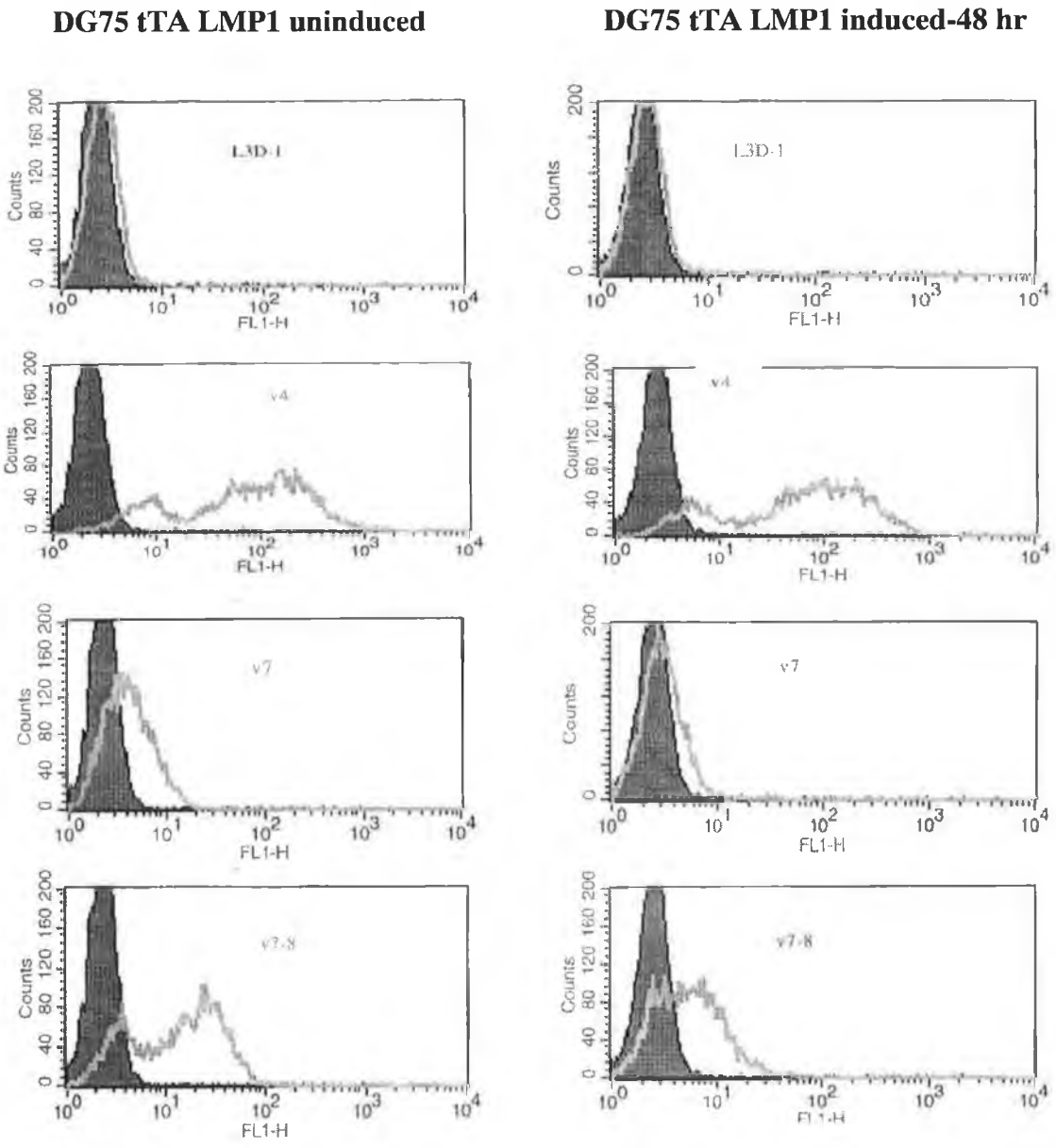


Fig. 3.14. Flow cytometry analysis of CD44 standard and variant expression in the tetracycline regulated cell line DG75 tTA LMP1 before and after induction of LMP1. Relative cell number is plotted on the Y axis and log fluorescence intensity on the X-axis. The antibodies used are indicated on each histogram.

It can be seen from figure 3.14 that CD44 standard expression was not detected in any experiment using the anti-standard CD44 antibody L3D-1. Once again failure to detect CD44 by this method may have been due to epitope masking caused by glycosylation or conformational changes due to the insertion of variant epitopes. CD44 variants were detected both before and after induction of LMP1. CD44v4 and CD44v6 were strongly expressed both before and after induction whereas the expression of CD44v3 and v4-5 was not detected (results not shown). The expression of CD44v7 and v7-8 appeared slightly downregulated after induction of LMP1.

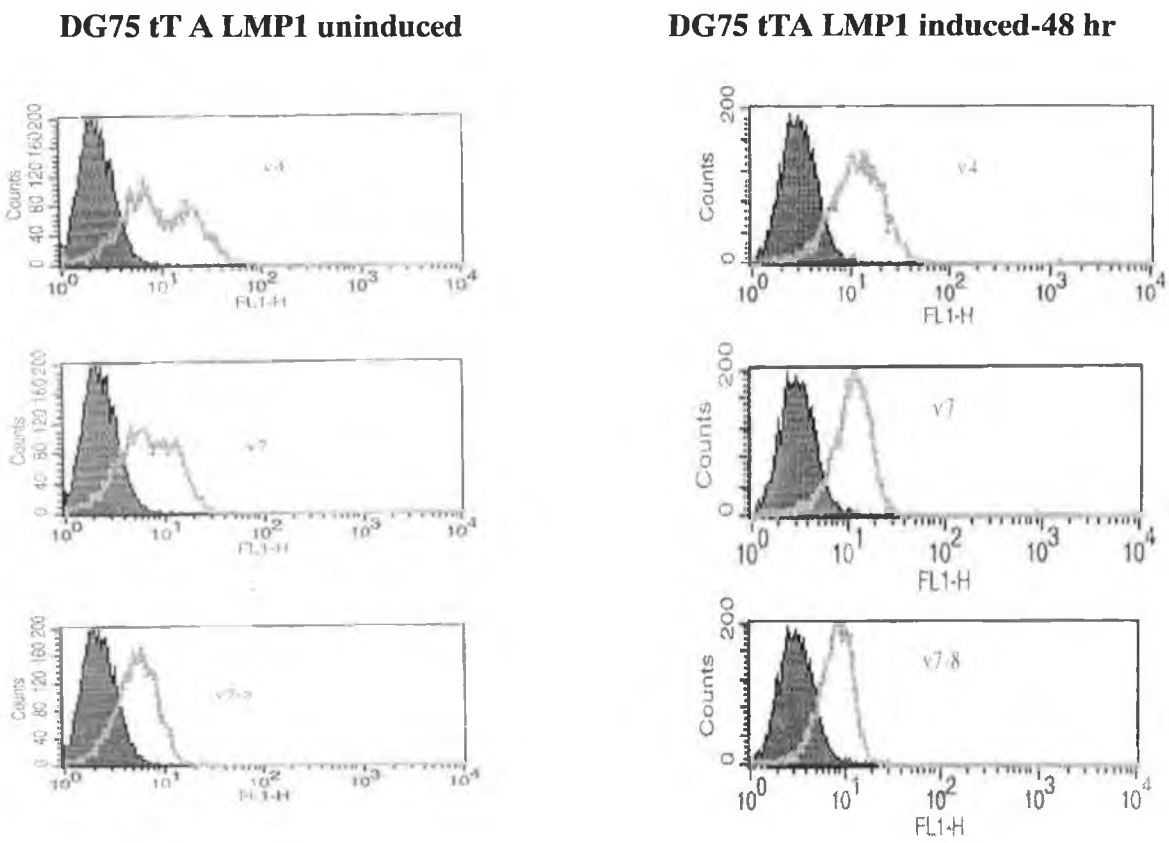


Fig. 3.15. Measurement of CD44 variant isoform expression in the tetracycline regulated cell line DG75 tTA LMP1 before and after induction of LMP1. Relative cell number is plotted on the Y-axis and log fluorescence intensity on the X-axis. The antibodies used are indicated on each histogram.

Two additional anti-standard antibodies, D2.1 or 44F.10.2 were included in a second round of flow cytometric analysis, however, CD44 expression was not detected with these anti-standard antibodies either (results not shown). CD44 variants were again detected but the pattern observed was different from the initial experiments with greater

v7 expression detected in both induced and uninduced cells with a slightly greater level of expression in the induced cells which contradicts the first experiment (see figure 3.14/3.15). CD44v7-8 expression did not change and v4 appeared downregulated in both induced and uninduced cells when compared with initial experiments.

It has been noted that the induction of LMP1 in the cell line DG75 tTA LMP1 causes a cytostatic effect in the cells after 48hr, thus the apparent inconsistencies in CD44 variant and standard isoform expression may have been attributable to this factor (Floettmann *et al.,* 1996). In order to investigate this possibility, the cells were examined 96 hr after removal of tetracycline from the media and analysed by flow cytometry. However, the pattern of CD44 variant expression was again irregular and CD44s was not detected. The pattern of CD44 standard and variant expression was examined in the cell line DG75 tTA EBNA2 before and after induction of EBNA2 (results not shown). Again CD44 standard was not detected however strong positive reaction using some anti-variant mAbs indicated the presence of variant isoforms. The patterns of CD44 variant expression before and after 24 hr and 48 hr induction were identical.

Due to the varied results seen with flow cytomety, western blot analysis was carried out, using the anti-standard antibody BRIC 238 which recognises CD44 standard and CD44 variant containing proteins.

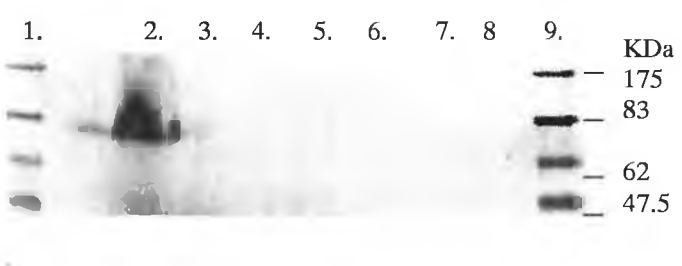


Fig. 3.16. Detection of CD44 expression by western blotting in the cell lines DG75 tTA EBNA2 and DG75 tTA LMP1. Lane no. 1. 9. Protein marker, 2. HuT 78, 3. DG75 tTA EBNA2 uninduced, 4. DG75 tTA EBNA2 24hr induction, 5. DG75 tTA EBNA2 48 hr induction, 6. DG75 tTA EBNA2 72 hr induction, 7. DG75 tTA LMP1 uninduced 8. DG75 tTA LMP1 48 hr induction.

Figure 3.16 illustrates clearly that the induction of LMP1 or EBNA2, in the EBV-negative BL cell line DG75 does not result in CD44 expression at the protein level. The ensemble of these results imply that (1) CD44s is not expressed in the EBV negative BL cell line DG75 and is not upregulated by either EBNA2 or LMP1 when expressed as sole viral proteins. (2) Given that CD44 expression is upregulated at the RNA level in Mutu III LMP1+ and not in Mutu III LMP1- this suggests that EBNA2 and LMP1 are both necessary for CD44 upregulation or that other EBV latent genes are required for upregulation of CD44 in BL. (3) The anti v4, v7 and v7-8 appear to crossreact with cellular proteins in DG75. It remains possible however that the v4 and v7-8 epitopes are expressed as part of novel CD44 molecules that do not react with the mAbs used in these experiments.

3.6 RIBONUCLEASE PROTECTION ASSAY

The ribonuclease protection assay (RPA) is a highly sensitive and specific method for the detection and quantitation of mRNA species. The procedure is based on the hybridization of the analysed RNA to a radioactively labelled RNA probe and subsequent digestion of unhybridized RNA with single-stranded-specific nucleases, typically a combination of RNases A and T1. The RPA procedure used in this study is outlined in section 2.9.

The pGEM 3Zf CD44v clone digested with *Nco* I was used to generate a riboprobe for RPA. *Nco* I cuts the CD44v cDNA within v7 resulting in a probe which contains the right end standard exons, plus v8, v9, v10 and a portion of v7 as illustrated below.

PGEM 3Zf CD44v

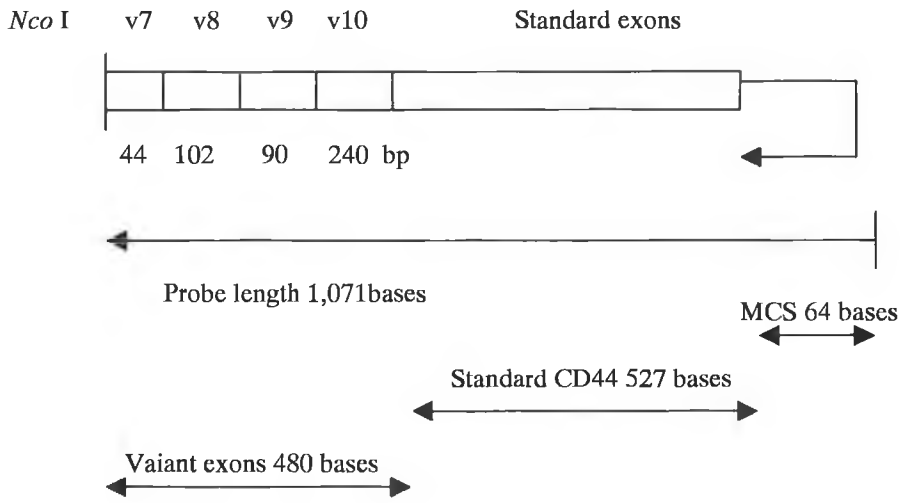


Fig. 3.17. Schematic representation of the pGEM 3Zf CD44v riboprobe construct.

The probe was generated by *in vitro* transcription as before, and used for RPA. The results obtained are shown in figure 3.18.

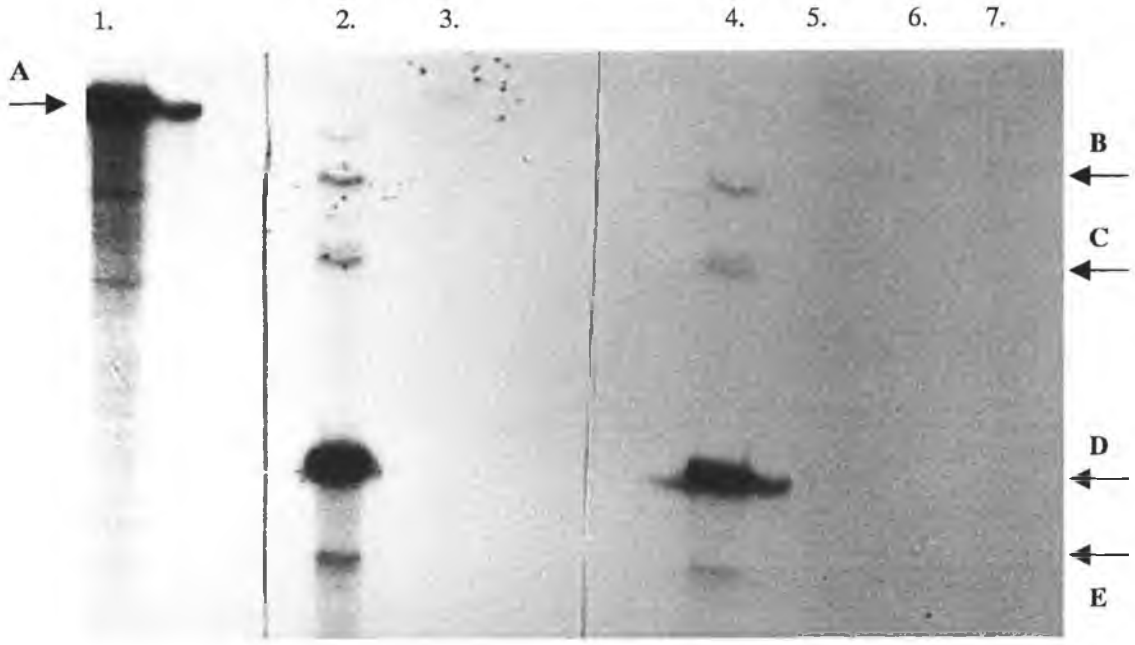


Fig. 3.18. RPA assay for the detection of CD44 transcripts. An RPA was carried out using 20 μg of total RNA and a ^{32}P-labelled CD44v riboprobe. The labelled probe is indicated as A and protected fragments are indicated as B-E. Lane No. 1. Labelled CD44v probe, 2. HuT 78, 3. Mutu I, 4. Mutu III (LMP1+), 5. Mutu III (LMP1-), 6. DG75 tTA LMP1 uninduced 7. DG75 tTA LMP1 induced 96 hr.

The labelled probe (A) was electrophoresed alongside the samples to indicate that the probe was predominately full length, it also served as a size marker. CD44 transcript was detected in the cell lines HuT 78 and Mutu III LMP1+, but not in Mutu III LMP1-, Mutu I or before/after induction of LMP1 or EBNA2 in the cell lines DG75 tTA LMP1 and DG75 tTA EBNA2 respectively (discussed in next section) (EBNA2 not shown). This experiment indicated that upregulation of CD44 was occurring at the level of transcription and confirmed the findings of the northern blot. Also EBNA2 or LMP1 are not sufficient when expressed alone to up-regulate transcription of the CD44 gene. The main transcript detected by RPA was the CD44s exons (D). Bands B and C may be the result of variant exon expression in conjunction with the standard exons. Band E may represent variant exons without the expression of standard, however this is unlikely as the sizes do not seem to correspond to any selection of variant exons.

3.7 DISCUSSION

CD44 is considered to be both structurally and functionally, one of the most variable surface molecules. Alternative splicing of variant exons as well as posttranslational modification of the molecule generates a wide repertoire of CD44 variant isoforms (Ponta *et al.,* 1997). BL cell lines displaying a type III latency program have been reported to express CD44 whereas EBV-negative and type-I latency BL cell lines do not express CD44 (Gregory *et al.,* 1990; Kryworckho *et al.,* 1995). Thus the expression of CD44 in BL cell lines correlates with the expression of the EBV latent proteins. The EBV latent proteins EBNA2 and LMP1 have been implicated in regulating CD44 expression in a BL background (Kryworckho *et al.,* 1995). CD44 expression has been implicated in metastasis and enhanced tumour formation in BL cells. One report demonstrated that over expression of CD44 standard but not CD44v8-10 isoforms in the BL cell line Namalwa enhanced tumour formation and metastatic behaviour in these cells (Sy *et al.,* 1991). Thus the upregulation of CD44 expression by EBV latent genes may result in increased metastatic and tumourigenic capabilities of BL cells. The aim of the work presented in this chapter was to examine the expression of CD44 isoforms in a BL cell background and in particular the effects (if any) of LMP1 and EBNA2.

Since mAbs to CD44 variants have been developed and made commercially available their use in the analysis of CD44 expression has become widespread (Naor *et al.,* 1997). In this study, we used a panel of anti-CD44 antibodies to determine the expression pattern of CD44 in the cell lines examined. Initial analysis was carried out by flow cytometry on the cell lines Mutu I and Mutu III, which display type-I and type-III latency phenotypes respectively. These cell lines were used to examine the collective effect of the EBV latent proteins on CD44 gene expression. Although CD44s and CD44v expression was detected in both cell lines by flow cytometry, using the mAb D2.1, this finding is contrary to a previous report by Gregory *et al.,* (1990), who described CD44s expression in Mutu III only. D2.1 recognises the 83kDa form of CD44 but also a smaller 45 kDa protein p45. Further analysis of Mutu I and Mutu III by western blotting using the anti-standard antibody D2.1 illustrated clearly that CD44s was not expressed in either cell line and the positive result obtained for CD44s

expression by flow cytometry was most likely due to the recognition of p45 by D2.1. This result was investigated further using a second anti-standard antibody BRIC 238, which confirmed the absence of CD44s from Mutu III.

Mutu III is a type-III latency BL cell line which is characterised by the expression of all EBV latent proteins with the concomitant up-regulation of B-cell activation antigens and cellular adhesion molecules such as CD44. A partial characterisation of the EBV latent genes expression in Mutu III clone c148D illustrated that LMP1 was not expressed and that this was a clone specific defect when compared with other Mutu III clones. The LMP1+ clone of Mutu III expressed CD44s were as the LMP1- Mutu III clone did not, as shown by western blot. This novel Mutu III clone now known as Mutu III LMP1- proved a useful tool in investigating the role of EBV latent genes on CD44 expression as it illustrated that the expression of LMP1 in the type-III latency BL cell line Mutu III is probably linked with CD44 expression. This also suggests that expression of all the EBV latent genes are required to upregulate CD44 expression. This was illustrated by Fitcher and colleagues who reported CD44 upregulation in a LCL, this group used an LCL which was immortalised through a mutant EBV in which the expression of EBNA2 was conditionally regulated, in this system CD44 expression coincided with the restoration of EBNA2 expression (Fitcher *et al.,* 1997). As type-III latency corresponds to EBV associated diseases such as IM and PTLD, it can be inferred that the expression of CD44 in these diseases may dependent on LMP1 or EBNA2 expression.

As LMP1 and EBNA2 are the principal effectors of phenotypic change in Burkitt's lymphoma, the effect of these EBV latent genes on CD44 expression in a EBV negative BL background, was examined using a inducible system (Floettmann *et al.,* 1996). CD44 expression was examined by flow cytometry before and after induction. The flow cytometric results were again highly varied, with the presence of positive peaks indicating the presence of CD44 variant epitopes without the presence of CD44 standard epitopes. This is unusual as all known variant isoforms contain standard CD44, however, the extensive post-traslational modification that CD44 undergoes may have interfered with the detection of CD44s. Also the insertion of variant exons and the

possible introduction of both N- and O-glycosylation sites may lead to the disruption of epitopes recognised by anti-CD44 antibodies. The positive signals observed by flow cytometry using some mAbs most likely represents non-specific cross-reactivity with cellular proteins other than CD44.

This possibility has been strengthened by a recent report that examined the used of mAbs for the detection of CD44 (Martegani *et al.,* 1999). In this study Martegani and colleagues investigated the immunoreactivity of a large panel of commercially available anti-standard and anti-variant CD44 antibodies with a set of stable cell lines expressing various combinations of CD44 variant exons. They examined the cells by immunohistochemistry, fluorescence activated cell sorting (FACS) and RT-PCR. The results demonstrated that the immunoreactivity of some mAbs directed to CD44 exon specific epitopes can be impaired by the structural variability of the molecule (Martegani *et al.,* 1999). Their findings demonstrated that (1) certain exon assortments and/or posttranslational modifications of CD44v molecules can mask CD44 exon specific epitopes; (2) glycosaminoglycan side chains, carried by some CD44v molecules of high molecular weight, may play a critical role in determining the exact conformation of the molecule, which is necessary for the detection of CD44 variant epitopes by specific mAbs, (3) in a panel of stable transfectants expressing CD44 N-glycosylation site-specific mutants, generated in the constant region of the extracellular domain, asparginine-isoleucine substitution is sufficient per se to impair the immunoreactivity of several mAbs to standard CD44. Thus, conformational changes due to alternative splicing of CD44 variant exons and/or posttranslational modification of the molecule (different degrees of glycosylation), which are cell-type specific, are likely to generate CD44 variants that elude immunodetection. These findings strongly suggest that immunohistochemical analysis of CD44 expression *in vitro* and *in vivo*, using mAb specific for CD44 variant encoded exons and epitopes, can potentially be impaired by false negative results (Martegani *et al.,* 1999). The results obtained in this study with the anti-CD44s antibodies argue that epitope masking is not responsible for the failure to detect CD44s as three different anti-standard CD44 antibodies were used.

Further investigation by western blotting revealed that the cell lines DG75 tTA LMP1 and DG75 tTA EBNA2 did not express CD44 at the protein level before or after the induction of LMP1 or EBNA2 respectively. This demonstrated clearly that the expression of LMP1 or EBNA2 alone in this EBV negative BL background is insufficient for the up-regulation of CD44 expression. Although the findings of Martegani and colleagues (1999) may explain some false negatives, the apparent structural complexity of CD44s and CD44v may also explain false positives with possible cross-reactivity with other molecules or a lack of specificity resulting in the false detection of CD44 expression. This appears to be a problem in this study as CD44 v7 and v7-8 were detected in DG75 tTA LMP1 before and after induction, however when RNA from these cell lines was analysed by RPA it was clear that mRNA transcript for either v7 or v8 were not present (see next paragraph). This indicates that the results obtained using the mAbs were due to cross-reactivity with an unrelated molecules and not an as yet unidentified CD44 variant. Western blot analysis was carried out using a few selected CD44 anti-variant mAbs, however, this resulted in a high degree of non-specific background bands and the failure to detect CD44 variants (results not shown).

In order to obtain a clear answer as to the expression of CD44s in the cell lines examined it was decided to look at mRNA transcripts by northern blotting and ribonuclease protection assay (RPA). CD44s expression was examined in Mutu I and Mutu III LMP1 + by northern blotting which gave three clear CD44 transcripts in Mutu III LMP1+ but none in Mutu I. These three major transcripts have been previously described (Goldstein *et al.,* 1989; Samenkovic *et al.,* 1989; Quackenbush *et al.,* 1990), and are thought to be due to utilization of mutiple polyadenylation signals (Harn *et al.,* 1991). RPA was also used to reinforce results obtained by northern blotting and to examine the mRNA transcript from the cell lines DG75 tTA LMP1 and DG75 tTA EBNA2 before and after the induction of the latent proteins. Results showed that EBNA2 or LMP1 when expressed alone in an EBV negative BL cell line are not sufficient to upregulate CD44 expression at the transcriptional level. Also LMP1 expression correlates with CD44 expression at the transcriptional level in the type-III

BL cell line Mutu III. The RPA also indicated the possible presence of splice variant transcripts in Mutu III LMP1+ and HuT 78.

3.8 CONCLUSIONS

In conclusion CD44 protein or transcript was not detected in the type-I BL cell line Mutu I. The drift to a type-III phenotype correlated with a strong up-regulation of CD44 expression. This up-regulation was at the transcriptional level. CD44 was not detected in the Mutu III LMP1- clone, thus providing evidence of a direct role for LMP1 in CD44 expression. In an EBV negative BL cell line background neither EBNA2 nor LMP1 alone were sufficient to up-regulate CD44 expression. Due to the widely varied and unreproducible results obtained for CD44 expression using variant specific mAbs by flow cytometry and western blotting, it was clear that mAbs alone were not sufficient to provide a definitive answer as to the presence or absence of CD44 standard/splice variants. Therefore an alternative method for the detection of CD44 expression is desirable. As the results obtained in this study by RPA clearly illustrated CD44 expression, it was decided to design a set of CD44 exon-specific probes which could be used to examine CD44 expression at the mRNA level in EBV-infected cells by RPA. This RPA would be used in conjunction with mAbs to determine the CD44 expression pattern of any cell line examined. This will be discussed fully in the next chapter.

CHAPTER 4

ANALYSIS OF CD44 EXPRESSION BY EXON-SPECIFIC RPA.

4.1 INTRODUCTION

Twenty exons are involved in the genomic organisation of the cell-surface transmembrane glycoprotein CD44. Ten of these exons encode the standard form of CD44, whereas the other ten exons can undergo alternative splicing resulting in variants which are inserted into the standard backbone of CD44 in various combinations (Screaton *et al.,* 1992). In normal tissue, the steady-state level of CD44 mRNA is low, and the variety of alternatively spliced transcripts produced from this complex gene is limited. However, increased and disorderly expression of CD44 isoforms has been observed in a number of cancers (Sneath and Mangham, 1998). CD44 isoforms have been implicated in tumour metastasis and progression and are considered to have a lot of potential as a possible marker for the evaluation of the metastatic capabilities of certain tumours. Also, evaluation of CD44 variant expression could provide valuable information on the prognosis of certain cancers (Sy *et al.,* 1997).

As the results described in chapter 3 have indicated, and as others have reported (Naor *et al.,* 1997; Martegani *et al.,* 1999), the use of mAbs alone does not always give a clear answer to the question of CD44 variant expression. Various other methods exist for CD44 analyses such as RT-PCR and exon junction analysis, all of which have both advantages and disadvantages, these will be discussed in detail later. The development of an assay for the detection of CD44 isoforms with the inherent versatility and sensitivity of an RT-PCR based method but without its disadvantages, is desirable. In this study a method was developed for the quantitative detection of human CD44 isoforms based on the ribonuclease protection assay (RPA) procedure, which was described by Zinn *et al.,* (1983). The ribonuclease protection assay (RPA) is a highly sensitive and specific method for the detection and quantitation of mRNA species. Thus, a cDNA fragment of interest can be subcloned into a plasmid that contains these bacteriophage promoters T7, SP6 or T3, and the construct can be used as a template for synthesising radiolabelled anti-sense RNA probes. The RPA procedure used in this study is outlined in section 2.9. The CD44 exon-specific RPA developed in this study is based on a panel of CD44 exon specific probes from which labelled riboprobes were prepared by *in vitro* transcription. These ^{32}P-labeled

riboprobes were in turn used in a hybridisation reaction with the RNA of interest followed by RNase digestion and electrophoresis in a denaturing gel of the protected probe fragments after (see figure 4.1). By comparing results obtained with each variant specific probe and calculation of fragment sizes it was possible to determine the CD44-exon composition of the cells examined.

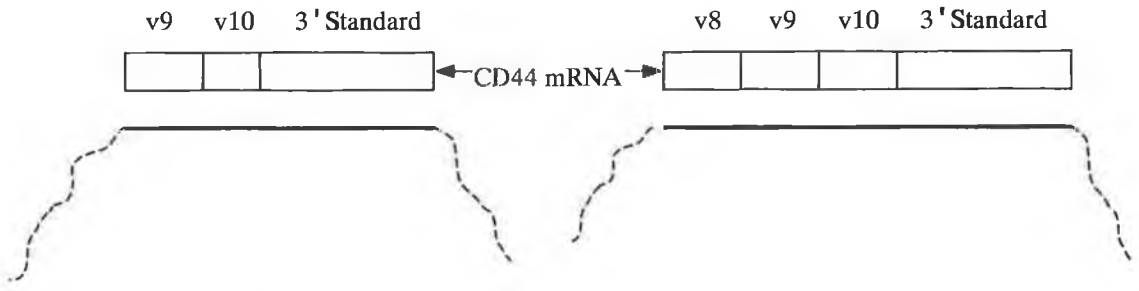


Fig. 4.1. Schematic representation of probe hybridization during RPA. CD44 mRNA segments are represented by open boxes. The labelled probe is represented as a black line, either unhybridized (dashed) or hybridised (solid line). Unhybridized probe and mRNA is then digested by RNase resulting in two fragments of defined length corresponding to different CD44 exon combinations.

Although not practical for the analysis of clinical samples, this assay, as it would be quantitative, would be useful in the analysis of factors that affect CD44v exon splicing and usage. The CD44 RPA was initially used to analyse CD44 variant expression in the colon carcinoma cell line HT29. This cell line was chosen as the CD44 exon profile of HT29 has been widely studied and established using other methods (Van Weering *et al.,* 1993; Woodman *et al.,* 1996; Goodison *et al.,* 1997; Reeder *et al.,* 1998). The exon-specific RPA was also used to examine the pattern of variant isoform expression in EBV-positive cell lines which exhibit type-I and type-III latency and compared with the CD44 expression pattern of EBV-positive lymphoblastoid cell lines (LCL). Type-III EBV-positive cell lines are derived from type-I BL cell lines which have drifted in culture to express all the EBV latent genes. An LCL is established by isolating spontaneously transformed latently infected B-lymphocytes from peripheral blood of patients previously infected with EBV. LCL represent type-III infections of resting B cells and do not possess the translocated *c-myc* seen in BL. As LCLs and BL type-III cell lines reflect EBV latent gene expression profile associated with different EBV-malignancies, then comparison of CD44 expression in both types of cell lines is of interest.

4.2 GENERATION OF CD44 PROBES FOR EXON-SPECIFIC RNASE PROTECTION ASSAY

Several CD44 exon-specific probes were constructed from a CD44 cDNA which contained both standard and variant CD44 exons. A defined length of the CD44 cDNA was excised from the recombinant pGEM 3Zf CD44v clone by a *Bam*HI/*Bcl* I double digest and subcloned into pGEM 7Zf as the MCS of pGEM 7Zf contained more useful restriction sites for this study (figure 4.2). *Bam* HI cuts the CD44v cDNA within standard exon 3 and *Bcl* I cuts at the start of standard exon 17 resulting in a CD44 cDNA fragment of 1,684 bp coding from exon 3 to exon 17 (see figure 4.2.B). *Bcl* I and *Bam* HI have compatible ends, thus the CD44 fragment was subcloned in to *Bam* HI digested pGEM 7Zf. After subcloning the *Bcl* I site is abolished.

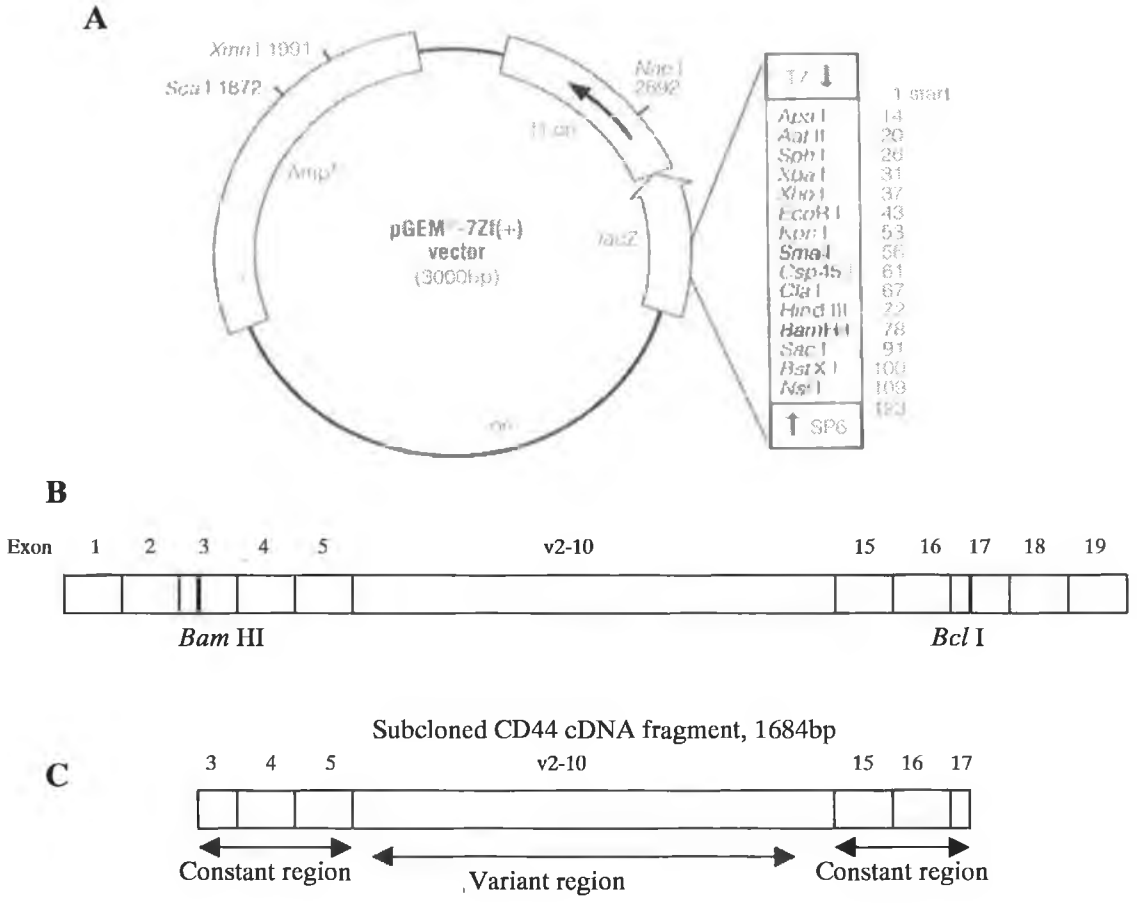


Fig. 4.2. A Schematic map of pGEM 7Zf. B Schematic representation of the CD44 cDNA. C. Schematic representation of subcloned portion of CD44 cDNA.

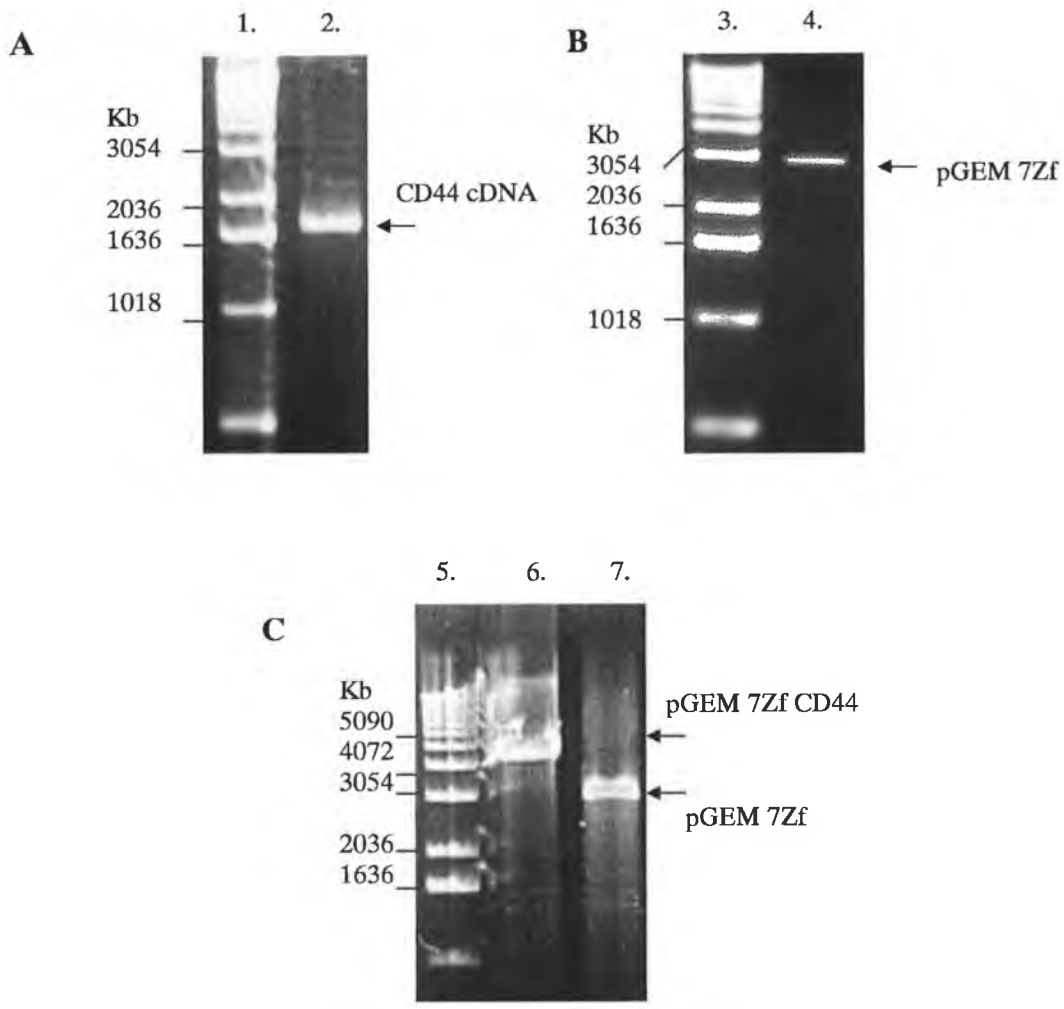


Fig. 4.3. Subcloning of CD44 cDNA into pGEM 7Zf. **A.** Purified CD44 cDNA fragment *Bam* HI/*Bcl* I digest. **B.** Purified *Bam* HI digested pGEM 7Zf. **C.** Recombinant pGEM 7Zf CD44 and pGEM 7Zf digested with *Bam* HI. Lanes no. 1, 3, 5, 1kb DNA ladder, 2. CD44 cDNA fragment, 4. *Bam* HI digested pGEM 7Zf, 6. *Bam* HI digested recombinant pGEM 7Zf CD44, 7. *Bam* HI digested pGEM 7Zf.

Restriction digests were carried out to determine the orientation of the pGEM 7Zf CD44 recombinant clones, using *Rsa* I and *Bam* HI/*Hind* III double digests as illustrated in figure 4.4.

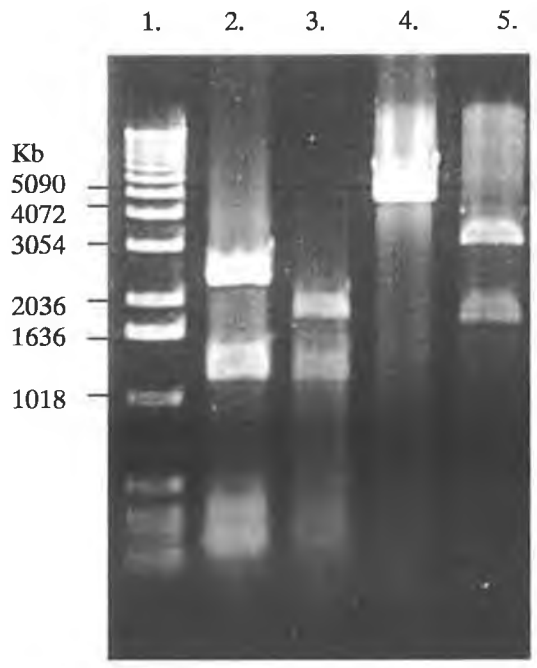


Fig. 4.4. Orientation restriction digestion of recombinant pGEM 7Zf CD44. The orientation of two recombinant pGEM 7Zf CD44 clones was determined by *Rsa* I and *Bam* HI/*Hind* III digestion. Lane no. 1. 1 kb DNA ladder, 2. Clone 1 *Rsa* I digest, 3. Clone 2 *Rsa* I digest, 4. Clone 1 *Bam*HI/*Hind* III digestion, 5. Clone 2 *Bam*HI/*Hind* III digestion.

It is clear from figure 4.4. that clones 1 and 2 are in different orientations, as restriction patterns corresponded with the predicted sizes for both orientations (not given). Clone 1 which was in the SP6 orientation (i.e. SP6 was the promoter from which a riboprobe antisense to the CD44 mRNA could be transcribed) and was used in further experiments. This clone was named pGEM 7Zf CD44. Riboprobe templates were generated from plasmids by digestion with restriction enzymes that resulted in a 5' overhang or a blunt end. Subsequent *in vitro* transcription of these yielded riboprobes which were antisense to the CD44 mRNA. Schematic representations of the various templates are outlined in figure 4.5.

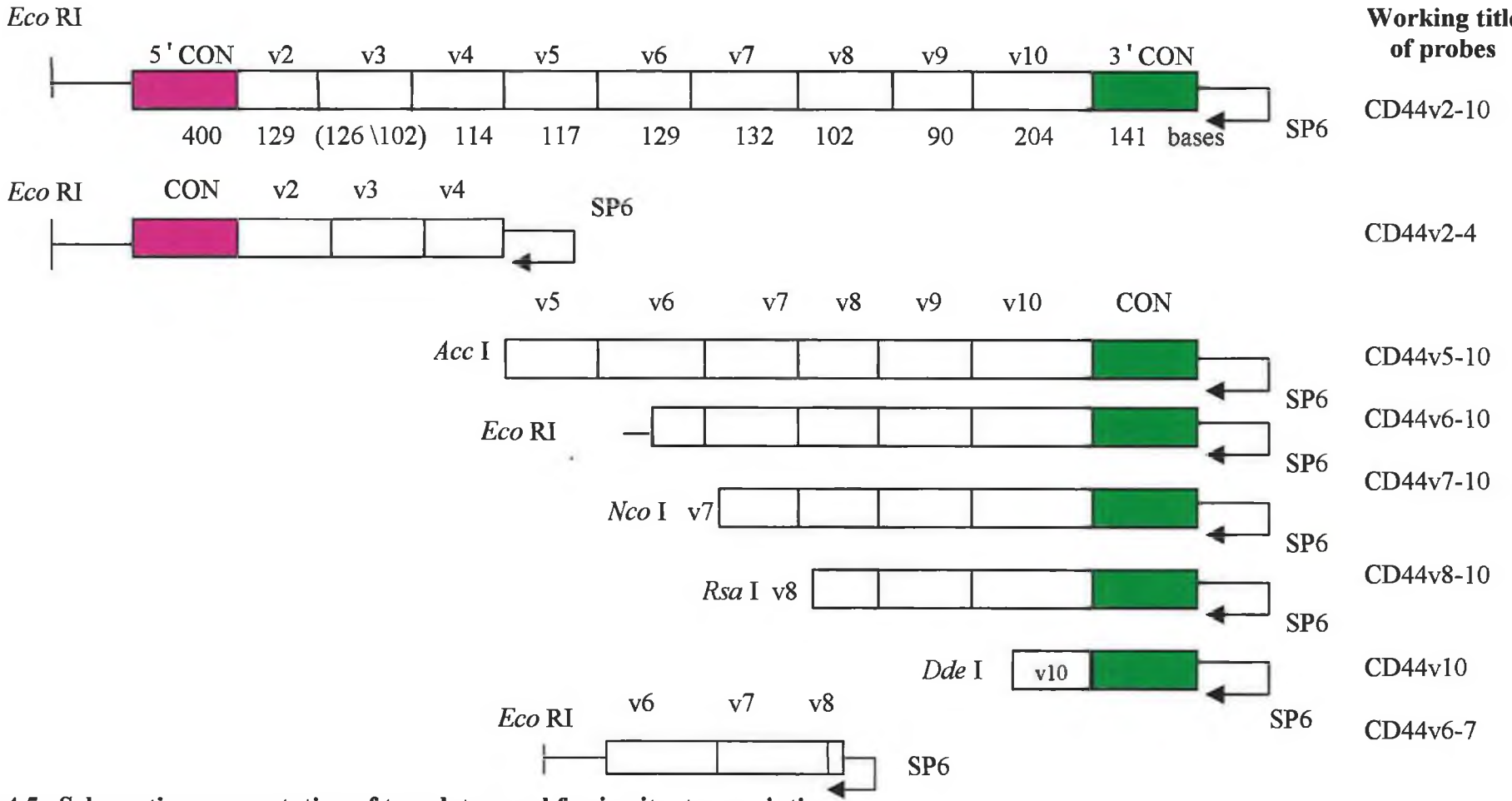


Fig. 4.5. Schematic representation of templates used for *in vitro* transcription.

4.3 GENERATION OF TEMPLATES FOR RIBOPROBE CONSTRUCTION

The full length pGEM7Zf CD44 clone, which contains both the 5' and the 3' standard exons as well as variant exons 2-10, was used to investigate the presence of CD44. pGEM 7Zf CD44 was linearized with *Eco* RI as illustrated in figure 4.7, this resulted in a template containing all variant exons and the resultant labelled probe was called CD44v2-10. The standard or constant (CON/C) regions of the probe will hybridise with 5' and 3' standard exons resulting in two protected fragments of defined size, in this case 400 bases and 141 bases respectively. The CD44v5-10 probe was produced by digestion of the CD44v2-10 probe with *Acc* I as illustrated in figure 4.7. The CD44v6-10 probe was generated by subcloning an *Eco* RV/*Bcl* I fragment from pGEM 3Zf CD44v (as described in section 3.1.3) into pGEM 7Zf which had been double digested with *Bam* HI and *Sma* I as illustrated in figure 4.6.A. Restriction analysis was used to confirm the size and orientation of the recombinant clone pGEM 7Zf v6-10. *Rsa* I digestion resulted in three fragments of 2311 bp, 1179 bp and 206 bp if cDNA inserts were in the orientation suitable for using SP6 as the promoter for riboprobe construction (see figure 4.6.B).

