Epstein Barr Nuclear Antigen 1 Induced Lymphoma in Transgenic Mice

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In memory of my mother, Rosie, whose courage and determination were an inspiration to me.

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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.

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Abbreviations

ATP - adenosine triphosphate
bp - base pairs
cpm - counts per minute
CTP - cytidine triphosphate
DMSO - dimethyl sulphoxide
DNA - deoxyribonucleicacid
dNTP - deoxyribonucleotides (dATP, dCTP, dGTP, dTTP)
DS - region of dyad symmetry
eBL - endemic Burkitt's lymphoma
EBNA 1-6 - Epstein Barr Nuclear Antigen 1-6
EBV - Epstein Barr virus
ECACC - European catalogue of animal cell culture
EDTA - ethylenediaminetetraacetic acid
Eμ - murine immunoglobulin heavy chain enhancer
ENU - N-ethyl-N-nitrosurea
Et Br - ethidium bromide
FCS - foetal calf serum
FR - family of repeats
g - gram
GAPDH - glyceraldehyde phosphate dehydrogenase
GTP - guanosine triphosphate
hr - hour
IgH - immunoglobulin heavy chain
kbp - kilo base pairs
LCL - Lymphoblastoid cell line
LP - Leader protein (EBNA 5)
M - molar
μg - microgram
min - minute
μl - microlitre
ml - millilitre
mM - millimolar
MoMLV - Moloney murine leukemia virus
NPC - nasopharyngeal carcinoma
ori P - epstein barr virus latent origin of replication
PCR - polymerase chain reaction
RACE - rapid amplification of cDNA ends
RAG - recombination activating genes
RNA - ribonucleicacid
sBL - sporadic Burkitt's lymphoma
SDS - sodium dodecyl sulphate
sec - second
SSCP - single stranded conformational polymorphism
TTP - thymidine triphosphate
UTP - uridine triphosphate
UV - ultra violet
V-D-J - variable-diversity-joining (immunoglobulin recombination)
YAC - yeast artificial chromosome

Abstract

Burkitt's lymphoma is a human B cell lymphoma which carries a characteristic translocation resulting in *c-myc* deregulation due to juxtaposition of an immunoglobulin locus. There is a strong association between endemic Burkitt's lymphoma (eBL) and Epstein Barr virus (EBV). EBNA-1 is the only EBV latent antigen consistently expressed in eBL biopsies. Although tissue culture assays have not attributed any oncogenic activity to EBNA-1, two lines of Eμ EBNA-1 transgenic mice succumb to monoclonal B cell lymphoma (Wilson and Levine, 1992, Wilson, Bell and Levine, 1996).

The experiments described in this thesis were designed to explore what cellular factors contribute to Eμ EBNA-1 induced lymphomas.

The latency and penetrance of the phenotype are different between the two Eμ EBNA-1 transgenic lines; 100% of line 26 mice succumb to lymphoma in 4-12months, 43% of line 59 mice succumb to lymphoma by 2 years of age. An analysis of the RNA expression of the transgene has identified complex patterns of expression in both lines, with distinct differences between the lines.

EBNA-1 is a functionally pleiotropic protein. The DNA binding activity of EBNA-1 is required for its essential role in viral DNA replication during latency, and also for its activity as a transcriptional transactivator. EBNA-1 DNA binding activity has been detected in line 26 tumour samples, but not in

line 59 spleens, although the level of protein detected by Western analysis is higher in line 59 samples than in line 26 samples (Wilson and Levine, 1992). This may indicate that the level of EBNA-1 DNA binding activity is an important determinant of oncogenicity.

Given that all the tumours in both lines are monoclonal, an attempt has been made to identify which oncogenes synergise with EBNA-1 in tumourigenesis. Several lines of evidence are strongly suggestive of a co-operation between EBNA-1 and deregulated *c-myc* in tumourigenesis, indicating that EBNA-1 may play a direct role in the pathogenesis of Burkitt's lymphoma.

Chapter 1

Introduction

1.1 Epstein Barr Virus and Human Disease

Epstein Barr Virus (EBV) is a human gamma herpes virus which is highly prevalent in all communities (for review see Epstein and Achong, 1986). The majority of people become infected before the age of 25. In children, initial infection is largely asymptomatic. Primary EBV infection begins in the pharyngeal epithelium, which is permissive for viral replication. From the epithelium the virus spreads to the subepithelial B lymphocytes where latent infection is established. After primary infection the virus persists for life within the host.

EBV causes a wide spectrum of acute and chronic infections in normal and immunocompromised individuals (Khanna *et al.*, 1995 and references therein) . Primary EBV infection is the usual cause of infectious mononucleosis (glandular fever). The virus is also tightly associated with a number of human malignancies including; endemic Burkitt's lymphoma (eBL), nasopharyngeal carcinoma, and the lymphomas to which immunosuppressed individuals are prone. Recently it has become apparent that there is a strong association between EBV and nasal T cell lymphomas (Meijer *et al.*, 1996). The potential involvement of the virus in a

number of other clinical syndromes has been proposed. These include Hodgkin's disease, chronic mononucleosis, an X-linked lymphoproliferative syndrome (Duncan's syndrome), oral hairy leukoplakia in AIDS patients and other epithelial malignancies including carcinoma of the parotid gland (Khanna *et al.*, 1995).

The EBV genome is a double stranded DNA molecule of 172kbp, the B95-8 prototype of which has been completely sequenced (Baer *et al.*, 1984). (Figure 1.1). Although most of the early and late lytic infection genes show consistent homology in different herpes viruses, the latent genes and a few lytic infection genes show no homology to other herpes virus genes. During lytic infection a large number (more than 80) of virus specific RNA species are expressed.

Normal, resting human B-lymphocytes infected *in vitro* with EBV are transformed into permanent lymphoblastoid cell lines (LCLs). These cell lines provide evidence of the ability of EBV alone to induce indefinite B-cell proliferation and provide a model system for viral latency. The virus is maintained as a multicopy episome. By contrast to lytic infection, in latent infection only eight viral protein encoding genes are expressed (Kieff and Liebowitz, 1990). Six of these encode EBV nuclear antigens [EBNA-1, 2, 3A, 3B, 3C and Leader Protein (alternatively known as EBNA-1, 2, 3, 4, 6 and 5 respectively)]. The other two genes encode latent membrane proteins [LMP 1 and LMP 2 (A and B)(alternatively known as LMP and TP (1 and 2) respectively)].

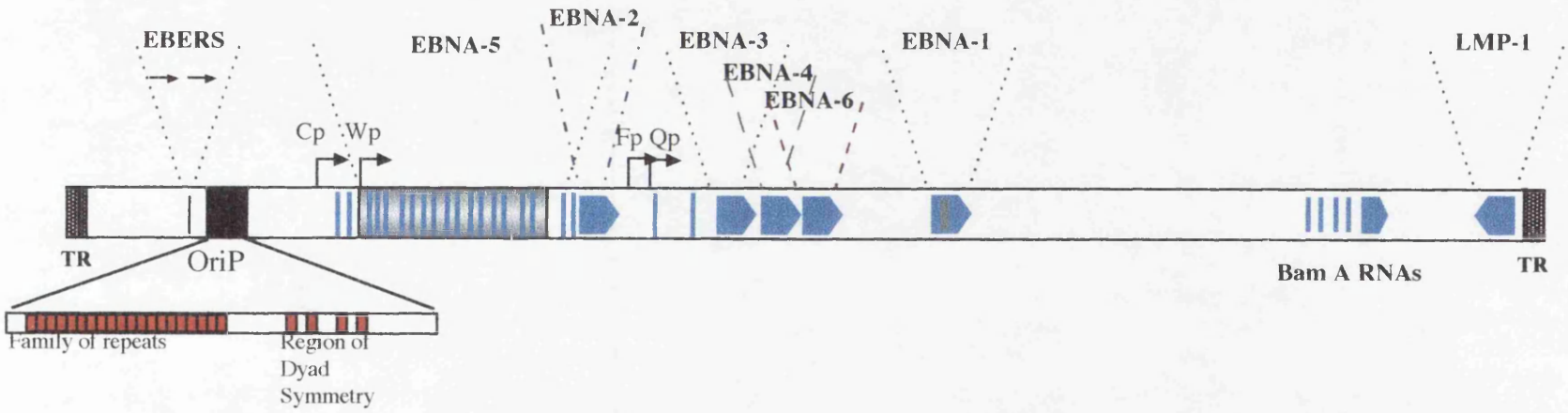


Figure 1.1

Simplified illustration of the B95-8 EBV genome. OriP, the latent origin of replication is indicated by a black box. Red boxes indicate the EBNA-1 binding sites within oriP. The five EBNA mRNAs are spliced from a single transcript, the location of exons encoding the latent antigens are indicated (blue). The position of the promoters which are utilised for EBNA gene expression in the different types of latency are indicated; Wp and Cp are active in group III latently infected cells, Qp is active in group I latently infected cells. Fp is active during the lytic cycle. Transcripts from Cp and Wp are spliced to yield mRNAs for all five EBNAs. Qp and Fp give rise to EBNA-1 expression only. The EBERs are two small, nonpolyadenylated, palindromic RNAs transcribed in both latent and lytic cycles. The open reading frame for LMP-1 lies in the opposite orientation to that of the EBNAs. During the latent cycle EBVcircularises to form an episome linked at the terminal repeats (stippled), the open reading frame for LMP 2a and b lies across the terminal repeats and is not shown. Bam A RNAs are a complex family of spliced polyadenylated RNAs which may give rise to one or more proteins.

Latency	Cell Type	EBV Gene Expression	Cell Phenotype
I	Burkitt's lymphoma biopsy samples, and type I cell lines	EBNA-1, EBERs	B cells which grow as single suspension and are sensitive to the induction of apoptosis. Expression of other latent antigens can be induced leading to transformation to latency III phenotype
II	Nasopharyngeal carcinoma biopsy samples, EBV Positive Hodgkins disease	EBNA-1, LMP1, LMP2, EBERs, BamHI A transcripts	Epithelial cells
III	EBV transformed lymphoblastoid cell lines (LCLs), and type III BL cell lines	EBNA-1-6, LMP1, LMP2, EBERs	B cells which express cell surface adhesion molecules, grow in tight clumps and are relatively resistant to apoptosis

Table 1.1

In addition two small untranslated RNAs (EBER1 and EBER2) are expressed. An unusual set of 3' coterminal spliced mRNAs transcribed through the BamHI A fragment of the virus were initially detected at high levels in nasopharyngeal carcinoma cells and subsequently found to be present at low levels in some latently infected B lymphocytes (Smith *et al.*,1993). Recently these transcripts have been shown to encode one or more proteins that are expressed in latently infected cells and in tumour tissue (Fries *et al.*, 1997).

However, different forms of EBV latency also exist (Klein, 1989). (Table 1.1). In biopsy samples from nasopharygeal carcinomas only EBNA-1, LMP1 and 2 and the EBERS are expressed (Brooks *et al.*,1992). Burkitt's lymphoma biopsy cells can also be maintained in culture. In their initial state these cells express only EBNA-1 and the EBERs (Rowe *et al.*, 1987). Certain stimuli can induce the expression of the other latent antigens, this is associated with a phenotypic change in the cells which become more like LCLs in appearance. An analysis of latent gene expression in peripheral blood lymphocytes has identified an EBNA-1 only pattern of latent gene expression like latency I (Qu and Rowe, 1992). A further EBV 'latency program' has been identified in vivo in resting Bcells, where LMP2A is the only latent antigen detectably expressed (Miyashita *et al.*, 1997).

EBNA 2 has been shown to play a central role in B cell transformation (Hammerschmidt and Sugden, 1989, Cohen *et al.*, 1989, Kempkes *et al.*, 1995). It is expressed together with EBNA 5 prior to the expression of other viral genes after infection of B cells, and acts as a transcriptional activator of latent viral and

cellular genes (eg CD21, CD23 and c-fgr) (Calendar *et al.*, 1987, Wang *et al.* 1987, Abbot *et al.* 1990, Cordier *et al.*, 1990, Fahraeus *et al.*, 1990, Ghosh and Kieff, 1990, Knutson, 1990, Sung *et al.* 1990, Woisetschlager *et al.*, 1991, Zimber-Strobl *et al.*, 1991,1993, Jin and Speck, 1992, Ling *et al.*, 1993, Laux *et al.*, 1994, Meitinger *et al.*, 1994). EBNA2 transactivation has been shown to be mediated via an interaction with a DNA binding protein called CBF1 or RBP-Jκ which serves to target EBNA2 to DNA (Zimber-Strobl *et al.*, 1994). The similarities in mechanism and outcome between this interaction and the interaction of CBF1 and the intracellular domain of the receptor Notch1, led to a model in which EBNA-2 mimics constitutive Notch signaling (Hseih *et al.*, 1996, 1997). A number of tumours have been identified in which mutation of Notch family genes is thought to result in constitutive Notch signalling (reviewed in Wilson,1997). By analogy EBNA-2 may have similar oncogenic action.

LMP 1 has potent transforming activity (Wang *et al.*, 1985, Baichwal and Sugden, 1988, Wilson *et al.*, 1990, Hu *et al.*, 1993, Fishraeus *et al.*, 1990). Expression of LMP 1 effects changes in expression of numerous cellular genes including upregulation of CD40, CD23 and cell adhesion molecules such as CD11a, CD54 and CD58, and downregulation of CD10, CD77, insulin-like growth factor receptor type 1 and possibly also the recombination activating genes 1 and 2 (RAG 1 and RAG 2) (Wang *et al.*, 1988, Wang *et al.* 1990, Peng and Lundgren, 1992, Fahraeus *et al.*, 1992, Kriauciunas *et al.*, 1993, Kuhn-Hallek *et al.*, 1995).

LMP 1 expression may also induce the expression of Bcl-2 and this could be one mechanism by which the protein inhibits cell death (Henderson *et al.*,1991, Finke *et al.* 1992). The mechanism of action of LMP 1 is beginning to be elucidated. Many of the actions of LMP 1 may be exerted through activation of the NF-κB transcription factor (Laherty *et al.*, 1992, Hammerskjöld and Simurda, 1992). Current evidence suggests that this effect is mediated by usurption of a cellular signalling pathway of the tumour necrosis factor (TNF) pathway (Mosialos *et al.*, 1995, Sandbeg *et al.*, 1997).

Both EBNA 2 and LMP 1 act to upregulate cellular expression of Bcl 2. In addition, it has also recently been shown that EBNA 4 can upregulate Bcl 2 expression (Silins and Sculley, 1995).

Whilst LMP 1 has demonstratable activity in several assays as an oncogene, this is not so for the other latent genes, even though some (EBNA-1, 2, 3, 6 and LMP-1) are required for transformation of B cells in culture by EBV (Hammerschmidt and Sugden, 1989, Mannick *et al.*, 1991, Swaminathan *et al.*, 1991, Allan *et al.*, 1992, Tomkinson *et al.*, 1993, Kaye *et al.*, 1993, 1995). EBNA-1 particularly has not been reported to act as an oncogene in tissue culture assays. However, given that EBNA-1 is essential for viral DNA replication, its role in B cell transformation cannot be tested using the deletion/recombinant virus approach utilised in assessing the role of the other latent proteins. Nevertheless, the oncogenic potential of EBNA-1 has now been clearly revealed *in vivo*, since Eμ

EBNA-1 transgenic mice are predisposed to B cell lymphoma (Wilson and Levine, 1992, Wilson, *et al.*, 1996).

1.2 Burkitt's lymphoma

Burkitt's lymphoma (BL) occurs throughout the world. It is one of the most frequent cancers in African children (endemic BL). In Europe and the USA it represents less than 3% of childhood cancers, but in all regions it represents a significant proportion of malignant non-Hodgkin's lymphomas (30-40%). BL is a malignant lymphoma comprised of a clonal population of undifferentiated lymphoid cells.

Malaria is thought to be a contributing factor in endemic BL. The high incidence of BL occurs in regions where malaria is also prevalent (equatorial Africa and New Guinea). It has been proposed that the immunosuppression resulting from malarial infection increases the likelihood of BL development. There is a strong association between BL and EBV. More than 95% of endemic BLs are EBV genome positive (Lenoir and Bornkamm, 1986), although less than 1% of the patients' total circulating B cell pool is detectably infected with EBV (Moss *et al.*, 1983). The association of the virus with sporadic BLs is less clear. Only approximately 15% of sporadic cases are EBV positive. However, integrated viral DNAs have been detected in some sporadic tumours possibly suggestive of a wider involvement of EBV in the onset of sBL than previously suspected (Razzouk *et al.*, 1996).

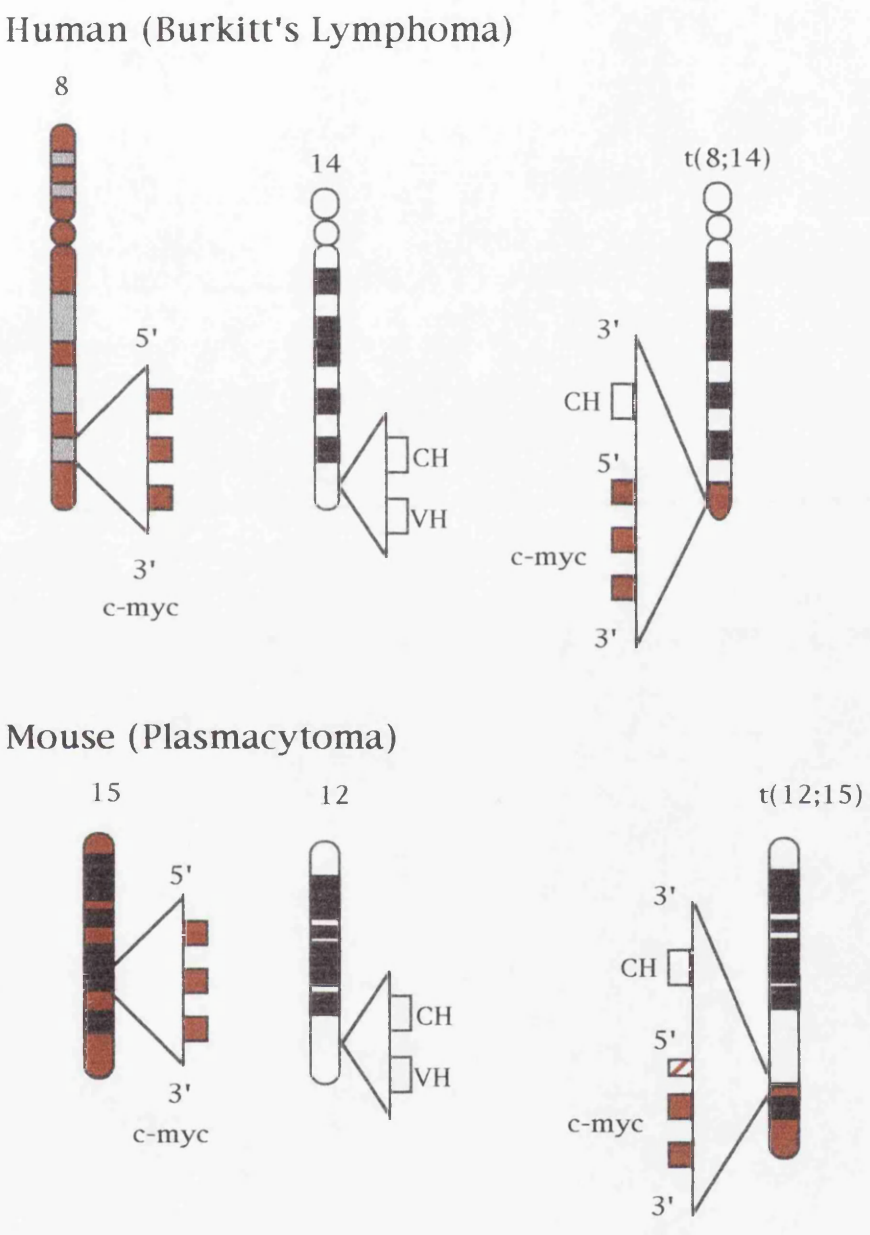
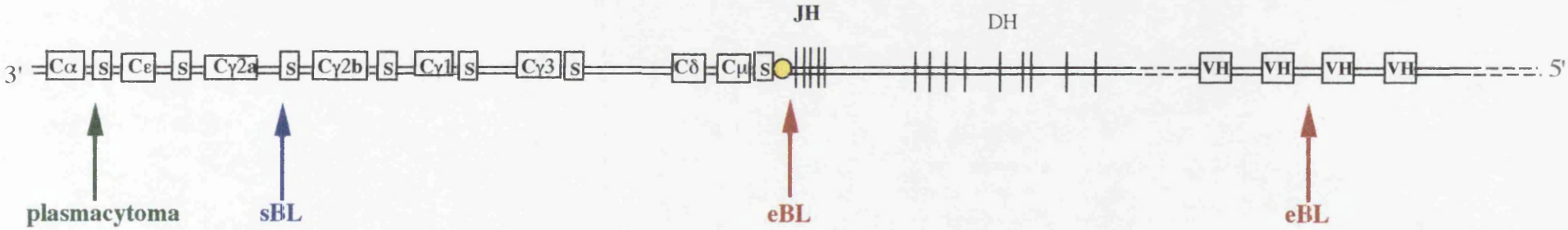
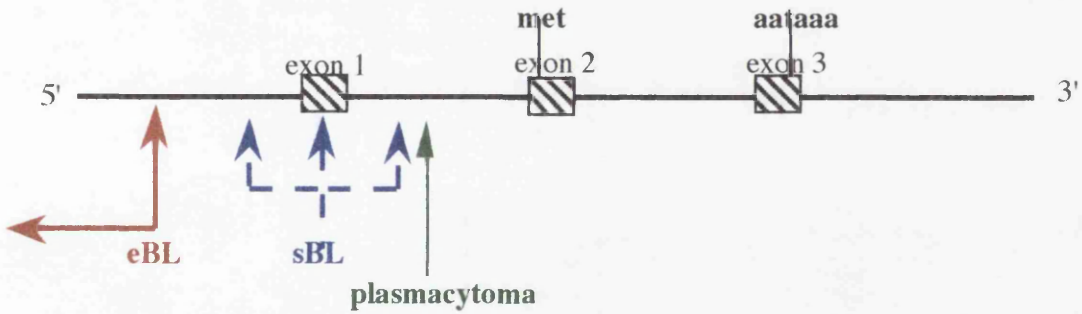


Figure 1.2
Diagram illustating the *c-myc*/immunoglobulin heavy chain locus translocations common to human Burkitt's lymphoma and murine plasmacytoma



Immunoglobulin heavy chain locus
human chromosome 14, mouse chromosome 12



***c-myc* locus**
human chromosome 8, mouse chromosome15

Figure 1.3
Diagram of the breakpoints in the immunoglobulin heavy chain and c-myc loci in human Burkitt's lymphoma and murine plasmacytoma. Common breakpoints are indicated by arrows; red- endemic BL, blue- sporadic BL, green- murine plasmacytoma. The position of the IgH enhancer is indicated by the yellow circle.

Characteristic of BL is a translocation involving the *c-myc* locus on chromosome 8 and one of the three immunoglobulin loci (reviewed in Cory, 1986). The most common translocation involves the heavy chain locus (IgH) on chromosome 14. In 10% of cases either of the light chain loci on chromosomes 2 and 22 are involved. (Figure 1.2). All of the translocatons result in the deregulation of *c-myc* expression. However the precise location of the breakpoints in the more frequent t(8;14) translocation differs between sporadic and endemic Bls. (Figure 1.3). In sporadic BL the breakpoint on chromosome 8 occurs in the region of exon or intron 1 of *c-myc*. Within this region are elements which regulate the normal expression of *c-myc*, and their disruption leads directly to the deregulation of *c-myc* expression. In these translocations the breakpoint on chromosome 14 is most often within one of the switch regions of the immunoglobulin gene. Murine plasmacytomas carry a *c-myc* translocation very similar in nature to that seen in sporadic BL, in the majority of tumours the immunoglobulin heavy chain enhancer on chromosome 12 becomes juxtaposed with a truncated *c-myc* gene on chromosome 15. In endemic BL, the breakpoint on chromosome 8 occurs at some distance (kilobases) 5' to the *c-myc* gene and within the constant or joining region at the immunoglobulin locus, such that the immunoglobulin heavy chain enhancer is on the same chromosome as *c-myc*. The difference in the site of breakpoint within the IgH locus may reflect the different stage of the cells involved in each tumour. Sporadic BLs appear to derive from cells involved in immunoglobulin class switching. The translocation in endemic BL may occur at the time of V-D-J

joining ie. in more immature B-cells. The fact that sporadic BL cells are more advanced along the differentiation pathway is supported by the observation that sporadic but not endemic BLs secrete IgM. The exact signifcance of the differences in the translocations and whether the presence of EBV is in any way related is unclear.

The precise role of EBV in the development of endemic BL is not known. It may be that the virus is directly involved in the transformation of B cells, or that through immortalization of B cells the virus produces a pool of cells more susceptible to further oncogenic changes. The known transforming capabilities of LMP1 may be important in the development of the disease. Other latent antigens may also play a role. However, in EBV positive Burkitt's lymphoma biopsies consistently EBNA-1 is the only viral antigen detectably expressed. If EBV plays a role in the maintenance of the tumour phenotype then this can only be through the action of EBNA-1 and/or the EBERs. However, tissue culture assays have shown that the EBERs are dispensible for transformation of B cells (Swaminathan *et al.*, 1991). It may also be that EBNA-1 performs a critical function in the onset or progression of Burkitt's lymphoma tumours.

1.3 EBNA-1

a. Viral Regulation of EBNA-1 expression

EBV adopts more than one form of latency in different cellular environments (reviewed in introduction section 1.1). EBNA-1 expression is consistent in all forms of viral latency; expression of the protein is essential for maintenance of the viral episome (Yates *et al.* 1984, Rawlins *et al.*, 1985). The use of different promoters is one mechanism by which the virus controls its gene expression. During the early stages of infection in B lymphocytes, transcription of six viral nuclear antigens (EBNA-1-6) is initiated from an early promoter (Wp). This is followed by a switch to an upstream promoter (Cp) (Woisetschlager *et al.*, 1989, 1990). In LCLs latent gene expression is from Wp or Cp. Wp and Cp are mutually exclusive promoters. The large transcripts are alternatively spliced to yield the individual EBNA mRNAs. Recent evidence suggests that the expression of EBNA-1 may be important in the switching from Wp to Cp (Schlager *et al.*, 1996). Cp activity is regulated by EBNA-1 and EBNA2. EBNA-1 binding to the multiple EBNA-1 binding sites in the family of repeats (FR) region of oriP leads to the upregulation of expression from Cp (Reisman and Sugden, 1986, Yates *et al.*, 1984) and an EBNA2 dependent enhancer is located in in the proximal region upstream of Cp (Sung *et al.*, 1992, Woisetsclager *et al.*, 1991).In latency I cells and NPCs the expression of EBNA-1 is from a distinct promoter located in the Bam HI Q region of the virus, designated Qp (Schaefer *et al.*, 1995). EBNA-1 again has

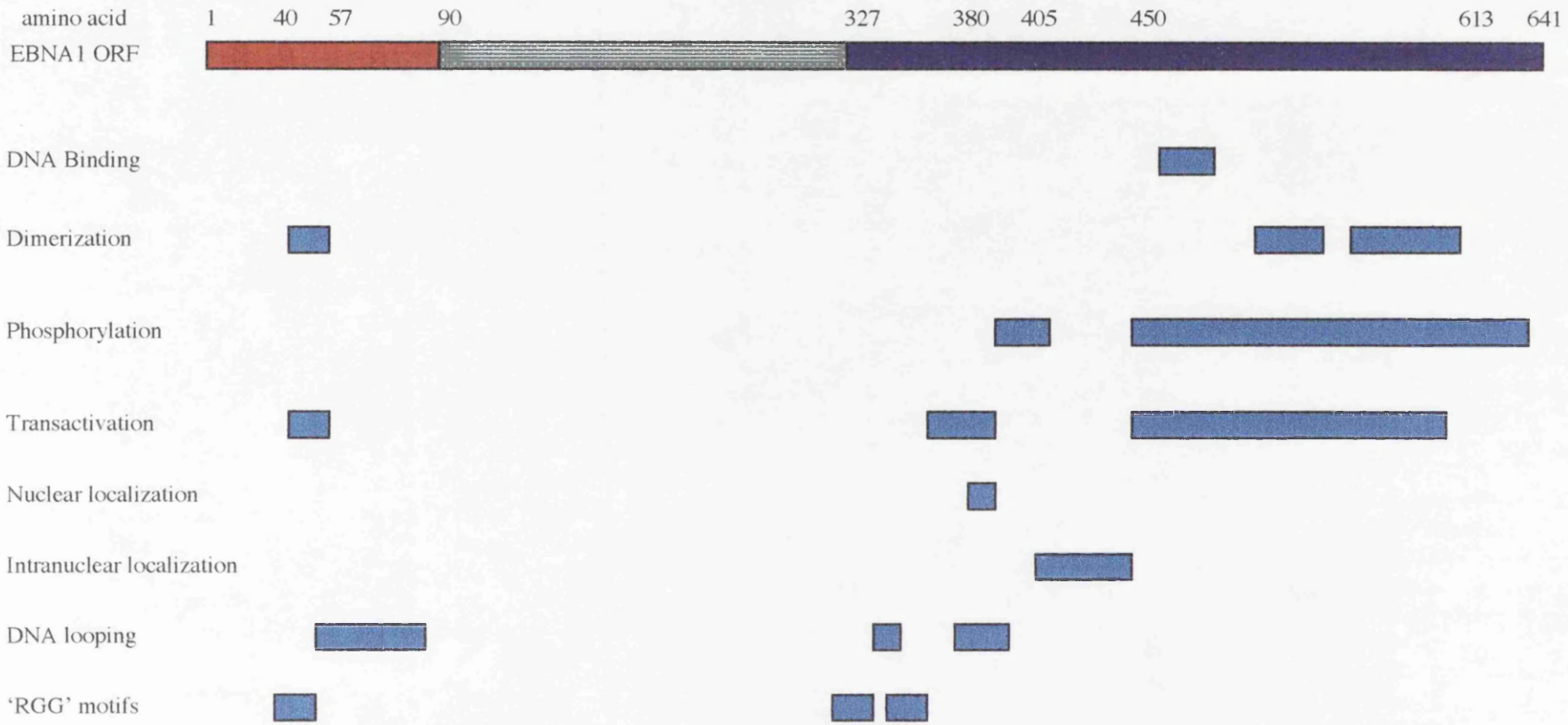


Figure 1.4
Diagram illustrating the functional domains of EBNA-1. The 641 amino acid sequence of EBNA-1 is divided into three domains; amino acids 1-89 forming the unique N-terminus (red), amino acids 90-327 forming the repetitve region (grey), and amino acids328-641 the unique C-terminus (purple). Indicated in blue are the location limits for known functions of EBNA-1. (References in text).

a role in the regulation of this promoter, however EBNA-1 binding to a region within BAM HI Q leads to downregulation of expression (Sample et at, 1992). These binding sites are located within the transcribed sequences and it has been postulated that the downregulation may involve EBNA-1 RNA binding. Another promoter, Fp, located immediately upstream of Qp was originally identified as the active promoter for EBNA-1 expression in type 1 latency. However, this promoter has subsequently been shown to be an exclusively lytic promoter (Lear *et al.*, 1992, Schaefer *et al.*, 1995).

b. EBNA-1 Structure and Function

In the B95-8 strain of EBV EBNA-1 is a 641 amino acid protein. The amino and carboxy terminal domains of the protein are separated by 235 amino acids of Gly-Gly-Ala repeats (figure 1.1) (Baer *et al.*, 1984). The number of these repeat regions varies between different strains of the virus, so the total size of the protein varies between ~69 to 94 kDa. This repeat region is thought to be important in the ability of EBNA-1 to evade recognition by the host's immune system. Insertion of the repeat region into EBNA-4 led to inhibition of Tcell recognition of the protein through disruption of peptide processing and presentation (Levitskaya *et al.*, 1995).

EBNA-1 has an established role in the replication and maintenance of the viral episome through the latent origin of replication ori P. EBNA-1 and oriP are the only viral contributions needed to mediate viral DNA replication in latently

infected cells (Yates *et al.*, 1985). Ori P consists of two regions containing multiple EBNA-1 binding sites. The region of dyad symmetry (DS) contains four EBNA-1 binding sites at which latent replication is initiated. Upstream of this is a repeat region, family of repeats (FR), containing 20 copies of the EBNA-1 binding site. In addition to its role in replication of the virus FR can act as a transcriptional enhancer in the presence of EBNA-1 (Reisman and Sugden, 1986). EBNA-1 binds to DNA as a homodimer, and this is essential for its activity both in replication and as a transcriptional transactivator (Polvino-Bodnar and Schaffer, 1992, Goldsmith *et al.*, 1993, Frappier *et al.*, 1994). Mutation and deletion analysis have determined that the portion of the protein required for these activities lies between aa 460 and 607 (Figure 1.4). Crystallization of this region of EBNA-1 both bound and unbound to DNA has indicated that the DNA binding region is composed of two regions. The C-terminal core domain, comprising residues 504-604, is virtually identical in structure to the DNA binding and dimerization domain of the E2 protein of bovine papilloma virus, despite no sequence similarities. Residues 461-504 comprise an N-terminal flanking domain (Barwell *et al.*, 1996, Bocharev *et al.*, 1996).

Within the amino terminal region of the protein are a number of 'RGG' motifs which are thought to be important in RNA binding. EBNA-1 has been demonstrated to bind RNA in vitro although no specificity of binding is apparent (Snudden *et al.*, 1994).

No enzymatic activities have been attributed to EBNA-1. A number of regions of the protein have been shown to be important for transactivation (figure 1.4) (Polvino-Bodnar *et al.*, 1988, Yates and Camiolo, 1988, Polvino-Bodnar and Scaffer, 1992).

EBNA-1 is phosphorylated on serine(s) in its carboxy terminal domain, but effects of phosphorylation on the protein's activities have not been reported (Hearing and Levine, 1985, Frappier and O'Donnell, 1991).

An additional activity of EBNA-1 which is likely to be important particularly in replication is the ability to link regions of DNA to which it binds specifically (figure 1.4). Three separate regions of the protein can independently link DNA to which they are bound (Mackey *et al.*, 1995). Electron microscopy has shown that EBNA-1 bound to oriP links DS (region of dyad symmetry) and FR(family of repeats) looping out the intervening DNA (Yates *et al.*, 1985).

Cellular factors have been identified which can bind to oriP *in vitro* (Wen *et al.*, 1990, Zhang and Nonoyama, 1994). By analogy, there may exist sites in the host genome to which EBNA-1 can bind *in vivo.*

The combination of DNA and RNA binding activities with the transactivation and repression capabilities provide EBNA-1 with great potential to disrupt cellular gene regulation.

1.4 Tumourigenesis and Transgenic Mouse Models

It is now generally accepted that cancers arise as the result of a number of genetic or epigenetic changes (reviewed in Hunter, 1991). The progression from normal to cancerous cell involves a series of events which allow the cell to evade the usual controls on proliferation. The specific nature of the changes involved varies greatly between different tumours. However the effects of the changes fall into classes common to the majority of tumours: acquisition of growth factor independence, ability to ignore restraining signals, escape from immunological surveillance, induction of angiogenesis, breach of surrounding tissues and metastatic invasion. Genes identified as having oncogenic potential encode proteins of widely varying function, from cell surface receptors and cytokines to nuclear transcription factors. The stages in the development of different cancers are similar (figure 1.5). Haemopoietic neoplasms may require fewer steps given that the cells are inherently migratory.

Transgenic systems provide a powerful tool for the analysis of the effects of specific genetic changes *in vivo*. The ability to investigate pre-neoplastic changes in cells allows the investigation of the process of tumourigenesis in discrete stages. This is an advantage over analysis of human tumour biopsies or cell lines where only the end point is available.

The inactivation of specific genes by homologous recombination can be used to assess candidate tumour suppressor genes. Introduction of mutated or inappropriately expressed genes allows assesment of their oncogenic potential.

1. Genetically altered cell

Tumour development begins when some cell (red) within a normal population (blue) sustains a genetic mutation(s) that increases its propensity to proliferate when it would normally rest, or in some cases a cell which would normally die is able to survive.

2. Hyperplasia

Proliferation of this cell gives rise to hyperplasia. After time further mutation(s) of one of these cells (lilac) gives rise to a cell less responsive to the normal controls on cell growth, for example through the gain of growth factor independence.

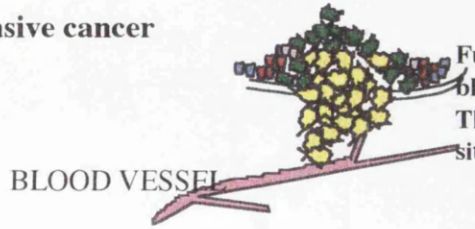
3. Dysplasia

The proliferating cells become more abnormal in appearance, further mutation generates a cell even more removed from normal (green) which continues to proliferate, the cell may now have become able to evade immunological surveillance.

4. In situ cancer

The cells continue to proliferate, but are still contained within tissue boundaries. However, further mutation may allow the cell (yellow) to breach tissue boundaries. Induction of angiogenesis by the tumour is important to sustain an adequate blood supply for the proliferating cells.

5. Invasive cancer



Further genetic changes may allow the tumour to shed cells into the blood or lymphatic system, the mass is now considered malignant. The renegade cells may establish new tumours (metatases) at other sites.

Figure 1.5
Diagram illustrating the stages in solid tumour development

Transgenic mouse models for many types of cancer now exist. Of specific relevance to this work are a number of models of haemopoietic malignancies (reviewed in Adams and Cory, 1991).

Characteristic of human Burkitt's lymphoma is a translocation in which the *c-myc* gene is linked to an immunoglobulin locus (reviewed in introduction section 1.2). Transgenic mice enabled direct testing of the hypothesis that deregulated *c-myc* expression is oncogenic (Adams *et al.*, 1985). Eμ-*c-myc* transgenic mice (the transgene consists of the immunuglobulin heavy chain enhancer, Eμ, linked to the *c-myc* gene) develop monoclonal, nodal pre-B or B cell lymphomas. However, it is clear that the structure of the transgene has a strong bearing on the accuracy of the disease model (reviewed in Wilson, 1997). A transgenic model even closer to BL in phenotype has been generated through the introduction of a large (220kb) IgH/*c-myc* translocation region carried on a yeast artificial chromosome (YAC) (Bützler *et al.* , 1997). These mice also succumb to monoclonal B-cell lymphoma at an early age, but the tumours in these mice arise at non-nodal sites and consist of sIgM+ B cells.

In the assessment of the oncogenic potential of genes, the ability to direct transgene expression in specific cell types or developmental stages is important. A given gene may be highly oncogenic in one cell type, but have little observable effect in another.

In developing a disease model it is critical that the gene of interest is linked to appropriate expression control sequences. The immunoglobulin heavy chain enhancer (Eμ) has been used to direct the expression of a number of genes in both B and T cells. Whilst Eμ-*c-myc* mice develop B cell lymphomas (Adams *et al.*,1985), Eμ N *ras* mice succumb to T cell lymphomas (Harris *et al.*, 1988) and Eμ H ras mice do not develop lymphomas but do develop lung adenocarcinomas (Suda *et al.*, 1987), presumably due to some sequence within H *ras* which directs expression in lung tissue. Varying the regulatory sequences attached to a transgene allows the investigation of effects of expression in different cell types, however cellular sequences flanking the transgene insertion point can modify the pattern of expression.

In addition, in transgenic models the strain background of the animals can have a significant bearing on the observed phenotype. On a C57BL/6, SLJ or BALB/c background Eμ-*c-myc* transgenes provoke B lymphoid tumours almost exclusively. However, in C3H/HeJ mice the transgene provokes predominantly T lymphomas (Yukawa *et al.*, 1989). The strain difference may reflect varying stromal environment.

Analysis of the pre-neoplastic state of transgenic tissues can yield information about the mechanism of action of an oncogene. In pre-tumourous Eμ-*c-myc* mice there are greatly elevated numbers of cycling early B lymphocytes (Adams and Cory, 1991, Morgenbesser and DePinho, 1994). This observation suggested that

c-myc expression favours proliferation over differentiation. Support for this theory is provided by the recent observation that Myc protein induces the expression of a key regulator of G1 cell cycle progression (Galaktionov *et al.*, 1996) and represses the expression of a growth arrest gene (Marhin *et al.*, 1997). Transgenic models of neoplasms have provided support for the multistep model of tumourigenesis. The tumours which develop in Eμ-*c-myc* transgenic mice are all monoclonal and arise in a stochastic fashion. The Eμ-*c-myc* transgene is expressed before birth in the vast majority of B lineage cells. Calculation from the rate of tumour onset suggests that a tumourigenic clone typically arises after 10^{10} divisions of B lymphoid cells (Harris *et al.*, 1988). Given this low frequency at least one precise, or more likely several, additional genetic changes are required to take a B lymphoid cell aberrantly expressing *c-myc* on to becoming tumourigenic. One of the greatest advantages of the use of transgenics in the study of oncogenesis is the ability to begin to address the sequence of genetic changes required for the development of a tumour (described in section 1.6).

1.5 Eμ EBNA-1 Transgenic mice

A number of Eμ EBNA-1 transgenic mouse lines have been generated by Joanna Wilson (Wilson and Levine, 1992, Wilson, et al., 1996). The transgene contains the EBNA-1 coding region linked to the polyomavirus early promoter and the

Summary of Eμ EBNA-1 Transgenic Lines

Line	Copy No.	Phenotype	RNA	Protein							Genotype
				Spleen	*Lymph Node*	*Thymus*	*Liver*	*Heart*	*Kidney*	*Brain*	
26	2	100% lymphoma by age 4-12 months	+++	+	+	-	-	-	-	-	+
59	1-2	~ 50% neoplastic lesions by age 24 months	++	+++	+++	+	-	-	-	+	++
60	1 (rearranged)	none	+/-	-	ND	-	-	-	-	-	++
61	10	none	-	-	ND	ND	ND	ND	ND	ND	+(Y)

(-) no expression detected

(+, ++, +++) relative levels of expression

ND not determined

(+) hemizygous

(++) homozygous

(Y) Y chromosome insert

Table 1.2

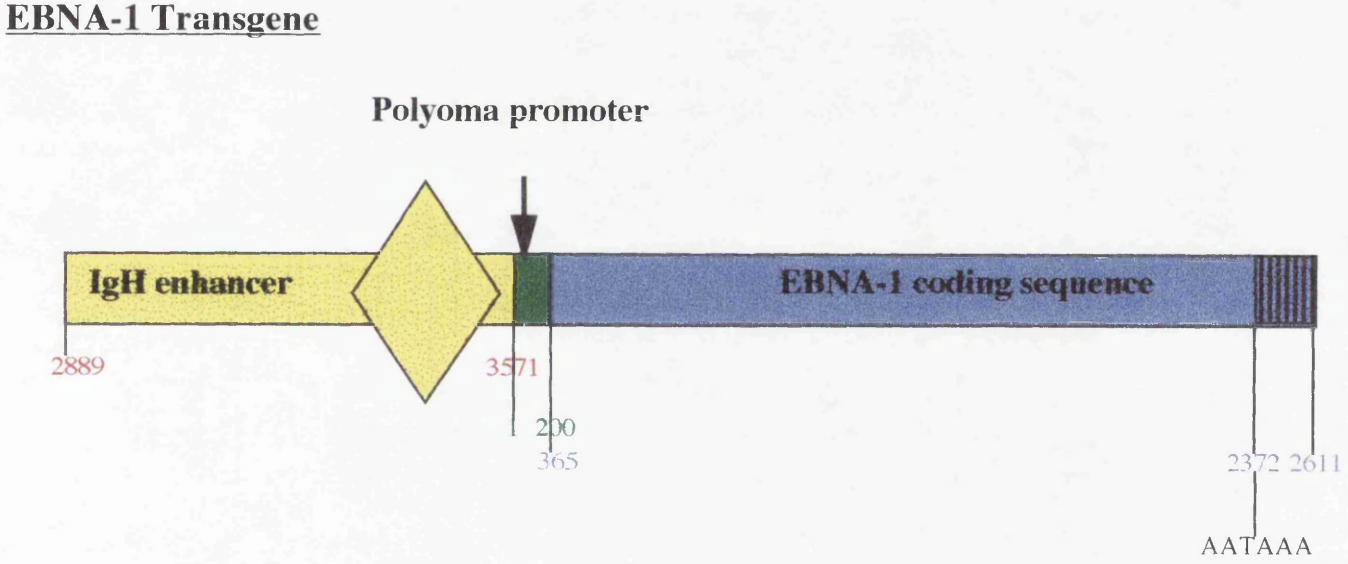


Figure 1.6
Structure of the Eμ EBNA-1 transgene.The EBNA-1 coding region from the B95-8 strain of the virus (blue) is linked to the polyomavirus early promoter (green) and immunoglobulin heavy chain sequences (yellow), the defined enhancer sequence is represented by the diamond shape (Wilson and Levine, 1992). Numbers indicate sizes in base pairs: red IgH enhancer (Banerji *et al*, 1983), green polyomavirus early promoter (Tooze,1981), blue EBV sequences (Baer *et al*, 1984).

mouse immunoglobulin heavy chain enhancer (Wilson *et al*, 1990), designed to enhance expression in B lymphocytes (figure 1.6). Mice of two Eμ EBNA-1 transgenic lines (designated line 26 and line 59) succumb to monoclonal B cell lymphoma, demonstrating that EBNA-1 is oncogenic *in vivo*.

In both line 26 and line 59 there are one or two copies of the transgene, and EBNA-1 protein is detectably expressed within lymphoid cells. Two other Eμ EBNA-1 transgenic lines are also relevant to this study, designated line 60 and line 61. Line 60 harbours a single rearranged transgene in which the EBNA-1 coding region is interrupted. Line 61 harbours multiple copies of the transgene (~ 10), with an inheritance pattern indicative of integration on the Y chromosome. There is no detectable expression of EBNA-1 protein in the spleens of animals of line 60 or 61, and neither line displays any pathological phenotype (summarized in table 1.2).

Although mice of both line 26 and line 59 succumb to monoclonal B cell lymphoma, the penetrance and latency of the disease is very different in each line. Whilst 100% of line 26 mice succumb to lymphoma between the ages 4-12 months, 43% of line 59 mice sampled at 2 years of age had neoplastic lesions in the spleen. This may be related to the different expression pattern of the transgene in each line (table 1.2 and chapter 3) or may be due to differences in the oncogenicity of the protein expressed in each of the lines (chapter 5). It may be that there are particular forms of EBNA-1 which are more oncogenic. In most

normal peripheral blood cells 2-3 subtypes of EBV are detected which have sequence differences in EBNA-1. In BL only a single subtype has been observed in each tumour and one specific subtype (V-leu) was found only in BL cells (Bhatia *et al.*, 1996).

Expression of EBNA-1 is not sufficient for tumourigenesis; the lymphomas in Eμ EBNA-1 transgenic mice are monoclonal. Identification of the cellular factors which co-operate with EBNA-1 may elucidate the mechanism by which EBNA-1 acts as an oncogene and provide an insight into the development of EBV associated lymphomas (chapter 6).

1.6 Identification of Synergising oncogenes in Tumourigenesis in Transgenic mice

a.Genomic rearrangements

Consistent DNA rearrangements in tumours in a number of transgenic mice may indicate co-operation with the transgene in the tumourigenic process. Most plasmacytomas in Eμ–*abl* transgenic mice carry a *c-myc* rearrangement (Rosenbaum *et al.*,1990), as do many of the lymphomas arising in Eμ–*bcl-2* mice (McDonnell *et al.*, 1989, Strasser *et al.*, 1993) indicative of the ability of *c-myc* to synergise with a number of other oncogenes.

Rearrangements at the chromosomal level; translocations, deletions, duplications, can be detected through karyotyping. Southern blotting can be used to detect

rearrangements at the gene level. Single stranded conformational polymorphism (SSCP) analysis can be used to screen for point mutations within a specific locus of interest. Whilst this approach can be used to identify common genetic alterations within tumours, one cannot then assess what role these alterations play in the process of tumourigenesis.

b.Transgenic Cross Breeding

Synergy between two candidate oncogenes can be assessed directly by cross breeding transgenic lines. Changes in the rate of onset, penetrance or nature of tumours which develop are indicative of a co-operation between the transgenes in oncogenesis. This approach has demonstrated the synergy between *c-myc* and *bcl-2* (Strasser *et al.*, 1990, introduction section 1.8) and between *c-myc* and *pim-1* in oncogenesis (Verbeek *et al.*, 1991, introduction section 1.8).

c.Proviral insertional mutagenesis

The Anton Berns laboratory has developed a powerful technique for the identification of synergising oncogenes (Berns, 1988). On post-natal infection in the mouse, Moloney murine leukaemia virus (MoMLV) proviral insertions occur relatively randomly throughout the genome. Some insertions may confer a growth or survival advantage to a cell through the (in)activation of cellular genes. The viral presence also serves to "tag" the gene. Using MoMLV infection of mice

transgenic for one oncogene allows identification of genes which synergise with the transgene in tumourigenesis. Such an approach further demonstrated the strong synergy between *pim-1* and *myc* family members in oncogenesis (reviewed in Berns *et al.*, 1994). Infection of Eμ *pim-1* mice greatly accelerated T lymphoma onset and every tumour bore a virus near the *c-myc* or *N-myc* genes.

This approach can also be used to identify candidate genes involved in later stages of tumour progression, such as angiogensis and metastatic potential, by comparing genetic alterations in primary and secondary tumours, for example two genes (*tiam-1* and *frat-1*) have been identified which seem to be involved in progression in T-cell lymphomas (Habets *et al.*, 1994, Jonkers *et al.*, 1997).

d.Chemical induction of tumours

Chemicals can be used to induce tumours. In susceptible strains of mice intraperitoneal injection of the mineral oil pristane induces plasmacytomas (Anderson and Potter, 1969). Although Eμ-*c-myc* transgenic mice do not develop spontaneous plasmacytomas, young Eμ-*c-myc* mice treated with pristane develop plasma cell tumours as well as lymphoma (Harris *et al.*, 1988). Plasmacytomas arose much faster in Eμ-*c-myc* transgenic mice than in pristane primed BALB/c or NZB mice suggesting that the rate limiting step in tumourigenesis in the latter strains is the chance occurrence of a *myc* translocation. IL-6 is produced by macrophages in response to pristane (Van Snick *et al.*, 1988) and is required for

plasmacytoma growth. The macrophage ridden, IL-6 enriched granuloma induced by injection of the mineral oil may nurture the nascent plasmacytoma cell.

The incidence of T lymphomas in Eμ *pim-1* mice is dramatically enhanced if the mice are treated with the carcinogen N-ethyl-N-nitrosourea (ENU) (Breuer *et al.*, 1989). ENU is a mutagen, all ENU induced tumours expressed high levels of *c-myc* RNA, although no evidence of myc mutation was found.

A clear disadvantage of this approach over viral insertional mutagenesis is that the precise mechanisms of the chemical induction are not known.

1.7 Mechanism of Action of EBNA-1 as an Oncogene

The role of EBNA-1 in the regulation of viral RNA expression and in viral DNA replication are well established, however the effects of the protein on the cellular machinery are less clearly defined. EBNA-1 may exert its oncogenic effect through binding to cellular DNA sequences and modifying transcription, through effecting the stability or translation of mRNAs through RNA binding, through protein-protein interactions or through a combination of activities. It may be that there are particular forms of EBNA-1 which are more oncogenic (Bhatia *et al.*, 1996, discussed in introduction section 1.5).

If EBNA-1 plays a role in Burkitt's lymphoma, it could act in a number of different ways;

i. EBNA-1 may act through deregulated c-myc *expression*

EBNA-1 could exert its oncogenic action through affecting the rate at which translocations occur, or through exerting influence over the expression of a translocated *c-myc* gene. The nature of the translocations detected in endemic and sporadic BL are different (discussed in section 1.2), EBNA-1 may be a factor in this. In this work some potential targets of EBNA-1 have been identified which might affect the process of translocation in B-cells;

- **1. RAG 1 and 2**

The recombination activating genes 1 and 2 (RAG 1and 2) are the only lymphoid specific genes required for V(D)J recombination of immunoglobulin loci (Oettinger *et al.*, 1990). Usually these genes are expressed in a narrow window of precursor lymphocyte development. However, EBNA-1 expression induces the expression of RAG 1 and 2 in cell lines (Srinivas and Sixbey, 1995). Identification of heptamer-nonamer recombination signal sequences at the breakpoint regions of several 8;14 translocations in BL cells have led to the speculation that the translocations arise due to aberrant recombination (Haluska *et al.*, 1987). The inappropriate expression of RAG1 and 2 induced by EBNA-1 could lead to a greater incidence of aberrant translocations.

- **2. LR1**

LR1 is a sequence specific DNA binding protein, identified as a potential regulator of immunoglobulin isotype switch recombination in activated B lymphocytes (Williams and Maizels,1991). There are multiple binding sites for the protein within the heavy chain intronic enhancer (Eμ) and also in the G rich switch

regions. In addition there are a number of binding sites for LR1 close to and within the *c-myc* gene. In vitro analysis has indicated that at least one of these sites has a role to play in *c-myc* expression (Brys and Maizels, 1994). Mutation of one site close to the P1 promoter of *c-myc* led to a 5.5-fold decrease in reporter gene expression in the EBV positive BL cell line Raji, and a 3.8-fold decrease in reporter gene expression in the EBV negative B cell lymphoma cell line BJAB. This evidence has led to the speculation that LR1 may have a dual role to play in transcription and recombination. Coupled with the distribution of LR1 sites within the immunoglobulin and *c-myc* loci it is tempting to postulate a role for LR1 in the aberrant translocations and deregulated *c-myc* expression in BL.

LR1 contains two polypeptides, one of 106kDa and one of 45kDa. The 106kDa component has recently been identified as nucleolin (Hanakahi *et al.*, 1997), an abundant protein found in nucleoli, the N terminus of which is homologous to histone H1. Nucleolin is thought to play a role in determining the architecture of the DNA within the nucleolus and may play an analogous role as a component of LR1 in B cells.

LR1 has been identified as a key regulator of transcription from the Fp promoter of EBV (Bulfone-Paus *et al.*, 1995). It follows that enhanced LR1 activity in B cells might be beneficial to the virus. One action of EBNA-1 may be to increase the levels of active LR1 within B cells, thus both enhancing viral transcription and contributing to *c-myc* deregulation.

ii. EBNA-1 may co-operate with deregulated c-myc expression.

Identification of which oncogenes synergise with EBNA-1 in tumourigenesis could be important in determining whether EBNA-1 acts through or in conjuction with *c-myc* in tumourigenesis (section 1.8). If EBNA-1 acts through *c-myc*, then it might be expected to co-operate with oncogenes with which *c-myc* synergizes. If the action of EBNA-1 is through some other mechanism then it might be expected to co-operate with deregulated *c-myc*.

1.8 Potential co-operating factors in EBNA-1 induced tumourigenesis

a. c-myc

The *c-myc* gene has an established role in lymphoid tumourigenesis. Of particular relevance to this study, the characteristic translocations of human BL link the gene to an immunoglobulin locus. Given the strong association between EBV and BL , *c-myc* is a likely candidate for co-operation with EBNA-1 in tumourigenesis.

Myc protein is a nuclear transcription factor, the expression of which is tightly regulated in normal cells. To exert its effects Myc protein must form a heterodimeric complex with Max, like Myc another basic region helix-loop-helix leucine zipper protein (Amati *et al.*, 1993). Deregulated *c-myc* expression is common in multiple tumour types. The potency of this oncogene is likely to be associated with its role in the progression of the cell cycle (reviewed in Henriksson and Lüscher, 1996). However, the role of Myc extends beyond the

induction of the cell cycle, Myc expression is maintained in proliferating cells and down regulated as cells become differentiated. In addition, where there is a block to cellular proliferation, deregulated *c-myc* expression is a potent inducer of programmed cell death (apoptosis) (Evan *et al.*, 1992). Few transcriptional targets of Myc have been identified. However one, the cell cycle phosphatase *cdc-25A*, may be an important effector of c-myc oncogenic activity (Galaktionov *et al.*, 1996). Cdc-25A is essential for the transition from G1 to S phase of the cell cycle (Jinno *et al.*,1994, Hoffman *et al.*, 1994). *cdc-25A* expression is stimulated by Myc, through Myc/Max binding to elements in the *cdc-25A* gene. *cdc-25A* is itself a protooncogene, which like *c-myc* co-operates with Ha-*ras* in transformation of normal rodent fibroblasts (Galaktionov *et al.*, 1995). In addition, Cdc-25A and Myc share the ability to induce p53-dependent apoptosis, and Myc driven apoptosis is inhibited by *cdc-25A* antisense oligonucleotides (Galaktionov *et al.*, 1996). Myc dependent inhibtion of growth cycle arrest may be mediated through the repression of the growth arrest gene *gadd*45 (Marhin *et al.*, 1997). This may well contribute to the tumourigenic potential of transformed cells, through precluding their entering a growth arrested state.

c-myc is a member of a family of homologous genes. Two other members were identified through their involvement in human tumourigenesis (reviewed in Marcu *et al.*, 1992). *N-myc* is commonly amplified in neuroblastomas, and *L-myc* in small cell lung carcinomas. All three genes elicit tumours when overexpressed in transgenic mice (reviewed in Morgenbesser and DePinho, 1994). The ability of the

genes to induce lymphomas differs, with *c-myc* the most potent, then *N-myc* and then *L-myc*. *c-myc* expression is in part self regulated; Myc protein feeds back to down regulate its own expression (reviewed in Marcu *et al.* ,1992). In tumours generated in Eμ N- or *L-myc* mice, no expression of endogenous *c-myc* was observed (reviewed in Adams and Cory,1991). This may indicate that negative crossregulation is operating and that N- and *L-myc* can substitute for *c-myc* function *in vivo*.

It may be that EBNA-1 acts through *c-myc* in lymphomagenesis by contributing to deregulation of its expression. The phenotype of line 26 bears a strong resemblance to that of Eμ-*c-myc* transgenic mice (Adams *et al.*, 1985). As a transcriptional transactivator it is conceivable that EBNA-1 may transactivate the *c-myc* gene, a regulator of the *c-myc* gene or a translocated *c-myc* gene. The exact nature of the translocations in EBV associated BL and sporadic BL are different (reviewed in section 1.2). EBNA-1 may have a causal role to play in the process of translocation, or the presence of EBNA-1 may allow for the selection for translocations which would not otherwise be oncogenic.

Although highly oncogenic, the deregulated expression of Myc proteins is insufficient to cause tumourigenesis. Indeed, no one oncogene has been found to be sufficient for a one step tumourigenic conversion of a normal cell. A number of oncogenes which co-operate with myc genes in tumourigenesis have been identified (discussed in section 1.8b-d). If the oncogenic action of EBNA-1 is

through deregulation of *c-myc* expression then factors which co-operate with *c-myc* in tumourigenesis should also co-operate with EBNA-1. If EBNA-1 co-operates with *c-myc* then some factors which would otherwise synergise with deregulated myc expression in tumourigenesis may be redundant where EBNA-1 is expressed.

b. bcl-2

The most common human lymphoma, follicular lymphoma, characteristically has a t(14;18) translocation, in which the *bcl-2* gene and immunoglobulin locus are juxtaposed, leading to an upregulation of *bcl-2* expression. Eμ *-bcl-2* transgenic mice display a polyclonal hyperplasia of non cycling follicular centre B-lymphocytes, which can progress to give rise to a long latency monoclonal malignant lymphoma (McDonnell *et al.*, 1989, 1990, 1991, Strasser *et al.*, 1991). Overexpression of *bcl-2* has been shown to promote cell survival and inhibit programmed cell death (apoptosis). bcl2 protein is localized mostly to the outer mitochondrial membrane, but also is found in other membranes. There is evidence that *bcl-2* prevents apoptosis through acting in an antioxidant pathway (Hockenberry *et al.*, 1993). *bcl-2* belongs to a family of genes. Other members of the gene family play important roles in both positive and negative regulation of programmed cell death. The members of the Bcl-2 family can form homo and heterodimers. The pro-apoptic member Bad can dimerize with Bcl-2 and Bcl-x_L, and if overexpressed can counter their survival promoting effects. Similarly Bax can form heterodimers with both Bcl-2 and Bcl-x_L. In transfected cells an excess of

Bax results in cell death, where Bcl-2 overexpression promotes cell survival. It is thought that the relative ratio of anti-apoptic dimers versus pro-apoptic dimers is important in determining the resistance of a cell to apoptosis. Post translational modifications also have a role to play in determining the effect of family members (reviewed in Gajewski and Thompson, 1996).

