The oncogenic activity of the latent membrane protein of EBV in transgenic mice

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The research reported in this thesis is my own original work
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any other degree.

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A historical comparison to the life of a Ph.D. student:-

Lapsed into a state of almost mindless depression and stupor, Robert Bruce king of Scots perceived that, although his wits had sunk into a dull vacancy, his senses had not. He had, in fact, been heavily watching a spider which was striving assiduously to attach it's slender thread to a point on the sheer rock wall of the cave.

Four times it had gone through the difficult and involved process without success. The fifth attempt again ended in failure. The sixth effort showed intelligence as well as determination and it looked as though this effort might succeed- but no.

"It is of no avail" the Bruce muttered, shaking his head. "Can you not see it?"

But the spider would not admit defeat. Undeterred it launched itself downwards with unabated resolve, and this time it managed to hold on to the cave wall.

"Now, by the saints,- here is a wonder!" Bruce exclaimed.
"Six efforts did not deter this spider. Shall I despair more easily?"

Adapted from The Path of the Hero King, by Nigel Tranter, 1970.

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Abstract

There is an ever increasing list of disease states which have been shown to have an association with EBV latent infection. In particular research has focused on the latent membrane protein (LMP-1). Study of this gene has largely been restricted to B-cells *in vitro* due to the inability to infect epithelial cells with EBV *in vitro*. This has resulted in little information concerning the effects of LMP-1 in an epithelial environment. As a consequence, the role of LMP-1 in nasopharyngeal carcinoma (NPC), with which EBV is most closely associated, is poorly understood.

Creation of transgenic mice where LMP-1 is directed to the epithelial cell compartment has therefore achieved two important goals. LMP-1 can now be studied in an epithelial cell *in vivo* whilst the mice provide the first step in a model for the disease state of NPC. In this study the mechanism of action of LMP-1 in the PyLMP-1 line 53 of transgenic mice has been investigated. In addition, a study on the progression of the transgenic LMP-1 induced hyperplastic phenotype to carcinoma has been conducted.

Firstly, using immunohistochemical techniques, the hyperplastic epidermal phenotype of the PyLMP-1 mice previously reported is shown to result from a 2-3 fold increase in the rate of cellular proliferation whilst differentiation continues unimpeded in the transgenic skin.

Secondly, the mouse skin model of multi-stage carcinogenesis is utilised to show that LMP-1 does not act as an initiator of carcinogenesis nor does it affect the conversion to a more malignant tumour. However, LMP-1 does function to increase both the rate and number of lesions forming during chemical tumour promotion, and more importantly LMP-1 acts as a weak or second stage promoter on it's own. This finding has significant implications for NPC.

Thirdly, by cross-breeding PyLMP-1 line 53 with other lines of transgenic or knockout mice it is shown that LMP-1 does not co-operate with activated Ha-*ras* or loss of p53 function in tumour progression. However, the combination of the PyLMP-1 transgene and K10-TGFβ1 transgene results in embryonic lethality.

Lastly, the EμLMP-1 line 39 transgenic mice which express LMP-1 in the B-cell compartment at very low levels have been studied for a 24 month period and shown to succumb to a long latency lymphoma.

Table of contents

Commonly used abbreviations

αα–	Amino acid
bp-	Base pair
BL (e or s)-	Burkitt's lymphoma (endemic or sporadic)
BrdU-	Bromo-deoxyuridine
C/N-terminus-	Carboxy or Amino terminus
CR2-	Complement receptor type 2
CST-	Complementary strand transcript
d7-	Day 7
DMBA-	Dimethylbenzanthracene
DNA-	Deoxy-ribonucleic acid
EBNA-	Epstein-Barr virus nuclear antigen
EBV-	Epstein-Barr virus
EGF/R-	Epidermal growth factor/Receptor
Eμ-	IgH heavy chain intronic enhancer
FCS-	Foetal calf serum
Ha-*ras*-	Harvey-*ras*
HD-	Hodgkin's disease
K (6)-	Keratin
LCL-	Lymphoblastoid cell line
LMP-	Latent membrane protein
LOH-	Loss of heterozygosity
mRNA-	Messenger RNA
NF-κB -	Nuclear factor κB
NPC-	Nasopharyngeal carcinoma
ORF-	Open reading frame
PKC-	Protein kinase C
Py-	Polyoma
RAR-	Retinoic acid receptor
RNA-	Ribonucleic acid
RS-	Reed-Sternberg
ST-	Stromelysin
TANK-	TRAF family member associated NF- κB activator
TGF-	Transforming growth factor
TNF/R-	Tumour necrosis factor/Receptor
TPA-	12-*O*- tetradecanoylphorbol-13-acetate
TRAF-	TNF receptor associated factor
ts-	Temperature sensitive
WT-	Wild-type

Index of headings

Index of figures

Index of tables

CHAPTER 1:- GENERAL INTRODUCTION

1A. EPSTEIN-BARR VIRUS.

Epstein-Barr Virus (EBV) is a human herpes virus which is common in all human populations (for reviews see Kieff, 1996; Rickinson and Kieff, 1996). Primary infection with EBV usually occurs at an early age, is clinically inapparent, and results in lifelong virus persistence in a latent state which the host immune system keeps under surveillance. However, if infection with EBV is delayed, infectious mononucleosis, a lymphoproliferative disorder, often ensues. Malignant diseases have also been identified in latently infected humans, in which EBV is thought to play a key role. These include Burkitt's Lymphoma (BL), immunoblastic B cell lymphomas, certain T cell lymphomas, Hodgkin's disease (HD), and Nasopharyngeal Carcinoma (NPC). The focus of this thesis concerns the oncogenic activity of one of the viral proteins expressed during latency, the latent membrane protein-1 (LMP-1), and its mechanism of action with particular respect to Nasopharyngeal Carcinoma (NPC).

1A.1 Genome organisation.

The genome of EBV within its virion is a linear duplex deoxyribonucleic acid (DNA) molecule of approximately 172,000bp (see figure 1.1). The ends of the linear virion DNA contain direct repeats, described as the terminal repeats, which fuse together allowing circularization of the molecule in the infected cell.

Figure 1.1:- Linear map of the EBV viral genome.

This linear map shows the relative positions of the coding exons of the latently transcribed genes. The genome circularizes in latently infected cells via fusion of the terminal repeats (TR), therefore the LMP2 transcripts are transcribed across this fused TR region. (Adapted from Rickinson and Kieff, 1996).
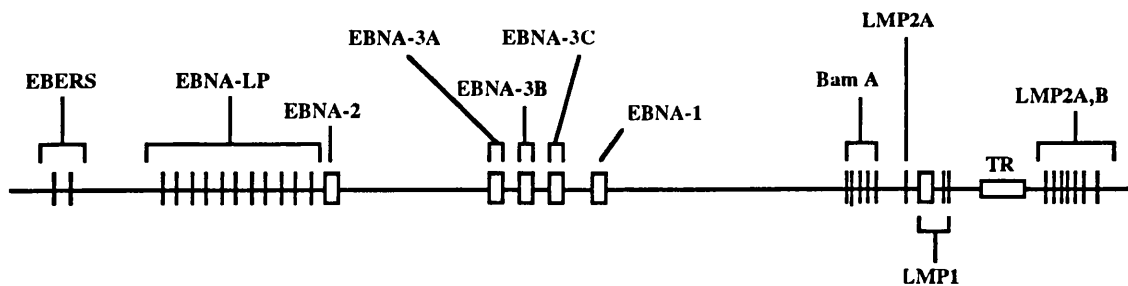
The size varies depending on the EBV strain, with the proto-type B95-8 strain (derived from an infectious mononucleosis patient and passaged in cotton top tamarins) being most commonly quoted. Two major polymorphisms are known, based on a heterogeneous sequence of the Epstein-Barr virus nuclear antigen-2 (EBNA-2) gene and the restriction enzyme polymorphisms observed in several parts of the viral genome. These have led to the term types 1 and 2 EBV (Abdel-Hamid *et al.*, 1992) where type 1 has a more efficient transforming ability than type 2. One of the restriction enzyme polymorphisms is characterised by an extra BAMH1 site within the BAMH1-F fragment of EBV, giving rise to a small fragment called BAMH1-f or simply the "f" variant.

The EBV linear map is organised according to the different size of fragments generated by *Bam* H1 digestion with most latent genes reading from left to right, and most lytic genes reading right to left. Initially many proteins were termed after the *Bam* H1 fragment harbouring the coding exons and the direction of their expression. For example, LMP-1 was originally referred to as BNLF-1 (*Bam* H1 fragment N , Left Frame number 1(Baichwal and Sugden, 1988)). LMP-1 has also been described as LYDMA (Lymphocyte-determined membrane antigen (Fennewald *et al.*, 1984)). It is now commonly referred to as LMP-1 and this is the nomenclature I will use.

1A.2   Infection and immortalization.

Primary infection is thought to occur in the stratified squamous epithelium of the nasopharynx where the virus is thought to replicate during it's "lytic phase". The B95-8 virus genome has been completely sequenced and demonstrated that 90% of the coding capacity of EBV is composed of genes expressed during the lytic phase of the viral life cycle. These are classed as immediate-early, early or late genes depending on when they are

expressed during productive infection, but are not the subject of this thesis and will not be dealt with in any detail.

The method of entry into the target epithelial cell is unknown, but from here EBV is believed to gain access into the circulating B cell population where infection is largely non-productive and characterises the "latent state". The interaction of EBV with B cells is initiated through the virus binding to the B cell surface molecule, complement receptor type 2, CR2 (Fingeroth *et al.*, 1984), also given the "cluster of differentiation" number CD21. This results *in vitro* in virus induced growth transformation and subsequent immortalization of the B cell. Cells expressing any class of surface immunoglobulin (sIg) are targets therefore EBV does not distinguish between subsets of B cells. The external envelope of EBV is dominated by the virus-encoded glycoprotein (gp) gp350/220 through which virus binding to the B lymphocyte receptor CR2 is mediated (Knox and Young, 1995). The lytic phase of the viral life cycle ends with cell lysis and the release of newly formed EBV virions.

Both the infection and immortalization of primary B lymphocytes with EBV can be efficient. More than 50% of sIg$^+$ B cells isolated from the peripheral blood of an adult human can be infected with EBV, and the efficiency with which the infected cells are immortalised is between 30-100% (Reviewed in Middleton *et al.*, 1991). The proliferating immortal B lymphocytes produced are called lymphoblastoid cell lines (LCLs) and harbour the viral genome as an autonomously replicating episome in a latent state. Only 10% of the coding capacity of EBV is represented by genes of the latent state of EBV.

1A.3   Latent gene expression.

There are 8 proteins expressed during latency (Reviewed by Kieff, 1996) and these include 6 nuclear antigens and 2 membrane antigens. Again there are different systems to term the proteins expressed during latency. The six nuclear proteins are termed Epstein-Barr Virus nuclear antigen (EBNA)-1, -2, -3a, -3b, -3c, and -LP in one system and EBNA-1, 2, 3, 4, 5 and 6 in the other, where EBNA-3a, b, and c are EBNA-3, 4, and 6 respectively, and EBNA-LP is EBNA-5. The two integral membrane proteins are commonly referred to as LMP-1 and LMP-2, although differential splicing of LMP-2 gives rise to two proteins -2a, and -2b (where 2a has an additional 5' exon). LMP-2a and -2b are also referred to as TP-1, and -2 (TP refers to Terminal Protein). In this thesis I

will use the notation of EBNA-1, -2, -3a, -3b, -3c, -LP and LMP-1, LMP-2a, -2b (as shown in figure 1.1). There are also two small non-coding RNAs called EBER-1 and -2 which are expressed during latency and a family of intricately spliced mRNAs which are complementary strand transcripts (CSTs) expressed from the *Bam* H1 A region of the viral genome (Smith *et al.*, 1993). These were originally identified in an NPC passaged tumour (Gilligan *et al.*, 1990) and now shown to contain open reading frames (ORFs). Functions have been identified for some of these proteins briefly described below (Raab-Traub,1996; Kieff, 1996).

EBNA-1 binds to the origin of replication of the plasmid form of the viral genome and allows replication of the viral episome by the host DNA polymerase. EBNA-1 is the only viral protein which is essential for transmission of the viral DNA to the progeny cells of a B cell.

EBNA-2 affects viral and cellular gene expression by binding to the cellular transcription factor CBF1. Notch, a gene family controlling cell fate determination in several tissues acts through CBF1 also. Thus EBNA-2 may act like activated Notch family members (Hunter, 1997). EBNA-2 is essential for growth transformation of lymphocytes (Hammerschmidt and Sugden, 1989).

The EBNA-3 proteins, -3a, -3b, and -3c, also bind to CBF1 and affect viral gene expression. Both EBNA-2 and EBNA-3 regulate the viral promoters for LMP-1 and LMP-2. In addition, EBNA-2 and -3 are responsible for inducing expression of B-cell activation markers.

EBNA-LP, along with EBNA-2, is synthesised first following lytic infection and they are required to transactivate the expression of subsequent latent genes. EBNA-LP is not required for immortalization of B cells (Hammerschmidt and Sugden, 1989).

LMP-1 is essential for growth transformation of B-lymphocytes *in vitro* (Kaye *et al.*, 1993). It is the only latent protein shown to be oncogenic in tissue culture assays (Wang *et al.*, 1985). It induces or inhibits the expression of multiple cellular genes. As the focus of this thesis LMP-1 will be discussed in more detail in later sections.

LMP-2 contains exons from both ends of the linear genome therefore can only be transcribed across the fused termini of the episome (Sample *et al.*, 1989). It has been shown to interact with the cellular tyrosine kinases fyn and lyn and thereby may interfere with signal transduction (Longnecker *et al.*, 1991).

The EBERs are the most abundant viral (ribonucleic acids) RNAs in EBV infected cells and are expressed in all of the malignancies associated with EBV. Despite this they are not required for lymphocyte transformation.

The mRNAs originally found expressed from the *Bam* Hl A fragment in passaged NPC cells (Gilligan *et al.*, 1990) have recently been shown to encompass the BARFO open reading frame at the 3' end of the transcripts. Rabbit antiserum raised against BARFO revealed the presence of a membrane associated protein (Fries *et al.*, 1997) which may be one of a family of such proteins expressed during latency. Their function is poorly understood.

There are three types of latency described in LCLs with each corresponding to a characteristic disease state. Only EBNA-1 is expressed in Latency 1. In Latency 2 EBNA-1 and the LMPs are expressed, and in Latency 3 all six nuclear antigens plus the LMPs are expressed. The EBERs are expressed in all types of latency.

1B.   EBV and Malignant Disease.

EBV is implicated in the pathogenesis of a number of human lymphoid and epithelial malignancies. The lymphoid tumours include the immunoblastic B lymphomas that arise in immunocompromised individuals, certain T cell lymphomas, Burkitt's lymphomas and a subset of Hodgkin's disease cases. The association is strongest in nasopharyngeal carcinoma where the virus is present in 100% of all poorly differentiated or undifferentiated cases and may also be present in all squamous cell NPC's (Reviewed in Karimi and Crawford, 1995; Rickinson and Kieff, 1996). EBV is also associated with other, but not all tumours, of the sinonasal region (Leung *et al.*, 1995). In particular there has recently been detected a specific association with nasal T cell lymphomas (Chiang *et al.*, 1996; and Wu *et al.*, 1996).

1B.1   Immunoblastic B cell lymphomas.

Immunocompromised patients such as recipients of immunosuppressive therapy, AIDS sufferers, or patients with primary immunodeficiency diseases are at increased risk for the development of EBV-positive immunoblastic B cell lymphomas (Reviewed in Karimi and Crawford, 1995; Rickinson and Kieff, 1996). These lymphomas express all of the latent genes and the same cell surface markers as LCLs of latency 3 and generally

resemble large-cell lymphomas. In around 50% of cases occurring in transplant patients there is evidence of a recent EBV infection. This strongly suggests that these tumours arise because the host immune system can no longer control EBV induced. B cell immortalization and proliferation. Extensive B cell immortalization by EBV might greatly enhance the likelihood of accumulation of oncogenic mutations, thereby leading to these tumours.

1B.2   T cell lymphomas.

EBV has been detected in various T cell lymphoproliferations and lymphomas such as nasal T cell lymphoma. A recent study showed that 100% of nasal lymphomas were EBV associated with expression of EBNA-1, LMP-1, LMP-2 and *Bam* H1 A transcription (Chiang *et al.*, 1996). This strong correlation between EBV and T cell lymphoma suggests a role for EBV in the pathogenesis of the disease but as yet the precise events remain unexplored.

1B.3   Burkitt's lymphoma.

The term "Burkitt's lymphoma" was originally coined to describe a tumour that accounts for about 50% of all childhood neoplasms in equatorial Africa. The lymphoma is generally discovered before the end of the teenage years and is distinguishable by it's geographical prevalence, although the disease in both areas is identical by histology and characterised by a "starry sky" appearance. The endemic form (eBL) is found in equatorial Africa largely limited to a broad band covering 15° either side of the equator. The sporadic form (sBL) occurs elsewhere in the world where the incidence is up to 50-fold less than for eBL. The two tumour forms can be distinguished via a variety of markers. For example, one of the characteristic features of eBL is the presence of jaw tumours, whereas jaw involvement in sBL is much less frequent. Also, EBV is associated with eBL in about 97% of cases, whereas the association of sBL with EBV is only of the order of 15-20% (Reviewed in Karimi and Crawford, 1995; Rickinson and Kieff, 1996). Presence of EBV can therefore be used to characterise or diagnose a BL but more importantly may also be involved in the pathogenesis of the endemic form of the disease.

One feature that all Burkitt's Lymphomas have in common is the presence of non-random chromosomal translocations which

juxtapose the c-*myc* proto-oncogene to one of the immunoglobulin (Ig) genes. (Reviewed in Karimi and Crawford, 1995; Rickinson and Kieff, 1996). The type of breakpoints and the position of the breakpoints on the involved chromosomes can also be used to define different types of BL. It is most likely that altered expression of c-*myc* resulting from it's positioning next to immunoglobulin genes is central to the pathogenesis of BL. Levels of c-*myc* in BL are similar to levels of c-*myc* in LCLs suggesting that it may be inappropriate expression rather than overexpression which is important in BL.

The only EBV latent protein consistently expressed in eBL biopsies and eBL phenotype cell lines is EBNA-1, the virus genome maintenance protein, although the EBERs are also consistently detected (Reviewed in Karimi and Crawford, 1995; Rickinson and Kieff, 1996). Indeed EBNA-1 expression in transgenic mice, where the transgene is directed to the lymphoid cell compartment, predisposes the mice to monoclonal B cell lymphomas (Wilson and Levine, 1992; Wilson *et al.*, 1996).

Two lines of EμEBNA-1 mice, lines 26 and 59 succumb to monoclonal B cell lymphoma, the indicator of which is a massive enlargement of the spleen concurrent with massive enlargement of the liver and/or lymph nodes. 100% of line 26 mice are overcome with an invasive lymphoma within the age range 4-12 months. Line 59 mice demonstrate a much longer latency to onset of disease, with 50% of mice succumbing to lymphoma by 24 months of age (Wilson and Levine, 1992; Wilson *et al.*, 1996). Expansion of the white pulp of the spleen and lymph nodes appears prior to the development of neoplasia suggesting that EBNA-1 is involved in the pre-neoplastic stage of tumorigenesis.

1B.4   Hodgkin's disease.

EBV has also been implicated in Hodgkin's disease (HD) with reports of up to 50% association in the USA and Europe, and 90-100% in Peru and Honduras (Reviewed in Karimi and Crawford, 1995; Rickinson and Kieff, 1996). EBV has been shown to be present in all 4 types of HD but the closest association is with the most aggressive types (>80%). The disease itself is characterised by multinucleated Reed-Sternberg (RS) cells which are present in very small numbers (2%) in the tumour mass and are probably of B cell lineage (Rickinson and Kieff, 1996). However the disease and its pathogenesis are confusing and poorly understood.

The association with EBV was noted when HD patients showed elevated antibody titres to EBV antigens (viral capsid antigen-VCA, early antigens of the lytic cycle and EBNA-1) at the time of presentation as well as some years prior to disease (Mueller *et al.*, 1989). A threefold increased risk of HD has also been documented in people who have had infectious mononucleosis. The EBV latent genes expressed in HD give it a latency 2 phenotype with expression of EBNA-1, LMP-1, LMP-2a, and -2b, and the EBERs. Immunohistochemical analysis showed that LMP-1 is expressed at high levels in RS cells of EBV positive HD tumours. The observation that LMP-1 transfected into EBV negative HD cells gives rise to the formation of a multinucleated morphology similar to that of RS cells raises the possibility that LMP-1 acts to promote such a multinucleated cell phenotype (Knecht *et al.*, 1996). In 30% of HD cases, polymorphisms of the LMP-1 gene have been identified (Reviewed in Karimi and Crawford, 1995; Kieff, 1996). This may give rise to a more oncogenic LMP-1 as has been suggested for NPC (see section 1E.2)

Although EBV is associated with HD, it is only a subset of cases which demonstrate this. For example, among young adult cases, the age group in which EBV serology is clearly positive generally has the lowest EBV-genome positive HD cases (Glaser *et al.*, 1996). This paradox adds further complexity to the role of EBV in HD, a story which is clearly unfinished.

Whilst EBV is found associated with all of these disease states, the strongest association of all the human malignancies is with Nasopharyngeal Carcinoma.

1C.   EBV and Nasopharyngeal Carcinoma.

Nasopharyngeal carcinoma (NPC) is an epithelial tumour showing world-wide incidence but a marked geographical and population bias. It develops with high incidence, particularly in adult males, in Southeast Asia in the coastal provinces of Guandong, Guangxi and Hunan where it may represent 20% of all cancer cases. Within China the incidence in variation is so dramatic that NPC was referred to as the "Canton Tumour". NPC also frequently develops in Eskimo populations and occurs with elevated incidence in North Africa. Elsewhere in the world NPC is uncommon (Reviewed in Raab-Traub, 1996)

NPC develops at the back of the post-nasal space in a discrete area called the Waldeyer's ring. The World Health

Organisation have classified NPC into 3 categories depending on the degree of differentiation shown by the tumour (Hording *et al.*, 1993):-
1) WHO1 NPC are highly differentiated squamous cell carcinomas with intercellular bridges and keratinization.
2) WHO2 NPC retain epithelial growth patterns and shape but show no evident squamous differentiation.
3) WHO3 NPC show no keratinization or distinctive growth pattern and have a syncytial appearance. They are completely undifferentiated.

The WHO2 and WHO3 undifferentiated NPCs are the type most frequently found and often contain numerous infiltrating lymphocytes among the tumour cells giving rise to the term "lymphoepithelioma." The tumour can metastasise and in fact NPC is unique among the head and neck cancers in that it has the highest rate of distant metastases. The various stages of disease have also been classified according to the site and spread of the tumour (Ho, 1978). Radiation therapy is the main treatment for NPC, but despite this a significant number of patients fail with distant metastases.

1C.1   Factors influencing NPC epidemiology.

Studies of NPC are hampered by the relative rarity of intact tumour material. Moreover it has not been possible to directly passage tumour cells in culture. Transplantation in nude mice represents a way of obtaining abundant, homogeneous tumour material, but this is frequently unsuccessful with this particular tumour. However one primary tumour (C15) and two metastasised tumours (C17 and C18) have been successfully transplanted (Busson *et al.*, 1988). It was only possible to propagate these tumours by passaging them through nude mice, and not possible to grow these tumours *in vitro* . An EBV-positive NPC (CAO) has been successfully propagated as a xenograft in nude mice (Hu *et al.*, 1991), whilst 3 NPC tumour lines (designated Xeno-1, -2, and -3) were successfully established from fresh biopsy material injected sub-cutaneously into athymic mice and then passaged in nude mice for many generations (Huang *et al.*, 1989). This further highlights the difficulty in working with NPC material.

These solid tumour lines provide a valuable tool for investigating NPC. However, much of the information about NPC has come from the C15 tumour alone (Chen, F., *et al*, 1995; Brooks

et al., 1992; Hu *et al.*, 1991; Gilligan *et al.*, 1990 - all discussed later) and so care should be taken when interpreting these results, bearing in mind that these tumour lines represent highly manipulated cases which might not typify the *in vivo* situation.

1C.1.1   Genetic susceptibility.

Various factors have been shown to play a role in the pathogenesis of NPC. It is likely that the genetic background may be a factor since the area of highest incidence is Southeast Asia. However, a comprehensive epidemiological study has not been conducted which might demonstrate heritable susceptibility. NPC afflicts both males and females although the incidence is higher in males.

1C.1.2   Diet and lifestyle.

Diet and lifestyle are likely to be involved and studies have shown that there is a higher incidence of NPC among people who live and work on boats and cook in the open. Similar studies have shown that people who consume salted fish during weaning, in childhood and throughout their adult life, also have a high relative risk of NPC. Moreover, extracts of salted fish have been shown to enhance transforming activity in cell culture, and more tumours are observed in rats fed salted fish than in controls (Zou and Landolph, 1991; Yu *et al.*, 1989). The salting process involves the use of crude salt containing nitrite and nitrate contaminants, the fish are not gutted, and are stored for 4-5 months before consumption. This combination allows nitrite reducing *Staphylococci* to carry out nitrosation, producing volatile N-nitrosamines which are mild tumour promoting agents. Indeed a correlation was found between the level of N-nitrosamines in salted fish and risk of NPC (Zou *et al.*, 1994). Thus the high levels of NPC may be attributed to the consumption of salted fish containing high levels of N-nitrosamines. The lifestyle and diet might perhaps explain the average age for NPC in Southern China, which is 45-50 years demonstrating long latency.

It therefore appears that the incidence of NPC may be influenced by a heritable susceptibility and also an environmental/dietary factor. However, of all EBV-associated malignancies the tightest association is with NPC. Therefore it may be the combination of factors:- EBV, genetic susceptibility and diet that combine to fully dictate incidence.

1C.1.3   Viral influence.

The most striking feature of NPC is the 100% association of all poorly differentiated or undifferentiated NPC's with EBV. Early seroepidemiologic studies revealed that patients with NPC had unusually high antibody titres to the EBV viral capsid antigen (VCA) and to a complex of early replicative proteins termed early antigen (EA) (Henle *et al.*, 1976). Detection of these antibodies is therefore an early indication of the onset of NPC. This was confirmed by polymerase chain reaction (PCR) and *in situ* hybridisation (Chang *et al.*, 1990; Niedobitek *et al.*, 1991). EBV DNA was shown to be present in pre-invasive neoplastic lesions taken from biopsies (Pathmanathan *et al.*, 1995). The viral DNA was clonal and in a latent state. Therefore EBV infection is critical to the pathogenesis of NPC.

Not only is EBV consistently found in NPC cases, but there may be a prevalence of specific variants of EBV (see section 1A.1) in distinct geographic locations which contribute to the endemic patterns of incidence. In a study of NPC biopsies from Southern China the vast majority were type 1 "f" variants (Lung *et al.*, 1991), whereas NPC cases from Caucasians in North America and in North Africa were predominantly the type 1 prototype "F" variant (Lung *et al.*, 1992; Bouzid *et al.*, 1994). Alaskan NPCs surprisingly are type 2, but with the "F" variant (Abdel-Hamid *et al.*, 1992). Therefore no one subtype predominates in all NPCs, but in Chinese NPC the BAMH1- "f" variant may be the most common variant in the region.

1C.2   Genetic alterations.

Whilst heritability, diet and viral influence may pre-dispose the nasopharynx to the development of NPC, the progression may be a consequence of alterations in chromosomes such as loss of tumour suppressor genes. Although alterations of the common tumour suppressor genes p53 and Rb are rare in NPC (Spruck *et al.*, 1992; Sun *et al.*, 1993), cytogenetic studies have shown that abnormalities on chromosomes 1,3,11,12, and 17 were present in some NPC samples (Huang *et al.*, 1989). A region of homozygous deletion was then identified at position p21-p23 on chromosome 9 (Huang *et al.*, 1994). This frequent allelic loss on chromosome 9 in NPC may suggest the presence of a tumour suppressor gene(s) in this area, loss of which could contribute to NPC progression. The region identified on chromosome 9 includes the putative tumour

suppressor gene p16 (as well as p15 and p19) which binds to and inhibits the catalytic activity of the CDK4/Cyclin D complexes (Serrano *et al.*, 1993). Loss of p16 function could therefore contribute to uncontrolled cell growth. Indeed, homozygous deletion of p16 was shown to be common in primary NPC tumours and NPC xenografts suggesting that inactivation of p16 may be involved in the pathogenesis of NPC (Lo *et al.*, 1995). Loss of heterozygosity on chromosome 11, which is a common genetic aberration in many tumours, was also identified in NPC. Two distinct regions of deletions were found at 11q13.3-11q22 and 11q22-11q24 suggesting the presence of multiple tumour suppressor genes on chromosome 11 (Hui *et al.*, 1996). Taken together, these findings indicate multiple complex chromosomal alterations could be responsible in part for the progression of NPC.

1C.3   EBV action.

1C.3.1   Lytic proteins.

Since elevated antibodies to EBV early antigens pre-date the development of NPC by several years, it is feasible that EBV lytic genes may be associated with the tumour. Many groups have looked at the viral state in NPC and could find no linear DNA, the lytic form of the virus, nor expression of ZEBRA (BZLF-1), a protein which switches EBV from latent to productive cycle (Gilligan *et al.*, 1990; Chao *et al.*, 1996). There is however controversy about the involvement of ZEBRA as one report cites 87% of NPC patients exhibiting antibodies to the ZEBRA protein, and in another, the gene encoding ZEBRA was shown to be expressed in all NPC specimens studied (Joab *et al.*, 1991; Cochet *et al.*, 1993). In another report the immediate early genes were present in only some of the NPC biopsies studied by a reverse transcribed PCR (RT-PCR) approach. A common feature however was the expression of ZEBRA (Martel-Renoir *et al.*, 1995). Two possibilities therefore exist. The EBV lytic cycle may be partially re-activated in NPC, or the lytic mRNAs may be present in only a few of the cells within the tumour thus detectable by RT-PCR, but not reflect expression throughout the tumour. The role of ZEBRA is definitely unclear.

1C.3.2   Latent proteins.

It is most likely that one or more of the EBV latent genes are responsible for the pathogenesis of NPC. Various methods have been used to determine expression of the latent genes in NPC the conclusion of which is that EBNA-1, LMP-1, LMP-2A and -2B, BARF0 and the EBERs are all expressed to some extent.

EBNA-1 has been shown to be present in 100% of NPC's by RT-PCR (Brooks *et al.*, 1992; Chen, F., *et al.*, 1995). Although EBNA-1 is expressed in all known forms of EBV latency due to it's requirement for episomal maintenance, different EBNA-1 promoters are used in different situations giving rise to distinguishable EBNA-1 transcripts. It is interesting therefore that the EBNA-1 mRNA species in NPC is of the Q/U/K spliced variety driven from the Fp/Qp promoter (Brooks *et al.*, 1992). This shows that EBNA-1 in NPC is associated with selective use of the Qp promoter as opposed to the Wp and Cp promoters which are used in LCLs. EBNA-1 has been ascribed no *in vitro* transforming ability and was not thought to play an oncogenic role *in vivo* in EBV associated disease. The recent demonstration that EBNA-1 predisposes transgenic mice to lymphoma (Wilson and Levine, 1992; Wilson *et al.*, 1996) revealed that EBNA-1 could act as an oncogene in B cells *in vivo*. In addition, EBNA-1 expression increases the tumorigenicity of EBV negative HONE-1 cells (of NPC origin) (Sheu *et al.*, 1996). In conclusion, the biological effects of EBNA-1 appear to be greater than just episomal maintenance, and it may be important to the development of NPC as well as B cell lymphomas.

The EBERs are also found in 100% of primary and metastatic NPCs. *In situ* hybridisation using EBER specific probes can thus be used to identify the presence of EBV in an NPC and to identify a metastasised tumour as being NPC in origin (Chao *et al.*, 1996). The EBERs are therefore useful markers of NPC although their potential role in the disease is still unclear

In NPC there is consistent transcription of the BamH1-A region of EBV, originally detected in the C15 xenograft (Busson *et al.*, 1988) but later shown to be present in tumour biopsies (Chen, H.L., *et al.*, 1992). Sequencing of cDNA clones from C15 revealed a number of rightward transcripts. The 3'-ends of all the clones terminated within a region containing two poly-adenylation sites. One thing that all the transcripts had in common was that they either partly or wholly encompassed the open reading frame (ORF) BARF0 (Chen, H.L., *et al.*, 1992), which is predicted to encode a

basic protein of about 20 kilo-daltons (kDa). Since the function of BARFO is as yet unknown, it's relevance to NPC is not yet clear.

The LMP-2 gene contains exons located at both ends of the linear EBV genome and therefore can only be transcribed across the fused termini of the episomal form. Northern blot and RT-PCR analysis have shown that the majority of NPC biopsies have LMP-2 message. Two studies (Busson *et al.*, 1992; Brooks *et al.*, 1992) showed that LMP-2A was detected in almost 100% (10/11 and 17/18 respectively) of the cases, whether primary NPC or metastatic tumour, whereas LMP-2B was expressed at much lower levels (3/11 and 7/18 respectively). The consistent expression of LMP-2 in all cases suggests that it may be involved in the onset or progression of NPC.

The detection of LMP-1 in NPC biopsies has varied according to the technique used. Total RNA from the nude mouse passaged cell line C15 (Busson *et al.*, 1988) contained two messages with sequences specific for the 5'-end of LMP-1. Sequence analysis of a cDNA library made from the same cell line showed that two forms of LMP-1, utilising the normal promoter and an internal promoter, were indeed present (Gilligan *et al.*, 1990). Western blot analysis of protein content from tumour biopsies showed that LMP-1 was present in 38-77% of NPC cases (Young *et al.*, 1988 (24 cases); Zheng *et al.*, 1994b (23 cases); Hu *et al.*, 1995 (74 cases); and Fu *et al.*, 1991 (13 cases) in order of increasing LMP-1 levels detected). Monoclonal antibody immunohistochemistry staining allows single cell expression to be detected. In this way 78% of NPC tumours were shown to be LMP-1 positive (Stewart and Arrand, 1993; Vera-Sempere *et al.*, 1996). RT-PCR amplification of the LMP-1 gene, using nested primers and total RNA from two NPC derived solid tumour lines, and from RNA extracted from 24 NPC biopsies, showed the presence of LMP-1 message in >90% of cases (Chen, F., *et al.*, 1995). Therefore it appears that LMP-1 message is present in the majority of NPC cases, but LMP-1 protein is only detected in about 65% of cases.

1D.   The Latent Membrane Protein-1.

1D.1   LMP-1 protein structure.

LMP-1 was cloned and sequenced in 1984 by Fennewald *et al.* The protein was shown to be 386αα long with a predicted

molecular weight of 42kDa. Based on amino acid sequence, the protein was shown to have:-

1) A short, 25αα long hydrophilic, cytoplasmic, amino terminal domain.

2) Six hydrophobic, 20αα long, alpha-helical transmembrane segments, separated by five 8 to 10αα long reverse turn loops.

3) A 200αα long carboxy terminal domain, rich in acidic, glycine and proline residues that projects into the cytoplasm.

A polyclonal rabbit antiserum was raised against the protein and this antiserum was used to identify a specific protein in every EBV infected B cell line tested (Hennessy *et al.*, 1984). This protein separated with the membrane/microsomal fraction and was not seen in the cytoplasmic supernatent. Furthermore, the antiserum specifically reacted with the plasma membrane of cells tested and stained in a characteristic patch/cap expression (Liebowitz *et al.*, 1986) where LMP-1 associated with vimentin intermediate filaments (Liebowitz *et al.*, 1987). This association with the cytoskeleton would explain why half or more of LMP-1 is resistant to non-ionic detergent extraction (Liebowitz *et al.*, 1987). LMP-1 does not require vimentin to form these patches however suggesting that it may interact with itself or other cell membrane proteins (Liebowitz *et al.*, 1989). This was therefore the first characterisation of a membrane bound protein found in latently EBV infected B cells.

Being a membrane protein it was initially assumed that LMP-1 would function as a receptor and it was shown that LMP-1 was structurally similar to members of the β adrenergic receptor family of signalling proteins. However, the amount of LMP-1 projecting out of the cell as a potential binding site is very small and no ligand has yet been identified. With the discovery that LMP-1 aggregates in the plasma membrane and associates with members of the Tumour Necrosis Factor Receptor (TNFR) Associated Factor (TRAF) family of signalling proteins (discussed later in section 1D.5.2) the current view is that LMP-1 is not a receptor in the classical sense, but rather the initiator of signalling events in the absence of ligand.

LMP-1 protein is characterised by serine/threonine phosphorylation at a ratio of 6:1 of the C-terminal domain and rapid turnover (half-life of 2-3hrs) due to specific proteolytic cleavage, which causes the release of a phosphorylated C-terminal fragment (p25) into the cytoplasm (Moorthy and Thorley-Lawson, 1993b). Phosphorylation has long been recognised as a mechanism for regulating the function of proteins so may be important in the

function of LMP-1. Indeed two important sites at S-313 and T-324 were identified therefore phosphorylation would seem to provide a mechanism for the regulation of LMP-1.

1D.2   Transforming ability of LMP-1.

1D.2.1   Rodent fibroblasts.