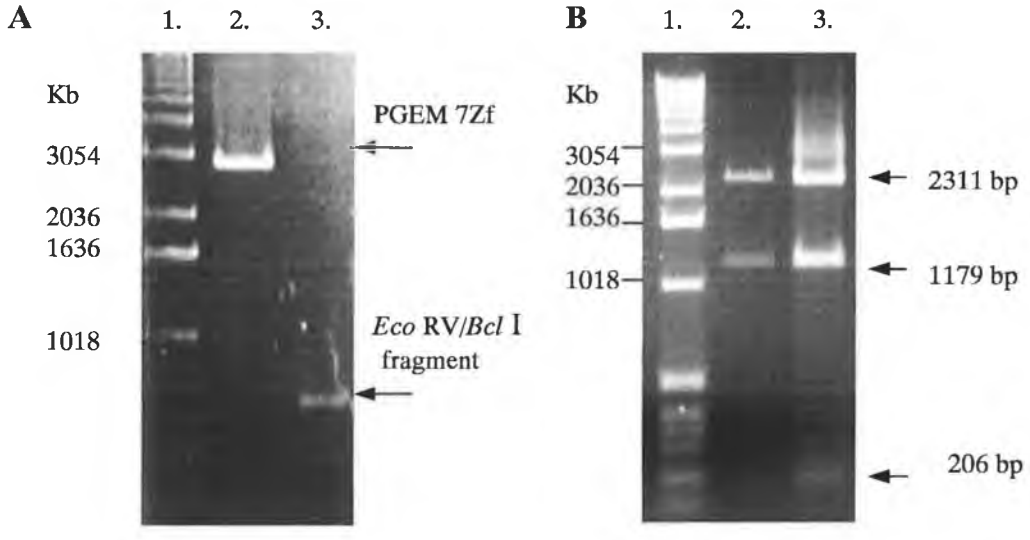


Fig. 4.6 Cloning and restriction analysis of pGEM 7Zf v6-10. A. The size and integrity of the purified *Eco* RV/*Bcl* I fragment from pGEM 3Zf CD44v, and the *Bam* HI/*Sma* I double digested pGEM 7Zf were verified by gel electrophoresis prior to ligation. Lane no. 1. 1 kb DNA ladder, 2. pGEM 7Zf *Bam* HI/*Sma* I, 3. pGEM 3Zf CD44v *Eco* RV/*Bcl* I fragment. **B.** DNA minipreps from recombinant

clones of pGEM 7Zf v6-10, were digested with *Rsa* I to determine their orientation. Lane no. 1. 1 kb DNA ladder, 2. Clone 1, 3. Clone 2.

Figure 4.6 illustrates that pGEM 7Zf v6-10 recombinant clones were of the correct size and in the correct orientation for the use of the SP6 promoter see figure 4.6.B.

The CD44v7-10, CD44v8-10 and CD44v10 templates were prepared by further restriction digests of pGEM 7Zf CD44 or pGEM 7Zf v6-10 (see figure 4.7. below).

CD44v7-10 = pGEM 7Zf v6-10 digested with *Nco* I.
CD44v8-10 = pGEM 7Zf v6-10 digested with *Rsa* I.
CD44v10 = pGEM 7Zf v6-10 digested with *Dde* I.

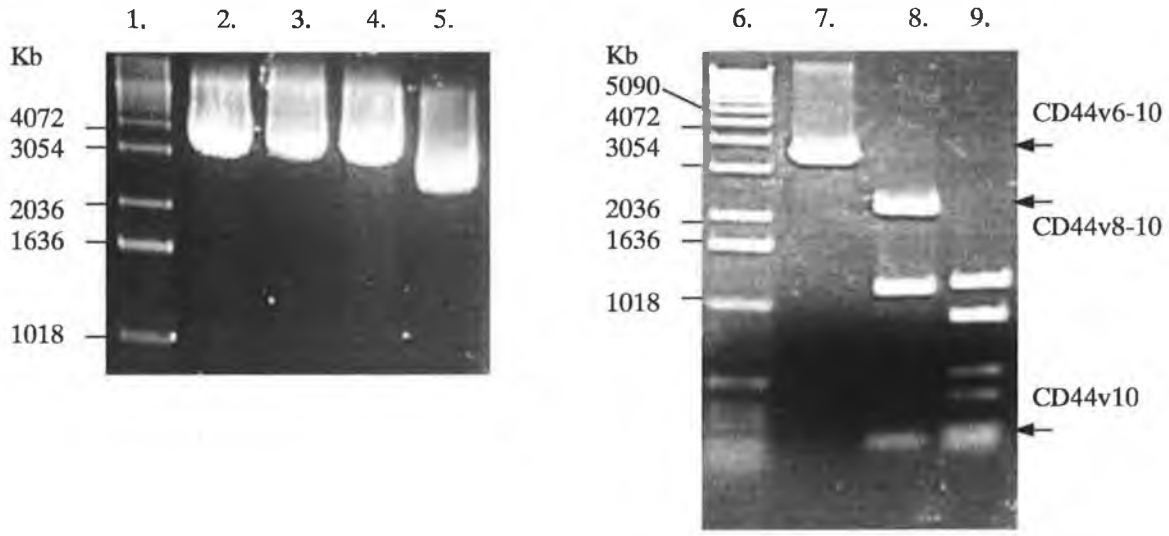


Fig. 4.7. Preparation of templates for CD44 riboprobes by restriction digestion. The recombinant clones pGEM 7Zf CD44 or pGEM 7Zf v6-10, were digested with various restriction enzymes, generating new CD44 exon-specific probes. Lane no. 1, 6, 1 kb DNA ladder, 2. pGEM 7Zf CD44/*Nco* I, 3. pGEM 7Zf CD44/*Eco* RI, 4. pGEM 7Zf CD44/*Acc* I, 5. pGEM 7Zf v6-7/*Eco* RI (described below). 7. pGEM 7Zf v6-10/*Eco* RI, 8. pGEM 7Zf v6-10/*Rsa* I, 9. pGEM 7Zf v6-10/*Dde* I.

The CD44v2-4 probe was prepared by digesting pGEM 7Zf CD44 with *Acc* I and *Sac* I, which removes a 925bp section of the CD44 cDNA from the end of the exon v4 to the

Sac I site in the multiple cloning site of pGEM 7Zf, (see figure 4.2.A). This fragment was removed and the remaining vector was religated creating pGEM 7Zf v2-4 see figure 4.8.A. DNA minipreps of recombinant pGEM 7Zf v2-4 clones were digested with *Eco* RI demonstrating that the clones were of the correct size, figure 4.8.B. *Rsa* I digests were also carried out to verify the constructs (results not shown).

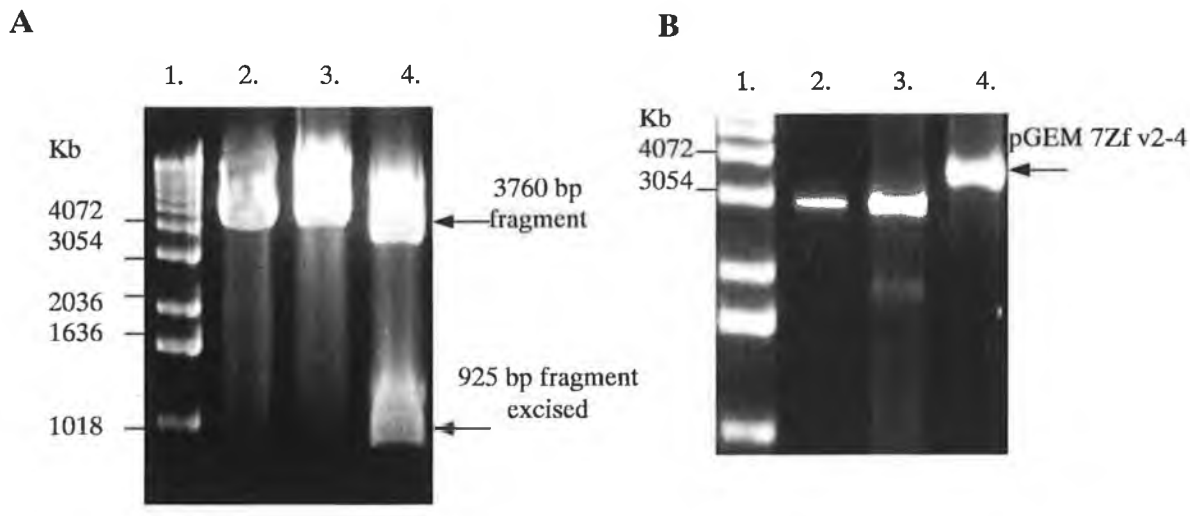


Fig. 4.8. Cloning of pGEM 7Zf v2-4. A. A fragment of 925 bp was excised from pGEM 7Zf CD44 and the remaining vector was religated to form pGEM 7Zf v2-4. Lane no. 1. 1 kb DNA ladder, 2. pGEM 7Zf CD44/*Nco* I, 3. pGEM 7Zf CD44/*Acc* I, 4. pGEM 7Zf CD44 *Acc* I/*Sac* I.
B. *Eco* RI digestion of pGEM 7Zf v2-4. Lane no. 1. pGEM 7Zf *Bam* HI, 2. pGEM 7Zf *Eco*RI, 3. pGEM 7Zf v2-4 *Eco* RI.

The final probe, CD44v6-7 was prepared by subcloning a PCR amplified fragment of CD44v6-7 cDNA into pGEM 7Zf. The forward primer was designed to hybridise at the start of variant exon 6, the reverse primer hybridised at the start of variant exon 8 thus ~20 bases from v8 were also amplified. The expected size of the PCR amplification product was 288 bp. Each primer incorporated a *Bam* HI sites at its 5' end. pGEM 7Zf CD44 was used as the DNA template for PCR amplification, the PCR protocol is outlined in section 2.5.5.

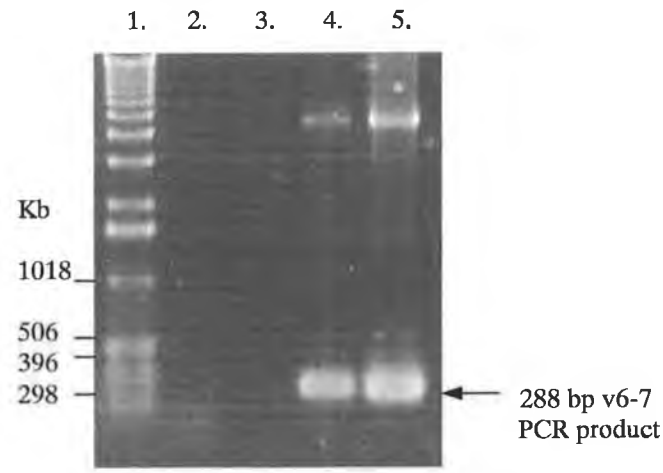


Fig. 4.9. PCR amplification of CD44v6-7. Lane no. 1. 1kb DNA ladder, 2, 3, negative controls, 4, 5, PCR amplification products.

After PCR amplification a PCR product of the correct size was obtained as illustrated in figure 4.9. This amplified product was isolated and digested with *Bam* HI, purified and subcloned into the *Bam* HI site of pGEM 7Zf. Two recombinant pGEM 7Zf v6-7 clones (a) and (b), were digested with *Bam* HI to ensure the inserts were of the correct size and *Eco* RV/*Hind* III to determine the orientation (see figure 4.10 below).

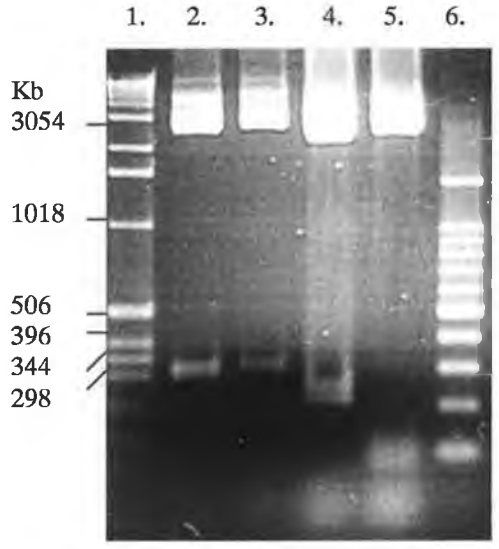


Fig. 4.10. Orientation digests of pGEM 7Zf v6-7 recombinant clones. Lane no. 1. 1 kb DNA ladder, 2. pGEM 7Zf v6-7 clone (a) *Bam* HI, 3. pGEM 7Zf v6-7 clone (b) *Bam* HI, 4. pGEM 7Zf v6-7 clone (a) *Eco* RV/*Hind* III, 5. pGEM 7Zf v6-7 clone (a) *Eco* RV/*Hind* III, 6. 100 bp DNA ladder.

Figure 4.10 illustrates that the inserts are the correct size but there is one in each orientation. Clone b (lane no. 5) was in the SP6 orientation, this clone was used for further experiments.

A summary of all CD44 exon-specific probes is given in table 4.1. Labelled probe sizes and protected fragment sizes are also indicated.

Table 4.1. CD44 exon-specific probes.

PROBE NAME	PROBE COMPOSITION	PROBE SIZE BASES	MAXIMUM PROTECTED SIZE BASES
CD44V2-10	C5'v2v3v4v5v6v7v8v9v10 C3'	1763	1684 Full length, 400 C5', 141 C3'
CD44v2-4	C5'v2v3v4	824	757
CD44v5-10	v5v6v7v8v9v10 C3'	962	914
CD44v6-10	Δv6v7v8v9v10 C3'	798	718
CD44v7-10	Δv7v8v9v10 C3'	629	581
CD44v8-10	Δv8v9v10 C3'	562	517
CD44v10	Δv10 C3'	260	215
CD44v6-7	v6v7vΔ8v	368	288

C5' = 5' CD44 standard exons, C3' = 3' CD44 standard exons, Δ=contains part of the indicated exon.

Prior to hybridizing the CD44 variant exon-specific probes with a given RNA the CD44v2-10 probe was first employed to determine if CD44 was expressed in that particular cell line. In each RPA experiment the labelled probes were also electrophoresed alongside the RNA samples to check their integrity. All probes were full length and intact for each experiment (not always shown). Twenty micrograms of total RNA was used in each RPA analysis, however only half of this reaction was loaded onto the gel. A probe for GAPDH was included for each sample to ensure equal loading. RPA was carried out as described in section 2.9. The RNA used in these experiments was first examined by gel electrophoresis for structural integrity, illustrated in figure 4.11.

4.4 RNA ANALYSIS.

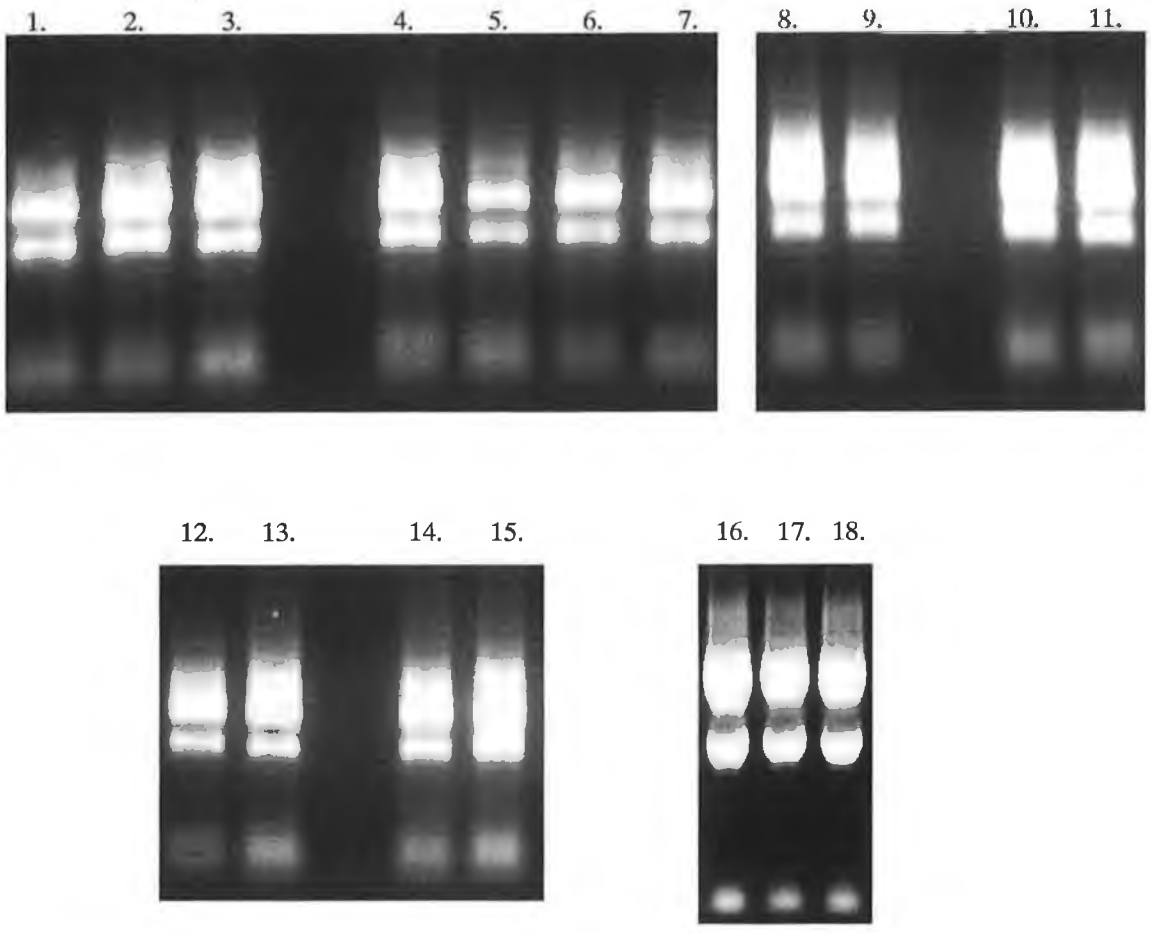


Fig. 4.11. Analysis of RNA structural integrity. Approximately 3μg of total RNA from each cell line was examined by agarose gel electrophoresis. Lane no. 1. BL41, 2. BL41 B958, 3. IARC 171, 4. Mutu I, 5. Mutu III c95, 6. HuT 78, 7. HT29, 8. Ag876, 9. Mutu III c62, 10. BL72, 11. IARC 307, 12. BL74, 13. IARC 290B, 14. X50-7, 15. RAEL BL, 16.C33A, 17. C33A Neo, 18. C33A LMP1.

Structurally intact RNA was present in all cases indicated by the 28S, 18S and 5S ribosomal bands. The first cell lines analysed using the CD44 exon-specific RPA included the colon carcinoma cell line HT29. This cell line was chosen to establish the assay as the pattern of CD44 expression in HT29 has been well established (discussed below).

4.5 CD44 EXON-SPECIFIC RPA ANALYSIS.

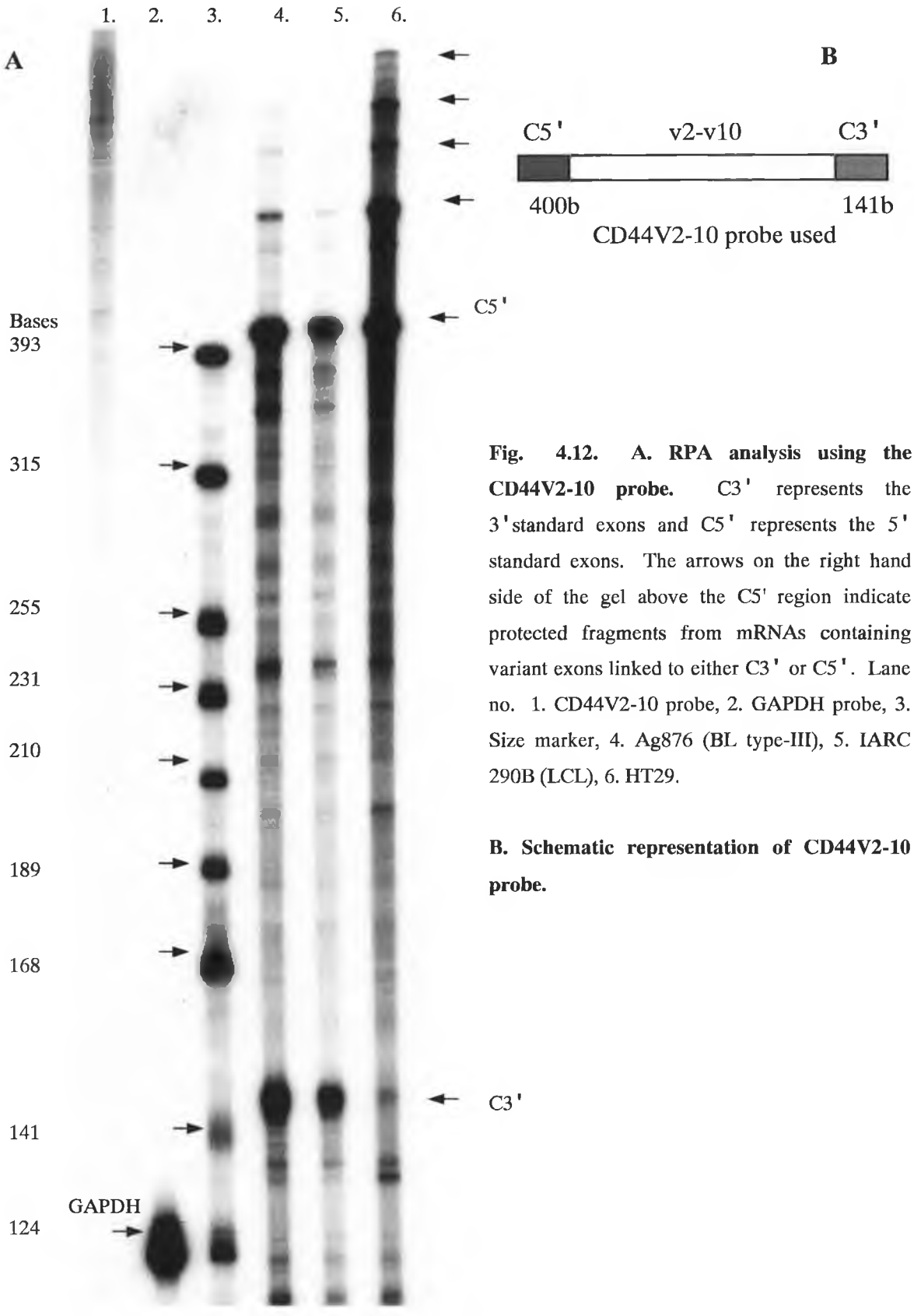


Fig. 4.12. A. RPA analysis using the CD44V2-10 probe. C3' represents the 3'standard exons and C5' represents the 5' standard exons. The arrows on the right hand side of the gel above the C5' region indicate protected fragments from mRNAs containing variant exons linked to either C3' or C5'. Lane no. 1. CD44V2-10 probe, 2. GAPDH probe, 3. Size marker, 4. Ag876 (BL type-III), 5. IARC 290B (LCL), 6. HT29.

B. Schematic representation of CD44V2-10 probe.

It is clear from figure 4.12 that all three cell lines express CD44 mRNA. The 5' and 3' constant exons are visible in Ag876 and IARC 290B (lanes 4 and 5). In contrast HT29 mRNA (lane 6) gave a very low level of C3' protected fragment (141b). This indicated that either these exons are weakly expressed in HT29 of else they are almost always found linked with the adjacent v10 exon thus increasing the molecular weight of the protected fragment. The higher molecular weight bands present in all three lanes indicated the presence of variant exon-containing mRNAs. HT29 appears to have a substantially greater proportion of higher molecular weight bands indicating the presence of several variant exon combinations. These bands are not a consequence of overloading as the level of GAPDH mRNA does not vary appreciably between samples. GAPDH mRNA levels were analysed as a control for the quantity of RNA loaded. Figure 4.13 A represents GAPDH mRNA from the same gel illustrated in figure 4.12.

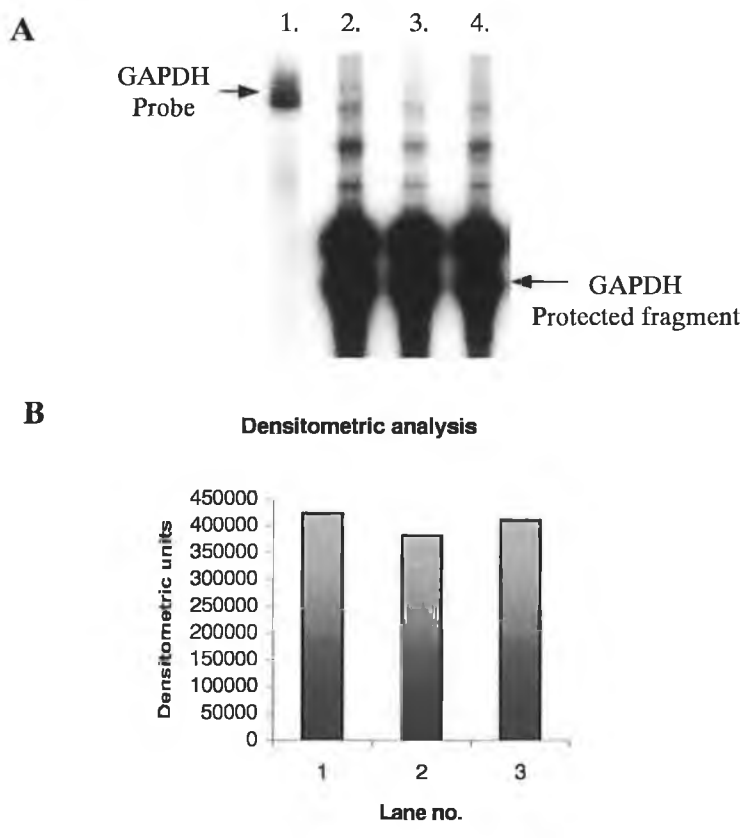


Fig. 4.13. A. GAPDH analysis of loading. Lane no. 1. GAPDH probe, 2. Ag876, 3. IARC 290B, 4. HT29. **B. Densitometric analysis of GAPDH.**

As the expression of CD44 standard in the cell line HT29 had been confirmed (figure 4.12) and the presence of many CD44 variants had been implied, the full range of CD44 variant exon-specific probes was used to investigate CD44 mRNA structure in this cell line. Size evaluation and comparison between probes was used to determine the composition of a given protected fragment.

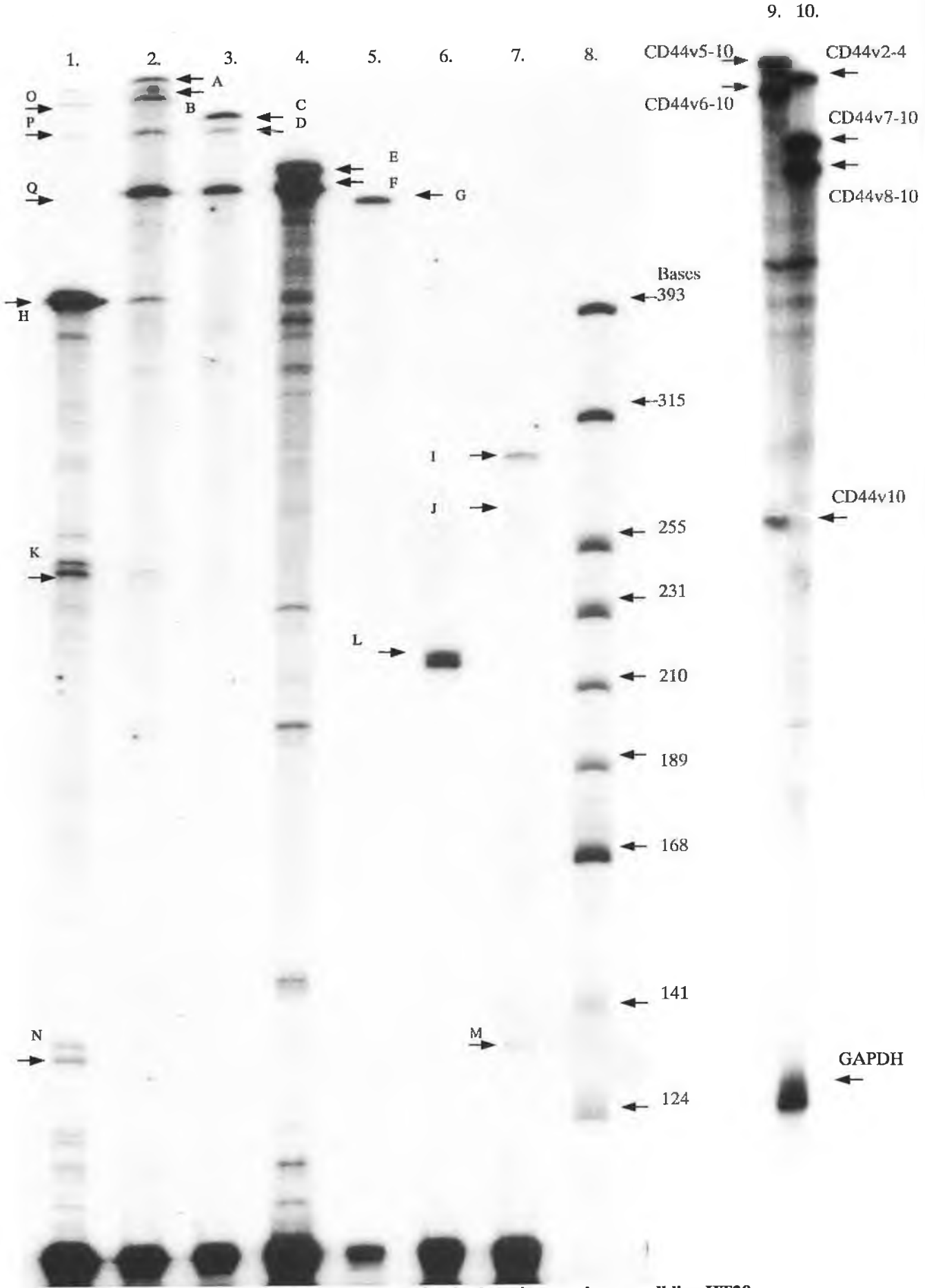


Fig. 4.14. RPA analysis of CD44 variant expression in the colon carcinoma cell line HT29.

Fig. 4.14. RPA analysis of CD44 variant expression in the colon carcinoma cell line HT29. Protected bands representing CD44 variant exons are indicted with an arrow from A-M. Lane no. 1. CD44v2-4, 2. CD44v5-10, 3. CD44v6-10, 4. CD44v7-10, 5. CD44v8-10, 6. CD44v10, 7. CD44v6-7, 8. Marker, 9, 10. Probes.

Fragment composition.

A. CD44v5-10C3'	914 b	H. CD44C5'	400 b (lane 1. only)	O. CD44v2-4C5' 769b
B. CD44v6-10C3'	798 b	I. CD44v6-8Δ	288 b	P. CD44v2-3C5' 655b
C. CD44vΔ6-10C3'	718 b	J. CD44v6-7	268 b	Q. CD44v2C5' 519b
D. CD44v7-10C3'	669 b	K. CD44v3-4	240 b (lane 1. only).	
E. CD44Δv7-10C3'	581 b	L. CD44v10C3'	215 b	
F. CD44v8-10C3'	537 b	M. CD44 v6 and/or v7		
G. CD44Δv8-10C3'	517 b	N. CD44 v2 and or/v3		

GAPDH mRNA levels were analysed as a control for the quantity of RNA in each lane. Figure 4.15 A represents GAPDH mRNA from the same gel illustrated in figure 4.14. Densitometric analysis revealed the relative intensities of the GAPDH bands.

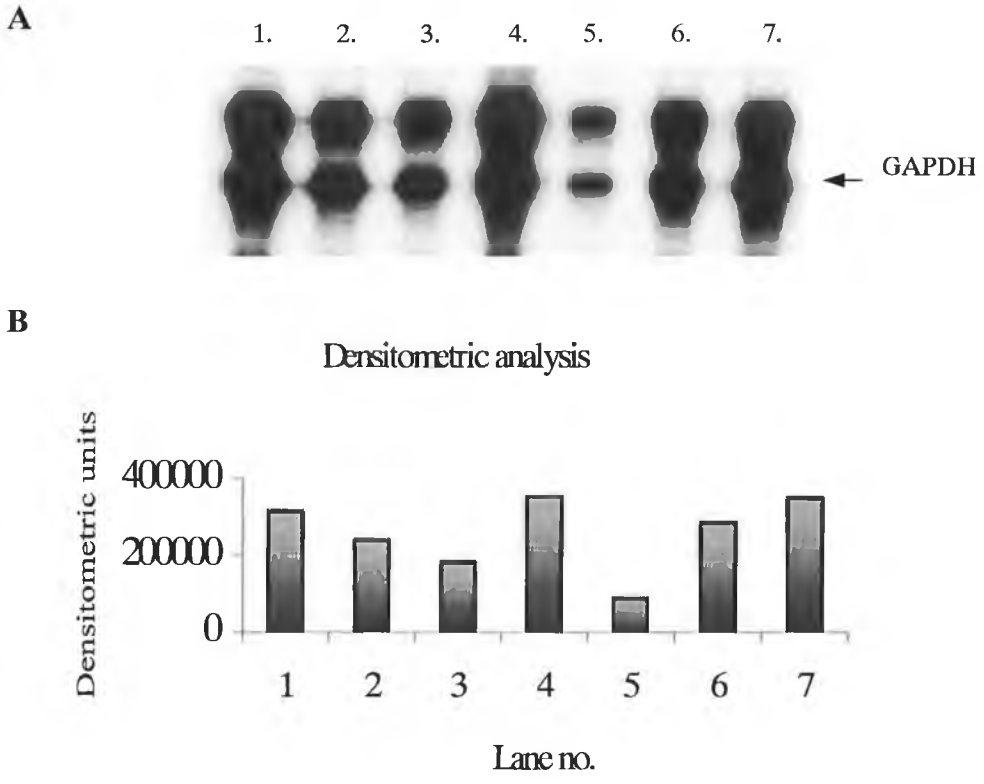
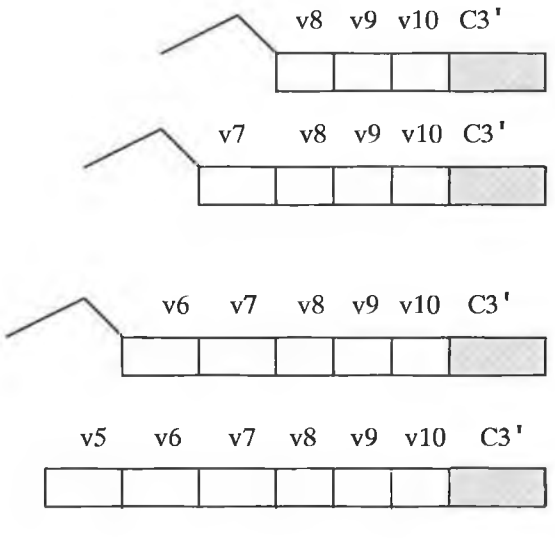


Fig. 4.15. A. Analysis of GAPDH transcript levels. B. Densitometric analysis of GAPDH bands. All lanes were loaded as illustrated in figure 4.14.

CD44 exon-specific RPA revealed the pattern of HT29 CD44 exon expression. All CD44 variant exons were detected in HT29 mRNA with the 5' standard exons and the variant exons v8-v10 as the predominantly used exons. V6 and v7 are more frequently joined to v8 than spliced on their own (figure 4.14 bands I and J). mRNA containing 9-10C3' or 10C3' are not detected in HT29. The 3' standard exons do not occur alone, but appear to be always spliced to v10. Thus the 3' splicing pattern of CD44 mRNA in HT29 appears to be as follows:



F. CD44v8-10C3'

D. CD44v7-10C3'

B. CD44v6-10C3'

A. The CD44v5-10C3' transcript occurs, however, this may be spliced to other exons 5' to v5.

Towards the 5' end of the CD44 mRNA, many combinations of exons are seen with the major signals obtained from the 5' constant exons. Higher molecular weight protected fragments are seen in very low levels corresponding to the 5'C linked to v2, v2-3, or v2-4, possibly bands Q, P and O respectively, indicating mRNA combinations containing theses variants are rare. Alternate donor/splice sites in this region may seem to confuse the issue by leading to an apparent under representation of the longer combinations. In this regard bands potentially corresponding to v2 and or v3 and v3-4 are also to be seen (lane 1. Bands N and K respectively).

4.6 CD44 EXON-SPECIFIC RPA ANALYSIS OF EBV-POSITIVE B-CELL LINES

The RPA was then used to examine CD44 exon-specific expression in a range of EBV-positive B-cell lines. Type-I and type-III latency EBV-positive BL cell lines along with lymphoblastoid cell lines (LCL) were investigated some of which were matched pairs (mp, i.e. arose from the same patient), these cell lines are summarised in table 4.2 below. A further description of cell lines is given table 2.1.

Table 4.2. Cell lines used in CD44 exon-specific RPA analysis.

Type-I latency	Type-III latency	LCL	Miscellaneous
			HT29
			HuT 78
BL41 (mp)	BL41 B958	IARC 171	
Mutu I (mp)	Mutu III c95 Mutu III c62 Mutu III LMP1-		
BL74 (mp)		IARC 290B	
	BL72 (mp)	IARC 307	
	Ag876		
		X50-7	
Rael BL			
Kem BL			
			C33A (mp) C33A Neo C33A LMP1

A representative BL type-III and LCL cell line (Ag876 and IARC290B respectively) were chosen initially and examined to determine the pattern of CD44 exon splicing. These cell lines were previously shown to express CD44 (illustrated in figure 4.12.) thus variant exon-specific probes were used to investigate CD44 mRNA structure in this cell line. (figure 4.16).

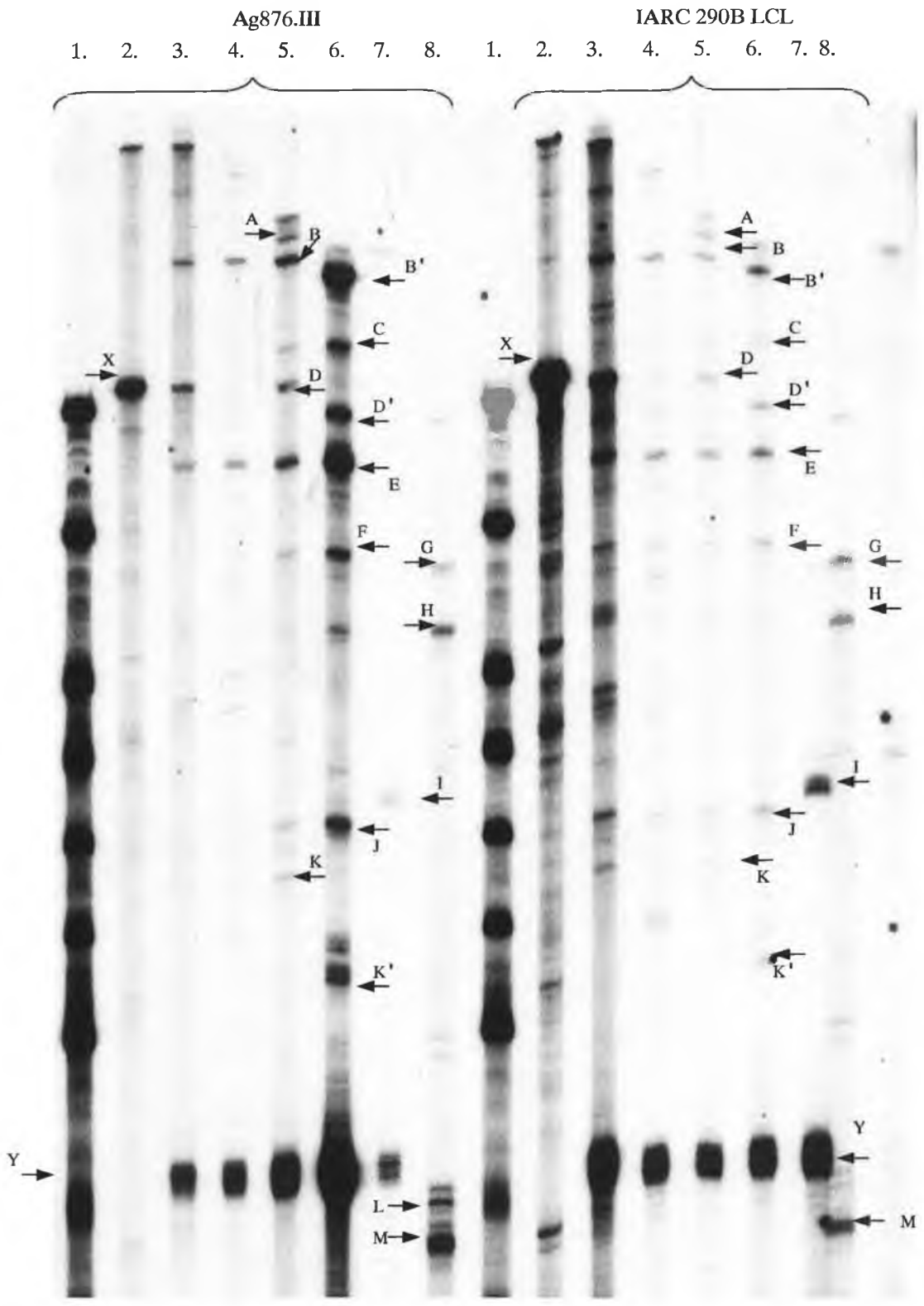


Fig. 4.16. RPA analysis using the CD44 variant exon-specific probes. Cell lines are indicated at the top of the figure, probes used are indicated by the lane number. Lane no. 1. Marker, 2. CD44v2-4, 3. CD44v5-10, 4. CD44v6-10, 5. CD44v7-10, 6. CD44v8-10, 7. CD44v10C, 8. CD44v6-7. Bands A-M represent variant exons, explanation on next page.

Fig. 4.16. RPA analysis using the CD44 variant exon-specific probes.

A. CD44vΔ7-10C3'	G. CD44v6-Δ8	X. D44C5'	Y. CD44 C3'
B. CD44v8-10C3'	H. CD44v6-7		
B'CD44Δv8-10C3'	I. CD44Δv10C3'		
C. CD44v9-10C3'	J. CD44v10		
D. CD44v8-10	K. CD44v8-9		
D'. CD44Δv8-10	K' CD44vΔ8-9		
E. CD44v10C3'	L. CD44v7		
F. CD44v9-10	M. CD44v6		

Band A (lane 5) corresponds to the expected molecular weight for CD44Δv7-10C3'and is only visible with the CD44v7-10 probe. This band is no longer present in lanes 3 and 4 using a probe containing the complete v7 exon. Band B (lane 6) corresponds to the expected molecular weight for CD44v8-10C3'. This band is present in lanes 3, 4, and 5. This band is no longer present in lane 6 using a probe containing a truncated exon 8 which results in the band B' which is only present in this lane. Band C represents v9-10C3' which should be visible using probes CD44v6-10, CD44v7-10 and CD44v8-10. However, band C is not observed using probes CD44v7-10 and CD44v8-10 in Ag876. Band D' (lane 6) corresponds to the expected molecular weight for Δ8-10 C3' and is only visible with the CD44v8-10C3' probe. This band is no longer present using a probe containing the complete v8 exon (lanes 3, 4, 5) but reappears as band D corresponding to v8-10 C3'. Band E corresponds to v10C C3' and will result from all probes containing v10C3' (lanes 3,4,5,6). This band drops down to I, when using the Δ10C3' probe containing only a portion of v10. Band K (lanes 4,5,6) corresponds to the expected molecular weight for v8-9 and is only visible with probes CD44v5-10, CD44v6-10 CD44v7-10, which contain a full length v8-9 exon. This band is no longer present in lane 6 using a probe containing a truncated exon 8 but reappears as band K' corresponding to Δ8-9.

The results obtained (figure 4.16), illustrate that a similar and very complex pattern of CD44 variant exon expression is used in both types of cells. Both the 3' and the 5' exons are widely expressed with CD44v8-10C3' being the predominant transcript. GAPDH analysis is represented in figure 4.17.

It can therefore be concluded that the following exon combinations are used in these two cell lines: v7-10C3', v8-10C3', v9-10C3', v10C3', v8-10, v8-9, v9-10, v6-7, v6, v7 and v10. The existence of v10 containing mRNAs that do not splice to the known C3' constant exon splice acceptor site has not previously been reported. It is possible that (a) a near by alternate splice acceptor site to the one at the start of the C3' exon is used, (b) splicing of v10 occurs to another downstream exon beyond the probes 5' end. In the LCLs v7 appears less frequently on its own (compare bands L and M lane 8 for both cell lines figure 4.25).

GAPDH mRNA levels were analysed as a control for quantity of RNA loaded. Figure 4.17.A, represents GAPDH mRNA from the same gel illustrated in figure 4.16. Densitometric analysis revealed the relative intensities of the GAPDH bands.

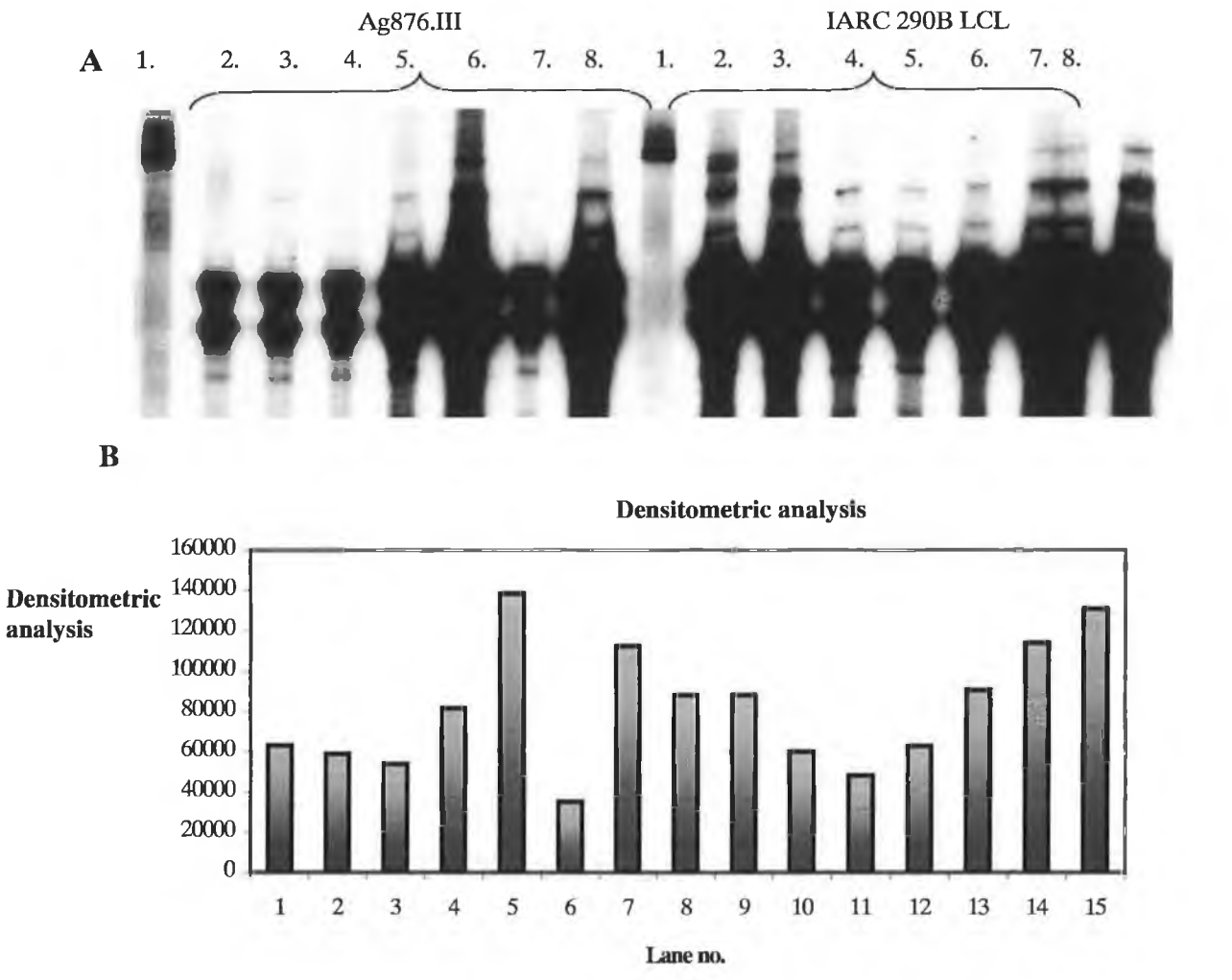


Fig. 4.17. RPA analysis of GAPDH transcript levels. Cell lines are indicated at the top of the figure, probes used are indicated by the lane number. Lane no. 1. Marker, 2. CD44v2-4, 3. CD44v5-10, 4. CD44v6-10, 5. CD44v7-10, 6. CD44v8-10, 7. CD44v10C, 8. CD44v6-7. **B. Densitometric analysis of GAPDH.**

Thus it appears that the complex pattern of CD44 variant exon splicing is similar in type-III EBV-positive BL cell lines and in LCLs. In order to investigate this further a panel of BL type-III and LCL cell lines were investigated by RPA using the CD44 variant exon-specific riboprobes as illustrated in figure 4.18-4.25.

