Mechanisms of Action of Epstein-Barr Virus Nuclear Antigen 1 as an oncogene

by

Penelope Tsimbouri

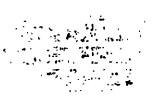
Thesis submitted to the Faculty of Science, University of Glasgow for the degree of Doctor of Philosophy.

Division of Molecular Genetics

May 2000

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

To those who mean the most,
my family and George



In the middle of every difficulty lies opportunity

Albert Einstein

Acknowledgements

I would like to thank my supervisor Dr Joanna Wilson for her advice and support throughout my PhD years and for her comments on the writing of this thesis.

In the lab, I would like to thank David Stevenson and Mark Drotar for their help and advice in the technical part of my PhD work. Thanks to David Stevenson and Catherine Winchester for their help in the completion of my thesis.

I would like to thank Dr. David Gillespie for his help and advice with my protein work. Thanks to Dr. Bill Cushley for his advice with the FACS analysis.

Thanks to everybody in the lab, Jenny, Marie Anne, Steve, Maria, Donald, Nooshin and Lynn for the nice time we have in the lab. It's nice to work with such people.

My gratitude to Robert MacNab, the girls in the Anderson, the ladies in the prep-room, the wash-room, and the store, for making life so much easier.

Most of all, I want to thank all my family for their love and support and 'daring' me not to give up when everything looked black. A special thanks to George, who has given me love and the strength I need to overcome difficult moments and shared his life with me the last 8 years. I would also like to thank Mr and Mrs Bakirtzis for their moral support.

I would also like to thank my friend Ms McGowan for the nice times we spent together and everything I have learned from her about Glasgow.

I would like to thank Picous, my cat, for his love and affection.

Finally, my grateful thanks to Leukaemia Research Fund for funding my work.

Declaration

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

Penelope Tsimbouri

Table of Contents

Chapter 2 Materials and Methods

List of Figures

Chapter 1 Introduction

Chapter 2 Materials and Methods

Chapter 3 Cell phenotype and growth characteristics

Chapter 5. Effects of EBNA 1 on cellular gene expression II: Differential expression

Chapter 6 Final discussion and future directions

List of Tables

Chapter 1 Introduction

Chapter 2 Materials and Methods

Chapter 3 Cell phenotype and growth characteristics

Chapter 4 Effects of EBNA 1 on cellular gene expression I: Selected genes

Chapter 5. Effects of EBNA 1 on cellular gene expression II: Differential expression

Abbreviations

ABC	Avidin–biotin complex
AP1	Activator protein 1
APC	Antigen-presenting cell
APS	Ammonium persulphate
ART	Aerosol resistant tips
Bak	Bcl2-homologous antagonist/killer
Bcl2	B cell lymphoma protein 2
BclxL	B cell lymphoma protein xL (long)
BCR	B cell receptor
BL	Burkitt's lymphoma
BSA	Bovine serum albumin
BPV	Bovine papilloma virus
CBS	Chromosome binding site
Cpm	Counts per minute
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulphoxide
DNA	Deoxiribonucleic acid
dNTP	Deoxy-ribonucleoside triphosphate
DTT	Dithiothreitol
GAPDH	Glyceraldehyde-P-dehydrogenase
GMCSF	Granulocyte-macrophage cell growth factor
EBER	EBV-encoded RNA
EBNA	Epstein-Barr nuclear antigen
EBF	Early B cell factor
EBP2	EBNA 1 binding protein 2
EBV	Epstein-Barr virus
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
Egr1	Early growth response 1
ERK	extracellular signal regulated kinase
EtBr	Ethydium bromide
EtOH	Ethanol
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
FC	Phenol/Chlorofom
FCS	Foetal calf serum
Fig.	Figure
FITC	Fluorescein isothiocyanate
Fra	Fos related antigen
GC	Germinal centre
HCK	human haemopoietic cell kinase
HD	Hodgkin's disease
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HHV	Human herpes virus
HIV	Human immunodeficiency virus

HMW	High molecular weight
hnRNPD	Human nuclear ribonucleoprotein D
HSV	Herpes simplex virus
Ig	Immunoglobulin
IκB	kappa B inhibitor
IRF	Interferon-stimulated response element
ISRE	Interferon-regulatory factor
IL	Interleukin
JAK	janus kinase
LB	Luria-Bertani medium
LCL	Lymphoblastoid cell line
LMP	Latent membrane protein
LN	Lymph node
MAPK	Mitogen activated protein kinase
MAR	Matrix attachment region
Mdm2	Murine double minute 2
MHC	Major histocompatibility complex
min	Minute
MIP1α	macrophage inflamatory protein 1 alpha
μg	Micrograms
μl	Microlitres
μM	Micromolar
MoMLV	Moloney murine leukaemia virus
MOPS	3-N-morpholinopropanesulphonic acid
mRNA	Messenger RNA
NB	Nuclear bodies
NFκB	Nuclear factor kappa B
ng	Nanograms
NK	Natural killer
nm	Nanometres
No.	Number
NPC	Nasopharyngeal carcinoma
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBC	Peripheral blood cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
pg	Picograms
PI	Propidium iodide
PML	Promyelocytic leukaemia gene
pmol	Picomoles
PMSF	Phenylmethylsulphonyl fluoride
Rag	Recombonation activating gene
RBC	Red blood cell
RNA	Ribonucleic acid
rpm	Revolutions per minute

RPMI	Roswell Park Memorial Institute
SAPK	Stress-activated protein kinase (JNK)
SATB1	Scaffold associated DNA-binding protein 1
SDS	Sodium dodecyl-sulphate
sec	Seconds
STAT	Signal transducer and activator of transcription
TdT	Terminal deoxyclucleotidyl transferase
TEMED	N,N,N',N'-tetramethyldiamine
TBS	Tris-buffered saline
TPA	12-O-tetradecanoyl phorbol-13-acetate
TRADD	TNF-receptor associated death domain
TRAF	TNF-receptor associated factor
TUNEL	TdT-mediated dUTP Nick End labelling
UV	Ultra violet
WBC	White blood cell
WT	Wild type
XC	Xylene cyanol

Abstract

Epstein-Barr Virus (EBV) is a human herpes virus associated with several malignancies including endemic Burkitt's lymphoma (eBL), nasopharyngeal carcinoma (NPC) and polyclonal B-cell lympho-proliferations in immunosuppressed individuals. Epstein-Barr virus nuclear antigen 1 (EBNA 1) plays a key role in the life cycle of the virus and is consistently expressed in all these tumour types. However, no oncogenic activities of EBNA 1 have been identified in cell cultures. Nevertheless, EBNA 1 is a viral oncogene when expressed *in vivo* in transgenic mice which succumb to monoclonal B cell lymphoma (Wilson and Levine, 1992, Wilson *et al.*, 1996). The experiments described in this thesis were designed to explore how EBNA 1 may act as an oncogene *in vivo*.

EBNA 1 was found to be redundant in terms of tumour latency with Bcl2 in transgenic crossbreeding experiments. Therefore, the ability of EBNA 1 to inhibit cell death or differentiation was examined by cell culture techniques. Moreover, cell surface markers examined by FACS. Flow cytometric analysis of spleen and bone marrow (BM) cells from EμEBNA 1 transgenic mice revealed that the percentage of sIg+ (and possibly CD23+) cells was statistically significantly higher when compared to the wild type control littermates. EμEBNA 1 spleen and bone marrow (BM) cell survival cultures showed no survival advantage over the wt cultures. However, a proliferation/survival assay revealed that EμEBNA 1 transgenic positive BM cells have statistically significant higher proliferation/ survival rate than the wt BM cells. These results may indicate that EBNA 1 drives cells to differentiate and supports their survival/proliferation leading to increased sIg.

Since EBNA 1 is a DNA-binding protein acting as a transcriptional transactivator the expression levels of cellular genes involved in cell death and differentiation were examined in transgenic mice at pre- and post-tumour stages. The antiapoptotic gene *BclxL* was found up-regulated and the pro-apoptotic gene *Bad* was found downregulated. This strongly supports the earlier phenotypic observation that EBNA 1 may act in a similar fashion or through *Bcl2* family genes. Moreover, *Rag1* and *Rag2* genes were also found to be up-regulated in EμEBNA 1 transgenic mouse spleens and this could lead to increased recombination, genome instability and possibly tumour development.

Further studies, using a macroarray for differential gene expression, suggest that several immediate early response genes such as *Jun* and *Fos* family members and *Egr1* may be affected by EBNA 1. If EBNA 1 does up-regulate these genes this action could lead to increased differentiation and/or proliferation of EBNA 1 positive B cells.

Thus, there are several lines of evidence suggesting that EBNA 1 supports B cell survival/proliferation and differentiation through de-regulation of genes responsible for the development of B cells. This may be the mechanism by which EBNA 1 causes tumourigenesis.

Chapter 1

Introduction

1.1 EBV associated diseases and latent genes

Epstein-Barr virus (EBV) is a B-lymphotropic gamma herpes virus which is widespread in all human populations and it is carried by the majority of individuals as a lifelong asymptomatic infection (Epstein and Achong, 1986). Most people become infected before they reach adulthood. In young children initial infection is usually asymptomatic, whilst post-puberty infection can lead to infectious mononucleosis (glandular fever). Primary EBV infection begins in the pharyngeal epithelium due to its permissive environment for viral replication. From there the virus spreads to the subepithelial B lymphocytes where it persists in a latent state (Faulkner *et al.*, 2000).

EBV is strongly implicated in the pathogenesis of several human malignancies (Baumforth *et al.*, 1999 and references therein; Kawa, 2000), notably in the endemic and in some cases of sporadic Burkitt's lymphoma (BL), in approximately 50% of Hodgkin's disease (HD) cases, nasal T-cell lymphomas and in undifferentiated nasopharyngeal carcinoma (NPC) (Magrath, 1990; Meijer *et al.*, 1996; Rickinson and Kieff, 1996). Possible association of EBV with breast and gastric cancers has been reported recently (Chu *et al.*, 1998; Bonnet *et al.*, 1999; Takada, 1999). EBV is also responsible for a wide spectrum of acute and chronic ailments in healthy and immunocompromised individuals. In addition, potential involvement of EBV in a number of other clinical syndromes such as Duncan's syndrome (X-linked lymphoproliferation) in oral hairy leukoplakia in AIDS patients and chronic mononucleosis, has been proposed (Khanna *et al.*, 1995).

The EBV genome is a linear double stranded 172Kbp DNA (Fig. 1.1). EBV (B95.8 strain) was the first herpes virus to be completely sequenced (Baer *et al.*, 1984; Parker *et al.*, 1990). Comparison of EBV with the other herpes viruses

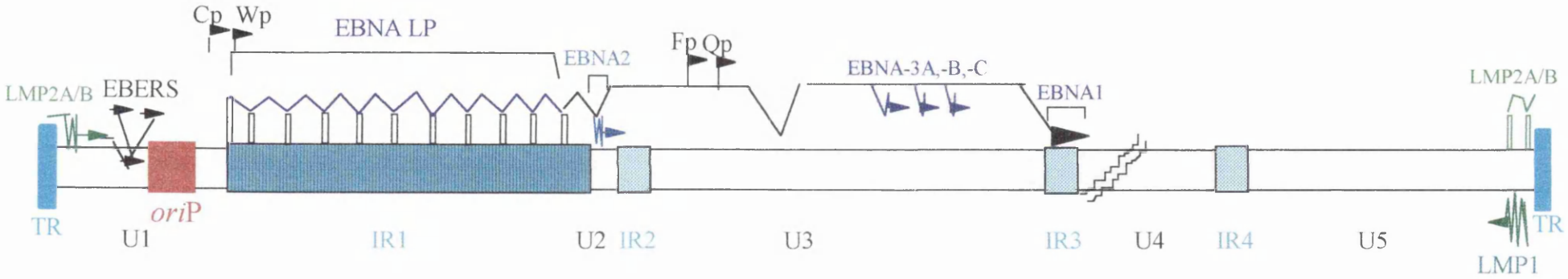


Fig. 1.1 Diagramatic representation of the B95-8 EBV genome (map not to scale), illustrating the promoters (arrowhead) and structures of the known latency associated mRNAs and the two non-coding small EBV RNAs (EBERs, transcribed in latent or productive infection) .The internal repeats (IR1 to IR4) (turquoise), and the largely unique sequence domains (U1 to U5) are noted, as are the terminal repeats (TR, blue). *ori*P, the latent origin of replication, is indicated by a red box. The LP and EBNA mRNAs (purple) are spliced from a single transcript which originates from the promoters Cp or Wp; the position of the EBNA 1-specific promoter Qp active during type I and II latency, and Fp promoter active in the lytic cycle, are represented by arrows. The open reading frame for LMP1 (dark green) lies in the opposite orientation to the EBNAs. LMP2A/B (light green) ORFs span the terminal repeats.

showed large regions with colinear, although distant, identity at the predicted protein level. The relatively conserved domains encode for EBV genes that function in early or late stages of lytic infection (Davison and Taylor, 1987). However, the EBV genes expressed in B lymphocyte latent infection have no detectable similarity to other herpes virus genes and may have arisen in part from cellular DNA (Heller *et al.*, 1985; Moore *et al.*, 1990; Henderson *et al.*, 1993). *In vitro*, EBV infection is largely restricted to B lymphocytes. At least 10% of the infected normal resting B cells are capable of long-term growth *in vitro* as lymphoblastoid cell lines (LCLs). Most of our knowledge of EBV latent and lytic infection is based on infection of B lymphocytes *in vitro*, while *in vitro* infection of epithelial cells is largely abortive (Sixbey, 1983).

A large number of viral mRNA species (~30 early mRNAs and ~30 late mRNAs) are expressed during lytic infection. In contrast, in LCLs EBV expresses six different nuclear proteins (EBV nuclear antigens, EBNAs): [EBNA 1, 2, 3A, 3B, 3C and LP (leader protein) (or alternative nomenclature EBNA 1, 2, 3, 4, 6 and 5)]; two different latent integral membrane proteins LMP1 and LMP2 (A and B forms which result from differential promoter usage) [or alternative nomenclature LMP1 and TP (1 and 2)]. Two small non-polyadenylated RNAs or EBERs (1 and 2) are also expressed. In addition, a set of 3' coterminal spliced mRNAs located at U5 and transcribed through the *Bam*HI A fragment of EBV have been detected at high levels in NPC. They have subsequently been found to be present at lower levels in latently infected B cells (Smith *et al.*, 1993). More recently, these transcripts were shown to encode a number of proteins that are expressed in latently infected cells and in tumours (Fries *et al.*, 1997).

EBV can establish different types of latency associated with malignancy, each of which is characterised by the differential expression of the group of latency genes and referred to as types I, II and III (Table 1.1). In type III latency, usually found in EBV infected LCLs and type III BL cell lines, all EBV latent genes are expressed (Hudson *et al.*, 1985; Bodescot *et al.*, 1987). Type III latency is characteristic of immunoblastic B cell lymphoma that arises in AIDS patients and artificially

Table 1.1 Epstein-Barr virus infection states, viral gene expression and associated disorders

Latency stage	**Associated disorders**	**EBV gene expression**
I	Burkitt's lymphoma	EBNA 1, EBERs
II	Nasopharyngeal Carcinoma, Hodgkin's disease and possibly nasal Tcell lymphoma	EBNA 1, LMP1, LMP2A, 2B *Bam*HI A transripts, EBERs
III	Immunoblastic B cell lymphomas of immuno-suppressed individuals	EBNA 1, 2, 3A, 3B, 3C, LMP1, LMP2, EBERs
Lytic (early) cycle	Oral hairy leukoplakia	EBNA 1, LMP1, LMP2A, 2B, BZLF1

immunosuppressed individuals. In type I and II latency, the expression of nuclear antigens is restricted to EBNA 1 (Rowe *et al.*, 1986). BL biopsy cells and type I cell lines (latency I) express only EBNA 1 and the EBERs (Rowe *et al.*, 1987). Type II latency (characterised by NPC and Hodgkin's disease) differs from latency I in the additional expression of variable combinations of LMP1, LMP2A and LMP2B and the *Bam*HI transcripts (Brooks *et al.*, 1992; Kerr *et al.*, 1992). However, more recently another type of EBV latency has been identified *in vivo*, in resting B lymphocytes of healthy individuals, in which LMP2A is the only EBV latent protein expressed and possibly EBNA 1 persists (Miyashita *et al.*, 1997).

During infection of B cells in culture EBNA LP and EBNA 2 are the first viral proteins to be expressed. EBNA LP makes an important, but not essential contribution to the outgrowth of B cells in culture (Hammerschmidt and Sugden, 1989). Association of EBNA LP with Rb and p53 has been suggested based on *in vitro* biochemical interactions (Szekely *et al.*, 1993). Expression of EBNA LP in transgenic mice, using the metalothionein promoter, was directed mainly to the heart, liver and kidney with no discernible effect on tumour development or incidence. However, the transgenic mouse heart phenotype was difficult to relate to gene function and even more to a possible role in EBV associated diseases (Huen *et al.*, 1993).

EBNA 2 transactivates viral genes (LMP1 and LMP2B) as well as upregulates certain cellular genes (*CD21*, *CD23* and *c-fgr*) (Abbot *et al.*, 1990; Calender *et al.*, 1990; Cordier *et al.*, 1990; Fahraeus *et al.*, 1990; Knutson, 1990; Woisetschlaeger, 1991; Zimber-Strobl *et al.*, 1991; Jin and Speck, 1992; Ling *et al.*, 1993; Zimber-Strobl *et al.*, 1993). EBNA 2 has been shown to exert its transactivating function through an interaction with the recombination binding protein RBP-Jk or CBF1, the homologue of *Drosophila* suppressor of Hairless (Henkel *et al.*, 1994; Zinber-Strobl e*t al.*, 1994; Hsieh and Hayward, 1995). Activated (as well as mutant) cellular Notch protein binds to and activates CBF1 (Hsieh *et al.*, 1997). The similarities between the Notch-CBF1 and EBNA 2-CBF1 interactions are suggestive of a model in which EBNA 2 could mimic constitutive Notch signalling to

block differentiation (Hsieh *et al.*, 1996; 1997). Moreover, mutations of *Notch* family genes that result in constitutive Notch signalling have been identified in a number of tumours (reviewed in Wilson, 1997). Therefore EBNA 2 may act in a similar way in tumour development. In addition, *c-myc* expression is also up-regulated in primary B-lymphocyte infection at the time EBNA LP and EBNA 2 are expressed (Alfieri *et al.*, 1991). Moreover, EBNA 2 was shown to transactivate *p53* through induction of NFκB activity (Chen and Cooper, 1996). In contrast, in BL cells heterologous EBNA 2 expression down-regulates expression of the translocated *IgH-c-myc* gene, presumably through the IgH sequences (Jochner *et al.*, 1996).

The EBNA 3 mRNAs are some of the least abundant EBNA mRNAs within the EBV infected B cell and have only a few molecules per latently infected cell (Petti, 1988; Robertson and Kieff, 1995; Kieff, 1996). EBNA 3A and 3C are essential for growth transformation and the immortalisation process of B cells in culture (Tomkinson and Kieff, 1992). EBNA 3C upregulates CD21 and may also be involved in the regulation of LMP1 expression (Allday *et al.*, 1994; Sample and Parker, 1994; Marshal *et al.*, 1995; Bain *et al.*, 1996). EBNA 3B although dispensible for growth transformation, upregulates the expression of the cytoskeletal protein Vimentin as well as CD40 and can downregulate the BL associated antigen CD77 (Silins *et al.*, 1994). EBNA 3 proteins were shown to repress EBNA 2 mediated transactivation of the viral LMP2 promoter and possibly LMP1 promoter (Allday *et al.*, 1994; Le Roux *et al.*, 1995). They were also shown to interact with RBP-Jk (Robertson *et al.*, 1996a).

LMP1, which is able to transform fully certain rodent fibroblast cell lines, is essential for immortalisation of resting B lymphocytes in culture and is required for continued proliferation of the resulting LCLs (Wang *et al.*, 1985; Baichwal and Sugden, 1988; Peng and Lundgren, 1992; Kaye *et al.*, 1993; Kilger *et al.*,1998). In addition, LMP1 induces epithelial hyperplasia in transgenic mice when expressed in the epidermis and induces B cell lymphoma when expressed in lymphoid tissues (Wilson *et al.*, 1990; Wilson, 1997; Kulwicht *et al.*, 1998). EBV negative B cells transfected *in vitro* with LMP1 expression vectors resemble B cells that are

proliferating in response to antigen or mitogen activation. They have increased expression of CD23, CD39, CD40, CD44 and cell adhesion molecules such as CD54 (ICAM1), LFA1 and LFA3, as well as increased cytokine production. LMP1 expression in B cells prevents apoptosis through the activation of anti-apoptosis proteins such as Bcl2, Bclx, Mcl1, p53 and A20 (Wang *et al.*, 1990; Henderson *et al.*, 1991; Laherty *et al.*, 1992; Rowe *et al.*, 1994; Chen and Cooper, 1996; Fries *et al.*, 1996; Wang *et al.*, 1996). However, ectopic overexpression of LMP1, using transfection assays in either B or epithelial cell lines, can inhibit differentiation or result in cytotoxic effects (Hammerschmidt *et al.*, 1989; Eliopoulos *et al.*, 1996; Floettman *et al.*, 1996; Lu *et al.*, 1996). The mechanism of action of LMP1 is beginning to be elucidated. LMP1 has been shown to engage similar signalling pathways to CD40 ligation (Eliopoulos *et al.*, 1996; Gires *et al.*, 1997; Kilger *et al.*, 1998). LMP1 activates NFκB, Janus kinase (JAK) and signal transducers and activators of transcription (STAT) and possibly mitogen activated protein kinase (MAPK) pathways all of which lead to changes in gene expression (Hammarskjold and Simurda, 1992; Herrero *et al.*, 1995; Kieser *et al.*, 1997; Eliopoulos and Young, 1998; Eliopoulos *et al.*, 1999; Roberts and Cooper, 1998). Nevertheless, it was recently demonstrated that LMP1 signalling is distinct from CD40 (Floettman *et al.*, 1998). LMP1 activation of the NFκB transcription factor was shown to be mediated through association with tumour necrosis factor receptor associated factors (TRAFs) and tumour necrosis factor receptor associated death domain (TRADD) (the latter of which does not associate with CD40, but rather another family member, TNFR) (Mosialos *et al.*, 1995; Devergne *et al.*, 1996; Izumi and Kieff, 1997).

LMP2 is important for viral persistence and efficient B cell immortalisation (Qu and Rowe, 1992, Chen *et al.*, 1995, Brielmeier *et al.*, 1996, Caldwell *et al.*, 1998). LMP2A is phosphorylated by Syk protein tyrosine kinase and Src family protein kinases and possibly by MAPK (Burhkardt *et al.*, 1992, Fruehling and Longhecker, 1997; Panousis *et al.*, 1997). LMP2A blocks B cell receptor (BCR) signal transduction and prevents activation of lytic replication in EBV-transfected B cells *in vitro* (Longnecker and Miller, 1996). It was recently demonstrated that LMP2A

mimics BCR signalling and provides a survival signal to progenitor and peripheral receptorless B cells in transgenic mice (Caldwell *et al.*, 1998). A possible function of LMP2A in epithelial cells was recently suggested where LMP2A interacts with cell adhesion-initiated signalling pathways involving Csk, a negative regulator of Src kinase (Scholle *et al.*, 1999).

Another two viral gene products in latent infection are BHRF1 and BARF0 RNAs. BHRF1 is abundantly expressed very early in EBV lytic replication cycle (where BHRF1 appears to have an antiapoptotic effect) (Austin *et al.*, 1988; Henderson *et al.*, 1993). Although BHRF1 is expressed during the lytic cycle, BHFR1 transcripts have been detected in latent infected cells (Pearson *et al.*, 1987; Austin *et al.*, 1988). BHRF1 has sequence similarity with the *bcl2* protooncogene and it has been postulated that it may possess Bcl2-like activities. Nevertheless, deletion mutants showed that BHRF1 is nonessential for growth transformation of B cells and for virus replication and release from these cells in culture (Marchini *et al.*, 1991; Lee and Yates, 1992).

BARF0 RNA and protein are expressed in EBV infected B cells and epithelial cells. The protein has also been detected in protein extracts of NPC and BL biopsies and in a latency type I BL cell line (Karran *et al.*, 1992; Fries *et al.*, 1997). Moreover, BARF0 like EBNA 1 is not recognised by HLA class I-restricted EBV specific cytotoxic T cells (CTLs) (Khanna *et al.*, 1992, Levitskaya *et al.*, 1995). EBV recombinants lacking the entire BARF0 RNA-encoding region suggested that it is dispensible for latent infection in primary B cells or for growth transformation of B cells into LCLs (Robertson *et al.*, 1994).

EBV-encoded RNAs 1 and 2 (EBERs) are small nuclear RNAs transcribed by the RNA polymerase III system (Clarke *et al.*, 1992). They are expressed abundantly in latently infected cells. To date, the biological functions of EBERs remain unclear, although they were found to be associated with cellular proteins (Clemens, 1994 and references therein). Nevertheless, EBV recombinants lacking the EBERs demonstrated that EBERs are dispensable for the B cell immortalising function of EBV (Swaminathan *et al.*, 1991). However, it was recently suggested that

EBERs have oncogenic functions in BL cells which are possibly mediated through up-regulation of Bcl2 protein (Komano *et al.*, 1999).

EBNA 1 has an established role in the replication and maintenance of the episomal form of the EBV genome (Lupton and Levine 1985; Yates *et al.*, 1985; Chittenden *et al.*, 1989; Leight and Sugden, 2000 and references therein). It was recently demonstrated that EBNA 1 is needed for efficient, stable infection by the virus (Lee *et al.*, 1999). EBNA 1 expression is detected in all EBV associated tumours. While EBNA 1 does not show classical oncogenic activities in cell culture assays, transgenic mice that express EBNA 1 in the B cell compartment develop monoclonal B cell lymphoma (Wilson *et al.*, 1992; 1996). The mechanism of its oncogenesis has now begun to be elucidated (the topic of this thesis).

1.2 BL

Burkitt's lymphoma (BL) is a childhood cancer, which occurs throughout the world. It is a malignant lymphoma composed of a clonal population of undifferentiated B lymphocytes, in which EBV was first described (Epstein and Achong, 1986). BL occurs in two forms: endemic and sporadic. The endemic BL (eBL) is found in equatorial Africa and New Guinea, areas with high incidence of malaria which is postulated to be a contributing factor in disease onset. It has been suggested that the immunosuppression caused by malarial infection is likely to increase the chances of BL development. The sporadic world-wide form of BL (sBL) represents less than 3% of childhood cancers in Europe and the USA. However, in all regions it represents about 35% of malignant non-Hodgkin's lymphomas.

There is a strong association between eBL and EBV. All patients with eBL have antibodies to the viral capsid antigen (VCA) indicating that all individuals with the tumour have been infected by the virus. The evidence implicating EBV as factorial in eBL is strong, in that whilst 97% of the BL tumours in highly endemic areas carry the EBV genome, less than 1% of the total B cell population from the malaria carriers is detectably infected (Moss *et al.*, 1983). EBV association with sBL is less clear with only approximately 20% of sBL cases being EBV positive.

However, recent studies on EBV association with sBL showed viral DNA integration in some sBL cases which is suggestive of a broader involvement of EBV in the onset of sBL than was previously assumed (Razzouk *et al.*, 1996).

BL tumours display characteristic translocations involving the *c-myc* locus on chromosome 8 (8q24) and either the IgH locus on chromosome 14 (the "common" translocation seen in up to 80% of tumours) or one of the light chain loci on chromosomes 2 or 22 (the "variant" translocations seen in 10-20% of cases) (reviewed in Cory, 1986). All these translocations result in the de-regulation of *c-myc* expression. Although t(8:14) translocation is the most common one, in eBL and sBL there is a great heterogeneity of breakpoints with respect to both loci. For example, in sBL the breakpoint on chromosome 8 occurs in the region of *c-myc* intron or exon 1, which contains regulatory elements of *c-myc* expression. Hence, disruption of the regulatory function directly leads to the de-regulation of c-Myc expression. In these translocations the position of the breakpoint within the IgH locus on chromosome 14 most often involves the switch (S) region of the gene. Rodent plasmacytomas carry a *c-Myc* translocation similar in nature to that seen in sBL in which case the IgH enhancer on chromosome 12 becomes juxtaposed with a truncated *c-myc* gene on chromosome 15. In eBL tumours, the breakpoint on chromosome 8 occurs at some distance 5' to the *c-myc* gene and translocations tend to involve the intronic enhancer/J region of the IgH locus on chromosome 14. This variation in the site of breakpoints within the IgH locus may reflect the different stage of cells involved in each tumour. For example, the translocations involving the IgH J region, as in eBL, are likely to occur in immature pro-B cells during V-D-J joining. The translocations involving S regions, as in sBL, imply a later event occurring in mature B cells involved in immunoglobulin class switching which is further supported by the observation that sBL B cells, but not eBL B cells, secrete IgM. In addition, BL tumours are monoclonal in terms of immunoglobulin rearrangements. Most of them are IgM+IgD$^-$ ($\mu\kappa$ or $\mu\lambda$) but a minority appear to be derived from cells which have already switched to IgG or IgA production albeit with appropriate light chain restrictions. These tumours display a remarkably consistent

cell surface profile characterised by the co-expression of CD10 (CALLA) and the glycolipid moiety CD77 (BLA) which are used as diagnostic markers. Moreover, they lack B cell activation antigens such as CD23, CD30, CD39 and CD70, as well as ICAMI and LFA3 which are needed in cell adhesion to T cells and B7 necessary for T cell activation (Rowe *et al.*, 1985; Gregory *et al.*, 1988, 1990).

BL has a complex multistep pathogenesis involving deregulated *c-myc* expression and EBV as major contributing factors. However, the relative contribution of these factors and their order in the sequence of events is still unclear.

The mechanisms whereby EBV contributes to eBL development are still not fully understood. EBV is directly involved in B cell transformation in culture. Therefore, the potent growth transforming ability of the virus suggests that it could act early in the lymphomagenic process by expanding the population of cells at risk for translocation (Klein and Raab-Traub, 1987; Crawford *et al.*, 1993). Alternatively, it could act on B cells carrying a translocation, increasing the likelihood of accumulation of further changes either by extending the survival capacity of the cells or by driving expansion of the translocation positive clone. The known transforming activities of LMP1 could be important in the development of eBL. However, EBNA 1 is the only viral protein, along with the EBERs, consistently expressed in EBV-positive BL biopsies. Therefore, the EBV role in the development or at least the maintenance of BL phenotype could be mediated through EBNA 1 action and/or the EBERs. However, genetic deletion assays have shown that the EBERs are dispensable for transformation of B cells (Swaminathan, 1991). Conversely, transgenic mice for EBNA 1 succumb to monoclonal B cell lymphoma (Wilson *et al.*, 1992; 1996). This renders EBNA 1 as the main viral candidate oncoprotein performing a critical function at least in the maintenance of BL.

1.3 EBNA 1

EBNA 1 is the EBV protein that is present in most infection states and necessary for latent EBV infection. EBNA 1 is consistently expressed in EBV associated malignancies as described above (Table 1.1).

EBNA 1 is a multifunctional protein which plays a crucial role in the latent phase of EBV infection, including activation of DNA replication and maintenance of the episomal form of the viral genome during cell division (Leight and Sugden, 2000). This is mediated by EBNA 1 protein binding sites at the origin of replication *ori*P. In addition, EBNA 1 influences the expression of latent gene products, including autoregulation (Yates *et al.*, 1985; Sample *et al.*, 1986).

I) Viral and Cellular regulation of EBNA 1 expression

The open reading frame for EBNA 1 as defined on the physical map of the viral DNA is the *Bam*HI K fragment right frame 1 (BKRF1, 107950-109872 map units of B95.8 strain) (Summers *et al.*, 1982; Baer *et al.*, 1984). An analysis of cDNAs encoding EBNA 1 indicated that the message is a highly spliced bicistronic mRNA of 3.7Kbp which is expressed from the latent promoters in *Bam*HI-C (Cp) and *Bam*HI-W (Wp) that lie 90 and 80 Kbp, respectively, upstream of the EBNA 1 ORF (Speck and Strominger, 1985) (Fig. 1.1). The *Bam*HI-W promoter is constitutively active in B cells and is used on initial infection to drive expression of all EBNAs with individual transcripts being generated through differential splicing (Schlager *et al.*, 1996). EBNA 1 and EBNA 2 then activate the *Bam*HI-C promoter which drives the latency III EBNA expression seen in infectious mononucleosis.

In latency I (exemplified by BL) and latency II (exemplified by NPC) cells Wp and Cp are inactive and EBNA 1 expression is driven from a distinct promoter lacking a TATA box (Qp), located in the *Bam*HI-Q region (Schaefer *et al.*, 1995). Methylation of Cp is the major factor in loss of Cp activity (Robertson *et al.*, 1996; Schaefer *et al.*, 1997). Qp activity is regulated by two important elements. First, an interferon-stimulated response element (ISRE) which lies upstream of the Qp transcriptional start site and is bound by members of the interferon regulatory factor (IRF) family (Nonkwelo *et al.*, 1997; Schaefer *et al.*, 1997; Zhang and Pagano, 1997). The second element is called the Q locus consisting of 2 (lower affinity relative to *ori*P) EBNA 1 binding sites, located immediately downstream of the start site (Ambinder *et al.*, 1990). Although binding of EBNA 1 to the family of repeats (FR)

region of *ori*P leads to the up-regulation of the gene expression from Cp, EBNA 1 binding to this region within *Bam*HI-Q leads to down-regulation of viral gene expression (Sample *et al.,* 1992; Tsai *et al.,* 1995), speculated to be due to binding at a site 3' of the RNA start possibly inhibiting transcriptional progress. This repression appears to override the effects of the positive regulatory elements that lie upstream of the Qp start site.

EBNA 1 mRNA expression from Qp during type I latency is also regulated by cell cycle proteins. A member of the E2F family of cellular transcription factors (E2F1) activates Qp in the presence of EBNA 1 by competing with it for the partially overlapping binding sites (EBNA 1 autorepression domain) on the Q locus (Sung *et al.*, 1994; Davenport and Pagano, 1999). However, Ruf and Sample (1999) proposed that the E2F1 on its own does not a play significant role in the regulation of EBNA 1 expression but its primary function may be to target transcriptional repressors, such as pRb to Qp, to silence EBNA1 expression in resting B cells. Repression of Qp by pRb is mediated through variant E2F binding sites QpI and QpII. Nevertheless, it was recently demonstrated that Qp is positively regulated by cellular JAKs and STATs (Leonard and O'Shea, 1998; Chen *et al.*, 1999).

EBNA 1 is the only EBNA that continues to be produced during lytic infection. The lytic mRNA begins at a *Bam*HI-F promoter (near 62.2kb map units of B95.8 strain) immediately upstream of Qp, which was previously thought to be the active promoter for EBNA 1 expression in type I and type II latency (Lear *et al.*, 1992; Schaefer *et al.*, 1991). However, the Fp promoter was recently characterised as an exclusive lytic promoter (Schaefer *et al.*, 1995). It is worth noting that a cellular protein LR1 which is a sequence specific DNA binding protein, has been identified as a key regulator of transcription from the Fp promoter (Bulfone-Paus *et al.*, 1995).

II) EBNA 1 structure

EBNA 1 is a phosphoprotein with a high proline content, is charged and has an apparent molecular weight of 76KDa (varies with different repeat lengths) (Hennessy *et al.*, 1983; Hennessy and Kieff, 1983). The EBNA 1 641 amino acid

(aa) polypeptide is composed of 3 distinct regions with functional subdomains (Fig. 1.2).

a) The N-terminal basic region (1-89aa) is a short sequence contributing to some of the functions of EBNA 1. Interestingly, the EBNA 1 N-terminus shows internal polypeptide similarities with the myelin basic protein, implicated in multiple sclerosis (aa 64-68 and 74-78 in the EBNA 1 sequence) (Sumaya *et al.*, 1980). Similarly it has been noted that EBNA 1 is identical over a doublet repetitive sequence between 40 and 49 aa in its N-terminus with a small ribonucleoprotein, the D polypeptide (SmD) (Sabbatini *et al.*, 1993).

b) This is followed by the glycine/alanine (Gly-Ala) repeat, (from 90 to 328 aa), a well conserved non-random gly-ala, gly-gly-ala and gly-gly-gly-ala array, that could form open coil tertiary structures and might participate in intramolecular interactions such as formation of multimeric viral and/or viral/cellular protein complexes. The size of EBNA 1 varies considerably in individual EBV-containing cell populations (from 69 to 94 KDa, by SDS-PAGE) and this depends on the number of these repeats within the protein. Importantly this region confers the ability to evade the host immune response. It was recently demonstrated that these internal repeats in EBNA 1 generate an inhibitory signal that acts in *cis* to interfere with antigen processing and MHC class I presentation (Levitskaya *et al.*, 1995; Mukherjee *et al.*, 1998). The majority of antigens presented via the MHC I pathway are subject to ATP-dependent ubiquitination and degradation by the proteasome. *In vitro* experiments using EBNA 1 deletion mutants and chimeric proteins EBNA 1/EBNA 4 containing the gly-ala repeats, suggested that the gly-ala repeat may affect MHC I restricted responses by inhibiting antigen processing via the ubiquitin/ proteasome pathway (Levitskaya *et al.*, 1997).

Flanking the Gly-Ala repeat, are Arg-Gly rich sequences that resemble the 'RGG' motifs found in some RNA-binding proteins (Snudden *et al.*, 1994).

c) The unique C-terminal region (328-641aa) contains most of the protein's functional domains. It contains a proline-arginine rich region (327 to 377 aa) adjacent to the Gly-Ala repeat, functionally defined also in the N-terminal region, which

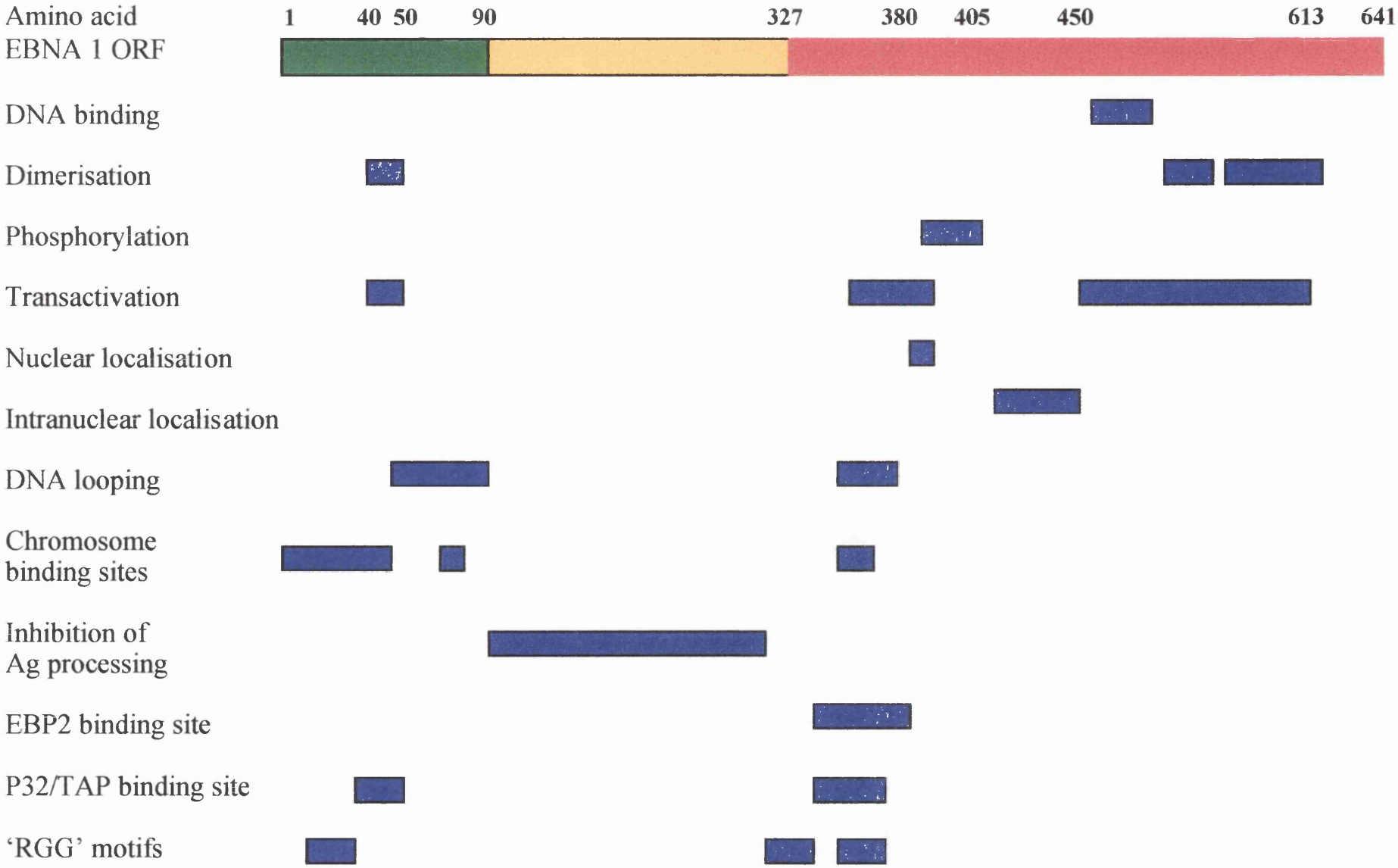


Fig. 1.2 Schematic representation of the functional domains of EBNA 1. The EBNA 1641 amino acid sequence is divided into three regions: the N-terminus, amino acids 1-89 (green); the Gly-Ala repeats, amino acids 90-327 (yellow) and the unique C-terminus, amino acids 328-641 (red). The blue boxes bellow indicate the location limits of known EBNA 1 functions or protein binding sites. (For gene nomenclature and references see text).

mediates interactions between EBNA 1 molecules bound to distant DNA binding sites (DNA looping or 'linking') (Goldsmith *et al.*, 1993; Mackey *et al.*, 1995; 1997; Avolio-Hunter and Frappier, 1998). Sequences between amino acid residues 379 and 387, adjacent to the one of the DNA looping domains, can function as a nuclear localisation signal (Ambinder *et al.*, 1991). It was recently demonstrated that a human cellular protein EBP2 (EBNA 1-binding protein 2) specifically interacts with EBNA 1 sequences important for plasmid maintenance. The EBP2 interacting region of EBNA 1 maps to aa 325-386 shown in Fig. 1.2 (Ambinder *et al.,* 1991) and an Arg-Gly region (determined using EBNA 1 deletion mutants) (Shire *et al.*, 1999). Another recently identified EBNA 1-interacting protein is the human cellular protein P32/TAP which specifically interacts with sequences important for DNA binding and transactivation. The C-terminal half of P32/TAP interacts with one N-terminal and one C-terminal region of EBNA 1 (aa 40-60 and aa 325-376) each of which contains arg-gly repeats (Wang *et al.*, 1997; Chen *et al.*, 1998).

Adjacent to this region there is a short serine-rich sequence that becomes phosphorylated (375-400aa) (Polvino-Boldnar, 1988). A long hydrophilic domain, from 459 to 607 aa, has sequence specific DNA binding and dimerisation activity. Mutational analyses of EBNA 1 showed that it has a bipartite dimerisation domain with residues located between 501 and 532 aa and between 554 to 598 aa, and DNA binding between residues 459 and 487 (Chen *et al.,* 1993).

The structure of the EBNA 1 DNA binding and dimerisation regions was recently solved by X-ray crystallography at 2.4Å resolution (Bochkarev *et al.*, 1995; 1996). EBNA 1 binds its DNA binding site as a dimer. The dimer is composed of two distinct DNA binding domains, the C-terminal or core domain and the flanking domain.

The core domain comprises residues 504-607 and contains the dimerisation interface. The structure of the EBNA 1 core domain is very similar to that of the DNA binding domain from the Bovine papilloma virus (BPV) protein E2, a transcriptional activator (Bochkarev *et al.*, 1995). The major difference between the proteins reside in the loop between the 2nd and the 3rd strands of the β sheet (9

additional residues in EBNA 1) which is referred to as the proline loop due to high proline content. Notably, this structure in other proteins allows heterodimerisation with other proteins of the same class and such interactions can significantly modify the function of the individual proteins involved. The EBNA 1 proline loop has sequence identity to the herpes simplex ICP4 protein and hBPM protein (human homologue of the yeast SNF2/SW12 gene product) well known transcriptional activators (Muchardt and Yaniv, 1993). EBNA 1 acts as a transcriptional activator when bound to its consensus sequence. However, EBNA 1 does not appear to belong to any previously recognised class of DNA-binding proteins (Inoue *et al.*, 1991) and has not been shown to heterodimerise.

The EBNA 1 flanking domain comprises residues 461-504 and is unique to EBNA 1 with no sequence or structural similarity to any other known proteins. It consists of a connector (aa 490-503), an α helix (aa 477-489) and an extended chain (aa 461-476). In the flanking domain α helix is oriented perpendicular to the DNA and is connected to the core domain by the connector. The extended chain enters the minor groove of the DNA and makes all of the sequence determining contacts with the DNA. *In vitro* studies using EBNA 1 truncation mutants have indicated that in addition to contributing to the DNA binding affinity and stability of EBNA 1, this minor groove extended chain inhibits the passage of replication forks through the FR element (Ermacova *et al*, 1996).

The core domain of the EBNA 1 DNA binding site undergoes very little structural change upon binding to DNA as compared to the protein's conformation in solution. In contrast, the flanking domain, although it adopts a similar structure in a dimer as it does in a monomer, its conformation changes dramatically upon DNA binding (Bochkarev *et al.*, 1996).

The DNA binding and dimerisation region is followed by a highly acidic region at the C-terminus of the protein which, although is not required for DNA replication or transactivation, appears to play a role in the segregation of the EBV episomes during cell division and thus the plasmid maintenance function of EBNA 1 (Yates and Camiolo, 1988).

III) *ori*P and EBNA 1 binding

The role of EBNA 1 in the replication and maintenance of the viral episome is mediated through the latent origin of replication *ori*P (Fig. 1.3) (Yates *et al.*, 1984; 1985; Aiyar *et al.*, 1998). *Ori*P consists of two regions containing multiple EBNA 1 binding sites. The first, and highest affinity site consists of 20 tandem direct 30bp repeats, called FR, and is mapped at about 7Kbp from the left end of the genome. The second highest affinity site, located 980bp to the right of the FR, contains two symmetrically positioned EBNA 1 binding sites within a 65bp dyad symmetry (DS) that can form a 31bp stem and a 3bp loop structure and two additional binding sites flanking the symmetrical sequence located at the base of the stem-loop sequence.

The FR and DS sequences comprise an EBNA 1 dependent *cis*-acting element for EBV episomal maintenance and replication in the latently infected cells, although initiation of the replication events have been detected external to *ori*P (Little and Schildkraut, 1995). Only 6-12 copies of the 30bp-repeated sequence and 114bp containing the DS region are required for these functions (Reisman *et al.*, 1985; Chittenden *et al.*, 1989).

FR is required for efficient replication *in vivo* and for the stable maintenance of plasmids in cell lines (Reisman *et al.*, 1985). In the presence of EBNA 1, FR functions as a transcriptional enhancer element in either orientation for RNA polII-transcribed genes (Reisman and Sugden, 1986). This enhancement appears to function in cells in which the Cp promoter is active, suggestive of an autoregulation mechanism for EBNA 1 expression (Middleton *et al.*, 1991). Cp also regulates the transcription of the other EBNAs during EBV latency and therefore EBNA 1-FR interaction may modulate viral gene expression. In addition, in cells in which the Fp promoter is active there appears to be a requirement for a *cis* regulatory element within EBNA 1 itself, again suggestive of autoregulation and thus probably strict expression control (Reisman and Sugden, 1986).

Gahn and colleagues (1989) found that FR acts both as a replication fork barrier and as a replication termination site. As a result, replication initiation from *ori*P although usually bi-directional proceeds predominantly unidirectionally. The

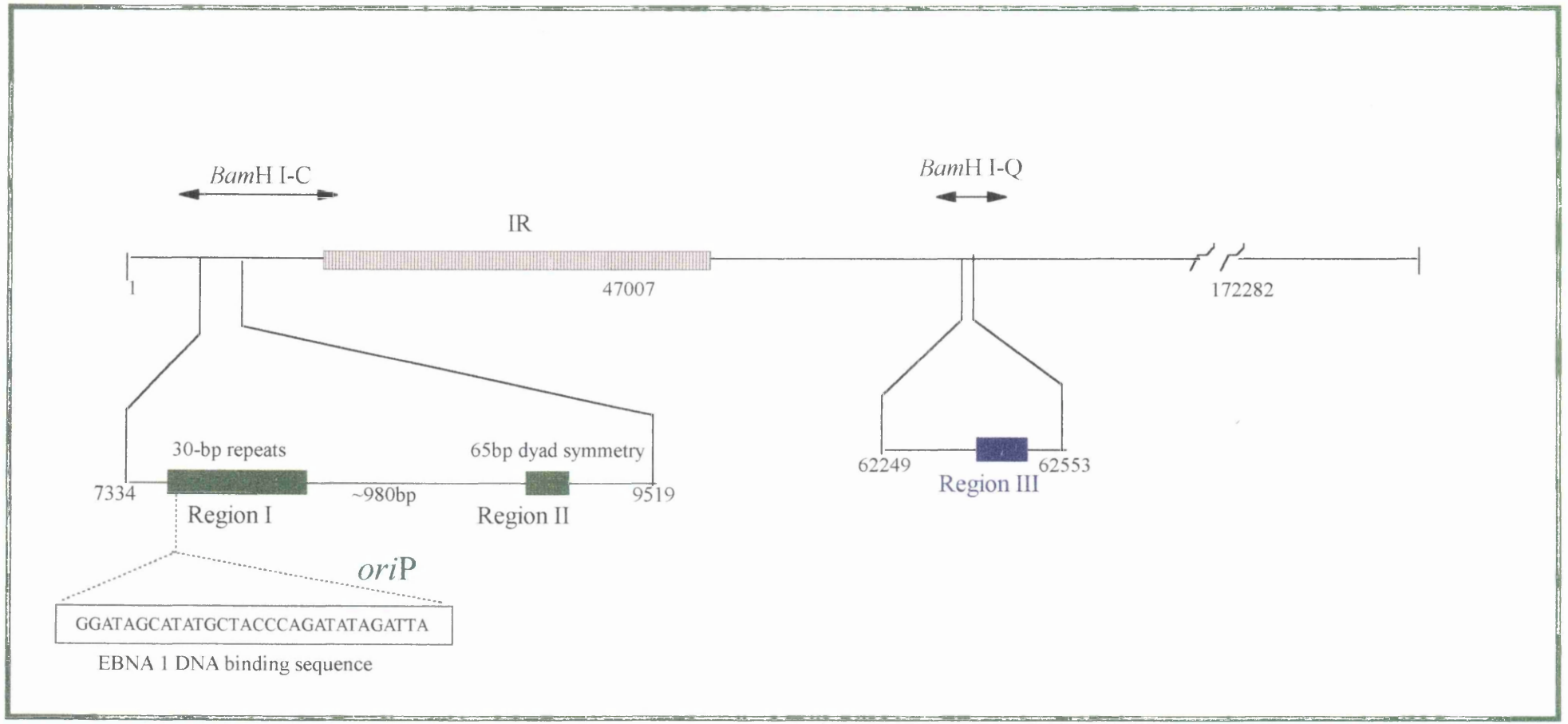


Fig. 1.3. Location of the EBNA 1 binding sites in the EBV genome (not to scale). *ori*P (green) located in the *Bam*HI-C region, contains two EBNA 1 binding sites, the family of repeats (FR, region I) and the dyad symmetry (DS, region II). The third EBNA 1 binding site (purple) is located in the *Bam*HI-Q region. The consensus sequence of the 30bp repeats is shown.

fork that moves towards the FR stops in the repeats, while the fork moving in the opposite direction progresses around the plasmid to meet the fork stalled in the FR repeats. In the presence of full length EBNA 1 protein, accumulation of replication forks stalled at the FR is enhanced. Deletion analyses revealed that the complete DNA-binding and dimerisation region of EBNA 1 is sufficient to elicit the pausing of replication forks, whereas deletion of a portion of the DNA binding domain abrogates the ability of EBNA 1 to stop replication forks (Platt *et al.*, 1993; Ermakova *et al.*, 1996). Replication forks stall at the FR in different EBV genomes with variable termination levels, among different cell lines. Thus, it can be suggested that different EBV strains may differ in the regulation of termination events (Little and Schildkraut, 1995).

Genetic and biochemical analyses have determined that DS contains, or is close to, the site at which DNA synthesis initiates (Wysokenskie and Yates, 1989). The dyad repeat is absolutely required for episomal replication. DS can partially denature, forming bubble and cruciform structures, as measured by nuclease sensitivity assays (Orlowski and Miller, 1991; Williams and Kowalski, 1993). Denaturation of the dyad region is effected by EBNA 1 binding (Hearing *et al.*, 1992). DS (as described above), contains 4 binding sites for EBNA 1 dimers termed 1-4 in order of their position, which differ in sequence and EBNA 1 binding affinity. Sites 1 and 4 have higher affinity for EBNA 1 than do sites 2 and 3 (Ambinder *et al.*, 1990; Summers *et al.*, 1996). However, all four binding sites together are not required for origin activity. Plasmids that lack binding site 1 replicate, although less efficiently than plasmids containing an intact DS region. Deletions impinging upon binding sites 2 and 4 abrogate the ability of mutated plasmids to replicate extra-chromosomally or result in abnormally large replication products of unknown structure (Chittenden *et al.*, 1989). Without the FR, the DS is inefficient in enhancing transcription or in enhancing plasmid replication or persistence in the presence of EBNA 1.

Two cellular DNA binding proteins (OBPs) have been reported to compete with EBNA 1 to bind *ori*P (Wen *et al.*, 1989; Zhang and Nonoyama, 1994). This

competition between EBNA 1 and OBPs may modulate the stability of the EBV genome as plasmid and /or regulate the expression of the EBNAs from Cp promoter.

IV) Properties and functions of EBNA 1

EBNA 1 is a double-stranded and single-stranded DNA-binding protein and is phosphorylated on serine residues with all the phosphorylation sites lying in the C-terminal domain. Protein phosphorylation-de-phosphorylation has for long been known to be a mechanism for regulating the function of proteins. However, the effects of phosphorylation of EBNA 1 on any of its activities have not been reported (Hearing *et al.*, 1985). Single stranded DNA-binding proteins have been implicated in key cellular processes such as DNA replication and genetic recombination. In general, this group of proteins binds to single stranded DNA irrespective of nucleotide sequence and binding results in a highly extended structure that may affect the ability of other proteins to interact with the nucleic acid. However, EBNA 1 has sequence-specific DNA binding properties.

Most of the known activities of EBNA 1 require that EBNA 1 maintain its ability to bind DNA and are mediated through EBNA 1 binding sites within the EBV genome (Yates and Camiolo, 1988; Polvino-Bodnar and Schaffer, 1992; Middleton and Sugden, 1994).

The interaction of EBNA 1 with the FR and the DS sites is co-operative, indicating facilitative alteration of the template and homotypic stabilising interaction between EBNA 1 molecules. Biochemical and electron microscopic studies indicate that EBNA 1 binding to the FR and the DS in the same *oriP*, results in such structures that lead to bending of the DNA, distortion of the duplex and looping out of the sequence between them bringing the two elements into close proximity (Frappier and O'Donnell, 1991a; 1992; Middleton and Sugden, 1992; Frappier *et al.*, 1994; Harrison *et al.*, 1994; Bochkarev *et al.*, 1996). One type of homotypic interaction involves interaction at a distance between EBNA 1 complexes assembled on the *oriP*. A similar EBNA 1-EBNA 1 interaction occurs between EBNA 1 molecules bound to FR and DS elements on different DNA molecules which causes

multiple DNA molecules to be linked together through a central EBNA 1 complex. The formation of this macromolecular complex is due to the ability of EBNA 1 to recognise both of these elements and to form homopolymers after associating with the templates (Frappier and O'Donnell, 1991b; Su *et al.*, 1991; Goldsmith *et al.*, 1993).

Initially, deletion analyses indicated that the amino acids (336 to 450) responsible for this EBNA 1 action are located on the N-terminal side of its dimerisation domain (Harrison *et al.*, 1994). Nevertheless, recent studies using overlapping internal deletion mutants indicated that two regions of EBNA 1 can cause DNA linking, aa 40-100 and aa 327-377. However, the stabilities of the linked complexes formed by the two regions vary dramatically; only complexes formed through the latter region are stable as shown by glycerol gradient sedimentation analysis (Mackey and Sugden, 1997; Avolio-Hunter and Frappier, 1998). However, the functional significance of the different stabilities of linked DNA complexes is not clear at present. Biochemical and EM studies suggest that only DNA-bound forms of EBNA 1 can interact through the DNA looping domain (Frappier and O'Donnell, 1991a; 1991b; Goldsmith *et al.*, 1993; Laine and Frappier, 1995). This may reflect a requirement for EBNA 1 to bind DNA, indicating that the looping domain must contact DNA and change its conformation in order to be active in mediating protein interactions.

DNA looping and linking mediated by the EBNA 1 looping domain (aa 327-377) has been shown to stabilise the EBNA 1 binding to the DS element, but the *in vivo* function of these interactions has not yet been determined (Su *et al.*, 1991; Goldsmith *et al.*, 1993; Frappier *et al.*, 1994).

To date, no known enzymatic activity has been associated with EBNA 1 (Frappier and O'Donnell, 1991b; Middleton and Sugden, 1992). Hence, EBNA 1 must recruit a number of cellular proteins essential for initiation of replication, such as proliferating cell nuclear antigen (PCNA), replication factor C (RFC), the DNA polymerase, polymerase-primase, replication protein A (RPA) and the cellular helicase. It was recently demonstrated that EBNA 1 interacts with RPA (hSSB), a

human single-stand DNA binding protein (Zhang *et al.*, 1998). RPA also interacts with a number of proteins involved in DNA metabolism including the viral proteins E2 of BPV (Li *et al.*, 1993) and EBNA 2 (Tong *et al.*, 1995) and DNA polymerase α-primase. Thus EBNA 1, through its interaction with RPA could recruit cellular replication proteins to replicate the EBV genome.

The intervening sequence between FR and DS seems to be less critical and the sequence can be altered without affecting *ori*P function. Deletions, insertions and inversions made within *ori*P indicate that neither a precise distance nor a particular orientation of the FR and DS components, relative to one another, are required for the activity of *ori*P (Reisman *et al.*, 1985; Wysokenski and Yates, 1989; Middleton *et al.*, 1994). Nevertheless, deletion mutants showed that there is a lower limit of the intervening sequence length that permits loop formation.

Determinants for the subnuclear distribution of EBNA 1, its ability to persist in cells and its ability to associate with chromosomes during mitosis reside in the C-terminal half of the protein (Yates and Camiolo, 1988; Marechal *et al.*, 1999). EBNA 1 has been shown to be invariably present on the chromosomes in metaphase preparations of mouse LTK cells and human Raji BL cells. In addition, circularised yeast artificial chromosomes containing *ori*P have been shown to interact in human cells expressing EBNA 1 (Grogan *et al.*, 1983; Harris *et al.*, 1985; Simpson *et al.*, 1996). Three independent chromosome binding sites (CBS-1, -2 and -3) on the EBNA 1 sequence that mediate EBNA 1 binding to metaphase chromosomes have been recently identified. Interestingly, two of them (CBS-1 and -2) mapped to a region that have been previously demonstrated to be required for long term maintenance of *ori*P-containing plasmids and are included in the linking/looping regions (CBS-1 aa 72-84, CBS-2 aa 328-365) (Shire *et al.*, 1999). The human cellular protein EBP2 (see section 1.3b) also interacts with specific EBNA 1 sequences, mapped within this same region (325-386 aa). Functional studies have shown that EBNA 1 mutants deficient in EBP2 binding can replicate *ori*P plasmids efficiently but are unable to maintain the plasmids after many cell generations. Therefore, EBNA 1 residues that mediate EBP2 interactions are required for the partitioning

function of EBNA 1. It can be postulated that the component of mitotic chromosomes with which EBNA 1 interacts might be EBP2. This is supported by the finding that mutations in the Arg region that disrupt EBP2 binding also have profound effects on the DNA looping or linking activity of EBNA 1 (Shire *et al.*, 1999). However, at present it is unknown whether the loss of the partition function is due to the abrogation of EBP2 binding or to loss of the DNA linking activity. This property of EBNA 1 may be important in the segregation of episomes into progeny nuclei. It is possible that through anchorage to host cell metaphase chromosomes, approximately equal numbers of EBV episomes could be segregated on each metaphase plate and hence into each daughter cell.

Using the yeast two hybrid system another EBNA 1 associated protein P32/TAP was identified (section 1.3b). This protein binds approximately the same region (aa 40-60 and aa 325-376) to which EBP2 binds and CBS-1 and -2 mapped (Wang *et al.*, 1997). P32/TAP is an evolutionary conserved eukaryotic protein that is found predominantly in the mitochondrial matrix and less in the nucleus (Matthews and Russell, 1998). P32/TAP has been implicated in splicing (Honore *et al.*, 1993), transactivation (Yu *et al.*, 1995a and b) and receptor function (Ghebrehiwet *et al.*, 1994; 1995). EBNA 1 acts as a transcriptional transactivator in the viral genome regulating transcription from EBV Qp and Cp and LMP1 promoters (Sugden and Warren, 1989; Sample *et al.*, 1992; Gahn and Sugden, 1995). In co-transfection assays, EBNA 1 can transactivate heterologous promoters when multiple EBNA 1 binding sites are present within the target plasmids (Reisman and Sugden, 1986; Wysokenski and Yates, 1989). In addition, it was recently demonstrated, using a reporter assay, that EBNA 1 promotes hepatitis C virus (HCV) replication via its transactivation function, albeit indirectly, possibly through induction of transcription of a cellular gene (Sugawara *et al.*, 1999). However, EBNA 1 does not have a specific transcriptional activation domain which was shown by different GAL4 reporter constructs (Horner *et al.*, 1995). Thus, other cellular proteins interacting with EBNA 1 may contribute to its transactivation ability. Deletion mutants lacking either of the two or both P32/TAP binding domains were unable to transactivate a

reporter containing *ori*P EBNA 1 binding sites. Therefore, it is likely that P32/TAP may contribute to EBNA 1-mediated transactivation.

EBNA 1 has been implicated in nuclear matrix attachment. It is notable that the *ori*P is contained within or immediately adjacent to the high affinity matrix attachment region (MAR) located within the *Bam*HI-C fragment of the EBV genome. This MAR consists of a ~3.5 kb region which corresponds to nucleotides 6160-9699 on the EBV B95-8 sequence (Baer *et al.*, 1984). This region contains the EBER genes (nucleotides 6629-7128) and *ori*P (nucleotides 7135-9317). It has been shown that degradation of matrix-associated DNA leaves the EBNA 1 binding sites in *ori*P still associated with the nuclear matrix (Jankelevich *et al.*, 1992). This property of EBNA 1 could be important to its role in both replication and transcriptional activation.

A further recently identified activity of EBNA 1 is that it can bind to RNA, at least *in vitro*. Arginine-glycine-containing ("RGG") motifs have been found in several proteins that interact with RNA, including those involved in post-transcriptional processing of RNA (Burd and Dreyfuss, 1994). EBNA 1 contains three potential "RGG" motifs (Fig. 1.2) located around the internal Gly-Ala-rich repetitive sequence of the protein. These motifs could be involved in the observed association between EBNA 1 and RNA (Snudden *et al*, 1994). This observation raises the possibility that EBNA 1 may function through post-transcriptional as well as transcriptional gene regulation.

EBNA 1 can therefore potentially play many roles in the cell. A combination of its DNA and RNA binding properties and repression and transactivation functions certainly qualify the protein as a potential oncogene. Despite the fact that cell culture experiments showed no evidence of EBNA 1 oncogenic activity, *in vivo* studies suggested that is highly likely that EBNA 1 plays a dominant role in cellular immortalisation, at least in B cells, and possibly even in tumourigenesis as experiments in transgenic mice suggest (Wilson and Levine, 1992; Wilson *et al.*, 1996). However, the mechanism of action of EBNA 1 is still to be elucidated.

1.4 B cell oncogenes and transgenic models of lymphoma

Tumourigenesis is a multistep process arising as a result of a number of genetic and epigenetic changes (Hunter, 1991). These changes, although they vary in nature in different tumours, allow the cell to evade cell cycle controls and their effects fall into common categories. These include: acquisition of growth factor independence, ability to avoid restraining signals, induction of angiogenesis, breach of surrounding tissues, escape from immunological surveillance and metastatic invasion.

Transgenic models of disease provide a powerful tool for the analysis of the effects of specific genetic changes *in vivo*. Analysis of tumour biopsies or cultured cells allow the investigation of only the endpoint events in the tumours. In contrast, transgenic models provide us with the ability to investigate pre-neoplastic changes in cells at specific stages during the process of tumourigenesis. In such systems overexpression or mutation of cellular and/or viral genes permit assessment of their oncogenic potential. In addition, inactivation of specific genes by homologous recombination is used to assess candidate tumour suppressor genes. Genes identified as having oncogenic potential encode a wide range of proteins with variable function, from cell surface receptors to cytokines and nuclear transcription factors (Adams and Cory, 1991a, 1991b; Berns, 1991; Fowlis and Balmain, 1993; Berns *et al.*, 1994; Brown and Balmain, 1995).

In assessing the oncogenic potential of cellular and viral genes it is very important to use the appropriate expression control sequences, in order to direct transgene expression in specific developmental stages or cell types. For example, the IgH (Eμ) enhancer has been used to direct expression of several genes in both B and in some cases T cells, or occasionally even other cell types (Dildrop *et al.*, 1989; Moroy *et al.*, 1990,). Eμ-*c-myc*, Eμ-*N-myc* and Eμ-*L-myc* mice develop B cell lymphomas (Adams *et al.*, 1985; Rosenbaum *et al.*, 1989; Sheppard *et al.*, 1998), Eμ-*N-ras* succumb to T cell lymphomas (Harris *et al.*, 1988) and Eμ-*H-ras* mice develop lung adenocarcinomas (Suda *et al.*, 1987). Although directed enhancer gene expression in different cell types allows the investigation of the effects of a gene in

those cells, cellular sequences flanking the transgene insertion site can affect its expression pattern.

In transgenic mouse models, the strain background is also important as it can influence the observed phenotype. For example, on a C57BL/6 or Balb/c background Eμ-*c-myc* transgenic mice develop mostly B cell lymphoma. However, in the C3H/HeJ strain, Eμ-*c-myc* mice develop predominantly T cell lymphomas (Yukawa *et al.*, 1989).

Analysis of pre-tumour tissues can yield information about the mechanism of action of the gene of interest. For example, pre-neoplastic changes observed in young mice positive for Eμ-*c-myc* agreed with c-Myc action as a nuclear transcription factor active in cell cycle progression (Adams and Cory, 1991b; Morgenhesse and DePinho, 1994; Henriksson and Luscher, 1996).

Transgenic mouse models for many tumour types are now available; amongst these are several B cell lymphomas which are particularly relevant to the work in this thesis (Adams and Cory, 1991a; Wilson, 1997 and references therein).

a) Eμ-*myc*. Many haematopoietic malignancies are characterised by specific chromosomal translocations e.g. *c-myc* translocation which juxtaposes the *c-myc* gene to an immunoglobulin locus in BL tumours, as well as in rodent plasmacytomas (reviewed in section 1.2). Evidence that the deregulated gene expression is oncogenic is derived from the transgenic mouse model. Eμ-*c-myc* mice succumb to monoclonal nodal, pre-B or B cell type lymphomas (Adams *et al.*, 1985). Recently, transgenic mice were generated in which a large (220Kb) IgH/*c-myc* translocation region carried in a yeast artificial chromosome (YAC) was introduced. Although these mice also develop monoclonal B cell lymphomas at an early age, the tumours in this case arise at non-nodal sites and consist of IgM+ B cells, similar to BL (Butzler *et al.*, 1997).

c-Myc plays an important role in promoting normal cell growth. It is a nuclear transcription factor, expressed under tight regulation in normal cells. c-Myc expression is maintained in proliferating cells and down regulated as cells pass through differentiation. The c-Myc protein exerts its function through heterodimerisation with Max, another basic region helix-loop-helix leucine zipper

protein (Amati *et al.*, 1993). Except for its role in cell cycle induction, c-Myc has been shown to induce apoptosis upon cell cycle arrest. As a nuclear transcription factor, c-Myc has a number of transcriptional targets and particularly genes involved in cell cycle regulation. One of these genes, *cdc-25A*, is essential for the transition from G1 to S phase (Hoffmann *et al.*, 1994). Its expression is stimulated by Myc/Max binding to regulatory elements in the *cdc-25A* gene. cdc-25A, is a phosphatase and display oncogenic properties in mammalian cells and like c-Myc, it co-operates with H-ras to transform normal rodent fibroblasts (Galaktionov *et al.*, 1995). Moreover, cdc-25A, like c-Myc, can induce p53-dependent apoptosis. In addition, c-Myc-driven apoptosis can be inhibited by cdc-25A antisense olinucleotides (Galaktionov *et al.*, 1996). c-Myc can also exert its oncogenic action through its ability to inhibit growth cycle arrest by repressing the expression of *gadd45*, a growth arrest promoting gene (Marhin *et al.*, 1997). A number of other cellular proteins including the transcriptional regulator YY1, the transcription factors AP2 and TFII-I and the zinc finger protein Miz1, were found to interact with the C-terminal domain of c-Myc. The N-terminal domain of c-Myc, in addition to interaction with the Rb family protein p107, also interacts with α-tubulin and the novel adaptor proteins Bin1, MM-1, Pam, TRRAP and AMY (Sakamuro and Pendergast, 1999 and references therein).

c-Myc belongs to a family of homologous proteins including also N-myc and L-myc. The other two members of the family were identified through their involvement in human tumourigenesis and by homology to c-Myc. *N-myc* is usually amplified in neuroblastomas whereas *L-myc* most commonly in small cell lung carcinomas (Marcu *et al.*, 1992). Like c-Myc, N-myc and L-myc cause tumourigenesis when overexpressed in the lymphoid compartment in transgenic mice. Eμ-*N-myc* mice also develop pre-B or B cell lymphomas and Eμ-*L-myc* mice develop B or T cell lymphomas with lower penetrance and longer latency (Dildrop *et al.*, 1989; Rosenbaum *et al.*, 1989; Moroy *et al.*, 1990; Morgenbesser and DePinho, 1994; Sheppard *et al.*, 1998).

b) Eμ-*Bcl2*: Another characteristic chromosomal translocation t(14;18), typifying human follicular lymphoma, results in the juxtaposition of the *bcl2* protooncogene and the IgH locus and leads to de-regulation of Bcl2 expression. Eμ–*Bcl2* transgenic mice develop monoclonal B cell lymphomas after long latency. These tumours result from the accumulation of excess non-cycling B cells of follicular origin and exhibit enhanced survival *in vitro* (McDonnell *et al.*, 1990, 1991, Strasser *et al.*, 1991). Bcl2 when overexpressed enhances cell survival without promoting proliferation. It prolongs the survival of haematopoietic cell in the absence of required factors and also in the presence of various stimuli inducing cellular death such as cytotoxic drugs or by apoptosis-inducing gene products (i.e. c-Myc) (Hockenbery, 1992; Strassser *et al.*, 1996 and references therein). Conversely, *Bcl2*-deficient mice demonstrate fulminant lymphoid apoptosis (Veis *et al.*, 1993).

Bcl2 belongs to a family of proteins (Bcl2, Bclx, Bad, Bak and Bax) which play important roles in cell death regulation (Yang and Korsmeyer, 1996). *Bclx* alternative splicing yields two forms of *Bclx* mRNA, a long form BclxL (233aa protein) and a short form BclxS (170 aa protein). The two Bclx forms have opposing effects: BclxL enhances viability i.e. can protect a growth factor-dependent cell line from apoptosis; in contrast BclxS acts in a dominant negative fashion to inhibit the protective effects of BclxL.

BclxL is an apoptosis inhibitor and upon growth factor deprivation promotes cell survival even more effectively than Bcl2. Moreover, BclxL can inhibit apoptosis in cells that have undergone Fas-induced protease activation (Boise *et al.*, 1993; 1997; Srivastava *et al.*, 1999). *BclxL* is the major *Bclx* mRNA form expressed during murine development and like Bcl2 its product localises to mitochondria (Gonzalez-Garcia *et al.*, 1994). Analyses of *BclxL*-deficient chimeric mice demonstrated significant alteration in the maturation stage of B cell development characterised by extensive clonal selection. Constitutive expression of *BclxL* promoted accumulation of B cell precursors and mature B cells in the mouse and enhanced B cell survival *in vitro*. Coexpression of *BclxL* and *Bcl2* leads to the enhanced accumulation of B cells in

transgenic mice and partially protected B cells from death, induced by IgD crosslinking using a specific anti-IgD antibody (Grillot *et al.*, 1996).

Bax accelerates cell death and can oppose the effects of Bcl2 and BclxL. However, there is no evidence that Bax has any activity in their absence. Three isoforms of Bax (alpha, beta and gamma) are generated by alternative splicing. Bax can form homodimers as well as heterodimers with Bcl2 and BclxL, and the relative amounts of Bcl2/BclxL and Bax determine viability during growth factor deprivation.

A recently identified *Bcl2*-related gene designated *Bak* (for Bcl2 homologous antagonist/killer), the product of which interacts with Bcl2 and BclxL, is functionally similar to Bax and enhances apoptotic cell death following apoptotic stimuli (Chittenden *et al.*, 1995; Kiefer *et al.*, 1995). However, Bak can inhibit cell death in an EBV-transformed cell line possibly by inducing viral antiapoptotic factors such as the Bcl2 viral homologue BHRF1 (Kiefer *et al.*, 1995).

Bad, a pro-apoptotic protein, can dimerise with Bcl2 and BclxL and when overexpressed can counter their survival promoting effects. It does so by displacing Bax from Bcl2/Bax and BclxL/Bax heterodimers, allowing more Bax/Bax homodimer formation, which promotes cell death (Yang *et al.*, 1995).

Most members of the family have sequence identity, clustered in two conserved regions BH1 and BH2 (Fig. 1.4). Site-directed mutagenesis of BH1 and BH2 in Bcl2, showed that these domains are important for binding to Bax. In addition, single amino acid substitutions (Gly142 with either Ala or Gla) in BH1 and (Trp188 to Ala) in BH2 completely disturb Bcl2 binding to Bax and abrogate the death repressor effect of Bcl2. However, its homodimerisation property is not affected (Yin *et al.*, 1994). The same effects were observed for BclxL (Sedlak *et al.*, 1995).

Bcl2 family members interact with each other in influencing cell death pathways (Craig, 1995). Bcl2 and BclxL form heterodimers with Bax and the relative amounts of Bcl2 or BclxL and Bax determine viability during growth factor deprivation with an increase in the amount of Bcl2 or BclxL relative to Bax promoting survival and an increase in the amount of Bax promoting death (Oltvai *et*

BCL2	136	ELFRDGV-NWGRIVAFFE--FGG	155	187	TWIQDN-GGWDAFVELY	202
BAX	98	DMFSDGNFNWGRVVALFY--FAS	118	150	GWIQDQ-GGWDGLLSYF	165
BCLXL	129	ELFRDGV-NWGRIVAFFS--FGG	148	180	PWIQEN-GGWDTFVELY	195
BAK	117	SLFESGI-NWGRVVALLG--FGY	136	169	RWIAQR-GGWVAALNLG	184
MCL1	252	HVFSDGVTNWGRIVTLIS--FGA	272	304	DWLVKQ-RGWDGFVEFF	319
A1	77	KEFEDGIINWGRIVTIFA--FGG	97	132	EWIRQN-GGWEDGFIKK	147
BAD	138	PPNLWAAQRYGRELRRMSDEFEG	160	182	GWTRIIQSWWDRN--LG	196
LMW5-HL (ASFV)	76	ELFRDLI-NWGRICGFIV--FSA	95	126	PWMISH-GGQEEFLAFS	141
BHRF1 (EBV)	89	EIFHRGDPSLGRALAWMA--WCM	109	142	GWIHQQ-GGWSTLIEDN	157
CED-9 (*C.elegans*)	159	AQTDQCPMSYGRLIGLIS--FGG	179	213	NWKEHN-RSWDDFMTLG	228

Fig. 1.4 Alignment of Bcl2 homologues in BH1 and BH2 domains. Numbers denote amino acid positions. Identical amino acids are outlined in red and conserved residues in blue. Dashes denote gaps in the sequence to maximise alignment.

al.,1993).Thus, the balance of Bcl2 family interactions is very fine and the slightest change could lead to either survival and hence tumour formation or apoptosis.

Both Bcl2 and BclxL are localised to mitochondria where they prevent apoptosis by blocking cytochrome c release and therefore caspase activation (Kharbanda *et al.*, 1997; Yang *et al.*, 1997). Mitochondria play a pivotal role in a cell death pathway as cytochrome c is released from mitochondria in cells undergoing apoptosis (Kluk *et al.*, 1997). Cytochrome c release results in caspase activation which in turn activates other death factors and leads to cell death (Bossy-Wetzel *et al.*, 1998).

The Bcl2 family genes also interact with other gene families e.g. Bcl2 has been shown to synergise with R-ras, c-Myc and Pim1 proteins (Fanidi *et al.*, 1992; Acton *et al.*, 1992). Transgenic cross breeding experiments have indicated a strong co-operation between c-Myc and Bcl2 in tumourigenesis. Eμ–*c-myc*/Eμ–*Bcl2* mice develop rapid onset tumours of a primitive haematopoietic cell type (Strasser *et al.*, 1990, 1996; Cory *et al.*, 1999).

c) Eμ-Pim1. *Pim1* is another lymphoid proto-oncogene which belongs to a gene family including *Pim2* and *Pim3*. *Pim1* was first implicated in T cell lymphomas due to its frequent activation by proviral insertion in murine MoMLV-induced T cell lymphomas and it was first identified by proviral tagging (reviewed in Wilson, 1997). Eμ–*Pim1* transgenic mice develop T cell lymphomas with a long latency, in a dose dependent manner. However, its role in human lymphomas is not yet clear. Transgenic cross breeding between Eμ–*c-myc* and Eμ–*Pim1* mice showed a dramatic synergy between the two genes. Eμ–*c-myc*/Eμ–*Pim1* foetuses succumb to lymphoblastic leukaemia perinataly (Verbeek *et al.*, 1991). In addition, proviral tagging showed that *Pim1* as well as *Pim2* are frequently activated by MoMLV in Eμ–*c-myc* mice (Haupt *et al.*, 1991; van Lohuizen *et al.*, 1991; Berns *et al.*, 1994). Similarly, either *c-myc* or *N-myc* is usually activated in Eμ–*Pim1* transgenic mouse tumours. This is suggestive of a strong co-operation between the two gene families.

Pim1 codes for a serine/threonine kinase which may regulate the expression of transcription factors such as c-Myc, cJun, cMyb. There is evidence that it may

inhibit apoptosis as well as differentiation (Allen and Berns, 1996). Lilly *et al.*, (1999) reported that Pim1 prolongs cell survival and inhibits apoptosis-related mitochondrial disfunction in part through a Bcl2-dependent pathway. Bcl2 expression is regulated by transforming proteins or signal transduction mediators which promote cell survival such as *vH-ras* gene product (Kinoshita *et al.*, 1995a), activated cMyb (Salomoni *et al.*, 1997), GMCSF (Kinoshita *et al.*, 1995b) and activated Jak2 (Sakai and Kraft, 1997). Pim1 is regulated by GMCSF (Polotskaya *et al.*, 1993; Sato *et al.*, 1993) and Jak2 (Sakai and Kraft, 1997). Some studies suggest that since *Pim1* and *Bcl2* share at least some upstream regulators it is possible that there is a linear relationship between the two genes while other studies are suggestive of a synergistic interaction between the two genes to enhance lymphomagenesis. Thus, the first assumption is supported by the findings that Pim1 supported *Bcl2* mRNA expression after IL3 withdrawal without a corresponding suppression of *Bax* mRNA expression (Lilly *et al.*, 1999). Moreover, Pim1 may act as a normal upstream regulator of *Bcl2* expression possibly through regulation of cMyb activity via the p100 transcription factor (Leverson *et al.*, 1998). However, other studies involving transgenic mice suggested that Pim1 inhibits apoptosis in a *Bcl2*-independent manner (Acton *et al.*, 1992).

d) Other B cell oncogenes. Proviral activation of the *Bmi1* gene has implicated Bmi1 as a collaborator of c-Myc in lymphomagenesis. The *Bmi1* gene encodes a zinc finger protein related to the *Drosophila Polycomb* gene family. Bmi1 is believed to be important in the regulation of the proliferation of haematopoietic cells throughout pre- and postnatal life (Berns *et al.*, 1994; Alkema *et al.*, 1997).

Gfi1 was isolated from the *pal1* locus. Proviral insertions in this locus lead to overexpression of *Gfi1* (Schmidt *et al.*, 1996; Scheijen *et al.*, 1997). The Gfi1 protein functions as a transcriptional repressor. Gfi1 overexpression can extend haematopoietic cell survival upon withdrawal of growth factors in culture, probably through its ability to inhibit the expression of Bax (an apoptosis promotor) (Grimes *et al.*, 1996a; 1996b; Zornig *et al.*, 1996).

A number of other oncogenes which co-operate with c-Myc in tumourigenesis (demonstrated by transgenic cross-breeding experiments) include N-ras (Harris *et al.*, 1988a; Alexander *et al.*, 1989) a cytoplasmic G protein (Harris *et al.*, 1988b), cyclinD1 (Lovec *et al.*, 1994), v-abl a tyrosine kinase (Rosenbaum *et al.*, 1990) as well as tumour suppressor gene mutations (usually loss of function) such as *p53*. Interestingly, *p53* mutations have been observed in sBL and eBL-derived cell lines (Farrell *et al.*, 1991).

1.5 EBNA 1 and complementation groups of co-operative genes in lymphomagenesis

A combination of the results of several studies of oncogene synergy in lymphomagenesis suggest that there are at least three different complementation groups of genes involved in tumour initiation and development (Fig. 1.5) (Wilson, 1997). One of the groups comprises the *Myc* family genes which are involved in cell proliferation. A second group contains the *Pim* family genes whose function is not very clear yet. The third group includes a number of different genes or factors which act to inhibit apoptosis, differentiation or senescence. The groupings are based on complementation studies in transgenic mice. For example, *c-myc* is never found activated in Eμ–*N-myc* or Eμ–*L-myc* tumours which suggest that they can substitute for c-Myc function *in vivo* and that their actions are redundant to some extent. Therefore these genes form one group (Adams and Cory, 1991b). In addition, crossing the strains of transgenic mice shows no synergy in lymphomagenesis, while a combination of transgenic cross breeding and proviral insertional mutagenesis helped considerably in the identification of co-operating genes between different complementation groups. For example, Bcl2 is an apoptosis inhibitor and Myc family proteins induce proliferation. Therefore, deregulated expression of both of these proteins could lead to accelerated tumour development (Strasser *et al.*, 1990; Fanidi *et al.*, 1992; Wagner *et al.*, 1993). However, the mono- or oligoclonality of these tumours suggests that these two proteins, even together, are not sufficient for complete tumourigenesis.

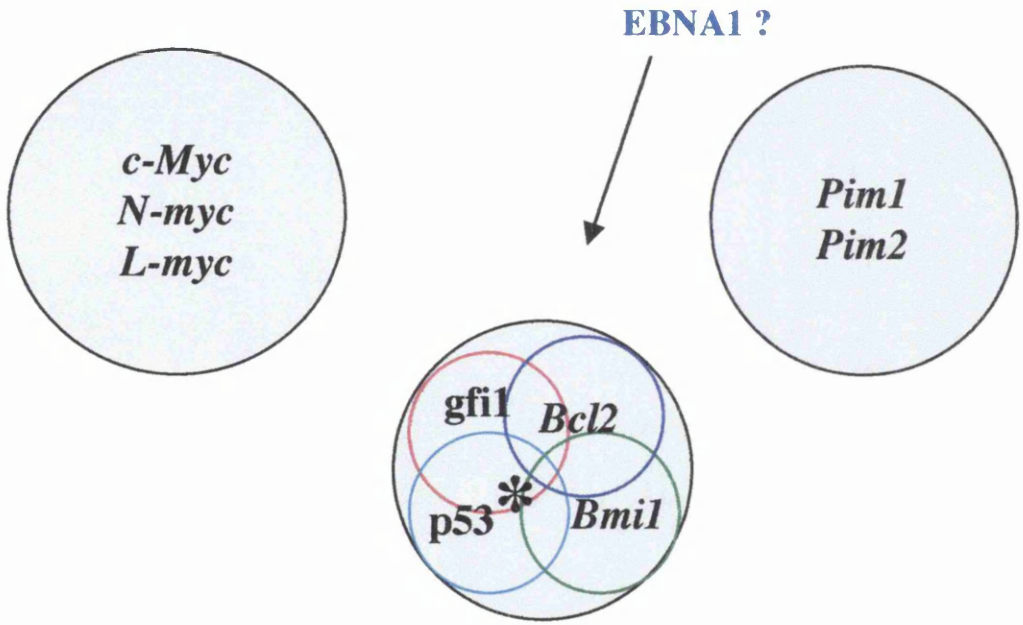


Fig. 1.5 Complementation groups in lymphomagenesis. Several proto-oncogenes and p53 loss (depicted by p53*) have been placed into groups depending on their ability to synergise in lymphomagenesis *in vivo*. The groupings are based on several tests of co-operativity, as detailed in section 1.5 (for example *c-Myc* can synergise with *Pim1* and *Bcl2*, but not with *N-myc* or *L-myc*). Transgenic crossbreeding experiments in our laboratory suggest that EBNA 1 belongs to the group of genes which act to repress apoptosis (indicated by arrow). Not all combinations have been analysed. (Figure adapted from Wilson, 1997).

Activation of *Bmi1* and *Gfi1* in the same tumour is never seen and thus these two genes can be placed in the same complementation group. However, in tumours induced by proviral insertion of MoMLV in Eμ–*Pim1* transgenic mice deregulated *c-myc* and *Gfi1* co-expression has been observed placing *Pim1, c-myc* and *Gfi1* in three different groups (Berns *et al.*, 1994; Schmidt *et al.*, 1996; Scheijen *et al.*, 1997).

Factors within a group could act in parallel pathways with redundant outcome. For example, MoMLV infection in either Eμ-*Bcl2* mice or *p53* null mice did not show a dramatic acceleration in tumour development, whereas MoMLV infection in Eμ-*c-myc* mice showed marked synergistic action. Therefore, MoMLV itself may lead to some apoptotic protection and is thus included in the same group as *bcl2* and *p53* loss or mutation (Acton *et al.*, 1992; Baxter *et al.*, 1996). Alternatively, the members of the same complementation group could act on the same signal transduction pathway but at different points. It has been suggested that Bcl2 and p53 can work as a repressor and effector in the same apoptosis pathway (Marin *et al.*, 1994).