The first report that LMP-1 could act as an oncogene came via the transfection of LMP-1 into NIH3T3 and Rat-1 fibroblasts (Wang *et al.*, 1985). In NIH3T3 cells LMP-1 expression led to an altered morphology and reduced sensitivity to low serum inhibition of cell growth. The effect of LMP-1 expression in Rat-1 cells was much more dramatic with LMP-1 expressing clones forming foci of heaped up cells in 10%FCS medium. LMP-1 expression also facilitated anchorage-independent growth in soft agar. Even more dramatically, LMP-1 expressing clones formed tumours when injected into nude mice. This was therefore the first demonstration that LMP-1 was indeed capable of oncogenicity and strengthened the hypothesis that it has a role in EBV associated human malignancies. This initial finding was substantiated and expanded when it was shown that LMP-1 also caused anchorage-independent growth in Balb/c 3T3 cells and LMP-1 expressing clones were capable of inducing tumours in athymic mice (Baichwal and Sugden, 1988). LMP-1 is therefore capable of transforming rodent fibroblasts.

1D.2.2   Epithelial cells.

The first demonstration that LMP-1 had an oncogenic effect in epithelial cells *in vivo* was demonstrated by Wilson *et al.*, 1990 by expression in transgenic mice. Expression of LMP-1 in the epidermis of transgenic mice resulted in mice which were runted and had in common a distinct phenotype of dermatosis giving a gross appearance of wrinkled, scurfy skin soon after birth. Histopathological examination revealed a thickening (hyperplasia) of the epidermis with a disorganisation of the differentiating layers, and this was maintained throughout the animals lifetime. This disruption of the epidermis by LMP-1 and the resultant hyperplasia mimics the initial stages of tumorigenesis, and provides clear evidence that LMP-1 is involved in epithelial transformation.

In support of the *in vivo* evidence that LMP-1 is oncogenic, LMP-1 was transfected into the SCC12F cell line which is an immortalised non-tumorigenic sub-clone of the human squamous cell carcinoma line SCC12 (Dawson *et al.*, 1990). SCC12F cells retain certain features of normal keratinocyte behaviour *in vitro*, including responsiveness to differentiation signals. Because the effects of LMP-1 in rodent cell transformation assays resemble those produced by activated Ras proteins, SCC12F cells were also transfected with activated c-Ha-*Ras* in parallel experiments.

The LMP-1 and Ras transfectants showed little evidence of normal cell stratification and were severely impaired in their ability to form crosslinked envelopes in response to calcium ionophore treatment. This suggests a reduced capacity for terminal differentiation. Indeed, compared to control transfectants, all LMP-1 expressing clones produced a much thicker, but less well organised epithelium with poor intercellular contacts (desmosomal junctions) when grown on collagen rafts. In the parallel Ras transfection studies, Ras produced an even thicker multilayer structure than LMP-1 with a similar absence of terminal differentiation markers. This study indicates that LMP-1 can induce changes in the surface phenotype and differentiation responsiveness of human epithelial cells and in certain aspects is functionally similar to activated Ras protein.

Similar studies using the immortalised non-tumorigenic cell line Rhek-1 as the target cell for LMP-1 transfection provided corroborative evidence for the last study. In these cases LMP-1 expressing Rhek-1 clones had a more fibroblastic morphology, with altered architecture and growth pattern (Fahraeus *et al.*, 1990; Zheng *et al.*, 1994a). Cells grew in tight, disorganised bundles that became multilayered and formed foci long before they reached confluence. This was different to control transfectant or parental Rhek-1 cells which grew as flat, polygonal colonies characterised by a "cobble-stone" appearance.

1D.2.3   B-cells.

The first observation that LMP-1 was required for the transformation of B cells came when the amino terminus of LMP-1 was deleted from a recombinant virus abolishing B cell transforming activity (Wang *et al.*, 1988). Subsequent studies have been done to analyse different regions of the LMP-1 protein using deletion mutants to attempt to discover the exact location of the transforming portion of the protein (See section 1D.6). All of these

studies confirmed that loss of LMP-1 function abolished the ability of EBV to transform B cells (Liebowitz *et al.*, 1992; Kaye *et al.*, 1993 and 1995). Although it is required in this function, it is not sufficient, since deletion of EBNA-2, EBNA-3a, -3c and EBNA-LP all result in loss of transforming activity by the virus in B cells in culture (EBNA-1 being not possible to delete in this assay).

1D.3   LMP-1 induced changes in gene expression.

1D.3.1   Epithelial cells.

The initial interest in the effects of LMP-1 in epithelium stems from the fact that the oral epithelium is thought to be the primary site of *in vivo* infection by EBV, and that of all the human malignancies associated with EBV, NPC has the strongest association. There is however a lack of a suitable culture system because it is not possible to infect epithelial cells *in vitro* with EBV without the prior introduction of the CR2 gene (Li *et al.*, 1992). This has therefore hampered studies into the effects of LMP-1 in epithelial cells and limited *in vitro* experiments largely to transfecting LMP-1 into various immortalised cell lines. Despite these problems the transfection system does allow observation of the effects of introduced viral or cellular genes on epithelial cell growth and differentiation.

For example, when LMP-1 was transfected into the SCC12F cell line (Dawson *et al.*, 1990) it was shown that CD40 and ICAM-1 levels were both increased. The levels of CD23, LFA-1, and LFA-3, which are effected in B cells, could not be assessed because either they are not normally present in epithelial cells or because they are already at very high levels in these cells. Further transfection studies have shown that the anti-apoptotic gene A20 is induced in the human cervical carcinoma derived epithelial cell line C33A upon LMP-1 transfection (Miller *et al.*, 1995). Furthermore the Epidermal Growth Factor Receptor (EGFR) has been shown to be overexpressed in NPC (Zheng *et al.*, 1994b), and was seen to be present at elevated levels in LMP-1 transfected C33A epithelial cells (Miller *et al.*, 1995). The EGFR induced was predicted to be functional because it became phosphorylated on Tyrosine residues when EGF was added to the medium. This led to phosphorylation of other proteins downstream in the EGFR signalling pathway, one of which may be p55Shc thus linking the EGFR pathway to the Ras pathway. LMP-1 expression also increased the proliferative response to EGF, as LMP-1 expressing C33A cells continued to

27

increase in number when plated in serum free media supplemented with EGF unlike controls. Therefore, LMP-1 may induce EGFR and thereby upregulate the EGFR signalling pathway. This may in part explain the similarities between transfected LMP-1 and Ras in growth transforming assays (Dawson *et al.,* 1990). The presence of increased EGFR in LMP-1 expressing NPC specimens (Zheng *et al.,* 1994b) indicates that LMP-1 may induce EGFR *in vivo* also and that upregulation of EGFR by LMP-1 may contribute to the development of NPC. The mechanisms by which LMP-1 upregulates EGFR and other proteins will be reviewed in section 1D.5)

LMP-1 expression *in vivo* in the epidermis of transgenic mice also caused changes in gene expression (Wilson *et al.,* 1990). Here, the cytokeratin K14 was shown to be induced in the suprabasal layers of the epidermis and not restricted to the basal layers as is normally the case. More dramatically the hyperproliferative keratin K6, normally found expressed only in hair follicles, was induced within the hyperplastic region of the epidermis. Thus LMP-1 induced changes in gene expression can be detected both *in vitro* and *in vivo.*

1D.3.2 B-cells.

Due to the severe problems limiting *in vitro* analysis of the effects of LMP-1 in epithelial cells, most of the information on LMP-1 induced gene expression alterations has come from *in vitro* investigations using B cells. Infection and immortalization of primary human B cells with EBV leads to a panoply of changes in gene expression (Reviewed in Kieff, 1996). Such immortalised EBV positive cell lines and also immortalised EBV positive and negative human B lymphoma or Burkitt's Lymphoma (BL) cell lines have been invaluable in determining the nature and cause of these changes in gene expression.

EBV negative human B lymphoma cells characteristically grow as single cells in culture and express low levels of activation and adhesion markers. EBV positive latency 3 type human B lymphoma cells on the other hand grow in clumps and show upregulated expression of activation and adhesion markers. Single gene transfer experiments where LMP-1 was transfected into EBV negative B lymphoma cell lines showed that LMP-1 had profound effects and was responsible for the increase in adhesion markers and subsequent growth in clumps. LMP-1 also caused increased

cell size, acid production, plasma membrane ruffling, and villous projections (Wang et al., 1988).

Expression of LMP-1 in a variety of cell lines has been examined including the EBV negative B lymphoma lines Louckes and BJAB, the EBV negative BL line BL41, and Daudi, an EBV positive cell line lacking LMP-1, EBNA-2 and part of EBNA-LP (Wang et al., 1988; Wang et al., 1990). Each cell type has a unique profile but in every case studied the BL marker CD10 was uniformly downregulated by LMP-1.

A summary of results from these cell lines showed that LMP-1 can induce the expression of the adhesion molecules ICAM-1 (CD54), LFA-1 (CD11a/CD18) and LFA-3 (CD58); CD21, the EBV receptor; CD23, the B cell activation marker; CD39, a lymphoid cell activation antigen recently shown to have ecto-apyrase activity; CD40, activation of which is critical for B-cell function, leading to activation and expression of cell surface markers, proliferation, immunoglobulin class switching and inhibition of programmed cell death; CD44, a molecule implicated in enhanced lymphoid tumour growth and dissemination; and CD71, the transferrin receptor.

Further analysis of CD23 upregulation by LMP-1 showed that LMP-1 specifically targets the type b CD23 mRNA (FcεRIIb) for upregulation (Wang et al., 1990). This is one of two CD23 mRNAs which is normally induced in B lymphocytes by IL-4, and would explain why EBV-infected LCL's have more FcεRIIb than FcεRIIa.

Other single gene transfection experiments involving LMP-1 have been invaluable in discovering further cellular gene expression changes induced by LMP-1. For example, only LMP-1 transfected EBV negative B lymphoma cells, and not cells transfected with any other latently expressed gene, showed elevated expression of the anti-apoptotic gene bcl- 2 (Henderson et al.,1991; Rowe et al.,1994). Another Bcl-2 family member, Mcl-1, was also shown to be rapidly induced in LMP-1 transfected EBV negative BL cell lines (Wang et al., 1996). One further anti-apoptotic protein, A20, was also shown to be induced in lymphocytes by LMP-1 (Laherty et al., 1992). The relevance of LMP-1 inducement of Bcl-2 family members, and other anti-apoptotic proteins is discussed in greater detail in section 1D.4.

LMP-1 may also induce interleukin 10 (IL-10) expression (Nakagomi et al., 1994) perhaps effecting the immunogenic response to latently EBV infected cells in vivo .

LMP-1 appears to also induce functional CaM kinase (Mosialos *et al.*, 1994) which might be responsible for aspects of the growth and transformation associated with LMP-1.

EBV transformed LCLs express high levels of human leukocyte antigen (HLA) which are efficient in antigen presentation. Latent gene transfection studies, where each latent gene was transfected alone into the EBV negative BL cell line Louckes, showed that LMP-1 was responsible for upregulating the HLA class II molecules (Zhang *et al.*, 1994). Quite why a viral gene should upregulate a component of one of the host cell's main defence mechanisms remains unclear.

LMP-1 co-localizes with and induces higher levels of vimentin in latently infected B-lymphocyte plasma membranes patches (Liebowitz *et al.*, 1987). Vimentin is not ordinarily found in plasma membrane patches therefore is presumably drawn there by LMP-1.

These effects on cell growth and activation, whilst varying from cell type to cell type, indicate that LMP-1 is an important mediator of EBVs effects on lymphocyte growth. Inducement by LMP-1 of Bcl-2 family members and also CD40 is likely to be important in cell survival and is discussed in the next section.

1D.4   LMP-1 expression and enhanced cell survival.

1D.4.1   B-cells.

Serial passage of cells with a BL phenotype can result in a broadening of EBV latent gene expression to that characteristic of LCLs (from type 1 to type 3). Comparing the two groups therefore allows analysis of the latent genes involved in the phenotypic changes that ensue. BL phenotype cells rapidly die by apoptosis when conditions that inhibit cell proliferation, such as serum starvation, prevail. On the other hand, LCLs retain their viability (Gregory *et al.*, 1991). This suggests that one or more of the latent genes other than EBNA-1 is responsible for this enhanced ability to survive in suboptimal conditions. It was subsequently shown that of the latent genes, LMP-1 could promote this cell survival, in part through upregulation of Bcl-2 (Henderson *et al.,* 1991; Martin *et al.,* 1993; and Rowe *et al.,* 1994). The increased cell survival induced by LMP-1 might allow a bypass of normal selection processes *in vivo* and thus allow the cell to enter the long lived memory B cell pool. Promoting cell survival also increases the chance of neoplastic alterations occurring.

If LMP-1 induced *Bcl* -2 expression was observed to take 48-72hrs then the cells would be at risk from apoptosis in the interim period. With this in mind it was thought that another Bcl-2 family member might play a role prior to *Bcl* -2 itself. Mcl-1, a rapidly inducible protein with a short half-life, was examined in EBV negative BL cell lines transiently or stably expressing LMP-1 (Wang *et al.,* 1996). It was shown that LMP-1 rapidly induces the expression Mcl-1 which can protect BL cells from apoptotic stimuli until such times as Bcl-2 levels are elevated. Thereafter Mcl-1 may be redundant and it's levels therefore decrease.

The A20 zinc finger protein is Tumour Necrosis Factor α (TNFα) inducible, and confers resistance to the cytotoxic effects of TNFα. Cells expressing A20 would therefore be protected from apoptosis and have a growth and survival advantage. It was shown that LMP-1 significantly induced the A20 transcript and was the single gene responsible for this upregulation (Laherty *et al.,*1992).

LMP-1 induction of CD40 could also protect B cells from apoptosis, not only as CD40 induces B cell proliferation and rescue from programmed cell death, but because CD40 activation induces the apoptosis inhibitor A20 (Sarma *et al.,* 1995).

As LMP-1 can induce Bcl-2 family members in certain cells thereby protecting the cells from apoptosis it was logical to determine if LMP-1 could also protect B cells from p53-triggered apoptosis. BL41 is an EBV-negative BL line carrying an endogenous mutant p53. These cells were transfected with LMP-1 and a temperature-sensitive (ts) p53 which would induce apoptosis at 32^{0}C where ts-p53 behaves as wild-type (WT) (but not at 37^{0}C). Both parental and LMP-1 transfectant cells grew at the permissive temperature (37^{0}C) and were actively cycling, but only LMP-1 expressing cells survived at 32^{0}C. However the cells were arrested in G1 of the cell cycle (Okan *et al.,* 1995). This suggests that LMP-1 protects B cells from p53 triggered apoptosis mediated at the non-permissive temperature, but not from p53 induced cell cycle arrest. The LMP-1 expressing cells also had increased levels of *Bcl* -2 compared to parental cells implying that an LMP-1 induced increase in *Bcl* -2 may be responsible for the resistance to p53 triggered apoptosis. Thus LMP-1 induction of *Bcl* -2 may protect B cells from two types of apoptotic stimuli, namely serum starvation and p53 induction, and so give these cells enhanced survival abilities and therefore increased neoplastic possibilities.

In vitro infection of primary human B cells by EBV results in activation of resting B cells and the synthesis of p53. The LCLs produced are very sensitive to chemotherapeutic agents resulting in apoptotic death (Allday *et al.*, 1995). In this example LMP-1 did not interfere with the activation of p53 in response to DNA damage, or it's function as a mediator of apoptosis. It would appear then, that LMP-1 can induce survival under certain conditions, but not under conditions where DNA damage occurs.

1D.4.2   Epithelial cells.

Although *Bcl-* 2 can protect B lymphocytes from p53 mediated apoptosis, *Bcl-* 2 levels are not altered in epithelial cells or NPC therefore *Bcl-2* levels do not appear to play a role in blocking apoptosis in epithelial cells. A20 can protect breast cancer cells and fibroblasts from TNF mediated apoptosis, and since A20 expression is induced in both lymphocytes and epithelial cells by LMP-1, it may be able to protect epithelial cells from apoptosis (Laherty *et al.*,1992).

Okan *et al* had shown that LMP-1 may protect B cells from p53 mediated apoptosis triggered by a non-permissive temperature, and similar findings were observed in the human lung carcinoma cell line H1299 (Fries *et al.*, 1996). In an epithelial cell line transfected with LMP-1, A20 levels were significantly increased (Miller *et al.*, 1995). When A20 was transfected into the H1299(ts)-p53 cells it protected the cells from apoptosis induced at 32^0C by serum withdrawal (Fries *et al.*, 1996). Therefore LMP-1 induces A20 which then protects cells from p53 mediated apoptosis in conditions of reduced temperature and reduced serum.

Just as in the B cell situation, LMP-1 has different effects in different epithelial cell lines tested and different effects at low, normal and high expression levels. For example, gene transfection studies involving LMP-1 often lead to low yields of stable transfectants from cultures in which transient transfection gave high LMP-1 expression. This has been shown on numerous occasions and was interpreted to mean that high level LMP-1 expression is actually toxic to cells. The possibility that the cell death could be due to apoptosis was investigated in Rhek-1 cells transfected with LMP-1 (Lu *et al.*, 1996). Under normal conditions, there was always more cell death in the LMP-1 transfectants compared to control transfectants or parental cells. Under conditions of serum starvation, the LMP-1 expressing cells showed

a dramatic increase in death, with the highest expressing clones exhibiting the greatest degree of cell death. This study therefore concludes that LMP-1 induces apoptosis in epithelial cells and obviously contradicts previous reports. The role of LMP-1 in protecting epithelial cells from apoptosis is therefore perhaps still open to question.

This section has described the different effects that LMP-1 has in B lymphocytes and epithelial cells. That many of the alterations in gene expression *in vitro* in tissue culture are mirrored *in vivo* in the various disease states suggests that these changes are important in the development and progression of malignancy. The mechanisms used by LMP-1 to introduce these shifts in gene expression patterns will now be discussed.

1D.5 Mechanisms of action of LMP-1.

1D.5.1 LMP-1 acts through NF-κB.

Nuclear Factor κB (NF-κB) is a heterodimeric member of the Rel-related family of transcription factors composed of a 50-kDa DNA binding subunit and a 65-kDa subunit containing a transactivation domain. NF-κB is normally bound in an inactive form by its cytoplasmic inhibitor IκB, but upon certain stimuli will dissociate, translocate to the nucleus and cause transcription of target genes by binding to κB sequences within their promoters (Reviewed in Opipari, 1990).

The A20 gene upregulated by LMP-1 contains two κB sequences, and NF-κB binding induces transcriptional activation. It has been shown that LMP-1 can upregulate the A20 promoter, through the NF-κB binding sites. Furthermore, gene induction through the first binding site is an early consequence of LMP-1 expression (Rowe *et al.*, 1994). Here LMP-1 was stably transefcted into an EBV negative BL cell line under the control of an inducible promoter, providing expression of LMP-1 at will. Induction of LMP-1 in these cells led to the induction of a co-transfected κB luciferase reporter construct. LMP-1 expression results in phosphorylation and proteolysis of IκB allowing the nuclear translocation of NF-κB in the form of p65 in conjunction with p50 and p52 (Herrero *et al.*, 1995). LMP-1 may therefore mediate its various effects on protein expression, including upregulation of adhesion molecules and enhancement of cell survival, through an NF-κB pathway. Indeed, various members of the adhesion

molecule family, including ICAM-1, have NF-κB responsive elements in their gene promoter region.

p53 has an NF-κB site in it's promoter region and as with the previous studies it was shown that LMP-1 induced NF-κB activation and subsequent upregulation of the p53 gene (Chen and Cooper, 1996). This is then another example of LMP-1 induced NF-κB activity and poses a very intriguing question which merits further investigation. Namely, why do the increased levels of functional p53 induced by LMP-1 fail to block cell division and/or induce apoptosis?

The various studies described so far have all involved transfection of LMP-1 into EBV negative B cell lines. Since LMP-1 has different effects in different cell types it may be predicted to have different effects on NF-κB activity in epithelial or fibroblastoid cells. Indeed, LMP-1 induced high levels of NF-κB activity in 293 cells, a transformed fibroblastoid cell line. In addition, a small but significant increase in NF-κB activity in HEp2 cells, an epithelial carcinoma cell line, was observed. However, no effect on NF-κB activity was noted in Balb/c 3T3 cells, a murine fibroblast cell line (Mitchell and Sugden, 1995). Thus, LMP-1 affects NF-κB in a cell type dependent manner.

LMP-1 expression alone is reported to induce many of the cell surface phenotype changes that are reminiscent of the activation process initiated by infection with EBV. To assess if these changes are a direct result of LMP-1's ability to activate NF-κB, the induction of two cell surface activation markers, CD54 and CD40, were compared to levels of activation of NF-κB. It was shown that high levels of NF-κB activity did not always efficiently translate into surface phenotype changes. This implies that other mechanisms must also be activated in concert with NF-κB to achieve the full extent of surface phenotype change. What these other mechanisms might be became clearer with the discovery that LMP-1 specifically interacts with members of the TNFR Associated Factor (TRAF) family of signalling molecules.

1D.5.2 LMP-1 interacts with members of the TRAF family of signalling molecules.

LMP-1 forms discrete patch/cap structures in the plasma membrane where it associates with the cytoskeletal protein vimentin (Liebowitz *et al.*, 1986) and is co-localized with LMP-2A (Longnecker *et al.*, 1991). Although LMP-2A has been shown to

be a substrate for src family tyrosine kinases (Longnecker *et al.*, 1991) a direct association with LMP-1 (for example using the yeast two-hybrid system) has not been shown.

LMP-1 is capable of forming patches in the plasma membrane in the absence of vimentin or any other EBV protein (Liebowitz and Kieff, 1989; Liebowitz *et al.*, 1992) suggesting that it may interact with itself, or with other cell membrane proteins to form large, noncovalently linked membrane complexes.

This hypothesis was substantiated when the yeast two-hybrid screening system was used to identify proteins which interact with the cytoplasmic C-terminal tail of LMP-1. An interaction was shown with a human protein with a ring finger and extended coiled-coil domain [LMP-associated protein (LAP1)], and with an EBV-induced protein EBI6 (Mosialos *et al.*, 1995). These proteins were found to be homologous to members of the recently discovered family of tumour necrosis factor receptor (TNFR)-associated factors (TRAFs) (Rothe *et al.*, 1994) which share a c-terminal homology region called the TRAF domain.

LAP1 was identical to the third member of the TRAF family, TRAF3, which was initially identified by several groups as binding to the C-terminus of the CD40 molecule and therefore alternatively called CD40 binding protein (CD40bp), CD40 Associated Factor (CRAF1) and (CAP-1), for CD40 Associated Protein-1, respectively (Hu *et al.*, 1994; Cheng *et al.*, 1995; and Sato *et al.*, 1995). EBI6 was shown to be the human homologue of the murine TRAF1.

This initial discovery was the first direct evidence that LMP-1 may be acting as a signalling molecule as it was the first report of LMP-1 directly interacting with signalling pathway factors, namely the TNF/CD40 pathway.

In transiently transfected cells, 30% of LMP-1 was shown to be associated with TRAF3, another 30% was associated with TRAF1 or TRAF1-TRAF2 complexes (Devergne *et al.*, 1996). In contrast, virtually all of the TRAF1 or TRAF3 observed was associated with LMP-1, and only 5% of TRAF2 likewise bound. Thus, the LMP-1 cytoplasmic domain interacts with and associates with cell proteins that are mediators of cytoplasmic signalling from the TNFR family.

In another study TRAF3 was used as the 'bait' in a yeast two-hybrid screen and a protein called TANK (TRAF family member associated NF-kB activator) was discovered (Cheng *et al.*, 1996). TANK is ubiquitously expressed, has no significant homologies and could interact with all three TRAFs. TANK, in

conjunction with TRAF2, activates NF-κB. However, in complete contrast, TANK inhibits NF-κB activation from the LMP-1 cytoplasmic tail. TANK competes with LMP-1 (and presumably TRAF1) for the binding of TRAF2, and so may inhibit NF-κB activity under these conditions by preventing TRAF2 association with aggregated LMP-1.

A model would therefore be that LMP-1 aggregates in the plasma membrane in association with TRAF's1, 2, and 3. TRAF1 is constitutively bound to LMP-1 and requires TRAF2 to bind and form a heterodimer for NF-κB activation. TANK is a negative regulator of LMP-1 mediated NF-κB activation. TRAF3 binds to LMP-1 with the greatest affinity, but its role is not clear, with no involvement in NF-κB activation.

This interaction of LMP-1 and TRAFs might lead to activation of the TNFR/CD40 pathways thereby causing constitutive cell growth and inhibiting apoptosis. One of the down stream effects of signalling through the TRAF molecules is also NF-κB activation (section 1D.5.1).

The focus of discussion so far has been the effects of LMP-1 on B-cells and epithelial cells both *in vitro* and *in vivo*. Intensive mutational analysis of all three domains of LMP-1, and in particular the carboxy terminus, has been carried out in an effort to characterise the regions required for the transforming ability of LMP-1 and the inducement of the numerous cellular gene expression changes occurring apon LMP-1 expression. A discussion of the important structural findings will form the basis for the next section.

1D.6   Mutational analysis of LMP-1.

1D.6.1   Functional analysis of the domain structure of LMP-1.

Once it was discovered that LMP-1 was oncogenic in rodent fibroblasts it became important to know which domains of the protein were responsible for the transforming events and which were responsible for the upregulation of cellular genes. The principal approach used involved construction of deletion mutants of the LMP-1 gene and then testing them for their transforming activity in rodent fibroblasts or effects on gene expression and transformation in B cells.

Mutants lacking sequences in the N-terminus or transmembrane (tm) domain had intermediate and no transforming ability respectively when transfected into Balb 3T3

cells (Hammerschmidt *et al.*, 1989). This suggested that the amino terminus and the transmembrane domain are necessary for the transforming effect of LMP-1 on rodent fibroblasts. In contrast, certain mutations in the C-terminus of LMP-1 were still able to transform Balb 3T3 fibroblasts.

The panel of mutants assessed was then extended. Both N-terminal mutants and transmembrane mutants are phosphorylated normally but are inefficiently cleaved or uncleaved respectively (Moorthy and Thorley-Lawson, 1993a). Furthermore, neither mutant could efficiently transform Rat-1 cells to anywhere near the levels of WT LMP-1. The first 30αα and last 23αα of the C-terminus were also shown to be essential for transformation of rodent fibroblasts. These results therefore indicate that all 3 domains of LMP-1 are required for the transforming ability of the protein.

In B cells, initial analysis indicated that the N-terminal plus the first 4 transmembrane loops are needed for functional activity of LMP-1 (Wang *et al.*, 1988). This region was further dissected by creating more N-terminal and transmembrane mutants (Liebowitz *et al.*, 1992) which revealed that the N-terminus, 1st, 3rd and 4th transmembrane domains each contribute to the effect of LMP-1 on B lymphocytes. N-terminal truncated proteins were created that could not initiate or maintain primary B cell transformation (Kaye *et al.*, 1993).

However, further mutational assessment of the role of the amino-terminal cytoplasmic domain (Izumi *et al.*, 1994) showed that the only mutant that was slightly retarded in its ability to transform primary B lymphocytes was one which had the transmembrane anchor sequence deleted. This result suggests that it is most likely that the N-terminal domain of LMP-1 is not essential to B lymphocyte transformation, but probably plays a structural role of anchoring the amino terminus of the 1st transmembrane domain of LMP-1 to the plasma membrane.

With this report, attention switched to focus on the carboxy-terminal domain of LMP-1 with regard to B cell transformation activity (Kaye *et al.*, 1995). Mutants with the whole of the C-terminus deleted could never be expressed without WT LMP-1 being present indicating that the C-terminus of LMP-1 is essential for B lymphocyte transformation. Using a panel of deletion mutants it was shown that the first 44αα are required for growth transformation and the last 155αα are required to provide a growth factor like effect. The C-terminal mutants are orientated correctly in the plasma membrane in wild-type LMP-1 fashion,

but are completely unable to induce ICAM-1 upregulation (Peng-Pilon *et al.*, 1995).

The conclusion from these results is therefore that the N-terminus plays a structural role in tethering the essential transmembrane domain to the plasma membrane. This is required to allow the C-terminus to give the transforming signals. The C-terminus will be further dissected in the following two sections to demonstrate the precise regions involved in cell transformation.

1D.6.2   Analysis of the C-terminus of LMP-1 for NF-κB and TRAF activating ability.

LMP-1 deletion mutants have been analysed to ascertain which domain(s) is required for activating NF-κB. The analyses revealed that the amino terminus held no intrinsic NF-κB stimulatory ability (Mitchell *et al.*, 1995). The membrane spanning domain and the carboxy terminal domain were however essential and more precisely, the last 55αα of the carboxy terminus were required for maximal stimulation. This implicates the C-terminal 55αα of LMP-1 as being the NF-κB inducing region.

Further analysis using similar LMP-1 deletion mutants confirmed the previous report and also noted that deletion of the first 38αα and the last 35αα of the C-terminal half severely inhibited NF-κB induction (Huen *et al.*, 1995). These regions were called CTAR-1(residues 194-232) and CTAR-2(residues 351-386) meaning 'C-terminal activating regions'. Both domains are required for maximal NF-κB stimulation but the CTAR-2 was shown to be the major effector.

Mutational analysis indicated that the first 44αα of the C-terminus of LMP-1 was sufficient to interact with TRAF3. The TRAF3 binding site was then pinpointed to the 12αα's 201-212. This region contains a core sequence of PxQxT (Devergne *et al.*, 1996). Systematic mutation of these 12αα revealed the importance of certain amino acids, but more importantly showed that all three TRAFs bind to this same core site.

This same proximal region of the LMP-1 carboxy terminus cytoplasmic tail is also known to activate NF-κB activity, albeit at much lower levels than the distal 45αα of the cytoplasmic tail. Mutation of P-204 and Q-206 of the core binding sequence, markedly reduced NF-κB activity. This strongly suggests that TRAF binding is linked to NF-κB activation in part, and also favours a role for TRAFs 1 and 2, since TRAF3 binding is only

slightly affected by mutations to P-204 or Q-206, but TRAF1 and 2 binding is greatly affected. Indeed TRAF3 repressed LMP-1 mediated NF-κB activity from this region, presumably by displacing TRAF1 and TRAF2 from the site.

This hypothesis was substantiated by experiments showing that co-transfection of TRAF1 and LMP-1(αα1-231) into EBV negative B cells, resulted in a several-fold increase of NF-κB activity, but TRAF2 had no such effect, however TRAF2 is constitutively expressed. When a dominant negative mutant of TRAF2 is co-transfected with LMP-1(αα1-231), 75% of the NF-κB activity is lost (Kaye *et al.*, 1996). This implicates TRAF2 also with an NF-κB activating role. Mutational analysis of TRAF1 and TRAF2 showed that the TRAF-N domain was essential for this increase in NF-κB activity (Devergne *et al.*, 1996; Kaye *et al.*, 1996). These findings suggest that either both TRAF1 and TRAF2 activate NF-κB independently, or more likely that a heterodimer of TRAF1 and TRAF2 activates NF-κB with TRAF1 associating directly with LMP-1.

An LMP-1 mutant lacking the proximal 64αα of the carboxy terminal cytoplasmic tail could not induce EGFR expression whilst a mutant lacking the distal 34αα of LMP-1 could (Miller *et al.*, 1997). This reveals two things. Firstly, that EGFR is unlikely to be induced by NF-κB activation because the mutant with the distal portion of LMP-1 intact could not induce EGFR and this region contains the majority of the LMP-1 NF-κB activating ability. Secondly, EGFR induction probably involves signalling via TRAF molecules since the TRAF interaction domain on LMP-1 must be intact for EGFR expression. The mutant with the TRAF interaction domain intact, but the distal portion of LMP-1 missing could still induce A20 in the absence of EGFR, indicating that although NF-κB activity is available, it does not account for EGFR expression. This also suggests that TRAF signalling from LMP-1 extends to activators of transcription other than NF-κB, giving two distinct signalling pathways.

The last sections have indicated the importance of the C-terminus in signalling by LMP-1. A TRAF interaction domain has been identified which binds all three TRAFs. This domain overlaps one of the two NF-κB activating sites and results imply that TRAF interaction and activation of NF-κB are intimately linked.

In conclusion LMP-1 is a viral oncogene capable of transforming different cell types and capable of inducing a wide range of cellular gene expression changes. LMP-1 enhances cell

survival, activates NF-κB, and interacts with the TRAF family of signalling proteins. That LMP-1 is involved in so many events and is expressed in 60% of NPC cases can be no co-incidence. The potential role of LMP-1 in NPC will now be discussed.

1E.   LMP-1 and NPC.

1E.1   Alterations in NPC LMP-1 variant.

Since LMP-1 is a likely candidate for involvement in the pathogenesis of NPC, research has focused on this protein and the role it might have in the nasopharngeal epithelium that predisposes it to tumorigenesis. The LMP-1 gene from a genomic library of a nude mouse propagated NPC biopsy was sequenced and found to differ from the prototype B95-8 LMP-1 sequence in certain important regions (Hu *et al.*, 1991). This NPC LMP-1 had lost many restriction sites including an informative *Xho*1 site in exon 1 of the gene. This site was also absent in 36 out of 37 Chinese NPC biopsies tested, but was intact in 17 out of 19 African NPC biopsies strengthening the idea that Chinese NPC is associated with a particular strain of EBV.

Substitutions at the N-terminus alter the overall charge of LMP-1. At the carboxy terminus the NPC LMP-1 isolate displayed a cluster of 7 perfect repeats of 11αα compared to the 4 imperfect repeats found in B95-8 LMP-1. Also at the C terminus, a 30bp deletion was present downstream from the 7 repeats. Many other single point mutations have resulted in the gain or loss or restriction sites. Overall the changes produce a 404αα protein compared to the 386αα B95-8 LMP-1 prototype version.

The LMP-1 gene from a Taiwanese NPC genomic library has also been sequenced and again showed differences compared to B95-8 LMP-1 (Chen, M.L., *et al.*, 1992). In the carboxy terminus there were 5 perfect 11αα repeats and a 33bp deletion in the same region as the 30bp deletion described above. There was also an 18bp insertion. This Taiwanese NPC LMP-1 is therefore distinct from B95-8 LMP-1 but closely resembles the Chinese NPC LMP-1 gene.

1E.2   NPC LMP-1 is more oncogenic and less immunogenic than the B95-8 prototype.

To test the theory that the LMP-1 variant in NPC might confer greater oncogenicity to the virus in respect to NPC, the NPC LMP-1 gene was transfected into Balb/c 3T3 cells or Rhek-1 cells (Chen, M.L., *et al.*, 1992; Zheng *et al.*, 1994b) and revealed greater oncogenic potential *in vitro* than the prototype B95-8 LMP-1 gene. Furthermore, the LMP-1 expressing cells were more tumourigenic in nude mice.

However, these results do not determine whether it is sequence differences within the NPC LMP-1 gene itself, or sequence differences in the promoter region of the NPC LMP-1 gene. This question was addressed by characterising the 5'-upstream region of NPC LMP-1 (Chen, M.L., *et al.*, 1995). It was shown that the NPC LMP-1 promoter is actually between 3x and 9x less active than the B95-8 promoter (Chen, M.L., *et al.*, 1995; Li *et al.*, 1996). Furthermore, promoter-gene hybrid constructs revealed that the increased oncogenicity resided within the NPC LMP-1 coding region. Interestingly, the NPC LMP-1 also appears to be less immunogenic than B95-8 LMP-1 (Trivedi *et al.*, 1994).

1E.3   10αα deletion is responsible for the enhanced oncogenicity.

The C terminus of NPC LMP-1 was shown to be sufficient to transform cells in hybrid constructs suggesting that it is the carboxy terminus of NPC LMP-1 that is important (Li *et al.*, 1996). Any change in the number of 11αα repeats in NPC LMP-1 had no effect on transforming ability. However, replacing the 30bp deleted in NPC LMP-1 with the matching 30bp sequence from B95-8 LMP-1 negates the enhanced transforming ability, and creating a 30bp deletion in B95-8 LMP-1 confers the same transforming potential as NPC LMP-1. This strongly suggests, that the absence of those 10αα gives NPC LMP-1 it's greater oncogenic potential.

1E.4   Sequence variation within the LMP-1 gene: Pertinence to disease.

It was reported that the 30bp deletion in the C-terminus of LMP-1 was prevalent in diseases other than NPC such as Hodgkin's Disease (Knecht *et al.*, 1993). This led to a more extensive search

for LMP-1 mutations in a variety of lymphoproliferative disorders (Knecht *et al.*, 1995). A high frequency of non-random point mutations at preferential sites within the 3' carboxy terminal region of LMP-1 was shown.

Further analysis of mutations within the C-terminus of LMP-1 revealed that the 30bp deletion was present at high incidence in aggressive Hodgkin's Lymphoma and malignant lymphomas but not in sporadic EBV associated lymphoid hyperplasia's suggesting that the deleted form may be preferentially selected in lymphomatous processes (Kingma *et al.*, 1996). A new 69bp deletion, in conjunction with the 4 common point mutations, was also reported (Klein *et al.*, 1995).

That these mutations in the C-terminus of LMP-1 are not uncommon was shown by a study of LMP-1 in healthy individuals and neoplastic conditions in the Japanese population (Itakura *et al.*, 1996) where 74% of cases carried the 30bp deletion. WT virus infected LCL's (Sandvej *et al.*, 1996) and all the LCL's from healthy individuals (Itakura *et al.*, 1996) contained the 30bp deletion also. Only one case had identical sequence to the B95-8 prototype LMP-1. Therefore, the 30bp deletion mutant may represent the most prevalent LMP-1 variant in Japan. Thus, the 30bp deletion mutant does not define a tumour specific EBV strain, but may however still be important for the tumorigenicity of LMP-1.

1E.5   Potential role of LMP-1 in NPC.

Immunohistochemical analysis showed that both the Epidermal Growth Factor Receptor (EGFR) and the cell cycle associated molecule Ki67 are both present in NPC biopsies (Zheng *et al.*, 1994b). EGFR was present in 100% of cases but was more strongly expressed in advanced tumour stages. Ki67 expression was likewise enhanced in these metastasised tumours. Interestingly the highest expression was seen in the LMP-1 positive cases implying a direct link between LMP-1 expression and increased Ki67 and EGFR expression. Since proliferative activity and EGFR expression have been linked to the size and stage of various neoplasm's (Brown and Gatter, 1990; Santini *et al.*, 1991), it could be concluded that LMP-1 expression may enhance proliferation and EGFR expression thus leading to a more malignant course.