CD44 mRNA expression was initially assessed by RPA using the CD44v2-10 probe.

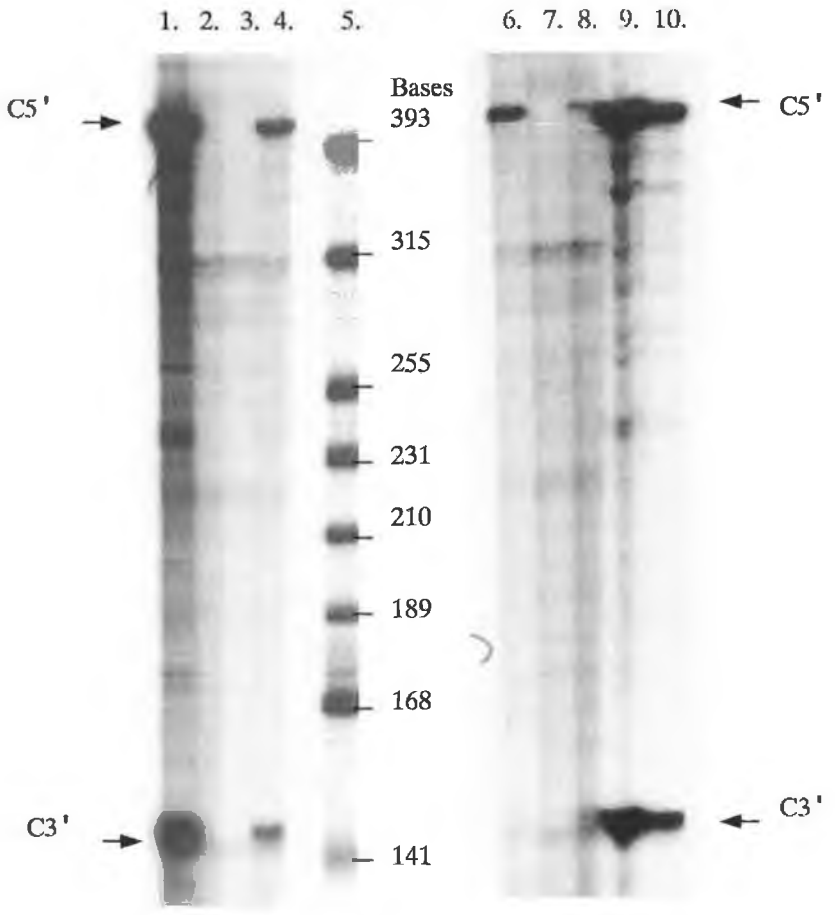


Fig. 4.18. RPA analysis of a range of cell lines using the CD44v2-10 probe. Lane no. 1. HuT 78, 2. Mutu I-, 3. Mutu III LMP1-, 4. Mutu III LMP1+,. 5. Marker,, 6. HT29, 7. BL41, 8. BL41 B958, 9, 10. IARC 171.

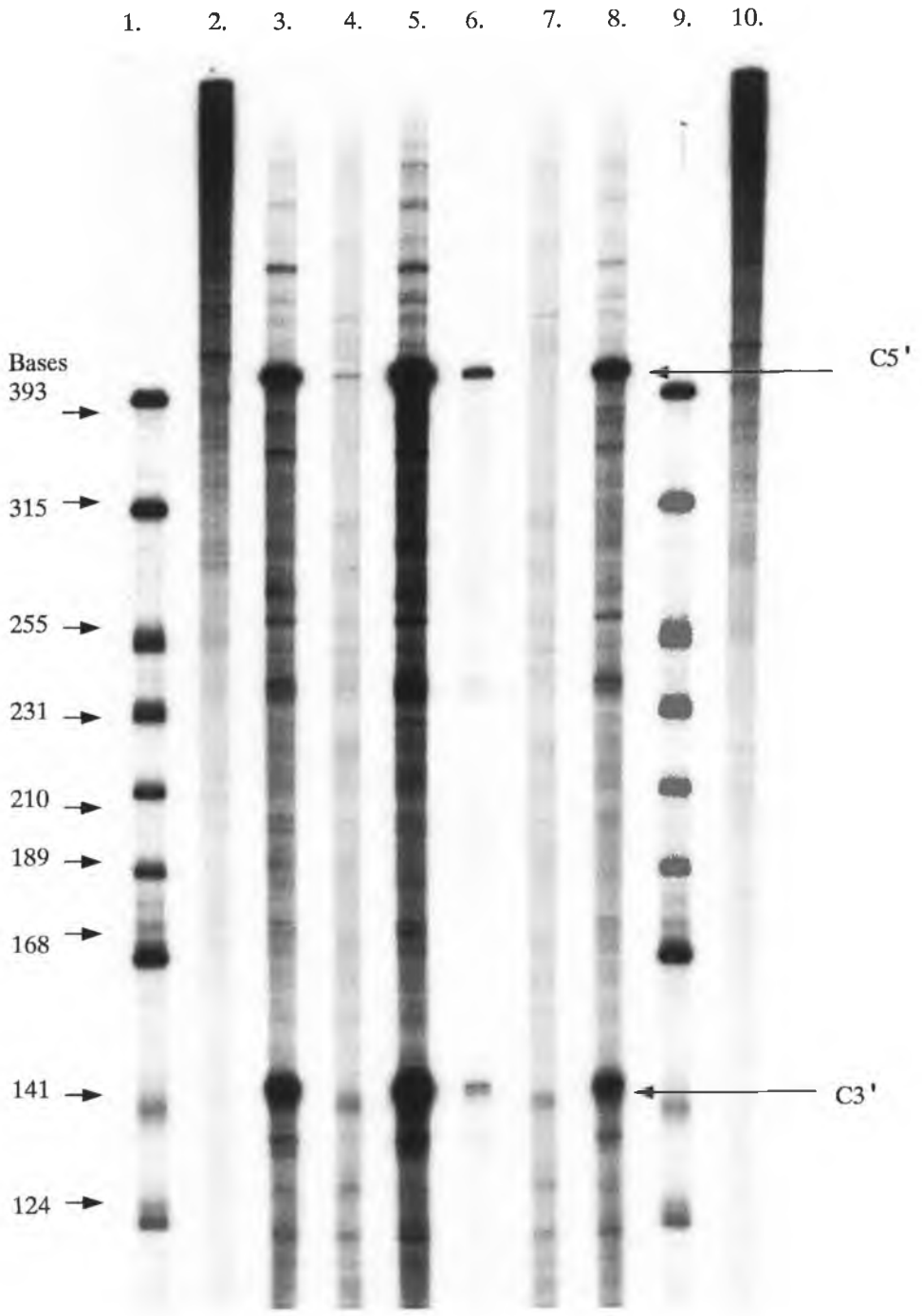


Fig. 4.19. RPA analysis of CD44 standard exon expression. Lane no. 1. Marker, 2. CD44V2-10 probe, 3. Ag876, 4. Mutu III c62, 5. BL72, 6. IARC 307, 7. BL74, 8. IARC 290B, 9. Marker, 10. CD44V2-10 probe.

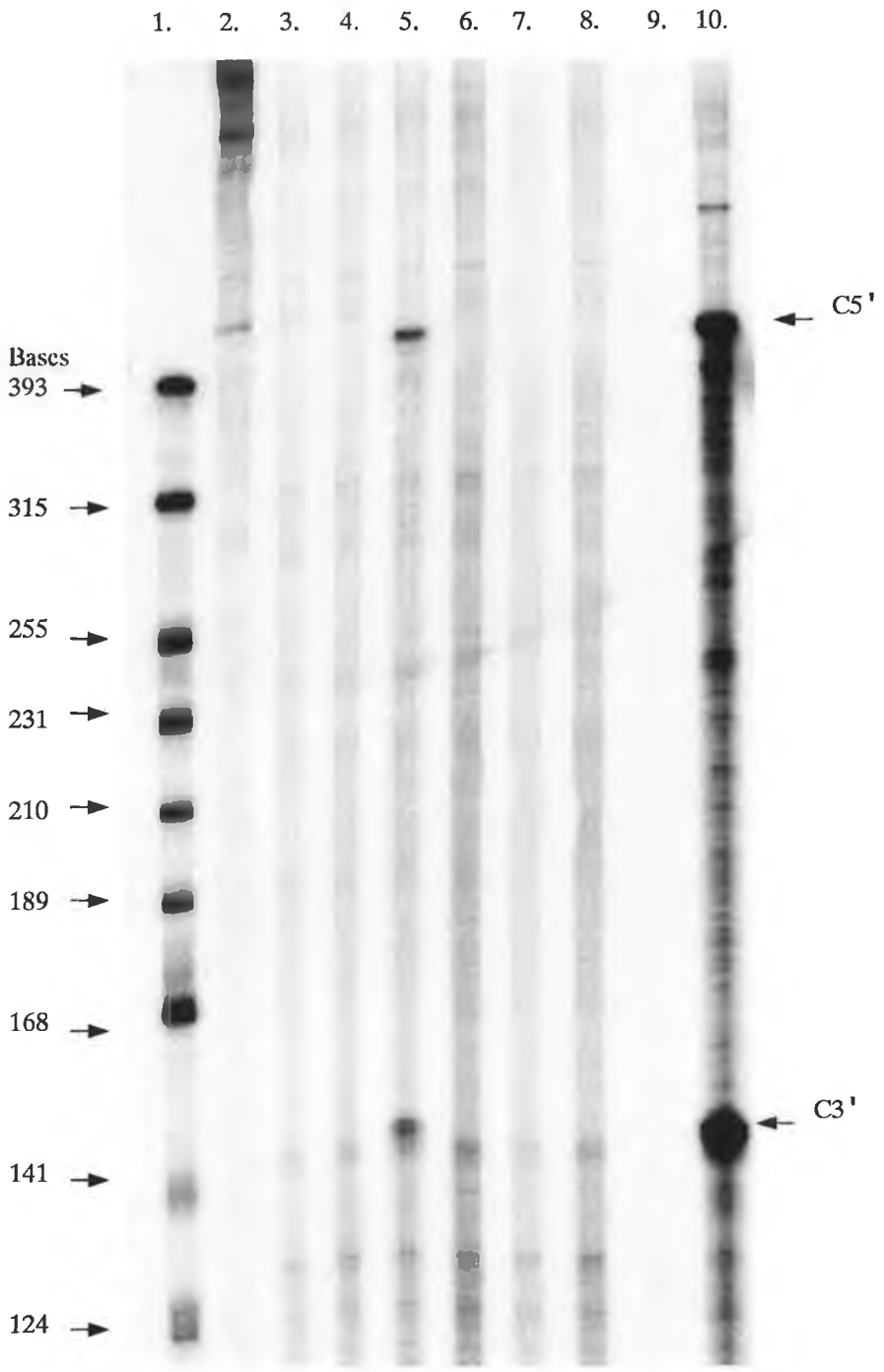


Fig. 4.20. RPA analysis of CD44 standard exon expression. Lane no. 1. Marker, 2. CD44V2-10 probe, 3. Kem BL, 4. Rael BL, 5. X50-7, 6. C33A, 7. C33A Neo, 8. C33A LMP1, 9. Jurkat, 10. HuT 78.

From figures 4.18-4.20. it is clear that CD44 C5' and C3' exons are transcribed in all type-III BLs and LCLs. The Mutu III clone c62 and the LCL X50-7 appear to express low levels of both C5' and C3' exons. CD44 was not detected in the type-I BL cell lines Mutu I, Rael BL and Kem BL. Figure 4.18 also confirms the absence of CD44 from Mutu III LMP1- as described before in chapter 3. The C33A cell lines were a group of stable epithelial cell lines one of which is stably transfected with LMP1. CD44 was not detected in any of these cell lines indicating that LMP1 expression alone is not sufficient to upregulate CD44 expression in this epithelial cell line. Although bands other than C3' and C5' are visible (figure 4.19 in particular), the presence of degraded probe fragments makes it difficult to conclude that variant exons are expressed. All cell lines that demonstrated CD44 expression were therefore analysed further using the CD44 variant exon-specific probes.

We then proceeded to compare the pattern of variant isoform expression in a range of type-III EBV positive BL cell lines and LCLs. In this set of experiments several cell lines were analysed simultaneously with one variant exon-specific probe, as indicated in figure 4.21-4.24.

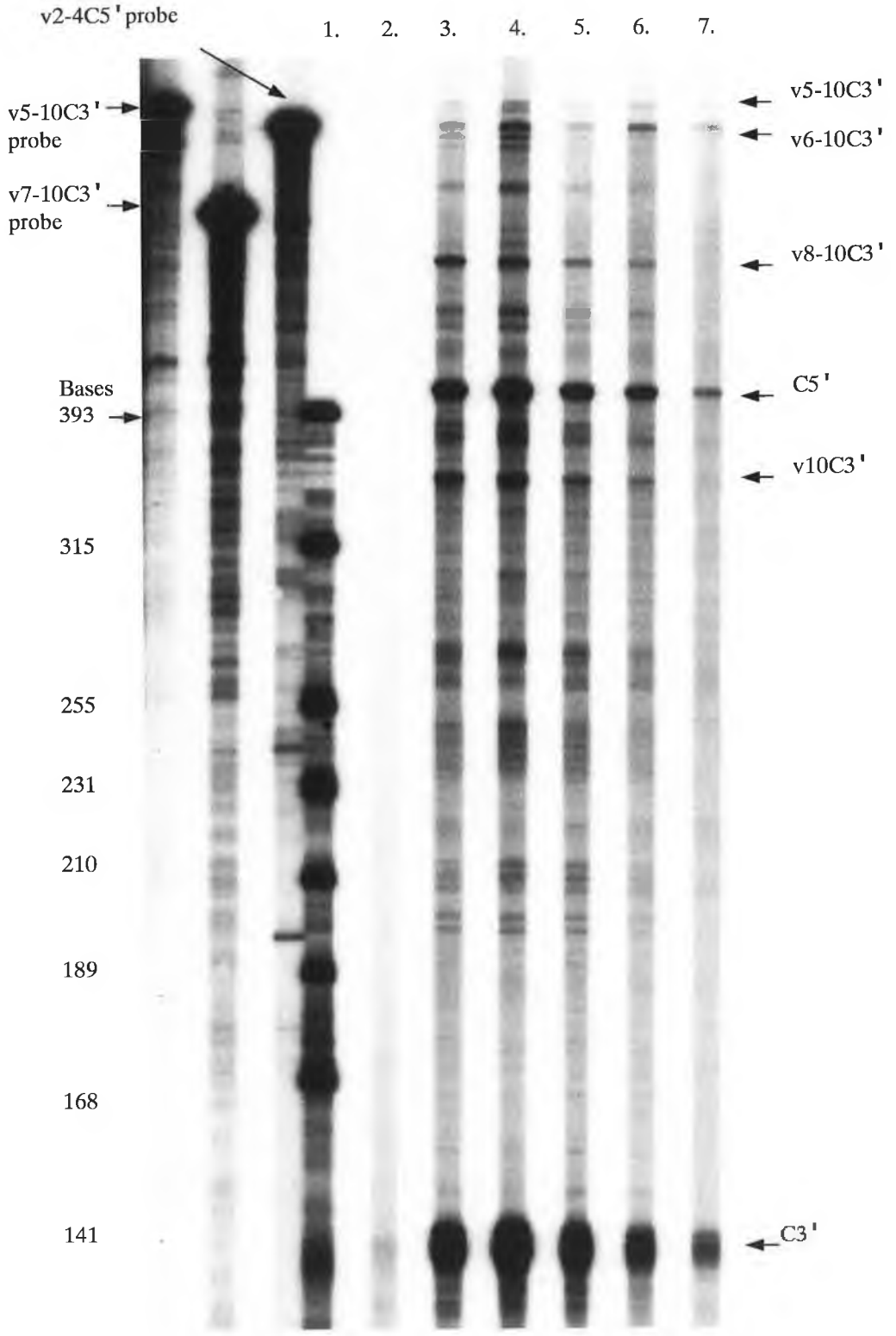


Fig. 4.21. RPA analysis using the CD44v5-10 variant exon-specific probe. Lane no. 1. Marker, 2. Mutu III c62, 3. Ag876 III, 4. BL72 III, 5. IARC 307 LCL, 6. IARC 290B LCL, 7. X50-7 LCL.

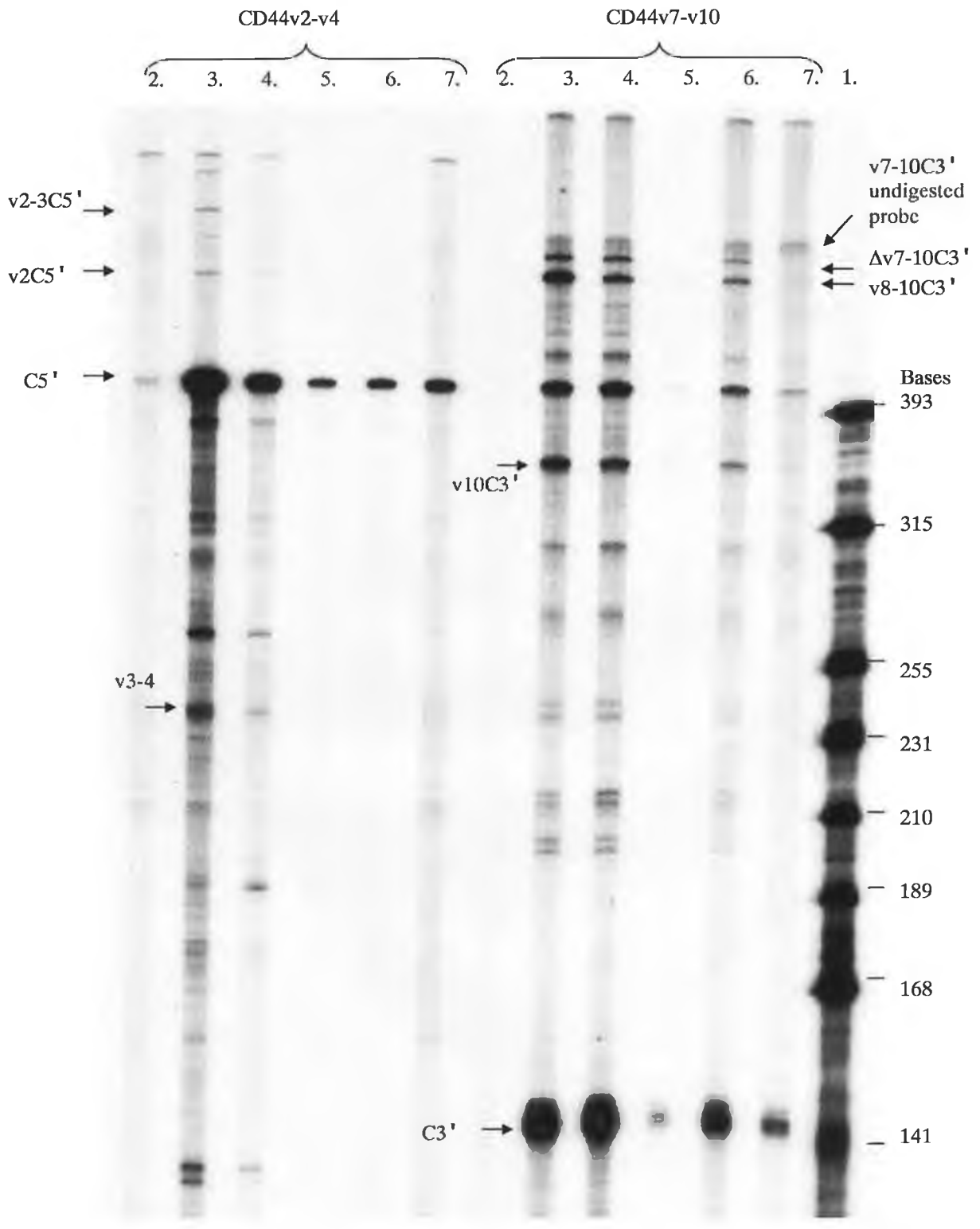


Fig. 4.22. RPA analysis using the CD44v2-4 and CD44v7-10 variant exon-specific probes. Lane no. 1. Marker, 2. Mutu III c62, 3. Ag876 III, 4. BL72 III, 5. IARC 307 LCL, 6. IARC 290B LCL, 7. X50-7 LCL.

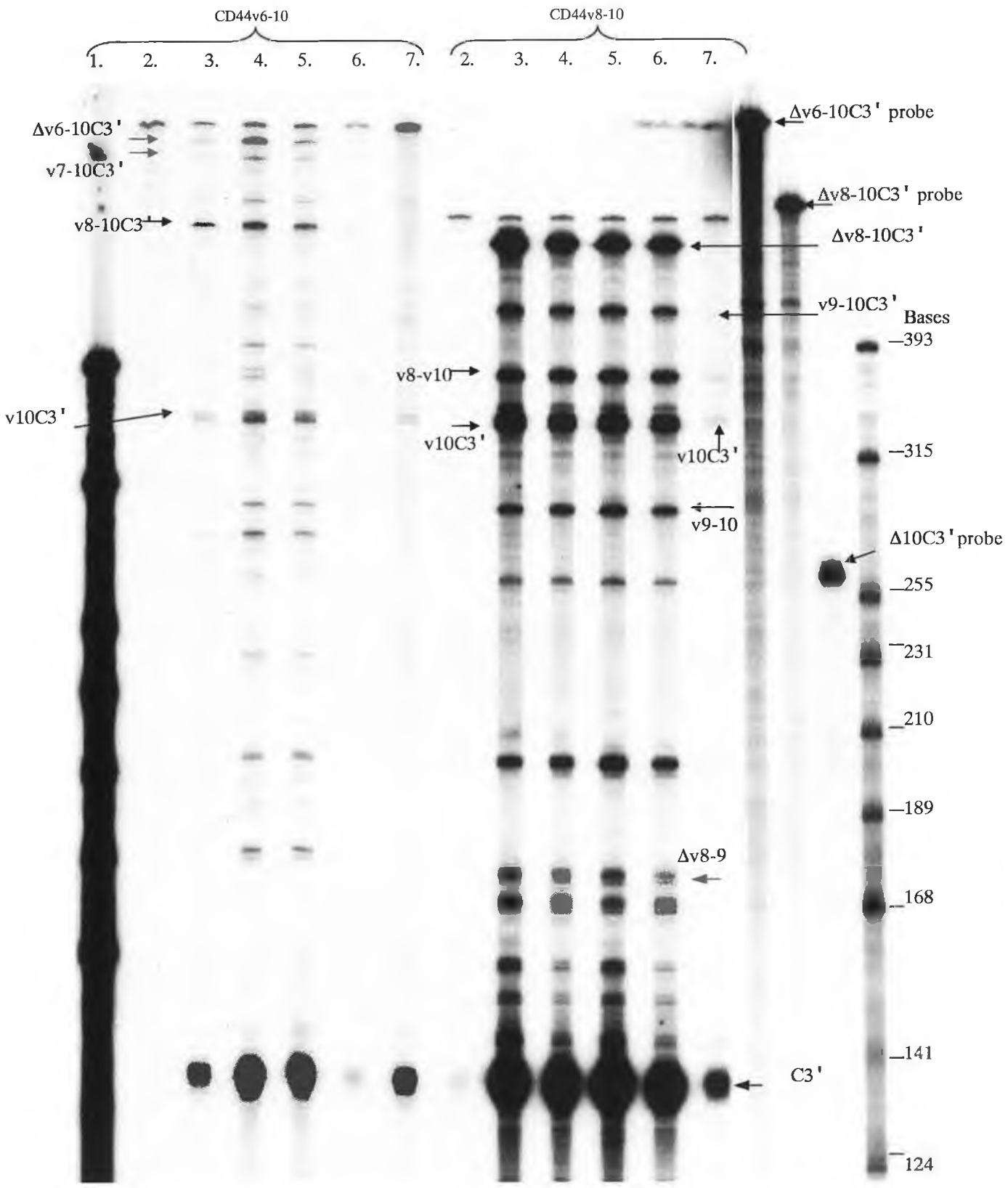


Fig. 4.23. RPA analysis using the CD44v6-10 and CD44v8-10 variant exon-specific probes. Lane no. 1. Marker, 2. Mutu III c62, 3. Ag876 III, 4. BL72 III, 5. IARC 307 LCL, 6. IARC 290B LCL, 7. X50-7 LCL.

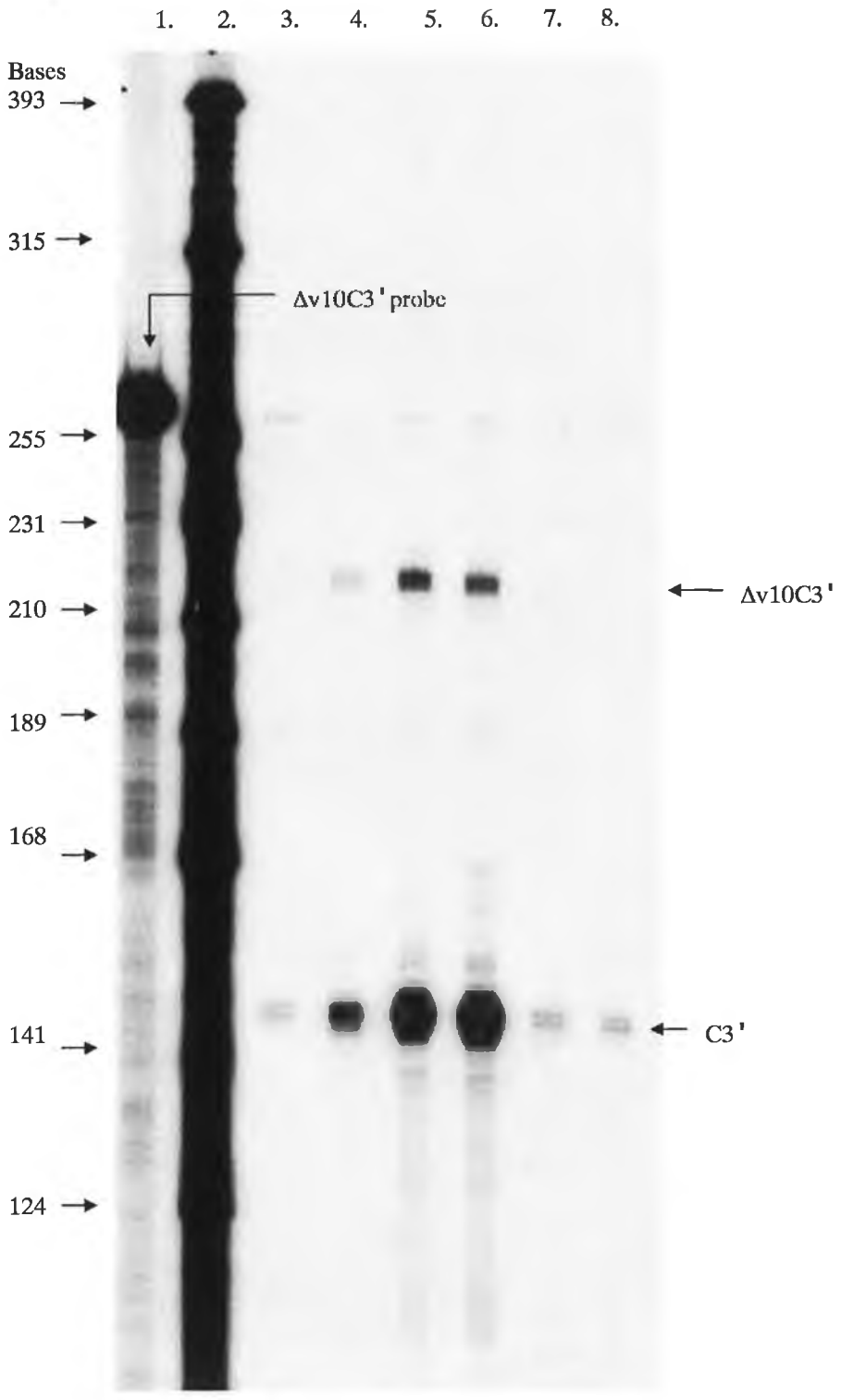


Fig. 4.24. RPA analysis using the CD44v10 variant exon-specific probe. Lane no. 1. CD44v10 probe, 2. Marker, 3. Mutu III c62, 4. Ag876 III, 5. BL72 III, 6. IARC 307 LCL, 7. IARC 290B LCL, 8. X50-7 LCL.

The results illustrated in figures 4.21-4.24, illustrate that type-III latency BL cell lines and LCLs demonstrate a similar pattern of CD44 variant exon splicing, with a preference for v8, v9 and v10 utilisation in various combinations. The level of variant exon expression is not equal in all cell lines as demonstrated by the considerably reduced level of variant exon expression in the type-III cell line Mutu III c62 and the LCL X50-7. In figure 4.21 and 4.22 there appears to be a certain amount of C5' exons present when the CD44v5-10 and CD44v7-10 probes are used, this may be due to incomplete digestion of pGEM 7Zf CD44 probe prior to probe labelling, this could possibly lead to other non-specific bands. The CD44 exon usage was examined in the BL type-III cell lines BL41B958 and the Mutu III LMP1+ clone c95 and was found to be similar to that reported above (results not shown).

As the expression of CD44v6 and CD44v6-7 has been implicated in metastasis it was considered important to examine specifically the expression of these variants in a range of EBV positive BL cell lines and LCLs. The results of this experiment are shown in figure 4.25. All type-III BL cell lines and LCLs clearly express v6 and v7. Levels of v6-7 transcript are similar in IARC 307 after normalising for GAPDH mRNA levels (figure 2.24). Faint bands were also observed in the cell lines X50-7 and Mutu III c62 indicating low levels of v6 and v7 transcript, this was not a consequence of loading as illustrated in figure 4.26. In the colon carcinoma cell line HT29 the majority of the v6 and v7 exons appear to be linked to v8 .

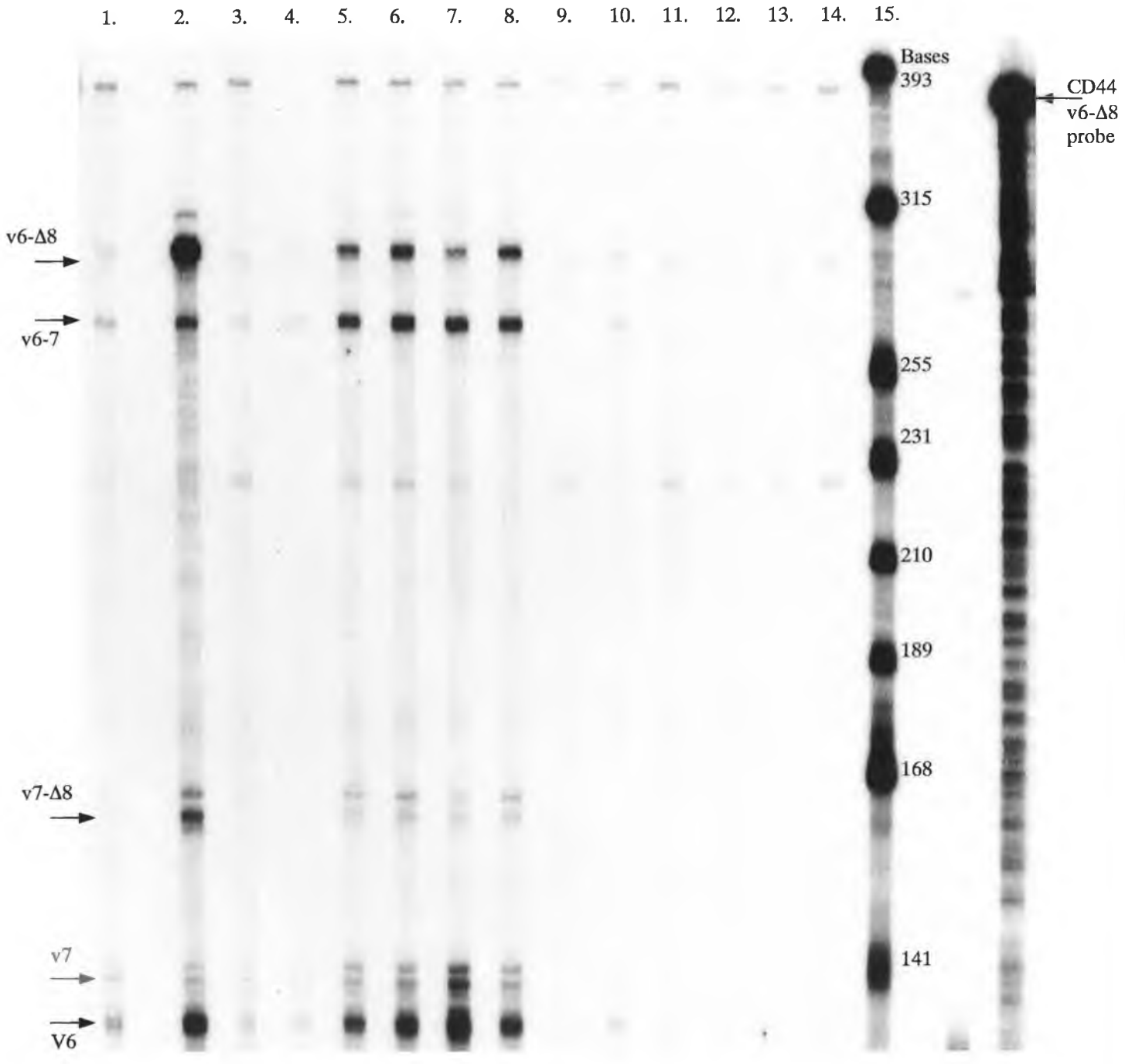


Fig. 4.25. RPA analysis using the CD44v6-7 variant exon-specific probe. Lane no. 1. HuT 78, 2. HT29, 3. X50-7 LCL, 4. IARC 307 LCL, 5. IARC 290B, 6. IARC 171, 7. Ag876, 8. BL72 III, 9. Mutu III c62, 10. Mutu III c95, 11. BL41 B958, 12. Mutu I, 13. BL74, 14.BL41, 15. Marker.

GAPDH mRNA levels were analysed as a control for quantity of RNA loaded. Figure 4.26.A, represents GAPDH mRNA from the same gel illustrated in figure 4.25. Densitometric analysis revealed the relative intensities of the GAPDH bands.

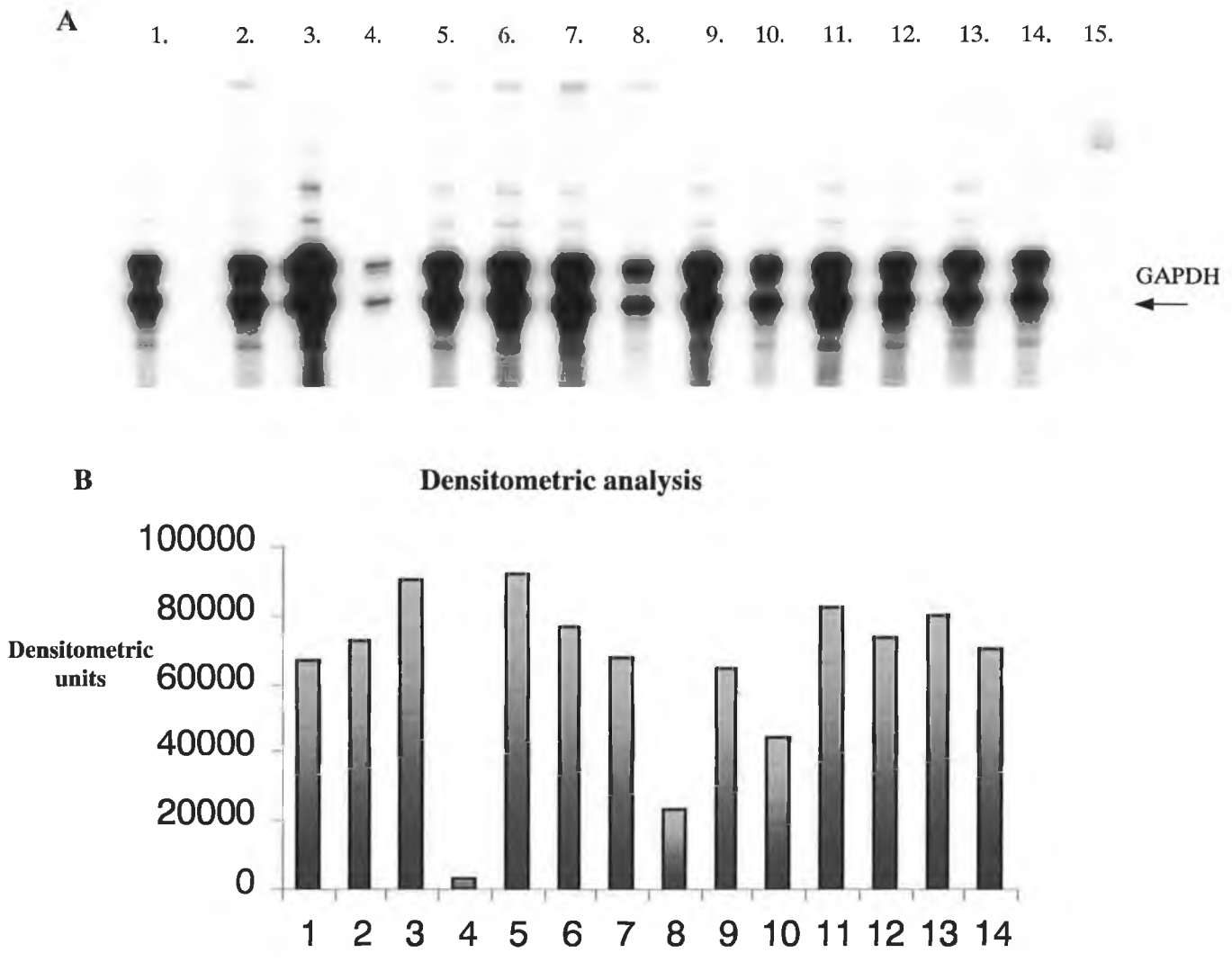


Fig. 4.26. A. RPA analysis of GAPDH transcript levels. Lane no. 1. HuT 78, 2. HT29, 3. X50-7 LCL, 4. IARC 307 LCL, 5. IARC 290B, 6. IARC 171, 7. Ag876, 8. BL72 III, 9. Mutu III c62, 10. Mutu III c95, 11. BL41 B958, 12. Mutu I, 13. BL74, 14.BL41, 15. Marker. **B. Densitometric analysis of GAPDH bands.**

4.7 WESTERN BLOT ANALYSIS OF CD44 EXPRESSION.

The RPA analysis yielded information on the profile of CD44 exon expression in the cell lines examined, however, the presence of transcript does not necessarily result in expression at the protein level. In order to investigate CD44 protein expression western blot analysis was carried out on all cell lines examined. The anti-standard CD44 antibody BRIC 238 was employed which recognises a CD44 epitope encoded by a standard exon, thus also theoretically recognising all variant containing isoforms which appear as an upward smear from the standard 83 kDa form.

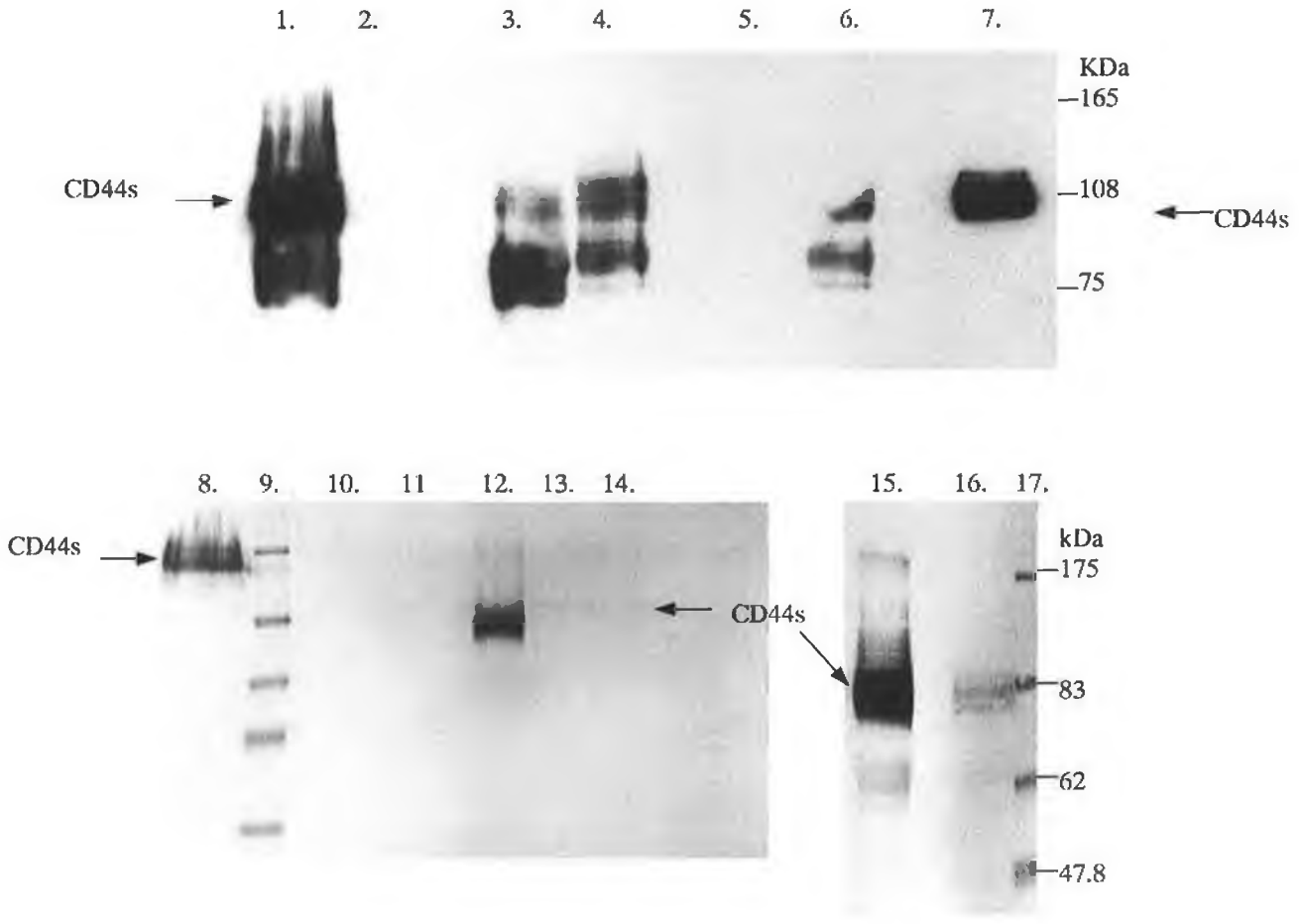


Fig. 4.27. Western blot analysis of CD44 expression. CD44 expression was determined by western blot analysis using the anti-standard antibody BRIC 238. Lane no. 1. Ag876, 2. Mutu III c62, 3. BL72 III, 4. IARC 307, 5. BL74, 6. IARC 290B, 7. IARC 171, 8. HT29, 9. Marker, 10. Mutu III c62, 11. Blank, 12, HuT 78, 13. BL41, 14, BL41 B958, 15. IARC 171, 16. X50-7, 17. Marker.

The cell lines Mutu I, Mutu III LMP1+ and Mutu III LMP1- were also examined for CD44 protein expression as described in figure 3.7. CD44 proteins were detected by western blot in all type-III BL cell lines and LCLs with the exception of Mutu III c62 and BL41 B958. Failure to detect CD44 protein in these cell lines is most likely due to the very low level of CD44 transcript present in these cell line. The low level of CD44 transcript detected by RPA, indicates that the mRNA is not translated or that the translated products are undetected. A low level of CD44 standard expression was detected in the cell line X50-7 when compared with other LCLs, this low level of expression is also observed at the transcriptional level. The CD44 protein detected by western blot in the cell line HT29 is of a higher molecular weight (figure 4.27 lower panel), this is also reflected by the RPA analysis which detected a large number of variant exon groups spliced to the standard exons with very low levels of the standard transcript detected. CD44s was not detected in the type-I cell lines BL74 and Bl41 as expected.

4.8 DISCUSSION

Several papers have reported the detection of certain alternatively spliced forms of CD44 in human metastatic tumours (Gunthert *et al.,* 1991; Matsumura and Tarin, 1992; Sneath Mangham *et al.,* 1998). As the precise combination of exons which are associated with the metastatic spread of a tumour cell have not all been completely defined a method for analysing CD44 expression in detail is required. Several methods have been described to analyse CD44 isoform expression in tumour cells and cell lines, including immunohistochemistry (IHC), RT-PCR followed by hybridisation, nested RT-PCR, exon run-off analysis and exon junction analysis. In the majority of studies to date the most frequently used methods of CD44 detection are immunohistochemistry using monoclonal antibodies, or RT-PCR. When compared to IHC, RT-PCR has advantages and disadvantages. RT-PCR is a more sensitive method then IHC and therefore, can be used to identify CD44 transcripts in exfoliated cells in stool and urine samples [this technique detects CD44 mRNA in as few as 10 tumour cells in 10^7 leukocytes (Matsumura and Tarin, 1992)]. In addition, RT-PCR can determine the exact composition of CD44 variants and therefore, is used to distinguish between CD44s and the other CD44 isoforms. However, it should be borne in mind that detection of CD44 mRNA does not imply that this transcript is expressed as a protein. RT-PCR is vulnerable to ribonucleases. Therefore not only must specimens be immediately frozen after removal, but they should not be stored, even at -70°C, for longer than 1 month. Ideally RT-PCR or at least the RNA extraction and cDNA RT step should be performed immediately (Matsumura *et al.,* 1994; Sugiyama *et al.,* 1995) and this is not always practical. Also strict precautions must be taken not to amplify trace contaminants to detectable levels.

The ability of IHC to discriminate between normal and neoplastic cells is an obvious advantage over RT-PCR, as the tumour samples contain a mixture of both types of cells. However the fact that antibodies are restricted to the recognition of accessible CD44 epitopes encoded by individual or limited number of exons is a disadvantage. Furthermore antibodies directed against the constant region of CD44 do not discriminate between standard and variant isoforms. Since various laboratories use a

range of mAbs to CD44 at different concentrations, and the percentage cut-off points of sample positively are determined arbitrarily be each research group, IHC is much more dependent on universal standardisation than is RT-PCR (Naor *et al.,* 1997). A recent study carried out by Martegani and colleagues investigated the reliability of immunodetection of CD44 isoforms using mAbs specific for CD44 variant exon products. They found that conformational changes due to the alternative splicing of CD44 variant exons and or posttranslational modification of the molecule generated variants that eluded immunodetection (Martegani *et al.,* 1999). Thus it appears that RT-PCR is a more reliable method for CD44 detection than mAb alone.

Van Weering and colleagues described an RT-PCR-based approach to analyse the composition of Human CD44 splice products (Van Weering *et al.,* 1993). In these experiments a CD44-specific oligonucleotide at the 3' side of constant exon 17 (C12A) was used in cDNA synthesis. This cDNA was subsequently amplified with two primers C13 (exon 5) and C2A (exon 16), located outside the variant part of the CD44 mRNA. This resulted in several amplification products depending on the cell line examined. To analyse the composition of each amplified band (representing one mRNA species), two different amplification methods were used, exon-specific runoff analysis and exon-specific PCR analysis (Van Weering *et al.,* 1993). In exon-specific runoff analysis, the PCR product from the amplification between primers C13 and C2A was amplified by linear PCR using ^{32}P-labeled oligonucleotide pv2-pv10 plus C13. This amplification yielded runoff products from that variant exon-specific primer to the PCR product. The runoff products from all the exon-specific primers were analysed on polyacrylamide gels to determine their exact length. In exon-specific PCR analysis the cDNA is amplified by exponential PCR between primer C2A and each of the variant exon-specific primers pv2-pv10. These products were analysed directly by agarose gel electrophoresis. Therefore in both methods the cDNA was amplified between one primer in the constant part of the mRNA and one primer in each variant exon. Determination of the length of the exon-specific amplification products from the agarose or polyacrylamide gel enabled the determination of the composition of the amplified bands. The colon carcinoma cell line HT29 was among those investigated in

this study, many transcripts were detected in HT29 the most abundant CD44 variant being CD44v8-10.