1.6 EBNA 1 transgenic mice

The important findings that EBNA 1 plays a key role in the life cycle of EBV, and is the only viral protein consistently expressed in EBV associated diseases, and in particular it is the only viral protein expressed in eBL, formed the basis for the construction of the EBNA 1 expressing transgenic mouse model (Wilson and Levine, 1992, Wilson *et al.*, 1996). Transgenic mice were generated harbouring a transgene designed to direct EBNA 1 expression to the B cell compartment. The transgene links the EBNA 1 coding region of the B95-8 strain of EBV to the polyoma virus (Py) early promoter and mouse IgH intronic enhancer (Eμ; Wilson and Levine, 1992, Fig. 1.6). From the ten lines generated only mouse lines 26 and 59 expressed the protein and succumbed to monoclonal B cell lymphoma, albeit with different disease latency periods. Mice of line 26 have a higher tumour incidence at an earlier age of onset (4-12 months) and expressed higher levels of EBNA 1 RNA than mice of line 59 (tumour onset 18-24 months). The differences in the tumour incidence may be

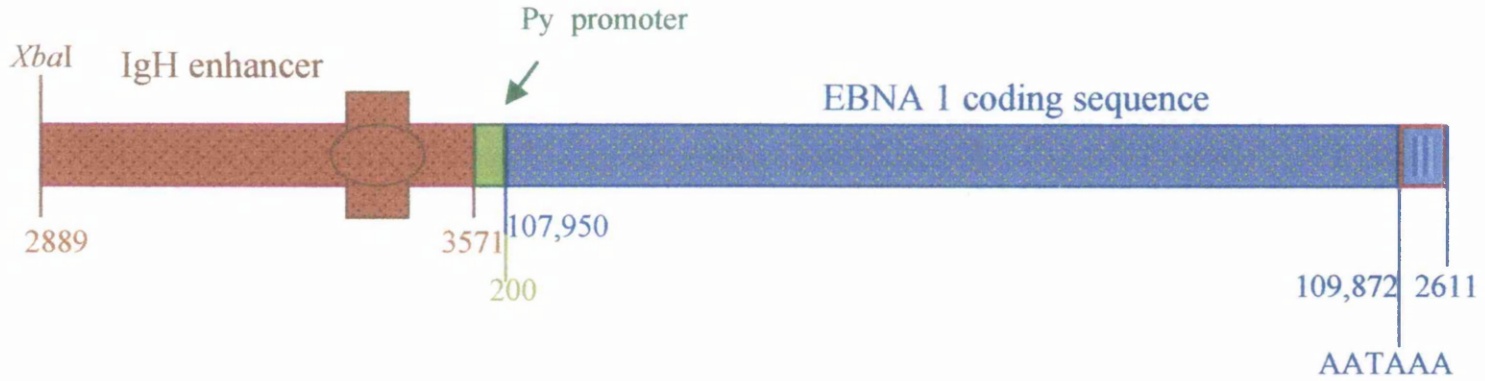


Fig. 1.6 EμEBNA 1 transgene structure. The EBNA 1 coding region from the B95-8 strain of EBV(blue) is linked to the polyoma virus early promoter (Py, green) and IgH enhancer sequences (mahogany) represented by a rectangular shape (adapted from Wilson and Levine, 1992). The numbers indicate the sizes of each part in base pairs: IgH enhancer in mahogany (Banerjii *et al.*, 1983), Polyoma early promoter in green (Tooze, 1981) and EBV sequences in blue (Baer *et al.*, 1984).

due to the different expression patterns of the transgene in each line. Alternatively, it might be that the expressed protein in each of the lines has different oncogenic abilities. Moreover, it is possible that the transgene may have picked up a mutation. However, sequence analysis of line 59 tumour DNA revealed that the transgene is wild type B95.8. In addition using a single strand confirmation polymorphism (SSCP) analysis no mutations were found in line 26 transgene although it has not been sequenced (JBW pers. com.).

The hallmark of the disease of transgenic positive mice of both lines is a massive enlargement of the spleen, liver and/or lymph nodes (LNs), which are replete with neoplastic cells. The morphology of the tumour cells from these lines varies at different stages of B cell development. Notably, all have IgH locus rearrangements as demonstrated by Southern blot analyses, which also revealed that these lymphomas are monoclonal (Wilson *et al.*, 1996). Although the latency differs between the two lines the lymphoma pathology is the same and is attributable to the EBNA 1 transgene. Moreover, the correlation of transgene expression and lymphoma phenotype is absolute, revealing that the expression of EBNA 1 can predispose B cells to lymphoma. As such, EBNA 1 can be regarded as a viral oncogene.

The fact that the lymphomas arising in these mice are monoclonal suggests that EBNA 1 is not sufficient for complete tumourigenic conversion of B cells. Therefore, other cellular genes may be involved, which either mediate the oncogenic action of EBNA 1 or they may co-operate with it in the onset of oncogenesis.

1.7 Mechanism of action of EBNA 1 and co-operative factors in oncogenesis

Although the major roles of EBNA 1 in the regulation of viral RNA expression and DNA replication are well characterised, the effects of the EBNA 1 protein on cellular components or factors are not well understood. EBNA 1 may exert its oncogenic effect through binding to cellular DNA sequences directly or indirectly and modifying transcription. Alternatively, EBNA 1 could modify DNA interactions (such as recombination), through binding to RNA and possibly effecting

translation, through other protein-protein interactions or through a combination of the above (reviewed in introduction section 1.3).

An obvious property of EBNA 1, which could be causal in its oncogenic function, is its ability to transactivate transcription. In transactivating cellular genes, EBNA 1 could directly regulate the expression of cellular proto-oncogenes known to be associated with lymphoproliferative disorders. Alternatively, EBNA 1 could modulate the expression of other genes which in turn effect the expression of proto-oncogenes or tumour suppressors.

Alterations of the *c-myc* gene structure and expression have been observed in many tumours including BL in which *c-myc* mRNA levels were higher than normal, and about 2 to 5 times increased relative to EBV-immortalised lymphocytes (Taub *et al.*, 1984). A very interesting fact is that the lymphoma phenotype described earlier (section 1.5) for both EBNA 1 expressing lines 26 and 59 has a striking resemblance to that described for Eμ-*c-myc* transgenic mice (Adams *et al.*, 1985). The phenotypic similarity of these lymphomas is suggestive of the possibility that the tumourigenic action of EBNA 1 is mediated through c-Myc. However, direct transactivation of *c-myc* by EBNA 1 has not been reported. Moreover, there are no consensus EBNA 1 binding sites found within the *c-myc* promoter and c-Myc has not been shown to be replaced by EBNA 1 in tissue culture assays. Furthermore, several lines of evidence from our laboratory indicate that EBNA 1 co-operates with c-Myc in lymphomagenesis and therefore does not act though it.

The other two members of the family, N-myc and L-myc, are also oncogenic (reviewed in section 1.4), although they differ in their oncogenic ability. Interestingly, in Eμ-*N-myc* and Eμ-*L-myc* transgenic tumours, no c-Myc expression was detected which is suggestive of a negative regulation of c-Myc expression and that N-myc and L-myc can substitute for c-Myc function *in vivo* (Adams and Cory, 1991a and 1991b). Thus it can be postulated that EBNA 1 may also co-operate with N-myc and L-myc in tumourigenesis.

As mentioned earlier *c-myc* strongly co-operates with *Bcl2* in transgenic cross-breeding experiments, (section 1.5). Since EBNA 1 co-operates with c-Myc,

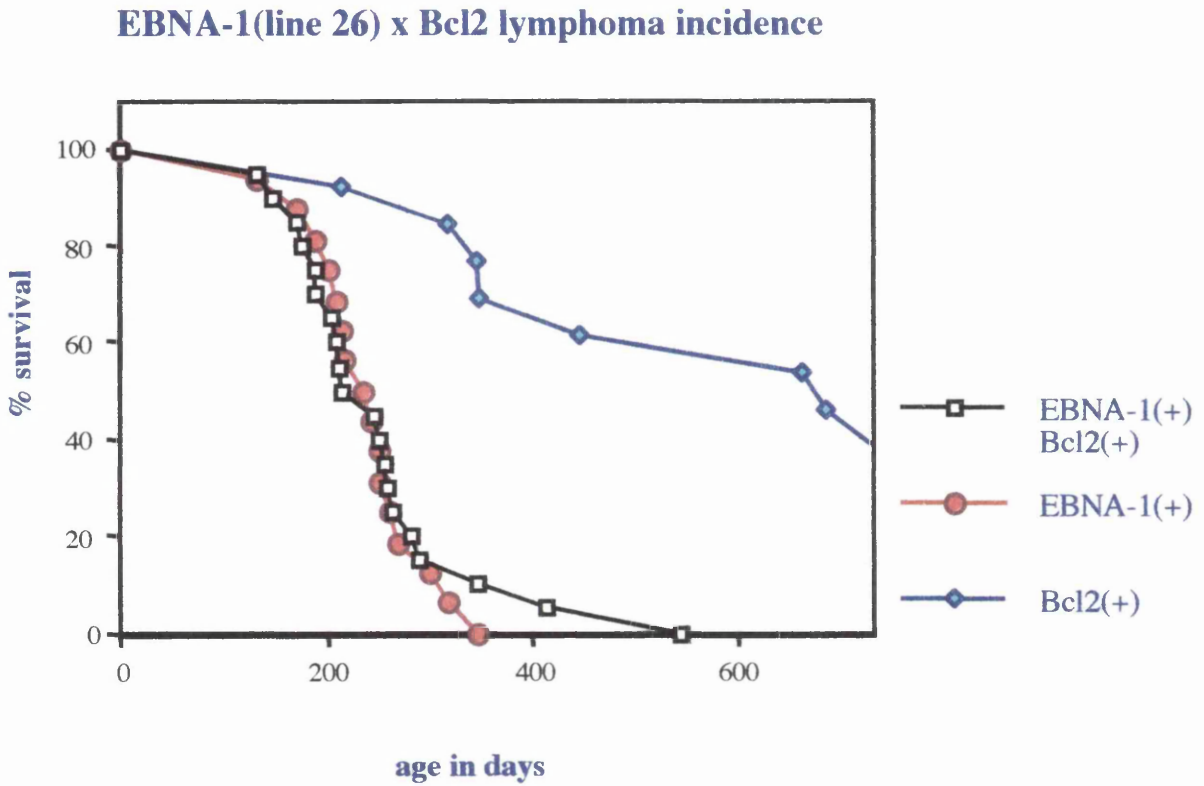


Fig. 1.7 Lymphoma incidence in single transgenic mice (red or cyan) and bi-transgenic (squares). Bi- transgenic mice show no reduction in latency to tumour onset, indicative of a degree of redundancy between EBNA 1 and Bcl2.

then factors which synergise with deregulated Myc expression in tumourigenesis could be redundant in the presence of EBNA 1. Transgenic cross-breeding experiments between EμEBNA 1 and Eμ-*Bcl2* mice showed that EBNA 1 is redundant with Bcl2 in that the survival or death rate between bi-transgenic mice and single transgenic controls was similar (Fig. 1.7). Therefore, EBNA 1 may act through it or in the same way as Bcl2 (conducted by M. Drotar and J. Coy in our laboratory, Tsimbouri *et al.*, in preparation).

Pim1 also co-operates with c-Myc in lymphomagenesis which makes it a likely candidate for co-operation with EBNA 1. Data from cross-breeding experiments between Eμ-*Pim1* and EμEBNA 1 in our laboratory are too preliminary to be able to interpret co-operative action.

Proviral insertional mutagenesis in Eμ-*c-myc* mice has identified a selection of genes which co-operate with c-Myc in tumourigenesis. These may also co-operate or be redundant with EBNA 1 in lymphomagenesis.

An alternative possible way in which EBNA 1 could exert its oncogenic effect is by modulating the cellular environment. One such mode of action could be through influencing the recombinogenic machinery which is active during B cell differentiation in generating functional immunoglobulin loci. This is supported by an observation which suggested that in EBV-positive BL cell lines, latent EBV gene expression (in the absence of LMP1 expression) correlates with increased transcription of the recombination activating genes *Rag1* and *Rag2* (Kuhn-Hallek *et al.*, 1995). The induction of *RAG* expression either directly or indirectly as a consequence of delayed cell maturity, may result in genomic instability. A very interesting observation is that, all of the EBNA 1 transgenic mouse lymphomas have undergone IgH rearrangement, as detected using a J-region probe (Wilson JB, personal communication).

Rag gene expression begins at the earliest stages of lymphocyte development (Hardy *et al.*, 1991; Turka *et al.*, 1991; Wilson *et al.*, 1994; Grawunder *et al.*, 1995b). In developing B cells, *Rag* transcript levels transiently decrease after successful immunoglobulin heavy chain gene rearrangement and increase again during light chain

gene rearrangement in pre-B cells (Grawunder *et al.,* 1995b). *Rag* gene transcription persists in surface IgM+ immature bone marrow B cells but is absent in mature IgD+ peripheral B cells (Grawunder *et al.,* 1995a). It was recently demonstrated that alteration of chromatin structure in the promoter region of *Rag1* gene, in addition to other control elements outside of the promoter region, is one of the mechanisms regulating tissue specific expression of the human *Rag1* (and possibly *Rag2)* gene(s) (Kitagawa *et al.,* 1996). Post-translational regulation is also important for regulating Rag expression. Rag2 is regulated in a cell cycle dependent fashion and accumulates at G0/G1. Upon entry into S-phase Rag2 is phosphorylated and targeted for degradation by a cell cycle-dependent kinase (Lin and Desiderio, 1993; Grawunder *et al.*, 1996). In addition, it has been suggested that Rag2 may influence the stability of Rag1 protein (Grawunder *et al.*, 1996). Using nuclear extracts from B cell lines, it was recently demonstrated that BSAP (PAX5, a B cell specific transcription factor) binds to a conserved sequence of *Rag2* promoter (Lauring and Schlissel, 1999) and regulates *Rag2* expression. In addition, Verkoczy and Berinstein (1998), using a differential display approach, cloned BSAP as a transcript whose expression correlated with high levels of *Rag2* transcription. Moreover, in this same region upstream of the *Rag2* promoter there are potential binding sites for Ikaros and Myb which are essential factors expressed at multiple stages of B and T cell development (Luscher and Eisenman, 1990; Georgopoulos *et al.,* 1997).

EBNA 1 is the only viral antigen which is consistently expressed in BL. However, other viral antigens could play a role in initiating and/or contributing in the tumour formation. Moreover, other cellular genes, either known or even completely novel ones could co-operate with EBNA 1 in the onset of tumourigenesis. Evidence from cross breeding experiments in our laboratory showed that EBNA 1 co-operates with c-Myc and is redundant with Bcl2 in lymphomagenesis. Therefore it can be postulated that EBNA 1 may be a member of the oncogenes which act to prevent cell death or cell differentiation such as *Bcl2* and thus it may not be expected to co-operate with other genes involved in apoptotic regulation.

1.8 Project Aims

This research project has involved the analysis of EμEBNA 1 transgenic mice at the pre-tumour stage in order to elucidate the mechanism of action of EBNA 1 as an oncogene *in vivo*. The problem was divided into two areas of study:

I) An examination of the effects of EBNA 1 action on the expression of key cellular genes in the B cell growth and differentiation pathways *in vivo*. Transgenic experiments in our laboratory demonstrated that EBNA 1 is an oncogene and responsible for the tumours arising in the EμEBNA 1 positive mice (Wilson and Levine, 1992; Wilson *et al.*, 1996). EBNA 1 is a transcriptional transactivator and it is likely that it can regulate the expression of cellular genes involved in key pathways, either directly or indirectly by exerting its oncogenic action through or in the same way as them.

II) The investigation of influence of EBNA 1 on the B cell phenotype and properties. EBNA 1 could affect B cell phenotype through deregulating the genes responsible for normal B cell maturation and/or differentiation.

Approaches

Chapter 3. B cell phenotype and growth characteristics

a) B cell phenotype

In order to evaluate the effects that EBNA 1 may have on B cell differentiation, spleen and bone marrow cells were collected from EμEBNA 1 mice and negative sibling control mice and fluorescence activated cell sorting (FACS) analysis using fluorescent conjugated antibodies was performed. Antibodies against B cell surface markers that are expressed differentially throughout differentiation were used and immunoglobulin gene expression characterised. This was conducted in order to evaluate the proportions of the various cell types within the tissue compartments as well as the stages of their maturation.

b) Transgenic B cell properties: Cell survival

In order to gauge any effects of EBNA 1 on cell survival and /or apoptosis spleen and bone marrow cells were cultured under standard conditions for lymphocytes with or without the presence of growth factors. Cell survival or apoptosis over time was monitored.

Chapter 4 Effects of EBNA 1 on cellular gene expression I: selected genes

To investigate if EBNA 1 effects cellular gene expression *in vivo* before the onset of tumourigenesis, slot blot analysis was performed using RNA samples. The slot blots were probed with selected genes that were postulated to be effected by EBNA 1, particularly members of the *Bcl2* family. The blots were quantified by phosphoimaging and the data were statistically analysed for significant differences using the statistics program Minitab.

Chapter 5 Effects of EBNA 1 on cellular gene expression II: Differential expression

A different approach used to investigate the effects of EBNA 1 on cellular gene expression was taken using the Mouse Atlas Expression array; a cDNA macro-array based technique very useful in profiling differential gene expression. Confirmation of gene de-regulation was subsequently done by slot blot analysis of pre-tumour and tumour samples. The data were statistically analysed for significance using the statistics program Minitab.

To test if the observed differential expression was apparent at the protein level, protein extract samples were western blotted with selected antisera and quantified by densitometric analysis. Furthermore, DNA binding activity (where appropriate) was assayed by band shift assays.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Antisera.

anti-BclxL	R&D systems, rabbit polyclonal anti-BclxL, cat.no. AF800
anti-CD40	Serotec, Rat anti-mouse CD40, cat.no.MCA 1143
anti-cFos	Santa Cruz, cat.no. SC-52-G
anti-pancFos	Santa Cruz, cat.no. SC-253
anti-FosB	Santa Cruz, cat.no. SC-1670
anti-Fra1	Santa Cruz, cat.no. SC-183
anti-Fra2	Santa Cruz, cat.no. SC-604
anti-cJun	made at the Beatson Institute, Bearsden, no. 730-5
anti-JunB	Santa Cruz, cat.no. SC-73
anti-JunD	Santa Cruz, cat.no. SC-74
anti-CD45R/B220	PharMingen, FITC conjugated rabbit polyclonal anti-mouse CD45R, cat.no. 01124A
anti-CD45R/B220	Sigma, R-Phycoerythrin conjugated monoclonal anti-mouse CD45R purified rat immunoglobulin,cat.no. P-3567.
anti-CD90(Thy1.2)	Sigma, FITC conjugated monoclonal anti-mouse Thy1.2, purified rat immunoglobulin, cat.no.F-7650
anti-IgD	Southern Biothech Associates Inc, FITC conjugated monoclonal anti-mouse IgD, cat.no.1120-02
anti-IgM-FITC	Sigma, FITC conjugated monoclonal anti-mouse IgM, cat.no. F-7122
anti-IgM-PE	Sigma, R-Phycoerythrin conjugated monoclonal anti-mouse, cat.no.P-5185
anti-sIg	Sigma, FITC conjugated goat anti-mouse polyvalent immuno-globulins (IgA, IgG, IgM) affinity isolated antibody, cat.no. F-1010
anti-Thb	Sigma, FITC conjugated monoclonal anti-mouse Thb, purified rat immunoglobulin, cat.no. F-3402
anti-rabbit-HRP	New England Biolabs, cat.no.7071
anti-active™ MAPK	Promega, (pTEpY) rabbit polyclonal antibody, cat.no. V8031
anti-ERK 1/2	Promega, rabbit polyclonal antibody, cat.no. V1141
	phospho-SEK1/
MKK4	NewEngland Biolabs, antibody (Thr223) cat.no. 9151S
SAPK/JNK	NewEngland Biolabs, antibody recognises total SAPK, cat.no. 9252

Isotype controls

FITC:	Serotec, Rat IgG2a negative control FITC, anti-mouse cat.no. MCA1212F
PE:	Serotec, Rat IgG2a negative control PE, anti-mouse cat.no. MCA1212PE

2.1.2 Cell lines

- Line 26 primary spleen and bone marrow cells
- 3959:48 LN cells derived from a lymphoma from a transgenic mouse harbouring EμEBNA 1 (line 59) and EμLMP1 (line 39)
- L 929 mouse fibroblasts: transfected with mouse CD40-ligand (K47)
  transfected with vector only control cells (K5)
- Rat 1 fibroblast cell line

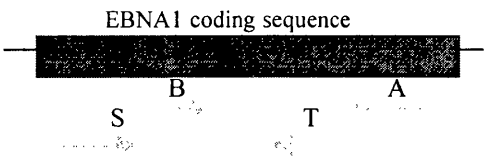
2.1.3 Growth Factors

IL2	Sigma, Recombinant mouse IL2, cat.no. I0523
IL4	Sigma, Recombinant mouse IL4, cat.no. I1020
IL6	Sigma, Recombinant mouse IL6, cat.no. I9646
IL7	Sigma, Recombinant mouse IL7, cat.no. I4892

2.1.4 Oligonucleotides MWG BIOTECH for EBNA 1 amplification:

oligoA	5'CCCTTCCTCACCCTCATCTCC3'
oligoB	5'GCTCTCCTGGCTAGGAGTCACG3'
oligoS	5'CCATATACGAACACACCGGC3'
oligoT	5'CATCCGGGTCTCCACCGCGC3'

Fig. 2.1 oligonucleotide position in the EBNA 1 coding sequence



2.1.5 Plasmids and probes

(For the methods used to generate and isolate all probe fragments see Methods)

Table 2.1 Epstein-Barr Virus derived sequences

Gene	Plasmid Lab.no.	Restriction digest	Fragment size	Reference
3'EBNA 1	114	BstXI/EcoRI	1 Kb	Wilson *et al.*, 1992

Table 2.2. Mouse derived sequences

Gene	Plasmid Lab.no.	Restriction digest	Fragment size	Reference
Gene Families				
Bcl2 family				
Bad	467	EcoRI	0.7Kb	Yang *et al.*, 1995
Bax	466	EcoRI/XhoI	0.765Kb	Oltvai *et al.*, 1993
Bcl2	67	EcoRI/HindIII	0.865Kb	Nunez *et al*,1989
$Bclx_L$	468	EcoRI	1Kb	Boise *et al.*,1993
Jun family				
cJun	504	BamHI	1.7Kb	Angel *et al.*, 1988
JunB	502	EcoRI	1.8Kb	Ryder *et al.*, 1988
JunD	503	EcoRI	1.7Kb	Ryder *et al.*, 1989
Myb family				
Amyb	488	SacI/PvuII	0.663KB	Facchinetti *et al*, 1997
cMyb	490	NcoI/MluI	1.9Kb	Badiani *et al.*, 1994
Rag family				
Rag1	294, 307	BamHI/XbaI	3.4Kb	Roman & Baltimore, 1996
Rag2	295	EcoRI	1.8Kb	Cuomo *et al.*, 1996
Genes				
Araf	277	EcoRI	2.6Kb	Huleihel *et al.*, 1986
B2	514	EcoRI/PstI	0.24Kb	White *et al*, 1989
Bmi1	325	EcoRI	2.2Kb	Haupt *et al*, 1991
c-Myc	56	XbaI	3.1Kb	Verbeek *et al.*, 1991,
Cyclin D1	272	EcoRI	1.3Kb	Nakagawa *et al.*, 1997
EBF	505	NcoI/NotI	0.48Kb	Hagman *et al.*, 1995
Egr1	510	EcoRI	2.5Kb	Caricasole *et al.*, 1996
cFos	269	BglII/PvuII	1.3Kb	Treisman, 1985
GAPDH	272	BamHI/SacI	0.316Kb	Sabath *et al.*, 1990
Gfi1	323	EcoRI	2.87Kb	Scheijen *et al.*, 1997
GMCSF	473	PstI	1.2Kb	Park *et al.*, 1992
HCK	515	EcoRI/NcoI	0.85Kb	Ziegler *et al.*, 1987
hnRNPD	522	NotI	2Kb	Dempsey *et al*, 1998
IgH (J region)	79	XbaI/XhoI	0.9Kb	Wilson *et al.*, 1992
Ikaros	506	PCR product	0.75Kb	Hahm, 1994
MDM2	278	EcoRI	1.5Kb	Chen *et al.*, 1993
MIP1α	474	EcoRI/XhoI	0.3Kb	Davatelis *et al.*, 1988
Nras	521	Pst1/Sst1	1.65Kb	Chang *et al.*, 1987
Pax5	509	SacII/SalI	0.373Kb	Adams *et al.*, 1992
Pic1	513	XbaI/XhoI	1.4Kb	Howe *et al.*, 1998
Pim1	264	BamHI	1Kb	Cuipers et *al.*, 1984
PML	515	EcoRI	1.6Kb	Goddard *et al.*, 1995
p16	454	XhoI	1Kb	Quelle *et al.*, 1995
p53	71	StuI/XhoI	1Kb	Oren & Levine, 1983
$SATB1_{12}$	511	HindIII/BamHI	1.2Kb	Nakagomi *et al.*, 1994
$SATB1_{13}$	512	HindIII/BamHI	1.1Kb	Nakogomi *et al.*, 1994
T cell receptor				
Jβ1	463	EcoRI/BamHI	3.3Kb	Palacios & Samarides, 1991
Jβ2	464	EcoRI/HindIII	1.8Kb	Palacios & Samarides, 1991

2.1.6 Reagents and Equipment

Acrylamide:	Biorad
Agarose:	SeaKem LE agarose, Flowgen Low melting point (LMP), Gibco
Animal supplies:	Mouse supplier, Harlan Olac Anaesthetic (Fluothane liquid), Zeneca Ltd Anaesthetising chamber, Fluotec Cauterising iron, International Market Supply Ear punch, ORME scientific Surgical instruments
Annexin V-FITC	Clontech, ApoAlertTM Annexin V-FITC cat.no. 8133-1 Pharmingen, Annexin V-FITC cat.no. 65874H
Antibiotics:	Ampicillin, Sigma Kanamycin, Sigma
Autoradiography:	
Cassettes:	Genetic Research Instrumentation (GRI) Ltd.
Film:	Kodak XAR
Film development:	X-ograph compactX2
Intensifying screens:	GRI
Phosphoimager:	Fuji
Intensifying screen:	Fuji
Bag sealer:	Vacuum bag sealer, Salton
Buffered phenol:	Buffer-saturated phenol, pH 7.5, Gibco
Buffers:	MOPS-E, Boehringer Tris-base, Boehringer
Camera:	UVP image processor, Mitsubishi video copyprocessor
Centrifuges:	Beckman centrifuge model J2-21 Eppendorf centrifuge 5415C Heraeus labofuge 400
Chemicals:	Acetic acid, Fisher Scientific Acetone, Fisher Scientific Biotin-16dUTP, Boehringer BSA, Sigma Chloroform, Fisher Scientific DAB, Sigma DEPC, BDH DMBA, Sigma Ethanol, Fisher Scientific Ethidium Bromide, Boehringer Formaldehyde, BDH Formamide, Sigma Gelatin, Sigma

	Hydrogen peroxide, Sigma Isopropanol, Fisher Scientific Methanol, Fisher Scientific Mercaptoethanol, Bio-rad Propidium Iodide, Molecular probes
Detergents:	SDS, Fisons/Sigma Triton X-100, Biorad Tween 20, Biorad
Electrophoresis:	
Electroblotter (DNA/RNA):	TE series TransphorElectroblotter, Hoeffer
Electrophoresis tank (DNA/RNA):	Kodak international biotechnologies inc
Power pack:	0-250V; 0.25A, Kikusui electronics
100bp ladder:	Gibco/Promega
1Kb ladder:	Gibco
Enzymes:	DNA restriction enzymes, Gibco Exo(-) Klenow, Stratagene Lysozyme, Sigma Proteinase K, Boehringer Terminal deoxynucleotidyl transferase TdT, Boehringer Trypsin, Sigma DNA T4 ligase, Boehringer
FACS machine:	Becton-Dickinson flow cytometer
Films:	Mitsubishi thermal paper K65HM FP4
Heating blocks:	Eppendorf thermomixer 5436
Homogeniser:	Kinematica polytron
Hotplates:	Fisons
DNA/RNA hybridisation:	
Hybridisation:	Hybaid midi oven and Hybaid hybridisation tubes
Pipette tips:	Aerosol Resistant Tips (ART), Gibco
Probe purification:	Push column beta shield device and Nuctrap probe purification columns, Stratagene.
Radio-isotopes:	α^{32}P dCTP (3000ci/mmol). Supplied in 0-4°C storage liquid Easytide form, NCN or Dupond. Amersham α^{32}P dATP (3000ci/mmol).
Random primers:	Stratagene Prime-IT II Kit (including buffers and Klenow)
Scintillation counter:	Beckman LS1801 liquid scintillation systems
Scintillation fluid:	Ecoscint scintillation solution, National Diagnostics.
Membranes:	Biodyne A+B, Pall biosupport membranes Imobillon P membrane, Millipore
Microscopy:	

Microscopes:	Olympus CK2 (cell counts and tissue culture) Leitz orthoplan (Fluorescence, Karyotyping and higher power magnification [x25-x100])
Slides:	Superfrost, Menzel-Glazer
PCR:	
Buffer+$MgCl_2$:	Gibco
dNTPs:	Boehringer
Oligonucleotides:	MWG BIOTECH
PCR cycler:	Appligene ONCOR CrocodileIII PTC-200, MJ Research
DNA purification columns:	Stratagene
Taq polymerase:	Gibco and Stratagene *Pfu turbo* DNA Polymerase Time saver^{TM} cDNA synthesis Kit (RT-PCR), Pharmacia
Protein protocols:	Aprotinin, Benzamidine, DTT, Dye Reagent Concentrate, Biolab Hepes, Leupeptin, o-Vanadate, Pepsatin, PMSF, Sigma
RNA protocols:	
Total RNA extraction:	TRI-REAGENT from Sigma was used for all total RNA extractions.
PolyA$^+$RNA extraction:	Oligotex mRNA Kit from Qiagen, used for the isolation of polyA$^+$ from total RNA.
Tubes:	6ml Falcon tubes, used to homogenise tissues and isolate RNA. 1.5ml RNAse-free Eppendorf tubes, used for the collection and storage of both total and polyA$^+$ RNAs.
Shaking platforms:	Rocking, rolling and shaking platforms, Hoeffer
Slot-blot apparatus:	Schleicher and Schuell minifold 2
Spectrophotometer:	Spectronic 601, Milton Roy
Stirrers:	Fisons
Tissue culture:	
Medium:	RPMI1640 without L-glutamate, Gibco
FCS:	Gibco
Fungizon:	Gibco
L-glutamine:	Gibco
Mytomycin:	Sigma
Penicillin:	Gibco
Streptomycin:	Gibco
DMSO:	Sigma
Freezing container:	Nalgene
Incubator:	Heraeus
Plasticware:	Corning

UV equipment:	
Stratalinker:	UV stratalinker 1800, Stratagene
Transilluminator:	UVP ultraviolet Transilluminator, GRI
Waterbaths:	Grant

2.2 Formulation of the most frequently used solutions

Annexin V-FITC	Annexin V-FITC buffered in: 50mM Tris, pH 8.0 80mM NaCl 1mM EDTA 0.09% (w/v) sodium azide 0.2% (v/v) BSA
AnnexinV-binding buffer	10mM Hepes/NaOH, pH 7.4 140mM NaCl 2.5mM $CaCl_2$, solution stored at 4°C
Church Buffer	1% (w/v) BSA 1mM EDTA 500mM $NaPO_4$ 7% (w/v) SDS at pH 7.2
Denaturing solution	0.6M NaCl 0.4M NaOH
2FC	49.45% (v/v) chloroform 0.1% (w/v) 8-hydroxyquinoline 1% (v/v) isoamyl alcohol 49.45% (v/v) re-distilled phenol Saturated with 1M Tris pH 8.0
Buffered neutral formalin (500ml)	50ml of 40% (v/v) formalhehyde 1.75g NaH_2PO_4 3.25g Na_2HPO_4 dH_2O up to 500ml
10x Goat wash	10% (v/v) Goat serum 1.3M NaCl 70mM $Na_2HPO_4.2H_2O$ 30mM $NaH_2PO_4.H_2O$ 0.5% (w/v) Sodium azide 2% (v/v) Triton-x-100 0.5% (v/v) Tween 20
KCl buffer (protein)	0.02 or 0.6M KCl 20mM Tris-Cl, pH 7.3 25% Glycerol 1.5mM $MgCl_2$ 0.2mM EDTA dH_2O
10x Loading buffer (DNA)	0.25% (w/v) Bromophenol blue 0.1M EDTA pH8.0 20% (v/v) Ficoll 25% (w/v) XC

Loading buffer (RNA)	17.8% (v/v) formaldehyde 50% (v/v) pure, deionised formamide 1x MOPS-E
10x Loading dye (DNA/RNA)	0.1% (w/v) Bromophenol blue 50% (v/v) glycerol (autoclaved) 0.1% (w/v) XC
MOPS-E buffer	1mM EDTA 20mM MOPS 5mM NaOAc pH/NaOH/7.0
NH_4Cl solution (ACT)	9 vols of NH_4Cl [0.83% (w/v) in H_2O] 1 vol of Tris [2.06% (w/v) in H_2O, pH7.65] check pH7.2, filter sterilisation
PBS	2.7mM KCl 1.4mM KH_2PO_4 137mM NaCl 4.3mM Na_2PO_4 pH/HCl/7.3
PBS.A	0.025% (w/v) KCl 0.025% (w/v) KH_2PO_4 1% (w/v) NaCl 0.14% (w/v) Na_2PO_4
Propidium Iodide staining sol.	5µg PI /ml 1xPBS 200µg/ml DNAse-free RNAse (Sigma) solution always made fresh before use
PI-1xPBS buffer:	8g NaCl 0.2g KCl 1.44g $Na_2HPO_4 \cdot 7H_2O$ 0.24g KH_2PO_4 H_2O to 1 lt, pH 7.2
<u>Protein buffers</u>:	
Blotting buffer:	25mM Tris 192mM Glycine 20% (v/v) Methanol (analytical grade)
Coomassie stain	10% (v/v) Acetic acid 0.006% (w/v) Coomassie stain
Gel fixing solution:	25% (v/v) Isopropanol 10% (v/v) Acetic acid
MENSA buffer:	62.5mM Tris-HCl, pH 6.8 2% (w/v) SDS 50mM Sodium 2-Mercaptoethanol
Nuclear protein extraction buffer:	20mM Hepes, pH 7.9 0.4M NaCl 1mM EDTA, pH 8 1mM EGTA, pH 8 1mM DTT 1mM PMSF freshly added

10x Protein binding buffer	100mM Hepes, pH 7.9 2mM EDTA 1M NaCl 1mg/ml BSA 10% (v/v) glycerol
Protein extraction buffer (total):	20mM Hepes, pH 7.9 5mM EDTA 10mM EGTA 0.4M KCl 10% (v/v) Glycerol 0.4% (v/v) Triton X-100
Proteinase inhibitor mix 200x:	100mM Benzamidine 200µg/ml Leupeptin 200µg/ml PepsatinA
4x Resolving buffer:	1.5M Tris.HCl pH 8.8 0.24% (v/v) TEMED 0.4% (w/v) SDS Store at 4°C for up to a month
Running buffer:	50mM Tris 380mM Glycine 0.1% (w/v) SDS
SDS-PAGE gel:	
10% (v/v) Resolving gel:	28ml H2O 14ml resolving buffer 14ml Bis Acrylamide 40% (v/v) 0.33ml 10% (v/v) APS
2x Stacking buffer:	0.25M Tris.HCl pH6.8 0.24% (v/v) TEMED 0.2% (w/v) SDS
Stacking gel:	15.5ml H_2O 19.5ml Stacking buffer 4ml Bis Acrylamide 40% (v/v) 0.585ml 10% (v/v) APS
Sucrose buffer (Nuclear extracts):	0.32M Sucrose 10mM Tris, pH 7.9 2mM MgAc. 3mM $CaCl_2$ 0.1% (v/v) Triton X-100
SSC	150mM NaCl 150mM Sodium citrate pH/NaOH/7.5
STE	1mM EDTA 10mM NaCl 10mM Tris.Cl pH7.5
TAE (50x stock soln.1Lt)	57.1ml Glacial acetic acid 37.2g $Na_2EDTA.2H_2O$ 242g Tris base, pH8.5

Tail solution	100mM EDTA pH8.0 150mM NaCl 1% (w/v) SDS 10mM Tris-HCl pH7.5
TBE (10x stock soln.1Lt)	55g Boric acid 40ml 0.5M EDTA 108g Tris base
TBS	140mM NaCl 20mM Tris pH/HCl/7.6
TE	1mM EDTA 10mM Tris base pH/HCl/7.6
Trypan blue staining solution:	Trypan Blue [0.4% (w/v)] 0.8% (w/v) NaCl 0.06% (w/v) KH_2PO_4
Trypsin solution:	0.25% (v/v) trypsin 0.02% (v/v) EDTA diluted in PBS
Washing buffer (Dynabeads)	1% (v/v) FCS final concentration in PBS

2.3 Formulation of most frequently used growth media

Agar plates	250ml dH_2O 10g Tryptone Oxoid L42 5g Yeast extract (Oxoid) 10g NaCl make up to 1lt with dH_2O pH 7.54 (NaOH)
Luria-Bertani (L-broth)	1% (w/v) Bacto-tryptone 0.5% (w/v) Bacto-yeast extract 1% (w/v) NaCl
Complete tissue culture medium	RPMI 1640 medium 5-20% (v/v) FCS 2% (v/v) L-glutamine (200mM stock) 2% (v/v) Penicillin/Streptomycin (10,000U/ml stock)
Methylcellulose medium	1% (v/v) methylcellulose in Iscove's MDM, 30% (v/v) FCS, 10^{-4}M 2-Mercaptoethanol, 2mM L- glutamine 10ng/ml rodent IL-7.
'Transfer' medium:	RPMI 1640 medium 1% (v/v) Fungizon (250mcg/ml stock) 5% (v/v) L-glutamine (200mM stock) 4% (v/v) Penicillin/Streptomycin (10,000U/ml stock) 1.5% (v/v) gentamicin (50mg/ml stock)

Freezing medium:	per ml of medium add 200µl FCS (20% final volume) and 100µl DMSO (10% final volume)

2.4. Methods

2.4.1 Animal procedures

All mouse procedures were performed according to the Home Office regulations and as detailed in the project licence. Unless stated, the procedures were conducted by me as described in my Home Office personal licence. Where the use of brief anaesthesia was required, a halothane/oxygen mixture was used in an anaesthesia chamber.

I. Breeding and numbering of transgenic mice

All food, water and bedding requirements were handled by the technicians in the animal house. Pairing of breeding animals and monitoring of litters was performed by the animal technicians and myself. At the age of three weeks, the pups were weaned and males and females were separated. The mice were given numbers (in consecutive sequence for each line), identified by ear punching, and a small biopsy (tail tip of 0.5-1cm) was taken under halothane anaesthesia. The biopsy sample was transferred to a Eppendorf tube for subsequent genomic DNA extraction and assessment of the transgenic status of the animal. The mouse tail tip was cauterised with a hot iron to stop bleeding and sterilise the wound.

II. Monitoring animal health status

According to the procedures as described in the Home Office project licence, all animals were monitored regularly for signs of ill health and discomfort. Any animal suffering (e.g. lymphoma) was euthanised without delay and a dissection was performed to examine the cause of illness.

III. Mouse lines

Line number	Transgene	Expression	Reference
26	EμEBNA1	B cells	Wilson and Levine, 1992; Wilson *et al.*,1996
59	EμEBNA1	B cells	Wilson and Levine, 1992; Wilson *et al.*,1996

IV. Tissue collection from transgenic mice

Animals were euthanised by a schedule 1 method according to guidelines. Mice were always dissected in the same way.

The fur of the animal was wetted with ethanol to exclude loose hair from the samples. Leaving the peritoneum intact the skin of the animal was cut back with fine scissors and forceps, and the peripheral lymph nodes (LN) collected. The peritoneum was then opened and the spleen, mesenteric LNs and liver were collected by careful excisions. The chest cavity was then opened and the thymus and cervical LNs collected. Any abnormalities in any of these or other tissues were noted and the lesion(s) collected. Part of the lesion was then put into 10% (v/v) buffered neutral formalin fixative and the rest snap frozen in liquid N_2 for -70°C storage. In every case a piece of tail and/or the brain was also frozen for further genomic analysis and confirmation of transgenic status if required.

Samples in fixative were sent for histopathological analysis to a pathologist contracted to the Glasgow Vet. School histopathology service where necessary.

2.4.2 Mammalian cell culture techniques

I. Explantation of lymphomas

The explantation of tissues was performed under sterile conditions. Surfaces and equipment were always cleaned with 100% (v/v) ethanol.

The lymphoma was excised, rinsed thoroughly in 1xPBS and cut in two pieces. The bigger piece was placed in explant transfer medium and placed on ice. The smaller piece was further cut in two, one piece was snap frozen and stored at -70°C and the other was placed in fixative for pathology. The lymphoma sample was transferred to a petri dish with 37°C warmed complete tissue culture medium with 20% (v/v) FCS.

The lymphoma was cut into small pieces using a sterile scalpel or mashed between two microscope slides with frosted ends, and then placed in a F25cm^3 culture flask. 5ml of warm complete tissue culture medium was added and the flask was incubated at 37°C, 5% (v/v) CO_2 for 3 to 5 days. Cell suspensions were then

transferred to a new F25cm^3 flask with 5-7ml complete tissue culture medium and incubated for further time. Cell growth was monitored and the suspension cells cultured in complete tissue culture 20% (v/v) FCS containing medium until their number expanded dramatically. At this point the growth medium was reduced to a 10% (v/v) FCS content. The cells were sub-cultured and then stored as frozen stocks as described bellow.

II. Sub-culturing of mammalian cells

When the cell populations were expanded they were sub-cultured to allow maintenance of the cell line. In the case of lymphoma or suspension cells, the cells were transferred to an appropriate volume falcon tube and pelleted in a Heraeus 400 centrifuge at 1000rpm/194x g/rotor no.8172 for 5 minutes. The cell pellet was then resuspended in fresh medium at the required density.

In the case of adherent cells, the medium was aspirated and the cells were washed in PBS.A/EDTA. Trypsin solution was gently added over the cells and incubated at 37°C for 5 minutes to dislodge the cells. The cells were then pelleted in exactly 194xg/5mins and then resuspended in the appropriate medium at the required density.

III. Storage of frozen cell stocks

After the cell population had expanded, the suspension cells were pelleted as above and resuspended in complete tissue culture medium with 20% (v/v) FCS and 10% (v/v) dimethyl sulphoxide (DMSO). 1ml aliquots of approximately 1x10^6 cells were placed in 1ml cryovials and transferred in Nalgene cryo 1°C freezing container which was placed in a -70°C freezer for 24 hours to ensure gradual (1°C/min) chilling of the cells. The vials were then transferred to a liquid N_2 container for long term storage.

IV. Defrosting of frozen cell stocks

Required cell stocks were removed and transferred to the culture room in a liquid N2 container, where they were quickly thawed in a 37°C water bath. The cells were washed in medium (minus FCS) by centrifugation 194xg for 5mins, to remove DMSO. They were then transferred to a F25cm^2 flask containing 5ml of appropriate medium.

V. Erythrocyte exclusion

Primary cells to be treated were transferred in an appropriate sized Falcon tube and pelletted at 194x g for 5 minutes. The cell pellet was resuspended at a concentration of 3x10^8 cells/ml in NH_4Cl buffer and incubated for 10 min at room temperature. This time is adequate to lyse erythrocytes because of their permeability to ammonium ions. 37°C does damage cells and at 4°C lysis is slower. Cells were immediately diluted with greater than a 5-fold excess of medium, mixed well by inverting the tube a few times and pelleted by spinning the tube at 194xg for 5 min. The supernatant was discarded, the cell pellet was resuspended in 10ml complete tissue culture medium or 1xPBS and a cell count was then undertaken.

VI. Viable cell counting by trypan blue exclusion

Immediately before counting, cells in a 10ml suspension were evenly distributed by gentle pipetting. 100µl of the cell suspension was mixed with 400µl of trypan blue staining solution in a Eppendorf tube. Cell counting was performed using a haemocytometer. An aliquot of the stained cells was transferred to one half of the cleaned chamber, sufficient to cover the semi-chamber area but not flood the surrounding trough. Cells were counted under 40x objective and only the non-blue cells wholly inside the area or touching the upper and left borders of the squares were scored. Cell number was calculated by multiplying the average count per 0.2mm^2 squares by the dilution factor (x5 in this case) and by 10^4 to give the viable cell count/ml. (Both live and dead cells can be scored using the trypan blue-exclusion method because dead cells take up the dye and are stained blue).

VII. B cell selection using DynaBeads, Dynal

Immunomagnetic cell isolation using Dynabeads Mouse panB (B220) is a fast and reliable method for isolation of B220 positive cells by positive selection.

i. Dynabeads washing procedure. The Dynabeads should be washed before use. The beads (already supplied in a buffer) were resuspended thoroughly in the vial and the desired amount was transferred to a Eppendorf tube. The tube was placed on a Dynal MPC magnetic device for 2 min and the fluid was pipetted off. 1-2ml of PBS/1% (v/v) FCS washing solution was added, the tube was removed from the magnetic device and the beads resuspended by inverting the tube. The wash was repeated and finally the beads were resuspended in an equal volume of washing buffer that was originally pipetted from the vial.

ii. Positive selection of B220 (+) mouse cells. Cells were isolated from spleen by squeezing the tissue between the frosted sides of two microscope slides releasing suspension splenocytes into PBS. Red blood cells (RBCs) were lysed, cells were washed in PBS and counted using the Trypan blue-exclusion method. All the subsequent steps were performed at 4°C to reduce any non-specific attachment of phagocytic cells to Dynabeads. Volumes were kept small for easier handling. After cell counting an estimation of the number of target cells was made and the required volume of pre-washed Dynabeads mouse panB (B220) was added (100µl= $4x10^7$ beads/ 10^7 target cells) directly to the cells suspension. The contents were mixed by gentle vortexing for 1 second and incubated for 20min at 4°C by gentle tilting and rotation. The tubes were then placed in the Dynal MPC magnetic device for 2 min to collect the beads/cells. The supernatant was decanted and the beads/cells were gently resuspended in 1ml 1x PBS/0.1% (v/v) FCS by gentle pipetting. The wash was repeated twice. At this point the isolated cells can be used for various research applications or stored at -70°C for later use.

Note: If detachment of the beads is required this can be achieved by overnight incubation of the rosetted cells at 37°C followed by agitation by pipetting. The detached cells are then removed in the supernatant when the tube is placed in the magnetic device.

VIII. FACS of primary cells

The spleen was excised and rinsed thoroughly in EtOH and was then placed in cold PBS solution. The femurs, collected for bone marrow (BM) extraction, were rinsed in EtOH and placed in cold PBS. The tissues were transported from the animal house to the laboratory, in PBS on ice. They were then transferred to 3ml (each) of cold fresh PBS in a petri dish in the culture hood. The spleen cells were released as described above. The BM was obtained from the femurs using a syringe and forcing PBS solution through the bone. The cells were collected into 15ml Falcon tubes containing 10ml PBS and centrifuged 194x g for 5min. Erythrocytes were excluded as described above. The cells were washed twice in cold PBS and counted, using the trypan blue exclusion method. $5x10^5$ cells were transferred to 1.5ml Eppendorf tubes for each staining. The cells were pelleted, in the bench top centrifuge, (300xg for 5min) and the cell pellet was resuspended in 0.5ml of PBS. 20μl of 1x "goat wash" was added, mixed by flicking the tube and incubated at 4°C for 20min. This blocking step was performed in order to minimise any non-specific binding. The cells were pelleted, washed in PBS, and resuspended in 0.5 ml PBS 2-4μl (approximately 1μg) of each antiserum was added, and incubated for 30min-1hour at 4°C (for antisera see Materials section 2.1.1). Samples were then pelleted, the supernatant was carefully removed, were washed in PBS once, to remove any residual unbound antibodies, and resuspended in 0.5ml PBS. Fluorescence was analysed using a Becton-Dickinson flow cytometer using 5-10 x 10^3 cells per sample. Negative staining controls were always used. Negative controls (isotype controls) establish background fluorescence and non-specific staining of the primary and/or secondary antibodies FITC or PE conjugated. They should not be reactive with the cells being analysed and should be used at the same concentration as the fluorophore conjugated specific antibody. The degree of autofluorescence or negative control reagent fluorescence will vary with the type of cells under study and sensitivity of the instrument used.

IX. Proliferation: Colony assay (methylcellulose cultures)

Methylcellulose culture was performed by using a modification of a technique described by Iscove *et al.* (1974). The complete methylcellulose medium for murine pre-B cell colony assays consists of: 1% (v/v) methylcellulose in Iscove's MDM, 30% (v/v) FCS, 10^{-4}M 2-Mercaptoethanol, 2mM L-glutamine and 10ng/ml rodent IL-7. $5x10^4$ BM or spleen cells were inoculated in a 1:10 (v/v) ratio to the methylcellulose medium. 0.5ml of cells were added to 4ml medium to yield quadruplicate 1.1ml cultures. Because methylcellulose medium is very viscous, after the cells were added, it was mixed well by shaking and left to sit for 10min to allow bubbles to rise to the top before dispensing. Dispensing into 22mm plates was done using a syringe and a 16 gauge blunt needle and then it was evenly distributed by gently tilting and rotating the plates. On day 7 of culture, aggregates consisting of approximately >40 cells were scored as a colony and aggregates consisting of approximately <40 cells were defined as clusters.

X. Apoptosis detection: AnnexinV-FITC/PI staining (Vermes *et al.*, 1995)

Cells were prepared as for the FACS analysis described in section VIII. This approach can be used directly in normal cultures after the induction of apoptosis. Cells were washed with cold PBS and then resuspended in 1x binding buffer (see solutions section) at a concentration of $1x10^6$ cells/ml. 100µl of the solution ($1x10^5$ cells) were then transferred to a FACS tube. 5µl of Annexin V-FITC and 10µl of PI were added per sample. The samples were gently vortexed and incubated for 15min at room temperature (20-25°C) in the dark. 400µl of 1x binding buffer was added to each sample and cells were analysed by FACS within 1 hr. The early apoptotic cells are Annexin V+/PI-. Cells that stain positive for both Annexin V-FITC and PI are either in the end stage of apoptosis, undergoing necrosis, or are already dead. Cells that stain negative for both Annexin V-FITC and PI are alive and not undergoing any measurable apoptosis.

XI. Rat 1 cells – MAPK and SAPK activation controls

For the MAPK activation controls, Rat 1 cells were grown to 70% confluence in 5% (v/v) Dulbeco's medium supplemented with penicilin/streptomycin (P/S) and L-glutamine. The medium was then aspirated and washed once with 1xPBS. The cells were then serum starved in medium containing only 0.25% (v/v) FCS +P/S+L-glutamine for 48hr. The serum concentration was then increased to 10% (v/v) and the cells incubated for only 15 min. Brief incubation is important because MAPK activation is transient and maximal at about 15min. The medium was then aspirated and cells were rinsed with ice cold 1xPBS. The cells were collected using a cell scraper, they were transferred and pelleted into a Eppendorf 1.5ml tube and snap frozen in LN_2 and stored at –70°C. Negative controls were Rat 1 cells grown in 5% (v/v) FCS containing medium that were then frozen as above.

For the SAPK activation Rat 1 cells were grown to 70% confluence with 5% (v/v) FCS containing medium. Anisomycin (a protein synthesis inhibitor) was then added to a concentration of 10μM for 15min incubation. The medium was then aspirated, cells washed in ice cold 1xPBS and frozen as above. Negative controls were Rat 1 cells grown in 5% (v/v) medium with no anisomycin treatment.

XII. CD40-ligand producer layer: Feeder layer preparation for B cell activation

CD40 ligand (CD40L) producing cells (K47) are L929 mouse fibroblasts transfected with aCD40L expressing construct. Control cells were L929 cells transfected with the vector only. All cells were cultured in RPMI medium supplemented with 5% (v/v) FCS, G418 (0.3mg/ml) (a selective agent permitting growth of those cells carrying the G418 resistant plasmid), L-glutamine and antibiotics. Cells were cultured until they reached confluence and washed with PBS to remove any residual FCS. They were then killed by adding mitomycin C at a concentration of 5μg/ml for 1hour and then washed 3 times with PBS to wash off any residual mitomycin. Primary cells could then be added to perform the planned assay.

2.4.3 Bacterial cell culture techniques

I. Bacterial cell cultures

The genotypes of all the strains of *Escherichia coli (E.coli)* are detailed by Maniatis *et al.*, (1982) as are the basic techniques used for the bacterial growth, plating and purification. E.coli DH5 was used for all plasmid propagation and was grown in Luria- Bertani (L-broth) medium. E.coli, Amp^+ resistant cells, were grown in L-broth supplemented with 50μg/ml Ampicillin. E.coli M15, used for the propagation of puC19 plasmid vectors, was grown in L-broth supplemented with 50μg/ml Ampicillin and 50μg/ml kanomycin. Glycerol stocks made and stored at -70°C.

II. Plasmid DNA transformation of *E.coli* DH5

For each transformation, E.coli DH5 competent cells were used. Cells were made competent according to the $CaCl_2$ method described in Current protocols in Molecular Biology (by Wiley Interscience) Lab mannual by a laboratory technician. An aliquot of lab stock competent cells were thawed and incubated for 10mins on ice. 100ng of plasmid DNA in a maximum volume of 80μl, was added to the cells and incubated for a further 15mins on ice. The cells were then heat-shocked at 42°C for 90secs and returned to ice for 2mins. 0.8ml of warmed L-broth was added and the cells incubated, with gentle agitation, at 37°C for 30mins. The culture was centrifuged at 9,000 xg for 1min to collect the cells and then, most of the medium was aspirated. The cells were resuspended, by gently tapping the tube, and plated on duplicate L-broth (+/- ampicillin as required) 1.1% (w/v) agar plates and incubated inverted at 37°C overnight.

2.4.4 DNA extraction and manipulation

I. Small scale preparation of plasmid DNA

The Wizard™ *Plus* Miniprep DNA purification system from Promega was used for small scale plasmid DNA isolation according to the manufacturers protocol (adapted from the method of Birnboim and Doly, 1979). 1.5ml of bacterial culture

was transferred into an Eppendorf tube and pelleted in a microcentrifuge for 1-2 min. The supernatant was removed completely and the cell pellet was resuspended in 0.2ml of cell re-suspension solution [50mM Tris (pH 7.5), 10μM EDTA and 100μg/ml RNAse A]. 0.2ml of cell lysis solution [0.2 M NaOH and 1% (w/v) SDS] was added and the contents were mixed by inverting the tubes a few times. (Some bacterial cells are more resistant to lysis and may require incubation for 3-5 min for efficient lysis). 0.2ml neutralisation solution (1.32M potassium acetate) was added and the contents were mixed by inverting the tube a few times. The lysate was centrifuged at 10,000xg in a microfuge for 15min. Meanwhile, one Wizard™ Minicolumn was prepared for each miniprep. The barrel from a disposable 3ml Luer-Lock^R syringe was attached to the extension of the minicolumn and 1ml of the resuspended resin was placed into the barrel. The cleared lysate from each miniprep was transferred into the barrel of the minicolumn/ syringe assembly containing the resin. The syringe plunger was inserted carefully and the slurry was gently pushed into the minicolumn. 2ml of Column Wash Solution [80mM potassium acetate, 8.3mM Tris-HCl (pH 7.5), 40μM EDTA and 55% (v/v) EtOH] was added into the barrel of the assembly and was gently pushed through the minicolumn. The syringe was then removed and the minicolumn was transferred to a 1.5ml Eppendorf and centrifuged at 10,000xg in a microcentrifuge for 2 min to dry the resin. The minicolumn was then transferred to a new tube, 50μl of TE (heated at 70°C) was added and minipreps were centrifuged at 10,000xg for 20 seconds to elute the DNA. The minicolumn was discarded and the plasmid DNA was stored at -20°C.

II. Large scale preparation of plasmid DNA (alkaline)

This protocol is a modification of the method of Birnboim and Doly (1979) and works well with all commonly used strains of *E.coli*.

200ml of selective LB medium were inoculated (with 100μl of a liquid mini culture) with the desired plasmid-containing bacteria and grown at 37°C for 12-16 hours with vigorous shaking (~300rpm). The bacteria were harvested by centrifugation at 6,000x*g* for 15mins at 4°C (in a Beckman JA-17 rotor). The

bacterial pellet was resuspended in 10ml of *SolI* (50mM glucose, 25mM TrisCl [pH 8.0], 10mM EDTA [pH8.0]), with 1ml of freshly prepared solution of lysozyme [10mg/ml in 10mM TrisCl (pH8.0)]. 20ml of freshly prepared Solution II [200mM NaOH, 0.1% (w/v) SDS] was added and the contents were mixed thoroughly by gently inverting the tube several times and incubated at room temperature for 10 mins. 15ml of ice cold Solution III (5M potassium acetate, glacial acetic acid [3M K, 5M acetate]) were added and the contents were mixed by shaking the tube several times. The samples were then incubated on ice for 10mins. A flocculent white precipitate formed at this stage consisting of chromosomal DNA, HMW RNA and K/SDS/protein/membrane complexes. The bacterial lysate was centrifuged at 11,000 xg (in a Becckman JA-17 rotor) for 15mins at 4°C. The supernatant was then filtered through a Whatman paper into a 250ml centrifuge tube. 28ml (0.6 volume) of isopropanol was added to the filtered supernatant, mixed well and incubated for 10min at room temperature. The plasmid DNA was recovered by centrifugation at 11,000 xg for 15mins at room temperature. The supernatant was carefully removed and the DNA pellet washed in 70% (v/v) EtOH at room temperature. The EtOH was removed by spinning the samples again at 11,000 xg for 15mins and aspiration. The pellet was air dried and resuspended in 3ml TE (pH8.0) buffer. The sample was then transferred to Eppendorf tubes.

The DNA was protein extracted once with equal volume of chloroform/isoamyl alcohol (24:1) and once with phenol/chloroform/isoamyl alcohol (25:24:1). The liquid phases where separated by centrifugation at 13,000x*g* for 15mins at 4°C in an Eppendorf centrifuge. The upper liquid phase containing the DNA was transferred to clean sterile tubes. The DNA was precipitated by adding equal volume of isopropanol and 1/10 volume (of the total) of 5M NaCl, at -20°C for 30 mins. The tubes were then centrifuged at full speed for 15 mins at 4°C, the DNA pellet washed with 70% (v/v) EtOH, air dried and resuspended in 1ml TE (pH 8.0). 10µl of RNase A added and incubated at 37°C for 30mins. The DNA pellet was protein extracted once more with equal volume of phenol and precipitated as before. The DNA pellet was washed with 70% (v/v) EtOH, air-dried and resuspended in

300µl of TE buffer. The DNA was then quantitated and digested with the appropriate enzymes to obtain the fragments of interest and analysed agarose gel electrophoresis.

III. High molecular weight/genomic DNA extraction

Genomic DNA was extracted from tissues or tails using a standard phenol/ chloroform/chloroform method (Wilson *et al.*, 1990). A tissue or tail segment was placed in a 1.5ml Eppendorf tube containing 0.7ml of tail solution. 35µl of proteinase K (0.47mg/ml final concentration) was added to each sample and incubated at 55°C in an Eppendorf thermomixer, shaking, overnight. At this stage, samples can be stored at 4°C indefinitely. The nucleic acids were separated from protein by adding an equal volume of buffer saturated phenol (pH7.5) and thoroughly mixing by inverting the tubes 15-20 times (no vortexing). The samples were then centrifuged in an Eppendorf centrifuge 13,000 xg for 5mins. The upper aqueous layer containing the DNA/RNA was carefully transferred into a fresh Eppendorf tube, without disturbing the interface, and protein further extracted by mixing with an equal volume of 2FC (see Solutions section). The samples were centrifuged in the Eppendorf centrifuge at 13,000 xg for 5mins. Again, the upper aqueous layer was carefully transferred into a fresh Eppendorf tube and mixed with 100µl of 10M NH_4OAc and 750µl of ice cold EtOH by shaking vigorously for several seconds (no vortexing). The samples were then centrifuged at 8,000 xg for 2mins to pellet the high molecular (HMW) DNA. The supernatant was removed without disturbing the pellet. The pellet was then washed with 1ml of 70% (v/v) EtOH, centrifuged for 2mins at 8,000 xg and air dried for few mins. The DNA was then resuspended in 215µl TE buffer (pH 7.6-8) by heating to 65°C for 10mins (or longer as required) with occasional agitation. The samples can then be stored at 4°C indefinitely.

IV. Quantitation of DNA

The optical density OD_{260} of DNA was measured by using aliquots of each DNA sample (OD=1=50mg/ml DNA). This allows the DNA concentration to be ascertained. The samples were then adjusted to the required concentration (usually

0.33μg/μl for genomic DNAs or 1mg/ml for plasmid DNAs) and stored at 4°C for HMW DNA or -20°C for plasmid DNA.

V. Restriction and electrophoresis of DNA

Restriction and electrophoresis of DNA was performed according to Wilson *et al.*, (1990). 5-10μg of genomic DNA was usually digested with restriction enzyme (1unit/μl) (0.5units/μg of DNA) and the manufacturers recommended buffer (1/10th of the total volume) at 37°C overnight for complete digestion. If necessary, further restriction enzyme was added and incubated for 2 hours at this point. The reaction was stopped by heating the samples for 10min at 65°C. 10% (v/v) loading buffer was added to each reaction and 3-5μg of the samples were loaded onto a 0.8% (w/v) agarose gel made up in 1xTAE. In a 300ml gel 10μl of 10mg/ml EtBr were added. The samples were electrophoresed in a horizontal tank in 1xTAE buffer for 5hrs at 100V. 1μg of 1Kb DNA ladder was also loaded to one of the wells for subsequent band size evaluation. The DNA was visualised on a short wave (280nm) UV transiluminator to ensure the DNA was intact and equally loaded. A photograph was always taken for records.

VI. Isolation of specific DNA fragments

Specific DNA fragments were isolated in two ways. The first was used for easily separable fragments and the second was used for less well resolvable DNA fragments and purer isolation.

A. 10-20μg of the plasmid containing the required DNA to be used as probe, were digested with appropriate restriction enzymes to generate the required DNA fragment(s). The digested DNA was electrophoresed at 50V for 4-6hrs in low melting agarose (LMP-agarose) in 1xTAE with 2μl of 10mg/ml EtBr/100ml. The DNA was visualised on a long wave transiluminator to estimate the DNA content of the desired fragment. The fragment was collected using a scalpel into a Eppendorf tube and was heated to 70°C to melt the agarose and the volume was estimated. The

fragment concentration was then adjusted to approximately 5ng/ml and stored at -20°C.

B. Purer DNA fragments were obtained using DEAE paper (NA45). The restricted DNA sample was electrophoresed in a 1% (w/v) agarose gel at 100V for 4hrs. A small piece of NA45 paper was inserted into a cut in the gel made in front of the desired DNA fragment. Another piece of paper was inserted behind the fragment to prevent contamination with the more slowly migrating DNA fragments or alternatively the gel above the fragment was cut off and discarded. The sample was run for a further 2hrs to transfer the DNA onto the paper, which was monitored under long wave UV to ensure successful transfer. The paper was rinsed in TE and the DNA eluted with 2x15mins incubations in 250µl 1M NaCl at 70°C. Eluates were pooled and extracted with 2FC and chloroform, the DNA was EtOH precipitated, dissolved in TE and stored at -20°C for further use.

VII. Southern blotting of DNA

The gel containing the DNA samples was trimmed to remove ladder tracks and was then treated with DNA denaturant (0.6M NaCl, 0.4M NaOH) for 45mins on a shaking platform. The gel was then washed 3x5mins in 1xTAE buffer. The DNA was transferred onto a nylon membrane (Biodyne B) by electroblotting for 3hrs 75V/1.5A in 1xTAE in a Hoeffer electroblotting tank as described by Wilson *et al.* (1990). The filter was then UV cross linked using a stratalinker and baked for 1hr at 80°C. The filter was stored at -20°C or probed directly (Wilson *et al.*, 1990).

VIII. Preparation of ^{32}P labelled DNA probe and hybridisation

Hybridisation probes for all blots were made using digested DNA fragments for plasmid DNA usually contained within LGT agarose. 25ng of the DNA fragment was used for each probe reaction. The Prime-It kit for Stratagene was used for probe preparation. 10µl of random 9-mer primers (27 OD units/µl) and 19µl of dH_2O were added to the DNA. The mixture was then heated for 5mins at 90-100°C to denature the dsDNA. Then, 10µl of 5xdCTP buffer, 5µl of α^{32}P dCTP (3000ci/mmol) and 1µl

of exo(-) Klenow polymerase were added to the reaction and incubated at 37°C for at least 15mins. Stratagene Nuctrap probe purification columns were used to remove any unincorporated nucleotides from the probe, in the Stratagene push column beta shield device, using STE buffer (according to the Stratagene Prime-It II random primer labelling kit instruction manual). 1µl of the purified probe was used to assess the specific activity of the labelled DNA (>5x10^{8}cpm/µg). The probe could either be stored at -70°C for few days or used directly.

For hybridisation, the blot was placed in a hybridisation tube with 10ml church buffer (see solutions section) in a hybridisation oven and rotated for at least 3hrs at 65-68°C for prehybridisation. The purified probe was denatured by heating for 10mins at 100°C, added directly to the hybridisation tube containing the membrane and rotated at 65-68°C overnight. The blot was then stringently washed 4x10mins in 2xSSC, 0.1% (w/v) SDS at room temperature, on a shaking platform, followed by 2x30mins in 0.1xSSC, 0.1% (w/v) SDS at 68°C in a shaking waterbath. The filter was then stored in polythene bag and taped into an autoradiography cassette with intensifying screen. The filter was exposed to Kodak XAR film at -70°C for the required time and the film was processed in a Kodak X-omat developer.

IX. DNA sequencing-preparation of samples

In house service using the MBSU-DNA sequencing in the Anderson complex, Molecular Genetics, Glasgow University.

2.4.5 RNA extraction and manipulation

To avoid RNA degradation form ribonucleases, all solutions were made up using autoclaved and diethyl-pyrocarbonate (DEPC)-treated H_2O. Eppendorf tubes and pipette tips were autoclaved and kept along with the RNA solutions in a designated RNAse-free cupboard. All surfaces and equipment to be used during the procedures were carefully cleared prior to use with 1% (w/v) SDS and then 75% (v/v) EtOH (made up with DEPC-treated H_2O). Gloves were worn at all times and changed frequently to avoid any contamination with RNAses.

I. Total RNA extraction

Frozen tissues were cut (if necessary) on dry ice using a sterile scalpel, without allowing the sample to thaw before preparation. The frozen sample was placed in a 7ml RNAse-free tube and homogenised in TRI-REAGENT (Sigma) (~1ml/ 50-100mg of tissue) using a polytron homogeniser [Chomczynski and Sacchi (1987) method slightly modified]. 0.2ml of chloroform per ml of TRI-REAGENT was added to the homogenate, the sample was shaken vigorously by hand for 15secs and allowed to stand for 2-15mins at room temperature. The resulting mixture was centrifuged at 11,000xg (10,000rpm in a JA-20 rotor) for 15mins at 4°C. Centrifugation separates the mixture into 3 phases: a red organic phase (containing protein), an interphase (containing DNA), and a colourless upper aqueous phase (containing RNA). The upper layer was carefully transferred into a fresh tube and 0.5ml of isopropanol/ml of TRI-REAGENT was added. The sample was mixed vigorously for 15secs and allowed to stand for 5-10mins at room temperature. The RNA sample was centrifuged at 11,000xg for 15mins at 4°C. The RNA precipitate formed a pellet on the side and bottom of the tube. The pellet was washed once with 75% (v/v) EtOH by vortexing and subsequent centrifugation at 7,500xg for 10mins at 4°C. The pellet was briefly air-dried, resuspended in 300µl TE/1% (w/v) SDS and transferred to an RNAse-free Eppendorf tube. The RNA was then precipitated by adding LiCl to a final concentration 2.5mM (10mM stock which preferentially precipitates RNA instead of DNA), mixed well and precipitated either -70°C for 30mins or -20°C overnight. The samples were then centrifuged at 13,000 xg for 30mins at 4°C. The supernatant was aspirated and the RNA pellet washed with 70% (v/v) EtOH and then resuspended in TE/0.1% (w/v) SDS and stored at -70°C.

II. mRNA extraction

mRNA (polyA+) was purified using oligo-dT cellulose columns (Stratagene or Qiagen, oligotex Quick polyA+ RNA extraction kit) according to the manufacturers protocol. This involves binding of the polyA+ fraction of RNA to a (0.5ml) cellulose oligo-dTcolumn, in a high salt (500mM NaCl) buffer (the flow-

through being the polyA- fraction). The column was then washed at low salt concentration (100mM NaCl) buffer and the mRNA eluted in a salt-free buffer. The mRNA was ethanol precipitated, quantitated and stored as described previously. Using this protocol, the mRNA yield is approximately 3% of the original total RNA sample used.

III. Quantitation and analysis of RNA

4µl of RNA were added to 16µl RNA loading buffer and heated to 65°C for 10mins, 2µl of 10x RNA loading dye was added and the samples were microfuged briefly. The samples were then loaded onto a formaldehyde gel. For a 50ml minigel, 0.7g agarose/40ml DEPC-H2O melted, 5ml MOPS-E (10x) added and cooled to 60°C before adding 2.6ml formaldehyde. The gel was electrophoresed at 100V for 1hr in 1xTAE buffer (DEPC-H_2O). The RNA was then viewed on a UV transiluminator to check the integrity of the RNA sample (presence of the three ribosomal bands). Degraded samples were not used further.

5µl of RNA was used to measure the OD260/280 at 1/60 dilution (OD_{260}=1= 40mg/ml). The OD280 was measured to estimate the purity of the sample. Proteins have an absorbance maximum at 280nm. An OD260/280 ratio of 1.8 or greater is taken as an acceptable level of purity. The RNA samples were adjusted to a concentration of 0.2-0.5mg/ml by adding TE/1% (w/v) SDS and absolute EtOH (in a final ratio of 1:2.5 aqueus:EtOH) and stored at -70°C. Required amounts of RNA for analysis were taken and precipitated with 3M NaOAc (1/35 volume).

IV. Northern blotting and detection of specific RNA bands

Gel tank and combs were treated with 3% (v/v) H_2O_2 for 10min and rinsed thoroughly with DEPC-H_2O to remove any RNAses. 20µg of each total RNA or 1-5µg mRNA sample]TE/0.1% (w/v) SDS/100% (v/v) EtOH] was aliquoted into fresh Eppendorfs and precipitated with 3M NaOAc (1/35 volume). The RNA pellet was resuspended thoroughly in 4µl DEPC-H_2O by heating to 65°C and vortexing. 16µl of RNA loading buffer was added and the sample was heated to 68°C for 10mins and

then transferred to ice. 2µl of 10x RNA loading dye was added and the samples were loaded on a formaldehyde gel. 1% (w/w) agarose in MOPS electrophoresis buffer (MOPS-E) was melted and cooled to 60°C before adding 17.8% (v/v) formaldehyde [of the 38% (w/v) stock solution] plus H_2O to the final volume for pouring. The gel was electrophoresed in circulating 1xMOPS-E buffer, slowly (50V) at 4°C for ~10hrs (Wilson *et al.*, 1990).

The gel was then trimmed and soaked 3x20mins in 0.5xTAE and electroblotted onto a nylon filter as described for southern blotting in section 2.4.4. The membranes were then treated and hybridised in the same way as described previously for Southern blots (section 2.4.4).

V. Slot blot analysis of total cell RNA

The slot blotting is a less time consuming technique than Northern blotting as it does not involve electrophoresis and separate blotting. It is particularly useful for quantifying expression levels of a specific gene, as all the sample is located at one point and not separated by electrophoresis (as in Northern).

The slot blot apparatus (Schieder and Schell) was treated with 3% H_2O_2 for 10mins and then thoroughly rinsed with DEPC-treated H_2O to remove any RNAses. (Slot blots were prepared in quadruplicate to have enough blots for all probes because each blot can be used approximately five times). 22µg of each RNA sample (5µg/slot) was resuspended in 44µl TE/0.1% (w/v) SDS. 132µl loading buffer was added to each sample, which was heated at 65°C for 10mins in an Eppendorf thermomixer. The tubes were then placed on ice and 44µl of cold 20xSSC was added in each. The slot blot apparatus was assembled with two pieces of pre-wetted 3mm paper underlying the nylon BiodyneB membrane (cut to size and pre-soaked in 1xTAE for 30 mins). The samples were loaded, 50µl/slot (~5µg) and then the vacuum was applied. When the samples had been sucked through, one drop (approximately 20µl) of 5xTAE was added to each slot and sucked through. The membrane was then UV cross linked and baked, and the blot probed and washed exactly as a Southern or a Northern blot membrane (Wilson *et al.*, 1990).

VI. cDNA synthesis

i. First strand cDNA synthesis

cDNA synthesis was performed using the SuperScript Pre-amplification system for first strand cDNA synthesis (Gibco) according to the manufacturers protocol. The procedure uses 1 to 5μg of total RNA to produce the first strand cDNA.

All solutions and equipment were RNase-free. Usually 2μg of total RNA (*n* μl) was mixed with 1μl oligo(dT)12-18 primers (0.5μg/μl) and made up to 12μl with DEPC-treated H_2O in a 0.2ml PCR tube. The mixtures were incubated at 70°C for 10mins, in a PCR cycler, and then placed on ice for at least 1min. In a separate Eppendorf tube the master reaction mix (2μl of 10xPCR buffer, 2μl of 25mM $MgCl_2$, 1μl of 10mMdNTP mix and 2μl of 0.1M DTT) was prepared. 7μl of the master mix were added to each RNA/primer mixture, mixed gently, and collected by brief centrifugation. The mixture was then incubated at 42°C for 5min. 1μl (20units) of SuperScript II RT was added to each tube, mixed and incubated at 42°C for a further 50min period. The reactions were terminated by heating at 70°C for 15min and chilled on ice. The reactions were then collected by brief centrifugation, 1μl of RnaseH (0.5mg/ml final concentration) was added to each tube and incubated at 37°C for 20min. The samples if not directly used can be stored at -20°C indefinitely.

ii. Second strand synthesis and amplification of target cDNA

10% of the first strand cDNA reaction was used for the amplification of the target cDNA. The following components were added in a thin walled 0.2ml PCR tube: 5μl of 10x PCR buffer, 3μl of 25mM $MgCl_2$, 1μl dNTP mix (0.2mM final concentration), 1μl of each specific amplification primer (10mM), 2μl of the first strand cDNA and 0.5μl of Taq DNA polymerase (2 units). Autoclaved distilled water was added up to a 50μl final volume. The reactions were incubated at 94°C for 5mins to denature the RNA/cDNA hybrid and 30 cycles of PCR of the optimised programme for each specific primer pair were performed in the PTC200 thermal

cycler. 10µl of the amplified sample was usually analysed using agarose gel electrophoresis.

2.4.6 Mouse Atlas cDNA Expression Array (Clontech Laboratories, Inc). (Methods are according the manufacturers instructions)

I. Preparation of cDNA probes

Two, duplicate, Atlas array membranes are provided with this kit, on which are duplicate spots of cDNAs isolated from 588 murine genes, including several house keeping genes to act as loading controls. The full list of genes included in this array is given in Appendix 5.1

i. cDNA synthesis

The 10µl reaction described below, converts 1µg of polyA+ RNA into ^{32}P labelled first strand cDNA. All solutions and equipment were RNAse free. The Master Mix for all labelling reactions (plus one extra reaction to ensure that there is sufficient volume) was prepared in a 0.5ml microfuge tube by combining the following (per reaction): 2µl of 5x reaction buffer (provided in the kit), 1µl of 10x dNTP mix (for dATP label), 3.5µl [$a^{32}P$]dATP (3,000Ci/mmol, 10µCi/ml, Amersham), 0.5µl DTT (100mM) and 1µl MMLV reverse transcriptase (50units/µl). The components were mixed well and the mix was stored on ice. 1µg (1-2µl) of polyA+ RNA sample was mixed with 1µl 10x CDC (cDNA cocktail) primer mix, in a 0.2ml PCR tube, by vortexing. The tubes were then centrifuged briefly in a microfuge and incubated in a preheated PCR thermal cycler (Peltier PTC-200 from MGR) at 70°C for 2min. The temperature of the thermal cycler was then reduced to 50°C and the samples were incubated for 2min. 8µl of the master mix was added to each reaction tube and the contents were mixed by gentle pipetting. The tubes were then incubated in the PCR thermal cycler at 50°C for 20min. The reaction was stopped by adding 1 µl of 10x termination mix.

ii. Column chromatography

Purification of the ^{32}P labelled cDNA fragments (>0.1kb) was performed using CHROMASPIN-200 DEPC-H_2O columns (supplied by the manufacturer).

After the gel matrix in the column was set, the sample was carefully applied in the centre of the gel bed's flat surface and allowed to be fully absorbed into the resin bed. The column was washed once with 40µl deionised H_2O and once with 250µl deionised H_2O (the washes were discarded in appropriate container for radioactive waste) and the column was then transferred to a clean 1.5ml Eppendorf tube. Four fractions were collected by adding 100µl of deionised H_2O to the column and allowing the H_2O to completely drain out of the column (each fraction was collected into a separate tube). The fractions were monitored for ^{32}P incorporation into the probe using the Cerenkov method (Reichert and French,1994) and were then stored at -70°C. In this method the entire sample can be counted without the addition of scintilation cocktail to the tubes. The samples are counted in the tritium (^3H) channel. Plotting the elution profile (which will give 2 peaks), the purified labelled probe is the first peak of the Cerenkov counts (usually fraction 2 and maybe 3), the second peak consists of the unincorporated nucleotides). The fractions used for the hybridisation of the Atlas membranes should be ~2-5x10^6 cpm.

II. Hybridisation of the mouse Atlas Array

A test hybridisation was always performed using the sample probe and a blank piece of Nytran-Plus membrane to assess the level of high background (indicative of poor quality probe) which could affect the hybridisation signals. Only low background probes were used.

A solution of ExpressHyb (supplied by the manufacturer) and sheared salmon testes DNA was freshly prepared: 15ml of ExpressHyb solution prewarmed at 68°C. 1.5mg of the sheared salmon testes DNA was denatured at 95-100°C for 5min, chilled quickly on ice and then mixed with the prewarmed ExpressHyb solution. The mixture was kept at 68°C until ready to use. The Atlas Array membrane was wetted with deionised H_2O and placed into a Hybridisation tube. 10ml of the prehybridisation solution was added and prehybridised for 30min with continuous agitation at 68°C.

The labelled cDNA probe (~200µl, 2-5x10^{6}cpm) was mixed with 1/10th of the total volume (about 22µl) of 10x denaturing solution (1M NaOH, 10mM EDTA) and incubated at 68°C for 20min. 5µl (1mg/ml) of C_0t-1 DNA (a mixture of highly repetitive DNA to reduce non-specific binding) was added and an equal volume (~225µl) of 2x neutralising solution (1M NaH_2PO_4 [pH 7.0]) was added. The probe was incubated for a further 10 min at 68°C (the final probe concentration was ~0.5-1x10^{6}cpm). The probe was then mixed with 5ml of the prewarmed solution prepared earlier. The prehyb solution was discarded and replaced with the solution containing the cDNA probe which was evenly distributed over the membrane and hybridised overnight with continuous agitation at 68°C.

Two washing solutions I and II were prepared and prewarmed at 68°C. The hybridisation solution was discarded in an appropriate container and replaced with 200ml of prewarmed solution I [2xSSC,1% (w/v) SDS]. The Atlas Array was washed four times for 20 mins at 68°C. Two additional 20min-washes were performed in 200ml of prewarmed solution II [0.1xSSC, 0.5% (w/v) SDS] with continuous agitation at 68°C. Using forceps, the Atlas Array membrane was removed from the bottle and wrapped in plastic wrap. The membrane was then exposed to a phosphoimaging screen at room temperature or to x-ray film at -70°C with an intensifying screen for different exposures of variable length of time. The image was analysed using phosphoimager.

2.4.7 Protein extraction and manipulation

I. Whole cell protein extraction (adapted from Marais *et al.*, 1993)

The following protocol can be used for protein extraction from either tissue culture cells, frozen cell pellets or homogenised tissue. Cells were collected, washed with TBS (Tris buffered saline) and pelletted by centrifugation at 1500xg for 5min. The pellet was resuspended in 1ml TBS and transferred into an Eppendorf tube and pelleted again by brief centrifugation (~15 seconds) in a microfuge. TBS was removed and cells were lysed by the gradual addition and flipping the tube, of protein extraction buffer (600µl in total). Cells were then lysed completely by pipetting up

and down (using a yellow tip) a few times. The lysate was then centrifuged at 14000xg for 15 min in a microfuge in the cold room. The supernatant was then transferred into a fresh Eppendorf tube. 5µl of the lysate was used for protein estimation and the remainder was stored at -70°C in aliquots.

II. Nuclear protein extraction

Nuclear protein extracts were obtained using a modification of the protocol described by Schreiber *et al.,* (1989). Fresh splenocytes were collected (as described above, section 2.4.2) in 5ml of ice cold PBS and then transferred into a 15ml Falcon tube on ice. Samples were harvested by centrifugation at 194xg (Heraeus 400 centrifuge rotor no. 8175) for 10min at 4°C. Cells were resuspended in 5ml Sucrose buffer and large debris was allowed to settle for a couple of minutes. Cells were then transferred (leaving debris behind) to a 7ml dounce homogeniser and cells lysed by 7 strokes using a loose pestle and transferred into a 15ml Falcon tube. Samples were then centrifuged at 3,500 rpm for 15min at 4°C to pellet nuclei, the cytoplasm was discarded (unless required) and the tubes were drained briefly on a tissue paper. The nuclear pellet was transferred into an Eppendorf tube and resuspended in 200µl nuclear protein extraction buffer containing proteinase inhibitors (Materials section 2.1.2), which was added dropwise with mixing, and then the tube was vigorously rocked at 4°C for 15min on a shaking platform. The samples were centrifuged for 5min in a microfuge at 4°C at 16,000xg and the supernatant (nuclear extract) stored in aliquots at -70°C.

III. Nuclear matrix protein extraction protocol

This is a combination of the Schreiber *et al.*, (1989) and Smith and Berezney, (1980), protocols with slight modifications. All steps were performed on ice.

Homogenisation of tissues or lysis of cells was performed with a dounce homogeniser using pestle A (the tight one, by 50 strokes) in 0.2ml (for cells) or 1ml (for tissue) of nuclear extraction buffer. The samples were centrifuged in a microfuge at 16,000xg for 30min at 4°C. The supernatant was then transferred into new tubes and stored at -20 or -70°C. The pellet was resuspended in isotonic buffer and 20µg of DNase1/ml was added. The samples were incubated for 16hours at 4°C. The next

day, the samples were sonicated 4 times for 30sec each, at 50 watt. The samples were then centrifuged at 1000xg for 10min at 4°C. (The supernatant is the DNAse fraction). The pellet was then re-extracted with 0.5ml high salt lysis buffer. The samples were pelleted by cetrifugation at 6,000xg for 10min at 4°C yielding the high salt supernatant which was transferred into a new tube and stored at -70°C. 2% (w/v) SDS and 1% (v/v) β-mercaptoethanol were added to the pellet and was boiled at 100°C for 5 min and then stored at –20°C or -70°C.

IV. Quantitation of proteins: Protein assay (microassay procedure 1-20μg protein/ml, BioRad)

5 dilutions of protein standard containing 1.25, 2.5, 5, 10 and 20 μg/ml BSA in TE buffer were prepared and used to generate a standard curve. 0.8ml of standards and appropriately diluted samples were placed in clean and dry plastic $1cm^3$ cuvettes. A "blank" was also included containing 0.8ml sample buffer. 0.2ml Dye Reagent Concentrate (BioRad) was added and samples were mixed several times by gentle inversion of the cuvettes. After a period of 5min to 1 hour, OD_{595} versus reagent blank was measured and OD_{595} values of the standards were plotted versus concentration. Test samples were read from the standard curve.

V. DNA-Protein binding assay (adapted from Frame *et al.*, 1991)

i. Acrylamide gel preparation for EMSAs. After assembling the apparatus and making sure that it was not leaking the non-denaturing polyacrylamide gel was prepared. 7ml of 30% (v/v) Acrylamide (30:1.579 acryl:Bis) was mixed with 2.25ml 10xTBE, 40ml dH2O, 350μl 10% (v/v) APS and 25μl TEMED. The gel was then polymerised for at least 1 hour before loading. The wells were numbered 1-14 (to be able to see them) on the outer glass plate prior to adding buffer (0.5x TBE) to reservoirs.

ii. Sample preparation. A master mix of the reaction components was first prepared by combining the following components: (for 15x30μl reactions) 45μl of 10xBinding buffer (see materials), 30μl poly-dI.dC (2μg/reaction), 2μl DTT (4mM

final concentration), 320µl ddH_2O. 25µl master mix was aliquoted in Eppendorf tubes on ice, 5µg of nuclear protein added and ddH_2O added (if required) to adjust final volume to 30µl. The binding assay was initiated by adding 1µl of ^{32}P labelled oligo probe (0.2-1ng/reaction) and samples incubated on ice for 15-30min. Meanwhile the gel [6% (v/v) non-denaturing polyacrylamide gel] was set up in the cold room and was pre-electrophoresed for 30min (150V). After 30min of binding, samples were loaded carefully into the bottom of each well using a round-bore sequencing tip. 10µl of loading tracking dye was added into one well to monitor progress. Samples were electrophoresed at 150V constant until the dye was approximately 3cm from the bottom of the gel. The running buffer was disposed into a radioactive sink (even though the probe was retained in the gel), glass plates were separated cautiously and the gel very carefully was placed into a glass dish with fixative [10% (v/v) acetic acid-10% (v/v) methanol] and gently agitated for 15-30min. The fixative was then drained off and a double thickness of Whattman 3MM paper was firmly pressed onto the gel. The gel was then lifted, covered with saran wrap and dried using a vacuum drier for 60min. The dried gel was exposed to X-AR Kodak film for the required time.

VI. Labelling oligonucleotide probes and purification

200ng of double-stranded oligo probe was mixed with 2µl of 10x PNK buffer (50mM Tris-HCl pH 7.6, 10mM $MgCl_2$, 5mM DTT, 0.1mM EDTA), 3µl (20µCi) [^{32}P] γATP, 13µl ddH_2O and 1µl T4 polynucleotide kinase. The reactions were incubated at 37°C for 30-60 min and then DNA was separated from unincorporated ATP by gel purification. The labelling reaction was resolved on neutral 15% (v/v) acrylamide/TBE gel, running the dye only 1/2 way to the bottom of the gel. The DNA was located by auto-radiography of the gel (2-5min exposure) and the DNA band was excised. The DNA was eluted by soaking the macerated gel slice in 200µl TE overnight at room temperature. The eluate was filtered through glass-wool and stored at -20°C.