This suggestion was substantiated when a clinical study of the features of LMP-1 expressing and non-expressing NPCs from Southern China was carried out (Hu *et al.*, 1995). The LMP-1

positive tumours grew faster and more expansively than the LMP-1 negative tumours. The LMP-1 positive tumours also tended to invade more frequently outside the nasopharynx, especially into the lymph nodes. However there were more LMP-1 negative samples in the highly advanced NPCs. This report therefore suggests that LMP-1 increases proliferation resulting in a more malignant tumour, but indicates that LMP-1 is either downregulated in some way or not involved at all when NPCs are at their most advanced.

Thus a great deal of information now exists concerning the action of LMP-1 in B cells. Also, in recent years several studies have begun to reveal the signal transduction pathways that are used by the protein to achieve it's effects. Never the less, the role of LMP-1 in NPC is still poorly understood. This project was begun in order to attempt to address this question using an *in vivo* model system- transgenic mice, in order to overcome the continuing difficulties of studying disease in humans and a tissue which is difficult to obtain.

1F. LMP-1 expression in transgenic mice.

Most of the experiments described so far have been carried out *in vitro* and in B cells and have highlighted many of the problems associated with extrapolating the results to NPC. For example, the dramatic variety of effects mediated by LMP-1 have not been shown in any one cell type but rather are a collection of results from a panel of cell types, each cell line tested giving different results. The results are most definitely cell line and cell lineage specific and are often confusing and contradictory. Because of this, analysis of the effects of LMP-1 expression *in vivo* in an appropriate cell type is vital. The technology involved in creating transgenic mice has facilitated such *in vivo* studies and provided a method of studying genes of interest at work in a living animal. For this reason transgenic mice were created to follow the consequences of the expression of LMP-1 in epithelial cells, as well as B lymphocytes *in vivo* (Wilson *et al.,* 1990).

Expression of the PyLMP transgene in stratified epithelium has the major advantage of allowing functional characterisation of the product through a complete sequence of differential states. Each differential layer of the epithelium expresses different pairs of keratins, and these sets of keratins vary between epithelia (Reviewed in Byrne *et al.,* 1994). The keratins are therefore

excellent markers of the differentiation/growth state of epithelial cells. As such, alterations in keratin expression can also indicate a disruption of differentiation or the induction of hyperplastic events.

Two separate transcriptional control elements, PyLMP and EμLMP, were used to express LMP-1 with the aim of directing expression to epithelial cells or B cells respectively. PyLMP consists of the B95-8 strain LMP-1 coding sequence preceded by the early region promoter and enhancer of the murine polyoma virus (Py), and EμLMP consists of the B95-8 strain LMP-1 coding sequence preceded by the polyoma virus promoter and the mouse immunoglobulin heavy chain (IgH) intronic enhancer (Eμ).

1F.1   PyLMP-1 transgenic mice.

The PyLMP-1 mice were created using the polyoma promoter and enhancer regions to drive expression of the LMP-1 transgene to the epithelial compartment (Wilson *et al.*, 1990). Lines of mice were generated in the C57Bl/6 strain of mice (one which is genetically well characterised). Two lines of mice from founders 5 and 53 displayed a common phenotype of epidermal hyperplasia and showed expression of the transgene in epidermis and at low level in tongue epithelium.

The PyLMP-1 transgenic mice from lines 5 and 53 were runted and had in common a distinct phenotype of dermatosis giving an overall appearance of wrinkled, scurfy skin soon after birth. This phenotype was always evident by d3 and was at it's most obvious by d7. Line 5 showed greater levels of LMP-1 expression than did line 53. Accordingly the skin phenotype in line 5 was more extensive. The epidermal hyperplasia in line 5 mice continued through to adulthood and in patches became very highly hyperplastic. In addition, the mice remained small (1/2 the body weight compared to negative mice) and produced small and infrequent litters (Wilson *et al.*, 1990). However, this line suffered an additional complication. The transgene integrated into the X-chromosome mutating a cellular locus leading to sex linked cleft palate (Wilson *et al.*, 1993). Finally, due to poor viability the line became extinct. Thereafter the focus of work in this thesis rests on experiments using mice of line 53.

Mice in line 53, after d12 quickly gained the weight and size of their LMP-1 negative littermates, and by weaning age the mice could not be distinguished by eye. The epidermal hyperplasia, marked in adults of line 5, is much less evident in adult mice of

line 53. However, as the animals aged a return to the obvious hyperplastic phenotype occurs in hairless regions, appearing as a "ring-tail" phenotype often also evident on paws and genitalia. LMP-1 positive mice of both lines also showed increased claw growth.

Keratin K6 is normally expressed in the hair follicles of the skin and in the proliferating epidermis of wounds, but has been seen to be expressed in the suprabasal epidermal layers in hyperplastic, neoplastic and psoriatic skin. It was therefore very interesting to observe the induction and aberrant expression of K6 in the suprabasal epidermal layers in the PyLMP-1 transgenic mice as well as extension of the expression of K14 (normally a marker of basal cell proliferation) to the suprabasal layers also.

Expression of LMP-1 RNA was restricted to the epidermis and levels of expression correlated with appearance of the phenotype where the only two lines to have readily detectable LMP-1 mRNA were the only two lines to develop hyperplasia. Despite the persistence of hyperplasia throughout the animals lifetime (notably in line 5), no evidence of skin papillomas or carcinomas was ever observed. This may reflect expression below a critical level or perhaps a requirement for other factors (viral or cellular) for progression to neoplasia.

1F.2   EμLMP-1 Transgenic mice.

The EμLMP-1 transgene includes the polyoma virus promoter and the mouse immunoglobulin heavy chain (IgH) enhancer in order to direct expression of the LMP-1 oncogene to the lymphoid compartment (Wilson et al., 1990). Of 15 pups carrying the transgene, eight viable EμLMP-1 mouse lines were generated from these mice. One EμLMP-1 founder displayed the phenotype of epidermal hyperplasia already described for the PyLMP-1 transgene series, however this founder did not breed to generate a line. Transgenically positive offspring from another line (Line 33) also demonstrated this phenotype, but only 4/52 LMP positive offspring were produced (demonstrating the founder was transgene mosaic) and these had low viability, were all dead within 24 weeks and were not able to breed and maintain the line. Another 3 founder mice were dead within 2 weeks. Together, the poor viability of mice in this series suggests a probable toxicity of the EμLMP-1 transgene (Wilson et al., 1990).

The three mRNA species which could be expressed from the PyLMP-1 and EμLMP-1 transgenes include the 2.5kb LMP-

encoding message; a 2.25kb message driven from an internal promoter at late times during the EBV lytic cycle; and a 0.6kb message driven from a second internal promoter at early times during the EBV lytic cycle. It was shown that the 2.5kb LMP-encoding message was abundantly expressed in a broad range of tissues, including skin, in the EμLMP-1 mouse line 33. Thus, the expression of the 2.5kb LMP-encoding transcript in the skin suggests that it may be the cause of the hyperplastic phenotype, but the broad range expression of this transcript may be relevant to the early lethality seen in the EμLMP-1 mice (Wilson *et al.*, 1990).

4 lines of mice in this series displayed initially a very mild phenotype, one of which EμLMP-1 line 39 is described in more detail and is the subject of study in chapter 5.

1G. Prologue and Aims.

The transgenic mice used in experiments presented in this thesis were of the PyLMP-1 line 53 and the EμLMP-1 line 39. Despite persistence of the phenotype throughout the life in some areas of skin (as described) in line 53, no progression to papilloma or carcinoma was noted. As the development of cancer is a multistage process other factors are likely to be required for the progression from hyperplasia to cancer. The aim of this work has been to address what these factors might be and how LMP-1 might be exerting it's oncogenic potential using this transgenic mouse epidermal model.

Given the potential role of LMP-1 in NPC, expression of the PyLMP-1 transgene in the epidermis of line 53 transgenic mice can be used as a model for the early stages of the disease (expression of the other viral genes involved in NPC may form subsequent stages in the development of the model). Although expression is not specifically directed to the nasopharynx, the epidermis is the most accessible and best studied epidermal tissue in the mouse providing the most appropriate *in vivo* model system in the first stages of study. The difficulties involved with investigating the effects of LMP-1 in epithelial cells *in vitro* can consequently be overcome with this transgenic mouse model. The experiments described here are therefore useful in determining the basic function of LMP-1 in epithelial cells *in vivo* whilst perhaps giving some insight as to what may be occurring in the EBV positive nasopharynx that can lead to NPC.

The investigative strategy is threefold.

Firstly an investigation into the expression pattern of PyLMP-1 in the transgenic epidermis and the effects of expression of the PyLMP-1 transgene has been conducted (Chapter 2). This procedure was designed to ascertain the cause of the observed phenotype in line 53 with respect to the mode of action of LMP-1 in otherwise normal epidermis. Accordingly, the carefully controlled processes of epidermal proliferation and differentiation in line 53 mice are examined in order to discover if LMP-1 has any effect on either process.

Secondly, transgenic PyLMP-1 line 53 mice have been subjected to the chemical multistage skin carcinogenesis model (Chapter 3). This model allows an examination of the consequences of LMP-1 expression during the evolution of malignancy. Such investigative procedure is invaluable in determining at what stage(s) during the carcinogenic process LMP-1 exerts its effects, and what these effects might result in. Therefore, LMP-1's ability to act as either initiator, promoter or enhancer of tumour formation is examined.

Thirdly, an examination of the co-operation of LMP-1 in tumourigenesis with other proteins of interest via cross-breeding experiments has been conducted (Chapter 4). Such cross-breeding experiments may reveal co-operation, counteraction, or even redundancy of two proteins, with a subsequent enhancement, ablation, or failure to alter an observed phenotype. Consequently, PyLMP-1 line 53 was crossbred with transgenic lines expressing Ha-*ras* and TGFβ1 epidermally, and with mice lacking p53 function, in order to check for alterations to the day 7 line PyLMP-1 phenotype, and thereafter progression from this to malignancy.

LMP-1 may play a role in the development of Hodgkin's disease and B-cell lymphomas arising in immunocompromised and AIDS patients. The EμLMP-1 line 39 transgenic mice, where expression is directed to the B-cell compartment, had no immediately apparent phenotype and no obvious phenotype by one year other than being less fit and fertile than negative siblings. However, expression of LMP-1 was so low as to be barely detectable and the mice had not been monitored beyond one year of age. The aim of the final part of this project, chapter 5, was therefore to monitor line 39 for a full 24 month period in order to address the possibility of late development of any abnormalities. In

conjunction with this study, cross-breeding line 39 mice with EμEBNA-1 (line 59) mice was carried out to reveal any potential co-operation between LMP-1 and EBNA-1 in lymphomagenesis.

CHAPTER 2:-   Investigation into the PyLMP-1 phenotype.

2A   Introduction

In this chapter the phenotype of the PyLMP-1 mice is further investigated. To set this into context the introductory section covers the biology of the skin, the changes in gene expression which occur during carcinogenesis and other transgenic models of skin carcinogenesis.

2A.1   Biology of the skin

The skin is the largest organ of the body and provides essential protection against ultraviolet radiation and other chemical and biological injury. The skin is composed of two major parts, namely the dermis and epidermis (Reviewed in Zinkel and Fuchs, 1994; Brown and Balmain, 1995). Fibroblasts, the main component of the dermis, secrete a dense network of collagen fibres which provide support for the epidermis and a protective cushion for the inner organs of the body. The dermis is highly vascularised, consisting of an extensive network of blood vessels necessary to supply both the dermis and the non-vascularized epidermis. The epidermis is separated from the dermis by a basement membrane and consists of stratified squamous epithelium, the primary cell type being the keratinocyte. Keratinocytes produce keratins, the cytoskeletal protein which forms networks within the epidermis giving it strength and flexibility. The epidermis is continually undergoing a process of terminal differentiation, where proliferating basal layer cells detach from the basement membrane to become terminally differentiated and, moving through the epidermis eventually are shed and replaced. This process is highly organised and characterised by sequential changes in gene expression.

The actively ¡proliferating keratinocytes of the basal layer co-express the cytokeratins (K) 5 and 14. An unknown signal initiates detachment of a proliferating keratinocyte from its contact with the basement membrane and exit from the cell cycle, whereby the keratinocyte now migrates upward into the suprabasal layer. The keratinocyte now changes morphology and flattens out with subsequent increased desmosomal contacts between neighbouring cells. This gives the appearance of spinous cells and the suprabasal layer can be divided into three categories based on the shape and characteristics of the keratinocytes

present. In the suprabasal layer K5 and K14 are downregulated and another pair of keratins, K1 and K10 become expressed. This switch is associated with an increase in bundling of keratin filaments in the cytoplasm.

The keratinocytes leave the spinous layer and enter the granular layer, where they synthesise a protein called filaggrin. Filaggrin associates with K1/K10 filament bundles, causing them to form large complex bundles of fibres and thus giving more strength to the epidermis. K1 and K10 are now downregulated and three other proteins, namely involucrin, loricrin and keratinocyte transglutaminase are upregulated. Involucrin is a substrate for transglutaminase enzyme mediated crosslinking and this croslinking adds strength to the cornified cell envelope. Loricrin is the last protein to be expressed, and just like involucrin, is crosslinked by transglutaminase action. Concomitant with this crosslinking is the massive destruction of cell integrity. The permeable cornified cell envelope of highly crosslinked proteins replaces the nuclear membrane in cells. The nuclei and cytoplasmic organelles are lost. The mature squames which reach the skin surface leave as empty shells full of keratin filament bundles. The whole differentiation process takes between 2 to 4 weeks to complete.

There are other proteins of the epidermis which are equally essential for the overall function of the organ, but are not expressed in a differentiation stage specific manner. These include the family of adhesion molecules called integrins (Reviewed in Varner and Cheresh, 1996). This family mediates cell-extracellular matrix and cell-cell interactions and are thought to regulate cell growth and differentiation. When integrins are specifically activated through contact with their corresponding matrix proteins, they influence regulation of gene expression, differentiation, growth control and cytoskeletal architecture. As well as adhesive properties integrins are also potent signalling molecules (Dedhar, 1995) and so provide many functions to the normal homeostasis of the skin.

Desmosomes also play an essential part in maintenance of a normal situation in the epidermis (Reviewed in Garrod, 1995). Desmosomes consist of a variety of proteins which serve amongst other things as adhesion molecules to keep cells tightly bound and organised. Two desmosome constituents are desmocollin and desmoglein. Desmosomes are essential for cell architecture as they provide the anchoring point for the keratin intermediate filament network of the keratinocytes. Desmosomal adhesion is homophilic and by maintaining the architecture of the epidermis, allows keratinocytes to communicate with each other and to receive signals. Thus the normal situation of proliferation and

differentiation which continues throughout a lifetime is extremely complex and relies on the co-operation and correct expression of certain proteins. An imbalance in this system could conceivably lead to a breakdown in the process and possibly contribute to malignant transformation.

2A.2   Cancer of the skin

The changes in gene expression during differentiation provide useful markers of the different stages of differentiation and identification of an intact/unaltered differentiation process. Alterations in this process could be identified as aberrant, absent or over-expression of these differentiation markers and would reflect a deregulated pathway of differentiation and pre-neoplastic events. For this reason analysis of these proteins in transgenic mice and human skin cancers has proved invaluable in investigating a role for them in aberrant differentiation (Reviewed in Christofori and Hanahan, 1994; Zinkel and Fuchs, 1994; and Brown and Balmain, 1995) .

2A.2.1   Alterations in keratins

Keratins, the structural backbone of the keratinocyte, have been shown to be aberrantly expressed in certain cases. In situations of faster cell turnover suprabasal expression of K6 and K16 has been shown in humans. These keratins are normally only expressed in hair follicles and sites of wound repair reflecting cell proliferation. As previously described, K6 is also found expressed in the suprabasal layers of the epidermis of PyLMP mice exhibiting hyperplasia (Wilson *et al.,* 1990).

K8 and K18 are characteristic of simple epithelia and early stages of embryogenesis. They are found in sweat glands but not expressed elsewhere in normal adult epidermis (Bosch *et al.,* 1988) however they are re-expressed in some human skin cancers (Laurijsen *et al.,* 1989). Transfection of a mutant human H-*ras* oncogene into the immortalised mouse epidermal cell line MCA3D induces the anomalous expression of K8 and K18 (Diaz-Guerra *et al.,* 1992) and it was shown that K18 was a direct target of the ras signal transduction pathway (Pankov *et al.,* 1994). Thus, oncogene activation provides an explanation for the unexpected expression of K18 in these skin carcinomas.

K13 is normally associated with terminal differentiation of internal stratified epithelia, but was shown to be consistently expressed in squamous cell carcinomas of the skin induced in mouse epidermis by chemical manipulation (Nischt *et al.,* 1988). The stage before carcinoma, i.e. papilloma, was heterogeneous for

K13 indicating that its aberrant expression may correlate with conversion to malignancy.

2A.2.2   Alterations in adhesion molecules

The importance of integrins and desmosomes in cancer is that, as mediators of cell adhesion, variation in their abundance or temporary or permanent modulation of their binding affinity, could result in changes of cell behaviour facilitating invasion and metastasis. As an example, normal epithelial cells express $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 6$, $\beta 1$ and $\beta 4$ integrin subunits whereas breast carcinoma cells show variable losses, disorganised expression or downregulation of these subunits (Varner and Cheresh, 1996).

Both basal and squamous cell carcinomas display a decrease in cell surface area occupied by desmosomes indicating that their deregulation may be an important factor in neoplasia (Garrod, 1995).

This brief look at some of the proteins of the epidermis has shown that they are often aberrantly expressed in human cancer and highlights the importance of a balance between proliferation and differentiation to maintain healthy skin. That more than 80% of tumours occurring in man are carcinomas and the incidence of skin cancer is steadily rising further strengthens the idea that the homeostasis of the epidermis is vitally important (Reichmann, 1994). Use of transgenic mouse models has dramatically promoted the study of candidate oncogenes in the epidermis providing a method of evaluating the roles they might play in carcinogenesis *in vivo*.

2A.3   Transgenic mouse models of carcinogenesis

The use of transgenic mouse models has shown that, whilst certain proteins or oncogenes are necessary for the transformation process, they are not sufficient alone to cause carcinogenesis and require additional events before tumours arise. For example, the H-*ras* oncogene (a potent oncogene in tissue culture assays) has been successfully over expressed in transgenic mouse skin by three groups and shown to cause marked hyperkeratosis (Bailleul *et al.*, 1990; Leder *et al.*, 1990; and Greenhalgh *et al.*, 1993a). The mice subsequently developed papillomas particularly at sites of wounding or abrasion such as the tail and ears suggesting that activation of *ras* is a necessary initiating step in carcinogenesis and that wounding acts as a promotion step.

Several other transgenic mice have been produced which exhibit an initiated phenotype similar to that seen for the Ha-*ras* transgenics. For example TGF-α overexpression in transgenic

mouse skin resulted in hyperkeratosis and hyperplasia with spontaneous papillomas occurring at sites of wounding or mechanical irritation (Wang *et al.*, 1994).

Individual integrin subunits α2, α5, and β1 were expressed in the epidermis of transgenic mice (Carroll *et al.*, 1995). β1 expression alone gave rise to epidermal hyperproliferation. By intercrossing the three lines of mice, bi-transgenics expressing α2β1 or α5β1 were created and these also exhibited epidermal hyperplasia. These findings also suggest that imbalance in integrin expression could be causal in skin carcinogenesis.

Various components of desmosomes have been overexpressed in transgenic mice or removed in knockout mice to investigate this possibility. For example, plakoglobin knockout mice presented with hyperkeratosis (Bierkamp *et al.*, 1996). Desmogleins are members of the cadherin superfamily and form the core of desmosomes. When mutant desmoglein was overexpressed in mouse epidermis the number of desmosomes was greatly reduced and the mice developed abnormalities in epidermal proliferation and differentiation (Allen *et al.*, 1996). Desmoglein associates with plakoglobin so these two studies alone demonstrate how imbalance in desmosomal protein levels can have severe consequences.

Overexpression of involucrin in transgenic mice produces an abnormal phenotype in both the epidermis and hair follicles (Crish *et al.*, 1993), presumably because it is an early scaffold protein essential for correct cornified envelope formation. Loricrin has also been overexpressed in transgenic mice but in this case all of the protein was incorporated into the cornified envelope and no phenotype was observed (Yoneda and Steinert, 1993). As the final protein expressed in differentiation it therefore does not appear to affect the flexible structure or function of the epithelial tissues.

Other transgenic mice which display a marked hyperplastic skin phenotype include v-*fos* (Greenhalgh *et al.*, 1993b), fibroblast growth factor receptor, FGFR1 (Werner *et al.*, 1993), keratinocyte growth factor, KGF (Guo *et al.*, 1993) and LMP-1(Wilson *et al.*, 1990). Thus, the over-expression of several known oncogenes and normal epidermal proteins results in a very similar phenotype in each case, and supports the idea that these oncogenes and growth factors do not act alone in the process of carcinogenesis, but rather are part of a multi-step pathway.

2A.4   PyLMP-1 transgenic lines 5 & 53

As described in section 1F, two lines of PyLMP-1 transgenic mice (lines 5 and 53) were shown to have in common the distinct phenotype of epidermal hyperplasia (Wilson *et al.*, 1990). The

viable line, line 53, has been used for study here, in which the phenotype is apparent soon after birth, most evident at d7, and then indistinguishable from negative littermates by three weeks of age, although often re-appearing in furless areas later in adulthood (noted on the tail as the "ring tail" phenotype).

In wild-type mice the cytokeratin K6 is expressed only in the continually proliferating hair follicles or in areas of proliferation brought on by wound repair. As such it is a useful marker for proliferating epidermis. The discovery of K6 expression in the epidermis of offspring from line 5 as early as at the day 1 old pup stage indicated disruption of normal epidermal homeostasis. Line 5 showed a 10 fold higher steady state level of K6 transcript than negative controls and line 53 a 2.5 fold increase.

The aberrant expression of this hyperproliferative keratin K6 and the hyperplastic thickening of the epidermis is strongly suggestive of aberrant cellular proliferation. The aim of this particular investigation was therefore to determine if increased proliferation was indeed responsible for the PyLMP-1 phenotype, since the phenotype may be a result of one of three events:-
1) Increased cell proliferation
2) Block in apoptosis
3) Disruption/alteration in the differentiation pathway

All of the above could conceivably give rise to an increase in overall cell number and therefore be responsible for the PyLMP-1 phenotype of hyperplasia.

2 B   RESULTS

2B.1   Bromodeoxyuridine (BrdU) labelling of line 53 epidermis.

Nuclear incorporation of Bromodeoxyuridine (BrdU) into the epidermis of mice provides an excellent marker of levels of cellular proliferation. Dividing cells *in vivo* incorporate BrdU supplied by intra-peritoneal injection. This technique was used to compare the proliferating capacity of line 53 transgenic epidermis (dorsal skin) with that of negative control (sibling) mice. As the line 53 transgenic phenotype is most apparent in d7 old pups, mice at this age were used in the study. Transgenic and negative control mice were injected with BrdU and tissues taken one hour later (as described in section 7B.1.7). BrdU uptake is visualised as a brown stained nucleus against a blue counterstain (figure 2.1). Cell counts were made (under x 312.5 power (see section 7B.4.5)),

counting the number of stained cells in an intact stretch of epidermis in one field of vision. 10 fields of view were counted for each tissue section and the mean number calculated (the results are presented in table 2.1). Where possible, counts were taken for epidermis from sections of dorsal skin and tail and the tongue epithelium. Where more than one section was stained and counted for the same tissue sample the counts are included in the same table box.

Tissue sections were stained for BrdU and analysed. Comparing stained sections of PyLMP-1 line 53 epidermis with those of control littermates it is clear that the transgenic mice display greater numbers of positively stained cells than the controls (table 2.1).

The relative numbers of cells showing BrdU uptake is elevated two to threefold in line 53 epidermis compared to negative controls. The mean number of stained cells in the 7d old dorsal skin epidermis group for the transgene positives is 25.53 (with a range of 14.3-31.8) compared to the negative controls 12.52 (range 6.6-17.1) (figure 2.2). Statistical analysis of this result with the two-sample T-test gives a highly significant T value of 6.57 with a P value of 0.0001 and 16 degrees of freedom.

For this and every other statistical analysis used in this thesis I have used a confidence limit of 95%. This means that for a result to be significant the P value must be less than 0.05. As the T value increases and the P value decreases the result becomes more and more significant and less likely to be due to chance. The degrees of freedom is calculated by subtracting one from the total number of counts used to calculate T. Therefore there were 17 counts used to calculate this particular example of a T-test. The statistical package Minitab was used to generate these and all other statistical analyses used in this thesis. (A more detailed explanation of the uses of the Minitab program can be found in section 3B.2.4).

Although relatively few adult samples were taken, the 2 fold difference seen with pup skins remains. In general the adult skins show much reduced proliferation (as would be expected) compared to 7d old pup skin. Nevertheless, the mean for the transgene positives is 4.24 compared to 2.1 for the negative control littermates (figure 2.2 and table 2.1). Using the two-sample T-test the difference between these two scores proved to be significant with a T value of 4.44 and P value of 0.0005 using 15 degrees of freedom. Therefore, this result is highly significant.

Therefore line 53 epidermis (dorsal skin) reveals 2x elevation in cellular proliferation compared to controls in both pup and adult dorsal skins.

Fig 2.1:-
Primary antibody was a monoclonal anti-BrdU (Sigma) Ab. Detection was via the Vectastain elite kit which uses an anti-mouse biotinylated IgG Ab, followed by an avidin/biotinylated horseradish peroxidase complex step. Final detection is with 3,3'-diaminobenzidine (DAB-Sigma) to reveal the brown stained nuclei. Counterstain is with haematoxylin.

Fig 2.3:-
Primary antibody was a rabbit polyclonal anti-human Ki67 (Dako). Secondary antibody was goat anti-rabbit IgG-FITC conjugated (Sigma).

Fig 2.4:-
Primary antibody was a rabbit polyclonal anti-loricrin Ab. Secondary antibody was goat anti-rabbit IgG-FITC conjugated (Sigma).

Fig 2.5:-
Primary antibody was a rabbit polyclonal anti-LMP-1 peptide Ab (gift from B.Sugden).
Secondary antibody was goat anti-rabbit IgG-FITC conjugated (Sigma).

	LMP-1 +ve	LMP-1 -ve
Fig 2.1 **BrDu**		
Fig 2.3 **Ki67**		
Fig 2.4 **Loricrin**		
Fig 2.5 **LMP-1**		
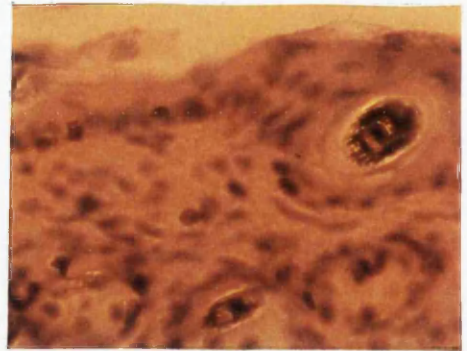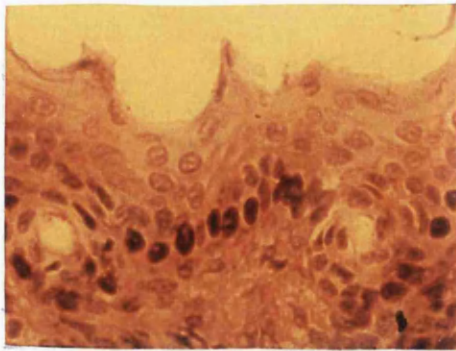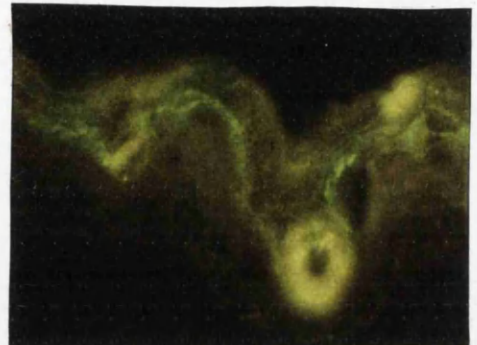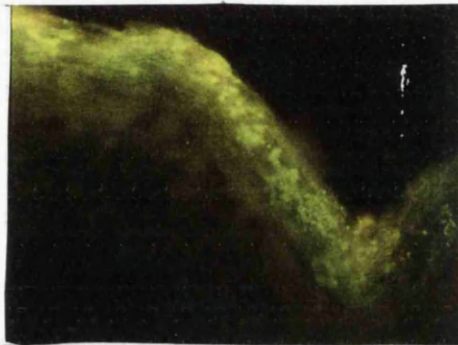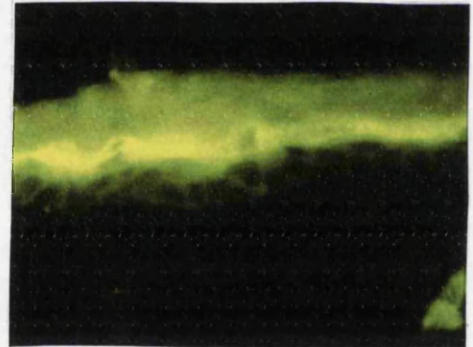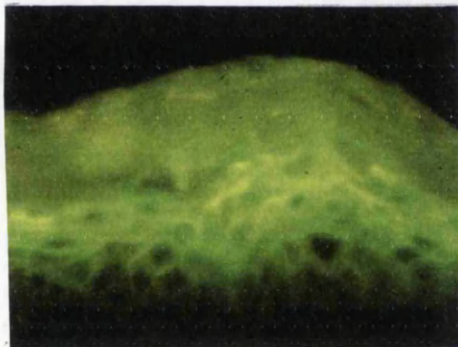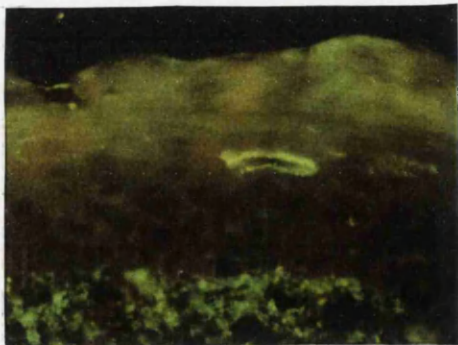
Figures 2.1, 2.3, 2.4, and 2.5:- Comparison of LMP-1 positive dorsal epidermis with negative control littermates. Detection details are given in sections 7B.4.5 and 7B.4.6

Table 2.1:

Table indicating the mice used to accumulate the BrdU totals and mean values for PyLMP-1 (line 53) transgenic positive mice and negative controls. Where counts were not possible due to bad sectioning or poor staining, this is indicated as Not Done (ND). 769 I.D. mice result from a cross breeding with Ras transgenic mice but are NOT Ras positive.

Counts= mean value from positive BrdU cells in 10 fields of view.

Mouse I.D.	LMP-1	Age	BrdU SKIN count(s)	BrdU TONGUE count(s)	BrdU TAIL count(s)
769.295	+	d7	28.4, 29.5, 26.4	29, 31, 34, 37	ND
769.361	+	d7	27.1, 25.1, 31.8	2 6	ND
53F.175	+	d7	24.6	21.5	ND
53F.177	+	d7	14.3	26.7	ND
53F.178	+	d7	19.7	26.8	36
53F.165	+	d7	ND	24	68
53F.166	+	d7	ND	ND	42.2
53F.168	+	d7	ND	ND	7 8

Mean= 25.53 SE= 1.6   Mean= 28.70 SE= 1.5   Mean= 52.00 SE= 8.8

Mouse I.D.	LMP-1	Age	BrdU SKIN count(s)	BrdU TONGUE count(s)	BrdU TAIL count(s)
769.331	-	d7	16.4, 17.1, 14.9	27, 22.5, 10.6	ND
769.335	-	d7	7.7, 9.6, 10	2.3	ND
769.356	-	d7	15.17	ND	ND
769.163	-	d7	10.2	15.6	ND
769.171	-	d7	9.3	ND	ND
769.173	-	d7	6.6	10	16.2
769.161	-	d7	ND	ND	13.9
769.170	-	d7	ND	ND	23.7

Mean= 12.52 SE= 1.1   Mean= 19.39 SE= 2.8   Mean= 17.50 SE= 2.1

Mouse I.D.	LMP-1	Age	BrdU SKIN count(s)	
53.716	+	Adult	2.9, 5.0, 2.9	Mean= 4.24
53.718	+	Adult	5.6, 3.9, 3.2	SE= 0.38
53.1039	+	Adult	6.0, 4.1, 4.6	
53.715	-	Adult	1.5, 3.0, 1.5	Mean= 2.1
53.719	-	Adult	3.1, 3.2, 2.1	SE= 0.30
53.1040	-	Adult	1.0, 1.0, 2.5	

57

Comparison of mean BrdU scores for line 53 PyLMP-1 positive dorsal skin versus negative controls
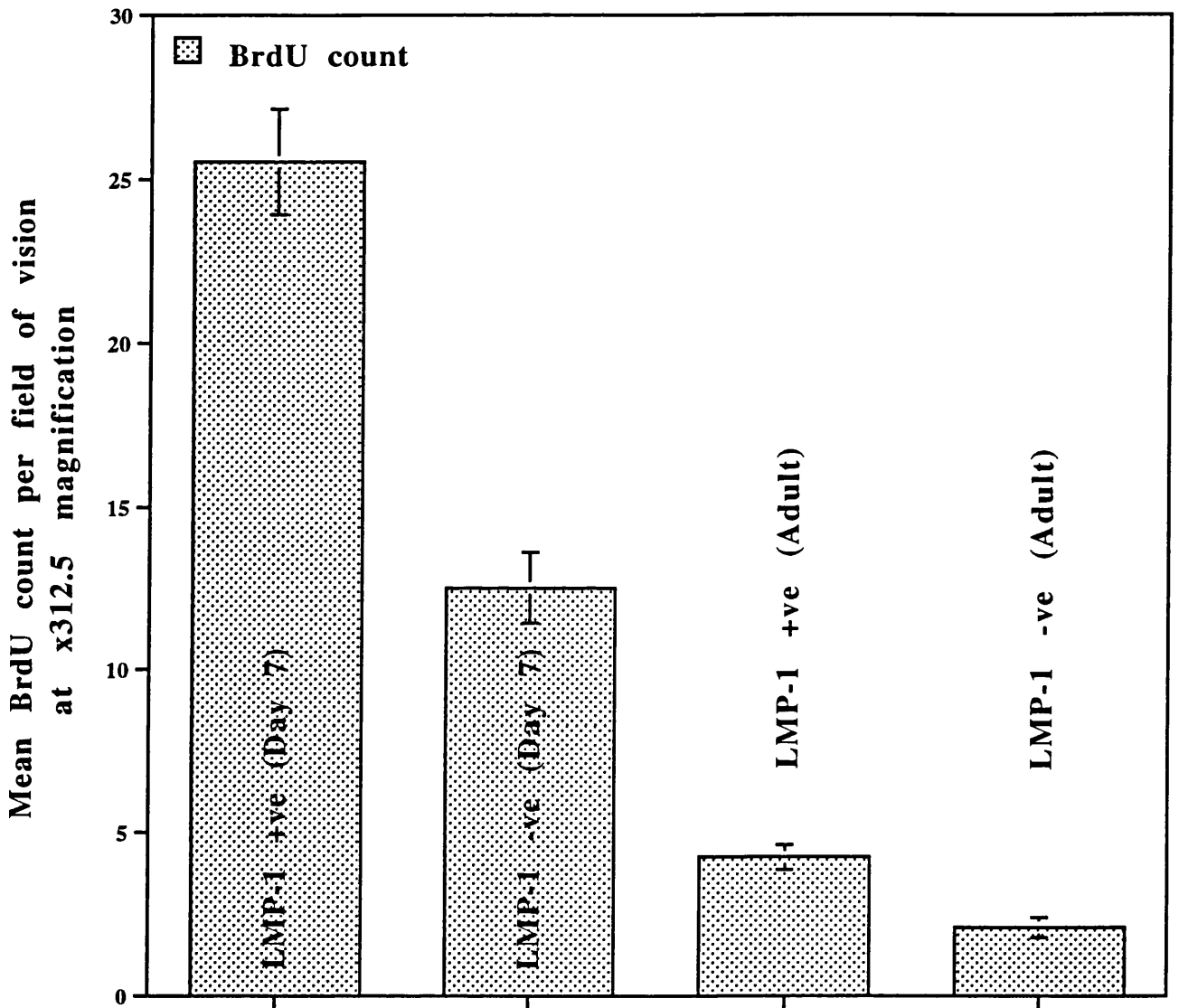
Figure 2.2

7μm sections of BrdU injected dorsal skin were stained with an anti-BrdU antibody (section 7B.4.5), and positive cells (brown) in the epidermis were counted per field of vision at x312.5 magnification. This figure shows the mean score for each transgenic group studied, at both day 7 and adult ages. Each mean was derived from at least 5 different sections from at least 3 different mice. Each section was counted for 10 fields of vision and then averaged (mean). Each average was then totalled to calculate the overall mean score for each group. Error bars represent the standard error of the mean.

Sections were also cut from tail and tongue and stained cell counts taken from epidermis and epithelium (respectively). Again the PyLMP-1 line 53 mice had higher BrdU labelling scores than their negative control littermates. The mean score for d7 LMP-1 positive tail epidermis was 52.00 compared to 17.50 for d7 controls. The mean score for d7 LMP-1 positive tongue epithelium was 28.70 compared to 19.39 for d7 controls. Both of these differences proved to be significant by two sample T-test analysis despite the sample size being small in these cases. The T-test result for tail was T=3.82 with a P=0.019 and 4 degrees of freedom. The T-test result for tongue was T=2.98 with P=0.015 and 9 degrees of freedom.