Exon-specific RT-PCR has also been used by other groups. Reeder and colleagues examined the effect of antisense CD44v6 expression on colorectal tumour cell line (HT29), metastasis and tumour growth in a wound environment (Reeder *et al.,* 1998). A recent paper by Lochart and colleagues describes RT-PCR followed by exon-specific PCR (Lochart *et al.,* 1999). The RT step is carried out with an oligo-$(dT)_{15}$ as opposed to a CD44-exon specific primer as described by Van Weering *et al.,* (1993). Exon-specific RT-PCR is a reliable procedure suitable for laboratories lacking resources to hybridise or work with radioactivity. Moreover the method is useful in screening large numbers of samples from solid tumours. When compared with RT-PCR followed by hybridisation RT-PCR exon-specific amplification showed similar sensitivity but required less time to perform. However strict operation procedures are required to avoid contamination and rigorous controls must be performed to detect false positive results. Exon-specific RT-PCR allows determination of exon composition of CD44 isoforms, however, the intensity of the bands obtained is not a reliable indicator of abundance due to the variation in primer sequence and non-linearity of PCR amplification. RT-PCR is also very sensitive to contamination as extremely small amounts of RNA or DNA contamination can lead to spurious results.

Goodison and colleagues evaluated the order of exon assembly, in the colon carcinoma cell line HT29, at the 5' boundary between standard and variant portions of CD44 (Goodison *et al.,* 1997). This was carried out in an effort to identify if there was a tumour-defining splicing pattern that could be diagnostically useful and provide an insight into the mechanism involved in the abnormal expression of CD44 in the neoplastic process. Initially they carried out PCR, using two primers one from a 5' constant exon and one from a 3' constant exon. This resulted in a CD44s PCR product and various higher molecular weight products if variant exons were present. Southern blot hybridisation was then carried out with exon-specific probes. From a diagnostic point of view RT-PCR followed by southern hybridisation, although successful, is technically difficult and laborious for routine clinical use. Thus they designed a new

assay, exon-junction analysis, in which blotting and hybridisation are unnecessary (Goodison *et al.,* 1997). The same group also performed exon junction analysis using a PCR-based approach. PCR amplification was carried out using a standard exon-anchored primer specific for a particular standard-variant exon junction. The overlapping primer was designed to have 18 bases complementary to the variant exon (X) and six bases of the primer complimentary with standard exon 5. By this method CD44 mRNA from the colon cancer cell line HT29 was shown to have every possible 5/X exon junction. The sensitivity of PCR allowed for the detection of relatively rare junctions. This technique therefore, identified the composition of CD44v exon products and revealed the splicing pattern. This technique is also based on RT-PCR and is liable to the concomitant problems associated with this method (Goodison *et al.,* 1997).

Thus it appears that the use of hybridization techniques is laborious and difficult to determine the exact composition of each CD44 mRNA species in the cell. Antibodies against epitopes encoded by each variant exon can only be used to show the presence of certain CD44 variant epitopes on cells but not the context in which they are present in the protein molecule. CD44 exon-specific RT-PCR is the most sensitive and versatile of the methods described to date however, it has many inherent disadvantages, principally its sensitivity to low levels of contaminating RNA and DNA and the fact that it is not quantitative. The aim of the work presented in this section was to analyse CD44 standard and CD44 variant exon usage in a range of EBV-positive B-cell lines. This was in part achieved by developing a qualitative and quantitative method for analysis of a portion of CD44 mRNAs in the cell.

A novel CD44 exon-specific RPA was designed for the detection of CD44 exon expression patterns. As RPA is a direct hybridization between the labelled probe and the RNA of interest it circumvents the two major problems of RT-PCR (1) RPA does not involve reverse transcription of RNA thus under representation of rare RNAs in the resultant cDNA cannot occur (2) PCR amplification is not required for RPA thus amplification of spurious PCR products due to contamination is also circumvented. RPA analysis is fully quantitative when factors such as RNA or labelled probe quantities are not limiting, as the levels of the resultant RNA:RNA hybrid are a direct

indication of the amount of specific mRNA present. The use of an internal control to analyse GAPDH mRNA levels allows for direct quantification and comparisons to be made between cell lines. As CD44 exons have been shown to be spliced preferentially in groups (Bell *et al.*, 1998), it was decided that the creation of probes that contained several variant exons would be more informative that those containing single exons, this is borne out when examining several variant exon probes simultaneously. The assay was initially carried out with the colon carcinoma cell line HT29 as the CD44 exon expression in this cell line has been previously analysed (Van Weering *et al.,* 1993; Reeder *et al.,* 1998). The results obtained in this study are in general agreement with others (Van Weering *et al.,* 1993; Reeder *et al.,* 1998), illustrating the presence of all CD44 variant exons and the predominant expression of CD44 exons v8-v10 and the C5' exons. The exon-specific RPA also revealed the distinct absence of the 3' constant exons 15, 16 and 17. The lack of CD44 standard cDNA in HT29 has been previously recorded by Van Weering *et al* (1993), and was suggested to be the result of infrequent use of the alternative splice donor site in the constant exon 5. It appears from this study that these C3' exons are always spliced along with variant exon 10 and do not occur on their own as illustrated in figure 4.13. Western blot analysis of CD44 expression in HT29 which revealed proteins of 160-180 kDa without the expression of the usual 83 kDa standard form which is predominant in most other cell lines (figure 4.27). This indicates that the majority of CD44 expressed in HT29 contains variant epitopes.

When employed to examine CD44 expression in EBV-positive cell lines the CD44-exon specific RPA revealed clearly that the type-I BL-cell lines examined in this study do not express CD44. This is in agreement with previous northern and western blots analysis carried out during the course of this study (illustrated in chapter 3) and with previous reports (Gregory *et al.,* 1990). The failure to express CD44 in the type-I BL cell lines is not understood. From the results obtained in this study (chapter 3 and 4) it can be suggested that CD44 expression is a result of the expression of all the EBV latent genes. Therefore the reduced pattern of EBV latent proteins observed in type-I BL cell lines would account for the loss of CD44 expression. As EBNA1 is the sole viral protein expressed in type-I BL cell lines it can be concluded that EBNA1, when expressed

alone in a BL cell line is not sufficient to upregulate CD44 expression at the RNA or protein level. When applied to type-III EBV-positive cell lines and LCLs the assay demonstrated the presence of CD44 standard and variant exon expression. Type-III EBV-positive cell lines are derived from type-I BL cell lines which have drifted in culture to express all the EBV latent genes. An LCL is established by isolating spontaneously transformed latently infected B-lymphocytes from peripheral blood of patients previously infected with EBV. As type III cell lines reflect the EBV latent gene expression profile associated with EBV-malignancies and LCLs reflect conditions in a spontaneously transformed cell derived from the blood of an infected individual, then comparison of CD44 expression in both types of cell lines is of interest. Type-III latency BL cell lines and LCLs demonstrate a similar pattern of CD44 variant splicing, with a preference for v8, v9 and v10. The pattern of variant expression is similar between cell lines but not the level of CD44 expression as exemplified by the type-III BL Mutu III c62 and the LCL X50-7, these cell lines express much lower levels of CD44 exons than other type-III and LCLs examined. Kryworuckho and colleagues examined the expression of CD44 in normal and transformed human B-cells by RT-PCR and IHC. Their results indicated that CD44s, CD44v8-10 and CD44v10 but not CD44v6-7 isoforms were detected in EBV-positive BL cell lines. The results obtained by RPA in this study show CD44v8-10 and CD44v10 as the main variant transcripts detected in EBV positive type-III BL cell lines. However, CD44v6-7 transcripts were also detected by RPA analysis using a CD44v6-7 specific probe as illustrated clearly in figure 4.23. This indicates clearly that v6-7 transcripts are transcribed in EBV-positive BL cell lines. Western blot analysis was carried out using an anti-v7 mAb, however, results obtained were uninterpretable due to the high level of background bands (results not shown).

In this study the detection of CD44 expression using the CD44v6-10 probe was less efficient than the use of other probes. This may have been due to secondary structure in the probe which prevented it binding effectively to the RNA. However the pattern of CD44v6 splicing can be deduced by combining the results received using the CD44v5-10, CD44 v7-10, CD44v8-10 probes and the v6-7 probe. These results indicated that in

HT29 v6 is predominantly joined to v7 and v8 and in the type-III cell lines and the LCLs v6 occurs both spliced to v7 and on its own.

It is clear that CD44 exon-specific RPA is a useful research tool which allows the simultaneous comparison of CD44 variant exon expression in different cell lines. However, RPA is a time consuming and labour intensive procedure thus its use as a routine diagnostic procedure for the detection of CD44 variant exons is at present not viable. A recent report has described a high through put RPA which employs formamide free buffers. It was found that the elimination of formamide from the hybridisation buffer not only shortens the hybridisation time to ~70 min, (as opposed to overnight) but also reduces the time for sample preparation because the buffer used constitutes as little as 30% of the reaction volume, so as there is no need to precipitate RNA prior to hybridisation (Mironov *et al.,* 1995). This group also reported that high specific-activity RNA-probes as old as one week can be successfully used for RPA, which means that at least 7 RPAs can be routinely performed in succession with the same probe, (as opposed to two overnight RPAs with the method employed in this study) (Mironov *et al.,* 1995). The use of ^{32}P in a routine diagnostic assay is not desirable due to safety considerations, thus developing an RPA which substituting ^{32}P with ^{33}P, or perhaps the development of a non-radioactive method would be beneficial. Thus it is clear that modification of the RPA used in this study may result in a high through put assay suitable for diagnostic purposes.

4.9 CONCLUSIONS

In conclusion the development of a novel qualitative and quantitative CD44 exon-specific RPA procedure allowed the analysis of exon usage in mRNA transcripts from the human CD44 gene. This assay was initially established using the colon carcinoma cell line HT29 in which the CD44 variant exon expression pattern had already been described. All CD44 variant exons were detected in HT29 mRNA v8-10 as the predominant variant exons present in mRNAs. Transcripts with the partial structure v8-10C3', v7-10C3', v6-10C3' were seen to occur in HT29. mRNA containing CD44v5-10C3' mRNA were also detected but these exons may additionally be spliced to more 5' variant exons. The 3' standard exons appear to be always spliced to v10. Towards the 5' end of the CD44 mRNA many combinations of variants were also observed with the predominant signal obtained from the C5' standard exons.

When used to examine the pattern of CD44 exon splicing in a range of EBV positive type-I and type-III BL and LCL cell lines it was observed that type-I cell lines do not express CD44. However, CD44 was detected in type-III BL cell lines and LCLs expressing LMP1. A similar but very complex pattern of CD44 variant exon expression was detected in both types of cells. In summary, variant exons v8, v9 and v10 are most often used, several combinations of which were detected. Transcripts containing v10 were not always seen to be spliced to the C3' exons indicating the usage of a alternative 3' splice acceptor site downstream of v10.

CHAPTER 5

EXAMINING THE EFFECTS OF EPSTEIN-BARR NUCLEAR ANTIGENS 1 AND 2 ON CELLULAR GENE EXPRESSION BY DIFFERENTIAL DISPLAY POLYMERASE CHAIN REACTION

5.1 INTRODUCTION

Establishing latent infection of the Epstein-Barr virus (EBV) requires the transcription of several viral genes, which are then translated into proteins. These proteins in turn have a role to play in establishing and maintaining latent viral infection, by transactivating both viral and cellular genes. The EBV nuclear antigen 2 (EBNA2) is one of the initial EBV encoded proteins expressed after primary infection of B lymphocytes (Alfieri *et al.,* 1991). Its expression is essential for immortalizing infected B-cells (Dambaugh 1984). EBNA2 is a transcription factor that can transactivate other viral genes as well as many cellular genes such as CD23 (see section 1.5.2). The EBV nuclear antigen protein 1 (EBNA1) encodes a sequence specific DNA binding protein which is the only virally encoded protein required for maintenance replication of the EBV viral episome. In EBV-positive cases of BL or NPC, every tumour cell normally expresses EBNA1. It is the only viral protein which is always present, thus suggesting it may confer a selective advantage on the tumour cell (Farrell, 1995). EBNA1 also activates lymphoid recombinase genes (RAGs) through an as yet unidentified mechanism (Rinivas and Sixbey, 1995). It is clear therefore that EBNA1 and EBNA2 are of great importance in establishing and maintaining EBV infection in B lymphocytes through their action on viral and cellular genes.

Changes in gene expression can lead to changes in the fundamental morphology and behaviour of a cell including differentiation, development and carcinogenesis. Thus any analytical method that can identify genes which are differentially expressed in different cell types is of great value. Differential display polymerase chain reaction (DD-PCR) is one such method first described by Liang and Pardee (1992). In this study DDRT-PCR was employed to examine a set of BL cell lines to assess the effects of the EBV latent proteins EBNA1 and EBNA2, on cellular gene expression. DG75 is an EBV negative BL cell line (Ben-Bassat *et al.,* 1977), DG75 EBNA1 and DG75 EBNA2 are stably transfected derivatives expressing EBNA1 and EBNA2 respectively (Welinder *et al.,* 1987). To examine further the effect of EBNA2 on cellular gene expression the tetracycline inducible cell line DG75 tTA EBNA2 described by Floettmann *et al.,* (1996) was included in the study. The expression of EBNA2 in this cell line was

induced by the removal of tetracycline from the growth medium. This system permitted the examination of the effect of EBNA2 in an isogenic background, eliminating any differences in cell lines due to clonal variation.

5.2 METHODS FOR GENE EXPRESSION ANALYSIS

The development of subtractive hybridization marked a significant breakthrough in the analysis of gene expression. Subtractive hybridization (SH) was first described by Hedrick *et al.,* in 1984. This method is based on hybridizing mRNA of one origin to mRNA of another origin; transcripts that do not find a match are then used in the construction of cDNA library. This technique has many applications, for example Lee *et al.,* (1991) describes its use as a positive selection method for the detection of candidate tumour suppressor genes. However, subtractive hybidization has some inherent disadvantages in that it is a laborious and lengthy procedure which requires a significant amounts of RNA. In recent years several methods have been described for large scale gene expression analysis, these include a DDRT-PCR. In addition to rapidity, differential display (DD) has many inherent advantages over SH. Subtractive hybridization only identifies genes which are differentially expressed in one of a pair of cell lines examined, DD-PCR in generating banding patterns on gels, allows simultaneous analysis of several cell lines. Significantly less mRNA is required for DD-PCR than for subtractive hybridisation thus allowing analyses to be carried out on smaller samples. Differential display has therefore found widespread applications. It has, for example, been used to identify genes that are differentially expressed in breast carcinoma cells (Liang *et al.*, 1992) and in preimplantation mouse embryos (Zimmermann and Schultz, 1994).

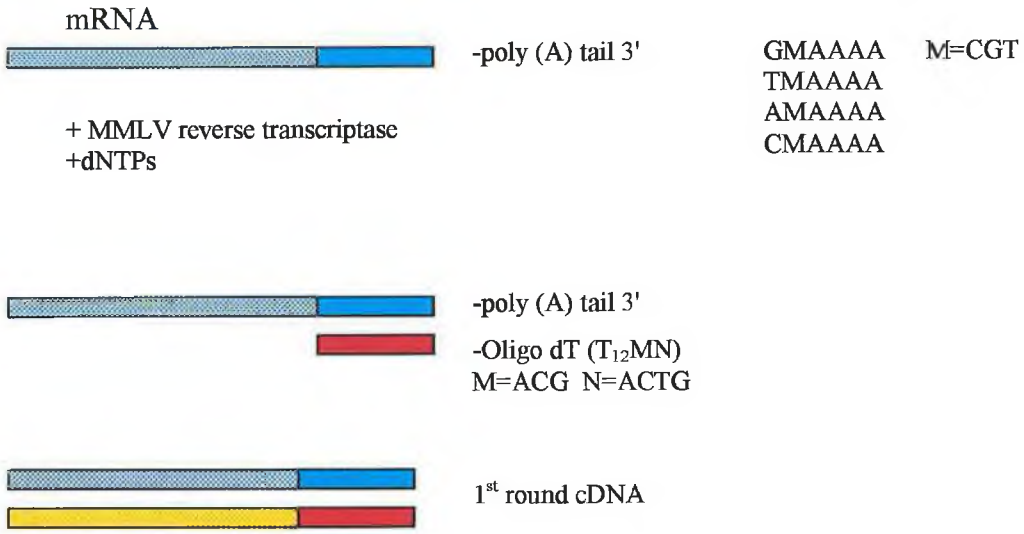
5.3 DIFFERENTIAL DISPLAY STRATEGY

The differential display strategy of the Liang and Pardee, which is often referred to as classical DD, was to amplify partial cDNA sequences from subsets of mRNAs by reverse transcription and PCR. These short sequences are then displayed on a sequencing gel. A simplified standard protocol for DDRT-PCR is outlined in figure

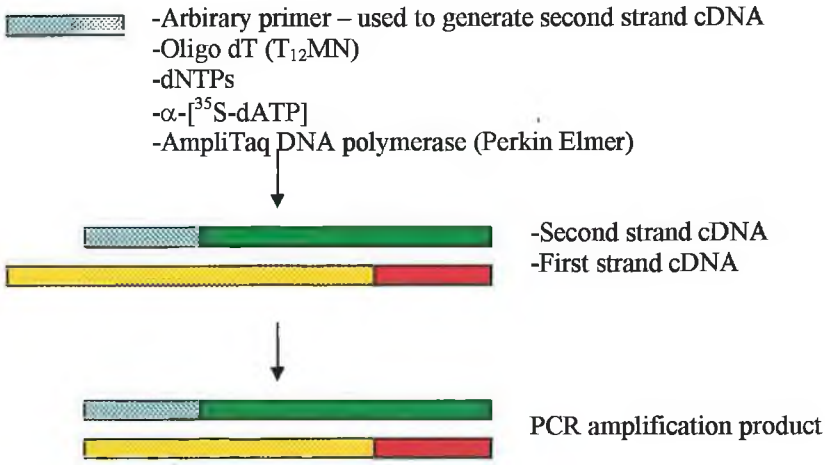
5.1. Polydeoxythymidilate (oligo dT) is employed as primer for the first strand cDNA synthesis which takes advantage of the fact that eukaryotic mRNAs have a 3' polyadenylated (poly(A)) tail to which the oligo (dT)can anneal (Liang and Pardee, 1992). Furthermore, the oligo (dT) is anchored by two 3' bases i.e. 5'-(T)n(A/G/C)N-3', where N is any base. There are twelve possible combinations of two bases not including T as the penultimate base. Each primer will be a perfect match with the 3' end of one twelfth of the total mRNA population. The resulting first strand cDNA can then be amplified by PCR using individual but arbitrarily chosen primers. The points at which a primer will anneal will be randomly distributed away from the poly (A) end of the cDNA. The 5' primer should in theory be short, to enable it to anneal frequently near the end of a cDNA strand. After numerous trials with primer sets and PCR conditions a combination of a 10-mer arbitrary primer and an anchored oligo (dT) was found to give specific DNA amplification (Liang and Pardee, 1992). Lowering of the dNTP concentration allowed for sufficient radiolabelling of the PCR product to give high resolution on a sequencing gel. This results in a set of cDNA fragments of varying length which when labelled by incorporating (α-^{35}S)dATP during amplification and separated on a DNA sequencing gel, produces a ladder-like pattern of bands (Liang and Pardee, 1992). A set of mRNAs possibly including differentially expressed mRNAs are thus represented as partial cDNAs. When a cDNA of interest is identified, it is isolated by elution from the dried gel, and reamplified by PCR. One or two rounds of reamplification PCR may be required, these PCR products in turn are cloned into a cloning vector (in this study a TA cloning vector from R&D systems was employed). The presence of inserts were confirmed by restriction enzyme analysis. Clones were then sequenced which revealed the orientation of the cDNA. This allowed construction of RNA probes to confirm differential expression by northern blotting and for isolation of the full-length gene. DDRT-PCR is not without its problems, difficulties have been encountered in reproducing differential gene expression patterns by northern blotting resulting in a very high incidence of false positives (Sun *et al.,* 1994). It has also been reported that DDPCR has a strong bias towards high copy number mRNAs, with rare transcripts not being detected (Bertolia *et al.,* 1995). Many modifications to the original protocol have been made which address these problems which are now discussed.

DIFFERENTIAL DISPLAY POLYMERASE CHAIN REACTION (DDRT-PCR)

1. Reverse Transcription



2. Polymerase chain reaction



3. Denaturing Polyacrylamide Electrophoresies

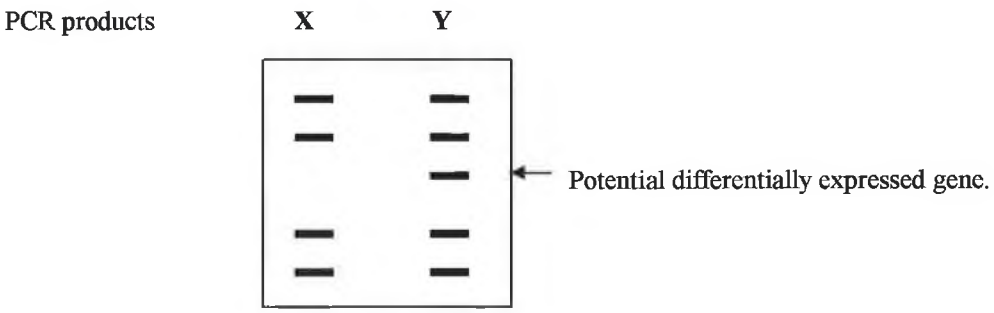


Fig. 5.1. Schematic representation of DDRT-PCR procedure.

5.4 MODIFICATIONS AND IMPROVEMENTS TO CLASSICAL DDRT-PCR

Since the original protocol was first published in 1992, modifications and improvements of classical differential display continue to appear in the literature. A method outlined by Bauer *et al.*, (1993) describes a mathematical model for primer design which produces an average of 70 bands at an annealing temperature of 42°C. They also described the application of the method to an automatic DNA sequencer and introduced non-denaturing gels which reduce the visual complexity of banding patterns (Bauer *et al.*, 1993). A rapid method for screening and cloning cDNAs generated by differential display has also been described. This method employs northern blots to affinity capture cDNAs which were radiolabelled by PCR prior to hybridization. Fragments of cDNA which demonstrated differences in mRNA levels were recovered from the membrane after hybridization and cloned (Li *et al.*, 1994).

A method termed arbitrary primed PCR fingerprinting of RNA or "RNA fingerprinting", was described whereby both double stranded cDNA synthesis and PCR are carried out using the same arbitrary primer (Welsh *et al.*, 1992). The problem of contamination of RNA preparations by chromosomal DNA was also addressed in this study. It was reasoned that because chromosomal DNA is double stranded, it would not be denatured prior to the synthesis of first cDNA strand, preventing primer annealing, and therefore DNA should not be able to participate in the first low stringency step. Thus as arbitrary primers are not introduced twice in opposite directions on a genomic DNA template it is not an efficient substrate for PCR. Thus it is unlikely that the presence of moderate amounts of dsDNA has an adverse effect on RNA fingerprinting (Welsh *et al.*, 1992). Enhanced Differential Display (EDD) is another recent modification of DDPCR (Linskens *et al.*, 1995). The principal novelty here lies in the introduction of a two step amplification procedure in which the first four PCR cycles are carried out at a lower annealing temperature with subsequent PCR cycles at a higher annealing temperature. This results in a more reproducible DD technique. However EDD involves the use of [α-^{32}P]dATP as a labelling nucleotide thus increasing the potential hazard to the user, it is also likely that EDD may not be suitable for the detection of rare genes (Linskens *et al.*, 1995).

A further advance on classical DD is a range of techniques which come under the heading of systematic differential display. All systematic DD techniques are designed on the presumption that the relative abundance of products in an amplified subset remains unchanged compared with the abundance of the original mRNA (Matz and Luckynoav, 1998). An example of systematic DD is a recently published procedure which describes the identification of differentially expressed genes by 'restriction endonuclease-based gene expression fingerprinting (GEF) (Ivanova and Belyavsky 1995). In this method, the first cDNA strand is synthesised using a 5'-biotinylated oligo dT-containing primer the resulting cDNA:RNA hybrid is then tailed using dGTP and a terminal transferase and the second strand of cDNA is synthesised using an oligo dC-primer. The cDNA is then digested with a frequently cutting restriction endonuclease. Three-prime terminal cDNA fragments are then selected by immobilization on streptavidin-coated microbeads and thus each mRNA species is represented by not more than one restriction fragment. An adapter is then ligated to one end of each restriction fragment which is then amplified by PCR with a biotinylated T^{13}-primer and an adaptor primer. The restriction products are then immobilised on to strepavidin microbeads at the biotinylated end, radiolabelled at the other end using ^{32}P-dATP, then sequentially treated with restriction enzymes. The resulting enzymatic products are then analysed by PAGE and fingerprints from different RNA samples are compared. One considerable disadvantage of this method is that due to its low sensitivity it allows only the analysis of the most abundant mRNAs (Ivanova and Belyavsky, 1995).

A technique termed restriction landmark cDNA scanning (RLCS) employs the same basic strategy as GEF whereby a biotinylated restriction fragment is selected by immobilization on streptavidin-coated beads and radiolabelled. The techniques differ at this stage as there is no further subdivision of fragments, instead they are resolved by two dimensional gel electrophoresis. As a radioactive label is only attached to one end of the 3' fragment each cDNA is represented by a single spot. This technique is quite difficult requiring skill to obtain reproducible results. Also the analysis of spot pattern is considerably more difficult than side by side analysis of band patterns, (Suzuki *et al.*, 1996; Kato, 1996).

Ordered differential display (ODD) is also based on the display of 3'-end restricted fragments of cDNA. Complimentary DNA (prepared from total RNA using a T-primer e.g. GTGAGTCGACCG$(T)_{13}$), was digested using the restriction enzyme *Rsa I* and a ^{32}P labelled adapter sequence is ligated at the 5' end. The 3' cDNA fragments were PCR-amplified by PCR suppression: 3' cDNA fragments were PCR-amplified, while the amplification of inner fragments [as well as poly A (-) cDNA fraction] was suppressed, as fragments flanked by inverted repeats cannot be amplified with the single primer corresponding to the outer part of the repeat. This results in samples containing fragments representing one transcript defined by flanking sequences at its termini: T-primer and an adapter sequence at the 5' end (Matz *et al.,* 1997). Such pools were then subdivided into simplified subsets by means of amplification with the primers annealing to the flanking sequences but extended by two bases at their 3' ends. Thus selecting 1/192 of the total pool. Both extended primers were designed for use under high stringency conditions to avoid the problem of decreased discrimination capabilities. Once a fragment is identified as differentially expressed it can be directly sequenced after PCR amplification. One disadvantage of ODD is that banding pattern obtained from complexed samples may be difficult to analyse on a sequencing gel thus leading to further complications when isolating a fragment of interest (Matz *et al.,* 1997). The advances made by systematic differential display are clear however these techniques require much more effort and special materials to produce a fingerprint. Also these techniques can be applied only to a comparison of samples of the same genetic background in order to avoid false positives originating from restriction fragment length polymorphism (Matz and Lukyanov, 1998).

5.5 NON-RADIOACTIVE DDRT-PCR

A study by Trentmann *et al.,* (1995) indicated that the use of [α-^{35}S]dATP, which was originally considered relatively safe, produces volatile decomposition products when subjected to high temperatures during PCR reactions in differential display. These products penetrate the PCR tubes and may prove dangerous to the researcher. In response to this Liang and Pardee (1995) confirmed the presence of ^{35}S contamination of the thermocycler used for DDRT-PCR experiments. They suggested that a PCR

machine be dedicated to DDRT-PCR alone, which was the case in this study. Alternatively a ^{33}P label could be used which has a sensitivity between that of ^{35}S and ^{32}P and does not appear to have the associated problems (Liang & Pardee, 1995). Many non-radioactive methods of DDPCR have now been developed in order to reduce the risk to the user.

A non-radioactive method involving the use of a fully degenerate hexamer to produce cDNA from mRNA has been described (Sokolov and Prockop, 1994). The cDNA is then amplified using various combinations of 2 or 3 arbitrary primers and the resulting bands are separated on a non-denaturing agarose gel, stained using ethidium bromide and viewed on a UV transilluminator. This technique, which avoids the use of radioisotopes, was used to identify a new brain-specific mRNA. Although this method is both rapid and sensitive, PCR amplification would have to be repeated with over 1000 different primers to obtain cDNA fragments representing each of the 15,000 different mRNAs present in a human cell (Sokolov and Prockop, 1994).

REN Display is a rapid and efficient method for nonradioactive DD and mRNA isolation. This procedure describes the use of horizontal polyacrylamide gels and visualisation of DNA bands by silver staining. Although REN display may not equal the sensitivity of classical DDPCR it has the inherent advantages of rapidity, safety and the ease of isolation of DNA fragments as they can be visualised on the gel (Lohmann *et al.,* 1995). One report described a non-radioactive DD method which employs a digoxigenin (DIG) conjugated poly-T degenerate primer (DIG-T_{12}VG) for reverse transcription and an arbitrary primer for PCR (Chen and Peck, 1996). After amplification, samples were separated on a 6% denaturing SDS-PAGE gel and transferred to a nylon membrane. The membrane was then blocked and incubated with an anti-DIG alkaline phosphatase antibody/enzyme conjugate. Following washing, a signal is generated on the membrane by the alkaline phosphatase colorimetric reaction with NBT BCIP. An advantage of this method is the fact that DNA amplification can be achieved from an isolated membrane strip containing a band of interest (Chen and Peck, 1996).

Fluorescent (DDRT-PCR FDDRT-PCR) partially circumvents the need for radioactivity. In FDDRT-PCR, modified 3'-anchoring oligo (dT) primers are employed to reverse transcribe total RNA such that a common 20mer sequence is introduced at the 5'-end of every cDNA. A fluorescent-labelled universal primer is then used in every PCR together with an arbitrary 10mer to generate 3' fluorescently-labelled cDNAs, which are analysed on an automated sequencer. When a differential cDNA is identified by FDDRT-PCR the PCR is repeated using the appropriate primers and a radioactive label (Smith *et al.,* 1997a). After elution of a cDNA of interest, the original 10mer and fluorescent primer are used for reamplification, this product is subjected to restriction enzyme digestion which yields a single fluorescently-labelled cDNA moiety corresponding to the 3' end of that mRNA. These are visualised on an automated DNA sequencer generating a restriction enzyme fingerprint of the gel eluted cDNA thus the presence of more than one cDNA fragment can be detected (Smith *et al.,* 1997b). Thus although this method does not avoid the necessity for radioactivity it does reduce its use significantly. A similar method described by Shohan and colleagues employed a fluorescently labelled PCR primer. This resulted in a dye-labelled PCR product which was resolved by electrophoresis for 30-40 min on a polyacrylamide capillary gel. Each product was automatically sized and quantified, these results were automatically stored on a data base which allowed retrospective analysis. Transcriptional specific DNA bands identified were then used to generate DNA-based probes for screening cDNA libraries (Shohan *et al.,* 1996).

Many other modifications have been cited in the literature. A report by Hadman *et al.,* (1995) describes the use of mRNA and a ^{32}P-labelled 3' primer which eliminated the problem of amplification due to two 5' primers as they will no longer be visible, yielding clean discrete labelled bands. Much attention has been placed on primer design in DDRT-PCR with many views as to how to improve the procedure by primer design. Graf and colleagues (1997) reported that rational primer design greatly improves DDPCR. They describe the design of primers of 12-14 bases in length with an A/T content of 60-80% which corresponds more closely to the A/T content of a typical 3'-UTR. Some primers also contained sequence motifs found in the 3'-UTR of immediate early genes. Although these primers might miss genes with non A/T rich 3'-

UTR this study illustrated the rapid isolation of many gene fragments. Further suggestions on primer design are given by Zhao *et al.,* 1995 and Afonina *et al.,* 1997.

The rapid evolution of RNA fingerprinting is set to continue. It has already been successfully applied to identify differentially expressed genes in different tissues and cancer cells (Chang *et al.,* 1997; Nelson *et al.,* 1998; Ulrix *et al.,* 1998). It may be used to investigate the response of a cell to a specific stimulus without cloning a single gene. Potential problems such as the under representation of rare mRNAs and the high percentage of false positives obtained remain to be fully addressed.

The research presented in the following section, aims to identify cellular genes whose expression is modulated by the key EBV latent proteins EBNA1 and EBNA2 in an EBV negative Burkitt's lymphoma cell line background by DDRT-PCR.

5.6 EBNA2 INDUCTION IN DG75 TTA EBNA2.

Before commencing DD experiments the induction of EBNA2 expression in the cell line DG75 tTA EBNA2 upon removal of tetracycline from the growth medium was examined by western blotting using the anti-EBNA2 antibody PE2 as described in section 2.6.7. The stably transfected cell line DG75 EBNA2 was included as a positive control. It can be seen from figure 5.2 that EBNA2 expression was detected in the cell line DG75 tTA EBNA2 after 24hr induction and in the cell line DG75 EBNA2.

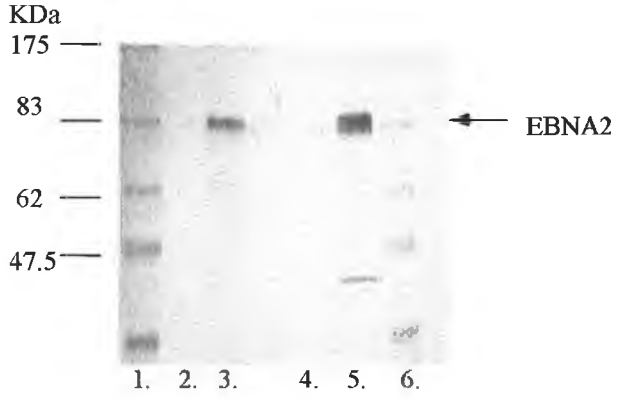


Fig. 5.2. Western Blot analysis of EBNA2 expression. The detection of EBNA2 expression using the anti-EBNA2 antibody PE2. Lane No. 1. Protein marker 2. DG75 tTA EBNA2 uninduced, 3. DG75 tTA EBNA2 induced (24hr), 4. DG75, 5. DG75 EBNA2, 6. Protein marker.

EBNA2 expression was also investigated by immunocytochemistry, as described in section 2.4.7. to investigate if EBNA2 was present in all cells in the induced population.

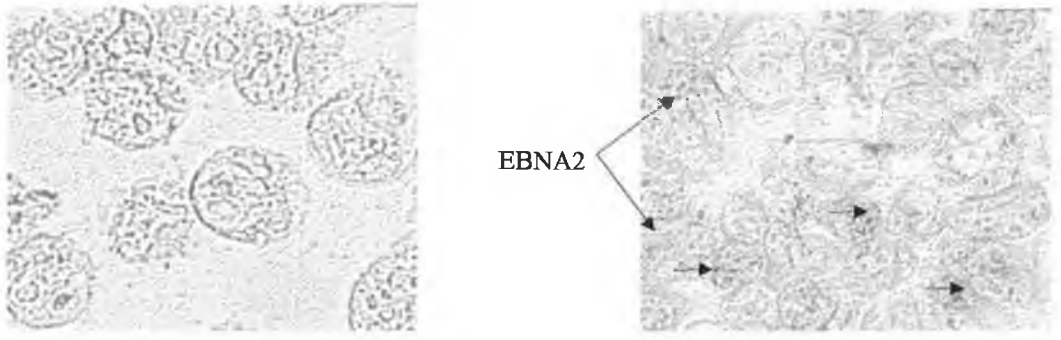


Fig. 5.3. Immunocytochemistry analysis of EBNA2 induction. Panel A represents DG75 tTA EBNA2 before tetracycline removal. Panel B represents DG75 tTA EBNA2 cells 24 hr after induction, the presence of EBNA2, indicated by purple staining(→), was detected in over 95% of the cell population.

5.7 ESTABLISHING THE STRUCTURAL INTEGRITY OF ISOLATED RNA.

The success of the differential display technique depends on the integrity of the RNA and on its being free of chromosomal DNA contamination. Total RNA is preferred over poly (A) RNA because of its cleaner background signal, easy purification, and integrity verification. RNA was isolated from all cell lines examined using RNA ISOLATOR as described in section 2.5.2. The resulting RNA was examined by electrophoresis through a formaldehyde gel as described in section 2.8.2.

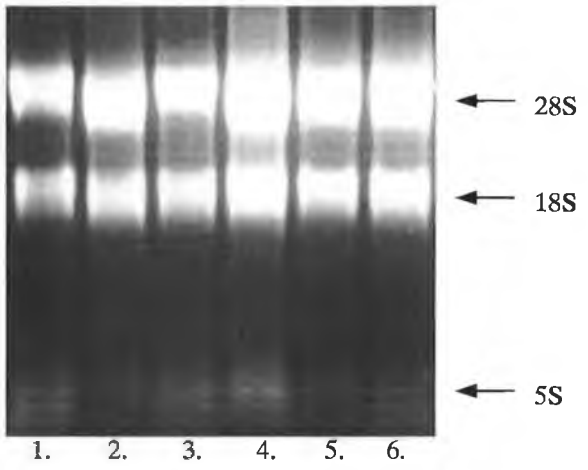


Fig. 5.4. Formaldehyde gel electrophoresis of RNA. Samples of total RNA were subjected to electrophoresis through a formaldehyde gel and viewed on a UV transilluminator. The 28S, 18S and 5S ribosomal bands are clearly visible indicating structurally intact RNA. Lane No. 1. DG75, 2. DG75 EBNA1, 3. DG75 EBNA2, 4. DG75 tTA, 5. DG75 tTA EBNA2 uninduced, 4. DG75 tTA EBNA2 induced.

It is clear from figure 5.4 that the RNA used for differential display analysis was structurally intact.

5.8 RT-PCR ANALYSIS OF RNA.

In order to ensure that the mRNA was intact and that contaminating DNA was not present RT PCR analysis of RNA from all cell lines investigated by differential display was carried out using primers for the house keeping gene β-actin (section 2.5.5). The β-actin primers used amplify a PCR product of 383 bp. The primers were designed to span an intron thus the presence of contaminating DNA can be detected by the presence of a 590 bp amplification product. The presence of a band of 383 bp only, in all cell lines indicates intact mRNA free from DNA contamination (figure 5.5).

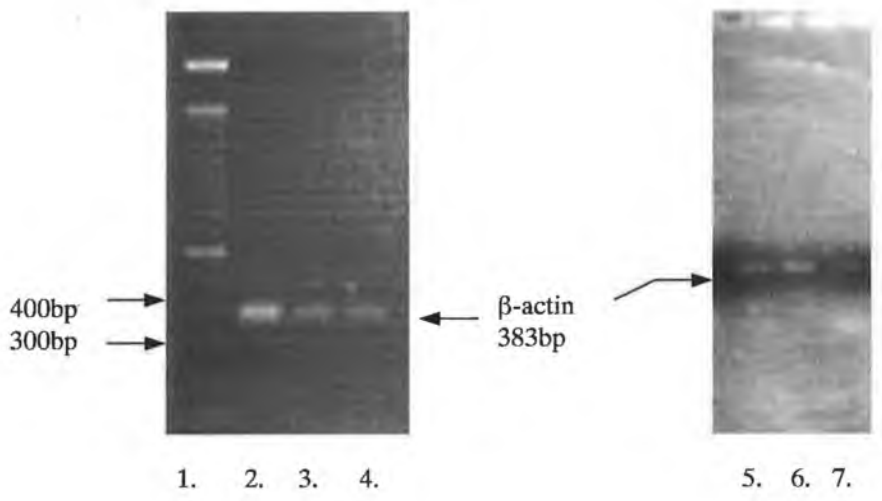


Fig. 5.5. RT PCR analysis of β-actin mRNA. Lane No. 1. DG75, 2. DG75 EBNA1, 3. DG75 EBNA2, 4. DG75 tTA, 5. DG75 tTA EBNA2 uninduced, 4. DG75 tTA EBNA2 induced..

5.9 DIFFERENTIAL DISPLAY INITIAL RESULTS.

Initial DDRT-PCR was carried out on RNA from the cell lines DG75, DG75 EBNA1, DG75 EBNA2, using various primer combinations. For example a primer such as 5'T_{12}GA allowed anchored annealing to mRNA containing TC located just upstream of their poly(A) tail (figure 5.1). Probability dictates that this primer recognises one twelfth of the total mRNA population (Liang and Pardee, 1992). Thus the primer designated T_{12}MN, where by M=ACG and N=ACGT will recognise all possible RNA populations. This primer combined with an arbitrary primer which binds randomly to the DNA, was selected so as to amplify DNA from 50 to 100 mRNAs because this is

the number optimal for display on the gel (Liang and Pardee, 1992). In total between 1000 and 2000 cDNAs were amplified from each cell line examined. Table 5.1 lists the bands which were initially identified. However, upon repeating all the DD experiments a large number of the bands were not reproducible and were eliminated as false positives. The bands which were repeatable are marked with a * in table 5.1.

Table 5.1. Potentially differentially expressed partial cDNA identified by DDRT-PCR.

CELL LINES COMPARED	SIZE (Bases).
DG75, DG75 EBNA1, DG75 EBNA2.	172
DG75, DG75 EBNA1, DG75 EBNA2.	310*
DG75, DG75 EBNA1, DG75 EBNA2.	292*
DG75, DG75 EBNA1, DG75 EBNA2.	195*
DG75, DG75 EBNA1, DG75 EBNA2.	132
DG75, DG75 EBNA1, DG75 EBNA2.	212
DG75, DG75 EBNA1	350*
DG75, DG75 EBNA1.	268
DG75, DG75 EBNA1.	198
DG75, DG75 EBNA1	160*
DG75, DG75 EBNA1	155*
DG75, DG75 EBNA1	141
DG75, DG75 EBNA1	144
DG75, DG75 EBNA1	142
DG75, DG75 EBNA1	144
DG75, DG75 EBNA1	142
DG75, DG75 EBNA1	140
DG75, DG75 EBNA2	402*
DG75, DG75 EBNA2	510
DG75, DG75 EBNA2	300*
DG75, DG75 EBNA2	150
DG75, DG75 EBNA2	150
DG75, DG75 EBNA2	148
DG75, DG75 EBNA2	145
DG75 EBNA1, DG75 EBNA2	212
DG75 EBNA1, DG75 EBNA2	355

Cell lines underlined in table 5.1 indicate cell lines in which the partial cDNA band appeared to be differentially expressed. The sections which are not underlined indicate cell lines in which the band was absent or did not appear to be differentially regulated.

Bands which were subjected to PCR within 48 hr were successfully cloned. Those bands are listed below, the primers used for their amplification are included in brackets:

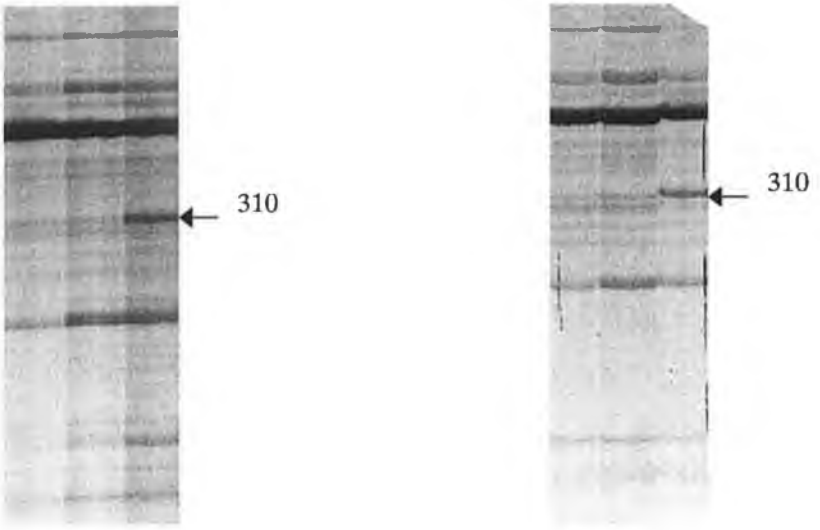
DG75, DG75 EBNA1, DG75 EBNA2.	(T_{12} MG, AP-14)	310*
DG75, DG75 EBNA1, DG75 EBNA2.	(T_{12} MT, AP-14)	195*
DG75, DG75 EBNA2	(T_{12} MC, AP-13)	402*
DG75, DG75 EBNA2	(T_{12} MA, AP-14)	300*

The tetracycline regulated cell line DG75 tTA EBNA2 was included in the study to examine more closely the regulation of cellular gene expression by EBNA2 in an isogenic background. EBNA2 was induced by the removal of tetracycline from the growth medium and differential display was carried out 9hr and 24 hr post-induction. DD was also carried out in the cell lines DG75 tTA and DG75 tTA EBNA2 before the removal of tetracycline. EBNA 2 induction was confirmed by western blotting and immunocytochemistry analysis as indicated in figures 5.2. Differential display experiments were repeated with the primers used to identify the reamplified cDNAs which are listed above. A band of 300 bases was down regulated in DG75 tTA EBNA2 at 9hr and 24hr post induction, results not shown. A band of 200 bases which appeared to be upregulated after induction of EBNA 2 was also identified figure 5.10.

DG75 tTA DG75 EBNA2 tTA (-), DG75 EBNA2 tTA(+)	(T_{12} MA, AP-14)	300b*
DG75 tTA DG75 EBNA2 tTA (-), DG75 EBNA2 tTA(+)	(T_{12} MC, AP-14)	200*

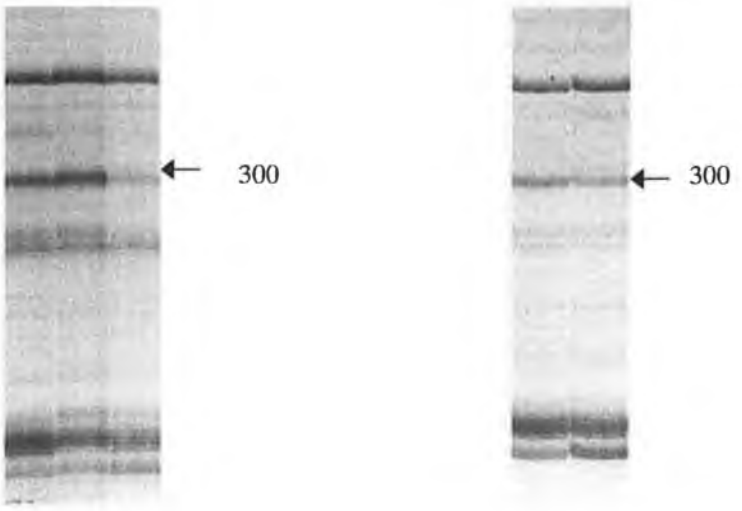
Differential display experiments are illustrated in figures 5.6-5.10.

5.9 DIFFERENTIAL DISPLAY EXPERIMENTS



A 1. 2. 3. B. 1. 2. 3.

Fig 5.6. Differential display analysis. The image shows the initial (A) and a repeat experiment (B) illustrating a potentially differentially expressed band of 310 bases. This band appears to be up regulated in the cell line DG75 EBNA2. **Lane No.** 1. DG75, 2. DG75 EBNA1, 3. DG75 EBNA2.



A. 1. 2. 3. B 1. 2

Fig 5.7. Differential display analysis. The figure shows the initial (A) and a repeat experiment (B) illustrating a potentially differentially expressed band of 300 bases. This band appears to be down regulated in the cell line DG75 EBNA2. **Lane No.** 1. DG75 2. DG75 EBNA1 3. DG75 EBNA2.