Radiolabelled AP1 probes were kindly prepared by Dr. David Gillespie.

VII. Western Blotting

75-100μg of whole or nuclear protein extracts were used per reaction. An equal volume of 2xGBS [125mM Tris-HCl pH6.8, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 4% (w/v) SDS, 0.004% (w/v) bromophenol blue] was added to each sample. The samples were then boiled for 5min at 100°C and transferred on ice until loaded. 20μl of prestained molecular weight markers (BenchMark™ pre-stained protein ladder, Gibco) were loaded along with the samples and separated on a 10% (v/v) SDS PAGE gel with 5% (v/v) stacking gel at 200V using. Proteins were then transferred to an Imobillon P membrane (pre-wet in methanol) for 2.5hours at 100V in blotting buffer. The membrane was then stained in Ponceau solution (protein stain) to check for the efficiency of the protein transfer and then blocked for 1hour in blocking buffer [5% (w/v) non-fat milk in TBS-T (Tris-Borate -Triton X100) buffer]. This blocking step helps to reduce non-specific binding and hence background signal at the later detection step. The membrane was then incubated overnight at 4°C with primary antibody (at the appropriate dilution usually 1:5000) in TBS-T buffer, then washed 4x in TBS-T buffer and reblocked [5% (w/v) non-fat milk in TBS-T buffer]. . The secondary antibody (anti-rabbit HRP-conjugated) was then added to the membrane and incubated for 1 hour at room temperature. The blot was then washed as above. 5ml of each buffer of the ECL luminol system kit were mixed together and added to the membrane, immediately, in the dark and gently shook by hand for 2 min. The solution was discarded and the membrane was immediately exposed to X-AR photographic film for the required time (usually 1-15 min).

VIII. Stripping and reprobing of western blots

The membrane was incubated at 55°C with pre-heated MENSA buffer [62.5mM Tris-HCl pH6.8, 2% (w/v) SDS, 50mM sodium 2-mercaptoethanol] for 30 min. The membrane was then rinsed with SDS wash buffer [62.5mM Tris-HCl pH6.8, 2% (w/v) SDS) and twice with PBS-T (1% (v/v) Tween®-20 in 10xPBS] and was then blocked [5% (w/v) non-fat milk in TBS-T buffer] and reprobed as per method described above. Membranes were stored in plastic wrap at –20°C.

Chapter 3

Cell phenotype and growth characteristics

3.1 Introduction

A series of experimental approaches have been employed to determine whether EBNA 1 has any effects on B cell phenotype. Initially (section 3.2), FACS analysis was used to examine any EBNA 1 effects on B cell differentiation using antibodies to B cell surface markers that are differentially expressed through B cell development. Using this method it was possible to determine the proportions and maturation stage of B cells in the lymphoid tissue compartments. Secondly (section 3.3), short term cultures (up to 3 weeks) of freshly explanted spleen and bone marrow (BM) cells were set up to examine cell survival and proliferation. Cell survival cultures were supplemented with different growth factors known to support B cell growth.

-IL2 is produced primarily by activated T helper 1 (Th1) cells. It acts through a specific, saturable, multisubunit IL-2 receptor complex found primarily on T cells, B cells and natural killer (NK) cells. The precise role of IL-2 *in vivo* is not known. However, transgenic mouse models suggest that it may play a role in the establishment of an anergic state at the T cell level (Kneitz *et al.*, 1995). It has also been reported that it augments B cell growth and Ig production (Ceuppens *et al.*, 1985).

-IL4 is produced by the Th2 subset of activated T cells but also from CD8+ T cells and is also known as B Cell Growth Factor (BCGF). IL4 induces IgM-stimulated B cells to enter S-phase (Howard *et al* 1982), upregulates IgG1, IgE and sIgM production by B cells (Snapper *et al*, 1988). IL4 also upregulates Fc receptor expression for IgE (CD23) on B cells and monocytes (Vercelli *et al.*, 1988) and MHC class II expression in resting B cells (Musso *et al.*, 1994). IL4 is known to support B cell growth in culture.

-IL6 is produced by a variety of cell types including lymphocytes and is also known as monocyte-derived human B cell growth factor and as B cell Stimulating Factor (BSF-2). IL6 is reported to exhibit multiple activities including induction of proliferation in a number of cells including EBV transformed B cells, and induction of B cell differentiation and stimulation of IgG secretion (Tosato *et al.*, 1988).

-IL7 is a product of BM stromal cells and is able to induce the growth and differentiation of precursor B lymphocytes, specifically B220+ pre-B cells (Williams *et al.*, 1990, Hayashi *et al.*, 1990).

-CD40 stimulation of different B cell lines has different effects on their growth, proliferation and differentiation depending on the activation status of the B cells. CD40 (αCD40) antibody/ligand stimulates entry of B cells to S-phase (Gordon, J., 1995; Parry *et al.*, 1994).

Cell population growth was monitored initially by counting viable cells at 24 hour intervals using trypan blue exclusion of dead cells. Normal splenocytes usually die by 2 weeks in explant culture and major changes to cell viability could be readily measured in this way. The effects of EBNA 1 on immature cell proliferation were detected by methylcellulose cultures of BM cells (section 3.4). The apoptosis rate of EμEBNA 1 positive cells versus wild type (wt) cells was assessed by a combination of AnnexinV and PI staining and FACS analysis (section 3.4). The cells used in these experiments were isolated from EBNA 1 transgenic positive and negative control sibling mice from line 26 (in which tumour incidence occurs between 4-12 months). At 2 months old, mice are young adults and developmentally mature and free of tumours. Therefore, the effects of EBNA 1 on adult B cell phenotype can be addressed without the influence of tumour progression.

3.2 Transgenic B cell phenotype

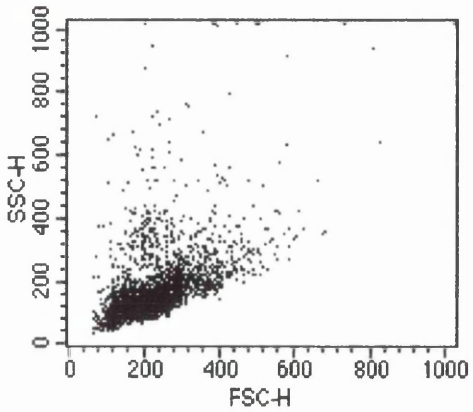
Splenocytes or BM cells were isolated from 2 month old EμEBNA 1 transgenic positive animals from line 26 and wt controls (as described in Methods section 2.4.2). After red blood cell (RBC) lysis, cells were stained with different cell markers and analysed by FACS using $5x10^5$cells/ sample.

3.2.1 Does EBNA 1 influence the size or proportion of the B cell component in the spleen cell population?

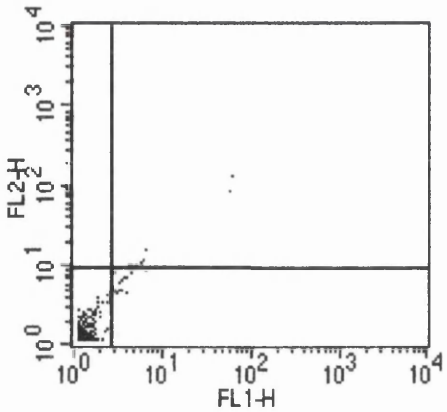
To determine the percentage of B and T cell populations in the spleens of transgenic positive and negative animals a flow cytometric analysis was performed on splenic cells from EμEBNA 1 positive and wt 2 month old mice from line 26. Phycoerythrin (PE)-conjugated (red fluorescence) antibodies, which recognise the panB cell surface marker CD45R/B220, were used to detect and estimate the percentage of B cells in the spleens of 21 transgenic positive and 20 wt mice. Fluorescein isothiocyanate (FITC)-conjugated (green fluorescence) antibodies which recognise the T cell (but also cross react with some activated B cells) surface marker Thy1.2, were used to detect and also estimate the percentage of T cells (also activated B cells) in the spleens from 7 transgenic positive and 7 wt mice. Staining controls were always included: an unstained control to measure and exclude the autofluorescence of the cells, and one single stained control for each different stain used. These controls are required to allow the flow cytometer to be calibrated and adjusted as necessary. Isotype controls for each type of antibody (when available) were used as a non-specific staining control. All FACS results were analysed as dot plots and representative data are shown in Fig. 3.1 (the markers used in this figure are discussed later). The mean fluorescence intensity was used as an alternative way of comparing two cell populations. The FACS collects the fluorescence intensity values of each individual cell and then calculates the average or mean intensity of the cell population in question which reflects the level of expression of a particular marker.

The results of the B cell/ T cell experiment are summarised in Table 3.1 and graphically and statistically depicted in Fig. 3.2. Statistical analysis was performed using the Two-sample T-test. Statistical significance with 95% confidence intervals is reached when $P<0.05$. A box plot displays the median, quartile values and range of the data set thereby providing more visual information concerning the data spread than a barr graph. The box plot also reads outlying data points. Minitab calculates outlying values depending on how concentrated are the groups of data analysed. The

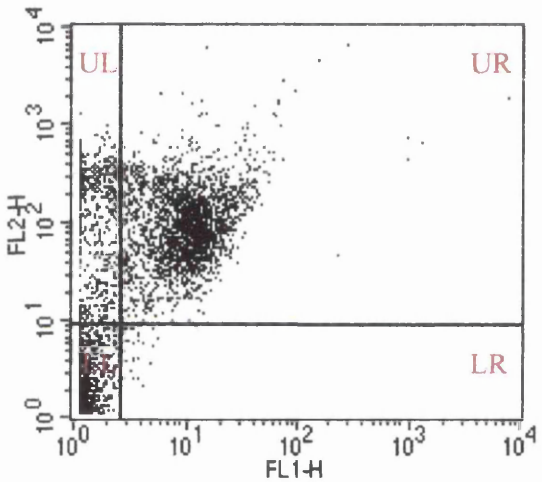
Forward and side scatter



Unstained FL2/FL1 control



PE/FITC stained cells



Log Data Units: Linear Values
Gated Events: 5000
X Parameter: FL1-H (Log)
Quad Location: 3, 9

Gate: No Gate
Total Events: 5000
Y Parameter: FL2-H (Log)

Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	622	12.44	12.44	1.72	186.88
UR	2198	43.96	43.96	17.22	146.61
LL	2150	43.00	43.00	1.27	2.03
LR	30	0.60	0.60	3.94	5.99

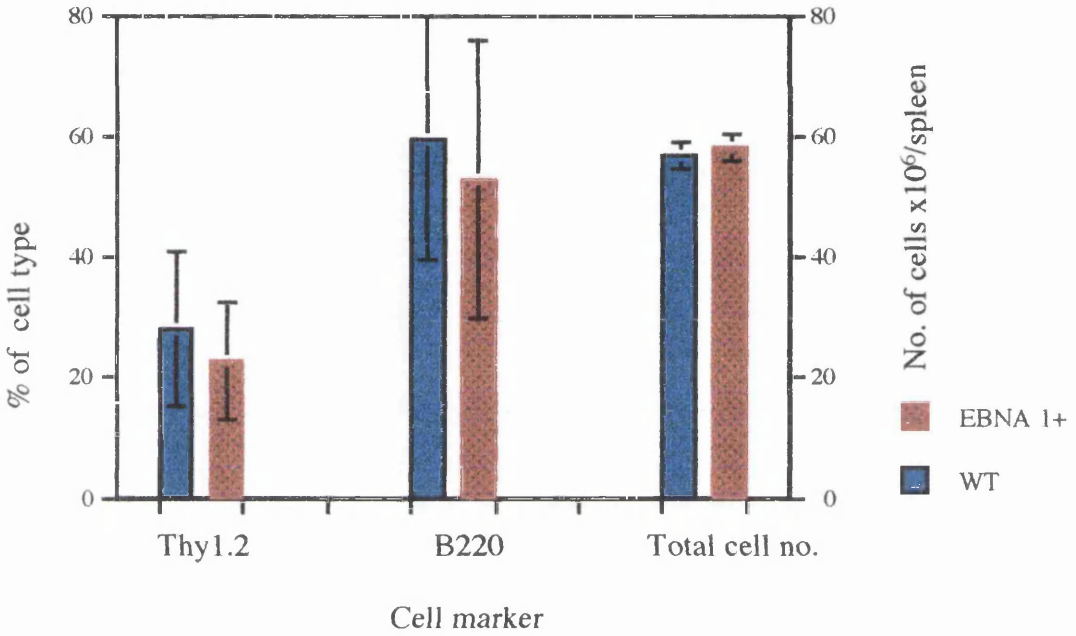
Fig. 3.1 Relative proportions of spleen and BM populations. The fluorescence from PE-conjugated antibodies is plotted on the Y axis (measured at the FL2 channel) and the fluorescence from the FITC-conjugated antibodies is plotted on the X axis (measured at the FL1 channel). Quadrants are set according to the autofluorescent unstained control. Compared samples always have the same quadrant for direct comparison. In the upper left (UL) quarter the PE-only stained cells are shown, in the lower left (LL) the unstained, the lower right (LR) the FITC-only stained cells and in the upper right (UR) the double (PE+/FITC+) positive cells are shown. The program conducts simple analytical tasks which give the percentage of each stained cell population as well as their mean fluorescence intensity. In the lower plot, B220-stained (PE-Y axis) cells are plotted against Thb stained (FITC-X axis) cells. The percentage of B cells that are positive for Thb is given by the following equation: UR% = (UR/UR+UL) x 100. In this case (44/44+12.5)x100=78% of B cells are positive for Thb.

Table 3.1 Proportion of B and T cells in spleen from EμEBNA 1 transgenic positive and wt 2 month old mice (line 26)

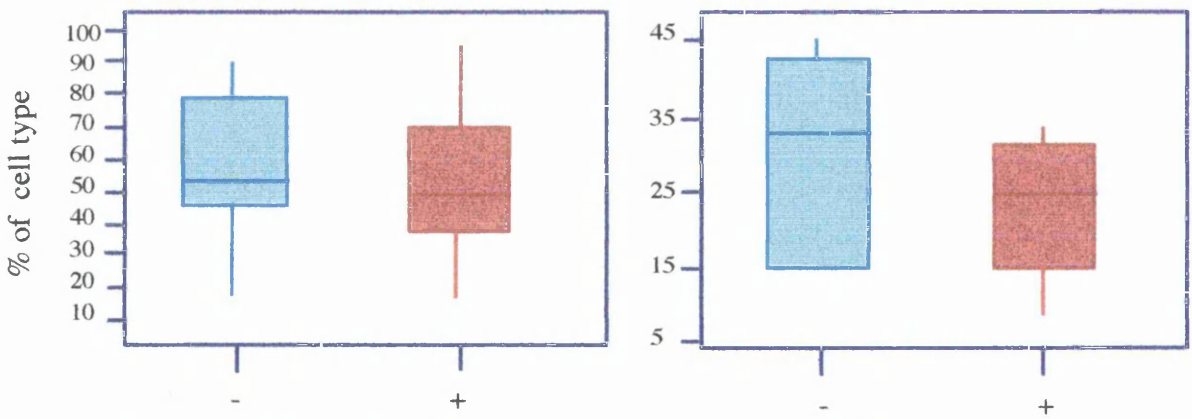
EBNA 1 + mice	Total no. of cells	% of B220	%Thy1.2 cells	WT mice	Total no. of cells	% of B220	% Thy1.2 cells
26.918 M	$4.32x10^7$	56.53	30.8	26.933M	$3.86x10^7$	63.54	44.3
26.941 M	$4.68x10^7$	93.53	31.15	26.940 F	$6.38x10^7$	86.56	32
26.943 M	$4.85x10^7$	94.13	23.36	26.942 M	$5.86x10^7$	94.4	41.4
26.944 M	$4.9x10^7$	68.76	33	26.945 M	$4.49x10^7$	76.3	34
26.948 F	$3.88x10^7$	71	19	26.947 M	$6.37x10^7$	85.3	15
26.951 F	$5.31x10^7$	83	7	26.949 F	$5.35x10^7$	83..3	17.5
26.952 F	$4.92x10^7$	87.66	14.4	26.950 F	$5.1x10^7$	81.4	15
Mean	**$4.7x10^7$**	**79.23**	**22.7**	**Mean**	**$5.34x10^7$**	**81.5**	**28.46**

Fig. 3.2 B and T cell number in the mouse spleen. A) Mean value of total cell number and mean % of B220+ cells in spleen of 21 EμEBNA 1 positive and 20 wt mice from line 26. The value for Thy1.2+ cells are means of 7 EμEBNA 1 positive and 7 wt mice. B) Statistical analysis of B220+ and Thy1.2+ cell populations in the mouse spleen. EμEBNA 1 transgenic mice represented by (+) and WT siblings by (-).

A) **B and T cell number in the mouse spleen**



B)



Two-sample T for B220 Spl

+/-	N	MEAN	STDEV	SEMEAN
+	21	53.0	23.0	5.0
-	20	59.5	20.7	4.6

95PCT CI FOR MU+ -MU-:(-20.4, 7.3)
TTEST MU +=MU- (VS NE):T=-0.96 P=0.35 DF=39
POOLED STDEV= 21.9

Two-sample T for Thy1.2 Spl

+/-	N	MEAN	STDEV	SEMEAN
+	7	22.67	9.77	3.7
-	7	28.2	12.9	4.9

95PCT CI FOR MU+ -MU-:(-18.8, 7.8)
TTEST MU +=MU- (VS NE):T=0.90 P=0.39 DF=12
POOLED STDEV= 11.4

symmetry of the results and any outlying observations can be seen. The total number of splenocytes isolated from the spleens of EμEBNA 1 positive and wt control animals was approximately $5x10^7$, with no significant difference between the two. However, occasionally an enlarged spleen, with greater cell number, was isolated from a transgenic positive animal. This was attributed to early neoplastic changes and these data were excluded from this pre-tumour analysis. 70-85% of the splenocytes were B220+ (B cells) and 20-28% were Thy1.2+ cells (T and some activated B cells). No significant difference is seen in the overall proportion of B cells in the spleens between EμEBNA 1 positive and wt animals. As expected no significant difference was observed in the proportion of Thy1.2+ cells (predominantly T cells) between transgenic positive and wt spleen populations.

3.2.2 Does EBNA 1 distort the proportion of B cell subtypes in the spleen cells?

To determine the effects of EBNA 1 expression on peripheral B cell populations, flow cytometric analysis for B cell surface markers which are expressed throughout B cell differentiation, was performed on primary splenic cells from EμEBNA 1 positive and negative siblings from 2 month old line 26 mice. In Fig. 3.3 the B cell maturation stages are illustrated and the surface markers expressed at each stage are indicated at the bottom of the figure by colour coded arrows. B cells differentiate from lymphoid stem cells into virgin/naive B cells and upon antigen stimulation they may become memory or plasma cells. The genes coding for antibodies are rearranged in the course of progenitor cell development. Pre-B cells express cytoplasmic μ chains only. The immature B cell has surface IgM, whereas mature B cells have other Ig molecules.

Thb was used as a marker for B cell immaturity, which is expressed in immature B cells and approximately 70% of thymocytes. As a marker of maturity, polyvalent surface immunoglobulin (sIg) (IgM, IgG and IgA) was used which is expressed on naive and mature differentiated B cells. Individual Igs were used as markers to examine the stage of the B cell differentiation. For example, IgD is expressed on naive B cells. IgM is expressed on B cells before isotype switching.

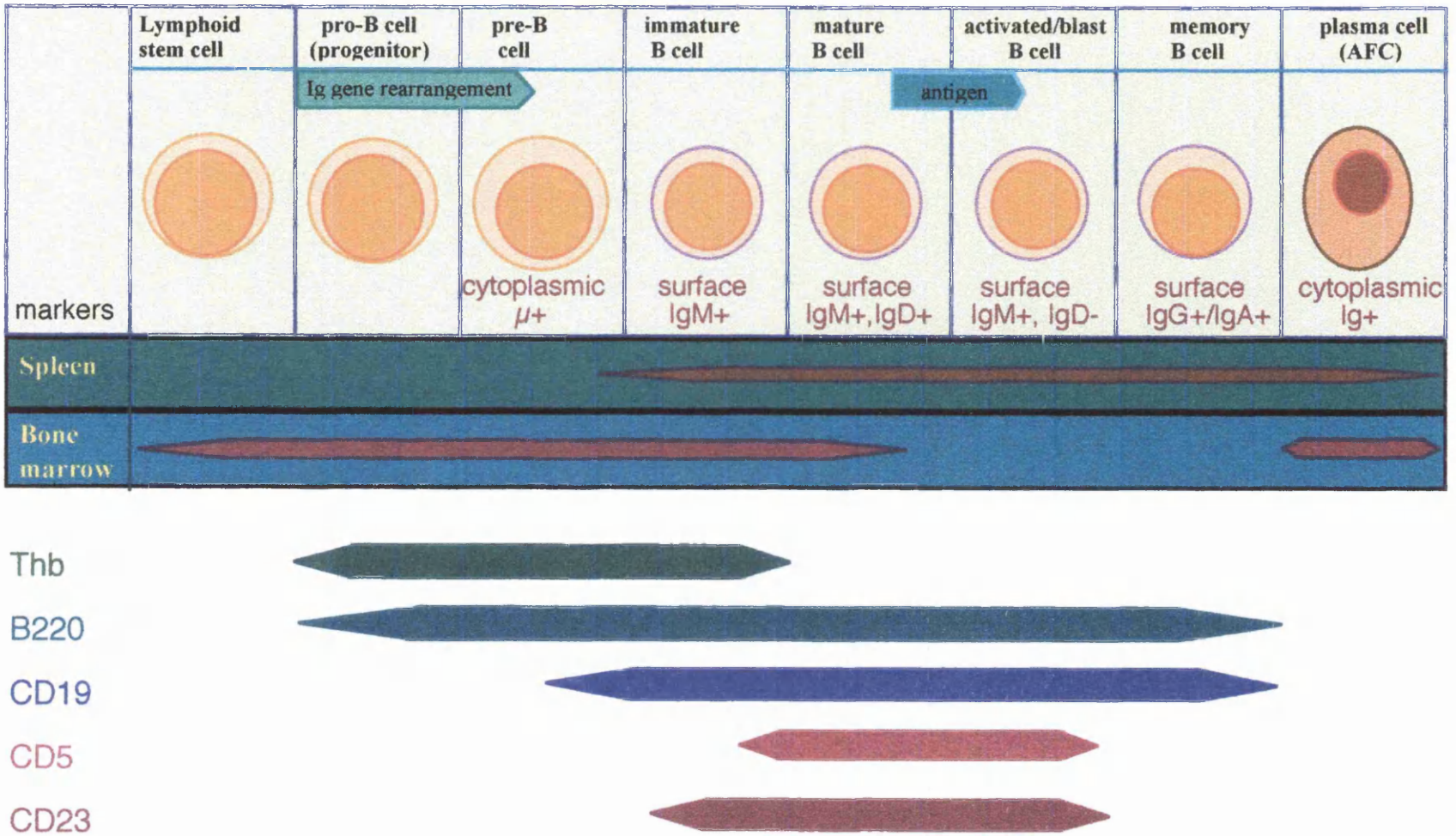


Fig. 3.3 B cell maturation/differentiation stages in the spleen (top) and bone marrow (BM , bottom). The cellular location of immunoglobulins is shown in purple and the individual sIg expressed are shown in red. The sequence of appearance of B cell surface markers that were tested is shown at the bottom of the figure by the colour coded arrows. B cell maturation in the bone marrow reaches the IgM+/IgD+ stage and then B cells enter circulation. Some plasma cells return to the BM.

CD23 is expressed on mature B cells and was used as marker of B cell activation state. CD5 is a marker for a minor sub-population of B cells referred to as B1 cells.

After RBC lysis, cells were counted and aliquots of $5x10^5$ cells/sample (for both test and control samples) were stained with PE-conjugated antibodies to B220 for total B cell population percentage and a FITC-conjugated second marker (those described above) and samples were analysed as in section 3.2.1. The percentage of B220+ cells and percentage of B cells expressing each of the secondary markers tested were calculated and are presented in Tables 3.2 and 3.3.

Representative dot plots for each staining are shown in Fig. 3.4. These dot plots are representatives from 4 separate experiments with at least 4 transgenic positive and 4 wt mice in each experiment. For each staining, the representative dot plots were chosen according to how close their percentage of positive cells was to the mean percentage of the positive cell population of the group. Equally, the mean fluorescence intensity of the dot plot chosen was as close as possible to the mean value of the group.

Statistical analysis was performed using Minitab to perform a Two-sample T-test in order to test if the values observed between transgenic positive and wt control mice were statistically significantly different. The results of the analysis are graphically illustrated and statistically depicted in Fig. 3.5. As it can be seen from Fig. 3.5b, there is no difference in the overall proportion and fluorescence intensity of most stains of B cells in the spleen between the EμEBNA 1 transgenic positive and wt mice, with the exception of sIg. Similar proportions of immature (Thb+) B cells as well as IgM+ and IgD+ B cells were obtained from both EμEBNA 1 positive and wt spleens. However, EμEBNA 1 transgenic samples showed a statistically significantly ($P=0.014$) higher proportion of sIg+ B cells compared to wt (by 1.5-fold), with 72% in EμEBNA 1 positives and 54% in wt. The sIg fluorescence intensity was also higher in the EμEBNA 1 positives with a relative value of 90% compared to 66% in wt. As can be seen more clearly in the histogram representation of the data in Fig. 3.6 the EμEBNA 1 B cells show 2 peaks of sIg staining of high and low intensity with roughly equal proportions. In contrast, the negative control shows a bias to low

Table 3.2 Proportion of B cells in spleen from EμEBNA 1 transgenic positive 2 month old mice from line 26

EμEBNA 1 + mice	Total no. of cells	% of B220 cells	% of ThB/ B220 cells	% of sIg§/ B220 cells	% of IgD/ B220 cells	% of IgM/ B220 cells	% of IgD/ IgM cells	% of CD23/ B220 cells	% of CD5/ B220 cells
26.918 M	$4.32x10^7$	56.53	58	-	-	-	-	-	-
26.941 M	$4.68x10^7$	93.53	54	-	-	-	-	-	-
26.943 M	$4.85x10^7$	94.13	47	-	-	-	-	-	-
26.944 M	$4.9x10^7$	68.76	70	-	-	-	-	-	-
26.948 F	$3.88x10^7$	71	57	-	-	-	-	-	-
26.951 F	$5.31x10^7$	83	40		-	-	-	-	-
26.952 F	$4.92x10^7$	87.66	33	-	-	-	-	-	-
26.1118 F	$9.84x10^7$	55.8	52.35 | 89.65	86.6 | 102	-	-	-	-	-
26.1119 F	$8.9x10^7$	58.75	34.5 | 92.1	62 | 83	-	-	-	-	-
26.1121 F	$9.8x10^7$	35.85	64.7 | 80.38	65.75 | 79	-	-	-	-	-
26.1268 F	$4.63x10^7$	12.83	66.53 | 41.59	77.4 | 126	-	-	-	-	-
26.1271 F	$7.95x10^7$	26.6	58.92 | 22.44	69.52 | 58.87	-	-	-	-	-
26.1571 M	$6.7x10^7$	37.21	-	-	86.6 | 11.82	-	-	54.25 | 6.94	6.88 | 26.67
26.1572 F	$2.0x10^7$	33.2	-	-	76.3 | 80.54	-	-	60.64 | 9.44	13.09 | 11.69
26.1573 F	$2.68x10^7$	34.82	-	-	77.25 | 47.1	-	-	63.32 | 8.67	15.76 | 12.01
26.1575 M	$7.5x10^7$	25.5	-	-	71.6 | 25.17	-	-	54.5 | 8.1	14.68 | 13.31
26.1576 M	$6.9x10^7$	46.5	-	-	84.14 | 13.35	-	-	77.2 | 9.16	6.27 | 11.56
26.1678 M	$7.925x10^7$	48.405	-	-	75.38 | 26.31	81.5 | 5.92	76.87 | 17.97	70.8 | 26.85	6.4 | 13.82
26.1679 M	$5.075x10^7$	48.53	-	-	77.94 | 25.63	81.25 | 6.00	75.21 | 14.5	68.87 | 21.89	7.9 | 12.82
26.1681 F	$5.6x10^7$	40.41	-	-	63.64 | 19.24	75.85 | 6.66	60.43 | 18.35	73.5 | 22.01	6.5 | 13.18
26.1682 M	$4.0x10^7$	51.21	-	-	77 | 17.73	81.1 | 5.51	73.66 | 17.94	67.8 | 13.13	4.7 | 11.42
Mean	**$5.8x10^7$**	**52.9**	**53.0 | 65.23**	**72.26 | 89.68**	**76.63 | 29.65**	**79.925 | 6.02**	**71.54 | 17.19**	**60.2 | 14.02**	**9.13 | 14.05**

- indicates the sample was not tested for that particular marker

The mean fluorescence intensity of double positive cells is shown in blue numbers next to the mean proportion (%) of B cell sub-populations

§ sIg ab recognises IgM, IgA, IgG

Table 3.3 Proportion of B cells in spleen from 2 month old wt mice from line 26

WT mice	Total no. of cells	% of B220 cells	% of ThB/ B220 cells	% of sIg§/ B220 cells	% of IgD/ B220 cells	% of B220/ IgM cells	% of IgD/ IgM cells	% of CD23/ B220 cells	% of CD5 /B220cells
26.933 M	3.86x10^7	63.54	33.6	-	-	-	-	-	-
26.940 F	6.38x10^7	86.56	66	-	-	-	-	-	-
26.942 M	5.86x10^7	94	51.7	-	-	-	-	-	-
26.945 M	4.49x10^7	76.5	72	-	-	-	-	-	-
26.947 M	6.37x10^7	85.3	48	-	-	-	-	-	-
26.949 F	5.35x10^7	83.3	36	-	-	-	-	-	-
26.950 F	5.1x10^7	81.4	38.73	-	-	-	-	-	-
26.1113 M	6.9x10^7	79.71	27.4 | 79.2	51.4 | 92	-	-	-	-	-
26.1120 F	9.72x10^7	53.66	50.5 | 83.25	59.5 | 83.6	-	-	-	-	-
26.1272 M	7.5x10^7	22.12	79.8 | 46.65	57.8 | 56.95	-	-	-	-	-
26.1273 M	9.8x10^7	37.85	67.86 | 29.58	47.63 | 31.07	-	-	-	-	-
26.1562 M	5.39x10^7	52.72	-	-	83.22 | 24.25	-	-	71.18 | 8.33	6.9 | 18.76
26.1564 F	2.1x10^7	43.75	-	-	78.67 | 37.25	-	-	60.14 | 10.24	13.9 | 12.6
26.1566 F	1.18x10^7	41.49	-	-	77.65 | 64.89	-	-	47.91 | 7.91	8.7 | 11.6
26.1568 F	4.9x10^7	50.25	-	-	79 | 16.97	-	-	68.17 | 8.94	9.67 | 11.7
26.1570 M	4.3x10^7	47.2	-	-	76.73 | 48.31	-	-	54.66 | 8.21	6.18 | 13.94
26.1676 M	3.82x10^7	53.944	-	-	83.7 | 17.02	63.25 | 5.67	80 | 9.96	45.4 | 10.21	5.86 | 16.94
26.1677 M	6.55x10^7	43.115	-	-	73.3 | 24.63	88.6 | 7.72	78 | 17.16	69.1 | 20.5	9.5 | 14.71
26.1680 M	6.925x10^7	44.225	-	-	72 | 26.12	85.85 | 6.53	74 | 21.86	65.45 | 27.04	14.7 | 16.21
26.1687 M	7.625x10^7	54.25	-	-	73.63 | 16.22	75.8 | 5.83	73.4 | 16.35	57 | 9.82	5.52 | 11.4
Mean	**5.7x10^7**	**59.7**	**51.96 | 59.67**	**54.08 | 65.9**	**77.54 | 30.63**	**78.375| 6.43**	**76.35| 16.33**	**59.89 | 12.35**	**8.99 | 14.2**

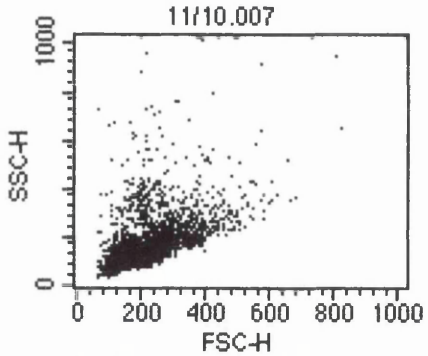
- indicates the sample was not tested for that particular marker

The mean fluorescence intensity of double positive cells is shown in blue numbers next to the mean proportion (%) of B cell sub-populations

§ sIg ab recognises IgM, IgA, IgG

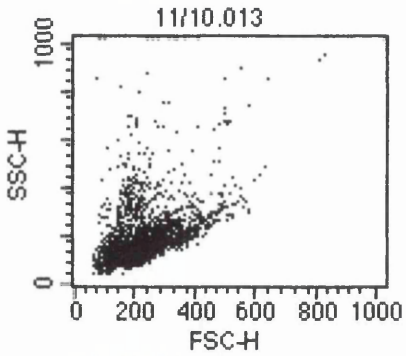
Fig. 3.4 FACS analysis of spleen cells stained by immunofluorescence and analysed by flow cytometry. Y axis: FL2-PE labelled antibody. X-axis: FL1-FITC labelled antibody. A forward and side scatter dot plot for each sample is also shown. Splenocytes from WT mice (A) are compared with splenocytes from EμEBNA 1 positive transgenic mice (B). The horizontal and vertical cursor lines are positioned based on autofluorescence of unstained cells. Based on this cut-off, the % of PE/FITC stained cells in each case was calculated by the number of double stained cells divided by the total number of PE-stained cells. The percentage and mean fluorescence intensity of individual quadrants are shown in red and green (respectively) inside each quadrant. A and B are each representative of 9 samples.

A: Representative WT control

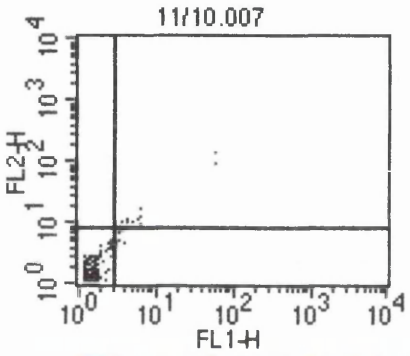


WT unstained forward and side scatter

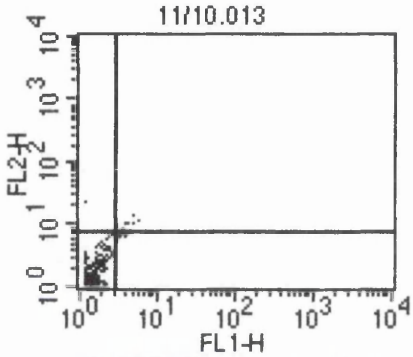
B: Representative EμEBNA 1+



EμEBNA 1+ forward and side scatter

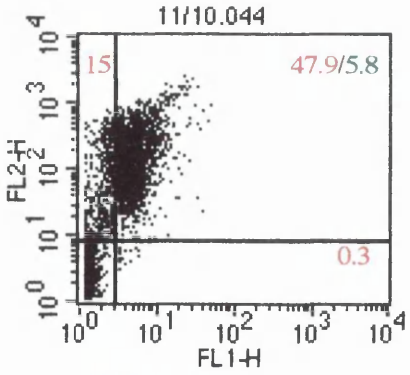


WT unstained FL1/FL2

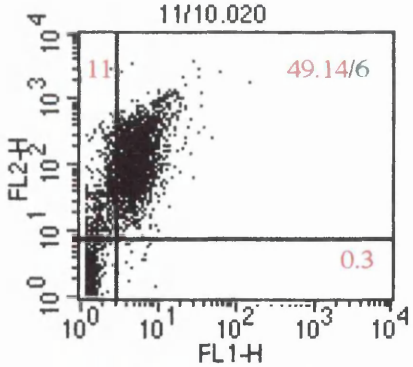


EμEBNA 1+ unstained FL1/FL2

CD19

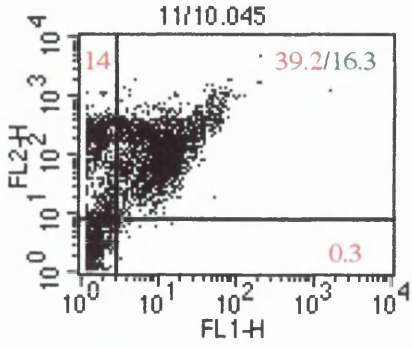


WT IgM

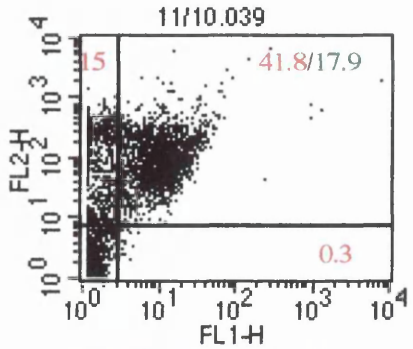


EμEBNA 1+ IgM

IgM



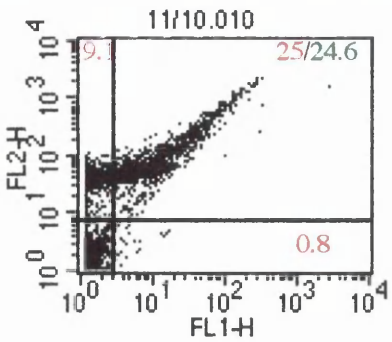
WT IgD



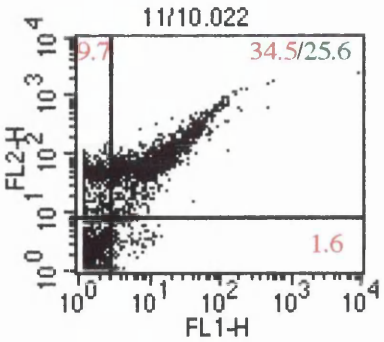
EμEBNA 1+ IgD

A: Representative WT control

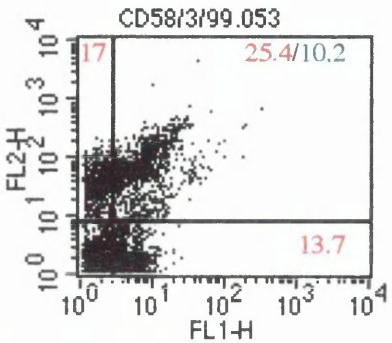
B: Representative EμEBNA 1+



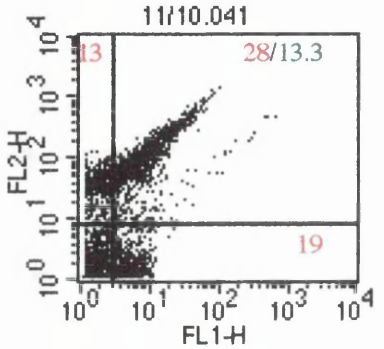
WT IgD



EμEBNA 1+ IgD

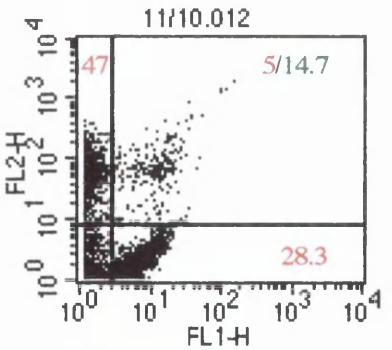


WT CD23

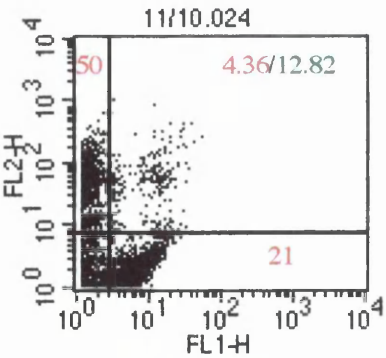


EμEBNA 1+ CD23

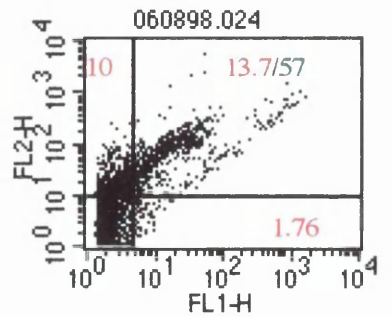
B220



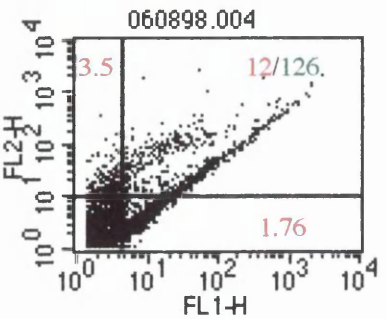
WT CD5



EμEBNA 1+ CD5

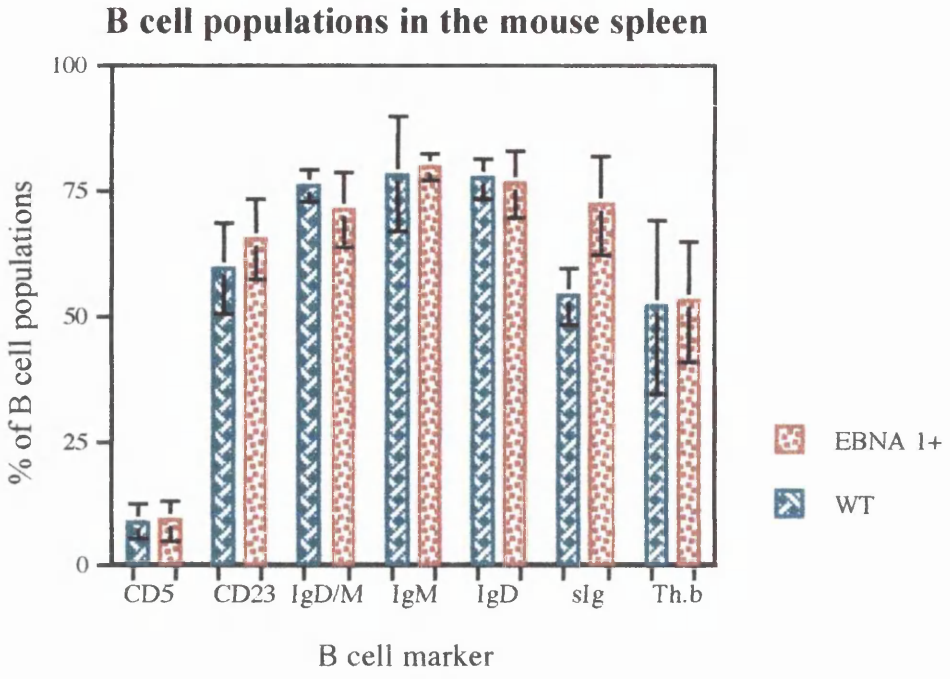


WT sIg



EμEBNA 1+ sIg

a)



b)

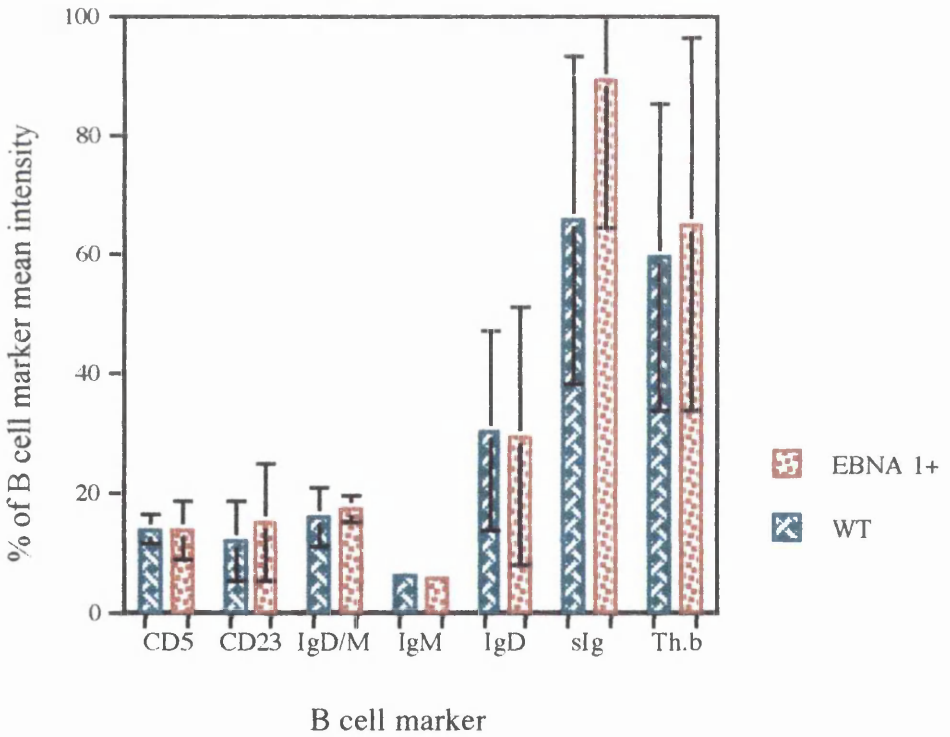
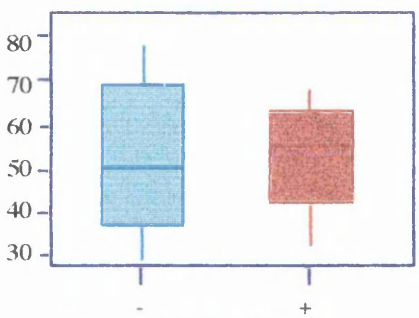


Fig. 3.5 B cell subpopulations in the mouse spleen. a) Graphical representation of the % of B220+ cells expressing secondary antigen. Values for CD5, CD23, IgD/M, IgD, IgM, sIg and ThB of EμEBNA 1 positive and wt control mice are means of 5-12 samples (Tables 3.2 and 3.3). b) Cell marker mean fluorescence intensity c) Statistical illustration of the FACS results for each individual staining as compared between EμEBNA 1 positive and wt control siblings.

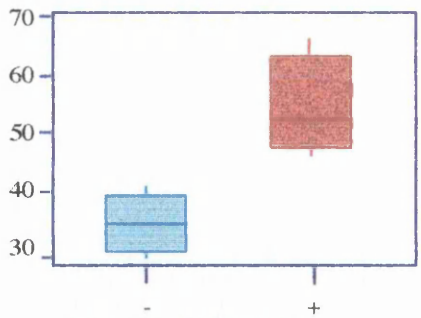
Fig. 3.5c



Two-sample T for ThB Spl

+/-	N	MEAN	STDEV	SEMEAN
+	12	53.0	12.2	3.7
-	11	52.0	17.4	5.2

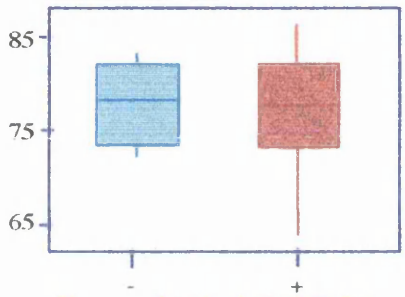
95PCT CI FOR MU+ -MU-:(-11.9, 14.0)
TTEST MU +=MU- (VS NE):T=0.17 P=0.87 DF=21
POOLED STDEV= 14.9



Two-sample T for sIg Spl

+/-	N	MEAN	STDEV	SEMEAN
+	5	72.26	9.84	4.4
-	4	54.08	5.54	2.8

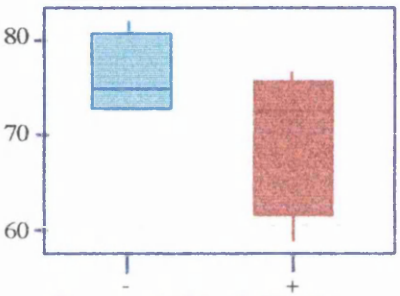
95PCT CI FOR MU+ -MU-:(5.0, 31.3)
TTEST MU +=MU- (VS NE):T=3.28 P=0.014 DF=7
POOLED STDEV= 8.27



Two-sample T for IgD/B220 Spl

+/-	N	MEAN	STDEV	SEMEAN
+	9	76.63	6.63	4.4
-	9	77.54	4.16	2.8

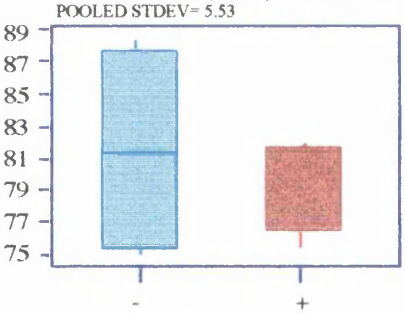
95PCT CI FOR MU+ -MU-:(-6.4, 4.6)
TTEST MU +=MU- (VS NE):T=-0.35 P=0.73 DF=16
POOLED STDEV= 5.53



Two-sample T for IgD/M Spl

+/-	N	MEAN	STDEV	SEMEAN
+	4	71.54	7.52	3.8
-	4	77.06	4.37	2.2

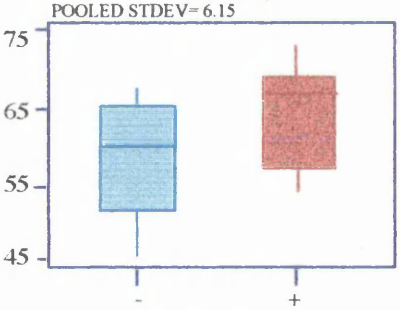
95PCT CI FOR MU+ -MU-:(-16.2, 5.1)
TTEST MU +=MU- (VS NE):T=-1.27 P=0.25 DF=6
POOLED STDEV= 6.15



Two-sample T for IgM Spl

+/-	N	MEAN	STDEV	SEMEAN
+	4	79.93	2.72	1.4
-	4	81.61	6.58	3.3

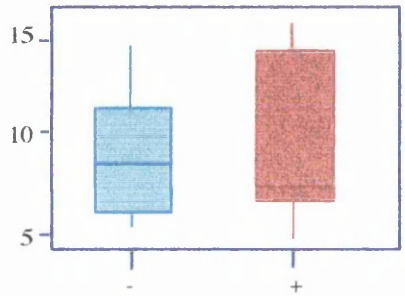
95PCT CI FOR MU+ -MU-:(-10.4, 7.0)
TTEST MU +=MU- (VS NE):T=-0.47 P=0.65 DF=6
POOLED STDEV= 5.03



Two-sample T for CD23 Spl

+/-	N	MEAN	STDEV	SEMEAN
+	9	65.65	8.08	2.7
-	9	59.89	9.03	3.0

95PCT CI FOR MU+ -MU-:(-2.5, 14.7)
TTEST MU +=MU- (VS NE):T=1.51 P=0.15 DF=16
POOLED STDEV= 8.57



Two-sample T for CD5 Spl

+/-	N	MEAN	STDEV	SEMEAN
+	9	9.13	4.17	1.4
-	9	8.99	3.38	1.1

95PCT CI FOR MU+ -MU-:(-3.7, 3.9)
TTEST MU +=MU- (VS NE):T=-0.08 P=0.94 DF=16
POOLED STDEV= 3.80

cell type (range)

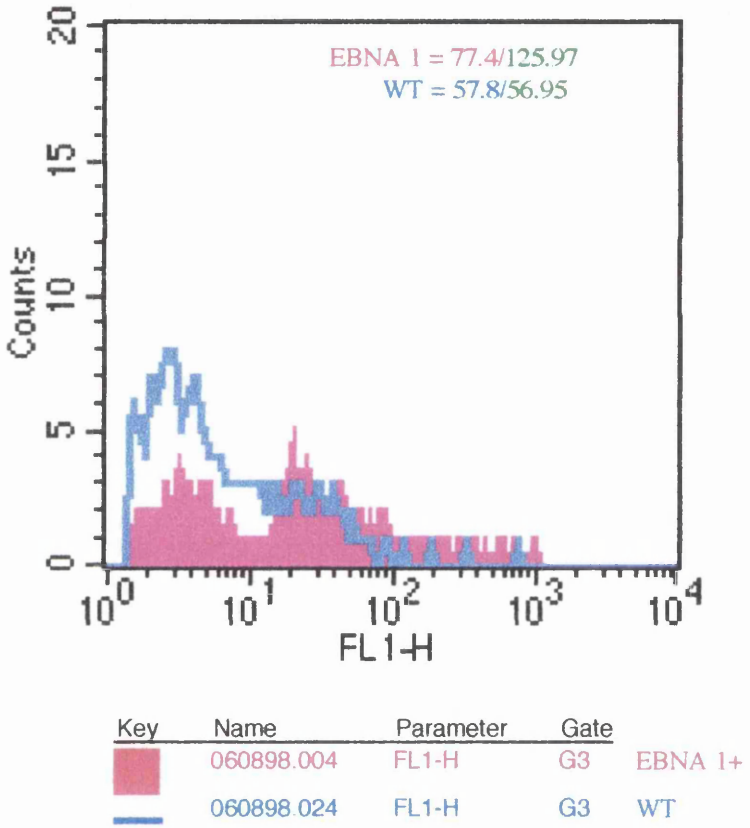


Fig. 3.6. Histogram illustrating the difference in the % of sIg+ cells between wt (blue) and EμEBNA 1 transgenic positive (magenta) spleen. The % of B cells positive for sIg and the mean intensity of sIg (green) are given in the top corner of the histogram. The results shown have been gated so is not to include the isotype match control peak.

intensity stain of cells. Nevertheless, since there is no obvious increase in the proportions of IgM+ or IgD+ cells, the increase in sIg may be due to increased proportion of IgA or IgG. This may suggest that there is an increase in the number of mature cells. However, this needs to be confirmed. Conversely, all spleen samples (both transgenic and wt) showed a high level of immature cells (average 51% of all cells were Thb+) suggesting perhaps, that this antigen may still be expressed on maturing B cells.

No difference in the proportion of B1 cells (CD5+) in EμEBNA 1 positive in comparison with wt spleen was observed. Results from 2 separate sets of experiments suggest that CD23 might be upregulated by EBNA 1. While the percentage of CD23+ cells was no different between the EμEBNA 1 and wt samples the fluorescence intensity was higher in EμEBNA 1 positive cells. That may indicate that more samples need to be tested in order to reach statistically significant levels.

3.2.3. Does EBNA 1 distort the population of B cell subtypes in BM cells?

To examine B cell development in EμEBNA 1 mice, BM cells from transgenic and non-transgenic animals were analysed for expression of Thb, IgD and sIg. BM cells were collected as described in methods section 2.4.2 and were treated in the same way as described earlier for the spleen cells. Aliquots of $5x10^5$ cells/sample were stained with different B cell surface markers and analysed by flow cytometry. The Two-sample T-test was used to statistically analyse the results of this experiment. The results are summarised in Table 3.4 and graphically illustrated in Fig. 3.7. Thb+ and IgD+ B cell populations were found to be similar between transgenic positive and negative mice. However, as in the spleen samples, the mature (sIg+) B cell population was found to be statistically significantly higher in the EμEBNA 1 mice when compared to wt controls (62.4% versus 46.6%).

The polyvalent antibody to sIgs recognises IgM, IgA and IgG. However, BM cells express only IgM and almost no IgG and IgA and therefore, the high percentage of mature B cells in the BM is likely to be due to the IgM marker. In order to

Table 3.4 Proportion of B cells in BM from EμEBNA 1 transgenic positive and wt 2 month old mice from line 26

EμEBNA 1 + mice	Total no. of cells	% of B220 cells	%of ThB/ B220 cells	% of sIg§ / B220 cells	% IgD/ B220 cells
26.1118	$2.63x10^7$	31.06	32.9	51.4	9.5
26.1119	$2.44x10^7$	25.19	36	75.7	42.4
26.1121	$3.3x10^7$	33.15	48.3	64.5	10
26.1268	$6.8x10^6$	15.43	56.84	62.1	31
26.1271	$8.56x10^6$	12.32	64.32	58.58	49
Mean	**$1.98x10^7$**	**23.43**	**47.67**	**56.456**	**28.38**
WT mice					
26.1113	$2.56x10^7$	25.67	24.3	43.1	14
26.1120	$3.33x10^7$	18.16	35.7	43.4	12.6
26.1272	$1.59x10^7$	27.88	65.1	54.13	36.84
26.1273	$9.8x10^6$	25.08	64.64	45.68	46.86
Mean	**$2.115x10^7$**	**24.2**	**47.43**	**46.57**	**27.57**

§ sIg ab recognises IgM, IgA and IgG.

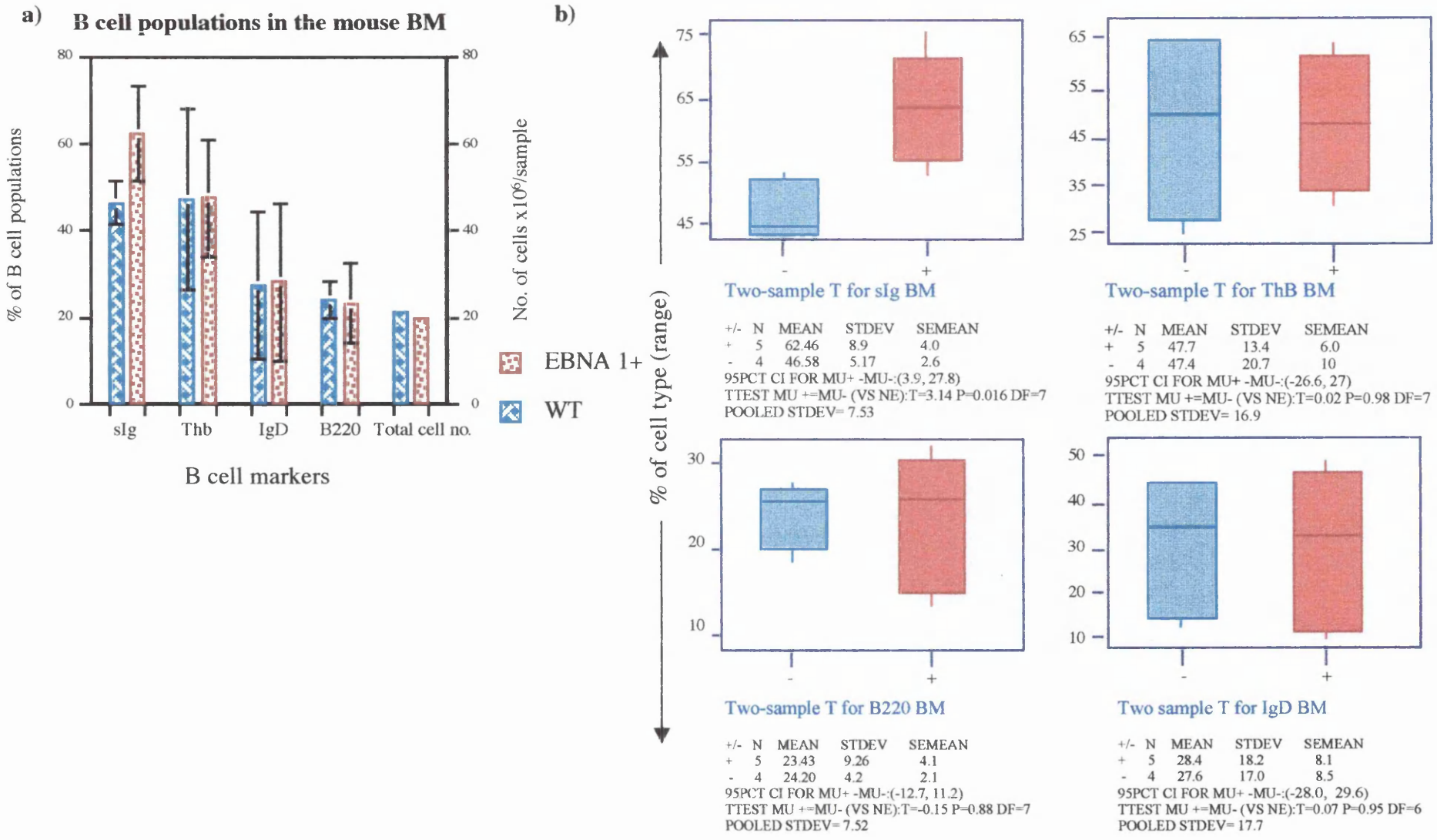


Fig. 3.7 B cell subpopulations in the mouse bone marrow. a) Graphical representation of the % of B220+ cells expressing secondary antigen. Values for sIg, ThB, IgD and B220 are the means of 5 EμEBNA 1 positive (red) and 4 wt (blue) control sibling mice 2 month old (Table 3.4) b) Statistical illustration of the FACS results for each individual staining as compared between EμEBNA 1 positive (+) and wt (-) siblings.

characterise the individual cell types stained with sIg, single staining using B220/IgM, B220/IgG and B220/IgA should be performed.

Based on these observations it can be postulated that EBNA 1 may promote maturation of B cells or allow mature B cells to survive longer or promote the expression of sIg in a manner independent of cell maturity. However, there was no increase in B cell numbers in the EμEBNA 1 positive tissues.

3.3. Transgenic B cell properties I: Cell survival

To address if EBNA 1 has any effects on B cell growth and survival, a series of cell culture experiments were performed using combinations of growth factors and different foetal calf serum (FCS) concentrations.

3.3.1 Does EBNA 1 influence Spleen and BM cell survival?

Spleen and BM cells from 3 EμEBNA 1 transgenic positive 2 month old mice (line 26), were cultured in parallel with splenocytes and BM cells from 2 wt siblings which served as controls. Splenocytes and BM cells were prepared as described in Methods section 2.4.2 and cultured in RPMI medium supplemented with 10% (v/v) FCS at an initial density of $4x10^6$cells/ml for BM and $3x10^6$cells/ml for spleen cells. Live cells were counted at 24hour-intervals by trypan blue exclusion (Fig. 3.8.).

BM cells from both EμEBNA 1 transgenic positive and negative animals showed an exponential decrease in their survival rate over 9 days of culture. Due to the contamination of the spleen cell cultures the results were incomplete but a comparable decrease in survival rate was observed in positive and negative samples. No differences were observed between EμEBNA 1 positive and negative samples.

3.3.2 FCS concentration influence on cell survival

Given that no advantage was evident in the survival of EμEBNA 1 positive cells under normal culture conditions, it was decided to employ conditions which might highlight any survival or growth advantage endowed by EBNA 1 under minimal growth conditions. Spleen and BM cells from 4 EμEBNA 1 positive and 4 wt 2

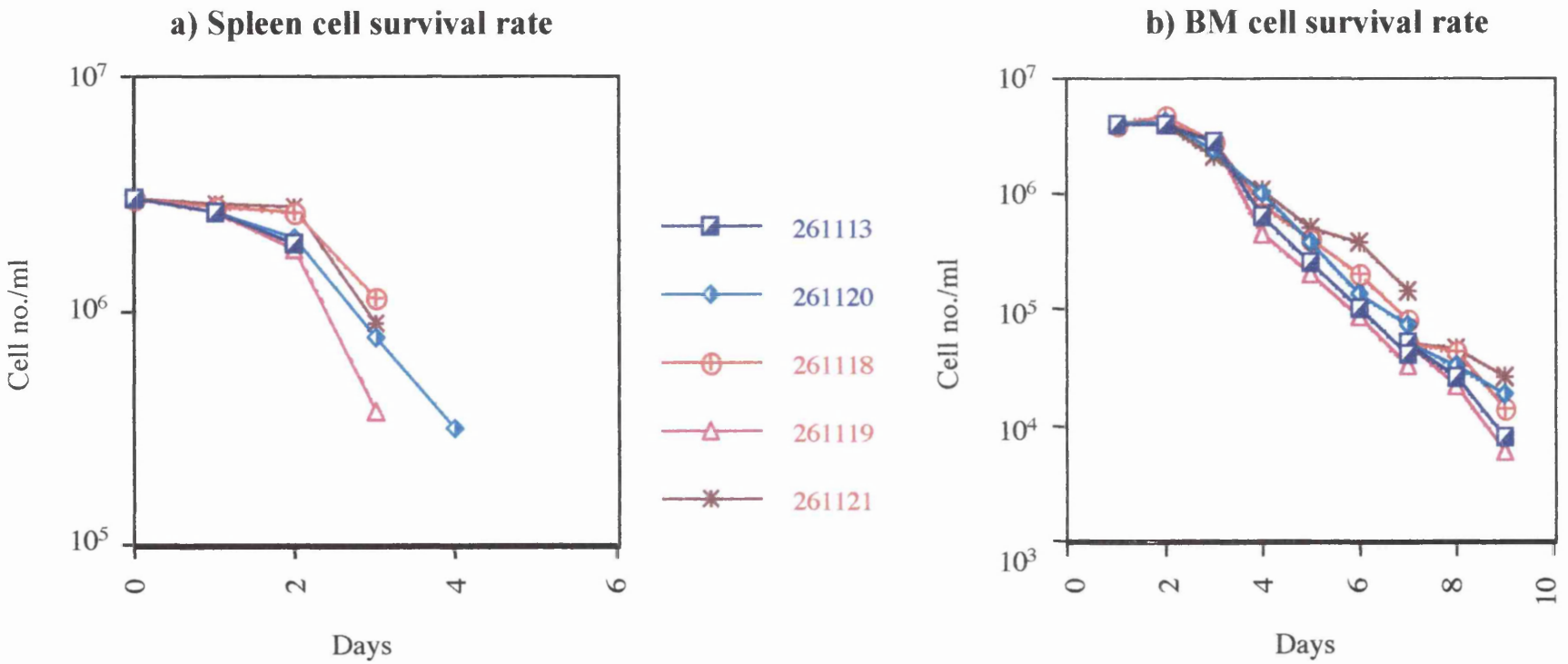


Fig. 3.8. Diagrammatic representation of a) spleen and b) BM cell survival in RPMI medium supplemented with 10% (v/v) FCS. Cells were cultured at an initial density of $4x10^6$ cells/ml for BM and $3x10^6$ cells/ml for spleen. Live cells were counted at 24hr intervals by trypan blue exclusion. EμEBNA 1 positive samples are shown in red and wt samples are shown in blue. Cell number is shown in logarithmic scale on the Y axis.

month old mice (line 26) were collected and cells cultured as described above. In this experiment, the cells were split in RPMI medium supplemented with either 1% (v/v) or 5% (v/v) FCS at an initial density of $5x10^6$cells/ml.

No difference was observed in the cell survival between the EμEBNA 1 positive and wt (Fig. 3.9). However, as expected, cells from cultures containing only 1% FCS showed significant reduction in the cell survival (few live cells left at 6 days) compared to the cell cultures with 5% FCS (14 days).

3.3.3 Growth factor influence on cell survival

To examine the possibility that EBNA 1 might co-operate with various cytokines/growth factors to stimulate cell survival, the experiment was repeated with some modifications. Amongst the growth factors that have specific activity on B cell proliferation, differentiation and Ig production are IL2, IL4, IL6, IL7 and CD40 ligand (see Introduction).

Splenocytes and BM cells from 2 EμEBNA 1 positive and 2 wt transgenic 2 month old mice (line 26) were prepared as before and cultured in RPMI medium supplemented with 10% (v/v) FCS at an initial density of $5x10^6$cells/ml. Aliquots were grown with (1ng/ml of each growth factor) αCD40+IL4 and with IL6+IL7 for spleen cells and with αCD40+IL4+IL6+IL7 for BM cells. Live cells were counted again at 24 hour intervals by trypan blue exclusion over a period of 24 days.

All cells showed a prolonged survival, in the presence of growth factors in comparison to the absence of cytokines (Fig 3.10 versus Fig. 3.8). From the two combinations of growth factors in the spleen cell cultures, IL4 and αCD-40 supported cell survival slightly more effectively compared to the spleen culture supplemented with IL6 and IL7 (Fig 3.10b). However, while the EμEBNA 1 transgenic positive cultures consistently showed a slightly better growth/survival pattern than the wt cultures (red lines always above blue ones), no dramatic difference was observed. In agreement with previous data (Armitage and Alderson, 1995 and references therein), the addition of growth factors promoted cell growth.

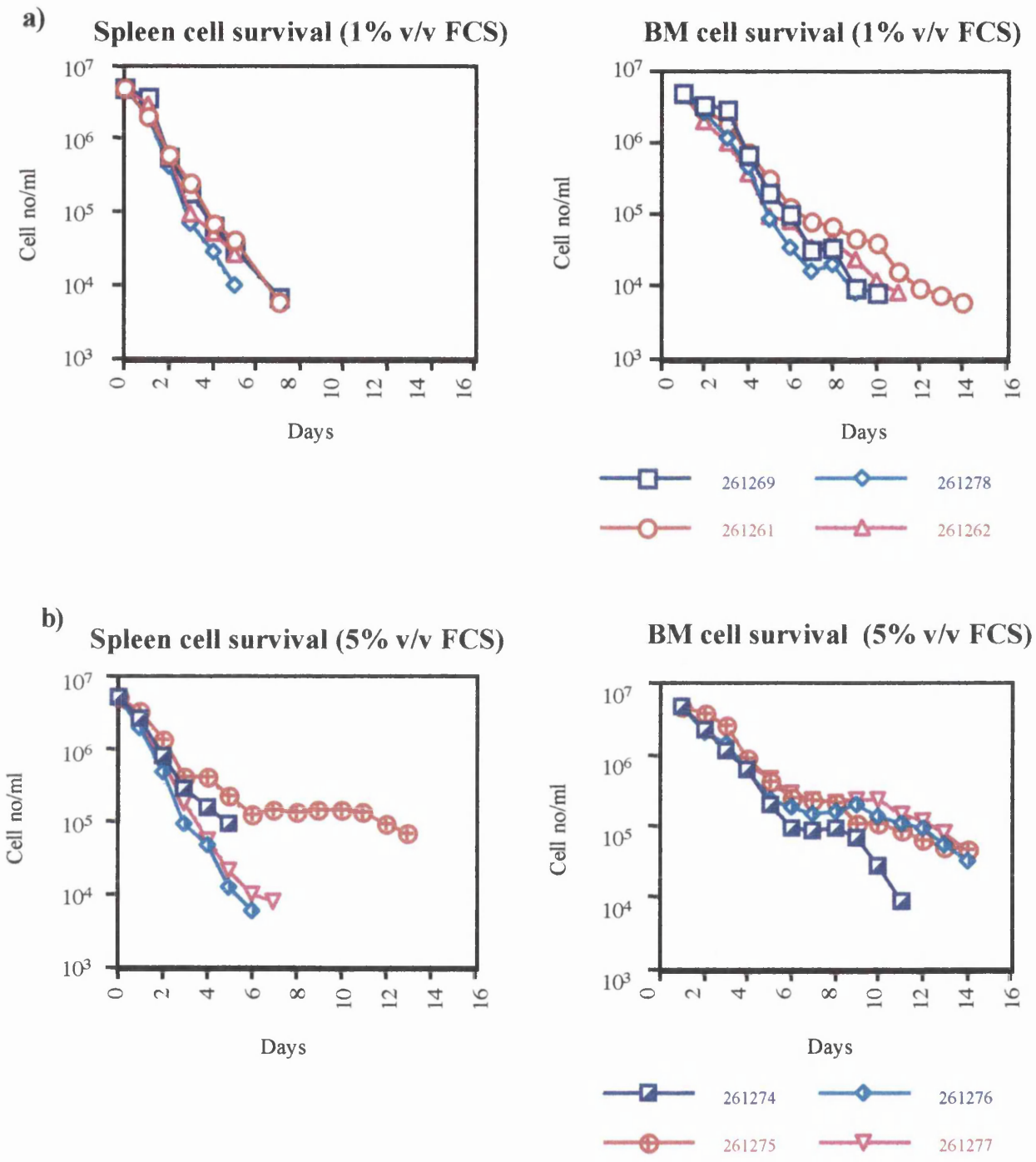
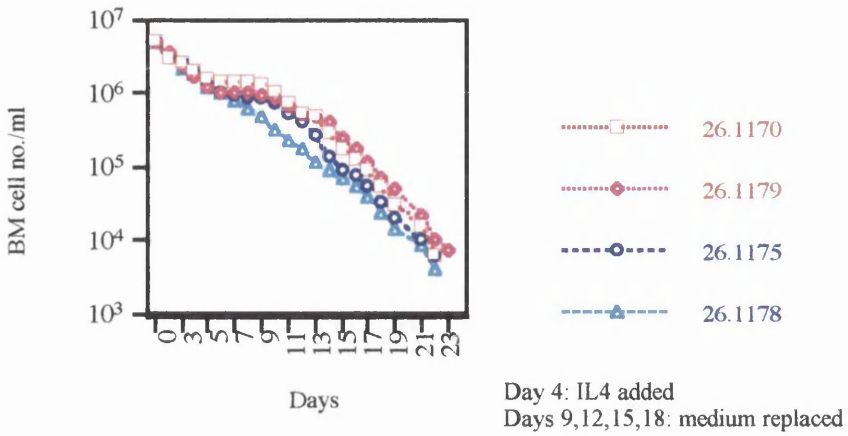
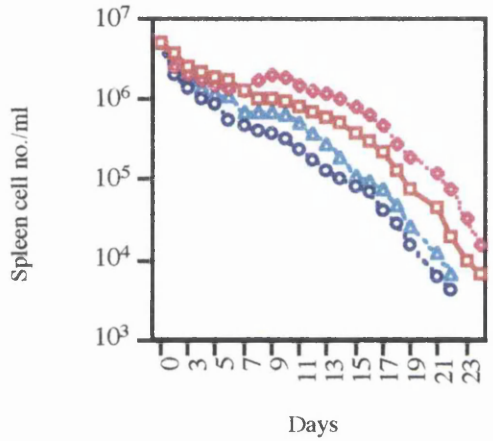


Fig. 3.9. The influence of FCS concentration on cell survival. Splenocytes and BM cells were grown in RPMI medium supplemented with antibiotics and either 1% (v/v) FCS (a) or 5% (v/v) FCS (b). Cells were seeded at a density of $5x10^6$ cells/ml and live cells were counted at 24 hour intervals by trypan blue exclusion. EμEBNA 1 positive samples are shown in red and wt samples in blue.

a) **BM cell (αCD40+IL4+IL6+IL7) survival curve**



b) **Spleen cell (αCD40+IL4) survival curve**



Spleen cell (IL6+IL7) survival curve

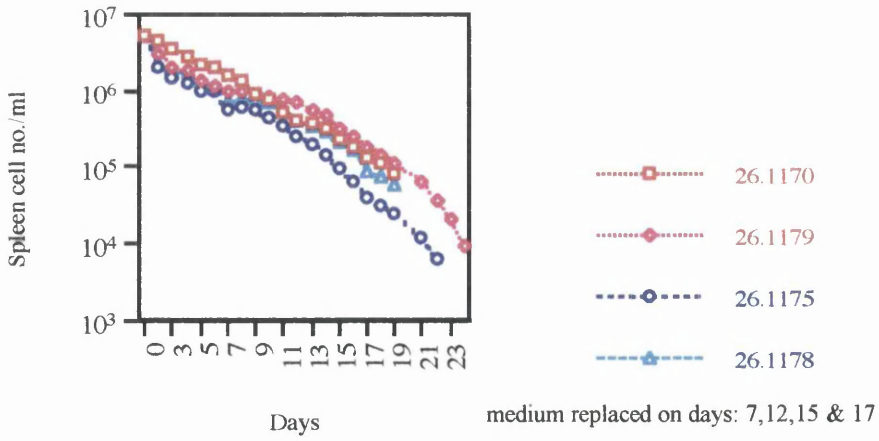


Fig. 3.10. Influence of growth factors on cell survival. Spleen and BM cells from 2 EμEBNA 1 positive and 2 wt 2 month old mice from line 26 were grown in RPMI medium supplemented with 10% (v/v) FCS. a) BM cells were grown in the presence of IL4, IL6, IL7 and αCD40. b) Spleen cells were grown in the presence of IL4 and αCD40 or IL6 and IL7. 1ng/ml of each growth factor was used in each case. Live cells were counted at 24 hour intervals by trypan blue exclusion. Medium replacement days are indicated at the bottom of each graph. EμEBNA 1 positive samples are shown in red and wt samples in blue.

To extend these observations we examined the effect of a combination of growth factors with low FCS concentration upon cell survival. Bone marrow and spleen cells from 4 EμEBNA 1 positive and 4 wt 2 month old mice were collected and cells cultured as described earlier. In this case, the cells were grown in RPMI medium supplemented with 1% (v/v) and 5% (v/v) FCS at an initial density of $5x10^6$cells/ml. For each FCS concentration no factors, IL4 only, αCD40 only and IL4+αCD40, were added (Fig. 3.11a and b and Fig. 3.12). Although no difference was observed in the survival between EBNA 1 positive and negative cell populations under each condition, the effects of the cytokines used were again obvious by the extended survival of all cultures in comparison to no growth factor addition.

CD40 antibodies in combination with IL4 have previously been shown to maintain proliferation of cells that are in active cell cycle (Armitage and Alderson, 1995). However, this was not evident here. Although IL4-containing cultures from both tissues and at both FCS concentrations showed better survival rates as compared to those without IL4, the CD40 antibody made no difference to the cell survival. A representative case is shown (Fig. 3.13). This may be due to antibody concentration, some laboratories recommend up to 100ng of growth factor/ml of culture while 1ng/ml of growth factor and antibody has been used in these assays. However, IL4 addition did provide a growth/survival advantage. Functionality of the growth factor is also important, for example, use of CD40 ligand instead of CD40 antibody may have been more effective in cell stimulation.

For this reason it was decided to repeat the experiment with slight modifications. In this case spleen cells were cultured in RPMI medium supplemented with 10% (v/v) FCS at an initial density of $5x10^6$ cells/ml. Each cell culture, which was derived from 2.5 mouse spleens, was split into 5 aliquots each with a different combination of growth factors including IL2, IL4 at a concentration of 1.5ng/ml of culture.

This time CD40 activation was supplied in the form of CD40 ligand (CD40L), which was derived from a CD40-producing cell line. The CD40L-producing cells (K47) are L929 mouse fibroblasts transfected with CD40L [or the

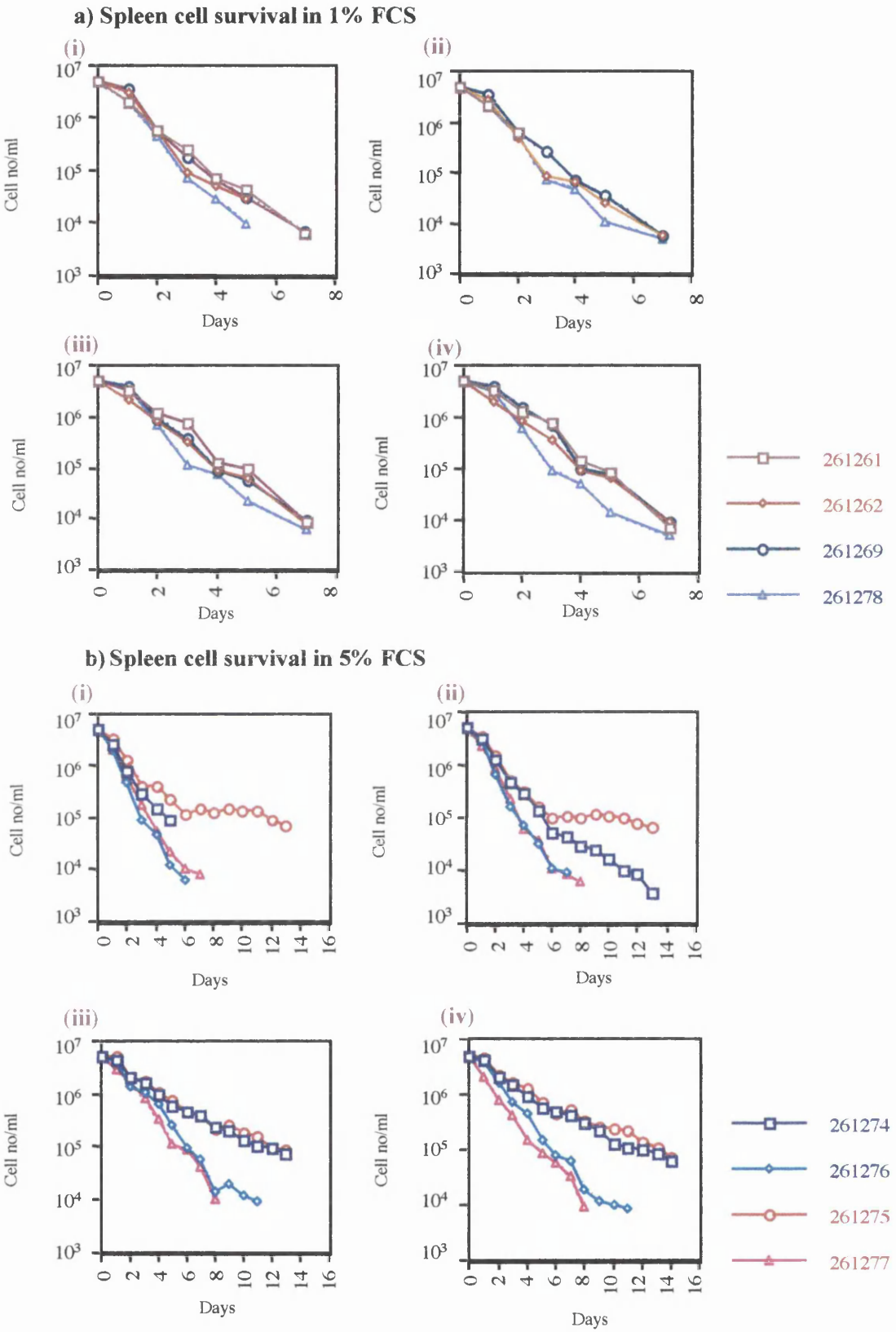


Fig. 3.11 Splenocyte survival in RPMI medium supplemented with 1% (v/v) (a) and 5% (v/v) (b) FCS. 2 EμEBNA 1 positive and 2 wt sibling mice were used in each case. Cells were seeded at a density of $5x10^6$ cells/ml in the absence of growth factors (i), with 1ng/ml of αCD40 (ii), with 1ng/ml of IL4 (iii) and 1ng/ml of each αCD40 and IL4 (iv). Live cells were counted at 24 hour intervals by trypan blue exclusion. EμEBNA 1 positive samples are shown in red and wt samples in blue.

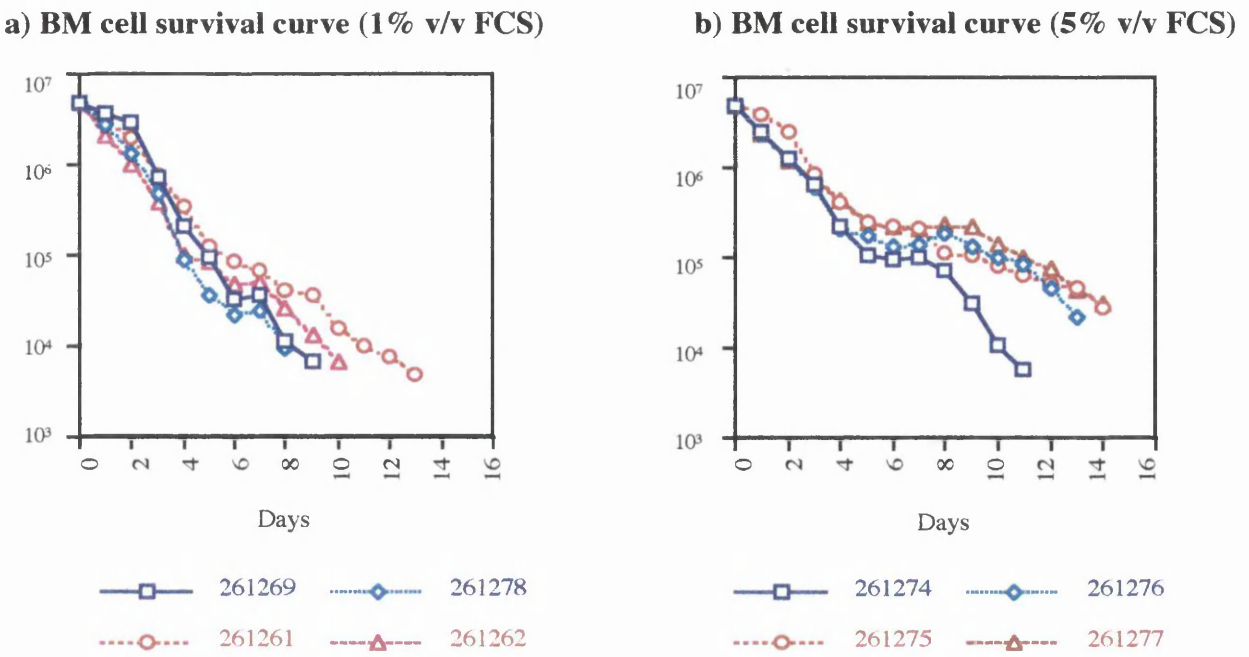


Fig. 3.12 Logarithmic representation of BM cell survival rate. Cells from 2 EμEBNA 1 positive and 2 wt mice were grown in culture medium containing either 1% (v/v) (a) or 5% (v/v) (b) FCS and was supplemented with αCD40 and IL4 at a concentration of 1ng/ml of each factor. Live cells were counted at 24 hour intervals by trypan blue exclusion. EμEBNA 1 positive samples are shown in red and wt samples in blue.

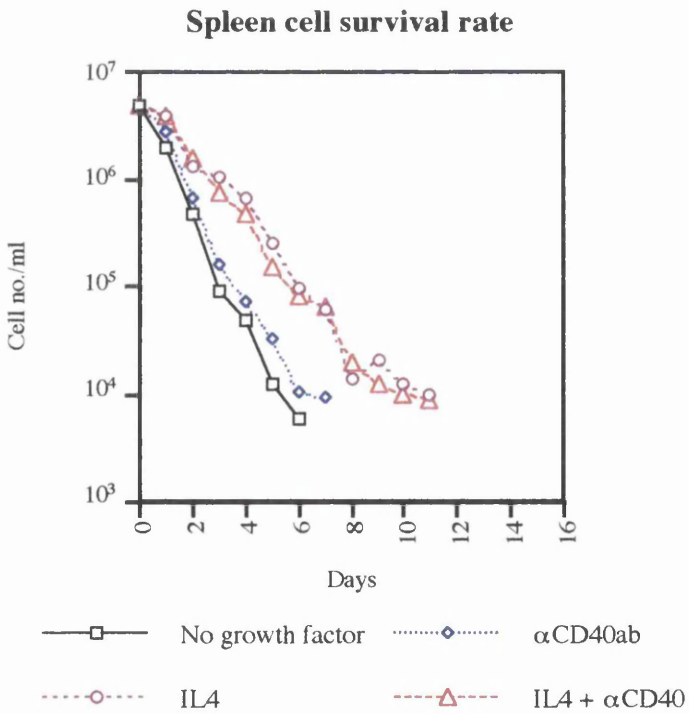


Fig. 3.13 Graphical representation of spleen cell survival rate from a wt control mouse in the presence or absence of growth factors. Cells were cultured for 11 days in medium containing 5% (v/v) FCS supplemented with different combinations of growth factors as indicated. Cells were seeded at an initial density of $5x10^6$ cells/ml and counted at 24 hour intervals by trypan blue exclusion.

vector alone (K5) as negative controls]. CD40 is not secreted but is attached to the cell surface of the CD40L-producing cells and therefore the cells were grown as a feeder layer and killed by adding mitomycin C.