It is notable that the tail in the d7 pups shows the largest increase (3x) when comparing transgene positives and negative control littermates. Indeed it is in the tail epidermis that the phenotype is most persistent in the adult ("ringtail"). However, this proved to be a difficult tissue to analyse, the bones making it problematic to obtain good perpendicular sections to obtain a suitable cross-section of epidermis and as such many stained sections were not readable. Indeed, the counts which were obtained came from tail sections cut by trained technicians at the pathology department of the Glasgow University Vet. School.

Nevertheless, all tissues analysed show a consistent elevation of BrdU stained cells in the epithelium reflecting cellular proliferation in the LMP-1 positive mice compared to controls.

It was also noted that there were some suprabasal stained nuclei in the PyLMP-1 line 53 transgene positive dorsal skin epidermis although accurate analysis was not carried out. No suprabasal staining was noted in the negative control littermates as expected.

2B.2 Immunofluorescent examination of epidermal proliferation.

The BrdU incorporation data indicate that there is increased proliferation in the LMP-1 positive epidermis which is probably responsible for the hyperplastic thickening of line 53 epidermis. To investigate this hypothesis further, immunostaining for Ki67, a marker of proliferative potential (Brown and Gatter, 1990), was carried out.

7µm sections of d7 line 53 transgenic and d7 negative control dorsal skin were cut and immunostained for Ki67 expression (as described in 7B.4.6). The proportion of stained (fluorescing) cells in the PyLMP-1 line 53 epidermal sections was much greater than the proportion of stained cells seen in control

sections (see figure 2.3). This is further evidence of increased proliferation in line 53.

Furthermore, the pattern of staining differed between LMP-1 positive and negative sections. The positive cells in the d7 negative control section were found in the basal layer as expected. However, as well as having more positive cells in the basal layer, the LMP-1 transgenic mice also displayed Ki67 staining in the suprabasal layers. Thus LMP-1 is also causing aberrant proliferation in the suprabasal layers (which should be terminally differentiating).

2B.3   Immunofluorescent examination of epidermal differentiation markers.

Several pieces of evidence indicate an increase in the proliferation rate is contributing to the line 53 phenotype. In order to address if the pattern of epidermal differentiation has been effected in this immunostaining for various protein markers of differentiation or normal epidermal homeostasis was carried out.

Antibodies to desmoplakin and desmocollin were used to stain epidermal sections. These were then sent to Prof. D.Garrod at the University of Manchester for expert examination. In Prof. Garrod's expert opinion, there was no difference in the expression or levels of expression of these two proteins of desmosomal complexes between line 53 positives and negative controls.

Antibodies against loricrin were used to stain sections of d7 line 53 epidermis. As described earlier, loricrin is the last protein to be expressed in the differentiating epidermis and staining occurs in the upper terminally differentiated layer. As line 53 epidermis is considerably thicker than control epidermis (due to the increase in proliferation) it initially appears as though there is an observable difference in staining pattern for loricrin. However, Prof. Garrod explained that in both cases loricrin was expressed in the upper suprabasal layers and that there was no difference in the immunostaining for loricrin (figure 2.4). At this level of detection the staining pattern was identical in both d7 line 53 epidermis and negative control.

The first markers of terminal differentiation expressed in the suprabasal layers, K1 and K10, have previously been examined (Wilson et al., 1990). Again no differences were observed between PyLMP-1 positives and negative control littermates.

Therefore it would appear from the markers of differentiation studied that the differentiation process in the epidermis of PyLMP-1 line 53 mice progresses unimpeded.

2B.4   Immunostaining   for   LMP-1.

Although LMP-1 has been shown to be expressed at both the RNA and protein levels in PyLMP-1 line 53 transgenic mouse epidermis, the exact cellular layer in which LMP-1 is expressed has not been established. The Polyoma virus promoter and enhancer were linked to the LMP-1 gene in order direct expression of the transgene to the epidermis. Unlike the keratin promoters K1, K10 and K14 used for other transgenic studies (Dominey *et al.*, 1993; Cui *et al.*, 1996; and Vassar *et al.*, 1992) where expression is directed to a known discrete epidermal compartment, the specificity of the Polyoma virus promoter activity has not been analysed. In order to establish the location of the LMP-1 protein within the various epidermal layers immunofluorescent staining for LMP-1 using the CS1-4 monoclonal Ab was attempted on d7 line 53 epidermis.

This revealed LMP-1 expression in an unexpected staining pattern in the upper suprabasal layers of the epidermis (figure 2.5). The staining appeared at regular intervals along the suprabasal layer but was not consistently detected throughout the layer. Predictably the positive cells showed membrane staining. It is possible that the inconsistent staining observed is due to the low levels of protein expressed or perhaps the staining procedure requires further optimisation. No staining was noted in any of the negative control sections.

It would therefore appear that LMP-1 protein is expressed in the upper suprabasal layers of the epidermis of the PyLMP-1 line 53 transgenic mice. It is not clear if lower levels of protein are expressed in the basal or other suprabasal layers.

2C.   Discussion of chapter 2.

The wrinkly, flaky, hyperplastic epidermis seen in line 53 was strongly suggestive of a disruption of the normal epidermal homeostasis. The aberrant expression of the hyperproliferative keratin K6 described previously (Wilson *et al.*, 1990) and the suprabasal layer staining of Ki67, a cell cycle associated molecule and also an excellent marker of proliferation, suggests that this disruption might be caused by an increase in cellular proliferation of the epidermis.

Direct evidence that LMP-1 is increasing cellular proliferation in this tissue comes from the BrdU staining results. It was immediately apparent just from looking at the stained line 53 sections that there were more stained nuclei in the LMP-1 positive sections than there were in the negative control sections.

When accurate counts were made, a 2 to 3-fold increase was found in BrdU labelling in the PyLMP-1 line 53 compared to negative controls. This difference was shown to be statistically significant by the student's two sample T-test. These results demonstrate that LMP-1 induces an increase in the proliferation of epidermal cells in these PyLMP-1 transgenic mice. Since line 53 expressed lower levels of LMP-1 than the extinct line 5 as well as displaying a less severe phenotype, it is possible that line 5 had greater increases in proliferative rates. This idea would be supported from the expression of K6 (10x increase in line 5, and 2.5x increase in line 53).

The lack of observable alterations to any of the differentiation markers studied suggests that the differentiation process has not been impeded. There were no gross alterations in desmosome staining pattern (desmoplakin and desmocollin). The staining pattern of K1 and K10 was similar to that of control mouse epidermis suggesting that the first stages of terminal differentiation proceed in the LMP-1 positive epidermis. The later stages of terminal differentiation also appear to be continuing since loricrin expression in line 53 epidermis was similar to that found in control epidermis.

Taken together the data indicate that the phenotype observed in line 53 PyLMP-1 transgenic mice is due to an increase in proliferation and not due to inhibition of differentiation. The visibly disorganised epidermis (seen in line 5) might be expected with increased proliferation without a concomitant increase in differentiation. Increased cellular proliferation with increased differentiation has been proposed (Cui et al., 1995) for K10-TGFβ1 transgenic mice where the net result was zero observable phenotype.

Dawson and colleagues (1990) suggested that LMP-1 inhibited differentiation as determined from assays in culture using a squamous cell carcinoma cell line. This differs from this study in that our work was conducted in vivo. More recently, Nicholson and co-workers (1997) furthered the work of Dawson et al., (1990). From these studies, no inhibition of differentiation was observed. Furthermore, suppression of LMP-1 expression resulted in an inhibition of proliferation (Mattia et al., 1997). As reported here, LMP-1 expression results in an increase in the expression of Ki67, a finding also suggested by correlation for the disease state of NPC.

The level of apoptosis in the epidermis of the PyLMP-1 mice was not investigated and it is possible that a block in apoptosis may also be partly responsible for the line 53 phenotype. LMP-1 has been shown to induce CD40 and A20 (Dawson et al., 1990; Miller et al., 1995) in epithelial cells both of which inhibit

programmed cell death (although CD40 also promotes proliferation). Furthermore, CD40 activation also induces A20 (Sarma *et al.,* 1995) potentially amplifying the anti-apoptotic signal. Both CD40 and LMP-1 operate through the TRAF family of signalling proteins implying that the pathways overlap. Although LMP-1 is capable of inducing A20 through its NF-κB activating ability, the use of the TRAF family may also allow LMP-1 to usurp the TNF/CD40 pathway causing constitutive cell growth and inhibition of apoptosis. Investigations into this possibility remain to be addressed.

The detectable expression of LMP-1 in the upper suprabasal layers by fluorescent staining is difficult to correlate with the impact of the phenotype in the lower layers and may reflect a detection problem. As BrdU and Ki67 staining was observed in the basal layer, LMP-1 expression in the basal layer might also be expected. The immunohistochemical detection of LMP-1 therefore requires verifying.

CHAPTER 3:-   Chemical carcinogenesis.

In this chapter the PyLMP-1 line 53 mice are subjected to protocols of chemical carcinogen treatment to establish what role LMP-1 may be playing in the mouse model of chemically induced carcinogenesis. The introductory section will therefore cover the history of this model and the known changes in gene expression which occur during the chemical treatment. This will lead into use of transgenic mouse models in the context of chemical carcinogen treatment to establish the exact role of each transgene, and in particular PyLMP-1, in the process of multi-stage skin carcinogenesis.

3A.1   Mouse skin carcinogenesis.

The mouse model of multi-step carcinogenesis has taken researchers a long way towards understanding the molecular events which underlie the transition from a normal cell to a transformed cell and neoplasia. As previously described, several oncogenes and growth factors have been expressed in the epidermis of transgenic mice to investigate the possibility that activation of specific genes may correlate with particular biological events. These transgenic mice have been shown to produce phenotypes reflecting the early stages of carcinogenesis, but in every case required further events to progress to a fully transformed phenotype. Carcinogenesis is therefore a multi-stage process, but the molecular analysis of tumorigenesis is often hampered by the unavailability of tissue specimens from the multiple stages. For this reason the study of mouse skin carcinogenesis using chemical carcinogens has been invaluable and shown that the process can be broken down into the stages of initiation, promotion and malignant conversion (fig 3.1).

Figure 3.1 :- **Summary of events during mouse skin chemical carcinogenesis treatment**

DMBA $\longrightarrow$ TPA $\longrightarrow$ Papilloma $\longrightarrow$ Squamous cell $\longrightarrow$ Spindle cell
(Initiator)   (Promoter)   (Conversion)   Carcinoma   Carcinoma

DMBA (Initiator)
- single application
- causes activating mutation in codon 61 of Ha-*ras* gene
- cells are 'primed'

TPA (Promoter)
- multiple applications
- persistant hyperplasia
- TGFβ1 induction
- TGFβ2 +β3 reduction
- activation of EGFR + erb2
- activation of PKC

Papilloma (Conversion)
- clonal benign tumours
- trisomy of chrm 6 + 7
- duplication of the mutant Ha-*ras* gene
- elevated TGFα, EGFR, IGF, IGFR, RXR levels
- Decrease in RARα+γ

- rare event, **5-10% only**
-subset of papillomas with increased risk of conversion

Squamous cell Carcinoma
- loss of TGFβ
- abberrant K13 expression
- maintenance of elevated TGFα, EGFR, IGF, IGFR
- altered patterns of cdk's
- increased ST-1
- loss of remaining WT Ha-*ras* allele
- LOH on chrm 11
- p53 gene mutated

Spindle cell Carcinoma
- very rare (0.07/mouse)
- more aggressive tumour
- amplification of mutant Ha-*ras* gene
- further LOH on chrm 4, 6, and 7
- altered expression pattern of ST-1
- loss of structure and cell adhesion molecules

65

3A.1.1   Tumour initiation.

Initiation occurs as a result of a single topical application of a mutagen to the dorsal skin of a susceptible strain of mouse. Initiated cells can remain dormant for a considerable period of time until activated by the promotional stage (Reviewed in Brown and Balmain, 1995). The initiated cell is phenotypically indistinct from normal cells but because it can remain initiated for such a period of time it is believed to belong to one of the population of long lived stem cells. At the molecular level, mutation of the Ha-*ras* proto-oncogene has been identified as a common initiating event in skin tumours produced by some carcinogens (Balmain and Brown, 1988) . Over 90% of mouse skin tumours initiated with Dimethylbenzanthracene (DMBA) display a transversion mutation (A-T) at codon 61 of the Ha-*ras* gene (Sukumar, 1989 & 1990; Brown *et al.*, 1990; Kemp *et al.*, 1994) with a smaller percentage also showing mutation in codon 12. Along with the Ha-*ras* transgenic mouse studies this is compelling evidence for the activation of the *ras* oncogene as a principle event in initiation. TGF-α transgenic mice can develop carcinomas upon tumour promotion and without prior chemical initiation (Wang *et al.*, 1994) indicating that TGFα does indeed operate through the Ras pathway.

3A.1.2   Tumour promotion:- immediate effects

The promotion step consists of repeated treatment of initiated mouse skin with a tumour promoter, a chemical carcinogen which is not necessarily a mutagen, and results in persistent general hyperplasia which is seen within 24h, is maximal at 48-72hrs, and can last for up one week (Esherick *et al.*, 1993). The most potent promoters of mouse skin are the phorbol esters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA).

TPA treatment of adult mouse skin produces a rapid induction of TGFβ1 mRNA in the suprabasal keratinocytes as early as 2-3Hrs post TPA (Akhurst *et al.*, 1988) with a concomitant increase in TGFβ1 protein levels, also localised to the suprabasal keratinocyes (Fowlis *et al.*, 1992). TPA treatment however causes a decrease in the levels of both TGFβ2 and TGFβ3 by 24hrs and 6hrs post TPA respectively (Esherick *et al.*, 1993). Thus, although TGFβ1 is induced in TPA treated mouse skin, the concomitant decrease in TGFβ2 and β3 may remove the overall growth

inhibitory effect of the TGFβ's and give rise instead to the observed hyperproliferative response.

TPA promotion of mouse epidermis has been shown to activate the epidermal growth factor receptor (EGFR) a member of the *erb* B family (Rho *et al.*, 1994; Xian *et al.*, 1995) and also another member of that family, *erb* B2 (Xian *et al.*, 1997). As a probable consequence of the elevated levels of activated EGFR and *erb* B2, c-*src* kinase activity was also observed to be elevated.

TPA is known to bind directly to protein kinase C (PKC) and cause its activation (Nishizuka 1984 & 1986). This activation of PKC by TPA must also be vitally important as it involves activation of such proteins as raf and mitogen activated protein kinase kinase (MAPKK) (Marquardt *et al.*, 1994). Since ras is already activated by the application of DMBA in these chemical carcinogen studies, and ras binds to raf, targeting raf to the membrane and activating it, this may amplify the PKC pathway further.

TPA appears to increase cross boundary communication between the dermis and epidermis (Kam and Pitts, 1988) contrary to *in vitro* reports of TPA blocking cell:cell communication. Therefore TPA is not promoting carcinogenesis by disrupting normal cell:cell interchange.

In cell culture experiments TPA treatment of keratinocytes has been shown to block the calcium-mediated induction of K1 and K10 (Dlugosz and Yuspa, 1993); induce both TGFβ1 (Fowlis *et al.*, 1992) and TGFα mRNA (Imamoto *et al.*, 1991); and induce sequential transcription of the *fos* and *myc* oncogenes (Balmain and Brown, 1988).

Thus, TPA promotion of susceptible mouse skin results in a plethora of events and the induction of the visible hyperplastic response. The events which occur after promotion are as yet unclear. One particular facet of phorbol ester treatment may be wholly responsible for papilloma formation, such as the induction of hyperplasia, or many of the resultant events may be required in tandem. Certainly the activation of PKC and the subsequent signal transduction pathways which will be stimulated may be important.

3A.1.3   Tumour promotion:- development of papillomas

Repeated applications of TPA to DMBA initiated mouse skin results in the formation of multiple discrete benign tumours called papillomas (figure 3.2). Papillomas are thought to be clonal, arising from a single initiated cell which has increased its rate of proliferation with a concomitant decrease in the rate of differentiation (Brown and Balmain, 1995).

A number of genetic alterations have been detected during the promotion stage in mouse skin tumours, including the frequent trisomy of chromosomes 6 and 7 (summarised in figure 3.1). The Ha-*ras* gene is located on chromosome 7 therefore trisomy of chromosome 7 results in the duplication of the mutant *ras* allele and suggests that increased activated *ras* might be further required (Bremner and Balmain, 1990). Duplication of the chromosome carrying the normal Ha-*ras* allele has never been detected, nor trisomy of chromosome 7 in tumours where Ha-*ras* is not mutated.

The benign papillomas formed by DMBA and TPA treatment display numerous alterations compared to normal mouse epidermis. Levels of both TGFα and its receptor EGFR were elevated in 100% and 90% respectively of papillomas tested (Rho *et al.*, 1994) at both mRNA and protein levels. Therefore the initial elevation of TGFα following TPA administration is maintained at high levels suggesting that this is an important event in the formation of papillomas.

Both insulin-like growth factor (IGF-1) and IGF-1 receptor (IGF-1R) were shown to be elevated in papillomas induced by TPA treatment of initiated mouse epidermis (Rho *et al.*, 1996). TPA treatment of normal epidermis failed to induce any increase in levels of either protein suggesting that these changes occur upon papilloma formation and not as a direct result of TPA induced proliferation.

Examination of the retinoic acid receptor (RAR) content of papillomas compared to normal epidermis showed a decrease in levels in the benign tumours (Darwiche *et al.*, 1995) whilst levels of another nuclear receptor, RXR, increased in the benign tumours. Close examination of the RAR transcripts in papillomas from TPA promoted skin showed a slight decrease in RARα levels with a complete absence of RARγ (Darwiche *et al.*, 1996). Since retinoids are essential for normal epidermal differentiation, reduction of their receptor levels may contribute to carcinogenesis. Transfection of primary keratinocytes with an activated Ha-*ras* construct also reduced RAR levels suggesting that this decrease might occur at the stage of initiation and activation of *ras* (Darwiche *et al.*, 1996).

Tumour promotion can be further divided depending on the type of promoter used. This led to the clasification of stage 1 promoters (also called full or conversion promoters) and stage 2 promoters (propagation promoters). TPA and wounding belong to stage 1 promoters, whereas chemicals such as mezerein or 12-retinoyl 13-acetate[i] (RPA) function as stage 2 promoters. Stage 2 promoters have only moderate or weak promoting activities but

can give rise to tumours following short treatment with a stage 1 promoter (Hennings and Boutwell, 1970).

3A.1.4   Tumour conversion:- papilloma to squamous cell carcinoma

The malignant conversion of a benign papilloma to a carcinoma is a relatively rare event with around 5-10% of papillomas converting to carcinomas. This conversion, although spontaneous, is not thought to be random as not all papillomas have an equal chance of conversion. Subsets of papillomas have been identified which display an increased risk of progression and these papillomas have differences in their TGF-β, $\alpha6\beta4$ integrin, K13 and RARα expression levels (summarised in figure 3.1) (Glick et al., 1993; Tennenbaum et al., 1993; Nischt et al., 1988; and Darwiche et al., 1996).

The majority of papillomas forming from TPA treatment of initiated mouse epidermis never progress to squamous cell carcinoma and these are classed as low risk. In low risk papillomas, 90% of the proliferating cells are confined to the basal cell compartment and the lesions express TGFβ1, whereas in most of the high risk papillomas TGFβ1 is downregulated in the lesions as soon as they arise with up to 40% of the proliferating cells in the suprabasal compartment (Fowlis et al., 1992; Glick et al., 1993). Squamous cell carcinomas are also devoid of TGFβ1 suggesting that they arise from the TGFβ1 deficient high risk papillomas (Glick et al., 1993).

High risk papillomas displayed basal and suprabasal $\alpha6\beta4$ expression which coincided with an expansion of the proliferating compartment into the suprabasal layer, whereas low risk papillomas demonstrated only basal $\alpha6\beta4$ expression and basal layer proliferation (Tennenbaum et al., 1993).

Keratin 13 (K13) is normally associated with terminal differentiation of internal stratified epithelia and is virtually absent from the entire body epidermis. However K13 has been shown to be present in a subset of papillomas arising from chemical carcinogen treatment (DMBA + TPA) and these papillomas represent a high risk group for malignant conversion (Nischt et al., 1988).

Papillomas have an overall decrease in levels of RAR's. However low risk papillomas have only a slight decrease in RARα levels whereas high risk papillomas have a much greater decrease

in RARα levels (Darwiche *et al.*, 1995). Therefore it is likely to be the RARα isoform that is most important in progression.

The squamous cell carcinomas that do arise demonstrate further alterations. The loss of TGFβ from high risk papillomas is maintained in carcinomas (Glick *et al.*, 1993) as is the aberrant expression of K13 (Nischt *et al.*, 1988). The elevated IGF-1, IGF-1R, EGFR, and TGFα levels seen in papillomas are maintained in the carcinoma stage (Rho *et al.*, 1994 and 1996). Furthermore, there are altered patterns of G_1 cyclins and cyclin-dependent kinases(cdk's) (Zhang *et al.*, 1997).

Stromelysin-1(ST-1) is a matrix-degrading metalloproteinase, a family of proteins thought to contribute to the process of invasion and metastasis via their ability to degrade the basement membrane and extracellular matrix barriers thus allowing tumour cells access to the lymphatic system. The levels of ST-1 were indeed increased from 8% in benign papillomas to 71% in malignant squamous cell carcinomas (Matrisian and Bowden, 1990). ST-1 expression was shown to be localised to the stromal tissue surrounding the tumour cells in both papilloma and carcinoma (Wright *et al.*, 1994).

Frequent loss of the remaining wild-type *ras* allele is often noted (Bremner *et al.*, 1990). In addition, loss of heterozygosity (LOH) on chromosome 11 (which harbours the p53 gene in mouse) is detected in 30% of cases. In every case where LOH on chromosome 11 was noted, the remaining p53 gene had suffered point mutations (Burns *et al.*, 1991). Most of these mutations resulted in loss of function of the gene. By comparison p53 heterozygous(+/-) and homozygous(-/-) knockout mice (Donehower *et al.*, 1992) were subjected to initiation with DMBA followed by 15 weeks of promotion with TPA (Kemp *et al.*, 1993a). Heterozygous knockout mice had equal numbers of papillomas compared to control mice, but p53 null mice strangely had fewer papillomas. However, the heterozygous knockout mice demonstrated a 3-fold increase in the progression frequency of papillomas to carcinomas, and loss of the remaining WT p53 allele was noted. The p53 null mice had a dramatically accelerated progression frequency despite having fewer papillomas to begin with. This clearly indicates that loss of one or both copies of functional p53 is associated with conversion of papillomas to carcinomas. With the absence of WT Ha-*ras* , the presence of two copies of activated Ha-*ras* , plus inactivated p53, this stage of the carcinogenic process produces highly malignant squamous cell carcinomas.

3A.1.5 Tumour conversion:- squamous cell carcinoma to spindle cell carcinoma

A further conversion step occurs with the progression from a squamous cell carcinoma to the more aggressive spindle cell tumour in which all organisation and markers of epithelial differentiation are completely lost. This conversion step is very rare with only 0.07 spindle cell carcinomas formed per mouse (of the SENCAR strain) following DMBA and TPA treatment (Klein-Szanto et al., 1989).

This conversion is associated with a further increase in the ratio of mutant to WT *ras* genes, caused by an amplification of the mutant (active) Ha-*ras* gene (Buchmann et al., 1991). Thus, from initiation right through to the most aggressive form of carcinoma, alterations in the Ha-*ras* play significant roles in this multistage progress towards carcinoma.

Additional genetic changes include LOH on chromosomes 4, 6, and 7 in areas thought to harbour tumour suppresser genes (Kemp et al., 1993b). It would appear therefore that in highly malignant spindle cell carcinomas, more and more homeostasis controlling mechanisms are found to be failing.

The levels of ST-1 are maintained at an elevated level in spindle cell carcinomas, but in contrast to the squamous cell carcinoma stage ST-1 is now expressed in the tumour cells as well as in the adjacent stroma (Wright et al., 1994). This suggests that the change in expression from surrounding stroma to the tumour itself is associated with the conversion of squamous to spindle carcinoma. The high levels of ST-1 expressed in both the tumour and surrounding tissue may allow these spindle cell tumours to invade and metastasise.

Spindle cell carcinomas have also been shown to lack expression of the cell adhesion molecules E- and P-cadherin and the desmosomal component desmoplakin (Stoler et al., 1993). The keratin filament network is also often completely absent in many spindle cell carcinomas and where it is still present, is very poorly organised. Expression of the integrin α6β4 is completely lost in spindle cell carcinomas (Gomez et al., 1992). The loss of structure and the loss of expression of these epidermal differentiation markers correlates with the squamous to spindle transition.

The ultimate stage of carcinogenesis is the ability of the tumour to invade the surrounding tissue and metastasise to distant sites. The increased expression of ST-1 may aid breakdown of membrane barriers and the loss of many adhesion molecules allow the tumour cells to detach from the tumour mass and enter the lymphatic system (reviewed in Portella et al., 1994).

71

3A.1.6 Chemical carcinogen treatment of transgenic mice.

The study of carcinogenesis in the mouse skin using chemicals has revealed a wealth of information concerning the complex stages and genetic events. This has been expanded apon using the same chemical treatment regime in mice which are already transgenic for an activated gene involved in the carcinogenic process, such as activated Ha-*ras* (Bailleul *et al.,* 1990). Treatment of these mice with tumour promoting agents resulted in the progression from papilloma to carcinoma further indicating that activation of *ras* is an important event but that other events are subsequently required for neoplasia. Just as with the Ha-*ras* mice, TGFα transgenic mice (Wang *et al.,* 1994) did not develop carcinoma but carcinomas could be induced following tumour promoter treatment of the mice.

Mice expressing TGFβ1 in the epidermis (described later in chapter 4) have been subjected to chemical carcinogen treatment and shown to be more resistant to the induction of benign papillomas than were controls (Cui *et al.,* 1996). The papillomas forming on the transgenic mice were however much more likely to convert to a malignant phenotype. Thus, chemical carcinogen studies of these transgenics has shown that TGFβ1 shows dual action in that it acts initially as a tumour suppresser but subsequently (later tumour stages) as a tumour enhancer.

Several groups have examined the role of TGFα in skin carcinogenesis using transgenic mice. K14-TGFα transgenic mice developed hyperplasia, hyperkeratosis and papillomas (Vassar and Fuchs, 1991; Vassar *et al.,* 1992) as did K1-TGFα transgenic mice (Dominey *et al.,* 1993; Wang *et al.,* 1994). TGFα transgenic mice have been subjected to chemical carcinogen treatment. The K1-TGFα transgenic mice were administered TPA treatment without prior DMBA initiation, and succumbed to papillomas, suggesting that TGFα overexpression can bypass the need for Ha-*ras* activation and thus act as an initiator of carcinogenesis (Wang *et al.,* 1994). Metallothionein promoter driven TGFα transgenic mice were initiated with a single dose of DMBA without subsequent promotion and virtually all of the TGFα transgenic mice succumbed to papillomas with some infrequent carcinomas (Jhappan *et al.,* 1994). Thus, chemical carcinogenesis studies of TGFα transgenic mice led to the discovery that enhanced TGFα expression can act in both the initiation and promotion of skin tumorigenesis.

Other transgenic mice to be challenged with chemical carcinogens include K1-v-*fos* mice. These mice developed

epidermal hyperplasia but only as a result of a promotion stimulus from wounding (Greenhalgh *et al.*, 1993). These mice were treated with TPA but developed papillomas only after a long latency (20-30 weeks of promotion) and in relatively few numbers per animal (Greenhalgh *et al.*, 1995). Furthermore these lesions were TPA independent as they persisted and converted to malignancy after TPA withdrawal. The tumours also harboured Ha-*ras* mutations in codons 12 and 61. Thus, chemical carcinogen studies of K1-v-*fos* transgenic mice revealed that v-fos may induce a sensitivity to TPA promotion, but that additional genetic events, such as mutation of Ha-*ras*, are required for tumorigenesis to progress.

3A.1.7   Rationale for chemical carcinogen studies in PyLMP-1 line 53 transgenic mice.

Since LMP-1 has been likened to mutant Ha-*ras* in tissue culture experiments (Dawson *et al.*, 1990) the question arises, can LMP-1 replace the function of mutant Ha-*ras* in carcinoma initiation and progression? DMBA can activate Ha-*ras* so can the expression of LMP-1 replace the function of DMBA? This question could be addressed by treating PyLMP-1 transgenic mice with TPA promotion only (See regime 2), and if LMP-1 could indeed replace the need for activated Ha-*ras*, it would be expected that papillomas and carcinomas would arise in the classical DMBA + TPA fashion.

In chapter 2 LMP-1 was shown to induce proliferation, so could LMP-1 expression in the PyLMP-1 transgenic mice substitute for the proliferative effects induced by TPA treatment? This question can be addressed by initiating the PyLMP-1 transgenic mouse skin with a singe application of DMBA (See regime 3). If inducing proliferation was the most important attribute of all the events induced by TPA in the promotional effect of the chemical, and if the level of proliferation induced by LMP-1 expression was appropriate, then it would be predicted that the LMP-1 transgenic mice would develop papillomas and subsequent carcinomas following DMBA initiation alone. Alternatively, LMP-1 may be able to act as a secondary (second stage) promoter following limited TPA treatment (See regime 4).

Administration of the PyLMP-1 mice with the standard DMBA + TPA protocol of treatment (Regime 1) could provide more information as to the role of LMP-1 in later stages of carcinogenesis, such as, does LMP-1 act to increase the conversion rate of papilloma to carcinoma and malignancy?

The use of chemical carcinogen studies on the PyLMP-1 mice is of particular relevance to the disease state of NPC. As

previously mentioned in section 1C.1.2, the salting process in areas of high incidence of NPC, gives rise to the production of volatile nitrosamines which have been shown to have tumour promoting effects. Thus it may be the combination of the presence of EBV and these tumour promoters that initiates NPC. Treating the PyLMP-1 mice with tumour promoting agents will determine if this hypothesis has any grounding, at least with respect to LMP-1 expression.

One single application of DMBA followed by twice weekly (for 20 weeks) applications of TPA leads to papilloma formation, 5-10% of which convert to carcinomas. This standard protocol was used as well as variant regimes to attempt to answer pertinent questions about the possible role for LMP-1 in carcinogenesis.

3B RESULTS

3B.1 Intercrossing to a carcinogen susceptible strain.

The PyLMP-1 mice were originally bred into a C57B1/6 strain background. It has been well documented that there are marked strain differences in response to epidermal tumour promotion using chemical carcinogens (reviewed in DiGiovanni, 1989 and 1991), most of this information having been accumulated from studying the effects of TPA. This led to an order of susceptibility to lesion formation being identified such that SENCAR> FVB> DBA/2> CD-1> C57B1/6 where C57B1/6 is the most resistant strain studied. Furthermore, C57B1/6 are also resistant to tumour promotion by full thickness skin wounding following initiation with DMBA (DiGiovanni *et al.,* 1993) a method shown to be previously tumour promoting in both K10-*ras* and K1-TGF-α transgenic mice (Bailleul *et al.,* 1990; Wang *et al.,* 1994). Therefore, the C57B1/6 mice are resistant to skin carcinogenesis involving more than one tumour promoting method. It was therefore necessary to cross the PyLMP-1 mice into a strain background which would respond to the protocol of DMBA and TPA treatment.

Initially the PyLMP-1 line 53 was intercrossed with stock DBA/2 mice but the F1 offspring proved to be poor breeders and too few mice were being born for the experiment to proceed with sufficient speed. The mice in the 50:50 C57B1/6:DBA/2 background had exactly the same phenotype as the 100% C57B1/6 mice and did not appear to suffer any greater phenotypic problems.

At this time the FVB strain of mice became available to the lab (both through importation and subsequently through the company Harlan Olac). FVB mice were found to breed well and also show greater susceptibility to chemical carcinogens than the DBA/2 strain. Therefore line 53 PyLMP-1 mice were intercrossed with mice of the FVB strain with greater success. Notably, again there were no differences in the LMP-1 induced phenotype between the strains C57Bl/6 and FVB. F1 positive offspring (backcross 1) from the original line 53 x FVB cross were identified by day 7 phenotype and subsequent southern blot of genomic tail DNA. These mice were backcrossed to FVB strain mice and positive offspring identified in the same manner (backcross 2). A further backcross to the FVB strain was conducted before mice were entered into the chemical carcinogen study. Mice of backcross 3 (87.5% FVB and 12.5% C57Bl/6) should be chemical carcinogen sensitive, since F1 mice have previously been demonstrated to be sensitive (Burns et al., 1991).

Mice were entered into the treatment protocols at 8 weeks of age and in the majority of cases the transgenic status was unknown at this point. This helped to eliminate any bias in assessment in the formation of lesions. Subsequently the transgenic status for all the mice was assessed and equivalent numbers of transgenic positive and negative siblings were entered into each study (except regime 1 where additional negatives were entered before status was ascertained). Four regimes of treatment were used with the following numbers of mice:-

Regime 1) DMBA + TPA-   29 LMP +ve and 31 LMP -ve. This
                        regime was conducted for 55
                        weeks which included 20 weeks of
                        DMBA + TPA treatment and 35 weeks
                        of observation

Regime 2) TPA alone-   15 LMP +ve and 20 LMP-ve. This
                        regime was conducted for 46 weeks
                        which included 20 weeks of TPA
                        treatment and 26 weeks of
                        observation

Regime 3) DMBA alone-   15 LMP +ve and 15 LMP-ve. This
                        regime was conducted for 35 weeks
                        which included a single application of
                        DMBA and 35 weeks of observation

Regime 4) DMBA followed- 7 LMP +ve and 6 LMP-ve. This

by 4 weeks only of TPA experiment was initiated towards the end of my Ph.D. studies so was subject to time constraints and thus was conducted by myself for only 20 weeks, of which 4 weeks were treatment and 16 weeks observation. Thereafter, Donald Campbell continued the observation.

As detailed in the methods (section 7B.1.4), DMBA at a concentration of $5x10^{-5}M$ ($25\mu g$) in acetone was applied to shaved dorsal skin followed one week later by twice weekly applications for twenty weeks of TPA also at a concentration of $5x10^{-5}M$ in acetone.

The mice were housed as mixed groups in the same cages to minimise possible environmental or treatment factors. Every week the mice were assessed in a blinded fashion (to avoid observer bias through knowledge of what the particular mouse showed the previous week) for the appearance of lesions. The number and size of papillomas and carcinomas formed were counted. Each lesion was designated a number from 1-4 depending on its diameter. Thus:-

$4 = > 1cm^2$

$3 = > 0.5cm^2$ but $< 1cm^2$

$2 = > 0.2cm^2$ but $< 1cm^2$

$1 = < 0.2cm^2$

After about 15 weeks of treatment the papilloma load became such that occasionally the larger papillomas masked some of the small papillomas and it was impossible to count the number of small size 1 papillomas accurately. For this reason they are not included in any calculations. All records and calculations therefore are based on the average number of papillomas of size 2-4 for both LMP-1 positive and LMP-1 negative mice per week.

3B.2 Chemical carcinogen regime 1:- DMBA plus 20 weeks of TPA

3B.2.1 Formation of papillomas

Small nodules began to appear during week 5 or later of TPA treatment and all mice had developed lesions by week 11. These lesions took on the recognisable papilloma shape which could be described as "cauliflower" like in that the papillomas had a large wrinkly head and were attached to the dorsal skin by a

thin "stalk" (Figure 3.2). The pattern of papilloma formation was unique for each mouse. Siblings housed within the same cage that had received identical doses of carcinogen at the same time, displayed wide variation in the number and size of papillomas formed indicating that initiation is a random event and that promotion of initiated cells may vary (Tables 3.1+3.2 and figures 3.2+3.3).

Figure 3.2

Figure 3.3
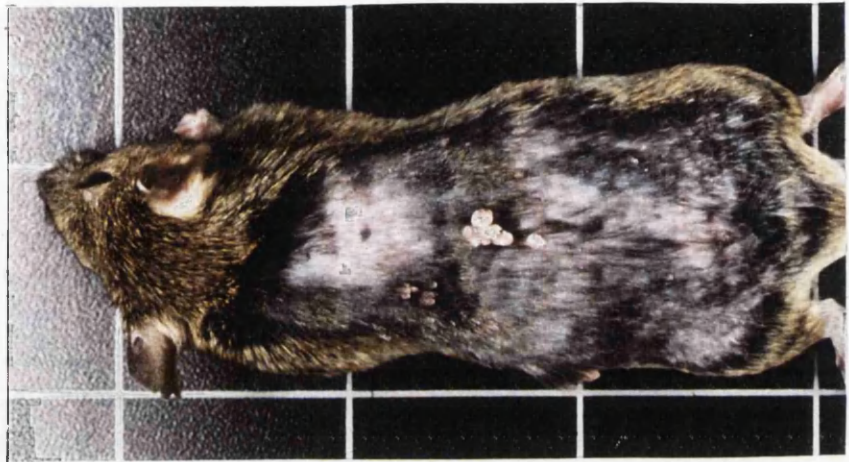
Figure 3.9

Figures 3.2, 3.3, and 3.9:- Figures 3.2 and 3.3 show the shape of papillomas which form and highlight the randomness of papilloma formation as the mice in these figures were housed within the same cage and received identical doses of chemicals. Figure 3.9 indicates the shape of a carcinoma and highlights the difference between papilloma and carcinoma.