Transgenic cross breeding experiments have indicated a strong co-operation between *c-myc* and *bcl-2* in tumourigenesis (Strasser *et al.*, 1990). Bitransgenic mice develop rapid onset tumours of a primitive haematopoietic cell type. If the oncogenic action of EBNA-1 is through deregulation of *c-myc* expression, then an equally strong co-operation between EBNA-1 and Bcl-2 might be predicted.

c. Pim-1

The *pim-1* proto-oncogene was first implicated in T cell lymphomagenesis through its frequent activation by proviral insertion in murine MoMLV induced T cell lymphomas (Cuypers *et al.*, 1984). Although it has not yet been shown to have a clear role in human lymphomagenesis, over expression of *pim-1* in Eμ *pim-1* transgenic mice induces T cell lymphomas in a dose dependent manner. Existing transgenic lines have relatively long tumour latencies, but the rate of tumourigenesis is greatly enhanced by mutagens able to activate collaborating oncogenes. *Pim-1* is a cytoplasmic serine threonine kinase, but as yet it is not known in which signal transduction pathway it functions. There is evidence that *Pim-1* may inhibit apoptosis and impair the differentiation of cells in which it is

expressed. *Pim-1* belongs to a family of genes, pim2 and pim 3 are highly homologous (reviewed in Allen and Berns, 1996).

Crossbreeding of Eμ-*c-myc* and Eμ *pim-1* transgenic mice demonstrates a dramatic synergy between these two oncogenes. Foetuses which express both transgenes succumb perinatally to lymphoblastic leukaemia (Verbeek *et al.*, 1991). This co-operation is further supported by proviral tagging experiments; *pim-1* is frequently activated by MuMLV in Eμ myc transgenic mice (Haupt *et al.*, 1991, van Lohuizen *et al.*, 1991), and in Eμ *pim-1* transgenic mice in nearly all of the tumours either *c-myc* or *N-myc* is activated by the integrated virus (van Lohuizen *et al.*, 1989). This synergy with *c-myc* makes *pim-1* a possible candidate for co-operation with EBNA-1 in tumourigenesis

d. Other potential factors

The factors discussed in 1.7a-c above represent areas where this study has focussed, however this does not represent an exhaustive investigation of potential co-operating factors in EBNA-1 induced tumours.

Proviral tagging of Eμ myc mice has identified a number of factors which co-operate with myc in tumourigenesis, and therefore may also co-operate with EBNA-1. In addition to *pim-1*(discussed above), two other loci, *bmi*-1(or *bla*-1), and *pal*-1, are frequently activated by proviral insertion (Haupt *et al.*, 1991, van Lohuizen *et al.*, 1991).

Proviral insertions in *bmi*-1 lead to the overexpression of the gene. The *bmi*-1 gene encodes a protein with zinc finger structures which belongs to the polycomb family and which seems to be important in regulating the proliferation of a number of haematopoeitic cells throughout pre and postnatal life (reviewed in Berns *et al.*, 1994).

The *pal-1* locus spans a region of about 50kb and contains a number of genes (Scheijen *et al.*, 1997). Most proviral insertions in the *pal*-1 locus lead to the high expression of one of these genes, *gfi*-1 (Schmidt *et al.*, 1996). *gfi*-1 encodes a protein which functions as a transcriptional repressor, one target of which is the Bax gene, a 'pro-death' member of the *bcl-2* family. In an IL2 dependent T cell line, IL2 withdrawal leads to an increase in Bax levels and subsequent apoptosis. However, if *gfi*-1 is overexpressed Bax transcription is repressed and cell death is retarded (Zörnig *et al.*, 1996, Grimes, To *et al.*, 1996, Grimes, Gilks *et al.*, 1996).

Further *in vivo* oncogenic partners of *c-myc* have been identified through the use of transgenic cross breeding experiments. H-*ras*, K-*ras* and N-*ras* encode cytoplasmic G proteins which are involved in the relaying of stimulatory signals from the cell surface to the nucleus. Mutations of K-*ras* are frequently involved in lung, ovarian, colon and pancreatic cancers. Mutations of N-*ras* are involved in leukaemias. Although Eμ N-*ras* mice do not develop B lymphoid tumours, *myc/ras* bitransgenic mice rapidly succumb to B lymphomas predominantly of early differentiation stages (Harris *et al.*, 1988).

The abl gene encodes a tyrosine kinase, mutation of which is associated with a number of haematologic malignancies (reviewed in Konopka and Witte,1985, De Klein *et al.*, 1988). Abelson murine leukaemia virus expresses a fusion protein (v-*abl*) which lacks the N terminal portion of the normal cellular *abl* gene product and has heightened tyrosine kinase activity. Eμ v-*abl* transgenic mice develop plasmacytomas, the majority of which harbour a rearranged *c-myc* gene. Crossbreeding of Eμ v-*abl* mice with Eμ-*c-myc* mice results in rapid development of oligoclonal plasmacytomas in the bitransgenic progeny (Rosenbaum *et al.*, 1990).

Translocation of the *cyclin* D1 (*bcl* 1) gene to an immunoglobulin locus is associated with some human lymphoid neoplasias. Cyclins and cyclin dependent kinases regulate the progression of the cell cycle. Eμ *cyclin* D1 transgenic mice do not develop lymphoid neoplasia. Only subtle alterations in the cycling behavior of B-cell populations are detected in the bone marrow of transgenic mice compared to normal. However, cross breeding of Eμ *cyclin*-D1 mice with Eμ *N-myc* or Eμ *L-myc* transgenic mice demonstrates a high level of co-operation between cyclin D1 and Myc in tumourigenesis (Lovec *et al.*, 1994).

Mutations of the tumour suppressor gene p53 are common in a wide range of human cancers. In normal cells DNA damage and other cellular stresses activate the transcription factor p53 resulting in growth arrest or apoptosis. Loss of p53 allows cells with unstable genomes (due to DNA damage) or in an abnormal

environment (ie located in a place with limited survival factors) to evade p53 induced apoptosis (reveiwed in Hall and Lane, 1997, Levine 1997). Homologous recombination has been used to generate mice that lack one or both copies of endogenous p53. Nullizygous mice all succumb to tumours of various types by the age of 10months. Heterozygous mice have a 50% incidence of cancer by the age of 18months; the cancers are predominantly lymphomas and sarcomas (Donehower *et al.*, 1993). Mutations of p53 have been observed in a number of BL derived cell lines of both endemic and sporadic origin (Farrell *et al.*, 1991). p53 loss synergises with *c-myc* in T-cell tumourigenesis in CD2-*myc* transgenic mice (Blyth *et al.*, 1995). Mutation or loss of p53 could therefore have a role to play in EBNA-1 induced lymphomas.

Other EBV antigens could interact with EBNA-1 in tumourigenesis. Both LMP1 and EBNA2 have been demonstrated to have oncogenic properties (reviewed in introduction section 1.1). Although EBNA-1 is the only EBV latent antigen whose expression is known to be consistently maintained in BL, other viral proteins may play a role in the initiation of tumourigenesis.

It is also concievable that the oncogenic action of EBNA-1 requires interaction with some completely novel proto-oncogene or tumour suppressor gene.

1.9 Complementation Groups of Synergizing Oncogenes in lymphomagenesis

Combination of the results of a number of studies of oncogene synergy in lymphomagenesis suggests that there are three complementation groups of oncogenes involved in the initial stages of tumour development (Wilson, 1997) (figure 1.7). One group contains the *myc* family members, at least one major function of which in oncogenesis is to drive cells towards proliferation. The second group contains the pim family genes, the exact function of which is not yet clear. The third group contains a number of genes which act to inhibit apoptosis, cell differentiation or senesence. For example, in MuMLV induced tumours in Eμ-pim-1 transgenic mice deregulated *c-myc* and gfi-1 expression may be concurrent in one tumour. However, *c-myc* and *N-myc* genes are never found to be co-activated in the same tumour cell clone and therefore these two genes constitute a complementation group. Similarly, simultaneous activation of gfi-1 and bmi-1 is rare (Berns *et al.*, 1994, Schmidt *et al.*, 1996, Scheijen *et al.*, 1997).

MoMLV falls into the third group because recent evidence suggests that the virus can promote cell survival through some other mechanism in addition to proto-oncogene insertional activation (Pak and Faller, 1996). In addition viral infection does not show an appreciable tumour acceleration in either Eμ blc2 mice or p53 null mice (Acton *et al.*, 1992, Baxter *et al.*, 1996). The placing of genes within a particular group does not necessarily represent functional redundancy of the members of that group (although this may be true of myc family members). Rather the genes within one grouping act in parallel pathways or at different

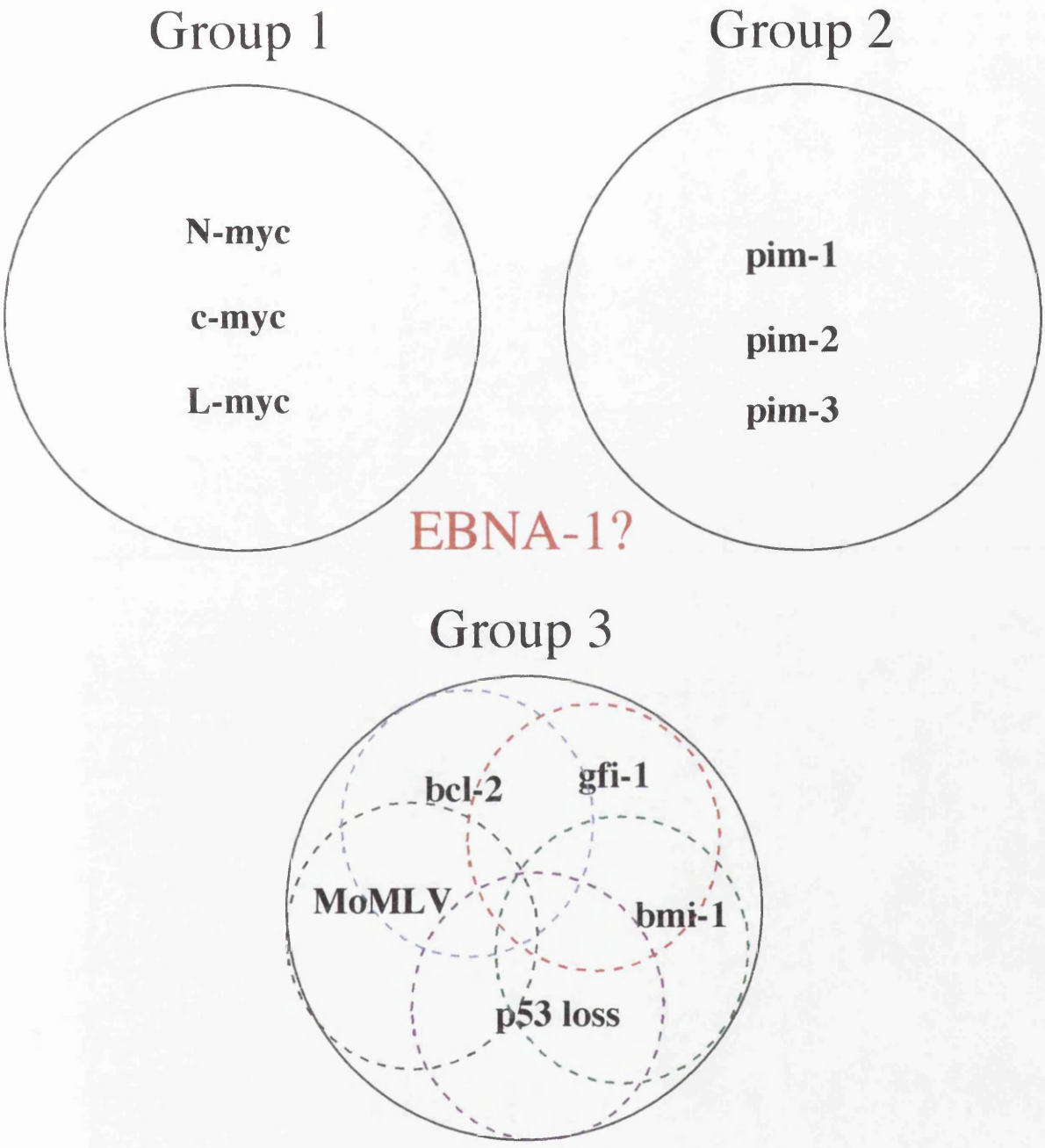


Figure 1.7
Complementation groups in lymphomagenesis. Several oncogenes, p53 loss and MoMLV have been grouped according to their ability to synergise in lymphomagenesis *in vivo* (reviewed in introduction section 1.6, 1.8-9). For example *myc* family members can synergise with *pim*-1 or *bcl*-2, but p53 loss and *bcl*-2 do not synergise. Not all combinations have been analysed. This work (chapter 6) was designed to address which complementation group, if any of these, EBNA-1 falls into. (Adapted fom Wilson, 1997).

points on the same signal transduction pathway. For example, some evidence suggests that *p53* and *bcl-2* serve as an effector and repressor function respectively of a common cell death pathway (Marin *et al.*, 1994).

The mechanisms of co-operation between different groups are beginning to become apparent. Myc family members induce cellular proliferation, but in a non permissive environment can also induce apoptosis. *Bcl-2* inhibits apoptosis, thus the inappropriate expression of both of these oncogenes could give rise to a cell which does not die in response to normal signals and is driven to proliferate. Both *in vivo* and *in vitro* experimentation have demonstrated the co-operation between *c-myc* and *bcl-2* in cell transformation (Strasser *et al.*, 1990, Faniidi *et al.*, 1992, Wagner *et al.*, 1993). However the combination of these two oncogenes is still not sufficient for tumourigenesis as the tumours which develop are mono or oligoclonal.

Analysis of the co-operation of EBNA-1 with other oncogenes may shed light on the mechanism by which it acts in tumourigenesis.

1.10 Aims of this research

This research project has involved the analysis of Eμ EBNA-1 transgenic mice to elucidate how EBNA-1 acts as an oncogene.

Approaches:

Chapter 3 ***Expression of EBNA-1 in Eμ EBNA1 Transgenic Mice***

A number of techniques have been used to analyse the expression of the Eμ EBNA-1 transgene at the RNA level. The aim was to assess whether the regulation of transgene expression is a contributing factor in the different latency and penetrance of the transgenic phenotype between line 26 and line 59.

Chapter 4 ***Cell Culture from Eμ EBNA-1 Induced Lymphomas***

Attempts have been made to generate cell lines from tumours in Eμ EBNA-1 transgenic mice. This approach allows a more in depth analysis of the nature of the tumours. Through the down regulation of EBNA-1 expression in cell lines *in vitro* it may be possible to assess whether continuous EBNA-1 expression is required for the maintenance of tumour phenotype. Analysis of genetic changes in cell lines *in vitro* (for example through karyotyping) allows investigation of potential secondary factors in tumourigenesis.

Chapter 5 ***Protein DNA Binding Activity of EBNA-1 and LR1 in Eμ EBNA-1 Transgenic Mice***

EBNA-1 is a pleiotropic protein and it is not clear what functions of the protein are oncogenic. DNA binding is required for the action of EBNA-1 in both DNA replication and transcriptional transactivation. Given this, an investigation of the DNA binding activity detectable in line 26 and line 59 was undertaken to address whether the differences in phenotype between the lines reflected different protein DNA binding activities.

LR1 was identified as a potential collaborator with EBNA-1 in tumourigenesis through its ability to contribute to *c-myc* deregulation and its implied role in *c-myc* translocations. However, at the time of this study the identity of the genes encoding LR1 were not known. To address whether LR1 plays a role in EBNA-1 induced tumourigenesis, it was attempted to compare the DNA binding activities of LR1 protein in line 26 and line 59 samples.

Chapter 6 *Co-operating Cellular Factors in Eμ EBNA-1 Induced Lymphomas*

Since EBNA-1 expression is insufficient to induce tumourigenesis in transgenic mice, a number of approaches have been used to identify synergising oncogenes. Particular emphasis has been placed on the role of Myc family members. Experimental approaches include: analysis of RNA expression patterns, screening for genomic rearrangements, plasmacytoma induction using pristane and transgenic cross breeding.

Chapter 2

Materials and Methods

2.1 Oligonucleotides

The oligonucleotides listed in table 2.1 were synthesised on an Applied Biosystems PCR Mate (departmental service). Oligonucleotides were removed from the column matrix and deprotected using the following protocol. The column was opened and the glass bead packing material poured into a Nunc 2ml tube. 1ml of 30% aqueous ammonia was added and the tube incubated at room temperature for 1 hour. The tube was then vortexed, briefly centrifuged and the supernatant removed to a fresh Nunc tube. A further 1ml of 30% aqueous ammonia was added and the tube incubated at 50^0C overnight. Ammonia stocks were stored at -20^0C. As required aliquots of oligonucleotides were precipitated with one fifth volume of 10M ammonium acetate and 2 volumes of absolute ethanol. The precipitate was washed with 70% ethanol and resuspended in distilled H_20 or TE as required.

The oligonucleotides listed in table 2.2 were ordered from commercial suppliers as shown.

EBNA-1 sequence nucleotide numbers indicated are with reference to the sequence of the BamHI K fragment of Epstein Barr virus, which has a total size of 5055bps. The sequence nucleotide numbers for the immunoglobulin

Oligonucleotide	*Oligonucleotide sequence (5'-3')*	*Description*
EB1 A	CCCTTCCTCACCCTCATCTCC	EBNA-1 coding sequence nucleotides 2281-2301 (reference in text)
EB1 B	GCTCTCCTGGCTAGGAGTCACG	EBNA-1 coding sequence nucleotides 1843-1864 (reference in text)
EB1 C	CCCCTCGTCAGACATGATTC	EBNA-1 coding sequence nucleotides 380-400 (antisense) (referencein text)
EB1 D	GAATCATGTCTGACGAGGGG	EBNA-1 coding sequence nucleotides 380-400 (sense) (reference in text)
EB1 E	CTTAAATTCACCTAAGAATG	EBNA-1 3' sequence nucleotides 2581-2600 (referencein text)
Py ORI F	CTTTTTCTCCAGAGTAAGC	Polyoma origin sequence nucleotides 16-34 (referencein text)
Myc2 5'A	ATGCCCCTCAACGTGAACTTC	Murine c-myc exon 2 5' primer 1 for SSCP (Bhatia *et al.*, 1993)
Myc2 5'B	TTCCCTTGGGGAGAAGGACGT	Murine c-myc exon 2 5' primer 2 for SSCP (Bhatia *et al.*, 1993)
Myc2 3'C	AGTGAGGATATCTGGAAG	Murine c-myc exon 2 3' primer 1 for SSCP (Bhatia *et al.*, 1993)
Myc2 3'D	GGTCTCGTCGTCAGGATC	Murine c-myc exon 2 3' primer 2 for SSCP (Bhatia *et al.*, 1993)
Myc3 5'E	GAAATTGATGTGGTGTCT	Murine c-myc exon 3 5' primer 1 for SSCP (Bhatia *et al.*, 1993)
Myc3 5'F	CTGCTTCAGGACCCTGCC	Murine c-myc exon 3 5' primer 2 for SSCP (Bhatia *et al.*, 1993)

Table 2.1

heavy chain enhancer are as described (Banerji *et al.*, 1983). Polyoma sequence nucleotide numbers are as described for the polyoma origin region (Tooze, 1981).

Oligonucleotide	*Oligonucleotide sequence (5'-3')*	*Description/supplier*
EB1 F	CCCCTCGTCAGACATG	EBNA-1 coding sequence nucleotides 384-400 (antisense) (referencein text) **Cruachem**
EB1 G	CATGTCTGACGAGGGG	EBNA-1 coding sequence nucleotides 384-400 (sense) (referencein text) **Cruachem**
EB1 H	TTCCAGGTCCTGTACC	EBNA-1 coding sequence nucleotides 404-418 (antisense) (referencein text) **Cruachem**
EB1 L	GGTACAGGACCTGGAA	EBNA-1 coding sequence nucleotides 404-418 (sense) (referencein text) **Cruachem**
EB1 I	GTTCCACCGTGGGTCC	EBNA-1 coding sequence nucleotides 634-650 (referencein text) **Cruachem**
EB1 J	CTTTGCAGCCAATGCAACTTG	EBNA-1 coding sequence nucleotides 614-634 (referencein text) **Cruachem**
EB1 K	GGGGTCTCCGGACACCATC	EBNA-1 coding sequence nucleotides 586-604 (referencein text) **Cruachem**
Anchor primer	CUACUACUACUAGGCCACGCGTCG ACTAGTACGGGIIGGGIIGGGIIG	5' RACE anchor primer **Gibco BRL**
UAP	CUACUACUACUAGGCCACGCGTCG ACTAGTAC	Universal Amplification Primer **Gibco BRL**
Myc 1	GAGCTGCCTTCTTAGGTCG	
IgH 1	CCAATAATCATAGAGCTC	IgH enhancer 4398-4415 (reference in text)
EB1bindA	GATCTAGGATAGCATATGCTACCC CGGGG	EBNA-1 DNA binding site sequence (Polvino-Bodnar and Scaffer, 1992) **Cruachem**
EB1bindB	GATCCCCCGGGGTAGCATATGCTA TCCTA	EBNA-1 DNA binding site sequence (Polvino-Bodnar and Scaffer, 1992) **Cruachem**
LR1bindA	GATCCTCCTGGGTCAAGGCTGAAT AGACGC	LR1 DNA binding site sequence (Williams and Maizels, 1991) **Cruachem**
LR1bindB	GATCTCCCCTGCGTCTATTCAGCC TTGACCC	LR1 DNA binding site sequence (Williams and Maizels, 1991) **Cruachem**
Sp1bind	-	Duplex oligonucleotide containing Sp1 consensus DNA binding site **Promega**

Table 2.2

2.2 DNA Probe Fragments

Table 2.3 lists the DNA fragments routinely used to make labelled probes in the following work. Enzymatic digestion of plasmid DNA and subsequent purification of probe fragments was carried out according to standard protocols listed later in this chapter. The EB13' probe was derived from the plasmid pEμ-EBNA-1 as described (Wilson and Levine, 1992). The EB15' probe was derived from plasmid pPy5'EB-1 as described (Wilson, et al., 1996).

Probe Fragment Name	*Description*
EB1 3'	Contains 3' region of EBNA-1 transgene (1kb) including EBNA-1 coding sequence (1645-2308) (referencein text)
EB1 5'	Contains Polyoma promoter sequences from EBNA-1 transgene (1-190) and 5' EBNA-1 coding sequence (365-554) (referencein text)
IgH1	XbaI- EcoRI fragment (682bps) from the murine immunoglobulin heavy chain enhancer
IgH2	BamH1- Xba1 fragment (1.3kb) from the murine immunoglobulin heavy chain enhancer
mmyc1	PstI fragment (420bps) from murine c-myc exon 2
mmyc2	XbaI fragment (3.1kb) containing murine genomic c-myc exon 2, intron 2 and exon 3
mbcl	HindIII-EcoRI fragment (865bps) containing murine bcl2 cDNA
mpim	BamH1 fragment (1kb) containing murine genomic pim1 exon 5, intron 5 and exon 6
hbcl	Sac1 fragment (550bps) containing human genomic bcl2 exon 1 3' region
mp53	murine p53 cDNA
mcycD1	murine cyclin D1 cDNA

Table 2.3

2.3 Protein Reagants

DNA restriction and modification enzymes and RNA modification enzymes

were obtained from a number of suppliers; BRL, Boehringer Mannheim, Promega, Pharmacia, Ambion.

Foetal calf serum was supplied by Sigma.

Anti EBNA-1 antibodies; monoclonal Aza2E8 was used as a direct tissue culture supernatant prepared by Joanna Wilson. The cells were grown as described (Hearing *et al.*, 1985). When the cells were confluent, supernatants were collected. The cells were spun down and the supernatant stored at -70^{0}C in aliquots until use then at 4^{0}C once defrosted. Anti EBNA-1 reactive human sera AM and WS were supplied by human donors A.M and W.S and stored at 4^{0}C. Different dilutions of these antisera were used according to the application.

All secondary antibodies used in cell staining experiments were supplied by Sigma.

2.4 Chemicals

CHEMICAL	*SOURCE*
General chemicals, biochemicals and organic solvents	BDH, May and Baker, Sigma
Media	Difco, Oxoid
Agarose	BRL
X-gal, IPTG	BRL
Radiochemicals	NEN, ICN
10x Restriction enzyme buffers	BRL, Boehringer Mannheim, Promega
Nucleotides	Boehringer Mannheim
Antibiotics	Sigma

Table 2.4

2.5 Bacterial Culture Media

L Broth: 10g tryptone, 5g yeast extract and 5g NaCl made up to 1litre in distilled water and adjusted to pH 7.5 with NaOH.

L Agar: as L Broth with the addition of 15g per litre of agar.

2.6 Sterilisation (Departmental service)

All growth media were sterilised at 120^{0}C for 15 minutes. Supplements and buffer solutions at 108^{0}C for 10 minutes and $CaCl_2$ at 114^{0}C for 10 minutes. Solutions for use in RNA work were autoclaved for at least 30 minutes.

2.7 Bacterial Growth Conditions

Liquid cultures for transformation, or DNA preparations were routinely grown in L broth at 37^{0}C with vigorous shaking. For plates L agar was used with antibiotic supplements as required. Plates were generally incubated overnight at 37^{0}C. Bacterial strains were stored in 50% L broth, 20% glycerol and 1% peptone at -70^{0}C.

2.8 Indicators

X-gal (5-bromo-4-chloro-3-indolyl-B-D-galactoside) was used in conjuction with TA cloning kit (InVitrogen) according to the manufacturer's instructions. Use of this indicator acts as a screen for plasmids with inserts in the polylinker region. Clones containing inserts were generally white (due to the disruption of the β-galactosidase gene), clones lacking inserts were blue. X-gal (40μg/ml in DMF) was stored at -20^{0}C.

2.9 Buffer Solutions

Stock Solution	*Components*
10X TBE	109g Tris base, 55g Boric Acid, 9.3g Na2EDTA.2H2O, made up to 1litre in distilled water
50X TAE	For 1L; 242g Tris base, 57.1ml glacial acetic acid, 100ml 0.5M EDTA pH8, made up to 1L with distilled water
10X gel loading dye (agarose gels)	20% ficoll, 0.25% bromophenol blue, xylene cyanol 0.25%, Tris HCl pH7.5 10mM, EDTA 1mM
10X restriction enzyme buffers	various as supplied by BRL, Boehringer Mannheim, Promega
TE buffer	10mM Tris/HCl, 1mM EDTA, pH 8.0
TE / 0.1%SDS	10mM Tris/HCl, 1mM EDTA, 0.1% SDS
20X SSC	3M NaCl, 0.3M trisodium citrate
0.5M EDTA pH8	EDTA plus distilled water titrated to pH8 with 10M NaOH
Church Buffer	28g SDS, 4g BSA, 0.8ml 0.5M EDTA pH8, made up to 300mls with distilled water, then add 100mls 1M Na_2HPO_4
'2FC' (buffered phenol chloroform)	50mls of phenol and 50mls of chloroform are mixed, then 1ml of isoamyl alcohol and 0.1g of hydroxyquinolene are added. The mixture is saturated and buffered with 1M Tris pH8.
Buffered phenol	Water saturated buffered phenol pH7.5 was supplied by Fisons
Tail Solution	50mM Tris pH8, 100mM EDTA pH8, 100mM NaCl, 1% SDS
STE buffer	100mM NaCl, 20mM Tris HCl pH7.5, 10mM EDTA
RNA loading buffer	12.5µl deionized formamide, 2.5µl 10 x MOPs buffer, 4µl 37% formaldehyde solution
RNA loading dye	50% glycerol, 0.1% bromophenol blue, 0.1% xylene cyanol, 50µM EDTA
10% Buffered Neutral Formalin	For 2.5L; sodium dihydrogen phosphate(anhydrous) 8.75g, disodium hydrogen phopsate(anhydrous) 16.25g, formaldehyde 250ml, made up to 2.5L with distilled water

Table 2.5

2.10 Plasmid DNA isolation

Medium Scale DNA Preparation 1 (basically as Birboim et al., 1979)

Solutions:

I. 50mM glucose, 25mM Tris/HCl pH8.0, 10mM EDTA.

II. 0.2M NaOH, 1% SDS (made fresh)

III. 5M Potassium acetate pH4.8; mix equal volumes of 3M CH_3COOK and 2M CH_3COOH

Method:

100ml cultures of stationary phase plasmid containing cells were harvested by centrifugation (12,400g, 10min at 4^0C). The pellet was resuspended in 4ml of solution I. 8ml of solution II was added and the mixture left on ice for 5 minutes. 6ml of solution III was then added, gently mixed and the cell debris and chromosomal DNA removed by centrifugation (39,200g, 30min at 4^0C). The plasmid DNA was precipitated from the supernatant with 12ml isopropanol for 15minutes at room temperature. The DNA was pelleted by centrifugation (27,200g, 15min at 20^0C), rinsed with 70% ethanol and then further purified by banding on a CsCl/EtBr gradient. The DNA was resuspended in 2.1ml of TE buffer and added to 270µl of a 15mg/ml ethidium bromide solution. 5g of CsCl was dissolved in 3ml of water and added to the DNA/EtBr solution. The gradients were centrifuged in a Beckman Ti70.1 fixed angle rotor at 200 000g for 16 hours at 20^0C. Where two bands were visible; a lower supercoiled plasmid band and an upper nicked DNA band, the lower band was removed using a 1ml syringe. The ethidium bromide was removed by repeated butanol extractions and the DNA recovered by ethanol precipitation. The DNA was washed in 70% ethanol and resuspended in 500µl TE.

Medium Scale DNA Preparation 2.

This was carried out using Qiagen Medium Scale Preparation kits for plasmid DNA, according to the manufacturer's instructions using 20mls of stationary phase plasmid containing cells.

Small Scale DNA Preparation .

The Birnboim, Doly (1979) miniprep method was used for small scale preparation of plasmid DNA as follows.

1.5ml of a stationary culture was harvested by centrifugation in an Eppendorf tube (12,000g, 30sec) and resuspended in 100µl of solution I (as described for large scale preparation above). 200µl of solution II was added and mixed by gentle inversion of the eppendorf tube. Following a 5 minute incubation on ice, 150µl of solution III was added and mixed by vortexing at the lowest setting of a Vortex Genie-2 (Scientific Instruments) for 10 seconds. Following a further 3 minutes on ice, the tube was centrifuged (12,100g, 5 min). The supernatant was collected and extracted against 400µl of 1:1 phenol: chloroform. Following centrifugation (12,000g, 5min) the supernatant was added to 2 volumes of ethanol. After a 5 minute incubation at room temperature and centrifugation (12,100g, 5min) the supernatant was carefully removed and the pellet washed with 70% ethanol, air dried and resuspended in 50ml TE buffer. 1µl of a 1mg/ml RNase A solution was added.

2.11 Isolation of Chromosomal DNA from Mouse Tissues

Preparation of DNA From Mouse Tail Samples

This was carried out basically as described in Wilson *et al.* 1990. Tissue samples were either taken fresh or stored at -70^{0}C prior to DNA preparation. 700µl of Tail solution (table 2.5) was added to the tail sample plus 35µl of a

10mg/ml solution of proteinase K. Digestion of the tissues was allowed to progress overnight at 55^{0}C. It is possible to store the samples at 4^{0}C following complete digestion with proteinase K for DNA purification at a later time point. To purify the DNA, the digested tail sample was subjected to sequential phenol, phenol/chloroform extractions. 700µl of phenol was added to the sample and mixed by hand. The sample was then centrifuged at approximately 9000rpm in an Eppendorf benchtop centrifuge for 15 minutes. The upper phase was removed to a fresh tube, 700µl of 2FC added, mixed and the centrifugation repeated. This step was then repeated. After a final centrifugation the upper aqueous phase was removed to a fresh tube and 1 volume of ice cold absolute ethanol and 100µl of 10M ammonim acetate added. Following gentle mixing by hand the sample was centrifuged briefly (2mins, 8000g 2mins). The supernatant was removed and the pellet washed with 70% ethanol, then air dried briefly and resuspended in TE. The majority of tail DNA samples were diluted to a final concentration of $^1/_3$ to $^1/_2$ µg per ml.

Preparation of Mouse Chromosomal DNA From Other Mouse Tissues

Basically the same protocol as above was used to prepare DNA from spleen, liver, lymph node and other tissues. Where necessary the tissue samples were physically mashed or cut to aid the initial digestion with proteinase K. Where the tissue sample was large, preparation solution volumes were increased accordingly.

2.12 Transformation of E.coli with plasmid DNA

This was carried out as described (Current Protocols). An overnight culture of the recipient strain (usually DH5) was diluted 1 in 100 into 20ml L broth and grown to a density of $2x10^8$ cells/ml (about 90mins).

The cells were harvested (21,100g, 1min, 4^0C) and resuspended in 10ml of 50mM $CaCl_2$. The cells were pelleted again, resuspended in 0.5ml ice cold 50mM $CaCl_2$ and kept on ice until use. 100µl aliquots of competent cells were added to DNA in TE buffer and, after gentle mixing left on ice for 15 minutes. 400µl of pre warmed L broth was added to the cell suspension and incubated at 37^0C for 90 minutes to allow expression of plasmid genes. 100µl aliquots of the transformation mixture were spread on to selective plates.

2.13 Restriction of DNA

For restriction of plasmid DNA 4-5 units of enzyme were used per µg of DNA. The appropriate restriction buffer was included and the reactions allowed to proceed for 1-3 hours at 37^0C (or the appropriate temperature according to the manufacturer's instructions).

The majority of mouse chromosomal DNA digests included 5µg of DNA in a total reaction volume of 20µl. An initial overnight incubation at 37^0C with 4-5 units of enzyme per µg DNA was followed by the addition of an extra 1-2 units of enzyme and a further 1-2 hour incubation at 37^0C.

The enzymes were inactivated by the addition of DNA loading buffer and heating to 70^0C. Alternatively, where subsequent manipulation of the DNA

was to be undertaken phenol extraction followed by ethanol precipitation was used to purify the DNA from the enzyme(s).

2.14 Ethanol Precipitation of DNA

For precipitation of small DNA fragments; $^1/_{10}$ volume of 3MNaOAc and 2 volumes of absolute ethanol were added to the DNA solution. After mixing, the DNA was precipitated at -20^{0}C for at least 30 minutes and pelleted for 15 minutes at 4^{0}C. The pellet was washed with 70% ethanol and dried. Chromosomal DNA was precipitated with $^1/_5$ volume of 10M Ammonium Acetate and 1 volume of cold absolute ethanol. After mixing the pellet was spun down at room temperature for 2 minutes and washed with 70% ethanol.

2.15 Ligation of DNA Fragments

Restriction fragments were ligated in reaction volumes of 10-20µl containing 1x ligation buffer and 0.5 units of T4 DNA ligase. Generally a 3:1 insert to vector ratio of fragments was used (10:1 for blunt end ligations). The reactions were incubated at 16^{0}C overnight. Aliquots of the ligation mix were used to transform competent cells.

2.16 Gel Electrophoresis of DNA

Agarose gels

0.8%-2% agarose gels were used. Agarose powder was dissolved in 0.5-1x TAE at 100^{0}C and then cooled to 55^{0}C before use. For some applications 1µl of 10mg/ml ethidium bromide was added to the gel prior to use. Otherwise the

gels were bathed in a 0.6mg/ml ethidium bromide solution after electrophoresis and then rinsed in water.

IBI gel kits were used. Running buffer was TAE at the same concentration as in the gel.

Visualisation of DNA was on a 254nm wavelength UV transilluminator except where the DNA was to be isolated from the gel, then a 365nm wavelength transilluminator was used.

Polyacrylamide Gels

- **1. Sequencing Gels (and gels for RNase Protection Assays)**

The gel kit used was made by IBI. A pre-made sequencing gel solution (6% polyacrylamide/8M urea) was used to pour the gels according to the manufacturer's instructions (Scotlab, Anachem). Gels were allowed to set for at least one hour and often left overnight. Gels were run in 1X TBE. Gels were pre-run for at least 45 minutes and run for 2-6 hours at 65W. After electrophoresis gels were either fixed in 10% acetic acid / 10% methanol for 15 minutes and then dried, or dried directly. Gels were dried onto Whatman 3MM chromatography paper under vacuum at 80^0C and then autoradiographed.

- **2. Non-denaturing gels for single stranded conformational analysis**

Various different conditions were used for gels for SSCP analysis (section 2.24). Gels were run on IBI sequencing gel apparatus. All the gels contained TBE at 0.5-1X and TBE was the running buffer. Both 30:1 and 19:1 acrylamide to bis-acrylamide ratios were used to optimise resolution of DNA

strands. In addition for some gels Hydrolink-MDE acrylamide solution was used (Hoeffer).

Gels were not pre run. Gels were run very slowly overnight (12-14hrs) either at room temperature with a fan to cool the gel, or at 4^0C. Gels were dried under vacuum at 80^0C and then autoradiographed.

- **3. Non-denaturing polyacrylamide gels**

These gels were used to separate protein-DNA complexes. Various concentrations of acrylamide were used according to the experimental requirements (section 2.31). All gels were made and run in 1X TBE buffer. Gel mixes were made according to a basic recipe (table 2.6).

Component	*Volume*
30% acrylamide / 0.8% bisacrylamide (w/v) solution (Biorad)	xml
10xTBE	5ml
10% APS (w/v)	200µl
TEMED	20µl
Made up to final volume of 50mls with distilled water	xmls

Table 2.6

A Biorad vertical gel electrophoresis apparatus was used. All gels were allowed to set for approximately 45 minutes and then pre-run for at least 45 minutes. Gels were run for variable lengths of time at room temperature at approximately 200V depending on the application.

2.17 Isolation of DNA Fragments after gel Electrophoresis

Isolation of DNA from Agarose gels

DNA fragments were cut from ethidium bromide stained gels and purified by one of two methods.

a. Spin X tubes (Costar) were used as recommended by the manufacturer. The Spin X tube contains a 0.22μC cellulose acetate filter. On centrifugation the DNA in solution flows through the filter into the collection tube, the agarose particles remain above the filter.

b.Qiaex II gel extraction kit (Qiagen) was used according to the manufacturer's instructions. The agarose is solubilised using a high concentration of a chaotropic salt which disrupts the hydrogen bonds between the sugars in the agarose. 40bp-50kb DNA fragments are adsorbed to Qiaex II silica particles in the presence of high salt. Impurities are removed by washing. The pure DNA is eluted from the matrix in water or TE (low salt/basic conditions).

Isolation of DNA from Low Melting Point Agarose gels

The band containing the DNA of interest was cut directly from ethidium bromide stained gels, mashed and TE or distilled water added to dilute the agarose. Prior to use the DNA was heated to 70^0C to melt the agarose and mixed thoroughly.

Isolation of DNA from Polyacrylamide gels

The ^{32}P labelled DNA of interest was identified using autoradiography and cut from the gel. The gel fragment was mashed and 0.5ml of elution buffer (0.5M ammonium acetate/ 1mM EDTA/ 10mM TRIS pH8) added. After an overnight incubation at 65^0C the DNA was ethanol precipitated and washed.

2.18 Southern Blotting of DNA

The method used was as described in the handbook for Pall Biodyne Membrane. Briefly, after electrophoresis gels were denatured in a solution of 1M sodium hydroxide/1.5M sodium citrate for 30 minutes. Capillary blotting was used to transfer the DNA from the gel to the membrane. 20 x SSC was used as the transfer solution. Transfer was allowed to occur overnight. Where the DNA fragments to be transferred were of large size an acid hydrolysis step (15 minutes in 0.25M hydrochloric acid) was included prior to denaturation to partially break down the fragments. On occasion Hybond N (Amersham) membrane was used, it was then necessary to include a neutralisation step after denaturation (15 minutes in 0.5MTris pH 7.4/1.5M sodium chloride). After complete transfer the membrane was baked for 1 hour at 80^0C and UV crosslinked.

2.19 Slot Blot Analysis of DNA

A vacuum slot blot apparatus (Schleicher and Schuell Minifold II) was used for the direct application of DNA to nylon membranes. To 5μg of DNA in 120μl, 40μl of 1M sodium hydroxide was added and the mixture vigorously vortexed to denature the DNA. After 10 minutes at room temperature 160μl of 10 x TAE buffer was added. The samples were vortexed, briefly spun down (2mins Eppendorf bench centirfuge, 14 000rpm) and put on ice. The membrane was pre soaked in 1 x TAE buffer. The samples were loaded without the vacuum. Vacuum was applied once all of the samples had been loaded. When the samples were through, a drop of 5 x TAE buffer was added

to each well. After complete transfer the membrane was baked for 1 hour at 80^0C and UV crosslinked.

2.20 Polymerase Chain Reaction (PCR)

The exact conditions used for PCR reactions varied depending on the application. Reaction cycles followed the format:

Cycle Description	*Number of Cycles*
Denaturation; 5minute, 95^0C	1
Denaturation; 1minute, 95^0C Anneal; 1minute, various Extension; 1-3minutes, 72^0C	30-35
Extension; 10minutes, 72^0C	1

Table 2.7

Reactions were carried out in volumes of 30-50µl. Exact ingredients varied with the application, but the basic reaction mix was as follows:

Reaction Component	*Concentration*
DNA (in H20 or TE)	various, approximately 0.1-0.5µg
10x reaction buffer (BRL or Promega)	$^1/_{10}$ total reaction volume
Magnesium Chloride solution	various, usually 2.5mM
dNTP mix(ATP, CTP,GTP,TTP)	various, usually 0.2mM
Primer oligonucleotide 1	various, usually 0.2µM
Primer oligonucleotide 2	various, usually 0.2µM
Taq DNA polymerase (BRL or Promega)	various, usually 0.4 units per reaction

Table 2.8

Thermal cyclers used were a Perkin Elmer Thermal Cycler and a Crocodile II (Appligene).

2.21 Cloning of DNA fragments

PCR products were cloned using the InVitrogen TA cloning system, basically according to the manufacturers instructions. This system utilises the overhanging thymidine nucleotide added to the end of PCR products generated by Taq polymerase. DNA products were visualised on ethidium bromide stained agarose gels and cut out as individual bands. The DNA was purified from the agarose using Qiaex II gel extraction kit (Qiagen) according to the manufacturer's instructions. Purified DNA was used directly in ligation reactions to the T- vector (InVitrogen). Transformation of the ligated DNA into competent E.coli was as described (section 2.12). The vector contains a copy of the β galactosidase gene at the polylinker site, expression of which is disrupted by the presence of an insert. This allowed for selection of colonies containing inserts (section 2.8). Several of the colonies obtained were picked, grown up in 1.5ml L broth and DNA prepared from them using the miniprep method (section 2.10). Plasmid DNA was subjected to an EcoRI digest, this enzyme cuts on either side of the polylinker and releases the cloned insert, and analysed by electrophoresis. Where the insert was of the expected size, the plasmid was subjected to further analysis eg restriction mapping and sequencing.

2.22 Sequencing of Plasmid DNA.

Two different protocols were used for the sequencing of miniprepped DNA.

Taq Terminator Sequencing (on ABI373A) at the CRC Beatson Institute for Cancer Research

Reactions were carried out using 1μg of double stranded plasmid DNA with 3.2pmoles of primer. The reaction mix and enzyme were supplied by Promega. The PCR reaction cycle was as follows; (96^0C 15 secs, 50^0C 1 sec, 60^0C 4mins) x 25 cyles (Perkin Elmer 9600 Thermal Cycler). Reactions were then extracted twice with phenol/chloroform, precipitated, dried and resupended in loading buffer. Reactions were carried out and gels run with the assistance of Rob McFaralane at the Beatson Institute.

Sequenase Quick Denature Sequencing Kit (USB)

Sequencing reactions were carried out with 1μg of double stranded plasmid DNA and 0.5pmoles of primer according to the manufacturers instructions. Samples were heated to 80^0C for 2 minutes prior to loading on 6% sequencing gels.

2.23 Probing of Southern, Northern and Slot Blots with Labelled DNA fragments.

25ng of appropriate DNA fragments were labelled using ^{32}PdATP or ^{32}PdCTP and the PrimeIt II (Stratagene) DNA labelling kit according to the manufacturer's instructions. This kit utilises Klenow DNA polymerase lacking the 3' exonuclease. Polymerisation reactions are primed from denatured template to which random nonamer oligonucleotides are annealed. Labelling reactions were allowed to proceed for 10 minutes to 1 hour, the reaction stopped and then unincorporated nucleotides separated from the probe

fragment using a Stratagene Nuctrap Push Column according to the manufacturer's instructions.

Prior to the addition of the labelled probe the membrane underwent a pre-hybridisation step.

Both pre-hybridisation and hybridisation were carried out in plastic tubes in a rotatory hybridisation oven. For the pre-hybridisation the membrane was soaked in Church Buffer at 65^0C for at least 1hour. Hybridisation was carried out in Church Buffer (approximately 8-15mls depending on the size of the membrane) at 65-68^0C for 12-16 hours. The labelled DNA probe fragment was denatured at 95^0C for 5 minutes prior to addition to the pre-warmed Church Buffer. This mixture was then added to the membrane (after removal of the pre-hybridisation mixture). Where extremely stringent hybridisation conditions were required, up to 20% formamide was included in the Church Buffer.

After hybridisation, membranes were washed for 20-60 minutes at room temperature in 2 x SSC, 0.1% SDS and then for 20-60 minutes at 68^0C in 0.1 x SSC, 0.1% SDS (stringent conditions).

Washed membranes were sealed in plastic bags and visualised by autoradiography.

2 .24 Single Stranded Conformational Analysis of DNA

SSCP analysis of the c-myc gene in transgenic tumour DNA was carried out using the protocols of Bhatia *et al.*, 1993. A selection of different genomic DNAs (tumour and non-tumour) from both positive and negative transgenic

mice were screened for mutations in c-myc exon 2. Each set of PCR reactions was electrophoresed on a range of different gels; 6% polyacrylamide (19:1 acrylamide to bis-acrylamide ratio) at room temperature and 4^0C, 6% polyacrylamide (30:1 acrylamide to bis-acrylamide ratio) at room temperature and at 4^0C, or Hydrolink-MDE (Hoeffer) at room temperature. All gels were run for 12-15 hours.

2.25 Preparation of RNA from Mouse Tissues

This was carried out essentially according to the method of Chomcznski and Sacchi (1987). Total RNA was prepared from snap frozen tissues by acid guanidinium thiocyanate-phenol-chloroform extraction using RNAsolB (Biogenesis) according to the manufacturer's instructions. The tissues were lysed using a poltron directly in a solution which denatures proteins and degrades DNA. Subsequent phenol/chloroform extraction is used to remove proteins and the RNA is precipitated with isopropanol or absolute ethanol. RNA was stored at -70^0C in 1 volume TE/0.1% SDS, and $2^1/_2$ volumes absolute ethanol. Prior to use in any assay RNA was precipitated with $^1/_{10}$ volume of 3M sodium acetate and washed with 70% ethanol. For RNase Protection assays and 5'RACE PCR any trace DNA was removed from the RNA by two further precipitations with lithium chloride: the RNA was resuspended in TE, 0.1% SDS and lithium chloride was added to a final concentration of 2.5M. The sample was mixed and incubated at -20^0C for 30minutes and then spun down at 4^0C for 30minutes. The precipitated RNA was then washed with 70% ethanol.

2.26 Northern Blotting of RNA

For Northern blots 10 or 20μg aliquots of RNA were run on 1% agarose gels containing $^1/_5$ volume formaldehyde and 1xMOPS E buffer. RNA samples were prepared in a formamide loading buffer and denatured at 65^{0}C for 5minutes prior to loading. Gels were electrophoresed for 5 hours at 150V at 4^{0}C. The gels were stained in ethidium bromide solution and visualised with UV irradiation. Transfer of the RNA to Pall Biodyne membrane was carried out by capillary blotting as described in the manufacturer's instructions (essentially as for Southerns section 2.18).

Subsequent probing of northern blots was as described (section 2.23).

2.27 RNA Slot Blot

Loading Buffer;

Component	*Volume*
Formamide	1ml
Formaldehyde (37% solution)	324μl
10 x MOPs buffer	200μl

Table 2.9

20 μg aliquots of lithium chloride precipitated RNA were resuspended in 40μl of TE/0.1%SDS. 120μl of loading buffer was added (table 2.9) and the sample incubated at 65^{0}C for 5 minutes then placed on ice. 40μl of cold 20 x SSC was added and then the samples loaded onto the slot blot apparatus. 5μg of RNA was loaded on to each membrane (50μl of sample). Pre soaked (10 x SSC) Hybond-N membrane (Amersham) was used. After application of the samples the membranes were baked at 80^{0}C for 1 hour and UV crosslinked.

2.28 RNase Protection Analysis

DNA fragments for riboprobe preparation were prepared from DNA purified by caesium chloride banding (section 2.10). Each plasmid was cut with the relevant enzyme to give a defined end point to the transcribed riboprobe. Digestion was allowed to proceed for three hours then additional enzyme was added for a further hour to ensure that the reaction had gone to completion. Proteinase K was added to the sample for 30 minutes at 37^0C to remove any contaminating RNase. Phenol/ chloroform extraction followed by ethanol precipitation ensured that the DNA was clean (RNase free)for use as a template in synthesis of the riboprobe.

Riboprobes were synthesised using a Maxiscript RNA polymerase transcription kit (AMBION) according to the manufacturer's instructions. All probes were labelled with ^{32}P UTP.

Riboprobe	*Plasmid/Source*	*Digestion/RNA polymerase*
EBNA-1 5'	pPy5'EB-1	EcoRI/T3
Polyoma/IgH 1	pIgHPy	PvuII/T3
Polyoma/IgH 2	pIgHPy	XbaI/T3
GAPDH	Ambion	DdeI/T7

Table 2.10

The riboprobes used are illustrated in figure 2.1.

Unincorporated labelled UTP was purified from the riboprobe using Stratagene Nuctrap Push Columns. The riboprobe was treated with RNase free DNase (BRL) and purified by phenol/chloroform extraction and subsequent ethanol precipitation. The probe was washed in 70% ethanol and resuspended in RNase Protection hybridisation buffer (Ambion).

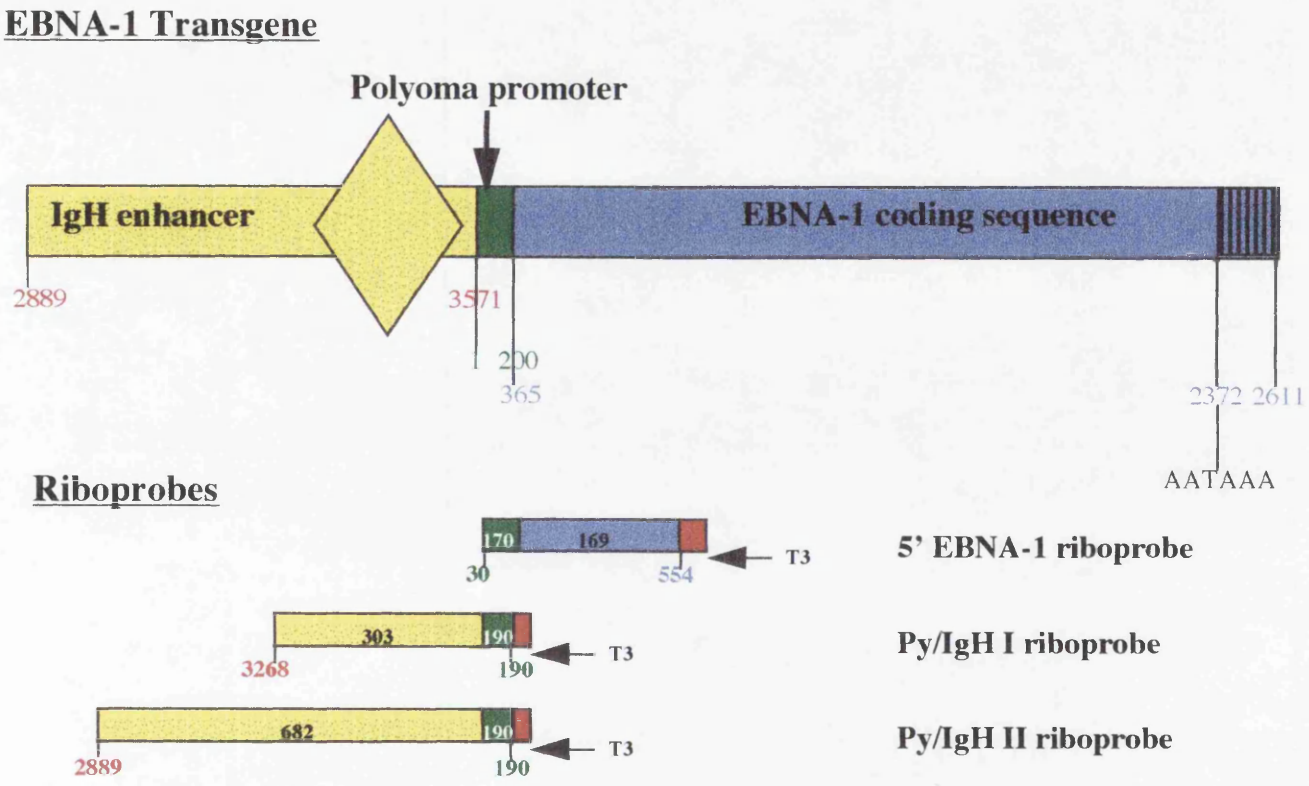


Figure 2.1
Cartoon illustrating riboprobes used in RNase protection assays. The numbers on boxes correspond to sizes in nucleotides. Yellow boxes represent IgH enhancer sequences, green boxes represent polyoma promoter sequences, blue boxes represent EBNA-1 sequences and red boxes represent plasmid vector sequences included in the riboprobe. Arrows labelled T3 indicate the direction of sythesis of the riboprobes. Coloured numbers correspond to transgene sequence positions: red IgH enhancer sequences (Banerji *et al.*,1983), green polyomavirus early promoter (Tooze,1981), blue EBVsequences (Baer *et al.*, 1984).

Hybridisation reactions and RNase digestion were carried out using reagents supplied with Ambion's RNase Protection Kit. The protocol was basically as described in the manufacturer's handbook as follows.

40-80μg samples of lithium chloride treated RNA were precipitated, washed and resuspended in hybridisation buffer containing $2x10^5$cpm of riboprobe per sample. Where a loading control was required, the GAPDH probe was added to the mixture in addition. In order to prevent the GAPDH signal from overwhelming the signal of interest, the riboprobe was sythesised with a greater concentration of unlabelled UTP(ratio of unlabelled to labelled UTP for GAPDH was 2:1, and for other probes was 3:5) such that the specific activity of the GAPDH probe was lower than that of the riboprobe of interest. After mixing the samples were heated to 95^0C and allowed to cool slowly to 65^0C. Hybridisation was allowed to proceed overnight at 65^0C. Samples were digested with RNaseA and RNaseT1 to remove single stranded RNA (Ambion, 1:50 dilution). After inactivation of the RNases and ethanol precipitation the samples were resuspended in 80% formamide loading buffer, denatured at 95^0C and separated on 6% polyacrylamide/urea gels. For probe alone samples $2x10^2$ cpm undigested probe was loaded. Protected fragments detected in RNase protection experiments were quanitified using phosphorimager analysis.

2.29 5'RACE PCR

5' RACE PCR reactions were carried out using a kit supplied by GIBCO BRL as depicted in figure 2.2.

All the RNA samples analysed with this technique had been twice precipitated with lithium chloride to remove any trace DNA. $^1/_3$ μg of each RNA was used in the initial cDNA synthesis. A gene specific primer (oligonucleotide EB1 I section 2.1, table 2.2) was used in all cDNA synthesis reactions. Following purification of the cDNA from the primer, the DNA was tailed with poly dC using terminal transferase. PCR amplification of the products was carried out using a second gene specific primer (oligonucleotide EB1 J, section 2.1, table 2.2) and the anchor primer (section 2.1, table 2.2) supplied with the kit. 50 μl reactions contained each primer at 0.2μM, dNTPs at 0.2mM, magnesium chloride at 1.5mM, 1x PCR reaction buffer(BRL) and 0.5 units per reaction of Taq DNA polymerase (BRL). Reactions denatured at 95^0C and then cooled to 80^0C prior to the addition of Taq polymerase. 30 cycles of amplification were performed with an annealing temperature of 60^0C and an extension time of 1 minute. 20μl aliquots of PCR reactions were electrophoresed in 2% agarose gels containing ethidium bromide. Gels were Southern blotted onto Pall Biodyne membrane (section 2.18) and probed with either probe EB1 5' containing 5' EBNA-1 and polyoma sequences, or probe IgH1 containing Eμ enhancer sequences.

For secondary amplification of PCR products a number of different protocols were used. In initial experiments, the primary PCR reaction was diluted 1:50 and 5μl used in a secondary PCR reaction using the same gene specific primer

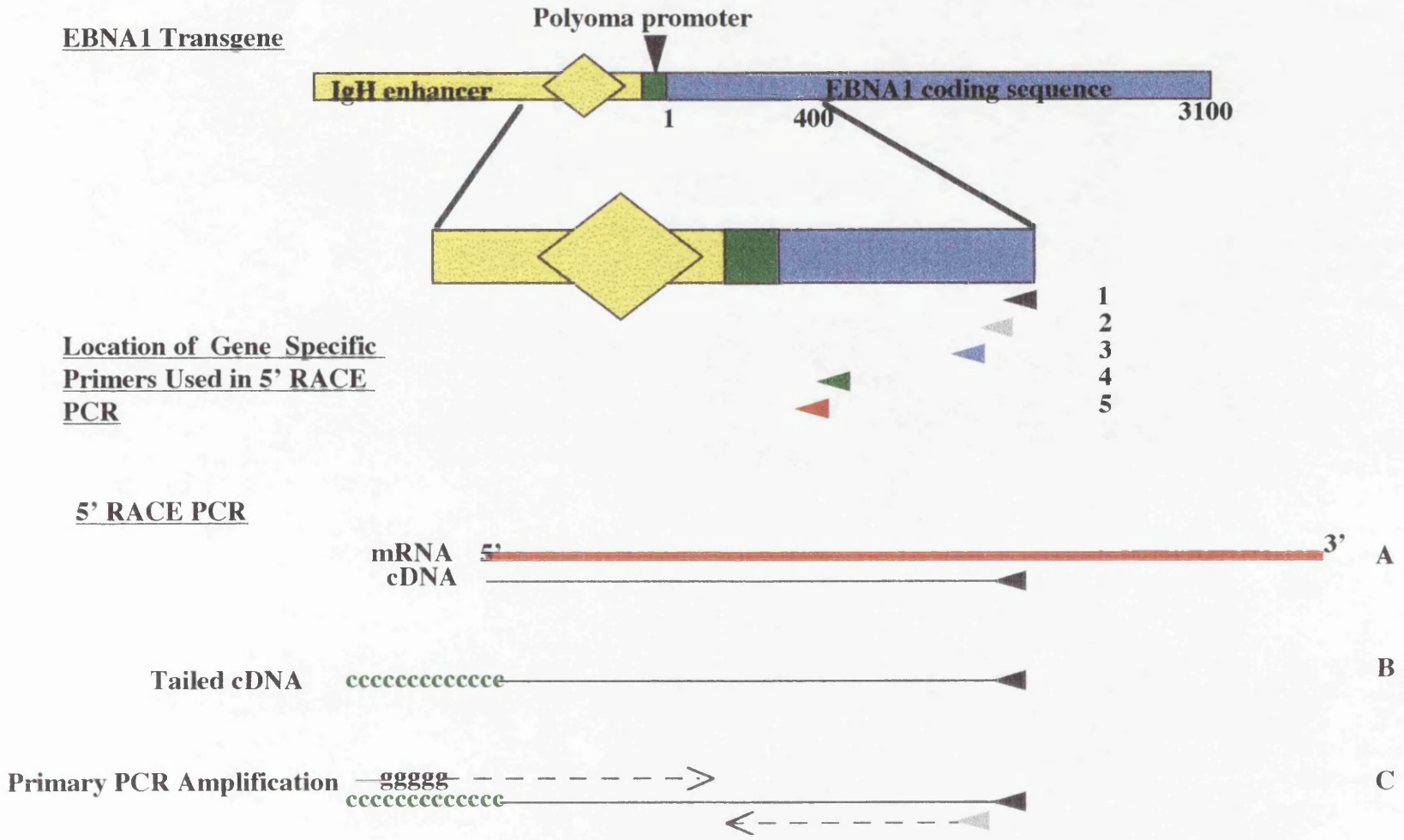


Figure 2.2

Cartoon of 5' Race Procedure. The numbered arrow heads represent the positions of the primers in the EBNA1 coding sequences. **1** is gene specific primer (EB1 I) used for cDNA synthesis. **2** is the gene specific primer used (EB1 J) in the primary PCR reaction. **3**, **4** and **5** are the gene specific primers (EB1 K, EB1 H, EB1 F) used in secondary amplification of RACE products.
The RACE procedure is diagrammed in three stages. **A** cDNA sythesis using gene specific primer on mRNA. **B** Tailing of cDNA with dC. **C** Primary PCR amplification of products with gene specific primer and anchor primer.