To eliminate any doubts that the observed effect was due to CD40L itself and not due to other possible growth factors produced by the fibroblasts themselves, cells transfected with only the K5 vector were used as controls. These cells were treated exactly in the same way as the K47 cells. Thus, the 5 combinations used in this experiment were K47-CD40L+IL4+IL2, K5+IL2+IL4, K47-CD40+IL4, K5+IL4, no growth factor added (Fig. 3.14). In this experiment the results were consistent with those described above.

Despite analysing cell growth/survival under these multiple conditions, there is no obvious difference in the survival rate between EμEBNA 1 transgenic positive when compared to wt control splenocyte samples (Fig. 3.14a). The presence of IL4 always enhanced cell growth/survival. CD40L provided under these conditions (or activation by antibodies) showed little or no effect upon these cells. However, the IL2+IL4 combination supported cell survival a little better as shown by the slight shift in the curve (Fig. 3.14b). Moreover, the combination of all three growth factors together was the most effective in supporting cell survival.

3.4 Transgenic B cell properties II: Cell differentiation and proliferation

3.4.1. Spleen and BM cell proliferation (methylcellulose cultures)

To study the capacity of BM B cells to proliferate and differentiate, EμEBNA 1 positive mouse cells were compared to negative ones. Two separate experiments were performed, the first using 2 EμEBNA 1 positive and 2 wt mice (shown in Fig. 3.16 and indicated by an asterisk) and the second using 4 transgenic positive and 4 wt controls. BM and spleen cells were cultured in quadruplicates in semisolid methylcellulose IL7-containing medium. In the presence of IL7 compact colonies often consisting of >100 small cells, were formed by the BM cells (Fig. 3.15). Mouse spleen cells formed very few, <30 cell clusters formed in the presence of IL7. The colonies were apparent by day 3 of culture and the size reached a plateau

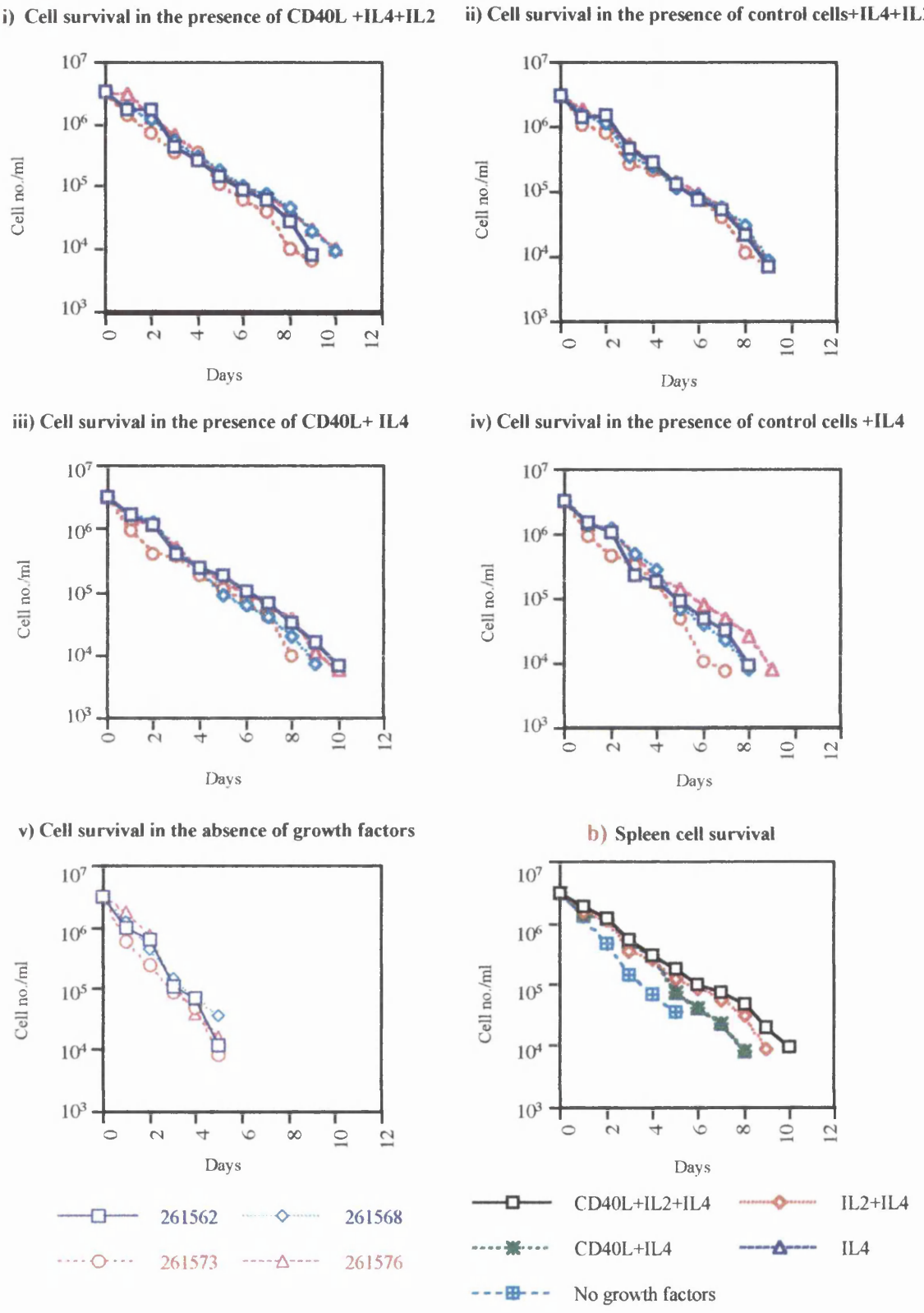


Fig. 3.14. Splenocyte survival in RPMI medium supplemented with 10% (v/v)FCS. 2 EμEBNA 1 positive (red) and 2 wt (blue) sibling mice were used. a) Cells were seeded at a density of 3.2×10^6 cells/ml in the presence of *CD40L* supplied by K47 cells (see text) +IL2+IL4 (i), K5 (control cells, see text) +IL2+IL4 (ii), CD40L+IL4 (iii), K5+IL4 (iv), or without growth factors (v). Each growth factor was used at a concentration of 1.5ng/ml of culture. b) effectiveness of each growth factor on cell growth within each culture.

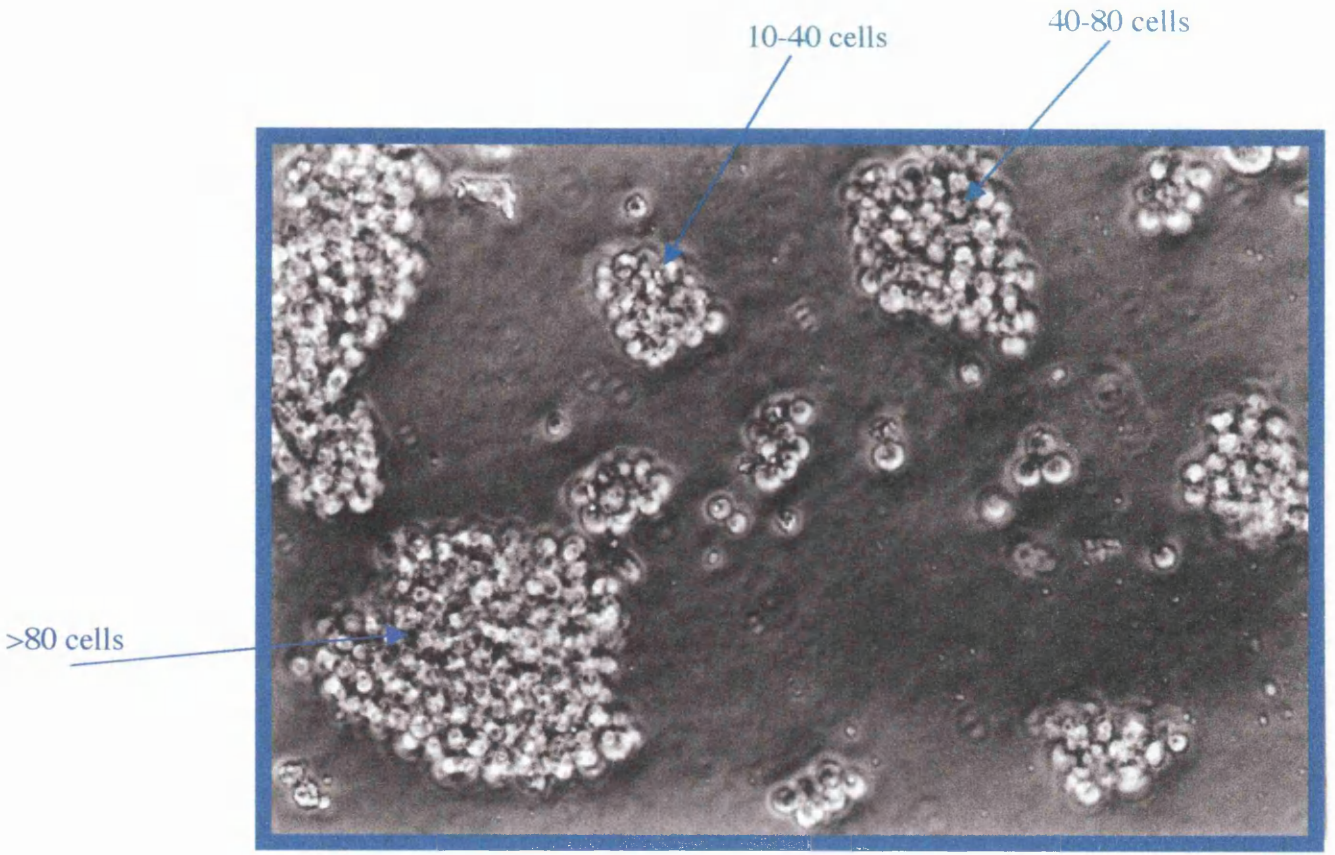


Fig. 3.15 B cell colonies formed by murine BM cells in the presence of IL-7 in methylcellulose culture medium. The different sized colonies are indicated by coloured arrows.

on day 7. For each sample (quadruplicate) the cell colonies were counted and their frequency calculated. Each colony number was placed into one of three subjective categories of approximately 10-40 cells, approximately 40-80 cells and >80 cells (Fig. 3.15). In all cultures there was a large number of single cells or very small clusters of 2-5 cells. Cell colony frequency was counted for each size category. Total colony numbers in these categories is shown in Fig. 3.16, while the proportion of the colony sizes are shown in Fig. 3.17 (where the colony number is taken as 100% for each culture).

As shown in Fig. 3.16a and c, it appeared that the overall number of colonies was lower in the first experiment (indicated by asterisk). Such differences are not unusual between two culture experiments. However, the proportion of the different colony sizes in experiment 1, shown in Fig. 3.17a is equivalent to the second experiment. From both experiments, it appeared that BM cells from EμEBNA 1 positive mice formed more colonies than the wild type control samples. The difference was apparent in the number of the larger colonies, with more large ones in the EμEBNA 1 positive cultures.

Statistical analysis was performed on all 6 EμEBNA 1 positive and 6 wt control samples, in order to test if the observed differences in the number and the frequency of the different colonies between the transgenic and wt cultures were significant. Highly statistically significant different number of colonies of the range of >80 cells was found in the transgenic cultures compared with wt (1.6-fold higher in EμEBNA 1 positive cultures, P=0.0006). Colonies in the range of 40-80 cells were also higher in the transgenic cultures. However, this is on the margin of statistical significance (P=0.051) (Fig. 3.16b). Figure 3.17 shows the relative proportions of these colony categories. The relative proportion of large colonies is greater in the EμEBNA 1 transgenic positive compared to wt mice (P=0.0000) and balancing this, the relative proportion of the small colonies (10-40 cells) is higher in the wt controls (P=0.0004).

The colony size classification is quite subjective and therefore a total cell count was performed for each separate sample using the trypan blue exclusion

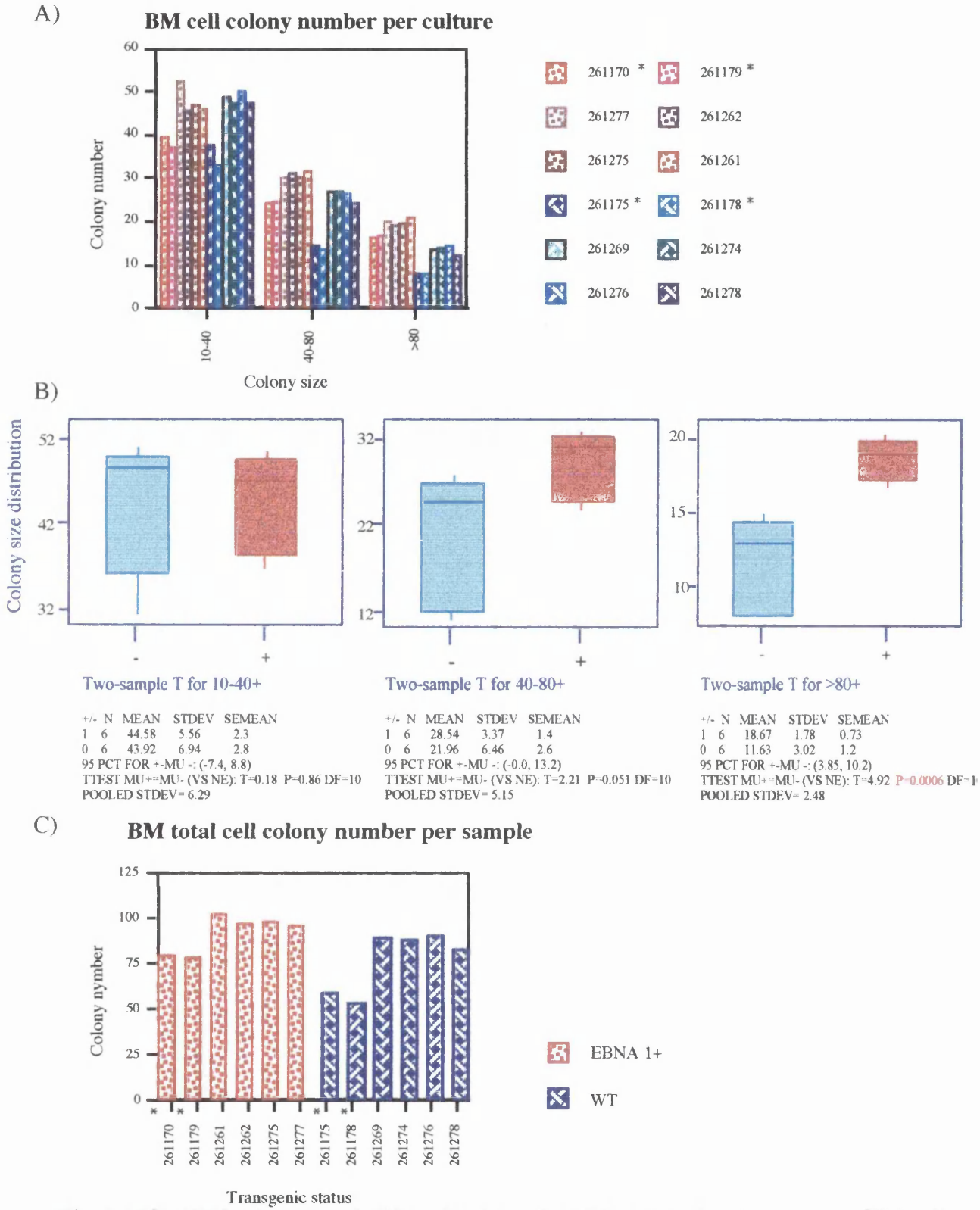


Fig. 3.16 EμEBNA 1 transgenic BM cells showed proliferative advantage over wt BM cells. Cells from 6 EμEBNA 1 positive (red) and 6 wt mice (blue) were cultured in methylcellulose medium (in quadruplicate) supplemented with IL7. Colony count and classification was performed on day 7. The number of B cell colonies per sample was calculated and each number was placed into 1 of 3 categories of colony size. The distribution of categories for transgenic and wt controls are plotted in A). B) Statistical illustration of the BM cell colony number results. Comparison between 6 EμEBNA 1 (+) positive and 6 wt (-) siblings. C) Total cell colony number per sample.

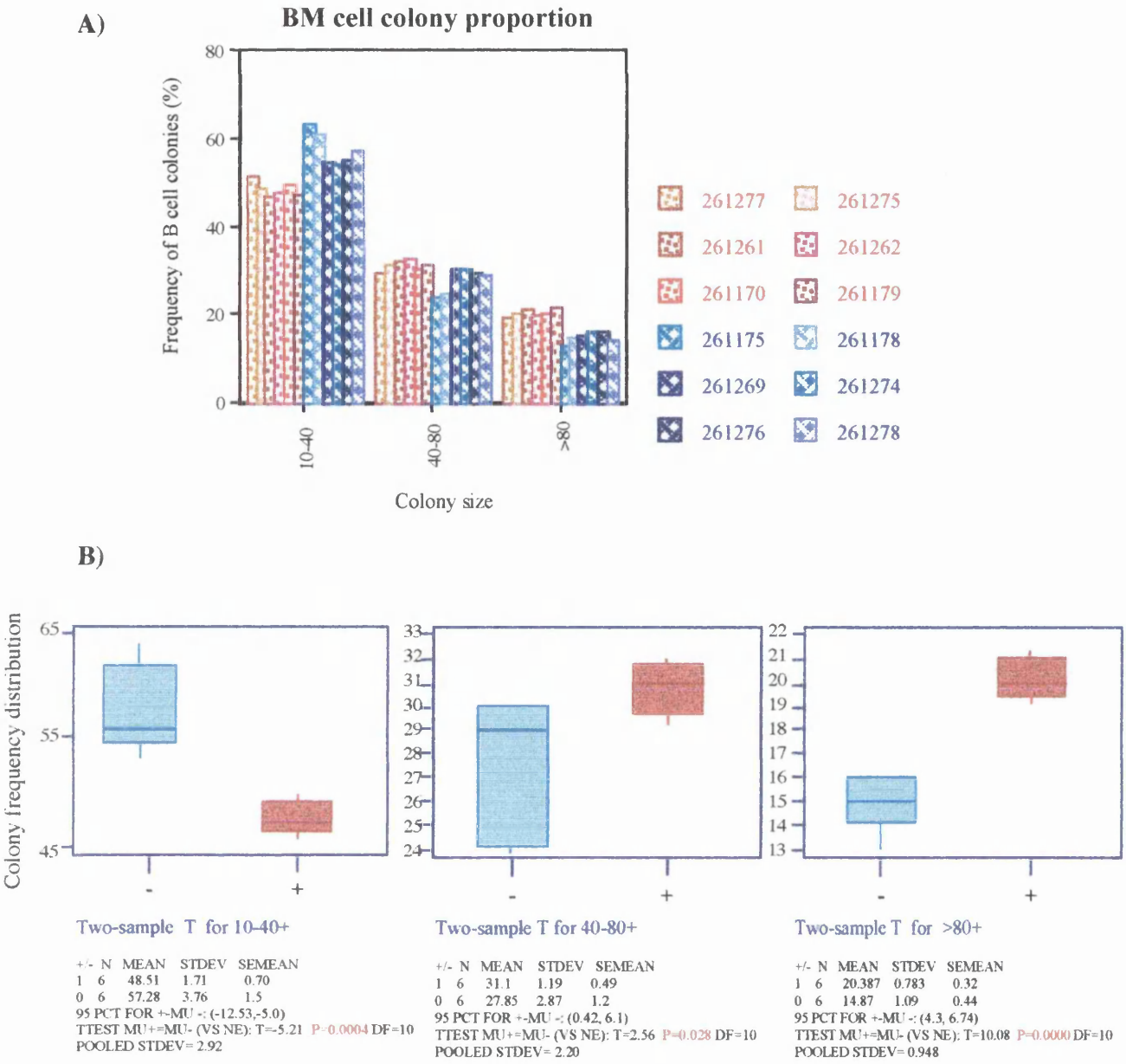


Fig. 3.17 EμEBNA 1 transgenic positive BM cells showed proliferative advantage over wt BM cells. Cells from 6 EμEBNA 1 positive (red) and 6 wt mice (blue) were cultured in methylcellulose medium supplemented with IL7. Colony count and classification was performed on day 7. Each % is calculated from the mean value of a quadruplicate from each individual mouse. The frequency of B cell colonies per sample was calculated and each frequency was placed into 1 of 3 categories of colony size. The distribution of categories for transgenic and wt controls are plotted in A). B) Statistical illustration of the BM cell colony frequency results. Comparison between 6 Eμ EBNA 1 positive (+) and 6 wt (-) siblings.

method, to determine if the total cell number present in each culture was consistent with the colony size classification. No significant difference was observed in the cell number between positive and negative for EμEBNA 1 positive BM cells (Fig. 3.18). However, in both positive and negative samples there was a large number of cells that only divided a few times (small clusters of cells). By calculating the total number of colonies per sample (on average 80) and comparing it with the initial number of cells inoculated per culture ($1x10^4$), then it is clear that only a small proportion of cells contribute to the colonies. As such, the final cell count merely reflects the bulk of the cells which did not divide, or only formed small colonies and the large colony cells are a very small proportion of this count, hence this count does not reflect the data presented in Figures 3.16 and 3.17.

Based on the above results it can be suggested that EBNA 1 may confer a survival or proliferative advantage to developing B cells in the transgenic BM cultures in comparison to the wt cultures.

3.4.2 Cell Apoptosis

To examine cell death specifically, AnnexinV/PI staining was performed on splenocytes from both EμEBNA 1 transgenic and wt siblings. Apoptosis is characterised by a variety of morphological features. Changes in the plasma membrane, such as the translocation of the membrane phosphatidyl serine (PS) from the inner to the outer cell membrane, are one of the earliest of these features. AnnexinV is a Ca^{2+}-dependent phospholipid binding protein that has high affinity for PS and binds to cells with exposed PS. Because externalisation of PS occurs in the earlier stages of apoptosis AnnexinV staining can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation. AnnexinV staining precedes the loss of membrane integrity, which accompanies the latest changes of cell death resulting from either apoptotic or necrotic processes. Therefore staining with AnnexinV in conjunction with PI allows the investigator to distinguish early apoptotic cells (AnnexinV+/PI-). In combination with FACS analysis it was

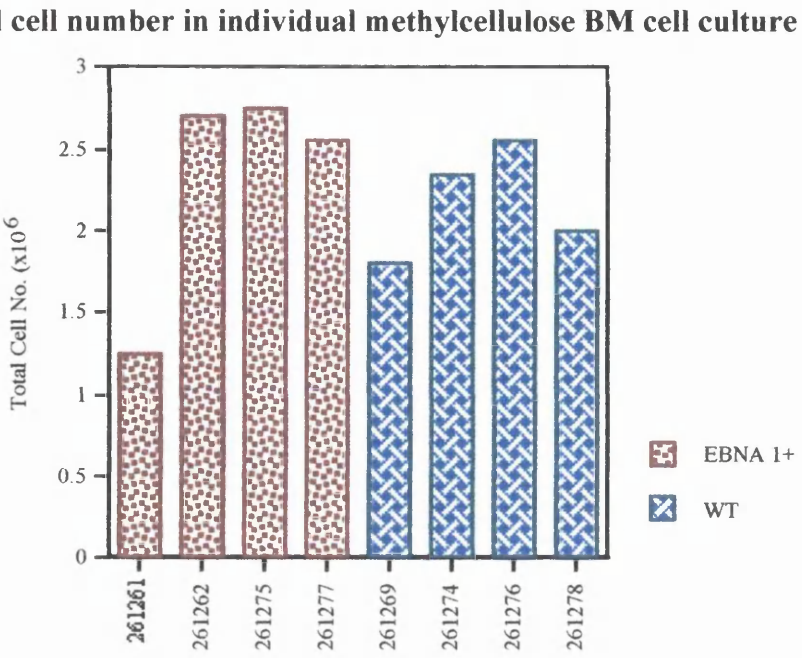


Fig. 3.18 Total cell number present in each individual BM cell culture. Each column represents the mean value of quadruplicates of each sample culture. 4 EμEBNA 1 transgenic positive and 4 wt control cultures were included in this assay.

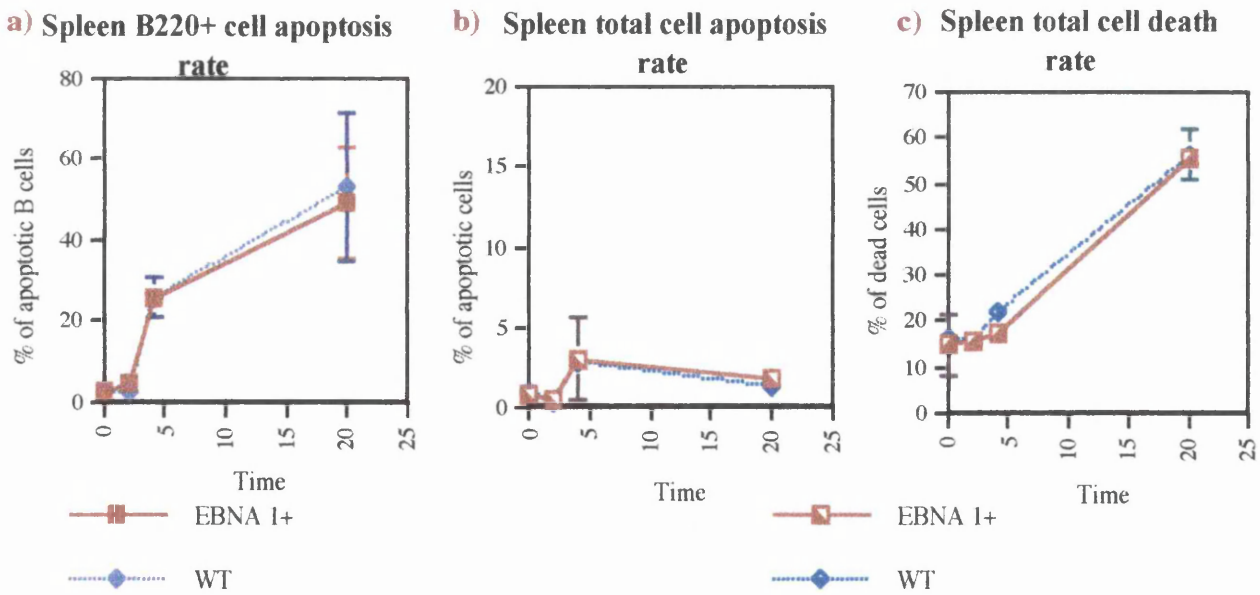


Fig. 3.19 Apoptotic death rate of spleen cells from 4 EμEBNA 1 positive and 4 wt mice at 2 month old. Cells were cultured at a density of $1x10^6$ cells/ml and samples collected at 0h, 2h, 4h, and 20h. FACS analysis was performed by staining the cells with AnnexinV-FITC/B220 to show the number of apoptotic B cells and AnnexinV-FITC/PI to show the total number of dead cells. a) B220+/AnnexinV-FITC+ cells, b) AnnexinV-FITC+/PI- total cells, c) AnnexinV-FITC+/PI+ total cells.

possible to calculate the percentage of apoptotic and dead cells in transgenic and wt cell cultures.

Splenocytes from 4 EμEBNA 1 positive mice and 4 wt controls were cultured in RPMI medium supplemented with 10% (v/v) FCS. Cell samples were taken at 0hr, 2hr, 4hr and 20hr of culture. Cells were stained with AnnexinV- FITC/B220-PE to quantitatively determine the percentage of B cells undergoing apoptosis. Cells were also stained with AnnexinV-FITC/PI to distinguish the early apoptotic cells (AnnexinV+/PI-). Cells that stain positive for both AnnexinV-FITC and PI are either in the end stage of apoptosis, undergoing necrosis, or are already dead. As shown in Fig. 3.19a the apoptotic rate of B cells (AnnexinV+/B220+) started to increase in the first 4hr and approximately 55% of the B cells were or started to apoptose within 20 hr. The total number of cells entering apoptosis in the total population (AnnexinV+/PI-) (Fig. 3.19b) was calculated by subtracting the AnnexinV+ only cells from the AnnexinV+/PI+ cell population. The number of cells entering apoptosis (AnnexinV+/PI+ cells) started to increase in the first 4hr and then started to decrease because in 20hr approximately 60% of all cells were dead (AnnexinV+/PI+). However, no significant difference was observed in the cell death rate between transgenic and wt spleen cells. These results are in accordance with the cell survival experiments described earlier.

While the data do not demonstrate any effect of EBNA 1 upon apoptosis, the negative data do not prove that EBNA 1 does not have any effect on B cell survival. It may be that EBNA 1 provides resistance to an apoptosis triggering factor, for example a strong cell cycle insult, or that any effect is only evident *in vivo*.

3.5 Discussion

A variety of approaches have been used to identify possible B cell characteristics that EBNA 1 may effect.

Flow cytometric analysis of splenic and BM cells from EμEBNA 1 transgenic mice revealed that the percentage of B220+/IgM+, B220+/IgD+, IgM+/IgD+, B220+/Thb+ and B220+/CD5+ cell populations was similar to that of control

littermates. The B220+/CD23+ cell population may be effected, but this was less conclusive. However, the percentage of B220+/sIg+ cells was higher in both spleen and BM from transgenic positive animals when compared to the wt control siblings. The fact that sIg+ B cell number was higher in both these tissues supports the result. Moreover, statistical analysis validates these results as the increased percentage of sIg+ B cells in the EBNA 1 transgenic positive spleen and BM was found to be statistically significant both for the proportion of sIg+ B cells and for the intensity of sIg staining.

As mentioned earlier in the text, the sIg polyvalent antibody used recognises IgM, IgG and IgA on the B cell surface. IgM is expressed in both immature and mature (naive and activated) B cells. Surface IgG and IgA are both expressed later during B cell maturation. Thus, by staining with B220/IgM, B220/IgG and B220/IgA separately it would be possible to calculate the percentage of immature or mature cells in the EμEBNA 1 positive and wt spleens and BM. In addition, after immunisation of the animals with a specific antigen and then measuring the secreted IgG in the serum by ELISA, it will be possible to monitor if EBNA 1 really effects B cell antibody production.

Further staining with markers that are expressed at certain stages of B cell maturation would help to distinguish the different B cell subtypes. CD38 is expressed in pro- and pre-B cells and then again later on the plasma cells surface whereas CD43 expression is restricted to the pro-B cells. In addition, PNA (peanut agglutinin) and GL-7 are two markers expressed on the surface of germinal centre B cells. CD25 would be another useful marker to examine since it is expressed only on activated B cells. Moreover, staining with CD25 antibody would indicate if CD25 expression is increased in the transgenic splenocytes of the BL model as has been reported in a Hodgkin's cell line expressing EBNA 1 (Kube *et al.*, 1999).

The cell survival/proliferation assay on developing B cells in the BM (methylcellulose colony assay) showed that EμEBNA 1 transgenic positive BM cells have a higher survival/proliferation rate than wt BM cells. Statistical analysis revealed that this survival/proliferation advantage, illustrated by the increased number and

proportion of larger colonies in the EμEBNA 1 transgenic cultures, was statistically significant (P=0.0006 and P=0.0000 respectively). However, it was not possible to characterise the cells of the colonies formed. In order to do that a FACS analysis could be performed using B cells surface markers that are expressed through development/differentiation such as those mentioned above. Nevertheless, the methylcellulose colony assay is quite subjective. Therefore, in order to make this assay more objective B cell selection could be performed prior to culture followed by measurement of the survival/proliferation of the sorted populations.

In order to examine proliferation directly, other approaches can be applied either in culture or *in vivo*. One such approach is to measure proliferation in culture by ^{3}H-thymidine incorporation. The addition of tritiated thymidine provides a labelled nucleotide that can be incorporated into replicating DNA. A specific activity of [^{3}H]-thymidine of 2Ci/mmol gives significant incorporation into DNA with a minimal amount of radiological damage.

Alternatively, proliferation can be assayed *in vivo*. This can be achieved by supplying BrdU to the EμEBNA 1 transgenic and wt animals. BrdU incorporates into replicating cells. Dividing B cells that will move from the BM to the spleen can either be sorted by FACS or seen *in situ* (in spleen sections) (Kline *et al.,* 1999). The effects of EBNA 1 may become obvious since in such experiments the cells are in their natural, permissive environment.

EμEBNA 1 cells did not show any survival advantage in culture when compared to wt ones under any conditions used. Although the results from these cell survival experiments were negative this does not definitively demonstrate that EBNA 1 does not effect cell survival. The results may not reflect what happens *in vivo* because the assays may be too stringent and not mimic the *in vivo* environment. There are many factors that may be responsible for the outcome of these results. First of all, because the cell cultures contained not only B cells but also other cell types i.e. T cells, macrophages and fibroblasts, there may be interactions between the different cell types which could influence the data. Within a cell culture of such

nature different cells may secrete factors (chemokines) which either induce cells to proliferate or to apoptose.

Other factors influencing the results may be the form, the functionality and the concentration of the growth factor used in the assay. IL4 supported cell growth far more effectively than any other growth factor used. In addition to its activities described earlier (Introduction), IL4 upregulates the expression of CD40 and also preferentially boosts the induction of DNA synthesis in B cells mediated by the combination of αCD40 and IgM. Thus, anti-CD40 or CD40L (ligand) were included in this assay and used in combination with IL4 to activate and support B cell growth. Either the CD40 antibody or the CD40L on their own were not very efficient in supporting the cell cultures. This could be due to the low concentration of CD40 antibody used or the feeder layer of CD40L-producing cells was not very effective in producing the CD40L required for B cell activation. Although IL2R (CD25) expression levels were found upregulated in a EBNA 1 transfected Hodgkin's cell line, supplement of cell cultures with IL2 marginally improved cell survival in both EμEBNA 1 positive and wt cell cultures.

To eliminate the influence of other cell types in culture a B cell selection prior to culture could be undertaken and greater concentrations of growth factors could be tried.

The apoptosis rate of EμEBNA 1 transgenic cells was similar to that of wt cells in culture. These results agree with the cell survival assay results in which case no differences were observed between transgenic and wt cell cultures under any culture conditions tested. Being negative, however, these results do not support but neither refute that EBNA 1 does effect B cell survival. Cell culture conditions may have been very stringent in comparison to the *in vivo* environment. The cell cultures used in the AnnexinV/PI staining contained other cells which could have influence the data. B220 staining was included in the assay to help distinguish the B cell population. However, as the staining was only in combination with AnnexinV/FITC it was not possible to say if the B220+ cells were at early apoptotic stages or if they

were dead. In approaching this problem, a B cell selection before the induction of apoptosis would be helpful and eliminate the staining problems for cell analysis.

Another limiting factor in this assay is that it did not distinguish between cells that have already undergone an apoptotic death and those that have died as a result of a necrotic pathway, because in either case the dead cells will be AnnexinV+/PI+. In approaching this problem, a strong and effective death insult, such as ionising radiation to trigger specific cell apoptosis pathways may be informative. In addition, taking samples at shorter time intervals might make it easier to gauge if EBNA 1 has any subtle effects on cell survival since the whole process would be faster.

Apoptotic cells characteristically exhibit DNA strand breaks and there is an alteration in their ability to scatter light at a forward angle reflecting cell size and at 90° reflecting cell granularity. Such changes can be detected using the terminal deoxinucleotidyl transferase TdT mediated dUTP Nick End Labelling (TUNEL) assay in combination with flow cytometry.

It is tempting to speculate that EBNA 1 promotes B cell differentiation and hence the increased sIg B cell population. If this is the case then the increase in methylcellulose colony numbers perhaps indicates that EBNA 1 drives more cells through differentiation and causes them to form more and larger colonies *in vitro*.

Alternatively, EBNA 1 may support the survival/proliferation of immature B cells, supported by the methycellulose colony numbers, and then drives them to differentiate and survive leading to increased sIg. However, at the moment we do not know the stage of B cell maturation in these colonies.

The accumulated data are therefore suggestive of a role for EBNA 1 in the survival or proliferation of immature or mature B cells. EBNA 1 has been shown to be redundant with Bcl2 in transgenic crossbreeding experiments, and consequently it is suggested that these genes act in the same pathway. Since *Bcl2* plays a critical role in blocking apoptosis and in the development of mature naive lymphocytes (Nakayama *et al.*, 1993) EBNA 1 may act directly or indirectly through deregulation of other cellular genes to effect the same pathway.

Chapter 4

Effects of EBNA 1 on cellular gene expression I: Selected genes

4.1 Introduction

The most up to date information concerning the transactivation function of EBNA 1 relates predominantly to the viral enhancer regions. The only cellular genes identified for which expression is up-regulated by EBNA 1 are *Rag1* and *2* (Kuhn-Hallek *et al.*, 1995; Srinivas and Sixbey, 1995). One of the aims of this project was to examine the possible effects of EBNA 1 on the expression of key cellular genes in the B cell differentiation and proliferation pathways *in vivo* at the pre-tumour stage, using the transgenic mouse model (Wilson *et al.*, 1992; 1996). The RNA expression level of different cellular genes in mouse tissues is variable depending on the developmental stage of the animal, the cell cycle point and the tissue that is analysed. A comparable study of cellular gene expression was therefore performed in age (siblings) and strain matched EμEBNA 1 positive and wt mice. It was important to collect tissues prior to the onset of neoplasia in this analysis to ensure that any observed effects were mediated by the EμEBNA 1 transgene and not by the secondary mutations that occur during tumourigenesis. In line 26, mice succumb to neoplasia at any age between 4 and 12 months. As such mice of 1 month (juvenile) and 2 month (young adults) were selected for this study. RNA samples were extracted from whole spleens and lymph nodes from EμEBNA 1 transgenic positive and wt sibling animals (see Methods section 2.4.5).

The spleen serves both a primary haemopoietic and lymphopoietic function in murine neonates but this is finished by 3-4 weeks of age. The total lymphoid content reaches adult levels at 8-10 weeks of age. Mechanical disruption of the spleen yields about $5\text{-}15\text{x}10^7$ cells including (about $2\text{x}10^5$ nucleated cells per mg fresh weight). The spleen is divided into red pulp and white pulp. The red pulp provides the emergency source of granulocytes and platelets. In addition it contains large numbers of macrophages, dendritic cells, scarce lymphocytes, endothelial cells and fibroblasts which usually act as accessory cells. The white pulp consists of lymphoid tissue, the periarteriolar lymphoid sheath, composed of T and B areas. T cells may be organised into either primary unstimulated follicles (surrounding aggregates of B cells) or secondary stimulated follicles, which possess a germinal centre (GC) (immature/naive B cell zone). Follicular dendritic cells and phagocytic macrophages are also found in germinal centres. Approximately 75% of the spleen cells are lymphocytes with about 50-55% being B cells and 25-30% T cells.

Lymph nodes (LNs) are small but numerous within the mouse body. The LN consist of a B cell area (cortex), T cell area (paracortex) and a central medulla with cellular cords containing T cells (CD4+ and scarce CD8+), B cells, plasma cells and abundant macrophages. In the cortex, primary follicles consist of mature resting B cells as well as macrophages and dendritic cells. In the secondary follicles germinal centres develop in response to antigenic stimulation and contain numerous large lymphocytes with phenotypic characteristics of activated B cells. The mechanical disruption of LN in a young adult mouse yields about 5-10x10^7 cells (without the mesenteric LN) (5x10^5 nucleated cells per mg wet weight). Approximately 75-80% of the lymphocytes is T cells and 20-25% B cells. Other cell types contribute in less than 1%.

The expression levels of several key cellular genes have been quantified by slot blotting total RNA samples from these tissues, probing and subsequent phosphoimaging analysis. Not knowing what expression level differences might be seen whole tissue was used as a first approach. Genes analysed in this approach include known B cell proto-oncogenes, apoptosis regulators and tumour suppressors. Due to the redundancy observed between EBNA 1 and Bcl2 in lymphomagenesis in transgenic cross-breeding experiments (see Introduction section 1.7) and the possibility that EBNA 1 could act through *Bcl2* or another member of the *Bcl2* gene family, the analysis started using probes for the *Bcl2* family members. As a positive control indicator the *Rag1* and *2* genes involved in immunoglobulin loci rearrangement were also analysed. Certain additional genes were included in this screen, as detailed below.

4.2 Gene expression analysis in juvenile (1 month) tissues by slot blots

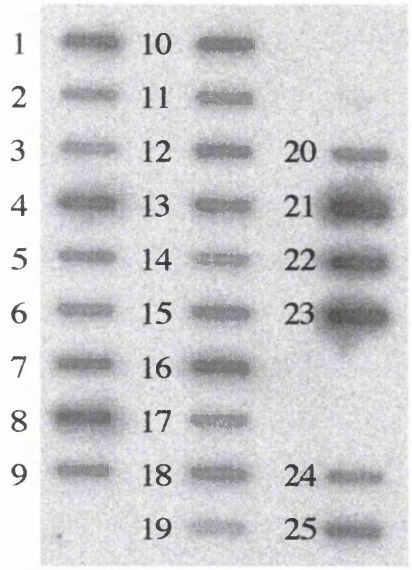
13 transgenic positive and 8 wt siblings at 1 month old from the EμEBNA 1 line 26 mice were studied. Total RNA was extracted from whole spleen and LN and slot blots prepared using 5μg of RNA per slot. Four duplicate blots were prepared with batch samples. The blots were hybridised with ^{32}P labelled probes generated by the random primed method from mouse-derived sequence plasmid cDNAs. The genes tested are listed in Table 4.1. Some tumour samples were also included in these slot blots as an indicator of over expression for certain transgenes. All slot blot signals were quantified by phosphoimage analysis. A representative slot blot autoradiogram is shown in Fig. 4.1 (phosphoimage values are given in Appendix 4.I); EμEBNA 1 positive and wt RNA samples are listed. The background value was subtracted from the test values. The values were normalised (quantified for the RNA amount present in each slot) by re-probing the slot blots with the house-keeping gene glyceraldehyde 3-P dehydrogenase (*GAPDH*) and calculating the ratio of the test signal value over

Table 4.1 Summary of comparative gene expression results in whole spleen and LN from line 26 1 month EμEBNA 1 transgenic mice versus wt

Gene	Spleen Mean tg+	Spleen Mean tg-	Spleen ratio +/-	Spleen P value *	LN Mean tg+	LN Mean tg-	LN ratio +/-	LN P value *
Bad	0.709	0.5874	1.2	(0.067)	0.588	0.7516	0.8	(0.0082)
Bak	0.0585	0.0565	1.0	(0.65)	0.1392	1.1363	1.0	(0.92)
Bax	0.467	0.264	1.77	(0.3)	2.182	2.49	0.87	(0.42)
Bcl2	8.47	7.21	1.2	(0.18)	2.306	2.687	0.86	(0.2)
BclxL	0.1976	0.1536	1.3	(0.011)	0.0609	0.0878	0.693	(0.14)
Bmi1	1.596	1.417	1.13	(0.32)	0.361	0.3824	0.95	(0.65)
Gfi1	1.917	1.604	1.2	(0.058)	2.398	2.121	1.13	(0.12)
cMyc	4.942	5.377	0.9	(0.25)	0.0917	0.0926	0.9	(0.85)
Pim1	5.988	7.16	0.8	(0.023)	0.0811	0.0876	0.92	(0.53)
CyclinD1	0.5916	0.624	0.95	(0.26)	0.1198	0.127	0.95	(0.45)
MDM2	3.38	2.56	1.3	(0.51)	4.21	4.05	1.0	(0.8)
GMCSF	4.49	4.102	1.1	(0.31)	0.952	0.833	1.15	(0.37)
MIP1α	1.794	2.032	0.88	(0.17)	0.325	0.3164	1.0	(0.84)
Rag1	1.943	1.539	1.3	(0.023)	1.77	1.757	1.1	(0.88)
Rag2	2.022	1.574	1.3	(0.018)	3.296	3.022	1.1	(0.21)

The ratio of EμEBNA 1 transgenic positive versus wt samples are given (ratio column). Statistical P value using the Two-sample T-test is given in parentheses for the data.

*P values of <0.05 are significantly different increased expression in EμEBNA 1+ mice
decreased expression in EμEBNA 1+ mice



1 26.818-	**10** 26.819+	
2 26.821-	**11** 26.820+	
3 26.823-	**12** 26.822+	**20** 26/ 85.8 Bcl2 tum control
4 26.825-	**13** 26.824+	**21** 59/ 87.8 Pim1 tum control
5 26.828-	**14** 26.827+	**22** 97.22 c-Myc tum control
6 26.832-	**15** 26.829+	**23** 97.32 c-Myc tum control
7 26.835-	**16** 26.830+	
8 26.817+	**17** 26.831+	
9 26.826-	**18** 26.833+	**24** 26.815+
	19 26.834+	**25** 26.816+

Fig. 4.1 Representative slot blot of RNA expression results. 5µg of whole spleen total RNA was loaded per slot and the blot was probed with *Rag2* radiolabelled sequence. The slot blot was probed, stripped and reprobed as described in Materials and Methods (section 2.D.4).

Slots 1-7, 9 wt control RNA from transgenic negative sibling mice
Slots 8,10-19, 24, 25 EBNA 1 positive RNA samples
Slots 20 EBNA1/ Bcl2 bi-transgenic tumour
21 Pim1/EBNA1 bi-transgenic tumour
22-23 97 c-Myc tumours

The slot blot phosphoimager values for *Rag2* before and after normalisation from 1 month spleen RNA are listed in Appendix 3.I.

the quantitative signal value. In some cases there are only 1 or 2 samples in the group (such as the Eμ-*c-Myc,* Eμ-*Pim1* and Eμ-*Bcl2* tumours), these can only give an indication of expression levels without statistical relevance and were used only as expression controls.

In order to gauge whether the observed differences in the expression levels of the genes tested were significant, statistical analysis was performed using the Two-sample T-test. Statistical significance with 95% confidence intervals is reached when $P<0.05$. In order to visualise the data box plots are shown in Fig. 4.2. Statistically significant differences ($P<0.05$) between EμEBNA 1 transgenic positive and wt control samples were found in the expression levels of several genes. Graphical data is shown in Fig. 4.2 for those results displaying significant differences. The data are summarised in Table 4.1.

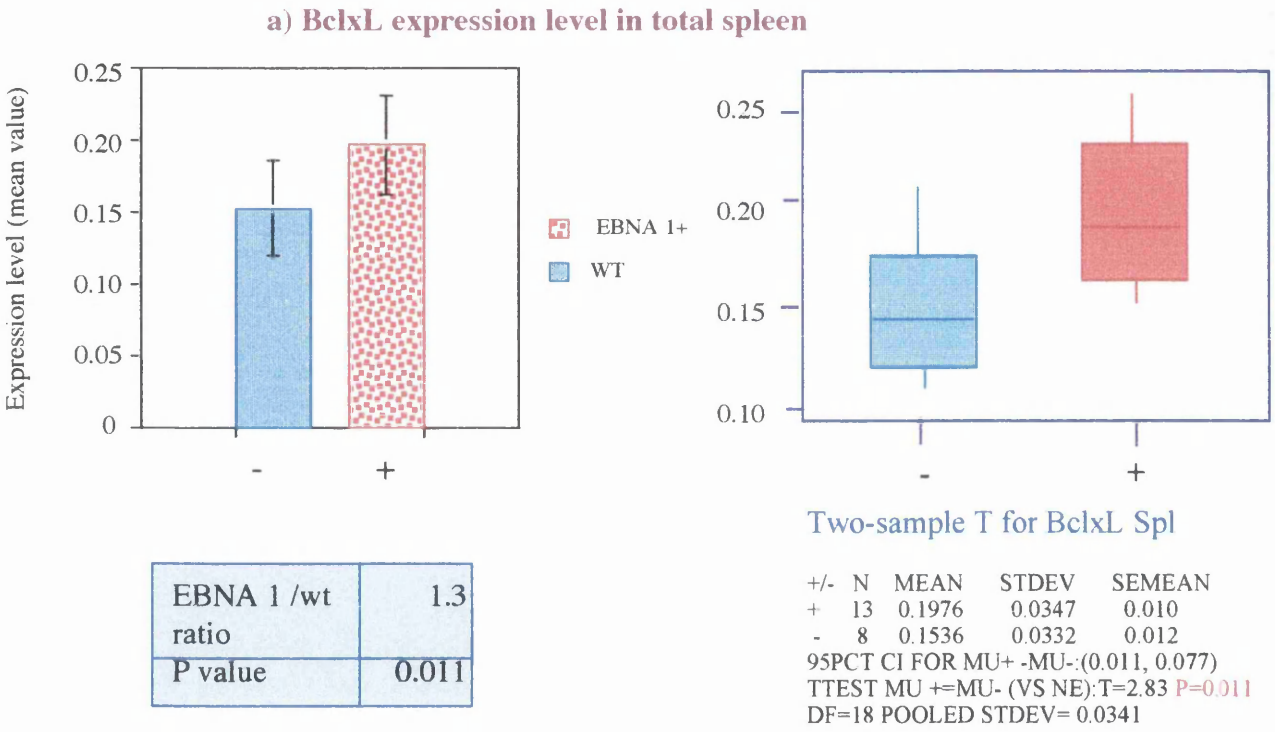
Of the genes tested in juvenile (1 month) spleen samples, from transgenic positive and wt mice, 4 displayed differential regulation. The expression levels of *BclxL*, *Rag1* and *Rag2* were found to be 1.3-fold higher in the EμEBNA 1 transgenic positives compared to wt controls, all to a significant degree. *Pim1* showed a lower expression level of 0.8-fold when compared to wt controls. This differs from the results obtained using LN samples where only the expression level of *Bad* (0.8x) was found to be significantly lower in the transgenic positive compared to the wt samples. This might be due to the different cellular compositions of these tissues as described in the introduction.

These data demonstrate that presenting a ratio between mean values is not sufficient for meaningful results, and that statistical analysis is required to support conclusions. For example, *Bax* expression (Fig. 4.3) shows the largest difference of the means between positive and wt mice with a ratio of 1.8. However, this difference is not statistically significant as indicated by the box plot, in which the spread of the data in the EμEBNA 1 positives shows a different pattern to that seen in the wt ones (Appendix 4.I), and a P value of 0.3. Even discounting the outlying values does not bring the P value to significance. This is also evident in the bar graphs with the error bars in each group of data. In contrast to *Bad*, *BclxL* showed a smaller (1.3-fold) but statistically significant increase because the data spread within each group was smaller, shown in the box plot and by the error bars in the bar graph.

4.3 Gene expression analysis in young adult (2 month) tissues

The analysis was extended to pre-neoplastic young adult tissues using mice at 2 months old. Whole spleen and LN total RNA samples from 8 EμEBNA 1 positive line 26 and 8 wt siblings were analysed using slot blots as described in section 4.2. EμEBNA 1 tumour samples from line 26 were also included within the slot blots (as

Fig. 4.2 Graphical and statistical illustration of statistically significant deregulated genes in 1 month whole spleen and LN total RNA. A) *BclxL* expression levels in spleen, b) and c) *Rag1* and *Rag2* expression level in spleen (respectively) d) *Bad* expression level in LN and e) *Pim1* expression in total spleen.The histograms illustrate the mean RNA expression level in the spleen or LN with standard deviation (bars) for EμEBNA 1 transgenic positive line 26 (red) and wt siblings (blue) at 1 month old. These values are shown in box plot (below). WT siblings are denoted - and EμEBNA 1 positives depicted as + on the X axis.

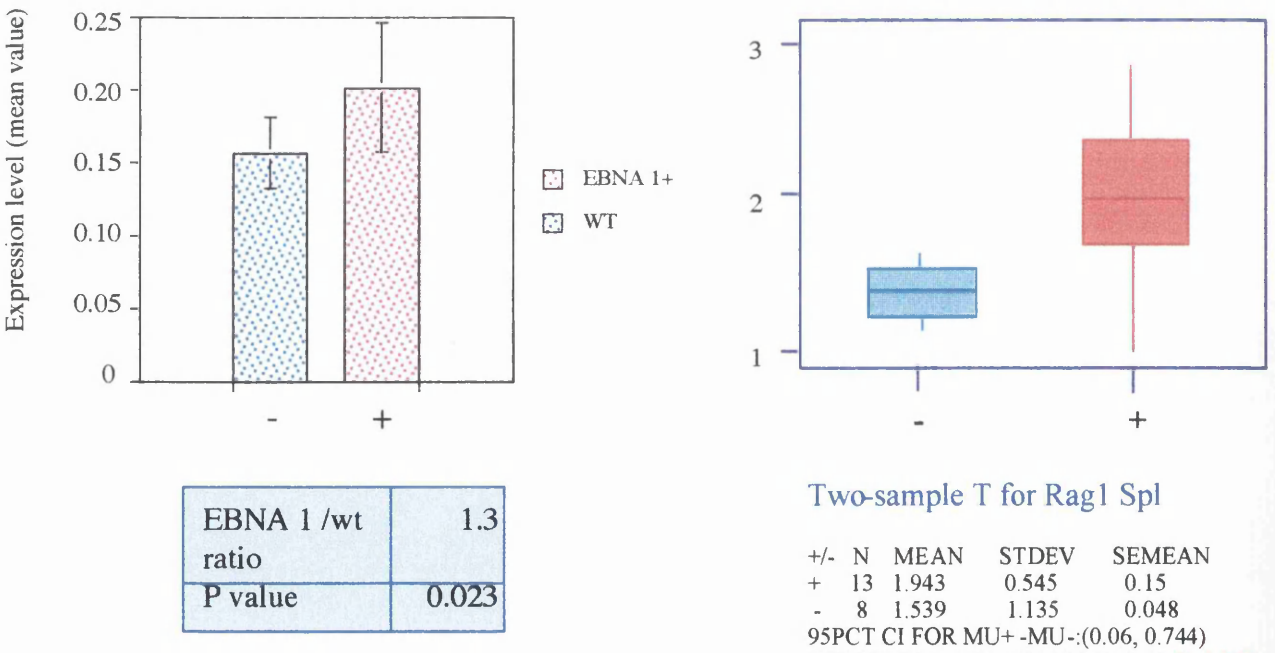


EBNA 1 /wt ratio	1.3
P value	0.011

Two-sample T for BclxL Spl

+/-	N	MEAN	STDEV	SEMEAN
+	13	0.1976	0.0347	0.010
-	8	0.1536	0.0332	0.012

95PCT CI FOR MU+ -MU-:(0.011, 0.077)
TTEST MU +=MU- (VS NE):T=2.83 P=0.011
DF=18 POOLED STDEV= 0.0341



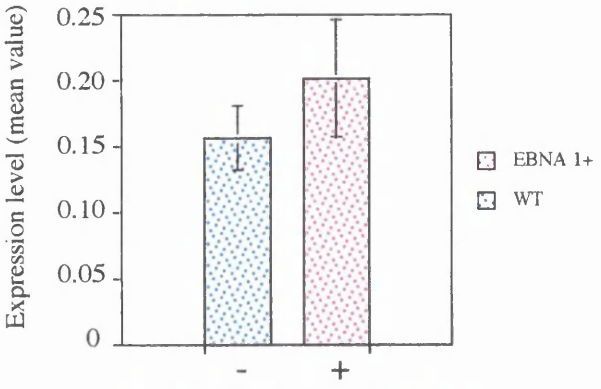
EBNA 1 /wt ratio	1.3
P value	0.023

Two-sample T for Rag1 Spl

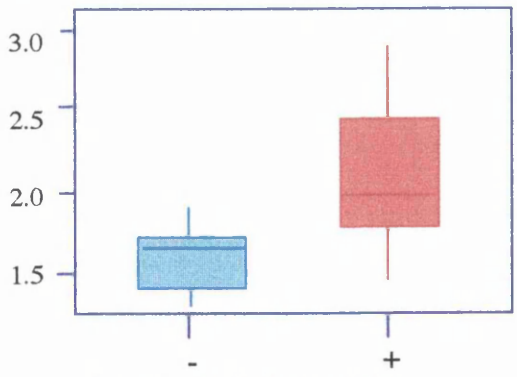
+/-	N	MEAN	STDEV	SEMEAN
+	13	1.943	0.545	0.15
-	8	1.539	1.135	0.048

95PCT CI FOR MU+ -MU-:(0.06, 0.744)
TTEST MU +=MU- (VS NE):T=2.83 P=0.023
DF=14 POOLED STDEV= 0.341

c) Rag2 expression level in total spleen



EBNA 1 /wt ratio	1.3
P value	0.018

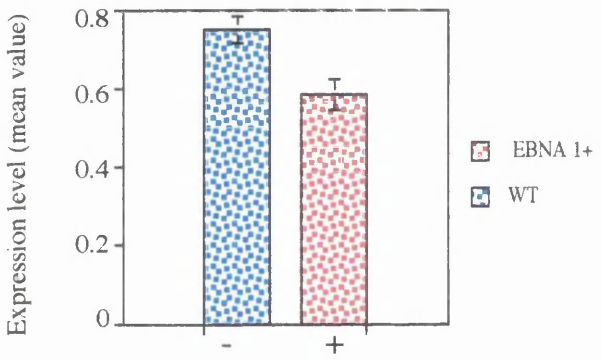


Two-sample T for Rag2 Spl

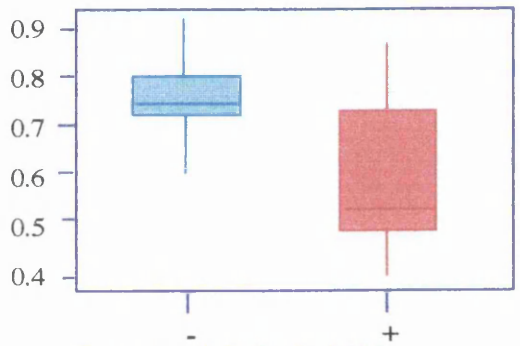
+/-	N	MEAN	STDEV	SEMEAN
+	13	2.022	0.447	0.12
-	8	1.574	0.248	0.088

95PCT CI FOR MU+ -MU-:(0.068, 0.810)
TTEST MU +=MU- (VS NE):T=2.58 P=0.018
DF=19 POOLED STDEV= 0.386

d) Bad expression level in total LN



EBNA 1 /wt ratio	0.8
P value	0.0082

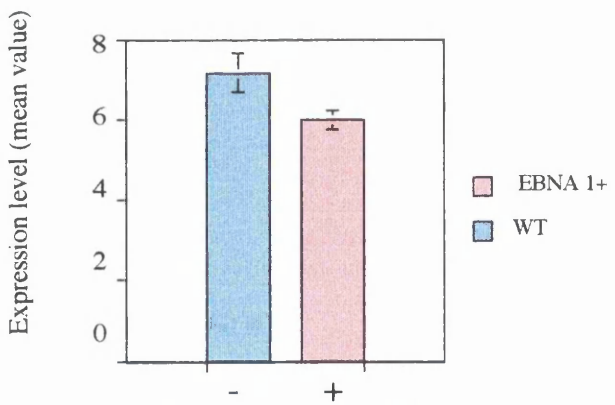


Two-sample T for Bad LN

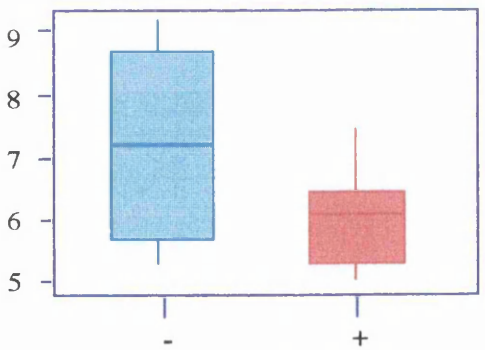
+/-	N	MEAN	STDEV	SEMEAN
+	13	0.588	0.136	0.038
-	8	0.7516	0.0963	0.034

95PCT CI FOR MU+ -MU-:(-0.279, -0.048)
TTEST MU +=MU- (VS NE):T=2.95 P=0.0082
DF=19 POOLED STDEV= 0.123

e) Pim1 expression level in total spleen



EBNA 1 /wt ratio	0.8
P value	0.023



Two-sample T for Pim1 Spl

+/-	N	MEAN	STDEV	SEMEAN
+	13	5.988	0.823	0.23
-	8	7.16	1.37	0.48

95PCT CI FOR MU+ -MU-:(-2.17, -0.18)
TTEST MU +=MU- (VS NE):T=-2.47 P=0.023
DF=19 POOLED STDEV= 1.06

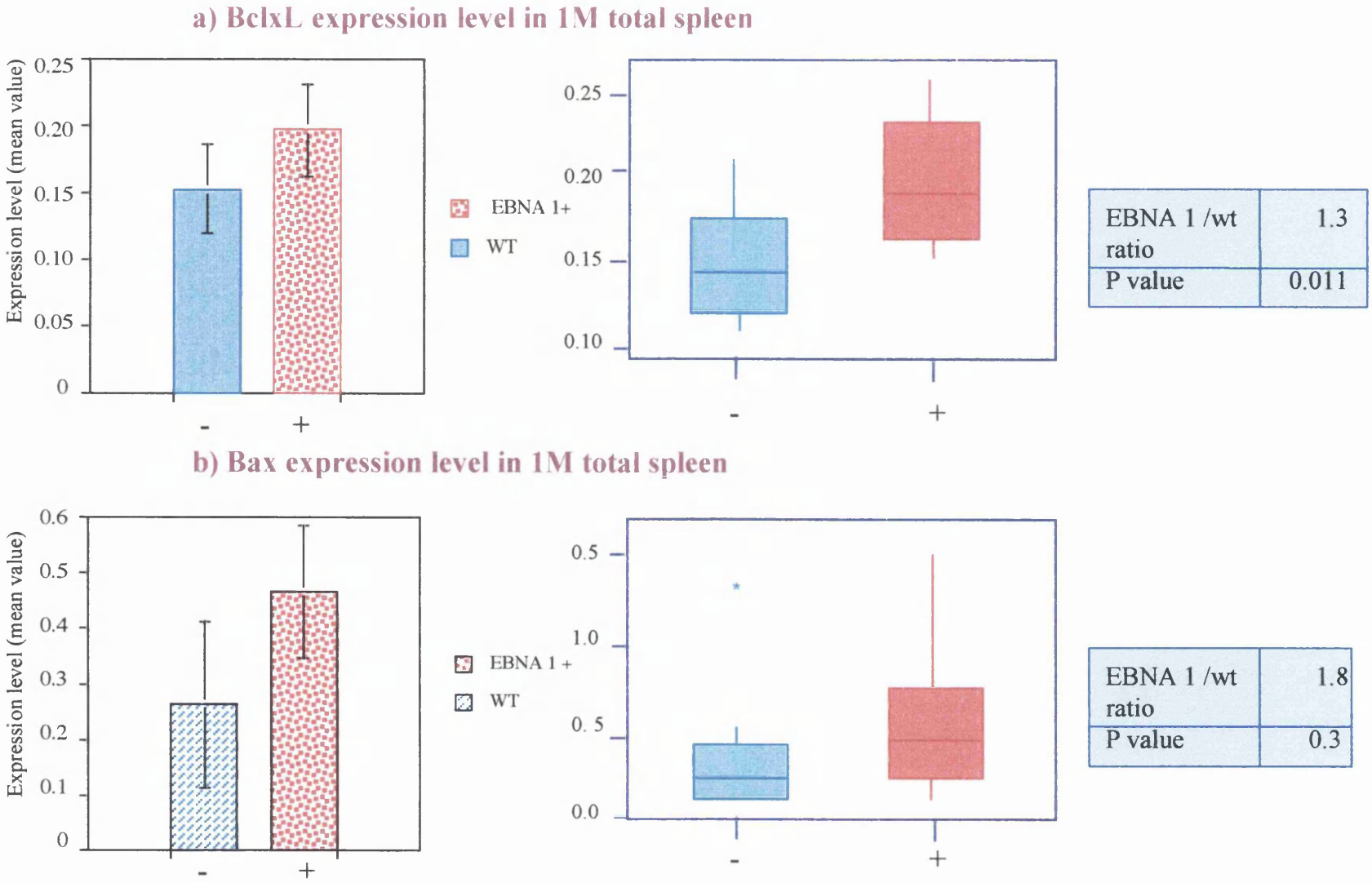


Fig. 4.3 Graphical and statistical illustration of *BclxL* (a) versus *Bax* (b) expression levels in 1 month whole spleen total RNA. The histograms show the mean of the expression level in the spleen cells with standard deviation (bars) for EμEBNA 1 transgenic positive line 26 (red) and wt siblings (blue) at 1 month old. These values are shown in box plot format on the right where wt siblings are denoted - and transgenic positive are depicted as + on the X axis. The ratio of means +/- and the P values of this ratio are given on the right.

described in section 4.2). Genes tested are listed in Table 4.2. The data obtained was analysed as described in section 4.2 and is summarised in Tables 4.2 and 4.3. Although some differences in the expression levels of some of the genes were observed, none reached statistical significance. This might be due to the spread of the values in each group of samples. This is apparent in the differences in the examples shown (Fig. 4.4 and 4.5) which both include an outlying value in the data set. The number of samples included in each group may also contribute to the outcome of the results. An outlyer may be present within a group of data, which highly influences the result when the sample size is small. For example, in the case of *Rag1* expression in whole spleen RNA an outlyer value in the wt samples gives a result that does not show a statistically significant difference between positive and wt samples (See Appendix 4.II and Fig. 4.4). When the outlyer is excluded the values are less spread and the result is statistically significant as shown in Fig. 4.4b. Similarly, in the case of *Mdm2* expression in the spleen an outlyer in the negative samples impacts the statistical analysis (Appendix 4.II). When the outlyer is excluded the result becomes significant as shown in Fig. 4.5a and b. The programme used for the statistical analysis considers as outlyer any value that is beyond 1.5-fold the inter-quartile range (the length of the box in the box plot). When a value is further away from the bulk of the values the programme does not exclude it but instead it is depicted as an asterisk. A question arises here: is it valid to exclude an outlyer, just because it changes the result? It may be appropriate to exclude it when there is a scientific explanation to support it. In the case of *Rag1* and *Mdm2*, since it is the same sample, it could be attributable to experimental error i.e. RNA sample extraction or poor hybridisation of this particular sample with *GAPDH.* Moreover, when one does this type of analysis, *in vivo,* variation in the samples is expected. This is because the analysis involves animals, which although grown under controlled conditions, they may differ as organisms especially in their immune reactions, behaviour (e.g. fighting) which impacts gene expression. However, because of the experimental approach e.g. animals grown in a controlled environment, same age, same protocols are used for RNA extraction and blot preparation and analysis, controllable variables are excluded.

4.4 Gene expression analysis in tumour samples

In order to investigate which genes are de-regulated in the EμEBNA 1 transgenic tumours; RNAs from several tumour samples were analysed as described in section 4.2. It would be anticipated that any changes in expression patterns detected would reflect mutational changes in tumour progression as well as the result of EBNA 1-directed de-regulation.

Total RNA samples were derived from whole spleen and LN tissues from 6

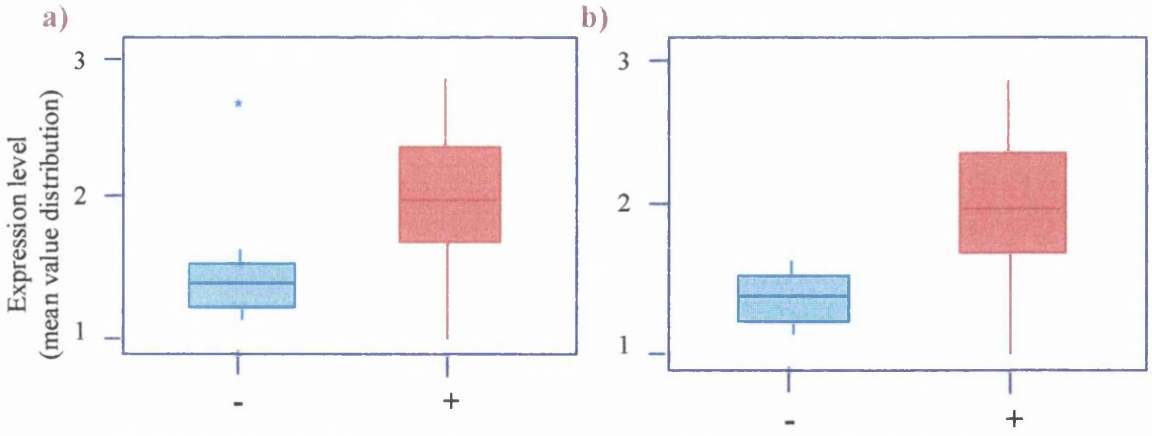
Table 4.2 Summary of comparative gene expression results in whole spleen and LN from line 26 2 month EμEBNA 1 transgenic mice versus wt

Gene	Spleen				LN			
	Mean tg+	Mean tg-	ratio +/-	P value *	Mean tg+	Mean tg-	ratio +/-	P value *
Bad	0.426	0.349	1.22	(0.62)	0.195	0.195	1.0	(0.99)
BclxL	0.1397	0.0943	1.45	(0.31)	0.336	0.755	0.44	(0.11)
gfi1	0.1222	0.1208	1.0	(0.97)	0.164	0.0733	2.3	(0.26)
cMyc	0.166	0.1272	1.3	(0.43)	0.477	0.323	1.5	(0.59)
Pim1	0.0477	0.0537	0.89	(0.72)	0.0489	0.116	0.42	(0.21)
cMyb	0.205	0.119	1.72	(0.073)	0.0982	0.236	0.4	(0.067)
MDM2	0.1660	0.117	1.4	(0.35)	0.191	0.194	1.0	(0.97)
	0.116	0.0759	2.2	(0.014)+				
GMCSF	1.087	0.751	1.45	(0.26)	0.628	0.72	0.873	(0.64)
MIP1α	0.1062	0.135	0.8	(0.49)	0.1157	0.19	0.6	(0.41)
Rag1	0.1353	0.0974	1.4	(0.2)	0.0835	0.0747	1.2	(0.52)
	0.1353	0.0802	1.7	(0.038)+				
Rag2	0.1014	0.1133	0.89	(0.63)	0.455	0.237	1.92	(0.3)

The ratio of EμEBNA 1 transgenic positive versus wt samples are given (ratio column). Statistical P value using the Two-sample T-test is given in parentheses for the data.

*P values of <0.05 are significantly different increased expression in EμEBNA 1+ mice
decreased expression in EμEBNA 1+ mice

+ values after outlyer has been excluded



Two-sample T for Rag1 Spl

+/-	N	MEAN	STDEV	SEMEAN
+	8	0.1353	0.0613	0.022
-	8	0.0974	0.0507	0.018

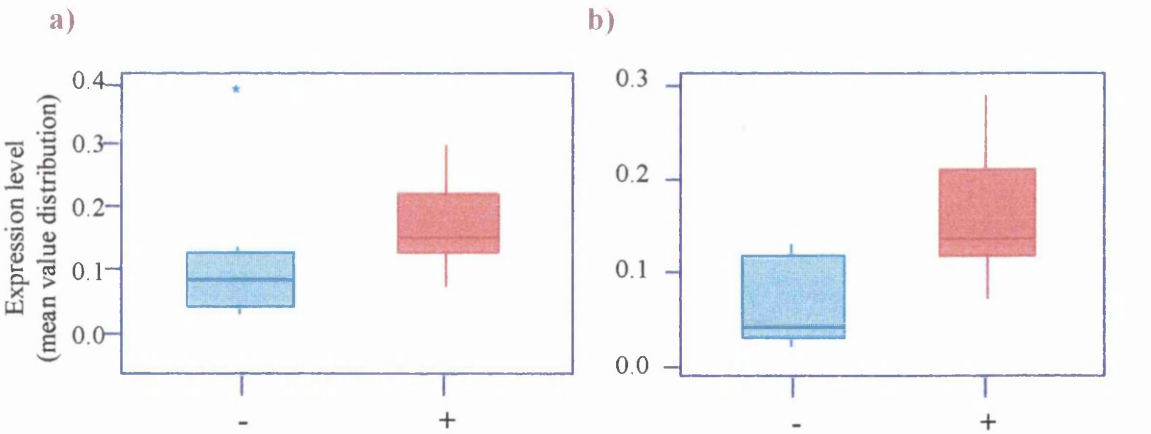
95PCT CI FOR MU+ -MU-:(-0.022, 0.098)
TTEST MU +=MU- (VS NE):T=1.35 P=0.20
DF=14 POOLED STDEV= 0.0562

Two-sample T for Rag1 Spl

+/-	N	MEAN	STDEV	SEMEAN
+	8	0.1353	0.0613	0.022
-	7	0.0802	0.0148	0.0056

95PCT CI FOR MU+ -MU-:(-0.022, 0.098)
TTEST MU +=MU- (VS NE):T=1.35 P=0.038
DF=14 POOLED STDEV= 0.0562

Fig. 4.4 Box plot representation, with numerical values bellow, of *Rag1* expression results in 2 month whole spleen total RNA from EμEBNA 1 positive (red) and wt (blue) mice. An outlyer in the group of the wt samples (represented by *) distorts the result and it renders it not statistically significant (a). When the outlyer is excluded the result becomes statistically significant (b).



Two-sample T for Mdm2 Spl

+/-	N	MEAN	STDEV	SEMEAN
+	8	0.1660	0.0728	0.026
-	8	0.117	0.123	0.044

95PCT CI FOR MU+ -MU-:(0.060, 0.158)
TTEST MU +=MU- (VS NE):T=0.97 P=0.35
DF=14 POOLED STDEV= 0.101

Two-sample T for Mdm2 Spl

+/-	N	MEAN	STDEV	SEMEAN
+	8	0.1660	0.0728	0.026
-	7	0.0759	0.0448	0.017

95PCT CI FOR MU+ -MU-:(0.021, 0.159)
TTEST MU +=MU- (VS NE):T=2.83 P=0.014
DF=13 POOLED STDEV= 0.0615

Fig. 4.5 Box plot representation, with numerical values bellow, of *Mdm2* expression results in 2 month whole spleen total RNA from EμEBNA 1 positive (red) and wt (blue) mice. An outlyer in the group of the wt samples (represented by *) distorts the result and it renders it not statistically significant (a). When the outlyer is excluded the result becomes statistically significant (b).

EμEBNA 1 mice with tumours in those tissues from line 26 and 5 EμLMP1 mice with tumours (line 39). The genes tested are listed in Table 4.3. A graph and a box plot of significantly different expression results are shown in Fig. 4.6. It is difficult to select an appropriate control to evaluate gene expression de-regulation in tumour samples. Whereas the tumour sample might be composed of 50-90% malignant cells, normal tissues include several cell types and cells at different stages of development. Thus, a gene expressed at a higher level, for example in normal T cells, would obscure this comparison. Moreover, the percentage of the tumour cells within the tissue varies between different tumours. As such, whole spleen, isolated splenocytes or selected B cells from wt control animals do not provide a cell type proportional control for tumour bearing tissue samples.

Initially, the line 26 tumour tissue samples were compared with 2 month old adult negative whole spleen or LN tissue. This data is summarised in Table 4.3. In the spleen tumours compared to wt young adult (2 month) whole tissues, a number of genes were found to be differentially expressed. Significantly higher expression levels were observed for *Bad* (3.2x), *BclxL* (4.7x), *cMyb* (4.65x), *Mdm2* (x4.2), *Mip1α* (2.7x) and *Pim1* (x1.95). However, the results obtained from LN tumours compared to normal young adult (2 month) whole tissue RNA, only *Gfi1* (9.45x) and *Rag1* (29x) showed considerable and statistically significant higher expression levels in these tumour samples.

Due to the cell content differences between the normal and the tumour tissues a comparison was also made with EμLMP1 transgenic tumours from line 39. In this case the data will reflect a compound of EBNA 1 and LMP 1 tumour progression characteristics, summarised in Table 4.4 and graphically depicted in Fig. 4.7. In the spleen EμEBNA 1 tumours from line 26 as compared to EμLMP1 tumours from line 39, *Gfi1* (1.4x) and *Rag2* (1.7x) were found statistically significantly higher in line 26 (EμEBNA 1). In contrast, *Bak* (0.85x), *Bax* (0.73x), *Bcl2* (x0.7) and *Mdm2* (x0.865) were found statistically significantly lower in line 26 in comparison to line 39 tumours. Quite different results were obtained with the LN EμEBNA 1 tumours (line 26) as compared to EμLMP1 tumours (line 39). In this case *Gfi1* (2.4x), *GMCSF* (2x), *Mdm2* (1.25x) and *MIP1a* (2x) were found higher in EμEBNA 1 versus EμLMP1 tumours whereas *Rag 1* (0.75x) was found lower in EμEBNA 1 tumours compared to EμLMP1 tumours.

4.5 Discussion

4.5.1 Gene expression in pre-neoplastic tissues

Using the transgenic mouse model for EBV associated BL an attempt was made to identify genes that either mediate EBNA 1 action or may co-operate with it in the onset of tumourigenesis. As a transcriptional regulator EBNA 1 may directly

Table 4.3 Summary of comparative gene expression results in spleen and LN tumours from line 26 EμEBNA 1 transgenic mice versus wt

Gene	Spleen				LN			
	Mean tg+	Mean tg-	ratio +/-	P value *	Mean tg+	Mean tg-	ratio +/-	P value *
Bad	1.116	0.349	3.2	(0.003)	0.195	0.195	1.0	(0.99)
BclxL	0.438	0.0973	4.6	(0.0009)	0.336	0.755	0.44	(0.11)
Amyb	0.259	0.183	1.4	(0.3)	0.164	0.0733	2.3	(0.26)
cMyb	0.553	0.119	4.65	(0.021)	0.0864	0.236	0.36	(0.12)
gfi1	0.0716	0.1208	0.6	(0.065)	0.693	0.0733	9.45	(0.0008)
cMyc	0.233	0.1272	1.83	(0.073)	0.1077	0.323	0.33	(0.088)
Pim1	0.1048	0.0537	1.95	(0.042)	0.0534	0.116	0.46	(0.34)
p16	0.0909	0.1331	0.7	(0.23)	0.207	0.1269	1.63	(0.38)
p53	0.349	0.313	1.1	(0.78)	0.513	0.379	1.35	(0.64)
MDM2	0.485	0.117	4.15	(0.0025)	0.171	0.194	0.88	(0.81)
GMCSF	1.055	0.751	1.4	(0.11)	0.443	0.72	0.61	(0.32)
MIP1α	0.364	0.135	2.69	(0.032)	0.171	0.19	0.9	(0.88)
Rag1	0.1589	0.0974	1.63	(0.099)	2.19	0.0747	29	(0.018)
Rag2	0.074	0.1133	0.86	(0.61)	0.284	0.237	0.83	(0.71)

The ratio of EμEBNA 1 transgenic positive versus wt samples are given (ratio column). Statistical P value using the Two-sample T-test is given in parentheses for the data.

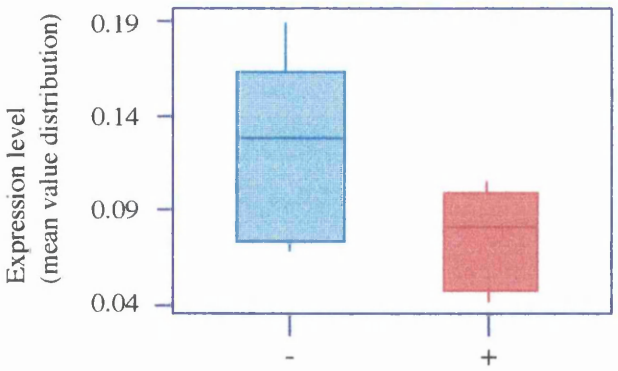
*P values of <0.05 are significantly different increased expression in EμEBNA 1+ mice
decreased expression in EμEBNA 1+ mice

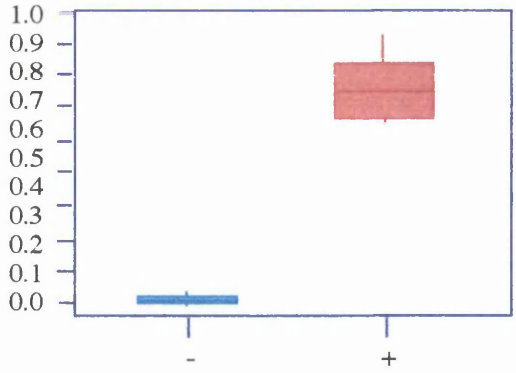
Table 4.4 Summary of comparative gene expression results in spleen and LN tumours from line 26 EμEBNA 1 transgenic mice versus line 39 EμLMP 1 mice

Gene	Spleen				LN			
	Mean 26	Mean 39	ratio 26/39	P value *	Mean 26	Mean 39	ratio 26/39	P value*
Bad	3.463	3.506	0.98	(0.84)	10.7	3.29	3.25	(0.32)
Bak	3.143	3.72	0.85	(0.045)	3.817	5.48	0.7	(0.054)
Bax	3.856	5.23	0.73	(0.027)	6.44	3.39	1.9	(0.22)
Bcl2	0.2497	0.3672	0.7	(0.037)	0.619	0.777	0.8	(0.073)
BclxL	4.54	2.74	1.65	(0.11)	5.66	2.744	2.0	(0.29)
Bmi1	0.418	0.4598	0.9	(0.16)	0.5355	0.526	1.0	(0.81)
gfi1	1.410	1.029	1.4	(0.0043)	2.059	0.866	2.4	(0.0005)
cMyc	4.44	1.37	3.24	(0.25)	0.7563	0.645	1.2	(0.17)
Pim1	3.7	1.47	2.5	(0.069)	2.129	1.85	1.15	(0.4)
CyclinD1	0.909	0.7758	1.2	(0.14)	0.895	0.928	0.96	(0.67)
MDM2	0.8772	1.013	0.865	(0.032)	0.9633	0.767	1.25	(0.0011)
GMCSF	3.431	3.211	1.0	(0.51)	6.14	3.116	2.0	(0.0014)
MIP1α	5.533	6.61	0.83	(0.18)	8.1	4.117	1.96	(0.029)
Rag1	1.408	1.872	0.75	(0.023)	2.88	2.063	1.4	(0.19)
Rag2	5.238	3.112	1.7	(0.0002)	10.07	6.92	1.45	(0.18)

The ratio of EμEBNA 1 transgenic positive versus wt samples are given (ratio column). Statistical P value using the Two-sample T-test is given in parentheses for the data.

*P values of <0.05 are significantly different increased expression in EμEBNA 1+ mice
decreased expression in EμEBNA 1+ mice





Two sample T for Gfi1 Spl Tum

+/-	N	MEAN	STDEV	SEMEAN
+	5	0.0716	0.0285	0.013
-	8	0.1208	0.0481	0.017

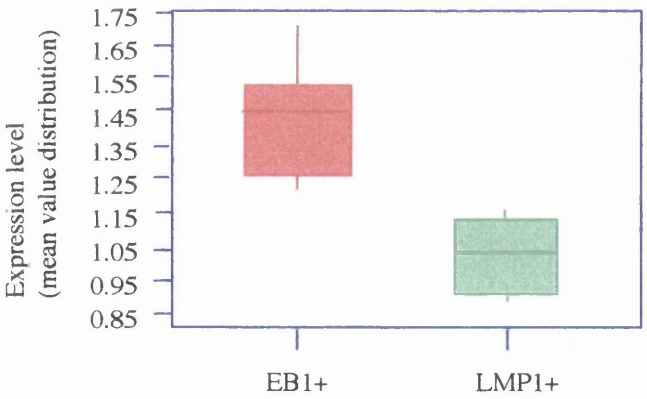
95PCT CI FOR MU+ -MU-:(-0.004, 0.102)
TTEST MU +=MU- (VS NE):T=2.05 P=0.065
DF=13 POOLED STDEV= 0.0615

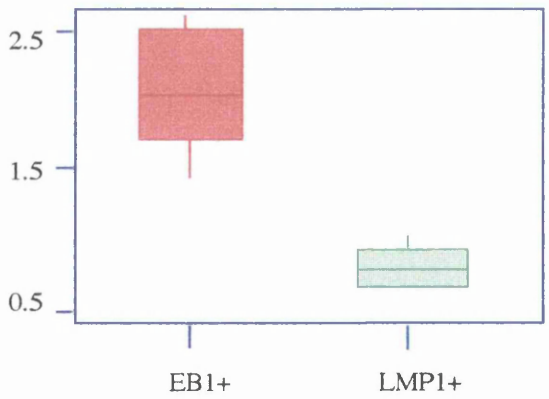
Two sample T for Gfi1 LN Tum

+/-	N	MEAN	STDEV	SEMEAN
+	5	0.693	0.150	0.067
-	8	0.0733	0.0191	0.067

95PCT CI FOR MU+ -MU-:(0.8068, -0.433)
TTEST MU +=MU- (VS NE):T=-9.22 P=0.0008
DF=4 POOLED STDEV= 0.0101

Fig. 4.6 Box plot representation of *Gfi1* expression results in line 26 spleen and LN tumours (red) as compared to wt whole spleen and LN total RNA (blue).





Two sample T for Gfi1 Spl Tum

+/-	N	MEAN	STDEV	SEMEAN
+	6	1.410	0.191	0.078
-	5	1.029	0.128	0.057

95PCT CI FOR MU+ -MU-:(0.154, 0.608)
TTEST MU +=MU- (VS NE):T=3.80 P=0.043
DF=9 POOLED STDEV= 0.166

Two sample T for Gfi1 LN Tum

+/-	N	MEAN	STDEV	SEMEAN
+	6	2.059	0.478	0.20
-	5	0.866	0.152	0.068

95PCT CI FOR MU+ -MU-:(0.68, 1.701)
TTEST MU +=MU- (VS NE):T=-5.31 P=0.0005
DF=9 POOLED STDEV= 0.371

Fig. 4.7 Box plot representation of *Gfi1* expression results in line 26 spleen and LN tumours (red) as compared to line 39 spleen and LN tumours (green).

effect the expression of cellular proto-oncogenes and tumour suppressor genes. Therefore, the expression levels of several key cellular genes that are often found de-regulated in lymphoid tumours were analysed.

The *Rag* genes are the only cellular genes suggested to be regulated by EBNA 1 (Kuhn-Hallek *et al.*, 1995; Srinivas and Sixbey, 1995). They were therefore included in this assay to investigate if this activity is present and measurable *in vivo* in murine cells and if so, to act as controls for this approach. Analysis of the level of *Rag1* and *2* expression in RNAs from EμEBNA 1 transgenic positive and wt control sibling mice revealed a consistent *Rag1* and/or *Rag2* up-regulation in EμEBNA 1 expressing cells at all developmental stages tested. At 1 month, the juvenile mouse tissues are still immature and undergoing developmental changes. As was mentioned earlier (section 4.1) the composition of lymphoid tissues reaches adult proportions at 8-10 weeks of age. Therefore, the *Rag* gene expression levels would be different according to the maturation stage of the cells. *Rag* expression is highly regulated during B lymphopoiesis. *Rag1* and *2* expression is primarily restricted to developing lymphocytes that are undergoing re-arrangement of their antigen receptor genes (Fig. 4.8). *Rag1* and *2* mRNA is first detected in pro-B cells but is down-regulated in early large B cells upon completion of IgH re-arrangements (Grawunder *et al.*, 1995a). The *Rag* genes are then transcribed again in small pre-B cells to allow light chain gene re-arrangements and later at Ig switching (Fig. 4.9). The fact that *Rags* were found up-regulated at the pre-tumour stage reflects that this *in vivo* approach is successful and useful in studying the mechanism of action of EBNA 1 as an oncogene.