Table 3.1:- LMP-1 positive mice used in chemical carcinogen treatment regime 1 (DMBA + TPA treatment), indicating the age of the mice when treatment commenced (weeks), the age of the mice when papillomas and subsequently carcinomas developed, the maximum numbers of each lesion appearing, and the conversion rates of papilloma to carcinoma.

Mouse i.d.	Age at start of treatment	LMP	Wk of treatment at death	1st paps (wk)	max. paps	1st carc. (wk)	max. carc.	% conversion
53F.183	16	+	53	8	24	34	1	4.2
53F.186	16	+	55	6	27	34	1	3.7
53F.191	16	+	35	7	26	21	5	19.2
53F.214	8	+	26	7	53	26	?	/
53F.221	8	+	34	7	50	29	4	8
53F.226	8	+	38	7	43	29	10	23.3
53F.234	8	+	36	8	52	23	5	9.6
53F.236	8	+	24	8	35	25	1	2.9
53F.238	8	+	35	8	50	27	7	14
53F.239	8	+	54	8	27	24	2	7.4
53F.243	8	+	53	6	26	33	2	7.7
53F.245	8	+	24	8	30	25	3	10
53F.246	8	+	38	8	33	27	6	18.2
53F.248	8	+	35	10	27	24	2	7.4
53F.252	8	+	54	7	24	30	2	8.3
53F.253	8	+	38	7	10	27	2	20
53F.255	8	+	36	7	26	29	5	19.2
53F.260	8	+	31	6	38	31	5	13.2
53F.268	8	+	19	6	33	12	1	3
53F.269	8	+	31	6	36	28	12	33.3
53F.278	10	+	43	5	35	37	2	5.7
53F.279	10	+	43	5	38	22	3	7.9
53F.287	10	+	34	5	40	24	2	5
53F.289	10	+	29	5	35	24	1	2.9
Total =24			Av. =7	Av. =34.5	Av. =27	Av. =3.65	Av. =11.05	

N.B. Mouse 53F.214 was found dead prior to the development of carcinomas and thus was excluded from conversion calculations

Table 3.2:- LMP-1 negative mice used in chemical carcinogen treatment regime 1 (DMBA + TPA treatment), indicating the age of the mice when treatment commenced (weeks), the age of mice when papillomas and subsequently carcinomas developed, the maximum numbers of each lesion appearing and the conversion rates of papilloma to carcinoma.

Mouse i.d.	Age at start of treatment	LMP	Wk of treatment at death	1st paps (wk)	max. paps	1st carc. (wk)	max. carc.	% conversion
53F.180	12	-	32	8	32	27	4	12.5
53F.181	15	-	26	8	25	23	1	4
53F.182	16	-	39	6	25	26	4	16
53F.184	12	-	54	8	22	39	3	13.6
53F.185	12	-	45	8	17	36	5	29.4
53F.187	12	-	40	9	1	27	1	100*
53F.188	16	-	31	8	48	?	?	/
53F.189	16	-	35	8	42	27	1	2.4
53F.194	16	-	32	8	31	26	4	12.9
53F.195	16	-	35	8	22	26	5	22.7
53F.196	16	-	39	9	40	32	5	12.5
53F.204	10	-	45	9	35	27	1	2.9
53F.205	10	-	35	10	20	24	6	30
53F.208	10	-	42	8	43	36	0	0
53F.210	10	-	43	9	31	31	2	6.5
53F.211	8	-	50	11	12	47	1	8.3
53F.212	8	-	25	7	28	18	1	3.6
53F.213	8	-	26	7	24	18	1	4.2
53F.215	8	-	45	11	17	37	2	11.8
53F.216	8	-	31	9	20	25	4	20
53F.217	8	-	47	7	24	37	4	16.7
53F.218	8	-	40	7	23	35	1	4.3
53F.219	8	-	12	7	15	?	?	/
53F.220	8	-	28	7	33	28	2	6.1
53F.222	8	-	42	7	27	33	6	14.8
53F.223	8	-	35	10	22	18	5	22.7
53F.225	8	-	41	7	36	28	4	11.1
53F.227	8	-	17	7	28	?	?	/
53F.228	8	-	30	7	41	29	2	4.9
53F.229	8	-	34	7	7	34	1	14.3
Total =30				Av. =8	Av. =26.37	Av. =29	Av. =2.81	Av. =15.40

N.B. Mice 53F.188, 53F.219 and 53F.227 were found dead before carcinomas had formed and thus were not included in the percentage conversion calculations.

*= Mouse 53F187 only ever developed one papilloma which subsequently converted to carcinoma. This gives the artificially high conversion rate of 100%. Removing this mouse from the calculation decreases the average conversion rate to **12.15%**

80

Comparison of mean papilloma load between LMP-1 positive mice and LMP-1 negative control groups (Regime 1)

Papillomas were first detected in the LMP-1 positive mice in week 5 of treatment. All mice in this group had papillomas by week 10, with an average time to onset of 7 weeks post DMBA treatment. The first papillomas to arise in the LMP-1 negative group were in week 6 of treatment. All mice in this group had papillomas by week 11, with an average time to onset of 8 weeks post DMBA. Thus, it would appear that the LMP-1 positive mice developed papillomas slightly faster than the LMP-1 negative mice. This difference proved to be statistically significant when the data was used in a two sample T-test. A T value of 3.45 was obtained with a P value of 0.0012 and 49 degrees of freedom.

As can be seen from figure 3.4, the average number of papillomas on the LMP-1 positive mice rose rapidly to 15 papillomas on average by week 10 of treatment, whereas at week 10 the LMP-1 negative mice had an average of only 12 papillomas. For the duration of the study (55 weeks), the average number of papillomas on LMP-1 positive mice was consistently higher than the average number of papillomas on LMP-1 negative mice.

After consultation with a scientific statistician and a mathematical statistician the most appropriate method of deciding whether there was significant difference in papilloma load between the two groups was reached. This involved taking each week at a time and conducting a Rankits plot. Rankits plots are a method of determining if values conform to a normal distribution. If normal distribution was found then a two sample T-test or Box-plot could be carried out to look for significant differences. All statistical analysis is discussed in greater depth in section 3B.2.4 whilst the results of the analysis are given here.

It was decided that the period between week 10 and week 25 showed the most differences between the groups and so attention was focused on this period. Analysis was carried out on weeks 10, 15, 20 and 25. At week 10 of treatment there was no significant difference between the two groups. At week 15 however, the two groups were significantly different with a T value of 2.15 a P value of 0.036 and 55 degrees of freedom. At week 20 no significant difference was noted. A significant difference between the groups at week 25 was however noted. A T value of 2.44 was obtained with a P value of 0.018 and 55 degrees of freedom.

After week 25 of the study, the average number of papillomas on both LMP-1 positive and LMP-1 negative mice declined at roughly the same rate. This reduction is due to some papillomas converting to carcinomas (and therefore being removed from the papilloma count) and some regressing.

However, the papilloma load on the LMP-1 positive mice, having reached an overall higher maximum average number, was maintained at higher levels than the LMP-1 negative mice for the remainder of the study. When mice were removed from the study, the number of papillomas at death for each mouse was included in all subsequent weekly counts. This was done because to remove the counts entirely as the mouse was sacrificed would artificially reduce the average papilloma load subsequently scored. Figure 3.4 therefore clearly shows that during chemical promotion, the average number of papillomas on the LMP-1 positive mice rose more quickly and to a higher level than the average number of papillomas on the LMP-1 negative mice.

In accordance with the Home Office licensing of this procedure, mice must be sacrificed when they reach a maximum acceptable papilloma/carcinoma load or when a carcinoma reaches 2cm in diameter. This maximum papilloma load is subjectively determined by experienced animal house staff and therefore is not subject to any bias of transgene status. As a consequence, from week 12 of the study onwards mice were removed from the study. This included 4 LMP-1 positive mice (53.F224, 230, 232, and 233) which were removed at week 18 of the study. Their papilloma scores at death were included in every subsequent count and therefore make up part of the average for the LMP-1 positive group. However, these mice (plus mouse 53.F254 which died of a prolapse at week 12 and whose scores were not included thereafter) were not included in table 3.1. Thus 29 LMP-1 positive mice started this program of treatment. Fig 3.5 shows the number of mice remaining in the study for each week. In accordance with the increased number of papillomas in LMP-1 positive mice compared to LMP-1 negative mice, the LMP-1 positive mice were removed from the study at earlier time points than the LMP-1 negative mice. It should be noted therefore that the maximum papillomas recorded has an artificial limit dictated by the requirement to set a limit for animal welfare. For example, at week 15 of the study there are >95% of the mice alive whereas after week 40 there are <25% of the mice alive in both groups. Nonetheless, up to week 25 (the main period of statistical analysis of the data), there are still >75% of both LMP-1 positive and LMP-1 negative mice left in the study.

From analysis of these results, the LMP-1 positive mice develop more papillomas at a faster rate than their LMP-1 negative counterparts and that this difference is statistically significant.

Death curves for LMP-1 positive mice and WT controls, both DMBA+TPA treated
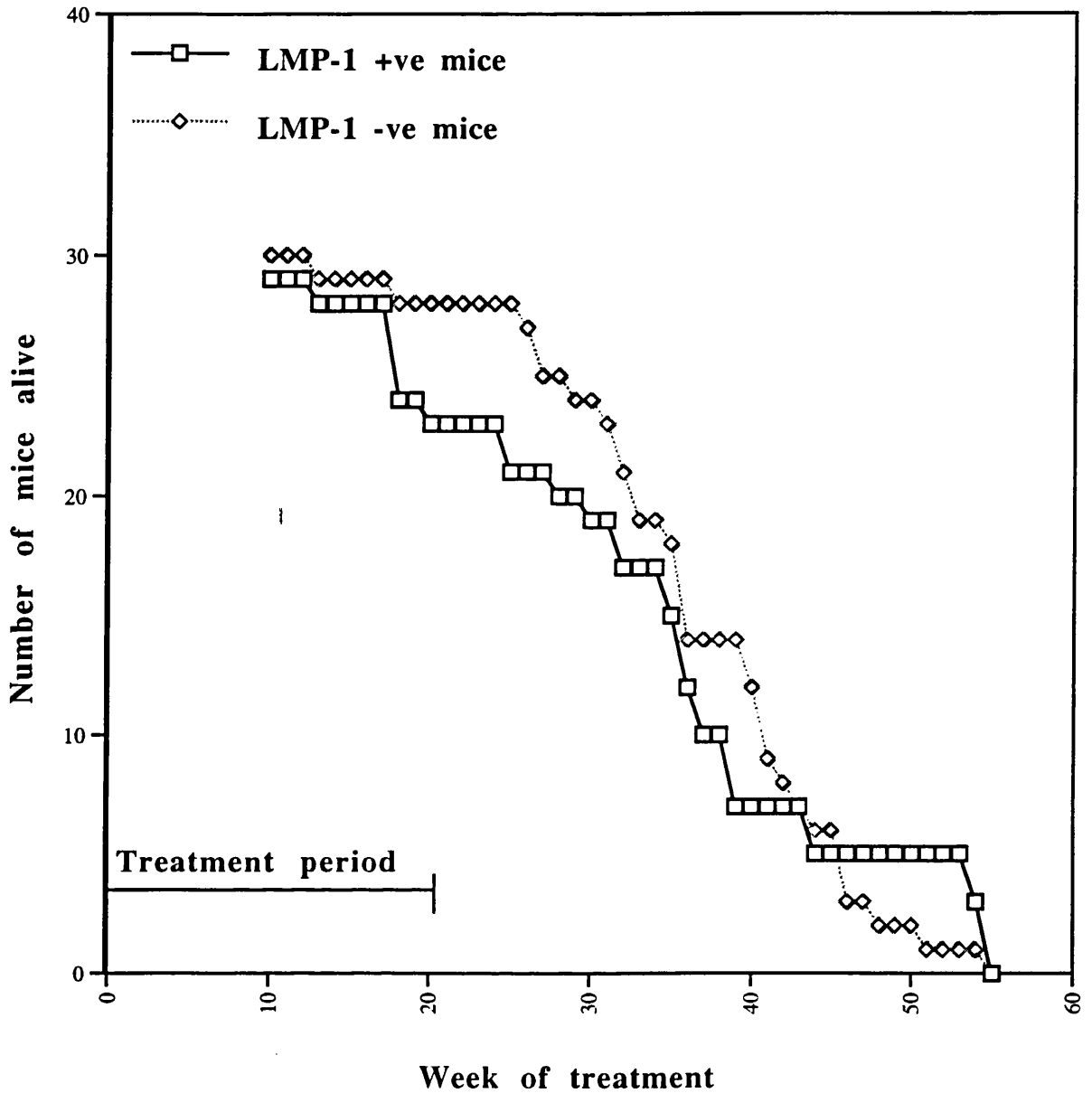
Figure 3.5

This graph indicates the rate of withdrawal from the study program for both LMP-1 positive and negative control littermates. Mice were removed from the study on the basis of advice given by the animal technicians. Causes of removal were prolapse, sickly looking mice, too large a papilloma load, large papillomas which hindered the animals movement, and formation of carcinoma.

Figures 3.6-3.8 show that there is also a difference in the average number of each size category of papillomas formed between the LMP-1 positive mice and their LMP-1 negative siblings. Figure 3.6 shows that the curves for the average number of size 2 papillomas are very similar to total numbers shown in figure 3.4. The LMP-1 positive mice develop more size 2 papillomas at a faster rate than do the LMP-1 negative mice. This difference is statistically significant. At week 10 there is no significant difference, but at weeks 15, 20 and 25 the differences become significant with T values of 3.03, 2.51, and 3.54, and P values of 0.0038, 0.015 and 0.0008 respectively. The degrees of freedom were 52, 55 and 54 respectively for these three time points. Again this separation arises at the start and is maintained for the duration of the study (a further 45 weeks). It should be noted that at 20 weeks of the study there are 21.68 size 2 papillomas on the PyLMP-1 positive mice. There are however only 2.04 size 3 papillomas and 0.64 size 4 papillomas at the same 20 week stage. Thus the graphs for figures 3.6-3.8 have different Y axis numberings with figure 3.6 ranging from 0-30 and figure 3.8 from 0-4.

Figure 3.7 reveals that the average number of size 3 papillomas is the same for both LMP-1 positive and LMP-1 negative mice. No statistically significant difference was observed at any time point. Figure 3.8 shows that there are on average, more size 4 papillomas on the LMP-1 negative mice than on the LMP-1 positive mice. Although this difference is easily apparent from figure 3.8, no statistically significant difference was observed until week 25 of the study. From week 15 of treatment to week 35 of treatment, there are consistently higher average numbers of size 4 papillomas on the LMP-1 negative mice compared to the LMP-1 positive mice. However, as can be seen in figure 3.5 there are fewer LMP-1 positive mice in the study during this period and certainly some of these mice had to be removed because of home office regulations regarding the size/number and positioning of papillomas.

Comparison of size 2 papilloma load between LMP-1 positive and LMP-1 negative control groups
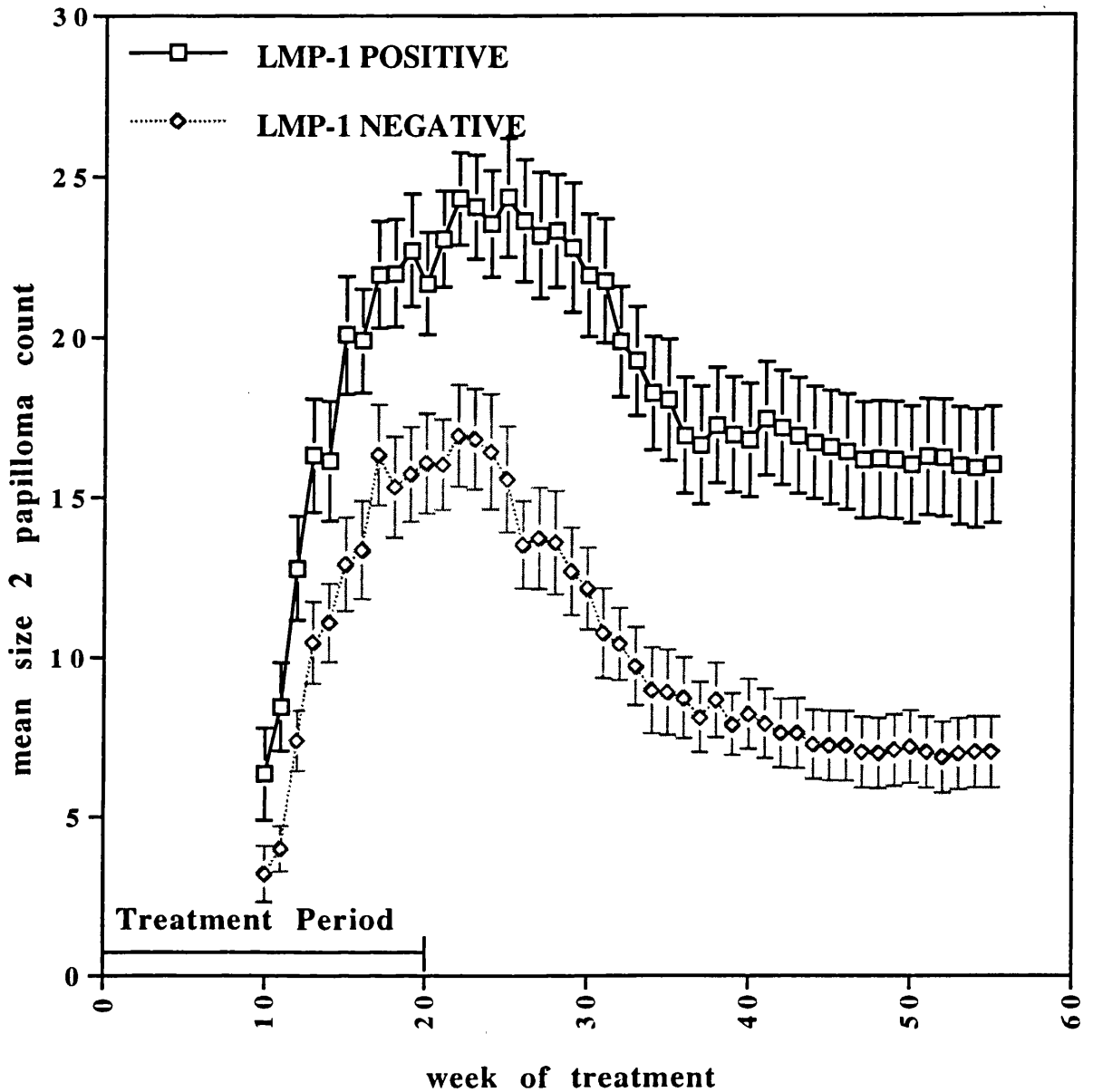
Figure 3.6

This graph shows the average number of papillomas of size 2 ($> 0.2\text{cm}^2 < 0.5\text{cm}^2$) for both of the experimental groups at each week of the study. Error bars show the standard error of the mean for these figures. The analysis was terminated at week 55 of the study.

Comparison of size 3 papilloma load between LMP-1 positive and LMP-1 negative control groups
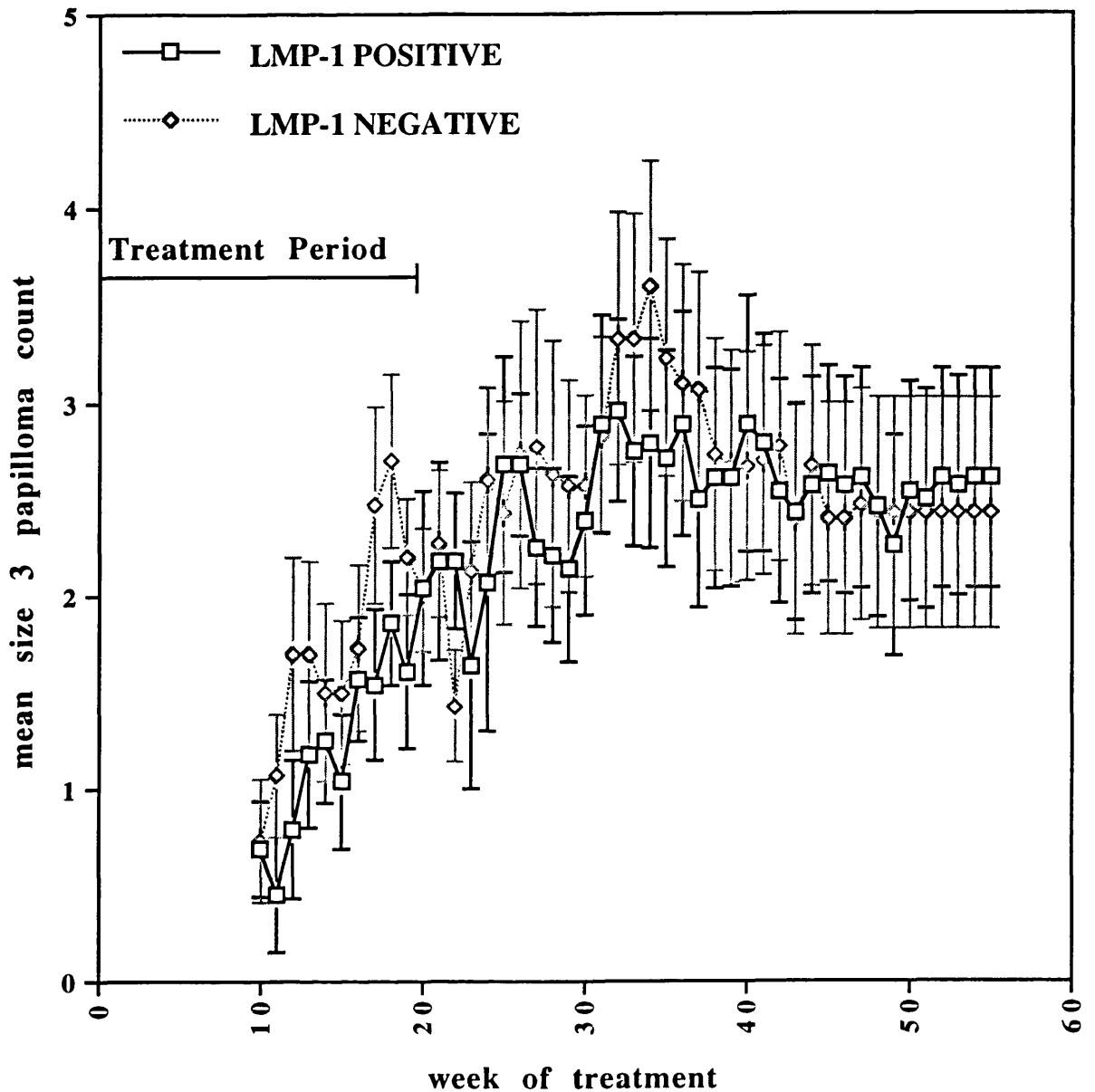
Figure 3.7

This graph shows the average number of papillomas of size 3

($> 0.5cm^2 < 1.0cm^2$) for both of the experimental groups at each week of the study.

Error bars represent standard error of the mean.

N.B. There were markedly fewer size 3 papillomas than size 2 in general, hence the expanded vertical axis.

Comparison of size 4 papilloma load between LMP-1 positive and LMP-1 negative control groups
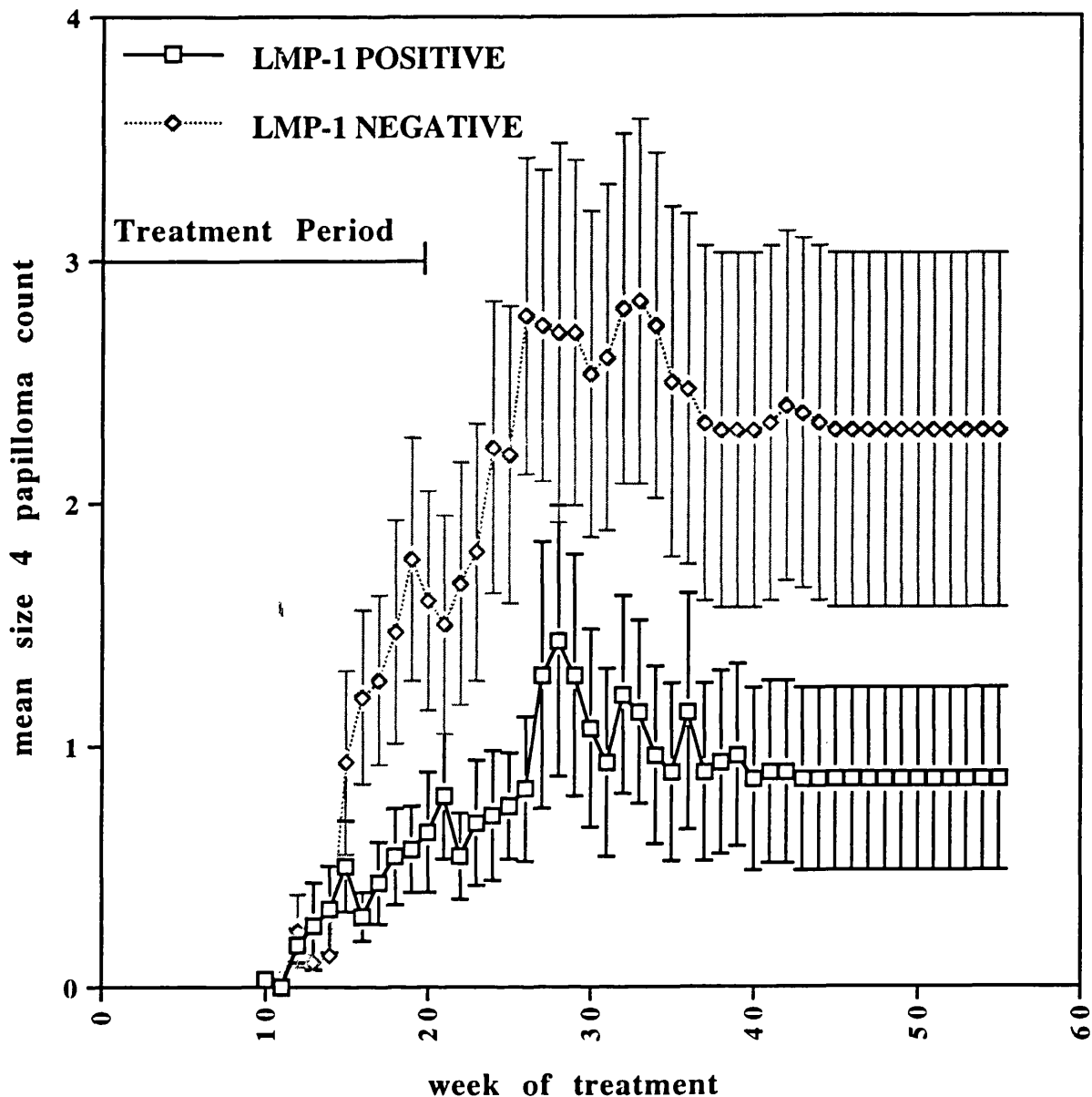
Figure 3.8

This graph shows the average number of papillomas of size 4

($> 1.0 cm^2$) for both of the experimental groups at each week of the study.

Error bars represent standard error of the mean.

N.B. There were markedly fewer size 4 papillomas than size 2, hence the expanded vertical axis.

3B.2.2 Conversion of papilloma to carcinoma

Whilst papillomas are attached to the dorsal skin via a "stalk" (easily moved by finger), carcinomas appear flat and are almost flush to the skin surface (figure 3.9). Papilloma and carcinoma were not always easily distinguishable by eye, but closer inspection of the attachment of the lesion facilitated distinguishing the type of lesion. For example, a lesion attached to the body by a "stalk" which could be "wobbled" was classed as a papilloma and this classification was confirmed by subsequent histopathology analyses of tissue sections. If the lesion was firmly attached over a larger area, in general this proved to be a carcinoma and was recorded as such. Most carcinomas were easily identified by a large area of necrosis on their surface some of which were open wounds. Mice were sacrificed on the advice of the animal technicians based on home office regulations.

Formation of carcinomas was recorded for all mice in the study. Figure 3.10 shows the average number of carcinomas per mouse through the test period. In general, soon after carcinoma formation it was necessary to remove the mice from the study, either because of excessive papilloma load or because of the size or location of the carcinoma. As such, the numbers of carcinomas formed is artificially low (for both LMP-1 +ve and LMP-1 -ve mice). Nevertheless, the average numbers formed in LMP-1 positive mice (3.65/mouse) is slightly higher than the LMP-1 negative mice (2.81/mouse).

Comparison of carcinoma load between LMP-1 positive mice and LMP-1 negative control groups
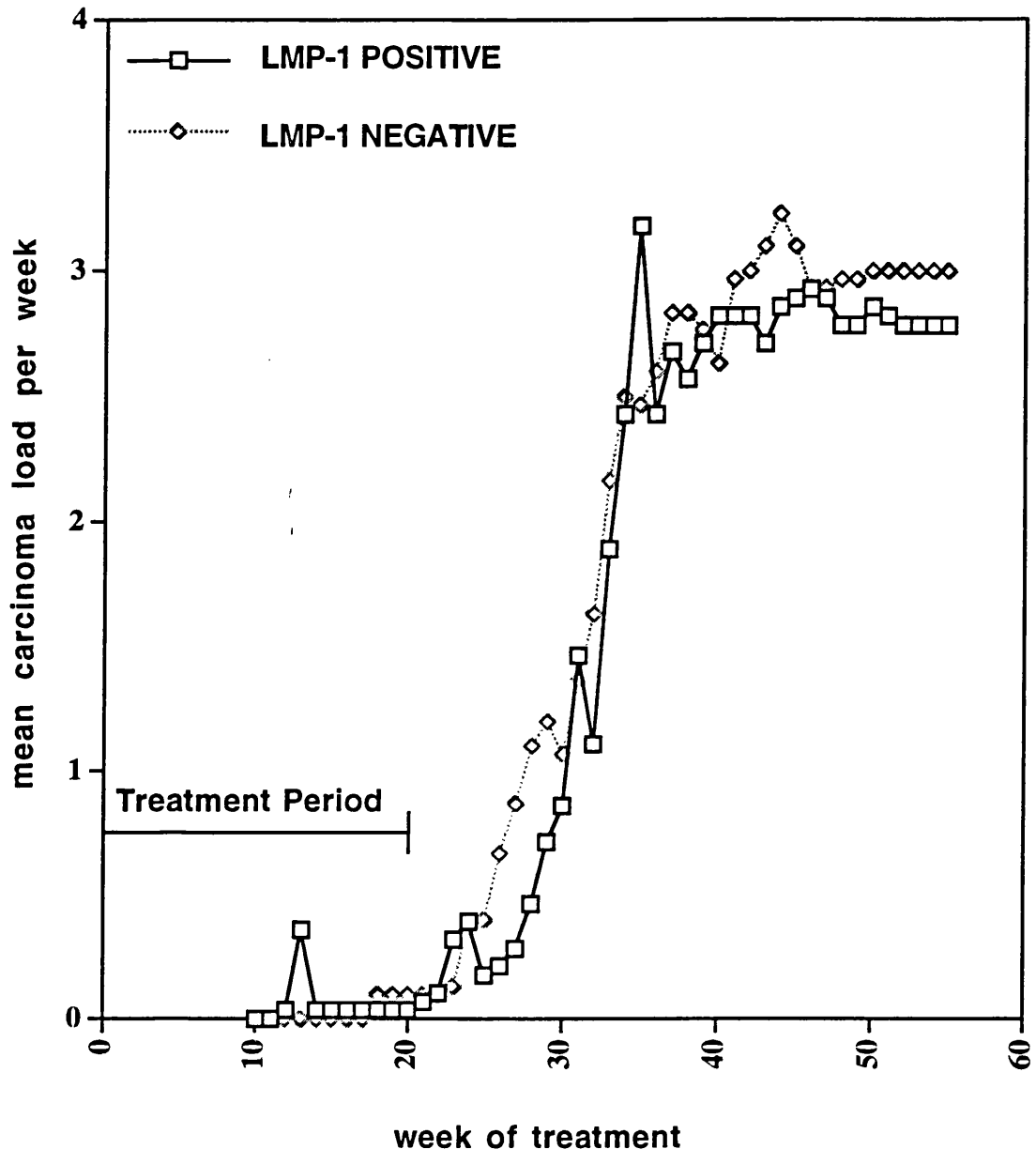
Figure 3.10

Each week the number of carcinomas arising on each individual mouse was counted. The mean carcinoma load for each week was then calculated by taking the total number of carcinomas in each group, including the last recorded carcinoma scores for mice sacrificed due to Home Office guidelines, and dividing by the number of mice in each group.

This graph indicates the average carcinoma load for both of the experimental groups at each week of the study. The graph shows that there is no clear difference between the two groups.

Error bars represent standard error of the mean.

In order to estimate the conversion rate of papilloma to carcinoma the carcinoma load was compared to the papilloma load. A note of the maximum number of papillomas formed on each individual mouse (which was generally greater than the number of papillomas on the mouse at death) as well the total number of carcinomas on that same individual when it was sacrificed was made. From this the conversion rate for each mouse was calculated and the mean is shown in tables tables 3.1 and 3.2. The average percentage conversion for LMP-1 negative controls was 15.40% and the percentage conversion for LMP-1 positive mice was 11.05%. Although the percentage conversion of benign papilloma to malignant carcinoma appears higher in the LMP-1 negative mice, much of this difference can be attributed to a single mouse, 53F.187, that only ever developed one single papilloma which subsequently converted to carcinoma. This gives a misleadingly high 100% conversion for this particular mouse. As an outlying value, it is statistically acceptable to consider the average figure whilst discounting this one mouse, giving a figure of 12.15% for LMP-1 negative mice compared to 11.05% in positive mice. There was no significant difference between these conversion rates when assesed by the two sample T-test. Both LMP-1 positive and LMP-1 negative mice have conversion rates apparently higher than the previously published rates of 5-10%.

3B.2.3   Study of carcinomas formed

As described in the materials and methods, carcinomas were excised immediately following sacrifice of the animal. Some of these were taken into tissue culture in order to derive cell lines (7B.2.1). Where possible, sections from all of the carcinomas and some of the papillomas were snap frozen and stored at -70^{0}C for molecular analysis and similarly sections from all of the carcinomas and some of the papillomas were placed in fixative for histopathological analysis.

The carcinomas in fixative were sectioned and haematoxylin and eosin (H+E) stained. The process of a papilloma converting to a carcinoma can be broken down into many stages based upon the appearance of the tumour. Papillomas have been assigned to four groups, from the well differentiated type 1 through to the less well differentiated type 4 with high proliferative activity, necrosis, and the beginnings of progression to Squamous Cell Carcinoma (SCC). Keratoacanthoma represents a separate group of lesions and resembles a cup or saucer shaped lesion with masses of overlaid keratin. Squamous cell carcinomas are divided into 4 groups also. Unlike papillomas there are no masses of keratin

produced and there are clear indications of a breakdown in normal epithelial tissue structure. Well differentiated SCC's are classified as type 1 and poorly differentiated as type 4 where there is very little evidence of differentiation and organisation. The amount of invasion into subcutaneous tissue and muscle increases from SCC type 1 to type 4. The spindle cell tumour represents the final stage of tumour progression where the tumour becomes more aggressively invasive and has lost markers of normal epithelium. The tumour takes its name from the "spindle" shape of the cells showing no epithelial organisation. A review of some of these stages can be found in Bogovski, 1994.

In total 111 suspected carcinomas were sectioned and H+E stained. These included 51 tumours from 14 LMP-1 positive mice, and 60 tumours from 20 LMP-1 negative control mice. LMP-1 positive mice had more definitive carcinomas amongst the 51 tumours counted than did the LMP-1 negative controls. Table 3.3 shows the distribution of tumour types after histopathological examination amongst those counted. As can be seen 70% of the tumours counted from the LMP-1 positive group proved to be carcinoma, whereas only 51% of the tumours counted from the LMP-1 negative group were classified as carcinoma. These lesions which were classified as carcinoma by gross observation and yet appeared to be papilloma by histopathology may have been in the process of converting. Most lesions contain mixtures of carcinoma grades and papillomatous areas, and it could be in these that a carcinoma region was missed in the diagnostic section. Alternatively they may have been late stage papilloma and misdiagnosed at the gross level. If this is the case, then the difference noted between the carcinoma averages (higher in the LMP-1 positive mice) would be greater, while the conversion rate difference (slightly higher in the LMP-1 negative group) would be less.

2/51 tumours were spindle cell carcinoma in the LMP-1 positive group with 1/60 spindle cell carcinoma in the LMP-1 negative control group. As previously mentioned, animal technicians have advised when a mouse was to be sacrificed and as a result full carcinogenic progression could not be followed in many cases. However, formation of this highly malignant variant of skin carcinoma is rare with reported incidences of 0.07/mouse in DMBA+TPA treated SENCAR mice (Klein-Szanto *et al.*, 1989). The frequency is not dramatically increased in LMP-1 mice compared to controls.

Table 3.3:- Summary of the types of tumour developing on LMP-1 positive and LMP-1 negative mice following chemical carcinogen regime 3 treatment (DMBA + TPA), indicating the number of tumours in each category and the percentage of the total.

	P	K	C1	C2	C3	C4	Sp.C
LMP-1 +	12 (24%)	2 (4%)	20 (40%)	9 (18%)	5 (10%)	1 (2%)	2 (4%)
LMP-1 -	27 (45%)	2 (3%)	16 (27%)	4 (7%)	6 (10%)	4 (7%)	1 (2%)

KEY:-

P= Papilloma

K= Keratoacanthoma

C1-4= Squamous cell carcinoma grade 1-4

Sp.C= Spindle cell carcinoma

Table 3.4:- Description of primary carcinoma cell lines and the carcinomas they were derived from.