(oligonucleotide EB1 J section 2.1, table 2.2) as the primary reaction and the Universal Amplification Primer (section 2.1, table 2.2). The same reaction conditions as for the primary reaction were applied. Subsequently, the primary PCR reactions were amplified in a second round of PCR using one of two different nested gene specific primers, either oligonucleotide EB1 F (section 2.1, table 2.2). or oligonucleotide EB1 H (section 2.1, table 2.2) in order to increase the specificity of products obtained. Ultimately, because the yield of products was low from either of the above approaches rather than dilute the primary PCR reaction it was purified using a Qiagen PCR clean up kit to remove any free primers. 5µl ($^1/_{10}$) of the resultant products were used in a secondary amplification using a nested gene specific primer (oligonucleotide EB1 K, section 2.1, table 2.2) and the Universal Amplification Primer. 20µl aliquots of products from secondary amplification reactions were electrophoresed on 2% agarose gels and Southern blotted and probed as above. The 5' RACE procedure is diagrammed in figure 2.2.

It was attempted to clone and sequence 5' RACE products from line 26 and line 59 samples. After two rounds of PCR using gene specific primer EB1 J, products from two 50 µl reactions for each line were pooled, precipitated and resuspended in 20µl then separated by electrophoresis in a 2% agarose gel. Fragments were cut from the gel and labelled according to size. Subsequent purification of the DNA fragments, cloning and sequencing was as described (sections 2.17,2.21,2.22).

2 .30 Preparation of Nuclear Protein Extracts from Mouse Tissues

The protocol used to prepare protein extracts from mouse spleens was essentially as described below. Tissues were snap frozen in liquid nitrogen and then stored at -70^{0}C. Tissues were pulverised in liquid nitrogen in a mortar and pestle and then resuspended in a hypotonic lysis buffer (0.6% NP40, 150mM NaCl, 10mM HEPES pH7.9, 1mM EDTA, 0.5mM PMSF). The cell suspensions were subjected to 5 strokes in a Dounce homogeniser (pestle B). After removal of tissue debris by centrifugation (30 secs at 2000rpm in a 15ml tube), the supernatant was incubated on ice for 5 mins. The nuclei were pelleted by centrifugation (5mins at 5000rpm) and then resuspended in 100-500µl of a buffer supplemented with a protease inhibitor cocktail (20mM HEPES pH7.9, 0.42M NaCl, 1.2mM MgCl2, 0.2mM EDTA, 0.5mM DTT, 25% glycerol, 0.5mM PMSF and 5µg/ml each of aprotinin, leupeptin, pepstatin A and 2mM benzamidine). The resuspended nuclei were incubated on ice for 20 mins for high salt extraction. The lysed nuclei were transferred to a microcentrifuge tube and cellular debris pelleted by a 15 secs centrifugation. The supernatant containing the DNA binding proteins was aliquoted in 30ml fractions, frozen in liquid nitrogen and stored at -70^{0}C. Protein concentrations were calculated using a Biorad assay.

2.31 Gel Retardation Assays for Protein DNA Binding Activity

1. EBNA-1 Gel Retardation Assays

The protocol used was essentially as described (Polvino-Bodnar and Schaffer, 1992). Complementary oligonucleotides EB1bindA and EB1bindB (section 2.1, table 2.2) were mixed in equimolar amounts, heated to 95^{0}C to denature

completely and then allowed to cool slowly to room temperature to anneal. A short non-complementary 5' extension (GATC) was included in each oligonucleotide to allow for labelling. Duplex oligonucleotide was labelled with α^{32}P dATP by filling in with Klenow. Labelled oligonucleotide was separated from unincorporated ^{32}P dATP using a Stratagene Nuctrap Push Column. To ensure that only duplex probe was used, the probe was purified by electrophoresis on a polyacrylamide gel and excision (section 2.17).

For the comparison of the binding activity of various protein extracts a master reaction mix was made containing the labelled oligonucleotide (1pmole per reaction), the binding buffer (150mM NaCl, 20mM HEPES, 10% glycerol, 1mM Mg Cl_2, 1mM β ME) and poly dI dC (1μg per reaction, Boehringer Mannheim). 25μl of reaction mix was added to 2μg of each protein of interest in a total volume of 5μl. The binding reaction was incubated for 20 minutes at room temperature prior to loading onto a 4% non denaturing polyacrylamide gel. Gels were run for $2^1/_2$-3hours at 200V.

For the determination of specificity of binding a reaction master mix was made as above, however for this experiment the mix contained the protein of interest. Labelled probe and the appropriate competitor were mixed and then the reaction mix added to them and incubated for 20minutes, as above the total reaction volume was 30μl.

Two different types of supershift experiment were performed. In the first the binding reactions were set up exactly as for the comparison experiment described above. After the initial 20 minute incubation 1μl of the appropriate

antibody (monoclonal Aza2E8, polyclonal human serum AM, or polyclonal human serum WS) was added to the reaction and incubation at room temperature continued for a further 20 minutes.

In the second type of binding reaction the protein was not pre-incubated with the probe prior to antibody addition, instead antibody and protein were added simultaneously and the reaction incubated for 40 minutes at room temperature.

2. LR1 Gel Retardation Assays.

The reaction conditions used for these experiments were as described previously (Williams and Maziels, 1991). Complementary oligonucleotides LR1bindA and LR1bindB (section 2.1, table 2.2) were mixed in equimolar amounts, annealed to form duplex and labelled with α^{32}P dATP as described above (section2.31.1).

Both comparison of protein activity experiments and specificity experiments were set up as described above. However the binding buffer used differed in composition (8mM HEPES, 60mM Kcl, 0.1% NP40, 10% glycerol) and the reactions were separated on 6% polyacrylamide gels.

3. SP1 Gel retardation assays.

Duplex oligonucleotide for use as a binding site probe was supplied by Promega, table 2.2. This was labelled with γ^{32}P dATP using T4 polynucleotide kinase and the probe separated on a Stratagene Nuctrap Push Column.

As described (section 2.31.1) binding reactions were set up in a volume of 30µl. The SP1 binding buffer used was (4mM $MgCl_2$, 0.6mM EDTA, 15mM

HEPES, 10% glycerol). Reactions were incubated at room temperature for 20 minutes and then separated by electrophoresis on either 4 or 6% polyacrylamide gels at 200V for 2-3hours.

2 .32 Tissue Culture

Maintenance of lymphocyte cell cultures

All cell lines were maintained in lymphocyte medium (RPMI 1640 medium (BRL), supplemented with 10-20% foetal calf serum (FCS), 4mM glutamine, Penstrep (BRL), 10^{-5}M β Mercaptoethanol and 7μg/ml dextran sulphate).

With the exception of primary lymphocyte cell lines all other cell lines were obtained through the ECACC catalogue.

Derivation of primary cell lines from transgenic spleen and lymph nodes

Spleen and lymph node tissue samples from mice were mashed in a small quantity of lymphocyte medium to yield a cell suspension. This was briefly spun down and the pelleted cells resuspended to a final concentration of $>1x10^8$ cells per ml in lymphocyte medium containing 20% FCS.

Where aliquots of cells were frozen, the freezing medium contained up to 50% FCS and 10% DMSO (dimethyl sulphoxide). Frozen cells were maintained in liquid nitrogen.

In attempts to derive cell lines from transgenic tissues the concentrated cell suspensions were monitored every day and gradually diluted by the addition of fresh medium to a concentration of $1x10^6$ cells per ml. Cell viability was addressed using trypan blue staining (Sigma) as directed by the manufacturer.

This dye is excluded by viable cells, but stains dead cells. Cells were counted using a haemocytometer.

Monitoring the effect of oligonucleotide addition to cell lines.

A number of different sense and antisense EBNA-1 oligonucleotides (EB1 C, EB1 D in table 2.1, EB1 F, EB1 G, EB1 H, EB1 L in table 2.2) were added to cell lines. 2 $x10^5$ cells of each type were aliquoted at the start of the experiment. 30μg of the appropriate oligonucleotide was added to the cells on day 1. At each interval of 24 hours an additional 5μg of oligonucleotide was added. After 72 hours 40μg additional oligonucleotide was added. From day 1, aliquots of cells were taken at 24 hourly intervals, stained with trypan blue and a count made of both viable and dead cells. At least 8 counts were made for each sample.

2 .33 Maintenance of Transgenic Mouse Lines

All animal work was carried out under Home Office Licence.

The transgenic mouse colony was monitored at least twice weekly, according to Home office requirements, for evidence of tumours or ill health. Routinely, samples of spleen, liver, lymph node, thymus and any abnormal looking tissues were collected in buffered formalin solution (table 2.10) for pathological examination. All pathological analysis was carried out by Dr. Sarah Toth at Glasgow University Veterinary Pathology Department. In addition samples of tissues were routinely snap frozen and stored at -70^0C for subsequent preparation of DNA, RNA and protein. For tissue culture,

samples were collected and rinsed briefly with 70% ethanol then immersed in FCS and taken directly into culture.

Monitoring of transgene status and transgenic lines

Tail genomic DNA was prepared as described in section 2.11.

Eμ EBNA-1 transgenic lines 26, 59, 60, 61, 26B and 59B were routinely screened by slot blot analysis and Southern blotting for transgene status (described in Wilson and Levine, 1992). For cross breeding experiments and pristane induction, Southern blotting was the method of choice to determine transgene status using probe EB1 3' (table 2.3). In addition a PCR approach was developed to screen tail DNA for the EBNA-1 transgene. Oligonucleotides EB1 A and EB1 B (table 2.1) were used as primers. The standard reaction used approximately $^1/_3$μg of DNA in a 50μl reaction containing 1x PCR reaction buffer (Promega), 2.5mM magnesium chloride, 0.2μM dNTPs, 0.2μM of each primer and 0.4units per ml Taq DNA polymerase (Promega). 30 amplification cyles were performed with an annealing temperature of 60^0C and an extension time of 1 minute. The combination of these two primers yields a 500bp product from transgenically positive DNA and no product from transgenically negative DNA.

Bcl2-Ig transgenic mice were imported from the laboratory of S. Korsmeyer (McDonnell *et al.*, 1989) and designated line 85. Tail DNA from these mice was screened by Southern analysis as described previously (McDonnell *et al.*, 1989). In addition slot blot analysis was used in some cases, but extreme

stringency was required in hybridisation (church buffer containing 20% formamide) and washing (70^0C, 0.1xSSC/0.1%SDS, 60minutes) to prevent cross hybridisation between the probe containing human Bcl2 sequences (probe hbcl, table 2.3) and the mouse genomic Bcl2.

Eμ-pim1 transgenic mice were imported from the laboratory of A. Berns (van Lohuizen *et al.*, 1989), designated line 87, and screened by Southern analysis as described (van Lohuizen *et al.*, 1989) using probe mpim (table 2.3).

Eμ myc transgenic mice were imported from the laboratory of A. Berns. The transgenic line was generated in that laboratory using the same constructs as described by Adams *et al.* (Adams *et al.*, 1985). The line was designated line 86. It was attempted to assess transgenic mice as described previously. It was not possible to determine the transgene status of these mice accurately. Definitively positive mice in this line were never obtained.

All mouse importations were overseen by the University Biological Services adhering to appropriate UK quarantine regulations according to licencing by the Home Office and Ministry of Agriculture.

Pristane Induction of Plasmacytomas.

Certain substrains of BalbC mice (including BalbCAnn) are susceptible to the induction of plasmacytomas by intraperitoneal injection of the mineral oil pristane (Anderson and Potter 1969). Both line 26 and line 59 (C57Bl6 strain background) were backcrossed to the BalbCAnn strain of mice. The new lines were called 26B and 59B respectively. After the second backcross to BalbCAnn all young mice in both line 26B and line 59B were injected with

0.5mls of pristane into the peritoneal cavity. This injection was repeated after 2months and again after four months. From the time of the first injection all mice were closely monitored for any outward appearance of lymphoma, plasmacytoma, or general ill health and euthanased on presentation of any of these symptoms according to Home Office guidelines.

2.34. Immunostaining of Transgenic Mouse Tissues for EBNA-1.

A number of different protocols were used to attempt to stain EBNA-1 protein in both frozen and paraffin embedded tissue sections.

Staining of Frozen tissue Sections

- **1. ExtrAvidin Peroxidase Staining with anti EBNA-1 antibodies**

The ExtrAvidin Peroxidase Staining Kit was supplied by Sigma. Endogenous peroxidase activity was blocked by a 5 minute incubation of the slides with 3% hydrogen peroxide. Slides were then washed in 1 x PBS buffer. Slides were incubated for 15 minutes with a 5% goat serum solution and then again washed with 1 x PBS. The anti EBNA-1 antibody was diluted for use in 1 x PBS containing 5% goat serum and 1% BSA. A number of different dilutions of both the human serum AM and the monoclonal Aza2E8 were used in attempts at staining. Where the monclonal Aza2E8 was used the slides were first incubated in a solution of anti mouse immunoglobulin antibodies to block the endogenous mouse immnuoglobulins present within the tissues used. The slides were incubated with the anti EBNA-1 antibody for 60 minutes and then washed with 1 x PBS. The biotinylated antibody was diluted 1:20 in 1 x PBS containing 1% BSA. When the human serum AM was used the secondary

antibody was biotinylated goat anti human immunoglobulin, when the monoclonal Aza2E8 was used the secondary antibody was biotinylated goat anti mouse immunoglobulin. The secondary antibodies were incubated on the tissues for 40 minutes and then the slides were washed with 1 x PBS. The ExtrAvidin peroxidase was diluted 1:20 in 1 x PBS containing 1% BSA and added to the slides for 30 minutes. Slides were then washed for 5 minutes in 1 x PBS. Staining of the slides was carried out using A.E.C substrate supplied by Sigma according to the manufacturer's instructions. Slides were counterstained with Mayer's hematoxylin (Sigma) and mounted using glycerol gelatin.

- **2. ExtrAvidin Alkaline Phosphatase Staining with anti EBNA-1 antibodies**

Essentially the protocol used was as above with the following exceptions.

a. After the addition of the ExtrAvidin Alkaline Phosphatse all washes of the slides were carried out in 1 x TBS (Sigma) rather then PBS buffer.

b. Staining of the slides was carried out using the Fast Red staining system (Sigma) according to the manufacturer's instructions.

Staining of Formalin Fixed Paraffin Embedded Tissues

Both of the peroxidase and phosphatase staining of tissues was attempted. The techniques were basically as described above with the addition of several steps.

- **Removal of Paraffin and Rehydration**

Slides were heated to 58^0C for 15 minutes and then transferred to a xylene bath for 5 minutes. The slides were removed to a fresh bath of xylene for a

further 5 minutes and then placed into fresh absolute ethanol for 3 minutes. The slides were removed to a fresh bath of absolute ethanol for a further 3 minutes and then placed into 90% ethanol for 3 minutes, then into 80% ethanol for 3 minutes and then rinsed gently with tap water.

- **Antigen " Unmasking" Techniques**

A number of different techniques were used to attempt to make the EBNA-1 protein more available for staining.

I. Trypsin digestion

After removal of paraffin the slides were incubated with a solution of 0.1% trypsin for 15 minutes at 37^0C and then rinsed with 1 x PBS.

II. Pressure Cooking

After removal of paraffin and blocking of endogenous peroxidase the slides are placed in citrate buffer (10mM sodium citrate buffer, pH6 Sigma) and cooked for 15 minutes at 15 psi. The slides are rinsed in 1 x PBS prior to staining.

In addition to the two anti EBNA-1 antibodies listed above attempts were also made to stain tissue sections using two antibodies supplied by Jaap Middeldorp, Organon Tekninka BV, The Netherlands. One antibody was a rabbit polyclonal, the other a mouse monoclonal. The protocols used were supplied by Jaap Middeldorp, but were basically as described above for frozen tissues and for formalin fixed tissues including a pressure cooking step as described.

No specific staining of the sections used was detected, however only transgenic tissues were used therefore the experiment did not contain a positive control.

Chapter 3

Expression of EBNA-1 in Eμ-EBNA-1 Transgenics

3.1 Introduction

The similarity in the nature of the tumours which develop in both line 26 and line 59 is strongly suggestive of a role in tumourigenesis for EBNA-1. However, the significant difference in the latency and penetrance of the phenotype between the two lines could be indicative of different expression patterns of the transgene. Indeed, previous work has confirmed that the detectable levels of EBNA-1 protein vary considerably between line 26 and line 59. Surprisingly, by Western analysis the detectable level of EBNA-1 protein is much higher in line 59 than in line 26 (Wilson and Levine, 1992).

In the following section I will present results from a number of experiments designed to analyse the nature of transgene transcription comparing the two lines.

3.2 Northern Analysis

Figure 3.1 shows a Northern blot of total RNAs from a selection of tissues of mice from both transgenic lines, from two control lines 60 and 61 which contain the Eμ-EBNA-1 transgene but have no phenotype and no detectable expression, and from some negative controls. The blot was probed with two EBNA-1 probes covering 5' and 3' EBNA-1 transcribed sequences to enhance the signal.

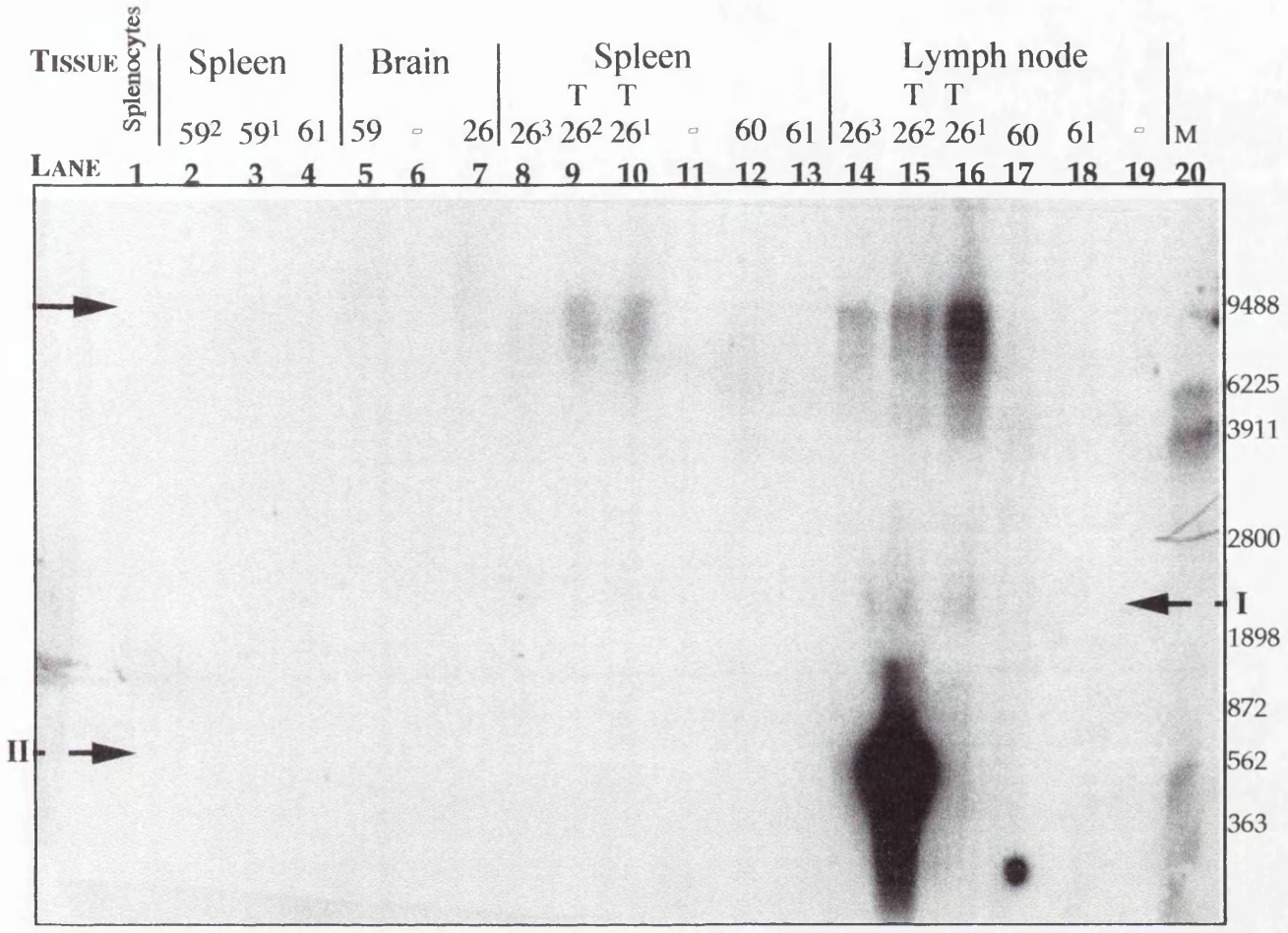


Figure 3.1

20μg of total tissue RNAs Northren blotted and probed with EBNA-1(probes EB1 5' and EB1 3') as described in Materials and Method .M-marker, sizes indicated in base pairs T - mouse had lymphoma. $59^1/59^2$ - splenic RNA from 2 different line 59 mice. $26^1/26^2/26^3$ - RNA from 3 different line 26 mice (spleen and lymph node). 60/61/ ▫ - Control RNAs from lines 60, 61 and transgenically negative mice respectively. Splenocytes- RNA prepared from cultured line 26 splenocytes.

Large EBNA-1 transcripts detected in line 26 samples are indicated by plain arrow, 2kb EBNA-1 transcript is indicated by dashed arrow I, truncated EBNA-1 transcript is indicated by dashed arrow II.

Only in the line 26 samples are any EBNA-1 containing transcripts detectable. This confirms original results using just the 3' probe (Wilson and Levine,1992, Wilson, et al., 1996). A large transcript is evident in the spleen and in the lymph nodes of line 26 samples (indicated by an arrow, figure 3.1). At greater than 9.5kb in size, this transcript is considerably larger than that predicted from transgene sequences. The inserted transgene sequences are completely contained within a 8.5kb Bcl1 fragment detected by Southern blotting and therefore the transcript must contain cellular sequences in addition to those derived from the transgene. The size of the predicted product using the transgene RNA start site and polyadenylation sequences would be 2.1kb. A faint band is evident in the three line 26 lymph node samples at approximately 2kb in size (figure 3.1, lanes14-16, band indicated by dashed arrow).

Lane 9 contains splenic RNA and lane 15 contains lymph node RNA from the same animal, 26.85, which had a malignant lymphoma. The large EBNA-1 containing transcript is evident in both samples, although more readily detectable in the lymph node sample. By ethidium bromide staining the levels of these two RNAs appears similar. In addition an abundant second transcript is detected in the lymph node sample which is not evident in the spleen sample (dashed arrow on figure 3.1). At less than 2kb this transcript is smaller than expected from the intact transgene. This smaller transcript has not been detected in any other line 26 samples and therefore its relevance to the progression of line 26 tumours in general is not clear, although its expression may have been involved in tumour development in this one animal. The DNA

from this lymph node was not analysed, therefore it is not known if this unique transcript is the product of a transgene rearrangement in these cells.

By this assay there is no detectable expression of EBNA-1 in any of the line 59 samples, nor predictably in the control samples from lines 60 and 61.

3.3 RNase Protection

As Northern analysis is not sensitive enough to detect low level expression, line 59 RNA samples were analysed further with more sensitive techniques. RNase protection is a more sensitive technique and also affords the ability to address transcript boundaries, such as initiation sites. The latter is of particular interest given that the line 26 transcripts detected by Northern analysis are not of the predicted size.

In initial experiments, a riboprobe containing the 5' region of EBNA-1 and the polyoma promoter sequences used in the transgene (figure 3.2) was used in protection experiments with total RNA from line 26, line 59 and transgenically negative spleen or lymph node samples. As had been previously shown (Wilson and Levine, 1992), in line 26 two major RNA products are protected with this probe of approximately 359 and 240 nucleotides in size. The larger product represents protection of the entire EBNA-1 and polyoma sequences in the probe and provides evidence that transcriptional initiation is occurring upstream of the predicted start site within the polyoma promoter of the transgene. It is not possible to determine from this experiment whether transcriptional initiation is occurring upstream of the probe, but within transgene sequences, or further upstream within cellular sequences. The

smaller product (approximately 240 nucleotides) represents protection of the entire EBNA-1 sequences and approximately 50 nucleotides of polyoma (within the promoter region, mapping close to the TATA box).

In the experiment shown in figure 3.3a, two different line 59 samples were compared with line 26 samples. In line 59, as in line 26, two major products are detected. The larger is identical in size to that detected in line 26 samples and correlates with protection of all the EBNA-1 and polyoma sequences contained within the probe. The smaller product is slightly shorter than that detected in line 26 (approximately 220 nucleotides), but is likely to correlate with the major viral start site within the polyoma promoter. Mouse 59.44 had a lymphoma which had greatly invaded the spleen. The spleen of mouse 59.58 was histologically normal. In order to determine the relative levels of expression between line 26 and line 59 samples, signals resulting from RNase protection analysis were normalised against GAPDH expression. Relative transcript levels (against GAPDH) are presented in table 3.1. It would appear from this analysis that there is little difference between total levels of 5' EBNA-1 containing transcripts in line 26 and line 59 samples, in contrast to the observations by Northern blotting. It is clear, however, that the relative usage of transcription initiation sites is different between the two lines (but consistent within lines).

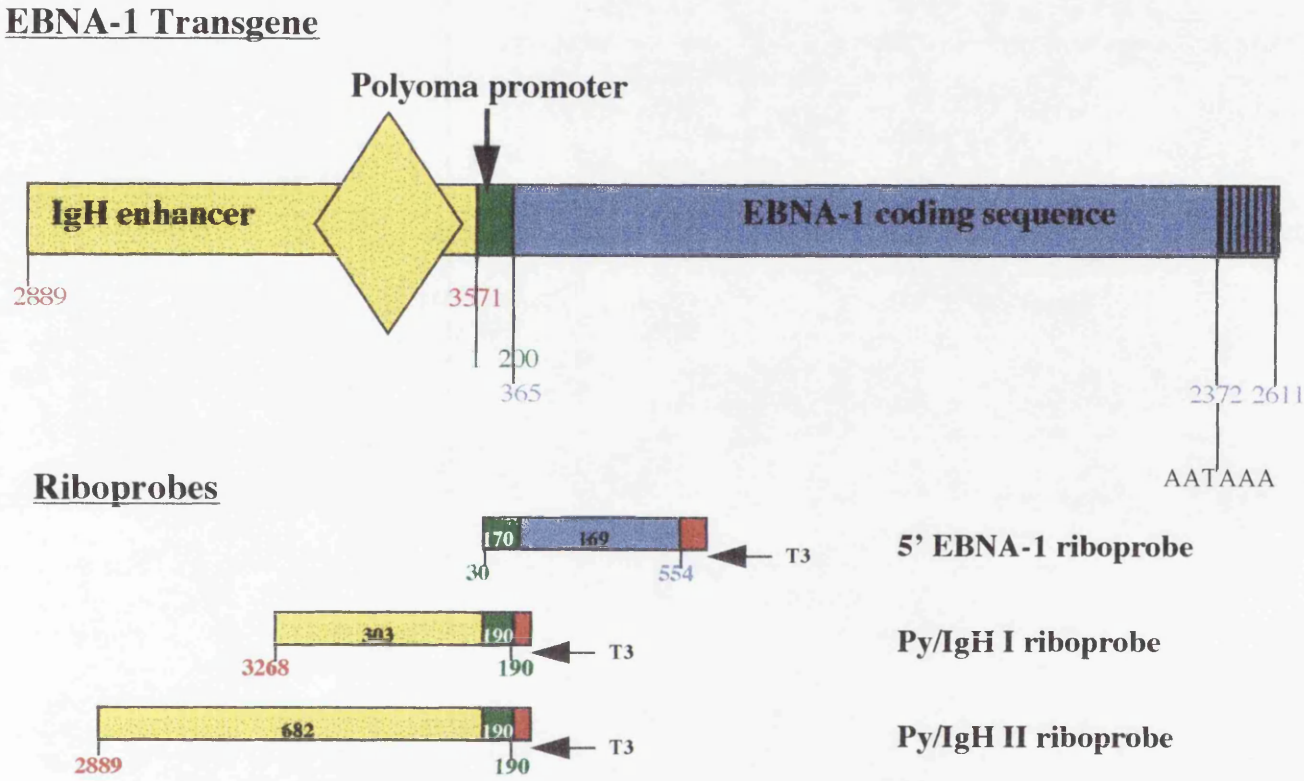


Figure 3.2

Cartoon illustrating riboprobes used in RNase protection assays. The numbers on boxes correspond to sizes in nucleotides. Yellow boxes represent IgH enhancer sequences, green boxes represent polyoma promoter sequences, blue boxes represent EBNA-1 sequences and red boxes represent plasmid vector sequences included in the riboprobe. Arrows labelled T3 indicate the direction of sythesis of the riboprobes. Coloured numbers correspond to transgene sequence positions: red IgH enhancer sequences (Banerji *et al.*,1983), green polyomavirus early promoter (Tooze,1981), blue EBVsequences (Baer *et al.*, 1984).

In the experiment shown in figure 3.3a, in addition to the two major products in line 26 and in sample 59.44 there are a series of other minor products of varying size. There are a number of potential explanations for these products. One is that there is extensive start site heterogeneity in the transcripts expressed. Another might be that the digestion of hybridised RNAs has not gone to completion. The fact that on a long exposure some undigested intact probe can still de detected suggests that this might be contributing to the banding pattern. It is also possible that the integrity of the RNA has been compromised at some stage in the experiment and that other than the two major bands, the majority of detected bands are due to partially degraded RNAs hybridising to the probe. The majority of the RNA in each sample was intact at the start of the experiment, evidenced by the clearly visible 28S RNA band on an ethidium stained gel. It is likely that all three of the above factors contribute to the pattern of products detected. The presence of a series of minor bands is a common observation in this type of assay.

To further characterise the larger 5' protected product detected in both lines, the RNase protection experiment conducted included two additional 5' extended probes, illustrated in figure 3.2. The results from this experiment can be seen in figure 3.3b. The probes were designed to protect sequences derived from the transgene, each probe extending differing degrees into the Eμ enhancer. Each probe was hybridised to splenic RNA from either line 26, line 59 (59.44), or a transgenically negative control. A number of products specific

ACTUAL VALUES	PEAKS				
	GAPDH	EBNA-1 1 (start upstream of Py promoter)	EBNA-1 2 (start in Py promoter)	EBNA-1 3 (start in Py promoter)	EBNA-1 Total
negative	3789				47
26	21 856	1588	959.6	738.6	3286.2
59.44	14 833	587.7	349	1053	1989.7
59.58	1967	112.9	92.2	98.89	303.99
60	2937				69.64
VALUES RELATIVE TO GAPDH	PEAKS				
	GAPDH	EBNA-1 1 (start upstream of Py promoter)	EBNA-1 2 (start in Py promoter)	EBNA-1 3 (start in Py promoter)	EBNA-1 Total
negative	1967				24.4
26	1967	142	86.36	66.47	294.83
59.44	1967	77.9	46.28	139.63	268.81
59.58	1967	112.9	92.2	98.89	303.99
60	1967				46.64

Table 3.1

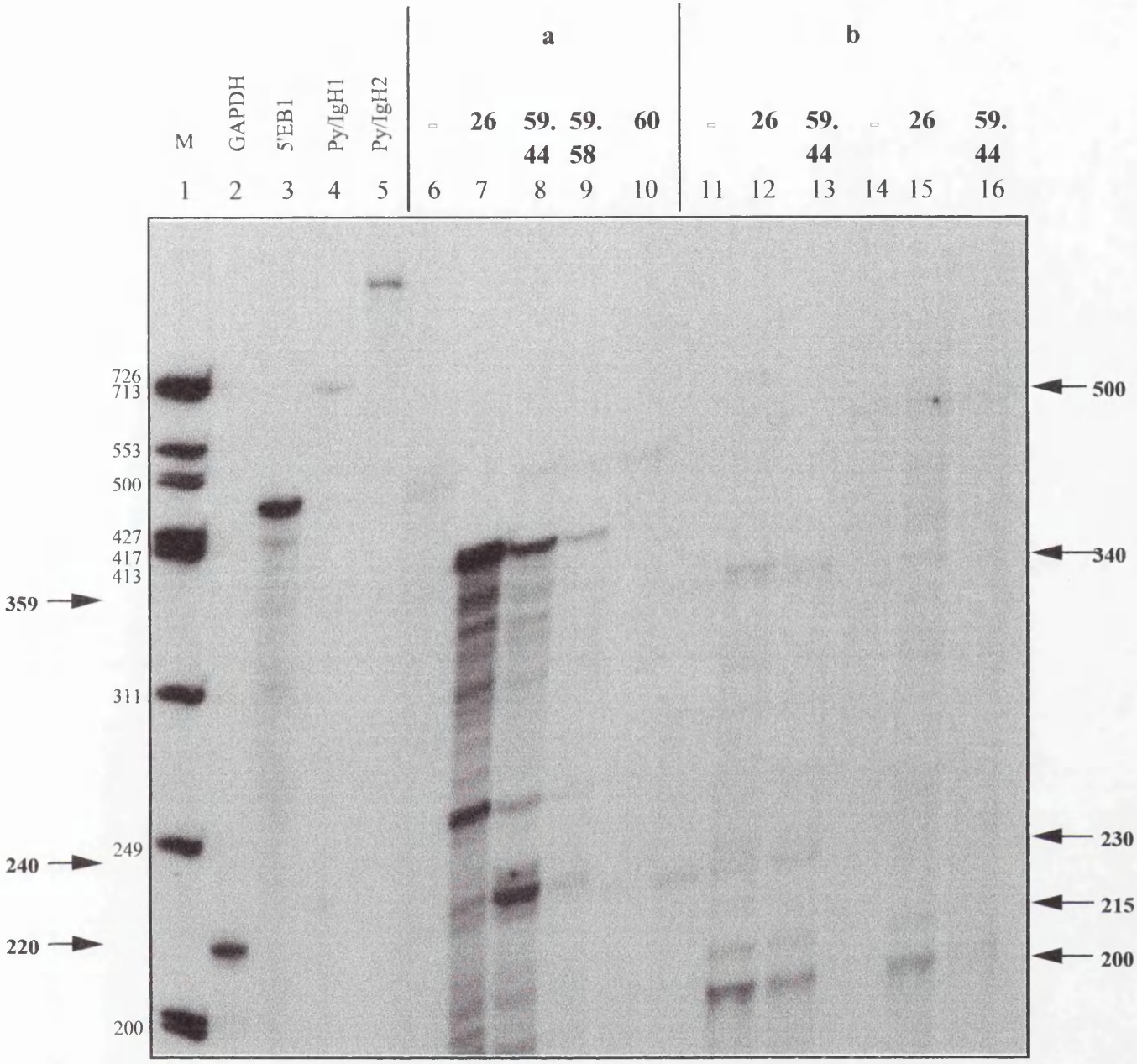


Figure 3.3

RNase protection experiment . Lane 1 - RNA marker ladder, sizes indicated in nuleotides. Lane 2 - GAPDH riboprobe. Lane 3 - 5'EB1 riboprobe. Lane 4 - Py/IgH1 riboprobe. Lane 5 - Py/IgH2 riboprobe. **a.** 50μg of various splenic RNAs hybridised to 5'EB1 riboprobe; lane 6 - negative RNA, lane 7 - line 26 RNA (tumour), lane 8 - line 59.44 RNA (tumour) lane 9 - line 59.58 RNA, lane 10 - line 60 RNA. **b.** Lane 11-13; 50μg each of negative, line 26, and line 59.44 RNAs respectively hybridised to Py/IgH1 riboprobe, lane 14-16; 50μg each of negative, line 26, and line 59.44 RNAs respectively hybridised to Py/IgH2 riboprobe. The major products with approximates sizes in nucleotides in experiment (**a**) are indicated on the left, and in experiment (**b**) are indicated on the right.

to the transgenic lines can be detected. In both lines a major band of approximately 200 nucleotides is protected as well as, at a lower level, a band of approximately 215 nucleotides with both probes. These products correlate with protection of RNAs containing all of the polyoma promoter sequence and probably initiating in the proximity of the junction with the IgH enhancer in the transgene. In addition, in both lines there is protection of a fragment about 340 nucleotides in size. This would correlate with a transcript which initiates within the IgH enhancer (still within the IgH1 probe). On close scrutinization of a long autoradiographic exposure it is also possible to detect other larger products in the line 26 samples (360 nucleotides, lane 15, 480 nucleotides, lane 15 and 16, and 500 nucleotides, lane 12 on figure 3.3b). The sensitivity of this experiment is insufficient to determine whether they are also present in line 59. A band of 230 nucleotides not specific to the transgene, as it is detected in the negative control, is also present with probe IgH1. This could be due to protection of endogenous sequences or to incomplete probe digestion.

It is clear from these results that the majority of the RNAs which protect the entire length of the first probe (5' EBNA-1, figure 3.3a) initiate within the transgene. At least two additional start sites can be postulated, which are used in both lines. The first and major of these upstream sites lies at the junction of the polyoma promoter and the IgH enhancer in the transgene. The second maps to within the enhancer itself. It is not possible from this experiment to exclude the possibility that some of the EBNA-1 containing transcripts initiate

even further upstream within the IgH enhancer or even in cellular sequences. Figure 3.4 presents a summary of the predicted start sites for transcripts from RNase protection data.

3.4 5' RACE PCR

From the results of Northern analysis and RNase protection assays, it is evident that the transcription pattern of the transgene in both lines is complex. Given that there are obvious differences between the two lines it is possible that the phenotypic differences seen correlate with transgene expression. To extend this analysis of expression, 5' RACE PCR was employed to characterise the nature of transcripts with different 5' ends in each of the transgenic lines.

A selection of line 26, line 59 and negative RNAs from spleen or lymph node samples were analysed by 5'RACE PCR. The primary 5'RACE PCR was carried out as described in Materials and Methods (chapter2, section 2.29). A gene specific primer (EB1 I, table 2.2) was used in the initial cDNA synthesis, and a nested gene specific primer (EB1 J, table 2.2) in the first round PCR reactions.

Figure 3.5 shows a Southern blot of primary PCR products, which were not visible by ethidium bromide staining, probed with 5' EBNA-1 (probe EB1 5'). Lane 1 contains products from a line 26 RNA made from a lymph node which

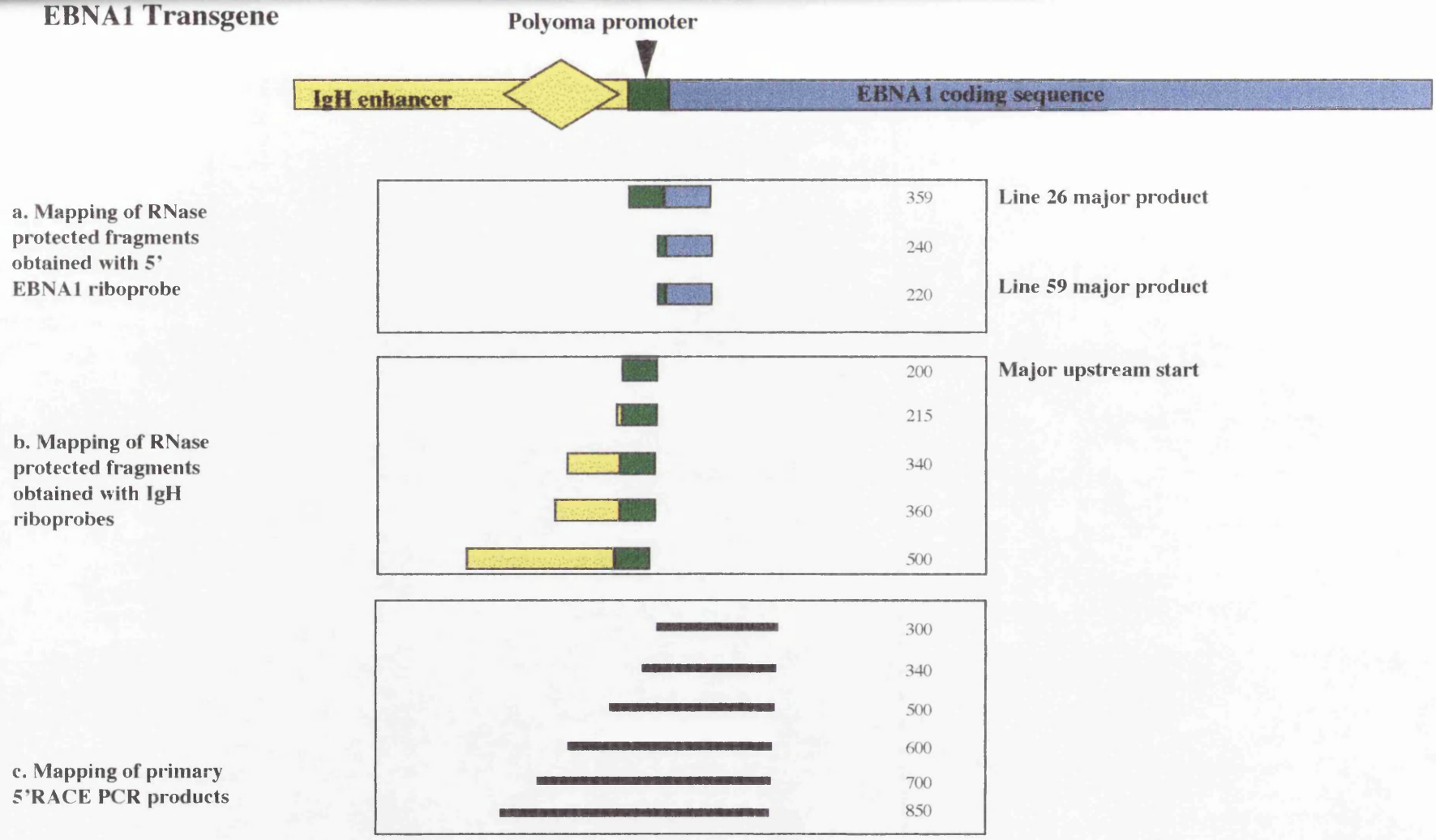


Figure 3.4
Summary of data obtained from RNase Protection and 5' RACE PCR assays. **a.**mapping of products obtained from RNase protection assay with 5' EBNA1 probe **b**. mapping of products obtained from RNase protection assays with IgH probes **c**. potential mapping of 5' RACE products. Numbers represent approximate sizes in nucleotides of products

was massively invaded by a lymphoma. A number of products are visible. These range in size from about 300bps to about 800bps. This correlates well with predicted sizes of products from RNase protection assays. A transcript initiating within the polyoma promoter (240 nucleotides band, figure 3.3a) would produce a RACE product of approximately 340bps in size. A transcript initiating at the junction between the polyoma promoter and the IgH enhancer (200 and 215 nucleotides bands, figure 3.3b) would produce a RACE product of approximately 480bps. Transcripts initiating from within the IgH enhancer could produce RACE products up to 1.2.kb in length. There are no products evident in the lane containing the line 59 sample, nor is it possible to detect anything in any of the negative controls.

In order to enhance the amount of products from the RACE to a more easily detectable level, a secondary amplification by PCR was performed. Products of the first round were diluted and subjected to a second round of amplification using universal amplification primer (GIBCO) and one of a selection of different EBNA-1 specific primers (oligonucleotides EB1 J, EB1 F, EB1 H, or EB1 K as described in Materials and Methods). Amplifications were performed a number of times on different cDNAs and isolates which had been tailed at different times. It is clear that the amount of product detected ultimately is acutely dependent on the quality of both cDNA sythesis and tailing reactions.

It proved extremely problematic to detect any products in initial reactions using two different line 59 RNAs; 59.93 and 59.102. After a

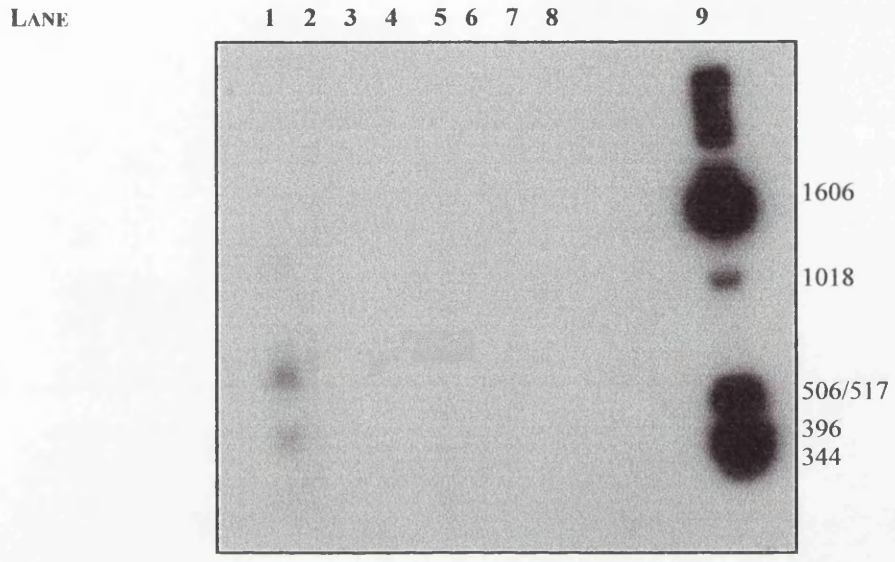


Figure 3.5
Southern blot of primary PCR products from 5'RACE PCR probed with EB1 5'. The reactions were carried out using the Gibco/BRL 5'RACE kit as described in Materials and Methods. Lanes 1-4; products of 5'RACE PCR reactions; cDNA synthesis primed with oligo EB1 I and, PCR reaction carried out with oligo EB1 J and the Anchor primer (Gibco). Lane 1 - 26.411 LN tailed cDNA, lane 2 -59.93 spleen tailed cDNA, lane 3 - 60.102 spleen tailed cDNA, lane 4 - negative spleen tailed cDNA. Lanes 5-8 ; PCR reaction as described above carried out on RNAs to control for DNA contamination of RNAs. Lane 5 - 26.411 LN RNA, lane 6 - 59.93 spleen RNA, lane 7 - 60.102 spleen RNA, lane 8 negative spleen RNA. Lane 9 - 1kb DNA marker ladder, sizes indicated in base pairs.

large scale secondary PCR reaction on products from the RACE procedure (Materials and Methods, section 2.29), two products from 59.102 RNA (approximately 1kb and 450bps in size) were detected on an ethidium stained gel (figure 3.6). However a negative control was not included in this experiment. Using RNA from a line 59 tumour (59.44 spleen) a wider range of products was obtained.

To address the specificity of products obtained, Southern blots of RACE products have been probed with EB1 5' (table 2.3) and with a probe to IgH enhancer sequences (IgH1, table 2.3). Primary PCR products were diluted and subjected to secondary amplification with a nested gene specific primer (EB1 F, table 2.2) and the universal amplification primer (GIBCO).The products were electrophoresed and then Southern blotted. A range of products is detected in both line 26 and line 59 samples with both the EB1 5' probe and the IgH1 probe (figure 3.7a and b). There is clearly overlap in the products hybridised to each probe, and as would be predicted, the IgH probe preferentially hybridises to the larger products. No products are detected in the negative controls included in this experiment (lanes 4 and 11, figure 3.7a and b), but this experiment also illustrates the variability in the amount of product obtained from EBNA-1 positive samples. Several line 59 and line 26 samples did not yield any detectable product in this experiment (lanes 3,6,12,13 and14, figure 3.7a). The same RNA can give different products depending on the cDNA sythesis and tailing reactions (compare lanes 1 and 2, and lanes 7, 8 and 14, figure 3.7a).

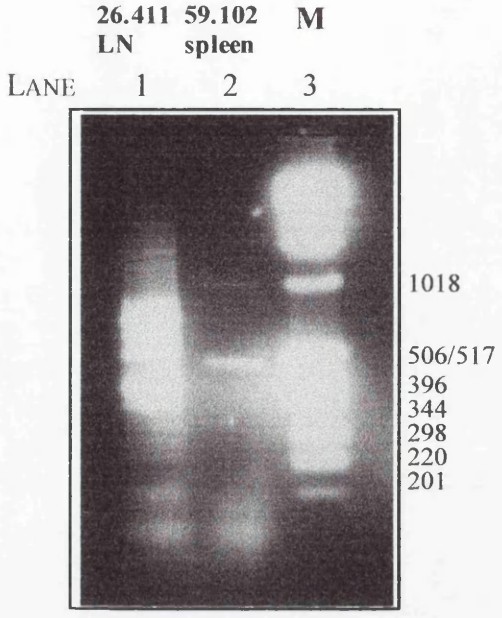


Figure 3.6
Ethidium bromide stained gel of PCR products from secondary amplification of 5'RACE products. Primary PCR products were diluted 1:50 and then 5µl was used per 50µl reaction as described in Materials and Methods. Re-amplification was carried out using the Universal amplification primer (Gibco) and oligo EB1 J. Lane 1 - Line 26. 411 LN, lane 2 - line 59.102 spleen, lane 3 - 1kb DNA marker ladder.

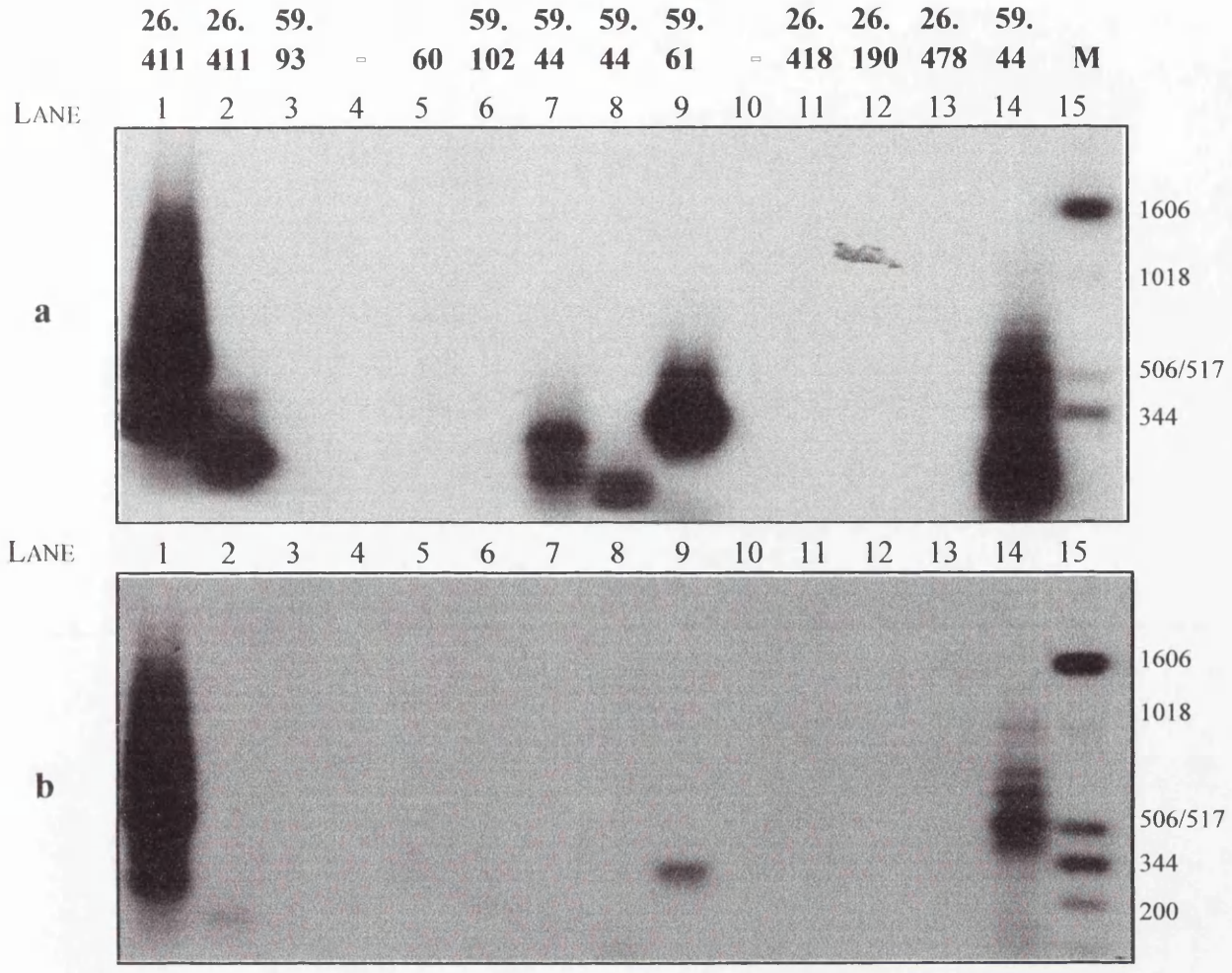


Figure 3.7

Southern blot of PCR products from secondary amplification of 5'RACE PCR products. The primary products were diluted , and re-amplified using oligo EB1 J and the universal amplification primer (Gibco) as primers. The origin of the RNA used for cDNA sythesis is indicated. In lanes 1 and 2, the RACE procedure was carried out on two different cDNA preparations of the same LN RNA from mouse 26.411. In lanes 7,8 and 14, the RACE procedure was carried out on different cDNA preparations of the same splenic RNA from mouse 59.44. **a.** Probed with EB1 5'. **b.** Probed with IgH1.

In order to maximise the levels of products from line 59 samples which had given variable results, a different approach was taken for secondary PCR reactions. In the experiment shown in figure 3.8a the primary RACE reactions were purified to remove any residual primers, rather than diluted. $^{1}/_{10}$ of each purified reaction was subjected to a second round of PCR using a nested gene specific primer (EB1 K) and the universal amplification primer (GIBCO). The reactions were electrophoresed on an ethidium stained agarose gel and Southern blotted and probed with EB1 5'. A variety of products were obtained in both line 26 samples and line 59 samples, visible on the ethidium stained gel (figure 3.8a). A range of products in line 26 and line 59 samples also hybridise strongly to EBNA-1 sequence (figure 3.8b). The most intense bands on the ethidium stained gel (figure 3.8a) do not necessarily represent the products which hybridise most strongly to EBNA-1. For example this is clearly evident with sample 26.478; on the ethidium stained gel there are strong bands of approximately 850 and 750 bps and a very vague shadow at about 450bps (lane 11, figure 3.8a). The band which hybridises most strongly to the EB1 5' probe is the band of 450bps. The 850bp product does not hybridise at all, and the 750bp product hybridises only very weakly. A strong band of approximately 190bps in size is detected in the line 60 sample on the ethidium stained gel(lane 12, figure 3.8a). This product does not hybridise strongly to EB1 5', two larger products (approximately 500bps and 380bps) hybridise to EB1 5' (lane 12, figure 3.8b) but are barely visible on the ethidium stained gel (lane 12, figure 3.8a).

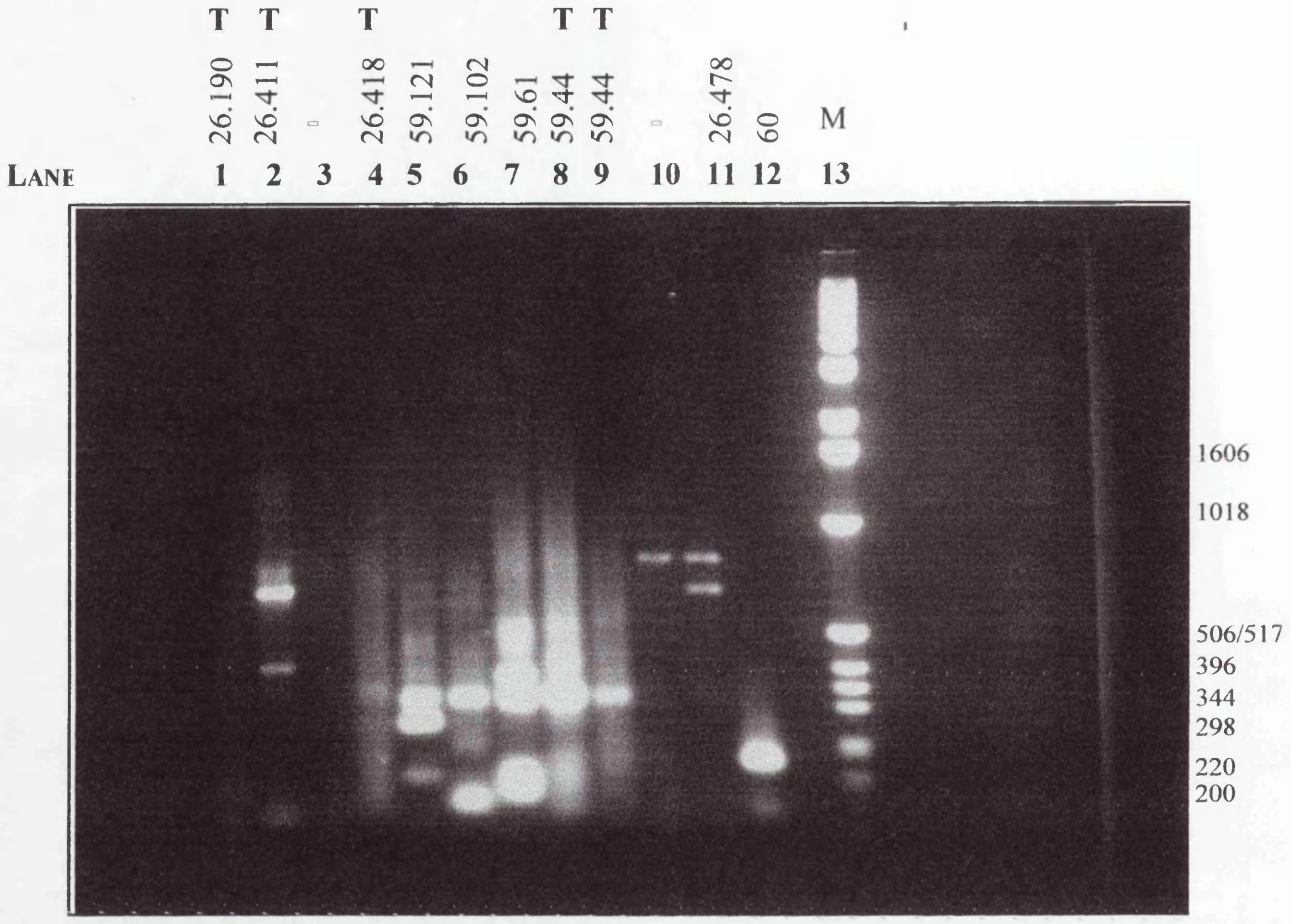


Figure 3.8a

Ethidium bromide stained agarose gel of PCR products from secondary amplification of 5' RACE PCR products. The 5' RACE products were purified to remove primers (see Materials and methods) and reamplified using EB1 K and the universal amplification primer (Gibco). Lane 13 - 1kb DNA marker ladder, sizes in base pairs are indicated. The animal from which the RNA was prepared is indicated. With the exception of 26.411, which was prepared from a lymph node, all the RNAs were prepared from spleens. T- indicates that the tissue was invaded by lymphoma.