In 1 month old whole spleen total RNA *BclxL* was found up-regulated in the EμEBNA 1 positive samples by 1.3 fold. Even though the ratio is low the result is significant, supported by the *Rag* control results which also showed a difference 1.3-fold. This result is consistent with observations from transgenic crossbreeding experiments between EμEBNA 1 and Eμ-*Bcl2* mice performed by M. Drotar and J. Coy in our laboratory. In this experiment *Bcl2* was found redundant with EBNA 1 in lymphomagenesis (as described in the introduction). Thus, EBNA 1 could act in a similar way to *Bcl2* or through a Bcl2 family member e.g. *BclxL* or *Bad*. However, this was not seen in the LN samples. Instead only *Bad* was found down-regulated by 0.8-fold. This difference may be due to the tissue composition e.g. spleen consists of more B cells and less T cells whereas LN have more T than B cells. Therefore, the effect of EBNA 1 on *BclxL* gene expression in B cells may be masked by the signal deriving from the T cells. The anti-apoptotic functions of *BclxL* are controlled in part by several interacting proteins such as *Bad* (an apoptosis promoter) which can heterodimerise with *BclxL* (Yang *et al.*, 1995). Therefore EBNA 1 could either directly or indirectly deregulate the expression of *Bcl2* or other family members.

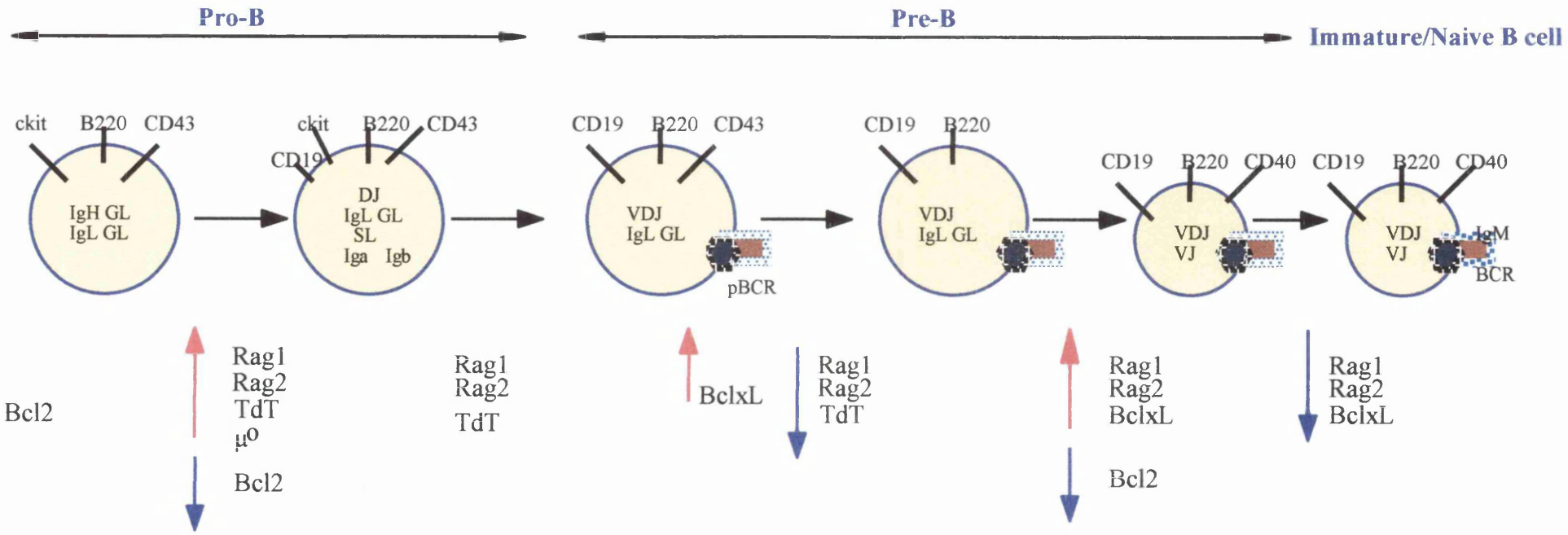


Fig. 4.8 Stages of primary B cell development. Characteristic B cell surface markers and expression patterns for genes are indicated. Large circles represent cycling cells, small circles represent non-dividing cells. Upward arrows represent induction of gene expression, downward arrows represent repression of gene expression. Genes without an arrow are expressed but their levels are not altered from the previous stage.
Abbreviations: B cell receptor (BCR). Germ line (GL), pre-B cell receptor (pBCR), surrogate light chain (SL), sterile μ transcript (μ^o). (Adapted from: Henderson A. and Calame K., 1998).

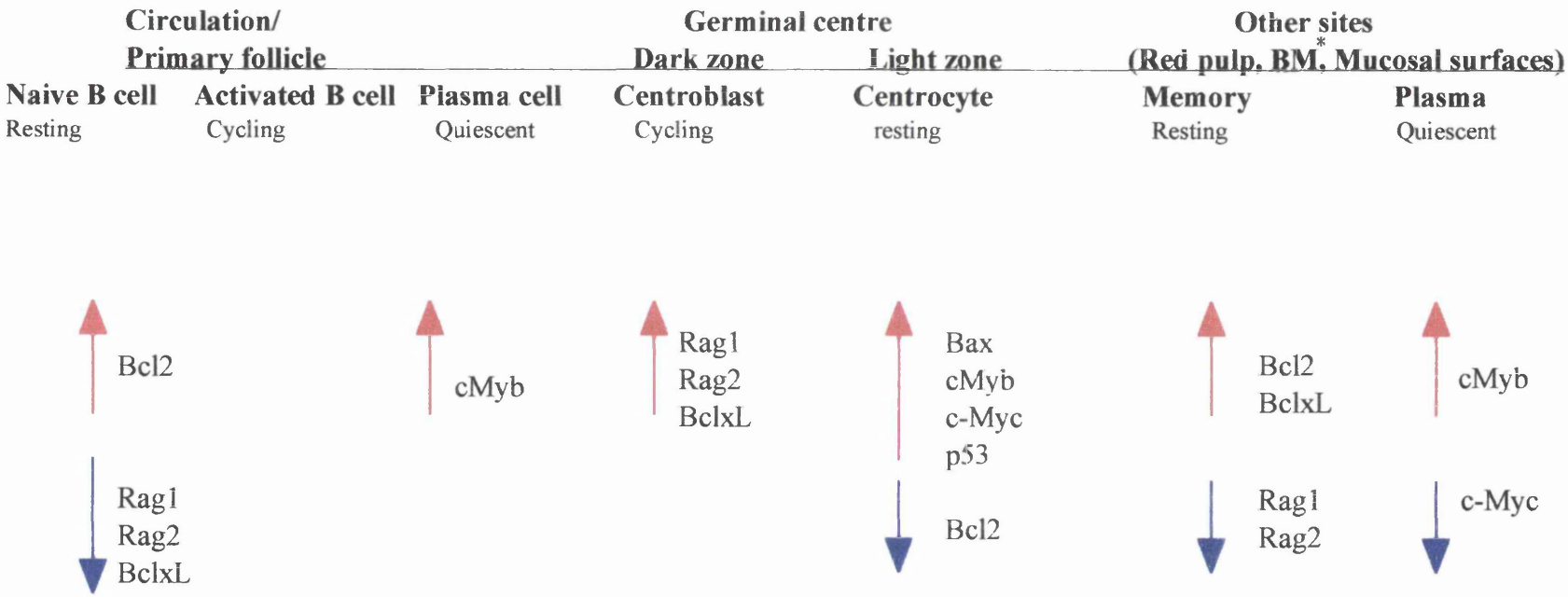


Fig. 4.9 Secondary changes (antigen dependent) of B cell development. Lines from top to bottom indicate the anatomical location, types of B cells, cell cycle status and expression patterns of genes known to increase (arrow up) or decrease (arrow down). (Adopted from: Henderson A. and Calame K., 1998).
Abbreviation: bone marrow (BM).

Nevertheless, these results are not apparent at the 2 month stage from whole spleen and LN, as none of the genes tested showed statistically significant differences. However, no changes were observed in *Rag 1* and *2* gene expression at this point.

This may be due to the different gene expression pattern in the spleen and LN from animals of different ages. In juvenile animals, which are still in the process of development, the immune system is not fully developed and therefore variation in gene expression would be expected. In contrast, the adult immune system is fully developed and unless there is an immunogenic factor that could trigger an immune reaction, gene expression is more stable.

The differences observed in the results obtained from the analysis of juvenile (1 month) and young adult (2 month) tissues may be attributable to age of the animal and or maturation stage of the cells as well as the differences between spleen and LN tissues. This assumption is consistent with recent observations in the *BclxL* expression patterns during mouse development. Expression analyses have shown that *BclxL* is the dominant *Bclx* gene mRNA transcribed in embryonic and postnatal tissues including primary lymphoid organs (Gonzalez-Garcia *et al.*, 1994; Fang *et al.*, 1994). *BclxL* is more abundant than *Bcl2* in adult tissues with the highest levels in adult brain, thymus and kidneys and to a lesser extent in spleen and LN. In the mouse embryo, *BclxL* mRNA is detected at day 8.5 through to 12.5 of development. *BclxL* expression at day 15.5 is more restricted to heart, brain, thymus and kidney with the highest level in the liver the major site of haematopoiesis in the mouse embryo. During B cell development *BclxL* is expressed predominantly in pre-B cells and provides a survival signal for the maintenance of pre-B cells and activated mature B cells. Transgenic mice over-expressing *BclxL* exhibit accumulation of immature and mature B cells implying that *BclxL* supports maturation of pre-B cells into B cells (Grillot *et al.*, 1996). However, in mature B cells *BclxL* expression is normally reduced. In addition *BclxL* protein has been detected at very low levels in pro-B cells, up-regulated in pre-B cells and down-regulated again in immature B cells (Fig. 4.8 and Fig. 4.9) (Grillot *et al.*, 1996). Interestingly *BclxL* and *Rags* are co-expressed during B cell development. Therefore, it can be postulated that EBNA 1 may act at a specific developmental stage, possibly resulting in de-regulation of these genes, indirectly.

c-Myc protooncogene expression levels were tested because *c-Myc* shows a co-operation with EBNA 1 in lymphomagenesis in transgenic cross-breeding experiments (introduction section 1.7). If EBNA 1 and *c-Myc* mutation/activation do co-operate in tumourigenesis, one would predict that EBNA 1 does not act through *c-Myc*. Consistent with this idea, no de-regulation of *c-Myc* by EBNA 1 was seen in the pre-tumour tissue.

Pim1 is a protooncogene that has been shown to co-operate with cMyc in lymphomagenesis in transgenic cross-breeding experiments (reviewed in Introduction section 1.4). In the RNA expression analysis in pre-tumour tissues *Pim1* was found to be statistically significantly lower in the 1 month EμEBNA 1 positive spleens compared to wt controls. *Pim1* is predominantly expressed in T cells. Therefore, since EBNA 1 is expressed in B cells, it could be that EBNA 1 might effect B cells which subsequently could effect T cells.

Since *BclxL* acts to inhibit apoptosis in a similar manner to *Bcl2* and is up-regulated in line 26, pre-tumour, EBNA 1 may act through *BclxL* (and/or *Bad*) to repress apoptosis. Therefore, EBNA 1 may act indirectly as an apoptosis regulator, factorial in oncogenesis. Moreover, EBNA 1 could act through de-regulation of *Pim1* resulting in up-regulation of the genes inhibited by it. EBNA 1 may de-regulate gene expression by binding to the promoter or other regulatory elements. However, a database search revealed no EBNA 1 binding sites in the promoters of any of the genes tested in this chapter. EBNA 1 was shown to bind the human cell protein P32/TAP which has been implicated in transcriptional regulation (see Introduction section 1.4). Thus, EBNA 1 could regulate gene expression by activating other transcription factors. However, a blast search needs to be undertaken to see if P32/TAP binding sites are present in the promoter of the gene tested in the current analysis.

4.5.2 Gene expression in tumours

The analysis of EBNA 1 effects on cellular gene expression was extended to the tumour stage where a number of genes were found up- or down-regulated in comparison to wt control samples. *Rag1* expression was consistently up-regulated in line 26 EμEBNA1 tumours by 29-fold (!) compared to wt controls and this change was statistically significant. In contrast, *Rag1* expression was significantly lower in EμEBNA 1 tumours (0.75x) when compared to EμLMP1 tumours which may suggest that *Rag1* is commonly up-regulated in human tumours, or also by LMP1. *Rag2* expression on the other hand did not show statistically significant changes in EμEBNA 1 positive tumours versus wt type controls. However, *Rag2* expression was statistically significant higher in the EμEBNA 1 positive tumours (1.7x) when compared to EμLMP1 positive tumours suggesting that LMP1 (in contrast to EBNA 1) down-regulates *Rag2* expression.

Adding together the results from the pre-neoplastic and tumour *Rag 1* and *2* gene expression assays, it is tempting to speculate that the oncogenic action of EBNA 1 might be exerted through enhanced *Rag* expression giving rise to genome instability and aberrant translocations. However, karyotypic analysis of EμEBNA 1 tumours showed that only approximately 10% of the tumours tested carried a

translocation (Wilson, JB personal communication). Translocations are not very common in mouse lymphomas compared to human lymphomas; instead mouse tumours more often display polysomy as was also seen in the EμEBNA 1 tumours.

Bcl2 family gene expression levels in the tumours were quite variable in comparison to the pre-neoplastic stage. In EBNA 1 positive spleen tumours *BclxL* and *Bad* expression were found to be significantly higher (4.6-fold and 3.2-fold respectively) in comparison to wt controls. In contrast, when compared to EμLMP1 tumours their expression, albeit higher, was not statistically significant. However, *Bak* (1.2x) *Bax* (1.4x) and *Bcl2* (1.43x) were found to be significantly down-regulated in line 26 tumours versus line 39 tumours which may suggest that LMP1 upregulates these genes. LMP1 has been shown to up-regulate Bcl2 (Henderson *et al.,* 1991). Therefore, both EBNA 1 and LMP1 may influence the balance between the Bcl2 family members by regulating their expression levels. The balance of the interaction of the *Bcl2* family members is very sensitive and thus the slightest effects from EBNA 1 or LMP1 could lead to prolonged cell survival and subsequently tumour formation.

Statistically significantly higher expression levels of *Gfi1* (9.45-fold) were observed in EμEBNA 1 LN tumours as compared to wt controls. However, this upregulation was not apparent in the spleen tumours. *Gfi1* was also found higher in the EμEBNA 1 spleen and LN tumour samples when compared to EμLMP1 positive tumours. Thus, *Gfi1* may be up-regulated in EBNA 1 tumours or comparatively down-regulated in the LMP1 tumours. However, as this de-regulation is not detected at the pre-tumour stage it might be a likely secondary mutation. *Gfi1* encodes a nuclear zinc-finger protein that carries a repressor domain, SNAG and functions as a position- and orientation-independent transcriptional repressor. Interestingly, it was recently demonstrated that Gfi1 down-regulates the expression of *Bax* and *Bak* (apoptosis promoters, *Bcl2* family) in established cell lines and transgenic *Gfi1*-positive primary cells (Grimes *et al.,* 1996b). Therefore, *Gfi1* may inhibit apoptosis by means of its repression of multiple pro-apoptotic regulators. The antiapoptotic properties of *Gfi1* may help to explain its strong collaboration with *c-Myc* during oncogenesis (Introduction section 1.4). Thus, if EBNA 1 up-regulates *Gfi1* this could lead to down-regulation of Bax or Bak in the EμENA 1tumours. This assumption is consistent with the gene expression results in the line 26 tumours described in section 4.4.

c-Myc is found to be de-regulated in BL tumours (discussed in Introduction sections 1.2 and 1.4). Preliminary data from our lab from transgenic cross-breeding experiments between EμEBNA 1 and Eμ-*c-Myc* mice is suggestive of a co-operative action between EBNA 1 and *c-Myc* de-regulation in tumourigenesis. Moreover, karyotypic analysis has revealed that most of the tumours arising in these mice have

trisomy of chromosome 15, the chromosome bearing *c-Myc*. If EBNA 1 and *c-Myc* activation/mutation co-operate in tumourigenesis, one would predict that EBNA 1 does not act through *c-Myc*. Consistent with this idea, no de-regulation of *c-Myc* by EBNA 1 was seen in the pre-tumour tissue and in tumours. This might be due to the wide variation in the level of *c-Myc* expression in transgenic and non-transgenic tissue (see Fig. 4.10 and Appendix 4.IV) regardless of tumour status which agrees with previous published work (van Lohuizen *et al.*, 1989). In addition, the variation in these samples could be due to the normal proliferative state of the spleen and LN. Moreover, it is possible that *c-Myc* may be mutated in some tumours and not in others.

Pim1 is another proto-oncogene that has been shown to co-operate with *cMyc* in transgenic cross-breeding experiments (reviewed in Introduction section 1.4). Although *Pim1* was found to be down-regulated in the 1 month pre-neoplastic spleen samples in the spleen tumours *Pim1* was found to be up-regulated. One possibility for this contrasting result could be that EBNA 1 may down-regulate *Pim1*, while, a secondary event in the tumours may overcome the EBNA 1 primary effect and upregulates *Pim1* expression. *Pim1* expression is highly induced by IL2, IL3 and GMCSF (Lilly *et al.*, 1992; Sato *et al.*, 1993; Buckley *et al.*, 1995; Rohwer *et al.*, 1996). *GMCSF* was found significantly higher in transgenic positive spleen compared to wt controls. Therefore up-regulation of *GMCSF* in these tumours could lead to up-regulation of *Pim1*. Another factor contributing to the *Pim1* expression results could be the differences in the cell composition between the normal and tumour tissues.

Mdm2 expression was found to be significantly higher in EμEBNA 1 spleen tumours (x4.2) compared to wt controls (but not in the LN samples). *Mdm2* was also found to be up-regulated (when outlyer is excluded) in the 2 month old RNAs. EBNA 1 could either directly or indirectly increase *Mdm2* expression. Karyotypic analysis in our lab of EμEBNA 1 tumours showed a high percentage (56%) of trisomy for chromosome 10. A potentially important locus on this mouse chromosome is *Mdm2* which negatively regulates p53. Mutated p53 has been found in some BL tumours (Farrell *et al.*, 1991). Therefore, it can be postulated that upregulation of *Mdm2* would lead to reduction of p53 function. Nevertheless, although *p53* gene expression analysis in EμEBNA 1 tumours showed no statistically significant changes of *p53* RNA expression levels, this does not reveal what is happening at the protein level.

The RNA source is important in the experiments described in this chapter. The EμEBNA 1 transgene expression has been directed to the B cell compartment. Thus, the effects of EBNA 1 regulation on the expression of cellular genes will be more obvious and the signals obtained more specific when the RNA samples are

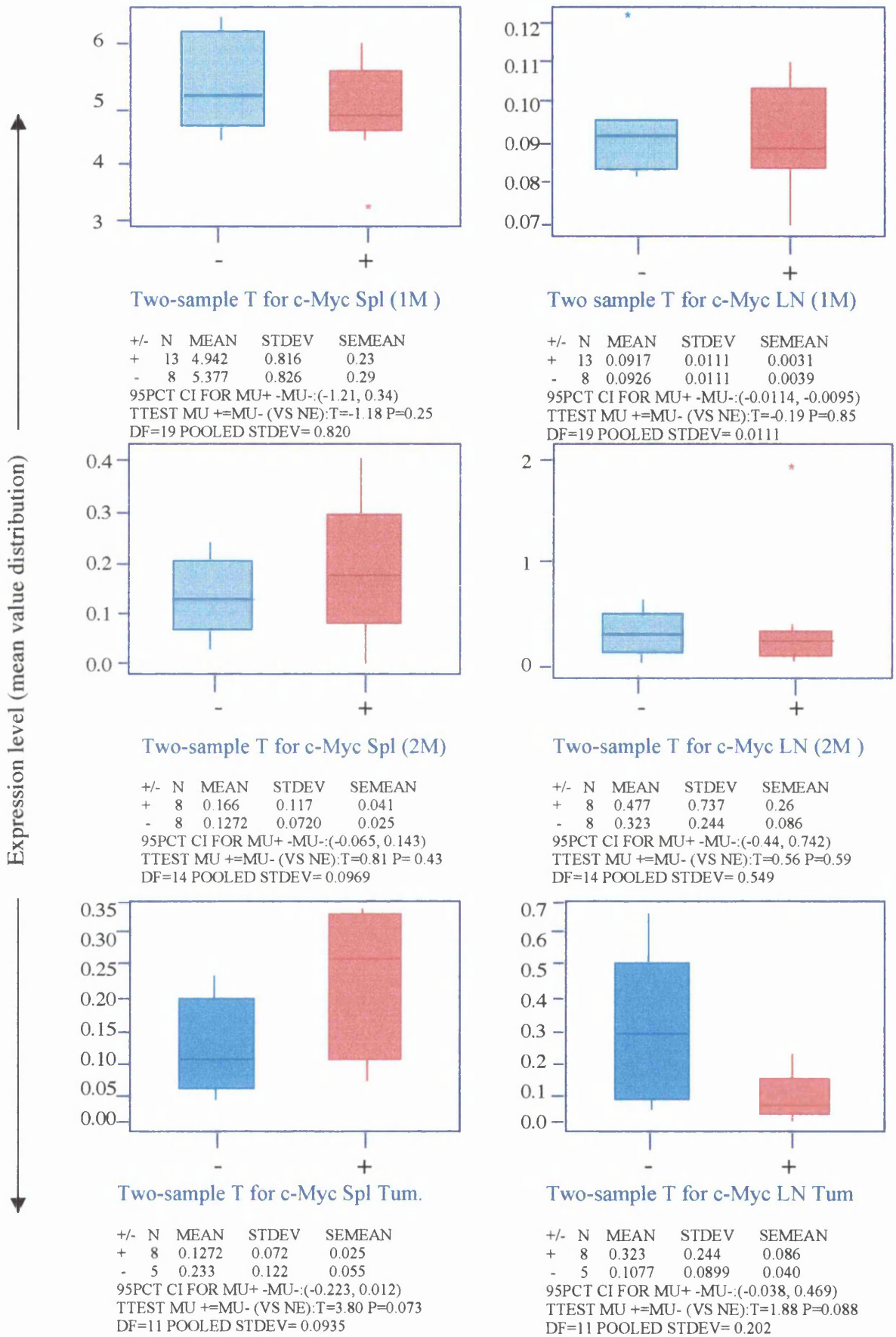


Fig. 4.10 *c-Myc* expression levels in 1 month and 2 month EμEBNA 1 transgenic positive (red) versus wt (blue) spleen and LN and in EμEBNA 1 positive spleen and LN tumours versus 2 month wt tissues.

extracted from selected B cells in comparison to total spleen or LN. For example, in the analysis of the cellular gene expression in juvenile (1 month) mice the RNA samples were extracted from total spleen and LN from line 26. Because the spleen is quite a heterogenous tissue consisting of many different cell types, the signal deriving from a gene that normally has higher expression in the T cells will mask the signal originating from the B cells. Therefore, the final observation may not reflect the actual effect of EBNA 1 action. However, the differences observed in the expression levels of *BclxL*, *Bad*, *Pim1*, *Rag1* and *2* and *Mdm2* may be due to EBNA 1 de-regulation since they were found de-regulated in both pre-tumour and in tumour tissue.

The RNA samples used in the gene expression analysis in tumour samples derive from monoclonal B cell tumours from line 26. Such tumours consist predominantly of B cells and therefore the signal obtained from the cellular gene expression is more specific. However, as these tumours are monoclonal (inferring further cellular transforming events), the differences observed in tumour tissues may be partly, but not necessarily due to EBNA 1. The changes in the expression levels of *Gfi1, MIP1α* and *GMCSF* may be secondary events in tumourigenesis as no significant differences were found in the pre-tumour samples. The high variation in gene expression in some tumour samples may be due to the fact that secondary mutations differ between tumours and therefore one may expect some higher values and some normal.

This kind of gene expression analysis, only reveals changes common to most or all tumours. Thus, comparing groups of data and doing statistics only give significance in the changes without specifying if the gene is de-regulated by EBNA 1 or is a common secondary mutation. Therefore a more sensitive assay for *c-Myc* expression would be more useful. For example, in order to detect any *c-Myc* de-regulation it would be more appropriate to compare *c-Myc* expression in cells (EBNA 1 positive and wt cells) which are synchronised with respect to their cell cycle stage. In normal cells *c-Myc* expression is highly regulated (Marcu *et al.*, 1992). Therefore, an assay which would assess cellular gene promoter usage in EBNA 1 induced tumours may be useful in detecting gene de-regulation.

Another factor, possibly contributing to the gene expression results in the tumours would be the controls that were used for the comparison. The problem is that we do not know what the percentage of each cell type is in the tumours as well as what the percentage of tumourous cells is. Therefore, the best solution to this problem would be to perform B cell selection from both tumour and normal tissues and then do a direct comparison of cell gene expression between the two sets of samples. Cell type purification and RNA extraction from pre-neoplastic tissues is described in Chapter 5.

A combination of the *BclxL* expression pattern throughout murine life and the findings that *BclxL* and *Rags* are up-regulated in EBNA 1 pre-neoplastic and tumour tissues compared to wt would suggest that EBNA 1 may effect B cell survival and delay B cells in certain stages. Alternatively, EBNA 1 could simply induce aberrant expression of genes that are not normally co-expressed.

Chapter 5

Effects of EBNA 1 on cellular gene expression II: Differential expression

5.1 Introduction

An alternative approach to investigate the effects of EBNA 1 on cellular gene expression was used next, employing the "mouse Atlas expression array", a comparative cDNA-based technique useful for profiling differential gene expression. This method can provide quantitative measurements of the expression levels of a large number of genes (588 genes in the array) in different samples. As such, levels may be compared between different samples, such as normal versus pathological tissues. Each Atlas array membrane contains 6 groups of genes encoding: a) oncoproteins, tumour suppressors and cell cycle regulators; b) stress response proteins, ion channel and transport proteins, intracellular signal transduction modulators and effectors; c) apoptosis-related proteins, DNA synthesis, repair and recombination proteins; d) transcription factors and general DNA binding proteins; e) receptors: growth factor and chemokine, interleukin and interferon, hormone and neurotransmitter, cell-surface antigens and cell adhesion proteins; f) growth factors, cytokines and chemokines, interleukins and interferons, cytoskeleton and motility proteins, protein turnover. The membranes also include some house-keeping genes and negative controls which serve as quantitative and qualitative controls. All genes included are listed in Appendix 5.I.

The assay as described here is more specific than those described in Chapter 4 since the RNA used was derived from selected B cells from 2 month old mice instead of using whole spleen or splenocyte samples. However, as an approach in general, it should be used only as an indicator of which genes should be analysed further and not as evidence of gene de-regulation. This is because the data can only show the ratio of difference between two compared sets, without the possibility of statistical analyses. Therefore, to test if any observed differential expression is real, by the criteria of statistical significance, the results obtained from the array must be confirmed. This was done here by returning to slot blot analyses.

The analysis was extended to the protein level for some of the genes that showed (or were close) to statistically significant differential expression levels.

5.2 Gene expression analysis in selected B cells from young adult mouse spleens: Gene expression array

Splenocytes were isolated separately from 4 transgenic positive and 4 wt (sibling controls) 2 month old mice from the EμEBNA 1 line 26. B cells were selected by a positive selection protocol employing the B220 (CD45R) antibody attached to magnetic beads. Total RNA was isolated from each of the 8 samples. 30μg of RNA from each of the EμEBNA 1 positives were pooled and similarly for the wt, so that parameters measured for each sample represent a mean of 4 positives and 4 wt. The positive and the wt RNA pools were then polyA+ selected. 1 μg of polyA$^+$RNA from each positive and wt RNA pool was used to make a cDNA probe for hybridisation to the mouse Atlas cDNA array membranes (see Methods section 2.4.5). Hybridisation signal intensity depends on the abundance in each RNA sample of transcripts from each of the 588 genes displayed on the macroarray.

The images of the 2 mouse Atlas membranes, one hybridised with the EμEBNA 1 positive 2 month RNA and the other hybridised with the 2 month wt RNA, were scanned by phosphoimager and are shown in Fig. 5.1. The intensity of the images differed due to the small difference in the activity of the radioactive probes (the EμEBNA 1 positive cDNA was better labelled than the wt cDNA). Thus the membranes were normalised by adding the phospho-image values of the house-keeping genes (included on each array) on each blot and calculating the ratio of the positive blot over the wt blot house-keeping gene average value (correction factor = EμEBNA 1+ cDNA/ wt cDNA of house-keeping gene mean value). The obtained correction factor was 2.4. The values from the wt test samples were normalised by multiplying them by the correction factor. The differences between transgenic positive and wt values were calculated by the ratio of the positive values compared to the corrected wt values (Ratio = EμEBNA 1+ cDNA value /wt corrected cDNA value).

The genes that showed the more obvious differential expression level are indicated by a number to the left or above the dots that represent each gene. Those genes showing differential levels of expression between EμEBNA 1 positive and wt

1 cJun
2 JunB
3 JunD
4 cFos
5 FosB
6 Fra2
7 EBF
8 Ikaros
9 Pax5
10 Egr1
11 cRel
12 POU
13 Nras
14 Araf
15 Elf1
16 INF inducible prot 1
17 Prothymosin α
18 SATB1
19 Grp78
20 Ets2
21 BST 1
22 HCK
23 HR21spA
24 IκB
25 INFγR2b chain
26 MAPKK1
27 PML
28 JAK3
29 STAT3

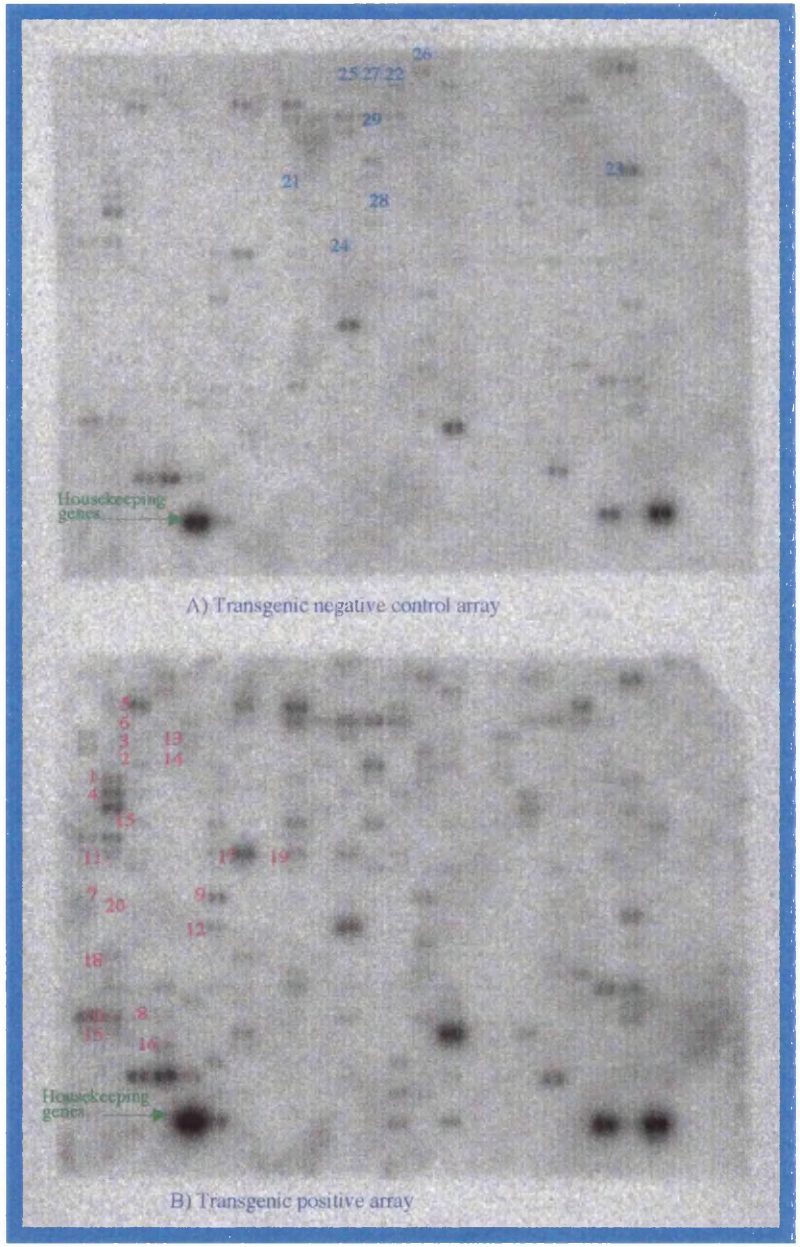


Fig. 5.1. Selected B cell mouse Atlas cDNA expression array results. Blot A was hybridised with wt control cDNA probe and blot B was hybridised with the EμEBNA 1 transgenic positive cDNA probe. The house-keeping genes used to calculate the correction factor are indicated by green arrow.
The genes showing a higher level in the transgenic positive B cells are indicated by magenta coloured numbers and those with the lower level are indicated by blue numbers to the left of each gene duplicate dot . For numbers 21, 24, 25, 26 and 29 the numbers are above the duplicate dots. The genes corresponding to each number are listed.

RNAs are presented in Table 5.1. The genes were selected according to their ratio difference, usually >2 fold, with a couple of exceptions because of their function. The transgenic positive and wt values before and after normalisation are also given. Those genes potentially up-regulated by EBNA 1, that is showing a higher level in the positive sample, include a number of transcription factors, several of which are proto-oncogenes, in contrast to those with a lower level which include a tumour suppressor gene, a lymphocyte differentiation antigen, a transcription factor inhibitor and an interferon pathway gene.

To support the above results the experiment was repeated using splenocyte RNA. Initially, total RNA was extracted from splenocytes of 11 EμEBNA 1 positive and 6 wt 2 month old mice from line 26. The total RNA samples were pooled and polyA+ mRNA was then extracted and radiolabelled cDNAs were then made. However, the large difference in the activity of the radiolabelled probes (as well as some areas of high background) made the data between positive and wt samples difficult to interpret. A similar problem was experienced when the experiment was repeated again using polyA+ mRNA from selected B cells of 1 month old mice from line 26, at which point the membranes were no longer usable.

5.3 Testing the candidate de-regulated genes

5.3.1 Slot blot analysis using splenocyte RNA

Having found that several cellular genes appear to be differentially expressed at the RNA level in the splenic B cells between EμEBNA 1 transgenic mice and wt controls, this observation must be rigorously tested. It is possible to obtain a large quantity of RNA from a mouse spleen, less from isolated splenocytes and a poor yield from selected B cells. The purity of B cells is in inverse proportion with the RNA yield. As a functional compromise, slot blots were prepared from total splenocyte RNA. The differentially expressed genes were quantified by a slot blot approach taking RNA samples from 5 positive and 5 negative (wt) mice for EμEBNA 1 line 26 (2 month old) and 3 positive and 3 negative (wt) mice for EμEBNA 1 line 59 (5-6 months old). 5 spleen tumours from line 26 transgenic positive animals were also assayed (mouse numbers are listed in Appendix 5.II).

Four duplicate slot blots were prepared simultaneously as described in Chapter 4. 5μg of total RNA was used per slot and the blots were hybridised with

Table 5.1 Summary of comparative cDNA expression Atlas array results in selected B cells from line 26 2 month old transgenic mice versus wt controls.

Gene	Higher expression tg+	tg-	tg- (x2.4)	ratio +/-	Gene	Lower expression tg+	tg-	tg- (x2.4)	ratio -/+
cJun AP1 component	1600	208	499.2	3.2	**BST1** lymphocyte diff. Ag CD38	80	170	408	5.1
junB //	162	23	55.3	2.93	**HCK** Y kinase	190	394	945	5.0
JunD //	230	34	81.6	2.8	**HR21spA** DNA repair protein	357	845	2028	5.7
cFos //	873	159	381.6	2.3	**IκB** NFkB inhibitor	121	164	393.6	3.3
FosB //	1101	268	643.2	1.7	**INFγR2b chain** (AF1)	130	315	756	5.8
Fra2 //	211	35	84	2.5	**MAPKK1**	519	437	1048.8	2.0
					PML tumour suppressor	136	324	777.6	5.7
EBF early B cell factor	108	17	40.8	2.65	**Jak3** Janus kinase 3	133	112	268.8	2.1
Ikaros DNA binding protein	250	37	88.8	2.8	**STAT3**	179	195	468	3.0
Pax5 B cell specific TF	594	168	403	1.5					
Egr1 Zn finger protein TF	976	176	422.4	2.3					
cRel NFκB complex TF	476	100	240	2.0					
Ets2 transcription factor	137	26	62.4	2.2					
POU domain assoc.factor	340	75	180	1.9					
Nras protooncogene	248	47	112.8	2.2					
Araf protooncogene	155	31	74.4	2.1					
Elf1 Y kinase ligand	660	147	352.8	1.9					
INF inducible protein 1	587	123	295.2	2.0					
Prothymosin α	1538	305	732	2.1					
SATB1 DNA binding nuclear matrix protein	325	33	79.2	4.1					
Grp78 glucose regulated prot78	392	76	182.4	2.1					

^{32}P radio-labelled probes generated by the random primed method from mouse-derived sequence plasmid cDNAs. The blots were hybridised with all obtainable gene sequences, from the list in Table 5.1. In addition, *BclxL* sequence was included following the results obtained in Chapter 4. The slot blot signals were quantified by phospho-image analysis. The values were normalised by probing the blots with *GAPDH* and subsequent calculation of the ratio of the test signal over the *GAPDH* signal value.

In determining whether the observed differences in the RNA expression levels of these genes were significant, statistical analysis was performed as described in Chapter 4. The results are summarised in Table 5.2 and representative graphs are shown in Fig. 5.2. In the 2 month splenocyte RNA samples from line 26 *Egr1* (2.5x) and *BclxL* (2.68x) showed statistically significant higher expression levels in the positive samples as compared to the wt control samples. Several of the other genes tested gave a ratio of means consistent with the Atlas results, showing 2-fold or greater elevation, but these did not reach statistical significance due to the high degree of variation between samples (see Appendix 5.II). Given that only 5 of each were tested, significance might be demonstrated if more samples were analysed.

In the tumours from line 26 as compared to wt 2 month splenocyte samples statistically significant higher expression was observed for *cJun* (2.45x), *cFos* (2.5x), *EBF* (3.25x), *Ikaros* (1.55x), *Egr1* (1.524x), *Nras* (2.62x), *BclxL* (2.0x), and *Pic1* (3.3x). A statistically significant lower expression level was found for *B2* (0.456x).

In the line 59 (slow tumour line) RNA sample slot blot analysis (EBNA 1 positive compared to wt samples) *cJun* (1.2x), *EBF* (1.4x) and *Araf* (2.56x) showed statistically significant higher expression levels. However, the small number of samples (only 3 samples) in each group compared reflect the outcome of these results as other genes such as *Ikaros* (1.3x), *BclxL* (2.12x) and *B2* (0.83x) were close to significance (see Table 5.2 and Appendix 5.II). Thus, more line 59 samples need to be tested in order to determine the relevance of changes in gene expression of these genes in the assay for line 59.

5.3.2 Slot blot analysis using selected B cell RNA

Further slot blot analyses were conducted, this time using total RNA extracted from selected B cells. Despite the low yield of selected B cell RNA it was

Table 5.2 Summary of comparative gene expression results in splenocytes from line 26 2 month old and line 59 5 month old transgenic mice and line 26 tumours versus wt controls.

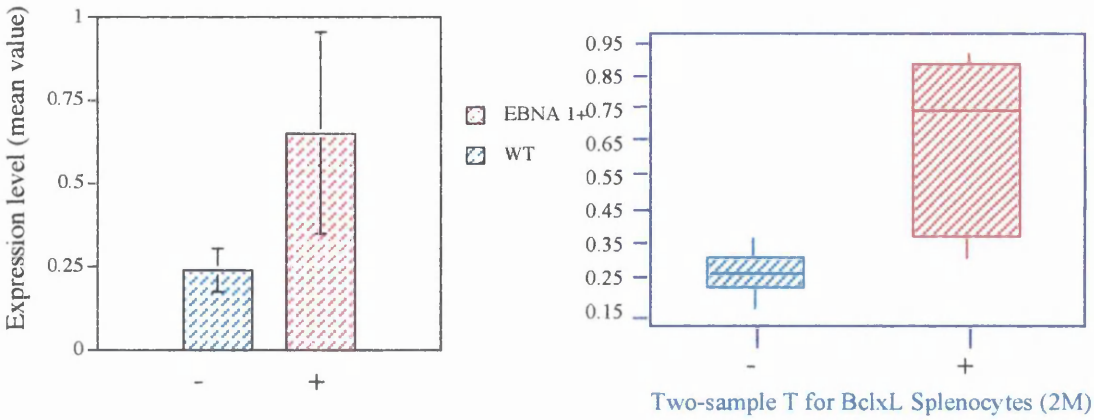
Gene	Line 26 2M vs 2M wt				Line 26 tumour vs 2M wt				Line 59 5M vs 5M wt			
	Mean tg+	Mean tg-	ratio +/-	P value *	Mean tg+	Mean tg-	ratio +/-	P value *	Mean tg+	Mean tg-	ratio +/-	P value *
cJun	0.88	0.3637	2.41	(0.097)	0.892	0.3637	2.45	(0.0019)	1.84	1.33	1.2	(0.019)
JunB	0.527	0.205	2.6	(0.19)	0.0382	0.205	0.186	(0.31)	0.639	0.373	0.43	(0.14)
JunD	0.337	0.174	2.0	(0.28)	0.1646	0.1744	1.0	(0.84)	0.509	0.43	0.2	(0.76)
cFos	0.254	0.128	2.0	(0.14)	0.316	0.1284	2.5	(0.003)	0.708	0.547	1.3	(0.66)
EBF	2.6	0.702	3.7	(0.14)	2.284	0.702	3.25	(0.0031)	4.279	3.05	1.4	(0.039)
Ikaros	0.917	0.517	1.77	(0.15)	0.801	0.517	1.55	(0.016)	1.996	1.556	1.3	(0.066)
Pax5	1.47	0.742	2.0	(0.18)	0.872	0.742	1.17	(0.34)	3.21	2.501	1.3	(0.45)
Egr1	0.873	0.353	2.5	(0.046)	0.538	0.3534	1.524	(0.042)	2.156	1.566	1.4	(0.26)
Nras	0.0577	0.0563	1.023	(0.93)	0.148	0.05638	2.62	(0.0003)	0.1947	0.2091	1.0	(0.54)
Araf	1.52	0.552	2.75	(0.16)	0.795	0.552	1.45	(0.087)	3.121	1.218	2.56	(0.02)
SATB1	1.81	0.917	2.0	(0.27)	0.844	0.917	0.9	(0.6)	1.87	1.663	1.125	(0.31)
HCK	2.27	1.78	1.3	(0.6)	1.411	1.787	0.78	(0.14)	3.354	2.484	9.35	(0.13)
PML	1.158	0.834	1.4	(0.38)	1.261	0.834	1.5	(0.084)	2.88	2.8	1.0	(0.92)
BclxL	0.653	0.243	2.68	(0.018)	0.486	0.243	2.0	(0.0079)	1.224	0.577	2.12	(0.084)
hnRNPD	0.79	0.222	3.56	(0.25)	0.564	0.222	2.54	(0.1)	1.15	0.753	1.53	(0.21)
B2	1.904	1.769	1.07	(0.63)	0.808	1.769	0.456	(0.0015)	1.2215	1.465	0.83	(0.082)
Pic1	0.0409	0.0207	1.97	(0.13)	0.0682	0.0207	3.3	(0.003)	0.1018	0.0628	1.62	(0.24)

The ratio of EμEBNA 1 transgenic positive versus wt samples are given (ratio column). Statistical P value using the Two-sample T-test is given in parentheses for the data.

*P values of<0.05 are statistically significantly different increased expression in EBNA 1 mice

decreased expression in EBNA 1 mice

a) ***BclxL* expression level in 2 month old splenocytes**



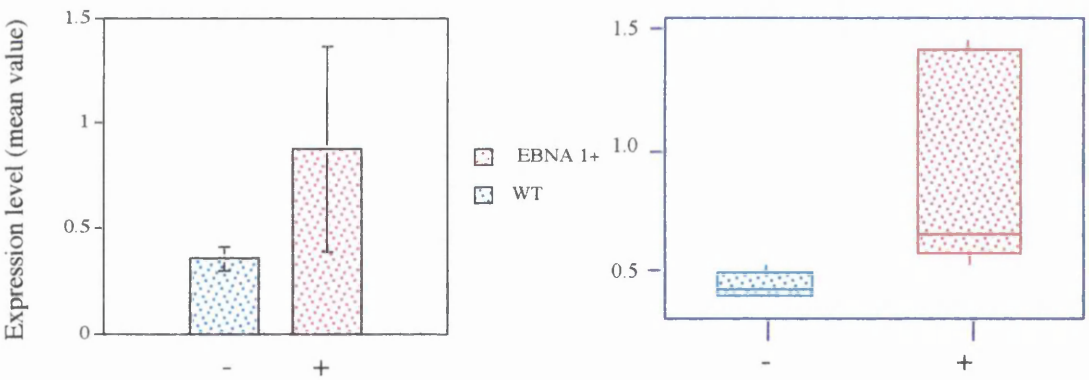
Ratio	2.68
P value	0.018

Two-sample T for BclxL Splenocytes (2M)

+/-	N	MEAN	STDEV	SEMEAN
+	5	0.653	0.302	0.14
-	5	0.2433	0.0653	0.029

95PCT CI FOR MU+ -MU-:(0.09,0.728)
TTEST MU +=MU- (VS NE):T=2.97 P=0.018 DF=8
POOLED STDEV= 0.218

b) ***Egr1* expression level in 2 month old splenocytes**



Ratio	2.47
P value	0.046

Two-sample T for Egr1 Splenocytes (2M)

+/-	N	MEAN	STDEV	SEMEAN
+	5	0.873	0.489	0.22
-	5	0.3534	0.058	0.026

95PCT CI FOR MU+ -MU-:(0.01,1.027)
TTEST MU +=MU- (VS NE):T=2.36 P=0.046 DF=8
POOLED STDEV= 0.348

Fig. 5.2 Graphical and statistical illustration of statistically significant deregulated genes *BclxL* (a) and *Egr1* (b) in 2 month splenocyte total RNA. The histograms show the mean of the expression level in the spleen cells with standard deviation (bars) for EμEBNA 1 transgenic positive line 26 (red) and wt siblings (blue) at 2 months old. These values are shown in box plot format on the right where wt siblings are denoted - and transgenic positive are depicted as + on the X axis. The ratio of means +/- and the P values of this ratio are given below.

decided to take the analysis to this high level of specificity. Total RNA was also extracted from the cells remaining after the B cell selection. These B cell depleted populations were used as controls in order to assess and compare the degree of the gene expression level contributing to the signal obtained from the total spleen and splenocyte RNA slot blots.

Total RNA was extracted from selected B cells from 8 EμEBNA 1 positive and 7 wt 2 month old control mice (line 26), 5 EμEBNA 1 positive and 5 wt 1 month old mice (line 26) and 11 EμEBNA 1 positive tumours from line 26 transgenic animals (the latter not B cell selected) (mouse numbers are listed in Appendix 5.III). Two duplicate slot blots were prepared simultaneously as described previously (Chapter 4). 5μg of each B cell selected and B cell depleted total RNA was used per slot and the blots were hybridised as above. The slot blot signals were quantified by phospho-image analysis and the values were normalised by *GAPDH* hybridisation as previously. The genes tested in this analysis were those that showed differential regulation at (or close to) statistically significant levels in the previous slot blot analyses and are listed in Table 5.3. *BclxL* and *Bad* were included in this assay to confirm the previous slot blot results in the whole spleen and splenocyte total RNA. The *Rag* genes were also included to test if the observed changes in their expression level observed at the 1 month old stage extends to the 2 month old stage. Statistical analysis was performed in order to determine if the observed differences in the RNA expression levels of these genes were significant. The results are summarised in Table 5.3.

Of the 13 genes tested, few were found to show statistically significant difference when comparing transgenic positives to wt controls. Again each group showed a high degree of variation between samples. Moreover, one of the EμEBNA 1 2 month old positive samples (mouse no. 26.1318) was very different from the rest of the group and contributed highly to the degree of variation within this group of samples. Although this RNA sample was from an age matched mouse, the mouse was from a different litter and the RNA was extracted on a different day from the rest. This sample was used to increase the number of samples tested for the statistical analysis but as an outlyer for most of the readings, it affected the results considerably. Nevertheless, as a set of valid data it must be included.

Table 5.3 Summary of comparative gene expression results in selected B cells from line 26 2 month old transgenic mice and line 26 tumours versus wt selected B cell controls.

Gene	Line 26 2M vs 2M wt				Line 26 tumour vs 2M wt			
	Mean tg+	Mean tg-	ratio +/-	P value *	Mean tg+	Mean tg-	ratio +/-	P value *
BclxL	1.999	1.267	1.6	(0.033)	1.687	1.267	1.3	(0.17)
Bad	5.51	7.49	0.73	(0.26)	5.54	7.49	0.78	(0.25)
Rag1	3.58	3.896	0.92	(0.77)	3.4	3.896	0.87	(0.39)
	2.7	3.896	0.69	(0.027)+				
Rag2	10.68	13.44	0.79	(0.4)	6.89	13.44	0.51	(0.0023)
cJun	6.91	6.08	1.13	(0.5)	4.77	6.08	0.78	(0.14)
					4.24	6.08	0.69	(0.0026)+
cFos	7.18	7.139	1.0	(0.98)	9.25	7.139	1.3	(0.11)
EBF	2.37	2.34	1.0	(0.97)	1.344	2.34	0.57	(0.0011)
	1.769	2.34	0.75	(0.03)+				
Ikaros	3.88	3.589	1.08	(0.76)	2.15	3.589	0.6	(0.0059)
Egr1	5.31	3.922	1.35	(0.2)	4.49	3.922	1.14	(0.36)
SATB1	4.22	2.812	1.5	(0.071)	2.406	2.812	0.85	(0.3)
hnRNPD	17.2	11.78	1.46	(0.28)	3.4	11.78	0.28	(0.000)
HCK	3.44	3.099	1.11	(0.66)	2.653	3.099	0.856	(0.16)

The ratio of EμEBNA 1 transgenic positive versus wt samples are given (ratio column). Statistical P value using the Two-sample T-test is given in parentheses for the data.

*P values of<0.05 are statistically significantly different increased expression in EBNA 1 mice
decreased expression in EBNA 1 mice

+P value when outlyer is excluded

When statistical analysis was performed at the 2 month old stage only *BclxL* (1.6-fold) was found to be statistically significantly up-regulated (Fig. 5.3). In contrast to all earlier results, *Rag1* showed statistically significant lower expression level (0.69x) in the transgenic B cells compared to wt cells but only when the outlyer was excluded (5.4a). In addition, *EBF* showed statistically significant lower expression level (0.75x) when an outlier was excluded (Fig. 5.4b and Appendix 5.III). At the tumour stage, *Rag2* (0.51x), *EBF* (x0.57), *Ikaros* (0.6x) and *hnRNPD* (0.28x) showed statistically significant lower expression levels in line 26 tumours as compared to 2 month old wt selected B cells.

Statistically significant increases in the expression levels were also found in the B cell depleted cells for *BclxL* (2.7x), *Egr1* (1.75x) and *SATB1* (2x) in 1 month old mice, and *hnRNPD* (2x) in 2month old mice (data not shown). The flow through represents a mix of cells from the spleen, including T cells and all the B cells that were not isolated by the protocol. As such it is difficult to attribute these differences to any one cell type. However, if these differences are due to the T cell expression patterns, it is possible that this is caused by B-T cell interactions. *In vivo*, a change in B cell phenotype or activity may well lead to changes in responsive T cells (and vice versa).

5.4 Analysis of protein function

While the slot blot and statistical analyses did not validate many of the observations made using the Atlas array, neither do they invalidate these results, indeed for several of the genes the 2-fold ratio was still apparent. In order to examine if the expression of a selection of these genes was affected at the protein or activity level, splenic protein extracts were assayed. For these studies, members of the AP1 transcription factor group (Jun and Fos) were focused on due to their prominence in the Atlas array study. In addition, the DNA binding protein SATB1 was examined since it showed the highest expression ratio (4.1-fold).

Protein extracts from splenocytes and selected B cells of 2 month old transgenic positive and wt control siblings from mice of line 26 were examined for protein levels using Western blot analysis. In addition, DNA binding activity (where appropriate) was assessed by band-shift assay. The levels of detected proteins were

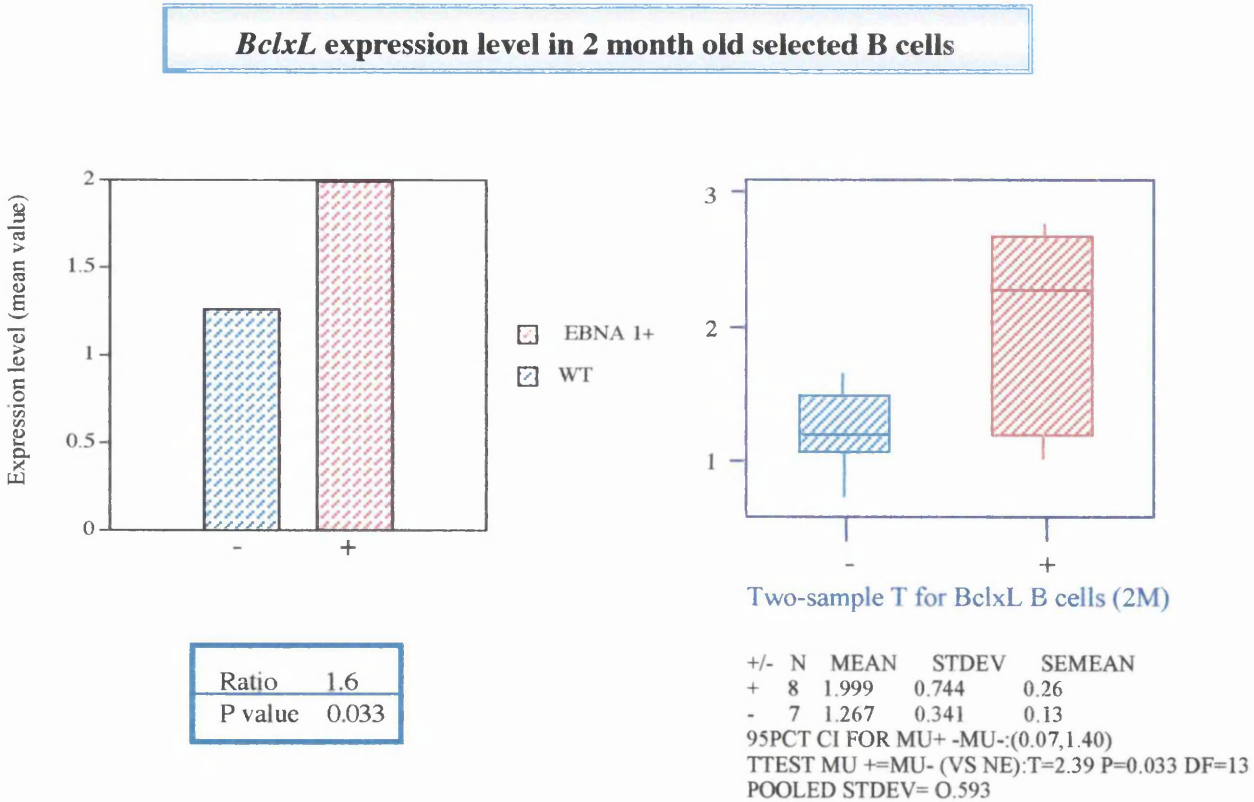
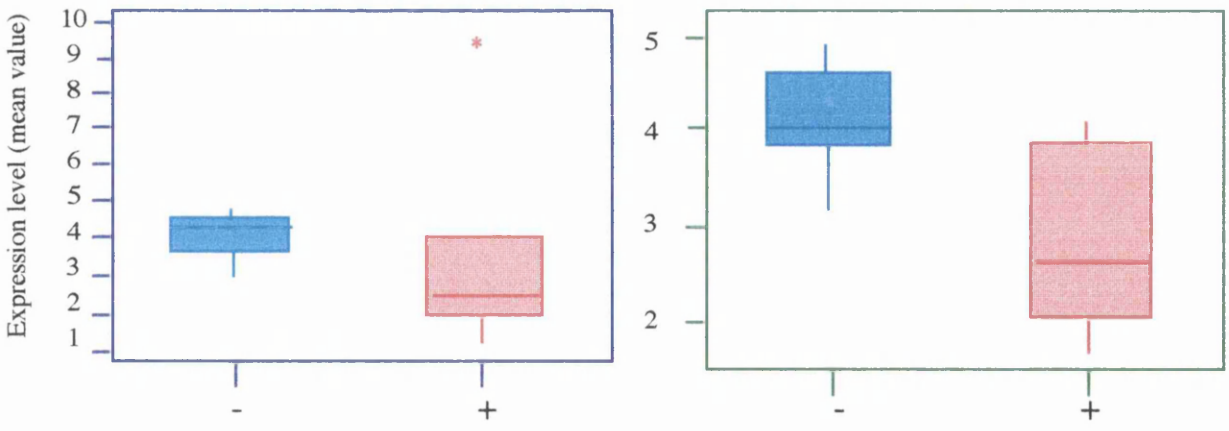


Fig. 5.3 Graphical and statistical illustration of statistically significant deregulated gene *BclxL* in 2 month selected B cell total RNA. The histogram shows the mean of the expression level in the splenic B cells with standard deviation (bars) for EμEBNA 1 transgenic positive line 26 (red) and wt siblings (blue) at 2 month old. These values are shown in box plot format on the right where wt siblings are denoted - and transgenic positive are depicted as + on the X axis. The ratio of means +/- and the P values of this ratio are given below.

a)



Two-sample T for Rag1 B cells (2M)

+/-	N	MEAN	STDEV	SEMEAN
+	8	3.58	2.66	0.94
-	7	3.896	0.71	0.27

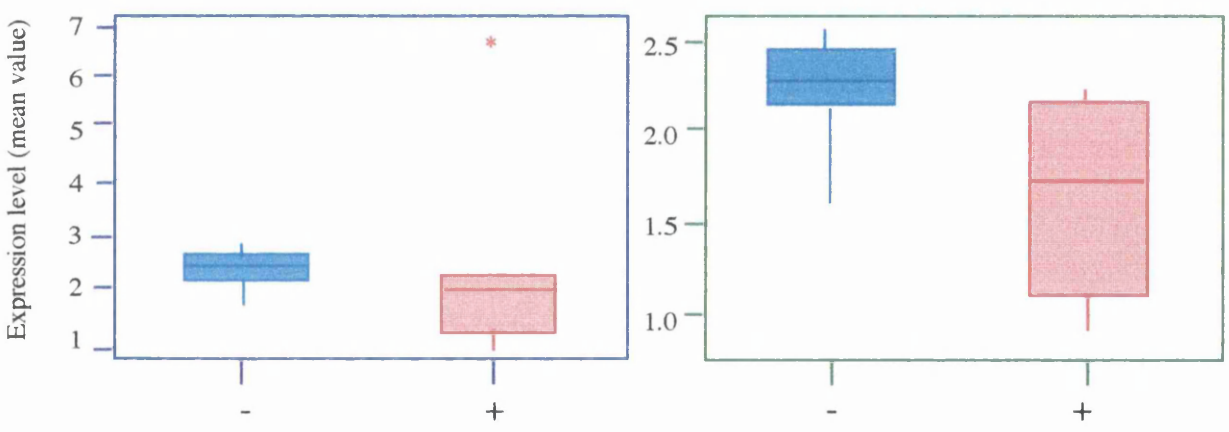
95PCT CI FOR MU+ -MU-:(-2.57, 1.93)
TTEST MU +=MU- (VS NE):T=-0.30 P=0.77 DF=13
POOLED STDEV= 2.01

Two-sample T for Rag1 B cells (2M)

+/-	N	MEAN	STDEV	SEMEAN
+	7	2.7	1.03	0.39
-	7	3.896	0.71	0.27

95PCT CI FOR MU+ -MU-:(-2.23,-0.16)
TTEST MU +=MU- (VS NE):T=-2.53 P=0.027 DF=12
POOLED STDEV= O.885

b)



Two-sample T for EBF B cells (2M)

+/-	N	MEAN	STDEV	SEMEAN
+	8	2.37	1.56	0.62
-	7	2.34	0.37	0.14

95PCT CI FOR MU+ -MU-:(-1.44, 1.5)
TTEST MU +=MU- (VS NE):T=-0.04 P=0.97 DF=13
POOLED STDEV= 1.31

Two-sample T for EBF B cells (2M)

+/-	N	MEAN	STDEV	SEMEAN
+	7	1.769	0.488	0.18
-	7	2.34	0.37	0.14

95PCT CI FOR MU+ -MU-:(-1.08,-0.07)
TTEST MU +=MU- (VS NE):T=-2.46 P=0.030 DF=12
POOLED STDEV= O.433

Fig. 5.4 Box plot representation with numerical values below of (a) *Rag1* and (b) *EBF* expression results in selected B cells from 2 month old mice. EμEBNA 1 transgenic positives represented by a + (red) and WT by a - (blue). An outlyer (*) in the group of positive samples distorts the results and renders it not statistically significant (blue box plot). When the outlyer is excluded the result becomes statistically significant (green box plot).

measured by densitometry of the autoradiographs. Finally, for those genes encoding kinases (i.e. JNK, MAPK), levels of active (phosphorylated) kinases were examined.

5.4.1 DNA/protein interactions: AP1

In order to examine AP1 components in splenocytes, whole cell protein extracts were prepared from primary splenocytes from 2 positive and 2 wt control 2 month old mice using a modification of the protocol of Marais *et al.*, (1993) in the presence of protease and phosphatase inhibitors. Protein concentration was then measured using the BioRad protein assay.

5μg of total protein from each sample was analysed in gel retardation assays using an oligonucleotide containing the TPA-responsive element (TRE or AP1 site) from the collagenase gene promoter (colTRE; Angel *et al.*, 1987). A positive control sample (sample 34, chicken fibroblast cell line protein extract) was included in all assays. However, the standard protocol for protein extraction and DNA binding had been optimised using cell lines. The bands obtained from the primary cell extracts were numerous and less well resolved giving smeary tracks making the results less clear. To demonstrate the specificity of the AP1 band corresponding to the one from the positive control, non-labelled double stranded TRE-oligonucleotide was added to the binding reactions (at a 50-fold molar excess over the probe) as a competitor for binding to the labelled probe. In the presence of competitor oligonucleotide the AP1 band was competed, demonstrating the specificity of the band (not shown).

To investigate the composition of the AP1 complex/band, antibody addition gel retardation analysis was performed on whole cell extracts from EμEBNA 1 transgenic positive and wt splenocytes. Under these conditions a specific interaction, between the test antiserum and a component of the DNA-binding complex is signified by disruption and/or further electrophoretic retardation (supershifting) of the complex. Supershift assays were conducted using antibodies for cFos, cJun, JunB, JunD, FosB and Fra1. Again the results were not very clear due to the numerous bands and smeary tracks in the lanes. Nevertheless, in a supershift assay using cFos, cJun, JunB, JunD, FosB and Fra1 antibodies, oligonucleotide retardation to the top of the gel was observed in the presence of cJun and JunD antibodies in both positive and negative samples suggesting that cJun and JunD are AP1 components in these cells (data not shown).

However, in general, in these first sets of experiments, band smearing, poor competition with specific cold competitor and poor resolution in antibody supershift assays may be due to several reasons. For example, splenocyte-derived samples contained red blood cells (RBC) as well as lymphocytes. Although RBCs do not have a nucleus they have cytoplasmic organelles with proteins such as NfE2, a haematopoietic factor, which can react with AP1.

In order to try and optimise the conditions using primary cells, several modifications in the protein extraction buffers and DNA binding reaction buffers were tried. The nuclear protein extraction buffer used [modified from Schreiber *et al.*, (1989)] was tested with or without Triton-X100 and with or without glycerol. The DNA binding buffer [modified from Marais *et al.*, (1993)] was tested with or without glycerol. Glycerol is usually used to aid subsequent gel loading, however, it was found that it interfered with the reaction and thus was excluded from the subsequent protein samples. Triton-X100 is used as a detergent to lyse the cells and as it did not interfere with the reaction it remained as one of the buffer components. It was also found that increasing the dIdC (non-specific competitor) concentration to 2μg/reaction was more effective in reducing the non-specific binding.

The experiment was repeated using nuclear protein extracts obtained from splenocytes from 5 EμEBNA 1 positive and 5 wt control mice 2 month old using the newly modified nuclear protein extraction protocol. 10μg of nuclear protein from each sample was analysed in a gel retardation assay using the TRE oligonucleotide probe.

The AP1 binding activity in some of these samples is illustrated in Fig. 5.5. In Fig. 5.6 only a pair of transgenic and wt samples is shown. The addition of the col-TRE competitor in Fig. 5.6a lanes 2, 5, 6 effectively competed the AP1 band showing high specificity of the probe used.

In the presence of cJun and Jun D antisera (and less for JunB) a shift of the AP1 band was observed in both EμEBNA 1 positive and wt samples. This was consistent in four different experiments using different pairs of transgenic positive and wt samples. As shown in Fig. 5.6b much of the AP1 complex/band was disrupted and supershifted by the cJun (lanes 3 and 4) and JunD (lanes 7 and 8) antisera but it was unaffected by cFos, FosB, Fra1 and Fra2 antisera (Fig. 5.5a/b). (In lane 2 of Fig. 5.6b, the band at the top of the gel was due to leak from lane 3

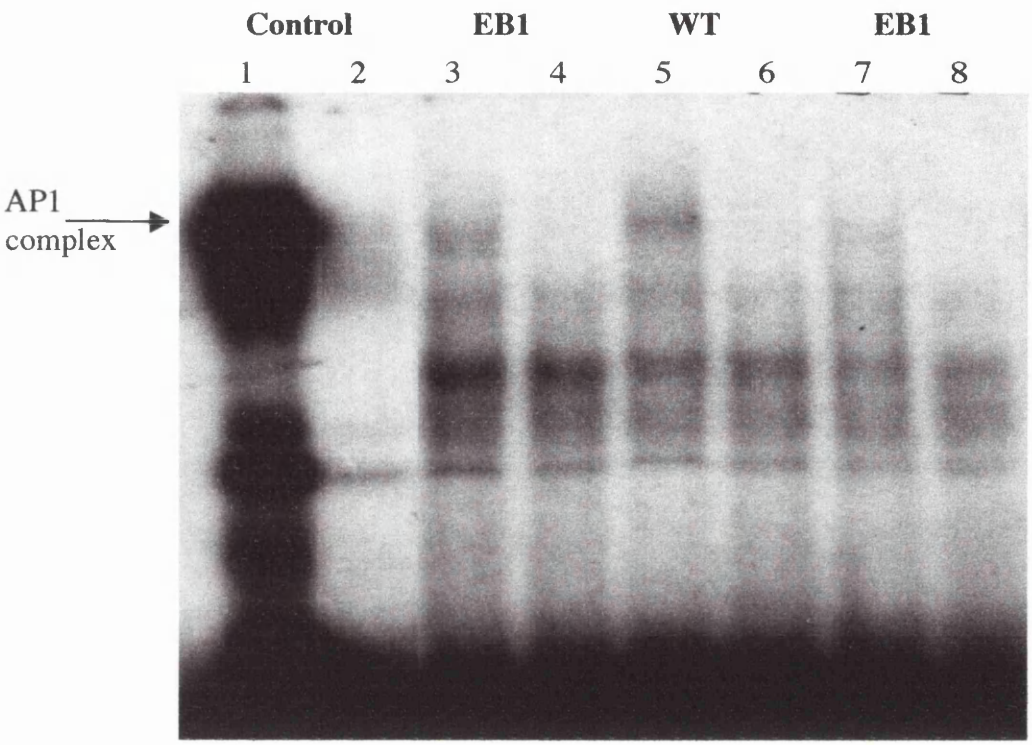
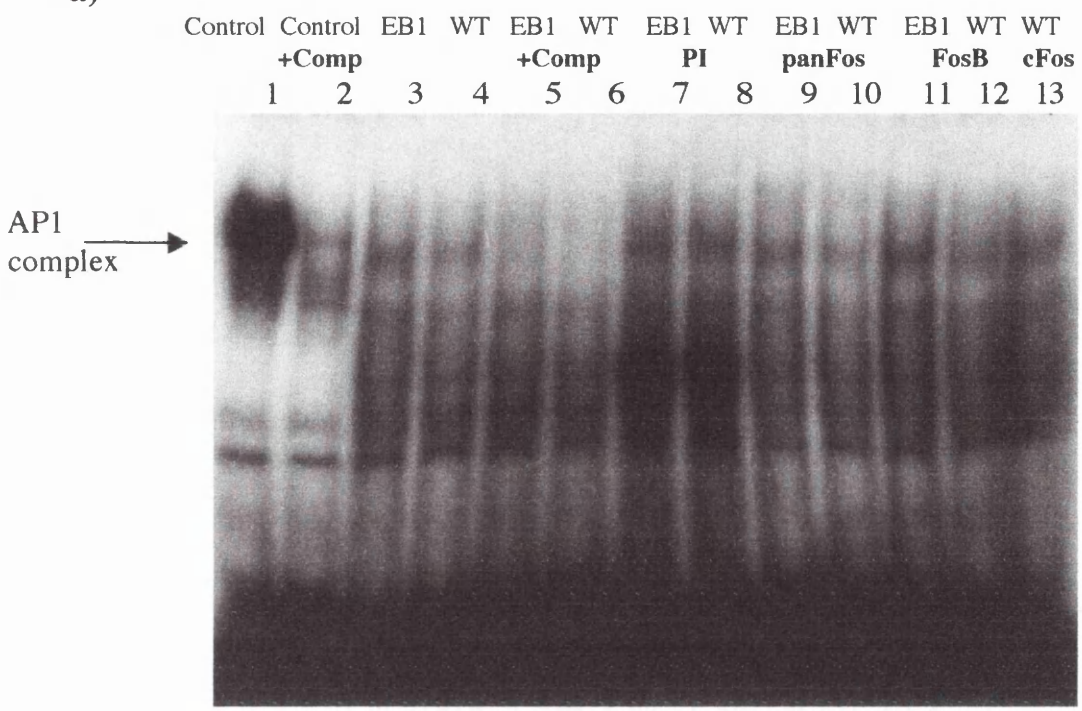


Fig. 5.5 Gel retardation assay of DNA/protein complex for AP1 band specificity. Nuclear extracts of splenocytes from transgenic positive (lanes 3-4,7-8) or wt mice (lanes 5, 6) or extracts of chicken fibroblasts (lanes 1-2, used as control) were mixed with radiolabelled oligonucleotide comprising a TRE sequence. In lanes 2,4,6,8 the unlabelled col-TRE oligo was added as a specific competitor for AP1 binding at 50-fold molar excess over probe.

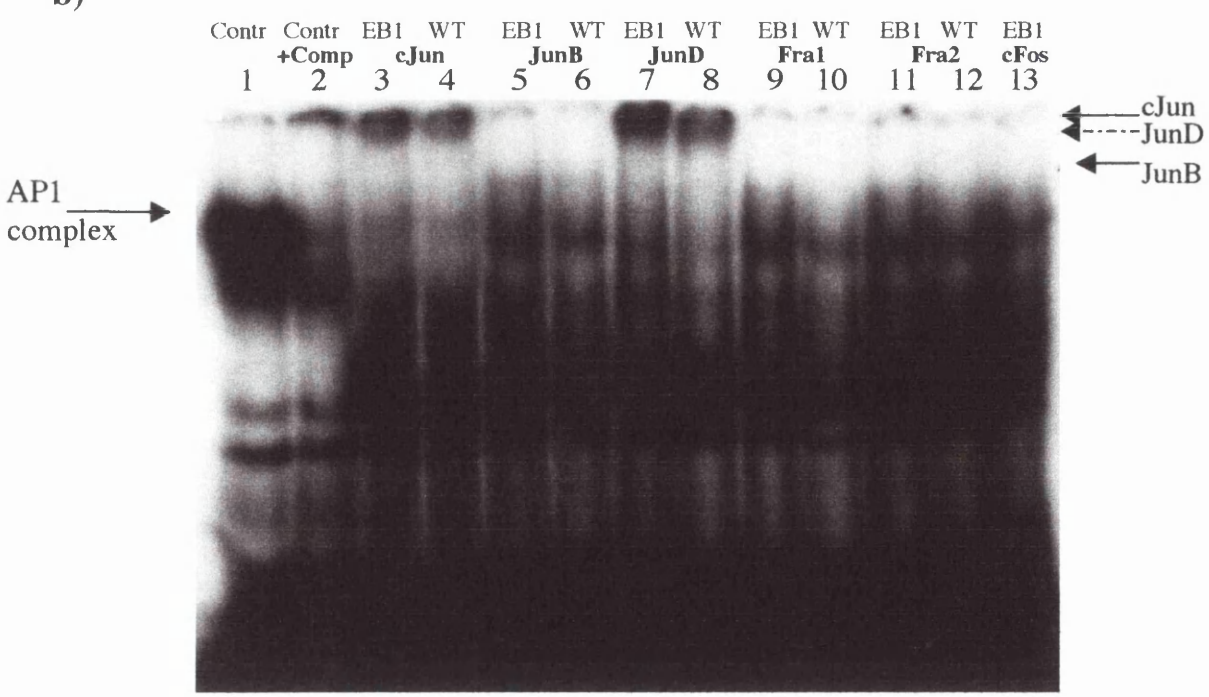
Fig. 5.6. EMSA of TRE oligonucleotides with EBNA 1 transgenic positive or wt tissue extracts and antibodies directed against Jun and Fos proteins. Nuclear cell extracts of splenocytes recovered from transgenic positive or wt mice, or extracts of chicken fibroblasts (5.6a/b lanes 1-2, used as controls) were mixed with radiolabelled oligonucleotides comprising a TRE sequence. Cold competitor oligonucleotides were added as a control (competitor 50x, lanes 2, 5, 6 in 5.6a and lane 2 in 5.6b). Alternatively, antibodies directed against either Fos (all), FosB, cFos, cJun, JunB, JunD, Fra1 or Fra2 were added to detect the presence of these factors as components of the AP1 complex in these cells. Pre-immune serum (PI) was added as a non-specific binding control (lanes 7, 8). EB1= protein extract from EμEBNA 1 transgenic positive mouse (26.1475) and WT= protein extract from a transgenic negative mouse (26.1476). The AP1 complex is indicated by an arrow to the left of the image. The bands supershifted by antisera to cJun, JunB and JunD are indicated by arrows to the right of the image.

Fig.5.6

a)


Control
Control +Comp
EB1
WT
EB1 +Comp
WT
EB1 PI
WT
EB1 panFos
WT
EB1 FosB
WT cFos
1 2 3 4 5 6 7 8 9 10 11 12 13
AP1 complex

b)


Contr
Contr +Comp
EB1
WT cJun
EB1
WT JunB
EB1
WT JunD
EB1
WT Fra1
EB1
WT Fra2
EB1 cFos
1 2 3 4 5 6 7 8 9 10 11 12 13
AP1 complex
cJun
JunD
JunB

during loading). These results indicate that the AP1 complex in these splenocytes consists of a cJun component, a JunD component and possibly a minor JunB component. What the Jun partners might be (homo- or heterodimers) is not clear. However, in general, the intensities of the bands containing the protein-DNA-binding complexes appeared similar between transgenic positive and wt control samples. Since the AP1 band contains many different proteins, subtle changes in the level or activity of one component could be masked.

5.4.2 Steady state protein levels

To examine if any potential differential expression between EμEBNA 1 transgenic and wt samples is apparent at the protein level, protein extracts from selected B cells of transgenic positive and wt controls were assayed by western blot. Line 26 tumour extracts were also analysed. The levels of selected proteins were measured by densitometry of the autorads.

Whole cell protein extracts were prepared from selected B cells and the B cell depleted cells from 7 EμEBNA 1 mouse spleens and 7 wt controls 2 month old. Protein extracts from each group were made after cells were pooled together to increase the protein available for each "sample" and to give an overview of the protein levels in EμEBNA 1 positive as compared to the wt control tissues.

Duplicate western blots were prepared by using 100μg of protein/lane. A representative western blot against BclxL is shown in Fig. 5.7. The antisera used were against BclxL, cJun, SATB1 and cFos.

Protein levels were measured by densitometry of the autoradiographs and they were normalised against non-specific bands from the compared samples. However, since no positive control was included, because no pre-immune sera or blocking peptide were available, an assumption of the correct band was made based on size. After calculating the ratio of the intensities of the BclxL bands, it was found that the protein level was higher in the positive B cells as compared to the wt controls by 1.2-fold. If this is indeed BclxL, this result is consistent with the RNA results described previously (Chapters 4 and 5).

Western blots of selected spleen B cell protein extracts (as described above) were probed with cJun and cFos antisera but these results were inconclusive (data not shown).

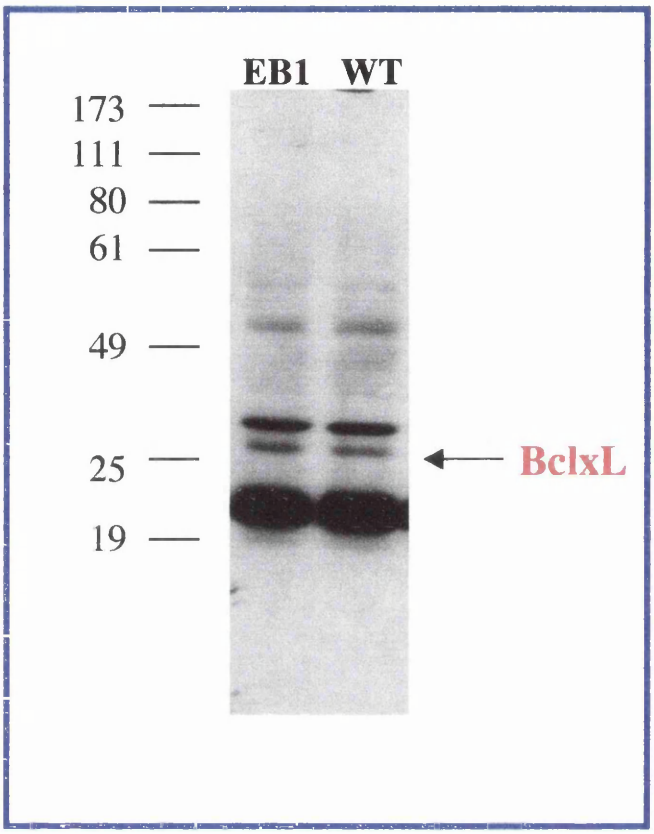


Fig. 5.7. Steady state BclxL protein level. Western blot analysis of BclxL in total cell extracts of selected B cells from 2 month old mice (line 26). Lane 1: EμEBNA 1 transgenic positive protein extracts (EB1), lane 2: wt protein extract (WT). The suspected BclxL band is indicated with an arrow. 100μg of total protein from selected spleen B cells was loaded per track. Rabbit anti-BclxL was used at a concentration of 0.3μg/ml and the secondary HRP-conjugated anti-Rabbit IgG at 1:2000 dilution. The detection system used was the luminol system ECL(Amersham # RNP2106).

SATB1 levels were also analysed. The previous blots were stripped using MENSA buffer and probed with the SATB1 antiserum (see Materials and Methods section 2.4.7). Although these are preliminary results and need further confirmation there was no difference in the protein level between positive and wt samples. SATB1 staining gave the expected size band of 85.9KDa which appeared as a doublet in both B cell enriched and depleted fractions (albeit fainter in the former) (not shown). However, since no positive and negative controls were included, it was not possible to say with certainty which band was the correct one and if there was an actual EBNA 1 effect on the protein level.

In line with the examination of Jun and Fos proteins, the activation status of SAPK and MAPK pathway members ERK and JNK was examined by western blot assay of the active, phosphorylated forms. No differences were found between transgenic positive and wt samples (not shown).

5.5 Discussion

To investigate the effects of EBNA 1 on cellular gene expression, a differential display approach was employed using the mouse Atlas cDNA expression array. Using this method the researcher can quantitate the expression levels of a large number of genes in two different samples in one experiment. The size of these arrays range from 588 (which was used here) up to 1200 genes with the new macroarray, or even more with microarrays either on glass or chips. The arrays also include different house-keeping genes which serve as quantitative/qualitative controls. This makes the normalisation easier, because subsequent hybridisation with a house-keeping gene (as in the slot blots) is not needed. However, because polyA+ RNA is used to make the cDNA probe there is a possibility that other, perhaps important mRNA messages will be lost during the purification and therefore may affect the final result.

As an approach, array analyses should not be used as absolute evidence of gene de-regulation but only as an indicator of which genes should be analysed further. This is because the data can only show the ratio of difference between two compared sets without the possibility of statistical analysis. However, this indication of gene expression may sometimes be misleading, and many genes that showed higher or lower expression in the Atlas, did not show any difference in the subsequent slot blot analysis or vice versa. For example, neither *BclxL* or *Rags* were selected candidates in

the Atlas approach but they showed statistically significant differential regulation in all of the slot blots. Moreover, subtle differences, such as the 1.3x, first observed for *BclxL* and *Rags*, may be easily disregarded in an array approach. Another negative feature of this approach is that the membranes are very sensitive to extensive handling and can only be used the maximum 2 or 3 times for the macroarrays and only once for the microchip arrays.

Analysis of the effects of EBNA 1 on cellular gene expression using the Atlas cDNA expression array has identified a number of genes which may be differentially expressed between the EμEBNA 1 positive splenic B cells and wt controls. Those showing higher expression levels in EμEBNA 1 positive samples (approximately 2-fold or more) include several *Jun* and *Fos* family members, and a number of B cell oncogenes and transcription factors such as *EBF, Egr1* and *Pax5*. Those showing lower expression levels include kinases such as haemopoietic cell kinase (*HCK*), *MAPKK1, JAK3* and the tumour suppressor *PML*. Interestingly, while *cRel* was amongst those genes showing higher expression levels, its inhibitor, *IκB* was amongst the most suppressed ones. Slot blot analysis was used to test the candidate gene expression in more samples.

Slot blot analysis using either splenocyte or selected B cell RNA in most cases did not confirm the differential regulation. However, although the slot blots did not confirm the Atlas results, this differential regulation may still be real since a lack of statistical significance in small sample numbers cannot refute this possibility. One of the reasons for the outcome of these results may be the large variation within the datasets. This variation is a characteristic of approaches using *in vivo* samples because unlike cell lines these samples derive from tissues consisting of different cell types that are not clonal. Different genes have different expression patterns at different cell cycle points and even more between different cell types and therefore this may contribute to the variation in these results. Alternatively, factors that effect the immune responses of the animals could also effect the outcome of the results. These mice derive from the same strain and they have the same genetic background. Therefore, one would expect that any changes at the genetic level would be similar. However, although these mice are kept in a controlled environment and treated in the same way, this environment is not completely free of pathogens. Thus, the presence of pathogens would trigger immune responses, different in each individual mouse.

Another factor influencing immune responses is aggressive behaviour (usually observed in male mouse cages). Consequently, since an immune response can be triggered by many factors the relative changes at the molecular level would be different. For example, different pathways would be triggered and therefore gene expression would be differentially affected in each case.

In conclusion, if the differential regulation observed in the Atlas approach is real, it may need many more samples to show statistical significance. As such, all the genes identified remain candidates for differential regulation, but further experiments are required to definitely support or refute the possibility. Implications regarding each candidate gene are considered below.

5.5.1 AP1

One third, 6 of the 20 genes that showed higher expression levels in the positive samples as compared to the wt controls are AP1 components (*cJun, JunB, JunD, cFos, FosB* and *Fra1*). This itself is strongly suggestive that these genes may be valid targets of de-regulation. This result is intriguing since these genes have been classified as immediate-early response genes, that is those genes that are induced in the absence of *de novo* protein synthesis and show an immediate change in their expression after cell stimulation. These genes serve as nuclear couplers of early cytoplasmic events to long term alterations in gene expression (Herschman, 1991). However, in subsequent slot blot analyses using RNA extracted from splenocytes or selected B cells, none of these genes showed any statistically significant differences in their expression levels between EμEBNA 1 positive and wt controls in pre-neoplastic tissues even though (for the genes tested) the increased ratio (of 2-fold) was still evident in the mean values derived from splenocytes. Nevertheless, two of these genes (*cJun* and *cFos*) showed statistically significant higher expression levels in line 26 tumours (by 2.4-fold and 2-fold respectively) as compared to the wt splenocyte RNA controls. However, *cJun* expression was later found statistically significant lower (1.45-fold or 0.69x) in the line 26 tumours when compared to the 2 month wt selected B cell RNA.

AP1/DNA binding assay revealed the presence of cJun, JunD and JunB in the AP1 complex in spleen cell nuclear protein extracts of both transgenic positive and wt controls while the Fos family members were not evident. Because band intensity

in bandshift assays varies considerably depending on the quality of the extract, densitometry is quite difficult. In addition, these results were much more ambiguous because protein was extracted from primary cells (comprising many different cell types and cell stages). Therefore this assay did not reveal information concerning the activity levels of these components in this system and was not examined further.

Of the 6 genes only *cJun* and *cFos* were tested for any differences at the protein level between EμEBNA 1 positive and wt samples. However, these results were inconclusive and further analyses of steady state protein might be informative.

AP1 is a transcription factor complex involved in cell growth, differentiation and stress signalling pathways (Angel and Karin, 1991; Karin and Hunter, 1995; Whitmarsh and Davis, 1996). AP1 consists of various combinations of Jun, Fos, ATF2, and possibly other, family members that dimerise via a leucine zipper domain and bind to DNA via an adjacent basic region (Gentz *et al.,* 1989; Kouzarides and Ziff, 1989). The Jun family consists of three gene products (cJun, JunB and JunD) while the Fos family consists of four gene products (cFos, FosB, Fra1 and Fra2). Jun proteins form heterodimers with Fos family proteins (Hai and Curran, 1991) and ATF2 and also form functional homodimers, although with reduced stability (Halazonetis *et al.,* 1988; Rauscher *et al.,* 1988). Fos proteins form heterodimers with Jun and ATF family proteins (Hai and Curran, 1991). AP1 binds to a specific target DNA sequence TGAC/GTCA, called TRE (tetradecanoyl phorbol acetate-responsive element, Angel and Karin, 1991) which was used as a probe in the band shift assay. The binding activity for a given TRE is determined by three main factors. Firstly, by the different combinations and the context of the surrounding sequences (Halazonetis, *et al.,* 1988; Ryseck and Bravo, 1991; Hawker *et al.,* 1996). Secondly, the expression of Jun/Fos proteins is temporarily co-ordinated in response to various stimuli with the result that the composition of AP1 may change in the cell depending on the cell cycle time point and stimulus (Curran and Morgan, 1987; Cohen *et al.,* 1989; Kovary *et al.*, 1991; 1992; Lallemand *et al.*, 1997). Thirdly, some Jun and Fos proteins possess transcriptional transactivation domains which are regulated by phosphorylation and thus different dimer combinations may exhibit different transactivation properties (Kouzarides and Ziff, 1989).