Cell Line	LMP	Carcinoma size	Carcinoma type	Cell Line Appearance
F191a	+	1.3cm^2	3	Spindly with very long pseudopodia (arm-like projections)
F204	-	1.2cm^2	2	Spindly with short pseudopodia extentions
F217	-	0.7cm^2	3	Spindly with very long pseudopodia
F226a	+	2.0cm^2	2	Mix of spindly cells with long pseudopodia, and cuboidal(non-spindly) cells
F226b	+	1.0cm^2	1	Cuboidal with single protrusion (sperm-like)
F226c	+	0.7cm^2	1	Cuboidal with long pseudopodia
F234a	+	1.2cm^2	3	Spindly but more cuboidal than long and thin
F278a	+	2.0cm^2	Sp	Spindly (sperm-like)
F278b	+	1.5cm^2	2	Spindly with long pseudopodia
F279	+	2.0cm^2	3	Spindly but more cuboidal than long and thin

NB:- All carcinomas were Squamous cell carcinomas except F278a which was a Spindle (Sp) cell carcinoma.

LMP-1 +ve   **LMP-1   -ve**

53F.191A   53F.204

53F.226A   53F.217

53F.278A   53F.220

Figure 3.11:- Examples of some of the primary cell lines generated from explanted carcinomas. The tumours were excised under sterile conditions into explant medium for transport from the animal house. These were then chopped up and left to adhere to tissue culture flasks (in growing medium) for 2 weeks. Thereafter cells began to grow out from the tumour and the tumour lumps could be discarded. Once the cell line was established it was maintained as described in section 7B.2.1, 7B.2.3 and 7B.2.4

8 LMP-1 positive and 2 LMP-1 negative carcinoma cell lines were established from excised carcinomas from eight different mice (See table 3.4). The original carcinomas varied in size from $0.7cm^2$ to $2cm^2$ and were subsequently shown to represent a range of SCC from type 1 to type 3 plus one spindle cell carcinoma. There were no obvious differences between cell lines derived from LMP-1 expressing or non-expressing tumours (figure 3.11). All grew rapidly in both the 20%FCS growing medium used to establish the cell line and the 10%FCS medium used to maintain the cell line, although proliferation rates were not determined. The cells were long and thin in the typical spindle morphology with numerous pseudopodia. Unlike normal epithelial cells which grow in the characteristic "cobblestone" appearance with distinct cell:cell boundaries, the carcinoma cell lines overlapped and overgrew each other clearly showing the loss of contact inhibition characterised by epithelial tumour cell lines. With insufficient time remaining to further characterise the properties of these carcinoma cell lines, they were frozen down for storage until analysis could be conducted and represent a useful source of material.

3B.2.4 Statistical analysis of Regime 1

Due to the nature of this experiment it was exceptionally difficult to find a statistical test which would allow me to analyse figure 3.4 as a whole e.g. looking at significant difference in rates of change. After discussion with two statisticians it was decided that the best approach was to analyse each time point separately. With Professor Wardlaw of the Cell Biology department, I used the Rankits program to determine if the data for each time point was randomly distributed. An example of a Rankit plot is given below, and for each point examined the data was found to be normally distributed.

As the data was normally distributed this allowed a two sample T-test to be applied (on the advice of Prof. Wardlaw). The data was entered into the Minitab computer program data spreadsheet. This is a statistical analysis program which is capable of applying many different statistical tests on raw data. Examples of the results from a two sample T-test are also given below.

Rankits plot:-
If the data does not deviate significantly from the straight line it can be assumed that the data is normally distributed. This is an

example of a rankits plot for the LMP-1 positive mouse group at week 15.

Figure 3.12:-
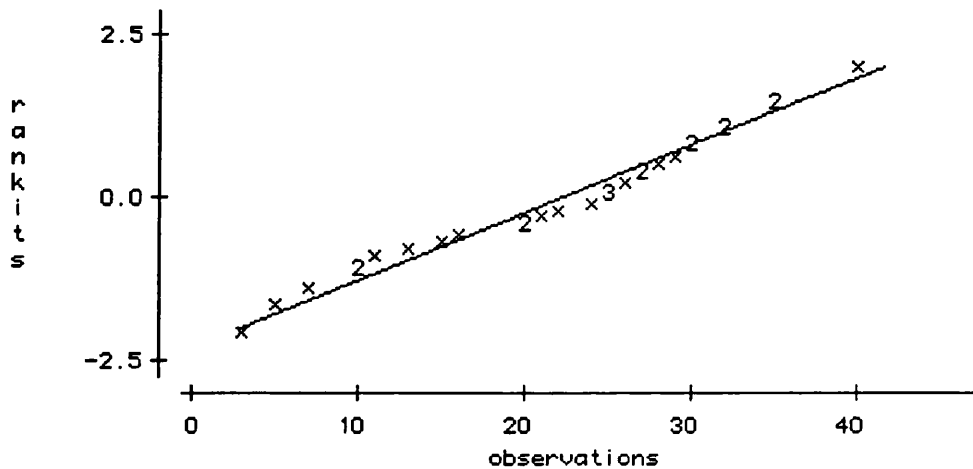
Basic Statistics + Two sample T-test:-

Minitab has a function that will give the following basic statistics on the data entered and the results of a two sample T-test analysis. These results are from week 15 data.

Two Sample T-test:-
For Wk15 LMP-1 positive v's Wk15 negative controls

	N	MEAN	STDEV	SE MEAN
Wk15 +	28	22.43	9.77	1.8
Wk15 -	30	16.70	8.32	1.5

Using 95% confidence limits, T-Test results :-
T= 2.40 P=0.020 DF= 53

Descriptive statistics for Week 15:-

	N	MEAN	MEDIAN	TRMEAN	STDEV	SEMEAN
Wk15 +	28	22.43	25.00	22.50	9.77	1.85
Wk15 -	30	16.70	17.50	16.77	8.32	1.52

	MIN	MAX	Q1	Q3
Wk15 +	3.00	40.00	13.75	29.75
Wk15 -	0.00	33.00	12.00	22.50

3B.3 Chemical carcinogen regime 2:-TPA for 20 weeks

To check if LMP-1 can substitute for DMBA induced Ha-*ras* mutation, mice were treated with TPA for twenty weeks without prior DMBA initiation.

Of the 35 mice that were put in the TPA treatment course, 11/15 LMP-1 positive and 17/20 LMP-1 negative mice remained 46 weeks after the first TPA treatment, when the experiment was terminated. Of the seven mice that were removed from the study, four had to be sacrificed due to anal prolapses and three died of unrelated causes (See tables 3.5a+b). Prolapses are quite common in topically challenged mice because the acetone facilitates spreading of the chemicals all over the treatment area (dorsal skin). However, some spreading to the anus is unavoidable and can cause eventual problems (such as prolapse). These mice (4 LMP-1 positives and 3 LMP-1 negatives) were sacrificed before the end of the 20 week treatment period and none of these mice had any signs of papillomas.

After 46 weeks none of the LMP-1 positive mice had developed any papillomas (table 3.5a). Surprisingly, 1/17 LMP-1 negative mice (53F.308) had one single size 2 papilloma on its dorsal skin at the termination of the experiment, which had appeared at week 29 of treatment and remained throughout the observation period (See table 3.5b). Two other LMP-1 negative control mice (53F.306 and 53F.315) had displayed single papillomas during the experiment, appearing at 16 and 14 weeks respectively, but these subsequently regressed and disappeared (See table 3.5b).

These results indicate that LMP-1 expression in these transgenic mice cannot substitute for DMBA treatment and hence Ha-*ras* activation.

98

Table 3.5a:- LMP-1 positive mice in chemical carcinogen treatment regime 2 (TPA promotion twice weekly for 20 weeks without prior DMBA initiation).

Mouse i.d.	Age at start of treatment	LMP	Wk of treatment at death	1st paps (wk)	max. paps
53F.294	9.5	+	46	/	/
53F.295	9.5	+	46	/	/
53F.300	9.5	+	10*	/	/
53F.309	8	+	46	/	/
53F.311	8	+	46	/	/
53F.317	8	+	40*	/	/
53F.322	8	+	44	/	/
53F.323	8	+	44	/	/
53F.327	8	+	44	/	/
53F.328	8	+	44	/	/
53F.349	8	+	38	/	/
53F.350	8	+	38	/	/
53F.352	8	+	38	/	/
53F.353	8	+	8*	/	/
53F.354	8	+	24*	/	/

* = mouse removed from study due to ill health
/ = Zero papillomas

Table 3.5b:- LMP-1 negative mice in chemical carcinogen treatment regime 2 (TPA promotion twice weekly for 20 weeks without prior DMBA initiation).

Mouse i.d.	Age at start of treat-ment	LMP	Wk of treat-ment at death	1st paps (wk)	max. paps
53F.298	9.5	-	46	/	/
53F.302	8	-	46	/	/
53F.303	8	-	46	/	/
53F.304	8	-	46	/	/
53F.305	8	-	46	/	/
53F.306	8	-	29*	16	1
53F.307	8	-	46	/	/
53F.308	8	-	46	29	1
53F.310	8	-	46	/	/
53F.312	8	-	46	/	/
53F.313	8	-	44	/	/
53F.314	8	-	44	/	/
53F.315	8	-	44	14	1
53F.316	8	-	35*	/	/
53F.319	8	-	44	/	/
53F.320	8	-	44	/	/
53F.321	8	-	44	/	/
53F.324	8	-	44	/	/
53F.325	8	-	15*	/	/
53F.326	8	-	44	/	/

* = mouse removed from study due to ill health
/ = Zero papillomas

3B.4   Chemical carcinogen regime 3:- DMBA alone

The PyLMP-1 transgenic mice have a phenotype of epidermal hyperplasia which is remarkably similar to that described for mouse dorsal skin following TPA treatment (DiGiovanni, 1992). TPA has many effects on mouse dorsal skin (described earlier), but if its principle function as a tumour promoter is to increase proliferation, then it might be possible that LMP-1 could substitute for the actions of TPA. To put this to the test LMP-1 positive mice and their LMP-1 negative control siblings were given a one off treatment with the initiator DMBA. This experiment was to address whether LMP-1 can act in the promotion stage of carcinogenesis, to promote tumour formation in conjunction with DMBA initiated cells.

Of the 30 mice which received the DMBA treatment, 14/15 LMP-1 positives and 12/15 LMP-1 negatives remained when the experiment was terminated 35 weeks after DMBA initiation. Of the 4 mice which were removed from the study (1 LMP-1 positive mouse and 3 LMP-1 negative mice), 3 had prolapses and 1 was found dead. None of these 4 mice had developed any signs of papillomas by the time they were removed from the sample population (See tables 3.6a+b).

None of the LMP-1 negative control mice showed any signs of papillomas forming throughout the observation period and no tumours were evident at the end of the experiment. In contrast, 4/14 LMP-1 positive mice had developed small (size 2- see section 3B.1) papillomas by the end of the experiment (See table 3.6a). 2 mice (53F.367 and 53F.388) had single papillomas, and the other two (53F.361 and 53F.368) had 5 and 2 papillomas respectively. Mice 53F.361 and 53F.388 also had the "ringtail" phenotype which often appears in the LMP-1 positive mice with maturity, and the development of this phenotype may have coincided with the appearance of the papillomas (date of appearance of ringtail phenotype not noted accurately). Some papillomas were also noted in the tail base region. Although the DMBA is not applied directly to the tail, some chemical will have reached the base of the tail because the acetone helps to spread the chemical all over the back thereby possibly initiating cells in the tail base region also.

Table 3.6a:- LMP-1 positive mice in chemical carcinogen treatment regime 3 (One single application of DMBA).

Mouse i.d.	Age at start of treatment	LMP	Wk of treatment at death	1st paps (wk)	max. paps
53F.355	8	+	35	/	/
53F.358	8	+	35	/	/
53F.359	8	+	35	/	/
53F.361	8	+	31	28	5
53F.362	8	+	23*	/	/
53F.365	8	+	31	/	/
53F.366	8	+	31	/	/
53F.367	8	+	31	31	1
53F.368	8	+	31	28	2
53F.375	8	+	30	/	/
53F.376	8	+	30	/	/
53F.377	8	+	30	/	/
53F.385	8	+	26	/	/
53F.387	8	+	26	/	/
53F.388	8	+	26	23	1

* = mouse removed from study due to ill health
/ = Zero papillomas

Table 3.6b:- LMP-1 negative mice in chemical carcinogen treatment regime 3 (One single application of DMBA).

Mouse i.d.	Age at start of treat- ment	LMP	Wk of treat- ment at death	1st paps (wk)	max. paps
53F.356	8	-	16*	/	/
53F.357	8	-	35	/	/
53F.360	8	-	35	/	/
53F.369	8	-	9*	/	/
53F.370	8	-	18*	/	/
53F.371	8	-	30	/	/
53F.372	8	-	30	/	/
53F.373	8	-	30	/	/
53F.374	8	-	30	/	/
53F.380	8	-	26	/	/
53F.381	8	-	26	/	/
53F.382	8	-	26	/	/
53F.383	8	-	26	/	/
53F.384	8	-	26	/	/
53F.386	8	-	26	/	/

* = mouse removed from study due to ill health
/ = Zero papillomas

3B.5   Chemical carcinogen regime 4:- DMBA and 1st stage (4 week) TPA treatment.

DMBA initiation and prolonged TPA promotion are required for papillomas and carcinomas to form in wild-type, susceptible strains of mice (Brown and Balmain, 1995). Limiting the promotion stage of chemical carcinogenesis to 4 weeks is insufficient for tumour formation. Promotion takes on average 8 weeks to yield papillomas in normal mice therefore the protocol of DMBA and limited TPA will not give rise to tumours in normal mice (Brown and Balmain, 1995). This treatment however forms the basis for identifying second stage promoters (as described in section 3A.1.3), which alone are not sufficient to induce lesions

The results from the analysis of the ability of LMP-1 to act as a promoter (regime 3- section 3B.4), perhaps suggest that LMP-1 in these mice can act as a weak or second stage promoter. In addition, while LMP-1 increases the papilloma load (in regime 1), no evidence that LMP-1 can act as an initiator was revealed (regime 2). Together this suggests that LMP-1 may act as a promoting agent of carcinogenesis. In a preliminary study to test if LMP-1 acts as a second stage promoting agent, 7 LMP-1 positive and 6 LMP-1 negative mice were subjected to a once only treatment of DMBA followed by 4 weeks of TPA treatment (defined as 1st stage promotion) (Hennings and Boutwell, 1970). This analysis was conducted at a late stage in this project, and it was not possible to conduct a more extensive program with more mice. The work was done with the co-operation of laboratory technician Donald H. Campbell (DHC).

None of the 6 LMP-1 negative mice developed any lesions by 16 weeks after DMBA treatment. In contrast, 7/7 of the LMP-1 positive mice developed papillomas by this time point (See table 3.7). The first papillomas to appear were noted at week 9 and the mean time to papilloma formation was 11 weeks. Taking week sixteen as example, four mice (53F.402, 404, 409, and 412) had one single small papilloma in size range 1. A further two mice (53F.410 and 411) had between 1 and 6 papillomas from size ranges 1-3. The final mouse (53F.401) had several (10) small size one papillomas and one size 2 papilloma. This particular mouse which displayed the greatest number of lesions also had the "ringtail" phenotype. The ringtail phenotype also correlated with increased number of papillomas in the DMBA alone regime. The number of papillomas on the LMP-1 positive group continued to rise during the observation period whilst no papillomas were ever noted on the LMP-1 negative group (figure 3.12). This increase in

papilloma load persists despite lack of continued TPA treatment. None of these papillomas showed conversion to carcinoma (as yet).

Table 3.7:- LMP-1 positive mice and LMP-1 negative mice in chemical carcinogen treatment regime 4 (One single application of DMBA followed by limited(4 weeks) TPA promotion).

Mouse i.d.	Age at start of treatment	LMP	Wk of treatment	1st paps (wk)	max. paps (so far)
53F.401	8	+	16	9	11
53F.402	8	+	16	10	1
53F.404	8	+	16	9	1
53F.409	8	+	16	9	1
53F.410	8	+	16	9	6
53F.411	8	+	16	9	3
53F.412	8	+	16	12	1
53F.403	8	-	16	/	/
53F.405	8	-	16	/	/
53F.406	8	-	16	/	/
53F.407	8	-	16	/	/
53F.408	8	-	16	/	/
53F.413	8	-	16	/	/

These records are shown up to 16 weeks of the study, which is still in progress.

/ = Zero papillomas

106

Comparison of mean papilloma load between LMP-1 positive and LMP-1 negative mouse groups following DMBA initiation and limited (4 week) TPA promotion.
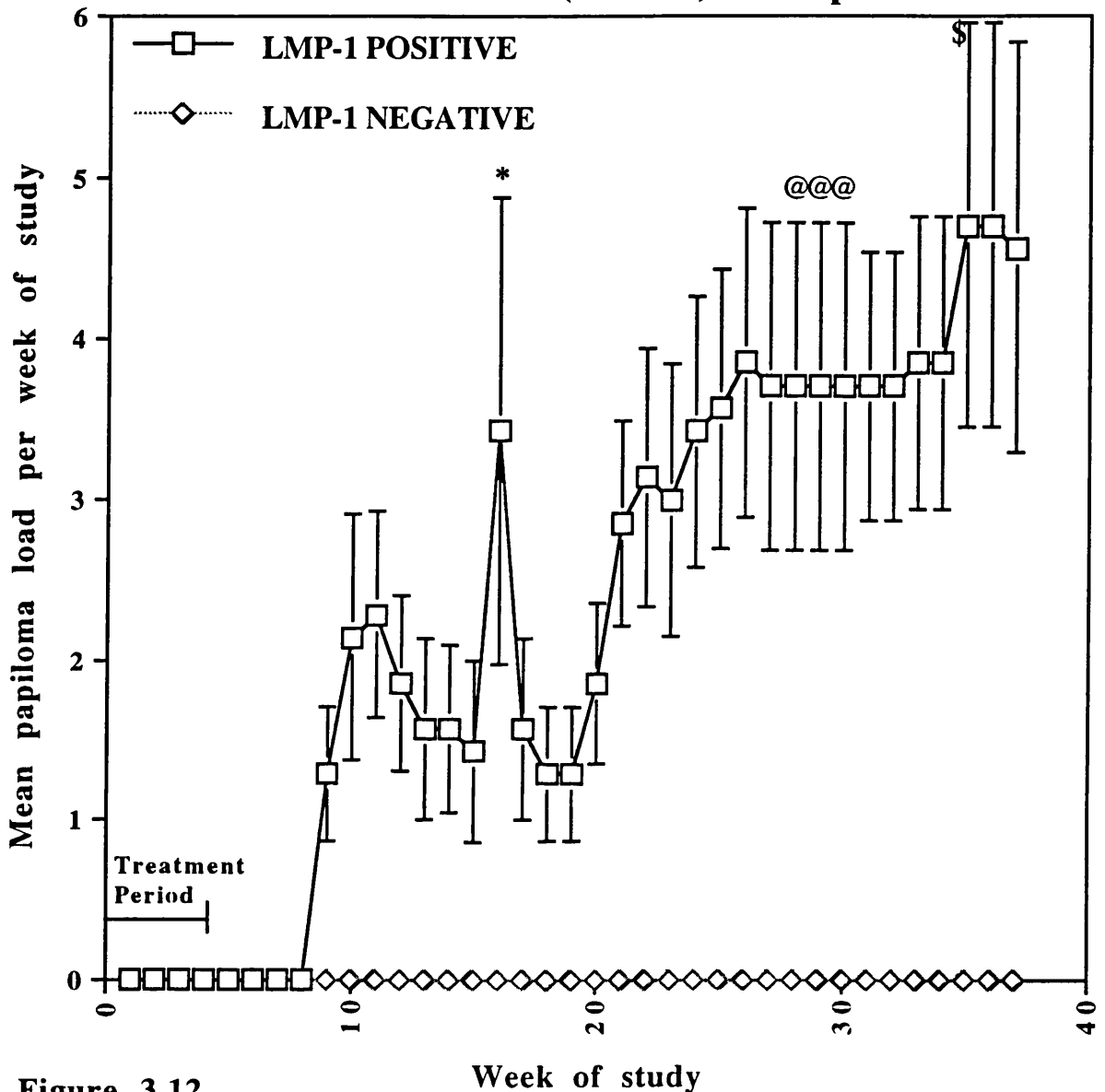
Figure 3.12

This graph shows the formation of papillomas on LMP-1 positive mice compared to the complete lack of lesions arising on LMP-1 negative mice. Every week (except week 16) Donald H. Campbell (DHC) counted the total number of papillomas on each individual mouse. From these, the mean papilloma score per week for each group was calculated and used to plot the above graph.

Error bars represent standard error of the mean

N.B.

* at week 16 the scoring was done by myself and not DHC, perhaps indicating that consistency in the scoring protocol is important.

@ at weeks 28-30 the mice under study were moved to a new animal house and scoring counts was not possible. The mean score for week 27 is shown for these weeks.

$ at week 35 the mice were shaved for the first time since week 27 revealing several more small papilomas which the fur had previously hidden. This explains the sudden rise in mean scores.

107

3C   Discussion of chapter 3.

Chemical carcinogen treatment regime 1 was designed to investigate the potential for LMP-1 to enhance the process of carcinogenesis. As the protocol of DMBA initiation followed by complete TPA promotion will yield papillomas and carcinomas on all susceptible strains of mice, this regime was used to analyse the role of LMP-1 in all stages of tumour progression, from early to late.

The first lesions appeared on the LMP-1 positive group one week before they were noted on the negative control group. When the mean time to lesion appearance was calculated for both groups the LMP-1 positive group achieved a mean time to onset of one week before the negatives. This suggests that LMP-1 may reduce the latency to lesion formation.

The number of papillomas on both groups rose for the duration of the TPA treatment. Here the LMP-1 positive mice developed more papillomas than their negative control littermates and this difference proved to be statistically significant. Therefore the LMP-1 positive group have an increased papilloma load compared to their negative control littermates.

These two results demonstrate that LMP-1 is acting to augment the action of the chemical carcinogens in lesion formation. Papilloma formation is a result of clonal expansion of an initiated cell which has increased its rate of proliferation with a concomitant decrease in differentiation (Brown and Balmain, 1995). Since the LMP-1 positive mice develop more papillomas at a faster rate than the LMP-1 negative mice, this could suggest that LMP-1 can act as an initiator, or a promoter. Results from chapter 2 gave direct evidence that LMP-1 increases proliferation which would support the idea that LMP-1 may act as a promoting agent.

Results from regime 2 demonstrate that LMP-1 (in these mice) cannot substitute for DMBA treatment (and therefore activation of Ha-*ras*) and is thus not acting as an initiating agent in carcinogenesis. This was perhaps unexpected given the similarities between LMP-1 and Ha-*ras* expressing SCC12F cells (Dawson *et al.,* 1990). It has also been suggested that LMP-1 (signalling through the TRAF family) upregulates the expression of the EGFR (Miller *et al.,* 1997). This pathway is linked to the ras pathway by p55Shc (Miller *et al.,* 1995).

Regime 3, where mice were initiated with DMBA but not followed up with TPA promotion, was designed to investigate the possibility that LMP-1 could act as a promoter. Here 4/14 LMP-1 positive mice developed papillomas. This result alluded to the

possibility that LMP-1 may be a tumour promoter, but would be classed as a weak or second (late) stage promoter. Taking this analysis further, mice were subjected to a treatment regime (initiation and limited (4 week) promotion) which is insufficient to yield papillomas in normal mice. The results from regime 4 demonstrates that LMP-1 can act as a second stage promoter. Here 100% of the LMP-1 mice developed papillomas whereas 0% of the negative control littermates did so. The overall number of papillomas formed in regimes 3 and 4 confirm that LMP-1 is a weak promoter.

Taken together these results demonstrate that LMP-1 is a weak promoter, and that LMP-1 can act as a second stage promoter. The level of promotion by LMP-1 might be enhanced if the NPC variant of LMP-1 was used to create the PyLMP-1 transgenic mice. This variant has already been shown to be more oncogenic than the B95-8 prototype LMP-1. Furthermore, the level of expression of LMP-1 in line 53 is known to be low and this might effect the level of promotion by LMP-1. Finally the observed suprabasal expression (chapter 2) may be relevant to the degree of promotional activity. Basal layer expression might have a stronger promoting effect. These points may also be relevant to the observed lack of initiating activity by LMP-1.

These results may have direct relevance to the disease state of NPC. Here an association between salted fish and incidence of NPC has been documented. The salting process gives rise to volatile nitrosamines which have been shown to have tumour promoting ability (Zou and Landolph, 1991; Yu et al., 1989). The combination of the tumour promoting ability of the nitrosamines and the tumour promoting ability of LMP-1 may be critical in the pathogenesis of NPC.

Regime 1 also revealed that LMP-1 did not influence the conversion rate of papilloma to carcinoma or squamous cell carcinoma to spindle cell carcinoma in these studies. These results suggest that LMP-1 does not alter the course of carcinogenesis once it has reached the papilloma stage. However, it may still be required to promote proliferation. Examination of LMP-1 expression in NPC cases shows that the protein is not consistently detected. It has been proposed that LMP-1 is active early on during disease progression but is then switched off. LMP-1 expressing NPC's were shown to grow faster than non-expressing NPC's (Hu et al., 1995) suggesting that LMP-1 increases proliferation early may not influence progression at later stages.

The demonstrations that LMP-1 is involved in the early stages of carcinogenesis may also be important concerning LMP-1 detection in NPC samples. Perhaps the reason less than 100% of

NPC cases express LMP-1 is because the action of LMP-1 has been superseded during progression.

CHAPTER 4:- Crossbreeding analysis of the interaction between LMP-1 and Ras, LMP-1 and TGFβ, and LMP-1 with loss of p53.

4A   Introduction

Because Ha-*ras* , TGFβ1, and p53 have been shown to play important roles in carcinogenesis, including chemically induced skin carcinogenesis, transgenic mice harbouring the activated *ras* gene, TGFβ1, and null p53 were chosen for cross-breeding studies with the LMP-1 mice. Therefore the general function of each protein is described in greater detail in the following sections.

4A.1   The Ha-Ras protein

Studies of chemical carcinogen induced carcinomas of mouse epidermis have shown that mutation in the Ha-*ras* gene at the cellular level is a major initiating event in carcinogenesis. Further loss of WT Ha-*ras* alleles coupled with duplication of mutant Ha-*ras* is also important in the progression from papilloma to squamous cell carcinoma to spindle cell carcinoma.

Ha-ras is one of four 21kDa proteins known collectively as the p21ras proteins. The other three *ras* family members are Ki-*ras* , N-*ras* , and R-*ras* (Reviewed in McCormick, 1989). The ras proteins bind guanine nucleotides with high affinity and act as GTPase's. Ras cycles between an active GTP-bound form and an inactive GDP-bound form and serves as a molecular switch in signal transduction. Therefore ras proteins play an important role in the normal growth of cells. However, many cancers have been identified where a mutation in *ras* has led to it being constitutively active indicating that the regulation of *ras* is carefully monitored under normal circumstances.

p21*ras* is activated upon receptor tyrosine kinase stimulation (Reviewed in Pronk, 1994). For example, both EGFR and IGF-1R stimulation activate p21*ras* measured by a shift from rasGDP to rasGTP. This activation is rapid and parallels the autophosphorylation of the receptor. The level and duration of *ras* activation depends on the cell type and the growth factor receptor that is activated.

The dissociation of GDP followed by the binding of GTP is slow as is the GTPase activity of ras in the hydrolysis of the bound GTP. Increasing the guanine nucleotide exchange activity and/or inhibiting GTPase activity would therefore result in activation of *ras*. Both these mechanisms of activating *ras* are therefore

carefully controlled (Reviewed in Downward, 1992). GTPase activating proteins (GAP's) increase the rate of hydrolysis of GTP bound ras, thus keeping ras in a GDP-bound, inactive state. These are therefore negative regulators of ras. (Reviewed in Tocque *et al*, 1997). The inability of mutant forms of ras to bind to ras-GAP would maintain *ras* in an active state. Inhibiting ras-GAP would also have the effect of maintaining the active state of *ras*. The common mutation in codon 61 of Ha-*ras* caused by DMBA treatment of mouse dorsal skin has the effect of reducing the rate of hydrolysis of GTP bound Ha-ras by blocking its stimulation by GAP's. Thus, Ha-*ras* becomes constitutively activated.

Nucleotide exchange factors such as ras-GDP releasing factor (ras-GRF), which control the rate of exchange of GDP for GTP, are positive regulators of the ras cycle. Increasing the activity of ras-GRF would create more active ras. Activated p21 ras associates with the serine threonine kinase raf (via the ras effector region) which is normally cytosolic, and translocates it to the plasma membrane (Leevers *et al*, 1994). Here raf becomes activated and phosphorylates either directly or indirectly, mitogen activated protein kinase-kinase (MAPKK) (Kyriakis *et al*, 1992). MAPKK is then activated and in turn phosphorylates MAP kinase (MAPK) causing it to become activated (Reviewed in Geilen *et al*, 1996). This pathway then conveys growth and differentiation signals to the nucleus, resulting in appropriate gene expression for example fos activation in the transcription factor complex AP1.

It has recently become clear that this linear model for ras mediated signalling is not the only mechanism by which activated ras can send information. The PKC pathway has been implicated with an involvement in ras signalling which leads to reorganization of the actin skeleton (Tocque *et al*, 1997). The ras story is therefore far from complete and awaits further work on downstream effectors of ras signalling.

Activated *ras* transgenics in a C57Bl/6 background were generated in the laboratory of A.Balmain utilizing a truncated K promoter resulting in expression of an activated Ha-*Ras* oncogene in the hair follicles of the epidermis. These mice display a phenotype of hyperkeratosis and developed sebacious adenomas at sites of irritation and wounding. Thus, benign tumours were frequently seen on the ears, base of tail and around the eyes. These lesions however did not progress to malignancy. When the mice were bred into an FVB strain background the phenotype became more severe (Personal communication-K. Brown). As early as day 1, some transgenic positives showed signs of papillomatous growth. This phenotype had a 30% penetrance and ranged from severe, where large areas of papillomatous growths developed and merged to cover the majority of the body (requiring sacrifice

of the animal), to mild where very discrete patches of papillomatous growth were observed. Other groups had similar results from their transgenic analysis of the *in vivo* effects of *ras* (Greenhalgh, D.A., *et al* , 1993 and Leder, A., *et al* , 1990). The conclusions drawn were that activated *ras* expression abrogated the need for an initiation step in carcinogenesis, but for tumours to form, promotion events such as wounding were still necessary.

4A.2   The TGFβl protein

The Transforming Growth Factor β (TGFβ) family consists of three multifunctional peptides with wideranging effects. They have been implicated in many cellular processes such as cell proliferation and differentiation, embryonic development, extracellular matrix formation, bone development, wound healing, hematopoiesis, and immune and inflammatory cell responses (Reviewed in Akhurst *et al*, 1992; and Okragly *et al*, Promega Notes No.47). Each of the three isoforms has been highly conserved throughout evolution, suggesting specific roles for each. However, they are often co-expressed and it has therefore been difficult to define a precise biological role for each individual isoform.

TGFβ's are synthesized as biologically inactive latent complexes. Active TGFβ is processed from the C-terminus of a larger peptide, but the remaining portion, known as the latency associated peptide, continues to associate non-covalently with TGFβ and only dissociation from this peptide results in full activity. The TGFβ proteins bind to sets of two membrane proteins called TGFβ receptors (TβRI and TβRII) (Reviewed in Massague, 1992). TβRI does not bind TGFβ when expressed alone but only when complexed to TβRII and it is thought that TGFβ functions to recruit TβRI into a stable complex with TβRII (Wrana *et al*, 1992 & 1994). Signalling occurs through TβRII but this fails to happen in the absence of TβRI. Thus both receptors are essential for both the binding and subsequent signalling action of TGFβ.

TGFβl knockout mice have been generated (Kulkarni *et al*, 1993). There was clear embryonic lethality, but 1/3 of the fetuses developed to term and appeared clinically normal at birth. However, at 3-4 weeks these mice died showing massive inflammatory lesions in many organs, thus suggesting a role for TGFβl in the homeostatic regulation of the immune response consistent with previous reports of TGFβl being a potent immunosuppressor.

There are also several reports of TGFβ1 transgenic overexpression with conflicting results. In one case an epidermal specific expression vector was used which did not express until d15 of development because TGFβ1 overexpression at early stages of embryogenesis can result in lethality *in utero* (Sellhayer *et al*, 1993). This gave rise to mice born with a very shiny and tautly stretched skin which affected their ability to move and breathe. BrdU analysis of the skin showed a marked reduction in the number of replicating cells in the epidermis and hair follicles. Death occurred within 24hrs and it was assumed that TGFβ1 expression was acting as a potent inhibitor of epithelial cell proliferation and so, when expression was turned on at d15 of embryogenesis the skin ceased to grow whilst the rest of the body continued to grow and stretch the skin.

The opposite result was seen by another group using the K10 promoter to direct expression of TGFβ1 to suprabasal keratinocytes (Cui *et al*, 1995). In these mice (termed line H) no phenotype was observed but BrdU analysis indicated a 2-3 fold elevation of DNA synthesis in the epidermis. This could be caused by a number of factors. Either TGFβ1 directly induces proliferation, or alternatively it induces epithelial differentiation and to compensate for this, proliferation must also increase, thus giving the net result of a normal phenotype. These K10-TGFβ1 transgenic mice did not exhibit the normal wave of basal keratinocyte DNA synthesis which normally occurs under conditions of perturbed homeostasis following TPA treatment. Therefore TGFβ1 is acting as expected in this case, as a negative regulator of growth when hyperplasia is induced. TβRII levels were shown to increase following TPA treatment and prior to the growth inhibitory response to TGFβ1. Its level of expression correlated with the growth sensitivity to TGFβ1 *in vivo* and *in vitro* suggesting that TGFβ1 and TβRII are part of the endogenous homeostatic regulatory machinery of the epidermis.

This was confirmed when a dominant-negative TβRII was expressed in the epidermis of transgenic mice (Wang *et al*, 1997). The transgenic mice displayed a thickened and wrinkled skin showing hyperplasia and hyperkeratosis. BrdU labellng showed a 2.5 increase in proliferating cells over controls. When the mice were bred to homozygosity, perinatal lethality occurred due to the hyperkeratotic phenotype. These results therefore confirm that TGFβ1 and TβRII are intimately involved in the maintenance of epidermal homeostasis.

To study the effect of inducible TGFβ1 expression rather than constitutive TGFβ1 expression further transgenic lines were

created using the K6 promoter. K6 is known to be expressed in an inducible manner in hyperplastic interfollicular epidermis, as well as being present in the outer root sheath keratinocytes of hair follicles. Seven lines of mice were created using an inducible bovine K6 promoter and either a constitutively activated form of TGFβ1 or WT TGFβ1 (Fowlis *et al*, 1996). In the lines produced it was intended that TGFβ1 should only be expressed following experimental manipulation such as TPA stimulation through the K6 promoter. Indeed there was no detectable epidermal expresssion of the transgene in any of the lines except line M5 which had constitutive, but low level, expression of TGFβ1. None of the lines, including M5, showed any adverse health effects as a consequence of the transgene and their skin and hair were morphologically and histologically identical to controls. Cell culture of K6-TGFβ1 transgenic keratinocytes and TPA treatment of intact dorsal skin both caused the induction of K6 and hence transgenic TGFβ1 expression, and this was highest in line M5. Line M5, like that of line H (Cui *et al*, 1995), showed a 2 fold increase in BrdU labelling of untreated dorsal skin compared to controls indicating a positive effect on epidermal proliferation. A positive effect on epidermal proliferation was also maintained up until 24hr post TPA treatment after which time the hyperplastic response was attenuated. Therefore, TGFβ1 induction in this instance is negatively regulating keratinocyte proliferation. Thus, TGFβ1 can act as a positive or negative epidermal growth regulator

These experiments have suitably demonstrated the multifunctional role of TGFβ1 in differentiation, proliferation, embryogenesis and immune/inflammatory responses. It is not altogether surprising therefore that imbalance of the homeostasis of the epidermis due to carcinogenesis should involve altered expression of TGFβ1. For example, TGFβ1 is rapidly induced in post TPA treated suprabasal keratinocytes (Akhurst *et al.*, 1988) but in high risk papillomas and the squamous cell carcinomas that arise from these papillomas, TGFβ1 is down regulated (Fowlis *et al.*, 1992; Glick *et al.*, 1993). Furthermore, TGFβ1 expression in K10-TGFβ1 transgenics acted initially as a tumour suppressor but latterly as a tumour enhancer (Cui *et al.*, 1996). Therefore expression of TGFβ1 is indeed altered during carcinogenesis.

4A.3   The p53 protein

p53 has been described as the "guardian of the genome" (Lane, 1992) because of its role following DNA damage to a cell leading to apoptosis or a pause in cell cycle and repair. When it

was first discovered it was classed as an oncogene because it could transform cells, but closer analysis showed that these p53 clones harbored mutations while WT p53 inhibited cell growth (Reviewed in Levine *et al*, 1991). Many human tumors have deletions of a small region of chromosome 17p which was later shown to include the p53 gene (Chromosome 11 for mouse). This led to the hypothesis that p53 was a tumour suppressor gene and indeed the p53 gene is the most commonly altered gene yet identified in human tumors suggesting that loss of WT function removes the protection provided by p53. So whilst functional WT p53 might indeed be the "guardian of the genome", mutated p53 is "a deadly inheritance" (Vogelstein, 1990).

The p53 protein is a DNA binding transcription factor that enhances the rate of transcription of several known genes that carry out, at least in part, the p53-dependent functions in a cell. Under normal circumstances p53 acts as a negative regulator of cell growth (Perry and Levine, 1993).