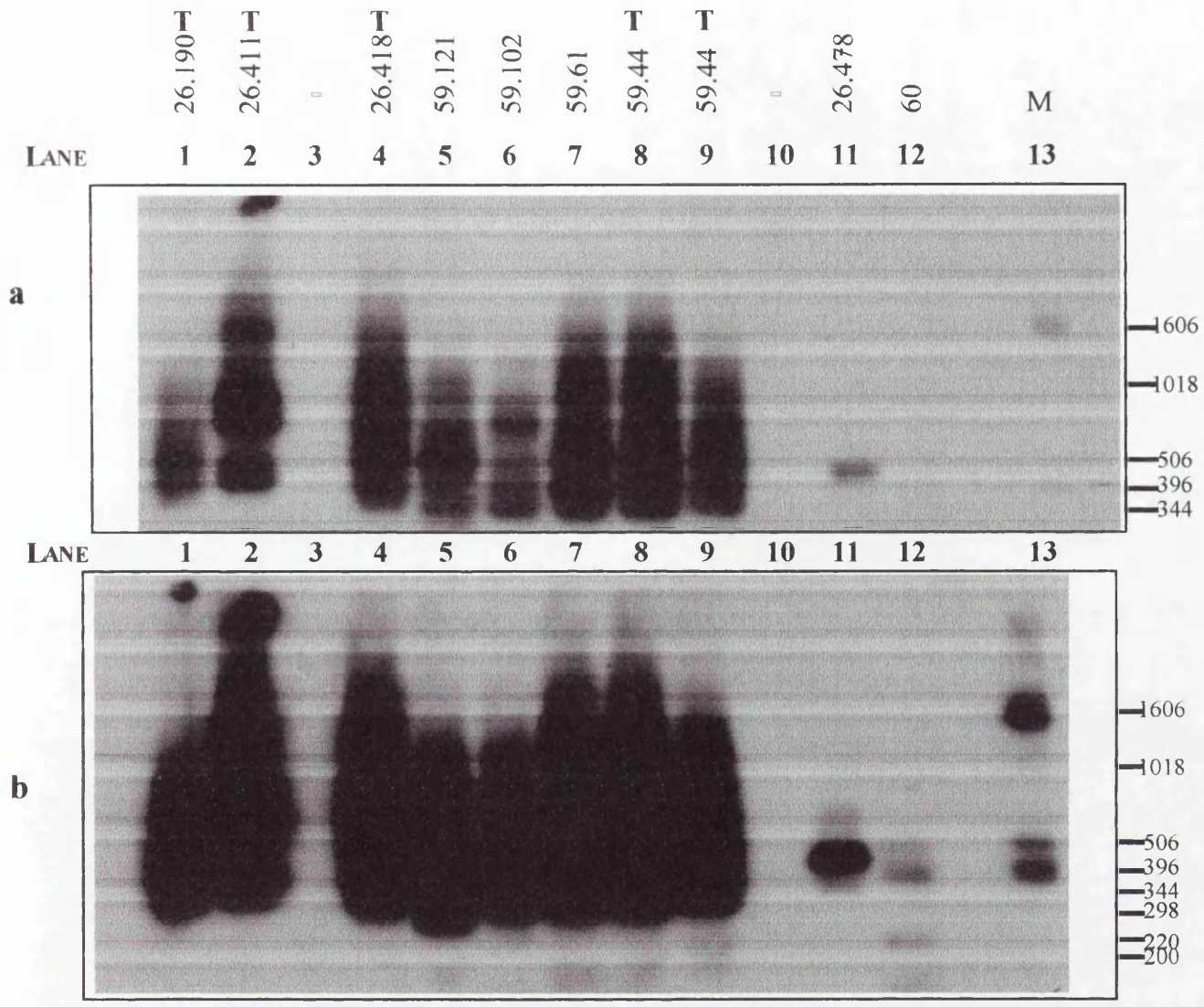


Figure 3.8b

Southern blot of 5' RACE PCR products generated as described in figure 3.8a legend and electrophoresed in an agarose gel. The gel was blotted as described in Materials and methods and probed with EB1 5'. (a) and (b) are two different exposures of the same blot.

That there is a low level of expression from the rearranged transgene in line 60 is in agreement with previous work (Wilson and Levine 1992, Wilson, *et al.*, 1996). Two different transgenically negative splenic RNAs were included in this experiment, 26.480 and 26.91 (lanes 3 and 10, figure 3.8a and b). Neither of these RNAs yielded products which hybridised to EB1 5'. However, RNA from 26.91 gave rise to a product of approximately 850bps in size (lane 10, figure 3.8a). A product of similar size was detected with 26.478 RNA (lane 11, figure 3.8a) which also did not hybridise to EB1 5'.The 850bp band is therefore not transgene specific and could be an artefact caused by the presence of some primer homology within mouse RNA sequences as it is clearly present in the negative control.

In order to locate start sites to the precise nucleotide, it was attempted to clone and sequence products detected in both lines. After the secondary PCR on RACE products, several reactions for line 26 and for line 59 were pooled, precipitated and run out on an ethidium stained gel. The major bands were cut out (approximately 850bps, 800bps, 650bps, 500bps 340bps and 300bps from the line 26 sample, and 450bps and 1kb from the line 59 sample) and the DNA purified and cloned. Clones containing inserts were then selected for subsequent sequencing on the basis of the size of the insert.

The presence of the poly G tail caused a block in many sequencing reactions, which proved to be problematic. Many of the sequences obtained did not contain any homology to EBNA-1. This might have been predicted from the experiment above since several of the most intense bands on the ethidium gel

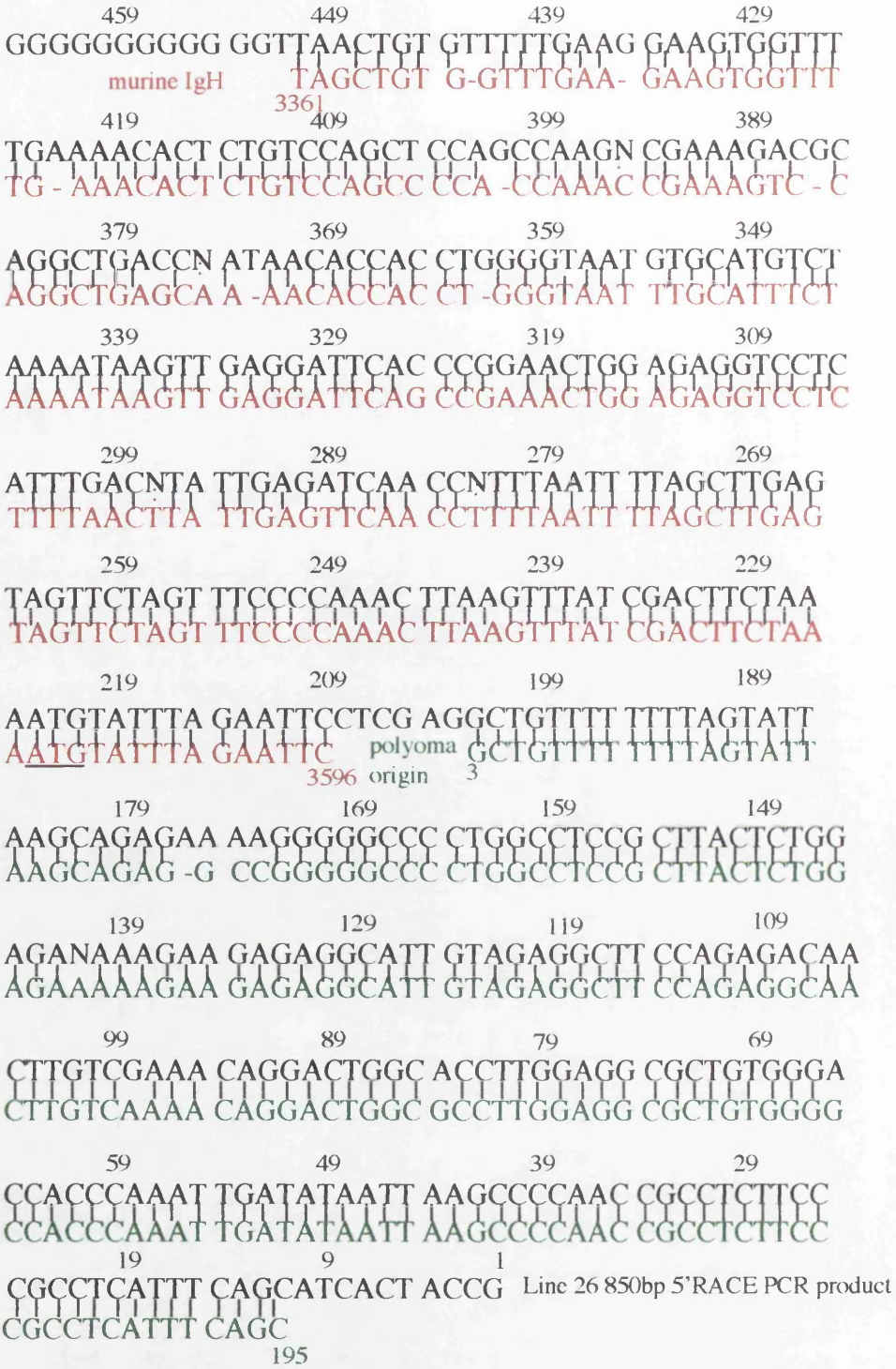


Figure 3.8c

DNA sequence of line 26 850bp 5'RACE PCR product (black). 5'RACE PCR products were cloned and sequenced as described in Materials and Methods. In this reaction the primer oligonucleotide was EB1F. The sequence has been aligned with murine immunoglobulin heavy chain enhancer sequence (red) (Banerji *et al.*,1983) and polyoma origin sequence (green)(Tooze *et al.*,1981).The ATG sequence contained within the enhancer is underlined.

do not hybridise well to EB1 5'. None of the 450bp fragments cloned from the line 59 sample contained any sequence resembling EBNA-1. Similarly, sequence obtained from the 300, 500, 650 and 800bp products (these sizes are an approximation taken from the position of the bands which were cut out for cloning) did not correlate with the expected sequence, although products of this size did hybridise to EBNA-1 on Southern blots of RACE reactions. These sequences did bear homologies to mouse and human sequences. The 650bp product had homologies to a large number of 5' ends of human cDNAs in the data base. The sequences do come from the mouse genome; when the 650bp product is used as a probe on a Southern blot of mouse tail DNA under stringent conditions a single band is detected in both transgenically positive and negative mice. The 300bp product had strong homology to mus musculus osteocalcin related gene.

One product sequence did give results as expected. The 850bp product from line 26 contains sequence from the Eμ enhancer, extending through the entirety of the polyoma promoter and into EBNA-1. This transcript starts in the middle of the enhancer at position 3361 (according to published sequence, Banerji *et al*, 1983). The start of this transcript is marked on the summary diagram (figure 3.4)

Southern blotting of RACE PCR products has demonstrated that a number of different transgene specific products were obtained. However, both line 59 and line 26 samples also gave rise to a number of products which did not hybridise to the EBNA-1 sequence and sequencing confirmed that a number of these

products contain murine sequences not transgene related. The origin of these products is not clear. That this technique can generate artefactual products is demonstrated by the detection of a product in one of the negative controls. Use of this technique did however allow confirmation of results obtained by RNase protection and precise mapping of one start site in line 26. In both line 26 and line 59 there is heterogeneity in start site usage. Also, products of greater than 1.2kb were detected by this method that did hybridise to EBNA-1 sequences. These products may therefore be bona fide, and would be predicted to start upstream of the transgene sequences, and possibly to contain murine cellular sequences.

3.5 Transgene Integration Site and Transgene Configuration

The regulation of transgene expression in both line 26 and line 59 is complex. In both lines transcriptional start sites other than within the polyoma promoter appear to be in use. It is possible that this is directed by the juxtaposition of the IgH enhancer and the polyoma promoter or that this is due to the influence of surrounding cellular sequences in both lines. In line 59, there appears to be a single complete copy of the transgene plus some additional incomplete transgene sequences inserted at an unkown site (Joanna Wilson, pers. comm.). In line 26, there appear to be two copies of the transgene, at least one of which is intact (figure 3.9). An EcoRI digest of line 26 genomic DNA probed with IgH sequences reveals a fragment of

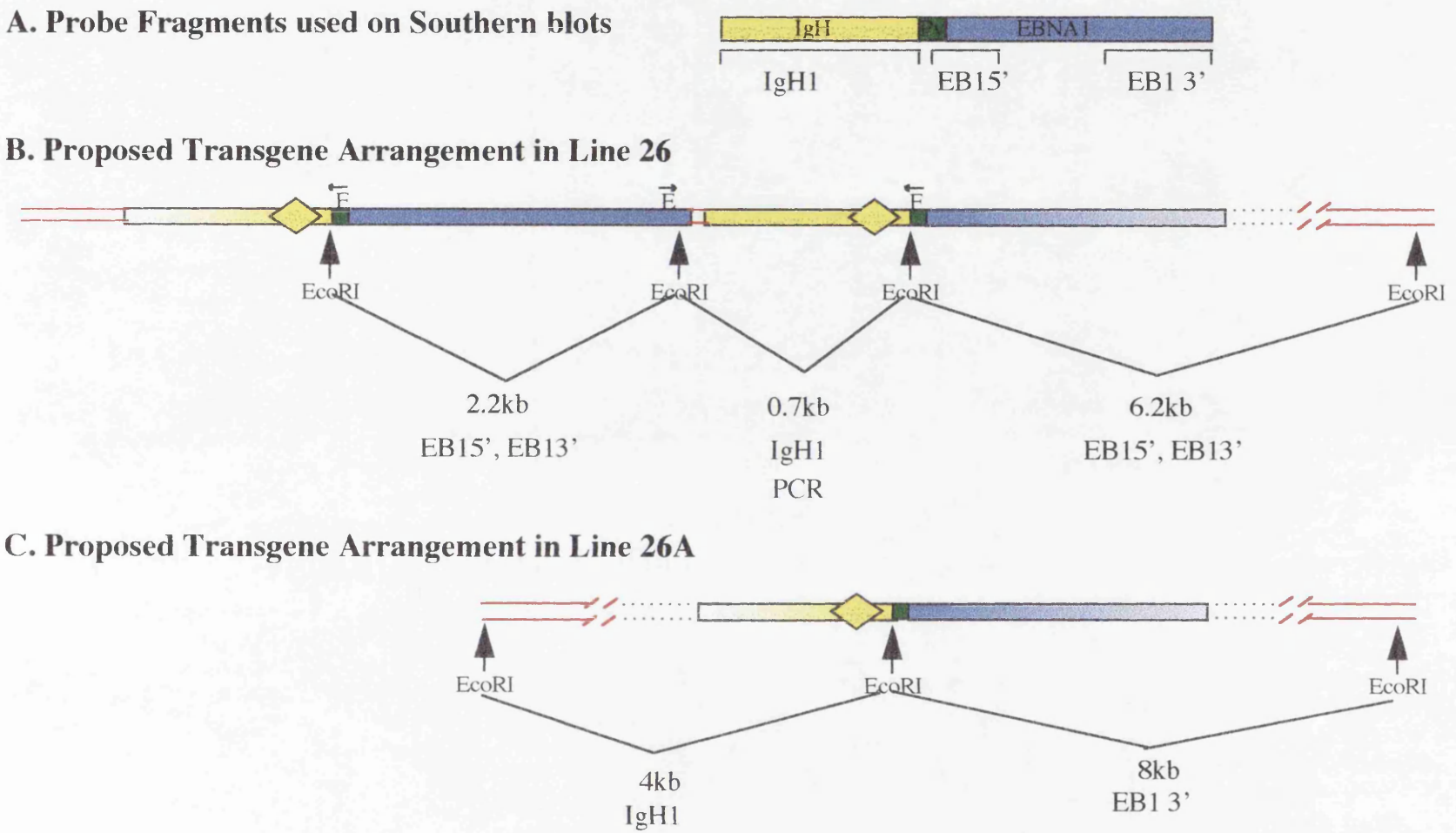


Figure 3.9

Cartoon of proposed configuration of transgene sequences in line 26 and line 26 A. **A.** Illustration of the probe fragments used on Southern blots of genomic DNA **B.** Proposed configuration of transgene sequences in line 26. **C.** Proposed configuration of transgene sequences in line 26 A. EcoRI sites are marked along with the fragment sizes detected on an Southern blot of EcoRI digested genomic DNA, below the fragment size it is indicated which probe fragment has been used. Red lines indicate the presence of cellular sequences of an unknown nature. Potential truncation of transgene sequences is indicated by shading. The postion of primers used in PCR are indicated by small arrows marked E and F.

approximately 700bps (data not shown). This fragment would be predicted if there are two head to tail tandem copies of the transgene in line 26. To test this hypothesis, it was attempted to use PCR to amplify between transgene sequences in line 26 and line 59. The PCR reaction used a sense primer to the 3' end of EBNA-1 (oligonucleotide EB1 E, table 2.1 and figure 3.9) and an antisense primer to the 5' end of the polyoma promoter (oligonucleotide Py ORI F, table 2.1 and figure 3.9). It was not possible to obtain any product from line 59 DNA or from a negative control DNA. However, in line 26 three major products were obtained of approximate size 700bps, 900bps and 1.2kb. The detection of a 700bp fragment is in agreement with the hypothesis that there are two directly abutting copies of the transgene in line 26 (figure 3.9). The detection of the two larger fragments is more difficult to explain. The PCR reaction was carried out at a relatively low stringency (annealing temperature of 45^0C) and so it is possible that these products are the result of mispriming from cellular DNA. However they are specific to line 26 and may indicate that the transgene integration is more complex than presented in figure 3.9.

On probing of a Southern blot of EcoRI digested standard line 26 tail DNA with EB1 3' (table 2.3), two major bands are detected, one of 2.2kb, as predicted, and a large band of about 6.2kb (figure 3.10). During the course of breeding of line 26, in one unusual case a germ line rearrangement involving the transgene has occurred. This rearrangement has created a copy of transgene sequences which now reside in an 8kb EcoRI DNA fragment (figure 3.10). This copy of the transgene is located at some distance to or is on a separate

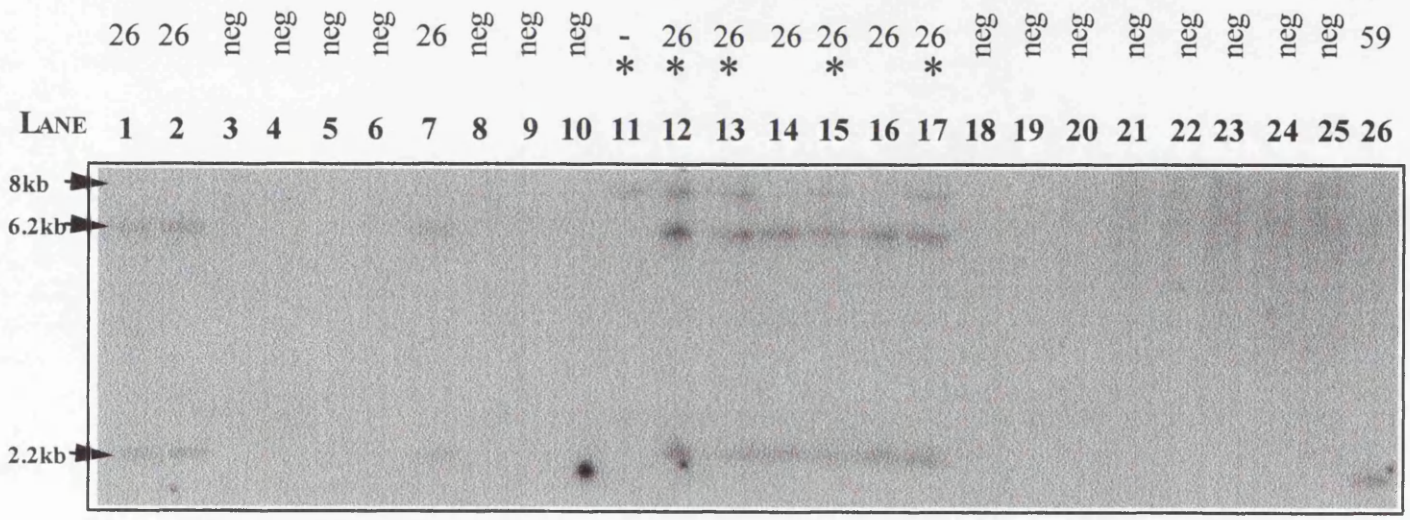


Figure 3.10

5µg of tail DNAs were digested with EcoRI, Southern blotted and probed with an EBNA-1 probe (EB1 3') as described in materials and methods.

26- line 26 positive DNAs, 59- line 59 positive DNAs, neg- transgenically negative DNAs, *- DNAs carrying rearranged Eµ EBNA-1 transgene

chromosome from the original transgene integration site and it has been possible to breed a line of transgenic mice harbouring only this copy, now designated line 26A. Both the 6.2kb EcoRI fragment and the 8kb fragment contain EBNA-1 sequences, but the extent and configuration is not clear. The 6.2kb fragment could be generated if the extreme 3' end of the transgene tandem was deleted and the EcoRI site lost (illustrated in figure 3.9). If the truncated transgene were copied to a new site within the mouse genome this could explain the 8kb fragment. Probing of a Southern blot of EcoRI digested DNA containing the line 26A rearrangement with IgH sequences highlights a band of 4kb (data not shown) which is attributable to the transgene. Line 26A is undergoing observation for phenotype. As yet no incidence of lymphoma has been observed, and the animals have been monitored over a two year period.

3.6 Summary and Discussion

From Northern analysis, it is clear that the total detectable level of EBNA-1 containing transcripts is higher in line 26 than in line 59. Although a large transcript is routinely detected in line 26 samples, consistently no EBNA-1 containing transcripts are detectable in line 59 samples. This correlates well with the observed phenotype. Mice of line 26 have a higher tumour incidence at an earlier age.

However, quantitation of the levels of transcripts containing 5' EBNA-1 by RNase protection suggests that the levels of initiated transcripts are fairly equivalent between the two lines. There are several potential explanations for the apparent difference in levels revealed by these two assays. The riboprobes in RNase protection assays cover only the 5' transgene sequences and therefore will not detect transcripts truncated at the 5' end. By contrast, the probes used on Northern analysis extend over a larger region of the transgene and therefore may detect a wider range of transcripts than detected by RNase protection. The large product detected by Northern analysis in line 26 may initiate downstream of the riboprobes used and may therefore not be represented in the results of the RNase protection assays. Northern analysis is a less sensitive technique than RNase protection and only fragments of consistent size (forming a band) can be detected. If the EBNA-1 containing transcripts vary considerably in size, then they would form a smear rather than a discrete band on a Northern blot which may not be detectable, particularly if the total level of transcripts is low as is the case in these transgenic lines. It is possible that the transcripts detected by RNase protection in line 59 have heterogeneous 3' ends (perhaps through inefficient poly A site recognition, discussed below) and therefore do not form a discrete band in Northern analysis.

It is clear that although the total level of 5' containing transcripts may be similar, the exact nature of the transcripts differs between the two lines. In line 26, the majority of transcripts detected start upstream of the polyoma

promoter (compare EBNA-1 peak 1 (upstream start) to EBNA-1 peaks 2 and 3 (polyoma start), table 3.1). The major start site of transcripts in line 59 maps to the major RNA start site of polyoma early viral transcripts within the polyoma promoter (compare EBNA-1 peak 3 to EBNA-1 peak 1 and 2, table 3.1).

RNase protection experiments indicate that the majority of transcripts initiate within the 5' enhancer/promoter region of the transgene, and there is little or no run on transcription from one transgene to the next in line 26. Therefore the large transcript in line 26 seen by Northern analysis is likely to extend 3' into cellular sequence.

The observation of a cellular-transgene hybrid transcript in line 26 is suggestive of a role for cellular sequences in the regulation of expression of the transgene in this line. The transgene integration site as well as the configuration of the integrated transgene in both lines may well play a role in the observed differences between the lines. If there is a copy of the transgene in line 26 which is truncated at the 3' end, then this may lack the polyadenylation site. The large transcript detected in line 26 could be due to the lack of this site. An alternative explanation is that the EBNA-1 polyadenylation site in the transgene may be weak, allowing a number of transcripts to run through it. This could give rise to transcripts with heterogeneous 3' ends.

Whilst the relative levels of the transcripts differ, in both lines it has been possible to map transcript start sites to regions upstream of the polyoma promoter. A number of different starts of transcripts within IgH enhancer

sequences have been identified. The RNase protection results reveal that multiple 5' start sites are used in both lines 26 and 59 splenic tissues, however the relative usage of these sites differs between the lines. If there are a number of different transcriptional initiation sites in use, then it is possible that different cell types or stages within the tissue use different initiation sites. The IgH enhancer contains binding sites for multiple different proteins which may differ in their importance for transcription in different cell types and cell stages. In a human pre-B cell line a number of messenger transcripts have been identified which initiate within the immunoglobulin intronic heavy chain enhancer and are extremely heterogeneous at the 5' end (Neale and Kitchingman, 1991). More than 50 different transcriptional initiation sites were identified mapping near to the Ig-specific octamer ATTTGCGT. An octanucleotide potential promoter element (ATTTGCAT) exists within the core mouse enhancer sequences contained within the transgene. It may be that the observed heterogeneity of 5' ends of transcripts from the transgene is due to multiple transcriptional start sites originating within the IgH enhancer.

Not all of the transcripts detected necessarily give rise to active EBNA-1 protein. The 850bp 5' RACE product sequenced contains an ATG upstream of the EBNA-1 translational start site, within the IgH enhancer. If translation occurs from this upstream ATG, this could reduce the efficiency of translation from the EBNA-1 ATG through competition for the translational machinery (Kozak, 1995). Since a greater proportion of line 26 transcripts initiate

upstream than for line 59, this may mean that translation of line 59 transcripts is more efficient.

From Southern blots of 5' RACE products, it appears that there may be products in excess of 1.2kb in size in both line 26 and line 59. However from RNase protection experiments, any starts upstream of the transgene are likely to be a very minor component of transgene expression. Such transcripts are likely to contain cellular sequence, although in line 26 they could be products resulting from read-through from one copy of the transgene to the next. The presence of differing cellular sequences in line 59 and line 26 EBNA-1 containing transcripts may be involved in the observed differences in phenotype between the two lines.

Work is ongoing in the laboratory to clone out and sequence the transgene insertion sites in each of the transgenic lines. This may provide valuable information as to the sequences directing transgene regulation and their potential role in the observed phenotypes of the lines. Although the differences in the expression pattern of the transgene sequences may in themselves explain the different phenotypes of line 26 and line 59 mice, it is still possible that in line 26 activation of a cellular proto-oncogene by the insertion of the transgene enhances the tumourigenecity of EBNA-1.

It is not known which domain(s) of EBNA-1 protein are required for the oncogenicity of the protein. An analysis has suggested however that certain EBNA-1 variant EBV strains are more common in tumours, and therefore may be more oncogenic (Introduction section 1.7 and Bhatia *et al.*, 1996). Sequence

analysis of the EBNA-1 transgene in different tumours could identify similar mutations in the transgenics which render EBNA-1 more oncogenic. In one line 26 tumour (26.85) a rearrangement has occurred in the metastasis which leads to a very high level of expression of a truncated EBNA-1 containing transcript. It is not clear whether this had a causal role in the metastasis of the tumour to the lymph node or if it is a subsequent rearrangement in a genomically unstable cell. Few line 26 tumours have been compared for EBNA-1 RNA expression between primary and metastatic lesions. Such an analysis might provide an insight into the significance of this rearrangement in the lymph node of 26.85. Given the differences in the RNA expression pattern between the transgenic lines and the potential role of cellular sequences, a measure of the activity of EBNA-1 protein in each of the transgenic lines may provide a clearer understanding of the differences in phenotype between line 26 and line 59 (chapter 5).

Chapter 4

Cell Culture from Eμ EBNA-1 induced Lymphomas

4.1 Introduction

It is not known whether continued EBNA1 expression is required within the transgenic tumour cells or if EBNA1 only sets up a predisposition to lymphoma and is subsequently no longer oncogenically required. In order to address this question, it was attempted to derive cell lines from line 26 tumours. Such cell lines could be used to investigate if EBNA1 continues to contribute to cell proliferation in culture and therefore, indirectly, whether it has a role in tumour maintenence in both the transgenic tumours and in the human disease. The effect of down regulation of EBNA1 expression in cell lines derived from tumours has been attempted here.

4.2 Derivation and Analysis of Primary Cell lines from line 26 Tumours

It was attempted to derive primary cell lines from line 26 tumours from both spleen and lymph node tissues from a number of different animals. The tissues were mashed in lymphocyte medium (chapter 2, section 2.32) and the resultant cell suspension seeded at a minimal cell density of 2 $x10^5$ live cells per ml. For the majority of tissues used the cells did not survive any longer than 1-2 weeks in culture, which is up to twice as long when compared to cells derived from negative controls (Joanna Wilson pers. comm.). Cells derived

from lymph node suspensions died sooner (within the first week) than cells derived from spleen suspensions. The majority of cultures monitored did not show an increase in cell number during culture. However one line 26 tumour, 26.305, did give rise to a cell line which was propagated in culture for more than 12 weeks. This line 26 mouse was unusual in that it did not succumb to lymphoma until it was over 1 year old (421 days old at death). Both spleen and lymph node tissues were massively invaded by the tumour and were used to derive cell suspensions, as described in materials and methods (chapter2, section 2.32). The spleen was mashed in 1 x PBS, the capsule removed and the cell suspension spun down briefly. Most of the supernatant was removed, and the cells resuspended in the remaining 2mls of PBS. 0.1mls aliquots of this concentrated suspension was diluted with 1ml of the media as described below (table 4.1). In addition to the components listed, all media contained glutamine and penstrep as described in materials and methods (chapter 2, section 2.32). The resultant confluent suspension culture was incubated overnight at 37^0C. The lymph node suspension was made using the same protocol, but the cells were ultimately at a lower concentration (approximately 3 x 10^6 live cells per ml) in 5 mls of the media as described below (table 4.2).

The cells were observed daily for any change in appearance or obvious outgrowth. None of the cells in DMEM medium (S4 - S6, LD, LE, LF) or the RPMI 1640/ DMEM mix (S7, S8) survived. Tables 4.3, 4.4, 4.5 and 4.6 list the observations made on the cell line over the 12 weeks it remained in

continuous culture. The cells in S1 and S2 were all dead after 12 days in culture.

Vial Name	*Medium Components*
S1	RPMI 1640, 10% FCS, 50μM βME
S2	RPMI 1640, 10% FCS, 50μM βME, 7μg/ml dextran sulphate
S3	RPMI 1640, 20% FCS, 50μM βME, 7μg/ml dextran sulphate
S4	DMEM, 10% FCS, 50μM βME
S5	DMEM, 10% FCS,
S6	DMEM, 10% FCS, 50μM βME, 7μg/ml dextran sulphate
S7	1:1 RPMI 1640:DMEM, 10% FCS, 50μM βME, 7μg/ml dextran sulphate
S8	1:1 RPMI 1640:DMEM, 15% FCS, 50μM βME, 7μg/ml dextran sulphate

Table 4.1 Spleen cell culture

Vial Name	*Medium Components*
LA	RPMI 1640, 10% FCS, 50μM βME
LB	RPMI 1640, 10% FCS, 50μM βME, 7μg/ml dextran sulphate
LC	RPMI 1640, 20% FCS, 50μM βME, 7μg/ml dextran sulphate
LD	DMEM, 10% FCS, 50μM βME
LE	DMEM, 10% FCS,
LF	DMEM, 10% FCS, 50μM βME, 7μg/ml dextran sulphate

Table 4.2 Lymph node cell culture

The survival of cells in S1, S2 and S3 is plotted on a graph (figure 4.1). After 9 days in culture an aliquot of S3 was removed and lipopolysaccharide added to it in an attempt to stimulate the growth of the cells. These cells were then labelled S3B and their growth monitored separately. The growth of the cells in S3 and S3B is plotted on a graph (figure 4.2). No obvious difference in the rate of cell growth in the presence and absence of lipopolysaccharide was noted.

Cells	*day 1*	*day 2*	*day 3*	*day 4*	*day 5*
S1	Medium yellow, resupended 10mls fresh medium	90% cell survival, 1.8 x 10^6 live cells/ml, 10mls fresh medium added	80% cell survival, 1.1 x 10^6 live cells/ml	85% cell survival, 1.2 x 10^6 live cells/ml	84% cell survival, 1.4 x 10^6 live cells/ml, 20mls fresh medium added
S2	Medium yellow, resupended 10mls fresh medium	60% cell suvival, 1.2 x 10^6 live cells/ml, 10mls fresh medium added	-	-	20mls fresh medium added
S3	Medium yellow, resupended 10mls fresh medium	75% cell survival, 3 x 10^6 live cells/ml, 10mls fresh medium added	-	-	Many smaller cells, 59% cell survival, 1.4 x 10^6 live cells/ml, 20mls fresh medium added
S4	Medium yellow, resupended 10mls fresh medium	-	Cells dying		
S5	Medium yellow, resupended 10mls fresh medium	-	Cells dying		
S6	Medium yellow, resupended 10mls fresh medium	-	Cells dying		
S7	Medium yellow, resupended 10mls fresh medium	-	Cells dying		
S8	Medium yellow, resupended 10mls fresh medium	-	Cells dying		

Table 4.3

Cells	*day 1*	*day 2*	*day 3*	*day 4*	*day 5*
LA	Medium yellow, 10mls fresh medium added	35% cell survival, 1 x 10^6 live cells/ml	Cells dying		
LB	Medium yellow, 10mls fresh medium added	30% cell survival, 1.32 x 10^6 live cells/ml	Cells dying		
LC	Medium yellow, 10mls fresh medium added	31% cell survival, 1.1 x 10^6 live cells/ml	Cells dying		
LD	-	-	Cells dying		
LE	-	-	Cells dying		
LF	-	-	Cells dying		

Table 4.4

Cells	*Day 9*	*Day 11*	*Day 12*	*Day 15*	*Day16*	*Day 17*
S1	-	very few live cells	cells dead			
S2	-	less than 8% cell survival, 8 x 10^4 live cells/ml	cells dead			
S3	Medium yellow, 72% cell survival, 2.2 x 10^6 live cells/ml, 5mls removed to separate flask (S3B), 15mls fresh medium added to remaining cells	56% cell survival, 1.4 x 10^6 live cells/ml	cells irregular shapes, but dense and medium yellow, 10 mls fresh medium added	48% cell survival, 1 x10^6 live cells/ml	60% cell survival, 1.14 x10^6 live cells/ml, 10 mls fresh medium added	40% cell survival, 8.4 x10^5 live cells/ml
S3B	Cells from S3, 5mls fresh medium added and LPS (2.5µg/ml)	65% cell survival, 0.8 x10^6 live cells/ml	-	38% cell survival, 5 x10^5 live cells/ml	-	33% cell survival, 1 x10^6 live cells/ml

Table 4.5

Cells	*Day 26*	*Day 34*	*Day 37*	*Day 47*	*Day54*			*Day 73*
S3	30% cell survival, 1.2 x 10^6 live cells/ml	Cells contaminated						
S3B	57% cell survival, 2 x 10^6 live cells/ml	8 x 10^5 live cells/ml, 20mls fresh medium added	1 x 10^6 live cells/ml, lots of dead cells, live cells growing in clumps	Cells split, 2 x15 mls removed and briefly centrifuged, supernatants removed and 5mls fresh medium added(S3Bsn), each pellet resuspended in 5mls fresh medium (S3Bp), 5mls medium added to original flask(S3Bo)	S3Bo lots of dead cells, 7 x 10^5 live cells/ ml	S3Bsn majority of cells dead	S3Bp 1 x 10^6 live cells/ ml	Aliquot of S3Bp sent to G. Klein laboratory

Table 4.6

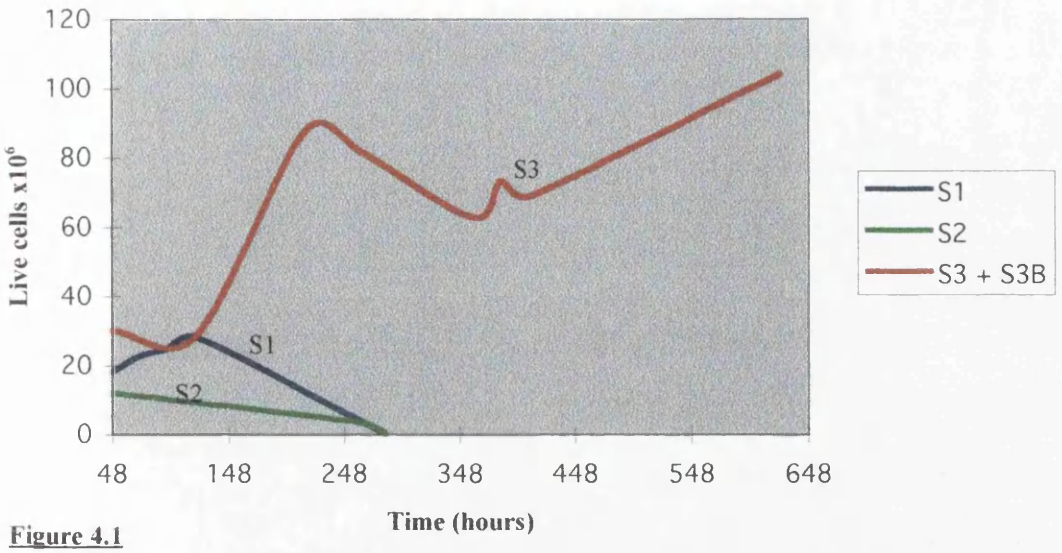


Figure 4.1

Figure 4.1
Graph showing cell survival of 26.305 splenocytes under different conditions. **S1** (RPMI 1640 + 10% FCS + 50μM βME), **S2** (RPMI1640 + 10% FCS + 50μM βME + 7μg/ml dextran sulphate), **S3** (RPMI 1640 + 20% FCS + 50μM βME + 7μg/ml dextran sulphate)

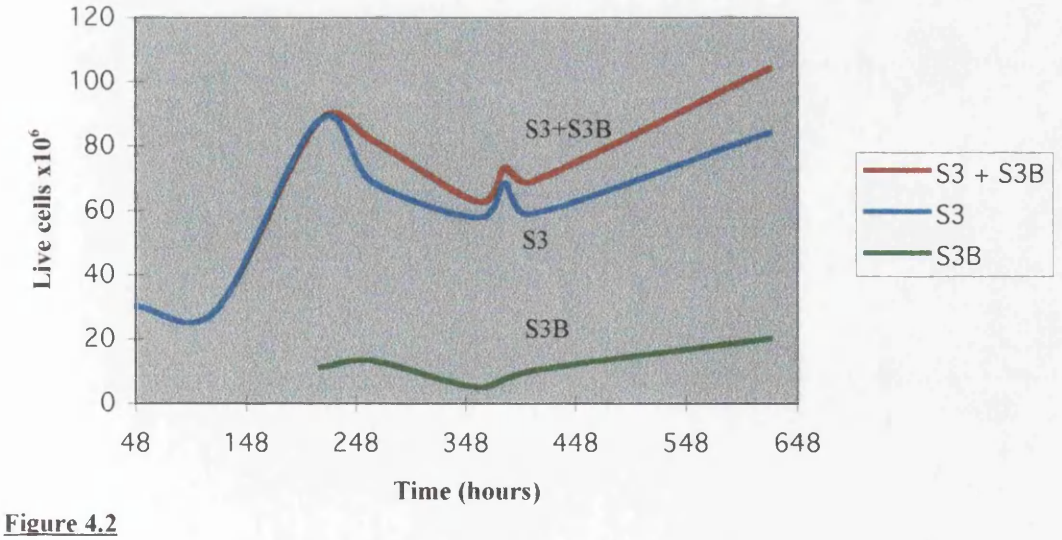


Figure 4.2

Figure 4.2
Graph showing cell survival of S3 splenocytes and the effect of LPS addition. LPS was added to an aliquot of S3 cells after 9 days in culture (S3B).

After 11 weeks in culture, the cell line derived from 26.305 splenocytes grew as a single cell suspension. An aliquot of the cells was sent to George Klein's laboratory for culture and karyotypic analysis. It was found by Western analysis to express readily detectable full length EBNA1 protein (Santiago Silva and George Klein, pers. comm.).

The cell line was maintained in continuous culture for more than three months, but ultimately could not be maintained indefinitely. It was attempted to re-establish the cell line from stocks of S3B frozen at 11 weeks, however this was unsuccessful.

4.3 Antisense Oligonucleotide inhibition of EBNA1 Expression

Analysis of the 26.305 cell line (by our collaborator Klein and colleagues) established that EBNA1 expression was maintained in the cells even after 11 weeks in culture. What function EBNA1 plays in tumourigenesis is not clear. To assess whether EBNA1 expression is required for the maintenance of tumour phenotype in culture (and therefore possibly also *in vivo*) it was attempted to use antisense oligonucleotides spanning EBNA1 coding sequences +1-+15 (EB1C) to reduce the expression of EBNA1 in cells taken from line 26 tumours. Two different line 26 spleens (26B63 and 26.463), both heavily infiltrated by tumour, were made into a cell suspension as described in materials and methods (chapter2, section2.32). After an overnight incubation, the cells were aliquoted and

oligonucleotides added directly to the growth medium as described in materials and methods (chapter2, section2.32). To control for possible non specific effects of the oligonucleotides, an EBV negative mouse plasmacytoma cell line MOPC31C was also included in the assay. In addition, the sense oligonucleotide (EB1D) should not effect EBNA1 expression within the cells and so the addition of this oligonucleotide also acts as a control for any non specific effect on cell growth or survival. The experiment was set up as illustrated in table 4.7. The survival of the cells was monitored daily. Both line 26 cell suspensions were replete with very large numbers of dead cells and cellular debris, as seen in earlier attempts to establish the tumour cells in culture. Trypan blue staining was used to ensure that only viable cells were counted. For each vial, the viable cell count was repeated at least 8 times at 24 hourly intervals and the results from the two duplicate vials were pooled and an average cell count calculated. Using the values thus obtained growth curves for each cell line have been plotted (figure 4.3). Although there is fluctuation in cell counts, there is no evidence that this is in response to the oligonucleotides in either sense or antisense orientation. The control cell line did not grow on a logarithmic scale as would have been predicted. All of the cell lines demonstrated slow growth initially. The addition of the antisense oligonucleotide may have retarded the growth of the 26B63 cells on day 3 and day 4. However this difference in growth is not at all evident by day 5 (figure 4.3b).The growth of the 26.463 cells may be marginally retarded by the presence of the antisense

VIAL NO.	A1	A2	A3	A4	A5	A6
CELLS	**MOPC31C**	**MOPC31C**	**MOPC31C**	**MOPC31C**	**MOPC31C**	**MOPC31C**
OLIGO	EB1C	EB1C	EB1D	EB1D	-	-
VIAL NO.	B1	B2	B3	B4	B5	B6
CELLS	**26B63**	**26B63**	**26B63**	**26B63**	**26B63**	**26B63**
OLIGO	EB1C	EB1C	EB1D	EB1D	-	-
VIAL NO.	C1	C2	C3	C4	C5	C6
CELLS	**26.463**	**26.463**	**26.463**	**26.463**	**26.463**	**26.463**
OLIGO	EB1C	EB1C	EB1D	EB1D	-	-

Table 4.7

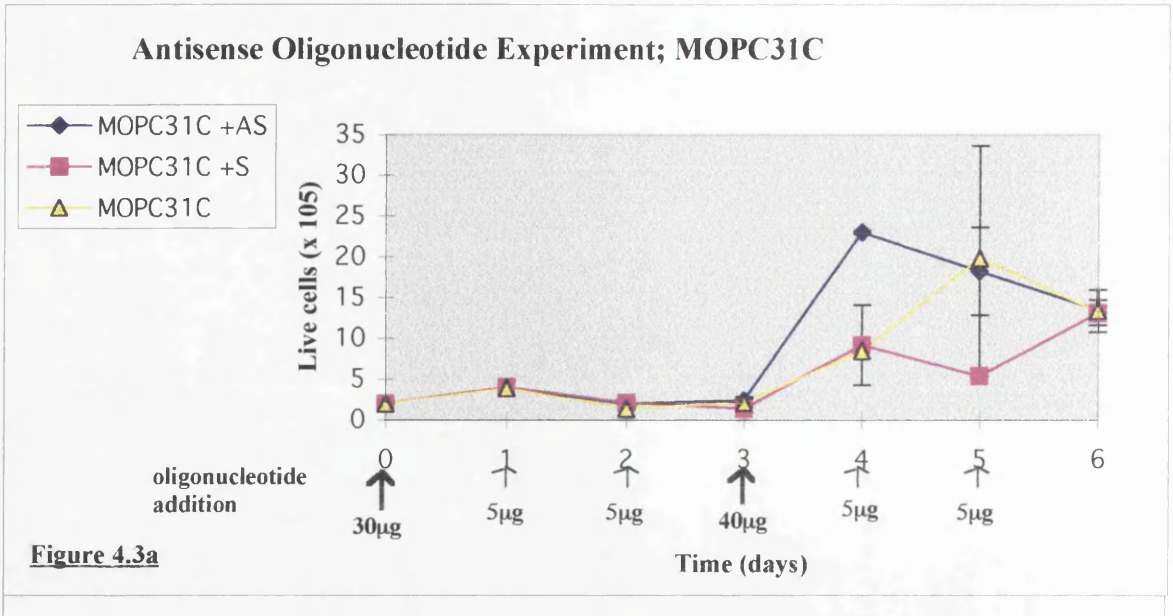


Figure 4.3a

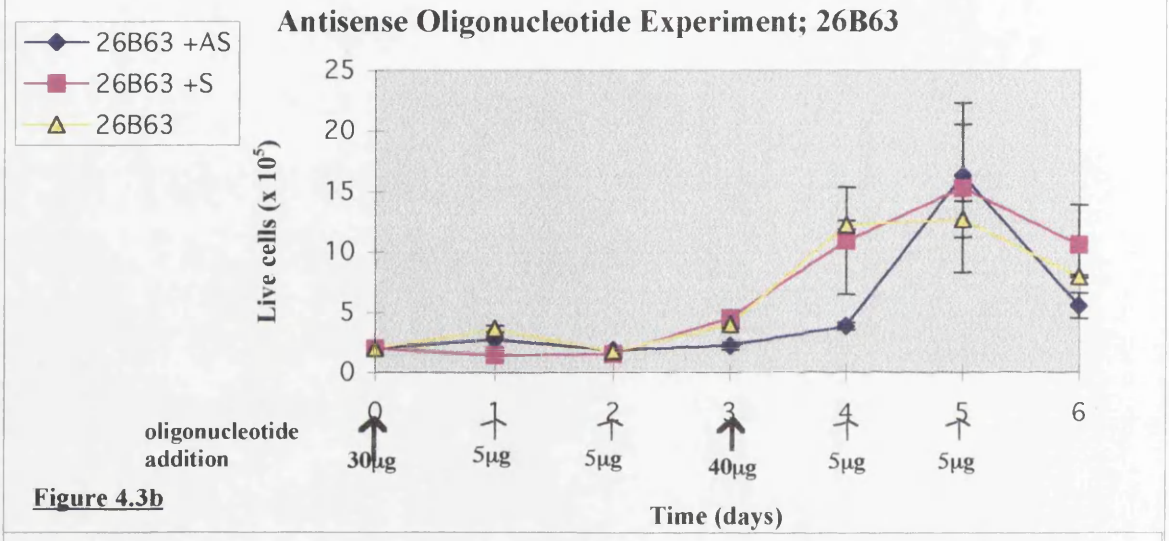


Figure 4.3b

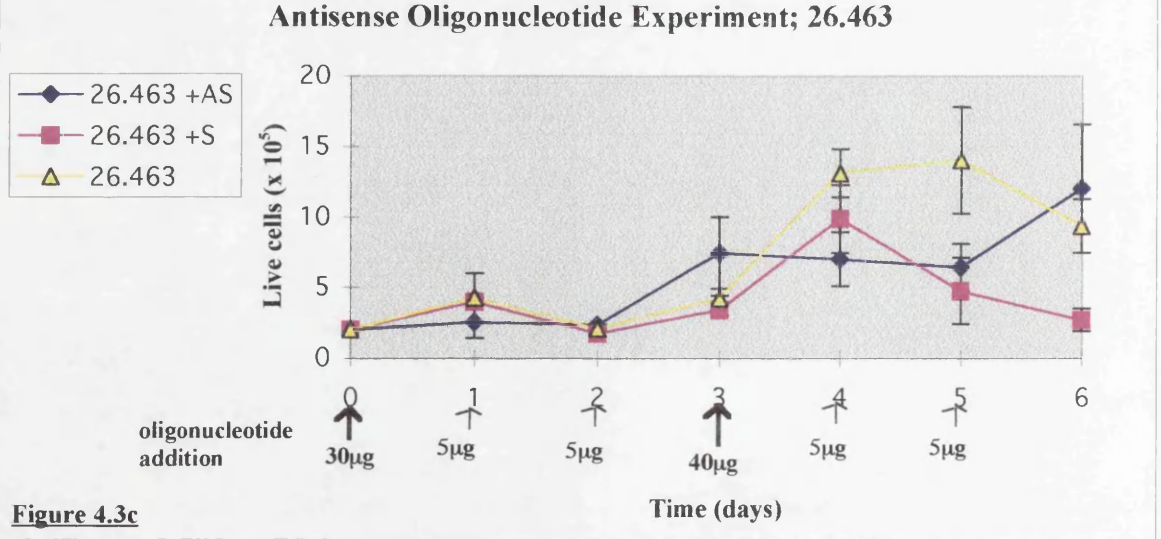


Figure 4.3c

Figure 4.3
Graphs of cell survival with and without the addition of oligonucleotides. Additions of oligonucleotides are indicated by arrows. **a** MOPC31C control cell line. **b** 26B63 splenocytes. **c** 26.463 splenocytes. **AS**; antisense oligonucleotide (EB1C). **S**; sense oligonucleotide (EB1D).

oligonucleotide on day 1 and day 4, (following large additions of the oligonucleotide) but not obviously at other time points (figure 4.3c).

4.4 Summary and Discussion

Cell lines from line 26 tumours cannot easily be established in culture using standard media and foetal bovine serum supplement. Cultures derived from the spleen survive more readily than cultures derived from lymph nodes. This may be due to the presence of additional supporting cells and growth factors in the splenic suspension. The fact that the 26.305 cells were only able to survive in 20% FCS is supportive of them requiring a high concentration of some factor present in the serum. Addition of supplementary cytokines may aid the establishment of cell lines from line 26 tumours. This approach will be used in future attempts to derive cell lines from line 26 (and line 59) tumours. Normal human B cells can be grown in culture through the addition of anti-CD40 and IL4 (Banchereau *et al.*, 1991). These could be used in future attempts to stimulate the growth of line 26 tumour cells, although anti-CD40 is not currently readily available to mouse.

Latency I Burkitt lymphoma cells, which express only EBNA1, are sensitive to apoptosis in culture. In contrast latency III Burkitt lymphoma cell lines, expressing all the EBV latent genes, are resistant to apoptosis (reviewed in introduction section 1.1) The additional expression of one or more of the other EBV latent antigens in line 26 tumour cells might increase their ability to

survive in culture. LMP-1 is an obvious target, particularly as current evidence suggests that LMP-1 may activate CD40 (reviewed in introduction section 1.1). Indeed, in the laboratory a cell line has recently been established from a tumour in a mouse carrying an Eμ-LMP-1 transgene in addition to the Eμ-EBNA1 transgene (Joanna Wilson pers comm.).

The pattern of growth of the 26.305 cell line is suggestive of additional changes having occurred in a subpopulation of cells to allow extended (if limited) survival in culture. The cells went through a number of crisis points where the number of live cells was decreasing before ultimately giving rise to a cell line growing steadily in culture. It is of interest that the expression of EBNA1 was maintained at a high level in these cells, as it is in *in vivo* passage (Wilson, Bell and Levine,1996), however, any continued function of EBNA-1 in these cells remains unclear. The tumour in mouse 26.305 was not detected until the mouse was well over a year old, older than most other line 26 mice. The tumour was well established; the spleen and lymph nodes were massively invaded. It may be that cells of this tumour carried additional genetic changes which allowed survival in culture where other line 26 tumour cells have died.

From the antisense oligonucleotide experiment attempted it is not possible to make any conclusion about the requirement for EBNA1 for cell survival *in vitro*. It is not clear whether the oligonucleotide used had a specific effect in the reduction of EBNA1 protein levels. The antisense oligonucleotide used here has been subsequently shown to have an inhibitory effect on the growth

of EBV positive cell lines (Roth, Curiel and Lacy, 1994), however this effect was only noted after seven days continuous culture in the presence of the oligonucleotide. Another oligonucleotide spanning nucleotides +16-+30 of the EBNA1 open reading frame was shown to have a much more dramatic effect on cell growth, although again only after seven days in culture. In any future experiments it might be possible to improve the downregulation of EBNA1 by using two oligonucleotides in combination. The primary difficulty in these experiments resided in the lack of established cell lines. The line 26 cell suspensions do not survive well for more than a week in culture. Even in the absence of oligonucleotide, a large proportion of the line 26 cells died over the course of the experiment. Furthermore, EBNA1 is a stable protein and prolonged exposure to the antisense oligonucleotide may be necessary to significantly decrease the absolute level of protein. To derive meaningful results from an experiment of this nature it would be necessary to use an established cell line such as that derived from 26.305 or from the crossbreeds (as mentioned above) and continue the experiment for a longer time period. Other methods of EBNA-1 inhibition could also be employed, for example the expression of potential dominant negative forms of the protein (Kirchmaier and Sugden, 1997).

Due to the difficulties in establishing a cell line from the tumours at this time and the ambiguities in the antisense experiments in primary cells, this line of investigation was not continued further.

Chapter 5

Protein DNA Binding Activity of EBNA1 and LR1 in Eμ EBNA1 Transgenic Lines

5.1 Introduction

Both protein and RNA expression analysis of EBNA1 in line 26 and line 59 have suggested that there are clear differences between the lines (chapter 3, Wilson and Levine, 1992, Wilson, Bell and Levine, 1996).

Although line 26 has the stronger phenotype, more protein is detected in line 59 by Western analysis. However, RNA expression in line 59 is not detected by Northern analysis whilst a large product is evident in line 26 samples. It is clear that the expression pattern of the transgene is complex in both lines, but not how this relates to tumourigenesis.

EBNA1 is a pleiotropic protein (see introduction section 1.3) and the mechanism by which it acts as an oncogene is not clear. It is likely that the tumourigenic action of EBNA1 requires the DNA binding activity of the protein, potentially to act as a transcriptional enhancer of cellular genes. Measuring the level of expression of EBNA1 either by protein or RNA analysis does not address the activity of the protein in the transgenic lines. Particularly given the ambiguity of expression data, it was attempted to address the activity of EBNA1 as a DNA binding protein in each of the

transgenic lines. The following section describes experiments designed to address the relative amounts of EBNA1 DNA binding activity between the different transgenic lines.

LR1 is a B cell specific, sequence specific DNA binding activity which regulates transcription in activated B cells. It consists of two polypeptides one of which has been identified as the ubiquitous protein nucleolin (Hanakahi *et al.*, 1997). Given the relationship between LR1 and c-myc in humans, and the role of LR1 in immunoglobulin isotype class switching (see introduction section 1.7b), this protein could well have a role in development of Burkitt's lymphoma. To investigate whether this protein has a role in EBNA1 induced tumours, its DNA binding activity in nuclear extracts from transgenic tissues has been investigated.

The levels of both EBNA1 and LR1 DNA binding activities were investigated using gel retardation assays. In these assays the protein of interest is allowed to form a complex with a labelled duplex oligonucleotide containing the DNA sequence of the protein binding site of interest. Complexes are separated by electrophoresis and analysed by autoradiography.

5.2 EBNA1 DNA Binding activity in Transgenic Mouse Lines

A series of preliminary experiments was carried out to address the nature and specificity of complexes detected using mouse splenic extracts on the EBNA1 DNA binding site oligonucleotide. This was particularly important given that

data published on EBNA1 binding activity has all been collected from human cell protein extracts, cell lines, or bacterially expressed EBNA1 and the binding activity of mouse tissue protein extracts on an EBNA1 binding site has not been addressed.

Protein extracts were prepared from whole spleens as described in Materials and Methods (chapter 2, section 2.30). The EBNA1 DNA binding site oligonucleotide used in binding assays contains a single copy of the EBNA1 binding site. All binding reactions contained poly dIdC as a competitor for non sequence specific DNA binding proteins.