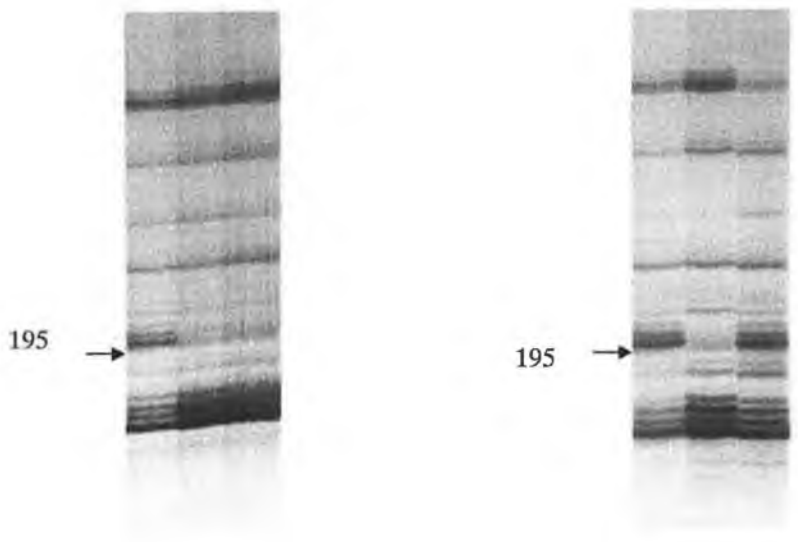


Fig 5.8. Differential display analysis. The figure shows the initial (A) and a repeat experiment (B) illustrating a potentially differentially expressed band of 195 bases. This band appears to be expressed in the DG75 cell line and DG75 EBNA2 but not in DG75 EBNA1. **Lane No.** 1. DG75, 2. DG75 EBNA1, 3. DG75 EBNA2.

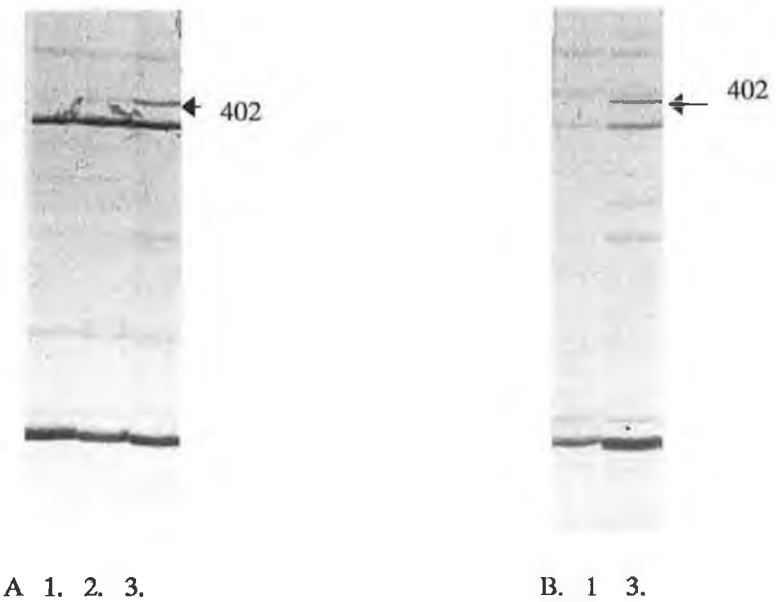


Fig 5.9. Differential display analysis. The image shows the initial (A) and a repeat experiment (B) illustrating a potentially differentially expressed band of 402 bases. This band appears to be up regulated in the cell line DG75 EBNA2. **Lane No.** 1. DG75, 2. DG75 EBNA1, 3. DG75 EBNA2.

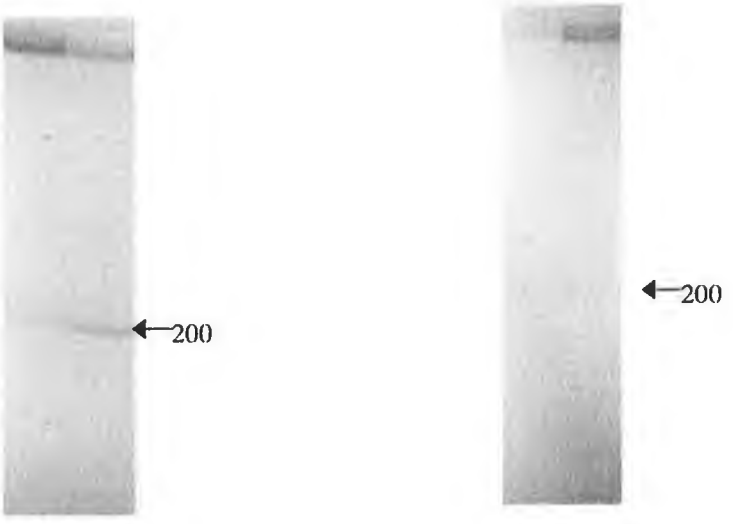


Fig 5.10. Differential display analysis. The figure shows the initial (A) and a repeat experiment (B) illustrating a potentially differentially expressed band of 195 bases. This band appears to be up regulated in the cell line DG75 tTA EBNA2 (induced). **Lane No.** 1. DG75 tTA, 2. DG75 EBNA tTA (Uninduced), 3. DG75 EBNA2 tTA (Induced).

5.10 PCR REAMPLIFICATION OF ISOLATED cDNA FRAGMENTS.

Upon identification cDNAs of interest were subsequently isolated from the polyacrylamide gel and reamplified by PCR. Reamplification was carried out using the same primer set and PCR conditions (with the exception of dNTP concentration) as described for DD-PCR (see section 2.7.6). Reamplification consisted of one or two rounds of PCR, if a PCR product was not detected by agarose gel electrophoresis after the initial PCR then 4 µl of a 1:100 dilution of the first round PCR was used as template for a second round of PCR. Some difficulty was encountered in the reamplification of a number of bands. Upon further investigation it was observed that bands which had been stored, after elution, prior to reamplification were difficult to reamplify, this may have been due to degradation caused by the presence of residual ^{35}S in the stored product.

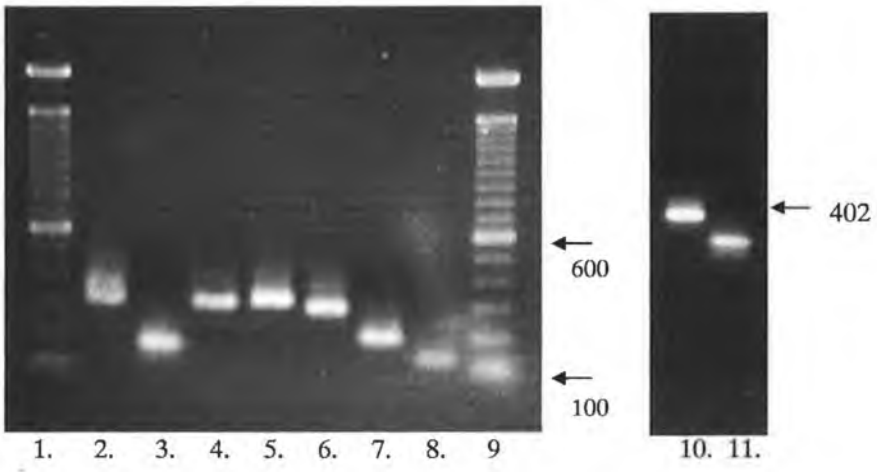


Fig. 5.11. Reamplification of differentially displayed cDNAs.

Lane No. PCR product.

1./9. 100 bp DNA marker,

2. B350; Not cloned due to the presence of two PCR products.
3. B212; Further experiments indicated that this band was not truly differentially regulated.
4. B300; Cloned.
5. B310; Cloned.
6. B292; Unable to clone.
7. B195; Cloned.
8. B132; Unable to clone.
10. B402; Cloned.
11. B172; Further experiments indicated that this band was not truly differentially regulated.

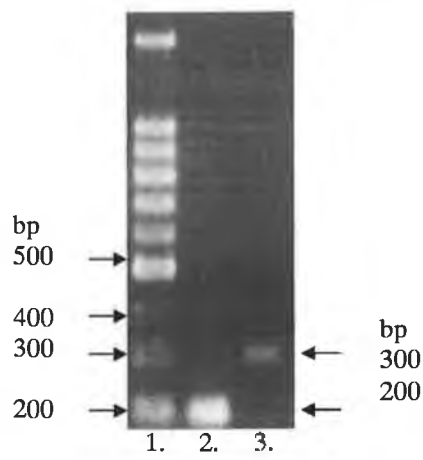


Fig. 5 12. Reamplification of differentially displayed cDNAs.

Lane No. PCR product.

1. 100 bp DNA marker.
2. B200; Cloned.
3. B300b; Cloned.

5.11 CLONING OF REAMPLIFIED DDPCR PRODUCTS.

Reamplified PCR products were cloned using the LigATor rapid cloning system from R&D systems into the pTAg cloning vector (see figure 5.13) as outlined in section 2.7.7. White colonies were chosen and DNA minipreparations were assessed for inserts by restriction analysis.

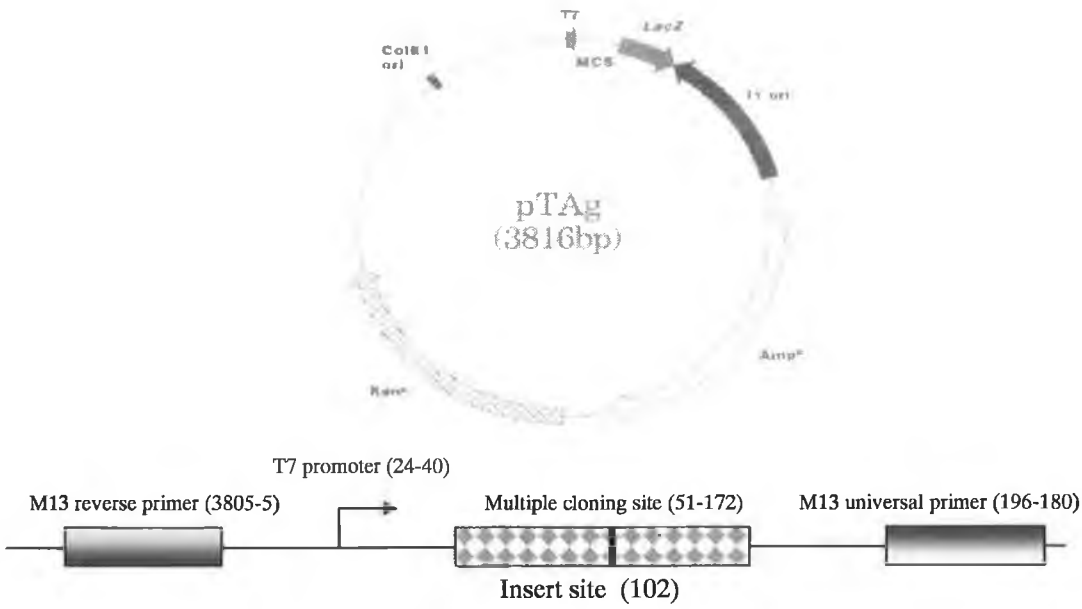


Fig. 5.13. Map of the cloning vector pTAg.

Restriction analysis was carried out on DNA minipreparations using the enzyme *Hinf I* or a double digestion with the enzymes *Bam HI* and *Hind III*. *Hinf I* cuts pTAg 13 times, one of the resulting fragments (459 bp fragment) spans the insertion site if an insert is present this fragment will become bigger, creating a new fragment of 459 plus the size of the cloned insert – only if the insert does not have a *Hinf I* site. *Bam HI* and *Hind III* are unique sites located either side of the insertion site in the MCS, thus a double digestion with these restriction enzymes results in the fragment being excised if the insert does not contain a *Bam HI* or a *Hind III* site.

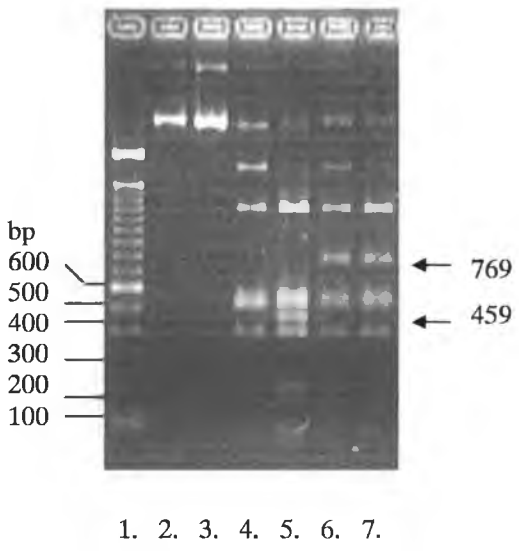


Fig. 5.14. Restriction analysis of PCR clones. Samples of DNA minipreps were digested with *Hinf I* and subjected to electrophoresis through a 1.5% agarose gel. Lane no. 1. 100 bp DNA marker, 2. pTAg empty vector uncut, 3. pTAg/control insert, 4. pTAg/control insert *Hinf I*, 5. pTAg *Hinf I*, 6. pTAg/B310 (a) *Hinf I*. 7. pTAg/B310 (b) *Hinf I*

From figure 5.14 it is clear that there is an insert present in the pTAg/B310 clones and the *Hinf I* digest illustrates that it is the correct size of 310 bp. *Hinf I* digest were carried out on other clones however the results obtained were not clear due to the presence of *Hinf I* sites with in the inserts, in such cases a *Bam HI* and *Hind III* double digest strategy was employed.

When pTAg is digested with *Bam HI* and *Hind III* 29 bp are added onto the insert thus B195 appears at approximately 224 bp (figure 5.15), B300 and B300b appears 329 bp (figure 5.15/5.16), B200 appears at 229 (figure 5.16) and B402 appears 431 bp (figure 5.15).

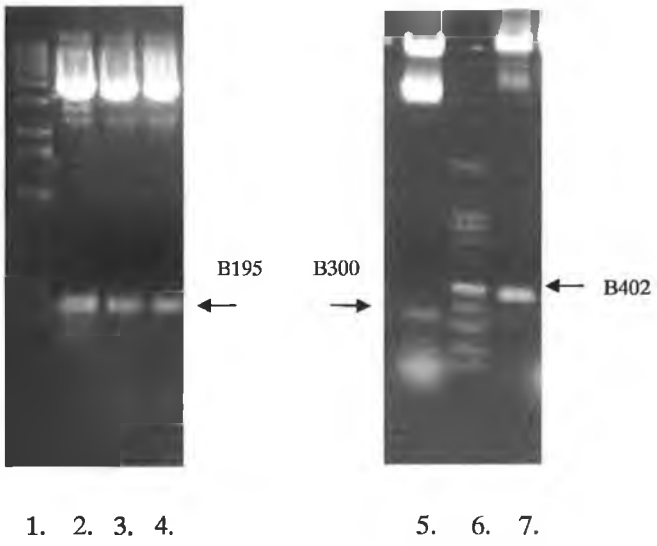


Fig 5.15. Restriction analysis of PCR clones. DNA minipreps digested with *Bam HI* and *Hind III* and subjected to electrophoresis through a 1.5% agarose gel. Lane no. 1. 1Kb DNA ladder, 2. pTAg/B195 3. pTAg/B195, 4. pTAg/B195 , 5 pTAg/B300, 6. 1Kb DNA ladder, 7. pTAg/B402.

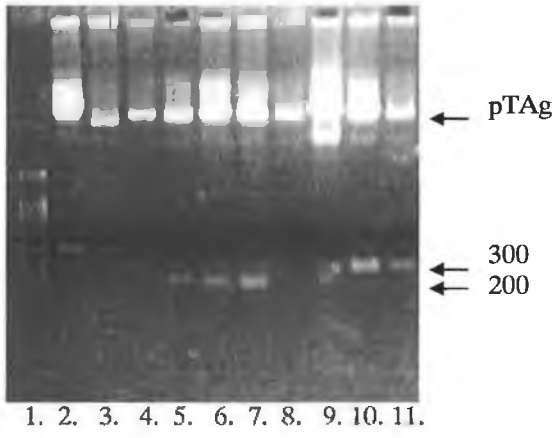


Fig 5.16. Restriction analysis of PCR clones. DNA minipreps digested with *Bam HI* and *Hind III* and subjected to electrophoresis through a 1.5% agarose gel. Lane no. 1. 100bp DNA ladder, 2-4. pTAg/β-actin (383bp), 5-9. pTAg/B200, 10-11. pTAg/B300b.

Some difficulties were encountered when cloning reamplified PCR products -this may have been due to the loss of A overhangs in the PCR products. PCR reactions were repeated in these cases but again cloning was unsuccessful. Thus a panel of six cloned partial cDNAs remained for analysis. These six partial cDNA clones were obtained from the analyses of one quarter of the total mRNA population (~3, 750 transcripts per cell line).

B310	Up regulated in DG75 EBNA2
B300	Down regulated in DG75 EBNA2
B195	Not expressed in DG75 EBNA1
B402	Up regulated in DG75 EBNA2
B300b	Down regulated in DG75 tTA EBNA2
B 200	Up regulated in DG75 tTA EBNA2

5.12 SEQUENCE ANALYSIS OF CLONED CDNAS.

Sequencing of cloned cDNA was carried out prior to northern blot analysis as DNA sequence analysis was taking place in the laboratory at that time and it allowed time to establish a panel of cloned cDNAs for northern blot analysis. Sequence analysis also revealed the orientation of the cloned cDNAs for the construction of riboprobes for northern blot analysis. A T7 Sequencing™ Kit from Pharmacia was used for sequencing the cloned cDNAs as described in section 2.7.8. Sequence analysis are illustrated in figure 5.17.

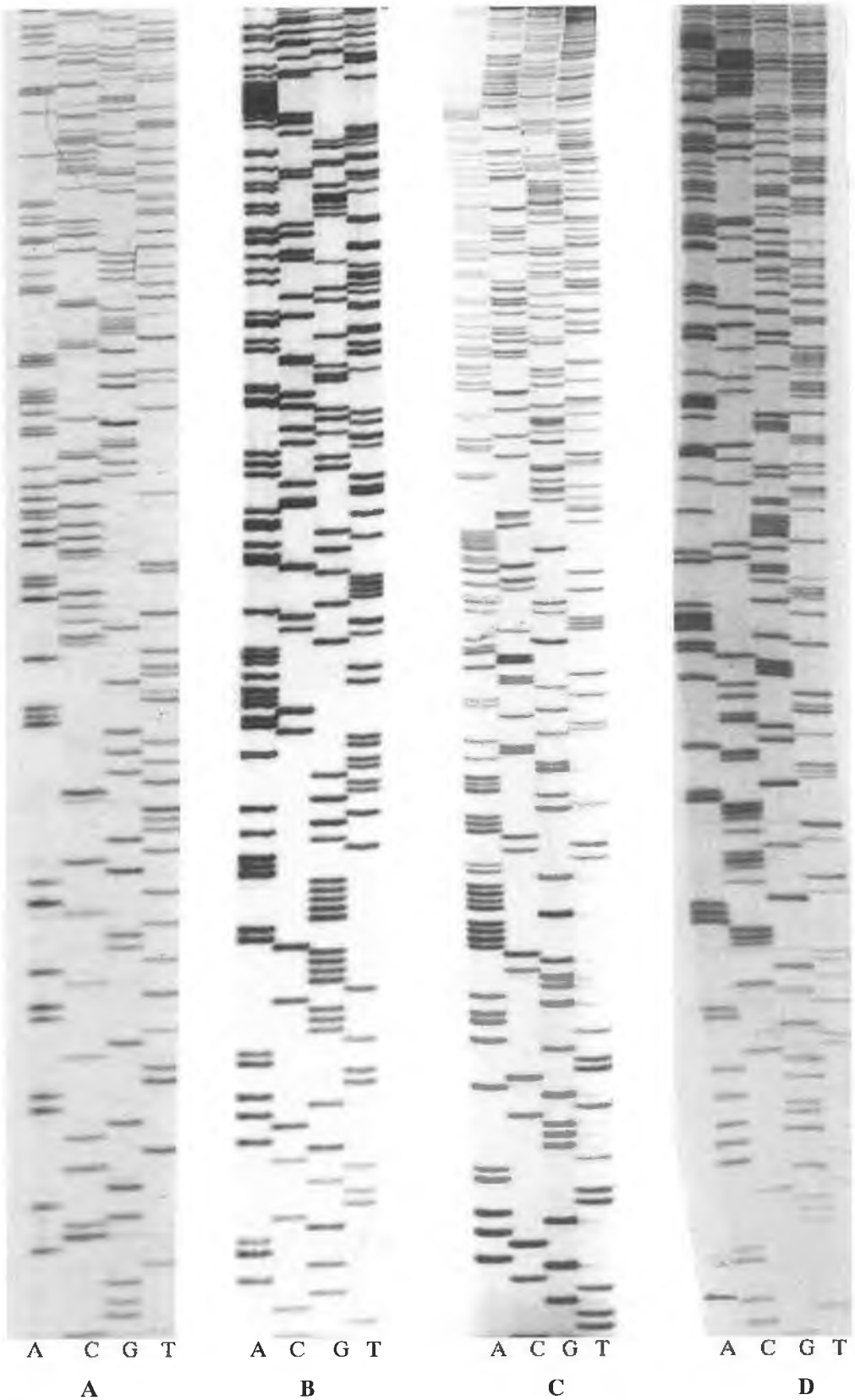


Fig 5.17. Photograph of DNA sequence autoradiogram. Sequencing reactions were run in the order ACGT from left to right. Set A: Control DNA, B: B195, C: B300, D: B310. Other sequence results not shown.

SEQUENCE ANALYSIS

A variety of search engines on the Internet were used to identify sequences obtained. The internet site used was Pedro's BioMolecular Research Tools – http://www.public.iasate.edu/~pedro/research-tools.html. BLASTN (Basic Local Alignment Research Tool Nucleotide) was the most frequently used search engine. The nucleotide sequence of the cDNAs of interest was entered into the BLASTN programme which carried out a search of data bases such as the EMBL and Genebank. The results were displayed as a list of sequences with decreasing alignment to the cDNA of interest.

SEQUENCE OF B310

GGATGAGCTTGAATGCTGTTCCCAAAGTCTCCCGTGTCCCAAGTTTCCAGTG
CCTTCTCAGGGCAATGAAAATAGTTTGTGGACAGCTGGGGGATGGTTTCAG
TGTCAAACTTTGGGAGTAAAATCTTTCATGTTTTTCTGAAGAACTTTGGCAT
TAGAATTTGTTGGTTCTGGAAAGTCAAGAACACAGTTGTTTTATTGAGAGA
NNNNNNN AAAAAAAAA.

Database Analysis:

A search of the INCB system at Trinity College was carried out and the results revealed greatest homology with homosapien cDNA clone.

SEQUENCE OF B 300 AND B300B.

CTTGATAAGAGGCGCAAAAGAAAAGAATCTCAAAGTGAAGGACCAGTTCG
AATTGCCTACCAAGACTTTGAGAATCACTACAAGAATAAACTCCTTGTGGTG
AAGGTTCTAAGCAGTGGGTCAGTTTCCGATAGGA... ... ...AAAAAAAA.

Sequence analysis revealed 100% homology between the sequence of B300 and B300b.

Database Analysis:

A search of the EMBL and Genebank databases revealed 100% homology with a section of the human ribosomal protein cDNA, S20 (RPS20).

SEQUENCE OF B195

GGCAAGGGGGAAATGAGTAGTTGTTATTCAACAAATATAAAGTCTCAGTTT
TGCAAGATGAATACCTTCTAGAGATCTGCTGTGCAACAAGGTGCCTATAGTT
AACATTGCTGTATTATGCCTTAACAACTTGAGAGGGTAGATCTCAGGTTAAG
TGTTCTTACCACAAAAAAAAAAAAAAAA.

Database analysis:

Sequence analysis revealed homology with (a) Human DNA sequence for cosmid N85E10 between markers D22S28-D22886 of chromosome 22q12, (b) Human DNA sequence for cosmid U96H1 DX5366-DX5877 on chromosome X and (c) Human Xp22 cosmid U106G8 and U26C6.

SEQUENCE OF B402

ATTATTCTTTGTATAGGTCCTCATGTTACGGTACGTTTTAAACGTCGCAGCC
GAACATCGTAGTATTAGACGCCTGATACAAGAC

Database Analysis:

Sequence analysis did not reveal any significant homology with any sequence in the database.

SEQUENCE OF B200

GTCGACAGATTAATGGGCTGGGGCAATGGGGAAATGAGTAGTTGTTATTCA
ACAAATATAAAGTCTCAGTTTTGCAAGATGAATACCTTCTAGAGACTGCTGT
GCAACAGGTGCCTATAGTTAACATTGCTGTATTATGCACTTAACAACTTGAG
AGGGGACTCAGGATGGCTCCGCAAAAAAAAAAAAAAAAA.

Database Analysis:

Database analysis did not reveal an exact match with any sequence. However sequence analysis using the BLASTn search showed that the sequence had homology with human DNA from two PAC cosmids and also to the human calbindin 27 gene at exon 10.

5.13 NORTHERN BLOT ANALYSIS

In order to confirm the differential expression of the cloned cDNAs northern blot analysis was carried out. RNA probes (riboprobes) were used to probe total RNA blotted onto a nitrocellulose membrane as described in section 2.8. Riboprobes were transcribed from the T7 promoter in the cloning vector pTAg as outlined in section 2.7.9.

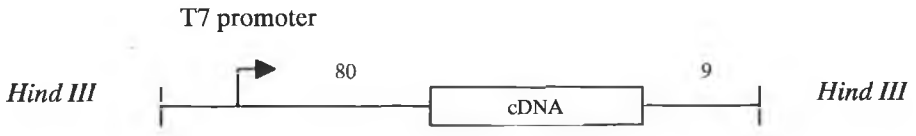


Fig. 5.18. Schematic representation of RNA probes. Each cloned cDNA was digested with *Hind III,* transcription started from the T7 promoter thus the full length riboprobe measured the length of the cloned fragment plus 89bp of the vector.

When labelling the first riboprobes B300 and B310, samples of both probes were run on a polyacrylamide gel along side a control reaction to ensure that the probes were full length and that degradation had not taken place. The gel was wrapped in cling film and exposed to X-ray film for 2 min and then developed. The bands appear quite close together as they were analysed on a high percentage gel over a short period of time.

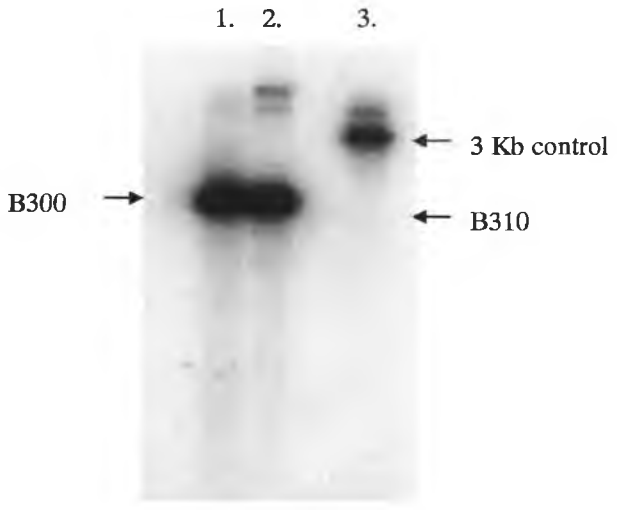


Fig 19. Verification of riboprobe quality. From the picture it is clear that the probes are full length and degradation has not take place. Lane no. 1. B300, 2. B310, 3. Control probe 3Kb.

Prior to blotting, the RNA of interest was run on a formaldehyde gel and viewed briefly under ultra violet light to control for loading. Ten micrograms of RNA was used in every lane. A representative photograph is shown below:

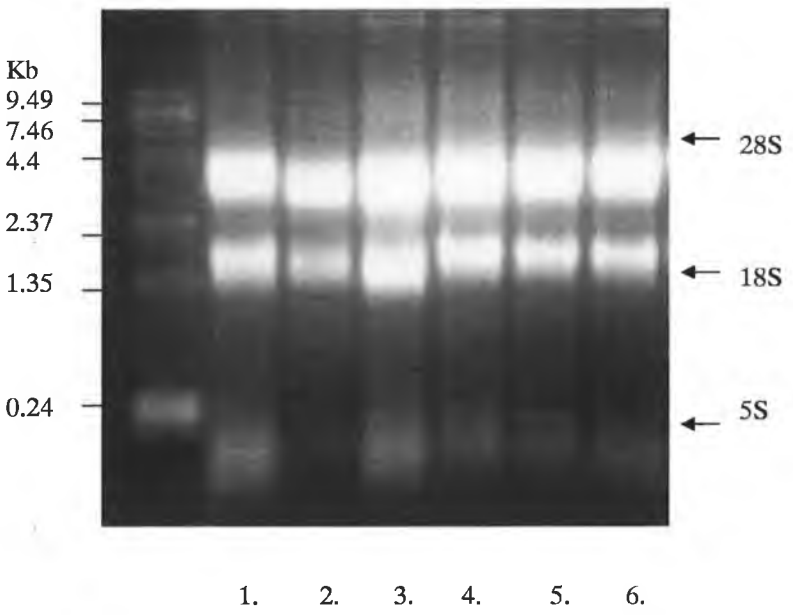


Fig. 5.20. Formaldehyde gel electrophoresis of RNA. Approximately 10μg of total RNA was subjected to electrophoresis through a formaldehyde gel and viewed on a UV transluminator. The 28S, 18S and 5S ribosomal bands are clearly visible indicating structurally intact RNA. Lane No. 1. DG75, 2. DG75 EBNA1, 3. DG75 EBNA2, 4. DG75 tTA, 5. DG75 tTA EBNA2 uninduced, 6. DG75 tTA EBNA2 induced.

Northern blotting was carried out by blotting 10μg of total RNA on to a nitrocellulose filter which was then probed using a ^{32}P-labelled riboprobe constructed from a cloned potentially differentially expressed partial cDNA. Ethidium Bromide was included in all RNA samples in order to view the RNA briefly under UV light to ensure equal loading of the samples on the gel. The gel shown in figure 5.21 was used in northern blot analysis of B200 as illustrated in figure 5.24. The approximate size of the bands obtained by northern blotting was estimated by comparison with the RNA size makers as shown in figure 5.20.

B310

In the experiment shown in figure 5.21, a riboprobe corresponding to the B310 cDNA was used to probe total RNA from a panel of cell lines, by northern blotting. The differential expression observed by DDRT-PCR (in this case downregulation coincident with EBNA2 expression) was not seen in the northern blot, thus indicating that the differential expression of B310 was false positive.

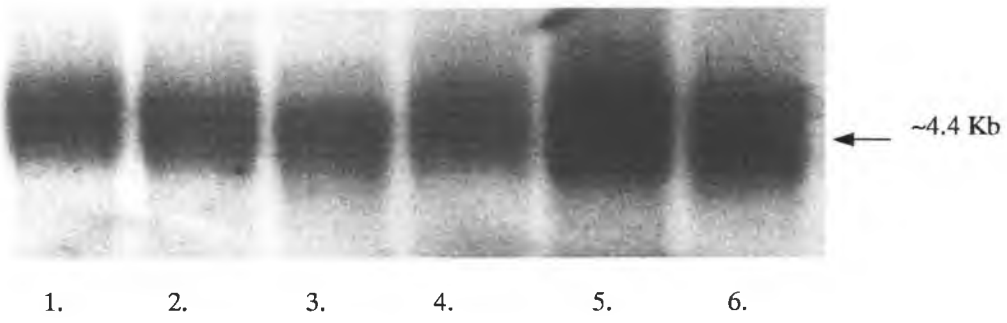


Fig. 5.21. Northern blot analysis of DD products. Lane No. 1. DG75, 2. DG75 EBNA1, 3. DG75 EBNA2, 4. DG75 tTA, 5. DG75 tTA EBNA2 (Uninduced), 6. DG75 tTA EBNA2 (Induced).

B300

In the experiment shown in figure 5.22, a riboprobe corresponding to the B300/B300b cDNA which corresponds to the human ribosomal protein S20 was used to probe total RNA from a panel of cell lines, by northern blotting. The differential expression observed by DDRT-PCR (in this case downregulation coincident with EBNA2 expression) was not seen in the northern blot. Thus indicating that the differential expression of B300 was false positive.

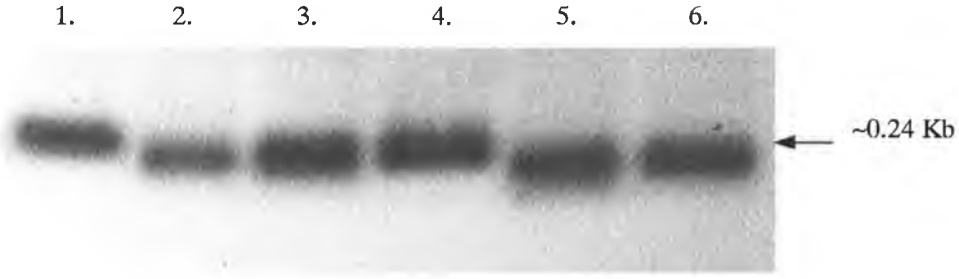


Fig. 5.22. Northern blot analysis of DD products. Lane No. 1. DG75, 2. DG75 EBNA1, 3. DG75 EBNA2, 4. DG75 tTA, 5. DG75 tTA EBNA2 (Uninduced), 6. DG75 tTA EBNA2 (Induced).

B195

In the experiment shown in figure 5.23, a riboprobe corresponding to the B195 cDNA was used to probe total RNA from a panel of cell lines, by northern blotting. The differential expression observed by DDRT-PCR (in this case downregulation coincident with EBNA1 expression) was not seen in the northern blot. The human S20 ribosomal protein was also included (B300) in this northern blot which indicates that there was approximately equal loading of RNA. Thus indicating that B195 was another false positive.

Fig. 5.23. Northern blot analysis of DD products. Lane No. 1. DG75, 2. DG75 EBNA1, 3. DG75 EBNA2, 4. DG75 tTA, 5. DG75 tTA EBNA2 (Uninduced), 6. DG75 tTA EBNA2 (Induced).

B200

In the experiment shown in figure 5.24, a riboprobe corresponding to the B200 cDNA was used to probe total RNA from a panel of cell lines, by northern blotting. The differential expression observed by DDRT-PCR (in this case upregulation coincident with EBNA2 expression) was not seen in the northern blot. Thus indicating that the differential expression of B200 was also false positive.

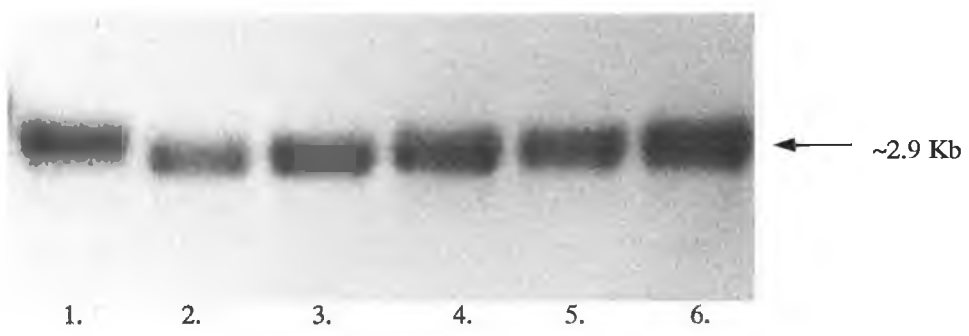


Fig. 5.24. Northern blot analysis of DD products. Lane No. 1. DG75, 2. DG75 EBNA1, 3. DG75 EBNA2, 4. DG75 tTA, 5. DG75 tTA EBNA2 (Uninduced), 6. DG75 tTA EBNA2 (Induced).

The RNA gel used in this northern blot is illustrated in figure 5.20. DG57 EBNA1 RNA is under-loaded in this gel however this is not important in this particular experiment as the expression of B200 as analysed by DDRT-PCR appeared to be upregulated with EBNA2 expression.

B402

In the experiment shown in figure 5.25, a riboprobe corresponding to the B402 cDNA was used to probe total RNA from a panel of cell lines, by northern blotting. The differential expression observed by DDPCR (in this case upregulation coincident with EBNA2 expression) was not seen in the northern blot. Thus indicating that the differential expression of B402 was also false positive.

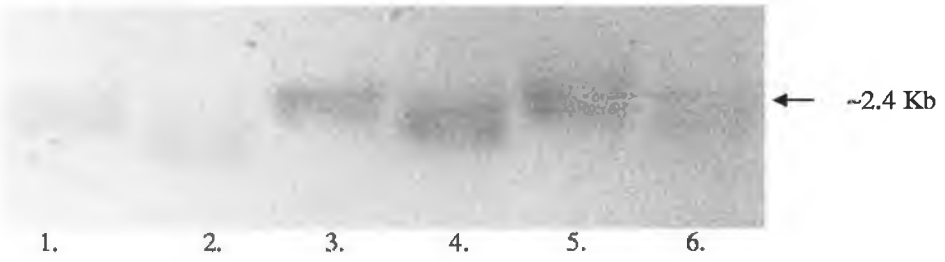


Fig. 5.25. Northern blot analysis of DD products. Lane No. 1. DG75, 2. DG75 EBNA1, 3. DG75 EBNA2, 4. DG75 tTA, 5. DG75 tTA EBNA2 (Uninduced), 6. DG75 tTA EBNA2 (Induced).

5.14 DISCUSSION AND CONCLUSIONS

The differential display method described by Liang and Pardee has many advantages over classical methods for analysis of gene expression (Liang and Pardee, 1992). These include its rapidity, the ability to display the message profile of many cell types simultaneously from a small amount of RNA, and its ability to identify both activated and repressed genes in the same reaction. Although simple in theory, utilization of this method is quite labour intensive (Hadmann *et al.,* 1995). Differential display has many steps and thus there are many areas were problems can arise. The initial step is the isolation of intact RNA, free from chromosomal DNA, ensuring that amplification of DNA does not occur during DD. Gel electrophoresis and β-actin RT-PCR, were used to show that the RNA was structurally intact and free from DNA contamination respectively, thus establishing the integrity of the RNA used in this study.

Problems can also be encountered in the isolation of cDNA fragments from the dried gel. An exact match must be obtained between the gel and the corresponding autoradiogram so as to eliminating the possibility of excising the wrong band or more than one band. Orientation markers were included in this study to avoid this problem, however occasionally more than one band resulted from PCR reamplification therefore these products were unsuitable for cloning. It was observed that immediate reamplification of eluted PCR products yielded best results. Partial cDNAs, which were stored for in excess of 48 hr after elution prior to reamplification, proved difficult or impossible to clone. This may have been the result of degradation caused by the presence of residual ^{35}S in the stored product. A major limitation of the procedure is that differences in gene expression observed by DD are false and often cannot be reproduced by northern blot analysis, these false positive cases can arise with a frequency of up to 70% (Sun *et al.,* 1994). The results of this study clearly illustrate that even after repeated DDPCR experiments and reamplification of PCR products of the correct size, false positives are indeed a major drawback of this particular approach. Northern blot analysis has clearly demonstrated that the alteration in band pattern observed in this study by differential display does not reflect real changes in mRNA levels but are artefacts of the procedure. False positives arise in a variety of ways such

as the amplification of contaminating DNA, the addition of an "A" to the end of an amplified cDNA fragment during PCR which appears as a differentially expressed band or the isolation of an incorrect cDNA fragment from a dried gel. In the method described in this study several cDNA fragments can result from the amplification of the same mRNA resulting in a complexed pattern of gene expression which may lead to false positives, additionally different recombinant clones with similar sized inserts will be generated.

The problem of false positives has been addressed by a number of groups in the form of modifications to the original protocol, some of which are discussed in this section. When using total RNA as a template Hadman and colleagues found that a large proportion (~40%) of total bands resulted from PCR amplification between two 5' primers. The number of bands amplified in this manner was reduced by using poly(A)– selected mRNA however this resulted in poor resolution. This lead to the introduction of an end labelled 3' primer, thus only bands which included this primer were visualised, (Hadman *et al.,* 1995). It has been suggested that insufficient starting material may be a factor in the relatively poor reproducibility seen in differential display. Even subtle differences in starting material results in a decrease in a transcript which can lead to a reproducible loss of a corresponding fragment in a fingerprint, so it will appear as a errant down regulation even in duplicates (Matz and Lukyanov, 1998). In order to reduce false positives caused by slight fluctuations in starting material McClelland *et al.,* (1995) suggested that fingerprints of at least two different concentrations of total RNA that differ by two fold are run in parallel to determine which bands are not reproducible. Making sure that there is enough starting material is a simpler solution which has also been suggested (Matz and Lukyanov, 1998). One study suggested when using an induction system, duplicate PCR products should be displayed (Sompayrac *et al.,* 1995). They also suggested displaying products over a time course of induction. This approach reduced the overall number of artifactual PCR or RT results. When possible they used cytoplasmic rather than total RNA to eliminate false positives due to nuclear RNA that is not transported to the cytoplasm (Sompayrac *et al.,* 1995).

Many of the DDRT-PCR products that appear as doublets and triplets (as is the case for B195), may represent two strands of the same fragment and molecules with and with out an additional A known to be added during PCR by Taq polymerase at the 3' end. This means that the true number of genes present are lower which may lead to false positives, as one transcript may have an additional "A" added and the corresponding gene in the next tube may not, thus appearing as a differentially expressed band. In order to avoid the complexity of the patterns on denaturing polyacrylamide gels caused by several bands derived from one DNA species, one study suggested electrophoresis through nondenaturing gels thus reducing their visual complexity and decreasing the probability of false positives (Bauer *et al.,* 1993).

Five of the six potentially differentially expressed bands identified in this study appeared to be up- or downregulated coincident with EBNA2 expression at this stage of the experiment, this was not surprising as EBNA2 has been shown to transactivate both viral and cellular gene expression (see section 1.5.2). EBNA2 transactivates gene expression by binding DNA through interaction with RBP-Jκ which is a downstream target gene of the cellular receptor Notch (Fortini and Artavanis-Tsakonas, 1994). Interaction with Notch converts the repressor RBP-Jκ into an activator that can then interact with EBNA2 (Waltzer *et al.,* 1995). The EBV latent genes EBNA3A, 3B and 3C have been shown to compete with EBNA2 for RBP-Jκ which result inhibition of the trancriptional activation of EBNA2 promoters (Le Roux *et al.,* 1994). EBNA2 has been shown to downregulate IgM and *c-myc* expression in BL cells but upregulate *c-myc* expression in LCLs. Thus it is clear that EBNA2 can both act as a transcriptional activator and a repressor.

Many of the problems of differential display have been addressed in the battery of modifications and improvements which have been published since the original protocol in 1992 (see section 5.4). When examining differential gene expression today one can make a much more informed decision, which would result in choosing a differential display method that is suitable for the system to which it is applied. The use of nondenaturing gels combined with the amplification of different RNA concentrations might therefore have reduced the number of false positives in this study. A systematic

approach (see section 5.4) to DD would reduce the complexity of bands as each mRNA would technically be represented by one cDNA as opposed to several in classical DD. A fluorescent DD approach which could be automated as described by Shohan and colleagues would both reduce the potential hazard to the user and ease identification of differentially expressed genes (Shohan *et al.,* 1996). The use of a GAPDH probe as a control for RNA loading, as opposed to visual estimation of loading, would be advised as this would provide a much more stringent method of control which can be quantified by densitometry thus allowing exact normalisation of banding patterns obtained. In this study the use of GAPDH or β-actin on northern blots would have helped, however, significant modulation of gene expression in the cases identified by DDPCR can still be ruled out.

Further developments in gene expression analysis such as Serial analysis of gene expression (SAGE), expressed sequence tags (ESTs), representational difference analysis (RDA), DNA microarrays and advances in subtractive hybridisation, are providing new alternatives for identifying genes which are differentially regulated. Expressed sequence tags (ESTs), are generated by large scale sequencing of randomly chosen cDNAs, this provides an individual fingerprint of the expression status of an analysed tissue or cell (Kozian and Kirchbaum, 1999). Differential gene expression can be identified by comparing the databases of EST of a given organ or cell type with sequence information from a different origin (Vasmatzis *et al.,* 1998). This method is not suitable for all laboratories however, as it requires large scale sequencing facilities. Serial analysis of gene expression (SAGE) was described by Velculescu *et al.,* in 1995. SAGE is another sequence-based approach to the identification of differentially expressed genes. Sequence tags are generated by the reverse transcription of mRNA using a biotinylated oligo (dT) primer. The cDNA is then digested with a frequently cutting restriction enzyme and then selected by binding to streptavidin coated beads. The cDNA is then split into two samples each of which are ligated to a primer (A' or B') containing a restriction site. The cDNA is digested using the appropriate restriction enzyme and the resulting DNA fragments are ligated, the resulting 'digitags' are amplified by PCR using primers A' and B'. Following PCR the primer sites A and B are removed and the sticky ends formed allow the DNA to form concatemers, which are

subsequently cloned. The structure of the concatamer has a typical pattern: between each anchoring site a digitag contains the sequence information of two independent cDNA tags (Kozian and Kirchbaum, 1999). The frequency of each tag in the cloned multimer directly reflects transcript abundance. In addition the short tags are long enough to uniquely identify the corresponding transcript in database searches. Based on the sequence information, comparative computational analysis for the presence and frequency of transcripts can be performed. When using an automated sequencer and computational support, SAGE is a powerful tool that allows rapid expression profiling of genes that are deposited in a gene bank. However this in itself is the greatest drawback of the procedure as only tags which are deposited in gene banks can be used for gene identification (Kozian and Kirchbaum, 1999). A further draw back of SAGE is the large amount of RNA required (2.5-5.0 μg of mRNA). Recently a modification of SAGE analysis called microSAGE has been described which enables SAGE to be carried out on very small quantities of RNA (500-5000 times less mRNA). In the modified protocol, all steps from RNA isolation to tag release are performed in a single tube in which the RNA, and later the cDNA, remain immobilised to the wall of the tube by means of steptavidin-biotin binding. This eliminates many of the steps which may lead to the loss of material. Furthermore, total RNA is used as opposed to poly(A) and thus the mRNA isolation step is eliminated. Also less PCR cycles are performed, after the PCR step the protocol is essentially the same as SAGE (Datson *et al.,* 1999).

Representational difference analysis was originally described as a method for the identification of particular differences between two complex genomes (Lisitsyn *et al.,* 1993). It has since been adapted to analyse differential gene expression by combining subtractive hybridization and PCR (Hubank and Schatz, 1994). In the first step, mRNA derived from two different populations, the tester (the population in which differential gene expression is expected to occur) and the driver, is reverse transcribed. The cDNA is then digested with a frequently cutting restriction enzyme and linkers are ligated to both ends. Following a PCR step the linkers of the tester and driver are digested and a new linker is ligated to the end of the tester cDNA. Tester and driver are allowed to hybridise, followed by PCR which exponentially amplifies only homoduplexes generated by the tester cDNA, via the priming site on both ends of the double-stranded

cDNA. The main advantage of RDA is the specific amplification of fragments exclusively present in one cDNA pool, owing to an enrichment of rarely expressed tester sequence (Kozian and Kirchbaum, 1999).

A new approach to the study of gene expression has been the development of DNA microarrays. Current cDNA microarrays are systematically gridded at high density. They are generated by using cDNAs, PCR products, cloned DNA or synthetic oligonucleotides which are linked to the surface of nylon filters glass slides or silicone chips (Schena, *et al.,* 1995). Differences in gene expression are determined by applying a labelled cDNA or oligomer to the microarray. If different fluorescent labels are used then two probes can be applied simultaneously and analysed at different wavelengths. The expression of up to 10,000 genes or more can be analysed on a single chip (Chee *et al.,* 1996). As hybridisation can be carried out in small volumes this may allow the detection of rare transcripts in probes of high sequence complexity. It should be emphasized that although these hybridization-based approaches seem likely to become the method of choice for large scale analysis of gene expression in humans, they cannot be readily applied to other organisms as complete cDNA sequences of other organisms are not available (Soares, 1997). Furthermore, this method cannot be used to identify unknown genes that are differentially regulated.