Crystallographic studies on p53 have shown that many of the frequently mutated amino acids in tumours have close contact with the DNA in a protein-DNA complex. Therefore disruption of the specific DNA-binding function is likely to be a critical event in the loss of WT p53 function.

p53 is mainly localized to the nucleus where levels are low in quiescent normal lymphocytes due to the protein's short half-life, but increase upon a cell's entry into the cell cycle. Overexpression of p53 in a variety of cell types results in arrest in the G_1 phase of the cell cycle or induction of programed cell death (apoptosis). Furthermore, DNA damage results in increased levels of p53 and a block in G_1, suggesting that p53 functions as a component of the cellular response to DNA damage, acting as a negative growth regulator (hence " genome guardian"). Upstream events which lead to p53 activation are mediated by stress, including DNA damage, hypoxia, heat shock, calcium phosphate treatment, and ribonucleotide depletion (Ruaro *et al*, 1997; and Levine, 1997).

p53 functions to activate p21 which then binds to a number of cyclin and cdk complexes to block cell cycle progression. p53 may also be involved in cell cycle regulation at two other checkpoints. These are the G_2/M phase checkpoint and the G_0-G_1-S phase transition although evidence for this is still accumulating.

Regulation of the cell cycle and the role of p53 in controlling it is obviously very complex. However it is apparent that a failure in the pathway brought about by p53 mutation/inactivation could lead to uncontrolled growth, and if the DNA was damaged in some way yet not repaired because of the unchecked growth, then neoplasia could result.

p53 activation gives rise to a second choice other than that of cell cycle arrest, namely apoptosis. Under circumstances where DNA damage is unrepairable, survival factors for the cells are limiting, or an activated oncogene is forcing the cell into a replicative cycle, p53-mediated apoptosis prevails. Therefore p53 inactivation is important on two counts with regard to the development of tumours. Firstly loss of p53 control of the cell cycle could increase the risk of malignant conversion, secondly, once tumours are established, loss of p53 would inhibit apoptosis and allow the tumour cell to survive.

p53 has been shown to bind to a number of cellular proteins. For example, the proto-oncogene nuclear product c-Abl is activated for its kinase activity following DNA damage, and c-Abl binds to p53 and enhances its transcriptional activity. This is therefore a positive regulatory role as it enhances the ability of p53 to induce cell cycle arrest at G_1. MDM2 represents the product of another proto-oncogene which binds to p53. MDM2 is actually transcriptionally activated by p53 and its function is to inactivate p53 mediated transcription. This therefore forms an autoregulatory loop so that p53 does not remain permanently activated longer than necessary and the cell can re-enter the cell cycle once the stress (DNA damage etc.) is removed.

The descriptions of p53 as the "Guardian of the genome" and the "Cellular gatekeeper for growth and division" are therefore well named. That 50-55% of human tumours display mutations in the p53 gene serves to illustrate the importance of maintaining two functional copies of the gene. Knockout mouse studies have further shown the importance of WT p53 in the inhibition of cancer (Donehower et al, 1992). Mice deficient for p53 developed normally. However these animals were prone to the spontaneous development of a variety of neoplasms. 74% of homozygous knockout (-/-) mice developed neoplasms by 6 months of age with a mean time to tumour onset of only 20 weeks. These mice succumbed to malignant lymphomas most frequently (70% of the tumours). Sarcomas were also identified. Heterozygous (+/-) knockout mice also succumbed to tumour formation but with a delayed onset compared to the -/- mice (Harvey et al, 1993). By 12 months of age only 8% of the +/- mice had developed tumours, but from 12 months onwards the rate of tumour development accelerated such that by 18 months of age, 50% of the +/- mice had developed tumours. The tumour type most commonly observed in this group of mice was different to the -/- mice, with 58% of the +/- mice developing osteosarcoma or sarcoma of soft tissues and only 32% developing lymphoma. Of the tumours arising in the p53+/- mice, 55% showed loss of the remaining WT allele which was not associated with a particular tumour type.

Thus it is clear that loss of one or both copies of p53 can quickly lead to tumour onset.

4A.4   Rationale for crossbreeding studies with LMP-1 transgenic mice.

Transgenic mice have enabled detailed study of the *in vivo* effects of individual proteins expressed in the epidermis during the process of skin carcinogenesis. Creating bi-transgenics then allows analysis of possible synergenistic effects during carcinogenic progression, where lack of synergy might indicate the oncogenes act in the same or parallel pathways. Bi-transgenics also allow identification of the stage of carcinogenesis where oncogene expression becomes causal, and allow determination of the nature of the events required to achieve malignancy.

PyLMP-1 mice were crossbred with K5-Ha*ras* , p53 null, K10-TGFβ1(line H), and K6-TGFβ1(line M5) mice in order to examine co-operation versus redundancy or interference.

Results from chapter 3 might predict that Ras and LMP-1 co-operate as an initiator and a promoter. Results from chapter 2 demonstrate that LMP-1 promotes proliferation. Therefore it was thought that it might be possible to mimic the carcinogenic treatment (e.g. DMBA induced *ras* mutation and TPA induced proliferation) instigated phenotype by mating activated *ras* transgenics with the PyLMP-1 mice. If LMP-1 was redundant with mutant ras (not seen in chemical carcinogen study regime 3) then no co-operation between these proteins would be expected. If however, the LMP-1 induced hyperplastic phenotype acted in conjunction with activated ras, enhancement of the carcinogenic process might be expected.

The potentially opposite effects of LMP-1 and TGFβ1 could predict opposite action to nullify the effects. The rationale behind the crossbreeding of LMP-1 with K6-TGFβ1 mice was threefold. Line M5 expresses TGFβ1 at very low levels (below those levels needed to detect the cellular source of the RNA by *in situ* hybridisation) and requires TPA induced hyperplasia to induce high levels of TGFβ1. Therefore, can the LMP-1 induced hyperplasia activate the expression of TGFβ1? or indeed enhance the expression of TGFβ1 via its ability to induce K6 expression? If TGFβ1 can block the proliferative response to TPA, can it also affect the hyperplastic phenotype of the LMP-1 mice via its role as a potent negative growth regulator?   If TGFβ1 is important in carcinogenesis, does it have an effect on the oncogenic potential of LMP-1? The question asked by the line H cross was, can

suprabasal expression of TGFβ1 exert a negative regulatory effect and abrogate the hyperplastic response generated by LMP-1?

If LMP-1 can act in part to inhibit p53 action (see section 1D.4), then this cross might show a degree of redundancy. Thus, the rationale behind cross-breeding PyLMP-1 mice with p53 knockout mice was to analyse the effects of the LMP-1 oncogene in the epidermis in the absence of tumour suppression from p53. Although mutations in p53 are not found in NPC, they have been shown to be a major component in the mouse skin model of multistage carcinogenesis

The experiments described in this chapter were therefore conducted to test these hypotheses.

4B   RESULTS

4B.1   Co-expression of LMP-1 and activated *Ras*

PyLMP-1 positive mice in a C57Bl/6 background (line 53) were bred with K5-Ha*ras* positive mice also in a C57Bl/6 background, and the offspring monitored. There was no difference between the LMP-1/*ras* transgene positives and the LMP-1 transgene positives at day 7, the time point where the LMP-1 skin phenotype is most pronounced. The mice which were positive for the *ras* transgene only had no phenotype at an early age and so were indistinguishable from the negative mice. Transgenic status was determined by southern blot analysis of tail-tipped DNA and the four categories of mice were then analysed for the duration of the study. These were:-
1) LMP-1/*ras* bi-transgenic positives =29 mice (only 19 used)
2) *ras* single transgene positives =63 mice (only 20 used)
3) LMP-1 single transgene positives (=16 mice)
4) Negative controls (=28 mice)

These mice were not born in the expected 1:1:1:1 ratio (with expected 34 mice per group) but instead were more like a 2:4:1:2 ratio. The bi-transgenics and negative control groups were slightly below expected whilst the *ras* single transgene positive group was almost double the total expected. The LMP-1 single transgene positive group was less than half the total expected.

At three months of age the first mice started to develop K5-Ha*ras* associated lesions around the eyes and ears (tables 4.1a+b), and these lesions were identified as sebacious adenomas.

Table 4.1a:- Table of mice positive for both the LMP-1 and Ras transgenes indicating the age of the mice when the first sebacious adenomas (polyps) appeared, and the accumulation of these lesions.

I.D.	1st polyps	1 mo	2 mo	3 mo	4 mo	5 mo	6 mo	7 mo	8 mo	9 mo	10 mo	11 mo	12 mo	13 mo	14 mo	15 mo	max
141	6	0	0	0	0	0	3	5	5	8	8	12	20	D			20
147	9	0	0	0	0	0	0	0	0	2	5	3	0	3	5	8	8
149	11	0	0	0	0	0	0	0	0	0	0	1	5	2	D		5
159	5	0	0	0	0	2	2	2	2	3	5	13	12	12	13	20	20
176	5	0	0	0	0	3	5	6	8	5	8	12	D				12
177	7	0	0	0	0	0	0	1	2	2	3	2	0	2	5	5	5
186	10	0	0	0	0	0	0	0	0	0	1	5	5	6	T		6
190	5	0	0	0	0	3	5	9	20	20	20	28	25	22	T		28
191	6	0	0	0	0	0	1	5	11	8	5	8	10	12	T		12
192	3	0	0	2	2	4	5	7	8	10	11	15	T				15
206	5	0	0	0	0	1	1	1	0	0	5	7	T				7
213	10	0	0	0	0	0	0	0	0	0	4	8	T				8
223	7	0	0	0	0	0	0	1	4	10	19	T					19
227	5	0	0	0	0	4	5	2	4	7	20	T					20
230	7	0	0	0	0	0	0	3	5	5	12	T					12
232	5	0	0	0	0	4	5	5	10	20	28	T					28
256	4	0	0	0	1	3	4	5	6	10	12	T					12
282	7	0	0	0	0	0	0	3	5	5	T						5
284	6	0	0	0	0	0	1	6	12	17	T						17

Av.= 6.47   Av.= 7.00   Av.= 13.63

Note:-   D= dead
           T= experiment terminated

Table 4.1b:- Table of mice positive for the Ras transgene only, indicating the age of the mice when the first sebacious adenomas (polyps) appeared, and the accumulation of these lesions

I.D.	1st polyps	1 mo	2 mo	3 mo	4 mo	5 mo	6 mo	7 mo	8 mo	9 mo	10 mo	11 mo	12 mo	13 mo	14 mo	15 mo	max
163	5	0	0	0	0	2	5	8	10	20	20	26	30	40	36	45	45
169	5	0	0	0	0	1	1	2	2	15	20	18	18	22	30	50	50
170	5	0	0	0	0	2	4	6	8	10	15	15	15	20	25	35	35
172	5	0	0	0	0	1	1	1	1	0	0	5	10	25	33	40	40
173	5	0	0	0	0	5	11	15	15	20	25	30	33	30	60	D	60
175	5	0	0	0	0	2	8	12	12	16	20	22	25	30	45	60	60
181	7	0	0	0	0	0	0	1	1	4	5	12	20	35	T		35
184	7	0	0	0	0	0	0	1	1	2	4	10	20	25	T		25
189	6	0	0	0	0	0	1	3	8	5	4	5	17	25	T		25
195	7	0	0	0	0	0	0	2	2	3	5	11	20	T			20
207	8	0	0	0	0	0	0	0	4	5	17	30	T				30
208	3	0	0	1	0	0	5	D									5
210	5	0	0	0	0	2	2	2	2	9	20	35	T				35
216	4	0	0	0	2	5	10	20	30	38	50	60	T				60
226	7	0	0	0	0	0	0	3	4	6	15	T					15
228	5	0	0	0	0	4	4	3	10	10	12	T					12
233	4	0	0	0	2	10	20	25	28	35	30	T					35
251	4	0	0	0	1	2	3	6	15	33	T						33
253	4	0	0	0	1	4	6	14	15	26	T						26
257	3	0	0	3	4	10	15	22	D								22

Av.= 5.20   Av.= 14.28   Av.= 33.40

Note:-   D= dead
              T= experiment terminated

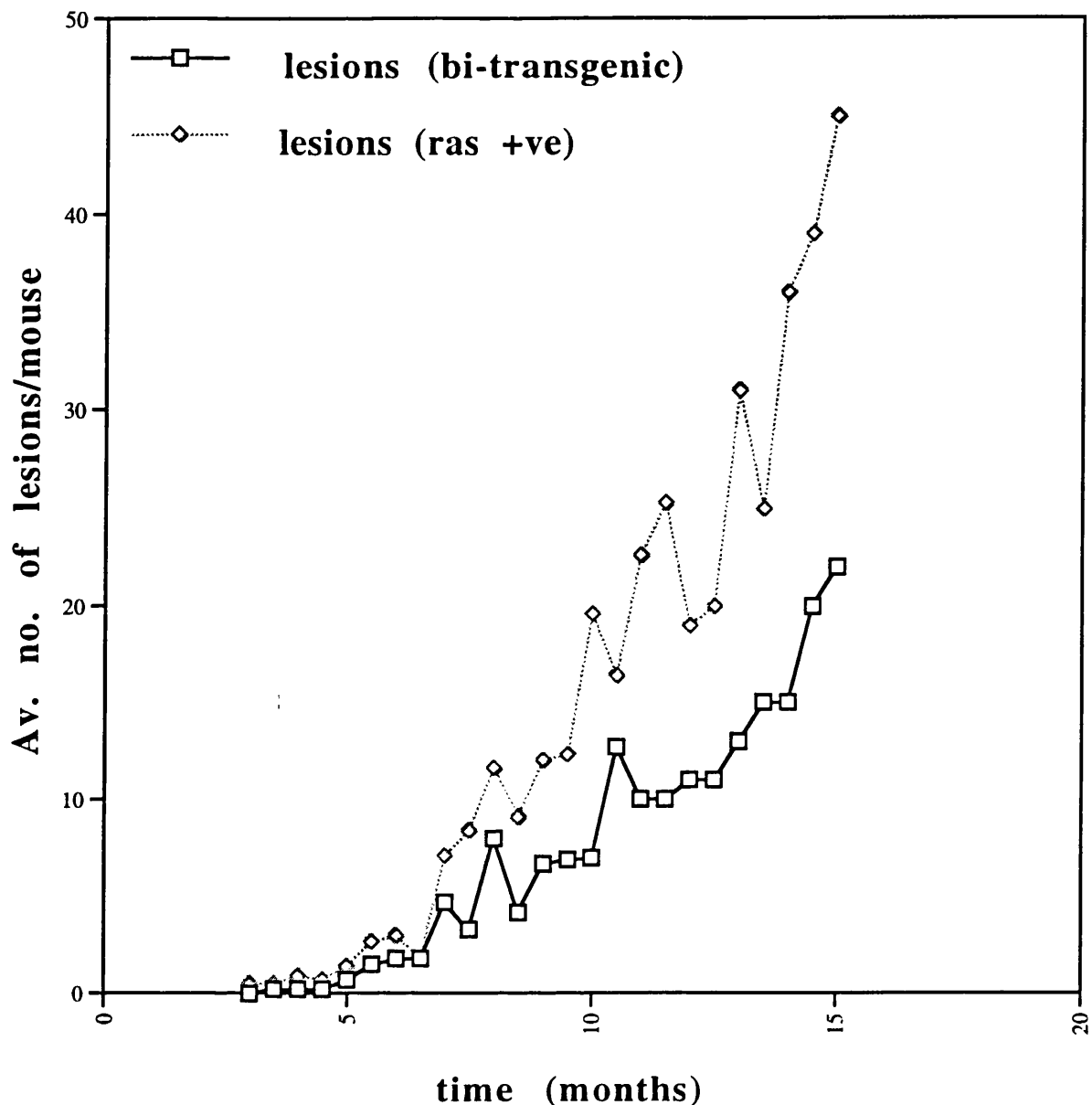Average number of lesions on ras
v's bi-transgenic mice.

Figure 4.1
Every two weeks the number of polyps (ears+eyes) on each individual mouse was counted. The counts for each group at each time point were totalled and the mean number of polyps per group per two week interval was calculated. These mean numbers were used to plot the above graph.

By counting the number of lesions on each mouse at two week intervals, the average number of lesions for each group of mice could be calculated and a graph of lesion formation against time could be plotted for each category (fig 4.1).

Neither the LMP-1 single transgene positive mice nor the negative control mice developed the K5-Ha*ras* associated lesions as expected. The LMP-1/*ras* bi-transgenic mice did not develop any more lesions than the *ras* single positive mice and instead developed fewer lesions overall. The average age to lesion formation for the bi-transgenic LMP-1/*ras* mice was 6.47 months and this group had an average of 7 lesions/mouse at 9 months of age. The *ras* single positive mice developed lesions slightly faster with an average age to onset of 5.20 months and this group had an average of 14.28 lesions/mouse at the age of 9 months. This difference in average numbers of lesions proved to be significant with a T value of 2.3, a P value of 0.030 and 25 degrees of freedom. Thus the *ras* single positive mice develop statistically significantly greater number of lesions than do the bi-transgenic mice. It should be noted that the data used to draw these conclusions covers a wide range. For example, at week 9 the bi-transgenic group has between 0-20 lesions and the *ras* transgenic group between 0-38 lesions.

Some of the lesions progressed from the white nodule appearance of the adenomas to a larger more papillomatous appearance. A small number of these adenomas and papilloma "like" lesions from both bi-transgenic and *ras* single positive mice were stored in fixative awaiting future analysis. None of the lesions progressed further to carcinoma. Furthermore, no skin papillomas in other areas were observed throughout the study.

In order to examine epidermal proliferation rates in these mice 7 day old bi-transgenic and *ras* single positive mouse pups were BrdU injected and skin, tongue and tail tissues collected and analysed as described in section 7B.4.5. The BrdU staining and counting revealed that the bi-transgenics had significantly higher numbers of proliferating cells in the dorsal skin epidermis than did the *ras* transgenics (see table 4.2). The mean BrdU score for bi-transgenic dorsal skin was 30.7 compared to 17.64 for K5-Ha*ras* positive transgenic dorsal skin. A two-sample T-test analysis of the data in table 4.2 gave a significant T value of 3.59 with a P value of 0.0012 and 30 degrees of freedom when comparing bi-transgenic dorsal skin BrdU counts with K5-Ha*ras* transgenic dorsal skin BrdU counts.

Table 4.2:-

Table indicating the mice used to accumulate the BrdU totals and mean values of K10-Ha-*Ras* transgenics and bi-transgenics (PyLMP-1 x K10-Ha-*Ras*). Where counts were not possible due to bad sectioning or poor staining, this is indicated as Not Done (ND).

Mouse I.D.	LMP-1	RAS	Age	BrdU SKIN count(s)	BrdU TONGUE count(s)
769.237	-	+	d7	18.9, 28	24, 24
769.298	-	+	d7	18	12
769.299	-	+	d7	12.9, 17	7
769.300	-	+	d7	12.4, 14	21, 28
769.329	-	+	d7	14.2	ND
769.330	-	+	d7	13.6	ND
769.334	-	+	d7	8	ND
769.336	-	+	d7	11.3	ND
769.338	-	+	d7	26	ND
769.339	-	+	d7	35	ND

Mean= **17.64** SE= 2.1   Mean= **19.33** SE= 3.3

Mouse I.D.	LMP-1	RAS	Age	BrdU SKIN count(s)	BrdU TONGUE count(s)
769.238	+	+	d7	42.6, 66	49
769.296	+	+	d7	38, 33.2, 41.4	56, 36
769.297	+	+	d7	23.2, 29.4	49, 25
769.332	+	+	d7	25.4, 34	ND
769.333	+	+	d7	22, 26.4	ND
769.340	+	+	d7	42.6, 33.5	ND
769.362	+	+	d7	15.8	ND
769.364	+	+	d7	19.4	ND
769.365	+	+	d7	16.2, 17.6, 17.8	ND
769.366	+	+	d7	50	ND

Mean= **30.7** SE= 2.9   Mean= **43.00** SE= 5.5

Comparison of mean BrdU scores for K10-Ha-*ras* positive dorsal skin versus K10-Ha-*ras* x PyLMP-1 bi-transgenics
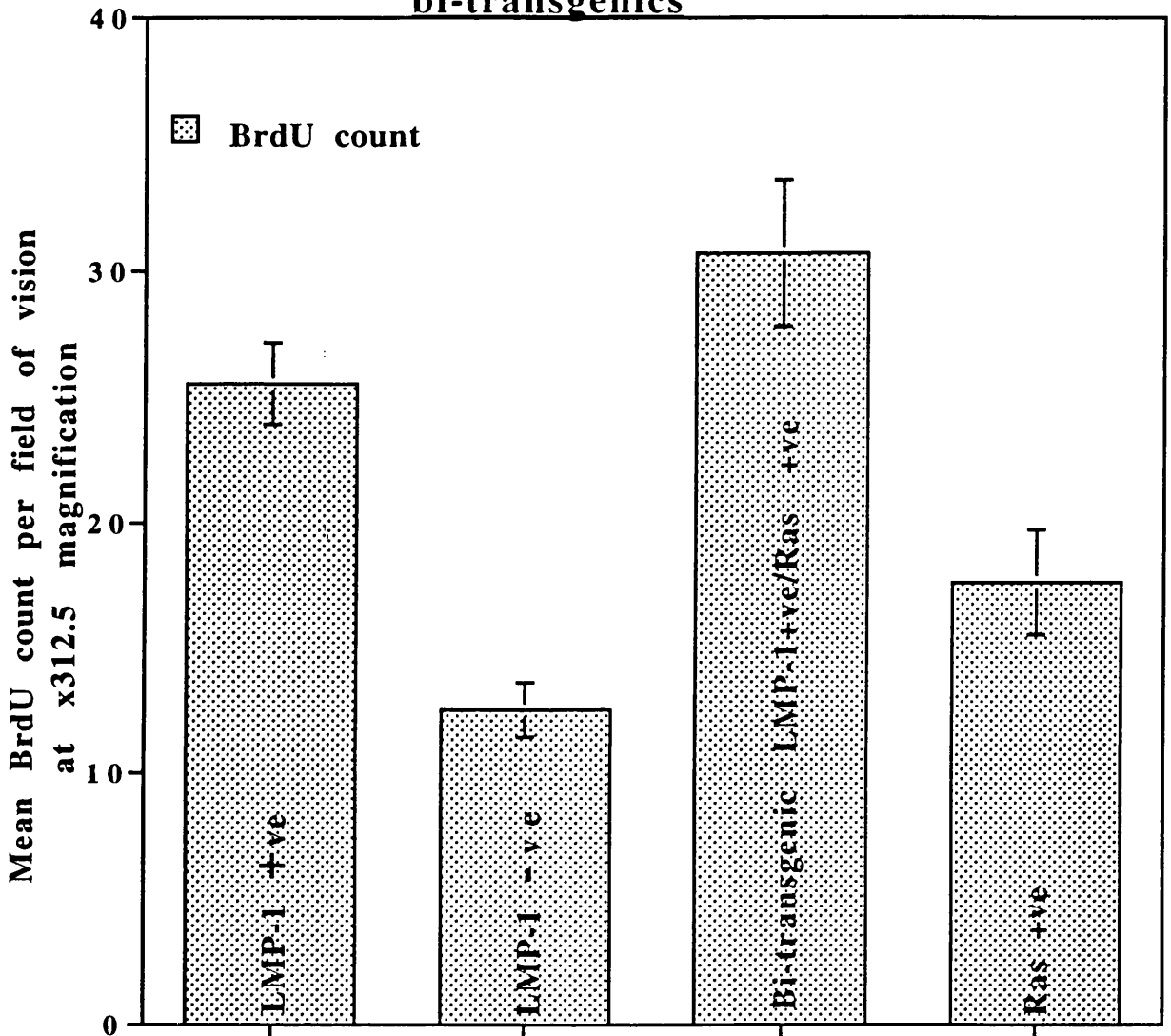
Figure 4.2

7μm sections of dorsal skin (from BrdU injected mice) were stained with an anti-BrdU antibody (section 7B.4.5), and positive cells (brown) in the epidermis were counted per field of vision at x312.5 magnification. This figure shows the mean score for each transgenic group studied. The BrdU counts for LMP-1 positive and negative control epidermis are included to allow a direct comparison with the mean BrdU scores for LMP-1/Ras bi-transgenics and Ras transgenics. Each mean was derived from at least 5 different sections from at least 3 different mice. Each section was counted for 10 fields of vision and then averaged (mean). Each mean was then totalled to calculate the overall mean score for each group. Error bars represent the standard error of the mean.

However, there was no significant difference between LMP-1 positive epidermal BrdU counts and bi-transgenic epidermal BrdU counts (see figure 4.2) suggesting that the increased levels of proliferation noted in the bi-transgenics is induced almost exclusively by LMP-1. The mean BrdU score for bi-transgenic dorsal skin of 30.7 was only slightly higher than that for the PyLMP-1 (line 53) dorsal skin mean BrdU score of 25.53. Comparing the BrdU scores for these two groups via the two-sample T-test resulted in a T value of 1.54 with P=0.14 and 27 degrees of freedom. The difference in mean scores between these two groups is therefore not significant.

PyLMP-1 dorsal skin did however have a significantly higher mean BrdU score than K5-Ha*ras* dorsal skin. The mean labelling score for line 53 dorsal skin was 25.53 compared to 17.64 for the K5-Ha*ras* transgenics. This gives a significant two-sample T-test T value of 2.95 with a P value of 0.0079 and 20 degrees of freedom. This therefore confirms that the increased levels of proliferation noted in the bi-transgenic epidermis are mostly accountable to the effects of the PyLMP-1 transgene.

Comparing the dorsal skin BrdU counts of the K5-Ha*ras* transgenic group (see table 4.2) with those of the LMP-1 negative control group (see table 2.1) shows a small but significant difference between these groups. A T value of 2.12 with P=0.048 and 18 degrees of freedom suggests that the K10-Ha-*ras* transgenic mice do indeed demonstrate a small increase in proliferation compared to negative controls and therefore may contribute a small effect to the bi-transgenic BrdU mean dorsal skin score.

K5-Ha*ras* mice in the FVB background were also mated with line 53 PyLMP-1 mice, but this was towards the end of my Ph.D. studies with inadequate time to achieve results.

4B.2   Co-expression of LMP-1 and activated TGFβ1.

It was decided to use both lines of TGFβ1 expressing mice because of the difference in their expression patterns. Line H TGFβ1 expression may overlap with Line 53 LMP-1 expression, therefore analysis of dual expression of the proteins could be achieved. LMP-1 has been shown to induce K6 expression (Wilson, J.B., *et al* , 1990) therefore might also be able to induce line M5 K6-TGFβ1 expression.

4B.2.1   LMP-1 crossed with K6-TGFβ1 line M5

Transgene homozygous K6-TGFβ1 line M5 were used to breed with PyLMP-1 line 53 mice, therefore 50% of the offspring should be LMP-1 positive/TGFβ1 hemizygous positive. To obtain the maximal effect of TGFβ1, these mice were then backcrossed to line M5 in an attempt to create LMP-1 positive mice in a TGFβ1 homozygous positive background.

Mice were examined between 4-7 days after birth where possible. No visible alteration or deviation from the typical d7 LMP-1 phenotype was detected, regardless of the TGFβ1 transgene status. The LMP-1 positive mice presented with their slightly runted size as judged by eye, and so were therefore at a disadvantage over their larger LMP-1 negative littermates, but this was no different to the previously observed line 53 appearance. The mice developed normally and no lesions were noted throughout the duration of the study (12 months). The only possible alteration to phenotype of the PyLMP-1 mice was in the time taken before the "ring-tail" phenotype manifested itself. This phenotype, which normally occurs in mature animals, was seen in mice carrying both transgenes at an earlier time point. However, this was not monitored closely and remains a subjective observation. Other than this observation, no differences were noted.

The procedure of BrdU injections was conducted in three groups of mice, with six mice per group. These groups were bi-transgenics with ringtail; bi-transgenics not showing ringtail; and LMP-1 negative/TGFβ1 positive (which show no phenotype). Dorsal skin, tongue, and tail tissues were harvested and paraffin embedded but time constraints meant the analysis could not be taken further. The results from this BrdU study might explain the apparent acceleration of the appearance of the ringtail phenotype.

4B.2.2   LMP-1 crossed with K10-TGFβ1 line H

Transgene homozygous line H mice were crossed with line 53 LMP-1 positive mice. 50% of the offspring are therefore expected to be LMP-1 positive/ TGFβ1 hemizygous. However, from 192 F1 mice born, only four LMP-1 positive mice were born (2%) and only 2 of these survived to weaning age and maturity (Figure 4.3). The large majority of litters were completely devoid of LMP-1 positive mice, showing no signs of the LMP-1 line 53 phenotype at d7, with transgene status later confirmed by southern blotting. The four LMP-1 positive mice all came from the same litter and were severely runted. The two positives that did not survive died

Fig 4.3:- Pedigree of PyLMP-1 (line 53) crossed with K10-TGFβ1 (line H)

KEY:-

□ Male
○ Female

(▪ LMP +ve
  ⊘ TGFβ +/+
  ⦿ LMP +/ TGFβ +/-
  ⊘ LMP -/ TGFβ +/-
  ⊞ LMP +/ TGFβ +/- OR +/-
  ⊠ LMP -/ TGFβ +/- OR +/+)
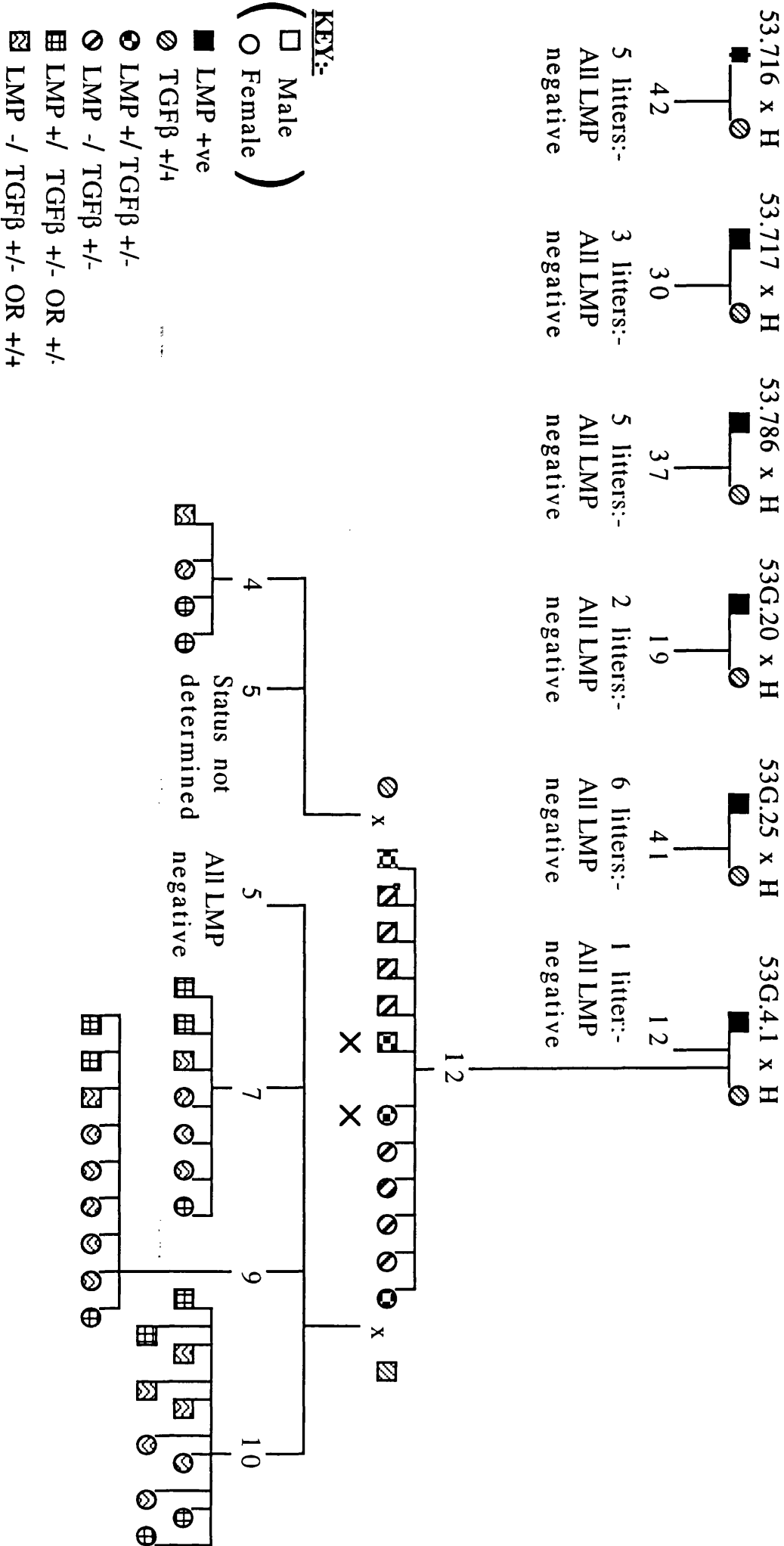
Table 4.3:- Analysis of transgenic embryos from PyLMP-1
(Line 53) crossed with K10-TGFβ1 (Line H).

Age taken	Male	Female	No. of embryos	LMP-1
postnatal d 2	53G.25	H stock	1 2	0
e 1 8	53G.25	H stock	3, 15	0,0
e 1 6	53G.20	H stock	8	0
e 1 4	53G.4.1	H stock	9	1

Footnote:- The designation 53G was given to line 53 mice bred in a separate animal house, compared to most studies described here. 53G mice were of the strain background 25% C57Bl/6/75% DBA

soon after birth. Thus it would appear that the majority of bi-transgenic LMP-1 +ve/ K10-TGFβ1 +ve mice have died *in utero* .

To investigate this embryonic lethality of the bi-transgenics, pregnant females from these crosses were sacrificed between embryonic (e)14 and e18 days of gestation (total gestation =20 days) and the embryo's analysed for transgene status. No LMP-1 positive foetuses were present after e16, with 1/9 LMP-1 positive present at e14 (Table 4.3) Surprisingly, with the exception of one extremely large and one extremely small litter, the litters were all within the normal size. One would expect a 50% reduction in litter size if none of the LMP-1 positive embryo's are born. Occasionally one or two of the foetuses appeared smaller than the rest but this did not correlate with LMP-1 transgene positivity.

The two mice which were both LMP-1 and TGFβ1 positive (Male 53H.110 & Female 53H.117) were much smaller than their LMP-1 negative littermates being half the size at d10 and still less than average weight by weaning age (13g & 11g respectively compared to an average weight of 17.4g for males and 15.6g for females of the same age). Nonetheless, the mice did not show signs of ill health and were fertile as adults (figure 4.3).

The only TGFβ1/ LMP-1 bi-transgenics (4 mice, 2 surviving) in the initial F1 crosses were all in one litter. This might suggest a heritable trait. The observation that LMP-1 positive mice are born in the F2 generation from these two mice (figure 4.3) would support this. The PyLMP-1 phenotype was not altered in these mice.

4B.3 Effect of LMP-1 expression in the absence of p53 function.

p53 knockout (p53-/-) mice (Line 92) in a mixed strain background were donated by A.Balmain at the CRC Beatson Institute for Cancer Research after permission from the creators (Donehower, L.A., *et al* , 1992). PyLMP-1 mice were crossed with these p53-/- mice and the offspring from this LMP-1 x p53-/- cross were heterozygous for p53. As such they are liable to an increased frequency of tumours compared to WT mice but slow forming compared to -/- mice. LMP-1 positive ofspring from this F1 cross were backcrossed to p53-/- mice in order to generate LMP-1 +ve/ p53 -/- mice.