Two different approaches were used to address the specificity of complexes detected. Firstly, specific and non specific unlabelled double stranded DNA oligonucleotides were included in the binding reactions as competitors for binding to the labelled probe (figure 5.1). In this experiment an extract from an EBNA-1 positive line 26 mouse spleen (26.505) which was invaded with lymphoma was used. A number of complexes are seen in the absence of a competitor oligonucleotide (lane 1, numbered 1-5). In lanes 2, 3 and 4, increasing amounts of the unlabelled EBNA1 binding site oligonucleotide was added as a specific competitor for binding. Even at the lowest concentration, this oligonucleotide clearly competes out complex 1. In addition, complex 2 is depleted by the presence of this competitor. In lanes 5,6 and 7, increasing amounts of the unlabelled oligonucleotide containing the LR1 binding site was used as competitor. This oligonucleotide does not compete well for complex 1 binding, although it does deplete complex 2 to a similar extent as the first

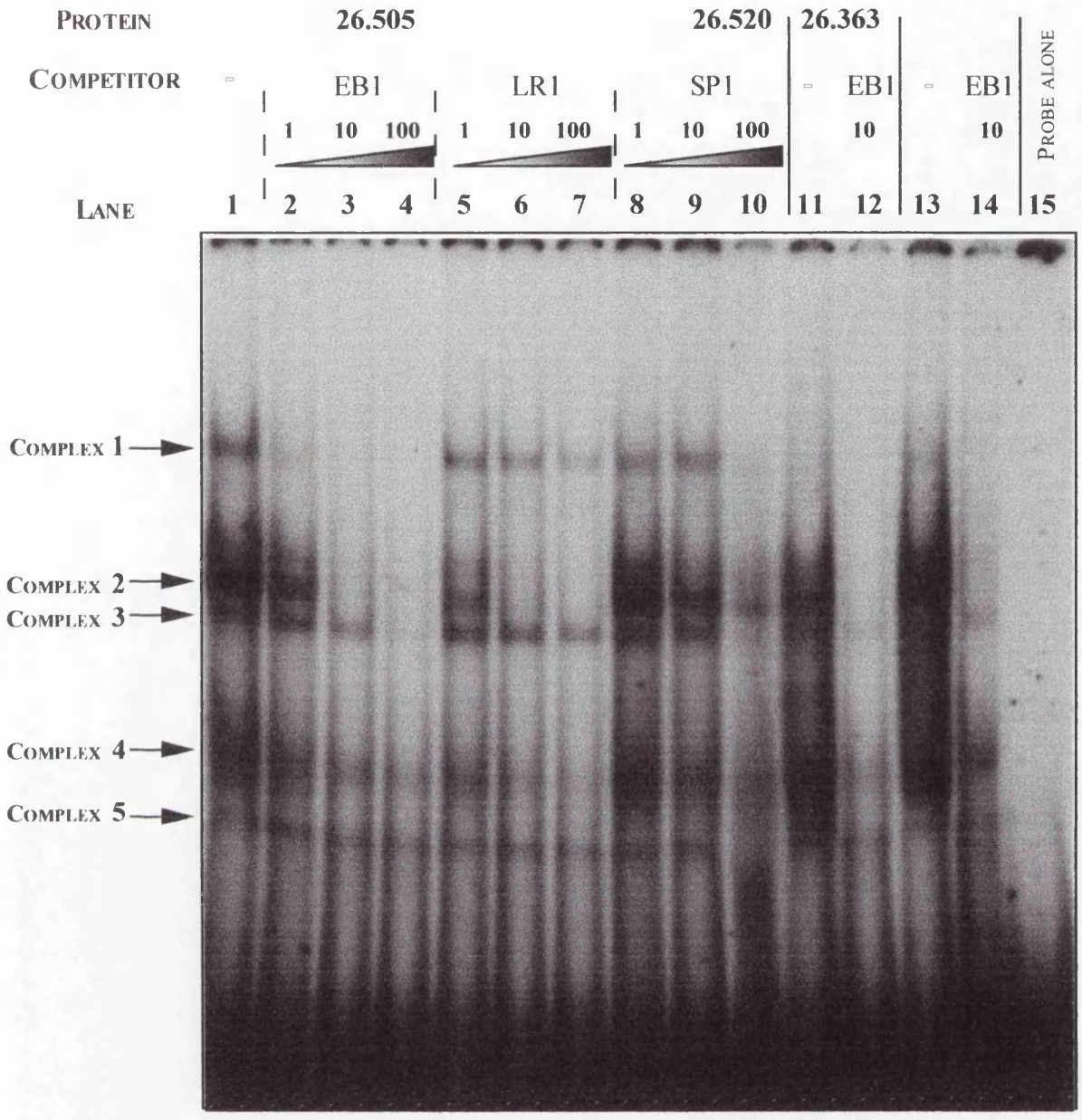


Figure 5.1

Gel retardation assay of DNA/protein complexes. Nuclear extracts from line 26 spleens were incubated with a labelled EBNA-1 binding site oligonucleotide and electrophoresed on a 4% polyacrylamide gel @200V, as described in Materials and Methods. Lanes 1-10 contain splenic protein extract from mouse 26.505, lanes 11-12 contain splenic protein extract from mouse 26.520, lanes 13-14 contain splenic protein extract from mouse 26.363. In lane 15 no protein extract was added. In lanes 2-4 the unlabelled EBNA-1 binding site oligonucleotide was added as a specific competitor for EBNA-1 binding at 1, 10 and 100 fold molar excess over probe respectively. In lanes 5-7 unlabelled LR1 binding site oligonucleotide was added at 1, 10 and 100 fold molar excess over probe respectively. In lanes 8-10 unlabelled SP1 binding site oligonucleotide was added at 1, 10 and 100 fold molar excess over probe. Arrows indicate protein DNA complexes detected.

oligonucleotide. In lanes 8,9 and 10, an oligonucleotide containing an Sp1 binding site was used as a non-specific competitor. This oligonucleotide only has an effect on the complexes at the highest concentration. At 100 fold excess (lane 10), the levels of all of the complexes appear reduced.. There is no apparent sequence homology between the EBNA1, LR1 and SP1 binding site oligonucleotides. Complexes 3,4 and 5 appear to be relatively non specific in their binding; they are only minimally affected by any of the competitors used. These results indicate that the most specific complex is complex 1. This complex is effectively competed for by the unlabelled probe fragment although it is not affected by either of the other two competitors used. The LR1 oligonucleotide competes as well as the EBNA1 oligonucleotide for complex 2, although this complex is not affected by the SP1 oligo.

In order to determine whether any of the complexes detected contain EBNA1, a second approach was taken. Antibodies to EBNA1 were included in the reaction mixture. EBNA1 containing complexes should be further retarded on electrophoresis.

As above, in the following assays an extract from a line 26 mouse spleen (26.505) was used, as well as two further line 26 EBNA-1 positive extracts (26.520 and 26.363). In initial experimients (figure 5.2), the antibody was added to the binding reaction after the protein had been exposed to the oligonucleotide. A further incubation period was allowed for antibody binding prior to electrophoresis of the reactions. Increasing amounts of the monoclonal antibody Aza2E8 does not appear to affect the position or intensity of any of

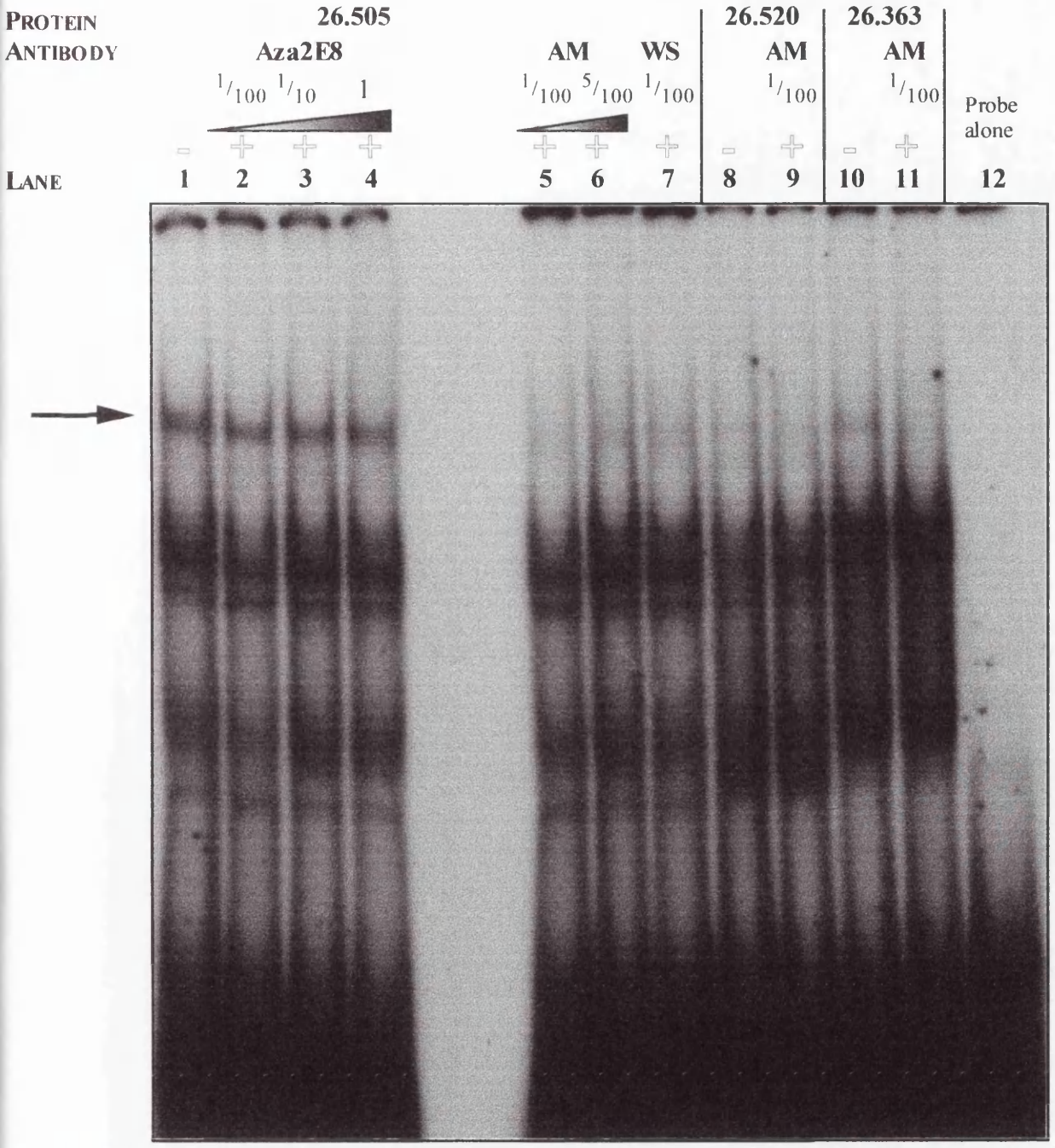


Figure 5.2

Supershift assay. Nuclear extracts from line 26 spleens were incubated with a labelled EBNA-1 binding site probe, after 20 mins anti EBNA-1 antibodies were added, after a further 20 mins the reactions were electrophoresed on a 4% polyacrylamide gel @ 200V, as described in Materials and Methods. Lanes 1-7 contain splenic protein extract from mouse 26.505, lanes 8-9 contain splenic protein extract from mouse 26.520, lanes 10-11 contain splenic protein extract from mouse 26.363. In lane 12 no protein extract was added. + -antibody added, - -no antibody. Aza2E8 -mouse monoclonal antibody, AM -human serum 1, WS -human serum 2. The various dilutions of antibodies used are indicated. The EBNA-1 specific complex is indicated by the arrow.

the detected complexes (lanes 2,3 and 4). However, increasing concentrations of the EBV positive human serum AM depletes complex 1 (lanes 5 and 6). A different EBV positive human serum, WS, was added to the reaction which also depletes the level of complex 1 (lane 7).

The fact that both human sera deplete complex 1 suggests that this complex contains EBNA1. It is possible that the sera react with a mouse protein specifically upregulated in line 26, but the apparent mobility of the complex correlates well with published data on EBNA1 binding (Polvino-Bodnar and Schaffer, 1992).

Aza2E8 does not appear to react with EBNA1 from this line 26 splenic extract. This could suggest that the protein is in a form that the monoclonal does not recognise, or that the antibody is unable to recognise EBNA1 which is bound to DNA. In order to address this the protein extract was incubated with the oligonucleotide probe and the antibody simultaneously (figure 5.3). In this case the presence of the antibody does deplete complex 1. This suggests that the monoclonal does not recognise EBNA1 which is already bound to DNA. It is believed that Aza2E8 may bind to EBNA1 at or near the DNA binding domain (Orlowski *et al.*, 1990).

Taken together the results of the above experiments suggest that complex 1 contains EBNA1.

Having determined that EBNA1 DNA binding activity is detectable in line 26 extracts, the relative levels of EBNA1 DNA binding activity between the different transgenic lines was compared (figure 5.4a and 5.4b). The extracts

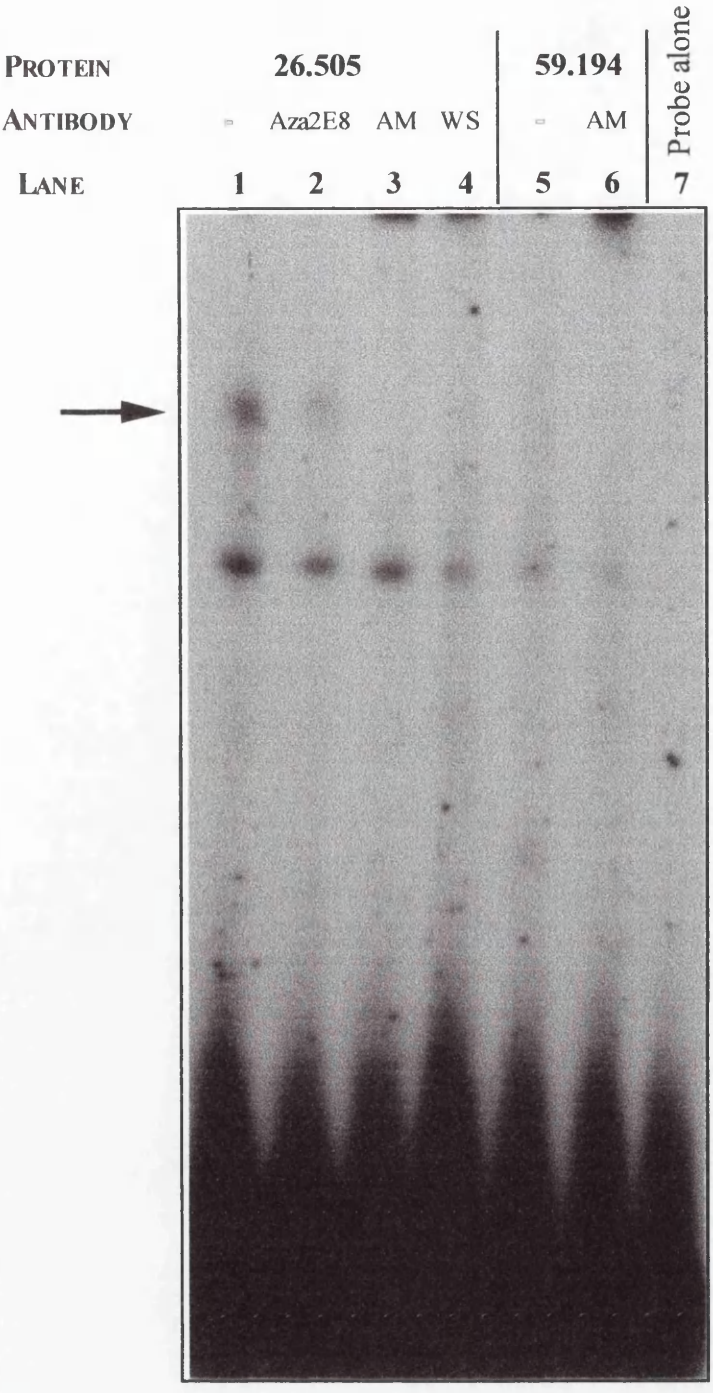


Figure 5.3

Supershift assay. Nuclear extracts and antibodies were incubated with a labelled EBNA-1 binding site probe for 40 mins and then electrophoresed @200V on a 4% polyacrylamide gel as described in Materials and Methods. Lanes 1-4 - splenic protein extract from mouse 26.505, lanes 5-6 -splenic protein extract from mouse 59.194, lane 7 -no protein extract. In lanes 1 and 5 no antibody was included. Antibody was included in lanes 2-4 and lane 7. Aza2E8 -mouse monoclonal antibody, undiluted; AM -human serum 1, 1:100 dilution; WS - human serum 2, 1:100 dilution. Arrow indicates EBNA-1 specific complex.

compared were each prepared from frozen spleen samples as above. An equivalent amount of protein was used in each reaction. In order to compare the integrity of the extracts, in addition to addressing the protein binding to the target EBNA1 binding site, extracts were analysed for their ability to bind to an Sp1 binding site. Sp1 was chosen because it is a ubiquitously expressed transcription factor and it was anticipated that the level of this protein is equivalent between the transgenic and normal spleens.

Figure 5.4a shows a direct comparison of a line 26 (26.460), a line 59 (59.196) and a negative (59.223) extract (lanes 1,2 and 3), respectively. Complex 1, the EBNA1 containing complex, is detectable in the line 26 sample albeit at a low level (marked with an arrow on figure 5.4a). No EBNA1 containing complex is detected in either the line 59 or the negative sample. This lack of detection of EBNA1 in the line 59 sample is unlikely to be due to poor integrity of the protein extract as the Sp1 binding activity in the line 59 extract is at least equivalent to that seen in the line 26 extract (figure 5.4b lanes 1 and 2).

Increasing the amount of protein added and the total reaction volume does not greatly enhance the binding detected (figure 5.4a lanes 4,5 and 6).

A selection of different line 26 extracts were also compared (figures 5.4a, lanes 7-10). In this experiment complex 1 is only detected in the sample 26.363 (lane 9). However, the samples 26.534 (lanes 7 and 8) and 26.520 (lane 10) have reduced Sp1 binding activity (figure 5.4b). In lanes 7 and 8 the amount of complex 1 appears reduced and the levels of complexes 3 and 4 increased, suggestive of protein degradation. Two different line 59 extracts, 59.140 and

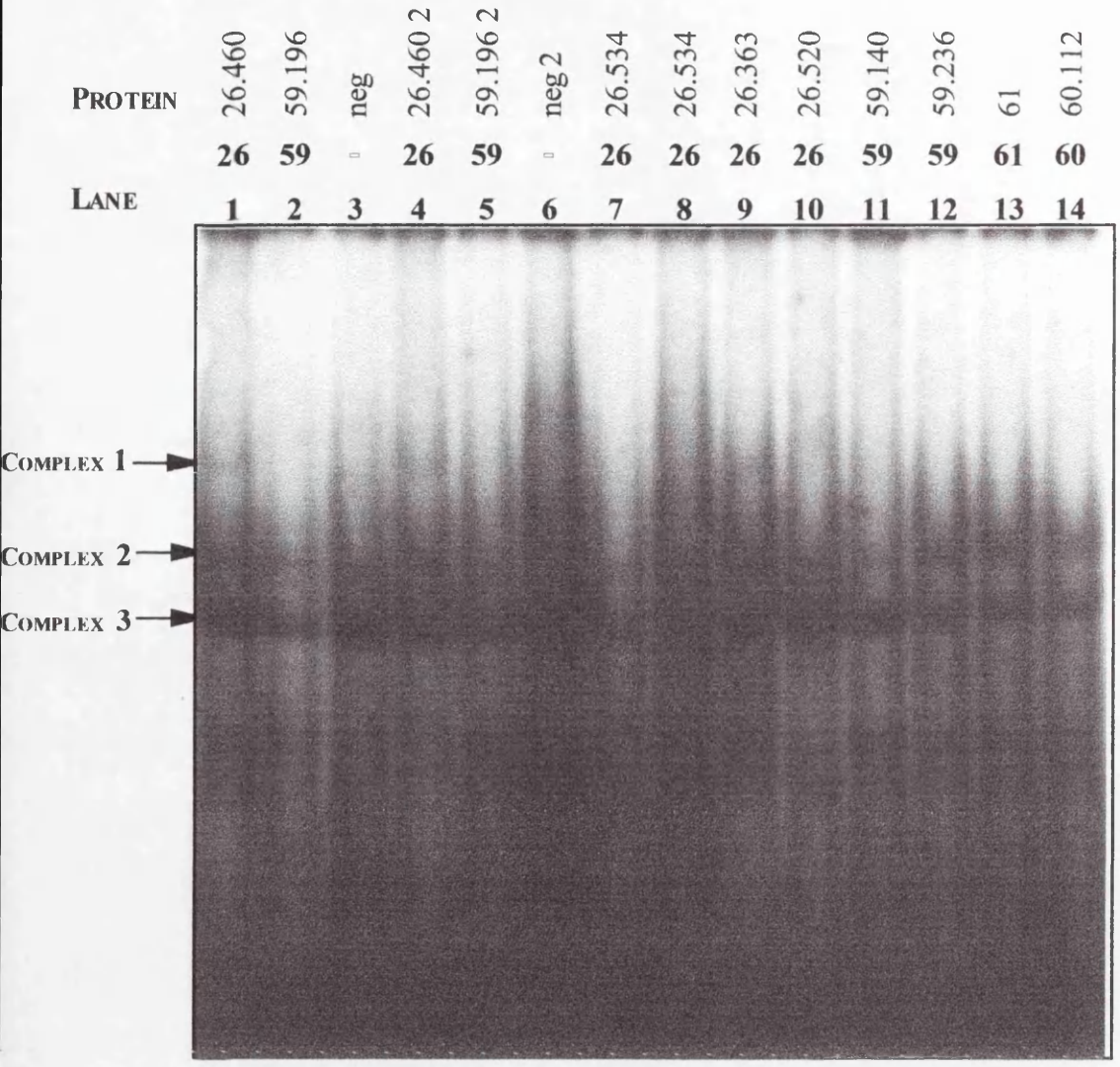


Figure 5.4a

Gel retardation assay. 2μg total protein of each of various splenic nuclear extracts were incubated with a labelled EBNA-1 binding site oligonucleotide and electrophoresed on a 4% polyacrylamide gel at 200V, as described in Materials and Methods. In lanes 4-6 the reaction volume was doubled, and 4μg total protein of each splenic extract as added. 26 - protein extract from line 26 mouse, 59 - protein extract from line 59 mouse, 60- protein extract from line 60 mouse, 61- protein extract from line 61 mouse, - - protein extract from a transgenically negative mouse. Arrows indicate protein DNA complexes detected.

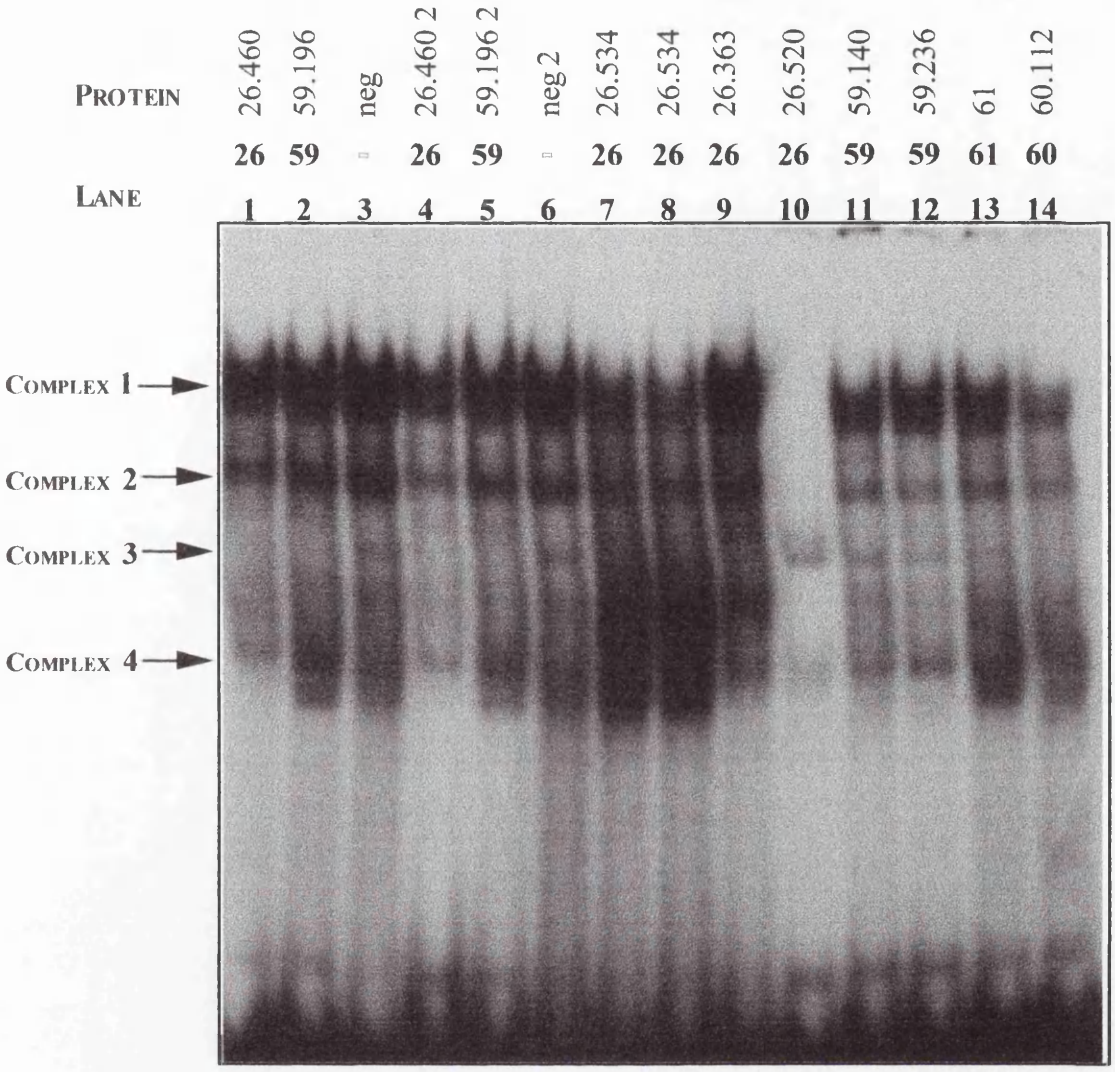


Figure 5.4b

Gel retardation assay. 2μg total protein of each of various splenic nuclear extracts (as in fig 5.4a) were incubated with a labelled SP1 binding site oligonucleotide and electrophoresed on a 4% polyacrylamide gel at 200V, as described in Materials and Methods. In lanes 4-6, 4μg of total protein of each extract was added. 26 - protein extract from line 26 mouse, 59 - protein extract from line 59 mouse, 60- protein extract from line 60 mouse, 61- protein extract from line 61 mouse, - protein extract from a transgenically negative mouse. Arrows indicate protein DNA complexes detected.

59.236 (lanes 11 and 12), a line 61 extract, 61.53(lane 13) and a line 60 extract, 60.112 (lane 14) were also assayed. None of these samples has any detectable EBNA1 DNA binding activity.Using several different extracts from different line 59 animals, no EBNA1 DNA binding activity has been detected. The level of EBNA1 binding activity detected in line 26 samples is low and is acutely dependent on the quality of the protein extract.

The tumour status of the tissues may also be relavent to the detection of EBNA-1 DNA binding activity. All of the line 26 tisssues used were invaded by lymphoma. The line 59 samples were pre-neoplastic.

5 .3. LR1 DNA Binding Activity in EBNA1 Transgenic Mice

The ability of the LR1 binding site oligonucleotide to compete for complex 2 bound to an EBNA1 binding site oligonucleotide (figure 5.1 lanes 5,6 and 7) prompted further investigation of LR1 DNA binding activity in transgenic protein extracts.

As yet no antibody to LR1 is available, therefore it has only been possible to address the specificity of complexes detected with an oligonucleotide containing an LR1 binding site by competition experiments. A line 26 protein extract from a mouse spleen, 26.460, which was invaded with lymphoma (figure 5.5) was used to address the specificity of complexes detected. A number of complexes are detected, labelled A to D (marked with arrows on figure 5.5). Complex C is a very diffuse band(s) in this experiment and has

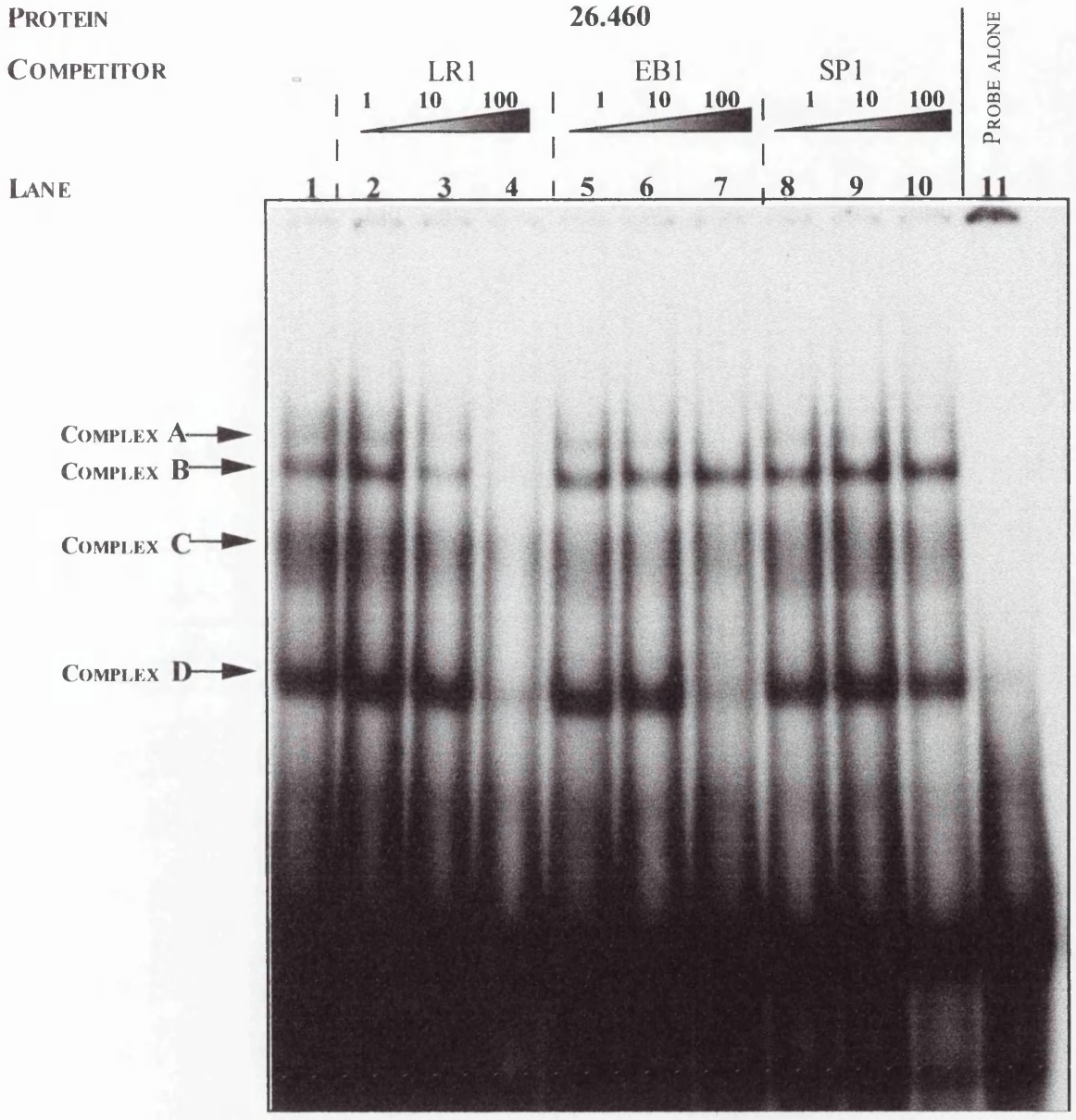


Figure 5.5
Gel retardation assay. Nuclear extract from line 26.460 spleen was incubated with a labelled LR1 binding site oligonucleotide and electrophoresed on a 6% polyacrylamide gel @200V, as described in Materials and Methods. In lane 11 no protein extract was added. In lanes 2-4 the unlabelled LR1 binding site oligonucleotide was added as a specific competitor for LR1 binding at 1, 10 and 100 fold molar excess over probe respectively. In lanes 5-7 unlabelled EBNA-1 binding site oligonucleotide was added at 1, 10 and 100 fold molar excess over probe respectively. In lanes 8-10 unlabelled SP1 binding site oligonucleotide was added at 1, 10 and 100 fold molar excess over probe. Arrows indicate protein DNA complexes detected.

been variably detected in other experiments. In lanes 2, 3, and 4 an increasing amount of the unlabelled LR1 binding site oligonucleotide has been added to the reactions. This appears to compete most effectively for complexes A and B, and for complexes C and D only at the highest concentrations of competitor. Increasing concentrations of the unlabelled EBNA1 binding site oligonucleotide was also included as a competitor in this assay (lanes 5, 6 and 7). Complex A and notably complex D appear to be depleted by high concentrations of this competitor. Complexes B and C do not appear to be effected. The addition of increasing amounts of the unlabelled Sp1 binding site oligonucleotide as a competitor appears to have a minimal, if any, effect on all of the complexes even at its highest concentration(lanes 8,9 and 10).

From this experiment it appears that the complex with the highest specificity for the LR1 binding site is complex B. It is probable that this complex contains LR1. The nature of the protein content of the other complexes is not known. From this result, in combination with the EBNA1 competition experiment (figure 5.1, lanes 5,6 and 7), it appears that there is a mouse protein or proteins (detectable in both transgenic and negative protein extracts), which bind semi-specifically to both EBNA1 and LR1 binding site oligonucleotides. The nature of this protein or proteins is not known, nor is the relevance to the observed transgenic phenotype.

If LR1 has a role to play in EBNA1 induced tumourigenesis, it may be that the overall level, or the activity of the protein, is increased in transgenically

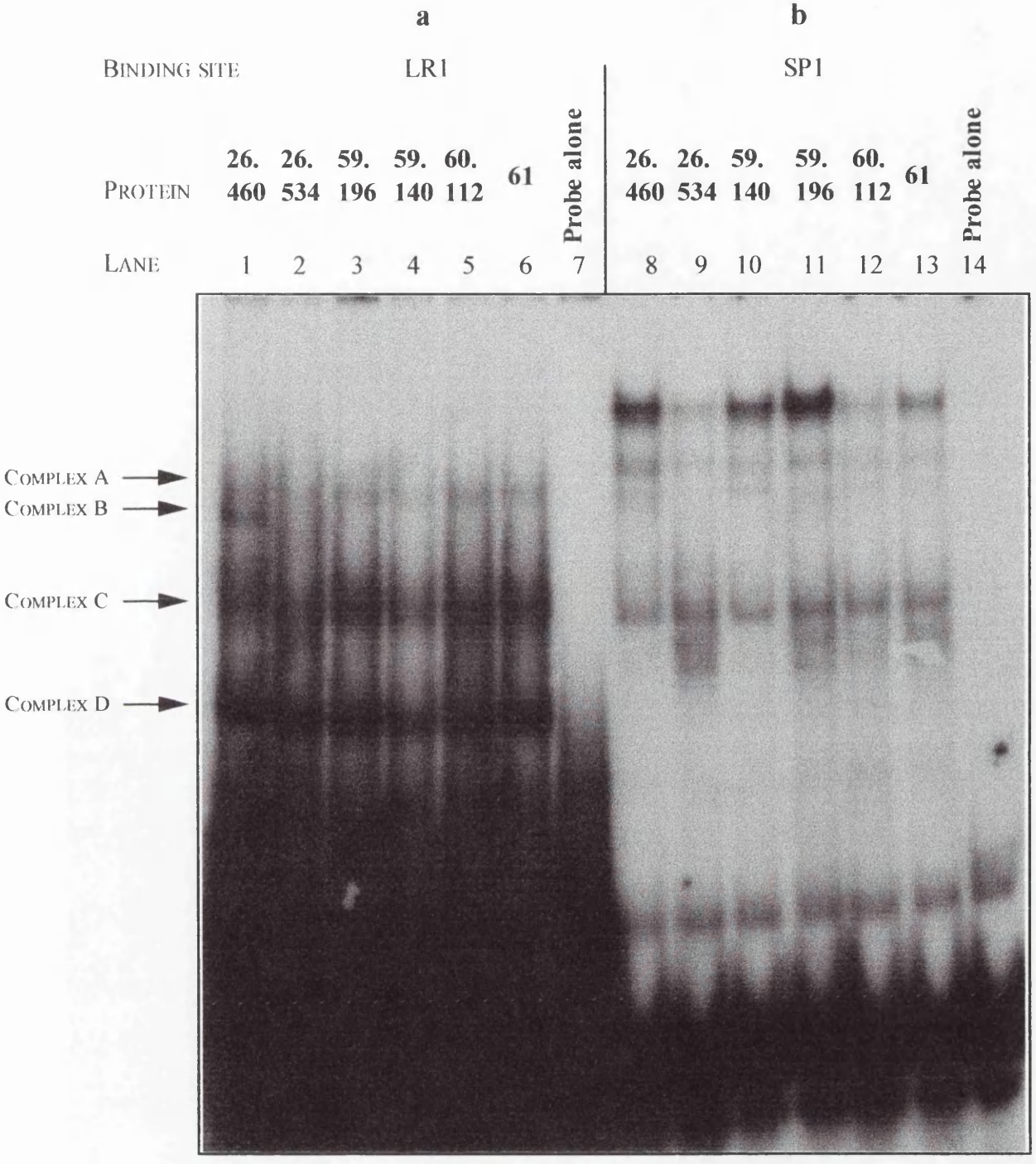


Figure 5.6

Comparison of the DNA binding activity of various splenic protein extracts to a labelled LR1 binding site probe (lanes 1-7) and a labelled SP1 binding site probe (lanes 8-14). 2μg total protein of each protein extract was incubated with the appropriate probe for 20mins and then electrophoresed in a 6% polyacrylamide gel at 200V, as described in Materials and methods. The mouse from which protein was prepared is indicated. Arrows indicate the complexes detected with the LR1 binding site probe.

positive animals. In order to address this a number of different splenic protein extracts from line 26, line 59 and negative animals have been compared for levels of LR1 DNA binding activity (figure 5.6a). As a control for the integrity of the protein extracts, Sp1 DNA binding activities have simultaneously been compared (figure 5.6b). As described above, Sp1 binding is used as a control because it is ubiquitously expressed. Although complexes are detected with the LR1 binding site using each extract, only the line 26 sample, 26.460 (figure 5.6a, lane 1) gives a clear complex B. The Sp1 activities of the two line 59 samples, 59.140 and 59.196, are similar to line 26.460 Sp1 activity, however there is no evidence of complex B with either extract (compare lanes 1, 3 and 4 with lanes 8, 10 and 11 in figure 5.6).

Mouse 26.460 had a lymphoma, none of the other tissues used in this experiment were neoplastic.

That LR1 DNA binding activity (complex B) is not detectable in any of the extracts other than 26.460 is suggestive that the protein is more active or present at a higher level in this spleen than in the others looked at.

5.4 Summary and Discussion

The protein/DNA binding studies using an EBNA-1 binding site allowed detection of a line 26 specific complex. The apparent mobility, specificty of binding and recognition of this complex by EBV positive human antisera suggest that it contains EBNA-1. Given this, it appears that the level of

EBNA1 protein DNA binding activity detected correlates directly with the degree of phenotype in the two lines. EBNA1 DNA binding activity is only detectable in line 26, in which 100% of positive mice succumb to monoclonal B- cell lymphoma, while any EBNA1 DNA binding activity in line 59 samples is below the resolution of the assay used. This result correlates with the level of EBNA1 RNA detected by Northern analysis in line 26 and line 59 (chapter 4, Wilson, Bell and Levine, 1996). However it should be noted that there was no evidence of tumour in any of the line 59 samples used in these experiments, whereas the line 26 spleens were all invaded by lymphoma. Therefore it may be that EBNA-1 DNA binding activity is enhanced in tumours, and this could also apply to line 59 tumours. Although normal peripheral blood cells carry 2-3 subtypes of EBV with EBNA-1 sequence differences, in Burkitt's lymphoma cells only one subtype is detected in each tumour, and an EBNA-1 variant V-leu is only detected in BL cells.(Bhatia *et al.*, 1996) These sequence variants may be more active in oncogenesis. Future experimental work should compare the EBNA1 DNA binding activities found in pre-tumour and tumour tissues of both lines. It would also be valuable to look at the sequence of EBNA-1 in pre-tumour and tumour tissues.

If the binding of EBNA1 to DNA is a required function for the protein in its tumourigenic role, then this difference in the detected activity may explain the phenotypic differences between the two lines.

The family of repeats unit of oriP has been shown to act as an enhancer in the presence of EBNA1. This activity requires the DNA binding activity of

EBNA1 (reviewed in introduction section 1.3). The oncogenic activity of EBNA1 could be due to transcriptional transactivation or repression of murine genes.

There is a clear discrepancy between the amount of protein detected in both lines by Western analysis (Wilson, *et al.*, 1996) and the amount of detectable DNA binding activity. There are a number of potential explanations for the discrepancy between detectable protein and detectable DNA binding activity. It may be that, when expressed to a high level, EBNA1 protein dimerises or multimerises even in the absence of DNA binding. EBNA1 binds to DNA co-operatively and linkage can occur between bound EBNA1 protein dimers (Frappier and O'Donnell, 1991, Middleton and Sugden, 1992, Su *et al.*, 1991, Mackey *et al.*, 1995). If this can also occur when the protein is in an unbound state, then it may be that EBNA1 protein in line 59 is formed into assemblies which are less readily available to bind to DNA.

In the experiments described here, nuclear protein extracts were prepared and used. It has been assumed that EBNA1 protein would be nuclear. There is no direct evidence for this. It may be that the protein expressed in line 59 is not imported into the nucleus, perhaps due to a mutation within the transgene sequences or to the effect of cellular sequences in the expressed transcripts (chapter 3). If this is the case, it may be possible to detect EBNA1 by Western analysis of the cytoplasmic fraction. It was attempted to address this using immunostaining of spleen and liver tissue sections from line 26, line 59 and negative mice. (See Materials and Methods). However, in a preliminary

analysis using a number of different techniques no EBNA1 staining of either line 26 or line 59 tissues was detected. These experiments did not include any slides of EBV positive cell lines as a positive control for the assay. Given the limitations of time for completion of the PhD thesis, this work was not completed. Further pursuit of this line of experimentation would be valuable.

It is possible that the difference in the amount of protein detected in tissues in each of the lines does not reflect the true level of protein expressed, but is a reflection on the ability of the anti EBNA1 antisera to recognize differing states of EBNA1. In order to detect EBNA1 protein on Westerns, it was first necessary to carry out an immunoprecipitation on cellular extracts. It may be that there is EBNA1 protein which is not recognized by the antisera used and therefore the apparent levels of EBNA1 may not be the true total levels.

Perhaps the results of the Westerns do give a true indication of the levels of EBNA1 protein, but the protein in line 59 is less active or alternatively, the protein in EBNA1 induced tumours is more active (since protein activity in pre-tumour line 26 samples has not been addressed). There may be differences in the phosphorylaion status of the protein in either line. The integrated transgene has not been sequenced in either transgenic line, therefore it is not possible to rule out the presence of mutations within the transgene in either line. However, a preliminary SSCP analysis of genomic DNA of the lines has been carried out to screen for mutations and none were detected (Gordon and Wilson, unpublished results).

In addition to EBNA1, the gel retardation assays performed revealed a number of mouse cellular factors which bind to the EBNA1 DNA binding site with varying degrees of specifity. In human cell extracts, cellular factors have been identified which bind to oriP and can compete with EBNA1 for binding (Wen *et al.*, 1990, Oh *et al.*, 1991, Zhang and Nonoyama, 1994). These proteins may play a role in the replication or maintenance of episomes of DNA from oriP, or in the transcriptional transactivation by EBNA1. The cellular factors detected in the current assays may represent the mouse equivalent of the human proteins.

There would appear to be a factor(s) present in mouse nuclear protein extracts which can bind both the LR1 DNA binding site and the EBNA1 DNA binding site (figure 5.1 complex 2, figure 5.5 complex D). This factor may have a role to play in the modulation of EBNA1 activity. Nucleolin, one of the components of LR1, has been proposed to play a role in the structural organisation of S region DNA during recombination (Hanakahi *et al.*,1997) . Perhaps nucleolin or a related factor has a role to play in the organisation of oriP. Supershift assays using an antibody to nucleolin would identify whether nucleolin is a component of any of the complexes detected with either the EBNA-1 or the LR1 binding site probes.

LR1 has been proposed to have a role in the deregulated expression of c-myc in Burkitt's lymphoma cells and has multiple binding sites within the immunoglobulin heavy chain locus (Brys and Maizels, 1994). If the expression or activity of this protein is altered by the presence of EBNA1

within cells, then this could lead to changes in c-myc expression which might be oncogenic. In at least one line 26 tumour there is an elevated protein binding activity to an LR1 binding site. LR1 is known to be induced by LPS stimulation of resting B-cells (Williams and Maizels, 1991). The induction is relatively slow and parallels the induction of switch recombination. It is not clear whether the presence of an LR1 binding activity in a line 26 tumour protein extract represents an activity required for tumour status or is a refection of the actively cycling or differential status of tumour cells. In the line 59 and negative extracts which are non tumour the number of actively cycling cells is likely to be lower. An analysis of LR1 binding activities in line 26 extracts from pre-neoplastic and early neoplastic tissues would indicate whether the detection of this activity is common in line 26 tissues and whether its presence precedes other neoplastic events.

LR1 activity has been shown to increase c-myc expression in both EBV positive and EBV negative lymphoma cell lines (Brys and Maizels, 1994). However, the degree of upregulation of expression is greater in the EBV positive cell line (5.5 fold in EBV positive cell line, 3.8 fold in EBV negative cell line). Perhaps the presence of EBNA1 is a factor in this difference.

The pattern of expression of LR1 protein has led to the proposal that it may be directly involved in the recombination of the immunoglobulin loci in B-cells (Williams and Maziels, 1991, Brys and Maizels, 1994). If this is in fact the case, it is possible to speculate, given the large concentration of LR1 binding sites at the c-myc gene locus as well as at the immunoglobulin loci, that this

protein may facilitate improper recombination events between these two loci giving rise to the translocations associated with Burkitt's lymphoma.

Chapter 6

Co-operating Cellular Factors in Eμ EBNA-1 induced Lymphomas

6.1 Introduction

Although two different lines of EBNA-1 transgenic mice succumb to B-cell lymphoma, all the tumours are monoclonal (Wilson and Levine, 1992, Wilson, *et al.*, 1996). The expression of EBNA-1 alone is not sufficient therefore for malignant conversion of a B cell. The following sections describe a number of experiments designed to investigate what cellular factors play a role in tumourigenesis and maintenance in concert with EBNA-1 in these transgenic mice. Identification of factors which co-operate with EBNA-1 may also shed light on the mechanism by which EBNA-1 acts in tumourigenesis.

Rearrangements of one or several known proto-oncogenes may be involved in the EBNA-1 induced lymphomas. Given the association between *c-myc* and EBNA-1 in Burkitt's lymphoma, the *c-myc* locus is a strong candidate for rearrangement in EBNA-1 induced lymphomas. The rearrangement could take a number of forms including translocation, chromosomal amplification or deletion and point mutations. Local DNA rearrangements can be detected by Southern analysis. Point mutations are often not detected by this technique. Single stranded conformational analysis (SSCP) is a powerful technique for the detection of point mutations. Karyotyping of tumour cells allows detection of

gross chromosomal rearrangements such as translocations and changes in ploidy.

Eμ EBNA-1 transgenic mice are remarkably similar in phenotype to those described in Eμ-*c-myc* transgenic mice (Adams *et al.*, 1985). This raises the possibility that the EBNA-1 phenotype is caused by a direct action of EBNA-1 on *c-myc* expression. In order to address this, the levels of *c-myc* RNA expression have been analysed in both pre-neoplastic and neoplastic tissues from line 26 and line 59 mice compared to transgenically negative controls.

To look directly for a co-operative effect in lymphomagenesis between deregulated *c-myc* expression and the presence of EBNA-1, an attempt was made to cross Eμ-*myc* transgenic mice with the EBNA-1 transgenic lines. In another approach, injection of mineral oil into certain substrains of Balb/c mice causes the development of plasmacytomas bearing a *c-myc* translocation similar in nature to that found in sporadic BL (introduction section 1.2, 1.6d). This protocol has been utilised to attempt to induce *c-myc* translocations *in vivo* in Eμ EBNA 1 mice. Both the rate of onset of tumourigenesis and the localisation of any *c-myc* translocations could be effected in transgenic versus negative animals.

A number of proto-oncogenes have been identified which cause lymphomagenesis in transgenic mice when the expression of the gene is directed by the immunoglobulin heavy chain enhancer (reviewed in Adams and Cory, 1991 and introduction section 1.4, 1.8). Both *bcl-2* and *pim-1* are proto-

oncogenes which have been shown to cooperate with *c-myc* when the transgenic mice in the separate lines have been crossbred (reviewed in introduction section 1.8). We have crossed both line 26 and line 59 mice with Eμ *bcl-2* mice and Eμ *pim-1* mice in order to investigate to which oncogene complementation group EBNA-1 might belong.

6.2 The Role of *c-myc* RNA expression in EBNA-1 Induced Lymphomas

In cell lines and tumours in which *c-myc* expression is deregulated, the level of *c-myc* RNA is upregulated compared to that in normal cells. A panel of transgenically positive total RNAs have been compared to negative total RNAs for the level of *c-myc* expression by slot blot analysis (figure 6.1). RNA loading was normalized by reprobing the blots with GAPDH (as described in section 2.x). The RNAs used were prepared from the spleens (or in some cases lymph node) of animals which varied in age and in tumour status. The transgenically negative RNAs examined exhibit an approximately three fold variation in the level of *c-myc* RNA detected. This probably reflects the variation in the age of the mice and normal proliferative state of the spleens. It does not appear that the level of *c-myc* RNA in line 26 or line 59 tumours is consistently higher than that detected in normal (non-neoplastic) spleens. Equally there is no obvious difference between transgenically positive and negative non tumour samples. Only one sample shows a greatly elevated level of *c-myc*, 26. 478 (number 17). This RNA was prepared

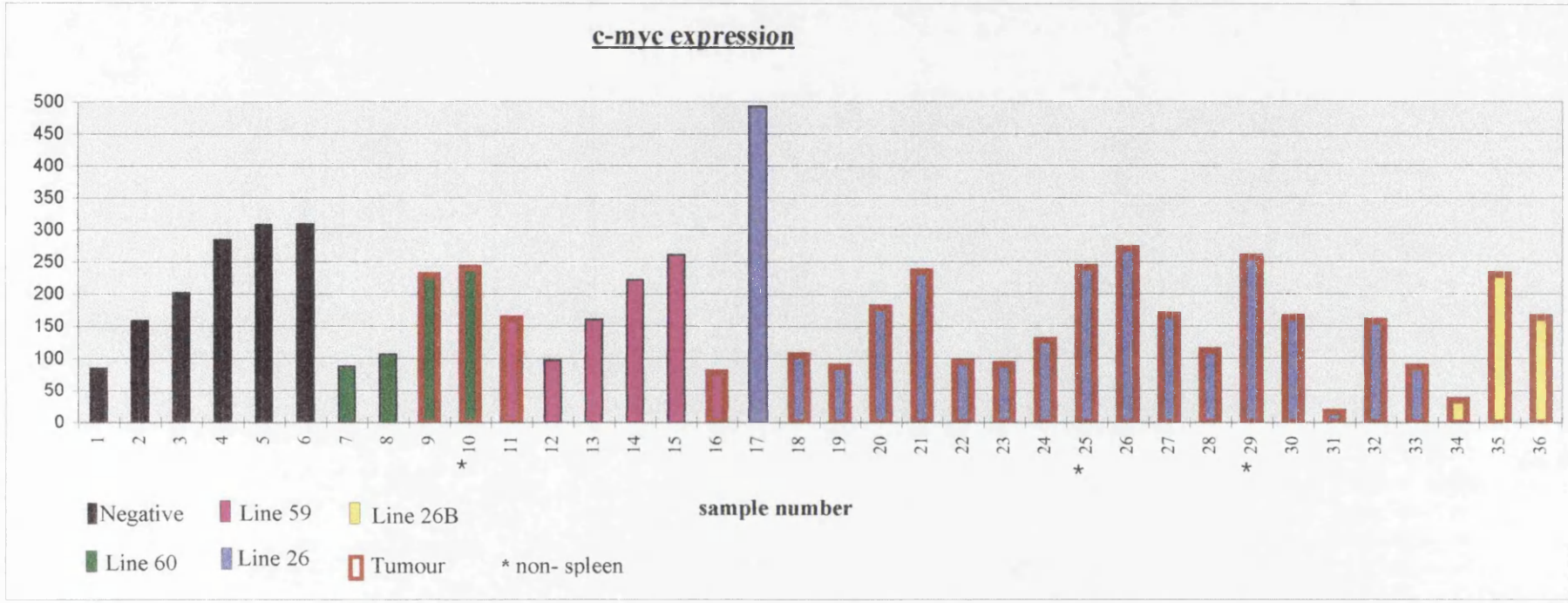


Figure 6.1a

Graph of c-myc RNA expression values obtained from an RNA slot blot of 5μg of various RNAs probed with probe mmyc1. GAPDH expression was used to control for loading variation. The majority of RNAs were prepared from spleens. There are three exceptions; samples 9 and 10 were prepared from the spleen and abdominal tumour from the same line 60 animal, samples 24 and 25 were prepared from the spleen and lymph node respectively of a line 26 line 26 animal, samples 28 and 29 were prepared from the spleen and lymph node respectively of a line 26 animal. A red outline indicates that the tissue was invaded by a tumour. The negative controls are illustrated in black. Line 60 samples are illustrated in green. Line 59 samples are illustrated in pink. Line 26 samples are illustrated in blue. Line 26B samples are illustrated in yellow.

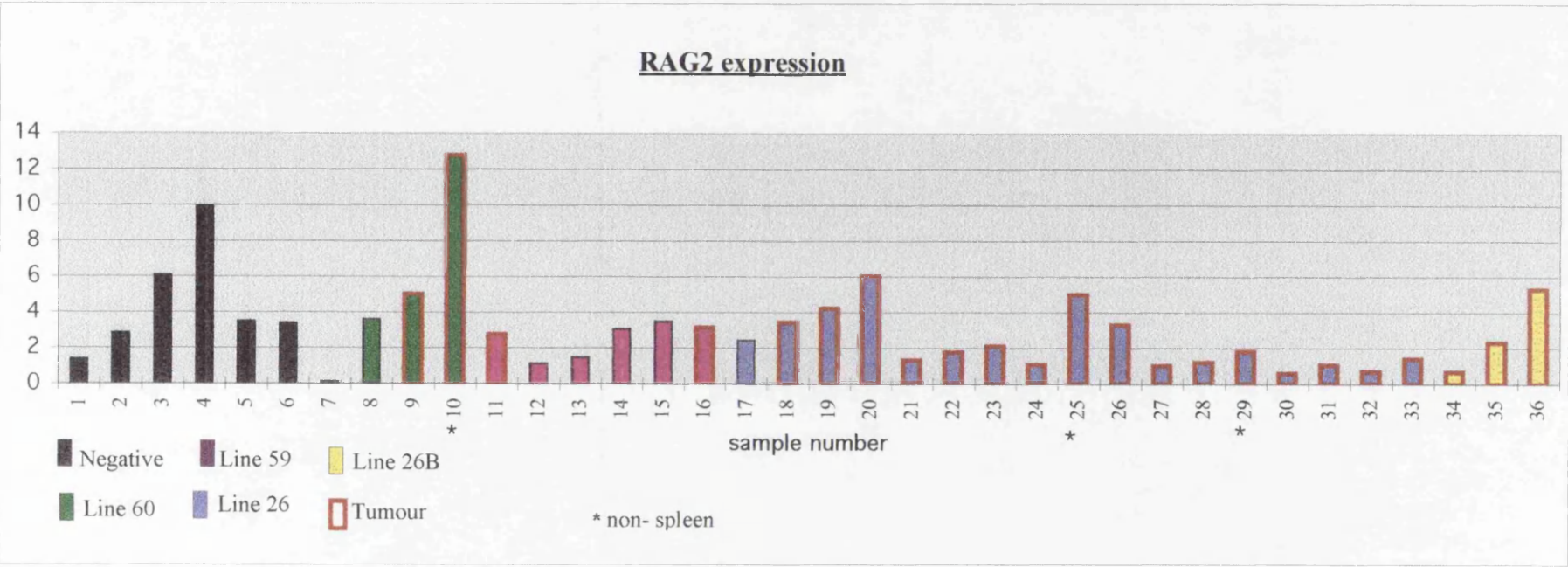


Figure 6.1b
RNA slot blot probed with RAG2 probe. GAPDH expression was used to control for loading variation.

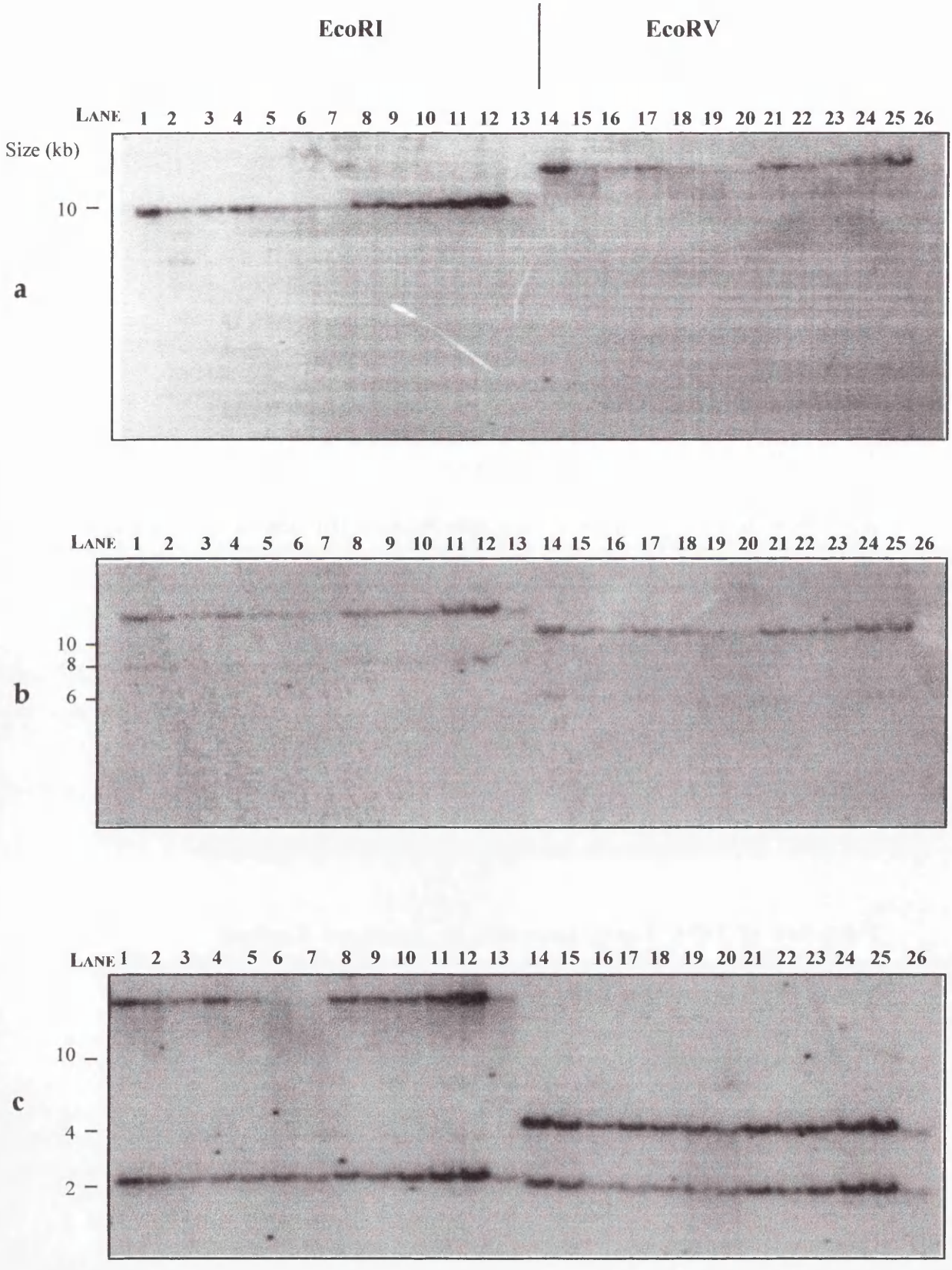
from the spleen of a 3 week old line 26 mouse. The higher level of *c-myc* expression detected in this sample may be due to the young age of the mouse, rather than an effect of EBNA-1. However, it is not possible to determine this as no negative control of the same age was included in this experiment. The spleen was histologically normal.

Recent evidence has suggested that EBNA-1 expression in cell lines in vitro leads to the induction of the recombinase activating genes 1 and 2 (Srinivas and Sixbey,1995). A probe for *RAG2* was used on the RNA slot blot described above and the results plotted on a graph (figure 6.1b). The level of *RAG2* expression was extremely low in all samples. Despite this, there is no evidence of an upregulation of *RAG2* in EBNA-1 transgenic tissues, whether or not they are neoplastic.