Some of these later phosphorylation events are catalysed by serine/threonine protein kinases activated via the MAP kinase and Jun kinase pathways.

Interestingly, the Atlas cDNA expression array analyses of EμEBNA 1 positive B cells indicated lower expression levels of MAPKK1 (which has pleiotropic effects).

TRE is found in the promoter of many genes including cell cycle regulators such as cyclin D1 and autocrine growth factors such as heparin-binding epidermal growth factor (HB-EGF) (Herber *et al.*, 1994; Albanese *et al.*, 1995; McCarthy *et al.*, 1997). Thus, if EBNA 1 does upregulate the AP1 group, one could expect the expression levels of genes regulated by AP1 components to be affected. Such genes include those involved in stress response, proliferation and differentiation. This could explain the cell proliferative advantage observed in the EμEBNA 1 positive bone marrow cell cultures in comparison to the wt ones, described in Chapter 3.

5.5.2 Other B cell transcription factors

The zinc finger transcription factor early growth response 1 (*Egr1*) is another immediate early response gene (Dinkel *et al.*, 1998) that was identified using the cDNA expression array approach. Its expression was higher in the EμEBNA 1 positive RNA by 2.3-fold in comparison to the wt controls. In subsequent slot blot analysis, using 2 month old splenocyte total RNA, *Egr1* up-regulation was consistent and statistically significant in both pre-tumour (2.5-fold) and tumour (1.5-fold) samples as compared to the 2 month old wt type splenocyte RNA controls. However, in the selected B cell slot blot although *Egr1* expression was still higher in the transgenic positive samples it was not statistically significant, possibly due to the high variation in this data set. These results in part support the array observation that *Egr1* is up-regulated by EBNA 1.

Egr1 transient expression in mature B cells is rapidly activated upon stimulation by B cell receptor (BCR) cross-linking (Seyfert *et al.*, 1989; McMahon and Monroe, 1995) whereas signals resulting from Fc receptor crosslinking inhibit induction (Klaus *et al.*, 1993; Gottschalk *et al.*, 1994). Little is known about the expression and function of Egr1 during the early steps of B cell differentiation. However, it was recently reported that *Egr1* expression can be detected in pro-B and pre-B (B220+/sIgM-) cells isolated from BM and foetal liver-derived pre-B cell cultures (Dinkel *et al.*, 1998). Thus it can be suggested that Egr1 might also have a regulatory function in early stages of B cell lymphopoiesis (Fig. 5.8 and 5.9). In the

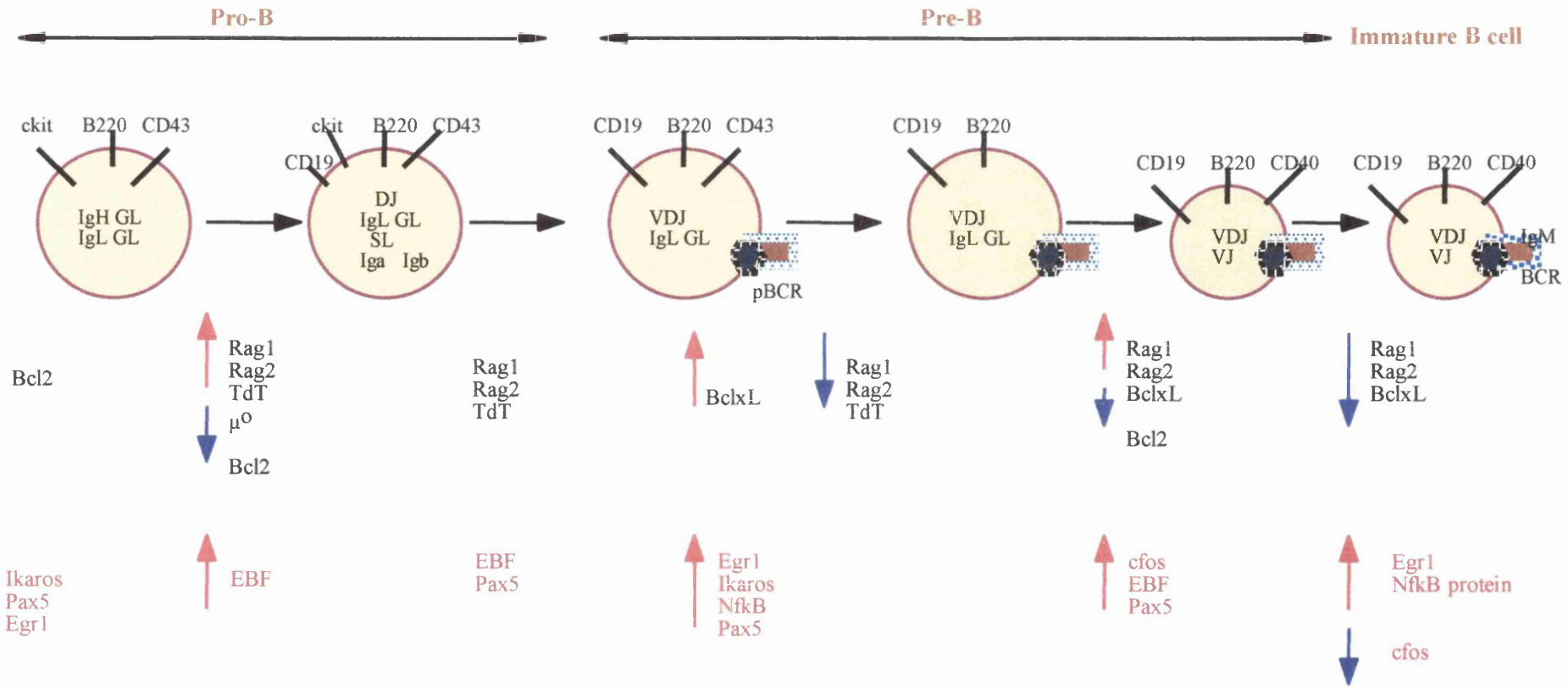


Fig. 5.8 Stages of primary B cell development. Characteristic B cell surface markers and normal expression patterns for genes are indicated. Large circles represent cycling cells, whereas small circles represent non-dividing cells. Upward arrows represent induction of gene expression, downward arrows represent repression of gene expression. Genes without an arrow are expressed but their levels are not altered from the previous stage. The genes shown in red are those showing differential regulation in the mouse Atlas cDNA array.

Abbreviations: germline (GL), surrogate light chain (SL), sterile μ transcript (μ^o).

(Adapted from: Henderson A. and Calame K., 1998).

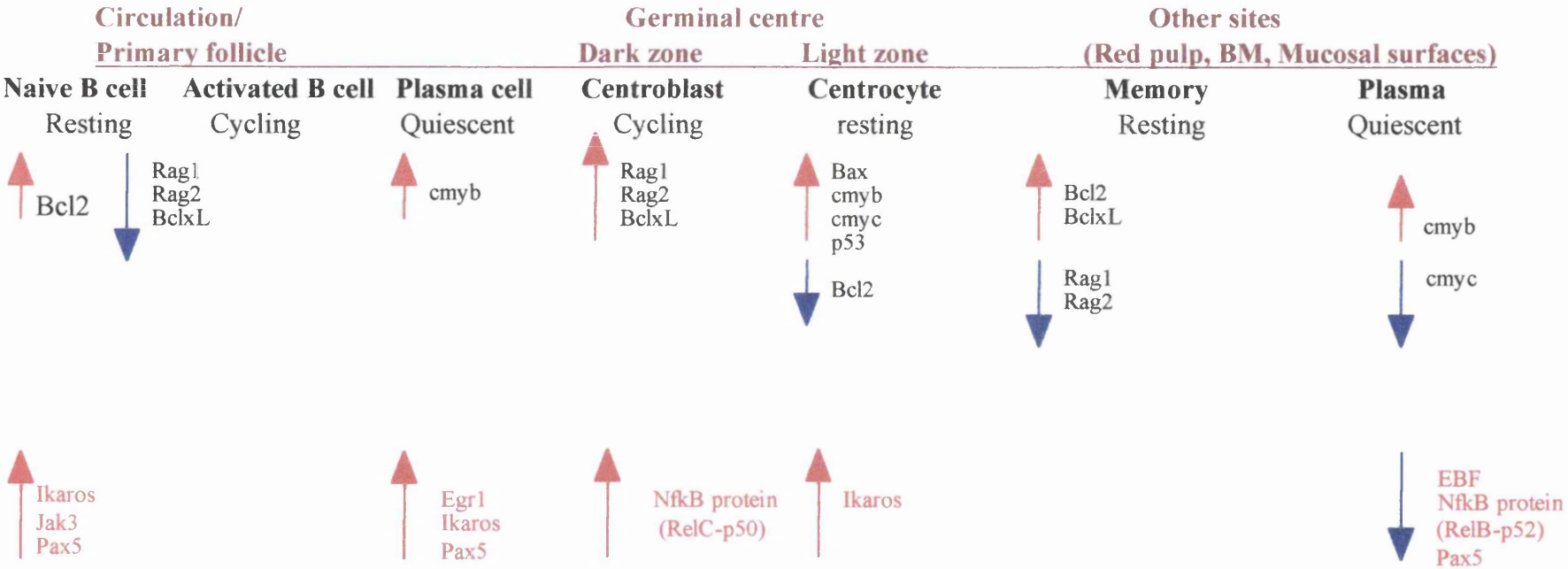


Fig. 5.9 B cell maturation. From top to bottom: anatomical location, maturity of B cells, cell cycle status and expression patterns of genes known to increase (arrow up) or decrease (arrow down). Genes at the bottom half of the figure are some of those that showed differential expression in the mouse Atlas cDNA array.

(Adapted from: Henderson A. and Calame K., 1998).

Abbreviation: bone marrow (BM)

same report, using transgenic mice, it was also demonstrated that overexpression of Egr1 appears to promote entry into the mature B cell stage even in the absence of Rag2 activity. Therefore if EBNA 1 does upregulate *Egr 1* then perhaps EBNA 1 would also promote B cell maturation. This could explain the flow cytometry results in Chapter 3 in which the percentage of sIg + B cells in the EμEBNA 1 positive spleens and BM was approximately 2-fold higher than that of the wt spleen and BM cells.

The early B cell factor (*EBF*) is a B cell specific transcription factor that was also identified in the mouse cDNA Atlas approach, showing 2.65-fold higher levels in EμEBNA 1 positive B cells. Its expression was further found to be statistically significantly higher in the tumour samples by 3.25-fold in comparison to the 2 month old wt splenocyte sample controls. Its expression was also found to be 3.7-fold higher (the highest difference of all genes tested) in the transgenic positive splenocytes *versus* wt ones, but this result was not statistically significant. However, the selected B cell results do not confirm the up-regulation. Although EBF is also expressed in other non-lymphoid tissues (Wang and Reed, 1993), experiments involving knockout EBF $^{+/-}$ or EBF $^{-/-}$ mice suggested that EBF plays a specific and important role in the transcriptional control of B cell differentiation at a stage before Ig gene rearrangement but after commitment of cells to the B lymphoid lineage (transition of pro-B to pre-B cells). These mice lacked transcripts from *Rag1* and *2* genes as well as other B cell specific genes such as *Pax5*, *mb-1, VpreB* and DJ_H-rearranged μ gene (Lin and Grosschedl, 1995; O'Riordan and Grosschedl, 1999). Thus, *EBF* is required at an earlier stage of B lymphopoiesis than *Pax5* and *Rags* and possibly regulates their expression. Interestingly, it was recently reported that EBF directly binds and activates the Pax5 promoter (O'Riordan and Grosschedl, 1999). If EBNA 1 does upregulate *EBF* then this could result in up-regulation of EBF target genes such as *Pax5* and possibly *Rags* which would promote B cell Ig rearrangements and maybe genomic instability that could ultimately lead to tumour.

Pax 5/BSAP expression level was also found higher in the EμEBNA 1 positive cDNA in the Atlas expression array by 1.5-fold versus the wt cDNA. However, although its expression was also higher in the splenocyte slot blot analysis (2-fold) it was not statistically significant. Pax5 is a member of the Pax family of transcription factors, which is expressed primarily in B cells, central nervous system (CNS) and

testis (Busslinger and Urbanek, 1995). Pax5 is found in all stages of B cell development, except plasma cells (Fig. 5.8 and 5.9). Knockout mice for *Pax5* lack pre-B, B and plasma B cells. V_HDJ_H recombination is also significantly reduced, suggesting an important role in B cell maturation and differentiation (Busslinger *et al.*, 1996). Several B cell specific genes have been implicated as targets for *Pax5* activation including CD19, *VpreB*, *λ5* and *mb1* and the germline IgH *Cε* promoter (Fitzsimmons *et al.*, 1996; Nutt *et al.*, 1997 and references therein) . It was recently reported that Pax5 binds to a conserved sequence in the *Rag2* promoter, critical for its activity (Lauring and Schlissel, 1999). Interestingly, many of these Pax5 regulated genes have multiple transcription start sites and lack a TATA box. This suggests that Pax5 may play a role in recruiting the basal transcription machinery to TATA-less promoters in B cells. In addition Pax5 has been suggested to act as a repressor at the heavy-chain 3' α enhancer (Singh and Birshtein, 1993; Neurath *et al.*, 1994), the κ light chain 3' enhancer (Roque *et al.*, 1996; Shaffer *et al.*, 1997) and the J chain promoter (Rinkenberger *et al.*, 1996). Therefore, if EBNA 1 leads to the up-regulation of Pax5 then it could affect B cell sub-population numbers in both spleen and BM by promoting B cell maturation. Since Pax5 binding sites exist within the *Rag2* promoter sequence, then up-regulation of *Pax5* by EBNA 1 could lead to increased recombination and genomic instability.

Ikaros, another lymphocyte specific transcriptional regulator, expression was found higher in the mouse Atlas expression array EμEBNA 1 positive RNA by a 2.8-fold in comparison to the wt control RNA. Slot blot analysis using either splenocyte or selected B cell RNA (2 month old) did not support the Atlas result. However, *Ikaros* expression was statistically significantly higher in the line 26 tumours versus wt controls. Thus, there is less evidence supporting *Ikaros* as a target of EBNA 1 de-regulation because its up-regulation was not supported by the slot blots. The *Ikaros* gene encodes a set of proteins, generated by alternative mRNA splicing, that contain kruppel-like zinc fingers organised in two functional domains, one for DNA binding and the other for dimerisation (Hahm *et al.*, 1994; Molnar and Georgopoulos 1994; Georgopoulos *et al.*, 1997). *Ikaros* is primarily expressed in haematopoietic tissues and its mRNA is present throughout B cell development. *Ikaros* gene expression appears to increase as B cells mature, but the relevance of absolute versus relative levels of Ikaros proteins is not understood (Georgopoulos *et al.*, 1997).

Ikaros null mutant mice lack foetal lymphocytes and adult B cells. The B cell developmental block is very early- prior to the pro-B cell stage (Wang *et al*, 1996). Ikaros binding sites have been described in promoters of a number of genes including *Rag1* and *2, IL2 receptor, Iga, VpreB, λ5* and *TdT* (Georgopoulos *et al.*, 1997). Interestingly, in pre-B cells *VpreB* and *λ5* receptor, along with IgH expression is required for pre-B cells to avoid negative selection. Therefore, up-regulation of *Ikaros* by EBNA 1 would affect the expression of Ikaros target genes and thus affect B cell maturation and differentiation.

The above findings are very interesting and consistent with *Rag1* and *2* regulation by EBNA 1 (Kuhn-Hallek *et al.*, 1995; Srinivas and Sixbey, 1995). As previously discussed in Chapter 4, analysis of the level of *Rag1* and *2* expression in RNAs from EμEBNA 1 transgenic positive and wt control sibling mice revealed a consistent *Rag1* and/or *Rag2* up-regulation in EBNA 1 expressing cells at all developmental stages tested. Based on the above it can be suggested that *Rag* gene expression appears to be regulated by a number of different transcription factors that are possibly regulated by EBNA 1. Therefore, it can be suggested that EBNA 1 may regulate *Rag* expression possibly through direct regulation of one of the above genes such as *Egr1* or other genes acting upstream of those tested. In addition, the fact that these genes were found de-regulated may suggest a B cell developmental stage at which EBNA 1 acts.

5.5.3 Proto-oncogenes

Other genes that were found up-regulated in the mouse Atlas expression array include the *Ets2, cRel* and the proto-oncogenes *Nras* and *Araf. Nras* de-regulation at 2 month stage was not supported by slot blots, but it was statistically significantly higher in the tumours by 2.65-fold versus wt splenocyte RNA. *Araf* was found up-regulated in both pre-tumour samples and in tumours but this change was not statistically significant.

Ets2 expression was 2.2-fold higher in the EμEBNA 1 transgenic versus wt array. *Ets2* is a nuclear protooncogene and is member of a large family of transcription factors known as the Ets family. Ets2 expression correlates with cell proliferation and differentiation and development (Bhat *et al.*, 1987; Boulukos *et al.*, 1990; Maroulakou *et al.*, 1994). It was recently demonstrated that Ets2 inhibits

apoptosis through binding to the *BclxL* promoter and upregulates its expression (Sevilla *et al.,* 1999). Therefore, since *BclxL* was up-regulated in the EBNA 1 positive samples, yet no EBNA 1 binding sites have been found in the *BclxL* promoter, it can be postulated that EBNA 1 may upregulate *BclxL* via *Ets2*. However, due to lack of probe availability *Ets2* expression results were not examined further.

cRel belongs to the NFκB/Rel family of transcription factors. There are at least 5 *Rel* family members that are involved in B cell development. These include *RelA* (p65), *RelB*, *cRel*, *NFκB1* (p105) and *NFκB2* (P100). The latter two are processed by proteolytic cleavage to generate the p50 and p52 subunits respectively, which can generate hetero- and homodimers. NFκB/rel dimers are located in the cytoplasm where they associate and are regulated by IκB proteins (Baeuerle and Henkel 1994; Siebenlist *et al.,* 1997; Thanos and Maniatis, 1995; Verma *et al.,* 1995). Interestingly, while *cRel* was expressed at a higher level in the EBNA 1 cDNA array, *IκB* (its opposing factor) was expressed at a lower level (3.3x reduced), suggesting a concerted action. However, due to the lack of plasmid probe availability the observed changes in the expression of both *cRel* and *IκB* cannot be confirmed at present. Nevertheless, it can be suggested that the changes in the expression of both these genes are not a coincidence and could be due to EBNA 1 action. In order to see if these changes are definitely due to EBNA 1 regulation, further expression analysis at the RNA and protein level should be pursued using material from selected B cells.

NFκB was originally characterised as a pre-B cell inducible transcription factor. Moreover, it is constitutively activated in mature B cells (Sen and Baltimore, 1986). A hierarchy of NFκB dimers during B cell development has been suggested; pre-B cells primarily have p50/p65 dimers, mature cells have cRel/p50 dimers and plasma cells have p52/RelB dimers (Fig. 5.8 and 5.9) (Liou *et al.,* 1994; Miyamoto *et al.,* 1994). Recent experiments in which a dominant-negative, non-degradable form of IκB was overexpressed in pre-B cells indicated that the NFκB/Rel proteins are necessary for B cell differentiation (Scherer *et al.*, 1996). In addition to the regulation of gene expression of genes important for immune and inflammatory responses, Rel/NFκB also controls the transcription of genes that confer resistance to death-inducing signals. These genes include those encoding the caspase inhibitors cIAP1 and cIAP2 and the TNF receptor-associated factors TRAF1 and TRAF2 (Wang *et al.,* 1998a). Interestingly, it was recently demonstrated that cRel upregulates *BclxL*

expression through binding and activating the *bclx* promoter (Chen *et al.*, 2000). These results are consistent with the increased expression of *BclxL* and *cRel* in the EμEBNA 1 transgenic positive samples. Therefore, if EBNA 1 upregulates *cRel* and down-regulates *IκB* then this would affect downstream targets of cRel transcriptional activation which would lead to increased cell survival and immunoglobulin gene rearrangement and possibly promote B cell differentiation. Increased Ig rearrangement in B cells at an earlier than usual, stage could cause these cells to be positively selected and lead to the accumulation of these cells in the spleen or other lymphoid tissues.

5.5.4 SATB1

The gene showing highest levels of expression (4.1-fold) in the EμEBNA 1 positive samples in comparison to wt controls in the cDNA array is *SATB1*. However, in the slot blot analysis using splenocyte RNA (2 month old) from pre-neoplastic and tumour tissues, *SATB1* de-regulation was not statistically significant. In the slot blot containing the 2 month old selected B cell RNA, *SATB1* expression was higher in the transgenic positive samples, but only close to statistical significance (with 26.1318 included, and statistically significant when this mouse is not included). This could be attributable to the high variation in the obtained values but also due to cell heterogeneity in the tissue. *SATB1* is predominantly expressed in T cells. Therefore, it can be postulated that EBNA 1 upregulates *SATB1* in B cells, but the signal originating from the T cells may be stronger and obscures that from the B cells. Alternatively, this result may be a false positive due to the very low expression of *SATB1* in B cells, where any small variation might show a significant difference. The results from the SATB1 protein level analysis were unclear since a positive SATB1 control (for the mammalian SATB1) was not included in the assay. For further experiments, since SATB1 is predominantly expressed in thymocytes, protein extracts from wt mouse thymocytes could provide the appropriate mammalian SATB1 positive protein control. Alternatively, *in vitro* expressed purified mammalian SATB1 protein could be used as positive control in this assay.

SATB1 is a cell type specific nuclear matrix or scaffold (MAR/SAR)-associated DNA-binding protein (Dickinson *et al.*, 1992). Experiments in transgenic mice and in transfected cells suggest that SATB1 generally exerts a negative

regulatory effect on gene expression (Kohwi-Shigematsu *et al.*, 1997; Liu *et al.*, 1997). Interestingly, the regulatory regions of several genes including $^{A}\gamma$-globin, CD8, Xlr nuclear protein and the long terminal repeat (LTR) of mouse mammary tumour virus, have been shown to contain matrix attachment regions (MARs) that bind SATB1 (Cunningham *et al.*, 1994; Banan *et al.*, 1997; Liu *et al.*, 1997; Escalier *et al.*, 1999). The nuclear matrix is an insoluble structural network of proteins within the nucleus and is involved in DNA replication, steroid hormone binding, gene transcription and hnRNA processing. Specific regions of cell DNA are attached to the nuclear matrix and called matrix attachment regions (MARs). The cell DNA located between the MAR sites form chromatin loops or domains representing functional units of replication or transcription (Nelson *et al.*, 1986; Bodnar, 1988).

Interestingly, EBNA 1 has been implicated in the nuclear matrix attachment (*ori*P is contained within or is immediately adjacent to a high affinity MAR) and also has a negative regulatory effect on gene transcription (see Introduction sections 1.3.III and 1.3.IV).

If EBNA 1 really upregulates *SATB1* this may have important secondary effects. Increased SATB1 expression may result in down-regulation of expression of genes responsive to SATB1. If some of these genes encode proteins that normally restrict expansion of B cells, this could partially explain the oncogenic role of EBNA 1 observed in the transgenic lines.

hnRNPD was included in this assay because it is a component (along with nucleolin) of the LR1 protein, another nuclear matrix binding protein. It is thought that LR1 binds near the *c-Myc* promoter and has roles in the transformation and regulation of c-Myc in B cell lymphomas (Brys and Maizels, 1994). It has also been shown to regulate EBNA 1 from the Fp promoter (Bulfone-Paus *et al.*, 1995). However, *hnRNPD* expression results were confusing. In the slot blots with the 2 month old splenocyte RNAs, *hnRNPD* showed a higher ratio, but was not statistically significant, in both pre-neoplastic tissues and in tumours. Moreover, in the slot blots with the selected B cell RNA, *hnRNPD* had still higher expression in the transgenic pre-neoplastic tissues but statistically significantly lower expression in the tumours. Down-regulation of *hnRNPD* may be a post-transformation event not necessarily mediated by EBNA 1.

5.5.5 BclxL confirmation

Table 5.4 BclxL RNA and protein expression results

	RNA level					Protein level
Gene	**Pre-Tumour stage**				**Spleen Tumour**	**2M Selected B cells**
	1M Spleen Ratio	2M spleen Ratio	2M Splenocytes Ratio	2M selected B cells Ratio	Ratio	Ratio
BclxL	1.3 (0.011)	1.5	2.68 (0.018)	1.6 (0.033)	4.7 (0.0009)	1.2

Analysis of the expression levels of the anti-apoptotic gene *BclxL* (a Bcl2 family member) showed an up-regulation of *BclxL* RNA which was supported at the protein level. These results are summarised in Table 5.4, where the ratios and the statistically significant P values are given (the latter in brackets). At the RNA level, in both 2 month old splenocyte and selected B-cell RNA, *BclxL* expression was consistently higher in the EμEBNA 1 positive samples when compared to the wt controls. The result from 2 month old spleen gave a 1.5 ratio without significance. This difference may be due to the specificity of the RNA used. Because *BclxL* is expressed in many different cell types in addition to B cells, the signal from the other cell types (e.g. T cells) might interfere with that from the B cells. In contrast, in the case of the splenocyte and selected B cell RNA the B cell signal is more enriched and the result more reliable. Interestingly, *BclxL* up-regulation was consistent at the tumour stage as well. Moreover, in addition to the RNA level, BclxL up-regulation was extended to the protein level by 1.2-fold, at a level comparable to that observed in the RNA studies. However, since there was no BclxL positive control included in the western blot it was difficult to say with certainty, which was the BclxL band. As control one could have used the peptide epitope that was used to produce the antibody for BclxL to block the antibody interaction. Alternatively, a recombinant BclxL expressed in transfected cells could have provided a positive control. However, neither the peptide or such a construct were available at the time the western blots were prepared.

These results strongly support the previous suggestion that EBNA 1 may act in the same way as Bcl2 and exerts its oncogenic action at least in part through up-regulation of *BclxL*. Eμ-*BclxL* transgenic mice show accumulation of immature and mature B cells, suggesting that BclxL supports the maturation of pre-B cells to mature B cells. Therefore, there is a possibility that EBNA 1 may upregulate *BclxL*

expression and drive cells into a more mature stage. This hypothesis is supported by the increased percentage of sIg+ B cells in the EBNA 1 transgenic positive samples.

5.5.6 Genes expressed at lower levels in EBNA 1 samples

A number of cellular genes showed lower expression levels in the mouse Atlas expression array including a tumour suppressor gene and several kinases. However, results were not confirmed in slot blot analysis for several of these genes in the cDNA array due to restriction of probe availability.

The human haemopoietic cell kinase (HCK) is a member of the Src family of protein tyrosine kinases. *HCK* expression was found to be 5-fold lower in the Atlas array probed with EμEBNA 1 positive cDNA. In the subsequent slot blot analysis in splenocyte and selected B cell RNA samples, *HCK* expression difference was not statistically significant. In tumours, *HCK* expression was found to be lower when compared to either 2 month old splenocyte (0.78x) or selected B cell (0.856x) wt controls, but again with no statistical significance. Therefore the Atlas finding was not confirmed. HCK is expressed in cells of the granulocytic and monocytic lineage and at low levels in B lymphocytes and NK cells. Its expression is regulated at the level of transcription and increases during myeloid differentiation of normal and leukaemic haemopoietic cells. Sp1 appears to play an essential role in the regulation of the differential gene expression of the *HCK* gene. Therefore, since HCK expression is normally low in B cells, the slightest variation in its expression level would appear quite dramatic as in the mouse Atlas. Moreover, in the slot blot analysis the high variation in the samples and also the heterogeneity in the cell population of the tissues from which the RNA was extracted, could also be responsible for the result.

The *PML* tumour suppressor gene expression level was found to be 5.7-fold lower in the EμEBNA 1 transgenic positive when compared to wt RNA by the mouse Atlas assay. This was not confirmed in the subsequent slot blot analyses. In the splenocyte 2 month old and tumour RNA, *PML* expression was found slightly higher (albeit not to statistical significance) in the transgenic positives. These contrasting results could be due to the difference in the RNA source. Again the presence of other cell types in the splenocyte samples may affect the results. Therefore, the signal originating from other cells may be stronger than that of B cells

and thus distort the final result. However, as *PML* expression was not tested in the selected B cell slot blots these results still need to be extended and therefore it is not possible to draw any final conclusions.

PML is a phosphoprotein associated with the nuclear matrix and is localised to the nuclear bodies (NB), (Dyck *et al.*, 1997). PML has been regarded as a growth regulator and shares many properties with tumour suppressors (Mu *et al.*, 1994; Guiochon-Mantel *et al.*, 1995; Liu *et al.*, 1995; He *et al.*, 1997). PML is expressed in most tissues, but its expression is regulated during normal haematopoiesis in a differentiation and lineage specific manner. PML is required in early haematopoiesis and erythropoiesis (Wang *et al.*, 1998b; Labbaye *et al.*, 1999). PML controls genes (LMP-2, LMP-7, TAP-1 and TAP-2) involved in MHC class I antigen presentation (Zheng *et al.*, 1998). This function is consistent with previous findings that PML expression is low in normal tissues with low levels of MHC class I (Gambacorta *et al.*, 1996) but also in tumours with reduced cell surface MHC class I molecules due to defective antigen presentation (Korkolopoulou *et al.*, 1996). Down-regulation of PML occurs in invasive and metastatic lesions in a large collection of tumours (Koken *et al.*, 1995; Gambacorta *et al.*, 1996) possibly due to disruption of the nuclear bodies in the tumour cells. Disruption of PML nuclear bodies is also caused by cytomegalovirus (Ahn and Hayward, 1997), herpes virus (HSV1) (Everett and Maul 1994; Everett *et al.*, 1997) and adenovirus infection (Rotem-Yehudar *et al.*, 1996), as a consequence of the interaction of viral proteins with PML NB protein complexes (Ahn *et al.*, 1998). Therefore, down-regulation of PML may enable a tumour or a virus to evade the immune defence of its host. Interestingly, EBNA 1 avoids immune recognition through its Gly-Ala repeat (Introduction section 1.3) which was shown to inhibit *in cis* the presentation of MHC class I restricted cytotoxic T cell epitopes probably by interfering with ubiquitin/proteasome-dependent protein degradation (Levitskaya *et al.*, 1995; 1997). If EBNA 1 downregulates *PML* this effect could lead to down-regulation of the genes devoted to MHC class I antigen presentation resulting in reduced expression of MHC class I molecules on the cell surface. Thus it can be suggested that EBV may have developed an additional route of escaping the host immune recognition through transcriptional regulation function of EBNA 1.

STAT3 is a member of the signal transducers and activators of transcription family of proteins and its expression level was lower in the EBNA 1 positive array in

comparison to the wt array. STAT3 is usually constitutively activated in cells transformed by oncogenic tyrosine kinases like Src and by human tumour viruses such as HTLV-1 (Migone *et al.,* 1995; Yu *et al.,* 1995). It was recently reported thatSTAT3 is constitutively activated in BM myeloma cells and that it contributes to this pathogenesis by preventing apoptosis through a BclxL-dependent mechanism (Catlett-Falcone *et al.*, 1999). However, since there was no probe fragment available, the analysis of STAT3 expression did not proceed further.

The rest of the genes that showed lower expression levels in the differential mouse Atlas expression array were not tested by the slot blot approach. Therefore, it is not clear at present if these are truly differentially regulated or if they are false positives from the Atlas.

5.5.7 Conclusions and future experiments

In general, the results from selected B cell RNA slot blots contrasted with the results from either the Atlas or splenocyte or total spleen RNA slot blots. Since the Rags did not show any statistically significant differences in these blots, this particular assay may have suffered from technical problems. Specifically, the blots were used at least 4 times each before hybridisation with the *Rag* probes, whereas *BclxL* was the first probe used. Therefore, it is possible that much of the RNA may have been lost, giving less reliable ratios. Thus, repeating hybridisation with certain probes on new slot blots using selected B cell RNA may be required to confirm the gene expression ratios obtained here.

There are cases where a gene may be up-regulated pre-tumour and down-regulated in the tumours and vice-versa. It can be postulated that certain events may occur pre-tumour to promote proliferation or cell survival but no longer be needed after secondary events have taken place because they may have been superseded by the latter. Thus an EBNA 1 effect pre-tumour could be masked by a secondary event in the tumour leading to the observed results.

Several observations suggest that EBNA 1 drives cells through development and differentiation by promoting, either directly or indirectly, the expression of genes involved in these pathways. In addition, it can be postulated that EBNA 1 may hold cells at a particular "normal" B cell phenotype. If these cells were normally negatively selected and destroyed by the host immune system, EBNA 1 may have

assisted these to escape negative selection and allow accumulation in the lymphoid tissues.

An alternative protocol to identify genes which may be de-regulated by EBNA 1 is subtractive hybridisation. In this method there is no restriction to the number of genes tested in contrast to the cDNA expression array where a fixed number of genes are tested. In this technique known or even novel genes may be isolated and identified which could be targets for EBNA 1 regulation. However, in this approach subtle changes are unlikely to be selected. Subsequent analysis will involve assays to examine if any de-regulated genes are direct or indirect targets of EBNA 1 action. This can be tested by transfection of B cells with a construct containing the promoter region of the gene of interest upstream of a reporter gene and a second construct expressing EBNA 1. Genes identified in this assay could be tested for steady state protein expression as well as protein activity by western blot and EMSAs respectively.

Alternatively, for example in the case of BclxL, protein localisation using FACS would be useful in assessing in which subtype of B cells BclxL expression is up-regulated.

Chapter 6

Final discussion and future directions

The occurrence of lymphoma in two different transgenic mouse lines for EBNA 1 is strong evidence that EBNA 1 is an oncogene *in vivo*. These mice develop monoclonal B cell lymphomas (Wilson *et al*, 1992; 1996) indicating that although EBNA 1 predisposes cells to tumour formation it is insufficient for lymphoma development and that secondary events are required.

EBNA 1 is a DNA binding protein and (at least in the viral genome) functions as a transcriptional transactivator and under some circumstances, as a transcriptional repressor. Furthermore it also binds to RNA and may regulate post-transcriptional events.

While EBNA 1 can act as an oncogene *in vivo*, the mechanism(s) by which EBNA 1 assists in the transformation of B cells is unclear. It has been the aim of this body of work to begin to address the role of EBNA 1 in lymphomagenesis by assessing the ability of EBNA 1 to modify gene expression in pre-tumour B cells, and thereby to modify the B cell phenotype. These phenomena have been addressed by a number of different approaches.

Differential expression analyses revealed that EBNA 1 up-regulates *BclxL, Rag1* and *Rag2* gene expression at both the pre-tumour stage and in tumours (Chapters 4 and 5). Results from a differential display cDNA expression array suggest that EBNA 1 possibly deregulates the expression of *Egr1, Ikaros, EBF, Pax5,* and *AP1* family members amongst others (Chapter 5). Flow cytometric analysis of splenic and BM cells from EμEBNA 1 transgenic mice revealed that the percentage of sIg+ (and possibly CD23+) cells was statistically significantly higher (73.3% versus 54% in the spleen and 64.5% versus 46.65% in the BM) when compared to the control littermates. Moreover, EμEBNA 1 transgenic positive BM cells have a statistically significant higher proliferation/ survival rate than the wt BM cells, as was shown by a cell survival/proliferation assay (Chapter 3).

BclxL de-regulation by EBNA 1 is consistent with our earlier observation that EBNA 1 is redundant with Bcl2 in a tumour latency assay and therefore it could act

through the Bcl2 family members to exert its oncogenic effects. The fact that these particular genes are (possibly) deregulated by EBNA 1 is very interesting because they are normally expressed during particular "windows' through B cell development and differentiation. *BclxL* is highly expressed in pre-B and germinal centre (GC) cells but it is down regulated as B cells mature (Fang *et al.,* 1996; Grillot *et al.,* 1996).

EBNA 1 as a transcriptional transactivator could up-regulate *BclxL* expression by direct action at the promoter of *BclxL* (by binding directly or via an adaptor protein). However, EBNA 1 binding sites are not evident in the *BclxL* promoter. Alternatively, EBNA 1 could indirectly up-regulate *BclxL* expression by up-regulating transcription factors, which then increase expression of *BclxL.*

BclxL expression is regulated at the transcriptional level, yet only three transcription factors (Ets2, Stat3 and cRel) have been shown to specifically activate this promoter. Recently Sevilla *et al.*, showed that the Ets2 transcription factor could inhibit apoptosis upon growth factor deprivation through transactivating *BclxL* (Sevilla *et al.*, 1999). Interestingly, *Ets2* expression was found to be higher by 2.2-fold in the EμEBNA 1 positive versus wt array. However, due to lack of probe sequence its expression was not analysed further. Catlett-Falcone *et al.,* (1999) showed that constitutive activation of Stat3 signalling by IL6 confers resistance to apoptosis in myeloma cells by induction of *BclxL* expression. However, the *Stat3* expression level was lower (0.3x) in the EμEBNA 1 positive compared to the wt Atlas expression array. Furthermore, IL6 in EμEBNA 1 transgenic and wt culture experiments supported both cultures equally (Chapter 3). Interestingly, it was recently demonstrated that cRel up-regulates *BclxL* expression by directly binding to the *Bclx* promoter (Chen *et al.,* 2000). *cRel* expression was found to be higher (2x) and the inhibitor *IκB* lower (3.3x) in the EμEBNA 1 positive Atlas array as compared to the wt array. Thus, Ets2 and/or NFκB (cRel) but probably not Stat3 may be possible candidates for indirect *BclxL* regulation by EBNA 1.

Rag1 and *Rag2* expression are primarily restricted to developing lymphocytes that are undergoing rearrangement of their antigen receptor genes (Fig. 4.8). *Rag1* and *Rag2* mRNA is first detected in pro-B cells but is down-regulated in early large B cells upon completion of IgH rearrangements (Grawunder *et al.,* 1995a). The *Rag* genes are then transcribed again in small pre-B cells to allow light chain gene rearrangements (Fig. 4.9) (Grawunder *et al.*, 1995b). It was more recently

demonstrated that *Rag1,2* are re-expressed in activated mature B cells both *in vivo* (in GCs) and *in vitro* (Hikida *et al.*, 1996).

Egr1 expression is detected in pro-B and pre-B (B220+/sIgM$^-$) cells isolated from BM and foetal liver-derived pre-B cell cultures (Dinkel *et al.*, 1998). *Ikaros* gene expression seems to increase as B cells mature (Georgopoulos *et al.*, 1997). *EBF* is expressed at the very early stages of B cell development. Finally, *Pax5* is expressed in all stages of B cell development except plasma cells.

The fact that all these genes were found deregulated in the EμEBNA 1 transgenic positive samples is very important and may indicate a particular B cell developmental stage at which EBNA 1 acts.

In vitro (Kuhn-Hallek *et al.*, 1995; Srinivas and Sixbey, 1995) and now *in vivo* (Chapter 4) *Rag* gene expression was found to be up-regulated by EBNA 1. *Rag* gene expression is regulated by different transcription factors that are possibly regulated by EBNA 1. Ikaros binding sites have been described in the promoters of a number of genes including *Rag1* and *Rag2* (Georgopoulos *et al.*, 1997). Moreover, Pax5 binding sites are found within the *Rag2* promoter and this binding is critical for its function (Lauring and Schlishel, 1999). Thus, EBNA 1 could up-regulate the *Rag* genes through up-regulation of either *Ikaros* or *Pax5*. However, *Pax5* expression is in turn regulated by EBF (O'Riordan and Grosschedl, 1999). Therefore, a cascade is formed consisting of EBF→Pax5→Rag1, 2, through which EBNA 1 could either directly or indirectly regulate *Rag* gene expression.

As a transcriptional regulator EBNA 1 may directly effect the expression of cellular proto-oncogenes and tumour suppressor genes. This could be achieved by DNA binding, EBNA 1 may bind to the cellular gene promoters. Alternatively, EBNA 1 may interact with cellular proteins that regulate gene expression. These possibilities can be addressed by the use of transient assay systems that are based on the use of reporter genes. In addition, as the EBNA 1 binding consensus is not absolute, and alternatively EBNA 1 may not directly interact with a given promoter, but perhaps with cellular proteins that do interact with the DNA sequence, potential interactions could be examined by mobility shift assays or the yeast 2-hybrid system.

Another approach in determining if EBNA 1 acts in a particular B cell subtype would be to test EBNA 1 expression levels in B cell fractions. The EμEBNA 1 transgene contains the IgH enhancer (Eμ), which should direct EBNA 1 expression in most B cells (after the pre-B cells stage). However, the Eμ activity in

each B cell compartment may differ and therefore EBNA 1 may be expressed more in a particular B cell type and less in another (and hence more effective in a particular B cell subtype). Protein extraction from sorted B cells and western blotting would help to detect EBNA 1 expression in the different B cell compartments.

Given that *BclxL* is up-regulated in EμEBNA 1 transgenic mice it is worthwhile comparing the phenotype results of EμEBNA 1 mice with Eμ-*BclxL* and Eμ-*Bcl2* transgenic mice. Both EμEBNA 1 and Eμ-*Bcl2* transgenic mice develop tumours whereas tumour development in Eμ-*BclxL* transgenic mice has not been reported. Tumour incidence differs with EμEBNA 1 developing tumours at 4-12 (sometimes earlier) months and Eμ-*Bcl2* mice with a later onset, usually of over 8 months. The difference in tumour incidence makes a direct comparison between EμEBNA 1 and Eμ-*Bcl2* transgenic mice difficult because there are many factors that need to be considered. For example, although both transgenes contain the IgH enhancer Eμ, we are not sure about the relative expression levels of the transgenes. EμEBNA 1 mice develop monoclonal tumours as do Eμ-*Bcl2* transgenic mice, which present a polyclonal hyperplasia, that then develops into monoclonal lymphoma (McDonnell and Korsmeyer, 1991). Both Eμ-*Bcl2* and Eμ-*BclxL* transgenic mice show modest splenomegaly at approximately 5 months of age (Fang *et al.*, 1996; McDonnell *et al.*, 1989). In this study mice were examined at 2 months of age when most did not show splenomegaly. The occasional EμEBNA 1 mouse with splenomegaly was excluded from the pre-tumour studies.

Splenomegaly in the Eμ-*Bcl2* and Eμ-*BclxL* transgenic mice was due to enlargement of the B cell compartment which was found to be due to a survival advantage conferred to the cells by the over-expression of either Bcl2 or BclxL. In the wt mice while Bcl2 plays a critical role in the survival of mature lymphocytes (Nakayama *et al.*, 1993), BclxL is important for the survival of immature lymphocytes and GC cells (Motoyama *et al.*, 1995). Thus, since EBNA 1 is redundant with Bcl2 in lymphoma onset and up-regulates *BclxL* it might be predicted that EμEBNA 1 transgenic cells would survive much better than the wt cell cultures. Eμ-*BclxL* transgenic explant B cell cultures and Eμ-*Bcl2* transgenic positive explant or primary cell cultures showed survival advantage over the control cells upon serum starvation (McDonnell *et al.*, 1989; Fang *et al.*, 1996; Takahashi *et al.*, 1999). In contrast, EμEBNA 1 transgenic positive cultures showed no difference to the wt

cultures under any conditions tested. An apoptosis assay using AnnexinV-FITC/PI staining showed similar results. Nevertheless, since culture conditions between the Eμ-*BclxL* or Eμ-*Bcl2* and EμEBNA 1 cells as well as the age of the mice were different, a direct comparison would not be appropriate. Whilst the Eμ-*BclxL* cultures consisted of selected B cells, the EμEBNA 1 cell cultures contained other cells such as T cells which would have undergone apoptosis and therefore any evidence of survival or delay in apoptosis of B cells may have been obscured. Therefore, to eliminate factors that may influence the B cell response to growth factors or FCS concentration and cell survival, a B cell selection and/or sorting prior to culture should be undertaken. Alternatively, cell sorting could be done on the cell cultures to analyse which B cell population EBNA 1 supports/effects. TUNEL assay on spleen cells or spleen sections from transgenic and control mice would be useful to determine if EBNA 1 inhibits apoptosis of B cells since it up-regulates *BclxL* or possibly by acting in the same way as Bcl2.

These approaches could also be performed on older animals. The cells tested in the experiments described in the previous chapters derived from 1 month and 2 month old mice. These mice are predisposed to tumours by EBNA 1 with a tumour incidence between 4-12 months. Thus there is a period between 2 and 4 months (some times more than 4 months) when mice may still be free of tumours. Thus, there is a possibility that changes in gene expression and hence cell phenotype due to EBNA 1 effect, may be more obvious in the older mice of this group. In this way a direct comparison with the Eμ-*BclxL* and Eμ-*Bcl2* (6 months old) would be possible.

Based on the redundancy between EBNA 1 and Bcl2 shown by transgenic crossbreeding, up-regulation of BclxL by EBNA 1 and the expression patterns of *BclxL* (Fig. 4.8 and 4.9), it can be postulated that EBNA 1 may increase B cell survival but nevertheless support B cell differentiation. This could explain the increase in the sIg+ B cell population in the EμEBNA 1 transgenic mouse spleens and the BM. In the BM, B cells are present up to naïve cell stage (IgM+) recognised by sIg antibodies. In addition, plasma cells may return to the BM, but these would express low sIg or are negative for sIg. As the sIg antibody recognises surface IgM, IgG and IgA, individual antibody staining for these markers is required to further characterise the increase in the sIg+B220+ cell population in both the EμEBNA 1 spleen and BM.

In contrast to Eμ-*Bcl2* and Eμ-*BclxL* transgenic mice with a 2 to 3-fold

increase of IgM+/B220+ B cells, the percentage of these cells were comparable between EBNA1 positive and wt sibling mice. Similar results were obtained for IgM+/IgD+ B cells in EBNA 1 and wt control mice in contrast to the 3 to 4-fold increase observed in the Eμ-*Bcl2* mice. However, by examining older EμEBNA 1 transgenic mice we may also see increase in the B cell number.

The increased IgM+/IgD+ B cell population in the Eμ-*Bcl2* transgenic spleen suggested that Bcl2 supports B cell differentiation. This was supported by the 2-fold increase in the serum Ig levels for IgG and IgA while IgM levels were comparable to those in normal littermates (McDonnell *et al,* 1989). In contrast, BclxL seemed to support antibody producing cells (APCs) but did not alter antibody production since serum Ig levels were comparable between Eμ-*BclxL* transgenics and wt controls (Takahashi *et al.,* 1999). Thus, by immunisation of the animals with a specific antigen and subsequent measurement of the secreted IgG in the serum by ELISA it would be possible to monitor if EBNA 1 effects B cell maturation and antibody production.

As mentioned earlier, *BclxL* is re-expressed in GC B cells with expression confined to the centrocyte subset in which (antigenic) selection is thought to occur (Tuscano *et al.*, 1996). Apoptotic cell death in GCs of Eμ-*BclxL* transgenic mice was significantly reduced and led to the persistence of many B cells carrying VDJ rearrangements that would normally be negatively selected (Takahashi *et al.*, 1999). Since EBNA 1 up-regulates *BclxL* it might be predicted to see a similar effect on the GC development and antibody responses of the EμEBNA 1 transgenic spleens. These could be tested initially by immunisation of the animals with an antigen followed by immunocytochemistry during the reactive period. Also looking at VDJ re-arrangements it would be possible to see if EμEBNA 1 transgenic positive cells have increased VDJ recombination. Increased VDJ recombination/hypermutation and increased survival might lead to decreased negative selection, retention of high (or even low) affinity B cells and accumulation of these "inappropriately" selected B cells in the GC. This is because GCs are sites where cells which are not positively selected undergo apoptosis and become phagocytosed. Therefore, since EBNA 1 upregulates *BclxL* the EμEBNA 1 positive cells will survive longer and accumulate in these compartments.

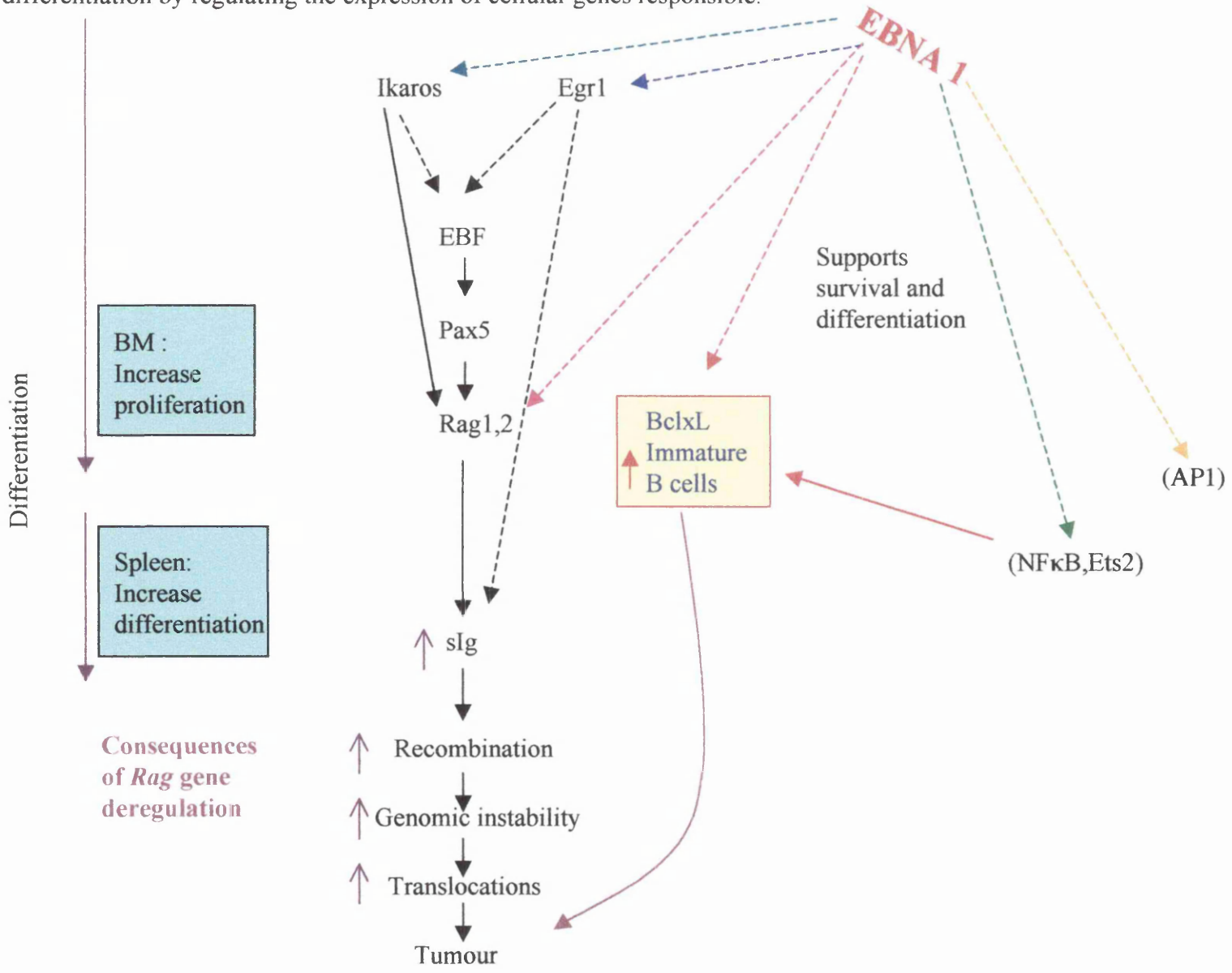
Eμ-*BclxL* B cells displayed some evidence of increased proliferation (using a BM cell culture assay) whereas Eμ-*Bcl2* cells were not tested (McDonell *et al.*, 1989;

Takahashi *et al.*, 1999). Similarly to Eμ-*BclxL* cells, EμEBNA1 transgenic BM cell cultures (grown in methylcellulose medium supplemented with IL-7 for selective growth) showed a statistically significant proliferative advantage over the wt control BM cell cultures (Fig. 3.16). However, the proliferating cell populations still need to be characterised. This can be done by pooling the methylcellulose colonies together and doing a FACS analysis using B cell surface markers. In addition, analysis of the morphology of the cells within the colonies would be informative. In addition the proliferative activity of the spleen GC compartments of both transgenic and control animals could be tested by BrdU incorporation *in vivo* followed by FACS. BrdU will only be incorporated into proliferating cells.

Based on the above observations and assumptions the diagram in Fig. 6.1 represents a possible model of EBNA 1 action which may lead to tumour development. A combination of the *Bcl2* and *BclxL* expression pattern throughout mouse life and the findings that EBNA 1 is redundant with Bcl2 and that *BclxL* and *Rag1, 2* are up-regulated in EBNA 1 pre-neoplastic and tumour tissues, as compared to the wt ones, would suggest that EBNA 1 may effect B cell maturation. Particularly, *BclxL* and *Rag1,2* genes apart from immature B cells (in both BM and spleen) are re-expressed in the germinal centres where B cell maturation takes place. In addition *Egr1* is expressed in both pre-B cells but also in mature/differentiated B cells. Therefore it can be postulated that EBNA 1 may drive cells through development and differentiation possibly indicated by the increased percentage of sIg+ B cells in both EμEBNA 1 transgenic spleen and BM. Alternatively, EBNA 1 may support the survival/proliferation of immature B cells supported by the methycellulose colony numbers and then drives them to differentiate and survive leading to increased sIg. However, the increased percentage of sIg may not be due to increased B cell differentiation *per se* but instead EBNA 1 may effect antibody production via the upregulation of the *Rag1,2* genes.

Alternatively, EBNA 1 could simply induce aberrant expression of genes that do not normally co-express or repress the expression of genes responsible for the control of immune response e.g. interferon responsive genes including those coding for JAKs and STATs. JAK3 and STAT 3 both showed lower expression levels in the EμEBNA 1 positive Atlas array. Binding of IFNα/βor IFNγ to their specific receptors, activates different sets of kinases within the JAK family which then leads to activation of different combinations of STAT proteins which then transactivate

Fig. 6.1 Model of the mechanism of action of EBNA 1. EBNA 1 may drive cells through development and differentiation by regulating the expression of cellular genes responsible.



downstream genes. Interferons are produced upon viral infection and prevent viruses from spreading efficiently, allowing the adaptive immune response enough time to develop and eliminate the virus. Particularly, IFNγ was found to inhibit both B cell proliferation and infection by EBV (Lotz *et al.,* 1985). Therefore, by down-regulating IFN pathway genes (i.e. those coding for JAKs/STATs) or by up-regulating their inhibitors (Hoey and Schindler, 1998; Leonard and O'Shea, 1998), EBNA 1 could counteract the anti-proliferative action of INFγ as well as weaken the host's immune response, therefore facilitating EBV maintenance in the B cells.

Moreover, EBNA 1 avoids immune recognition through its Gly-Ala repeat which was shown to inhibit *in cis* the presentation of MHC class I restricted cytotoxic T cell epitopes probably by interfering in the ubiquitin/proteasome-dependent protein degradation (Levitskaya *et al.*, 1995; 1997). Interestingly, PML a regulator of genes devoted to MHC class I antigen presentation, is possibly down-regulated by EBNA 1 (Chapter 5) and if this is true it could result in reduced expression of MHC molecules on the cell surface. Thus, it can be suggested that EBV may have developed an additional route of escaping the host immune recognition through EBNA 1 transcriptional regulation function.

Based on the results discussed in this thesis which are suggestive of an apoptosis inhibition function by EBNA 1 and the possibility that EBNA 1 may decrease negative selection in the GCs through BclxL up-regulation, it can be suggested that the virus takes advantage of the host cell environment in order to survive. By extending the survival of the virally infected cells and reducing the immune response to virally infected cells the virus can persist. EBNA 1 alone may perform many other functions.

References

Abbot, S. D., Rowe, M., Cadwallader, K., Ricksten, A., Gordon, J., Wang, F., Rymo, L. & Rickinson, A. B. (1990). Epstein-Barr virus nuclear antigen 2 induces expression of the virus- encoded latent membrane protein. *J Virol* **64**, 2126-34.

Acton, D., Domen, J., Jacobs, H., Vlaar, M., Korsmeyer, S. & Berns, A. (1992). Collaboration of pim-1 and bcl-2 in lymphomagenesis. *Curr Top Microbiol Immunol* **182**, 293-8.

Adams, B., Dorfler, P., Aguzzi, A., Kozmik, Z., Urbanek, P., Maurer-Fogy, I. & Busslinger, M. (1992). Pax-5 encodes the transcription factor BSAP and is expressed in B lymphocytes, the developing CNS, and adult testis. *Genes Dev* **6**, 1589-607.

Adams, J. M. & Cory, S. (1991). Transgenic models of tumor development. *Science* **254**, 1161-7.

Adams, J. M., Harris, A. W., Pinkert, C. A., Corcoran, L. M., Alexander, W. S., Cory, S., Palmiter, R. D. & Brinster, R. L. (1985). The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. *Nature* **318**, 533-8.

Ahn, J. H., Brignole, E. J., 3rd & Hayward, G. S. (1998). Disruption of PML subnuclear domains by the acidic IE1 protein of human cytomegalovirus is mediated through interaction with PML and may modulate a RING finger-dependent cryptic transactivator function of PML. *Mol Cell Biol* **18**, 4899-913.

Ahn, J. H. & Hayward, G. S. (1997). The major immediate-early proteins IE1 and IE2 of human cytomegalovirus colocalize with and disrupt PML-associated nuclear bodies at very early times in infected permissive cells. *J Virol* **71**, 4599-613.

Aiyar, A., Tyree, C. & Sugden, B. (1998). The plasmid replicon of EBV consists of multiple cis-acting elements that facilitate DNA synthesis by the cell and a viral maintenance element. *Embo J* **17**, 6394-403.

Albanese, C., Johnson, J., Watanabe, G., Eklund, N., Vu, D., Arnold, A. & Pestell, R. G. (1995). Transforming p21ras mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions. *J Biol Chem* **270**, 23589-97.

Alexander W.S., Bernard O., Cory S. and Adams ,J.M (1989). Lymphomagenesis in Emu-myc transgenic mice can involve ras mutations. *Oncogene* **4**,575-81.

Allday, M. J. & Farrell, P. J. (1994). Epstein-Barr virus nuclear antigen EBNA3C/6 expression maintains the level of latent membrane protein 1 in G1-arrested cells. *J Virol* **68**, 3491-8.

Alfieri, C., Birkenbach, M. & Kieff, E. (1991). Early events in Epstein-Barr virus infection of human B lymphocytes [published erratum appears in Virology 1991 Dec;185(2):946]. *Virology* **181**, 595-608.

Alkema, M. J., Bronk, M., Verhoeven, E., Otte, A., van't Veer, L. J., Berns, A. & van Lohuizen, M. (1997). Identification of Bmi1-interacting proteins as constituents of a multimeric mammalian polycomb complex. *Genes Dev* **11**, 226-40.

Allen, J. D. & Berns, A. (1996). Complementation tagging of cooperating oncogenes in knockout mice. *Semin Cancer Biol* **7**, 299-306.

Amati, B., Brooks, M. W., Levy, N., Littlewood, T. D., Evan, G. I. & Land, H. (1993). Oncogenic activity of the c-Myc protein requires dimerization with Max. *Cell* **72**, 233-45.

Ambinder, R. F., Mullen, M. A., Chang, Y. N., Hayward, G. S. & Hayward, S. D. (1991). Functional domains of Epstein-Barr virus nuclear antigen EBNA-1. *J Virol* **65**, 1466-78.

Ambinder, R. F., Shah, W. A., Rawlins, D. R., Hayward, G. S. & Hayward, S. D. (1990). Definition of the sequence requirements for binding of the EBNA-1 protein to its palindromic target sites in Epstein-Barr virus DNA. *J Virol* **64**, 2369-79.

Angel, P., Allegretto, E. A., Okino, S. T., Hattori, K., Boyle, W. J., Hunter, T. & Karin, M. (1988). Oncogene jun encodes a sequence-specific trans-activator similar to AP- 1. *Nature* **332**, 166-71.

Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J., Rahmsdorf, H. J., Jonat, C., Herrlich, P. & Karin, M. (1987). Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. *Cell* **49**, 729-39.

Angel, P. & Karin, M. (1991). The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim Biophys Acta* **1072**, 129-57.

Armitage, R. J. & Alderson, M. R. (1995). B-cell stimulation. *Curr Opin Immunol* **7**, 243-7.

Austin, P. J., Flemington, E., Yandava, C. N., Strominger, J. L. & Speck, S. H. (1988). Complex transcription of the Epstein-Barr virus BamHI fragment H rightward open reading frame 1 (BHRF1) in latently and lytically infected B lymphocytes. *Proc Natl Acad Sci U S A* **85**, 3678-82.

Avolio-Hunter, T. M. & Frappier, L. (1998). Mechanistic studies on the DNA linking activity of Epstein-Barr nuclear antigen 1. *Nucleic Acids Res* **26**, 4462-70.

Badiani, P., Corbella, P., Kioussis, D., Marvel, J. & Weston, K. (1994). Dominant interfering alleles define a role for c-Myb in T-cell development. *Genes Dev* **8**, 770-82.

Baer, R., Bankier, A. T., Biggin, M. D., Deininger, P. L., Farrell, P. J., Gibson, T. J., Hatfull, G., Hudson, G. S., Satchwell, S. C., Seguin, C. & et al. (1984). DNA sequence and expression of the B95-8 Epstein-Barr virus genome. *Nature* **310**, 207-11.

Baeuerle, P. A. & Henkel, T. (1994). Function and activation of NF-kappa B in the immune system. *Annu Rev Immunol* **12**, 141-79.

Bahr, G. F., Mikel, U. & Klein, G. (1975). Localization and quantitation of EBV-associated nuclear antigen (EBNA) in Raji cells. *Beitr Pathol* **155**, 72-8.

Baichwal, V. R. & Sugden, B. (1988). Transformation of Balb 3T3 cells by the BNLF-1 gene of Epstein-Barr virus. *Oncogene* **2**, 461-7.

Bain, M., Watson, R. J., Farrell, P. J. & Allday, M. J. (1996). Epstein-Barr virus nuclear antigen 3C is a powerful repressor of transcription when tethered to DNA. *J Virol* **70**, 2481-9.

Banan, M., Rojas, I. C., Lee, W. H., King, H. L., Harriss, J. V., Kobayashi, R., Webb, C. F. & Gottlieb, P. D. (1997). Interaction of the nuclear matrix-associated region (MAR)-binding proteins, SATB1 and CDP/Cux, with a MAR element (L2a) in an upstream regulatory region of the mouse CD8a gene. *J Biol Chem* **272**, 18440-52.

Banerji, J., Olson, L. & Schaffner, W. (1983). A lymphocyte-specific cellular enhancer is located downstream of the joining region in immunoglobulin heavy chain genes. *Cell* **33**, 729-40.

Baumforth, K. R., Young, L. S., Flavell, K. J., Constandinou, C. & Murray, P. G. (1999). The Epstein-Barr virus and its association with human cancers. *Mol Pathol* **52**, 307-22.

Baxter, E. W., Blyth, K., Donehower, L. A., Cameron, E. R., Onions, D. E. & Neil, J. C. (1996). Moloney murine leukemia virus-induced lymphomas in p53-deficient mice: overlapping pathways in tumor development? *J Virol* **70**, 2095-100.

Berns, A. (1991). Tumorigenesis in transgenic mice: identification and characterization of synergizing oncogenes. *J Cell Biochem* **47**, 130-5.

Berns, A., van der Lugt, N., Alkema, M., van Lohuizen, M., Domen, J., Acton, D., Allen, J., Laird, P. W. & Jonkers, J. (1994). Mouse model systems to study multistep tumorigenesis. *Cold Spring Harb Symp Quant Biol* **59**, 435-47.
Bhat, N. K., Fisher, R. J., Fujiwara, S., Ascione, R. & Papas, T. S. (1987). Temporal and tissue-specific expression of mouse ets genes. *Proc Natl Acad Sci U S A* **84**, 3161-5.

Birnboim, H. C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* 7, 1513-23.

Bochkarev, A., Barwell, J. A., Pfuetzner, R. A., Furey, W., Jr., Edwards, A. M. &
Frappier, L. (1995). Crystal structure of the DNA-binding domain of the Epstein-Barr virus origin-binding protein EBNA 1. *Cell* **83**, 39-46.

Bochkarev, A., Barwell, J. A., Pfuetzner, R. A., Bochkareva, E., Frappier, L. & Edwards, A. M. (1996). Crystal structure of the DNA-binding domain of the Epstein-Barr virus origin-binding protein, EBNA1, bound to DNA. *Cell* **84**, 791-800.

Bodescot, M., Perricaudet, M. & Farrell, P. J. (1987). A promoter for the highly spliced EBNA family of RNAs of Epstein-Barr virus. *J Virol* **61**, 3424-30.

Bodnar, J. W. (1988). A domain model for eukaryotic DNA organization: a molecular basis for cell differentiation and chromosome evolution. *J Theor Biol* **132**, 479-507.

Boise, L. H., Gonzalez-Garcia, M., Postema, C. E., Ding, L., Lindsten, T., Turka, L. A., Mao, X., Nunez, G. & Thompson, C. B. (1993). bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* **74**, 597-608.

Boise, L. H. & Thompson, C. B. (1997). Bcl-x(L) can inhibit apoptosis in cells that have undergone Fas-induced protease activation. *Proc Natl Acad Sci U S A* **94**, 3759-64.

Bonnet, M., Guinebretiere, J. M., Kremmer, E., Grunewald, V., Benhamou, E., Contesso, G. & Joab, I. (1999). Detection of Epstein-Barr virus in invasive breast cancers [see comments]. *J Natl Cancer Inst* **91**, 1376-81.

Bossy-Wetzel, E., Newmeyer, D. D. & Green, D. R. (1998). Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD- specific caspase activation and independently of mitochondrial transmembrane depolarization. *Embo J* **17**, 37-49.

Boulukos, K. E., Pognonec, P., Sariban, E., Bailly, M., Lagrou, C. & Ghysdael, J. (1990). Rapid and transient expression of Ets2 in mature macrophages following stimulation with cMGF, LPS, and PKC activators. *Genes Dev* **4**, 401-9.

Brielmeier, M., Mautner, J., Laux, G. & Hammerschmidt, W. (1996). The latent membrane protein 2 gene of Epstein-Barr virus is important for efficient B cell immortalization. *J Gen Virol* **77**, 2807-18.

Brooks, L., Yao, Q. Y., Rickinson, A. B. & Young, L. S. (1992). Epstein-Barr virus latent gene transcription in nasopharyngeal carcinoma cells: coexpression of EBNA1, LMP1, and LMP2 transcripts. *J Virol* **66**, 2689-97.

Brown, K. & Balmain, A. (1995). Transgenic mice and squamous multistage skin carcinogenesis. *Cancer Metastasis Rev* **14**, 113-24.

Brys, A. & Maizels, N. (1994). LR1 regulates c-myc transcription in B-cell lymphomas. *Proc Natl Acad Sci U S A* **91**, 4915-9.

Buckley, A. R., Buckley, D. J., Leff, M. A., Hoover, D. S. & Magnuson, N. S. (1995). Rapid induction of pim-1 expression by prolactin and interleukin-2 in rat Nb2 lymphoma cells. *Endocrinology* **136**, 5252-9.

Bulfone-Paus, S., Dempsey, L. A. & Maizels, N. (1995). Host factors LR1 and Sp1 regulate the Fp promoter of Epstein-Barr virus. *Proc Natl Acad Sci U S A* **92**, 8293-7.

Burd, C. G. & Dreyfuss, G. (1994). Conserved structures and diversity of functions of RNA-binding proteins. *Science* **265**, 615-21.

Burkhardt, A. L., Bolen, J. B., Kieff, E. & Longnecker, R. (1992). An Epstein-Barr virus transformation-associated membrane protein interacts with src family tyrosine kinases. *J Virol* **66**, 5161-7.

Busslinger, M. & Urbanek, P. (1995). The role of BSAP (Pax-5) in B-cell development. *Curr Opin Genet Dev* **5**, 595-601.

Busslinger, M., Klix, N., Pfeffer, P., Graninger, P. G. & Kozmik, Z. (1996). Deregulation of PAX-5 by translocation of the Emu enhancer of the IgH locus adjacent to two alternative PAX-5 promoters in a diffuse large- cell lymphoma. *Proc Natl Acad Sci U S A* **93**, 6129-34.

Butzler, C., Zou, X., Popov, A. V. & Bruggemann, M. (1997). Rapid induction of B-cell lymphomas in mice carrying a human IgH/c- mycYAC. *Oncogene* **14**, 1383-8

Caldwell, R. G., Wilson, J. B., Anderson, S. J. & Longnecker, R. (1998). Epstein-Barr virus LMP2A drives B cell development and survival in the absence of normal B cell receptor signals. *Immunity* **9**, 405-11.

Calender, A., Cordier, M., Billaud, M. & Lenoir, G. M. (1990). Modulation of cellular gene expression in B lymphoma cells following in vitro infection by Epstein-Barr virus (EBV). *Int J Cancer* **46**, 658-63.

Caricasole, A., Duarte, A., Larsson, S. H., Hastie, N. D., Little, M., Holmes, G., Todorov, I. & Ward, A. (1996). RNA binding by the Wilms tumor suppressor zinc finger proteins. *Proc Natl Acad Sci U S A* **93**, 7562-6.

Catlett-Falcone, R., Landowski, T. H., Oshiro, M. M., Turkson, J., Levitzki, A., Savino, R., Ciliberto, G., Moscinski, L., Fernandez-Luna, J. L., Nunez, G., Dalton, W. S. & Jove, R. (1999). Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. *Immunity* **10**, 105-15.

Ceuppens, J. L., Meurs, L. & Van Wauwe, J. P. (1985). Failure of OKT3 monoclonal antibody to induce lymphocyte mitogenesis: a familial defect in monocyte helper function. *J Immunol* **134**, 1498-502.

Chang, H. Y., Guerrero, I., Lake, R., Pellicer, A. & D'Eustachio, P. (1987). Mouse N-ras genes: organization of the functional locus and of a truncated cDNA-like pseudogene. *Oncogene Res* **1**, 129-36.

Chen, W. & Cooper, N. R. (1996). Epstein-Barr virus nuclear antigen 2 and latent membrane protein independently transactivate p53 through induction of NF-kB activity. *J Virol* **70**, 4849-53.

Chen, C., Edelstein, L. C. & Gelinas, C. (2000). The Rel/NF-kappaB family directly activates expression of the apoptosis inhibitor bcl-x(L) [In Process Citation]. *Mol Cell Biol* **20**, 2687-95.

Chen, F., Hu, L. F., Ernberg, I., Klein, G. & Winberg, G. (1995). Coupled transcription of Epstein-Barr virus latent membrane protein (LMP)-1 and LMP-2B genes in nasopharyngeal carcinomas. *J Gen Virol* **76**, 131-8.

Chen, H., Lee, J. M., Wang, Y., Huang, D. P., Ambinder, R. F. & Hayward, S. D. (1999). The Epstein-Barr virus latency BamHI-Q promoter is positively regulated by STATs and Zta interference with JAK/STAT activation leads to loss of BamHI-Q promoter activity. *Proc Natl Acad Sci U S A* **96**, 9339-44.

Chen, J., Marechal, V. & Levine, A. J. (1993). Mapping of the p53 and mdm-2 interaction domains. *Mol Cell Biol* **13**, 4107-14.

Chen, M. R., Yang, J. F., Wu, C. W., Middeldorp, J. M. & Chen, J. Y. (1998). Physical association between the EBV protein EBNA-1 and P32/TAP/hyaluronectin. *J Biomed Sci* **5**, 173-9.

Chittenden, T., Flemington, C., Houghton, A. B., Ebb, R. G., Gallo, G. J., Elangovan, B., Chinnadurai, G. & Lutz, R. J. (1995). A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions. *Embo J* **14**, 5589-96.

Chittenden, T., Lupton, S. & Levine, A. J. (1989). Functional limits of oriP, the Epstein-Barr virus plasmid origin of replication. *J Virol* **63**, 3016-25.

Chomczynski, P. & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate- phenol-chloroform extraction. *Anal Biochem* **162**, 156-9.

Chu, J. S., Chen, C. C. & Chang, K. J. (1998). In situ detection of Epstein-Barr virus in breast cancer. *Cancer Lett* **124**, 53-7.

Clarke, P. A., Sharp, N. A. & Clemens, M. J. (1992). Expression of genes for the Epstein-Barr virus small RNAs EBER-1 and EBER-2 in Daudi Burkitt's lymphoma cells: effects of interferon treatment. *J Gen Virol* **73**, 3169-75.

Clemens, M. J., Laing, K. G., Jeffrey, I. W., Schofield, A., Sharp, T. V., Elia, A., Matys, V., James, M. C. & Tilleray, V. J. (1994). Regulation of the interferon-inducible eIF-2 alpha protein kinase by small RNAs. *Biochimie* **76**, 770-8.

Cohen, D. R., Ferreira, P. C., Gentz, R., Franza, B. R., Jr. & Curran, T. (1989). The product of a fos-related gene, fra-1, binds cooperatively to the AP- 1 site with Jun: transcription factor AP-1 is comprised of multiple protein complexes. *Genes Dev* **3**, 173-84.

Cordier, M., Calender, A., Billaud, M., Zimber, U., Rousselet, G., Pavlish, O., Banchereau, J., Tursz, T., Bornkamm, G. & Lenoir, G. 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**, 1002-13.

Cortes, P., Ye, Z. S. & Baltimore, D. (1994). RAG-1 interacts with the repeated amino acid motif of the human homologue of the yeast protein SRP1. *Proc Natl Acad Sci U S A* **91**, 7633-7.

Cory, S. (1986). Activation of cellular oncogenes in hemopoietic cells by chromosome translocation. *Adv Cancer Res* **47**, 189-234.

Cory, S., Vaux, D. L., Strasser, A., Harris, A. W. & Adams, J. M. (1999). Insights from Bcl-2 and Myc: malignancy involves abrogation of apoptosis as well as sustained proliferation. *Cancer Res* **59**, 1685s-1692s.

Craig, R. W. (1995). The bcl-2 gene family. *Semin Cancer Biol* **6**, 35-43.

Crawford, D. H. & Catovsky, D. (1993). In vitro activation of leukaemic B cells by interleukin-4 and antibodies to CD40. *Immunology* **80**, 40-4.

Cunningham, J. M., Purucker, M. E., Jane, S. M., Safer, B., Vanin, E. F., Ney, P. A., Lowrey, C. H. & Nienhuis, A. W. (1994). The regulatory element 3' to the A gamma-globin gene binds to the nuclear matrix and interacts with special A-T-rich binding protein 1 (SATB1), an SAR/MAR-associating region DNA binding protein. *Blood* **84**, 1298-308.

Cuomo, C. A., Mundy, C. L. & Oettinger, M. A. (1996). DNA sequence and structure requirements for cleavage of V(D)J recombination signal sequences. *Mol Cell Biol* **16**, 5683-90.

Curran, T. & Morgan, J. I. (1987). Memories of fos. *Bioessays* **7**, 255-8.

Cuypers, H. T., Selten, G., Quint, W., Zijlstra, M., Maandag, E. R., Boelens, W., van Wezenbeek, P., Melief, C. & Berns, A. (1984). Murine leukemia virus-induced T-cell lymphomagenesis: integration of proviruses in a distinct chromosomal region. *Cell* **37**, 141-50.

Davatelis, G., Tekamp-Olson, P., Wolpe, S. D., Hermsen, K., Luedke, C., Gallegos, C., Coit, D., Merryweather, J. & Cerami, A. (1988). Cloning and characterization of a cDNA for murine macrophage inflammatory protein (MIP), a novel monokine with inflammatory and chemokinetic properties [published erratum appears in J Exp Med 1989 Dec 1;170(6):2189]. *J Exp Med* **167**, 1939-44.

Davenport, M. G. & Pagano, J. S. (1999). Expression of EBNA-1 mRNA is regulated by cell cycle during Epstein- Barr virus type I latency. *J Virol* **73**, 3154-61.

Davison, A. J. & Taylor, P. (1987). Genetic relations between varicella-zoster virus and Epstein-Barr virus. *J Gen Virol* **68**, 1067-79.

Dempsey, L. A., Hanakahi, L. A. & Maizels, N. (1998). A specific isoform of hnRNP D interacts with DNA in the LR1 heterodimer: canonical RNA binding motifs in a sequence-specific duplex DNA binding protein. *J Biol Chem* **273**, 29224-9.

Devergne, O., Hatzivassiliou, E., Izumi, K. M., Kaye, K. M., Kleijnen, M. F., Kieff, E. & Mosialos, G. (1996). Association of TRAF1, TRAF2, and TRAF3 with an Epstein-Barr virus LMP1 domain important for B-lymphocyte transformation: role in NF-kB activation. *Mol Cell Biol* **16**, 7098-108.

Dickinson, L. A., Joh, T., Kohwi, Y. & Kohwi-Shigematsu, T. (1992). A tissue-specific MAR/SAR DNA-binding protein with unusual binding site recognition. *Cell* **70**, 631-45.

Dildrop, R., Ma, A., Zimmerman, K., Hsu, E., Tesfaye, A., DePinho, R. & Alt, F. W. (1989). IgH enhancer-mediated deregulation of N-myc gene expression in transgenic mice: generation of lymphoid neoplasias that lack c-myc expression. *Embo J* **8**, 1121-8.

Dinkel, A., Warnatz, K., Ledermann, B., Rolink, A., Zipfel, P. F., Burki, K. & Eibel, H. (1998). The transcription factor early growth response 1 (Egr-1) advances differentiation of pre-B and immature B cells. *J Exp Med* **188**, 2215-24.

Dyck, J. A., Warrell, R. P., Jr., Evans, R. M. & Miller, W. H., Jr. (1995). Rapid diagnosis of acute promyelocytic leukemia by immunohistochemical localization of PML/RAR-alpha protein. *Blood* **86**, 862-7.

.Eliopoulos, A. G., Blake, S. M., Floettmann, J. E., Rowe, M. & Young, L. S. (1999). Epstein-Barr virus-encoded latent membrane protein 1 activates the JNK pathway through its extreme C terminus via a mechanism involving TRADD and TRAF2. *J Virol* **73**, 1023-35.

Eliopoulos, A. G., Dawson, C. W., Mosialos, G., Floettmann, J. E., Rowe, M., Armitage, R. J., Dawson, J., Zapata, J. M., Kerr, D. J., Wakelam, M. J., Reed, J. C., Kieff, E. & Young, L. S. (1996). CD40-induced growth inhibition in epithelial cells is mimicked by Epstein-Barr Virus-encoded LMP1: involvement of TRAF3 as a common mediator. *Oncogene* **13**, 2243-54.

Eliopoulos, A. G., Gallagher, N. J., Blake, S. M., Dawson, C. W. & Young, L. S. (1999). Activation of the p38 mitogen-activated protein kinase pathway by Epstein-Barr virus-encoded latent membrane protein 1 coregulates interleukin-6 and interleukin-8 production. *J Biol Chem* **274**, 16085-96.

Eliopoulos, A. G. & Young, L. S. (1998). Activation of the cJun N-terminal kinase (JNK) pathway by the Epstein- Barr virus-encoded latent membrane protein 1 (LMP1). *Oncogene* **16**, 1731-42.

Epstein, M.A. and Achong, B.Y., eds (1986). The Epstein-Barr virus: Recent advances, John Wiley and Sons, New York

Ermakova, O. V., Frappier, L. & Schildkraut, C. L. (1996). Role of the EBNA-1 protein in pausing of replication forks in the Epstein-Barr virus genome. *J Biol Chem* **271**, 33009-17.

Escalier, D., Allenet, B., Badrichani, A. & Garchon, H. J. (1999). High level expression of the Xlr nuclear protein in immature thymocytes and colocalization with the matrix-associated region-binding SATB1 protein. *J Immunol* **162**, 292-8.

Everett, R. D. & Maul, G. G. (1994). HSV-1 IE protein Vmw110 causes redistribution of PML. *Embo J* **13**, 5062-9.

Everett, R. D., Meredith, M., Orr, A., Cross, A., Kathoria, M. & Parkinson, J. (1997). A novel ubiquitin-specific protease is dynamically associated with the PML nuclear domain and binds to a herpesvirus regulatory protein [corrected and republished article originally printed in EMBO J 1997 Feb 3;16(3):566-77]. *Embo J* **16**, 1519-30.

Facchinetti, V., Loffarelli, L., Schreek, S., Oelgeschlager, M., Luscher, B., Introna, M. & Golay, J. (1997). Regulatory domains of the A-Myb transcription factor and its interaction with the CBP/p300 adaptor molecules. *Biochem J* **324**, 729-36.

Fahraeus, R., Rymo, L., Rhim, J. S. & Klein, G. (1990). Morphological transformation of human keratinocytes expressing the LMP gene of Epstein-Barr virus. *Nature* **345**, 447-9.

Fang, W., Mueller, D. L., Pennell, C. A., Rivard, J. J., Li, Y. S., Hardy, R. R., Schlissel, M. S. & Behrens, T. W. (1996). Frequent aberrant immunoglobulin gene rearrangements in pro-B cells revealed by a bcl-xL transgene. *Immunity* **4**, 291-9.

Fang, W., Rivard, J. J., Mueller, D. L. & Behrens, T. W. (1994). Cloning and molecular characterization of mouse bcl-x in B and T lymphocytes. *J Immunol* **153**, 4388-98.

Fanidi, A., Harrington, E. A. & Evan, G. I. (1992). Cooperative interaction between c-myc and bcl-2 proto-oncogenes. *Nature* **359**, 554-6.

Farrell, P. J., Allan, G. J., Shanahan, F., Vousden, K. H. & Crook, T. (1991). p53 is frequently mutated in Burkitt's lymphoma cell lines. *Embo J* **10**, 2879-87.

Faulkner, G. C., Krajewski, A. S. & Crawford, D. H. (2000). The ins and outs of EBV infection. *Trends Microbiol* **8**, 185-189.

Fitzsimmons, D., Hodsdon, W., Wheat, W., Maira, S. M., Wasylyk, B. & Hagman, J. (1996). Pax-5 (BSAP) recruits Ets proto-oncogene family proteins to form functional ternary complexes on a B-cell-specific promoter. *Genes Dev* **10**, 2198-211.

Floettmann, J. E., Eliopoulos, A. G., Jones, M., Young, L. S. & Rowe, M. (1998). Epstein-Barr virus latent membrane protein-1 (LMP1) signalling is distinct from CD40 and involves physical cooperation of its two C- terminus functional regions. *Oncogene* **17**, 2383-92.

Floettmann, J. E., Ward, K., Rickinson, A. B. & Rowe, M. (1996). Cytostatic effect of Epstein-Barr virus latent membrane protein-1 analyzed using tetracycline-regulated expression in B cell lines. *Virology* **223**, 29-40.

Fowlis, D. J. & Balmain, A. (1993). Oncogenes and tumour suppressor genes in transgenic mouse models of neoplasia. *Eur J Cancer* **4**, 638-45.

Frame, M. C., Wilkie, N. M., Darling, A. J., Chudleigh, A., Pintzas, A., Lang, J. C. & Gillespie, D. A. (1991). Regulation of AP-1/DNA complex formation in vitro. *Oncogene* **6**, 205-9.

Frappier, L., Goldsmith, K. & Bendell, L. (1994). Stabilization of the EBNA1 protein on the Epstein-Barr virus latent origin of DNA replication by a DNA looping mechanism. *J Biol Chem* **269**, 1057-62.

Frappier, L. & O'Donnell, M. (1991a). Epstein-Barr nuclear antigen 1 mediates a DNA loop within the latent replication origin of Epstein-Barr virus. *Proc Natl Acad Sci U S A* **88**, 10875-9.

Frappier, L. & O'Donnell, M. (1991b). Overproduction, purification, and characterization of EBNA1, the origin binding protein of Epstein-Barr virus. *J Biol Chem* **266**, 7819-26.

Frappier, L. & O'Donnell, M. (1992). EBNA1 distorts oriP, the Epstein-Barr virus latent replication origin. *J Virol* **66**, 1786-90.

Fries, K. L., Miller, W. E. & 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**, 8653-9.

Fries, K. L., Sculley, T. B., Webster-Cyriaque, J., Rajadurai, P., Sadler, R. H. & Raab-Traub, N. (1997). Identification of a novel protein encoded by the BamHI A region of the Epstein-Barr virus. *J Virol* **71**, 2765-71.

Fruehling, S. & Longnecker, R. (1997). The immunoreceptor tyrosine-based activation motif of Epstein-Barr virus LMP2A is essential for blocking BCR-mediated signal transduction. *Virology* **235**, 241-51.

Gahn, T. A. & Schildkraut, C. L. (1989). The Epstein-Barr virus origin of plasmid replication, oriP, contains both the initiation and termination sites of DNA replication. *Cell* **58**, 527-35.

Gahn, T. A. & Sugden, B. (1993). Marked, transient inhibition of expression of the Epstein-Barr virus latent membrane protein gene in Burkitt's lymphoma cell lines by electroporation. *J Virol* **67**, 6379-86.

Galaktionov, K., Chen, X. & Beach, D. (1996). Cdc25 cell-cycle phosphatase as a target of c-myc. *Nature* **382**, 511-7.

Galaktionov, K., Lee, A. K., Eckstein, J., Draetta, G., Meckler, J., Loda, M. & Beach, D. (1995). CDC25 phosphatases as potential human oncogenes. *Science* **269**, 1575-7.

Gambacorta, M., Flenghi, L., Fagioli, M., Pileri, S., Leoncini, L., Bigerna, B., Pacini, R., Tanci, L. N., Pasqualucci, L., Ascani, S., Mencarelli, A., Liso, A., Pelicci, P. G. & Falini, B. (1996). Heterogeneous nuclear expression of the promyelocytic leukemia (PML) protein in normal and neoplastic human tissues. *Am J Pathol* **149**, 2023-35.

Gentz, R., Rauscher, F. J. d., Abate, C. & Curran, T. (1989). Parallel association of Fos and Jun leucine zippers juxtaposes DNA binding domains. *Science* **243**, 1695-9.

Georgopoulos, K. (1997). Transcription factors required for lymphoid lineage commitment. *Curr Opin Immunol* **9**, 222-7.

Ghebrehiwet, B., Kew, R. R., Gruber, B. L., Marchese, M. J., Peerschke, E. I. & Reid, K. B. (1995). Murine mast cells express two types of C1q receptors that are involved in the induction of chemotaxis and chemokinesis. *J Immunol* **155**, 2614-9.

Ghebrehiwet, B., Lim, B. L., Peerschke, E. I., Willis, A. C. & Reid, K. B. (1994). Isolation, cDNA cloning, and overexpression of a 33-kD cell surface glycoprotein that binds to the globular "heads" of C1q. *J Exp Med* **179**, 1809-21.

Gires, O., Zimber-Strobl, U., Gonnella, R., Ueffing, M., Marschall, G., Zeidler, R., Pich, D. & Hammerschmidt, W. (1997). Latent membrane protein 1 of Epstein-Barr virus mimics a constitutively active receptor molecule. *EMBO J.* **16**, 6131-40.

Goddard, A. D., Yuan, J. Q., Fairbairn, L., Dexter, M., Borrow, J., Kozak, C. & Solomon, E. (1995). Cloning of the murine homolog of the leukemia-associated PML gene. *Mamm Genome* **6**, 732-7.