The combination of subtractive hybridisation (SH) and differential display would in theory result in a powerful strategy for cloning up- and down-regulated genes. A combination of these techniques have been used to examine altered gene expression in rat liver after 70% hepatacytomy, however the resulting pattern of gene expression did not show significant simplification of banding pattern (Hakvoort *et al.,* 1994). A reciprocal subtractive differential RNA display (RSDD) has been reported which again combines SH and DD. In this case DD was performed directly with reciprocally subtracted cDNA libraries that had not been subjected to PCR. Three single anchored oligo (dT) 3' primers were used for subsequent amplification prior to display. Reamplified cDNA identified using RSDD were analysed using reverse northern blots. RSDD resulted in a clear delineation of differentially expressed amplified bands using a reduced number of primers. Also as the SH round eliminates abundant cDNAs present

in both populations this technique is more sensitive to detecting rare transcripts which may be differentially expressed as they will no longer be masked (Kang *et al.,* 1998). The requirement for small quantities of RNA has always been cited as an advantage of DD and the need for large amounts of mRNA for SH has always been quoted as a disadvantage. This no longer appears to be so with a report by Ghosh (1996) describing a novel ligation mediated-PCR based strategy for construction of subtraction libraries from less than 0.5 μg of mRNA as opposed to 10 μg as previously required (Ghosh, 1996). Therefore SH is more suitable for combination with DD. A further advancement of SH, suppression subtractive hybridisation (SSH), has also been recently described which is based on a technology similar to RDA but with modification to normalise for mRNA abundance. A report by Yang *et al.,* (1999), proposes the combination of SSH and cDNA microarray for identification of differentially expressed genes (Yang *et al.,* 1999).

In the past few years highly sophisticated tools have been developed for the analysis of differential gene expression. Each of these techniques has a number of unique advantages, such as simplicity (DD) or range (SAGE) of analysis. Conversely there are also limitations including the unidirectional analysis of RDA, the high expense of DNA microarrays or the analysis only of known genes with SAGE. Differential display provides a fast and technically simple method for the identification of differentially expressed mRNAs and has become one of the most widely used methods for the analysis of gene expression. Sequences-based approaches such as SAGE or oligonucleotide arrays are excellent tools for high-throughput screening of expression profiles, but once differentially expressed genes are identified, the corresponding cDNA must be cloned. The choice of gene expression analysis technology used by a particular research group will depend on a number of factors including the equipment and the cost and the needs of a particular laboratory. Thus there is no universal superior method of gene analysis. However, it is highly desirable to have an ideal tool, which would allow the unambiguous identification of differentially expressed genes in a simple and parallel manner and be suited to the detection of low-abundance mRNA species in a single experiment. Thus the evolution of gene expression analysis is set to continue.

CHAPTER 6

FINAL SUMMARY

FINAL SUMMARY

The ensemble of the work presented in chapters 3 and 4 may be summarised as follows:

- CD44 protein or transcript was not detected in the type-I BL cell lines Mutu I, Rael BL, Kem BL, BL41, BL74.
- The drift to a type-III phenotype correlated with a strong up-regulation of CD44 expression at the cell surface.
- Upregulation was seen to be due to *de novo* transcription of the CD44 gene.
- CD44 was not detected in the Mutu III LMP1- clone, thus providing evidence of a direct role for LMP1 in CD44 expression.
- In an EBV negative BL cell line background neither EBNA2 nor LMP1 alone were sufficient to up-regulate CD44 expression.
- The use of mAbs by flow cytometry and western blotting yielded highly varied results indicating that mAbs alone were not sufficient to provide a definitive answer as to the presence or absence of CD44 standard/splice variants.

A novel set of CD44-exon specific probes were designed and used to examine CD44 variant exon splicing in EBV-infected cells by RPA. This assay was established by applying the CD44 exon-specific probes to the colon carcinoma cell line HT29.

- All CD44 variant exons were detected in HT29, with mRNAs containing the 5' standard exons and the variant exons v8-10 predominating. Transcripts encoding the CD44 exons v8-10C3', v7-10C3', v6-10C3' were also seen to occur in HT29. CD44v5-10C3' mRNA was also detected but these species may contain additional 5' variant exons.
- The 3' standard exons appear to be always spliced to v10.
- Towards the 5' end of the CD44 mRNA many combinations of variants were also observed with the predominant signal being obtained from the C5' standard exons.
- When used to examine the pattern of CD44 exon splicing in a number of EBV positive type-I and type-III BL and LCL cell lines it was observed that type-I cell

lines do not express CD44. However, CD44 was detected in type-III BL cell lines and LCLs.

- A similar but very complex pattern of CD44 variant exon expression was detected in both of these cell types of. The variant exons v8, v9 and v10 are most often used, several combinations of which were detected. Transcripts containing v10 were not always seen to be spliced to the C3' exons indicating the usage of a alternative 3' splice acceptor site downstream of v10.

In chapter 5 the effect of the EBV latent proteins EBNA1 and EBNA2 on cellular gene expression was examined by Differential display PCR. DD provides a fast and technically simple method for the identification of differentially expressed mRNAs and has become one of the most widely used methods for the analysis of gene expression. One of the original DDPCR methods was employed in this study, one which has been subsequently shown to be prone to false positives as was borne out by the results obtained in chapter 5.

CHAPTER 7

BIBLIOGRAPHY

Abbasi, A.M, Chester, K.A., Talbot, I.C., Macpherson, A.S., Boxer, G., Forbes, A., Malcolm, A.D. and Begent R .H. (1993). CD44 is associated with proliferation in normal and neoplastic human colorectal epithelial cells. *Eur J Cancer.* 29, 1995-2002.

Abbot, S.D., Rowe, M., Cadwallader, K., Ricksten, A., Gordon, J., Wang, F., Rymo, L., and Rickinson, A.B. (1990). Epstein-Barr virus nuclear antigen 2 induces expression of the virus encoded latent membrane protein. *J. Virol.,* 64, 2126-2134.

Adams, A. (1987). Replication of latent Epstein-Barr virus genomes in Raji cells. *J Virol.* 61, 1743-1746.

Afonina, I., Zivarts, M., Kutyavin, I., Lukhtanov, E., Gamper, H. and Meyer, R.B. (1997). Efficient priming of PCR with short oligonucleotides conjugated to a minor groove binder. *Nucleic Acids Res.* 25, 2657-2660.

Aitken, C., Sengupta, S.K., Aedes, C., Moss, D.J., and Sculley, T.B. (1994). Heterogeneity within the Epstein-Barr virus nuclear antigen 2 gene in different strains of Epstein-Barr virus. *J Gen Virol* 75, 95-100.

Aldwine, J.C., Kemp, D.J. and Stark, G.R. (1977). Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. *Proc Natl. Acad. Sci. U.S.A.* 74, 5350-5354.

Alfieri, C., Birkenbach, M. and Kieff, E. (1991). Early events in the Epstein-Barr Virus infection of human B lymphocytes. *Virology* 181, 595-608.

Allday M.J., Crawford D.H. and Griffin B.E. (1989). Epstein-Barr virus latent gene expression during the initiation of B cell immortaliation. *J Gen Virol.* 70, 1755-1764.

Allday, M.J, Crawford, D.H, and Thomas, J.A. (1993). Epstein-Barr virus (EBV) nuclear antigen 6 induces expression of the EBV latent membrane protein and an activated phenotype in Raji cells. *J Gen Virol* 74, 361-369.

Ambinder, R.F., Shah, W.A., Rawlins, D.R., Hayward, G.S., and Hayward, S.D. (1991). Definition of the sequence requirements for the binding of the EBNA1 protein to it's palindromic target sites in Epstein-Barr virus DNA. *J Virol.* 64, 2369-2379.

Ames, E.D., Conjalka, M.S., Goldberg, A.F., Hirschman, R., Jain, S., Distenfeld, A. and Metroka,C.E. (1991). Hodgkin's disease and AIDS. Twenty-three new cases and a review of the literature. *Hematol Oncol Clin North Am.* 5, 343-56.

Anagnostopoulos, I., Hummel, M., Kaudewitz, P., Korbjuhn, P., Leoncini, L. and Stein, H. (1996) Low incidence of Epstein-Barr virus presence in primary cutaneous T-cell lymphoproliferations. *Br. J. Dermatol.,* 134, 276-281

Anderson , M.C., Stam, N.J., Klein, G., Ploegh, H.L. and Masucci, M.G. (1991). Abberant expression of HL class antigens in BL cells. *Int. J. Cancer.* 47, 544-550.

Arch, R., Wirth, K., Hofmann, M., Ponta, H., Matzku, S., Herrlich, P., Zolle,r M. (1992) Participation in normal immune responses of a metastasis-inducing splice variant of CD44. *Science,* 257, 682-5

Artavanis-Tsakonas, S., Matsuno, K. and Fortini, M.E., (1995). Notch signalling. *Science*, 268, 225-232.

Aruffo, A., Stamenkovic, I., Melnick, M., Underhill, C.B. and Seed, B. (1990). CD4 is the principal cell surface receptor for Hyaluronate. *Cell.* U 61,U 1303-1313.

Aster, J.C., Robertson, E.S., Hasserjian, R.P., Tuner, J.R., Kieff, E. and Sklar, J. (1997). Oncogenic forms of NOTCH1 lacking either the primary binding site for RBP-Jκ or nuclear locatization sequences retain the ability to associate with RPB-Jκ and activate transcription. *J. Biol. Chem.,* 272, 11336-11343.

Austin, P.J., Flemington, E., Yandava, C.N., Strominger, J.L. and Speck, S.H. Complexed transcription of the Epstein-Barr virus Bam HI fragment H rightward open reading frame 1 (BHRF1) in latently and lytically infected B lymphocytes. *Proc. Nat. Acad. Sci. USA.* 85, 3678-3682.

Baer, R., Bankier, A.T., and Biggin, M.D. (1984). .DNA sequence and expression of the B95-8 Epstein-Barr virus genome. *Nature* 310, 207-211.

Ballerini, P., Gaidano, G., Gong, JZ., Tassi, V., Saglio, G., Knowles, DM. and Dalla-Favera, R. (1993). Multiple genetic lesions in immunodeficiency syndrome-related non-Hodgkin's lymphoma. *Blood* 81, 166-176

Bajorath, J., Greenfield, B., Monro, S.B., Day, A.J. and Aruffo, A. (1998). Identification of CD44 residues important for hyaluronan binding and delineation of the binding site. *J. Biological Chemistry,* 273, 338-343.

Bartolazzi, A., Nocks, A., Aruffo, A., Spring, F. and Stamenkovic, I. (1996). Glycosylation of CD44 is implicated in CD44-mediated cell adhesion to hyaluronan. *J. Cell Biology,* 132, 1199-1208.

Bauer, D., Muller, H., Reich, J., Riedel, H., Ahnerkid, V., Warthoe, P. and Strauss, M. (1993). Identification of differentially expressed mRNA species by an improved display technique (DDRT-PCR). *Nucleic Acids Res.,* 21, 4272-4280.

Bell, M.V., Cowper, A.E., Lefranc, M., Bell, J.I. and Screaton, G.R. (1998). Influence of Intron length on alternative splicing of CD44. *Mol, Cell. Biol,* 18, 5930-5941.

Ben-Bassat, H., Goldblum, M., Mitrani, S., Goldblum, E., Woffey, J.M., Cohen, M.M., Bentwich, Z., Ramot, B., Klein, E., and Klein, G. (1977). Establishment in continuous culture of a new type of lymphocyte from a "Burkitt like malignant lymphoma (line DG.-75). *Int. J. Cancer* 19, 27-33.

Benjamin, D., Magrath, I.T., Maguire, R., Janus, C,. Todd, H.D. and Parsons, R.G. (1982). Immunoglobulin secretion by cell lines derived from African and American undifferentiated lymphoma of Burkitt's and non-Burkitt's type. *J. Immuno.,* 129, 1336-1342.

Bennett, K.L., Modrell, B., Greenfield, B., Bartolazzi, A., Stamenkovic, I., Peach, R., Jackson, D.G., Spring, F. and Aruffo, A. (1995). Regulation of CD44 binding to hyaluronan by glycosylation of variably spliced exons. *J Cell Biol.* 131, 1623-33.

Beral, V., Peterman, T., Berkelman, R., and Jaffe, H., (1991). AIDS-associated non-Hodgkin lymphoma. Lancet 337, 805.

Berger R., and Bernheim A., (1985). Cytogenetics of Burkitt's lymphoma-leukemia: a review. In: *Burkitt's lymphoma, a human cancer model.* Lyon, France: IARC Scientific Publication 60, 65-80.

Bernheim, A., Berger, R. & Lenoir, G.M. (1981) Cytogenetic studies on African Burkitt's lymphoma cell lines: t(8;14), t(2;8), and t(8;22) translocations. *Cancer Genet. Cytogenet.,* 3, 307-315.

Bertioli DJ, Schlichter UH, Adams MJ, Burrows PR, Steinbiss HH, Antoniw JF (1995). An analysis of differential display shows a strong bias towards high copy number mRNAs. *Nucleic Acids Res.* 23, 4520-3.

Bhatia, K., Spangler, G., Gaidano, G., Hamdy, N., Dalla-Favera, R. & Magrath, I. (1994) Mutations in the coding region of c-myc occur frequently in acquired immunodeficiency syndrome-associated lymphomas. *Blood*, 84, 883-888

Billaud, M., Busson, P., Huang, D., MuelleLantzch, N., Rousselet, G., Pavlish, O., Wakasugi, H., Seigneurin, JM., Tursz, T. and Lenoir, GM., (1989). Epstein-Barr virus (EBV)-containing nasopharyngeal carcinoma cells express the B cell activation andtigen Blast2/CD23 and low levels of the EBV recptor CR2. *J Virol* 63: 4121-4128.

Birkenbach, M., Josefsen, K., Yalamanchili, R., Lenoir, G. and Kieff, E., (1993). Epstein-Barr virus induced genes: First lymphocyte-specific G protein-coupled peptide receptors. *J. Virol.,* 67, 2209-2220.

Birkenbach, M. Liebowitz, D., Wang, F., Sample, J. and Kieff, E., (1989). Epstein-Barr virus infection or the latent infection membrane protein induces vi-mentin expression. *J. Virol.,* 63,4079-4084

Borland, G., Ross, J.A. and Guy, K., (1998). Forms and Functions of CD44. *Immunology,* 93, 139-148.

Bornkamm, G.W., Delius, H., Zimber, U., Hudewentz, J., and Epstein, M.A., (1980). Comparison of Epstein-Barr virus strains of different origin by analysis of the viral DNAs. *J Virol.* 35, 603-618.

Bornkamm, G.W., Polack, A. and Eick, D., (1988). c-myc deregulation by chromosomal translocation in Burkitt's Lymphoma. In: Klein, G., ed., *Cellular Oncogene ActivationI,* New York M. Dekker, pp. 223-273.

Bourguignon LY, Lokeshwar VB, Chen X, Kerrick WG (1993). Hyaluronic acid induced lymphocyte signal transduction and HA receptor (GP85/CD44)-cytoskeleton interaction. *J Immunol.* 151, 6634-44

Brattsand, G., Cantrell, D.A., Ward, S., Ivars, F., and Gullberg, M., (1990). *J. Immunol.* 40, 3651-3658.

Brielmeier, M., Mautner,J., Laux,G. and Hammerschmidt, W., 1996). The latent membrane protein 2 gene of Epstein-Barr virus is important for efficient B cell immortalization. *J. Gen. Virol.,* 77, 2807-2818.

Brooks, L., Yao, Q.Y., Rickinson, A.B., and Young, L.S. (1992). Epstein-Barr virus latent gene transcription in nasopharyngeal carcinoma cells: co-expression of EBNA1, LMP1, and LMP2 transcripts. *J Virol.* 66, 2689-2697.

Brooks, L., Niedobitek, G., Agathanggelou, A. and Farrell, P.J., (1995). The expression of variant CD44 in nasopharyngeal carcinoma is unrelated to expression of LMP-1. *Am. J. Pathol.,* 146, 1102-1112.

Brousset, P., Schlaifer, D., Meggetto, F., Bachmann, E., Rothenberger, S., Pris, J., Delsol, G & Knecht, H (1994) Persistence of the same iral strain in early and late relapses of Epstein-Barr virus-associated Hodgkin's disease. *Blood,* 84, 2447-2451

Brown, N.A., Liu, C., Garcia, C.R., Wang, Y-F., Griffith, A., Sparkes, R.S. and Calame, K.L., (1986). Clonal origins of lymphoproliferative disease induced by Epstein-Barr virus. *J Virol.* 58, 975-978.

Burkhardt, A.L., Bolen, J.B., Kieff, E. and Longnecker, R., (1992). An Epstein-Barr virus transformation-associated membrane protein interacts with *src* family tyrosine kinases. *J. Virol.,* 66, 5161-5167.

Burkitt, D.P., (1958). A sarcoma involving the jaws in African children. *Brt. J. Surg.,* 46, 218-223.

Burkitt, D.P., (1962a). Determining the climatic limitations of a childrens cancer common in Africa. *Brt Med J.* 2, 1019-1023.

Burkitt, D.P. (1962b). A children's cancer dependent on climatic factors. *Nature,* 194, 232-234.

Burkitt, D.P. (1967). Burkitt's lymphoma outside the known endemic areas of Africa and New Guinea. *Int. J. Cancer,* 2, 562-565.

Burkitt, D.P. (1969). Etiology of Burkitt's lymphoma - An alternative hypothesis to a vectored virus. *J. Natl Cancer Inst.,* 42, 19-28.

Burkitt, D.P. (1970a). General features and facial tumours. In: Burkitt, D.P. & Wright, D.H., eds, *Burkitt's Lymphoma,* Edinburgh, E. & S. Livingstone, 186-197.

Burkitt, D.P. (1970b). Geographical distribution. In: Burkitt, D.P. & Wright, D.H., eds, *Burkitt's Lymphoma,* Edinburgh, E. & S. Livingstone, 186-197.

Calender A., Billaud M., Aubry J., Banchereau J., Vuillaume M. and Lenoir G. (1987). Epstein-Barr virus induces expression of B cell activation markers on in vitro infection of Epstein-Barr virus negative B lymphoma cells. *Proc Natl Acad Sci USA* 84, 8060-8064.

Calender A., Cordier M.m Billaud M., and Lenoir G.M. (1990). Modulation of cellular gene expression in B lymphoma cells following in vitro infection by Epstein-Barr virus(Epstein-Barr virus). *Int J Caner* 46, 658-663.

Camp, R.L., Scheylius, A., Johansson, C., and Pure E. (1993). CD44 is necessary for optimal contact alergic reponces but is not required for normal leucocyte extravasation. *J. Exp. Med* 178, 497.

Campbell S, Swann HR, Aplin JD, Seif MW, Kimber SJ, Elstein M. (1995). CD44 is expressed throughout pre-implantation human embryo development. *Hum Reprod* 10, 425-30

Carel, J.C., Myones, B.L., Frazier, B. and Holers, V.M. (1990). Structural requirements for C3d,g/Epstein-Barr virus receptor (CR2/CD21) ligand binding, in-ternalization, and viral infection. *J. Biol. Chem.,* 265, 12293-12299.

Carter, W.G. and Wayner, E.A. (1988). Characterization of class III colagen receptors, a phosphorylated transmembrane glycoprotein expressed in nucleated human cells. *J. Biol. Chem.* 263, 4193-4201.

Chang, G.T.G., Bloke, L.J., Steenbeek, M., Veldscholte, J., van Weerden, W.M., van Steenbrugge, G.J. and Brinkmann, A.O. (1997). Differentially expressed genes in

androgen dependent and -independent prostate carcinomas. *Cancer Res.* 57, 4075-4081.

Chao, T.-Y., Chow, K.-C., Chang, J-W., Wang, C.C., Tsao, T.-Y., Harn, H.-J & Chi, K.-H. (1996) Expression of Epstein-Barr vius-encoded RNAs as a marker formetastatic undifferentiated nasopharyngeal carcinoma. *Cancer* 78, 24-29

Chee, M., Yang, R., Hubbell, E., Berno, A., Huang, X.C., Stern, D., Winkler, J., Lockhart, D.J., Morris, M.S. and Fodor, S.P. (1996). Accessing genetic information with high-density DNA arrays. *Science* 274, 610-614.

Chen, J.J.W. and Peck, K. (1996). Non-radioactive differential display method to directly visualise and amplify differential bands on nylon membrane. *Nucleic Acids Res.* 24, 793-794.

Cho, M.S., Bornkamm, G.W. and zur Hausen, H. (1984). Structure of defective DNA molecules in Epstein-Barr virus preparations from P3HR-1 cells. *J. Virol.,* 51, 199-207.

Chong, J.M. Fukayama, M., Hayashi, Y., Takizawa, T., Koike, M., Konishi, M., Kikuchi-yanoshita, R., and Mikaki, M. (1994). Microsatellite instability in the progression of gastric carcinoma. *Cancer Res.* 54, 4595-4597.

Chong, J., Fukayama, M., Hayashi, Y., Funata, N., Takizawa, T., Koike, M., Muraoka, M., Kikuchi-Yanoshita, R., Miyaki, M. and Mizuno, S. (1997). Expression of CD44 variants in gastric carcinoma with or without Epstein-Barr virus. *Int. J. Cancer,* 74, 450-454.

Cobbold, S., Hale, G. and Waldmann, H., (1987). Non-linear LFA-1 family and leucocyte common antigens: new and previously defined clusters. *In Leucocyte Typing II: White Cell Differentiaon Antigens* (ed. McMicheal, A.J.) pp. 788-803. Oxford University Press, Oxford.

Cochet, C, Martel-Renoir, D., Grunewald, V., Bosq, J., Cochet, G., Schwaab, G., Bernaudin, J-F. and Joab, I. (1993). Expression of Epstein-Barr virus immediate early gene, BZLF1, in nasopharyngeal carcinoma tumor cells. *Virology* 197, 358-365.

Cohen, J.H.M., Revillard, J.P., Magaud, J.P., Lenoir, G., Vuillaume, M., Manel, A.M., Vincent, C. and Bryon, P.A. (1987). B cell maturation stages of Burkitt's lymphoma cell lines according to Epstein-Barr virus status and type of chromosome translocation. *J. Natl. Cancer. Inst.,* 78, 235-242.

Combaret, V., Lasset, C., Farppaz, D., Eouvier, R., Thiesse, P., Rebillard, A.C. (1995). Evaluationof CD44 prognostic value in neruroblastoma: comparisom with other prognostic factors. *Eur. J. Cancer* 31a, 545-549.

Cooper D.L., Dougherty G.J. (1995). To metastasize or not? Selection of CD44 splice sites. *Nat Med.* 7, 635-7.

Cordier, M., Calender, A., Billaud, M., Zimber, U., Rousselet, G., Pavlish, O., Banchereau, J., Tursz, T., Bornkamm, G. and Lenoir, M. (1990). Stable transfection of Epstein-Barr virus (EBV) nuclear antigen 2 in lymphoma cells containing the EBV P3HR1 genome induces expression of B-cell activation molecules CD21 and CD23. *J. Virol.* 64, 1002-1013.

Correa, P. and O'Conor, G.T. (1971) Epidemiologic patterns of Hodgkin's disease. *Int. J. Cancer,* 8, 192-201

Croce, C.M., Shander, M., Martinis, J., Cicurel, L., D'Ancona, G.G., Dolby, T.W. & Koprowski, H. (1979) Chromosomal location of the genes for human immunoglobulin heavy chains. *Proc. Natl Acad. Sci. USA,* 76, 3416-3419

Culty, M., Miyake, K., Kincade, P.W., Silorski, E., Butcher, E.C. and Underhill C. (1990). The hyaluronate receptor is a member of the CD44 (H-CAM) family of cell surface glycoproteins. *J. Cell Biol.* 111, 2765.

Dalchau, R., Kirkley, J., Fabre, J.W. (1980). Monoclonal antibody to a human leucocyte-specific membrane glycoprotein probably homologous to the leucocytre-common (l-C) antigen of the rat . *Eur J, Immunol* 10, 737-744.

Dalla-Favara R., Martinetti S., Gallo R., Erikson J. and Croce C. (1983). Translocation and rearrangements of the c-myc oncogene locus in human undifferentiated B Cell lymphomas. *Science* 219, 963-967.

Dales S. and Chardonet Y. (1973). Early events in the interaction of adenoviruses with Hela cells. IV. Association with microtubules and the nuclear pore complex during vectorial movement of the inoculum. *Virology* 56, 465-483.

Dambaugh, T., Raab-Traub, N., Heller, M., Beisel,C., Hummel, M., Cheung,A., Fennewaid, S., King, W., and Kieff, E. (1980). Variations among isolates of Epstein-Barr virus. *Ann. N.Y. Acad. Sci.* 354, 309-325.

Dambaugh, T., Hennessy, K., Chamnaukit, L., and Kieff, E. (1984). U2 region of Epstein-Barr virus DNA may encode Epstein-Barr nuclear antigen 2. *Proc Natl. Acad. Sci. U.S.A.* 81, 7632-7636.

Dambaugh, T., Wang, F., Hennessy, K., Woodland, E., Rickinson, A.B. and Kieff, E. (1986). Expression of the Epstein-Barr virus nuclear protein 2 in rodent cells. *J. Virol.* 59, 453-462.

Dammrich J., Vollmers H.P., Heider K.H., Muller-Hermelink HK. (1995). Importance of different CD44v6 expression in human gastric intestinaland diffuse type cancers for metastatic lymphogenic spreading. *J Mol Med* 73, 395-401

D'Amore, F., Johansen, P., Houmand, A., Weisenburger, D.D. and Mortensen, L.S. (1992) Epstein-Barr virus genome in non-Hodgkin's lymphomas occuring in immunocompetent patients: Highest prevalence in nonlymphoblastic T-cell lymphoma and correlation with a poor prognosis. *Blood*, 87, 1045-1055

Datson, N.A., van der Perk-de Jong, J., van der Berg, M.P., de Kloet, Verugdenhil, E. (1999). MicroSAGE: a modified proceedure for serial analysis of gene expression in limited amounts of tissue. *Nuc. Acids Res.* 27, 1300-1307.

Deacon E.M., Pallesen G., Niedobitek G., Crocker J., Brooks L., Rickinson A.B. and Young L.S. (1993). Epstein-Barr virus and Hodgkin's disease: Transcriptional analysis of virus latency in the malignant cells. *J. Exp. Med.* 177, 339-349.

Delecluse, J-H., Raphael, M., Magaud, JP., Felman, P., Alsamad, IA., Bornkamm, GW. and Lenoir, GM. (1993). Variable morphology of human immunodeficiency virus-associated lymphomas with c-myc rearrangements. The french study group of pathology for human-immunodeficiency virus-associated tumours. I. *Blood* 82: 552-563.

Delecluse, H-J., Rouault, J.-P., Ffrench, M., Dureau, G., Magaud, J.P. & Berger, F. (1995b) Post-transplant lymphoproliferative disorders with genetic abnormalites commonly found in malignant tumours. *Br. J. Haematol.*, 89, 90-97

Delsol, G., Brousset, P., Chittal, S. and Rigal-Huguet, F. (1992). Correlation of the expression of Epstein-Barr virus latent membrane protein and in situ hybridisation with biotinylated BamH1-W probes in Hodgkin's disease. *Am J Patol* 140: 247-252.

Devergne, O., Hatzivassiliou, E., Izumi, K.M., Kaye, K.M., Kleijnen, M.F. Kieff,E. and Mosialos, G. (1996). Association of TRAF1, TRAF2, and TRAF3 with an Epstein-Barr virus LMP1 domain important for B-lymphocyte transformation: role in NF-κB activation. *Mol. Cell. Biol.*, 16, 7089-7108.

Devergne, O., McFarland, E.C., Mosialos, G., Izumi, K.M., Ware, C.F. and Kieff, E. (1998). Role of the TRAF binding site and NF-κB activation in Epstein-Barr virus latent membrane protein 1-induced cell gene expression. *J. Virol.* 72, 7900-7908.

Dougherty, G.J., Lansdorp, E.M., Kooper, D.L., and Humperies R.K. (1991). Molecular cloning oc CD44 R1 and CD44 R2, two novl isoforms of the human CD44 lymphocyte "homing" receptor expressed by hematopoietic cells. *J. Exp. Med.* 174, 1.

Epstein, M.A., Achong, B.G., and Barr, Y.M. (1964). Virus particles in cultured lymphoblasts from Burkitt's lymphoma. *Lancet* 1, 702-703.

Fåhraeus, R., Fu, HL., Ernberg, I., Finke, J., Rowe, M., Klein, G., Falk, K., Nilsson, E., Yadav, M., Busson, P., Tursz, T., and Kallin, B. (1988). Expression of Epstein-Barr virus-encoded proteins in nasopharyngeal carcinoma. *Int J Cancer* 42:329-338.

Fåhraeus, R., Jansson, A., Sjoblom, A., Nilsson, T., Klein, G. and Rymo, L. (1993). Cell phenotype-dependent control of Epstein-Barr virus latent membrane protein 1 gene regulatory sequences. *Virology,* 195, 71-80.

Falk, K., Ernberg, I., Sakthivel, R., Davis, J., Christensson, B., Luka, J., Okano, M., Grierson, H.L., Klein, G. and Purtilo, D.T. (1990). Expression of Epstein-Barr virus encoded proteins and B-cell markers n fatal infectious mononucleosis. *Int. J. Cancer,* 46, 976-984.

Farrell, P.J., Bankier, A., Seguin, C., Deininger, P. and Barrell B.G. (1983). Latent and lytic cycle promoters of the Epstein-Barr virus. *EMBO J.* 2, 1331-1338.

Farrell, P.J., Rowe, D,T,, Rooney C.M. and Kouzarides, T. (1989). Epstein-Barr virus BZLF1 transactivator specifically bins to a consensus AP-1 site and is related to c-fos. *EMBO J* 8, 127-133.

Farrell, P.J. Epstein-Barr virus. In :O'Briens SJ ed. *Genetic maps.* New York; Cold Spring Harbor Press; 1992:120-133.

Farrell, P.J. (1995). Epstein-Barr virus immortalizing genes. *Trends in Microbiol.,* 3, 105-109.

Fennewald, S., van Santen, V. and Kieff, E. (1984). The nucleotide sequence of a messenger RNA transcribed in latent growth transforming virus infection indicates that it may encode a membrane protein. *J. Virol.,* 51, 411-419.

Fichter, M., Hinrichs, R., Eissner, G., Scheffer, B., Classen, S. and Ueffing, M. (1997). Expression of CD44 isoforms in neuroblastoma cells is regulated by PI 3-kinase and protein kinase C. *Oncogene,* 14, 2817-2824.

Floettmann, J.E., Ward, K., Rickinson, A.B. and Rowe, M. (1996). Cytostatic effects of EBV LMP-1 analysed using tetracycline regulated expression in B cell lines. *Virology,* 223, 29-40.

Floettmann, J.E. and Rowe, M. (1997). Epstein-Barr virus latent membrane protein-1 (LMP1) C-terminus activation region 2 (CTAR2) maps to the far C-terminus and requires oligomerisation for NF-kappaB activation. *Oncogene.* 15, 1851-8

Fortini, M.E. and Artavanis-Tsakonas, S. (1994). The suppressor of hairless protein particapates in Notch receptor signalling. *Cell,* 79, 273-282.

Fox S.B., Gatter K.C., Jackson D.G., Screaton G.R., Bell M.V., Bell J.I., Harris A.L., Simmons D., Fawcett, J. (1993). CD44 and cancer screening. *Lancet*, 342, 548-9

Frappier, L. and O'Donnell, M. (1991). Overproduction, purification and characterisation of EBNA1, the origin binding protein of Epstein-Barr virus. *J Biol Chem.* 266, 7819-7826.

Frappier, L. and O'Donnell, M. (1992). EBNA1 distorts *oriP*, the Epstein-Barr virus latent replication origin. *J Virol.* 66, 1786-1790.

Fraser, J.R.E., Kimpton, W.G., Laurent, T.C., Cahill, R.P. and Vakasis, N. (1988). Uptake and degredation of hyuronate in lymphatic tissues. *Biochem. J.* 256, 153-158.

Fraser, J.R.E., Kimpton, W.G., Laurent, T.C., (1989). Turnover and metabolisim of hyuronan. In *The Biology of Hyaluronan CIBA Fdn Symp.* 143. (ed. Laurent T.C.). pp41-59. Wiley, Chichester.

Friedrichs K, Franke F, Lisboa BW, Kugler G, Gille I, Terpe HJ, Holzel F, Maass H, Gunthert U . (1995). CD44 isoforms correlate with cellular differentiation but not with prognosis in human breast cancer. *Cancer Res.* 55, 5424-33.

Fries, K.L., Miller, W.E. and Raab-Traub, N. (1996). Epstein-Barr virus latent membrane protein 1 blocks p53-mediated apoptosis through the induction of the A20 gene. *J. Virol.*, 70, 8653-8659.

Fuckayma, M., Hayashi, Y., Iwasaki, Y., Chong, JM., Ooba, T., Takisawa, T., Koike, M., Misutani, S., Miyaky, M. and Harai, K. (1994). Epstein-Barr virus-associated gastric carcinoma and Epstein-Barr virus in infection of the stomach. *Lab. Invest.* 71, 73-81.

Gahn, T.A. and Sugden, B. (1995). An EBNA-1-dependent enhancer acts from a distance of 10 kilobase pairs to increase expression of the Epstein-Barr virus LMP gene. *J Virol.*, 69, 2633-2636

Gaidano, G. & Dalla-Favera, R. (1995) Molecular pathogenesis of AIDS-related lymphomas. *Adv. Cancer Res.*, 67, 113-153

Galibert, L., Durand, I., Banchereau, J. and Rousset, F. (1994). CD40-activated surface IgD-positive lymphocytes constitute the long term IL-4-dependent proliferating B cell pool. *J. Immunol.*, 152, 22-29.

Gallagher T. (1992). The protein and proteoglycan guise of Hermes/CD44. *Glycobiology*. U2,U 93-97.

Gerber p., Nkrumah F.K., Pritchett R. and Kieff E. (1976). Comparative studies of Epstein-Barr virus strains from Ghana and the United States. *Int. J. Cancer*. 17, 71-81.

Geser, A., Lenoir, G., Anvret, M., Bornkamm, G.W., Klein, G., Williams, E.H., Wright, D.H., and de Thé, G. (1983). Epstein-Barr virus markers in a series of Burkitt's lymphoma from the West Nile District of Uganda. *Eur J Cancer Clin Oncol* 19, 1394-1404.

Ghosh, D. and Kieff, E. (1990). *cic*-Acting regulatory elements near the Epstein-Barr virus latent-infection membrane protein transcriptional start site. *J. Virol.,* 64, 1855-1858.

Ghosh, S. (1996). A novel ligation mediated-PCR based strategy for construction of subtraction libraries from limiting amounts of mRNA. *Nucleic Acids Res.* 24, 795-796.

Gilligan, K., Rajadurai, P., Resnick, L. and Raab-Traub, N., (1990a). Epstein-Barr virus small nuclear RNAs are not expressed in permissively infected cells in AIDS-associated leukoplakia. *Proc. natl Acad. Sci. USA,* 87, 8790-8794.

Gilman-Sachs, A., (1994). Flow Cytometry. *Analytical Chemistry,* 66, 700-707.

Gires, O., Zimber-Strobl,U., Gonella, R., Ueffing, M., Marshall, G., Zeilder, R., Pich, D. and Hammerschmidt, W. (1997). Latent membrane protein 1 of Epstein-Barr virus mimics a constitutively active receptor molecule. *EMBO. J.,* 16, 6131-6140.

Givehchian M, Woerner SM, Lacroix J, Zoller M, Drings P, Becker H, Kayser K, Ridder R, von Knebel Doeberitz M. (1996). Expression of CD44 splice variants in normal respiratory epithelium and bronchial carcinomas: no evidence for altered CD44 splicing in metastasis. *Oncogene* 12, 1137-44

Glickman, J.N., Howe, J.G., and Steitz, J.A. (1988). Structural analysis of EBER1 and EBER2 ribonucleoprotein articles present in Epstein-Barr virus-infected cells. *J Virol.* 62, 902-911.

Goldstein, L.S., Zhou, D.F.H., Picker, L.J., Minty, C.N., Bargatze, R.F., Ding, J.F. and Butcher, E.C. (1989). A human lymphocyte homing receptor, the Hermes antigen, is related to cartilage proteoglycan core and link prroteins. *Cell,* 56, 1063.

Goodison, S, Yoshida, K., Sugino, T., Woodman, A., Gorham, H., Bolodeoku, J., Kaufmann, M. and Tarin, D. (1997). Rapid analysis of distinctive CD44 RNA splicing preferences that characterize colonic tumours. *Cancer Research,* 57, 3140-3144.

Gossen, M. and Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci U S A.* 89,5547-51.

Graf, D., Fisher, A.G. and Merkenschlager, M. (1997). Rational promer design greatly improves differential display-PCR (DD-PCR). *Nucleic Acids Res.* 25, 2239-2240.

Green, S.J., Tarone, G. and Underhill C.B. (1988). Aggregation of macrophages and fibroblasts is inhibited by a monoclonal antibody to the HA receptor. *Exp. Cell Res.* 178, 224-232.

Greenfield, B., Wang, W., Marquardt, H., Piepkorn, M., Wolff, E.A., Aruffo, A. and Bennett, K.L. (1999). Characterization of the heparan sulfate and chondroitin sulfate assembly sites in CD44. *J. Bioll Chem,* 274, 2511-2517.

Greenspan, J.S., Greenspan, D., Lennette, E.T., Abrams, D,I., Conant, M.A., Petersen, V. and Freese, U.K. (1985). Replication of Epstein-Barr virus within the epithehial cells of oral 'hairy' leukoplakia, an AIDS-associated lesion. *New Engl. J. Med.,* 313, 1564-1571.

Gregory, C.D., Murray, R. J., Edwards, C.F. and Rickinson, A.B. (1988). Downregulation of cell adhesion molecules LFA-3 and ICAM-1 in Epstein-Barr virus-positive Burkitt's lymphoma underlies tumour cell escape from virus-specific T cell surveillance. *J. Exp. Med.,* 167, 1811-1824.

Gregory, C.D., Rowe, M., and Rickinson, A.B. (1990). Different Epstein-Barr virus-B cell interactions in phenotypically distinct clones of a burkitt's lymphoma cell line. *J. Gen. Virol.* 71, 1481-1495.

Grossman, S.R., Johannsen, E., Tong, X., Yalamnchili, R., and Kieff, E. (1994). The Epstein-Barr virus nuclear antigen 2 transactivator is directed to response elements by the Jκ recombination signal binding protein. *Proc Natl. Acad. Sci. U.S.A.* 91, 7568-7572.

Gunthert, U., Hofmann, M., Rudy, W., Reber, S., Zoller, M., Haubmann, I., Matzku, S., Wenzel, A., Ponta H. and Herrlich, P. (1991). A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. *Cell,* 65, 13-24.

Gunthert, U., Stauder, R., Mayer, B., Terpe, H.J., Finke, L., Friedrichs, K. (1995). Are CD44 variant isoforms involved in human tumour progression? *Cancer Surv.* 24, 19-42

Gutierrez, M.I., Bhatia, K., Barriga, F., Diez, B., Muriel, F.S., de Andreas, M.L., Epelman, S., Risueno, C & Magrath, I.T. (1992) Molecular epidemiology of of Burkitt's lymphoma from South America: Differences in Breakpoint location and Epstein-Barr virus association from tumors in other world regions. *Blood,* 79, 3261-3266

Haag, E. and Ranman, V. (1994). Effects of primer choice and source of *Taq* DNA polemerase on banding patterns of differential display RT-PCR. *Biotechniques* 17, 226-228.

Haddow, A.J. (1963). An improved map for the study of Burkitt's lymphoma syndrome in Africa. *E. Afr. med. J.,* 40, 429-432.

Hadman, M., Adam, B.L., Wright, G.L. and Bos, T. (1995). Modifications to the differential display technique reduce background and increase sensitivity. *Anal. Biochem.* 226, 383-386.

Hakvoort, B.M., Leegwater, A.C.J., Michiels, F.A.M., Chamuleau, R.A.F.M. and Lamers, W.H. (1993). Identification of enriched sequences from a cDNA subtraction-hybridization procedure. *Nucleic Acids Res.* 22, 878-879.

Hamada, J., Sawamura, Y. and Van Meir, E.G. (1998). CD44 Expression and growth factors. *Frontiers in Biosci.*

Hammarskjold, M.-L. and Simurda, M.C. (1992). Epstein-Barr virus latent membrane protein transactivates the human immunodeficiency virus type 1 long terminal repeat through induction of NF-κ B activity. *J. Virol.,* 66, 6496-6501.

Hammerschmidt, W. and Sugden, B. (1988). Identification and characterization of *oriLyt*, a lytic origin of DNA replication of Epstein-Barr virus. *Cell.,* 55, 427-433.

Hammerschmidt, W. and Sugden, B. (1989). Genetic analysis of immortalizing functions of Epstein-Barr virus in human B lymphocytes. *Nature* 340, 393-397.

Hardingham, T.E., Fosang, A.J. (1992) Proteoglycans: many forms and many functions. *FASEB J* 6, 861-70

Harn HJ, Isola N, Cooper DL. (1991). The multispecific cell adhesion molecule CD44 is represented in reticulocyte cDNA. *Biochem Biophys Res Commun.* 178, 1127-34

Harn H.J., Ho L.I., Chang J.Y., Wu C.W., Jiang S.Y., Lee H.S., Lee W.H. (1995) Differential expression of the human metastasis adhesion molecule CD44V in normal and carcinomatous stomach mucosa of Chinese subjects. *Cancer*, 75, 1065-71

Harris, A., Young, B.D., and Griffin, B.E. (1985). Random association of Epstein-Barr virus with host cell metaphase chromosomes in Burkitt's lymphoma-derived cell lines. *J Virol.* 56, 328-332.

Harris, NL., Jaffe, ES., Stein, H., Banks, PM., Chan, JKC., Cleary, ML., Delsol, G., De Wolf-Peeters, C., Falini, B., Gatter, KC., Grogan, TM., Isaacson, PG., Knowles, DM., Mason, DY., Muller-Hermelink, H-K., Pileri, SA., Piris, MA., Ralfkiaer, E. and Warnke, RA. (1994). A revised European-American classification of lymphoid neoplasms: A proposal from the International Lymphoma Study Group. *Blood* 84: 1361-1392.

He, Q., Lesley, J., Hyman, R., Ishihara, K., Kincade, P.W. (1992). Molecular isoforms of murine CD44 and evidence that the membrane proximal domain is not critical for hyaluronate recognition. *J Cell Biol.* 119, 1711-9.

Heider, K.H., Dammrich, J., Skroch-Angel, P., Muller-Hermelink, H.K., Vollmers, H.P., Herrlich, P., Ponta, H. (1993) Differential expression of CD44 splice variants in intestinal- and diffuse-type human gastric carcinomas and normal gastric mucosa. *Cancer Res* 53, 4197-203

Heldin, P., Asplund, T., Ytterberg, D., Thelin, S., Laurent, T.C. (1992) Characterization of the molecular mechanism involved in the activation of hyaluronan synthetase by platelet-derived growth factor in human mesothelial cells. *Biochem J*, 283, 165-70

Hedrick S.M., Cohen D.I., Nielsen E.A., Davis M.M. (1984). Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. *Nature* 308, 149-53.

Heller M., Dambaugh T., Kieff E. (1981). Epstein-Barr virus DNA:IX. Variation among viral DNAs from producer and nonproducer infected cells. *J Virol* 38, 632-648.

Henderson, S., Rowe, M., Gregory, C., Croom-Carter, D., Wang, F., Longnecker, R., Kieff, E., and Rickinson, A., (1991). Induction of *bcl*-2 expression by Epstein-Barr virus latent membrane protein 1 protects infected B cells from programmed cell death. *Cell* 65, 1107-1115.

Henk D., Baas PD, Bos JL., (1993). A PCR-based method for the analysis of human CD44 splice products. *PCR Methods Appl.* 3, 100-106.

Henkel, T., Ling, PD., Hayward, SD. and Peterson, MG. (1994). Mediation of Epstein-Barr virus EBNA2 tansactivation by recombination signal-binding protein Jκ. *Science* 265, 92-95.

Henle, G. and Henle, W. (1970). Observations on childhood infctions with the Epstein-Barr virus. *J. Infect. Dis.,* 121, 303-310.

Henle, G., Henle, W. and Klein, G., (1971a). Demonstration of two distinct components in the early antigen complex of Epstein-Barr virus-infected cells. *Int. J. Cancer,* 8, 272-282.

Henle, G., Henle, W., Klein, G., Gunven, P., Clifford, P., Morrow, R.H. and Ziegler, J.L. (1971b). Antibodies to early Epsten-Barr virus-induced antigens in Burkitt's lymphoma. *J. Natl. Cancer Inst.,* 46, 861-871.

Henle, G. and Henle, W. (1976). Epstein-Barr virus-specific IgA serum antibodies as an outstanding feature of nasopharyngeal carcinoma. *Int J Cancer* 17: 1-7.

Henle, W. and Henle, G. (1979). Seroepidemiology of the virus. In: Epstein, M.A. and Achong, B.G., eds, *The Epstein-Barr Virus,* Berlin, Springer-Verlag, 61-78.

Hennessy, K. and Kieff, E. (1983). One of two Epstein-Barr virus nuclear antigens contains a glycine-alanine copolymer domain. *Proc Natl. Acad. Sci. U.S.A.* 80, 5665-5669.

Hennessy, K. and Kieff, E. (1985). A second nuclear protein is encoded by Epstein-Barr virus in latent infection. *Science* 227, 1238-1240.

Hennessy, K., Wang, F., Bushman, E.W. and Kieff, E. (1986). Definitive identification of a member of the Epstein-Barr Virus nuclear protein 3 family. *Proc. Natl. Acad. Sci. USA.,* 83, 5693-5697.

Herbst, H., Dallenbach, F., Hummel, M., Niedobitek, G., Pileri, S., Muller-Lantzsch, N. & Stein, H. (1991a) Epstein-Barr virus latent protein expression in Hodgkin and Reed Sternberg cells. *Proc. Natl Acad. Sci. USA,* 88, 4766-4770

Hitt, M.M., Allday, M., Hara, T., Karran, L., Jones, M.D., Busson, P., Tursz, T., Ernberg, I., and Griffin, B. (1989). Epstein-Barr virus gene expression in an NPC-related tumour. *EMBO J.* 8, 2639-2651.

Ho, J.H.C. (1971) Genetic and environmental factors in nasopharyngeal carinoma. In: Nakahara, W., Nishioka, K., Hirayama, T. & Ito, Y., eds, *Recent Advances in Human Tumor Virology and Immunology,* Tokyo, University of Tokyo Press, pp. 275-295

Ho, J.H.C., Ng, M.H., Kwan, H.C., and Chau,J.C.W. (1976). Epstein-Barr virus specific IgA and IgG serum antibodies in nasopharyngeal carcinoma. *Br J Cancer 34*: 655-659.

Ho, J.H.C. (1978). An epidemiologic and clinical study of nasopharyngeal carcinoma. *Int J Radiat Oncol Biol Phys* 4: 183-197.

Hofman, M., Ruddy, W., Zoller M., Togl, C., Ponta, H., Herrlich, P. and Gunthert, U. (1991). CD44 splice variants confer metastatic behavior :homologus sequences are expressed in human tumour cell lines. *Cancer Res,* 51, 5292.

Honda, A., Noguchi, N., Takehara H., Ohashi, Y., Asuwa N. and Mori Y. (1993) Cooperative enhancement of hyaluronic acid synthesis by combined use of IGF-I and EGF, and inhibition by tyrosine kinase inhibitor genistein, in cultured mesothelial cells from rabbit pericardial cavity. *FASEB J* 7, 1233-41

Horwitz, C.A., Henle, W., Henle, G., Rudnick, H. and Latts, E. (1985). Long-term serological follow-up of patients for Epstein-Barr virus after recovery from infectious mononucleosis. *J. infect. Dis.,* 151, 1150-1153.

Howe, J.G., and Steitz, J.A. (1986). Localisation of Epstein-Barr virus-encoded small RNAs by in situ hybridisation. *Proc Natl. Acad. Sci. U.S.A.* 83, 9006-9010.

Howe, J.G. and Shu, M.D. (1989). Epstein-Barr virus small RNA (EBER) genes: unique transcription units that combine RNA polymerase II and III promoter elements. *Cell* 57, 825-834.

Hsieh, J.J.-D. and Hayward, S.D. (1995). Masking of the CBF1/RBPJκ transcriptional repression domain by Epstein-Barr virus EBNA2. *Science,* 268, 560-563.

Hsieh, J.J.-D., Henkel, T., Salmon, P., Robey, E., Peterson, M.G. and Hayward, S.D. (1996). Truncated mammalian Notch1 activates CBF1/RBPJk-repressed genes by a mechanism resembling that of Epstein-Barr virus EBNA2. *Mol. Cell. Biol.,* 16, 952-959.