6.3 DNA Rearrangements in EBNA-1 induced Lymphomas

Detection of DNA Rearrangements by Southern Analysis

Tumours in line 26 and line 59 have been routinely screened for the presence of DNA rearrangements of the *c-myc* gene by Southern blot analysis. In figure 6.2a, DNAs from 12 different tumours digested with *Eco*RI and *Eco*RV reveal no rearrangements of *c-myc*. The same result was obtained from 13 other line 26 tumour DNA samples (Wilson, et al., 1996). This would suggest that there are no local rearrangements of the *c-myc* locus in these tumours. However, it is possible that digestion with further enzymes could reveal rearrangements not


EcoRI
EcoRV
LANE 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
Size (kb)
10
a
LANE 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
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8
6
b
LANE 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
10
4
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c

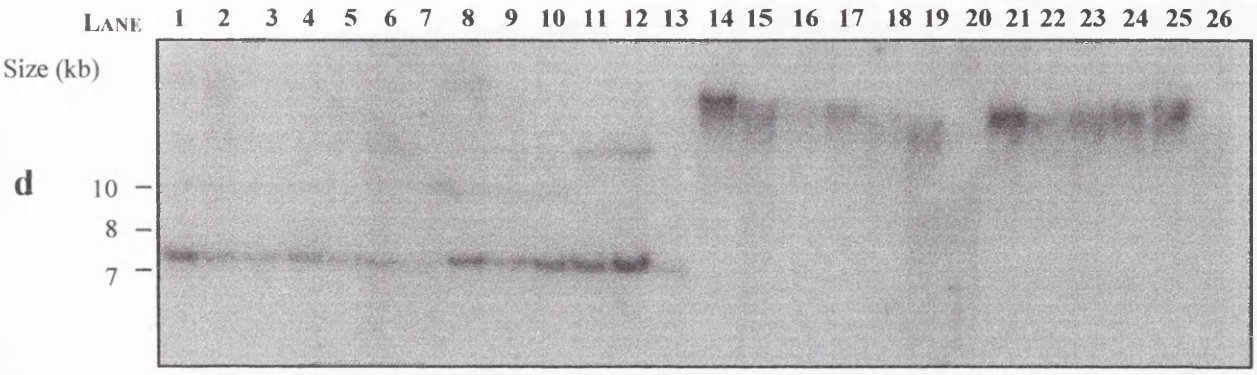

Figure 6.2

5μg of tissue DNAs digested with EcoRI or EcoRV as shown, and Southern blotted and probed, stripped and reprobed as described in Materials and Methods.
Lane 1 and 14 -tumour, 26.3 spleen; lane 2 and 15 -tumour, 26.4 spleen; lane 3 and 16 -tumour, 26.7 lmph node; lane 4 and 17- tumour, 26.16 spleen; lane 5 and 18 -tumour, 26.17 liver; lane 6 and 19 -tumour, 26.20 liver; lane 7 and 20 -tumour, 26.21 spleen; lane 8 and 21 -tumour, 26.24 lymph node; lane 9 and 22 -tumour, 26.30 liver; lane 10 and 23 -tumour, 26.41 lymph node; lane 11 and 24 -tumour, 59.21 lymph node; lane 12 and 25 -MoMLV induced tumour, 26.175 thymus; lane 13 and 26 -normal transgenically negative spleen.
a. c-myc probe (mmyc11, table 2.3); **b**. Bcl-2 probe (mbcl1, table 2.3); **c.** p53 probe (mp531, table 2.3); **d**. cyclin D1 probe (mcycD1, table 2.3)

discernible here due to the large size of the fragments and consequent poor resolution of similar sized bands. The Southern blot described above has been reprobed with probes to a number of other genes thought possibly to have a role in lymphomagenesis; *bcl-2* (figure 6.2b), *p53* (figure 6.2c), *cyclinD1* (figure 6.2d), *pim-1* (data not shown). No rearrangements in these genes have been detected using this approach.

Detection of DNA Rearrangements by SSCP Analysis

It has been shown that in many Burkitt's lymphomas and mouse plasmacytomas there are mutations within the coding sequence of the expressed *c-myc* allele (Bhatia *et al.*, 1993) using single stranded conformational polymorphisms analysis (SSCP)in clonal cell lines. It has been proposed that these mutations may lead to a more active *c-myc* protein. SSCP has been utilised to screen line 26 and 59 tumour DNAs for the presence of mutations within the *c-myc* coding sequence. Bhatia *et al.* found that the majority of mutations detected were located within residues 1-80 (*c-myc* exon 2, 5'). Tumour DNAs from line 26 and line 59 were screened by SSCP for the presence of mutations within residues 1-85 (figure 6.3a). Each PCR reaction was repeated at least twice and the products analysed under a number of different gel electrophoresis conditions. The DNAs were prepared from tissue samples which contained a range of cell types, even tumour bearing samples would contain a significant proportion of 'normal' cells. Moreover any DNA mutations might only effect one of the copies of *c-myc* within the cell.

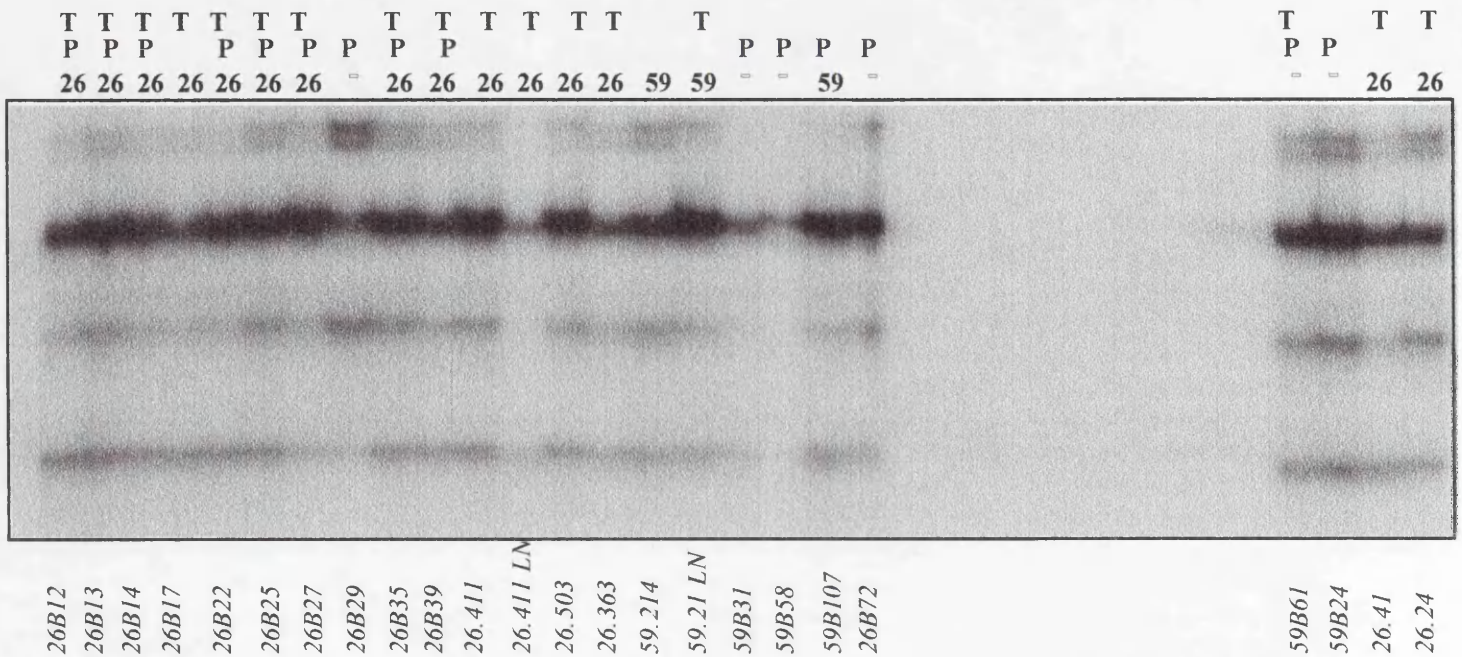

Figure 6.3a

SSCP analysis, *c-myc* exon 2 5'. PCR amplification of the DNAs was carried out using oligonucleotides Myc2 5' A and Myc2 5' B as described in Materials and methods. The products were then electrophoresed on a 19:1 polyacrylamide: bisacrylamide gel at 5W for 15hours. The numbers in italics at the bottom of the figure indicate the mouse from which DNA was prepared. With two exceptions all the DNAs were prepared from spleens, 26.411 LN and 59.21 LN were prepared from lymph nodes. **26** -indicates a line 26 EBNA-1 positive DNA, **59** - indicates a line 59 EBNA-1 positive DNA, - - indicates a transgenically negative DNA , **P** - indicates that the animal was injected with pristane (see text), **T** - indicates that the tissue was invaded by a tumour.

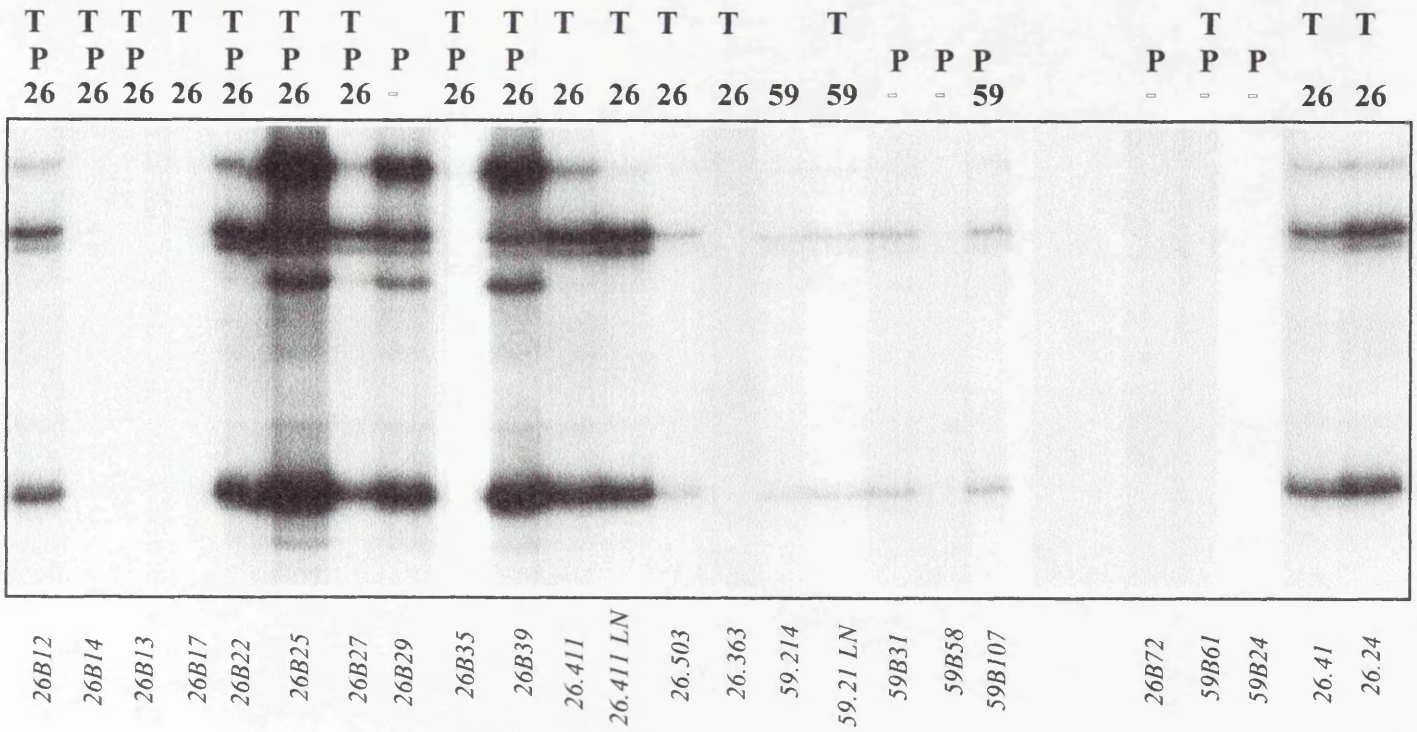

Figure 6.3b

SSCP analysis, *c-myc* exon 2 3'. PCR amplification of the DNAs was carried out using oligonucleotides Myc2 3' C and Myc2 3' D as described in Materials and methods. The products were then electrophoresed on a 0.6% MDE gel at 6W for 13hours. The numbers in italics at the bottom of the figure indicate the mouse from which DNA was prepared. With two exceptions all the DNAs were prepared from spleens, 26.411 LN and 59.21 LN were prepared from lymph nodes. **26** -indicates a line 26 EBNA-1 positive DNA, **59** - indicates a line 59 EBNA-1 positive DNA, - - indicates a transgenically negative DNA , **P** - indicates that the animal was injected with pristane (see text), **T** - indicates that the tissue was invaded by a tumour.

Therefore 'normal' DNA forms are likely to be the most prevalent even in tumour samples and tumour specific mutations would be expected as additional, perhaps faint, bands. In some samples additional faint bands were detected, however, variably some transgenically negative non tumour samples also contained these bands and they therefore may reflect alternative conformations. No additional bands could be unambiguously assigned specifically to tumour bearing DNA. Tumour DNAs have also been screened for mutations within exon2, 3' (residues 46-124) , again no unambiguous additional bands were apparent (figure 6.3b).

Therefore, under the conditions described here using SSCP with tissue samples, it was not possible to determine if point mutations were present within any of the *c-myc* sequences or not. A method to overcome this (without generating cell lines) would be to sort the cells in the tissues first (FACS) in order to enrich for B cells.

Detection of DNA Rearrangements by Karyotyping

Many of the *c-myc*/IgH translocations found in endemic Burkitt's lymphoma occur distal to the *c-myc* locus and therefore are not easily detected by Southern analysis. Karotypic analysis of tumour cells is a method to detect gross chromosomal abnormalities in EBNA-1 induced lymphomas. Karyotypic analysis of EBNA-1 tumours is being carried out by our collaborators in Stockholm (Santiago Silva and George Klein). Cell suspensions were prepared from line 26 tumours and frozen as described in materials and

methods (chapter 2, section 2.32). Aliquots were subsequently transported to Stockholm. An attempt was made to passage some of the cell samples in SCID mice prior to karyotypic analysis. For the majority of cell suspensions this technique was unsuccessful. However, using this approach 2 line 26 tumours (C57Bl/6 background) and 3 tumours from mice double positive for the EBNA-1 transgene and an N-*myc* transgene (section 6.2) have been successfully karyotyped (figure 6.4 a-e). A further 13 EBNA-1 positive tumours have been karyotyped directly from cell suspensions without freezing or passage in SCID mice. The most common changes are illustrated in table 6. 1. Trisomy 10 was present at a variable incidence (percentage of plates counted) in 10 of the 18 tumours analysed. Trisomy 15 was present in 63% of the tumours positive for the EBNA-1 transgene only and in 100% of tumours carrying both the EBNA-1 and N-*myc* transgenes. However trisomy 15 has not been detected in any of 5 tumours positive for both EBNA-1 and c-*myc* transgenes. Trisomy 10 and trisomy 15 were concurrent in 6 of the tumours. Other abnormalities were also detected, but they vary from tumour to tumour. Only 2 translocations were detected, one thought to be t(2;5;12) in a line 26 tumour, and one t(12;15) in a line 26/ N-*myc* double positive tumour.

6.4 Crossbreeding of EBNA-1 and *myc* Transgenic Mice

Assessment of the rate of tumour development and tumour types in mice positive for both the EBNA-1 transgene and the Eμ-*myc* transgene (Adams *et al.*, 1985) could give valuable information about any

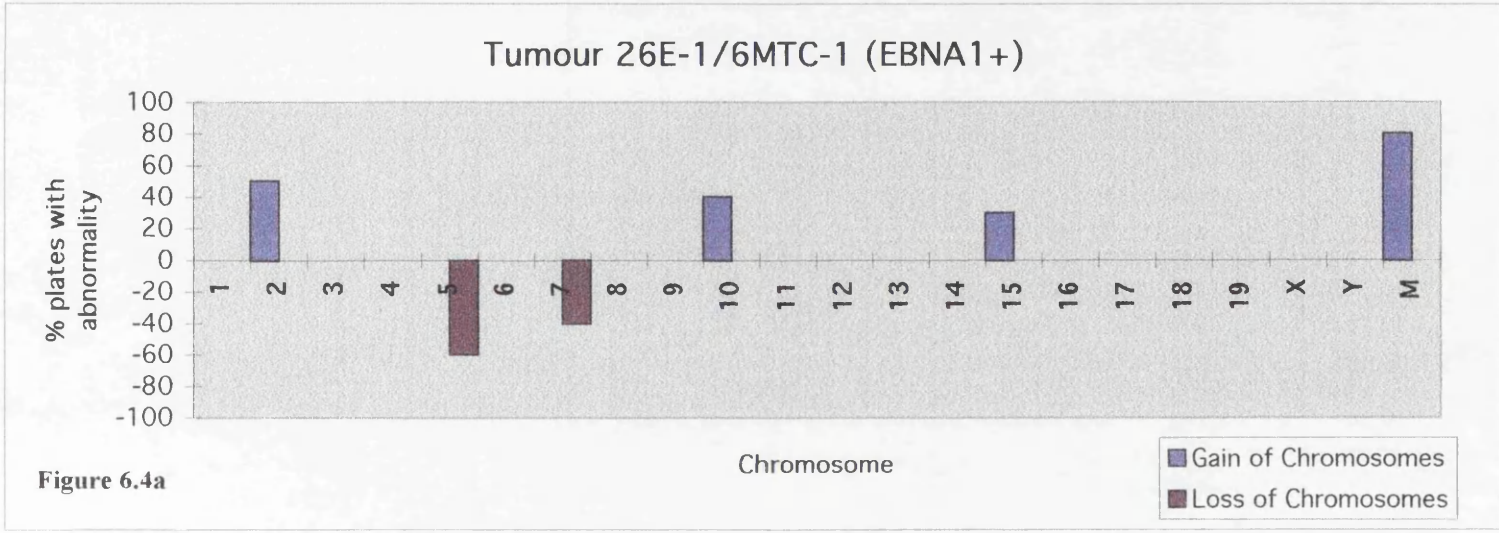

Figure 6.4a

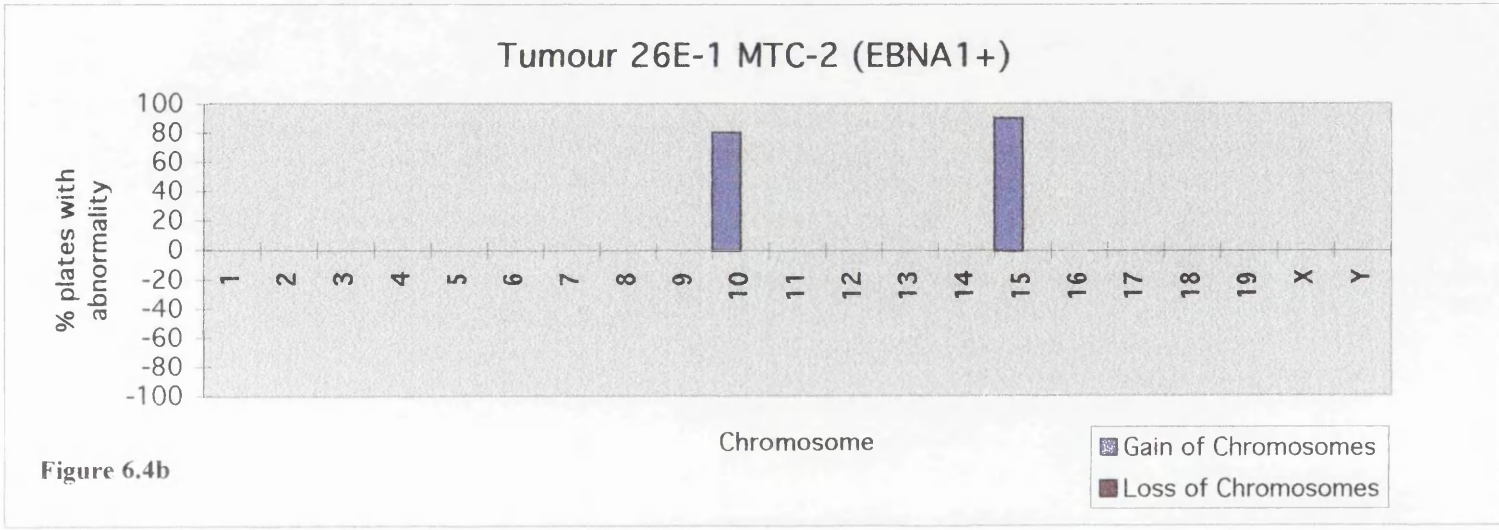

Figure 6.4b

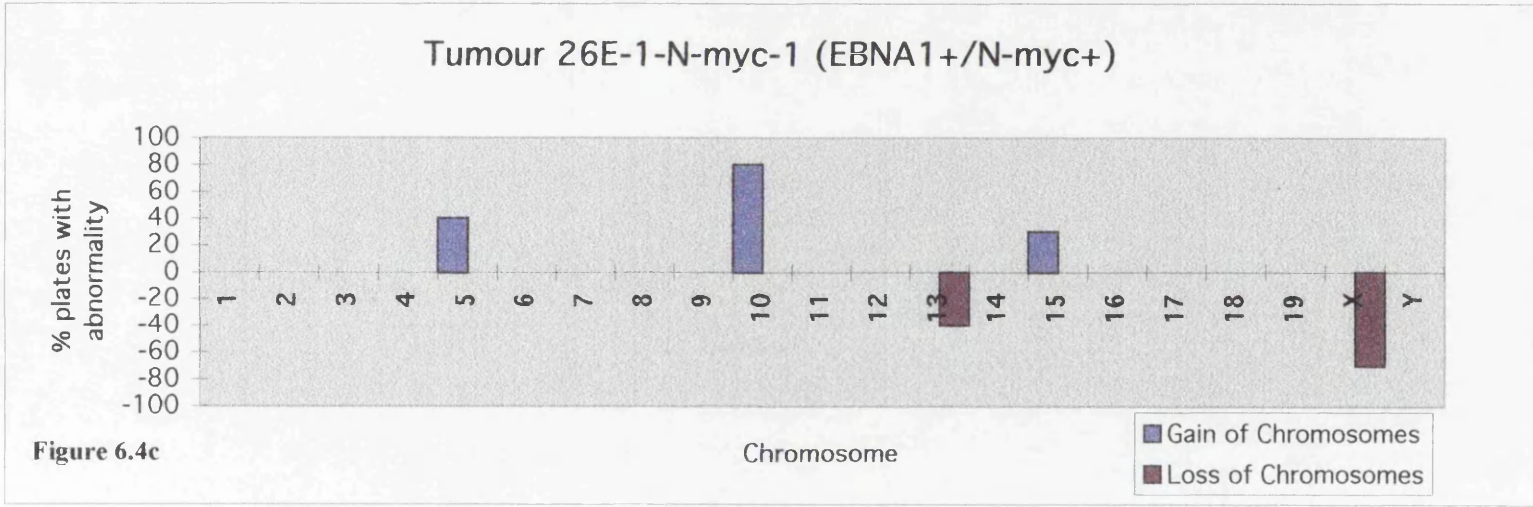

Figure 6.4c

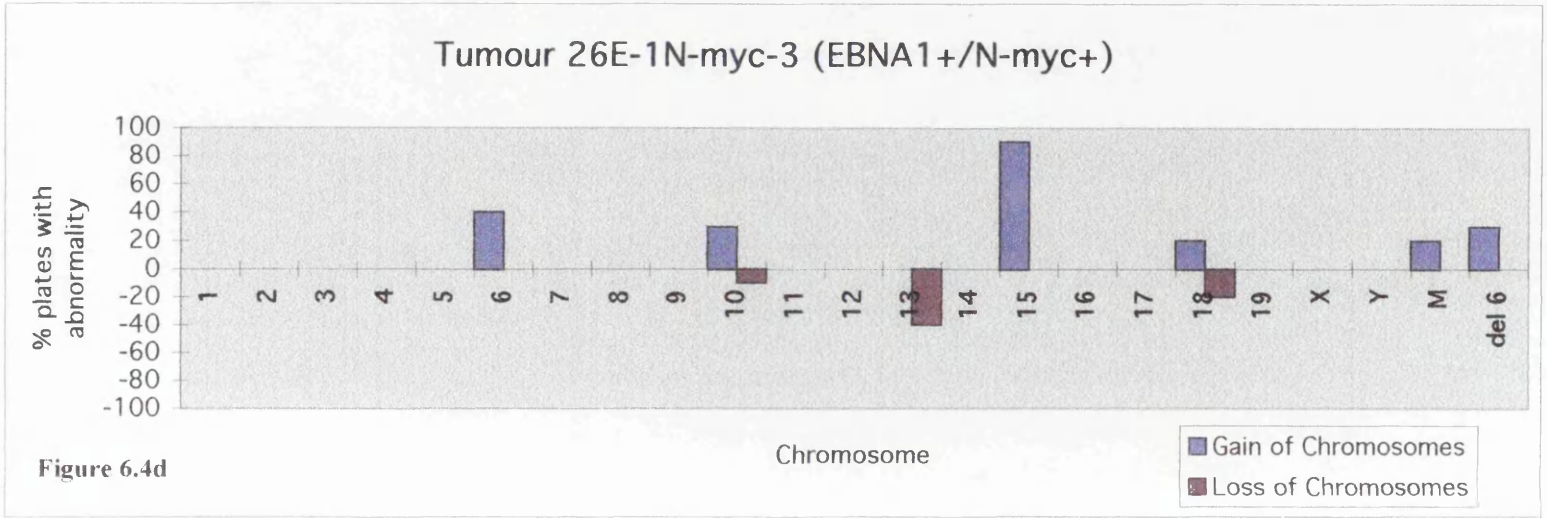

Figure 6.4d

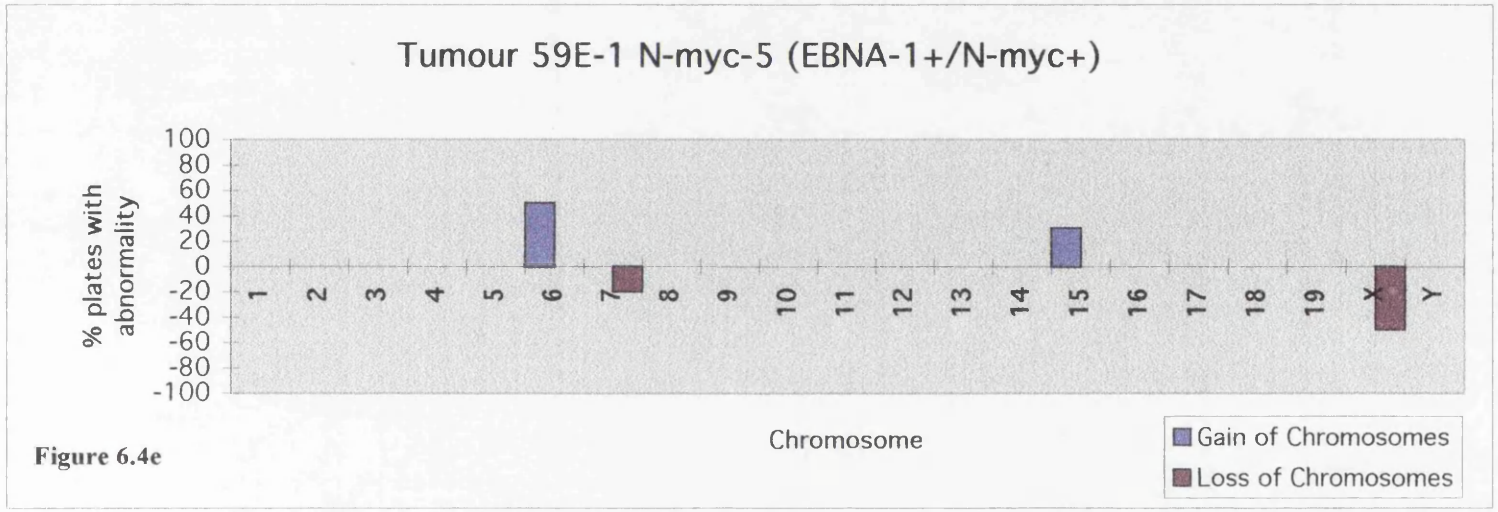

Figure 6.4e

Figure 6.4
Illustration of the karyotpes determined for 5 different EBNA1 induced tumours. **a** and **b** show the karyotpes of tumours from two different line 26 EBNA1positive mice. **c**,**d** and **e** show the karyotypes from three tumours in EBNA1 positive mice which were also positive for the N-myc transgene. **c** and **d** were from line 26 mice, **e** was from a line 59 mouse. Gain and loss of individual chromosomes is shown in terms of the % of plates counted showing that abnormality. M indicates the presence of an unidentifiable chromosome (**a** and **d**). Del 6 indicates the presence of a chromosome 6 carrying a deletion (**d**).

Transgenic status	*EμEBNA-1 line*	*Number of tumours*	*Cytogenetical change*				
			+6	+10	+15	+17	Translocations
EBNA-1 +	26	8	2 (25%)	5 (63%)	5 (63%)	2 (25%)	1- t(2;5;12)
EBNA-1/*c-myc*	26	5	0	2 (40%)	0	2 (40%)	0
double positive	59	1	0	0	1 (100%)	0	0
EBNA-1/N-*myc*	26	3	2 (67%)	3 (100%)	3 (100%)	1 (33%)	1- t(12;15)
double positive	59	1*	1*	0	1*	0	0
All EBNA-1 +	Both	18	5 (28%)	10 (56%)	10 (56%)	5 (28%)	2 different

Table 6.1

Common cyotogenetic changes detected in EBNA-1 positive tumours by karyotyping. * - EBNA-1 status being reconfirmed (G. Klein laboratory).

co-operative effect of the two oncogenes. It was therefore attempted to crossbreed Eμ-*myc* transgenic mice with Eμ EBNA-1 mice of both line 26 and line 59. Eμ-c-*myc* transgenic mice in the FVB strain background were imported from Anton Berns' laboratory in Amsterdam. These mice were generated in the laboratory of Professor Berns using the same transgene as originally described by Adams *et al.* (1985). The construct contains the immunoglobulin heavy chain enhancer inserted 5' to the murine *c-myc* gene. A tag of 600bps of ϕx174 DNA was included in the 3' untranslated region of *c-myc* exon 3. At the time of import it was noted in Professor Bern's laboratory that there were difficulties in maintaining the line. These mice were bred in quarantine and their progeny used to form a colony of mice designated line 86. Initially, tail DNA from line 86 mice was screened by slot blot analysis using a probe fragment supplied by the Berns laboratory (chapter2, section 2.33). Mice identified as apparently positive by this screen were used in subsequent breedings to line 26 and line 59. This gave rise to 76 offspring in the line 26 background, designated line 26/86, and 61 offspring in the line 59 background, designated line 59/86. It was attempted to determine the Eμ-*c-myc* transgene status of these mice using Southern blot analysis. Using the probe fragment and conditions supplied by the Berns laboratory it was not possible to detect any mice positive for the Eμ-*c-myc* transgene. The line 86 mice used in the initial round of breeding were retested for transgene status by Southern blot analysis and no positives were detected. In addition, Southern blots of line 86 and line 26/86 DNAs were hybridised with a probe to the IgH enhancer and to

mouse *c-myc* exon 2 and 3, but no evidence was found that any of the mice harboured Eμ-*c-myc* transgene sequences. Finally a PCR approach was attempted to assess the transgene status. Primers were designed to amplify transgene sequences between the IgH enhancer and *c-myc* exon 1 (Myc 1 and IgH 1, chapter 2, table 2.2). A product of 1kb in size should only have been obtained from transgenically positive DNA. As a control for the PCR reaction, primers to *c-myc* exon3 were included (MycE 3 5' and MycF 3 5') which should yield a product of 200bps in size. A range of line 86 and line 26/86 DNAs was used. In all the reactions the control 200bp product was detected, but no additional products were detected (data not shown). The DNAs used were intact and the Southern blots were in order as these have been reprobed using an EBNA-1 probe and the EBNA-1 transgene status has been unambiguously determined. It has therefore been concluded that none of the mice used to establish line 86 was in fact positive for the Eμ-*c-myc* transgene. All of the 26/86 and 59/86 progeny have been closely monitored for tumour appearance. The data collected on these mice (transgene status and tumour phenotype) is presented in tabular form (Appendix 1.1 and figure 6.5). The Eμ-EBNA-1 positive mice of cross 26/86 succumbed to monoclonal B cell lymphoma indistinguishable in appearance to the usual line 26 lymphoma pathology, but with an increased latency when compared to standard line 26 mice in C57Bl/6 background (figure 6.5a and b). The strain background of the 26/86 and 59/86 mice was F1 FVB / C57Bl/6. Of the EBNA-1 negative mice in the 26/86 cross (n=36), only 3 suffered from suspected lymphoma (16,18, and

36), and in the 59/86 cross (n=42) only 2 (23 and 26). The lack of lymphoma phenotype in the EBNA-1 negative mice is also supportive of the above conclusion that the mice did not harbour a *c-myc* transgene. It is not clear whether the lymphoma phenotype in the five EBNA-1 negative mice was due to any potential cryptic *myc* transgene sequences. The mice were old at presentation with the phenotype (2 years) and the spontaneous tumour rate in this hybrid strain background is not known. This incidence is within the spontaneous frequency that might be detected in old mice in normal colonies (although satistics on this particular F1 strain are not available).

As it was not possible to establish a clearly *c-myc* transgene positive stock from line 86, a further attempt to import Eμ-*c-myc* mice was made, this time from Professor Klein's stock established from the original line generated by Adams *et al.*. Due to Home Office and Ministry of Agriculture regulations (with UK quarantine restrictions) it is a good deal faster to export than import mice. During the process of importing Eμ *c-myc* mice (from the original Adams *et al.* stock) and Eμ N-*myc* (Dildrop *et al.*, 1989), lines 26 and 59 were exported to the Klein laboratory. Dr. S Silva has initiated the crossbreeding of both line 26 and line 59 with Eμ N-*myc* mice and Eμ *c-myc* mice. The Eμ c-*myc* mice succumb to rapid onset lymphomas, the tumour incidence in this line of Eμ N-myc mice is low as is the level of transgene expression. The first progeny in these crossbreeds are described. The first litter of mice from the

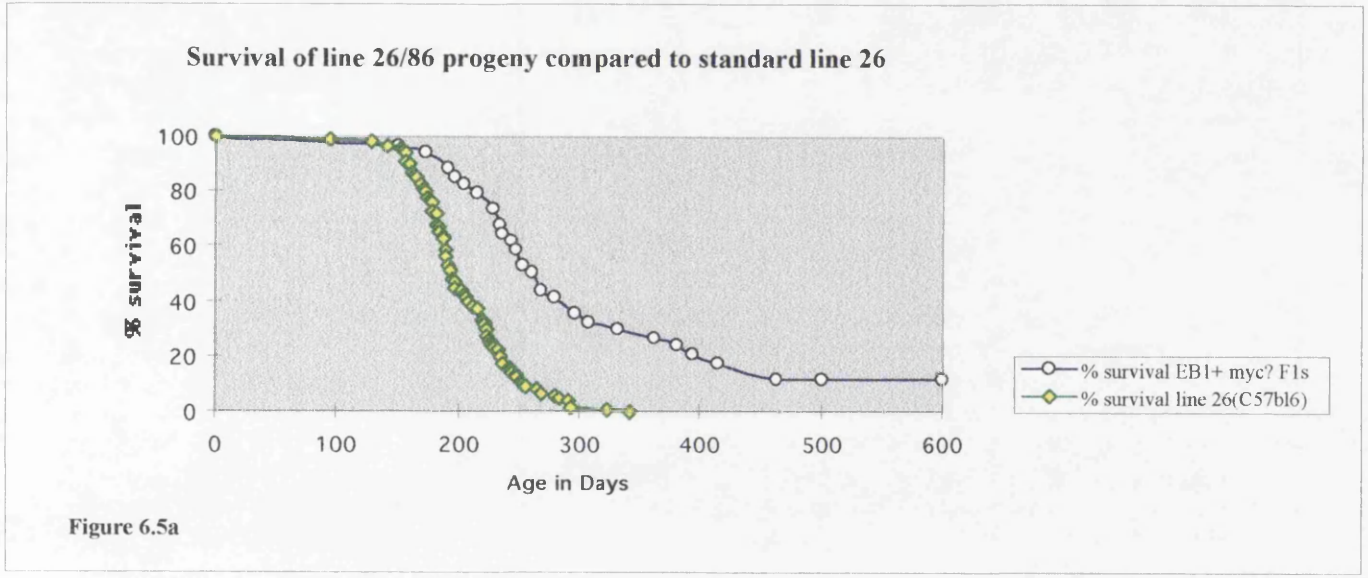

Figure 6.5a

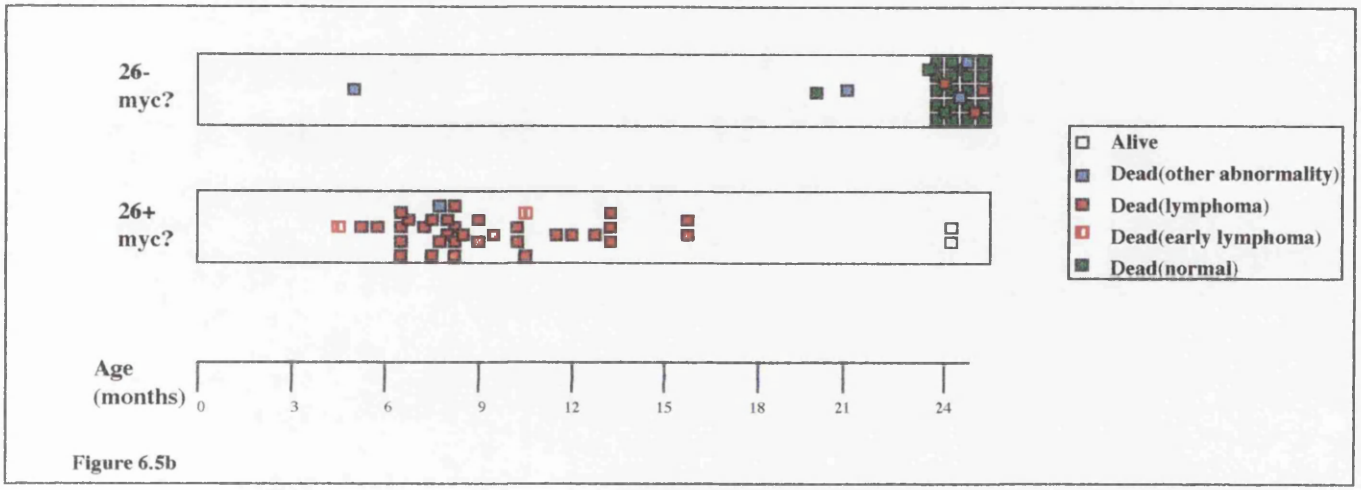

Figure 6.5b

Figure 6.5

Survival of 26/86 progeny. **a.** Graph showing percentagesurvival of EBNA1 positive 26/86 progeny compared to line 26 in the C57Bl/6 strain background. **b.** Diagram illustrating incidence of neoplasia in 26/86 progeny. White boxes indicate mice still survivng. Red boxes indicate death due to lymphoma, stripes indicates an early stage of neoplasia. Green boxes indicate end of experiment with no abnormalities detected. Blue boxes indicates death due to some abnormality other than lymphoma.

line 26 cross with Eμ N-*myc* mice were followed until they were approximately 170 days old (figure 6.6a). 4 out of 5 double positives all succumbed to lymphoma between 90 and 120 days of age. Only 1 of 4 EBNA-1 positive/N-*myc* negative mice succumbed to lymphoma within this time period (at 140 days of age). For a number of mice included in this study ,the N-*myc* transgene status is unclear (tested by S.Silva); on the figure these animals have been included as N-*myc* negative. One mouse which was EBNA-1 negative and N-*myc* status unclear succumbed to lymphoma at around 150 days of age. The first litter from the line 59 cross with the Eμ N-*myc* mice have been monitored to approximately 330 days of age (figure 6.6b). 2 of 5 double positive mice have succumbed to lymphoma within this time period (one at about 300 days of age and one at about 330 days of age). None of the single positive mice or the negative mice have succumbed to tumours within this time period. Whilst preliminary, these results (a shorter latency to tumour onset in double positive mice) are suggestive of a co-operation between EBNA-1 and N-*myc* in tumourigenesis. In the cross between line 26 and Eμ *c-myc* mice, 11 out of 11 double positive mice have succumbed to tumours, the majority in less than 75 days (figure 6.6c). In a 300 day observation period, only 2 of 6 EBNA-1 positive/*c-myc* negative mice have succumbed to tumours, one at 59 days and 1 at 83 days. 2 of 4 EBNA-1 negative/*c-myc* positive mice succumbed to tumours, both at 77 days. In the cross between line 59 and Eμ *c-myc* mice, sixteen double positive mice have been obtained

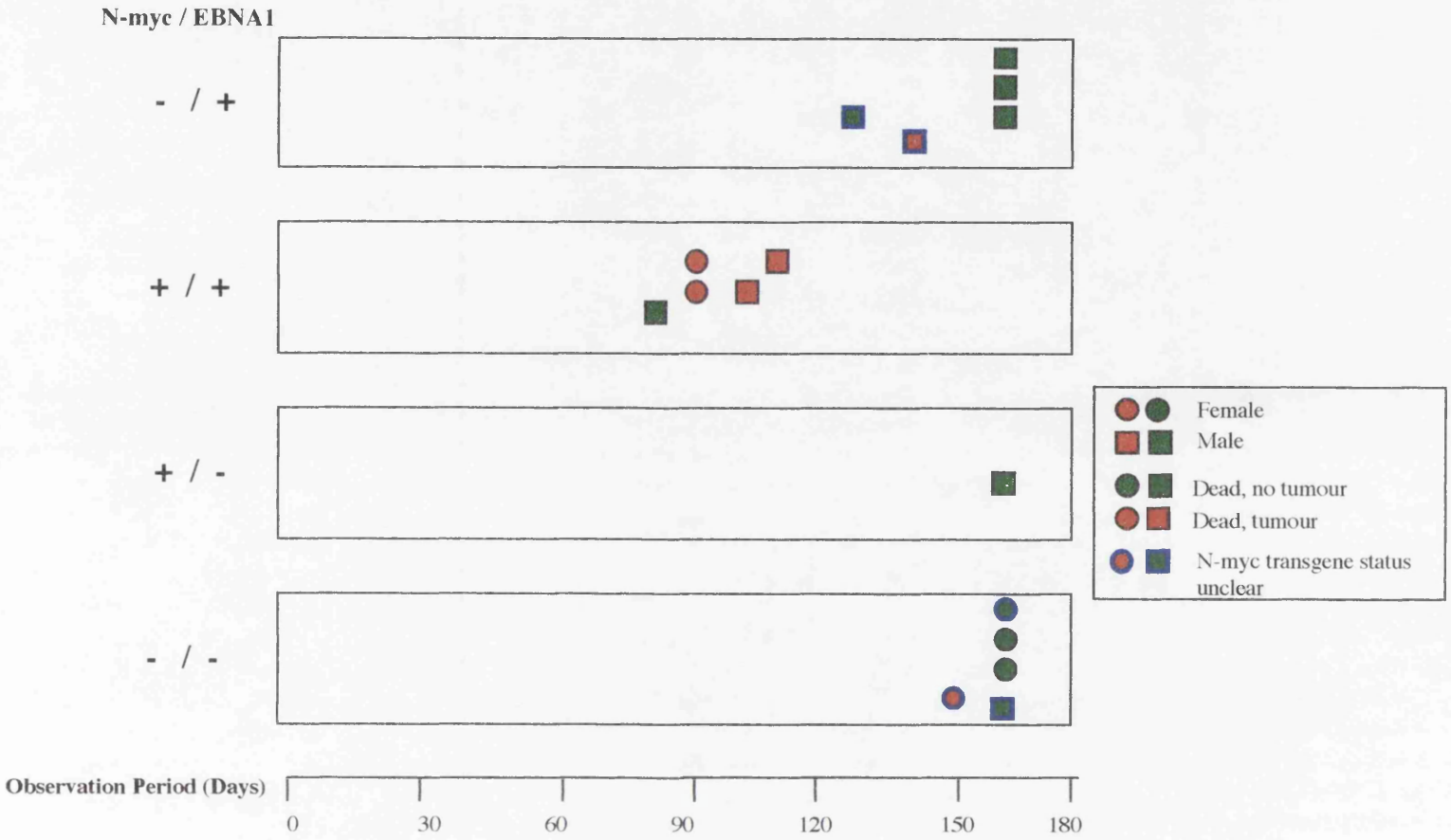

Figure 6.6a

Diagram indicating incidence of neoplasia in progeny from line 26 cross with N-*myc* transgenic mice. Mice which succumbed to lymphoma are indicated in red, green indicates that the mouse was found to be normal. For some mice the N-myc transgene status was ambiguous (tested by S.Silva), these are indicated with a blue outline.

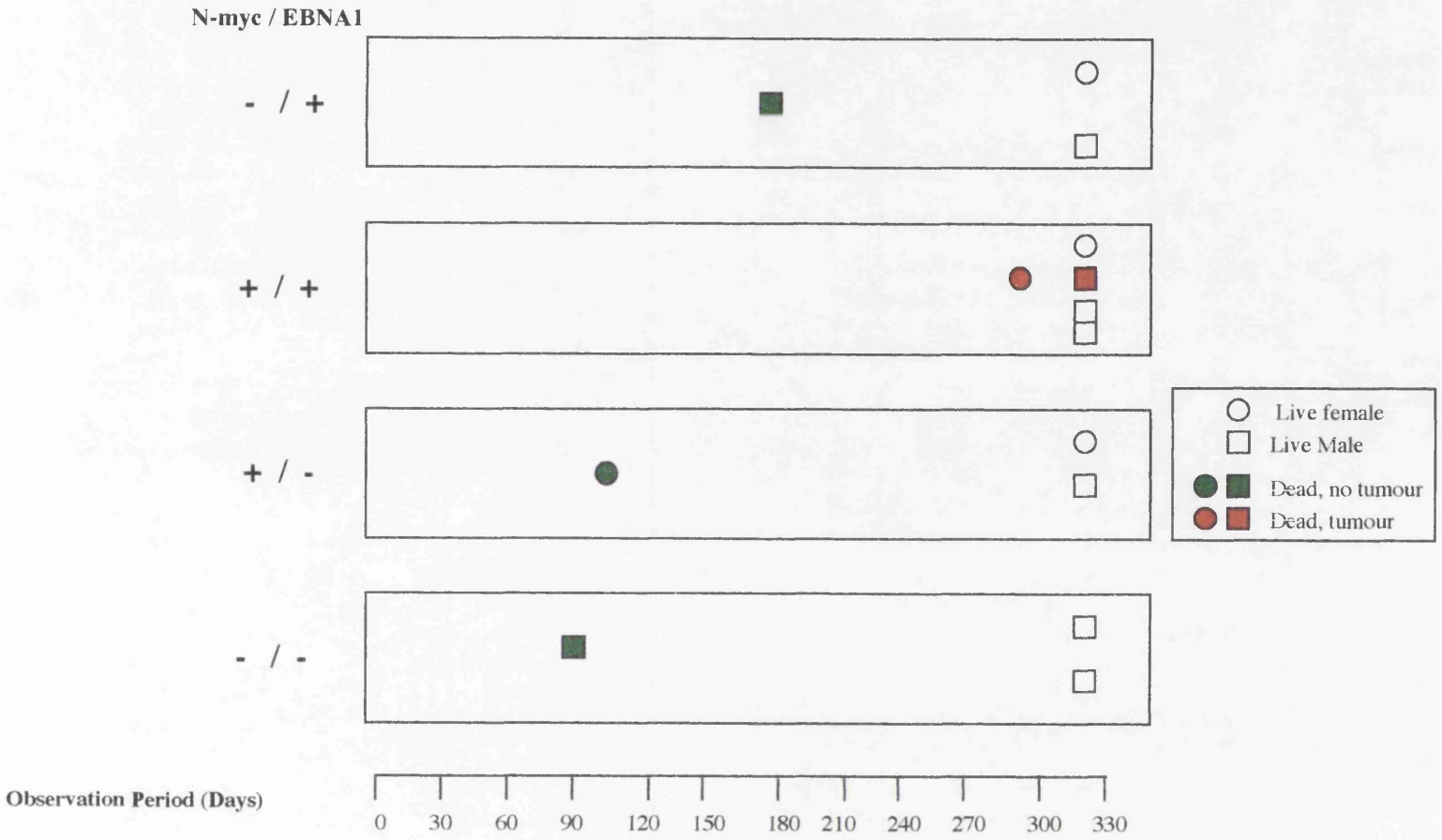

Figure 6.6b
Diagram indicating incidence of neoplasia in progency from line 59 cross with N-myc transgenic mice. Incidence of lymphoma is indicated in red, green indicates that the mice were normal when sacrificed. White symbols indicate that the animals were still surviving at the time the data was compiled.

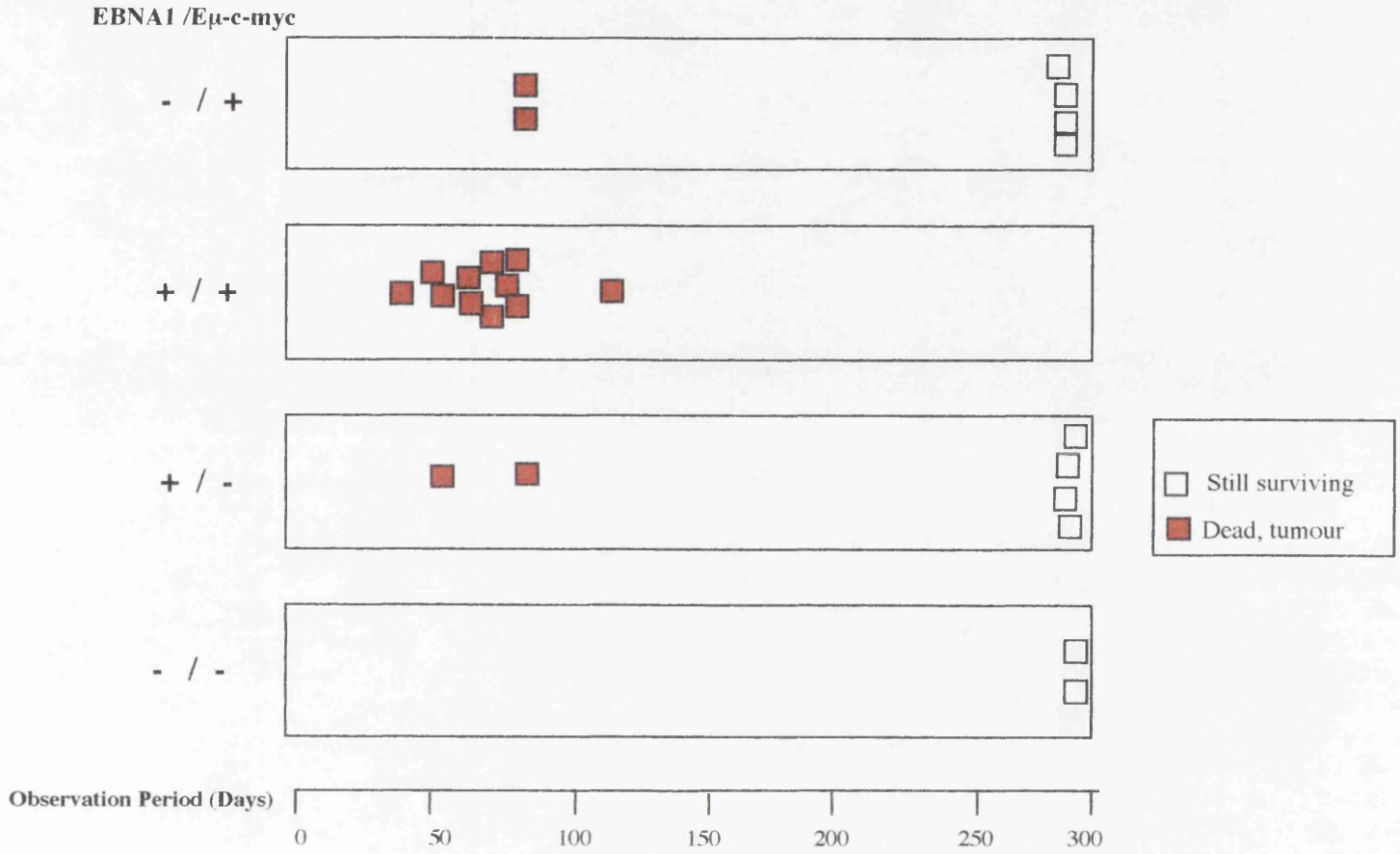

Figure 6.6c
Diagram indicating incidence of neoplasia in progeny from line 26 crosses with Eμ-*cmyc* transgenic mice. Red boxes indicate incidences of lymphoma. White boxes indicate that the mice were still surviving at the time the data was compiled.

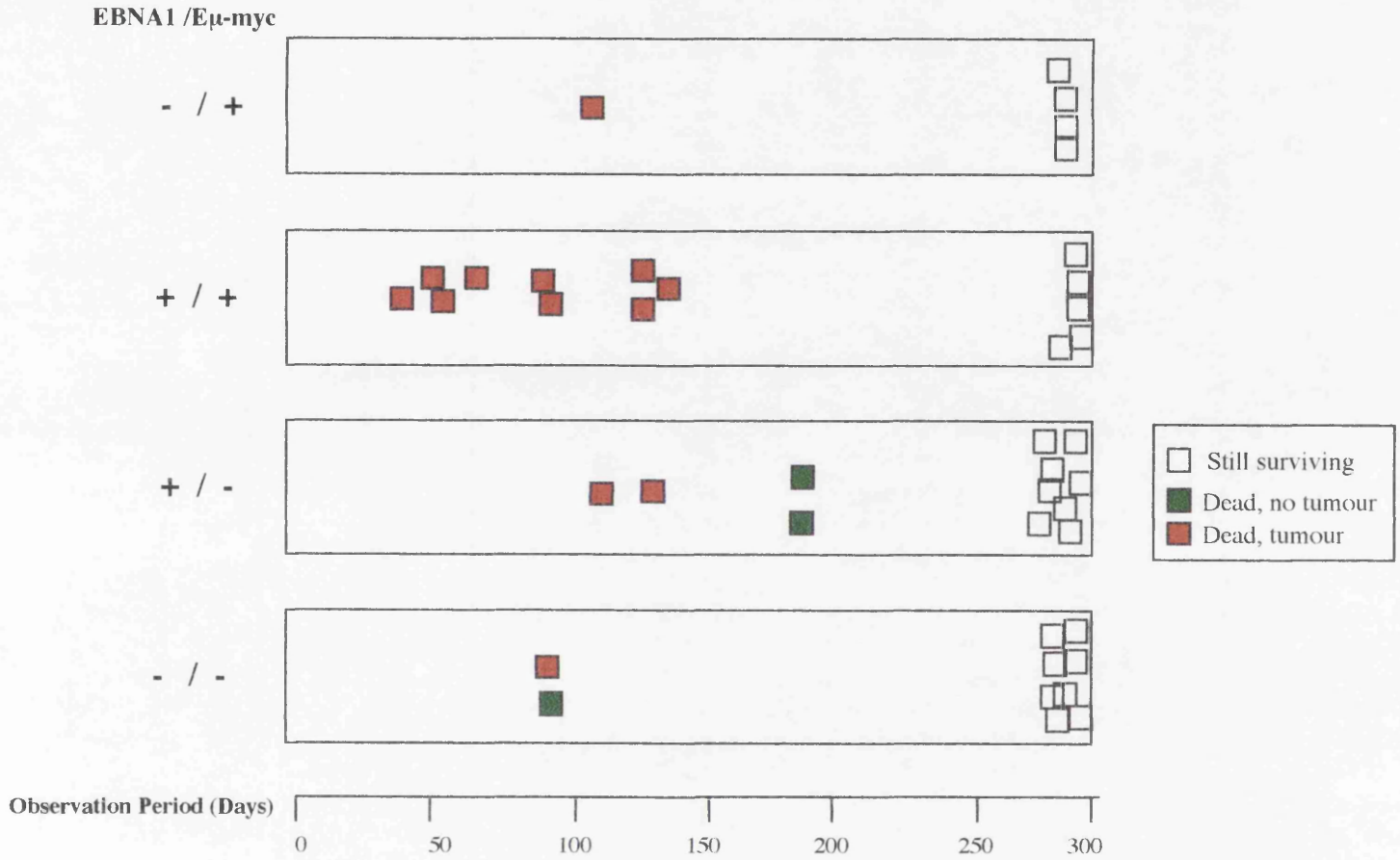

Figure 6.6d
Diagram indicating incidence of neoplasia in progeny from line 59 crosses with Eμ-*c-myc* transgenic mice. Incidence of lymphoma is indicated in red. Green indicates that the mouse was normal when sacrificed, white boxes indicate that the mouse was still surviving at the time the data was compiled.

(figure 6.6d). Over a 300 day observation period 9 tumours have arisen in these mice, the majority of which have arisen in less than 100 days. Few tumours have arisen in EBNA-1positive/*c-myc* negative mice; 2 in 12 mice, and both after 100 days. Only one tumour has arisen in the EBNA-1 negative/*c-myc* positve mice, at 112 days. The results of the crosses of both Eμ EBNA-1 transgenic lines with Eμ *c-myc* transgenic mice are strongly suggestive of a co-operation between EBNA-1 and *c-myc* in tumourigenesis.

6.5 Induction of *c-myc* Rearrangement *in vivo*

In another approach to investigate the effect and mechanism of *c-myc* translocation in EBNA-1 induced lymphomas, the susceptibility of some substrains of Balb/c mice to plasmacytomas by induction with the mineral oil, pristane, was utilised. This investigation was designed to address whether the induction of *c-myc* translocations co-operates with EBNA-1 in tumourigenesis, and also (in the longer term) whether the nature of any translocations detected differs from those found in plasmacytomas in negative mice. This experiment is similar to the cross breeding with Eμ *c-myc* transgenic mice in that it addresses whether EBNA-1 expression and deregulated *c-myc* can co-operate in tumourigenesis. In addition however this experiment can address whether the presence of EBNA-1 can effect the translocation process *in vivo*. Differences in the precise location of the DNA breakpoints in translocations have been found between endemic Burkitt's lymphoma (99%

EBV positive) and sporadic Burkitt's lymphoma (70% EBV negative) (Introduction section 1.2). Both line 26 and line 59 mice were bred into a Balb/cAnN background, generating line 26B and line 59B respectively. Due to the limitations of time it was not possible to breed the lines into a pure Balb/cAnN background. However the mice used in the pristane induction experiment were from at least the second backcross into the Balb/c background (on average 75% or more Balb/c).