Goldsmith, K., Bendell, L. & Frappier, L. (1993). Identification of EBNA1 amino acid sequences required for the interaction of the functional elements of the Epstein-Barr virus latent origin of DNA replication. *J Virol* **67**, 3418-26.

Gonzalez-Garcia, M., Perez-Ballestero, R., Ding, L., Duan, L., Boise, L. H., Thompson, C. B. & Nunez, G. (1994). bcl-XL is the major bcl-x mRNA form expressed during murine development and its product localizes to mitochondria. *Development* **120**, 3033-42.

Gordon, J. (1995). CD40 and its ligand: central players in B lymphocyte survival, growth, and differentiation. *Blood Rev* **9**, 53-6.

Gottschalk, A. R., Joseph, L. J. & Quintans, J. (1994). Fc gamma RII cross-linking inhibits anti-Ig-induced erg-1 and erg-2 expression in BCL1. *J Immunol* **152**, 2115-22.

Grawunder, U., Leu, T. M., Schatz, D. G., Werner, A., Rolink, A. G., Melchers, F. & Winkler, T. H. (1995a). Down-regulation of RAG1 and RAG2 gene expression in preB cells after functional immunoglobulin heavy chain rearrangement. *Immunity* **3**, 601-8.

Grawunder, U., Rolink, A. & Melchers, F. (1995b). Induction of sterile transcription from the kappa L chain gene locus in V(D)J recombinase-deficient progenitor B cells. *Int Immunol* **7**, 1915-25.

Grawunder, U., Schatz, D. G., Leu, T. M., Rolink, A. & Melchers, F. (1996). The half-life of RAG-1 protein in precursor B cells is increased in the absence of RAG-2 expression. *J Exp Med* **183**, 1731-7.

Gregory, C. D., Murray, R. J., Edwards, C. F. & Rickinson, A. B. (1988). Downregulation of cell adhesion molecules LFA-3 and ICAM-1 in Epstein- Barr virus-positive Burkitt's lymphoma underlies tumor cell escape from virus-specific T cell surveillance. *J Exp Med* **167**, 1811-24.

Gregory, C. D., Rowe, M. & 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**, 1481-95.

Grillot, D. A., Merino, R., Pena, J. C., Fanslow, W. C., Finkelman, F. D., Thompson, C. B. & Nunez, G. (1996). bcl-x exhibits regulated expression during B cell development and activation and modulates lymphocyte survival in transgenic mice. *J Exp Med* **183**, 381-91.

Grimes, H. L., Chan, T. O., Zweidler-McKay, P. A., Tong, B. & Tsichlis, P. N. (1996a). The Gfi-1 proto-oncoprotein contains a novel transcriptional repressor domain, SNAG, and inhibits G1 arrest induced by interleukin-2 withdrawal. *Mol Cell Biol* **16**, 6263-72.

Grimes, H. L., Gilks, C. B., Chan, T. O., Porter, S. & Tsichlis, P. N. (1996b). The Gfi-1 protooncoprotein represses Bax expression and inhibits T-cell death. *Proc Natl Acad Sci U S A* **93**, 14569-73.

Grogan, E. A., Summers, W. P., Dowling, S., Shedd, D., Gradoville, L. & Miller, G. (1983). Two Epstein-Barr viral nuclear neoantigens distinguished by gene transfer, serology, and chromosome binding. *Proc Natl Acad Sci U S A* **80**, 7650-3.

Guiochon-Mantel, A., Savouret, J. F., Quignon, F., Delabre, K., Milgrom, E. & De The, H. (1995). Effect of PML and PML-RAR on the transactivation properties and subcellular distribution of steroid hormone receptors. *Mol Endocrinol* **9**, 1791-803.

Hagman, J., Gutch, M. J., Lin, H. & Grosschedl, R. (1995). EBF contains a novel zinc coordination motif and multiple dimerization and transcriptional activation domains. *Embo J* **14**, 2907-16.

Hahm, K., Ernst, P., Lo, K., Kim, G. S., Turck, C. & Smale, S. T. (1994). The lymphoid transcription factor LyF-1 is encoded by specific, alternatively spliced mRNAs derived from the Ikaros gene. *Mol Cell Biol* **14**, 7111-23.

Hai, T. & Curran, T. (1991). Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proc Natl Acad Sci U S A* **88**, 3720-4.

Halazonetis, T. D., Georgopoulos, K., Greenberg, M. E. & Leder, P. (1988). c-Jun dimerizes with itself and with c-Fos, forming complexes of different DNA binding affinities. *Cell* **55**, 917-24.

Hammerschmidt, W. & Sugden, B. (1989). Genetic analysis of immortalizing functions of Epstein-Barr virus in human B lymphocytes. *Nature* **340**, 393-7.

Hammerschmidt, W., Sugden, B. & Baichwal, V. R. (1989). The transforming domain alone of the latent membrane protein of Epstein- Barr virus is toxic to cells when expressed at high levels. *J Virol* **63**, 2469-75.

Hammarskjold, M. L. & Simurda, M. C. (1992). Epstein-Barr virus latent membrane protein transactivates the human immunodeficiency virus type 1 long terminal repeat through induction of NF-kappa B activity. *J Virol* **66**, 6496-501.

Hardy, R. R. & Hayakawa, K. (1991). A developmental switch in B lymphopoiesis. *Proc Natl Acad Sci U S A* **88**, 11550-4.

Harris, A., Young, B. D. & Griffin, B. E. (1985). Random association of Epstein-Barr virus genomes with host cell metaphase chromosomes in Burkitt's lymphoma-derived cell lines. *J Virol* **56**, 328-32.

Harris, A. W., Pinkert, C. A., Crawford, M., Langdon, W. Y., Brinster, R. L. & Adams, J. M. (1988a). The E mu-myc transgenic mouse. A model for high-incidence spontaneous lymphoma and leukemia of early B cells. *J Exp Med* **167**, 353-71.

Harris, B. A. (1988b). Complete cDNA sequence of a human stimulatory GTP-binding protein alpha subunit. *Nucleic Acids Res* **16**, 3585.

Harrison, S., Fisenne, K. & Hearing, J. (1994). Sequence requirements of the Epstein-Barr virus latent origin of DNA replication. *J Virol* **68**, 1913-25.

Haupt, Y., Alexander, W. S., Barri, G., Klinken, S. P. & Adams, J. M. (1991). Novel zinc finger gene implicated as myc collaborator by retrovirally accelerated lymphomagenesis in E mu-myc transgenic mice [see comments]. *Cell* **65**, 753-63.

Hawker, K. L., Vass, J. K. & Ozanne, B. W. (1996). Isolation of novel, transcriptionally active AP-1 binding sites: implications for cellular transformation. *Oncogene* **13**, 283-92.

Hayashi, S., Kunisada, T., Ogawa, M., Sudo, T., Kodama, H., Suda, T. & Nishikawa, S. (1990). Stepwise progression of B lineage differentiation supported by interleukin 7 and other stromal cell molecules. *J Exp Med* **171**, 1683-95.

He, D., Mu, Z. M., Le, X., Hsieh, J. T., Pong, R. C., Chung, L. W. & Chang, K. S. (1997). Adenovirus-mediated expression of PML suppresses growth and tumorigenicity of prostate cancer cells. *Cancer Res* **57**, 1868-72.

Hearing, J. C., Lewis, A. & Levine, A. J. (1985). Structure of the Epstein-Barr virus nuclear antigen as probed with monoclonal antibodies. *Virology* **142**, 215-20.

Hearing, J., Mulhaupt, Y. & Harper, S. (1992). Interaction of Epstein-Barr virus nuclear antigen 1 with the viral latent origin of replication. *J Virol* **66**, 694-705.

Heller, M., Flemington, E., Kieff, E. & Deininger, P. (1985). Repeat arrays in cellular DNA related to the Epstein-Barr virus IR3 repeat. *Mol Cell Biol* **5**, 457-65.

Henderson, A. and Calame, K. (1998). Transcriptional regulation during B cell development. *Annu. Rev. Immunol.* 16,163-200.

Henderson, S., Huen, D., Rowe, M., Dawson, C., Johnson, G. & Rickinson, A. (1993). Epstein-Barr virus-coded BHRF1 protein, a viral homologue of Bcl-2, protects human B cells from programmed cell death. *Proc Natl Acad Sci U S A* **90**, 8479-83.

Henderson, S., Rowe, M., Gregory, C., Croom-Carter, D., Wang, F., Longnecker, R., Kieff, E. & 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**, 1107-15.

Henkel, T., Ling, P. D., Hayward, S. D. & Peterson, M. G. (1994). Mediation of Epstein-Barr virus EBNA2 transactivation by recombination signal-binding protein J k. *Science* **265**, 92-5.

Hennessy, K., Heller, M., van Santen, V. & Kieff, E. (1983). Simple repeat array in Epstein-Barr virus DNA encodes part of the Epstein-Barr nuclear antigen. *Science* **220**, 1396-8.

Hennessy, K. & 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**, 5665-9.

Henriksson, M. & Luscher, B. (1996). Proteins of the Myc network: essential regulators of cell growth and differentiation. *Adv Cancer Res* **68**, 109-82.

Herber, B., Truss, M., Beato, M. & Muller, R. (1994). Inducible regulatory elements in the human cyclin D1 promoter [published erratum appears in Oncogene 1994 Jul;9(7):2105-7]. *Oncogene* **9**, 1295-304.

Herrero, J. A., Mathew, P. & Paya, C. V. (1995). LMP-1 activates NF-kappa B by targeting the inhibitory molecule I kappa B alpha. *J Virol* **69**, 2168-74.

Herschman, H. R. (1991). Primary response genes induced by growth factors and tumor promoters. *Annu Rev Biochem* **60**, 281-319.

Hikida, M., Mori, M., Takai, T., Tomochika, K., Hamatani, K. & Ohmori, H. (1996). Reexpression of RAG-1 and RAG-2 genes in activated mature mouse B cells. *Science* **274**, 2092-4.

Hockenbery, D. M. (1992). The bcl-2 oncogene and apoptosis. *Semin Immunol* **4**, 413-20.

Hoey, T. & Schindler, U. (1998). STAT structure and function in signaling. *Curr Opin Genet Dev* **8**, 582-7.

Hoffmann, I., Draetta, G. & Karsenti, E. (1994). Activation of the phosphatase activity of human cdc25A by a cdk2-cyclin E dependent phosphorylation at the G1/S transition. *Embo J* **13**, 4302-10.

Honore, B., Madsen, P., Rasmussen, H. H., Vandekerckhove, J., Celis, J. E. & Leffers, H. (1993). Cloning and expression of a cDNA covering the complete coding region of the P32 subunit of human pre-mRNA splicing factor SF2. *Gene* **134**, 283-7.

Horner, D., Lewis, M. & Farrell, P. J. (1995). Novel hypotheses for the roles of EBNA-1 and BHRF1 in EBV-related cancers. *Intervirology* **38**, 195-205.

Howard, M. C. (1982). Antigen-induced B lymphocyte differentiation. *CRC Crit Rev Immunol* **3**, 181-208.

Howe, K., Williamson, J., Boddy, N., Sheer, D., Freemont, P. & Solomon, E. (1998). The ubiquitin-homology gene PIC1: characterization of mouse (Pic1) and human (UBL1) genes and pseudogenes. *Genomics* **47**, 92-100.

Hsieh, J. J. & Hayward, S. D. (1995). Masking of the CBF1/RBPJ k transcriptional repression domain by Epstein-Barr virus EBNA2. *Science* **268**, 560-3.

Hsieh, J. J., Henkel, T., Salmon, P., Robey, E., Peterson, M. G. & 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**, 952-9.

Hsieh, J. J., Nofziger, D. E., Weinmaster, G. & Hayward, S. D. (1997). Epstein-Barr virus immortalization: Notch2 interacts with CBF1 and blocks differentiation. *J Virol* **71**, 1938-45.
Hudson, G. S., Gibson, T. J. & Barrell, B. G. (1985). The BamHI F region of the B95-8 Epstein-Barr virus genome. *Virology* **147**, 99-109.

Huen, D. S., Fox, A., Kumar, P. & Searle, P. F. (1993). Dilated heart failure in transgenic mice expressing the Epstein-Barr virus nuclear antigen-leader protein. *J Gen Virol* **74**, 1381-91.

Huleihel, M., Goldsborough, M., Cleveland, J., Gunnell, M., Bonner, T. & Rapp, U. R. (1986). Characterization of murine A-raf, a new oncogene related to the v-raf oncogene. *Mol Cell Biol* **6**, 2655-62.

Hunter, T. (1991). Cooperation between oncogenes. *Cell* **64**, 249-70.

Inoue, N., Harada, S., Honma, T., Kitamura, T. & Yanagi, K. (1991). The domain of Epstein-Barr virus nuclear antigen 1 essential for binding to oriP region has a sequence fitted for the hypothetical basic- helix-loop-helix structure. *Virology* **182**, 84-93.

Iscove, N. N., Sieber, F. & Winterhalter, K. H. (1974). Erythroid colony formation in cultures of mouse and human bone marrow: analysis of the requirement for erythropoietin by gel filtration and affinity chromatography on agarose-concanavalin A. *J Cell Physiol* **83**, 309-20.

Izumi, K. M. & 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- kappaB. *Proc Natl Acad Sci U S A* **94**, 12592-7.

Jankelevich, S., Kolman, J. L., Bodnar, J. W. & Miller, G. (1992). A nuclear matrix attachment region organizes the Epstein-Barr viral plasmid in Raji cells into a single DNA domain. *Embo J* **11**, 1165-76.

Jin, X. W. & Speck, S. H. (1992). Identification of critical cis elements involved in mediating Epstein-Barr virus nuclear antigen 2-dependent activity of an enhancer located upstream of the viral BamHI C promoter. *J Virol* **66**, 2846-52.

Jochner, N., Eick, D., Zimber-Strobl, U., Pawlita, M., Bornkamm, G. W. & Kempkes, B. (1996). Epstein-Barr virus nuclear antigen 2 is a transcriptional suppressor of the immunoglobulin mu gene: implications for the expression of the translocated c-myc gene in Burkitt's lymphoma cells. *Embo J* **15**, 375-82.

Karin, M. & Hunter, T. (1995). Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus. *Curr Biol* **5**, 747-57.

Karran, L., Gao, Y., Smith, P. R. & Griffin, B. E. (1992). Expression of a family of complementary-strand transcripts in Epstein- Barr virus-infected cells. *Proc Natl Acad Sci U S A* **89**, 8058-62.

Kawa, K. (2000). Epstein-Barr virus--associated diseases in humans [In Process Citation]. *Int J Hematol* **71**, 108-17.

Kaye, K. M., Izumi, K. M. & Kieff, E. (1993). Epstein-Barr virus latent membrane protein 1 is essential for B- lymphocyte growth transformation. *Proc Natl Acad Sci U S A* **90**, 9150-4.

Kerr, B. M., Lear, A. L., Rowe, M., Croom-Carter, D., Young, L. S., Rookes, S. M., Gallimore, P. H. & Rickinson, A. B. (1992). Three transcriptionally distinct forms of Epstein-Barr virus latency in somatic cell hybrids: cell phenotype dependence of virus promoter usage. *Virology* **187**, 189-201.

Khanna, R., Burrows, S. R., Kurilla, M. G., Jacob, C. A., Misko, I. S., Sculley, T. B., Kieff, E. & Moss, D. J. (1992). Localization of Epstein-Barr virus cytotoxic T cell epitopes using recombinant vaccinia: implications for vaccine development. *J Exp Med* **176**, 169-76.

Khanna, R., Burrows, S. R. & Moss, D. J. (1995). Immune regulation in Epstein-Barr virus-associated diseases. *Microbiol Rev* **59**, 387-405.

Kharbanda, S., Pandey, P., Schofield, L., Israels, S., Roncinske, R., Yoshida, K., Bharti, A., Yuan, Z. M., Saxena, S., Weichselbaum, R., Nalin, C. & Kufe, D. (1997). Role for Bcl-xL as an inhibitor of cytosolic cytochrome C accumulation in DNA damage-induced apoptosis. *Proc Natl Acad Sci U S A* **94**, 6939-42.

Kiefer, M. C., Brauer, M. J., Powers, V. C., Wu, J. J., Umansky, S. R., Tomei, L. D. & Barr, P. J. (1995). Modulation of apoptosis by the widely distributed Bcl-2 homologue Bak. *Nature* **374**, 736-9.

Kieff, E., (1996). Epstein-Barr virus and it's replication. Fields Virology 3rd Eddition, Edited by Field, D.N., Knippe, D.M., Howley, P.M. *et al.* Lippincott-Raven Publishers, Philadelphia, 2343-96

Kieser, A., Kilger, E., Gires, O., Ueffing, M., Kolch, W. & Hammerschmidt, W. (1997). Epstein-Barr virus latent membrane protein-1 triggers AP-1 activity via the c-Jun N-terminal kinase cascade. *EMBO J.* **16**, 6478-85.

Kilger, E., Kieser, A., Baumann, M. & Hammerschmidt, W. (1998). Epstein-Barr virus-mediated B-cell proliferation is dependent upon latent membrane protein 1, which simulates an activated CD40 receptor. *EMBO J.* **17**, 1700-9.

Kinoshita, T., Yokota, T., Arai, K. & Miyajima, A. (1995a). Regulation of Bcl-2 expression by oncogenic Ras protein in hematopoietic cells. *Oncogene* **10**, 2207-12.

Kinoshita, T., Yokota, T., Arai, K. & Miyajima, A. (1995b). Suppression of apoptotic death in hematopoietic cells by signalling through the IL-3/GM-CSF receptors. *Embo J* **14**, 266-75.

Kitagawa, T., Mori, K., Kishi, H., Tagoh, H., Nagata, T., Kurioka, H. & Muraguchi, A. (1996). Chromatin structure and transcriptional regulation of human RAG-1 gene. *Blood* **88**, 3785-91.

Klaus, S. J., Phillips, N. E. & Parker, D. C. (1993). Effects of IL-4 and Fc gamma receptor II engagement on Egr-1 expression during stimulation of B lymphocytes by membrane immunoglobulin crosslinking. *Mol Immunol* **30**, 1553-8.

Klein, C. & Raab-Traub, N. (1987). Human neonatal lymphocytes immortalized after microinjection of Epstein- Barr virus DNA. *J Virol* **61**, 1552-8.

Kline, G. H., Hayden, T. A. & Klinman, N. R. (1999). B cell maintenance in aged mice reflects both increased B cell longevity and decreased B cell generation. *J Immunol* **162**, 3342-9.

Kluck, R. M., Bossy-Wetzel, E., Green, D. R. & Newmeyer, D. D. (1997). The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis [see comments]. *Science* **275**, 1132-6.

Kneitz, B., Herrmann, T., Yonehara, S. & Schimpl, A. (1995). Normal clonal expansion but impaired Fas-mediated cell death and anergy induction in interleukin-2-deficient mice. *Eur J Immunol* **25**, 2572-7.

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**, 2530-6.

Kohwi-Shigematsu, T., Maass, K. & Bode, J. (1997). A thymocyte factor SATB1 suppresses transcription of stably integrated matrix-attachment region-linked reporter genes. *Biochemistry* **36**, 12005-10.

Koken, M. H., Linares-Cruz, G., Quignon, F., Viron, A., Chelbi-Alix, M. K., Sobczak-Thepot, J., Juhlin, L., Degos, L., Calvo, F. & de The, H. (1995). The PML growth-suppressor has an altered expression in human oncogenesis. *Oncogene* **10**, 1315-24.

Komano, J., Maruo, S., Kurozumi, K., Oda, T. & Takada, K. (1999). Oncogenic role of Epstein-Barr virus-encoded RNAs in Burkitt's lymphoma cell line Akata. *J Virol* **73**, 9827-31.

Korkolopoulou, P., Kaklamanis, L., Pezzella, F., Harris, A. L. & Gatter, K. C. (1996). Loss of antigen-presenting molecules (MHC class I and TAP-1) in lung cancer. *Br J Cancer* **73**, 148-53.

Kouzarides, T. & Ziff, E. (1989). Leucine zippers of fos, jun and GCN4 dictate dimerization specificity and thereby control DNA binding. *Nature* **340**, 568-71.

Kovary, K. & Bravo, R. (1991). Expression of different Jun and Fos proteins during the G0-to-G1 transition in mouse fibroblasts: in vitro and in vivo associations. *Mol Cell Biol* **11**, 2451-9.

Kovary, K. & Bravo, R. (1992). Existence of different Fos/Jun complexes during the G0-to-G1 transition and during exponential growth in mouse fibroblasts: differential role of Fos proteins. *Mol Cell Biol* **12**, 5015-23.

Kube, D., Vockerodt, M., Weber, O., Hell, K., Wolf, J., Haier, B., Grasser, F. A., Muller-Lantzsch, N., Kieff, E., Diehl, V. & Tesch, H. (1999). Expression of epstein-barr virus nuclear antigen 1 is associated with enhanced expression of CD25 in the Hodgkin cell line L428. *J Virol* **73**, 1630-6.

Kuhn-Hallek, I., Sage, D. R., Stein, L., Groelle, H. & Fingeroth, J. D. (1995). Expression of recombination activating genes (RAG-1 and RAG-2) in Epstein-Barr virus-bearing B cells [see comments]. *Blood* **85**, 1289-99.

Kulwichit, W., Edwards, R. H., Davenport, E. M., Baskar, J. F., Godfrey, V. & Raab-Traub, N. (1998). Expression of the Epstein-Barr virus latent membrane protein 1 induces B cell lymphoma in transgenic mice. *Proc Natl Acad Sci U S A* **95**, 11963-8.

Labbaye, C., Valtieri, M., Grignani, F., Puglisi, R., Luchetti, L., Masella, B., Alcalay, M., Testa, U. & Peschle, C. (1999). Expression and role of PML gene in normal adult hematopoiesis: functional interaction between PML and Rb proteins in erythropoiesis. *Oncogene* **18**, 3529-40.

Laherty, C. D., Hu, H. M., Opipari, A. W., Wang, F. & Dixit, V. M. (1992). The Epstein-Barr virus LMP1 gene product induces A20 zinc finger protein expression by activating nuclear factor kappa B. *J Biol Chem* **267**, 24157-60.

Laine, A. & Frappier, L. (1995). Identification of Epstein-Barr virus nuclear antigen 1 protein domains that direct interactions at a distance between DNA-bound proteins. *J Biol Chem* **270**, 30914-8.

Lallemand, D., Spyrou, G., Yaniv, M. & Pfarr, C. M. (1997). Variations in Jun and Fos protein expression and AP-1 activity in cycling, resting and stimulated fibroblasts. *Oncogene* **14**, 819-30.

Lauring, J. & Schlissel, M. S. (1999). Distinct factors regulate the murine RAG-2 promoter in B- and T-cell lines. *Mol Cell Biol* **19**, 2601-12.

Lear, A. L., Rowe, M., Kurilla, M. G., Lee, S., Henderson, S., Kieff, E. & Rickinson, A. B. (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**, 7461-8.

Lee, M. A., Diamond, M. E. & Yates, J. L. (1999). Genetic evidence that EBNA-1 is needed for efficient, stable latent infection by Epstein-Barr virus. *J Virol* **73**, 2974-82.

Lee, M. A. & Yates, J. L. (1992). BHRF1 of Epstein-Barr virus, which is homologous to human proto- oncogene bcl2, is not essential for transformation of B cells or for virus replication in vitro. *J Virol* **66**, 1899-906.

Leight, E. R. & Sugden, B. (2000). EBNA-1: a protein pivotal to latent infection by epstein-barr virus [In Process Citation]. *Rev Med Virol* **10**, 83-100.

Leonard, W. J. & O'Shea, J. J. (1998). Jaks and STATs: biological implications. *Annu Rev Immunol* **16**, 293-322.

Leverson, J. D., Koskinen, P. J., Orrico, F. C., Rainio, E. M., Jalkanen, K. J., Dash, A. B., Eisenman, R. N. & Ness, S. A. (1998). Pim-1 kinase and p100 cooperate to enhance c-Myb activity. *Mol Cell* **2**, 417-25.

Levitskaya, J., Coram, M., Levitsky, V., Imreh, S., Steigerwald-Mullen, P. M., Klein, G., Kurilla, M. G. & Masucci, M. G. (1995). Inhibition of antigen processing by the internal repeat region of the Epstein-Barr virus nuclear antigen-1. *Nature* **375**, 685-8.

Levitskaya, J., Sharipo, A., Leonchiks, A., Ciechanover, A. & Masucci, M. G. (1997). Inhibition of ubiquitin/proteasome-dependent protein degradation by the Gly-Ala repeat domain of the Epstein-Barr virus nuclear antigen 1. *Proc Natl Acad Sci U S A* **94**, 12616-21.

Li, R., Yang, L., Fouts, E. & Botchan, M. R. (1993). Site-specific DNA-binding proteins important for replication and transcription have multiple activities. *Cold Spring Harb Symp Quant Biol* **58**, 403-13.

Lilly, M., Le, T., Holland, P. & Hendrickson, S. L. (1992). Sustained expression of the pim-1 kinase is specifically induced in myeloid cells by cytokines whose receptors are structurally related. *Oncogene* **7**, 727-32.

Lilly, M., Sandholm, J., Cooper, J. J., Koskinen, P. J. & Kraft, A. (1999). The PIM-1 serine kinase prolongs survival and inhibits apoptosis- related mitochondrial dysfunction in part through a bcl-2-dependent pathway. *Oncogene* **18**, 4022-31.

Lin, H. & Grosschedl, R. (1995). Failure of B-cell differentiation in mice lacking the transcription factor EBF. *Nature* **376**, 263-7.

Lin, W. C. & Desiderio, S. (1993). Regulation of V(D)J recombination activator protein RAG-2 by phosphorylation. *Science* **260**, 953-9.

Ling, P. D., Rawlins, D. R. & Hayward, S. D. (1993). The Epstein-Barr virus immortalizing protein EBNA-2 is targeted to DNA by a cellular enhancer-binding protein. *Proc Natl Acad Sci U S A* **90**, 9237-41.

Liou, H. C., Sha, W. C., Scott, M. L. & Baltimore, D. (1994). Sequential induction of NF-kappa B/Rel family proteins during B-cell terminal differentiation. *Mol Cell Biol* **14**, 5349-59.

Little, R. D. & Schildkraut, C. L. (1995). Initiation of latent DNA replication in the Epstein-Barr virus genome can occur at sites other than the genetically defined origin. *Mol Cell Biol* **15**, 2893-903.

Liu, J., Bramblett, D., Zhu, Q., Lozano, M., Kobayashi, R., Ross, S. R. & Dudley, J. P. (1997). The matrix attachment region-binding protein SATB1 participates in negative regulation of tissue-specific gene expression. *Mol Cell Biol* **17**, 5275-87.

Liu, J. H., Mu, Z. M. & Chang, K. S. (1995). PML suppresses oncogenic transformation of NIH/3T3 cells by activated neu. *J Exp Med* **181**, 1965-73.

Longnecker, R. & Miller, C. L. (1996). Regulation of Epstein-Barr virus latency by latent membrane protein 2. *Trends Microbiol* **4**, 38-42.

Lotz, M., Tsoukas, C. D., Fong, S., Carson, D. A. & Vaughan, J. H. (1985). Regulation of Epstein-Barr virus infection by recombinant interferons. Selected sensitivity to interferon-gamma. *Eur J Immunol* **15**, 520-5.

Lovec, H., Sewing, A., Lucibello, F. C., Muller, R. & Moroy, T. (1994). Oncogenic activity of cyclin D1 revealed through cooperation with Ha- ras: link between cell cycle control and malignant transformation. *Oncogene* **9**, 323-6.

Lu, J. J., Chen, J. Y., Hsu, T. Y., Yu, W. C., Su, I. J. & Yang, C. S. (1996). Induction of apoptosis in epithelial cells by Epstein-Barr virus latent membrane protein 1. *J Gen Virol* **77**, 1883-92.
Lupton, S. & Levine, A. J. (1985). Mapping genetic elements of Epstein-Barr virus that facilitate extrachromosomal persistence of Epstein-Barr virus-derived plasmids in human cells. *Mol Cell Biol* **5**, 2533-42.

Luscher, B. & Eisenman, R. N. (1990). New light on Myc and Myb. Part II. Myb. *Genes Dev* **4**, 2235-41.

Mackey, D., Middleton, T. & Sugden, B. (1995). Multiple regions within EBNA1 can link DNAs. *J Virol* **69**, 6199-208.

Mackey, D. & Sugden, B. (1997). Studies on the mechanism of DNA linking by Epstein-Barr virus nuclear antigen 1. *J Biol Chem* **272**, 29873-9.

Magrath, I. (1990). The pathogenesis of Burkitt's lymphoma. *Adv Cancer Res* **55**, 133-270.

Maniatis T., Fritsch, E. and Sambrook, J. (1982). Molecular cloning, a laboratory manual. Publ. Cold Spring Harbour, USA

Marais, R., Wynne, J. & Treisman, R. (1993). The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell* **73**, 381-93.

Marchini, A., Tomkinson, B., Cohen, J. I. & Kieff, E. (1991). BHRF1, the Epstein-Barr virus gene with homology to Bc12, is dispensable for B-lymphocyte transformation and virus replication. *J Virol* **65**, 5991-6000.

Marcu, K. B., Bossone, S. A. & Patel, A. J. (1992). myc function and regulation. *Annu Rev Biochem* **61**, 809-60.

Marechal, V., Dehee, A., Chikhi-Brachet, R., Piolot, T., Coppey-Moisan, M. & Nicolas, J. C. (1999). Mapping EBNA-1 domains involved in binding to metaphase chromosomes. *J Virol* **73**, 4385-92.

Marhin, W. W., Chen, S., Facchini, L. M., Fornace, A. J., Jr. & Penn, L. Z. (1997). Myc represses the growth arrest gene gadd45. *Oncogene* **14**, 2825-34.

Marchini, A., Tomkinson, B., Cohen, J. I. & Kieff, E. (1991). BHRF1, the Epstein-Barr virus gene with homology to Bc12, is dispensable for B-lymphocyte transformation and virus replication. *J Virol* **65**, 5991-6000.

Marin, M. C., Hsu, B., Meyn, R. E., Donehower, L. A., el-Naggar, A. K. & McDonnell, T. J. (1994). Evidence that p53 and bcl-2 are regulators of a common cell death pathway important for in vivo lymphomagenesis. *Oncogene* **9**, 3107-12.

Maroulakou, I. G., Papas, T. S. & Green, J. E. (1994). Differential expression of ets-1 and ets-2 proto-oncogenes during murine embryogenesis. *Oncogene* **9**, 1551-65.
Marshall, D. & Sample, C. (1995). Epstein-Barr virus nuclear antigen 3C is a transcriptional regulator. *J Virol* **69**, 3624-30.

Matthews, D. A. & Russell, W. C. (1998). Adenovirus core protein V interacts with p32--a protein which is associated with both the mitochondria and the nucleus. *J Gen Virol* **79**, 1677-85.

McCarthy, S. A., Chen, D., Yang, B. S., Garcia Ramirez, J. J., Cherwinski, H., Chen, X. R., Klagsbrun, M., Hauser, C. A., Ostrowski, M. C. & McMahon, M. (1997). Rapid phosphorylation of

Ets-2 accompanies mitogen-activated protein kinase activation and the induction of heparin-binding epidermal growth factor gene expression by oncogenic Raf-1. *Mol Cell Biol* **17**, 2401-12.

McDonnell, T. J., Deane, N., Platt, F. M., Nunez, G., Jaeger, U., McKearn, J. P. & Korsmeyer, S. J. (1989). bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. *Cell* **57**, 79-88.

McDonnell, T. J. & Korsmeyer, S. J. (1991). Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14; 18). *Nature* **349**, 254-6.

McDonnell, T. J., Nunez, G., Platt, F. M., Hockenberry, D., London, L., McKearn, J. P. & Korsmeyer, S. J. (1990). Deregulated Bcl-2-immunoglobulin transgene expands a resting but responsive immunoglobulin M and D-expressing B-cell population. *Mol Cell Biol* **10**, 1901-7.

McMahon, S. B. & Monroe, J. G. (1995). A ternary complex factor-dependent mechanism mediates induction of egr- 1 through selective serum response elements following antigen receptor cross-linking in B lymphocytes. *Mol Cell Biol* **15**, 1086-93.

Meijer, C. J., Jiwa, N. M., Dukers, D. F., Oudejans, J. J., de Bruin, P. C., Walboomers, J. M. & van den Brule, A. J. (1996). Epstein-Barr virus and human T-cell lymphomas. *Semin Cancer Biol* **7**, 191-6.

Middleton, T., Gahn, T. A., Martin, J. M. & Sugden, B. (1991). Immortalizing genes of Epstein-Barr virus. *Adv Virus Res* **40**, 19-55.

Middleton, T. & Sugden, B. (1992). EBNA1 can link the enhancer element to the initiator element of the Epstein-Barr virus plasmid origin of DNA replication. *J Virol* **66**, 489-95.

Middleton, T. & Sugden, B. (1994). Retention of plasmid DNA in mammalian cells is enhanced by binding of the Epstein-Barr virus replication protein EBNA1. *J Virol* **68**, 4067-71.

Miyamoto, S., Schmitt, M. J. & Verma, I. M. (1994). Qualitative changes in the subunit composition of kappa B-binding complexes during murine B-cell differentiation. *Proc Natl Acad Sci U S A* **91**, 5056-60.

Miyashita, E. M., Yang, B., Babcock, G. J. & Thorley-Lawson, D. A. (1997). Identification of the site of Epstein-Barr virus persistence in vivo as a resting B cell. *J Virol* **71**, 4882-91.

Molnar, A. & Georgopoulos, K. (1994). The Ikaros gene encodes a family of functionally diverse zinc finger DNA-binding proteins. *Mol Cell Biol* **14**, 8292-303.

Moore, K. W., Vieira, P., Fiorentino, D. F., Trounstine, M. L., Khan, T. A. & Mosmann, T. R. (1990). Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein- Barr virus gene BCRFI [published erratum appears in Science 1990 Oct 26;250(4980):494]. *Science* **248**, 1230-4.

Morgenbesser, S. D. & DePinho, R. A. (1994). Use of transgenic mice to study myc family gene function in normal mammalian development and in cancer. *Semin Cancer Biol* **5**, 21-36.

Moroy, T., Fisher, P., Guidos, C., Ma, A., Zimmerman, K., Tesfaye, A., DePinho, R., Weissman, I. & Alt, F. W. (1990). IgH enhancer deregulated expression of L-myc: abnormal T lymphocyte development and T cell lymphomagenesis. *Embo J* **9**, 3659-66.

Mosialos, G., Birkenbach, M., Yalamanchili, R., VanArsdale, T., Ware, C. & Kieff, E. (1995). The Epstein-Barr virus transforming protein LMP1 engages signaling proteins for the tumor necrosis factor receptor family. *Cell* **80**, 389-99.

Moss, D. J., Burrows, S. R., Castelino, D. J., Kane, R. G., Pope, J. H., Rickinson, A. B., Alpers, M. P. & Heywood, P. F. (1983). A comparison of Epstein-Barr virus-specific T-cell immunity in malaria-endemic and -nonendemic regions of Papua New Guinea. *Int J Cancer* **31**, 727-32.

Motoyama, N., Wang, F., Roth, K. A., Sawa, H., Nakayama, K., Negishi, I., Senju, S., Zhang, Q., Fujii, S. & et al. (1995). Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. *Science* **267**, 1506-10.

Mu, Z. M., Chin, K. V., Liu, J. H., Lozano, G. & Chang, K. S. (1994). PML, a growth suppressor disrupted in acute promyelocytic leukemia. *Mol Cell Biol* **14**, 6858-67.

Muchardt, C. & Yaniv, M. (1993). A human homologue of Saccharomyces cerevisiae SNF2/SWI2 and Drosophila brm genes potentiates transcriptional activation by the glucocorticoid receptor. *Embo J* **12**, 4279-90.

Mukherjee, S., Trivedi, P., Dorfman, D. M., Klein, G. & Townsend, A. (1998). Murine cytotoxic T lymphocytes recognize an epitope in an EBNA-1 fragment, but fail to lyse EBNA-1-expressing mouse cells. *J Exp Med* **187**, 445-50.

Musso, T., Varesio, L., Zhang, X., Rowe, T. K., Ferrara, P., Ortaldo, J. R., O'Shea, J. J. & McVicar, D. W. (1994). IL-4 and IL-13 induce Lsk, a Csk-like tyrosine kinase, in human monocytes. *J Exp Med* **180**, 2383-8.

Nakagawa, H., Wang, T. C., Zukerberg, L., Odze, R., Togawa, K., May, G. H., Wilson, J. & Rustgi, A. K. (1997). The targeting of the cyclin D1 oncogene by an Epstein-Barr virus promoter in transgenic mice causes dysplasia in the tongue, esophagus and forestomach. *Oncogene* **14**, 1185-90.

Nakagomi, K., Kohwi, Y., Dickinson, L. A. & Kohwi-Shigematsu, T. (1994). A novel DNA-binding motif in the nuclear matrix attachment DNA-binding protein SATB1. *Mol Cell Biol* **14**, 1852-60.

Nakayama, K., Negishi, I., Kuida, K., Shinkai, Y., Louie, M. C., Fields, L. E., Lucas, P. J., Stewart, V., Alt, F. W. & et al. (1993). Disappearance of the lymphoid system in Bcl-2 homozygous mutant chimeric mice. *Science* **261**, 1584-8.

Nelson, W. G., Pienta, K. J., Barrack, E. R. & Coffey, D. S. (1986). The role of the nuclear matrix in the organization and function of DNA. *Annu Rev Biophys Biophys Chem* **15**, 457-75.

Neurath, M. F., Strober, W. & Wakatsuki, Y. (1994). The murine Ig 3' alpha enhancer is a target site with repressor function for the B cell lineage-specific transcription factor BSAP (NF- HB, S alpha-BP). *J Immunol* **153**, 730-42.

Nonkwelo, C., Ruf, I. K. & Sample, J. (1997). The Epstein-Barr virus EBNA-1 promoter Qp requires an initiator-like element. *J Virol* **71**, 354-61.

Nunez, G., Seto, M., Seremetis, S., Ferrero, D., Grignani, F., Korsmeyer, S. J. & Dalla-Favera, R. (1989). Growth- and tumor-promoting effects of deregulated BCL2 in human B- lymphoblastoid cells. *Proc Natl Acad Sci U S A* **86**, 4589-93.

Nutt, S. L., Urbanek, P., Rolink, A. & Busslinger, M. (1997). Essential functions of Pax5 (BSAP) in pro-B cell development: difference between fetal and adult B lymphopoiesis and reduced V-to-DJ recombination at the IgH locus. *Genes Dev* **11**, 476-91.

O'Riordan, M. & Grosschedl, R. (1999). Coordinate regulation of B cell differentiation by the transcription factors EBF and E2A. *Immunity* **11**, 21-31.

Oltvai, Z. N., Milliman, C. L. & Korsmeyer, S. J. (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* **74**, 609-19.

Oren, M. & Levine, A. J. (1983). Molecular cloning of a cDNA specific for the murine p53 cellular tumor antigen. *Proc Natl Acad Sci U S A* **80**, 56-9.

Orlowski, R. & Miller, G. (1991). Single-stranded structures are present within plasmids containing the Epstein-Barr virus latent origin of replication. *J Virol* **65**, 677-86.

Palacios, R. & Samaridis, J. (1991). Rearrangement patterns of T-cell receptor genes in the spleen of athymic (nu/nu) young mice. *Immunogenetics* **33**, 90-5.

Panousis, C. G. & Rowe, D. T. (1997). Epstein-Barr virus latent membrane protein 2 associates with and is a substrate for mitogen-activated protein kinase. *J Virol* **71**, 4752-60.

Park, L. S., Martin, U., Sorensen, R., Luhr, S., Morrissey, P. J., Cosman, D. & Larsen, A. (1992). Cloning of the low-affinity murine granulocyte-macrophage colony- stimulating factor receptor and reconstitution of a high-affinity receptor complex. *Proc Natl Acad Sci U S A* **89**, 4295-9.

Parker, B. D., Bankier, A., Satchwell, S., Barrell, B. & Farrell, P. J. (1990). Sequence and transcription of Raji Epstein-Barr virus DNA spanning the B95-8 deletion region. *Virology* **179**, 339-46.

Parry SL, Hasbold J, Holman M, Klaus GG (1994). Hypercross-linking surface IgM or IgD receptors on mature B cells induces apoptosis that is reversed by costimulation with IL-4 and anti-CD40. *J Immunol.* **152**,2821-9.

Pearson, G. R., Luka, J., Petti, L., Sample, J., Birkenbach, M., Braun, D. & Kieff, E. (1987). Identification of an Epstein-Barr virus early gene encoding a second component of the restricted early antigen complex. *Virology* **160**, 151-61.

Peng, M. & 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**, 1775-82.

Petti, L., Sample, J., Wang, F. & Kieff, E. (1988). A fifth Epstein-Barr virus nuclear protein (EBNA3C) is expressed in latently infected growth-transformed lymphocytes. *J Virol* **62**, 1330-8.

Platt, T. H., Tcherepanova, I. Y. & Schildkraut, C. L. (1993). Effect of number and position of EBNA-1 binding sites in Epstein-Barr virus oriP on the sites of initiation, barrier formation, and termination of replication. *J Virol* **67**, 1739-45.

Polotskaya, A., Zhao, Y., Lilly, M. L. & Kraft, A. S. (1993). A critical role for the cytoplasmic domain of the granulocyte- macrophage colony-stimulating factor alpha receptor in mediating cell growth. *Cell Growth Differ* **4**, 523-31.

Polvino-Bodnar, M., Kiso, J. & Schaffer, P. A. (1988). Mutational analysis of Epstein-Barr virus nuclear antigen 1 (EBNA 1). *Nucleic Acids Res* **16**, 3415-35.

Polvino-Bodnar, M. & Schaffer, P. A. (1992). DNA binding activity is required for EBNA 1-dependent transcriptional activation and DNA replication. *Virology* **187**, 591-603.

Qu, L. & Rowe, D. T. (1992). Epstein-Barr virus latent gene expression in uncultured peripheral blood lymphocytes. *J Virol* **66**, 3715-24.

Quelle, D. E., Ashmun, R. A., Hannon, G. J., Rehberger, P. A., Trono, D., Richter, K. H., Walker, C., Beach, D., Sherr, C. J. & Serrano, M. (1995). Cloning and characterization of murine p16INK4a and p15INK4b genes. *Oncogene* **11**, 635-45.

Rauscher, F. J. d., Sambucetti, L. C., Curran, T., Distel, R. J. & Spiegelman, B. M. (1988). Common DNA binding site for Fos protein complexes and transcription factor AP-1. *Cell* **52**, 471-80.

Razzouk, B. I., Srinivas, S., Sample, C. E., Singh, V. & Sixbey, J. W. (1996). Epstein-Barr Virus DNA recombination and loss in sporadic Burkitt's lymphoma. *J Infect Dis* **173**, 529-35.

Reichert W.L. & French B. (1994). ^{32}P-Postlabeling Protocols for Assaying Levels of Hydrophobic DNA Adducts in Fish, NOAA-NWFSC Tech Memo-14,)

Reisman, D. & Sugden, B. (1986). trans activation of an Epstein-Barr viral transcriptional enhancer by the Epstein-Barr viral nuclear antigen 1. *Mol Cell Biol* **6**, 3838-46.

Reisman, D., Yates, J. & Sugden, B. (1985). A putative origin of replication of plasmids derived from Epstein-Barr virus is composed of two cis-acting components. *Mol Cell Biol* **5**, 1822-32.

Rickinson, A. B. & Kieff, E. (1996). Epstein-Barr Virus. *Fields Virology, 3rd ed. Fields, B.N., Knipe, P.M. and Howley, P.M. et al.*, Pub. Lippincott-Raven.

Rinkenberger, J. L., Wallin, J. J., Johnson, K. W. & Koshland, M. E. (1996). An interleukin-2 signal relieves BSAP (Pax5)-mediated repression of the immunoglobulin J chain gene. *Immunity* **5**, 377-86.

Roberts, M. L. & Cooper, N. R. (1998). Activation of a ras-MAPK-dependent pathway by Epstein-Barr virus latent membrane protein 1 is essential for cellular transformation. *Virology* **240**, 93-9.

Robertson, E. & Kieff, E. (1995). Reducing the complexity of the transforming Epstein-Barr virus genome to 64 kilobase pairs. *J Virol* **69**, 983-93.

Robertson, E. S., Lin, J. & Kieff, E. (1996a). The amino-terminal domains of Epstein-Barr virus nuclear proteins 3A, 3B, and 3C interact with RBPJ(k). *J Virol* **70**, 3068-74.

Robertson, E. S., Tomkinson, B. & Kieff, E. (1994). An Epstein-Barr virus with a 58-kilobase-pair deletion that includes BARF0 transforms B lymphocytes in vitro. *J Virol* **68**, 1449-58.

Robertson, K. D., Manns, A., Swinnen, L. J., Zong, J. C., Gulley, M. L. & Ambinder, R. F. (1996b). CpG methylation of the major Epstein-Barr virus latency promoter in Burkitt's lymphoma and Hodgkin's disease. *Blood* **88**, 3129-36.

Rohwer, F., Todd, S. & McGuire, K. L. (1996). The effect of IL-2 treatment on transcriptional attenuation in proto- oncogenes pim-1 and c-myb in human thymic blast cells. *J Immunol* **157**, 643-9.

Roman, C. A. & Baltimore, D. (1996). Genetic evidence that the RAG1 protein directly participates in V(D)J recombination through substrate recognition. *Proc Natl Acad Sci U S A* **93**, 2333-8.

Roque, M. C., Smith, P. A. & Blasquez, V. C. (1996). A developmentally modulated chromatin structure at the mouse immunoglobulin kappa 3' enhancer. *Mol Cell Biol* **16**, 3138-55.

Rosenbaum, H., Harris, A. W., Bath, M. L., McNeall, J., Webb, E., Adams, J. M. & Cory, S. (1990). An E mu-v-abl transgene elicits plasmacytomas in concert with an activated myc gene. *Embo J* **9**, 897-905.

Rosenbaum, H., Webb, E., Adams, J. M., Cory, S. & Harris, A. W. (1989). N-myc transgene promotes B lymphoid proliferation, elicits lymphomas and reveals cross-regulation with c-myc. *Embo J* **8**, 749-55.

Rotem-Yehudar, R., Groettrup, M., Soza, A., Kloetzel, P. M. & Ehrlich, R. (1996). LMP-associated proteolytic activities and TAP-dependent peptide transport for class 1 MHC molecules are suppressed in cell lines transformed by the highly oncogenic adenovirus 12. *J Exp Med* **183**, 499-514.

Rowe, M., Rowe, D. T., Gregory, C. D., Young, L. S., Farrell, P. J., Rupani, H. & Rickinson, A. B. (1987). Differences in B cell growth phenotype reflect novel patterns of Epstein-Barr virus latent gene expression in Burkitt's lymphoma cells. *EMBO J.* **6**, 2743-51.

Rowe, M., Peng-Pilon, M., Huen, D. S., Hardy, R., Croom-Carter, D., Lundgren, E. & Rickinson, A. B. (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**, 5602-12.

Rowe, M., Rooney, C. M., Edwards, C. F., Lenoir, G. M. & Rickinson, A. B. (1986). Epstein-Barr virus status and tumour cell phenotype in sporadic Burkitt's lymphoma. *Int J Cancer* **37**, 367-73.

Rowe, M., Rooney, C. M., Rickinson, A. B., Lenoir, G. M., Rupani, H., Moss, D. J., Stein, H. & Epstein, M. A. (1985). Distinctions between endemic and sporadic forms of Epstein-Barr virus-positive Burkitt's lymphoma. *Int J Cancer* **35**, 435-41.

Ruf, I. K. & Sample, J. (1999). Repression of Epstein-Barr virus EBNA-1 gene transcription by pRb during restricted latency. *J Virol* **73**, 7943-51.

Ryder, K., Lanahan, A., Perez-Albuerne, E. & Nathans, D. (1989). jun-D: a third member of the jun gene family. *Proc Natl Acad Sci U S A* **86**, 1500-3.

Ryder, K., Lau, L. F. & Nathans, D. (1988). A gene activated by growth factors is related to the oncogene v-jun. *Proc Natl Acad Sci U S A* **85**, 1487-91.

Ryseck, R. P. & Bravo, R. (1991). c-JUN, JUN B, and JUN D differ in their binding affinities to AP-1 and CRE consensus sequences: effect of FOS proteins. *Oncogene* **6**, 533-42.

Sabath, D. E., Broome, H. E. & Prystowsky, M. B. (1990). Glyceraldehyde-3-phosphate dehydrogenase mRNA is a major interleukin 2- induced transcript in a cloned T-helper lymphocyte. *Gene* **91**, 185-91.

Sabbatini, A., Bombardieri, S. & Migliorini, P. (1993). Autoantibodies from patients with systemic lupus erythematosus bind a shared sequence of SmD and Epstein-Barr virus-encoded nuclear antigen EBNA I. *Eur J Immunol* **23**, 1146-52.

Sakai, I. & Kraft, A. S. (1997). The kinase domain of Jak2 mediates induction of bcl-2 and delays cell death in hematopoietic cells. *J Biol Chem* **272**, 12350-8.

Sakamuro, D. & Prendergast, G. C. (1999). New Myc-interacting proteins: a second Myc network emerges. *Oncogene* **18**, 2942-54.

Salomoni, P., Perrotti, D., Martinez, R., Franceschi, C. & Calabretta, B. (1997). Resistance to apoptosis in CTLL-2 cells constitutively expressing c-Myb is associated with induction of BCL-2 expression and Myb-dependent regulation of bcl-2 promoter activity. *Proc Natl Acad Sci U S A* **94**, 3296-301.

Sample, C. & Parker, B. (1994). Biochemical characterization of Epstein-Barr virus nuclear antigen 3A and 3C proteins. *Virology* **205**, 534-9.

Sample, J., Henson, E. B. & Sample, C. (1992). The Epstein-Barr virus nuclear protein 1 promoter active in type I latency is autoregulated. *J Virol* **66**, 4654-61.

Sample, J., Lancz, G. & Nonoyama, M. (1986). Mapping of genes in BamHI fragment M of Epstein-Barr virus DNA that may determine the fate of viral infection. *J Virol* **57**, 145-54.

Sandberg, M., Hammerschmidt, W. & Sugden, B. (1997). Characterization of LMP-1's association with TRAF1, TRAF2, and TRAF3. *J Virol* **71**, 4649-56.

Sato, N., Sakamaki, K., Terada, N., Arai, K. & Miyajima, A. (1993). Signal transduction by the high-affinity GM-CSF receptor: two distinct cytoplasmic regions of the common beta subunit responsible for different signaling. *Embo J* **12**, 4181-9.

Schaefer, B. C., Woisetschlaeger, M., Strominger, J. L. & Speck, S. H. (1991). Exclusive expression of Epstein-Barr virus nuclear antigen 1 in Burkitt lymphoma arises from a third promoter, distinct from the promoters used in latently infected lymphocytes. *Proc Natl Acad Sci U S A* **88**, 6550-4.

Schaefer, B. C., Paulson, E., Strominger, J. L. & Speck, S. H. (1997). Constitutive activation of Epstein-Barr virus (EBV) nuclear antigen 1 gene transcription by IRF1 and IRF2 during restricted EBV latency. *Mol Cell Biol* **17**, 873-86.

Schaefer, B. C., Strominger, J. L. & Speck, S. H. (1995). Redefining the Epstein-Barr virus-encoded nuclear antigen EBNA-1 gene promoter and transcription initiation site in group I Burkitt lymphoma cell lines. *Proc Natl Acad Sci U S A* **92**, 10565-9.

Scheijen, B., Jonkers, J., Acton, D. & Berns, A. (1997). Characterization of pal-1, a common proviral insertion site in murine leukemia virus-induced lymphomas of c-myc and Pim-1 transgenic mice. *J Virol* **71**, 9-16.

Scherer, D. C., Brockman, J. A., Bendall, H. H., Zhang, G. M., Ballard, D. W. & Oltz, E. M. (1996). Corepression of RelA and c-rel inhibits immunoglobulin kappa gene transcription and rearrangement in precursor B lymphocytes. *Immunity* **5**, 563-74.

Schlager, S., Speck, S. H. & Woisetschlager, M. (1996). Transcription of the Epstein-Barr virus nuclear antigen 1 (EBNA1) gene occurs before induction of the BCR2 (Cp) EBNA gene promoter during the initial stages of infection in B cells. *J Virol* **70**, 3561-70

Schmidt, T., Zornig, M., Beneke, R. & Moroy, T. (1996). MoMuLV proviral integrations identified by Sup-F selection in tumors from infected myc/pim bitransgenic mice correlate with activation of the gfi-1 gene. *Nucleic Acids Res* **24**, 2528-34.

Scholle, F., Longnecker, R. & Raab-Traub, N. (1999). Epithelial cell adhesion to extracellular matrix proteins induces tyrosine phosphorylation of the Epstein-Barr virus latent membrane protein 2: a role for C-terminal Src kinase. *J Virol* **73**, 4767-75.

Schreiber, E., Matthias, P., Muller, M. M. & Schaffner, W. (1989). Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res* **17**, 6419.

Sears, A. E. & Roizman, B. (1990). Amplification by host cell factors of a sequence contained within the herpes simplex virus 1 genome. *Proc Natl Acad Sci U S A* **87**, 9441-4.

Sedlak, T. W., Oltvai, Z. N., Yang, E., Wang, K., Boise, L. H., Thompson, C. B. & Korsmeyer, S. J. (1995). Multiple Bcl-2 family members demonstrate selective dimerizations with Bax. *Proc Natl Acad Sci U S A* **92**, 7834-8.

Sen, R. & Baltimore, D. (1986). Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism. *Cell* **47**, 921-8.

Sevilla, L., Aperlo, C., Dulic, V., Chambard, J. C., Boutonnet, C., Pasquier, O., Pognonec, P. & Boulukos, K. E. (1999). The Ets2 transcription factor inhibits apoptosis induced by colony-stimulating factor 1 deprivation of macrophages through a Bcl-xL- dependent mechanism. *Mol Cell Biol* **19**, 2624-34.

Seyfert, V. L., Sukhatme, V. P. & Monroe, J. G. (1989). Differential expression of a zinc finger-encoding gene in response to positive versus negative signaling through receptor immunoglobulin in murine B lymphocytes. *Mol Cell Biol* **9**, 2083-8.

Shaffer, A. L., Peng, A. & Schlissel, M. S. (1997). In vivo occupancy of the kappa light chain enhancers in primary pro- and pre-B cells: a model for kappa locus activation. *Immunity* **6**, 131-43.

Sheppard, R. D., Samant, S. A., Rosenberg, M., Silver, L. M. & Cole, M. D. (1998). Transgenic N-myc mouse model for indolent B cell lymphoma: tumor characterization and analysis of genetic alterations in spontaneous and retrovirally accelerated tumors. *Oncogene* **17**, 2073-85.

Shire, K., Ceccarelli, D. F., Avolio-Hunter, T. M. & Frappier, L. (1999). EBP2, a human protein that interacts with sequences of the Epstein-Barr virus nuclear antigen 1 important for plasmid maintenance. *J Virol* **73**, 2587-95.

Siebenlist, U., Franzoso, G. & Brown, K. (1994). Structure, regulation and function of NF-kappa B. *Annu Rev Cell Biol* **10**, 405-55.

Simpson, K., McGuigan, A. & Huxley, C. (1996). Stable episomal maintenance of yeast artificial chromosomes in human cells. *Mol Cell Biol* **16**, 5117-26.

Silins, S. L. & Sculley, T. B. (1994). Modulation of vimentin, the CD40 activation antigen and Burkitt's lymphoma antigen (CD77) by the Epstein-Barr virus nuclear antigen EBNA- 4. *Virology* **202**, 16-24.

Singh, M. & Birshtein, B. K. (1993). NF-HB (BSAP) is a repressor of the murine immunoglobulin heavy-chain 3' alpha enhancer at early stages of B-cell differentiation. *Mol Cell Biol* **13**, 3611-22.

Sixbey, J. W., Vesterinen, E. H., Nedrud, J. G., Raab-Traub, N., Walton, L. A. & Pagano, J. S. (1983). Replication of Epstein-Barr virus in human epithelial cells infected in vitro. *Nature* **306**, 480-3.

Smith, H. C. & Berezney, R. (1980). DNA polymerase alpha is tightly bound to the nuclear matrix of actively replicating liver. *Biochem Biophys Res Commun* **97**, 1541-7.

Smith, P. R., Gao, Y., Karran, L., Jones, M. D., Snudden, D. & Griffin, B. E. (1993). Complex nature of the major viral polyadenylated transcripts in Epstein- Barr virus-associated tumors. *J Virol* **67**, 3217-25.

Snapper, C. M., Finkelman, F. D. & Paul, W. E. (1988). Differential regulation of IgG1 and IgE synthesis by interleukin 4. *J Exp Med* **167**, 183-96.

Snudden, D. K., Hearing, J., Smith, P. R., Grasser, F. A. & Griffin, B. E. (1994). EBNA-1, the major nuclear antigen of Epstein-Barr virus, resembles 'RGG' RNA binding proteins. *Embo J* **13**, 4840-7.

Speck, S. H. & Strominger, J. L. (1985). Analysis of the transcript encoding the latent Epstein-Barr virus nuclear antigen I: a potentially polycistronic message generated by long-range splicing of several exons. *Proc Natl Acad Sci U S A* **82**, 8305-9.

Srinivas, S. K. & Sixbey, J. W. (1995). Epstein-Barr virus induction of recombinase-activating genes RAG1 and RAG2. *J Virol* **69**, 8155-8.

Srivastava, R. K., Sasaki, C. Y., Hardwick, J. M. & Longo, D. L. (1999). Bcl-2-mediated drug resistance: inhibition of apoptosis by blocking nuclear factor of activated T lymphocytes (NFAT)-induced Fas ligand transcription. *J Exp Med* **190**, 253-65.

Strasser, A., Harris, A. W., Bath, M. L. & Cory, S. (1990). Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2. *Nature* **348**, 331-3.

Strasser, A., Harris, A. W. & Cory, S. (1991). bcl-2 transgene inhibits T cell death and perturbs thymic self- censorship. *Cell* **67**, 889-99.

Strasser, A., O'Connor, L., Huang, D. C., O'Reilly, L. A., Stanley, M. L., Bath, M. L., Adams, J. M., Cory, S. & Harris, A. W. (1996). Lessons from bcl-2 transgenic mice for immunology, cancer biology and cell death research. *Behring Inst Mitt*, 101-17.

Su, W., Middleton, T., Sugden, B. & Echols, H. (1991). DNA looping between the origin of replication of Epstein-Barr virus and its enhancer site: stabilization of an origin complex with Epstein-Barr nuclear antigen 1. *Proc Natl Acad Sci U S A* **88**, 10870-4.

Suda, Y., Aizawa, S., Hirai, S., Inoue, T., Furuta, Y., Suzuki, M., Hirohashi, S. & Ikawa, Y. (1987). Driven by the same Ig enhancer and SV40 T promoter ras induced lung adenomatous tumors, myc induced pre-B cell lymphomas and SV40 large T gene a variety of tumors in transgenic mice. *Embo J* **6**, 4055-65.

Sugawara, Y., Makuuchi, M., Kato, N., Shimotohno, K. & Takada, K. (1999). Enhancement of hepatitis C virus replication by Epstein-Barr virus- encoded nuclear antigen 1. *Embo J* **18**, 5755-60.

Sugden, B. & Warren, N. (1989). A promoter 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**, 2644-9.

Sumaya, C. V., Myers, L. W. & Ellison, G. W. (1980). Epstein-Barr virus antibodies in multiple sclerosis. *Arch Neurol* **37**, 94-6.

Summers, H., Barwell, J. A., Pfuetzner, R. A., Edwards, A. M. & Frappier, L. (1996). Cooperative assembly of EBNA1 on the Epstein-Barr virus latent origin of replication. *J Virol* **70**, 1228-31.

Summers, W. P., Grogan, E. A., Shedd, D., Robert, M., Liu, C. R. & Miller, G. (1982). Stable expression in mouse cells of nuclear neoantigen after transfer of a 3.4-megadalton cloned fragment of Epstein-Barr virus DNA. *Proc Natl Acad Sci U S A* **79**, 5688-92.

Sung, N. S., Wilson, J., Davenport, M., Sista, N. D. & Pagano, J. S. (1994). Reciprocal regulation of the Epstein-Barr virus BamHI-F promoter by EBNA-1 and an E2F transcription factor. *Mol Cell Biol* **14**, 7144-52.

Swaminathan, S., Tomkinson, B. & Kieff, E. (1991). Recombinant Epstein-Barr virus with small RNA (EBER) genes deleted transforms lymphocytes and replicates in vitro. *Proc Natl Acad Sci U S A* **88**, 1546-50.

Szekely, L., Selivanova, G., Magnusson, K. P., Klein, G. & Wiman, K. G. (1993). EBNA-5, an Epstein-Barr virus-encoded nuclear antigen, binds to the retinoblastoma and p53 proteins. *Proc Natl Acad Sci U S A* **90**, 5455-9.

Takada, K., Komano, J. & Imai, S. (1999). [The role of Epstein-Barr virus in oncogenesis]. *Tanpakushitsu Kakusan Koso* **44**, 2565-74.

Takahashi, Y., Cerasoli, D. M., Dal Porto, J. M., Shimoda, M., Freund, R., Fang, W., Telander, D. G., Malvey, E. N., Mueller, D. L., Behrens, T. W. & Kelsoe, G. (1999). Relaxed negative selection in germinal centers and impaired affinity maturation in bcl-xL transgenic mice. *J Exp Med* **190**, 399-410.

Taub, R., Moulding, C., Battey, J., Murphy, W., Vasicek, T., Lenoir, G. M. & Leder, P. (1984). Activation and somatic mutation of the translocated c-myc gene in burkitt lymphoma cells. *Cell* **36**, 339-48.

Thanos, D. & Maniatis, T. (1995). NF-kappa B: a lesson in family values. *Cell* **80**, 529-32.

Tomkinson, B. & Kieff, E. (1992). Use of second-site homologous recombination to demonstrate that Epstein- Barr virus nuclear protein 3B is not important for lymphocyte infection or growth transformation in vitro. *J Virol* **66**, 2893-903.

Tong, X., Drapkin, R., Reinberg, D. & Kieff, E. (1995). The 62- and 80-kDa subunits of transcription factor IIH mediate the interaction with Epstein-Barr virus nuclear protein 2. *Proc Natl Acad Sci U S A* **92**, 3259-63.

Tooze, J. ed. (1981). DNA tumour viruses, in molecular biology of tumour viruses aprt 2. Cold spring Harbor Laboratory, New York.

Tosato, G., Gerrard, T. L., Goldman, N. G. & Pike, S. E. (1988). Stimulation of EBV-activated human B cells by monocytes and monocyte products. Role of IFN-beta 2/B cell stimulatory factor 2/IL-6. *J Immunol* **140**, 4329-36.

Treisman, R. (1985). Transient accumulation of c-fos RNA following serum stimulation requires a conserved 5' element and c-fos 3' sequences. *Cell* **42**, 889-902.

Tsai, C. N., Liu, S. T. & Chang, Y. S. (1995). Identification of a novel promoter located within the Bam HI Q region of the Epstein-Barr virus genome for the EBNA 1 gene. *DNA Cell Biol* **14**, 767-76.

Tsimbouri, P., Coy, J.L., Drotar, M.E., and Wilson, J.B. (2000). EBNA 1 upregulates *BclxL* and Rags *in vivo*. Manuscript in preparation.

Turka, L. A., Schatz, D. G., Oettinger, M. A., Chun, J. J., Gorka, C., Lee, K., McCormack, W. T. & Thompson, C. B. (1991). Thymocyte expression of RAG-1 and RAG-2: termination by T cell receptor cross-linking. *Science* **253**, 778-81.

Tuscano, J. M., Druey, K. M., Riva, A., Pena, J., Thompson, C. B. & Kehrl, J. H. (1996). Bcl-x rather than Bcl-2 mediates CD40-dependent centrocyte survival in the germinal center. *Blood* **88**, 1359-64.

van Lohuizen, M., Verbeek, S., Krimpenfort, P., Domen, J., Saris, C., Radaszkiewicz, T. & Berns, A. (1989). Predisposition to lymphomagenesis in pim-1 transgenic mice: cooperation with c-myc and N-myc in murine leukemia virus-induced tumors. *Cell* **56**, 673-82.

van Lohuizen, M., Verbeek, S., Scheijen, B., Wientjens, E., van der Gulden, H. & Berns, A. (1991). Identification of cooperating oncogenes in E mu-myc transgenic mice by provirus tagging [see comments]. *Cell* **65**, 737-52.

Veis, D. J., Sorenson, C. M., Shutter, J. R. & Korsmeyer, S. J. (1993). Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. *Cell* **75**, 229-40.

Verbeek, S., van Lohuizen, M., van der Valk, M., Domen, J., Kraal, G. & Berns, A. (1991). Mice bearing the E mu-myc and E mu-pim-1 transgenes develop pre-B-cell leukemia prenatally. *Mol Cell Biol* **11**, 1176-9.

Vercelli, D., Jabara, H. H., Lee, B. W., Woodland, N., Geha, R. S. & Leung, D. Y. (1988). Human recombinant interleukin 4 induces Fc epsilon R2/CD23 on normal human monocytes. *J Exp Med* **167**, 1406-16.

Verkoczy, L. K. & Berinstein, N. L. (1998). Isolation of genes negatively or positively co-expressed with human recombination activating gene 1 (RAG1) by differential display PCR (DD RT-PCR). *Nucleic Acids Res* **26**, 4497-507.

Verma, I. M., Stevenson, J. K., Schwarz, E. M., Van Antwerp, D. & Miyamoto, S. (1995). Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation. *Genes Dev* **9**, 2723-35.

Vermes, I., Haanen, C., Steffens-Nakken, H. & Reutelingsperger, C. (1995). A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J Immunol Methods* **184**, 39-51.

Wagner, A. J., Small, M. B. & Hay, N. (1993). Myc-mediated apoptosis is blocked by ectopic expression of Bcl-2. *Mol Cell Biol* **13**, 2432-40.

Wang, C. Y., Mayo, M. W., Korneluk, R. G., Goeddel, D. V. & Baldwin, A. S., Jr. (1998a). NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c- IAP2 to suppress caspase-8 activation. *Science* **281**, 1680-3.

Wang, D., Liebowitz, D. & Kieff, E. (1985). An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. *Cell* **43**, 831-40

Wang, F., Gregory, C., Sample, C., Rowe, M., Liebowitz, D., Murray, R., Rickinson, A. & 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 cooperatively induce CD23. *J Virol* **64**, 2309-18.

Wang, J. H., Nichogiannopoulou, A., Wu, L., Sun, L., Sharpe, A. H., Bigby, M. & Georgopoulos, K. (1996a). Selective defects in the development of the fetal and adult lymphoid system in mice with an Ikaros null mutation. *Immunity* **5**, 537-49.

Wang, M. M. & Reed, R. R. (1993). Molecular mechanisms of olfactory neuronal gene regulation. *Ciba Found Symp* **179**, 68-72.

Wang, S., Rowe, M. & Lundgren, E. (1996b). Expression of the Epstein Barr virus transforming protein LMP1 causes a rapid and transient stimulation of the Bcl-2 homologue Mcl-1 levels in B-cell lines. *Cancer Res* **56**, 4610-3.

Wang, Y., Finan, J. E., Middeldorp, J. M. & Hayward, S. D. (1997). P32/TAP, a cellular protein that interacts with EBNA-1 of Epstein-Barr virus. *Virology* **236**, 18-29.

Wang, Z. G., Delva, L., Gaboli, M., Rivi, R., Giorgio, M., Cordon-Cardo, C., Grosveld, F. & Pandolfi, P. P. (1998b). Role of PML in cell growth and the retinoic acid pathway. *Science* **279**, 1547-51.

Wen, L. T., Tanaka, A. & Nonoyama, M. (1989). Induction of anti-EBNA-1 protein by 12-O-tetradecanoylphorbol-13- acetate treatment of human lymphoblastoid cells. *J Virol* **63**, 3315-22.

White, R. J., Stott, D. & Rigby, P. W. (1989). Regulation of RNA polymerase III transcription in response to F9 embryonal carcinoma stem cell differentiation. *Cell* **59**, 1081-92.

Whitmarsh, A. J. & Davis, R. J. (1996). Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. *J Mol Med* **74**, 589-607.

Williams, D. E., Namen, A. E., Mochizuki, D. Y. & Overell, R. W. (1990). Clonal growth of murine pre-B colony-forming cells and their targeted infection by a retroviral vector: dependence on interleukin-7. *Blood* **75**, 1132-8.

Williams, D. L. & Kowalski, D. (1993). Easily unwound DNA sequences and hairpin structures in the Epstein-Barr virus origin of plasmid replication. *J Virol* **67**, 2707-15.

Wilson, A., Held, W. & MacDonald, H. R. (1994). Two waves of recombinase gene expression in developing thymocytes. *J Exp Med* **179**, 1355-60.

Wilson, J. B. (1997). Transgenic mouse models of disease and Epstein-Barr virus. *EBV Report* **4**, 63-72.

Wilson, J. B., Bell, J. L. & Levine, A. J. (1996). Expression of Epstein-Barr virus nuclear antigen-1 induces B cell neoplasia in transgenic mice. *EMBO J.* **15**, 3117-26.

Wilson, J. B. & Levine, A. J. (1992). The oncogenic potential of Epstein-Barr virus nuclear antigen 1 in transgenic mice. *Curr Top Microbiol Immunol* **182**, 375-84.

Wilson, J. B., Weinberg, W., Johnson, R., Yuspa, S. & Levine, A. J. (1990). Expression of the BNLF-1 oncogene of Epstein-Barr virus in the skin of transgenic mice induces hyperplasia and aberrant expression of keratin 6. *Cell* **61**, 1315-1327.

Woisetschlaeger, M., Jin, X. W., Yandava, C. N., Furmanski, L. A., Strominger, J. L. & Speck, S. H. (1991). Role for the Epstein-Barr virus nuclear antigen 2 in viral promoter switching during initial stages of infection. *Proc Natl Acad Sci U S A* **88**, 3942-6.

Wysokenski, D. A. & Yates, J. L. (1989). Multiple EBNA1-binding sites are required to form an EBNA1-dependent enhancer and to activate a minimal replicative origin within oriP of Epstein-Barr virus. *J Virol* **63**, 2657-66.

Yang, E. & Korsmeyer, S. J. (1996). Molecular thanatopsis: a discourse on the BCL2 family and cell death. *Blood* **88**, 386-401.

Yang, E., Zha, J., Jockel, J., Boise, L. H., Thompson, C. B. & Korsmeyer, S. J. (1995). Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell* **80**, 285-91.

Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T. I., Jones, D. P. & Wang, X. (1997). Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked [see comments]. *Science* **275**, 1129-32.

Yates, J.L. and Camiolo, S.M. (1988). Dissection of DNA replication and enhancer activation functions of Epstein-Barr nuclear antigen 1. *Cancer cells,* **6**, 812-5

Yates, J. L., Warren, N. & Sugden, B. (1985). Stable replication of plasmids derived from Epstein-Barr virus in various mammalian cells. *Nature* **313**, 812-5

Yin, X. M., Oltvai, Z. N. & Korsmeyer, S. J. (1994). BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax [see comments]. *Nature* **369**, 321-3.

Yu, L., Loewenstein, P. M., Zhang, Z. & Green, M. (1995a). In vitro interaction of the human immunodeficiency virus type 1 Tat transactivator and the general transcription factor TFIIB with the cellular protein TAP. *J Virol* **69**, 3017-23.

Yu, L., Zhang, Z., Loewenstein, P. M., Desai, K., Tang, Q., Mao, D., Symington, J. S. & Green, M. (1995b). Molecular cloning and characterization of a cellular protein that interacts with the human immunodeficiency virus type 1 Tat transactivator and encodes a strong transcriptional activation domain. *J Virol* **69**, 3007-16.

Yukawa, K., Kikutani, H., Inomoto, T., Uehira, M., Bin, S. H., Akagi, K., Yamamura, K. & Kishimoto, T. (1989). Strain dependency of B and T lymphoma development in immunoglobulin heavy chain enhancer (E mu)-myc transgenic mice. *J Exp Med* **170**, 711-26.

Zhang, D., Frappier, L., Gibbs, E., Hurwitz, J. & O'Donnell, M. (1998). Human RPA (hSSB) interacts with EBNA1, the latent origin binding protein of Epstein-Barr virus. *Nucleic Acids Res* **26**, 631-7.

Zhang, L. & Pagano, J. S. (1997). IRF-7, a new interferon regulatory factor associated with Epstein-Barr virus latency. *Mol Cell Biol* **17**, 5748-57.

Zhang, S. & Nonoyama, M. (1994). The cellular proteins that bind specifically to the Epstein-Barr virus origin of plasmid DNA replication belong to a gene family. *Proc Natl Acad Sci U S A* **91**, 2843-7.

Zheng, P., Guo, Y., Niu, Q., Levy, D. E., Dyck, J. A., Lu, S., Sheiman, L. A. & Liu, Y. (1998). Proto-oncogene PML controls genes devoted to MHC class I antigen presentation. *Nature* **396**, 373-6.

Ziegler, S. F., Marth, J. D., Lewis, D. B. & Perlmutter, R. M. (1987). Novel protein-tyrosine kinase gene (hck) preferentially expressed in cells of hematopoietic origin. *Mol Cell Biol* **7**, 2276-85

Zimber-Strobl, U., Kremmer, E., Grasser, F., Marschall, G., Laux, G. & 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**, 167-75.

Zimber-Strobl, U., Strobl, L. J., Meitinger, C., Hinrichs, R., Sakai, T., Furukawa, T., Honjo, T. & Bornkamm, G. W. (1994). Epstein-Barr virus nuclear antigen 2 exerts its transactivating function through interaction with recombination signal binding protein RBP-J k, the homologue of *Drosophila* Suppressor of Hairless. *EMBO J.* **13**, 4973-82.

Zimber-Strobl, U., Suentzenich, K. O., Laux, G., Eick, D., Cordier, M., Calender, A., Billaud, M., Lenoir, G. M. & Bornkamm, G. W. (1991). Epstein-Barr virus nuclear antigen 2 activates transcription of the terminal protein gene. *J Virol* **65**, 415-23.

Zornig, M., Schmidt, T., Karsunky, H., Grzeschiczek, A. & Moroy, T. (1996). Zinc finger protein GFI-1 cooperates with myc and pim-1 in T-cell lymphomagenesis by reducing the requirements for IL-2. *Oncogene* **12**, 1789-801.