Huang, D.P, Ho, J.H.C. and Webb, KS. (1981). Volatile nitosamines in salt-preserved fish before and after cooking. *Food Cosmet Toxicol* 19: 167-171.

Hubank, M. and Schatz, D.G. (1994). Identifying differences in mRNA expression by representational difference analysis of cDNA). *Nucleic Acids Res.,* 22, 5640-5648.

Hudson D.L., Speight P.M., Watt F.M. (1996). Altered expression of CD44 isoforms in squamous-cell carcinomas and cell lines derived from them. *Int J Cancer.* 66, 457-63.

Huen, D.S., Henderson, S.A., Croom-Carter, D. and Rowe, M. (1995). The Epstein-Barr virus latent membrane protein-1 (LMP1) mediates activation of NF-κB and cell surface phenotype via two effector regions in its carboxy-terminal cytoplasmic domain. *Oncogene,* 10, 549-560.

Huet, S., Groux, H., Caillou, B., Valentin, H. and Prieur, A.M. (1989). CD44 contributes to T cell activation. *J Immunol*, 143, 798-801

Hummel, M., and Kieff, E. (1982). Mapping of polypeptides encoded by the Epstein-Barr virus genome in productive infection. *Proc. Natl Acad. Sci. USA.* 79, 5698-5702.

IARC Monographs (1997). Epstein-Barr Virus and Kaposi's Sarcoma Herpesvirus/Human Herpesvirus 8. IARC Working group on the evaluation of carcinogenic risks to humans. Volume 70. Lyon, France. ISBN 92 832 1270 3

Idzerda RL, Carter WG, Nottenburg C, Wayner EA, Gallatin WM, St John T. (1989). Isolation and DNA sequence of a cDNA clone encoding a lymphocyte adhesion receptor for high endothelium. *Proc Natl Acad Sci U S A.* 86, 4659-63.

Imai S., Koizumi S., Sugiura M., Tokunaga M., Uemura Y., Yamamoto N., Tanaka S., Sato E. and Osato T. (1994). Gastric carcinoma: Monoclonal epithelial malignant cells expressing Epstein-Barr virus latent infection protein. *Proc. Natl Acad. Sci. USA* 91, 9131-9135.

Imoto, S., Kameya, T., Sato, Y., Sato, B., Inoue, K. and Oya, M. (1995). Localization of virus in liver biopsy specimens of acute EBV hepatitis using non-isotopic in situ hybridization procedure: Presence of EBV in hepatocytes and lymphocytes. *Int. Hepatol. Commun.*, 3, 53-56.

Ivanova, N.B. and Belyavsky, A.V. (1995). Identification of differentially expressed genes by restriction endonuclease-based gene expression fingerprinting. *Nucleic Acids Res.* 23, 2954-2958.

Izumi, K.M., Kaye, K.M. and Kieff, E. (1994). Epstein-Barr virus recombinant molecular genetics analysis of the LMP1 amino-terminal cytoplasmic domain reveals a probable structural role, with no component essential for primary B-lymphocyte growth transformation. *J. Virol.* ,68, 4369-4376.

Izumi, K.M., Kaye, K.M. and Kieff, E.D. (1997). The Epstein-Barr virus LMP1 amino acid sequence that engages tumor necrosis factor receptor associated factors is critical for primary B lymphocyte growth transformation. *Proc. Natl Acad. Sci. USA.*, 94,1447-1452.

Izumi, K.M. and Kieff, E.D. (1997). The Epstein-Barr virus oncogene product latent membrane protein 1 engages the tumor necrosis factor receptor-associated death domain protein to mediate B lymphocyte growth transformation and activate NF-κB. *Proc. Natl. Acad. Sci. USA.*, 94, 12592-12597.

Jackson, D.G., Buckley, J., and Bell, J.I. (1992). Multiple variants of the human lymphocyte homing receptor CD44 generated by insertions at a single site in the extra cellular domain. *J. Biochem.* 267, 4732.

Jalkanen, S. and Jalkanen, M., Bargatze, R., Tammi, M. and Butcher, E.C. (1988). Biochemical properties of proteins involved in lymphocyte recognition of High endothelial venules in man. *J. Immuno.* 141, 1615-1623.

Jalkanen, S. and Jalkanen, M. (1992). Lymphocyte CD44 binds the COOH-terminal heparin-binding domain of fibronectin. *J. Cell. Biol.* 116, 817-825.

Jarrialt, S., Brou, C., Logeat, F., Schroeter, E.H., Kopan, R. and Isreal, A. (1995). Signalling downstream of activated mammalian Notch. *Nature*, 377, 355-358.

Jiang W.Q., Szekely L., Wendel-Hansen V., Ringertz N., Klein G. and Rosen A. (1991). Co-localization of the retinoblastoma protein and the Epstein-Barr virus-encoded nuclear antigen EBNA-5. *Exp. Cell Res.* 197, 314-318.

Jiang WG. (1994). Membrane ruffling of cancer cells: a parameter of tumour cell motility and invasion. *Eur J Surg Oncol.*, 21, 307-9

Jochner, N., Eick, D., Zimber-Strobl, U., Pawlita, M., Bornkamm, G.W. and Kempkes, B. (1996). Epstein-Barr virus nuclear antigen 2 is a tanscriptional suppressor of the immunoglobulin μ gene: Implications for the expressio of the translocated c-myc gene in Burkitt's lymphoma cells. *EMBO J.* 15, 375-382.

Johannsen, E., Koh, E., Mosialos, G., Tong, X., Kieff, E. and Grossman, S.R. (1995). Epstein-Barr virus nuclear protein 2 transactivation of the latent membrane protein 1 promoter is mediated by Jκ and PU.1. *J Virol.* 69, 253-262.

Johannsen, E., Miller, C.L., Grossman, S.R. and Kieff, E. (1996). EBNA-2 and EBNA-3C extensively and mutually exclusively associate with RBP-Jκ in Epstein-Barr virus transformed B lymphocytes. *J. Virol.* 70, 4179-4183.

Joske, D. and Knecht, H. (1993). Epstein-Barr virus in lymphomas: a review. *Blood Reviews* 7, 215-222.

Kaiser, C., Laux, G., Eick, D., Jochner, N., Bornkamm,G.W. and Kempkes, B. (1999). The proto-oncogene c-*myc* is a direct target gene of Epstein-Barr virus nuclear antigen 2. *J. Virol.,* 73, 4481-4484.

Kallin, B., Dillner, J., Ernberg, I., Ehlin-Henriksson, B., Rosen, A., Henle, W., Henle, G. and Klein, G. (1986). Four virally determined nuclear antigens are expressed in Epstein-Barr virus-transformed cells. *Proc. Natl Acad. USA,* 83, 1499-1503.

Kang, D., LaFrance, R., Su, Z. and Fisher, P.B. (1998). Reciprocal subtraction differential RNA display: An efficient and rapid procedure for isolating differentially expressed gene sequences. *Proc. Nat. Acad. Sci.* 95, 13788-13793.

Kaplan, HS. (1980). Hodgkin's disease: Unfolding concepts concerning it's nature, management and prognosis. *Cancer* 45, 2439-2474.

Karran, L., Gao, Y., Smith, PR., and Griffin, BE. (1992). Expression of a family of complementary strand transcripts in Epstein-Barr virus infected cells. *PNAS USA* 89: 8058-8062.

Kato, K. (1996). RNA fingerprinting by molecular indexing. *Nucleic Acids Res.* 24, 394-395.

Katoh S, Zheng Z, Oritani K, Shimozato T, Kincade P.W. (1995). Glycosylation of CD44 negatively regulates its recognition of hyaluronan. *J Exp Med* 182, 419-29

Kaufmann M, Heider KH, Sinn HP, von Minckwitz G, Ponta H, Herrlich P. (1995). CD44 variant exon epitopes in primary breast cancer and length of survival. *Lancet* 345, 615-9.

Kaye, K.M., Izumi, K.M. and Kieff, E. (1993). Epstein-Barr virus latent membrane protein 1 is essential for B-lymphocyte growth transformation. *Proc. Natl Acad. Sci. USA.*, 90, 9150-9154.

Kelleher, D. and Long, A. (1992). Development and characterization of a protein kinase C beta-isozyme-deficient T-cell line. *FEBS Lett.* 301, 310-4.

Kenney, S., Kamine, J., Holley-Guthrie, E., Lin, J., Mar, E. and Pagano, J. (1989). The Epstein-Barr virus BZFL-1 immediate-early gene product differentially affects latent versus productive Epstein-Barr virus promoters. *J. Virol.* 63, 1729-1736.

Kersten, M.J., Van Gorp, J., Pals S.T., Boon, F. and Van Oers, M.H.J. (1998). Expression of Epstein-Barr virus latent genes and adhesion molecules in AIDS-related non-Hodgkin's lymphomas: Correlation with Histology and CD4-cell number. *Leukemia and Lymphoma,* 30, 515-524.

Kieff, E. (1996). Epstein-Barr virus and it's replication. In Fields, B.N., Knipe, D.M., Howley, P., Chanock, R.M., Melnick, J.L., Month, T.P., Roizman, B., and Straus, S.E., eds, *Fields Virology*, Philadelphia, Lippincott-Raven, 2343-2396.

Kimble, J., Henderson, S. and Crittenden, S. (1998). Notch/LIN-12 signaling: transduction by regulated protein splicing. *TIBS*, 23, 353-357.

Klein, G. (1979). Lymphoma development in mice and humans: diversity of initiation is followed by convergent cytogenetic evolution. *PNAS USA* 76, 2442-2446.

Klein, G. (1987). In defence of the "old" Burkitt lymphoma scenario. In: Klein, G., (ed.), *Advances in viral oncology* 7, 207-211. Raven press, New York.

Knowles, DM., Chamulak, GA., Subar, M., Burke, JS., Dugan, M., Wernz, J., Slywotzky, C., Pelicci, G., Dalla-Favera, R., and Raphael, B. (1988). Lymphoid neoplasia associated with the acquired immunodeficiency syndrome (AIDS). *Ann Int Med* 108, 744-753.

Knowles, DM., (1993). Biologic asects of AIDS-related non-Hodgkin's lymphoma. *Curr Opin Oncol* 5, 845-851.

Koopman, G., Heider, K.H., Horst, E., Adolf, G.R., Van den Berg, F., Ponta, H., Herrlich, P., Pals, S.T. (1993) Activated human lymphocytes and aggressive non-Hodgkin's lymphomas express a homologue of the rat metastasis-associated variant of CD44. *J Exp Med*, 177, 897-904

Kozian, D.H., and Kirschbaum, B.J., (1999). Comparative gene expression analysis. *TIBTECH* 17, 72-78.

Knudson C.B., Knudson W. (1990). Hyaluronan Binding proteins in development, tissue homeostasis, and disease. *Exp Cell Res* 187, 224-33

Knutson, J.C. (1990). The level of *c-fgr* RNA is increased by EBNA-2, an Epstein-Barr virus gene required for B-cell immortalization. *J. Virol.,* 64, 2530-2536.

Korabiowska, M., Brinck, U., Honig, J., Mora, O., Bartkowski, S. and Schauer, A. (1995). CD-44: a marker of metastases of head and neck melanomas. *In Vivo.* 9, 253-6

Kripalani-Joshi, S. & Law, H.Y. (1994) Identification of integrated Epstein-Barr virus in nasopharyngeal carcinoma using pulse field gel electrophoresis. *Int. J. Cancer,* 56, 187-192.

Krogh-Jensen, M., D'Amore, F., Jensen, M.K., Christensen, B.E., Thorling, K., Pedersen, M., Johansen, P., Boesen, A.M. and Andersen, E. (1995). Clinicopathological features, survival and prognostic factors of primary central nervous system lymphomas: trends in incidence of primary central nervous system lymphomas and primary malignant brain tumors in awell-defined geographical area. Population-based data from the DanishLymphoma Registry, LYFO, and the Danish Cancer Registry. *Leuk Lymphoma.* 19, 223-33.

Kryworuckho, M., Diaz-Mitoma, F. and Kumar, A. (1995). CD44 isoforms containing exons V6 and V7 are differentially expressed on mitogenically stimulated normal and Epstein-Barr virus-transformed human B cells. *Immunology,* 86, 41-48.

Kryworuckho, M., Gee, K., Diaz-Mitoma, F. and Kumar, A. (1999). Regulation of CD44-Hyaluronan inteactions in Burkitt's lymphoma and Epstein-Barr virus-transformed lymphoblastoid B cells by PMA and interleukin-4. *Cellular Immunology,* 194, 54-66.

Kurozumi, K., Nishida, T., Nakao, K., Nakahara, M. and Tsujimoto, M. (1998). Expression of CD44 variant 6 and lymphatic invasion: Importance to lymph node metastasis in gastric cancer. *World J. Surg.*, 22, 853-858.

Labarriere N, Piau JP, Otry C, Denis M, Lustenberger P, Meflah K, Le Pendu J. (1994). H blood group antigen carried by CD44V modulates tumorigenicity of rat colon carcinoma cells. *Cancer Res* 54, 6275-81.

Laherty, C.D., Hu, H.M., Opipari, A.W., Wang, F. and Dixit, V.M. (1992). The Epstein-Barr virus LMP1 gene product induces A20 zinc finger protein expression by activating nuclear factor-κB. *J. Biol. Chem.,* 267, 24157-24160.

Laux, G., Perricaudet, M. and Farrell, P.J. (1988a). A spliced Epstein-Barr virus Gene expressed in immortalized lymphocytes is created by circularization of the linear viral genome. *EMBO. J.,* 7, 769-774.

Laux, G., Dugrillon, F., Eckert, C., Adam, B., Zimber-Strobl, U., and Bornkamm, G.W. (1994). Identification and characterisation of an Epstein-Barr virus nuclear antigen 2-responsive *cis*-element in the bidirectional promoter region of latent membrane protein and terminal protein 2 genes. *J Virol.* 68, 6947-6958.

Lazaar, A.L., Pure, E. (1995). CD44: A model for regulated adhesion function. *The Immunologist,* 3, 19-25.

Lear, A.L., Rowe, M., Kurilla, M.G., Lee, S., Henderson, S., and Kieff, E. (1992). The Epstein-Barr virus (EBV) nuclear antigen 1 BamHI F promoter is activated on entry of EBV transformed B cells into the lytic cycle. *J Virol.* 66, 7461-7468.

Lee, S.W., Tomasetto, C. and Sager, R. (1991). Positive selection of candidate tumor suppresser genes by subtractive hybridization. *Proc. Natl. Acad. Sci. USA.* 88, 2825-2829.

Lee, M.A. and Yates, J.L. (1992). BHRF1 of Epstein-Barr virus, which is homologous to human proto-oncogene bcl-2, is not essential for transformation of B-cells or for virus replication in vitro. *J. Virol.* 66, 1899-1906.

Lee, S.P., Morgan, S., Skinner, J., Thomas, W.A., Jones, S.R., Sutton, J., Khanna, R., Whittle, H.C., and Rickinson, A.B. (1995). Epstein-Barr virus isolates with the major HLA B35.01-restricted cytotoxic T lymphocyte epitope are prevalent in a highly B35.01-positive African population. *Eur J Immunol* 25, 102-110.

Lenoir, G.M., Preud'homme, J.L., Bernheim, A & Berger, R. (1982) Correlation between immunoglobulin light chain expression and variant translocation in Burkitt's lymphoma. *Nature,* 298, 474-476

Leoncini, L., Vindigni, C., Megha, T., Funto, I., Pasenti, L., Musaro,M., Remieri, A., Seri, M., Anagnostopoulos, J. and Tosi, P. (1993). Epsetin-Barr vius ingastric canser: data and unanswered questions. *Int. J. Cancer.* 53, 898-901.

Le Roux, A., Kerdiles, B., Walls, D., Dedieu, J.F. and Perricaudet, M. (1994). The Epstein-Barr virus determined nuclear antigens EBNA-3A, 3B, 3C repress EBNA-2-mediated transactivation of the viral terminal protein 1 gene promoter. *Virology,* 205, 596-602.

Lesley, J., Schulte, R., Hyman, R. (1991) Binding of hyaluronic acid to lymphoid cell lines is inhibited by monoclonal antibodies against Pgp-1. *J Biol Chem*, 266, 17983-9

Lesley.J., He, Q., Miyake, K., Hyman, R. and Kincade, P.W. (1992). Requirments for hyuronic acid binding by CD44: A role for the cytoplasmic domain and activation by antibodies. *J. Exp. Med.*175, 257-266.

Lesley.J., Hyman, R. and Kincade, P.W. (1993). CD44 and its interaction with extracellular matrix. *Adv Immunol.* 54, 271.

Levine, P.H & Connelly, R.R. (1985). Epidemiology of nasopharyneal cancer. In: Wittes, R.E., ed., *Head* and Neck Cancer, New York, John Wiley & Sons, pp.13-34

Li, F., Barnathan, E.S. and Kariko, K. (1994). Rapid method for screening and cloning cDNAs generated in differential mRNA display: application of northern blot for affinity capturing of cDNAs. *Nucleic Acids Res.* 1764-1765.

Liang, P. and Pardee, A.B. (1992). Differential Display of eukaryotic messenger RNA by means of Polymerase Chain Reaction. *Science*, 257, 967-971.

Liang, P., Averboukh, L., Keyomarsi, K., Sager, R. and Pardee, A.B. (1992). Differential Display and cloning of messenger RNAs from human breast cancer *versus* mammary epithelial cells. *Cancer Res.*, 52, 6966-6968.

Liang, P. and Pardee, A.B. (1995). Responce: Alternatives to ^{35}S as a label for the differential display of eukaryotic messenger RNA. *Science* 267, 1186.

Liebowitz, D., Wang, D. and Kieff, E. (1986). Orientation and patching of the latent infection membrane protien encoded by Epstein-Barr virus. *J. Virol.*, 58, 233-237.

Liebowitz, D., Mannick, J., Takada, K. and Kieff, E. (1992). Phenotypes of Epstein-Barr virus LMP1 deletion mutants indicate transmembrane and amnio terminal cytoplasmic domains necessary for effect in B-lymphoma cells. *J. Virol,* 66, 4612-4616.

Linskens, M.H.K., Feng, J., Andrews W.H., Enlow, B.E., Saati, S.M., Tonkin, L.A., Funk,W.D. and Villeponteau, B. (1995). Cataloging altered gene expression in young and senescent cells using enhanced differential display. *Nucleic Acids Res.* 23, 3244-3251.

Lisitsyn, N., Lisitsyn, N., Wigler, M. (1993). Cloning the difference between two complex genomes. *Science* 259, 946-951.

Lockhart, M.S., Gravisaco, M.J., Mongini, C., Waldner, C., Alvarez, E. and Hajos, S.,E. (1999). Altenative exon-specific PCR method for the analysis of human CD44 isoform expression. *Oncology Reports,* 6, 219-224.

Lokeswar, V.B. and Bourguignon, L.Y.W. (1991). Post-traslational protein modifiation and expression of ankrin-binding sites in GP85 (pgp-1/CD44 and in biosynthetic precursors during T-lymphoma membrane biosynthesis. *J. Biol. Chem.* 266, 17983-17989.

Lohmann, J., Hanspeter, S. and Bosch, T.C.G. (1995). REN display, a rapid and efficient method for nonradioactive differential display and mRNA isolation. *BioTechniques* 18, 200-202.

Longnecker, R. and Kieff, E. (1990). A second Epstein-Barr virus membrane protein (lmp2) is expressed in latent infection and colocalizes with LMP1. *J. Virol.,* 64, 2319-2326.

Longnecker, R., Durker, B., Roberts, T.M. and Kieff, E. (1991). An Epstein-Barr virus protein associated with cell growth transformation interacts with tyrosine kinase. *J. Virol.,* 65, 3681-3692.

Longnecker, R., Miller, C.L., Miao, X.-Q., Marchini, A. and Kieff, E. (1992). The only domain which distinguishes Epstein-Barr virus latent membrane protein 2A (LMP2A) from LMP2B is dispensable for lymphocyte infection and growth transformation *in vitro*; LMP2A is therefore nonessential. *J. Virol.,* 66, 6461-6469.

Luka, J. Kallin, B. and Klein, G. (1979). Induction of the Epstein-Barr virus (EBV) cycle in latently infected cells by *n*-butyrate. *Virology.,* 94, 228-231.

Luka, J., Deeb, ZE., Hartmann, DP., Jenson, B. and Pearson, GR. (1988). Detection of antigens associated with Epstein-Barr virus replication in extracts from biopsy specimens of nasopharyngeal carcinomas. *J Natl Cancer Inst* 80, 1164-1167.

Lukes, R.J. and Butler, J.J. (1992). The pathology and nomenclature of HD. *Cancer* Res., 26, 1063-1081.

Mackay C.R., Terpe, H.J., Stauder, R., Marston, W.L., Stark,H., and Gunthert, U. (1994). Expression and modulation of CD44 its variant isoforms in humans. *J. Cell. Biol.* 124, 71-82.

MacMahon, B. (1957). Epidemiological evidence on the nature of Hodgkin's disease. *Cancer,* 10, 1045-1054

MacMahon, E.M.E., Glass, J.D., Hayward, S.D., Mann, R.B., Scott-Becker, P., Charache, P., McArthur, J.C., and Ambinder, R.F. (1991).Epstein-Barr virus in AIDS-related primary central nervous system lymphoma. *Lancet* 338, 969.

Magrath, I.T. (1990). The pathogenesis of Burkitt's lymphoma. *Recent Adv. Cancer Res.,* 55, 133-270.

Magrath, I.T. (1991). African Burkitt's lymphoma. History, biology, clinical features and treatment. *Am. J. pediatr. hematol. Oncol.,* 13, 222-246.

Magrath, I., Jain, V., and Bhatia, K. (1993). Molecular epidemiology of Burkitt's lymphoma. In: Tursz,T., *et al.*, (eds.), The Epstein-Barr virus and associated diseases. *Colloque INSERM* 225, 377-396.

Magrath, I. (1997) Small noncleaved cell lymphomas. In: Magrath, I., ed., *The Non-Hodgkin's Lymphomas,* 2nd Ed., London, Edward Arnold(in press)

Maniatis T., Hardison R.C., Lacy E., Lauer J., O'Connell C., Quon D., Sim G.K., Efstratiadis A. (1978). The isolation of structural genes from libraries of eucaryotic DNA. *Cell* 15, 687-701.

Mann, K.P., Staunton, D. and Thorley-Lawson, D. (1985). Epstein-Barr virus-encoded protein found in plasma membranes of transformed cells. *J. Virol.,* 55, 710-720.

Manolov, G. and Manolova, Y. (1972). Marker band in one chromosome 14 from Burkitt lymphomas. *Nature,* 237, 33-34.
Manolova, Y., Manolov, G., Kieler, J., Levan, A. and Klein, G. (1979). Genesis of the 14q+ marker in Burkitt's lymphoma. *Hereditas*. 90, 5-10

Manten-Horst, E., Danen, E.H., Smit, L., Snoek, M., Le Poole, I.C., Van Muijen, G.N., Pals, S.T., Ruiter, D.J. (1992) Expression of CD44 splice variants in human cutaneous melanoma and melanoma cell lines is related to tumor progression and metastatic potential, *Lance,t* 340, 1053-8

Marchini, A., Tomkinson, B., Cohen, J.L., and Kieff, E. (1991). BHRF1, the Epstein-Barr virus gene with homology to *bcl2*, is despensible for B-lymphocyte transformation and virus replication. *J Virol.* 65, 5991-6000.

Marchini A, Cohen J., Wang F., and Kieff E. (1992a). A selectable marker allows investigation of a non-transforming Epstein-Barr virus mutant. *J Virol.* 66, 3214-3219.

Marchini A., Longnecker R. and Kieff E. (1992b). Epstein-Barr virus (Epstein-Barr virus) negative B-lymphoma cell lines for clonal isolation and replication of Epstein-Barr virus ecombinants. *J Virol* 66, 4972-4981.

Mark W. and Sugden B. (1982). Transformation of lymphocytes by Epstein-Barr virus requires only one-fourth of the viral genome. *Virology.* 122, 431-443.

Martegani, M.P., Del Prete, F., Gasbarri, A., Natali, P.G. and Bartolazzi, A. (1999). Structural variability of CD44v molecules and reliability of immunodetection of CD44 isoforms using mAbs specific for CD44 variant exon products. *Am. J. Pathol,* 154, 291-300.

Martin, J.M. and Sugden, B. (1991). Transformation by the oncogenic latent membrane protein correlates with its rapid turnover, membrane localization, and cytoskeletal association. *J. Virol.,* 65, 3246-3258.

Martin, J.M., Veis, D., Korsmeyer, S.J. and Sugden, B. (1993). Latent membrane protein of Epstein-Barr virus induces cellular phenotypes independently of expression of Bcl-2. *J. Virol.*, 67, 5269-5278.

Marschall, M., Schwarzmann, F., Leser, U., Okr, B., Alliger, P, Mairhofer, H. and Wolfe, H. (1991). The BL'F4 transactivator of Epstein-Barr virus is modulated by type and differentiation of the host cell. *Virology*. 181, 172-179.

Mason, R.M., Crossman, M.V. and Sweeney, C. (1989). Hyaluronan and hyaluronan-binding proteins in cartilaginous tissues. *Ciba Found Symp.* 143, 107-16; discussion 117-20, 281-5.

Masucci, MG., Torsteinsdottir, S., Colombani, J., Brautbar, C., Klein, E. and Klein, G. (1987). Down-regulation of HLA class I antigens and of the Epstein-Barr virus-encoded latent membrane protein in Burkitt lymphoma lines. *PNAS USA* 84, 4567-4571.

Matsumura, Y., Tarin, D., Mason, R.M., Crossman, M.V., and Sweeny, C. (1989). Significance of CD44 gene products for cancer diagnosis and disease evaluation. *Ciba Found. Symp.*, 143, 107-120.

Matsumura, Y. and Tarin, D. (1992). Significance of CD44 gene products for cancer diagnosis and disease evaluation. *Lancet.* 340, 1053-8.

Matsumura, Y., Hanbury, D., Smith, J., Tarin, D. (1994) Non-invasive detection of malignancy by identification of unusual CD44 gene activity in exfoliated cancer cells, *BMJ,* 308, 619-24

Matz, M.V., Usman, N., Shagin, D., Bogdanova, E. and Lukyanov, S.A. (1997). Ordered differential display: a simple method for systematic comparison of gene expression profiles. *Nucleic Acids Res.* 25, 2541-2542.

Matz, M.V. and Lukyanov, S.A. (1998). Different strategies of differential display: areas of application. *Nucleic Acids Res.* 24, 5537-5543.

Mayer B, Jauch KW, Gunthert U, Figdor CG, Schildberg FW, Funke I, Johnson JP. (1993). De-novo expression of CD44 and survival in gastric cancer. *Lancet* 342, 1019-22.

Mayer, H.B., Wanke, C.A., Williams, M., Crosson, A.W., Federman, M. and Hammer, S.M. (1996). Epstein-Barr virus-induced infectious mononucleosis complicated by acute renal failure: Case report and review. *Clin. Infect. Dis.*, 22, 1009-1018.

McBride, O.W., Hieter, P.A., Hollis, G.F., Swan, M.C. &Leder, P. (1982) Chromosomal location of human kappa and lambda immunoglobulin light chain constant region genes. *J. exp. Med.* 155, 1480-1490

McCarthy, N.J., Hazlewood, S.A., Huen, D.S., Rickinsn, A.B. and Williams, G.T. (1996). The Epstein-Barr virus gene BHRFI, a homologue of the cellular oncogene bcl-2, inhibits apoptosis induced by gamma radiation and chemotherapeutic drugs. *Adv. Exp. Med. Biol.,* 406, 83-97.

McClelland, M., Mathieu-Daude, F. and Welsh, J. (1995). RNA fingerprinting and Differential Display using arbitrarily primed PCR. *TIG.* 11, 242-246.

Middleton, T., Gahn, T.A., Martin, J.M., and Sugden, B. (1991). Immortalising genes of Epstein-Barr virus. *Adv Virus Res.* 40, 19-55.

Miller, G., Rabson, M. and Heston, L. (1984). Epstein-Barr virus with heterogenous DNA disrupts latency. *J. Virol.,* 50, 174-182.

Miller, N. and Hutt-Fetcher, L.M. (1988). A monoclonal antibody to glycoprotein gp85 inhibits fusion but not attachment of Epstein-Barr virus. *J Virol* 62, 2366-2372.

Miller,G. (1990). Epstein-Barr virus; Biology, Pathogenesis, and Medical Aspects. In Fields, BN., Knipe, DM., *et al.*, (ed.), Virology, Raven Press., New York.

Miller, C.L., Lee, J.H., Kieff, E. and Lngnecker, R. (1994). An integeral membrane protein (LMP2) blocks reactivation of Epstein-Barr Virus from latency following surface immunoglobulin crosslinking. *Proc. Natl. Acad. Sci. USA.,* 91, 772-776.

Miller, W.E., Shelton-Earp, H., and Raab-Traub, N. (1995a). The Epstein-Barr virus latent membrane protein 1 induces expression of the epidermal growth factor receptor. *J. Virol.* 69, 4390-4398.

Miller, W.E., Mosial os, G., Kieff, E. and Raab-Traub, N. (1997). Epstein-Barr virus LMP1 induction of epidermal growth factor receptor is mediated through a TRAF signaling pathway disinct from NF-κB activation. *J. Virol.* 71, 586-594.

Mironov, V.N., Van Montagu, M. and Inze, D. (1995). High throughput Rnase protection assay. *Nucleic Acids Research,* 23, 3359-3360.

Mitchell, T. and Sugden, B. (1995). Stimulation of NF-κB mediated transcription by mutant derivatives of the latent membrane protein of the Epstein-Barr virus. *J. Virol.,* 69, 2968-2976.

Moll, J., Schmidt, A., Van der Putten, H., Plug, R., Ponta, H., Herrlich, P., Zoller, M. (1996) Accelerated immune response in transgenic mice expressing rat CD44v4-v7 on T cells. *J Immunol*, 156, 2085-94

Montone, K.T., Litzky, L.A., Wurster, A., Kaiser, L., Bavaria, J., Kotloff, R., Palevsky, H., Pietra, G.G. & Tomaszewski, J.E. (1996a) Analysis of Epstein-Barr virus-associated

post-transplantation lymphoproliferative disorder after lung transplantation. *Surgery,* 119, 544-551

Moore, K.W., Vieira, P., Fiorentino, D.F., Trounstine, M.L., Khan, T.A. and Mosmannm T.R. (1990). Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein-Barr virus gene BCRFI. *Science,* 248, 1230-1234.

Moore K.W., O'Garra A, de Waal M., Vieira P., Mosmann T.R. (1993). Interleukin-10. *Annu Rev Immunol.* 11, 165-190.

Moorthy, R. and Thorley-Lawson, D.A., (1990). Processing of the Epstein-Barr virus-encoded latent mambrane protein p63/LMP. *J. Virol.,* 64, 829-837.

Moorthy, R. and Thorley-Lawson, D.A. (1993a). All three domains of the Epstein-Barr virus-encoded latent membrane protein LMP-1 are required for transformation of rat-1 fibroblasts. *J. Virol.,* 67, 1638-1646.

Moorthy, R. and Thorley-Lawson, D.A. (1993b). Biochemical, Genetic, and functional analyses of the phosphorylation sites on the Epstein-Barr virus-encoded oncogenic latent membrane protein LMP-1. *J. Virol.,* 67, 2637-2645.

Moran, CA., Tuur, S., Angritt, P., Reid, AH., and O'Leary, TJ. (1992). Epstein-Barr virus in Hodgkin's disease from patients with human immunodeficiency virus infection. *Mod Pathol* 5, 85-88.

Morgenlander, J.C. (1996). A syndrome of concurrent central and peripheral nervous system involvement due to Epstein-Barr virus infection. *Muscle Nerve,* 19, 1037-1039.

Mosialos, G., Birkenbach, M., Yalamanchili, R., VanArsdale, T., Ware, C. and Kieff, E. (1995). The Epstein-Barr virus transforming Protein LMP1 engages signaling proteins for the tumor necrosis factor receptor family. *Cell,* 80, 389-399.

Moss D.J., Misko I.S., Burrows S.R., Burman K., McCarthy R., and Sculley T.B. (1988). Cytotoxic T-cell clones discriminate between A- and B-type Epstein-Barr virus transformants. *Nature.* 331, 719-721.

Mueller-Lantzsch, N., Georg, B., Yamamoto, N. and zur Hausen, H. (1980). Epstein-Barr Virus-induced proteins. III. Analysis of polypeptides from P3HR-1-EBV-superinfected NC37 cells by immunoprecipitation. *Virology.*, 2, 231-233.

Mulder, J.W., Kruyt, P.M., Sewnath, M., Oosting, J., Seldenrijk, C.A., Weidema, W.F., Offerhaus, G.J. and Pals, S.T. (1994). Colorectal cancer prognosis and expression of exon-v6-containing CD44 proteins. *Lancet.* 344, 1470-2.

Munoz, A., Schrager, L.K., Bacellar, H., Speizer, I., Vermund, S.H., Detels, R., Saah, A.J., Kingsley, L.A., Seminara, D. and Phair, J.P (1993) Trends in the incidence of

outcomes defining acquired immunodeficiency syndrome (AIDS) in the Multicenter AIDS Cohort Study: 1985-1991. *Am. J. Epidemiol.,* 137, 423-438

Nakagomi, H., Dolcetti, R., Bejarano, M.T., Pisa, P., Kiessling,R. and Masucci, M.G. (1994). The Epstein-Barr virus latent membrane protein-1 (LMP1) induces interleukin-10 production in Burkitt lymphoma lines. *Int. J. Cancer,* 57, 240-244.

Nalesnik, M.A. and Starzl, T.E. (1994). Epstein-Barr virus, infectious mononucleosis, and posttransplant lymphoproliferative disorders. *Transplant Sci.* 4, 61-79.

Nanoyama, M. and Pagano, J.S., (1973). Homology between Epstein-Barr virus DNA and viral DNA from Burkitt's lymphoma and nasopharyngeal carcinoma determined by DNA-DNA reassociation kinetics. *Nature* 242, 44-47.

Naor, D., Vogtsionov, R., Ishshalom, D. (1997). CD44 structure , function and association with the metastatic process. *Adv. Cancer. Res.* 71, 241-319.

Nelson, P.S., Plymate, S.R., Wang, K., True, L.D., Ware, J.L., Gan,L. and Hood, L. (1998). Hevin an antiadhesion extracellular matrix protein, is down regulated in metastatic prostate adenocarcinoma. *Cancer Res.* 58, 232-236.

Nemerow, G.R. and Cooper, N.R. (1984). Early enents in infection of human B lymphocytes by Epstein-Barr virus: internalization process. *Virologyy,* 132, 186-196.

Nemerow, G.R., Mold, C., Schwend, V.K., Tollefson, V., and Cooper, N.R. (1987). Identification of gp350 as the viral glycoprotein mediating attachment of Epstein-Barr virus (EBV) to the EBV/C3d receptor at B cells: sequence homology of gp350 and C3 complement fragment C3d. *J Virol.* 61, 1416-1420.

Nemerow G., Houghton R., Moore M. and Cooper N. (1989). Identification of an epitope in the major envelope protien of Epstein-Barr virus that mediates viral binding to the B lymphocyte Epstein- Barr virus receptor (CR2). *Cell.* 59, 369-377.

Niedobitek, G., Agathanggelou, A., Herbst, H., Whitehead, L., Wright, D.H., and Young, L.S. (1997b). Epstein-Barr virus (EBV) infection in infectious mononucleosis: virus latency, replication and phenotype of EBV-infected cells. *J. Pathol.,* 182, 151-159.

Niswander, L., Tickle, C., Vogel, A., Booth, I., Martin, G.R. (1993) FGF-4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb. *Cell,* 75, 579-87

Nonkwelo, C., Henson, E.B.D. and Sample, J. (1995). Characterization of the Epstein-Barr virus Fp promoter. *Virology,* 206, 183-195.

Nonkwelo, C., Skinner, J., Bell, A., Rickinson, A. and Sample, J. (1996). Transcriptional start site downstream of the Epstein-Barr virus (EBV) Fp promoter in

early-passage Burkitt's lymphoma cells define a fourth promoter for expression of the EBV EBNA-1 protein. *J. Virol.,* 70, 623-627.

O'Conor, GT. (1970). Persistent immunologic stimulation as a factor in oncogenesis with special reference to Burkitt's tumour. *Am J Med* 48, 279-285.

O'Neill, H.C. (1989) Antibody which defines a subset of bone marrow cells that can migrate to thymus. *Immunology*, 68, 59-65

Ohshima, K., Kikuchi, M., Masuda, Y., Sumiyoshi, Y., Eguchi, F., Mohtai, H., Takeshita, M. & Kimura, N. (1991) Epstein-Barr viral genomes in carcinoma metastatic to lymph nodes. Association with nasopharyngeal carcinoma. *Acta. Pathol. Jpn.,* 41, 437-443

Olurin, O. and Williams, A.O. (1972). Orbiti-ocular tumors in Nigeria. *Cancer,* 30, 580-587.

Orkin, R.W., Underhill,C.B. and Toole, B.P. (1982). Hyuronate degredation in 3T3 and simian virus transformed 3T3 cells. *J. Biol. Chem.* 257, 5821-5826.

Orlowski, R. and Miller, G. (1991). Single-stranded structures are present within plasmids containing the Epstein-Barr virus latent origin of replication. *J Virol.* 65, 677-686.

Osada, A., Nakashima, H., Furue,M., and Tamaki, K. (1995). Up-regulation of CD44 expression by tumour necrosis factor α is neutralised by interleukine-10 in vangerhans cells. *J. Invest, Dermatol.* 105, 124-127.

Pallesen, G., Hamilton-Dutoit, S.J., Rowe, M., Lisse, I., Ralfkiaer, E., Sandvej, K. and Young, L.S. (1991). Expression of Epstein-Barr virus replicative proteins in AIDS-related non-Hodgkin's lymphoma cells. *J Pathol.* 165, 289-99

Parkin, D.M., ed. (1986) *Cancer Occurrence in Developing Countries* (IRAC Scientific Publications No.75), Lyon, IRAC

Parkin, DM., Whelan, SL., Ferlay, J., Raymond, L. and Young, J., (eds.), (1997). Cancer Incidence in Five Continents, Vol. VII (IARC Scientific Publications No. 143), Lyon, IARC.

Patarroyo, M., Prieto, J., Ernberg, I and Gahmbergl, T. (1988). Absence or low expression of leucocyte adhesion molecules CD11 and CD18 on BL cells. *Int. J. Cancer.* 41, 901-907.

Patel, D.D., Hale, L.P., Whichard, L.P., Radcliff, G., Mackay, C.R. and Haynes, B.F. (1994). Expression of CD44 molecules and CD44 ligands during human thymic fetal development: expression of CD44 isoforms is developmentally regulated.

Peach, R.J., Hollenbaugh, D., Stamenkovic, I., Aruffo, A. (1993). Identification of hyaluronic acid binding sites in the extracellular domain of CD44. *J Cell Biol.* 122, 257-64

Pearson, G.R., Vroman, B., Chase, B., Sculley, T., Hummel, M. and Kieff, E. (1983). Identification of polypeptide components of the Epstein-Barr virus early antigen complex with monoclonal antibodies. *J. Virol.* 47, 193-201.

Pedersen, C., Gerstoft, J., Lundgren, J.D., Skinhoj, P., Bottzauw, J., Geisler, C., Hamilton-Dutoit, J., Thorsen, S., Lisse, I., Ralfkiaer, E. & Pallesen, G. (1991). HIV-associated lymphoma: Histopathology and association with Epstein-Barr virus genome related to clinical, immunological and prognostic features. *Eur. J. Cancer,* 27, 1416-1423

Peng, M. and Lundgren, E. (1992). Transient expression of the Epstein-Barr virus LMP1 gene in human primary B cells induces cellular activation and DNA synthesis. *Oncogene,* 7, 1775-1782.

Pessac, B., and Defendi, V. (1972). Cell aggregation: role of acid mucopolysaccarides. *Science.* 175, 898-890.

Petti, L. and Kieff, E. (1988). A sixth Epstein-Barr virus nuclear protein (EBNA3B) is expressed in latently infected growth-transformed Lymphocytes. *J. Virol.,* 62, 2173-2178.

Petti, L., Sample, C., and Kieff, E. (1990). Subnuclear localisation and phophorylation of Epstein-Barr virus latent infection nuclear proteins. *Virology* 176, 563-574

Platt, T.H., Tcherepanova, I.Y., Schildraut, C.L. (1993). Effects of number and position of EBNA1 binding sites in Epstein-Barr virus oriP on the sites of initiation, barrier formation, and termination of replication. *J. Virol.* 67, 1739-1745.

Polack, A., Delius, H., Zimber, U. and Bornkamm, G.W. (1984a). Two deletions in the Epstein-Barr virus genome of the Burkitt lymphoma nonproducer line Raji. *Virology,* 133, 146-157.

Ponta, H., Wainwright, D. and Herrlich, P. (1998). The CD44 protein family. *Int. J. Biochemistry & Cell Biology,* 30, 299-305.

Power, C. and Walls, D. (1993). Epstein-Barr virus infection. *J. Biomed. Sci.,* 4, 10-16.

Provisor, A.J., Iacuone, J.J., Chilcote, R.R., Neiburger, R.G., Crussi, F.G. and Baehner R.L. (1975). Acquired agammaglobulinemia after a life-threatening illness with clinical

and laboratory features of infectious mononucleosis in three related male children. *New Engl. J. Med.,* 293, 62-65.

Pulvertaf, R.J.V. (1965). A study of malignant tumours in Nigeria by short term tissue culture. *J. Clin. Path.* 18, 261-273.

Purtilo, D.T. (1976). Pathogenesis and phenotypes of an X-linked recessive lymphoproliferative syndrome. *Lancet,* 2 882-885.

Quackenbush, E.J., Vear S., Greves A., and Letare, M. (1990). Conformation by peptide sequence and co-expression on various cell types of the idenity of CD44 and p85 glycoprotein. *Mol Immunol* 27, 947.

Raab-Traub, N., Dambaugh, T. & Kieff, E. (1980). DNA of Epstein-Barr virus VIII: B95-8, the previous prototype , is an unusual deletion derivative. *Cell,* 22, 257-267

Raab-Traub, N., Hood, R., Yang, C.-S., Henry, B. & Pagano J.S. (1983). Epstein-Barr virus transcription in nasopharyngeal carcinoma. *J. Virol.,* 48, 580-590

Raab-Traub, N. and Flynn, K. (1986). The structure of the termini of the Epstein-Barr virus as a marker of clonal cellular proliferation. *Cell* 47, 883-889.

Reber S, Matzku S, Gunthert U, Ponta H, Herrlich P, Zoller M. (1990). Retardation of metastatic tumor growth after immunization with metastasis-specific monoclonal antibodies. *Int J Cancer.* 46, 919-27.

Reeder, J.A., Gotley, D.C., Walsh, M.D., Fawcett, J. and Antalis, T.M. (1998). Expression of antisense CD44 variant 6 inhibits colorectal tumor metastasis and tumor growth in a wound environment. *Cancer Research,* 58, 3719-3726.

Resnick, L., Herbst, J.S., Ablashi, D.V., Atherton, S., Frank, B., Rosen, L. and Horwitz, S.N. (1988). Regression of oral hairy leukoplakia after orally administered acyclovir therapy. *J. Am. med. Assoc.,* 259, 384-388.

Rickinson, A.B., Young, L.S., and Rowe, M. (1987). Influence of the Epstein-Barr virus nuclear antigen EBNA 2 on the growth phenotype of virus-transformed B cells. *J Virol.* 61, 1310-1317.

Ricksten, A., Kallin, B., Alexander, H., Dillner, J., Fahraeus, R., Klein, G., Lerner, R. and Rymo, L. (1988). *Bam*HI E region of the Epstein-Barr virus genome encoded three transformation- associated nuclear protiens. *Proc. natl Acad. Sci. USA,* 85, 995-999.

Rincon, J., Prieto, J. and Patarroyo, M. (1992). Expression of integrins and other adhesion molecules in Epstein-Barr virus-transformed B lymphoblastoid cells and Burkitt's lymphoma cells. *Int. J. Cancer,* 51, 452-458.

Ring, C. (1994). The B cell-immortalizing function of Epstein-Barr virus. *J. Gen. Virol.*, 75, 1-13.

Rinivas, SK. and Sixbey, J. (1995). Epstein-Barr virus induction of recombinase-activating genes RAG1 and RAG2. *J Virol.* 69: 8155-8158.

Robertson, E.S., Grossman, S., Johannsen, E., Miller, C., Lin, J., Tomkinson, B., and Kieff, E. (1995). Epstein-Barr virus Nuclear Protein 3C Modulates Transcription through Interaction with the Sequence-Specific DNA-Binding Protein Jκ. *J. Virol.* 69, 3108-3116.

Robertson, E.S., Lin, J., and Kieff, E. (1996). The amino-terminal domains of Epstein-Barr virus nuclear proteins 3A, 3B and 3C interact with RBPJκ. *J Virol.* 70, 3068-3074.

Roca X., Mate, J.L., Ariza, A., Munoz-marmol, A.M., Uexkull-Guldeband,C., Pellicer, I., Navas-Palacios, J.J., Isamat, M. (1998). CD44 isoform expression follows two alternative splicing pathways in breast tissue. *American J. Path.* 153, 183-190.

Rogers, R.P., Woisetschlaeger, M., and Speck, S.H. (1990). Alternative splicing dictates translational start in Epstein-Barr virus transcripts. *EMBO J.* 9, 2273-2277.

Rogers, R.P., Strominger, J.L., and Speck, S.H. (1992). Epstein-Barr virus in B lymphocytes: Viral gene expression and function in latency. *Adv Cancer Res.* 58, 1-26.

Romaris, M., Bassols, A., David, G. (1995) Effect of transforming growth factor-beta 1 and basic fibroblast growth factor on the expression of cell surface proteoglycans in human lung fibroblasts. Enhanced glycanation and fibronectin-binding of CD44 proteoglycan, and down-regulation of glypican. *Biochem J,* 310, 73-81

Rooney C. M., Gregory, C.D., Rowe, M., Finerty, S., Edwards, C. and Rupani, H. (1986). Endemic Burkitt's lymphoma: phenotypic analysis of tumour biopsy cells and of the derived tumour cell lines. *J. Natl. Cancer Inst.* 77, 681-687.

Rooney, C., Howe, J.G., Speck, S.H. and Miller, G. (1989). Influences of Burkitt's lymphoms and primary B cells on latent gene expression by the nonimmortalizing P3J-HR-1 strain of Epstein-Barr virus. *J Virol.* 63, 1531-1539.

Rosa, M.D., Gottlieb, M., Lerner, M.R., Steitz, J.A. (1981). Striking similarities are exhibited by two small Epstein-Barr virus encoded ribonucleic acids and the adenovirus associates ribonucleic acids VA_1 and VA_2. *Mol Cell Biol.,* 1, 785-796.

Rowe, D., Heston, L., Metlay. J. and Miller, G. (1985). Identification and expression of a nuclear antigen from the genomic region of the Jijoye strain of Epstein-Barr virus which is missing in its non-immortalizing deletion mutant P3HR-1. *Proc. Nat Acad Sci U. A. S. 82, 7429-7433.*

Rowe, D.T., Rowe, M., Evan, G.I., Wallace, L.E., Farrell, P.J., and Rickinson, A.B. (1986). Restricted expression of EBV latent genes and T-lymphocyte-detected membrane antigen in Burkitt's lymphoma cells. *EMBO-J.* 5, 2599-2607.

Rowe, M., Rowe, D.T., Gregory, C.D., Young, L.S., Farrell, P.J., Rupani, H., and Rickinson, A.B. (1987). Differences in B cell growth phenotype reflect novel patterns of Epstein-Barr virus latent gene expression in Burkitts lymphoma cells. *EMBO-J.* 6, 2743-2751.

Rowe, M., Young, L.S., Cadwallader, K., Petti, L., Kieff, E., and Rickinson, A.B. (1989). Distinction between Epstein-Barr virus type A (EBNA 2A) and type B (EBNA 2B) isolates extends to the EBNA3 family of nuclear proteins. *J Virol.* 63, 1031-1039.

Rowe, D.T., Hall, L., Jaob, I. and Laux, G. (1990). Identification of Epstein-Barr virus terminal protein gene products in latently infected B-lymphocytes. *J Virol.* 64, 2866-2875.