Line 26B and line 59B mice were injected with pristane and their subsequent lymphoma development closely monitored. The protocol requires 3 injections of pristane, 2 months apart, as described in Materials and Methods. Very few overt plasmacytomas were detected in either of the lines. However, the pathology of the pristane injected mice was complicated. The incidence of neoplasia was low in the negative mice (figure 6.7). Incidence of lymphoma was usually grossly obvious due to tumour invasion of the spleen, lymph nodes and/or liver. Diagnosis of plasmacytoma was more subjective. Samples of spleen, liver, lymph node, thymus and any other tissue of abnormal appearance were sent for pathological analysis from all animals included in this study. Pristane is an irritant which causes granulomas and the production of ascites fluid in the peritoneal cavities of injected mice. Animals in this study were euthanased on any outward sign of ill health, or on gross swelling of the abdomen (which was most often as a result of the build up of ascites fluid). Previous studies of plasmacytoma incidence have assigned a diagnosis of plasmacytoma on the basis of the appearance of cells in one drop of the

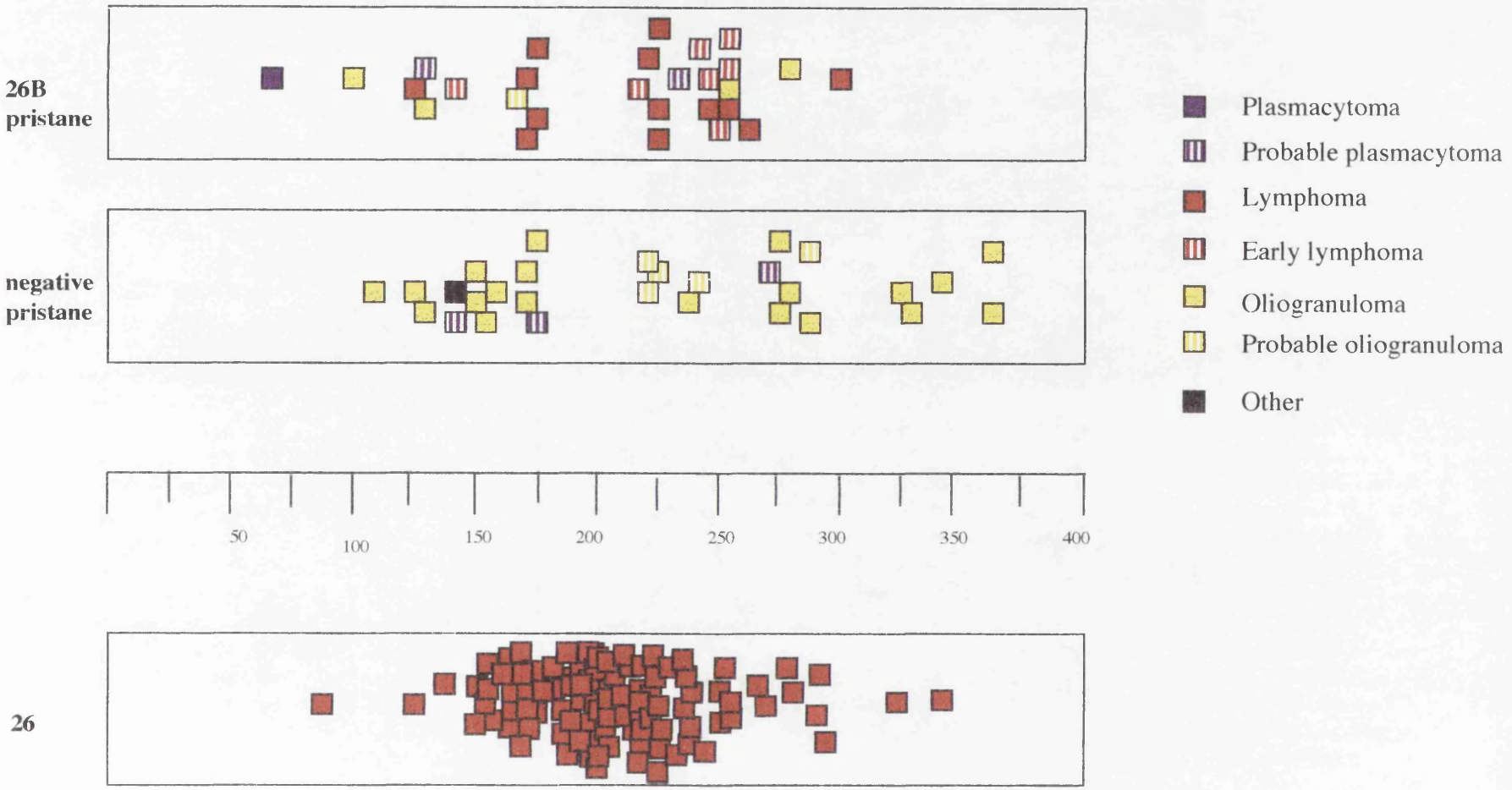

Figure 6.7
Diagram illustrating incidence of *neoplasia in pristane injected* EBNA1 positive mice compared to negative mice and compared to standard line 26 (C57Bl/6). The colour coded boxes indicate the phenotype of the mice when sacrificed (see key).

ascites fluid. The presence of 10 'characteristic plasmacytoma cells' per slide led to the diagnosis of plasmacytoma (Potter *et al.*, 1985). In this study diagnosis was on the basis of the appearance of solid tissues and hence incidence of plasmacytoma may be under-represented as a result. An analysis of all of the 26B pristane injected mice does not indicate any clear co-operation (in terms of tumour onset) between EBNA-1 and pristane in tumourigenesis (figure 6.7 and 6.8). However there were two variables in this study; the mice injected ranged in age at the first injection of pristane and also in their backcross status into the Balb/c background, both of which may influence tumour onset. At the start of the study mice of backcross 2 (on average 75% Balb/c) received the first injection of pristane at different ages : 7 mice at 162 days old, 9 mice at 105 days old, 10 mice at 78 days old, 9 mice at 50 days old. Subsequently mice of backcross 3 (87.5% Balb/c) and backcross 4 (93.75% Balb/c) all received the first injection between 24 and 34 days of age (25 mice in total). When these two groups of mice are analysed separately, a clear difference in tumour onset is observed (figure 6.9a). Taking age only into account the curve of the graph for those mice under 2 months of age at the first injection (pink) is less steep than when taking age and backcross into account (figure 6.9b, red). This suggests that both variables probably influence tumour onset. Comparing the phenotypes of mice of backcross 2 injected at different ages (figure 6.9c, blue boxes) there is no striking difference in tumour onset. However, tumour onset is accelerated in the mice of backcross 3 and 4 (figure 6.9c red box). The numbers of mice involved in these comparisons are small,

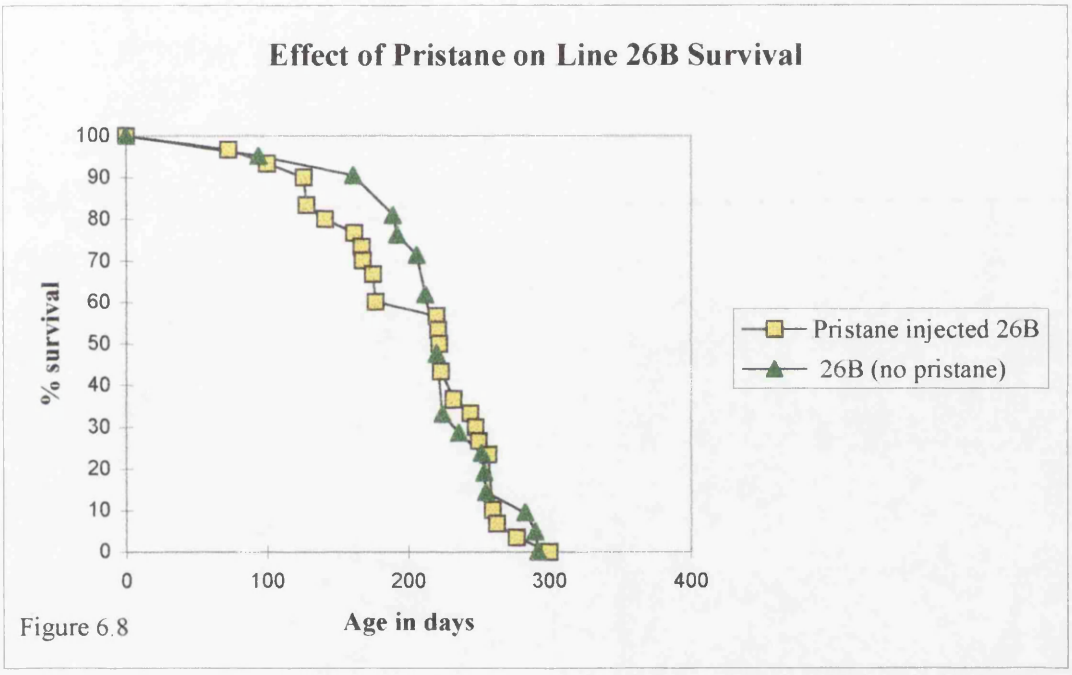

Figure 6.8
Graph showing death curve of line 26B mice compared to pristane injected line 26B mice

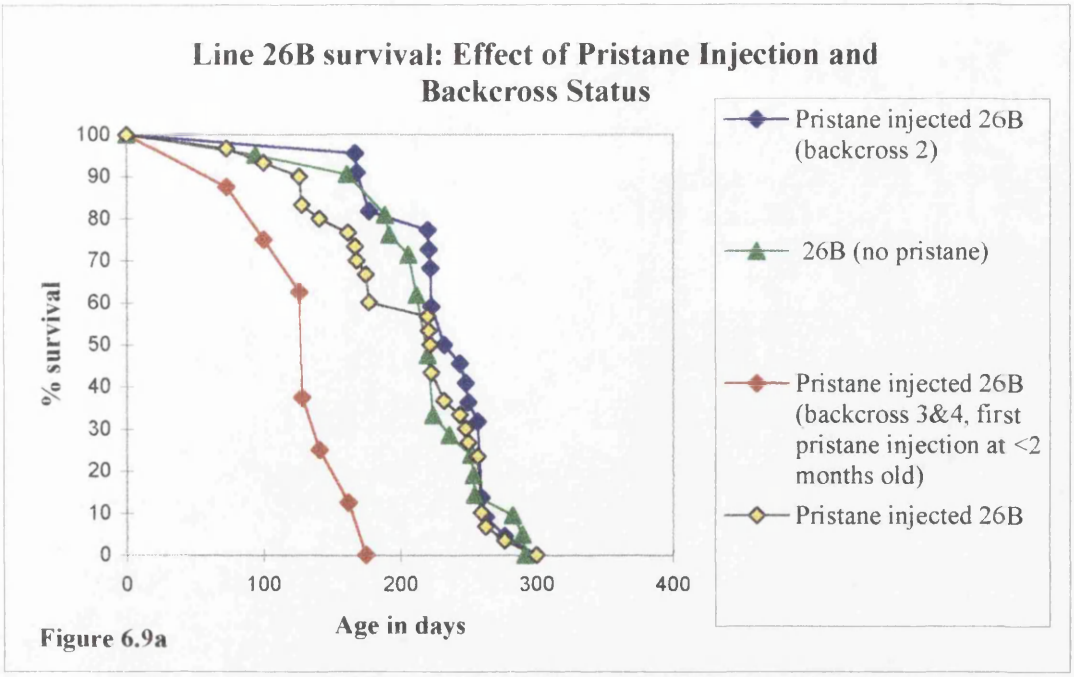

Figure 6.9a

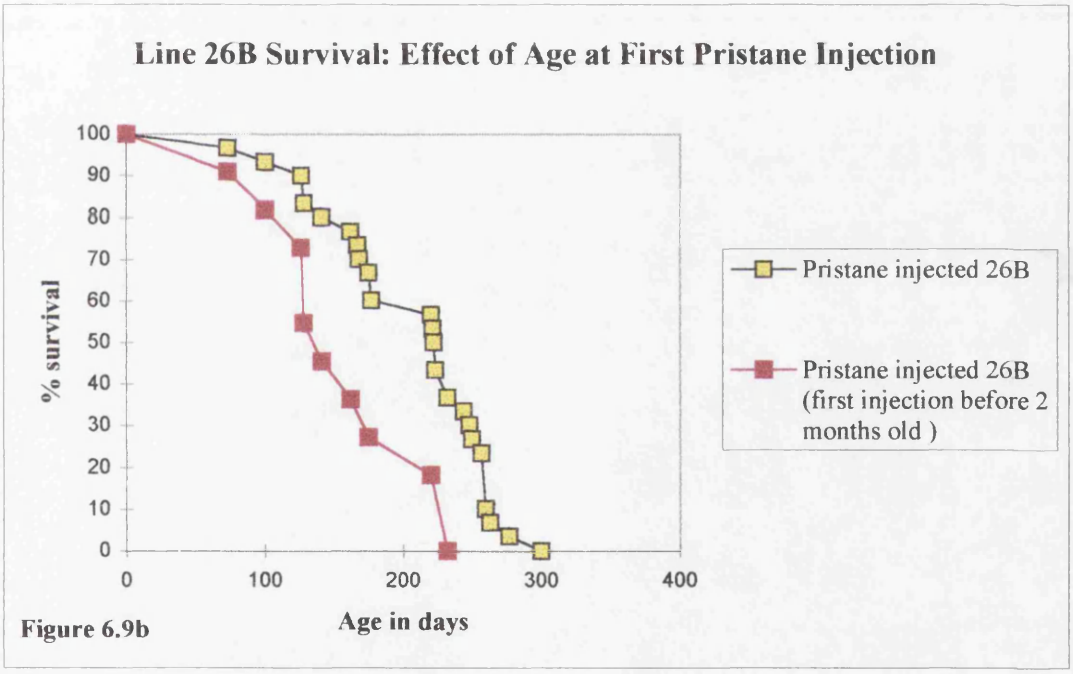

Figure 6.9b

Figure 6.9

Graphs illustrating the effects that backcross stage to BalbcAnN and age at first pristane injection have on the survival of line 26B mice injected with pristane. **a.** Illustrates the effect of backcross status and age at first injection of pristane. The survival of pristane injected mice of backcross 2 (blue) and of backcross 3&4 (red) have been plotted separately. Mice of backcross 2 varied in age at first injection of pristane. Mice of backcross 3&4 were all less than two months old when first injected with pristane. For comparison the survival of 26B mice without pristane injection has also been plotted (green) and with pristane injection (yellow). **b.** Illustrates the effect of age at first injection of pristane on survival. The survival of 26B mice of all backcrosses which were less than two months old when first injected with pristane (pink) is compared to the survival of all pristane injected 26B mice (yellow).

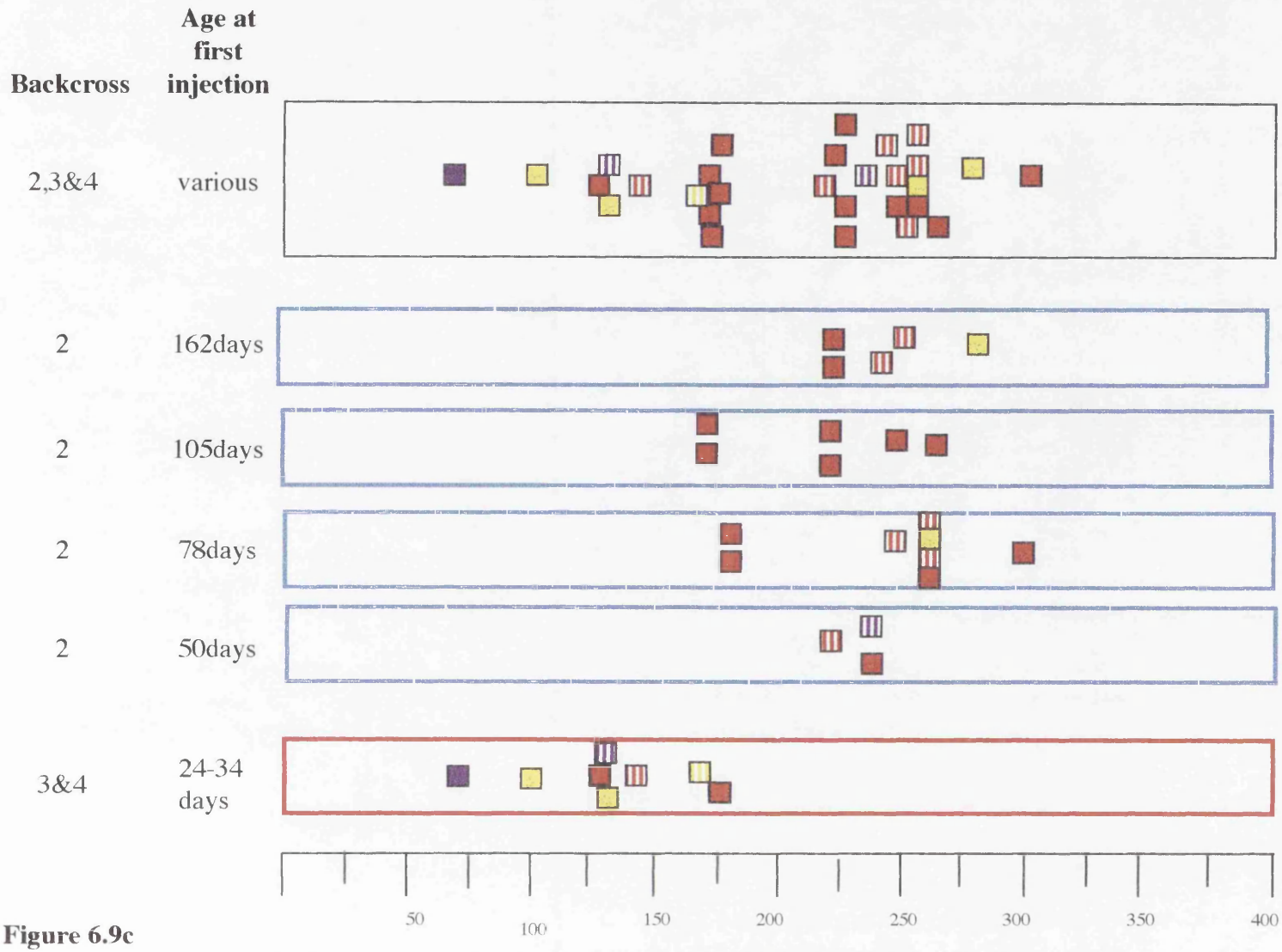

Figure 6.9c

Diagram illustrating the incidence of neoplasia in pristane injected EBNA1positive mice. In the top black outlined box all positive mice are included.The blue outlined boxes illustrate the phenotypes of mice of backcross 2. The mice have been separated according to their age at first injection of pristane. The red outlined box illustrates the phenotypes of mice of backcross 3&4 all of which were first injected between 24 and 34 days of age.

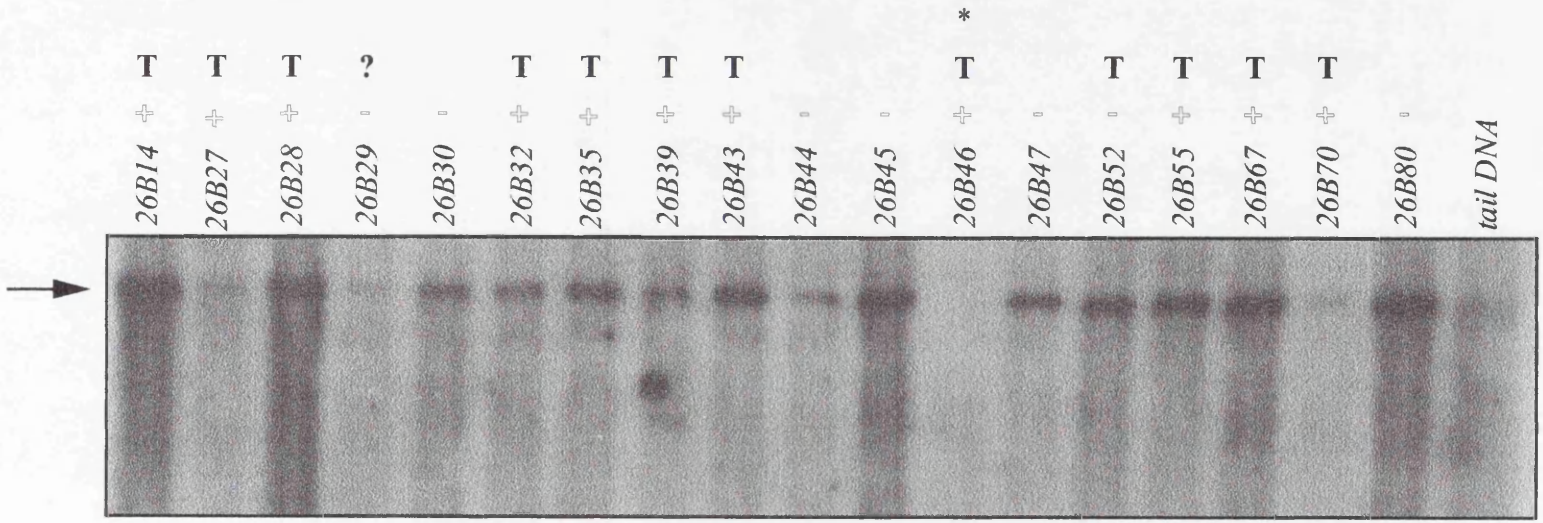

Figure 6.10

Southern blot of EcoRI digested splenic DNAs from pristane injected mice, probed with mmyc1. The arrow indicates the single unrearranged *c-myc* band detected in all DNAs analysed. The numbers in italics indicate the mouse DNA was prepared from. + - EBNA-1 positive mouse, - - transgenically negative mouse. **T** - indicates that the tissue was invaded by a tumour. Tail DNA was included as a control for unrearranged *c-myc*. **?** - indicates that there may have been a developing plasmacytoma. * - indicates that the DNA was partially degraded

and it is not possible to separate the effects of these two variables. Given that susceptibiltiy to pristane induction is known to be tightly strain dependent, and that heterogeneity in genetic background can greatly influence the onset of tumour development (as is clear from the effect of crossing C57Bl/6 line 26 to FVB mice, figure 6.5), it is more relevant to use the data set from backcross 3 and 4 in making conclusions about the effect of pristane induction. By this criteria, the current evidence indicates that pristane induction in the Balb/c strain background does accelerate the development of tumours in line 26B, compared to non injected in C57Bl/6 strain background. All of the line 59B mice were of backcross 2 and were first injected between 30 and 50 days of age (appendix 1.2). As yet the numbers of mice which have developed any phenotype are small, but there is no observable difference between the transgenically positive and negative mice.

Splenic DNA has been prepared from the majority of pristane injected mice. By Southern analysis no rearrangements involving the *c-myc* locus have been detected in any of the DNAs (figure 6.10). However, only an *Eco*RI digest of the DNA has been used. Digestion with further enzymes could reveal rearrangements not discernible here due to the large size of the fragments and consequent poor resolution of similarly sized bands. A number of cell suspensions were prepared from tissues (spleen, lymph node or thymus) of line 26B pristane injected mice as described in materials and methods (chapter 2, section 2.32). These were sent to George Klein's laboratory in Stockholm and are awaiting karyotypic analysis.

6.6 Cross breeding to Eμ-*bcl-2* Transgenic Mice

The oncogene *bcl-2* is commonly involved in follicular lymphoma in humans. Mice carrying an Eμ-*bcl-2* transgene develop monoclonal B-cell lymphomas with a very long latency to tumour development (reviewed in intoduction section 1.7d). To address whether there is any co-operative effect between EBNA-1 and *bcl-2* in tumourigenesis, both line 26 and line 59 mice have been crossed with Eμ-*bcl-2* transgenic mice. The *bcl-2* transgenic mice have been designated line 85. In the line 26 cross there are 68 offspring (tabulated in appendix 1.3), of which it would be expected that 25% would be double positives if there were no detremental developmental consequences. The actual representation of double positves was slightly lower than expected (19%), but this was the same as the percentage of mice EBNA-1 negative/*bcl-2* positive (figure 6.11). In the line 59 cross there are 40 offspring (tabulated in appendix 1.4). Again, the representation of double positive mice is lower than expected (11%) and lower than either transgene singly (figure 6.12). However, the total number of mice involved in this study is still small and therefore these differences may not be significant, as the mice of lines 59 and 85 are long lived with a long latency period to tumour onset. The study of the line 59/85 crossbreed is ongoing. As yet there is no observable increase in the rate of lymphoma onset between the single positive mice and the double positive mice. Over a two year observation period, no tumours have arisen in 4 double positive mice. Of the 7 EBNA-1 positive/bcl-2 negative mice, 3 have

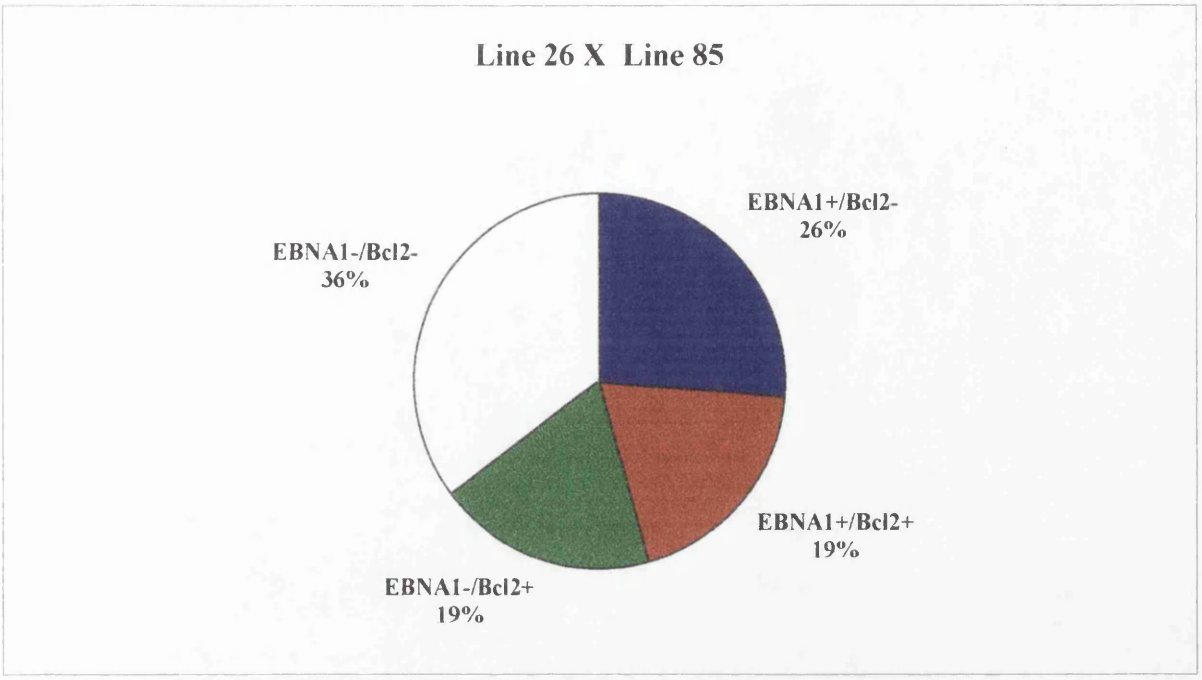

Figure 6.11
Pie chart showing percentage distribution of transgene status of offspring in line 26/line 85 cross

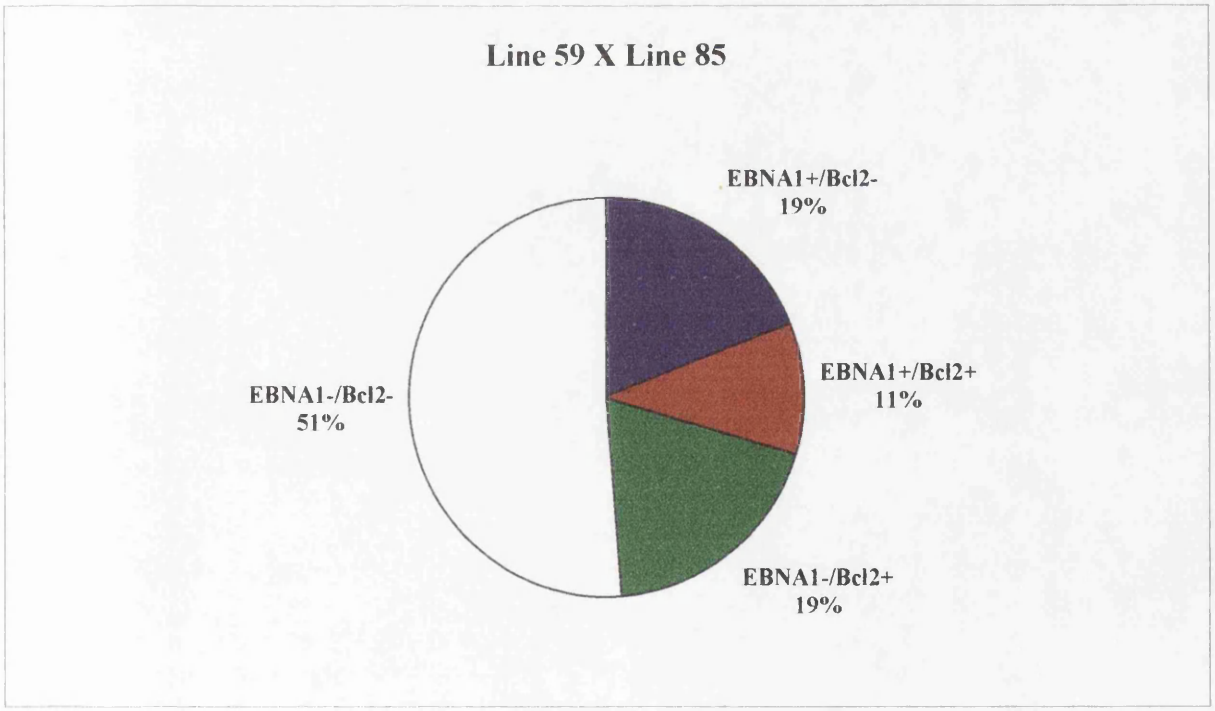

Figure 6.12
Pie chart showing percentage distribution of transgene status of offspring in line 59/line 85 cross

succumbed to lymphoma, and the cause of death of two further mice is not known. 4 of 10 EBNA-1 negative/bcl-2 positive mice have succumbed to lymphoma, and the cause of death of 3 further mice is not known (fig 6.13).

The majority of the EBNA-1 positive mice in the 26/85 cross have succumbed to lymphoma (figure 6.14a). Comparing littermates, there is very little difference between the lymphoma incidence of EBNA-1 positive mice and that of the double positive mice (figure 6.14a and b). The numbers of mice involved in these comparisons are still relatively small; 14 mice EBNA-1 positive/*bcl-2* negative and 10 mice EBNA-1 positive/*bcl-2* positive. Unfortunately, the date of death of 3 double positive mice was not recorded. All three mice survived beyond 280 days of age, but died at some point in the following 60 days and are represented at the mid point of this peroid (310 days) (marked in purple on figure 6.14a). As the total numbers of mice are small, the inclusion of these 3 mice does alter the death curve (figure 6.14c, red). In addition, the shape of the EBNA-1positive/*bcl-2* negative death curve is altered by the inclusion of another 3 mice. One of these mice did not succumb to lymphoma and survived for two years. For the other two of these mice, the exact date of death was not known, although they survived beyond 365 days of age (figure 6.14c, dark blue). If the survival of the double positive mice is compared to the survival of standard line 26 (figure 6.14b and c, pale blue) ,then there is no difference detectable in the rate of tumour onset (figure 6.14a,b and c). The strain background of the standard line 26 and the line 85 mice is predominantly C57Bl/6, thus there should be little or no strain

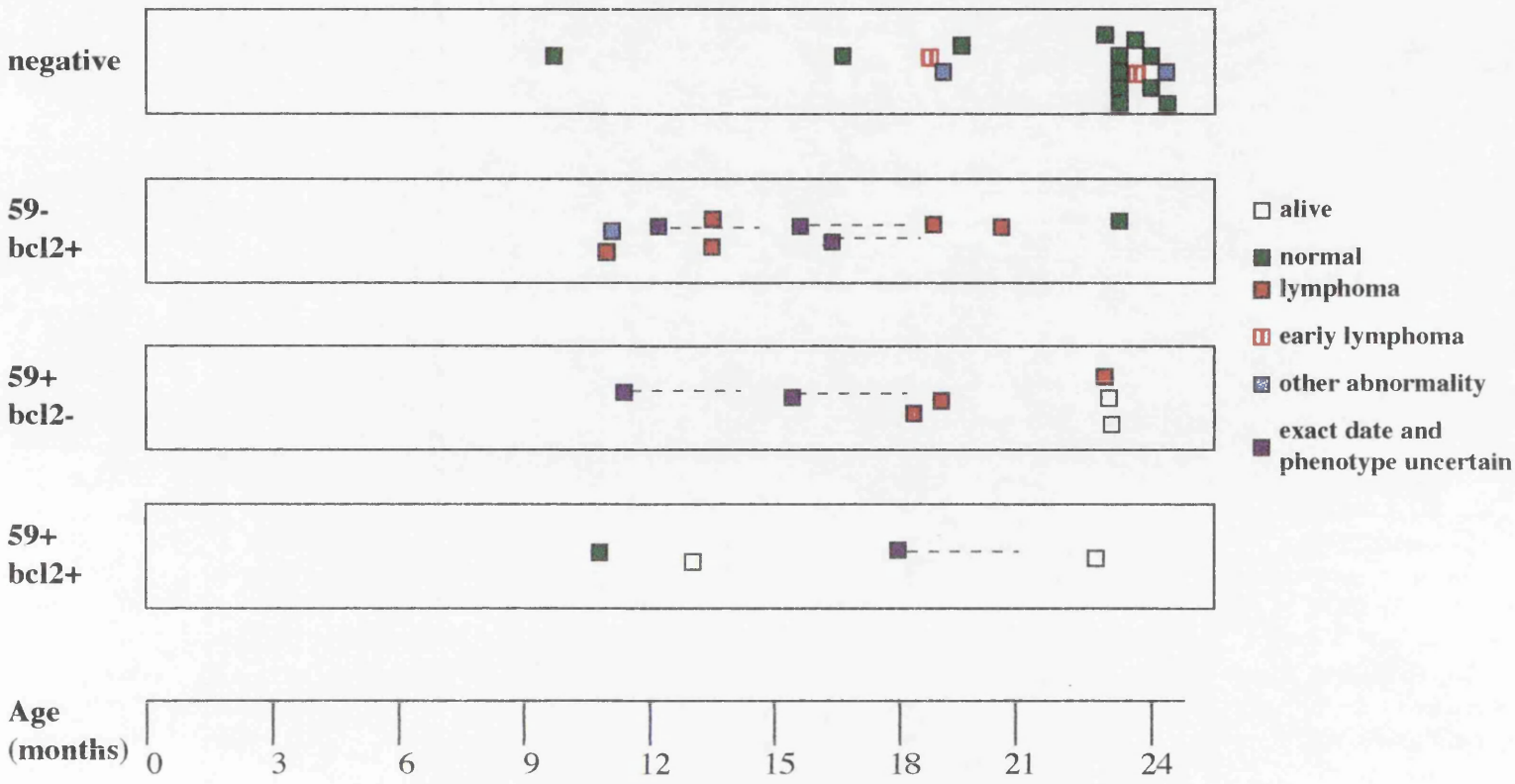

Figure 6.13
Diagram indicating incidence of neoplasia in the 59/85 progeny. The coloured boxes indicate the phenotype of the mice at death (see key). The exact cause of death of some mice is not known, this is indicated in purple. Where the date of death was not recorded, this is indicated by a dashed line.

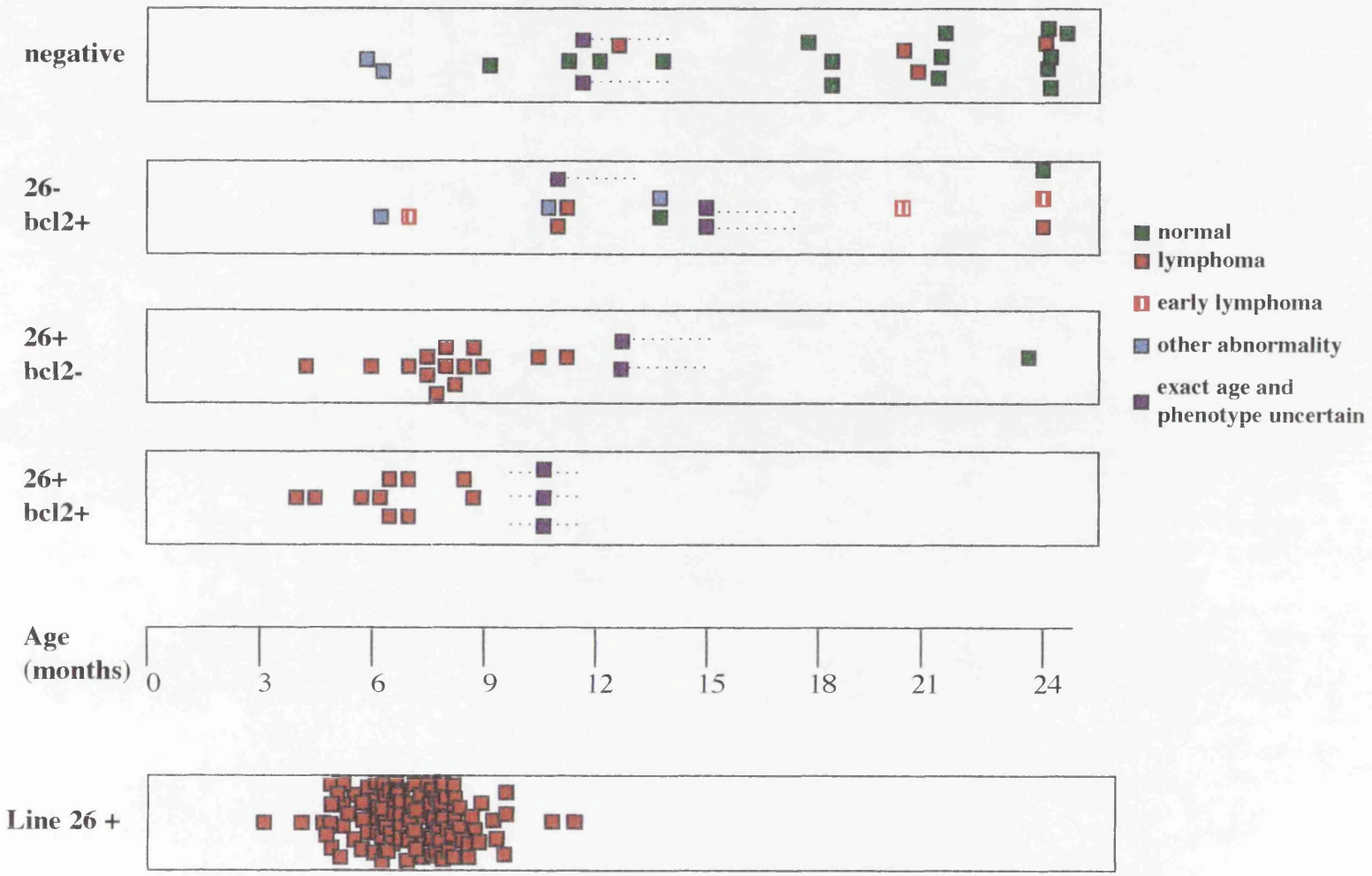

Figure 6.14a

Diagram illustrating the incidence of neoplasia in 26/85 progeny. Coloured boxes indicate the age at death and phenotype of the mice (see key). Included are a number of mice for which there is no precise information on when they died or what the phenotype was, these are indicated in purple. The three double positive mice survived beyond 280days of age but died at some point in the following 60 days without being recorded. For the other mice indicated in purple the date of death may have been up to 12 months after the time point shown. As a comparison the survival of line 26 mice from predominantly C57Bl/6 background, but from outwith this cross is included (line 26+ on figure).

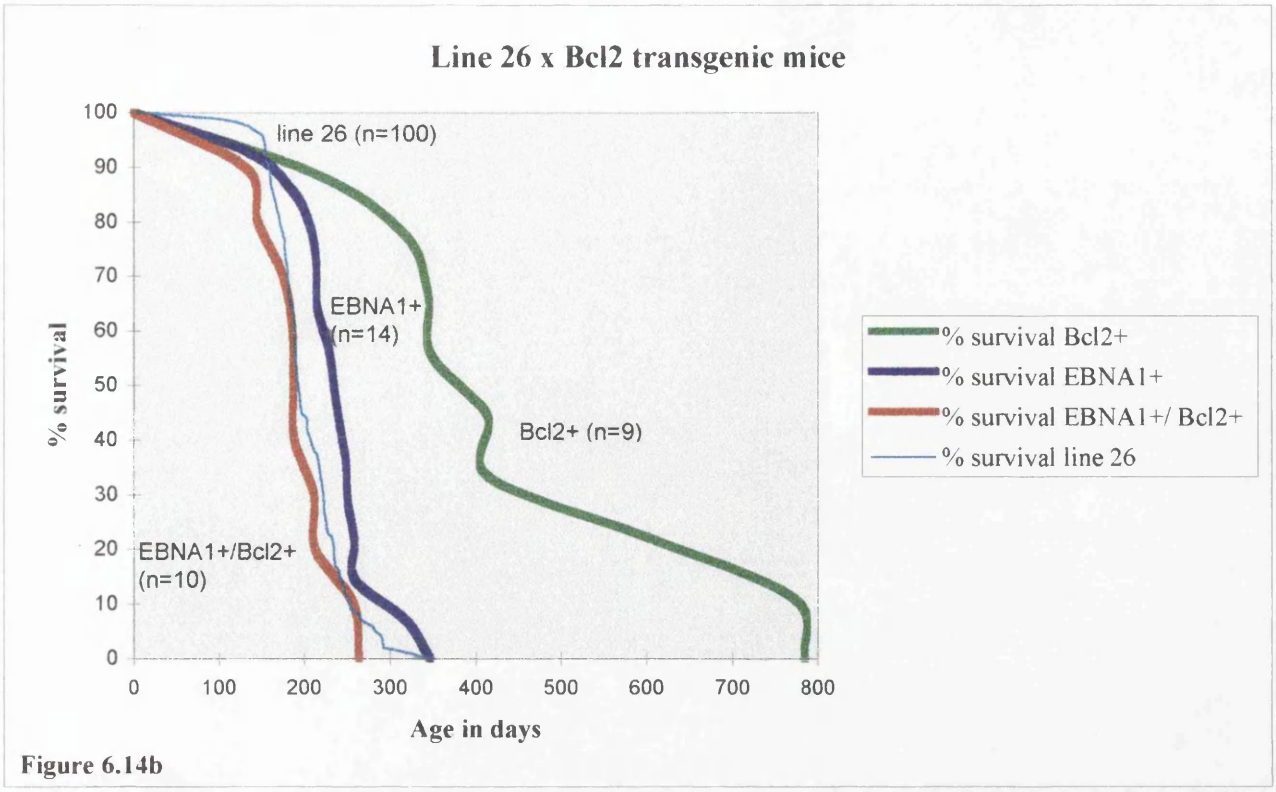

Figure 6.14b

Figure 6.14b

Graph illustrating the incidence of lymphoma in progeny from the cross between line 26 and Bcl2 transgenic mice. For comparison the survival of line 26 mice from outwith this cross (100 mice of predominantly C57Bl/6 strain background) has also been plotted.

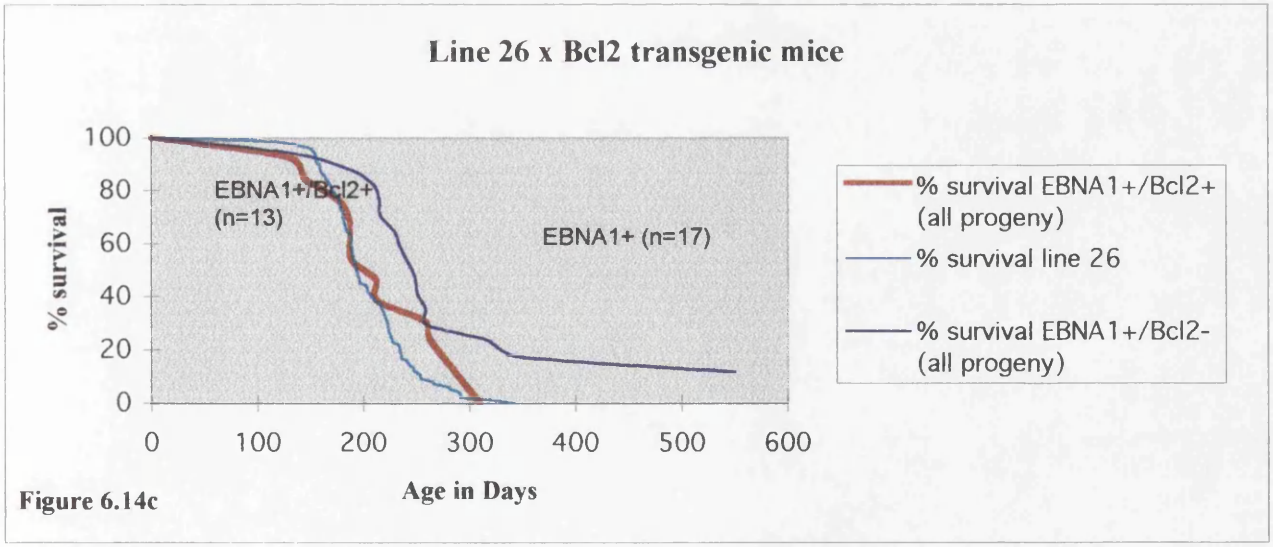

Figure 6.14c

Figure 6.14c

Graph illustrating the incidence of lymphoma in progeny from the cross between line 26 and Bcl2 transgenic mice. These curves include some mice for which the age at death was not recorded. Three EBNA1+/Bcl2+ mice survived for more than 280days, but died at some point in the following 60 days without being recorded and have been plotted at 310days. Two EBNA-1 positive mice survived for more than 370 days and died at some point within the following 12 months without being recorded and have been plotted at 550 days. In addition one EBNA-1 positive mouse did not succumb to lymphoma but was normal when sacrificed at 2 years of age. For comparison the survival of line 26 mice from outwith this cross (100 mice of predominantly C57Bl/6 strain background) has also been plotted.

difference between the two transgenic lines. The average age to death of the EBNA-1positive/*bcl-2* negative mice in this study is 239 days. The average age to death of the standard line 26 mice is 204 days. The average age to death of the double positive mice is 223 days bearing in mind that the 3 mice for which the exact date of death is unknown are included, assuming death at 310 days. The overall conclusion of this study is that there is very little, if any, difference in the onset of tumourigenesis between line 26 EBNA-1 positive mice and line 26 EBNA-1positive/*bcl-2* positive mice. These results indicate that there is no co-operation between EBNA-1 and *bcl-2* in tumourigenesis.

6.7 Cross breeding to Eμ-*Pim-1* Transgenic Mice

Eμ-*pim-1* transgenic mice were imported from Professor Bern's laboratory in Amsterdam. 5-10% of Eμ-*pim-1* transgenic mice develop T-cell lymphomas of by 7 months of age (van Lohiuzen *et al*, 1989). Overexpression of *pim-1* co-operates very strongly with deregulated *c-myc* expression in tumourigenesis (reviewed in introduction section 1.7e). A cross breeding experiment between both EBNA-1 transgenic lines and Eμ-*pim-1* transgenic mice (designated line 87) was set up to investigate whether there is any co-operative effect between these two oncogenes *in vivo*. In the line 26 cross, there are 22 offspring (appendix 1.5). The representation of double positives at 18% is less than the 25% which would be predicted. The 26/87 offspring were derived from a cross where one parent was hemizygous for the Eμ EBNA-1 transgene (C57Bl/6

strain background) and the other parent was hemizygous for the Eμ-*pim-1* transgene (FVB strain background). Given that the number of mice positive for the Eμ-*pim-1* transgene only is also lower than expected (14%), this may well be due to a low rate of transmission of the Eμ-*pim-1* transgene, rather than to a co-operative detremental developmental effect between the two transgenes (figure 6.15). In the line 59 cross there are 35 offspring, all of which are positive for the EBNA-1 transgene (since the line 59 mice used as parents in this cross were homozygous for the EBNA-1 transgene) and 13 of which are also positive for the Eμ-*pim-1* transgene (appendix 1.6). The representation of double positive mice is less than might be predicted (37%), however, given than the representation of *pim-1* positive mice in the line 26 cross is also low this may be due to a low rate of transmission of the Eμ-*pim-1* transgene (figure 6.16). The line 87 mice were of the FVB strain background. Both line 26 and line 59 mice used in this study were of C57Bl/6 strain background. All the progeny of these crosses were therefore F1 hybrids of these two strains. All of the 26/87 EBNA-1 positive mice succumbed to lymphoma. The double positive mice were all euthanased with lymphoma symptoms before the age of 12 months, whereas the EBNA-1 positive/*pim-1* negative mice survived longer (figure 6.17 a and b). The average age to death of the double positives is 284 days compared to 369 days for the EBNA-1 positive/*pim-1* negative mice. However, the numbers of mice involved in this study are still small. The EBNA-1 positive mice from the line 26 cross with

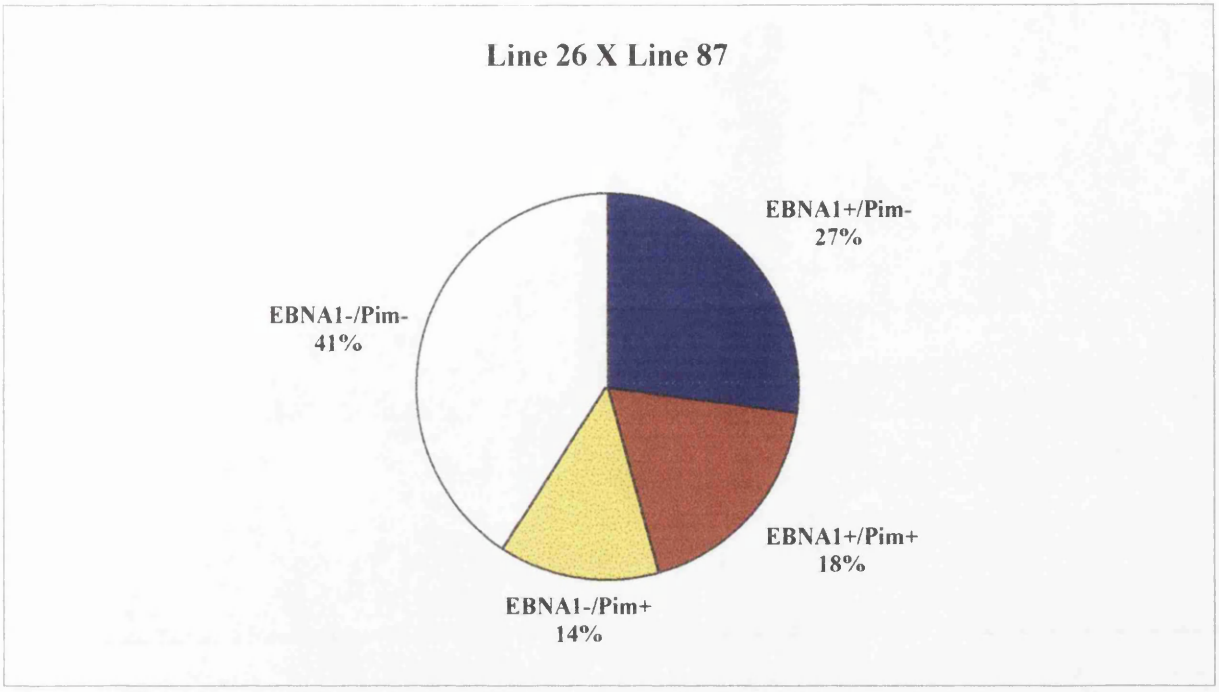

Figure 6.15
Pie chart showing percentage distribution of transgene status of offspring in line 26/ line 87 cross

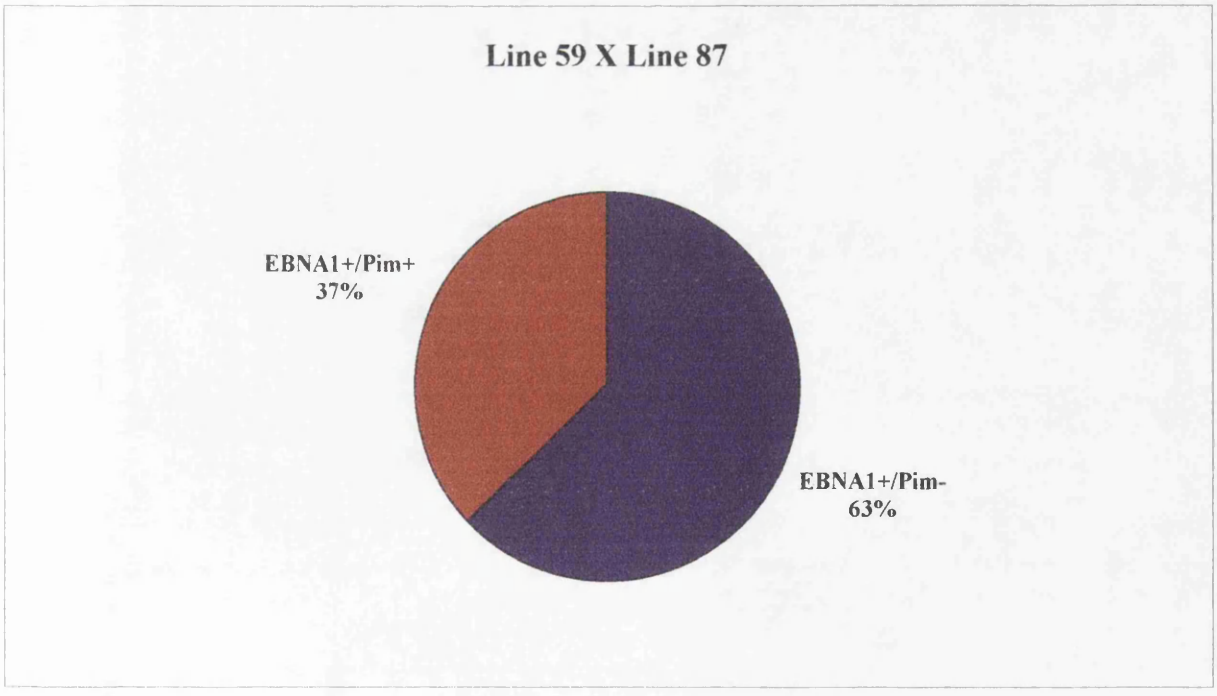

Figure 6.16
Pie chart showing percentage distribution of transgene status of offspring in line 59/ line 87 cross

line 86 (section 6.4) have the same strain background to the 26/87 offspring. Given the conclusion that the 26/86 mice do not harbour an Eμ-*myc* transgene, these mice can be used as a control for the line 26 phenotype in this F1 strain background (figure 6.17 a and b). The average age to lymphoma of the 26/86 EBNA-1 positive mice is 297days. Given the larger number of animals in this group it is probably more valid to use this as a control group (for strain background influence). Even using these mice as a control for the line 26 phenotype, it is difficult to determine if there is any co-operation between the two transgenes, with only 4 mice in the double positive group. Only by increasing the numbers of mice in this study will it be possible to make a firm conclusion about any co-operation between the two transgenes. However, in the line 59 cross with the Eμ-*pim-1* transgenics the latency to tumour onset appears to be less in the double positive mice than in mice with either transgene alone (figure 6.18). 54% of the double positive mice have succumbed to lymphoma by two years of age (average age of onset 466days), compared to 33% of the EBNA-1 positive/*pim-1* negative mice (average age of onset 671 days). This could be suggestive of a co-operative effect between the two oncogenes.

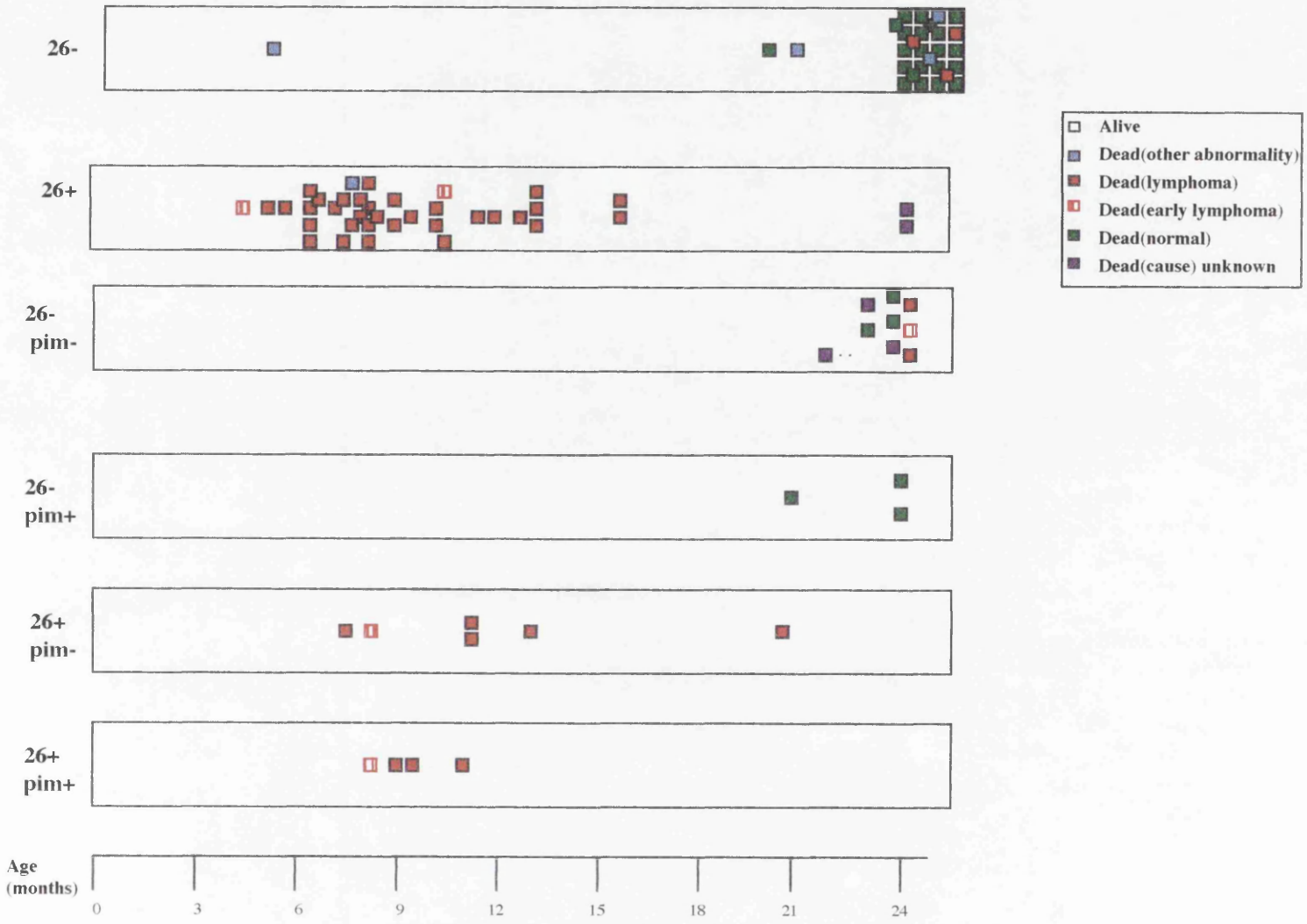

Figure 6.17a

Diagram illustrating survival of 26/87 progeny. The survival of the EBNA1+/myc? mice which have the same strain background are included for comparison. Coloured boxes indicate phenotypes of mice at death (see key). For one mouse the exact date of death was not recorded this is indicated by a dashed line.