Chapter 4 APPENDIX I

Pim-1 1month Spleen RNAs

Animal No.	Spleen +	GAPDH	Ratio	Animal No.	Spleen -	GAPDH	Ratio
26815	2277	311	7.3215	26818	4060	516	7.868
816	2475	329	7.5228	821	1598	178	8.978
817	6507	997	6.5266	823	1760	208	8.462
819	2769	485	5.7093	825	5375	660	8.144
820	1893	371	5.1024	826	3066	469	6.537
822	2581	454	5.685	828	2103	394	5.338
824	2686	539	4.9833	832	1516	269	5.636
827	1943	390	4.9821	835	4857	766	6.341
829	3657	600	6.095				
830	5864	968	6.0579				
831	2127	350	6.0771				
833	4155	642	6.472				
834	1073	202	5.3119				

Bad 1month LN RNAs

Animal No.	LN +	GAPDH	Ratio	Animal No.	LN -	GAPDH	Ratio
26815	494	988	0.5	26818	1383	1678	0.824
816	530	964	0.5498	821	542	733	0.739
817	558	872	0.6399	823	830	1440	0.576
819	483	598	0.8077	825	535	586	0.913
820	879	1058	0.8308	826	270	362	0.746
822	476	654	0.7278	828	802	1088	0.737
824	260	394	0.6599	832	568	805	0.706
827	537	984	0.5457	835	476	614	0.775
829	267	635	0.4205				
830	225	543	0.4144				
831	300	601	0.4992				
833	239	490	0.4878				
834	358	635	0.5638				

Rag1 1month Spleen RNAs

Animal No.	Spleen+	GAPDH	Ratio	Animal No.	Spleen -	GAPDH	Ratio
26815	773	382	2.0236	26818	1236	757	1.633
816	943	480	1.9646	821	512	326	1.571
817	938	678	1.3835	823	593	342	1.734
819	1178	677	1.74	825	940	621	1.514
820	833	494	1.6862	826	820	502	1.633
822	1114	547	2.0366	828	556	428	1.299
824	1297	771	1.6822	832	578	386	1.497
827	315	391	0.8056	835	885	617	1.434
829	1128	517	2.1818				
830	1666	582	2.8625				
831	962	366	2.6284				
833	1322	522	2.5326				
834	402	233	1.7253				

Rag2 1month spleen RNAs

Animal No.	Spleen+	GAPDH	Ratio	Animal No.	Spleen -	GAPDH	Ratio
26815	484	202	2.396	26818	994	718	1.384
816	795	288	2.7604	821	382	305	1.252
817	1392	790	1.762	823	320	250	1.28
819	906	652	1.3896	825	995	572	1.74
820	524	364	1.4396	826	530	333	1.592
822	727	408	1.7819	828	504	296	1.703
824	732	407	1.7985	832	426	253	1.684
827	422	147	2.8707	835	863	440	1.961
829	690	379	1.8206				
830	1176	541	2.1738				
831	476	244	1.9508				
833	649	309	2.1003				
834	228	112	2.0357				

BclxL 1month Spleen RNAs							
Animal No.	Spleen+	GAPDH	Ratio	Animal No.	Spleen -	GAPDH	Ratio
26815	93	353	0.2635	26818	95	589	0.161
816	60	400	0.15	821	31	227	0.137
817	120	601	0.1997	823	29	236	0.123
819	80	511	0.1566	825	76	412	0.184
820	72	345	0.2087	826	58	335	0.173
822	97	447	0.217	828	56	269	0.208
824	90	385	0.2338	832	30	261	0.115
827	53	272	0.1949	835	70	534	0.131
829	80	442	0.181				
830	110	583	0.1887				
831	59	325	0.1815				
833	72	448	0.1607				
834	42	180	0.2333				
Bax 1month Spleen RNAs							
Animal No.	Spleen +	GAPDH	Ratio	Animal No.	Spleen -	GAPDH	Ratio
26815	0	202	0	26818	179	718	0.249
816	0	288	0	821	0	305	0
817	59	790	0.0747	823	0	250	0
819	638	652	0.9785	825	255	572	0.446
820	135	364	0.3709	826	414	333	1.243
822	319	408	0.7819	828	23	296	0.078
824	306	407	0.7518	832	0	253	0
827	206	147	1.4014	835	45	440	0.102
829	110	379	0.2902				
830	120	541	0.2218				
831	172	244	0.7049				
833	94	309	0.3042				
834	21	112	0.1875				

Chapter 4 APPENDIX II

Rag1 2 month Spleen RNA

Animal No.	Spleen+	GAPDH	Ratio	Animal No.	Spleen-	GAPDH	Ratio	Animal No.	Spleen Tum	GAPDH	Ratio
26908	392	1638	0.23932	26911	239	1095	0.218	26569	322	1305	0.25
909	331	2800	0.11821	913	208	2105	0.099	26572	189	920	0.21
910	121	632	0.19146	915	82	1336	0.061	26574	142	862	0.16
912	170	4640	0.03664	919	79	877	0.09	26583	113	999	0.11
916	106	756	0.14021	920	150	2305	0.065	26584	67	1084	0.06
917	69	485	0.14227	923	49	674	0.073				
921	94	1050	0.08952	924	67	861	0.078				
927	110	879	0.12514	926	68	714	0.095				

Rag1 2 month LN RNA

Animal No.	LN +	GAPDH	Ratio	Animal No.	LN -	GAPDH	Ratio	Animal No.	LN Tum	GAPDH	Ratio
26908	415	5351	0.07756	26911	289	2220	0.13	26569	282	249	1.13
909	442	4726	0.09353	913	307	5713	0.054	26572	501	131	3.82
910	406	5727	0.07089	915	226	2707	0.083	26574	301	176	1.71
912	560	4601	0.12171	919	234	2508	0.093	26583	458	147	3.12
916	563	4553	0.12365	920	209	3384	0.062	26584	298	258	1.16
917	65	1081	0.06013	923	210	3492	0.06				
921	263	4355	0.06039	924	258	4756	0.054				
927	256	4236	0.06043	926	174	2864	0.061				

Mdm2 2 month Spleen RNA

Animal No.	Spleen+	GAPDH	Ratio	Animal No.	Spleen-	GAPDH	Ratio	Animal No.	Spleen Tum	GAPDH	Ratio
26908	236	780	0.30256	26911	121	299	0.405	26569	139	249	0.56
909	210	1570	0.13376	913	238	1244	0.191	26572	107	131	0.82
910	83	577	0.14385	915	98	829	0.118	26574	67	176	0.38
912	254	4159	0.06107	919	63	526	0.12	26583	67	147	0.46
916	87	534	0.16292	920	100	1929	0.052	26584	57	258	0.22
917	57	243	0.23457	923	20	525	0.038				
921	88	660	0.13333	924	20	545	0.037				
927	56	360	0.15556	926	22	626	0.035				

c-Myb 2 month Spleen RNA

Animal No.	Spleen+	GAPDH	Ratio	Animal No.	Spleen-	GAPDH	Ratio	Animal No.	Spleen Tum	GAPDH	Ratio
26908	101	780	0.12949	26911	59	299	0.197	26569	85	249	0.34
909	146	1570	0.09299	913	148	1244	0.119	26572	127	131	0.97
910	107	577	0.18544	915	94	829	0.113	26574	84	176	0.48
912	208	4159	0.05001	919	99	526	0.188	26583	92	147	0.63
916	153	534	0.28652	920	190	1929	0.098	26584	90	258	0.35
917	97	243	0.39918	923	40	525	0.076				
921	138	660	0.20909	924	39	545	0.072				
927	104	360	0.28889	926	55	626	0.088				

c-Myb 2 month LN RNA

Animal No.	LN +	GAPDH	Ratio	Animal No.	LN -	GAPDH	Ratio	Animal No.	LN Tum	GAPDH	Ratio
26908	164	1094	0.14991	26911	118	209	0.565	26569	93	418	0.22
909	161	748	0.21524	913	201	1813	0.111	26572	58	728	0.08
910	126	1345	0.09368	915	181	391	0.463	26574	134	2264	0.06
912	114	1431	0.07966	919	119	397	0.3	26583	145	6032	0.02
916	139	1393	0.09978	920	111	1108	0.1	26584	64	1373	0.05
917	9	221	0.04072	923	72	989	0.073				
921	110	1580	0.06962	924	125	1073	0.116				
927	61	1651	0.03695	926	107	666	0.161				

Gfi1 2 month LN RNA

Animal No.	LN +	GAPDH	Ratio	Animal No.	LN -	GAPDH	Ratio	Animal No.	LN Tum	GAPDH	Ratio
26908	99	5351	0.0185	26911	233	2220	0.105	26569	167	282	0.59
909	160	4726	0.03386	913	262	5713	0.046	26572	274	501	0.55
910	287	5727	0.05011	915	174	2707	0.064	26574	281	301	0.93
912	2156	4601	0.46859	919	190	2508	0.076	26583	322	458	0.7
916	2444	4553	0.53679	920	202	3384	0.06	26584	206	298	0.69
917	68	1081	0.0629	923	240	3492	0.069				
921	297	4355	0.0682	924	340	4756	0.071				
927	322	4236	0.07602	926	274	2864	0.096				

BclxL 2 month Spleen RNA

Animal No.	Spleen +	GAPDH	Ratio	Animal No.	Spleen -	GAPDH	Ratio	Animal No.	Spleen Tum	GAPDH	Ratio
26908	174	780	0.22308	26911	68	299	0.227	26569	249	514	0.48
909	160	1570	0.10191	913	117	1244	0.094	26572	131	572	0.23
910	43	577	0.07452	915	71	829	0.086	26574	176	276	0.64
912	38	4159	0.00914	919	107	526	0.203	26583	147	621	0.24
916	84	534	0.1573	920	59	1929	0.031	26584	258	429	0.6
917	70	243	0.28807	923	16	525	0.03				
921	42	660	0.06364	924	27	545	0.05				
927	72	360	0.2	926	21	626	0.034				

Bad 2 month Spleen RNA

Animal No.	Spleen +	GAPDH	Ratio	Animal No.	Spleen -	GAPDH	Ratio	Animal No.	Spleen Tum	GAPDH	Ratio
26908	467	780	0.59872	26911	345	299	1.154	26569	278	249	1.12
909	500	1570	0.31847	913	412	1244	0.331	26572	218	131	1.66
910	204	577	0.35355	915	248	829	0.299	26574	210	176	1.19
912	288	4159	0.06925	919	253	526	0.481	26583	130	147	0.88
916	223	534	0.4176	920	237	1929	0.123	26584	186	258	0.72
917	230	243	0.9465	923	97	525	0.185				
921	195	660	0.29545	924	30	545	0.055				
927	148	360	0.41111	926	104	626	0.166				

MIP1a 2 month Spleen RNA

Animal No.	Spleen +	GAPDH	Ratio	Animal No.	Spleen -	GAPDH	Ratio	Animal No.	Spleen Tum	GAPDH	Ratio
26908	124	780	0.15897	26911	109	299	0.365	26569	129	249	0.52
909	92	1570	0.0586	913	113	1244	0.091	26572	91	131	0.69
910	45	577	0.07799	915	69	829	0.083	26574	50	176	0.28
912	69	4159	0.01659	919	73	526	0.139	26583	29	147	0.2
916	47	534	0.08801	920	77	1929	0.04	26584	32	258	0.12
917	37	243	0.15226	923	69	525	0.131				
921	64	660	0.09697	924	52	545	0.095				
927	72	360	0.2	926	85	626	0.136				

Pim1 2 month Spleen RNA

Animal No.	Spleen +	GAPDH	Ratio	Animal No.	Spleen -	GAPDH	Ratio	Animal No.	Spleen Tum	GAPDH	Ratio
26908	153	1776	0.08615	26911	187	1330	0.141	26569	205	1481	0.14
909	151	3530	0.04278	913	176	2794	0.063	26572	77	750	0.1
910	48	1075	0.04465	915	61	1937	0.031	26574	204	1332	0.15
912	72	6076	0.01185	919	84	1385	0.061	26583	128	1731	0.07
916	97	1187	0.08172	920	108	2644	0.041	26584	90	1608	0.06
917	36	859	0.04191	923	32	997	0.032				
921	42	1173	0.03581	924	42	1119	0.038				
927	46	1236	0.03722	926	30	1281	0.023				

Chapter 4 APPENDIX III

mBcl-2 Tumour Spleen RNAs

Animal No.	LMP+/EBNA1-	GAPDH	Ratio	Animal No.	LMP+/EBNA1+	GAPDH	Ratio	Animal No.	LMP-/EBNA1+	GAPDH	Ratio	Animal No.	c-Myc	GAPDH	Ratio	Animal No.	Bcl-2+/EBN	GAPDH	Ratio
3959.1	173	428	0.404206	3959.22	123	265	0.4642	26537	158	685	0.23066	9722	280	532	0.526316	2685.32	101	141	0.7163
3959.21	220	948	0.232068	3959.33	194	704	0.2756	26569	110	561	0.19608	9732	107	126	0.849206				
3959.35	70	158	0.443038	5939.22	154	490	0.3143	26572	103	302	0.34106								
5939.14	92	207	0.444444	5939.96	160	387	0.4134	26574	74	356	0.20787								
5939.84	176	561	0.313725	5939.97	165	411	0.4015	26583	103	305	0.3377								
								26584	110	305	0.36066								

hBak Tumour Spleen RNAs

Animal No.	LMP+/EBNA1-	GAPDH	Ratio	Animal No.	LMP+/EBNA1+	GAPDH	Ratio	Animal No.	LMP-/EBNA1+	GAPDH	Ratio	Animal No.	c-Myc	GAPDH	Ratio	Animal No.	Bcl-2+/EBN	GAPDH	Ratio
3959.1	1725	428	4.030374	3959.22	1107	265	4.1774	26537	1949	685	2.84526	9722	2303	532	4.328947	2685.32	810	141	5.7447
3959.21	2773	948	2.925105	3959.33	2603	704	3.6974	26569	1626	561	2.8984	9732	920	126	7.301587				
3959.35	631	158	3.993671	5939.22	1898	490	3.8735	26572	1740	302	5.76159								
5939.14	879	207	4.246377	5939.96	1776	387	4.5891	26574	1049	356	2.94663								
5939.84	1912	561	3.4082	5939.97	1622	411	3.9465	26583	1177	305	3.85902								
					810			26584	1015	305	3.32787								

Bax Tumour Spleen RNAs

Animal No.	LMP+/EBNA1-	GAPDH	Ratio	Animal No.	LMP+/EBNA1+	GAPDH	Ratio	Animal No.	LMP-/EBNA1+	GAPDH	Ratio	Animal No.	c-Myc	GAPDH	Ratio	Animal No.	Bcl-2+/EB1+	GAPDH	Ratio
3959.1	2020	321	6.292835	3959.22	1456	210	6.9333	26537	2540	551	4.6098	9722	2520	594	4.242424	2685.32	614	181	3.3923
3959.21	3925	714	5.497199	3959.33	1868	482	3.8755	26569	1519	349	4.35244	9732	482	143	3.370629				
3959.35	1002	169	5.928994	5939.22	1353	399	3.391	26572	1238	385	3.21558								
5939.14	1262	249	5.068273	5939.96	1413	423	3.3404	26574	752	202	3.72277								
5939.84	2349	698	3.36533	5939.97	1485	476	3.1197	26583	1089	283	3.84806								
								26584	894	264	3.38636								

Gfi1 Tumour Spleen RNAs

Animal No.	LMP+/EBNA1-	GAPDH	Ratio	Animal No.	LMP+/EBNA1+	GAPDH	Ratio	Animal No.	LMP-/EBNA1+	GAPDH	Ratio	Animal No.	c-Myc	GAPDH	Ratio	Animal No.	Bcl-2+/EB1-	GAPDH	Ratio
3959.1	446	370	1.205405	3959.22	254	185	1.373	26537	642	444	1.44595	9722	458	612	0.748366	2685.32	265	135	1.963
3959.21	775	839	0.923719	3959.33	485	408	1.1887	26569	414	294	1.40816	9732	172	167	1.02994				
3959.35	215	197	1.091371	5939.22	478	426	1.1221	26572	489	390	1.25385								
5939.14	286	276	1.036232	5939.96	641	488	1.3135	26574	278	192	1.44792								
5939.84	653	733	0.890859	5939.97	639	598	1.0686	26583	381	323	1.17957								
								26584	413	239	1.72803								

Mdm2 Tumour Spleen RNAs

Animal No.	LMP+/EBNA1+	GAPDH	Ratio	Animal No.	LMP+/EBNA1+	GAPDH	Ratio	Animal No.	LMP-/EBNA1+	GAPDH	Ratio	Animal No.	c-Myc	GAPDH	Ratio	Animal No.	Bcl-2+/EB1-	GAPDH	Ratio
3959.1	348	321	1.084112	3959.22	206	210	0.981	26537	464	551	0.84211	9722	656	594	1.104377	2685.32	224	181	1.2376
3959.21	654	714	0.915966	3959.33	438	482	0.9087	26569	277	349	0.7937	9732	153	143	1.06993				
3959.35	195	169	1.153846	5939.22	399	399	1	26572	316	385	0.82078								
5939.14	228	249	0.915663	5939.96	428	423	1.0118	26574	183	202	0.90594								
5939.84	696	698	0.997135	5939.97	500	476	1.0504	26583	259	283	0.91519								
								26584	261	264	0.98864								

Rag2Tumour Spleen RNAs

Animal No.	LMP+/EBNA1+	GAPDH	Ratio	Animal No.	LMP+/EBNA1+	GAPDH	Ratio	Animal No.	LMP-/EBNA1+	GAPDH	Ratio	Animal No.	c-Myc	GAPDH	Ratio	Animal No.	Bcl-2+/EB1-	GAPDH	Ratio
3959.1	1237	370	3.343243	3959.22	849	185	4.5892	26537	2277	444	5.12838	9722	2265	612	3.70098	2685.32	883	135	6.5407
3959.21	2177	839	1.474374	3959.33	1734	408	2.0809	26569	1731	294	7.7449	9732	1001	167	13.56287				
3959.35	683	197	11.05076	5939.22	1673	426	4.0704	26572	1809	390	4.43846								
5939.14	890	276	2.474638	5939.96	1990	488	3.4283	26574	1005	192	9.42188								
5939.84	1887	733	1.214188	5939.97	2040	598	3.3278	26583	1385	323	3.11146								
								26584	1494	239	5.79498								

Mdm2 Tumour LN RNAs

Animal No.	LMP+/EBNA1+	GAPDH	Ratio	Animal No.	LMP+/EBNA1+	GAPDH	Ratio	Animal No.	LMP-/EBNA1+	GAPDH	Ratio	Animal No.	c-Myc	GAPDH	Ratio	Animal No.	Bcl-2-/EB1+	GAPDH	Ratio
3959.1	95	132	0.719697	3959.22	199	201	0.99	26537	290	263	1.10266	9722	138	231	0.597403	2685.8	192	230	0.8348
3959.21	127	154	0.824675	3959.33	187	180	1.0389	26569	88	91	0.96703	9732	174	681	0.255507				
3959.35	116	155	0.748387	5939.22	145	149	0.9732	26572	34	36	0.94444								
5939.14	99	129	0.767442	5939.96	163	158	1.0316	26574	172	172	1								
5939.84	140	180	0.777778	5939.97	295	284	1.0387	26583	300	321	0.93458								
								26584	112	120	0.93333								

GMCSFTumour LN RNAs

Animal No.	LMP+/EBNA1+	GAPDH	Ratio	Animal No.	LMP+/EBNA1+	GAPDH	Ratio	Animal No.	LMP-/EBNA1+	GAPDH	Ratio	Animal No.	c-Myc	GAPDH	Ratio	Animal No.	Bcl-2-/EB1+	GAPDH	Ratio
3959.1	293	67	4.373134	3959.22	521	93	5.6022	26537	1217	244	4.9877	9722	539	301	1.790698	2685.8	958	202	4.7426
3959.21	336	105	3.2	3959.33	554	102	5.4314	26569	324	55	5.89091	9732	1203	752	1.599734				
3959.35	336	108	3.111111	5939.22	410	81	5.0617	26572	78	9	8.66667								
5939.14	264	108	2.444444	5939.96	598	120	4.9833	26574	845	141	5.99291								
5939.84	390	159	2.45283	5939.97	1087	241	4.5104	26583	1888	325	5.80923								
								26584	826	150	5.50667								

Mip1a Tumour LN RNAs

Animal No.	LMP+/EBNA1+	GAPDH	Ratio	Animal No.	LMP+/EBNA1+	GAPDH	Ratio	Animal No.	LMP-/EBNA1+	GAPDH	Ratio	Animal No.	c-Myc	GAPDH	Ratio	Animal No.	Bcl-2-/EB1+	GAPDH	Ratio
3959.1	294	67	4.38806	3959.22	655	93	7.043	26537	1338	244	5.48361	9722	706	301	2.345515	2685.8	1228	202	6.0792
3959.21	442	105	2.8	3959.33	731	102	7.1667	26569	397	55	7.21818	9732	1682	752	2.236702				
3959.35	450	108	4.092593	5939.22	686	81	8.4691	26572	133	9	14.7778								
5939.14	426	108	4.166667	5939.96	862	120	7.1833	26574	1160	141	8.22695								
5939.84	617	159	2.679245	5939.97	144	241	0.5975	26583	2079	325	6.39692								
								26584	976	150	6.50667								

Rag1 Tumour LN RNAs

Animal No.	LMP+/EBNA1-	GAPDH	Ratio	Animal No.	LMP+/EBNA1+	GAPDH	Ratio	Animal No.	LMP-/EBNA1+	GAPDH	Ratio	Animal No.	c-Myc	GAPDH	Ratio	Animal No.	Bcl-2-/EB1+	GAPDH	Ratio
3959.1	172	67	2.567164	3959.22	324	93	3.4839	26537	564	244	2.31148	9722	308	301	1.023256	2685.8	428	202	2.1188
3959.21	253	105	2.409524	3959.33	396	102	3.8824	26569	143	55	2.6	9732	717	752	0.953457				
3959.35	234	108	2.166667	5939.22	293	81	3.6173	26572	47	9	5.22222								
5939.14	209	108	1.935185	5939.96	340	120	2.8333	26574	391	141	2.77305								
5939.84	197	159	1.238994	5939.97	510	241	2.1162	26583	776	325	2.38769								
								26584	294	150	1.96								

Gfi1 Tumour LN RNAs

Animal No.	LMP+/EBNA1-	GAPDH	Ratio	Animal No.	LMP+/EBNA1+	GAPDH	Ratio	Animal No.	LMP-/EBNA1+	GAPDH	Ratio	Animal No.	c-Myc	GAPDH	Ratio	Animal No.	Bcl-2-/EB1+	GAPDH	Ratio
3959.1	95	106	0.896226	3959.22	209	112	1.8661	26537	408	208	1.96154	9722	443	303	1.462046	2685.8	534	419	1.2745
3959.21	122	140	0.871429	3959.33	227	106	2.1415	26569	154	60	2.56667	9732	1011	1103	0.916591				
3959.35	106	144	0.736111	5939.22	205	100	2.05	26572	42	16	2.625								
5939.14	102	140	0.728571	5939.96	265	137	1.9343	26574	396	190	2.08421								
5939.84	196	178	1.101124	5939.97	435	217	2.0046	26583	806	467	1.72591								
								26584	347	249	1.39357								

Chapter 4 APPENDIX IV

c-Myc 1month Spleen RNAs

Animal No.	Spleen +	GAPDH	Ratio	Animal No.	Spleen -	GAPDH	Ratio
26815	1842	353	5.2181	26818	3314	589	5.6265
816	1741	400	4.3525	821	984	227	4.3348
817	3737	601	6.218	823	1080	236	4.5763
819	2313	511	4.5264	825	2679	412	6.5024
820	1627	345	4.7159	826	1863	335	5.5612
822	2494	447	5.5794	828	1755	269	6.5242
824	2301	385	5.9766	832	1294	261	4.9579
827	1303	272	4.7904	835	2636	534	4.9363
829	2408	442	5.448				
830	3032	583	5.2007				
831	1497	325	4.6062				
833	2111	448	4.7121				
834	534	180	2.9667				

c-Myc 1month LN RNAs

Animal No.	LN +	GAPDH	Ratio	Animal No.	LN -	GAPDH	Ratio
26815	541	6170	0.0877	26818	753	8294	0.0908
816	495	4811	0.1029	821	381	4672	0.0815
817	416	4010	0.1037	823	894	10110	0.0884
819	236	2763	0.0854	825	368	3166	0.1162
820	533	6018	0.0886	826	135	1662	0.0812
822	289	4197	0.0689	828	817	8533	0.0957
824	185	1821	0.1016	832	553	5759	0.096
827	728	8483	0.0858	835	376	3994	0.0941
829	287	3515	0.0817				
830	219	2297	0.0953				
831	351	3187	0.1101				
833	316	3366	0.0939				
834	425	4997	0.0851				

c-Myc 2 month Spleen RNAs

Animal No.	Spleen +	GAPDH	Ratio	Animal No.	Spleen -	GAPDH	Ratio	Animal No	Spleen Tum	GAPDH	Ratio
26908	254	3270	0.0777	26911	293	1313	0.2232	26569	328	2182	0.1503
909	372	4530	0.0821	913	306	2757	0.111	26572	98	1457	0.0673
910	181	1138	0.1591	915	289	1978	0.1461	26574	340	974	0.3491
912	110	6847	0.0161	919	39	931	0.0419	26583	396	1165	0.3399
916	204	961	0.2123	920	144	2720	0.0529	26584	320	1238	0.2585
917	176	461	0.3818	923	166	700	0.2371				
921	159	1110	0.1432	924	79	679	0.1163				
927	166	640	0.2594	926	68	763	0.0891				

c-Myc 2month LN RNAs

Animal No.	LN +	GAPDH	Ratio	Animal No.	LN -	GAPDH	Ratio	Animal No	LN Tum	GAPDH	Ratio
26908	217	5439	0.0399	26911	41	1626	0.0252	26569	172	2153	0.0799
909	346	4034	0.0858	913	564	6891	0.0818	26572	810	8236	0.0983
910	827	5038	0.1642	915	524	3750	0.1397	26574	367	6483	0.0566
912	863	2834	0.3045	919	490	2298	0.2132	26583	347	8651	0.0401
916	577	2418	0.2386	920	1207	2813	0.4291	26584	452	1715	0.2636
917	248	109	2.2752	923	926	1942	0.4768				
921	759	1778	0.4269	924	1010	2002	0.5045				
927	462	1663	0.2778	926	792	1105	0.7167				

c-Myc Tumour LN RNAs

Animal No.	LMP+/EBNA1-	GAPDH	Ratio	Animal No.	LMP+/EBNA1+	GAPDH	Ratio	Animal No	LMP-/EBNA1+	GAPDH	Ratio	Animal No	c-Myc	GAPDH	Ratio	Animal No.	Bcl-2-/EB1+	GAPDH	Ratio
3959.1	150	106	1.4151	3959.22	318	112	2.8393	26537	588	208	2.8269	9722	325	303	1.073	2685.8	641	419	1.53
3959.21	211	140	1.5071	3959.33	272	106	2.566	26569	200	60	3.3333	9732	1082	1103	0.981				
3959.35	185	144	1.2847	5939.22	275	100	2.75	26572	252	16	15.75								
5939.14	150	140	1.0714	5939.96	305	137	2.2263	26574	359	190	1.8895								
5939.84	280	178	1.573	5939.97	519	217	2.3917	26583	763	467	1.6338								
								26584	306	249	1.2289								

c-Myc Tumour Spleen RNAs

Animal No.	LMP+/EBNA1-	GAPDH	Ratio	Animal No.	LMP+/EBNA1+	GAPDH	Ratio	Animal No	LMP-/EBNA1+	GAPDH	Ratio	Animal No	c-Myc	GAPDH	Ratio	Animal No.	Bcl-2+/EB1+	GAPDH	Ratio	Animal No.	Pim1+/EB1+	GAPDH	Ratio
3959.1	231	370	0.6243	3959.22	196	185	1.0595	26537	358	444	0.8063	9722	476	612	0.778	2685.32	121	135	0.896	5987.8	475	307	1.5472
3959.21	404	839	0.4815	3959.33	312	408	0.7647	26569	242	294	0.8231	9732	232	167	1.389								
3959.35	175	197	0.8883	5939.22	258	426	0.6056	26572	271	390	0.6949												
5939.14	182	276	0.6594	5939.96	308	488	0.6311	26574	152	192	0.7917												
5939.84	418	733	0.5703	5939.97	298	598	0.4983	26583	193	323	0.5975												
								26584	197	239	0.8243												

Chapter 5 Appendix I

Genes on the Atlas Mouse cDNA Expression Array

Oncogenes & Tumor Suppressors

Tumor Suppressors & Related Proteins

-APC; Adenomatous Polyposis Coli protein
-BRCA1; Breast/ovarian cancer susceptibility locus 1 product BRCA2; Breast cancer susceptibility locus 2 product
-DCC; netrin receptor; immunoglobulin gene superfamily member; former tumor suppressor protein candidate
-EB1 APC-binding protein
-Ezrin; Villin 2; NF-2 (merlin) related filament/plasma membrane associated protein
-Madr1; mSmad1; Mothers against dpp protein (Mad) murine homologue; TGF-beta signaling protein-1 (bsp-1); candidatetumor suppressor gene
-Mdm2; p53-regulating protein
-NF2; Merlin (moesin-ezrin-radixin-like protein); shwannomin, murine neurofibromatosis type 2 susceptibility protein
-p107; RBL1; Retinoblastoma gene product-related protein p107 (cell cycle regulator)
-p130; Retinoblastoma gene product-related protein Rb2/p130 (cell cycle regulator)
-p53; tumor suppressor; DNA-binding protein
-Rb; pp105; Retinoblastoma susceptibility-associated protein (tumor suppressor gene; cell cycle regulator)
-TSG101 tumor susceptibility protein
-Tumor suppressor maspin
-VHL; Von Hippel-Lindau tumor suppressor protein
-WT1; Wilms tumor protein; tumor suppressor
-ZO-1; Tight junction protein; discs-large family member, partially homologous to a dIg-A tumor suppressor in Drosophila

Proto-Oncogenes & Transcription Factor-Related Oncogenes

-A-myb proto-oncogene; myb-related protein A
-B-myb proto-oncogene; myb-related protein B
-c-ErbA oncogene; thyroid hormone receptor
-c-Fos proto-oncogene; transcription factor AP-1 component. fos cellular oncogene
-c-Jun proto-oncogene (transcription factor AP-1 component), RNA polymerase I termination factor TTF-1
-c-myb proto-oncogene protein
-c-myc proto-oncogene protein
-c-rel proto-oncogene
-Ear-2; v-erbA related proto-oncogene
-Elk-1 ets-related proto-oncogene
-Fli-1 ets-related proto-oncogene
-Fos-B; c-fos-related protein fos B
-Fra-2 (fos-related antigen 2)
-Gli oncogene; zinc finger transcription factor
-Jun-B; c-jun-related transcription factor
-Jun-D; c-jun-related transcription factor
-L-myc proto-oncogene protein
-Net; ets related transcription factor; activated by Ras
-N-myc proto-oncogene protein

Serine/Threonine Protein Kinase Oncogenes

-A-Raf proto-oncogene
-B-Raf proto-oncogene
-Cot proto-oncogene
-Casein kinase II (alpha subunit)
-Pim-1 proto-oncogene

Tyrosine Protein Kinase Oncogenes: Receptor Protein Tyrosine Kinase Oncogenes

-c-Fms proto-oncogene (macrophage colony stimulating factor 1 (CSF-1) receptor)
-c-Kit proto-oncogene (mast/stem cell growth factor receptor tyrosine kinase)
-Met protooncogene
-PDGFRa; platelet-derived growth factor alpha-receptor
-Ret proto-oncogene (Papillary thyroid carcinoma-encoded protein)
-Ski proto-oncogene
-Sky proto-oncogene (Tyro3; Rse; Dtk)
-Tie-2 proto-oncogene
-Vegfr1; Vascular endothelial growth factor receptor 1/Fms-related tyrosine kinase 1 (Flt1)

Tyrosine Protein Kinase Oncogenes: Non-Receptor Tyrosine-Protein Kinases

-c-Abl proto-oncogene
-c-Fes proto-oncogene
-c-Fgr proto-oncogene
-c-Src proto-oncogene
-Lymphocyte-specific tyrosine-protein kinase LCK

Intracellular Signal Transduction-Related Oncogenes

-c-Cbl proto-oncogene (Adaptor protein)
-H-ras proto-oncogene; transforming G-protein
-Lfc proto-oncogene
-N-ras proto-oncogene; transforming G-protein
-Shc transforming adaptor protein; Src homology 2 (SH2) protein, SHB-related

Growth Factor-Related Oncogenes

-CSF-1; M-CSF; colony stimulating factor-1
-Int-3 proto-oncogene; NOTCH family member; NOTCH4
-Preproglucagon
-Beta-protachykinin a

Non-Tyrosine Kinase Receptor Oncogenes

-c-Mpl; thrombopoietin receptor; hematopoietic growth factor receptor superfamily member
- Mas proto-oncogene (G-protein coupled receptor)

Other Oncogenes & Related Genes

-IGFBP-2; insulin-like growth factor binding protein 2; autocrine and/or paracrine growth promoter
-Tiam-1 invasion inducing protein; GDP-GTP exchanger-related

Cell Cycle Regulators

Cyclins

-Cyclin A (G2/M-specific)
-Cyclin A1 (G2/M-specific)
-Cyclin B1 (G2/M-specific)
-Cyclin B2 (G2/M-specific)
-Cyclin C (G1-specific)
-Cyclin D1 (G1/S-specific)
-Cyclin D2 (G1/S-specific)
-Cyclin D3 (G1/S-specific)
-Cyclin E (G1/S-specific)
-Cyclin F (S/G2/M-specific)
-Cyclin G (G2/M-specific)
-Cyclin G2 (G2/M-specific)

Cell Cycle-Regulating Kinases

-Cdk4; cyclin-dependent kinase 4
-Cdk5; cyclin-dependent kinase 5
-Cdk7; MO15; cyclin-dependent kinase 7 (homologue of Xenopus MO15 cdk-activating kinase)
-p58/GTA; galactosyltransferase associated protein kinase (cdc2-related protein kinase)

Cdk Inhibitors

-p18ink4; cdk4 and cdk6 inhibitor
-p19ink4; cdk4 and cdk6 inhibitor
-p21/Cip1/Waf1; cdk-inhibitor protein 1
-p27kip1; G1 cyclin-Cdk protein kinase inhibitor, p21-related

-p57kip2; cdk-inhibitor kip2 (cyclin-dependent kinase inhibitor1B) member of the p21CIP1 -Cdk inhibitor family, candidate tumor suppressor gene
-Wee1/p87; cdc2 tyrosine 15-kinase

Other Cell Cycle-Regulating Proteins

-Cdc25 phosphatase; guanine nucleotide releasing protein
-Cdc25a; cdc25M1; MPI1 (M-phase inducer phosphatase 1)
-Cdc25b; cdc25M2; MPI2 (M-phase inducer phosphatase 2)
-Myeloblastin; trypsin-chymotrypsin related serine protease
-Prothymosin alpha
-Tob antiproliferative factor; interacts with p185erbB2

Stress Response Proteins

-HSP27; heat shock 27kD protein 1
-HSP60; heat shock 60 kDa protein 1 (chaperonin, GroEL homologue); mitochondrial matrix protein P1
-HSP84; heat shock 84kD protein
-HSP86; heat shock 86kD protein
-MTJ1; DnaJ-like heat-shock protein from mouse tumor
-Osp94 osmotic stress protein; APG-1; hsp70-related
-MDR1; P-glycoprotein; multidrug resistance protein; efflux pump
-HMG1-related VDJ recombination signal binding protein
-MmMre11a putative endo/exonuclease
-C3H cytochrome P450; Cyp1b1
-ERp72 endoplasmic reticulum stress protein; protein disulfide isomerase-related protein
-Etoposide induced p53 responsive (EI24) mRNA
-Glucose regulated protein, 78kD; Grp78
-Oxidative stress-induced protein mRNA
-P-1-450; dioxin-inducible cytochrome P450

Ion Channel & Transport Proteins

-ATP-binding casette 8; ABC8; homolog of Drosophila white CCHB3; calcium channel (voltage-gated; dihydropyridine-sensitive; L-type) beta-3 subunit)
-Golgi 4-transmembrane spanning transporter; MTP
-Glucose transporter-1, erythrocyte; Glut1
-Voltage-gated sodium channel

Intracellular Signal Transduction Modulators & Effectors

Receptors & Cell Surface Proteins

-B7-2; T lymphocyte activation antigen CD86; CD28 antigen ligand 2, B7-2 antigen; alternative CTLA4 counter-receptor
-BST-1; lymphocyte differentiation antigen CD38
-C-C CKR-1; CCR-1; C-C chemokine receptor type 1, macrophage inflammatory protein-1 alpha receptor; MIP-1alpha-R; RANTES-R
-Cf2r; coagulation factor II (thrombin) receptor
-Eph3 (Nuk) tyrosine-protein kinase receptor
-Etk1 (Mek4; HEK) tyrosine-protein kinase receptor HEK
-Frizzled-3; Drosophila tissue polarity gene frizzled homologue 3; dishevelled receptor
-Hek2 murine homologue; Mdk5 mouse developmental kinase; Eph -related tyrosine-protein kinase receptor
-Htk; Mdk2 mouse developmental kinase; Eph -related tyrosine-protein kinase receptor
-IFNgR2; interferon-gamma receptor second (beta) chain; interferon gamma receptor accessory factor-1 (AF-1)
-Interleukin-6 receptor beta chain; membrane glycoprotein gp130
-LCR-1; CXCR-4; CXC (SDF-1) chemokine receptor 4; HIV coreceptor (fusin); G protein-coupled receptor LCR1 homologue
-LFA1-alpha; integrin alpha L; leukocyte adhesion glycoprotein LFA-1 alpha chain; antigen CD11A (p180)
-Prostaglandin E2 receptor EP4 subtype
-Tie-1 tyrosine-protein kinase receptor
-Transferrin receptor protein (p90, CD71)
-uPAR1; urokinase plasminogen activator surface receptor (CD87)
-Vegfr2; KDR/flk1 vascular endothelial growth factor tyrosine kinase receptor

Transcription Factors

-Beta2-RAR; retinoic acid receptor beta-2
-CRE-BP1; cAMP response element binding protein 1
-I-kappa B alpha chain
-I-kappa B beta
-NF-kappa-B transcription factor p65 subunit; rel-related polypeptide
-Pml; Murine homologue of the leukemia-associated PML gene
-RXR-beta cis-11-retinoic acid receptor
-Stat1; signal transducer and activator of transcription
-Stat3; APRF; acute phase response factor
-Stat5a; mammary gland factor
-Stat6; signal transducer and activator of transcription 6; IL-4 Stat; STA6
-TANK; I-TRAF; TRAF family member associated NF-kB activator
-Transcription factor A10
-Transcription factor TF II D
-Tristetraproline

-Receptor-Associated Proteins & Adaptors

-Cas; Crk-associated substrate; focal adhesion kinase substrate
-Crk adaptor protein
-Csk; c-Src-kinase and negative regulator
-Fyn proto-oncogene; Src family member
-Hck tyrosine-protein kinase
-SLAP; src-like adapter protein; Eck receptor tyrosine kinase-associated
-Syk tyrosine-protein kinase (activated p21cdc42Hs kinase [ack])
-Syp; SH-PTP2; adaptor protein tyrosine phosphatase

Intracellular Kinase Network Members

-CamK IV; Ca2/calmodulin-dependent protein kinase IV (catalytic chain)
-cAMP-dependent protein kinase type I-beta regulatory chain
-Erk1; extracellular signal-regulated kinase 1; p44; Ert2
-Inhibitor of the RNA-activated protein kinase, 58-kDa
-Jak3 tyrosine-protein kinase; Janus kinase 3
-Jnk stress-activated protein kinase (SAPK)
-LIMK; LIM serine/threonine kinase
-MAPK; MAP kinase; p38
-MAPKAPK-2; MAP kinase-activated protein kinase; MAPKAP kinase 2
-MAPKK1; MAP kinase kinase 3 (dual specificity) (MKK1)
-MAPKK3; MAP kinase kinase 3 (dual specificity) (MKK3, MEK3)
-MAPKK4; MAP kinase kinase 4; Jnk activating kinase 1; (JNKK1; SEK1; MKK4)
-MAPKK6; MAP kinase kinase 6(dual specificity) (MKK6)
-PKC-alpha; protein kinase C alpha type
-PKC-beta; protein kinase C beta-II type
-PKC-delta; protein kinase C delta type
-PKC-theta; protein kinase C theta type
-Rsk; ribosomal protein S6 kinase

Non-Protein Kinase Effectors

-PI3-K p110; phosphatidylinositol 3-kinase catalytic subunit

-PI3-K p85; phosphatidylinositol 3-kinase regulatory subunit; phosphoprotein p85; PDGF signaling pathway member
-PLC beta; phospholipase C beta 3
-PLC gamma; phospholipase C gamma

G-Proteins

-G-alpha-13 guanine nucleotide regulatory protein
-Gem; induced, immediate early protein; Ras family member
-Rab-2 ras-related protein
-Rac1 murine homologue
-R-ras protein, closely related to ras proto-oncogenes
-Transducin beta-2 subunit
-Vav; GDP-GTP exchange factor; proto-oncogene

Other Related Proteins

-14-3-3 protein eta
-Cortactin; protein tyrosine kinase substrate
-Dvl2; dishevelled-2 tissue polarity protein
-GapIII; GTPase-activating protein
-IRF1; interferon regulatory factor 1
-PTPRG; protein-tyrosine phosphatase gamma
-WBP6; pSK-SRPK1; WW domain binding protein 6 serine kinase for SR splicing factors
-Zyxin; LIM domain protein; alpha-actinin binding protein

Apoptosis-Related Proteins

Cysteine Proteases

-Caspase-11; ICH-3 cysteine protease; upstream regulator of ICE
-Caspase-3; Nedd2 cysteine protease (positive regulator of programmed cell death ICH-1 homologue)
-Caspase-7; Lice2; ICE-LAP3 cysteine protease

Bcl-2 Family

-Bad; heterodimeric partner for Bcl-XL and Bcl-2; promotes cell death
-BAG-1; bcl-2 binding protein with anti-cell death activity
-Bak apoptosis regulator; Bcl-2 family member
-Bax; Bcl-2 heterodimerization partner and homologue
-Bcl-2; B cell lymphoma protein 2, apoptosis inhibitor
-Bcl-W apoptosis regulator; Bcl-2 family member
-Bcl-xL apoptosis regulator (bcl-x long); Bcl-2 family member
-BID; apoptic death agonist

Glutathione Peroxidases, Transferases, etc.

-Glutathione peroxidase (plasma protein); selenoprotein
-Glutathione reductase
-Glutathione S-transferase A
-Glutathione S-transferase (microsomal)
-Glutathione S-transferase Mu 1
-Gluthathione S-transferase (theta type1); phase II conjugation enzyme
-GST Pi 1; glutathione S-transferase Pi 1; preadipocyte growth factor

Other

-A20 zinc finger protein; apoptosis inhibitor
-Adenosine A1M receptor
-Adenosine A2M2 receptor
-Adenosine A3 receptor
-ALG-2; calcium binding protein required for programmed cell death
-Blk; B lymphocyte kinase; Src family member
-c-Akt proto-oncogene; Rac-alpha; proteine kinase B (PKB)
-CD27; lymphocyte-specific NGF receptor family member
-CD 30L receptor (Lymphocyte activation antigene CD 30, Ki-1 antigene)
-CD40L; CD40 ligand
-Chop10; murine homologue of Gadd153 (growth arrest and DNA-damage-inducible protein)
-Clusterin; complement lysis inhibitor; testosterone-repressed prostate message 2; apolipoprotein J; sulfated glycoprotein-2
-CRAF1; TNF receptor (CD40 receptor) associated factor; TRAF-related
-DAD-1; defender against cell death 1
-FAF1; Fas-associated protein factor, apoptosis activator
-Fasl receptor (Fas antigen, Apo-1 antigen)
-Fasl; Fas antigen ligand; generalized lymphoproliferation disease gene (gld) in mice
-FLIP-L; apoptosis inhibitor; FLICE-like inhibitory protein
-Fms-related tyrosine kinase 3 Flt3/Flk2 ligand
-Gadd45; growth arrest and DNA-damage-induible protein
-IGFR II; insulin-like growth factor receptor II, cation-independent mannose-6-P receptor; elevated in Wilms's tumor cells
-IGF-I-R alpha; insulin-like growth factor I receptor alpha subunit
-iNOSl; nitric oxide synthase (inducible)
-Interleukin-1 receptor
-NADPH-cytochrome P450 reductase
-Neuronal death protein
-Nm23-M2; nucleoside diphosphate kinase B; metastasis-reducing protein; c-myc-related transcription factor
-Nur77 early response protein; thyroid hormone (TR3) receptor
-p55cdc; cell division control protein 20
-PD-1 possible cell death inducer; Ig gene superfamily member
-Protein tyrosine phosphatase
-PS-2; homologue of the Alzheimer's disease gene
-Relaxin
-RIP cell death protein; Fas/APO-1 (CD95) interactor, contains death domain
-Sik; Src-related intestinal kinase
-SPI3; serpin; similar to human proteinase inhibitor 6 (placental thrombin inhibitor) serine proteinase inhibitor
-STAM; signal transducing adaptor molecule
-Stromelysin-3; matrix metalloproteinase-11 (MMP-11)
-TDAG51; couples TCR signaling to Fas (CD95) expression
-TNF 55; tumor necrosis factor 1 (55kd)
-TRAIL; TNF-related apoptosis inducing ligand; Apo-2 ligand
-Tumor necrosis factor receptor 1; TNFR-1

DNA Synthesis, Repair & Recombination Proteins

-Activator -1 140 KD subunit (replication factor C 140KD)
-AP endonuclease; apurinic/apyrimidinic endonuclease (Apex)
-Atm; ataxia telangiectasia murine homologue
-ATP-dependent DNA helicase II 70 kDa subunit; thyroid Ku
-(p70/p80) autoantigen p70 subunit; p70 Ku)
-ATP-dependent DNA helicase II 80 kDa subunit; thyroid Ku (p70/p80) autoantigen p80 subunit; p80 Ku)
-DNA ligase I
-DNA ligase III
-DNA polymerase alpha catalytic subunit (p180)
-DNA topoisomerase I (Top I)
-DNA topoisomerase II (Top II)
-PA6 stromal protein; RAG1 gene activator
-DNA-polymerase delta catalytic subunit
-DNAse I
-ERCC-1; DNA excision repair protein

-ERCC3 DNA repair helicase; DNA-repair protein complementing XP-B cells (XPBC)
-ERCC5 excision repair protein; DNA-repair protein complementing XP-G cells (XPG)
-GTBP; G/T-mismatch binding protein; MSH6
-HR21spA; protein involved in DNA double-strand break repair; PW29; calcium-binding protein
-MHR23A; Rad23 UV excision repair protein homologue; xeroderma pigmentosum group C (XPC) repair complementing protein
-MHR23B; Rad23 UV excision repair protein homologue; xeroderma pigmentosum group C (XPC) repair complementingprotein
-MLH1 DNA mismatch repair protein; MutL homologue
-MmLim15; RecA-like gene; DMC1 homologue; meiosis-specific homologous recombination protein
-MmRad51; yeast DNA repair protein Rad51 and E coli RecA homologue
-MmRad52; yeast DNA repair protein Rad52 homologue
-MSH2 DNA mismatch repair protein; MutS homologue 2
-PCNA; proliferating cell nuclear antigen; processivity factor
-Photolyase/blue-light receptor homologue
-PMS2 DNA mismatch repair protein; yeast PMS1 homolog 2
-Pur-alpha transcriptional activator; sequence-specific ssDNA-binding protein
-Rad50; DNA repair protein
-RAG-1; V(D)J recombination activating protein
-RAG-2; V(D)J recombination activating protein
-ShcC adaptor; Shc-related; brain-specific
-Translin; recombination hotspot binding protein
-Ubiquitin-conjugating enzyme, yeast Rad6 homologue; murine HR6B
-Ung1; uracil-DNA glycosylase
-XPAC; xeroderma pigmentosum group A correcting protein
-XRCC1 DNA-repair protein, affecting ligation

Transcription Factors & General DNA Binding Proteins

-Ablphilin-1 (abi-1) similar to HOXD3
-Activating transcription factor 4 (mATF4)
-Adipocyte differentiation-associated protein
-AT motif-binding factor ATBF1
-Basic domain/leucine zipper transcription factor
-Brain factor 1 (Hfhbf1)
-Brain specific transcription factor NURR-1
-Brn-3.2 POU transcription factor
-Butyrate response factor 1
-CACCC Box- binding protein BKLF
-CCAAT- Binding transcription factor (C/ EBP)
-Caudal type Homeobox 1 (Cdx1)
-Caudal type Homeobox 2 (Cdx2)
-Sim transcription factor
-Early B cell factor (EBF)
-Engrailed protein (En-1) homolog
-Engrailed protein (En-2) homolog
-Erythroid transcription factor NF-E2
-DNA-binding protein SATB1
-DNA-binding protein SMBP2
-DP-1 (DRTF-polipeptide 1) cell cycle regulatory transcription factor
-E2F-5 transcription factor
-Egr-1 Zn-finger regulatory protein
-Elf-1 Ets family transcription factor
-Epidermal growth factor receptor kinase substrate EPS8
-ERA-1 Protein (ERA-1-993)
-Erf (Ets-related transcription factor)
-Erythroid kruppel-like transcription factor
-Ets-related protein PEA 3
-Ets-2 transcription factor
-Ets-related protein Sap 1A
-GA binding protein beta-2 chain
-GATA binding transcription factor (GATA-4)
-GATA-3 transcription factor
-Gbx 2
-Glial cells missing gene homolog (mGCM1)
-Gut-specific Kruppel-like factor GKLF
-Heat shock transcription factor 2 (HSF 2)
-Hepatocyte nuclear factor 3/forkhead homolog 8 (HFH-8)
-HMG-box transcription factor from testis (MusSox17)
-HMG-14 non histone chromosomal protein
-Homeo Box protein 1.1 (Hox-1.1)
-Homeo Box protein 2.1 (Hox-2.1)
-Homeo Box protein 2.4 (Hox-2.4)
-Homeo Box protein 2.5 (Hox-2.5)
-Homeo Box protein 3.1 (Hox-3.1)
-Homeo Box protein 4.2 (Hox-4.2)
-Homeo Box protein 7.1 (Hox-7.1)
-Homeo Box protein 8 (Hox-8)
-Homeobox protein HOXD-3
-Ikaros DNA binding protein
-Sp4 zinc finger transcription factor
-Interferon inducible protein 1
-Interferon regulatory factor 2 (IRF 2)
-Kruppel-like factor LKLF
-Lbx 1 transcription factor
-Mph-1 nuclear transcriptional repressor for hox genes
-MRE-binding transcription factor
-Myocyte nuclear factor (MNF)
-Myogenic factor 5
-Neuronal helix-loop-helix protein NEX-1
-NF-1B protein (transcription factor)
-NF-kappa B binding subunit (nuclear factor) (TFDB5)
-Nuclear factor related to P45 NF-E2
-Nuclear hormone receptor ROR-ALPHA-1
-Nucleobindin
-Octamer binding transcription factor (Oct 3)
-PAX-8 (paired box protein PAX 8)
-Split hand/foot gene
-SRY-box containing gene 3 (Sox3)
-PAX-5 (B cell specific transcription factor)
-PAX-6 (paired box protein)
-POU domain, class 2, associated factor 1
-PSD-95/SAP90A
-Retinoic acid binding protein II cellular (CRABP-II)
-Retinoic acid receptor RXR- gamma
-Retinoid X receptor interacting protein (RIP 15)
-T-lymphocyte activated protein
-Transcription factor 1 for heat shock gene
-Transcription factor BARX1 (homeodian transcription factor)
-Transcription factor C 1
-Transcription factor CTCF (11 zinc fingers)
-Transcription factor LIM-1
-Transcription factor LRG - 21
-Transcription factor NFAT 1, isoform alpha
-SRY-box containing gene 4
-Transcription factor RelB
-Transcription factor S-II (transcription elongation factor)
-Transcription factor SEF2
-Transcription factor SP1P (POU domain transcription factor)
-Transcription factor SP2
-Transcription factor UBF

-Transcriptional enhancer factor 1 (TEF-1)
-YB1 DNA binding protein
-YY1 (UCRBP) transcriptional factor
-Zinc finger Kruppel type Zfp 92
-Zinc finger transcription factor RU49
-Zinc finger X-chromosomal protein (ZFX)

Growth Factor & Chemokine Receptors

-Activin type I receptor
-Orphan receptor
-Bone morphogenetic protein receptor
-C-C chemokine receptor (Monocyte chemoattractant protein 1 receptor (MCP-1RA)
-CD4 receptor (T cell activation antigene)
-CD 40L receptor (TNF receptor family)
-C5A receptor
-Corticotropin releasing factor receptor
-b receptor [Ednrb]
-Granulocyte colony-stimulating factor receptor
-Monotype chemoattractant protein 3
-D-Factor/LIF receptor
-ERBB-2 receptor (c-neu, HER2 protein tyrosine kinase)
-ERBB-3 receptor
-Erythropoietin receptor
-Fibroblast growth factor receptor 4
-Fibroblast growth factor receptor Basic (b FGF-R)
-G-protein-coupled receptor
-Granulocyte-macrophage colony-stimulating factor receptor
-Growth factor receptor
-Limphotoxin receptor (TNFR family)
-Macrophage mannose receptor
-Pre-platelet-derived growth factor receptor
-snoN; ski-related oncogene
-TGF-beta receptor type 1

Interleukin & Interferon Receptors

-Interferon alpha-beta receptor
-Interferon-gamma receptor
-Interleukin-1 receptor type II
-Interleukin-10 receptor
-Somatostatin receptor 2
-Interleukin-2 receptor gamma chain
-Interleukin-3 receptor
-Interleukin-4 receptor (membrane-bound form)
-Interleukin-5 receptor
-Interleukin-7 receptor
-Interleukin-8 receptor
-Interleukin-9 receptor

Hormone Receptors

-Androgen receptor
-Calcitonin receptor 1b
-Estrogen receptor
-Glucocorticoid receptor form A
-Growth hormone receptor
-Insulin receptor
-Insulin receptor substrate-1 (IRS-1)
-Prolactin receptor PRLR2
-Low density lipoprotein receptor

Neurotransmitter Receptors

-5-Hydroxytryptamine receptor [Serotonin receptor type 2 (5HT2)]
-5-Hydroxytryptamine (serotonin) receptor 1b
-5-Hydroxytryptamine (serotonin) receptor 1c
-5-Hydroxytryptamine (serotonin) receptor 1e beta
-5-Hydroxytryptamine (serotonin) receptor 2c
-5-Hydroxytryptamine (serotonin) receptor 3
-5-Hydroxytryptamine (serotonin) receptor 7
-Acetylcholine receptor delta submit
-Adrenergic receptor, beta 1
-Cannabinoid receptor 1 (brain)
-Cannabinoid receptor 2 (macrophage, CB2)
-Dopamine receptor 4
-G-protein coupled receptor
-GABA-A receptor alpha-1 submit
-GABA-A transporter 1
-GABA-A transporter 3
-GABA-A transporter 4
-Glutamate receptor, ionotropic AMPA 1
-Glutamate receptor, ionotropic NMDA2A (epsilon 1)
-Glutamate receptor, ionotropic NMDA2B (epsilon 2)
-Nicotinic acetylcholine receptor

Cell-Surface Antigens & Cell Adhesion Proteins

-P-selectin (glycoprotein ligand-1)
-Catenin alpha
-CD18 antigen beta subunit (leukocyte adhesion LFA-1) (CD3, P150, 95)
-CD2 antigen
-CD28 (receptor for B71)
-CD3 antigen, delta polypeptide
-CD31 (Platelet endothelial cell adhesion molecule 1)
-CD44 antigen
-CD45 associated protein (CD 45-ap, LSM-1)
-CD7 antigen
-CD14 antigen
-CD22 antigen
-Cell surface glycoprotein MAC-1 alpha subunit
-Desmocollin 2
-Dystroglycan 1
-Glutamate receptor channel subunit gamma
-Integrin alpha 2 (CD49b)
-Integrin alpha 4
-Integrin alpha 5 (CD51)
-Integrin alpha 6
-Integrin alpha 7
-Dipeptidyl peptidase IV
-Integrin beta
-Integrin beta 7 subunit
-Intercellular adhesion molecule-1
-Lamimin receptor 1
-Neuronal-cadherin (N-cadherin)
-Neuronal cell surface protein F3
-Vascular cell adhesion protein 1
-VLA-3 alpha subunit

Growth Factors, Cytokines & Chemokines

-Basic Fibroblast growth factor (b-FGF)
-Bone morphogenetic protein 1
-Bone morphogenetic protein 2 (BMP-2) (TGF-beta family)
-Bone morphogenetic protein 4 (BMP-4) (TGF-beta family)
-Bone morphogenetic protein 7 (BMP-7) (osteogenic protein 1)
-Bone morphogenetic protein 8a (BMP-8a) (TGF-beta family)
-Cek 5 receptor protein tyrosine kinase ligand
-Cek 7 receptor protein tyrosine kinase ligand
-Endothelial ligand for L-selectin (GLYCAM 1)
-Epidermal growth factor (EGF)
-Fibroblast growth factor 9
-Follistatin
-Gamma interferon induced monokine (MIG)
-Glial cell line-derived neurotrophic factor
-Granulocyte colony-stimulating factor (G-CSF)
-Growth/ diffferentiation factor 1 (GDF-1) (TGF- beta family)
-Growth/ diffferentiation factor 2 (GDF-2)
-Heparin-binding EGF-like growth factor (Diphtheria toxin receptor)
-Hepatocyte growth factor (hepapoitein)
-Hepatoma transmembrane kinase ligand

-Inhibin alpha subunit
-Inhibin beta A subunit (TGF beta family)
-Insulin-like growth factor binding protein -6 (IGFBP 6)
-Insulin-like growth factor binding protein-1 (IGFBP-1)
-Insulin-like growth factor binding protein-3 (IGFBP-3)
-Insulin-like growth factor binding protein-4 (IGFBP-4)
-Insulin-like growth factor binding protein-5 (IGFBP-5)
-Insulin-like growth factor-2 (somatomedin A)
-Insulin-like growth factor-IA
-Keratinocyte growth factor FGF-7
-K-fibroblast growth factor
-Leukemia inhibitory factor (LIF) (cholinergic differentiation factor)
-Macrophage inflammatory protein
-Macrophage inflammatory protein 1 beta (Act 2)
-Macrophage inflammatory protein 2 alpha (MIP 2 alpha)
-Mad related protein 2 (MADR2)
-Mast cell factor
-Mothers against DPP protein (mad homolog Smad 1, transforming growth factor beta signaling protein)
-Nerve growth factor alpha (alpha-NGF)
-Nerve growth factor beta (beta-NGF)
-Neuroleukin
-Oncostatin M
-Placental ribonuclease inhibitor (Angiogenin)
-Platelet- derived growth factor (A chain) (PDGF-A)
-Prepro-endothelin-3
-Thrombomodulin
-Thrombopoietin
-Transforming growth factor beta
-Transforming growth factor beta 2
-Tumor necrosis factor beta TNF-beta (Lymphotoxin-alpha)
-Uromodulin
-Vascular endothelial growth factor (VEGF)

Interleukins & Interferons

-Interleukin 1 beta
-Interleukin 10
-Interleukin 11 (adipogenesis inhibitory factor)
-Interleukin 12 (p40) beta chain
-Interleukin 15
-Interleukin 4
-Interleukin 6 (B cell differentiation factor)
-Interleukin 7

Cytoskeleton & Motility Proteins

-Alpha cardiac myosin heavy chain
-CamK II; Ca2+/calmodulin-dependent protein kinase II (beta subunit)
-CDC42 GTP-binding protein; G25K
-Cytoskeletal epidermal keratin (14 human)
-Cytoskeletal epidermal keratin (18 human)
-Cytoskeletal epidermal keratin (19 human)
-Epidermal keratin (1 human)
-Fetal myosin alkali light chain
-Kinesin family protein KIF1A
-Kinesin heavy chain
-Kinesin like protein KIF 3B
-Non-muscle myosin light chain 3
-Rab-3b ras-related protein
-Vimentin
-Unconventional myosin VI

Protein Turnover

Proteases & Inhibitors

-Angiotensin-converting enzyme (ACE) (clone ACE.5.)
-Cathepsin B
-Cathepsin D
-Cathepsin H
-Cathepsin L
-Collagenase type IV
-Cytotoxic cell protease 2 (B10)
-Cytotoxic T lymphocyte-specific serine protease CCP I gene (CTLA-1)
-Gelatinase B
-Interleukin-converting enzyme (ICE)
-Mast cell protease (MMCP) -4
-Membrane type matrix matalloproteinase
-Protease nexin 1 (PN-1)
-Tissue plasminogen activator
-Urokinase type plasminogen activator

Inhibitors

-Alpha-1 protease inhibitor 2
-Plasminogen activator inhibitor
-Plasminogen activator inhibitor-2
-Serine protease inhibitor 2 (spi-2)
-Serine protease inhibitor 2.4
-Serine protease inhibitor homolog J6
-TIMP-2 tissue inhibitor of metalloproteinases-2
-TIMP-3 tissue inhibitor of metalloproteinases-3

Housekeeping Genes

-Glyceraldehyde 3-phosphate dehydrogenase (G3PDH)
-Myosin 1
-Murine ornithine decarboxylase (MOD)
-Beta-actin
-Calcium-binding protein Cab45
-Ribosomal protein S29
-Ubiquitin
-Phospholipase A2
-Hypoxanthine phosphoribosyl transferase (HPRT)

Negative Controls

-M13 mp18(+) STRAND DNA
-Lambda DNA
-pUC18

Chapter 5 Appendix II

BclxL Spleen RNA

Animal No.	Spleen 26 2M+	GAPDH	Ratio	Animal No.	Spleen 26 2M-	GAPDH	Ratio	Animal No.	Spleen 59+	GAPDH	Ratio	Animal No.	Spleen 26T	GAPDH	Ratio
261339	5217	6755	0.7723	261361	1341	5690	0.2357	59416	2190	1278	1.713615	26569	1732	3681	0.4705
261356	2307	2494	0.925	261362	881	5497	0.1603	59417	1409	1711	0.823495	26572	1557	4980	0.3127
261360	4707	12690	0.3709	261374	1085	4445	0.2441	59421	1165	1027	1.134372	26574	1287	2210	0.5824
261363	758	835	0.9078	261376	862	3696	0.2332					26583	1362	2056	0.6625
261381	1978	6823	0.2899	261377	1171	3410	0.3434					26584	1322	3306	0.3999
				59418	938	1968	0.4766								
				59419	990	2157	0.459								
				59420	937	1180	0.7941								

Egr1 Spleen RNA

Animal No.	Spleen 26 2M+	GAPDH	Ratio	Animal No.	Spleen 26 2M-	GAPDH	Ratio	Animal No.	Spleen 59+	GAPDH	Ratio	Animal No.	Spleen 26T	GAPDH	Ratio
261339	3332	5818	0.5727	261361	1748	3915	0.4465	59416	1494	708	2.1101695	26569	1711	3640	0.4701
261356	2065	1463	1.4115	261362	2151	7064	0.3045	59417	2039	1358	1.5014728	26572	1916	4173	0.4591
261360	5299	11280	0.4698	261374	1807	4892	0.3694	59421	1428	500	2.856	26574	1374	1804	0.7616
261363	1469	1048	1.4017	261376	940	3026	0.3106					26583	1037	1613	0.6429
261381	3736	7379	0.5063	261377	637	1897	0.3358					26584	1116	3117	0.358
				59418	1994	1240	1.6081								
				59419	1395	1196	1.1664								
				59420	1087	565	1.9239								

cJun Spleen RNA

Animal No.	Spleen 26 2M+	GAPDH	Ratio	Animal No.	Spleen 26 2M-	GAPDH	Ratio	Animal No.	Spleen 59+	GAPDH	Ratio	Animal No.	Spleen 26T	GAPDH	Ratio
261339	3737	6755	0.5532	261361	1802	5690	0.3167	59416	2846	1278	2.2269171	26569	2343	3681	0.6365
261356	2938	2494	1.178	261362	1975	5497	0.3593	59417	3028	1711	1.7697253	26572	3380	4980	0.6787
261360	6427	12690	0.5065	261374	1594	4445	0.3586	59421	2181	1027	2.1236611	26574	2777	2210	1.2566
261363	1517	835	1.8168	261376	1393	3696	0.3769					26583	2120	2056	1.0311
261381	2344	6823	0.3435	261377	1388	3410	0.407					26584	2834	3306	0.8572
				59418	2423	1698	1.427								
				59419	2398	2157	1.1117								
				59420	1721	1180	1.4585								

cFos Spleen RNA

Animal No.	Spleen 26 2M+	GAPDH	Ratio	Animal No.	Spleen 26 2M-	GAPDH	Ratio	Animal No.	Spleen 59+	GAPDH	Ratio	Animal No.	Spleen 26T	GAPDH	Ratio
261339	836	5818	0.1437	261361	614	3915	0.1568	59416	298	708	0.420904	26569	1120	3640	0.3077
261356	640	1463	0.4375	261362	593	7064	0.0839	59417	465	1358	0.3424153	26572	701	4173	0.168
261360	1293	11280	0.1146	261374	444	4892	0.0908	59421	680	500	1.36	26574	659	1804	0.3653
261363	453	1048	0.4323	261376	411	3026	0.1358					26583	659	1613	0.4086
261381	1046	7379	0.1418	261377	331	1897	0.1745					26584	1029	3117	0.3301
				59418	640	1240	0.5161								
				59419	504	1196	0.4214								
				59420	397	565	0.7027								

Ikaros Spleen RNA

Animal No.	Spleen 26 2M+	GAPDH	Ratio	Animal No.	Spleen 26 2M-	GAPDH	Ratio	Animal No.	Spleen 59+	GAPDH	Ratio	Animal No.	Spleen 26T	GAPDH	Ratio
261339	6925	9361	0.7398	261361	2990	7946	0.3763	59416	5347	2519	2.1226677	26569	8197	9072	0.9035
261356	5378	3854	1.3954	261362	3803	9238	0.4117	59417	4924	2899	1.6985167	26572	5756	8304	0.6932
261360	7467	17720	0.4214	261374	3514	6869	0.5116	59421	3361	1551	2.166989	26574	4004	3881	1.0317
261363	2393	1490	1.606	261376	3158	5506	0.5736					26583	2962	4086	0.7249
261381	3716	8817	0.4215	261377	3579	5039	0.7103					26584	4154	6400	0.6491
				59418	4486	2788	1.609								
				59419	3978	2889	1.3769								
				59420	3062	1822	1.6806								

EBF Spleen RNA

Animal No.	Spleen 26 2M+	GAPDH	Ratio	Animal No.	Spleen 26 2M-	GAPDH	Ratio	Animal No.	Spleen 59+	GAPDH	Ratio	Animal No.	Spleen 26T	GAPDH	Ratio
261339	5949	5824	1.0215	261361	3418	5087	0.6719	59416	4896	1079	4.5375348	26569	5290	3106	1.7032
261356	5829	1751	3.329	261362	4179	6854	0.6097	59417	5095	1388	3.6707493	26572	6052	3401	1.7795
261360	8818	8501	1.0373	261374	2386	4374	0.5455	59421	3934	850	4.6282353	26574	5161	1497	3.4476
261363	4013	592	6.7787	261376	1926	2549	0.7556					26583	3730	1297	2.8759
261381	4545	5560	0.8174	261377	3041	3272	0.9294					26584	4573	2832	1.6148
				59418	5037	1625	3.0997								
				59419	4023	1568	2.5657								
				59420	3171	910	3.4846								

Araf Spleen RNA

Animal No.	Spleen 26 2M+	GAPDH	Ratio	Animal No.	Spleen 26 2M-	GAPDH	Ratio	Animal No.	Spleen 59+	GAPDH	Ratio	Animal No.	Spleen 26T	GAPDH	Ratio
261339	5038	5824	0.865	261361	2375	5087	0.4669	59416	4149	1079	3.8452271	26569	3446	3106	1.1095
261356	3552	1751	2.0286	261362	2708	6854	0.3951	59417	4628	1388	3.3342939	26572	2569	3401	0.7554
261360	4106	8501	0.483	261374	1851	4374	0.4232	59421	1855	850	2.1823529	26574	1160	1497	0.7749
261363	2187	592	3.6943	261376	1805	2549	0.7081					26583	1085	1297	0.8365
261381	2886	5560	0.5191	261377	2513	3272	0.768					26584	1413	2832	0.4989
				59418	2050	1625	1.2615								
				59419	1484	1568	0.9464								
				59420	1316	910	1.4462								

Nras Spleen RNA

Animal No.	Spleen 26 2M+	GAPDH	Ratio	Animal No.	Spleen 26 2M-	GAPDH	Ratio	Animal No.	Spleen 59+	GAPDH	Ratio	Animal No.	Spleen 26T	GAPDH	Ratio
261339	380	9361	0.0406	261361	381	7946	0.0479	59416	467	2519	0.185391	26569	1203	9072	0.1326
261356	266	3854	0.069	261362	445	9238	0.0482	59417	500	2899	0.1724733	26572	943	8304	0.1136
261360	470	17720	0.0265	261374	409	6869	0.0595	59421	351	1551	0.2263056	26574	780	3881	0.201
261363	160	1490	0.1074	261376	328	5506	0.0596					26583	622	4086	0.1522
261381	398	8817	0.0451	261377	336	5039	0.0667					26584	894	6400	0.1397
				59418	573	2788	0.2055								
				59419	537	2889	0.1859								
				59420	430	1822	0.236								

B2 Spleen RNA

Animal No.	Spleen 26 2M+	GAPDH	Ratio	Animal No.	Spleen 26 2M-	GAPDH	Ratio	Animal No.	Spleen 59+	GAPDH	Ratio	Animal No.	Spleen 26T	GAPDH	Ratio
261339	13710	6755	2.0296	261361	12890	5690	2.2654	59416	1643	1278	1.2856025	26569	2470	3681	0.671
261356	3668	2494	1.4707	261362	6680	5497	1.2152	59417	2101	1711	1.2279369	26572	3243	4980	0.6512
261360	20030	12690	1.5784	261374	9500	4445	2.1372	59421	1182	1027	1.150925	26574	1925	2210	0.871
261363	1611	835	1.9293	261376	5922	3696	1.6023					26583	2008	2056	0.9767
261381	17140	6823	2.5121	261377	5539	3410	1.6243					26584	2884	3306	0.8724
				59418	2834	1968	1.44								
				59419	2825	2157	1.3097								
				59420	1943	1180	1.6466								

Pic1 Spleen RNA															
Animal No.	**Spleen 26 2M+**	**GAPDH**	**Ratio**	**Animal No.**	**Spleen 26 2M-**	**GAPDH**	**Ratio**	**Animal No.**	**Spleen 59+**	**GAPDH**	**Ratio**	**Animal No.**	**Spleen 26T**	**GAPDH**	**Ratio**
261339	189	5818	0.0325	261361	97	3915	0.0248	59416	85	708	0.1200565	26569	154	3640	0.0423
261356	94	1463	0.0643	261362	107	7064	0.0151	59417	94	1358	0.0692194	26572	189	4173	0.0453
261360	135	11280	0.012	261374	76	4892	0.0155	59421	58	500	0.116	26574	164	1804	0.0909
261363	75	1048	0.0716	261376	61	3026	0.0202					26583	152	1613	0.0942
261381	179	7379	0.0243	261377	53	1897	0.0279					26584	215	3117	0.069
				59418	103	1240	0.0831								
				59419	106	1196	0.0886								
				59420	95	565	0.1681								

Chapter 5 Appendix III

BclxL B cells

Animal No.	Spleen 26 2M	GAPDH	Ratio	Animal No	Spleen 26 2M-	GAPDH	Ratio	Animal No	Spleen 261M+	GAPDH	Ratio	Animal No.	Spleen 261M-	GAPDH	Ratio	Animal No.	Spleen 26tun	GAPDH	Ratio
265917	165	71	2.32394	261523	380	295	1.2881	261599	163	186	0.8763	261598	57	45	1.2667	26569	583	290	2.0103
265919	202	91	2.21978	261532	125	105	1.1905	261601	102	92	1.1087	261600	142	70	2.0286	26572	295	138	2.1377
261521	265	180	1.47222	261537	274	154	1.7792	261606	49	81	0.6049	261602	164	180	0.9111	26574	207	126	1.6429
261528	68	67	1.01493	261540	244	155	1.5742	261608	119	138	0.8623	261604	45	89	0.5056	26583	275	283	0.9717
261529	129	56	2.30357	261545	187	148	1.2635	261609	138	164	0.8415	261610	58	19	3.0526	26584	162	125	1.296
261530	142	51	2.78431	261553	188	171	1.0994									261114	328	259	1.2664
261538	316	110	2.87273	261559	122	173	0.7052									261124	532	657	0.8097
261318	258	258	1													261162	288	102	2.8235
																261174	520	518	1.0039
																261235	454	192	2.3646
																261305	524	235	2.2298

BclxL B-cell depleted cells

Animal No.	Spleen 26 2M	GAPDH	Ratio	Animal No	Spleen 26 2M-	GAPDH	Ratio	Animal No	Spleen 261M+	GAPDH	Ratio	Animal No.	Spleen 261M-	GAPDH	Ratio
265917	344	67	5.13433	261523	508	135	3.763	261599	144	25	5.76	261598	57	31	1.8387
265919	128	61	2.09836	261532	509	120	4.2417	261601	220	45	4.8889	261600	167	116	1.4397
261521	181	53	3.41509	261537	458	93	4.9247	261606	198	24	8.25	261602	18	44	0.4091
261528	195	51	3.82353	261540	330	55	6	261608	171	30	5.7	261604	137	37	3.7027
261529	218	41	5.31707	261545	351	66	5.3182	261609	299	73	4.0959	261610	65	20	3.25
261530	299	46	6.5	261553	302	51	5.9216								
261538	335	59	5.67797	261559	315	65	4.8462								
261318	734	310	2.36774												

SATB1 B cells

Animal No.	Spleen 26 2M	GAPDH	Ratio	Animal No	Spleen 26 2M-	GAPDH	Ratio	Animal No	Spleen 261M+	GAPDH	Ratio	Animal No.	Spleen 261M-	GAPDH	Ratio	Animal No.	Spleen 26tun	GAPDH	Ratio
265917	488	71	6.87324	261523	593	295	2.0102	261599	402	186	2.1613	261598	285	45	6.3333	26569	773	290	2.6655
265919	278	91	3.05495	261532	280	105	2.6667	261601	273	92	2.9674	261600	337	70	4.8143	26572	517	138	3.7464
261521	409	180	2.27222	261537	450	154	2.9221	261606	195	81	2.4074	261602	409	180	2.2722	26574	237	126	1.881
261528	274	67	4.08955	261540	461	155	2.9742	261608	392	138	2.8406	261604	322	89	3.618	26583	424	283	1.4982
261529	284	56	5.07143	261545	472	148	3.1892	261609	338	164	2.061	261610	187	19	9.8421	26584	322	125	2.576
261530	297	51	5.82353	261553	508	171	2.9708									261114	503	259	1.9421
261538	574	110	5.21818	261559	514	173	2.9711									261124	867	657	1.3196
261318	365	258	1.41473													261162	441	102	4.3235
																261174	742	518	1.4324
																261235	510	192	2.6563
																261305	570	235	2.4255

SATB1 B-cell depleted cells

Animal No.	Spleen 26 2M	GAPDH	Ratio	Animal No	Spleen 26 2M-	GAPDH	Ratio	Animal No	Spleen 261M+	GAPDH	Ratio	Animal No.	Spleen 261M-	GAPDH	Ratio
265917	541	67	8.07463	261523	566	135	4.1926	261599	315	25	12.6	261598	177	31	5.7097
265919	301	61	4.93443	261532	475	120	3.9583	261601	391	45	8.6889	261600	376	116	3.2414
261521	219	53	4.13208	261537	537	93	5.7742	261606	309	24	12.875	261602	219	44	4.9773
261528	291	51	5.70588	261540	463	55	8.4182	261608	331	30	11.033	261604	210	37	5.6757
261529	456	41	11.122	261545	543	66	8.2273	261609	484	73	6.6301	261610	138	20	6.9
261530	364	46	7.91304	261553	512	51	10.039								
261538	402	59	6.81356	261559	533	65	8.2								
261318	744	310	2.4												

cJun B cells

Animal No.	Spleen 26 2M	GAPDH	Ratio	Animal No	Spleen 26 2M-	GAPDH	Ratio	Animal No	Spleen 261M+	GAPDH	Ratio	Animal No.	Spleen 261M-	GAPDH	Ratio	Animal No.	Spleen 26tun	GAPDH	Ratio
265917	682	71	9.60563	261523	1359	295	4.6068	261599	864	186	4.6452	261598	430	45	9.5556	26569	1158	290	3.9931
265919	508	91	5.58242	261532	694	105	6.6095	261601	550	92	5.9783	261600	529	70	7.5571	26572	840	138	6.087
261521	632	180	3.51111	261537	1031	154	6.6948	261606	364	81	4.4938	261602	644	180	3.5778	26574	643	126	5.1032
261528	318	67	4.74627	261540	971	155	6.2645	261608	658	138	4.7681	261604	767	89	8.618	26583	804	283	2.841
261529	387	56	6.91071	261545	909	148	6.1419	261609	672	164	4.0976	261610	315	19	16.579	26584	641	125	5.128
261530	551	51	10.8039	261553	1011	171	5.9123									261114	1108	259	4.278
261538	1167	110	10.6091	261559	1097	173	6.341									261124	1711	657	2.6043
261318	911	258	3.53101													261162	1033	102	10.127
																261174	1424	518	2.749
																261235	1036	192	5.3958
																261305	986	235	4.1957

cJun B-cell depleted cells

Animal No.	Spleen 26 2M	GAPDH	Ratio	Animal No	Spleen 26 2M-	GAPDH	Ratio	Animal No	Spleen 261M+	GAPDH	Ratio	Animal No.	Spleen 261M-	GAPDH	Ratio
265917	699	67	10.4328	261523	1127	135	8.3481	261599	510	25	20.4	261598	249	31	8.0323
265919	462	61	7.57377	261532	904	120	7.5333	261601	605	45	13.444	261600	451	116	3.8879
261521	494	53	9.32075	261537	908	93	9.7634	261606	460	24	19.167	261602	556	44	12.636
261528	620	51	12.1569	261540	704	55	12.8	261608	441	30	14.7	261604	572	37	15.459
261529	692	41	16.878	261545	819	66	12.409	261609	848	73	11.616	261610	215	20	10.75
261530	759	46	16.5	261553	817	51	16.02								
261538	780	59	13.2203	261559	917	65	14.108								
261318	1752	310	5.65161												

Egr1 B cells

Animal No.	Spleen 26 2M	GAPDH	Ratio	Animal No	Spleen 26 2M-	GAPDH	Ratio	Animal No	Spleen 261M+	GAPDH	Ratio	Animal No.	Spleen 261M-	GAPDH	Ratio	Animal No.	Spleen 26tun	GAPDH	Ratio
265917	734	71	10.338	261523	713	295	2.4169	261599	681	186	3.6613	261598	416	45	9.2444	26569	1327	290	4.5759
265919	543	91	5.96703	261532	340	105	3.2381	261601	668	92	7.2609	261600	646	70	9.2286	26572	949	138	6.8768
261521	617	180	3.42778	261537	662	154	4.2987	261606	341	81	4.2099	261602	780	180	4.3333	26574	644	126	5.1111
261528	250	67	3.73134	261540	720	155	4.6452	261608	681	138	4.9348	261604	634	89	7.1236	26583	840	283	2.9682
261529	249	56	4.44643	261545	724	148	4.8919	261609	698	164	4.2561	261610	356	19	18.737	26584	595	125	4.76
261530	297	51	5.82353	261553	762	171	4.4561									261114	1038	259	4.0077
261538	738	110	6.70909	261559	613	173	3.5434									261124	1584	657	2.411
261318	524	258	2.03101													261162	647	102	6.3431
																261174	1391	518	2.6853
																261235	978	192	5.0938
																261305	1072	235	4.5617

Egr1 B-cell depleted cells

Animal No.	Spleen 26 2M	GAPDH	Ratio	Animal No	Spleen 26 2M-	GAPDH	Ratio	Animal No	Spleen 261M+	GAPDH	Ratio	Animal No.	Spleen 261M-	GAPDH	Ratio
265917	1096	67	16.3582	261523	773	135	5.7259	261599	413	25	16.52	261598	231	31	7.4516
265919	557	61	9.13115	261532	680	120	5.6667	261601	653	45	14.511	261600	547	116	4.7155
261521	630	53	11.8868	261537	723	93	7.7742	261606	491	24	20.458	261602	363	44	8.25
261528	662	51	12.9804	261540	602	55	10.945	261608	461	30	15.367	261604	428	37	11.568
261529	593	41	14.4634	261545	768	66	11.636	261609	932	73	12.767	261610	270	20	13.5
261530	602	46	13.087	261553	70	51	1.3725								
261538	569	59	9.64407	261559	832	65	12.8								
261318	1191	310	3.84194												

EBF B cells

Animal No.	Spleen 26 2M	GAPDH	Ratio	Animal No	Spleen 26 2M-	GAPDH	Ratio	Animal No	Spleen 261M+	GAPDH	Ratio	Animal No.	Spleen 261M-	GAPDH	Ratio	Animal No.	Spleen 26tun	GAPDH	Ratio
265917	466	71	6.56338	261523	477	295	1.6169	261599	339	186	1.8226	261598	129	45	2.8667	26569	312	290	1.0759
265919	203	91	2.23077	261532	245	105	2.3333	261601	239	92	2.5978	261600	175	70	2.5	26572	226	138	1.6377
261521	222	180	1.23333	261537	339	154	2.2013	261606	184	81	2.2716	261602	324	180	1.8	26574	168	126	1.3333
261528	115	67	1.71642	261540	397	155	2.5613	261608	295	138	2.1377	261604	205	89	2.3034	26583	192	283	0.6784
261529	95	56	1.69643	261545	411	148	2.777	261609	485	164	2.9573	261610	60	19	3.1579	26584	146	125	1.168
261530	112	51	2.19608	261553	415	171	2.4269									261114	244	259	0.9421
261538	248	110	2.25455	261559	450	173	2.6012									261124	433	657	0.6591
261318	273	258	1.05814													261162	263	102	2.5784
																261174	562	518	1.0849
																261235	416	192	2.1667
																261305	345	235	1.4681

EBF B-cell depleted cells

Animal No.	Spleen 26 2M	GAPDH	Ratio	Animal No	Spleen 26 2M-	GAPDH	Ratio	Animal No	Spleen 261M+	GAPDH	Ratio	Animal No.	Spleen 261M-	GAPDH	Ratio
265917	172	67	2.56716	261523	258	135	1.9111	261599	102	25	4.08	261598	52	31	1.6774
265919	106	61	1.7377	261532	244	120	2.0333	261601	168	45	3.7333	261600	188	116	1.6207
261521	110	53	2.07547	261537	232	93	2.4946	261606	99	24	4.125	261602	111	44	2.5227
261528	107	51	2.09804	261540	175	55	3.1818	261608	96	30	3.2	261604	160	37	4.3243
261529	104	41	2.53659	261545	193	66	2.9242	261609	199	73	2.726	261610	60	20	3
261530	136	46	2.95652	261553	179	51	3.5098								
261538	138	59	2.33898	261559	197	65	3.0308								
261318	491	310	1.58387												

Bad B cells

Animal No.	Spleen 26 2M	GAPDH	Ratio	Animal No	Spleen 26 2M-	GAPDH	Ratio	Animal No	Spleen 261M+	GAPDH	Ratio	Animal No.	Spleen 261M-	GAPDH	Ratio	Animal No.	Spleen 26tun	GAPDH	Ratio
265917	267	39	6.84615	261523	495	96	5.1563	261599	514	103	4.9903	261598				26569	627	130	4.8231
265919	125	65	1.92308	261532	327	31	10.548	261601	277	40	6.925	261600	284	23	12.348	26572	837	70	11.957
261521	129	78	1.65385	261537	378	46	8.2174	261606	184	23	8	261602	448	52	8.6154	26574	357	57	6.2632
261528	85	8	10.625	261540	415	47	8.8298	261608	231	29	7.9655	261604	404	34	11.882	26583	704	125	5.632
261529	148	67	2.20896	261545	392	69	5.6812	261609				261610	165	8	20.625	26584	320	117	2.735
261530	157	13	12.0769	261553	371	54	6.8704									261114	371	143	2.5944
261538	296	48	6.16667	261559	443	62	7.1452									261124	634	316	2.0063
261318	280	108	2.59259													261162	381	51	7.4706
																261174	991	328	3.0213
																261235	681	103	6.6117
																261305	526	67	7.8507

Bad B-cell depleted cells

Animal No.	Spleen 26 2M	GAPDH	Ratio	Animal No	Spleen 26 2M-	GAPDH	Ratio	Animal No	Spleen 261M+	GAPDH	Ratio	Animal No.	Spleen 261M-	GAPDH	Ratio
265917	357	41	8.70732	261523	318	55	5.7818	261599	376	19	19.789	261598			
265919	426	15	28.4	261532	336	49	6.8571	261601	354	36	9.8333	261600	240	44	5.4545
261521	169	14	12.0714	261537	320	38	8.4211	261606	378	24	15.75	261602	334	16	20.875
261528	107	23	4.65217	261540	398	45	8.8444	261608	230	21	10.952	261604	368	21	17.524
261529	179	19	9.42105	261545	446	49	9.102	261609	328	60	5.4667	261610	193	13	14.846
261530	245	25	9.8	261553	482	48	10.042								
261538	302	19	15.8947	261559	530	53	10								
261318	417	123	3.39024												

cFos B cells

Animal No.	Spleen 26 2M	GAPDH	Ratio	Animal No	Spleen 26 2M-	GAPDH	Ratio	Animal No	Spleen 261M+	GAPDH	Ratio	Animal No.	Spleen 261M-	GAPDH	Ratio	Animal No.	Spleen 26tun	GAPDH	Ratio
265917	304	39	7.79487	261523	510	96	5.3125	261599	574	103	5.5728	261598				26569	1081	130	8.3154
265919	391	65	6.01538	261532	193	31	6.2258	261601	350	40	8.75	261600	391	23	17	26572	799	70	11.414
261521	322	78	4.12821	261537	311	46	6.7609	261606	228	23	9.913	261602	553	52	10.635	26574	667	57	11.702
261528	118	9	13.1111	261540	360	47	7.6596	261608	367	29	12.655	261604	411	34	12.088	26583	880	125	7.04
261529	153	67	2.28358	261545	461	69	6.6812	261609				261610	416	8	52	26584	902	117	7.7094
261530	173	14	12.3571	261553	415	54	7.6852									261114	1025	143	7.1678
261538	488	52	9.38462	261559	460	62	7.4194									261124	1590	316	5.0316
261318	258	108	2.38889													261162	762	51	14.941
																261174	1724	328	5.2561
																261235	1077	103	10.456
																261305	792	67	11.821

cFos B-cell depleted cells

Animal No.	Spleen 26 2M	GAPDH	Ratio	Animal No	Spleen 26 2M-	GAPDH	Ratio	Animal No	Spleen 261M+	GAPDH	Ratio	Animal No.	Spleen 261M-	GAPDH	Ratio
265917	416	41	10.1463	261523	562	55	10.218	261599	284	19	14.947	261598			
265919	151	15	10.0667	261532	516	49	10.531	261601	489	36	13.583	261600	451	44	10.25
261521	309	14	22.0714	261537	488	38	12.842	261606	343	24	14.292	261602	260	16	16.25
261528	351	23	15.2609	261540	437	45	9.7111	261608	349	21	16.619	261604	257	21	12.238
261529	344	19	18.1053	261545	458	49	9.3469	261609	872	60	14.533	261610	212	13	16.308
261530	381	25	15.24	261553	457	48	9.5208								
261538	280	19	14.7368	261559	587	53	11.075								
261318	744	123	6.04878												

hnRNPD B cells

Animal No.	Spleen 26 2M	GAPDH	Ratio	Animal No	Spleen 26 2M-	GAPDH	Ratio	Animal No	Spleen 261M+	GAPDH	Ratio	Animal No.	Spleen 261M-	GAPDH	Ratio	Animal No.	Spleen 26tun	GAPDH	Ratio
265917	674	39	17.2821	261523	1153	96	12.01	261599	746	103	7.2427	261598				26569	263	130	2.0231
265919	906	65	13.9385	261532	542	31	17.484	261601	1070	40	26.75	261600	566	23	24.609	26572	475	70	6.7857
261521	704	78	9.02564	261537	630	46	13.696	261606	562	23	24.435	261602	594	52	11.423	26574	298	57	5.2281
261528	382	9	42.4444	261540	456	47	9.7021	261608	832	29	28.69	261604	559	34	16.441	26583	77	125	0.616
261529	574	67	8.56716	261545	704	69	10.203	261609				261610	438	8	54.75	26584	290	117	2.4786
261530	384	14	27.4286	261553	552	54	10.222									261114	505	143	3.5315
261538	584	52	11.2308	261559	574	62	9.2581									261124	198	316	0.6266
261318	634	108	5.87037													261162	317	51	6.2157
																261174	358	328	1.0915
																261235	376	103	3.6505
																261305	344	67	5.1343

hnRNPD B-cell depleted cells

Animal No.	Spleen 26 2M	GAPDH	Ratio	Animal No	Spleen 26 2M-	GAPDH	Ratio	Animal No	Spleen 261M+	GAPDH	Ratio	Animal No.	Spleen 261M-	GAPDH	Ratio
265917	598	41	14.5854	261523	601	55	10.927	261599	435	19	22.895	261598			
265919	662	15	44.1333	261532	618	49	12.612	261601	572	36	15.889	261600	510	44	11.591
261521	707	14	50.5	261537	488	38	12.842	261606	616	24	25.667	261602	551	16	34.438
261528	1054	23	45.8261	261540	820	45	18.222	261608	578	21	27.524	261604	720	21	34.286
261529	1691	19	89	261545	878	49	17.918	261609	408	60	6.8	261610	472	13	36.308
261530	440	25	17.6	261553	887	48	18.479								
261538	779	19	41	261559	875	53	16.509								
261318	462	123	3.7561												

Ikaros B cells

Animal No.	Spleen 26 2M	GAPDH	Ratio	Animal No	Spleen 26 2M-	GAPDH	Ratio	Animal No	Spleen 261M+	GAPDH	Ratio	Animal No.	Spleen 261M-	GAPDH	Ratio	Animal No.	Spleen 26tur	GAPDH	Ratio
265917	292	39	7.48718	261523	222	96	2.3125	261599	245	103	2.3786	261598				26569	239	130	1.8385
265919	265	65	4.07692	261532	140	31	4.5161	261601	211	40	5.275	261600	135	23	5.8696	26572	149	70	2.1286
261521	149	78	1.91026	261537	180	46	3.913	261606	169	23	7.3478	261602	198	52	3.8077	26574	128	57	2.2456
261528	53	9	5.88889	261540	186	47	3.9574	261608	194	29	6.6897	261604	161	34	4.7353	26583	153	125	1.224
261529	70	67	1.04478	261545	229	69	3.3188	261609				261610	174	8	21.75	26584	179	117	1.5299
261530	84	14	6	261553	212	54	3.9259									261114	210	143	1.4685
261538	164	52	3.15385	261559	205	62	3.3065									261124	330	316	1.0443
261318	158	108	1.46296													261162	167	51	3.2745
																261174	428	328	1.3049
																261235	324	103	3.1456
																261305	296	67	4.4179

Ikaros B-cell depleted cells

Animal No.	Spleen 26 2M	GAPDH	Ratio	Animal No	Spleen 26 2M-	GAPDH	Ratio	Animal No	Spleen 261M+	GAPDH	Ratio	Animal No.	Spleen 261M-	GAPDH	Ratio
265917	161	41	3.92683	261523	146	55	2.6545	261599	230	19	12.105	261598			
265919	110	15	7.33333	261532	146	49	2.9796	261601	241	36	6.6944	261600	172	44	3.9091
261521	83	14	5.92857	261537	138	38	3.6316	261606	184	24	7.6667	261602	180	16	11.25
261528	90	23	3.91304	261540	211	45	4.6889	261608	141	21	6.7143	261604	197	21	9.381
261529	121	19	6.36842	261545	256	49	5.2245	261609	215	60	3.5833	261610	184	13	14.154
261530	106	25	4.24	261553	254	48	5.2917								
261538	76	19	4	261559	276	53	5.2075								
261318	193	123	1.56911												

Rag1 B cells

Animal No.	Spleen 26 2M	GAPDH	Ratio	Animal No	Spleen 26 2M-	GAPDH	Ratio	Animal No	Spleen 261M+	GAPDH	Ratio	Animal No.	Spleen 261M-	GAPDH	Ratio	Animal No.	Spleen 26tun	GAPDH	Ratio
265917	691	71	9.73239	261523	775	295	2.6271	261599	524	186	2.8172	261598	221	45	4.9111	26569	757	290	2.6103
265919	382	91	4.1978	261532	397	105	3.781	261601	309	92	3.3587	261600	319	70	4.5571	26572	545	138	3.9493
261521	383	180	2.12778	261537	668	154	4.3377	261606	248	81	3.0617	261602	493	180	2.7389	26574	436	126	3.4603
261528	144	67	2.14925	261540	746	155	4.8129	261608	400	138	2.8986	261604	415	89	4.6629	26583	561	283	1.9823
261529	109	56	1.94643	261545	651	148	4.3986	261609	562	164	3.4268	261610	582	19	30.632	26584	411	125	3.288
261530	155	51	3.03922	261553	640	171	3.7427									261114	818	259	3.1583
261538	430	110	3.90909	261559	624	173	3.6069									261124	1088	657	1.656
261318	396	258	1.53488													261162	660	102	6.4706
																261174	1123	518	2.168
																261235	865	192	4.5052
																261305	976	235	4.1532

Rag1 B-cell depleted cells

Animal No.	Spleen 26 2M	GAPDH	Ratio	Animal No	Spleen 26 2M-	GAPDH	Ratio	Animal No	Spleen 261M+	GAPDH	Ratio	Animal No.	Spleen 261M-	GAPDH	Ratio
265917	303	67	4.52239	261523	578	135	4.2815	261599	599	25	23.96	261598	296	31	9.5484
265919	162	61	2.65574	261532	504	120	4.2	261601	710	45	15.778	261600	568	116	4.8966
261521	238	53	4.49057	261537	496	93	5.3333	261606	569	24	23.708	261602	428	44	9.7273
261528	262	51	5.13725	261540	419	55	7.6182	261608	588	30	19.6	261604	570	37	15.405
261529	243	41	5.92683	261545	567	66	8.5909	261609	1010	73	13.836	261610	499	20	24.95
261530	323	46	7.02174	261553	582	51	11.412								
261538	344	59	5.83051	261559	869	65	13.369								
261318	1025	310	3.30645												

Rag2 B cells

Animal No.	Spleen 26 2M	GAPDH	Ratio	Animal No	Spleen 26 2M-	GAPDH	Ratio	Animal No	Spleen 261M+	GAPDH	Ratio	Animal No.	Spleen 261M-	GAPDH	Ratio	Animal No.	Spleen 26tun	GAPDH	Ratio
265917	828	39	21.2308	261523	897	96	9.3438	261599	1018	103	9.8835	261598				26569	758	130	5.8308
265919	676	65	10.4	261532	698	31	22.516	261601	738	40	18.45	261600	453	23	19.696	26572	450	70	6.4286
261521	258	78	3.30769	261537	686	46	14.913	261606	573	23	24.913	261602	710	52	13.654	26574	400	57	7.0175
261528	172	9	19.1111	261540	575	47	12.234	261608	636	29	21.931	261604	766	34	22.529	26583	471	125	3.768
261529	172	67	2.56716	261545	698	69	10.116	261609				261610	221	8	27.625	26584	618	117	5.2821
261530	215	14	15.3571	261553	661	54	12.241									261114	833	143	5.8252
261538	470	52	9.03846	261559	796	62	12.839									261124	1214	316	3.8418
261318	477	108	4.41667													261162	769	51	15.078
																261174	1557	328	4.747
																261235	1089	103	10.573
																261305	492	67	7.3433

Rag2 B-cell depleted cells

Animal No.	Spleen 26 2M	GAPDH	Ratio	Animal No	Spleen 26 2M-	GAPDH	Ratio	Animal No	Spleen 261M+	GAPDH	Ratio	Animal No.	Spleen 261M-	GAPDH	Ratio
265917	497	41	12.122	261523	730	55	13.273	261599	531	19	27.947	261598			
265919	308	15	20.5333	261532	824	49	16.816	261601	598	36	16.611	261600	414	44	9.4091
261521	217	14	15.5	261537	624	38	16.421	261606	297	24	12.375	261602	320	16	20
261528	273	23	11.8696	261540	504	45	11.2	261608	217	21	10.333	261604	382	21	18.19
261529	267	19	14.0526	261545	477	49	9.7347	261609	488	60	8.1333	261610	218	13	16.769
261530	356	25	14.24	261553	454	48	9.4583								
261538	348	19	18.3158	261559	549	53	10.358								
261318	981	123	7.97561												

HCK B cells

Animal No.	Spleen 26 2M	GAPDH	Ratio	Animal No	Spleen 26 2M-	GAPDH	Ratio	Animal No	Spleen 261M+	GAPDH	Ratio	Animal No.	Spleen 261M-	GAPDH	Ratio	Animal No.	Spleen 26tun	GAPDH	Ratio
265917	521	71	7.33803	261523	960	295	3.2542	261599	394	186	2.1183	261598	253	45	5.6222	26569	934	290	3.2207
265919	450	91	4.94505	261532	352	105	3.3524	261601	166	92	1.8043	261600	224	70	3.2	26572	465	138	3.3696
261521	464	180	2.57778	261537	480	154	3.1169	261606	111	81	1.3704	261602	438	180	2.4333	26574	347	126	2.754
261528	114	67	1.70149	261540	494	155	3.1871	261608	221	138	1.6014	261604	475	89	5.3371	26583	470	283	1.6608
261529	104	56	1.85714	261545	492	148	3.3243	261609	433	164	2.6402	261610	262	19	13.789	26584	236	125	1.888
261530	199	51	3.90196	261553	480	171	2.807									261114	558	259	2.1544
261538	400	110	3.63636	261559	464	173	2.6821									261124	1334	657	2.0304
261318	411	258	1.59302													261162	431	102	4.2255
																261174	1176	518	2.2703
																261235	592	192	3.0833
																261305	594	235	2.5277

HCK B-cell depleted cells

Animal No.	Spleen 26 2M	GAPDH	Ratio	Animal No	Spleen 26 2M-	GAPDH	Ratio	Animal No	Spleen 261M+	GAPDH	Ratio	Animal No.	Spleen 261M-	GAPDH	Ratio
265917	618	67	9.22388	261523	735	135	5.4444	261599	188	25	7.52	261598	203	31	6.5484
265919	257	61	4.21311	261532	618	120	5.15	261601	412	45	9.1556	261600	409	116	3.5259
261521	324	53	6.11321	261537	583	93	6.2688	261606	376	24	15.667	261602	421	44	9.5682
261528	248	51	4.86275	261540	361	55	6.5636	261608	320	30	10.667	261604	502	37	13.568
261529	213	41	5.19512	261545	474	66	7.1818	261609	590	73	8.0822	261610	473	20	23.65
261530	408	46	8.86957	261553	437	51	8.5686								
261538	274	59	4.64407	261559	890	65	13.692								
261318	1465	310	4.72581												