Rowe, M., Lear, AL., Croom-Carter, D., Davies, AH., and Rickinson, AB. (1992). Three pathways of Epstein-Barr virus gene activation from EBNA1-positive latency in B lymphocytes. *J Virol* 66, 122-131.

Rowe, M., Peng-Pilon, M. and Huen, D. (1994). Upregulation of bcl-2 by the Epstein-Barr virus latent membrane protein LMP1: a B-cell specific response that is delayed relative to NF-kB activation and to induction of cell surface markers. *J. Virol.,* 68, 5602-5612.

Rymo, L. (1979). Identification of transcribed regions of Epstein-Barr virus DNA in Burkitt Lymphoma-derived cells. *J Virol.* 32, 8-18.

Saeland, S.,Duvert, V., Moreau, I. and Banchereau, J. (1993). Human B cell precursors proliferate and express CD23 after CD40 ligation. *J. Exp. Med.,* 178, 113-120.

Saemundsen, A.K., Kallin, B. and Klein, G. (1980). Effect of *n*-butyrate on cellular and viral DNA synthesis in cells latently infected with Epstein-Barr virus. *Virology.,* 107, 557-561.

Salles, G., Zain, M., Jiang, W.M., Boussiotis, V.A., Shipp, M.A. (1993). Alternatively spliced CD44 transcripts in diffuse large-cell lymphomas characterization and comparison with normal activated B cells and epithelial malignancies. *Blood,* 82, 3539-47

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular cloning a laboratoy manual. Second edition by Cold Spring Harbour Press.

Sample, J., Hummel, M., Braun, D., Birkenbach, M., and Kieff, E. (1986). Neucleotide sequences of of mRNAs encoding Epstein-Barr virus nuclear proteins: probable transcriptional initiation site. *Proc. Natl. Acad. Sci. USA* 83, 5096-5100.

Sample, J., Liebowitz, D. and Kieff, E. (1989). Two related Epstein-Barr virus membrane proteins are encoded by separate genes. *J. Virol.,* 63, 933-937.

Sample, J. and Kieff, E. (1990). Transcription of the Epstein-Barr virus Genome during latentcy in growth-transformed lymphocytes. *J. Virol.,* 64, 1667-1674.

Sample, J., Young, L., Martin, B., Chatman, T., Rickinson, A., Kieff, E.F., and Kieff, E.D. (1990). Epstein-Barr virus types 1 and 2 differ in their EBNA3A, EBNA3B and EBNA3C genes. *J Virol.* 64, 4084-4092.

Sample, J., Brooks, L., Sample, C., Young, L., Rowe, M, Rickinson, A., and Kieff, E. (1991). Restricted Epstein-Barr virus protein expression in Burkitt's lymphoma is due to a different Epstein-Barr nuclear antigen 1 transcriptional initiation site. *Proc. Natl. Acad. Sci. USA* 88, 6343-6347.

Sample, J., Henson, E.B.D. and Sample, C. (1992). The Epstein-Barr virus nuclear protein I promoter active in type-I latency is autoregulated. *J. Virol.,* 66, 4654-4661.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A.* 74, 5463-7

Schena, M., Shalon, D., Davis, R.W. and Brown, P.O. (1995). Quantitaive monitoring of gene expression patterns with a complimentary DNA microarray. *Science* 270, 467-470.

Screaton, G.R., Bell M.V., Jackson,D.G., Cornellis, F.B., Gerth, E.U., and Bell,J.I. (1992). Genomic structure of DNA encoding the lymphocyte homing receptor CD44 receals at least twelve alternatively spliced exons. *Proc. Nat. Acad. Sci. USA.* 89, 12160.

Screaton, G.R., Bell M.V., Jackson,D.G. (1993). The identification of a new alternative exon with highly restricted tissue expression in transcripts encoding the mouse Pgp-1 (CD44) homing receptor:comparison of all ten variable exons between mouse human and rat. *J. Biol. Chem.* 268, 12235.

Seiter S, Arch R, Reber S, Komitowski D, Hofmann M, Ponta H, Herrlich P, Matzku S, Zoller M. (1993). Prevention of tumor metastasis formation by anti-variant CD44. *J Exp Med.* 177, 443-55.

Shoham NG, Arad T, Rosin-Abersfeld R, Mashiah P, Gazit A, Yaniv A. (1996). Differential display assay and analysis. *Biotechniques* 20, 182-4.

Shtivelman, P., and Bishop, J.M. (1991). Expression of CD44 is repressed in neuroblastoma cells. *Mol Cell Biol.* 11, 5446.

Siaw M.F.W., Nemerow G.R., Cooper N.R. (1986). Biochemical and analysis of the Eptein-Barr virus/C3d receptor (CR2). *J Immunol* 136, 4146-4151.

Sinclair, A.J., Brimmell, M. and Farrell, P.J. (1992). Recipricol antagonisim of steroid hormones and BZLF1 in switch between Epstein-Barr virus latent and productive cycle gene expression. *J. Virol.* 66, 40-77.

Sinclair, A.J., Palermo, I., Peter, G., Farrell, P.J. (1994). EBNA-2 and EBNA-LP co-coperate to cause G_0 to G_1 transition during immortalisation of resting human B lymphocytes by Epstein-Barr virus. *EMBO J.* 13, 3321-3328.

Sinn HP, Heider KH, Skroch-Angel P, von Minckwitz G, Kaufmann M, Herrlich P, Ponta H. (1995). Human mammary carcinomas express homologues of rat metastasis-associated variants of CD44. *Breast Cancer Res Treat.* 36, 307-313

Sixbey J.W., and Pagano J.S. (1985). Epstein-Barr virus transformation of human B lymphocyte despite inhibition of viral polymerase. *J. Virol.* 53, 299-301

Sjoblom, A., Nerstedt, A., Jansson, A. and Rymo, L. (1995a). Domains of the Epstein-Barr virus nuclear antigen 2 (EBNA2) involved in the transactivation of the latent membrane protein 1 and the EBNA Cp promoters. *J. Gen. Virol.,* 76, 2669-2678.

Sliutz, G., Tempfer, C., Winkler, S., Kohlberger, P., Reinthaller, A., Kainz, C. (1995) Immunohistochemical and serological evaluation of CD44 splice variants in human ovarian cancer. *Br J Cance,r* 72,1494-7

Sleeman, J., Moll, J.,Sherman, l., Dall, P., Pals, S.T., Ponta, H., Herrlich,P. (1985). The role of CD44 splice variants in human metastatic cancer. *CIBA Found Symp.* 189, 142-151.

Sleeman, J., Moll, J.,Sherman, l., Dall, P., Pals, S.T., Ponta, H., Herrlich, P. (1995). The role of CD44 slpice variants in human metastatic cancer. In *Cell Adhesion and Human diseases.* Chister: Wiely, 142-156.

Smith, N.R., Aldersley, M., Li, A., High, A.S., Moynihan, T.P., Markham, A.F. and Robinson, P.A. (1997a). Automated differential display using a fluorescently labeled universal primer. *Biotechniques* 23, 274-279.

Smith, N.R., Li, A., Aldersley, M., High, A.S., Markham, A.F. and Robinson, P.A. (1997b). Rapid determination of the complexity of cDNA bands extracted from DDRT-PCR polyacrylamide gels. *Nucleic Acids Res.,* 25, 3552-3554.

Sneath, R.J.S. and Mangham, D.C. (1998). The normal structure and function of CD44 and its role in neoplasia. *Clin Pathol: Mol Patholo.* 51, 191-200.

Snudden, D.K., Hearing, J., Smith, P.R., Grasser, F.A. and Griffin, B.E. (1994). EBNA-1, the major nuclear antigen of Epstein-Barr vieus, resembles 'RGG' RNA binding proteins. *EMBO J.*, 13, 4840-4847.

Snug, N. S., Wilson, J., Davenport, M., Sista, N.D. and Pagono, J.S. (1994). Reciprocal regulation of the Epstein-Barr *Bam*HI-F promoter by EBNA-1 and an E2F transcription factor. *Mol. Cell. Biol.*, 147, 7144-7152.

Soares, M.B. (1997). Identification and cloning of differentially expressed genes. *Cur. Opin. Biotech.* 8, 542-546.

Sokolov,B. and Prokop, D. (1994). A rapid and simple PCR based method for isolation of cDNAs from differentially expressed genes. *Nucleic Acids Res.*, 22, 4009-4015.

Sompayrac, l., Jand, S., Burn, T.C., Tenen, D.G. and Danna, K.J., (1995). Overcoming limitations of mRNA differential display technique. *Nucleic Acids Res.* 22, 4738-4739.

Speck, SH., Pfitzner, A. and Strominger, Jl. (1986). An Epstein-Barr virus transcript from a latently-infected, growth transformed B-cell line encodes a highly repetitive polypeptide. *Proc of the Nat Acad Sci. U.S.A.* 83, 9298-9302.

Spencer, C.A. and Groudine, M. (1991). Control of *c-myc* in normal and neoplastic cells. *Adv. Cancer Res.*, 56, 1-48.

Sriskandan, S., Labrecque, L.G. and Schofield, J. (1996). Diffuse pneumonia associated with infectious mononucleosis: Detection of Epstein-Barr virus in lung tissue by in-situ hybridization. *Clin. infect. Dis.*, 22, 578-579.

Stamenkovic, I., Amiot, M., Pesanto, J.M.P. and Seed, B. (1989). A lymphocyte molecule involved in lymph node homing is a member of the cartilage link family. *Cell* 56, 1057.

Stamenkovic, I., Arufo, A., Amist, M. and Seed, B. (1991). The hematopoetic and epithelial forms of CD44 are distinct polypeptides with different adhesion potentials for hyaluronan-bearing cells. *EMBO J.* 10, 343-347.

Stauder, R. and Gunthert, U. (1995). CD44 Isoforms: Impact on lymphocyte activation and differentiation. *The Immunologist,* 3, 78-83.

Stevens., R.L., Kamada, M.M., Serafin, W.E. (1989). Structure and function of the family of proteoglycans that reside in the secretory granules of natural killer cells and other effector cells of the immune response. *Curr Top Microbiol Immunol,* 140, 93-108

Stevens J.W, Palechek P.L, Griebling T.L, Midura R.J, Rokhlin O.W., Cohen M.B. (1996). Expression of CD44 isoforms in human prostate tumor cell lines. *Prostate.* 28, 153-61.

Sugden, B. and Warren, N. (1989). A promter of Epstein-Barr virus that can function during latent infection can be transactivated by EBNA-1, a viral protein required for viral DNA replication during latent infection. *J. Virol.*, 63, 2644-2649.

Sugiyama, M., Woodman, A., Sugino, T., Crowley, S. Ho, K. Smith, J., Matsumura, Y. and Tarin, D. (1995). *J clin pathol* 48 m142-m147

Suh, S.I., Baek, W.K., Park, J.W., Bae, O.S., Suh, M.H., Choe, B.K. (1995). Identification of CD44 splice variant in Korean colorectal cancers and cell lines. *J Korean Med Sci* 10, 169-75.

Sun, Y., Hegamyer, G., Colburn, N.H. (1994). Molecular cloning of five messenger RNAs differentially expressed in preneoplastic or neoplastic JB6 mouse epidermal cells: one is homologous to human tissue inhibitor of metalloproteinases-3. *Cancer Res.* 54, 1139-1144.

Suzuki, H., Yaoi, T., Kawai J., Hara, A., Kuwajima, G. and Watanabe, S. (1996). Restriction landmark cDNA scanning (RLCS): a novel cDNA displaysystem using two-dimensional gel electrophoresis. *Nucleic Acids Res.* 24, 289-294.

Sy, M.S., Guo, Y.J. and Stamenkovic, I. (1991). Distinct effects of two CD44 isoforms on tumour growth *in vivo. J. Exp. Med.,* 174, 859-866.

Sy, M.S., Guo, Y.J., Stamenkovic, I. (1992) Inhibition of tumor growth in vivo with a soluble CD44-immunoglobulin fusion protein. *J Exp Med*, 176, 623-7

Sy M.S., Mori, H. and Liu, D. (1997). CD44 as a marker in human cancers. *Current Opin. Oncology,* 9, 108-112.

Szekely, L., Selivanova, G., Magnusson, KP., Klein, G. and Wiman, KG. (1993). EBNA-5, an EBV-encoded nuclear antigen, binds to the Rb and p53 proteins. *Proc. Nat. Acad. Sci.. U.S.A.* 90, 5455-5459.

Takada, K. (1984). Cross-linking of cell surface immunoglobulins induces Epstein-Barr virus Burkitt lymphoma lines. *Int. J. Cancer.,* 33, 27-32.

Takada, K. and Ono, Y. (1989). Synchronous and sequential activation of latently infected Epstein-Barr virus genomes. *J. Virol.,* 63, 445-449.

Takahashi K, Stamenkovic I, Cutler M, Saya H, Tanabe K. (1995). CD44 hyaluronate binding influences growth kinetics and tumorigenicity of human colon carcinomas. *Oncogene* 11, 2223-32.

Tanaka, Y., Adams, D.H., Hubscher, S., Hirano, H., Siebenlist, U., Shaw, S. (1993). T-cell adhesion induced by proteoglycan-immobilized cytokine MIP-1 beta. *Nature*, 361, 79-82

Tanner, J., Weis, J., Fearon, D., Whang, Y., and Kieff, E. (1987). Epstein-Barr virus gp350/220 binding to the B lymphocyte C3d receptor mediates adsorption, capping, and endocytosis. *Cell* 50, 203-213.

Tax F.E., Yeargers J.J., Thomas J.H. (1994). Sequence of C. elegans lag-2 reveals a cell-signalling domain shared with Delta and Serrate of Drosophila. *Nature* 368, 150-4.

Teder, P., Bergh, J., Heldin, P. (1995). Functional hyaluronan receptors are expressed on a squamous cell lung carcinoma cell line but not on other lung carcinoma cell lines. *Cancer Res*, 55, 3908-14

Ten Seldam, R.E.J., Cooke, R. and Atkinson, L. (1966). Childhood lymphoma in the territories of Papua and New Guinea. *Cancer*, 19, 437-446.

Terpe, H.J., Stark, H., Prehm, P., Gunthert, U. (1994). CD44 variant isoforms are preferentially expressed in basal epithelial of non-malignant human fetal and adult tissues. *Histochemistry*, 101, 79-89

Thomas, J.A., Hotchin, N.A., Allday, M.J., Amlot, P., Rose, M., Yacoub, M. and Crawford, D.H. (1990). Immunohistology of Epstein-Barr virus-associated antigens in B cell disorders from immunocompromised individuals. *Transplantation.* 49, 944-53.

Thomas, J.A., Felix, D.H., Wray, D., Southam, J.C., Cubie, H.A. & Crawford, D.H. (1991a). Epstein-Barr virus gene expression and epithelial cell differentiation in oral hairyplakia. *Am. J. Pathol.,* 139, 1369-1380

Thorley-lawson, D.A., Geilinger, K. (1980). Monoclonal antibodies against the major glycoprotein ($_{gp}$350/220) of Epstein-Barr virus neutralize infectivity.*Proc. Natl Acad. Sci. USA,* 77, 5307-5311.

Thorley-Lawson, D.A. (1980). The suppression of Epstein-Barr virus infection *in vitro* after infection but before transformation of the cell. *J. Immuno.,* 124, 745-751.

Thorley-Lawson, D.A. (1981). The transformation of adult but not newborn human lymph by Epstein-Barr virus and phytohemagglutinin is inhibited by interferon: The early activation by T cells of Epstein-Barr infection is mediated by interferon. *J. Immunol.*, 120, 828 -833.

Thorley-lawson, D.A. and Israelsohn, E.S, (1987). Generation of specific cytotoxic T-cells with a fragment of the Epstein-Barr virus-encoded p63/latent membrane protein. *Proc. Nat. Acad. Sci.. U.S.A.* 84, 5384-5388.

Thorley-lawson, D.A. and Mann, K. P. (1985). Early events in Epstein-Barr infection provide a model for B-cell activation. *J. Exp. Med.,* 162, 45-59.

Tierney, R.J., Steven, N., Young, L.S. and Rickinsn, A.B. (1994). Epstein-Barr virus latency in blood mononuclear cells: Analysis of viral gene transcription during primary infection and in the carier state. *J. Virol.,* 68, 7374-7385.

Tirelli, U., Errante, D., Dolcetti, R., Gloghini, A., Serraino, D., Vaccher, E., Franceschi, S., Boiocchi, M. and Carbone, A. (1995). Hodgkin's disease and human immunodeficiency virus infection: clinicopathologic and virologic features of 114 patients from the Italian Cooperative Group on AIDS and Tumors. *J Clin Oncol.* 13, 1758-67.

Tolg,C., Hofmann, M., Herlick, P., and Ponta H. (1993). Splicing choice from ten variant exons establishes CD44 variability. *Nuc. Acids. Res.* 21, 1225.

Tomkinson, B. and Kieff, E. (1992a). Use of second-site homologous recombination to demomstrate that Epstein-Barr virus nuclear protein 3B is not important for lymphocyte infection or growth transformation *in vitro*. *J. Virol.* 66, 2893-2903.

Tomkinson, B., Robertson, E. S. and Kieff, E. (1993). Epstein-Barr virus nuclear proteins EBNA-3A and EBNA-3C are essential for B-lymphocyte growth transformation. *J. Virol.* 67, 2014-2025.

Touitou, R., Cochet, C. and Joab, I.. (1996). Transcriptional analysis of the Epstein-Barr virus interleukin-10 homologue during the lytic cycle. *J. Gen. Virol.,* 77, 1163-1168.

Toyama-Sorimachi, N., Sorimachi, H., Tobita, Y., Kitamura, F., Yagita, H., Suzuki, K. Miyasaka, M. (1995) A novel ligand for CD44 is serglycin, a hematopoietic cell lineage-specific proteoglycan. Possible involvement in lymphoid cell adherence and activation. *J Biol Chem* 270, 7437-44

Trentmann, S.M. and van der Knaap, E. (1995). Alternatives to ^{35}S as a label for the differential display of eukaryotic messenger RNA. *Science* 267, 1186.

Trochon, V., Mabilat, C., Bertrand, P., Legrand, Y., Smadja-Joffe, F., Soria, C., Delpech, B., Lu, H. (1996). Evidence of involvement of CD44 in endothelial cell proliferation, migration and angiogenesis in vitro. *Int J Cancer*, 66, 664-8

Tsang, S.-F., Wang, F., Izumi, K.M. and Kieff, E. (1991). Delineation of thr *cic*-acting element mediating EBNA-2 transactivation of latent infection membrane protein expression. *J. Virol.,* 65, 6765-6771.

Turley, E.A., Austen, L., Vandelight, K. and Clary, C. (1991). Hyaluronan and a cell-associated hyaluronan binding protein regulate the locomotion of ras-transformed cells. *J. Cell Biol.* 112, 1041-1048.

Uhl-Steidl, M., Muller-Holzner, E., Zeimet, A.G., Adolf, G.R., Daxenbichler, G., Marth, C., Dapunt, O. (1995) Prognostic value of CD44 splice variant expression in ovarian cancer. *Oncology*, 52, 400-6

Ulrix, W., Swinnen, J.V., Heyns, W. and Verhoeven, G. (1998). Identification of the phosphatic acid phosphatase Type 2a isoenzyme as an Androgen-regulated gene in human prostatic adenocarcinoma cell line LNCaP. *J. Biol. Chem.* 273, 4660-4665.

Underhill, C.B. and Dorfman, A. (1978). The role of hyuronic acid in intracellular adhesion of cultured mouse cells. *Exp. Cell Res.* 117, 155-164.

Underhill, C.B. andToole, B.P. (1980). Physical characterisation of hyuronate binding to the surface of siminian virus 40 transfromed cells. *J. Biol. Chem.* 255, 4555-4549.

Underhill, C.B. andToole, B.P. (1981). Receptors for HA on the surface of parent and virus-transformed cell lines: Binding and aggregation studies. *Exp. Cell Res.* 131, 419-423.

Underhill C.B., Green S.J., Comoglio P.M., Tarone G. (1987). The hyaluronate receptor is identical to a glycoprotein of Mr 85,000 (gp85) as shown by a monoclonal antibody that interferes with binding activity. *J Biol Chem.,* 262, 13142-6.

Underhill, C. (1992). CD44: The hyaluronan receptor. *J. Cell Science,* 103, 293-298.

Van Weering, D.H.J., Baas, P.D. and Bos, J.L. (1993). A PCR-based method for the analysis of human CD44 splice products. *PCR Methods Appl.* 3, 100-106.

Vasmatzis, G., Essand, M., Brinkmann, U., Lee, B. and Pastan, I. (1998). Discovery of three genes specifically expressed in human prostate by expressed sequence tag database analysis. *Proc. Nat. Acad. Sci. USA* 95, 300-304.

Velculescu, V.E., Zhang, L., Vogelstein, B. and Kinzler, K.W. (1995). Serial analysis of gene expression. *Science* 270, 484-487.

Vieira, P., de Waal-Malefyt, R., Dang, M.-N., Johnson, K.E., Kastelein, R., Fiorentino, D.F., deVries, J.E., Roncarolo, M.-G., Mosmann, T.R. and Moore, K.W. (1991). Isolation and expression of human cytokine synthesis inhibitory factor cDNA clones: Homology to Epstein-Barr virus open reading frame BCRFI. *Proc. Natl Acad. Sci. USA,* 88, 1172-1176.

Wainwright, D., Sherman, L., Sleeman, J., Ponta, H., Herrlich, P. (1996) A splice variant of CD44 expressed in the rat apical ectodermal ridge contributes to limb outgrowth. *Ann N Y Acad Sci*, 785, 345-9

Walls, D. and Perricaudet, M. (1991). Novel downstream elements upregulate transcription from an Epstein-Barr virus latent promoter. *EMBO J.* 10, 143-151.

Walter, J., Schirrmacher, V. and Mosier, D. (1995). Induction of CD44 Expression by the Epstein-Barr virus latent membrane protein LMP1 is associated wth lymphoma dissemination. *Int. J. Cancer,* 61, 363-369.

Waltzer, L., Logeat, F., Brou, C., Isreal, A., Sergeant, A. and Manet, E. (1994). The human J Kappa recombination signal sequence binding protein (RBP-J Kappa) targets the Epstein-Barr virus EBNA2 protein to its DNA responsive elements. *EMBO J.* 13, 5633-5638.

Waltzer, L., Bourillot, P.Y., Sergeant, A. and Manet, E. (1995). RBP-Jκ repression activity is mediated by a co-repressor and antagonised by the Epstein-Barr virus transcription factor EBNA2. *Nucl. Acids Res.* 23, 4935-4939.

Waltzer, L., Perricaudet, M., Sergeant, A. and Manet, E., (1996). Epstein-Barr virus EBNA3A and EBNA3C proteins both repress RBP-Jκ-EBNA2-activated transcription by inhibiting the binding of RBP-Jκ to DNA. *J. Virol.,* 70, 5909-5915.

Wang, D., Liebowitz, D. and Kieff, E. (1985). An EBV membrane protein expressed in Immortalized lymphocytes transforms established rodent cells. *Cell,* 43, 831-840.

Wang, F., Gregory, C.D., Rowe, M., Rickinson, A.B., Wang, D., Birkenbach, M., Kikatani, H., Kishimoto, T. and Kieff, E. (1987). Epstein-Barr Virus nuclear antigen 2 specifically induces expression of the B-cell activation antigen CD23. *Proc. Natl. Acad. Sci. USA*. 84, 3452-3456.

Wang, D.,Liebowitz, D. and Kieff, E. (1988a). The truncated form of the Epstein-Barr virus latent-infection membrane protein expressed in virus replication does not transform roden fibroblasts. *J. Virol.,* 62, 2337-2346.

Wang, D.,Liebowitz, D., Wang, F., Gregory, C., Rowe, M., Rickinson, A. Larson, R., Springer, T. and Kieff, E. (1988b). Epstein-Barr virus latent infection membrane alters the human B Lymphocyte phenotype: deletion of the amino terminus abolishes activity. *J. Virol.,* 62, 4173-4184.

Wang, F., Gregory, C.D., Sample, C., Rowe, M., Liebowitz, D., Murray, R., Rickinson, A.B., and Kieff, E. (1990). Epstein-Barr virus latent membrane protein (LMP1) and nuclear proteins 2 and 3C are effectors of phenotypic changes in B lymphocytes: EBNA-2 and LMP1 co-operatively induce CD23. *J Virol.* 64, 2309-2318.

Wang, F., Tsang, S-F., Kurilla, M.G., Cohen, J.I., and Kieff, E. (1990a). Epstein-Barr virus nuclear antigen 2 transactivates latent membrane protein LMP1. *J Virol.* 64, 3407-3416.

Weber, G.F., Ashkar, S., Glimcher, M.J., Cantor, H. (1996). Receptor-ligand interaction between CD44 and osteopontin (Eta-1). *Science* 271, 509-12.

Weidlinder, C., Larsson, N., Szigeti, R., Ehlin-Henriksson, B., Henle, G., Henle, W., Klein, G., Ricksten,A., Rymo, L., and Sulitzeanu, D. (1987). Strable transfection of a human lymphoma line by sub-genomic fragments of Epstein-Barr virus DNA to meassure humoral and cellular immunity to the corresponding prioteins. *Int. J. Cancer* 40, 389-395.

Weiss, L.M., Strickler, J.G., Warnke, R.A., Purtilo, D.T. and Sklar, J. (1987). Epstein-Barr viral DNA in tissues of Hodgkin's disease. *Am J Pathol.* 129, 86-91.

Welsh, J., Chad, K., Dalal, S.S., Cheng, R., Ralph, D. and McClelland, M. (1992). Arbitrarily primed PCR fingerprinting of RNA. *Nucleic Acids Res.* 20, 4965-4970.

Wielenga, V.J., Heider, K.H., Offerhaus, G.J., Adolf, G.R., Van den Berg, F.M., Ponta, H., Herrlich, P., Pals, S.T. (1993) Expression of CD44 variant proteins in human colorectal cancer is related to tumor progression. *Cancer Res,* 53, 4754-6.

Williams, D.L., and Kowalski, D. (1993). Easily unwound DNA sequences and hairpin structures in the Epstein-Barr virus origin of plasmid replication. *J. Virol.,* 67, 2707-2715.

Wilson, J.B. and Levine, A.J. (1992). The oncogenic potential of Epstein-Barr virus nuclear antigen-1 in transgenic mice. *Curr. Top. Microbiol. Immunol.* 182: 374-384.

Wirth, K., Arch, R., Somasundaram, C., Hofmann, M., Weber, B., Herrlich, P., Matzku, S., Zoller, M. (1993) Expression of CD44 isoforms carrying metastasis-associated sequences in newborn and adult rats. *Eur J Cancer,* A, 1172-7

Woisetschlaeger, M., Yandava, C.N., Furmanski, L.A., Strominger, J.L., and Speck, S.H. (1990). Promoter switching in Epstein-Barr virus during initial stages of infection of B-lymphocytes. *Proc. Natl. Acad. Sci. USA* 88, 3942-3946

Woodman, A.C., Sugiyama, M., Yoshida, K., Sugino, T., Borgya, A., Goodison, S., Matsumura, Y. and Tarin, D. (1996). Analysis of anamolous CD44 gene expression in human breast, baldder, and colon cancer and corelation of observed mRNA and protein isoforms. *Am. J. of Pathol.,* 149, 1519-1530.

Yamaguchi A, Saito M, Gio T, Iida A, Takeuchi K, Hirose K, Nakagawara G, Urano T, Furukawa K, Shiku H. (1995). Expression of CD44 variant exons 8-10 in gastric cancer. *Jpn J Cancer Res.* 86, 116-71.

Yamaguchi A, Urano T, Goi T, Saito M, Takeuchi K, Hirose K, Nakagawara G, Shiku H, Furukawa K. (1996). Expression of a CD44 variant containing exons 8 to 10 is a useful independent factor for the prediction of prognosis in colorectal cancer patients. *J Clin Oncol.* 14, 1122-27

Yang, B., Yang, B.L., Savani, R.C., Turley, E.A. (1994) Identification of a common hyaluronan binding motif in the hyaluronan binding proteins RHAMM, CD44 and link protein. *EMBO J,* 13, 286-96

Yang, G.P., Ross, D.T., Kuang, W.P., Brown,P.O. and Weigel, R.J. (1999). Combining SSH and cDNA micoarrays for rapid identification of differentially expressed genes. *Nucleic Acids Res.* 27, 1517-1523.

Yates, J.L. and Guan, N. (1991). Epstein-Barr virus-derived plasmids replicate only once per cell cycle and are not amplified after entry into cells. *J Virol.* 65, 483-488.

Yoshida, K., Bolodeoku, J., Sugino, T., Goodison, S., Matsumura, Y., Warren, B.F., Toge, T., Tahara, E., Tarin, D. (1995) Abnormal retention of intron 9 in CD44 gene transcripts in human gastrointestinal tumors. *Cancer Res*, 55, 4273-7

Young, L.S., Yao, Q.Y., Rooney, C.M., Sculley, T.B., Moss, D.J., Rupani, H., Laux, G., Bornkamm, G.W., and Rickinson, A.B. (1987). New type B isolates of Epstein-Barr virus from Burkitt's lymphoma and from normal individuals in endemic areas. *J Gen Virol* 68, 2853-2862.

Young, L.S.,Alfieri, C., Hennessy, K., Evans, H., O'Hara, C., Anderson, K. C., Ritz, J., Shapiro, R.S., Rickinson, A., Kieff, E. and Cohen, J.I. (1989). Expression of Epstein-Barr virus transformation-associated genes in tissues of patients with EBV lymphoproliferative disease. *New Engl. J. Med.,* 321, 1080-1085.

Young, L.S., Lau, R., Rowe, M., Niedobitek, G., Packham, G., Shanahan, F., Rowe, D.T., Greenspan, D., Greenspan, J.S., Rickinson, A.B. and Farrell, P.J. (1991). Differentiation-associated expression of the Epstein-Barr virus BZLF1 transactivator protein in oral hairy leukoplakia. *J. Virol.,* 65, 2868-2874.

Yu, Q. and Toole, B.P. (1996). A new alternatively spliced exon between v9 and v10 provides a molecular basis for synthesis of soluble CD44. *J. Biol. Chem.* 271, 20603.

Zagouras, P., Stifani, S., Blaumueller, C.M., Carcangiu, M.L., Artavanis-Tsakonas, S. (1995). Alterations in Notch signaling in neoplastic lesions of the human cervix. *Proc Natl Acad Sci.* 92, 6414-8.

Zetter B.R. (1993). Adhesion molecules in tumor metastasis. *Semin Cancer Biol.*4, 219-29.

Zhang, P-f., Klutch, M., Armstrong, G., Qualtiere, L., Pearson, G., and Marcus-Sekura, C.J. (1991). Mapping of the epitopes of Epstein-Barr virus gp350 using monoclonal antibodies and recombinant proteins expressed in *Escherichia coli* defines three antigenic determinants. *J Gen Virol* 72, 2747-2755.

Zhang, Q., Brooks, L., Busson, P. (1994a). Epstein-Barr virus (Epstein-BarrVirus) latent membrane protein 1 increases HLA class II expression an Epstein-Barr vius-negative B cell line. *Eur. J. Immunol.,* 14, 1467-1470.

Zhao, S., Ooi, S.L. and Pardee, A.B. (1995). New primer stategy improves precision of differential display. *BioTechniques*, 18, 842-850.

Zhao, B., Marshall, D.R. and Sample, C.E. (1996). A conserved domain of the Epstein-Barr virus nuclear antigens 3A and 3C binds to a discrete domain of Jκ. *J. Virol.,* 70, 4228-4236.

Zhou, D.F.H., Ding, J.F., picker, L.J., Bargatze, R.F., Butcher, B.C. and Goeddel, D.V. (1989). Molecular cloning and expession of Pgp-1. The mouse homologue of the human H-CAM (Hermes) lymphocyte homing receptor. *J. Immunol.* 143, 3390.

Zimber-Strobl, U., Kremmer, E., Grasser, F., Marschall,G., Laux, G. and Bornkamm, G.W. (1993). The Epstein-Barr virus nuclear antigen 2 interacts with an EBNA2 responsive *cis*-element of the terminal protein 1 gene promoter. *Embo. J.,* 12, 167-175.

Zimber-Strobl, U., Kempkes, B., Marschall, G., Zeilder, R., Van Kooten, C., Banchereau, J., Bornkamm, G.W. and Hammerschmidt, W. (1996). Epstein-Barr virus latent membrane protein (LMP1) is not sufficient to maintain proliferation of B cells but both it and activated CD40 can prolong their survival. *EMBO.* 15, 7070-7078.

Zimmermann, J. and Schultz, R.M. (1994). Analysis of gene expression in the preimplantation mouse embryo : Use of mRNA Differential Display. *Proc. Natl. Acad. Sci. USA.* 91, 5456-5460.

Zinn K, DiMaio D, Maniatis T (1983). Identification of two distinct regulatory regions adjacent to the human beta-interferon gene. *Cell* 34, 865-79.

Zoller, M. (1995). CD44: physiological expression of distinct isoforms as evidence for organ-specific metastasis formation. *J Mol Med*, 73, 425-38

APPENDIX A

SOLUTIONS FOR DNA MANIPULATION

TE buffer

10 mM	Tris
1 mM	EDTA pH 8.0

Solutions for mini-preparation of plasmid DNA

Solution I

50 mM	Glucose
25 mM	Tris.Cl (pH 8.0)
10 mM	EDTA (pH 8.0)

Solution II (Prepared fresh)

0.2 N	NaOH
1 % (w/v)	SDS

Solution III

60 ml	5 M potassium acetate
11.5 ml	Glacial acetic acid
28.5 ml	Distilled water

The resulting solution is 3M with respect to potassium and 5M with respect to acetate.

DNAse-free RNAse

RNAse A (1 mg/ml) in sterile water.

100^{o}C 30 min. Cool slowly, store -20^{o}C

Solutions for Maxipreparations of DNA - Qiagen Buffers

Buffer P1 (Resuspension buffer)

50 mM	Tris CL, pH 8.0
10 mM	EDTA
100 µg	RNase A

Store at 4°C after the addition of RNase A.

Buffer P2 (Lysis buffer)

200 mM Sodium Hydroxide

1% (w/v) SDS

Prepared fresh and stored at room temperature.

Buffer P3 Neutralization buffer)

3.0 M Potassium acetate pH 5.5

Stored at 4°C.

Buffer QBT (Equilibriation buffer)

750 mM NaCL

50 mM MOPS pH 7.0

15% (v/v) Isopropanol

0.15% (v/v) Triton X®-100

Stored at room temperature.

Buffer QC (Wash buffer)

1.0 M NaCL

50 mM MOPS pH 7.0

15% (v/v) Isopropanol

Stored at room temperature.

Buffer QF (Elution buffer)

1.25 M NaCL

50 mM Tris CL, pH 8.5

15% (v/v) Isopropanol

Stored at room temperature.

50% (v/v)Glycerol

25 ml Distilled H_2O

25 ml Glycerol

Autoclaved and stored at room temperature.

0.5 M EDTA

186.1 g	EDTA
800 ml	Distilled water
6 g	NaOH pellets

pH to 8.0 with 5 M NaOH

Volume was adjusted to 1 L with water

50X TAE

242 g	Tris
57.1 ml	Acetic acid.
100 ml	0.5 M EDTA pH 8.0

Adjusted to 1L with water

5X TBE

54 g	Tris
27.5 g	Boric acid
20 ml	0.5 M EDTA pH 8.0

Adjusted to 1L with water.

Ethidium bromide

0.1 g/ 10 ml water (10 mg/ml)

Stored in dark at room temperature.

Agarose gel loading dye

40% (w/v) sucrose

0.25% (w/v) bromophenol blue

BACTERIAL GROWTH MEDIA

LB agar

10 g	Tryptone
5 g	Yeast extract
5 g	NaCl
15 g	Agar technical

Autoclaved and plates stored at 4°C.

LB agar plus ampicillin

Ampicillin was added to a final concentration of 100 μg/ml to LB agar (50 °C).

Plates were stored at 4 °C.

LB broth (per L)

10 g	Bacto-tryptone
5 g	Yeast extract
5 g	NaCl

Autoclaved and stored at 4 °C.

LB broth plus ampicillin

Ampicillin was added to a final concentration of 100 μg/ml to LB broth and stored at 4 °C.

SOB medium (per L)

20 g	Tryptone
5 g	Yeast extract
0.5 g	NaCl
10 ml	KCl (250 mM)

Adjusted pH to 7.0 with 5 M NaOH

Autoclaved, cooled to ~5°C and added :

10 ml 1 M $MgCl_2$

Stored at 4 °C.

SOC medium (per L)

1L	SOB
7.5 ml	50% glucose (filter sterilised)

Stored at 4 °C.

IPTG stock solution (100 mM)

24 mg IPTG per ml of sterile H_2O

Filter sterilised and kept on ice until ready to use.

X-Gal stock solution (5%(w/v))

This solution was prepared fresh for each use

50 mg of X-Gal per ml of N,N' dimethyl-formamide in a sterile tube.

Protected from light and stored on ice until ready to use.

Ampicillin stock solution (50 mg/ml)

50 mg of ampicillin per ml of sterile H_2O

Filter sterilised and stored at -20 °C.

LB plates with antibiotics and IPTG/X-Gal.

To 1L of autoclaved LB agar (cooled to 50 °C) the following were added

0.5 μM	IPTG (5ml IPTG 100mM stock solution)
80 μg/ml	X-Gal (1.6 ml 5% (w/v) X-Gal stock solution)
50 μg/ml	Ampicillin (1ml of 50mg/ml solution)

Plates were stored at 4 °C protected from light.

CELL CULTURE MEDIA/SOLUTIONS

Supplemented RPMI (200 ml)

176 ml	RPMI 1640
20 ml	Foetal calf Serum (Decomplemented - 50°C for 30 min)
2 ml	200 mM L-glutamine
2 ml	Penicillin/Streptomycin (1000 U/ml-1000 μg/ml)

Supplemented McCOY'S 5A (200 ml)

178 ml	MACOY'S 5A with L-glutamine
20 ml	Foetal calf Serum (Decomplemented; 50°C for 30 min)
2 ml	Penicillin/Streptomycin (1000 U/ml-1000 μg/ml)

Supplemented DMEM High Glucose

178 ml	DMEM high glucose with L-glutamine
20 ml	Foetal calf Serum (Decomplemented; 50°C for 30 min)
2 ml	Penicillin/Streptomycin (1000 U/ml-1000 μg/ml)

10X Phosphate Buffered Saline (PBS)

14.24 g	$Na_2HPO_4.2H_2O$ (8 mM)
2.04 g	KH_2PO_4 (1.5 mM)
80.0 g	NaCL (137 mM)
2.0 g	KCL (2.7 mM)

pH 7.5 and make up to 1 litre.

Diluted 1 in 10 in sterile distilled water and used at a 1X working concentration.

Trypsin EDTA 1X (0.25% (v/v))

2.5 ml	10X trypsin (2.5%(v/v))
0.5 ml	1% (w/v) EDTA
22 ml	1X PBS

The solution was mixed well, aliquoted and stored at -20°C.

Thiol supplements. The following were added to 200 ml of supplemented media:

200 μl	α-Thiolglycerol
2 ml	Sodium pyruvate
2 ml	HEPES

Bathocuproine disulfonic acid (BCS - 10 mM stock solution)

36.4 mg BCS

10 ml 1X PBS

Dissolved by vortexing, filter sterilised using a 0.2 micron filter, aliquoted and stored at -20 °C.

α-Thiolglycerol

A stock solution of 50 mM in PBS containing 20 μM BCS was prepared.

20 μl 10 mM BCS

10 ml 1X PBS

43.3 μl 100% α-thiolglycerol

Filter sterilised using a 0.2 micron filter, aliquoted and stored at -20 °C.

Sodium pyruvate

100 mM stock solution in 1X PBS (Gibco BRL). Stored at 4°C.

HEPES

1 M stock solution pH7.5 (Gibco BRL). Stored at room temperature.

Microphenolic acid/Xanthine supplements

200 ml Supplemented RPMI

0.5 μg/ml Microphenolic acid

50 μg/ml Xanthine

Microphenolic acid stock solution 2.5 mg/mL

2.5 mg Microphenolic acid

1 ml Sterile d.H_2O

Two micro litres per ml of media was added giving a final concentration of 0.5 μg/ml.

Xanthine stock solution of 25 mg/mL

25 mg Xanthine

1 ml Sterile d.H_2O

Twenty micro litres per ml of media was added to give a final concentration of 50 μg/ml.

Geneticin G418 (stock solution 50 mg/ml) for tetracycline inducible cell lines

0.1 g Geneticin

2 ml RPMI 1640

Filter sterilised using a 0.2 micron filter, aliquoted and stored at -20 °C. 20 μl of the stock solution was added per ml of media to give a final concentration of 1000 μg/ml.

Hygromycin (stock solution 50 mg/ml supplied)

Ten micro litres of the stock solution was added per ml of media to give a final concentration of 500 μg per ml. Stored at 4 °C.

Tetracycline (stock solution 5 mg/ml)

5 mg Tetracycline

1 ml 100% Ethanol

Stored at -20 °C, 1 μl of tetracycline was added to 5 ml of media to give a final concentration of 1 μg per ml.

Geneticin G418 (stock solution 600 mg/ml) For transfected epithelial cell lines C33A Neo and LMP1

0.6 g Geneticin

1 ml 1 M Hepes pH 7.5

Filter sterilised using a 0.2 micron filter, aliquoted and stored at -20 °C. One μl of the stock solution was added per ml of media to give a final concentration of 600 μg per ml.

Flow Cytometry-Wash buffer

100 ml 10X PBS

Sprinkle of BSA

0.01% (w/v) Na Azide (0.1g/1000ml)

Made up to 1L with distilled water and stored at 4°C.

2% (w/v) Paraformaldehyde

2 g	Paraformaldehyde
100 ml	1X PBS

Heated to 65°C, allowed to cool, filtered, and store at 4°C.

SOLUTIONS FOR PROTEIN ISOLATION

Suspension buffer

0.1 M	NaCL
0.01 M	Tris CL (pH 7.6)
0.001 M	EDTA (pH 8.0)
1μg/ml	Apoprotinin
100μg/ml	PMSF

Stored at 4 °C.

2X SDS gel loading buffer

100 mM	Tris CL
200 mM	DTT
4% (w/v)	SDS
0.2% (w/v)	Bromophenol blue
20% (v/v)	Glycerol

Two X loading buffer was prepared with out DTT and stored at room temperature. DTT was added just prior to use from a 1M stock

Protease Inhibitors

2 mg/ml	Leupeptin
0.1 mM	PMSF (phenylmethylsulfonyl flouride)

1% Nonidet p40 (Np40)

100 ml	PBS
0.038 g	EGTA
946 μl	Nonidet p40

Stored at 4 °C.

BSA Stock Solution

50 mg BSA

This solution was made up to 50 ml with a 1:1 mixture of PBS and 1% (v/v)Np40.

SOLUTIONS FOR SDS PAGE/WESTERN BLOTTING

1 M Tris.Cl pH 6.8

1.5 M Tris.Cl pH 8.8

10% (w/v) SDS

10% (w/v) Ammonium persulphate (APS)

Acrylagel

Bis-acrylagel

TEMED

1 M Dithiothreitol

10X Tris glycine running buffer (500 ml)

15.138 g Tris

71.125 g Glycine

5.0 g SDS

Made up to 500 ml with distilled water.

Destain

100 ml Acetic acid

400 ml Methanol

500 ml Distilled water

Coomassie blue stain

1 g Coomassie blue R

200 ml Destain

Transfer Buffer (10X stock solution)

30.3g Tris

144.2g Glycine

Adjusted to pH 8.3, made up to 1 litre with distilled water, stored at room temperature.

1X working Soluiton

100 ml 10X Stock solution

200 ml Methanol

700 ml Distilled H_2O

Stored at 4°C. Methanol was not used in the transfer buffer when protein of 120Kda or more were transfered.

TBS (1X)

6.1 g Tris

8.8 g NaCl

Made up to 1 L with distilled water and adjusted to pH 7.5 with HCl.

Autoclaved and stored at room temperature.

TBST (0.1%, v/v)

1 L TBS (as above)

1 ml Tween 20

Blotto

50 ml 1X TBS (as above)

25 µl 0.05% (v/v)T ween 20 (0.5 ml/L)

2 g 5% (w/v) non-fat dry milk 50 g/L (Marvel)

0.5g NaN_3

Sodium azide (5%) (w/v)

50 mg NaN_3

950 µl Distilled water

REAGENTS FOR SEQUENCING

Six percent denaturing polyacrylamide gel

Six percent denaturing polyacrylamide gel was prepared for sequence analysis. The following formula was employed to determine the amount of acrylamide and bis-acrylamide required:

Va = volume of acrylamide

Vb = volume of bis-acrylamide

Vt = total volume of gel mix 150 ml

C = % crosslinking 5.2 %

A = % gel 6/8 %

$$Va = \frac{Avt}{30} \qquad Vb = \frac{ACVt}{200}$$

Va = 6*150/30 = 30 ml Vb = 6*5.2*150/ = 24 ml

6 % Denaturing PAG

63 g Urea

30 ml Acrylamide

24 ml Bisacrylamide

15 ml 10X TBE

Made up to 150 ml with UP H_2O

Six hundred and fifty micro litres of 10% (w/v) APS and 150 µl TEMED were added, and mixed briefly, directly before pouring.

10X TBE (per 500 ml)

54 g	Tris base
27.5 g	Boric acid
20 ml	0.5 M EDTA (pH 8.0)

One X concentration was used for polyacrylamide gel preparation.

10% (w/v)Ammonium persulphate

0.1 g APS/ml ultra pure H_2O

Developer (5 L)

1.50 L	H_2O
1.25 L	Developer
2.25 L	H_2O

Stirred for 2 min

Fixer (5.125 L)

3.625 L	H_2O
1.250 L	A fixer
0.250 L	B fixer

Stirred for 2 min

REAGENTS FOR RNA ANALYSIS

RNA sample buffer

50 % (v/v)	Deionized formamide
8.3 % (v/v)	Formaldehyde
0.027 M	MOPS pH 7.0
6.7 mM	Sodium acetate

RNA loading buffer (containing ethidium)

50% (v/v)	High grade glycerol
1 mM	EDTA (pH 8.0)

0.4% (w/v) Bromophenol blue

0.1 μg/ml Ethidium bromide

Aliquoted and stored at -20°C.

5X MOPS Buffer (Formaldehyde gel running buffer)

0.1 M MOPS (pH 7.0)

40mM Sodium Acetate

5 mM EDTA (pH 8.0)

20.6g of 3 -(*N*-morpholino)propanesulfonic acid (MOPS) was dissolved in 800 ml of DEPC treated 50 mM sodium acetate. The pH of the solution was adjusted to 7.0 using 2 M NaOH. 10 ml of DEPC-treated 0.5 M EDTA (pH 8.0) was added and the volume of the solution was adjusted to 1L using DEPC-treated H_2O. The 5X solution was filter sterilised through 0.2 micron filters prior to use.

Formaldehyde gel

1 part Formaldehyde

3.5 parts agarose in DEPC H_2O

1.1 parts 5X MOPS buffer

0.68g Agarose

35ml DEPC H_2O

The agarose solution was boiled until fully dissolved and allowed to cool to 60 °C. The following were then added.

11 ml 5X MOPS buffer

10 ml Formaldehyde

56 ml Final volume

The gel was cast in a fume hood and allowed to set for approximately 45 min. The gel was electrophoresed in 1X formaldehyde gel running buffer.

RNA loading buffer

50%(v/v)	High grade glycerol
1 mM	EDTA (pH 8.0)
0.25% (w/v)	Bromophenol blue
0.25% (w/v)	Xylene cyanol FF

DEPC treated overnight, autoclaved and stored at room temperature.

20XSSC

175.3g	NaCL
88.2g	NaCitrate

pH to 7.0 using a few drops of 10M NaOH and made up to 1 litre using up H_2O. DEPC treated and autoclaved. Stored at room temperature.

Hybridization Buffer A

50 % (v/v)	Deionized formamide
6X	SSC
1% (w/v)	SDS
0.1% (v/v)	Tween 20
100μg/ml	tRNA

Prepared fresh prior to use stored at room temperature.