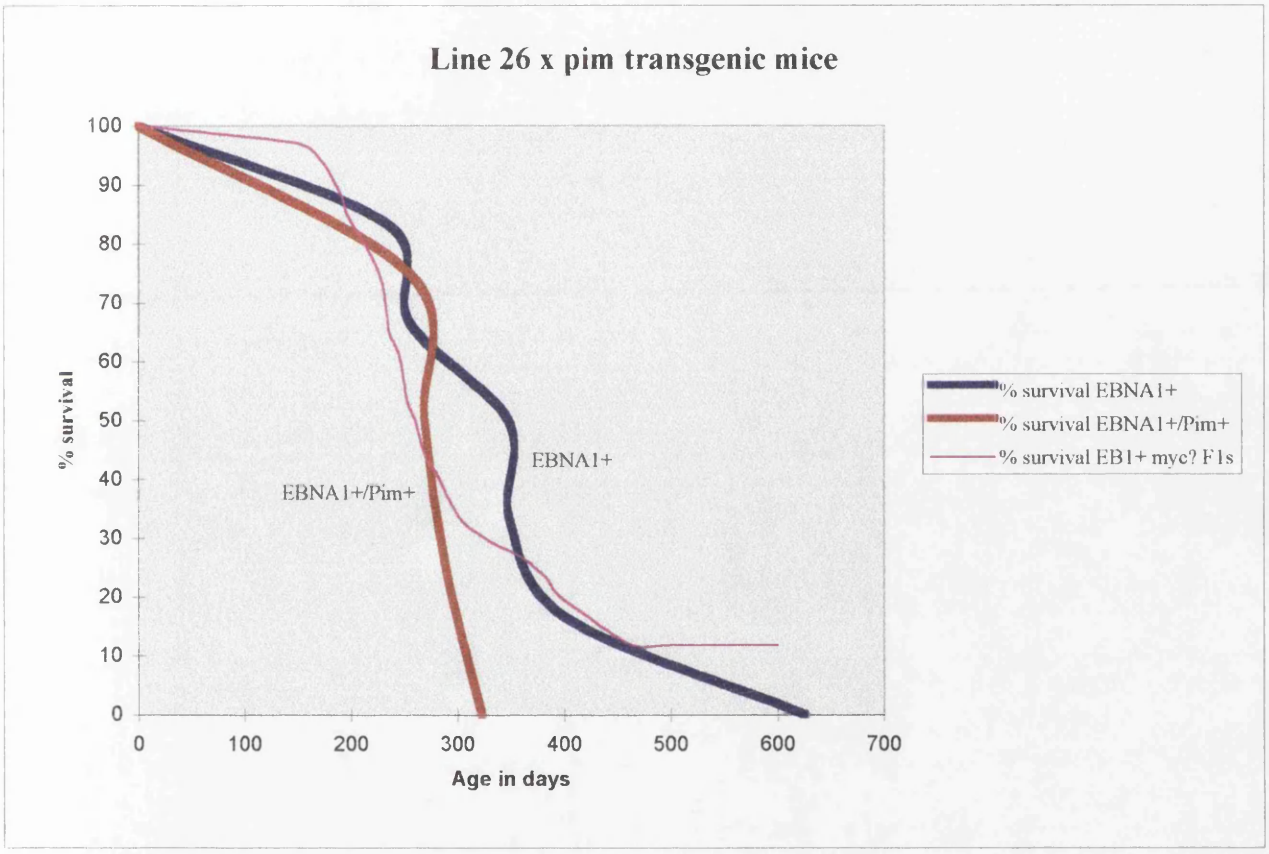

Figure 6.17b
Graph of survival of 26/87 progeny. For comparison the survival of the EBNA1+/myc? mice which have the same strain background are included.

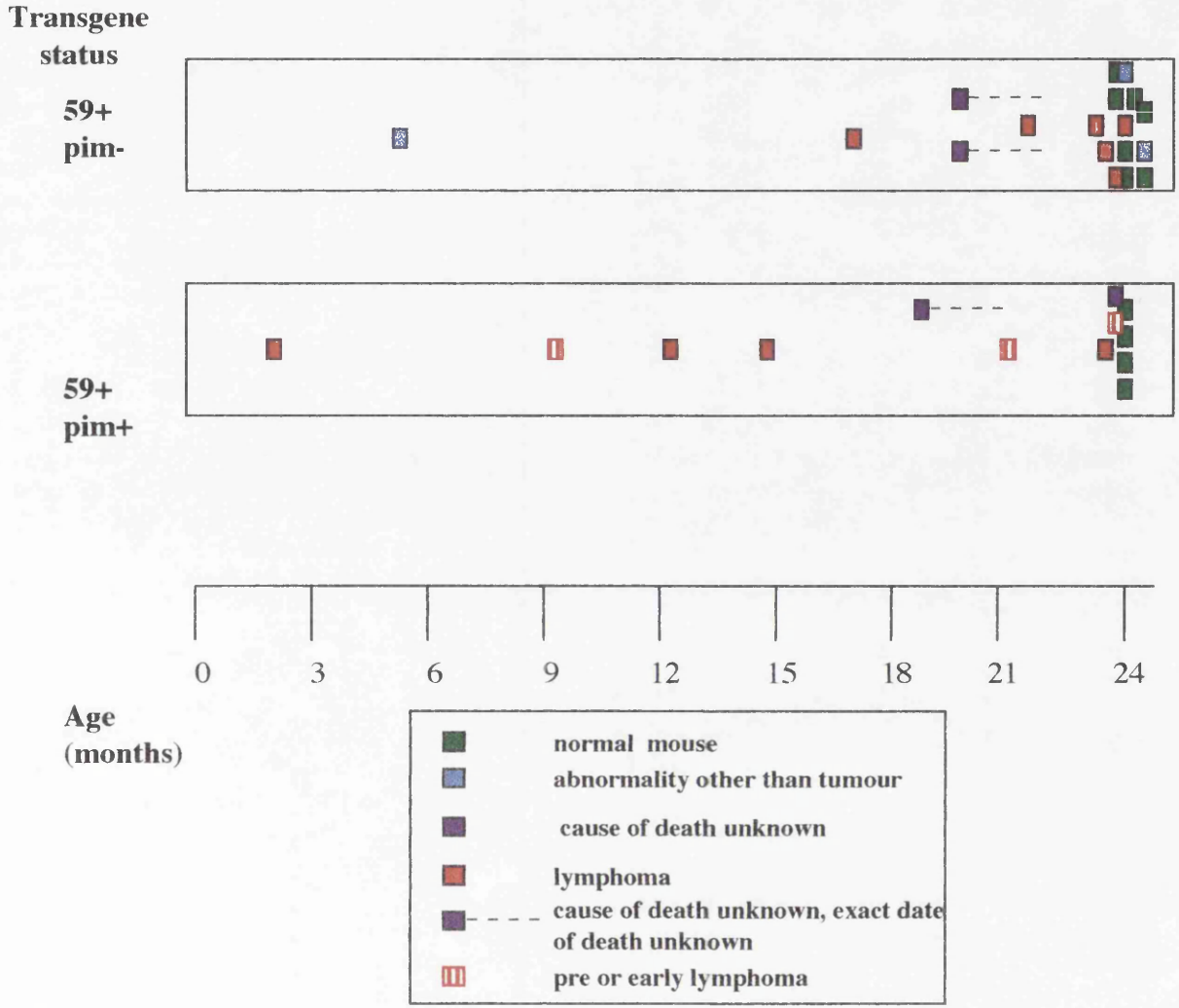

Figure 6.18
Diagram illustrating survival of 59/87 progeny. Coloured boxes indicate phenotypes of mice when sacrificed (see key). Where the exact date of death was not recorded this is indicated by a dashed line.

6.8 Summary and Discussion

A variety of approaches have been used to identify factors which co-operate with EBNA-1 in tumourigenesis.

An analysis of the level of RAG2 expression in RNAs from EBNA-1 transgenics has not revealed any consistent RAG2 upregulation in EBNA-1 expressing cells. It is tempting to speculate that, given that EBNA-1 induces RAG1 and RAG2 expression in tissue culture cells (Srinivas and Sixbey,1995), its oncogenic action might be exerted through enhanced RAG expression giving rise to an increase in aberrant translocations. However the evidence presented here does not support this. Not only is there no apparent consistent RAG2 upregulation, but of 18 EBNA-1 positive tumours karyotyped, only two have been found to carry a translocation. This is a common observation in mice compared to men: human lymphomas often carry translocations, while in mouse tumours translocations are infrequent and polysomy more common (S. Silva, pers.comm).

The strong association between EBNA-1 expression and *c-myc* deregulation in Burkitt's lymphoma leads to the speculation that EBNA-1 may act through, or in contrast in concert with, *c-myc* in tumourigenesis. It is possible that EBNA-1 can contribute to the deregulation of *c-myc* expression through directly transactivating the translocated *c-myc* gene (for example through an effect on the IgH enhancer), or indirectly through transactivation of another gene which subsequently contributes to the deregulation of *c-myc* (for example LR1, introduction section 1.7 and chapter 5). If any one of these is the

mechanism by which EBNA-1 acts as an oncogene, then it might be expected that deregulated *c-myc* expression would be routinely detected in tissues in which EBNA-1 is expressed. Analysis of the levels of *c-myc* expression in tumour and non-tumour samples from Eμ EBNA-1 mice does not provide any evidence that *c-myc* expression is consistently upregulated. RNA was prepared from a number of different positive and negative animals. It is clear that there is a wide variation in the level of *c-myc* expression in non transgenic tissue as well as transgenic tissue regardless of tumour status. This is in agreement with published results (van Lohuizen *et al.*, 1989). The RNA slot blot may not be a sensitive enough assay to detect subtle differences in *c-myc* expression between transgenic and non transgenic tissues. It may be that a relatively small upregulation of *c-myc* expression (perhaps a two fold change) can have a dramatic effect in the appropriate cell type. A comparative study of *c-myc* expression in age matched, strain matched positive and negative mice may yield more information, for example by preparing RNA from an entire litter of mice sacrificed at the same time. However it might be necessary to compare expression in cells which are synchronised with respect to their cell cycle stage to detect any deregulation of *c-myc* expression in cells expressing EBNA-1. Alternatively an assay which assesses *c-myc* promoter usage may be useful in detection of deregulation of the gene. In normal cells, *c-myc* expression is tightly regulated. It can be transcribed from either of two promoters P1 or P2. In fibroblasts and EBV immortalised B cells P2 is utilised preferentially (reviewed in Marcu *et al.* 1992), in Burkitt's lymphoma cells P1

is used at least as frequently (Eick and Bornkamm, 1989). An analysis of *c-myc* promoter usage (for example using an RNase protection assay) in EBNA-1 induced lymphomas might provide evidence of *c-myc* deregulation.

If EBNA-1 acts in concert with *c-myc* in tumourigenesis, it might also be expected to detect an upregulation of *c-myc* expression in EBNA-1 induced tumours. This is not evident from the RNA slot blot results, but again this may be due to an inadequacy in the assay. The levels of expression are being compared between different tissues, taken from different animals of varying ages and genetic background, and so the comparison is not necessarily being made between similar cell types. To address whether *c-myc* upregulation is important in tumourigenesis, it might be more valid to compare levels of expression between pre-neoplastic and neoplastic tissue from Eμ EBNA-1 mice.

Although still preliminary, the results from the crossbreeding of Eμ EBNA-1 lines with N-*myc* and *c-myc* transgenic lines are strongly suggestive of a co-operative effect between the two oncogenes. The latency to tumour onset in EBNA-1/N-*myc* and in EBNA-1/Eμ-*c-myc* double positive mice is considerably reduced when compared to mice carrying either transgene alone. Induction with pristane appears to have decreased the latency to tumour onset in line 26, and also to have led to some incidence of plasmacytoma rather than lymphoma. If one of the effects of pristane is to increase the likelihood of a translocation involving *c-myc* occurring in cells of the immune system, then the decreased latency to tumour onset may be evidence of a co-operation between

EBNA-1 and *c-myc*. Whilst Southern analysis has not detected any rearrangements involving *c-myc* in DNA from pristane injected animals, this may be due to a failing in the assay rather than an absolute lack of *c-myc* locus rearrangements. Many of the DNAs used in Southern analysis were prepared from tissues (usually spleen) in which the tumour may only have been a small part. A genomic rearrangement in a few cells might not be detectable from a background of predominantly normal cells. An *Eco*RI digestion of the DNA was used for the majority of Southerns, it may be that use of different enzyme digestions would aid the detection of *c-myc* rearrangements. The karyotypic analysis being carried out in Prof. Klein's laboratory will be important in determining whether there are any gross chromosomal rearrangements involving the *c-myc* locus (or other loci) in the pristane induced tumours.

It may be that the enhanced rate of tumour induction in pristane injected mice is due to some effect other than, or in addition to, *c-myc* rearrangements. For example it has been found that pristane induction allows the development of plasmacytomas in Eμ *c-myc* transgenic mice (in the Balb/c strain background) at a rate much faster than in transgenically negative mice (Harris *et al.*, 1988). In the absence of pristane these transgenic mice develop lymphomas exclusively. One postulated explanation is that macrophages treated with pristane produce IL-6, which is a factor needed for plasmacytoma growth (Hirano *et al.*, 1990, Kawano *et al.*, 1988). In the absence of pristane there may be insufficient IL-6 to allow plasmacytoma development. IL-6 transgenic mice develop spontaneous plasmacytomas carrying a *c-myc* translocation

when bred into a Balb/c genetic background (Suematsu *et al.*, 1992) .Whilst the induction of a *c-myc* translocation may be the rate limiting factor in the development of plasmacytomas, other factors have a role to play.

63% (5 of 8) of the EBNA-1 positive tumours analysed by karyotyping have been shown to carry trisomy chromosome 15, on which the murine *c-myc* gene is located. Whilst it may be that there is some other locus on chromosome 15 which co-operates with EBNA-1 in tumourigenesis, it is tempting to suggest that the additional copy of *c-myc* is the essential co-operating oncogene. Three tumours from mice doubly positive for the EBNA-1 transgene and the N-*myc* transgene also carried trisomy 15. It could be argued that the deregulated expression of *c-myc* due to trisomy chromosome 15 would be redundant in a cell already over-expressing N-*myc*. However, the N-*myc* transgenic line used in the cross breeding experiment expresses the transgene to a very low level and the tumour phenotype is of low penetrance and long latency in this line. It is therefore possible that further expression of *c-myc* is important in the development of tumours. Further weight is added to the argument that the *c-myc* locus is important by the observation that none of the line 26/Eμ-*c-myc* double positive tumours carried trisomy 15. In these tumours the high level of *c-myc* expression provided by the transgene may have supplanted the need for trisomy 15 in oncogenesis.

Taken as a whole the evidence to date is strongly suggestive of a co-operative role for *c-myc* deregulation in EBNA-1 induced tumourigenesis.

The identification of other oncogenes which are able to co-operate with EBNA-1 may aid in the understanding of what function EBNA-1 plays in tumourigenesis. Using Southern analysis to detect DNA rearrangements of a number of known oncogenes (*bcl-2*, *p53*, *cyclinD1*, *pim-1*) none have been detected in EBNA-1 tumours.

The numbers of mice in the crossbreeding with Eμ *pim-1* transgenics is still small and so the results are difficult to interpret, particularly due to the added complication of strain background differences. It is clear from the analysis of line 26 that the genetic strain background of the mice can greatly influence the transgenic phenotype. Line 26 EBNA-1 positive mice that are F1 hybrids between C57Bl/6 strain and FVB strain develop lymphomas at a reduced rate when compared to mice of the C57Bl/6 background (figure 6.5a and b). For Eμ *c-myc* transgenic mice, genetic background has been found to have a profound effect not only on the latency to tumour onset, but also on the nature of the tumour cells. In some strain backgrounds tumours were predominantly of B lymphoid origin; in C3H/HeJ mice tumours were predominantly of T lymphoid origin (Yukawa *et al.*, 1989).

Current results from the crossbreeding of line 59 and Eμ *pim-1* transgenics are suggestive of a co-operation between EBNA-1 and *pim-1*. The latency to tumour onset is reduced and the percentage of mice succumbing to lymphoma is higher among double positives. However, the result from the line 26 Eμ *pim-1* cross is less clear cut. The latency to tumour onset in double positive mice is only slightly reduced when compared to mice positive for EBNA-1 alone,

although the deaths of the mice are all clustered at an early time point. Only through increasing the numbers of mice in this study will it be possible to determine if there is some degree of co-operation between the transgenes.

It is possible that the different pattern of expression of the EBNA-1 transgene in line 26 and line 59 is a factor in the apparent different level of co-operation with the Eμ *pim-1* transgene. The EBNA-1 transgene in line 59 is expressed more widely (introduction table 1.2), including within the thymus. The Eμ *pim-1* transgene alone induces lymphomas of a T cell origin. The more obvious co-operation between line 59 Eμ EBNA-1 and Eμ *pim-1* may be due to a greater degree of overlap in the expression of the two transgenes. Determination of the cell type of tumours in the double positives from both line 26 and line 59 may provide clarification on this point.

Crossbreeding of the Eμ EBNA-1 transgenics to Eμ *bcl-2* mice indicates that there is no co-operation between EBNA-1 and *bcl-2*. By comparison, there is a very strong co-operation between Eμ *c-myc* and Eμ *bcl-2* in transgenic mice (Strasser *et al.*, 1990). This is further evidence that the oncogenic effect of EBNA-1 is not exerted through deregulation of *c-myc* (figure 6.19). It may be that EBNA-1 expression in a cell provides a similar function to the upregulation of *bcl-2* to render the co-expression of these two oncogenes redundant in lymphomagenesis. It is thought that *bcl-2* exerts its oncogenic effect through allowing cells to avoid death by apoptosis (reviewed in introduction section 1.8b). EBNA-1 may act on some other gene product involved in an anti-apoptic pathway. The mechanism by which EBNA-1

protects from apoptosis may not be through *bcl-2*. Burkitt's lymphoma cell lines which retain the cellular phenotype of freshly isolated tumour cells (group I EBV latency) are sensitive to apoptosis induced by a number of triggers(Gregory *et al.*, 1991). Over-expression of bcl-2 in group I BL cell lines following gene transfer affords protection from apoptosis (Milner *et al.*, 1992). However, in this *in vivo* assay (lymphoma onset), the upregulation of bcl-2 is redundant.

A high percentage of the EBNA-1 positive tumours (56%) analysed carry trisomy chromosome 10. One potentially important locus on chromosome 10 is *mdm-2* a proto-oncogene which is a negative regulator of *p*53. Mutation of *p*53 is found in some Burkitt's lymphomas (Farrell *et al.*, 1991). Upregulation of *mdm-2* provides an alternative route to reduce *p*53 function. *Mdm-2* is located on human chromosome 12, and trisomy 12 is the most common numerical chromosomal abnormality in human B-cell malignancies (Offit *et al.*, 1991).

By analogy with other systems, it is possible to speculate that if EBNA-1 fufills an anti-apoptic role in tumourigenesis *in vivo,* then it may not co-operate with other oncogenes which fufill the same role (introduction section 1.8, 1.9). So, EBNA-1 might not be expected to co-operate with other genes involved in apoptic regulation (figure 6.19).

The evidence suggests that EBNA-1 co-operates with *c-myc* in tumourigenesis, and may also co-operate with *pim-1*. The use of murine MoMLV proviral tagging experiments (intoduction section 1.6c) may identify

further oncogenic partners of EBNA-1 (work ongoing in this laboratory).

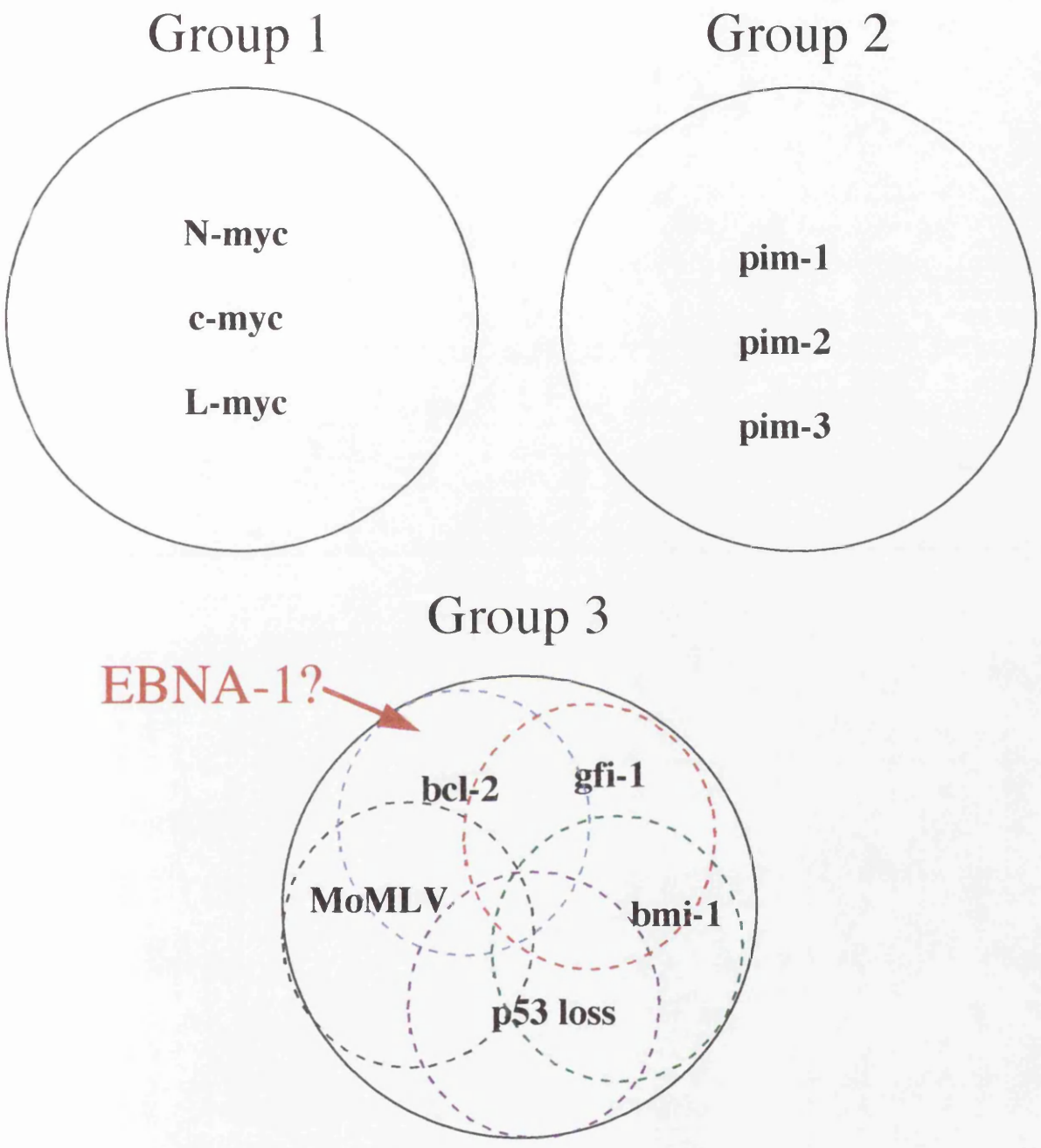

Figure 6.19
Complementation groups in lymphomagenesis. Several oncogenes, p53 loss and MoMLV have been grouped according to their ability to synergise in lymphomagenesis *in vivo* (reviewed in introduction section 1.6, 1.8-9). For example *myc* family members can synergise with *pim*-1 or *bcl*-2, but p53 loss and *bcl*-2 do not synergise. Not all combinations have been analysed. Given that there is no apparent co-operation between EBNA-1 and *bcl*-2(section 6.6, 6.8), and there is mounting evidence for co-operation between EBNA-1 and *myc*, EBNA-1 has been placed in group 3. (Adapted from Wilson, 1997).

Summary and Future Directions

The incidence of lymphoma in two different lines of Eμ EBNA-1 transgenic mice is strong evidence that EBNA-1 is an oncogene. However, differences in penetrance and latency to onset of disease between the lines lead to a number of questions. Influences of the local cellular environment, transgene copy number, or mutation of transgene sequences could be contributing factors in the different phenotypes of the lines.

In both lines the pattern of transcription of the transgene is complex (chapter 3) , however, there are clear differences between the lines. In addition, in both lines there are transcripts which may contain cellular sequences. An analysis of the transgene insertion sites will be important in understanding the contribution of local cellular sequences to the observed transgenic phenotypes. Sequences analysis of the inserted transgenes could identify point mutations which may contribute to the different expression patterns, or which effect EBNA-1 protein activity.

EBNA-1 protein expression was maintained in a cell line derived from a line 26 tumour even after 12 weeks in culture (chapter 4). Establishment of further cell lines from EBNA-1 induced tumours may be valuable in the investigation of the role of EBNA-1 in tumour maintenance.

The total level of transgenic RNA is higher in line 26 than in line 59 (chapter 3), as is the detectable level of EBNA-1 DNA binding activity in line 26 tumour samples (chapter 5). However, Western analysis has detected more

EBNA-1 protein in line 59 tumour and pre-tumour samples (Wilson and Levine, 1992). Immunostaining of EBNA-1 protein in transgenic tissues from both lines at different stages of tumour development may aid the comparison of the relative levels and distribution of the protein in each of the lines at different stages of tumourigenesis.

The steady state level of EBNA-1 protein may not reflect protein activity. The level of protein activity (DNA binding) may be a better criterion for measurement of oncogenicity. Therefore, the study of EBNA-1 DNA binding should be extended to compare the activities in both line 26 and line 59 at different stages of tumourigenesis. It may be that the level of EBNA-1 DNA binding activity is enhanced in line 59 tumours. Comparative sequencing of DNA from tumour and pre-tumour tissues could identify mutations which 'activate' EBNA-1 in oncogenesis.

Gel retardation assays have identified a factor(s) which bind both to EBNA-1 and LR1 DNA binding sites. Supershift assays using an antibody to nucleolin would identify whether this component of the transcription factor LR1 binds to both sites.

A number of lines of evidence indicate that deregulated *c-myc* and EBNA-1 co-operate in tumourigenesis (chapter 6). Therefore, EBNA-1 may play a direct role in the pathogenesis of Burkitt's lymphoma in conjuction with *c-myc*.

The crossbreeding of transgenic mice suggests that EBNA-1 and *bcl-2* do not synergise in lymphomagenesis. *In vivo* EBNA-1 may have an anti-apoptic like activity, which renders the co-expression of *bcl-2* redundant *in vivo* in

tumourigenesis. Transgenic crossbreeding experiments in combination with the use of MuMLV pro-viral tagging experiments may identifiy other oncogenic partners of EBNA-1, and further elucidate the mechanism by which it acts as an oncogene.

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Line 26 x Line 86

mouse number	date of birth	date of death	Age at death	Transgene status	Phenotype
1	26.3.94	3.7.95	464ds	EB1+ myc?	ill looking, swollen abdomen, LNs and spleen large, liver slighlty large, white spots on spleen and liver
2	26.3.94	26.3.96	730ds	EB1- myc?	normal
3	26.3.94	26.3.96	730ds	EB1- myc?	normal
4	26.3.94			EB1+ myc?	
5	26.3.94			EB1- myc?	
6	26.3.94	3.7.95	464ds	EB1+ myc?	sacrificed due to difficulty with walking, LNs large, spleen large with white spots, liver normal size but virtually white
7	26.3.94			EB1- myc?	
8	26.3.94	11.4.95	381ds	EB1+ myc?	massive lymphoma, spleen and liver ++++, LNs+
9	26.3.94	17.5.96	782ds	EB1- myc?	Animal was very slow moving, spleen, liver, LNs and thymus appeared normal
10	26.3.94			EB1- myc?	
11	26.3.94	19.8.94	146ds	EB1- myc?	Thinner than littermates and hunched, spleen and LNs slighlty large
12	26.3.94	15.11.94	234ds	EB1+ myc?	massive lymphoma, spleen and LNs huge, liver normal-slightly large
13	26.3.94	29.11.94	248ds	EB1+ myc?	Swollen abdomen, massive lymphoma, spleen enormous, liver huge, LNs and thymus enlarged
14	26.3.94	10.5.96	775ds	EB1- myc?	tumour in large bowel, thymus spleen and LNs appeared normal, liver appeared pale
15	26.3.94	6.11.95	590ds	EB1- myc?	swollen abdomen, very fat otherwise normal
16	26.3.94	26.4.96	761ds	EB1- myc?	animal looked ill, spleen was normal size but had small tumour attached to it, liver normal size but abnormal appearance, thymus slightly large
17	26.3.94	12.12.95	626ds	EB1- myc?	paralysed back legs, possibly due to blocked bladder, spleen, liver, LNs all normal
18	26.3.94	26.4.96	761ds	EB1- myc?	left axillary LNs were enormous, other LNs normal, spleen x2, thymus x2, liver normal
19	26.3.94	2.8.94	129ds	EB1+ myc?	mouse very thin looking, coat patchy & uneven coloured, quite hunched, LNs, spleen, thymus slightly enlarged
20	26.3.94			EB1- myc?	
21	26.3.94	26.3.96	730ds	EB1- myc?	normal
22	26.3.94	30.12.94	279ds	EB1+ myc?	massive lymphoma, spleen and liver huge, LNs slightly enlarged
23	26.3.94	26.3.96	730ds	EB1- myc?	normal
24	26.3.94			EB1- myc?	
25	26.3.94			EB1- myc?	
26	26.3.94		?	EB1+ myc?	?
27	26.3.94	26.3.96	730ds	EB1+ myc?	normal
28	28.3.94			EB1+ myc?	

Line 26 x Line 86

mouse number	date of birth	date of death	Age at death	Transgene status	Phenotype
29	28.3.94			EB1- myc?	
30	28.3.94	28.3.96	730ds	EB1+ myc?	normal
31	28.3.94	28.3.96	730ds	EB1- myc?	normal
32	28.3.94	17.5.95	414ds	EB1+ myc?	unwell appearance, spleen and liver large and mottled, large red mass attached to gut possibly lymphoid origin, kidneys slightly enlarged, LNs normal
33	28.3.94	7.12.94	253ds	EB1+ myc?	paralysed back legs, abdomen swollen, spleen large, liver slightly large and mottled appearance, kidneys grossly enlarged and mass below them possibly lymphoid or ovary, LNs slightly large
34	28.3.94	28.3.94	730ds	EB1- myc?	normal
35	23.4.94	23.4.96	730ds	EB1- myc?	normal
36	23.4.94	29.5.96	766ds	EB1- myc?	spleen x10, liver x3, thymus and LNs normal
37	23.4.94	13.12.94	234ds	EB1+ myc?	swollen abdomen, huge spleen and liver, LNs slightly large
38	23.4.94	23.4.96	730ds	EB1- myc?	normal
39	23.4.94			EB1- myc?	
40	23.4.94	20.3.95	331ds	EB1+ myc?	found dead, spleen and liver huge
41	23.4.94			EB1- myc?	
42	24.4.94	14.2.95	296ds	EB1+ myc?	swollen abdomen, spleen+++, liver +++, LNs normal
43	24.4.94	14.2.95	296ds	EB1+ myc?	swollen abdomen, spleen and liver gigantic, LNs normal
44	24.4.94	24.4.96	730ds	EB1- myc?	normal
45	24.4.94	13.10.94	172ds	EB1+ myc?	unwell appearance, swollen abdomen, huge lymphoma, spleen and liver enormous, LNs slightly enlarged
46	24.4.94	1.11.94	191ds	EB1+ myc?	Swollen abdomen, spleen enormous, liver and LNs enlarged
47	24.4.94	24.4.96	730ds	EB1- myc?	normal
48	24.4.94	24.4.96	730ds	EB1- myc?	normal
49	24.4.94	21.12.94	241ds	EB1+ myc?	quite fat but otherwise normal
50	24.4.94	11.4.95	362ds	EB1+ myc?	massive lymphoma, spleen and liver ++++, LNs +
51	24.4.94	16.12.94	236ds	EB1+ myc?	massive lymphoma, spleen and liver huge, LNs enlarged
52	24.4.94	24.4.96	730ds	EB1- myc?	normal
53	24.4.94	24.4.96	730ds	EB1- myc?	normal
54	24.4.94	1.11.94	191ds	EB1+ myc?	swollen abdomen, spleen enormous, liver and LNs enlarged
55	16.5.94	16.5.96	730ds	EB1- myc?	normal
56	16.5.94	13.6.95	393ds	EB1+ myc?	massive lymphoma, spleen and liver enormous, LNs enlarged

mouse number	date of birth	date of death	Age at death	Transgene status	Phenotype
57	16.5.94	16.5.96	730ds	EB1- myc?	normal
58	16.5.94	7.12.94	215ds	EB1+ myc?	swollen abdomen, spleen huge, liver huge, LNs slightly large
59	16.5.94	13.10.94	150ds	EB1+ myc?	Swollen abdomen, enormous liver and spleen, LNs slightly large
60	16.5.94	7.12.94	205ds	EB1+ myc?	swollen abdomen, spleen enormous, liver enlarged
61	16.5.94	24.1.95	253ds	EB1+ myc?	swollen abdomen, massive lymphoma, spleen and liver huge, LNs enlarged
62	16.5.94	31.1.95	260ds	EB1+ myc?	massive lymphoma, spleen enlarged, LNs huge, liver normal
63	16.5.94	20.3.95	308ds	EB1+ myc?	massive lymphoma, spleen and LNs huge, liver enlarged
64	20.5.94	20.5.96	730ds	EB1- myc?	normal
65	20.5.94			EB1+ myc?	
66	20.5.94	20.3.95	304ds	EB1+ myc?	?
67	20.5.94	20.5.96	730ds	EB1- myc?	normal
68	20.5.94	20.5.96	730ds	EB1- myc?	normal
69	20.5.94	2.12.94	196ds	EB1+ myc?	swollen abdomen, huge liver and spleen, LNs normal-slightly large
70	20.5.94	20.5.96	730ds	EB1- myc?	normal
71	20.5.94	13.2.95	269ds	EB1+ myc?	unwell appearance, massive lymphoma, spleen, liver LNs all large
72	20.5.94	20.5.96	730ds	EB1- myc?	normal
73	20.5.94	13.2.95	269ds	EB1+ myc?	found dead, carcass canablised
74	20.5.94	4.1.95	229ds	EB1+ myc?	Enormous liver and spleen, LNs slightly enlarged
75	20.5.94	19.1.95	244ds	EB1+ myc?	massive lymphoma, spleen, liver LNs enlarged
76	20.5.94	4.1.95	229ds	EB1+ myc?	huge lymphoma, spleen enormous, liver enormous, LNs enlarged

Appendix 1.1a
Data collected on 26/86 progeny. Mice positive for the EBNA1 trangene are labelled as EB1+ myc?. Notes in the phenotype section indicate the gross observations made on the animal at the time of its death

Line 59 x Line 86

mouse number	date of birth	date of death	Age at death	Transgene status	Phenotype
59/86 1	4.4.94	4.4.96	730ds	EB1- myc?	normal
2	4.4.94	4.4.96	730ds	EB1- myc?	normal
3	4.4.94	4.4.96	730ds	EB1+ myc?	normal
4	4.4.94	4.4.96	730ds	EB1+ myc?	normal
5	4.4.94	4.4.96	730ds	EB1- myc?	normal
6	4.4.94	4.4.96	730ds	EB1- myc?	normal
7	4.4.94	4.4.96	730ds	EB1+ myc?	normal
8	4.4.94	4.4.96	730ds	EB1- myc?	normal
9	4.4.94	4.4.96	730ds	EB1- myc?	normal
10	4.4.94	4.4.96	730ds	EB1+ myc?	normal
11	4.4.94	4.4.96	730ds	EB1- myc?	normal
12	6.4.94	10.5.96	730ds	EB1- myc?	normal
13	6.4.94	17.11.95	590ds	EB1- myc?	Back very scarred, possibly due to fighting or mites, spleen, liver, LNs normal
14	6.4.94	6.4.96	730ds	EB1- myc?	normal
15	6.4.94	14.2.95	314ds	EB1- myc?	Back very scarred, possibly due to fighting or mites, left S.I LN slightly enlarged, otherwise normal
16	6.4.94	6.4.96	730ds	EB1- myc?	normal
17	6.4.94	6.4.96	730ds	EB1- myc?	normal
18	6.4.94	15.4.96	740ds	EB1- myc?	tumour on lung, other tissues appeared normal
19	6.4.94	6.4.96	730ds	EB1+ myc?	normal
20	30.4.94	30.4.96	730ds	EB1- myc?	normal
21	30.4.94			EB1- myc?	
22	30.4.94	30.4.96	730ds	EB1- myc?	normal
23	30.4.94	30.4.96	730ds	EB1- myc?	lymphoma evident, LNs huge, spleen x10, thymus x10, liver normal
24	30.4.94	30.4.96	730ds	EB1- myc?	normal
25	30.4.94	30.4.96	730ds	EB1- myc?	normal
26	30.4.94	30.4.96	730ds	EB1- myc?	spleen x5, other tissues appeared normal
27	21.5.94	21.5.96	730ds	EB1- myc?	normal
28	21.5.94	21.5.96	730ds	EB1- myc?	normal

Line 59 x Line 86

mouse number	date of birth	date of death	Age at death	Transgene status	Phenotype
29	21.5.94	21.5.96	730ds	EB1- myc?	normal
30	21.5.94	22.4.96	337ds	EB1- myc?	Animal swollen. Abdomen blood filled, liver pale coloured, spleen 2x normal
31	21.5.94	21.5.96	730ds	EB1- myc?	normal
32	21.5.94	21.5.96	730ds	EB1- myc?	normal
33	14.6.94			EB1- myc?	
34	14.6.94			EB1- myc?	
35	14.6.94			EB1- myc?	
36	14.6.94			EB1- myc?	
37	14.6.94			EB1- myc?	
38	14.6.94			EB1- myc?	
39	14.6.94			EB1- myc?	
40	13.6.94			EB1+ myc?	
41	13.6.94			EB1+ myc?	
42	13.6.94			EB1+ myc?	
43	13.6.94			EB1+ myc?	
44	13.6.94	13.6.96	730ds	EB1- myc?	spleen, LNs, thymus normal, large white tumour mass attached to liver
45	13.6.94			EB1- myc?	
46	13.6.94			EB1- myc?	
47	13.6.94			EB1+ myc?	
48	13.6.94			EB1+ myc?	
49	13.6.94			EB1+ myc?	
50	13.6.94			EB1- myc?	
51	13.6.94			EB1+ myc?	
52	17.6.94			EB1- myc?	
53	17.6.94			EB1- myc?	
54	17.6.94	5.6.96	718ds	EB1+ myc?	spleen enlarged (x5), all other tissues appeared normal
55	17.6.94			EB1- myc?	
56	17.6.94	5.6.96	718ds	EB1+ myc?	spleen enlarged (x3), all other tissues normal

57	17.6.94			EB1+ myc?	
58	17.6.94	26.4.96	679ds	EB1+ myc?	axillary and lateral axillary LNs were huge (left side), other LNs were normal, spleen was slightly large (x2), liver normal
59	17.6.94			EB1+ myc?	
60	17.6.94			EB1- myc?	
61	17.6.94			EB1+ myc?	

Appendix 1.1b
Data collected on 59/86 progeny. Mice positive for the EBNA1 transgene are labelled as EB1+ myc?. Notes in the phenotype section indicate the gross observations made on the animal at the time of its death.

mouse number	date of birth	date of death	Age at death	Transgene status	Phenotype
59B 92	23.3.95	17.11.95	208ds	EB1-	huge abdomen, masses of fluid in peritoneal cavity, spleen 4-5x normal, liver 2-3x normal, some nodules, thymus enlarged
93	23.3.95			EB1+	
94	23.3.95			EB1+	
95	23.3.95	4.3.96	346ds	EB1-	LNs and liver normal, spleen 2x normal, lots of fluid and nodules in peritoneal cavity
96	23.3.95			EB1+	
97	23.3.95	20.11.95	211ds	EB1-	spleen, stomach and pancreas fused, masses of fluid in peritneal cavity, liver fused to diapragm, thymus huge, LNs normal
98	23.3.95	17.11.95	208ds	EB1+	huge abdomen, masses of fluid in peritoneal cavity, spleen 4-5x normal, liver 2-3x normal, some nodules, thymus huge
99	23.3.95			EB1-	
100	23.3.95	20.3.96	363ds	EB1+	spleen 10x normal, thymus 10x normal, lots of fluid and nodules in peritoneal cavity
101	23.3.95	6.11.95	197ds	EB1+	enlarged abdomen, spleen 3x normal, liver 2x normal, lots of nodules in abdomen, very little fluid, LNs 2-3x normal, thymus enlarged
102	17.4.95	22.4.96	392ds	EB1+	spleen 2x normal, bloody fluid in abdomen, liver pale with white spots
103	17.4.95	22.4.96	392ds	EB1-	animal swollen and ill looking, spleen 4x normal with pale patches, tumour attached to liver
104	17.4.95	2.6.97		EB1-	liver slightly large, small tumour attached, spleen 2x with faint white spots
105	17.4.95			EB1-	
106	17.4.95			EB1+	
107	17.4.95	6.6.95	40ds	EB1+	Severly injured genitailia due to fighting, spleen 3-4x normal, LNs at site of injury large(5-6xnormal), other LNs normal to slightly large
108	17.4.95			EB1+	
109	17.4.95	20.11.96		EB1+	spleen 3x, tumour growing on liver
110	17.4.95			EB1-	
111	17.4.95	20.3.96	338ds	EB1+	spleen enlarged, thymus enormous, lots of fluid and nodules in peritoneal cavity
112	17.4.95			EB1-	
113	17.4.95	10.12.96		EB1+	spleen 3x
114	17.4.95			EB1-	

Appendix 1.2

Data collected on pristane injected 59B progeny. Notes in the phenotype section indicate the gross observations made on the animal at the time of its death.

Line 26 x Eμbcl-2

mouse number	date of birth	date of death	Age at death	Transgene status	Phenotype
1	30.4.93	11.4.94	346ds	EB1+ bcl2-	mouse euthanased, probable lymphoma
2	30.4.93	3.5.94	368ds	EB1- bcl2-	euthansed due to slight unwell appearance, normal
3	30.4.93	25.11.93	209ds	EB1+ bcl2-	found dead
4	30.4.93	2.12.93	216ds	EB1+ bcl2-	swollen abdomen, enlarged spleen, liver, LNs
5	30.4.93	29.11.93	213ds	EB1- bcl2+	unwell appearance,thin,LNs slightly enlarged,spleen slightly enlarged, liver normal colouring possibly slightly large
6	30.4.93	29.11.93	213ds	EB1+ bcl2+	mouse very ill,thin,appeared partially paralysed, LNs slightly enlarged, spleen slightly enlarged, large white nodules on liver
7	30.4.93	10.6.93	41ds	EB1- bcl2-	found dead, carcass cannabalised
8	13.6.93	27.5.94	348ds	EB1- bcl2+	massive abdominal tumour attached to gut (mesenteric LN?) spleen enlarged, fluid in peritoneal cavity, LNs slighlty big, liver normal
9	13.6.93	2.8.94	415ds	EB1- bcl2-	Very fat, otherwise normal
10	13.6.93	24.5.94	345ds	EB1- bcl2+	huge abdominal tumorattached to gut ct (mesenteric LN?) liver and spleen normal, other LNs possibly slightly large
11	13.6.93	2.8.94	415ds	EB1- bcl2+	very fat , otherwise normal
12	13.6.93	14.1.94	215ds	EB1+ bcl2-	mouse found dead, externally nothing obvious, spleen enlarged, liver enlarged and discoloured
13	13.6.93	25.7.95	772ds	EB1- bcl2+	massive abdominal masses (mesenteric LNs?), spleen 4x normal, liver enlarged,pale, some clear fluid in abdominal cavity, other LNs 2-3x normal
14	13.6.93	28.2.94	260ds	EB1+ bcl2-	lymphoma, enlarged spleen, liver and LNs
15	13.6.93	19.7.95	766ds	EB1- bcl2-	Swollen mass at neck, pus filled,enlarged superficial cervical LNs, spleen 3x normal some clear whitish fluid in abdominal cavity
16	13.6.93	20.5.94	341ds	EB1- bcl2-	appeared fat and unwell, spleen and LNs 2x normal
17	29.7.93	22.9.95	785ds	EB1- bcl2+	Box flooded, very ill, very thin, spleen small difficult to find, liver normal size apart from uneven nodule on one lobe, large white mass attached to gut (mesenteric LN?), thymus huge
18	29.7.93	6.4.94	251ds	EB1+ bcl2-	Probable lymphoma found dead
19	29.7.93	17.11.95	841ds	EB1- bcl2-	normal
20	12.8.93	11.4.94	242ds	EB1+ bcl2-	lymphoma, found dead
21	12.8.93	6.6.95	602ds	EB1- bcl2+	swollen abdomen, spleen enlarged, large white mass attached to gut(mesenteric LN?)
22	12.8.93	3.5.94	264ds	EB1+ bcl2+	Lymphoma, spleen, liver, LNs all huge, thymus large
23	12.8.93	28.2.94	259ds	EB1+ bcl2-	lymphoma, enormous liver, huge spleen huge LNs
24	11.3.94	21.11.95	620ds	EB1- bcl2-	liver mottled and enlarged, whitish mass at top of middle lobe, LNs slightly large(1.5-2x), spleen 2x normal
25	11.3.94	15.11.94	249ds	EB1+ bcl2-	lymphoma, spleen and LNs enlarged
26	11.3.94	4.8.94	146ds	EB1+ bcl2+	massive lymphoma, spleen, liver, LNs all huge
27	11.3.94	21.11.95	620ds	EB1- bcl2-	normal

Line 26 x Eμbcl-2

mouse number	date of birth	date of death	Age at death	Transgene status	Phenotype
28	11.3.94	1.11.94	235ds	EB1+ bcl2-	LNs huge, spleen huge, liver normal, thymus huge
29	11.3.94	21.11.95	620ds	EB1- bcl2-	normal
30	4.4.94	23.5.95	414ds	EB1- bcl2+	mouse found dead, spleen, liver,Lns all normal, haemorrage at neck, source not clear
31	4.4.94	4.4.96	730ds	EB1- bcl2-	normal
32	9.5.94	1.11.94	176ds	EB1+ bcl2+	lymphoma, unwell appearance, swollen abdomen, greying fur, huge spleen, liver enlarged, LNs slightly large
33	9.5.94	9.5.96	730ds	EB1- bcl2-	normal
34	9.5.94	9.5.96	730ds	EB1- bcl2+	normal
35	9.5.94	3.7.96	730ds+	EB1- bcl2-	normal
36	9.5.94	1.11.94	176ds	EB1- bcl2-	unwell appearance, greying fur, spleen slightly large 1-2x normal, LNs 3x normal
37	9.5.94	9.8.96	730ds+	EB1- bcl2-	normal
38	9.5.94		?	EB1+ bcl2-	?
39	9.5.94	7.12.94	212ds	EB1+ bcl2+	swollen LNs and abdomen, spleen huge, liver enlarged, LNs huge, thymus enlarged
40	17.5.95	29.4.97	712ds	EB1+ bcl2-	normal
41	17.5.95	?		EB1- bcl2-	
42	17.5.95	?		EB1- bcl2+	
43	17.5.95	21.11.95	188ds	EB1+ bcl2+	very thin and hunched, spleen 2-3x normal, liver normal size but very yellow coloured, LNs 5x normal, fluid filled sac around thymus and enlarged lobes
44	17.5.95	20.11.95	187ds	EB1+ bcl2-	spleen liver and LNS grossly enlarged
45	17.5.95	?	200ds+	EB1- bcl2+	Animal missing from colony 18.1.96
46	17.5.95	29.3.96	317ds	EB1- bcl2+	small swollen mass on head, spleen slightly enlarged (2x)
47	17.5.95	26.9.95	132ds	EB1+ bcl2+	swollen abdomen, massive liver and spleen, LNs slightly enlarged
48	6.6.95	4.3.96	272ds	EB1- bcl2-	spleen 1.5x normal
49	6.6.95	20.4.96	318ds	EB1+ bcl2-	found dead
50	6.6.95	?	280ds+	EB1+ bcl2+	animal missing 25.7.96, survived to march 1996
51	6.6.95	?	280ds+	EB1+ bcl2+	animal missing 25.7.96, survived to march 1996
52	6.6.95	19.2.96	258ds	EB1+ bcl2+	bad loss of hair, cervical LNs and superficial inguinal LNs enlarged
53	6.6.95	18.10.95	134ds	EB1+ bcl2-	found dead, probable lymphoma
54	6.6.95	?	280ds+	EB1+ bcl2+	animal missing 25.7.96, survived to march 1996
55	6.6.95	12.12.95	189ds	EB1+ bcl2+	LNs grossly enlarged, spleen 5-6x normal, liver enlarged

mouse number	date of birth	date of death	Age at death	Transgene status	Phenotype
56	6.6.95	17.1.97		EB1- bcl2-	spleen 3x, thymus enlarged, cervical LN enlarged
57	16.8.95	?		EB1+ bcl2-	
58	16.8.95	11.6.96	229ds	EB1+ bcl2-	LNs large, spleen 10x, thymus 5x
59	16.8.95	26.4.96	183ds	EB1- bcl2-	Liver appeared abormal, thymus slightly enlarged, spleen and LNs normal
60	16.8.95	?		EB1- bcl2+	
61	16.8.95	30.4.96	187ds	EB1+ bcl2+	All LNs hage, spleen grossly enlarged (x20) and infiltrated by tumour, thymus enlarged (x10)
62	17.10.95	17.3.97		EB1- bcl2-	normal
63	17.10.95	?		EB1+ bcl2-	
64	17.10.95	25.11.96		EB1- bcl2-	spleen 6x, liver slightly large
65	17.10.95	?		EB1- bcl2-	
66	17.10.95	6.6.97		EB1- bcl2-	spleen 4x, LNs enlarged
67	17.10.95	6.5.97		EB1- bcl2-	normal
68	17.10.95	31.3.97		EB1- bcl2-	normal

Appendix 1.3

Data collected on progeny from line 26 cross with Eμ-bcl2 transgenic mice (line 85). Notes in the phenotype section indicate the gross observations made on the animal at the time of its death.

Line 59 x Eμbcl-2

Mouse Number	Date of birth	Date of death	Age at death	Transgene status	Phenotype
59/85 1	27.9.94	3.5.96	554ds	eb1- bcl2-	Liver slightly enlarged (x2), thymus enlarged (x5), otherwise normal
2	27.9.94	20.8.96	692ds	eb1- bcl2-	spleen enlarged x7, thymus x5, animal appeared sickly, LNs normal
3	27.9.94	11.9.96	714ds	eb1- bcl2+	normal
4	27.9.94	11.9.96	714ds	eb1- bcl2-	normal
5	18.8.94	?	500ds+	eb1- bcl2+	animal missing 25.7.96
6	18.8.94	21.8.96	730ds+	eb1- bcl2-	liver slightly enlarged, otherwise normal
7	16.8.94	9.6.95	297ds	eb1- bcl2-	very fat, spleen normal, liver enlarged and pale
8	16.8.94	2.8.96	716ds	eb1- bcl2-	eye infection, spleen slightly enlarged but not infiltrated
9	15.12.94	?		eb1- bcl2-	
10	15.12.94	4.3.97	730ds+	eb1- bcl2-	spleen 3x, liver pale
11	15.12.94	11.9.96	635ds	eb1- bcl2-	normal
12	6.1.95	4.3.97	730ds+	eb1- bcl2-	normal
13	6.1.95	16.8.96	587ds	eb1- bcl2+	Lymphoma, spleen 10x, liver enlarged x2 and mottled, LNs normal
14	6.1.95	4.3.97	730ds+	eb1- bcl2-	normal
15	6.1.95	?		eb1+ bcl2+	
16	6.1.95	5.12.95	333ds	eb1- bcl2+	found dead, carcass part eaten, large mass on gut, difficult to distinguish gut from mass, LNs ~2x and dark, spleen ~3x
17	6.1.95	2.12.96	696ds	eb1- bcl2-	spleen slightly large, liver slightly large with white spots
18	6.1.95	17.11.95	315ds	eb1+ bcl2+	swollen abdomen, mostly fat, spleen normal-2x
19	6.1.95	24.3.97	730ds+	eb1- bcl2-	testicular growth, otherwise normal
20	6.1.95	?		eb1- bcl2+	
21	6.1.95	22.8.96	593ds	eb1- bcl2-	animal scabby (mites?), tissues normal
22	15.5.95	12.11.96	545ds	eb1+ bcl2-	enlarged LN at throat, otherwise normal
23	15.5.95	17.2.97	643ds	eb1- bcl2-	normal
24	15.5.95	29.4.97	714ds	eb1- bcl2-	normal
25	15.5.95	23.1.97	617ds	eb1- bcl2+	sac due to fighting, blind, other tissues normal
26	15.5.95	?		eb1+ bcl2-	
27	1.8.95	?		eb1- bcl2+	
28	1.8.95	3.3.97	579ds	eb1- bcl2-	liver slightly large, other tissues normal
29	1.8.95	?		eb1+ bcl2-	

Mouse Number	Date of birth	Date of death	Age at death	Transgene status	Phenotype
30	21.9.95	14.8.97	692ds	eb1+ bcl2-	spleen 3x and almost white, LNs enlarged, Liver slightly enlarged
31	21.9.95	6.5.97	561ds	eb1+ bcl2-	small abdominal tumour, other tissues normal
32	21.9.95	10.9.96	354ds	eb1- bcl2+	animal appeared ill, tissues looked normal
33	21.9.95			eb1+ bcl2-	
34	21.9.95	10.12.96	415ds	eb1- bcl2+	spleen enlarged, with attached mass
35	21.9.95	13.12.96	418ds	eb1- bcl2+	spleen enlarged
36	21.9.95			eb1+ bcl2+	
37	21.9.95	2.12.96	407ds	eb1+ bcl2+	normal
38	21.9.95	27.3.97	521ds	eb1- bcl2-	normal
39	21.9.95			eb1+ bcl2-	
40	21.9.95			eb1- bcl2-	

Appendix 1.4
Data collected on progeny from line 59 cross with Eμ-bcl2 transgenic mice (line 85). Notes in the phenotype section indicate the gross observations made on the animal at the time of its death.

Mouse Number		Date of birth	Date of death	Age at death	Transgene status	Phenotype
26/87	1	26.6.94	14.2.94	235ds	eb1+ pim-	Lymphoma, Lns obvious, swollen abdomen, spleen +++, liver normal, LNs +++, thymus ++, animal quite fat, fat deposits on abdominal organs
	2	26.6.94	9.6.95	348ds	eb1+ pim-	Lymphoma, spleen enormous, Lns huge, liver enlarged, large mass in abdominal cavity (mesenteric LN?) thymic LNs and possibly also thymus enlarged
	3	26.6.94	4.7.96	730ds+	eb1- pim-	normal
	4	26.6.94	13.3.96	626ds	eb1+ pim-	LNs enlarged, liver 2x, spleen 4x
	5	26.6.94	4.7.96	730ds+	eb1- pim+	normal
	6	25.7.94	24.7.96	730ds	eb1- pim-	spleen 3x, thymus 3x, liver and LNs normal
	7	25.7.94	24.7.96	730ds	eb1- pim-	normal
	8	25.7.94	20.4.95	269ds	eb1+ pim+	massive lymphoma, spleen and liver +++, LNs +, thymus+
	9	25.7.94	15.4.96	630ds	eb1- pim+	normal
	10	25.7.94	10.5.95	289ds	eb1+ pim+	Mouse found dead, tissue badly degraded, spleen and liver enlarged 5x normal, but not huge
	11	25.7.94	24.7.96	730ds	eb1- pim-	spleen 3x, other tissues normal
	12	25.7.94	24.7.96	730ds	eb1- pim-	spleen 5x, thymus 2x, other tissues normal
	13	25.7.94	24.7.96	730ds	eb1- pim+	normal
	14	25.7.94	31.8.95	402ds	eb1+ pim-	massive lymphoma, spleen, liver and LNs huge
	15	25.7.94	13.6.95	323ds	eb1+ pim+	mouse very thin, spleen 3x normal, LNs enlarged
	16	25.7.94	2.5.96	646ds	eb1- pim-	found dead
	17	27.9.94	9.6.95	255ds	eb1+ pim+	Swollen abdomen, animal very fat, spleen 2x normal and mottled appearance, liver normal, LNs normal-slightly enlarged
	18	27.9.94	9.6.95	255ds	EB1+ pim-	Swollen abdomen, mouse very fat, spleen enlarged, LNs slightly large and dark red, thymus possibly slightly enlarged but hard to distinguish from surrounding fat
	19	27.9.94	?	600ds+	EB1- pim-	animal missing 25.7.96
	20	27.9.94	21.8.96	703ds	eb1- pim-	normal
	21	27.9.94	18.7.96	721ds	eb1- pim-	found dead
	22	27.9.94	11.9.95	349ds	eb1+ pim-	massive swelling of abdomen, spleen and liver both huge

Appendix 1.5
Data collected on 26/87 progeny. Notes in phenotype section refer to gross appearance at death.

Line 59 x Eμpim-1

Mouse Number	Date of birth	Date of death	Age at death	Transgene status	Phenotype
59/87 1	11.6.94	4.7.96	730ds+	eb1+ pim+	liver and spleen normal, small tumour mass attached to lung
2	11.6.94		730ds+	eb1+ pim+	
3	11.6.94	31.8.95	446ds	eb1+ pim+	massive abdomen, liver huge and discoloured, spleen enlarged, LNs enlarged, some bloody fluid in peritoneal cavity
4	11.6.94	26.4.96	685ds	eb1+ pim-	massive abdominal tumour, spleen x3, thymus x2
5	28.6.94	20.4.95	297ds	eb1+ pim+	bad scarring on back, ?mites?, spleen slightly large, LNs slightly large
6	28.6.94	25.11.94	150ds	eb1+ pim-	very fat, otherwise normal
7	9.7.94	12.7.96	730ds+	eb1+ pim-	
8	9.7.94	20.6.96	711ds	eb1+ pim+	liver enlarged (x3) with pale spots, spleen enlarged (x3)
9	9.7.94	20.6.96	710ds	eb1+ pim-	Liver slightly large (x1.5) and pale with white spots, spleen enlarged with white spots (x4), Lns huge
10	9.7.94	12.7.96	730ds+	eb1+ pim-	normal
11	9.7.94	12.7.96	730ds+	eb1+ pim-	normal
12	9.7.94	12.7.96	730ds+	eb1+ pim-	normal
13	9.7.94	12.7.96	730ds+	eb1+ pim+	normal
14	9.7.94	22.1.96	562ds	eb1+ pim-	Liver enlarged 2.5x,spleen enlarged1.5x, abdominal cavity filled with fliud
15	25.7.94	24.7.96	729ds	eb1+ pim-	normal
16	25.7.94	?	550ds+	eb1+ pim+	animal missing 20.5.96
17	25.7.94	24.7.96	729ds	eb1+ pim-	normal
18	25.7.94	26.4.96	635ds	eb1+ pim+	animal sickly looking, having difficulty walking, spleen, liver, LNs and thymus looked normal
19	25.7.94	3.5.96	642ds	eb1+ pim-	Lns and thymus normal, spleen huge (x20), liver normal, large abdominal tumour
20	9.8.94	21.8.96	730ds+	eb1+ pim+	all tissues normal
21	9.8.94	20.8.96	730ds+	eb1+ pim-	Small growth on back all other tissues normal
22	9.8.94	21.8.96		eb1+ pim-	normal
23	9.8.94	9.7.96	699ds	eb1+ pim-	spleen 20x, thymus 20x, huge abdominal tumour filling peritoneal cavity, liver and LNs normal
24	9.8.94	21.8.96		eb1+ pim-	spleen 5x, small abdominal tumour, LNs normal
25	11.9.94	11.9.96		eb1+ pim-	normal
26	24.10.94	20.11.96		eb1+ pim+	normal
27	24.10.94	20.11.96		eb1+ pim-	normal
28	24.10.94	21.11.96		eb1+ pim+	normal
29	24.10.94	30.10.96		eb1+ pim-	normal
30	24.10.94	21.11.96		eb1+ pim-	rectal prolapse, otherwise normal

Mouse Number	Date of birth	Date of death	Age at death	Transgene status	Phenotype
un-numbered female	24.10.94	9.1.95	77ds	eb1+ pim+	found dead, spleen, LNs and thymus enlarged
31	14.11.94	17.11.95	368ds	eb1+ pim+	hairless mouse from birth, bad scarring on back of neck,?mites?, spleen4-5x normal, liver 2-3x normal, LNs slightly large and dark
32	14.11.94	?		eb1+ pim-	
33	14.11.94	?		eb1+ pim-	
34	14.11.94	21.11.96		eb1+ pim-	normal

Appendix 1.6
Data collected on line 59/87 progeny