Due to the early onset of tumours in the p53-/- mice it was difficult to establish a line of mice that was LMP-1 +ve in a p53 -/- background. Most information was gained from p53 +/- mice as detailed in appendix 1. However, no tumours other than those published (Donehower, L.A., *et al* , 1992; Harvey, M., *et al* , 1993)

Figure 4.4:-Incidence and type of tumours arising in PyLMP-1 (line 53) transgenic mice crossed with p53 -/- (line 92) knockout mice.
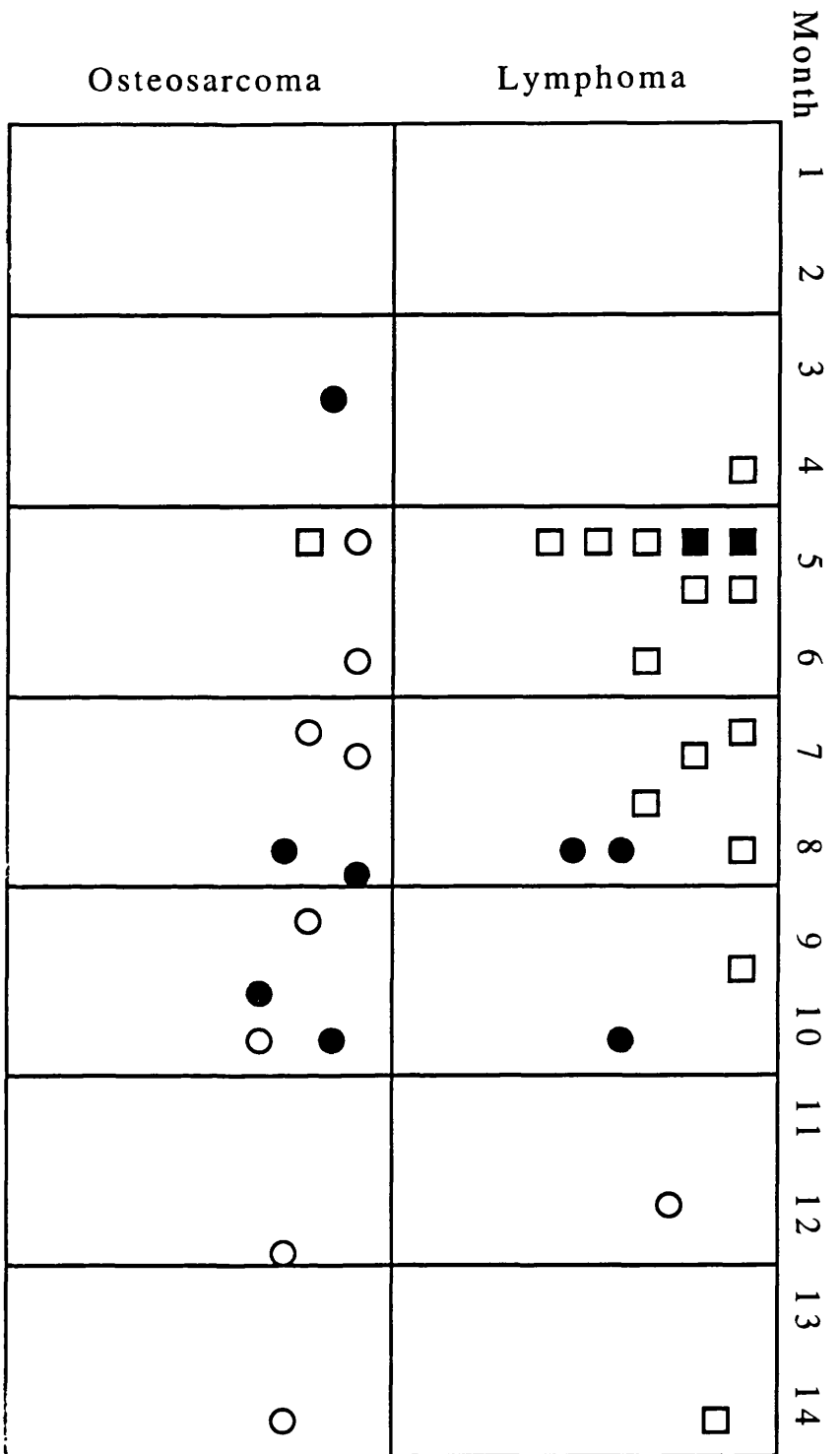
Key:-
- ■ p53 -/- LMP-1 +ve
- □ p53 -/- LMP-1 -ve
- ● p53 +/- LMP-1 +ve
- ○ p53 +/- LMP-1 -ve

131

arose. In addition, tumour onset did not differ between LMP-1 positive and LMP-1 negative siblings. The majority of the mice developed a hunched appearance with paralysed or restricted hind limb movement which proved to be due to sarcomas pressing on the spine (figure 4.4). No carcinomas were observed.

4C   Discussion of chapter 4

4C.1   Ras x LMP-1

K5-Ha*ras* transgenic mice developed lesions around the ears and eyes slightly earlier than did the bi-transgenic LMP-1/*ras* mice. In addition the accumulation of these sebacious adenomas was decreased in the bi-transgenic mouse group with half the numbers of lesions at 9 months compared to the single transgene K5-Ha*ras* group (7.00 v's 14.28). Although fewer mice contributed to the data at later stages, the difference in these mice continued to increase.

From these results it would appear that co-expression of LMP-1 and activated *ras* reduces the number of benign lesions compared to activated *ras* alone. This study would also imply that there is no co-operation between these oncogenes. Indeed the reverse, LMP-1 seems to be inhibiting the effects of *ras* in generating this type of lesion, giving rise to a significant difference as judged by a two sample T-test. This finding was contrary to expectations especially given the tumour promoting effects of LMP-1 noted in chapter 3 where LMP-1 could act as a weak or second stage promoter providing Ha-*ras* was already activated. How LMP-1 might inhibit the *ras* induction of benign lesions is unclear and requires further analysis.

The lack of co-operation might be due to the expression patterns of both transgenes. Ha-*ras* is expressed in the hair follicles and the results from chapter 2 imply that PyLMP-1 may have a suprabasal expression pattern. It is possible that the effects of LMP-1 are not influenced by *ras* because of the different areas that they exert their effect.

Another consideration is the mouse strain background in which these mice are bred. Neither the Ha-*ras* or the bi-transgenics develop carcinoma but both lines of mice are bred in a C57Bl/6 background which is not susceptible to chemically induced carcinogenesis (DiGiovanni, 1991). It is therefore possible that the strain background "masks" any co-operative effect between LMP-1 and *ras* . Crossing the PyLMP-1 mice in the chemical carcinogen sensitive FVB background with the K5-Ha*ras*

mice also in an FVB background might highlight co-operation between these two proteins. This experiment was initiated but could not be completed due to time restraints.

BrdU analysis of day 7 old bi-transgenic and LMP-1 transgenic epidermis revealed no significant difference in mean scores between these two groups. Furthermore, the K5-Ha*ras* mice only showed a very slight significant increase in BrdU scores when compared to negative controls. This shows that activated *ras* (in these mice) does not significantly contribute to, or inhibit the LMP-1 induced epidermal proliferation described in chapter 2. This suggests that the amplification of mutant *ras* alleles seen in chemical carcinogenesis does not contribute to the hyperproliferation noted.

4C.2   TGFβ1 x LMP-1

TGFβ1 attenuates the hyperplastic response provided by TPA treatment of line M5 within 24Hrs of application but does not negate the hyperplastic response in the bi-transgenic mice induced by continual LMP-1 expression.

It is possible that the LMP-1 induced hyperplasia does not activate the K6-TGFβ1 transgene. In this case the two-fold increase in BrdU labelling of untreated line M5 dorsal skin (Fowlis *et al.*, 1996) might be expected to add to the increased proliferation induced by LMP-1 (from chapter 2).

Alternatively, the LMP-1 induced hyperplasia may be sufficient to activate or enhance the activation of the K6-TGFβ1 transgene but the negative regulation by TGFβ1 is insufficient to overcome the effects of LMP-1. Given that the two lines of mice individually show increased epithelial proliferation, yet together do not appear to show an additive effect (by phenotypic examination), may suggest that LMP-1 does indeed activate K6-TGFβ1 which in turn suppresses the proliferative effect seen in untreated line M5 dorsal skin. This proposed suppression must be insufficient to negate the PyLMP-1 induced phenotype. However, the BrdU injected tissues from these mice were not analysed due to time constraints and may yet show an increased proliferative rate. Also, levels of TGFβ1 expression were not looked at.

The combination of LMP-1 and TGFβ1 as tested here does not result in carcinogenic co-operation but no chemical progression studies were carried out to investigate this further. LMP-1 and TGFβ1 may be co-operating, or indeed negating the effects of each other, but in a manner not detectable by this experiment. Chemical carcinogen treatment of bi-transgenics could provide further information. It is known that TGFβ1 is

downregulated in papillomas at high risk for malignant conversion to carcinoma, and in the carcinomas which arise from these high risk papillomas. Furthermore, chemical carcinogen treatment of the K10-TGFβ1 mice revealed that TGFβ1 inhibited papilloma formation. If LMP-1 is activating TGFβ1 in the PyLMP-1/K6-TGFβ1 cross the prediction would therefore be that no carcinogenic progression would be observed. However, I did observe the PyLMP-1 skin phenotype with potential acceleration of the adult "ring-tail" appearance.

Bi-transgenic mice from the PyLMP-1 x K10-TGFβ1 cross appear to be disadvantaged *in utero* such that embryonic death results. The TGFβ1 transgene is driven by the K10 promoter in line H mice to direct expression to the differentiating suprabasal cell compartment. K10 mRNA is first detected in developing embryo's at e13.5 but only in the nasal region. This expression spreads slightly to encompass the whisker pads and paws by e14.5, but there is still no dorso-lateral epidermal expression. Expression of K10 however rises dramatically at e15.5 to cover most of the embryo surface (Byrne *et al.*, 1994). This confirms earlier reports and infers that active TGFβ1 will not be expressed in the epidermis before e15. This allows development to proceed without the potentially lethal effects of overexpressed TGFβ1 to embryogenesis.

A previous study using the K1 promoter (also expressed at e15.5) to drive TGFβ1 transgene expression to the suprabasal layer of the epidermis resulted in the birth of mice with extremely taught skin (Sellheyer *et al.*, 1993). These mice had no mobility and died at day1. The differences between the K10-TGFβ1 and the K1-TGFβ1 studies probably reflects a difference in the level of TGFβ1 protein expressed during embryogenesis in the different lines of mice.

Our bi-transgenic mice appear to have an even more severe phenotype than the K1-TGFβ1 mice, with death *in utero* before e16 rather than at day1. It is possible that TGFβ1 is being switched on before e15.5. A suggestion that this might be the case was provided by analysis of K10 mRNA levels in new born PyLMP-1 line 5 transgenic mice. At day 8 mRNA levels for K10 are increased 3-4 fold in the PyLMP-1 mice over their negative siblings (unpublished results from our lab.). Although the levels of K10 in the PyLMP-1 mice are comparable with controls at day10, it is possible that the elevation noted at day8 also occurs during embryogenesis. If this was the case then LMP-1 could be upregulating the expression of K10 and thus TGFβ1 in the bi-transgenics during a critical stage of development, resulting in

embryonic death. Direct evidence for this theory awaits analysis of K10 mRNA from bi-transgenic or PyLMP-1 embryo's younger than e15. Alternatively there may be lethal consequences from the dual expression of these transgenes.

The litter which provided 4 LMP-1 positive pups, 2 of which survived to breed and produce further LMP-1 positive offspring may represent a line of mice which have escaped the lethality of early TGFβ1 expression.

4C.3   p53 Knockout x LMP-1

No epidermal tumours arose in LMP-1 +ve mice in either a p53 +/- or p53 -/- background before they succumbed to p53 deficiency associated tumours. This suggests several possiblities.

It may be that even with two 'hits', LMP-1 expression plus lack of p53, this is still insufficient to promote progression in the skin. Since LMP-1 is a weak or late stage tumour promoter perhaps acting to augment more powerful tumour promoters (Chapter 3), it may need prior oncogenic activation before it can operate. The absence of p53 is not an early event in carcinogenesis but associated with later stages. Therefore no co-operation is noted at early stages of carcinogenesis in this case because LMP-1 may require activation of Ha-*ras* and some tumour promotion before it can exert its own tumour promoting/augmenting effects. Subjecting LMP-1 +ve/ p53 -/- mice to chemical carcinogenesis, or intercrossing LMP-1/ *ras* mice with p53 deficient mice might provide additional information in this respect.

It is possible that LMP-1 may overcome WT p53 action as it protects B-cells and epithelial cells from p53 triggered apoptosis mediated at non-permissive temperatures (Okan *et al.*, 1995; Fries *et al.*, 1996). This would imply a degree of redundancy with p53-/- in this assay as far as skin carcinogenesis.

Since p53 is apparently not mutated in NPC it is more likely that its tumour suppressing effects are overcome by other means (or perhaps it is not normally expressed in the NPC cell type). Although p53 appears unaltered, the tumour suppressor gene p16 has been shown to be mutated in NPC (Lo *et al.*, 1995) and in other tumours of epithelial origin. Creating LMP-1 transgenic mice with oropharyngeal expression and crossing them with p16 knockout mice might give rise to a more NPC like situation in the oropharynx.

CHAPTER 5 : Characterisation of line 3 9.

In this chapter the EμLMP-1 transgenic mice created by Wilson *et al.*, 1990, are investigated further. This series was not previously monitored for an extended period of time (over 1 year of age). This chapter therefore briefly describes the history of these EμLMP-1 mice before detailing the analysis of one particular EμLMP-1 line over a 24 month time course.

5 A   EμLMP-1 Transgenic mice.

Expression of the EμLMP transgene mostly gave rise to non-viable mouse lines. Three founder mice were dead within 2 weeks of birth and a further founder female, EμLMP.33, gave rise to only 4 LMP positive offspring from several litters, and these were not viable past 6 months. These 4 offspring did however display the hyperplastic dermatitis phenotype. This left only eight viable mouse lines (Wilson *et al.*, 1990). Of these lines 39, 44, 48, and 4 9 were bred to homozygosity. All displayed a mild phenotype which manifested as ruffled, greying fur as the mice grew older. These mice were less dynamic than negative animals with reduced fertility (fewer, smaller litters). The order of phenotypic intensity was:-

Line 33>>> 44 > 48 > 49 > 39
(lethal)

Expression was found not to be restricted to B-lymphocytes in line33 mice, but detectable in a broad array of tisues. Expression in line 39 was also analysed by northern blotting. On a long exposure LMP-1 transcript expression could be detected in spleen and thymus, with none or below detectable levels in other tissues, perhaps accounting for the viability of line 39. Subsequently, only line 39 was maintained and it's further characterisation and long latency phenotype is the subject of this chapter.

In the consideration of animal house space and in accordance with Home Office preferences, most mouse experiments (including transgenic mouse lifespan) are limited to 1 year of study. However there are several reports indicating that transgene induced tumour onset can have a long latency. It was decided to investigate any long latency phenotype in line 39 (up to 24 months) and simultaneously to investigate any synergy with EBNA-1 predisposition to lymphoma (Wilson and Levine, 1992; Wilson *et al.*, 1996).

EμEBNA-1 transgenic mice have been described previously in section 1B.3. Two lines of mice, lines 26 and 59, expressing the EμEBNA-1 transgene, succumbed to monoclonal B cell lymphomas with latencies of 4-12 months and 18-24 months respectively. The tumour phenotype is a dramatic enlargement of the spleen along with enlargements of the liver and/or lymph nodes. For the purpose of this study EμEBNA-1 line 59 was crossed with EμLMP-1 line 39 because of the long latency to lymphoma of line 59. Co-operation may be easier to follow in this cross because a change in tumour latency might be more obvious than in a line 26 cross where the mice are overcome with EBNA-1 induced lymphoma as early as 4 months of age.

Evidence that two oncogenes can co-operate to induce lymphomagenesis is provided by other transgenic cross breeding studies. For example, Eμ–*pim* -1 transgenic mice demonstrate very low incidence of tumour development which can be enhanced with transgene optimisation (Lohuizen *et al.,* 1989). Eμ-c-*myc* transgenic mice are predisposed to monoclonal lymphomas of pre-B or B-cell type (Adams *et al.,* 1985). However, when these two lines of mice are crossed, the Eμ-c-*myc* / Eμ-*pim* -1 bi-transgenics develop B-cell lymphomas *in utero* demonstrating dramatic co-operation (Verbeek *et al.,* 1991).

5B   RESULTS

5B.1   Cross-breeding EμLMP-1 with EμEBNA-1:-

Low level LMP-1 expression is sufficient alone to induce lymphomagenesis.

Homozygous EμLMP-1 (line 39) transgenic mice were cross-bred with hemizygous EμEBNA-1 mice of line 59. All the offspring from the crosses should be EμLMP-1 positive (hemizygous), whereas 50% of the mice should be EμEBNA-1 positive. Thus there were two groups under observation, one with mice harbouring EμLMP-1 alone (line 39), and another with mice bi-transgenic for both EμLMP-1 and EμEBNA-1 (line 39/59.)

37 mice of EμLMP-1 line 39 were born (See appendix 2). Unfortunately, during this extended period of study spanning more than 4 years, there have been several moves of the animals (between different animal houses) and several changes in animal house staff. As a consequence the time of death of 9 mice was not accurately recorded. These have been indicated as ? (for age at death). Except for mouse 39/59.1 , it was not possible to ascertain

cause of death. In addition, for 3 mice, although the age at death is known, it was not possible to ascertain cause of death from the carcasses. For 25 mice, age at death/sacrifice due to ill health and cause of death are known.

15/20 (75%) mice succumbed to lymphoid neoplasia (fig 5.1) with the gross appearance of lymphoma between the ages of 16.5 and 24 months (with the end point of the experiment at 24 months of age). The average latency to lymphoma was 20.1 months. 5 mice were sacrificed at the experiment end point (22-24 months old) without obvious pathological abnormality. Mice sacrificed before the age of 22 months due to ill health or otherwise (e.g. wounds from fighting) which was not attributable to lymphoma were not included in the final totals. For this reason 5 mice were withdrawn from the study (at months 11,12,14,20 & 21), hence the final total of 20 EµLMP-1 line 39 mice (Summarised in appendix 2). As many of the 12 mice which died/went missing without record are likely to have also succumbed to lymphoma (e.g. mouse 39/59.1), the total of 75% is probably an underestimate.

The mice were generally healthy up to the age of 16 months, after which point signs of illness became evident. The mice had either a massive swelling of the abdomen or were hunched and slow moving. When examined, the mice displayed a massive enlargement of the mesenteric lymph nodes (MLN) and spleen, with variable involvement of the liver and peripheral lymph nodes. The enlargement of the spleen consistently correlated with the swollen abdominal appearance, whereas the mice that looked sickly and were hunched/ slow moving generally had a MLN lymphoma which was probably constricting the gut and causing discomfort.

Of the mice diagnosed with lymphoma 12/15 (80%) mice displayed MLN involvement, 12/15 (80%) showed spleen enlargements, with 9/15 (60%) showing enlargements of both the MLN and the spleen (See appendix 2). No single mouse had lymphoma which did not involve either the MLN or the spleen. In addition, occasional involvement of other organs was noted (liver 27%, peripheral lymph nodes 27%, peyer's patches 20%).

Pathological diagnosis on a section of tissues from affected mice consistently indicated lymphoblastic lymphoma, often with multi-organ involvement. However, there were also three examples of Histiocytic sarcoma (mice 39/59.57, 59/39.13, and 59/39.23- see appendix 2).

138

Death from lymphoma in line 39 mice
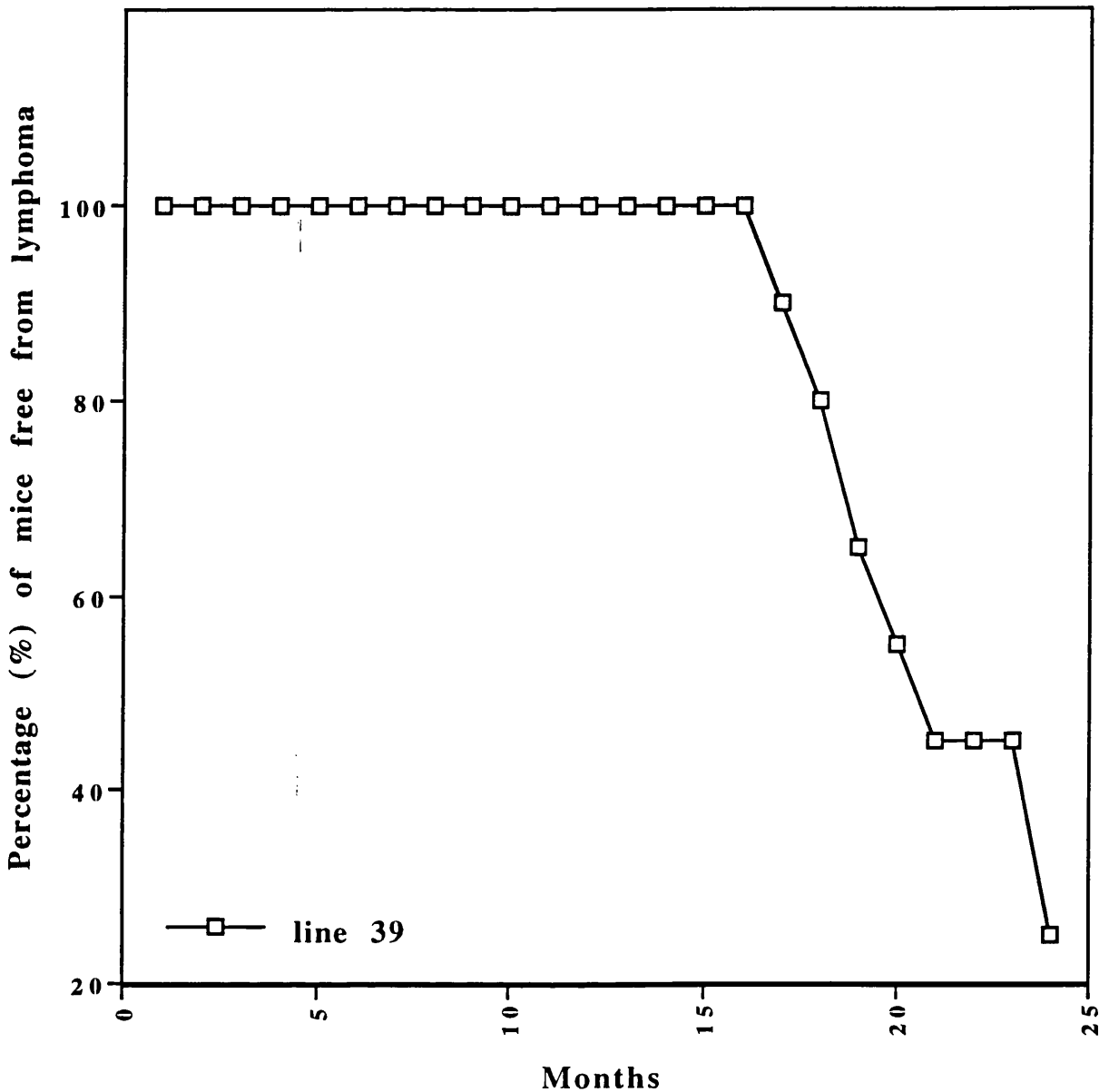
Figure 5.1

Incidence of lymphoma in line 39 mice. From 16 months onwards mice began to develop lymphoma of the mesenteric lymph node and/or spleen characterised by a massive enlargement of those organs. There was also variable involvement of other tissues such as peripheral lymph nodes, liver or thymus.

5B.2   IgH and TcR rearrangements of Line 39 tumours

In order to investigate the cell lineage and clonality of the lymphoid tumours, rearrangements of the endogenous immunoglobulin and T-cell receptor loci were analysed. DNA from line 39 tumour tissues was extracted as described in the methods section 7B.3.1. Where possible, DNA was extracted from three different tissues with tumour involvement per mouse, generally including MLN, spleen and one other tissue. This allows comparison of tissues from the same mouse and comparison of tissues between mice. The DNA was digested with *Eco* R1 and electrophoresed for southern blotting. Two different probes were used in order to investigate the cell lineage and clonality of these line 39 tumours. The murine immunoglobulin heavy chain and T-cell receptor loci were analysed for rearrangements by using a heavy chain joining region (J_H) DNA fragment and a T-cell β receptor joining region (TcR $J_{\beta2}$) DNA fragment respectively as probes.

Of the 8 mice tested which were diagnosed as having lymphoblastic lymphoma, 5/8 (63%) showed clonal rearrangements of the IgH locus (figure 5.2a,b,c). The germ line 6.5kb *Eco* R1 IgH-J fragment was present in all of the samples as expected whether neoplastic or not because the tumour tissues still contain normal cells within them. However, many tumour samples revealed one or two other bands indicating an IgH rearrangement(s). The rearranged fragments detected were identical in the neoplastic tissues from a single mouse and only ever 1 or 2 bands per tumour were detected. This demonstrated that the tumours were monoclonal. For those tissues which were initially observed as being enlarged but for which no IgH rearrangements were detected, it may be that the neoplastic component in the sample was too minor for detection of the rearranged band, or, that they do not harbour IgH rearrangements.
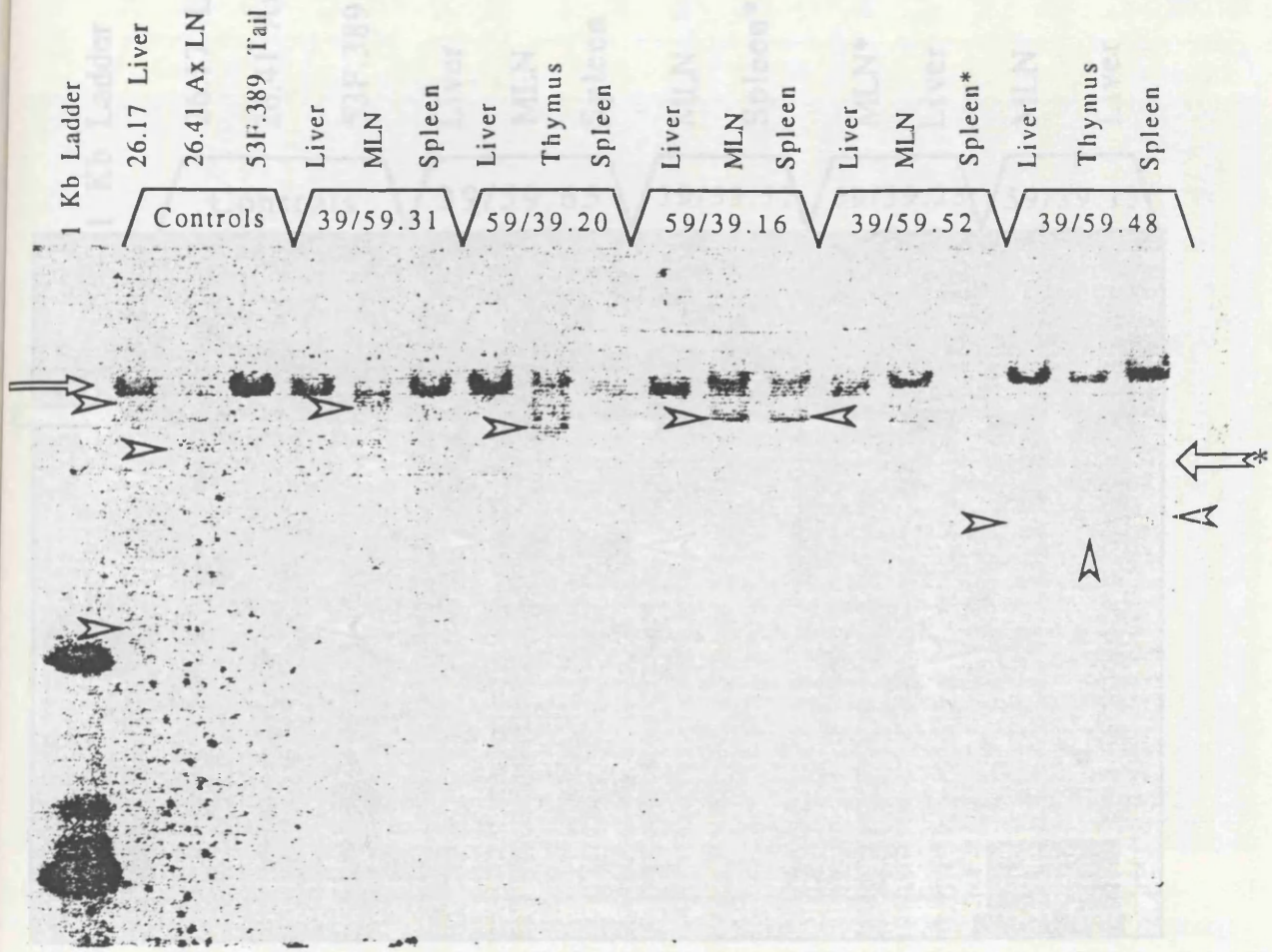
Figure 5.2a:- Clonal immunoglobulin heavy chain re-arrangements
DNA extracted from tumour tissue was *Eco* R1 digested and electrophoresed for southern blotting. A heavy chain joining region (JH) DNA fragment was used as a probe to analyse the murine immunoglobulin heavy chain locus. The germ line 6.5kb *Eco* R1 IgH-J fragment is present in all of tl samples because of the presence of normal cells within the tumour mass. However, many of the tumour samples also contain additional bands representive of IgH rearrangements.

KEY:-

⇒=6.5kb germ line *Eco* R1 IgH-J fragment

▷=Rearrangements of the IgH locus

*⇒= Loading dye contamination

Spleen*= denotes underloaded lane
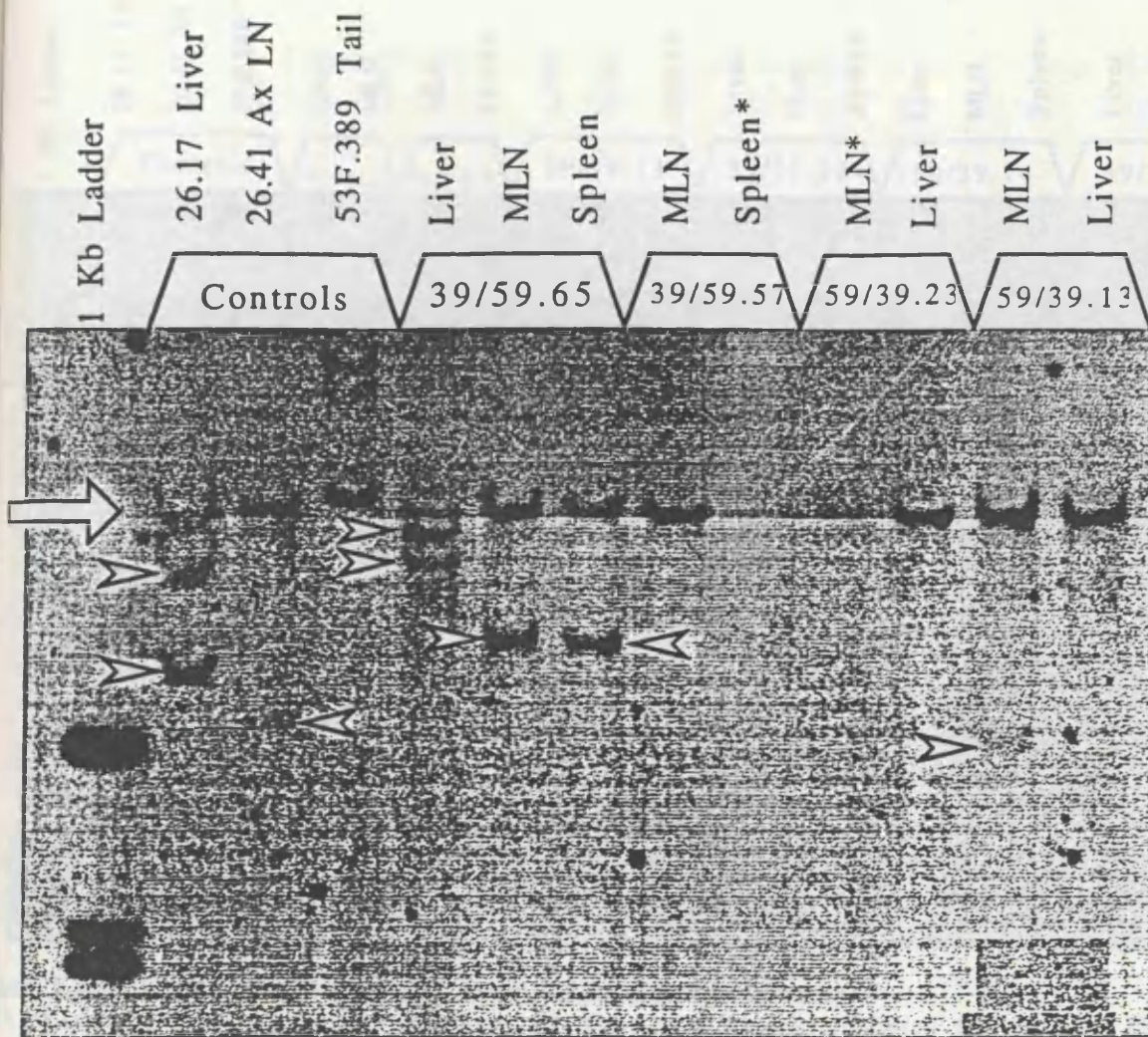
Figure 5.2b:- Clonal immunoglobulin heavy chain re-arrangements
DNA extracted from tumour tissue was *Eco* R1 digested and electrophoresed
for southern blotting. A heavy chain joining region (JH) DNA fragment was
used as a probe to analyse the murine immunoglobulin heavy chain locus. The
germ line 6.5kb *Eco* R1 IgH-J fragment is present in all of the samples because
of the presence of normal cells within the tumour mass. However, many of the
tumour samples also contain additional bands representive of IgH
rearrangements.

KEY:-

=6.5kb germ line *Eco* R1 IgH-J fragment

=Rearrangements of the IgH locus

*= denotes underloaded lane

142
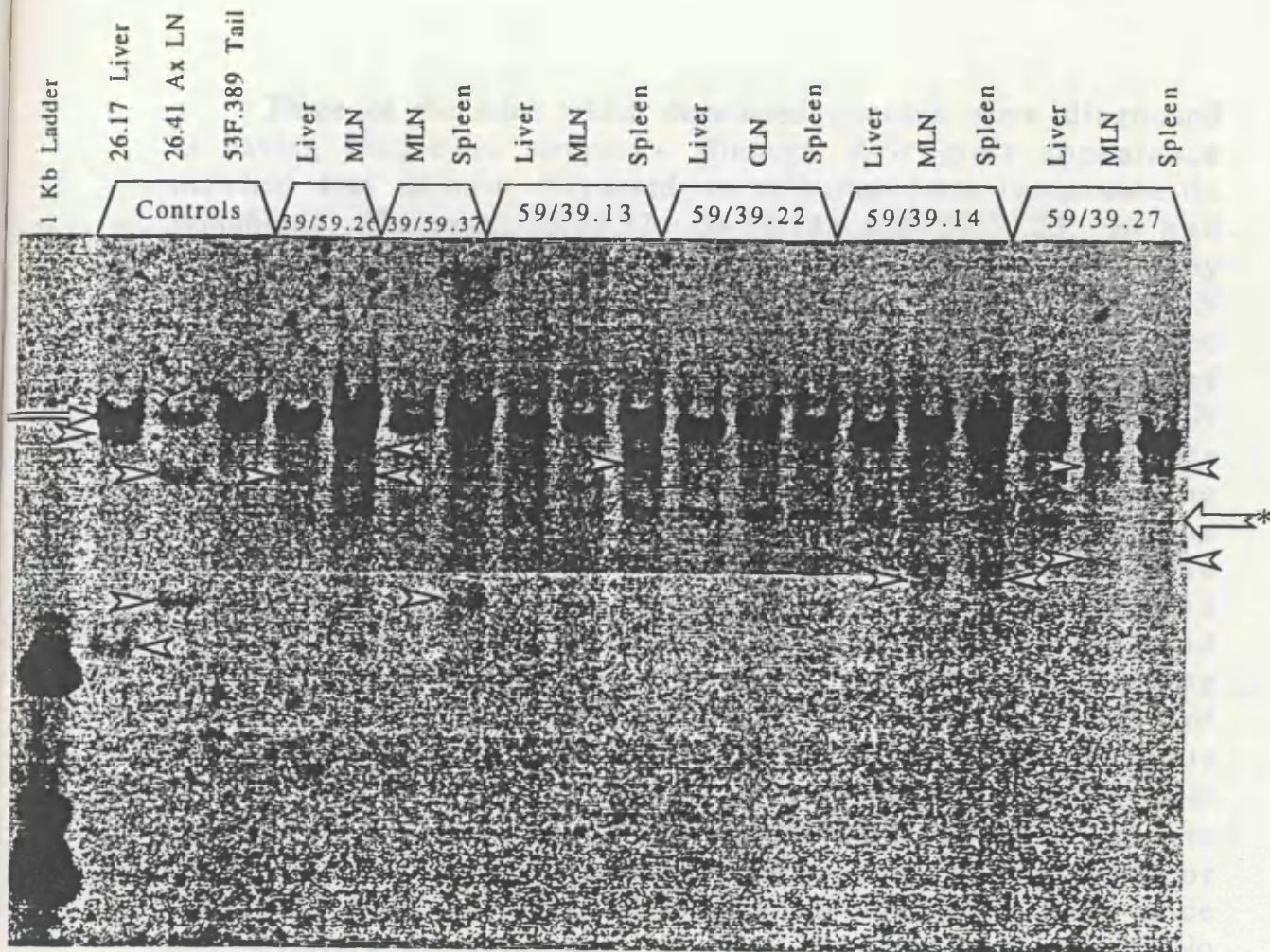
Figure 5.2c:- Clonal immunoglobulin heavy chain re-arrangements
DNA extracted from tumour tissue was *Eco* R1 digested and electrophoresed for southern blotting.
A heavy chain joining region (JH) DNA fragment was used as a probe to analyse the murine immunoglobulin heavy chain locus. The germ line 6.5kb *Eco* R1 IgH-J fragment is present in all of the samples because of the presence of normal cells within the tumour mass. However, many of the tumour samples also contain additional bands representive of IgH rearrangements.

=6.5kb germ line *Eco* R1 IgH-J fragment

=Rearrangements of the IgH locus

= Loading dye contamination

Three of the mice which developed tumours were diagnosed as having histiocytic sarcoma's although their gross appearance matched that of mice diagnosed as suffering from lymphoblastic lymphoma. The mice, 39/59.57, 59/39.13, and 59/39.23, all had slightly enlarged and highly discoloured spleens with a "lumpy texture" or obvious tumour in one area, and all had MLN involvement. The tumours from these mice were investigated further. IgH rearrangements were not detected in the tumours of mice 39/59.57 or 59/39.23, but were for mouse 59/39.13 (MLN tissue) (figure 5.2b) When tumours from these mice were analysed for TcR rearrangements, one set of samples from the mouse 59/39.13 revealed two additional bands along with the expected 2.0kb germline band indicating that rearrangements had occurred at both TcR alleles (figure 5.3). Although the upper band could potentially be a product of partial digestion, the lower band cannot. Both neoplastic tissues analysed had the same banding pattern. However, since IgH rearrangements were also noted for this tumour, it is not clear if both IgH and TcR rearrangements reside within the same neoplastic cells or if there are two cellular components to the tumour. No TcRβ rearrangements were seen in tumour tissues from the other two mice with histiosarcoma or from the 5 mice with lymphoblastic lymphoma. These three mice may therefore represent a subgroup of tumours from line 39. No other tumour samples revealed detectable alterations in the TcRβ locus (figure 5.4)

Thus, in this line, low level LMP-1 expression in the lymphoid compartment of transgenic mice results in the neoplastic conversion to lymphoma which are predominantly B cell in origin and monoclonal.
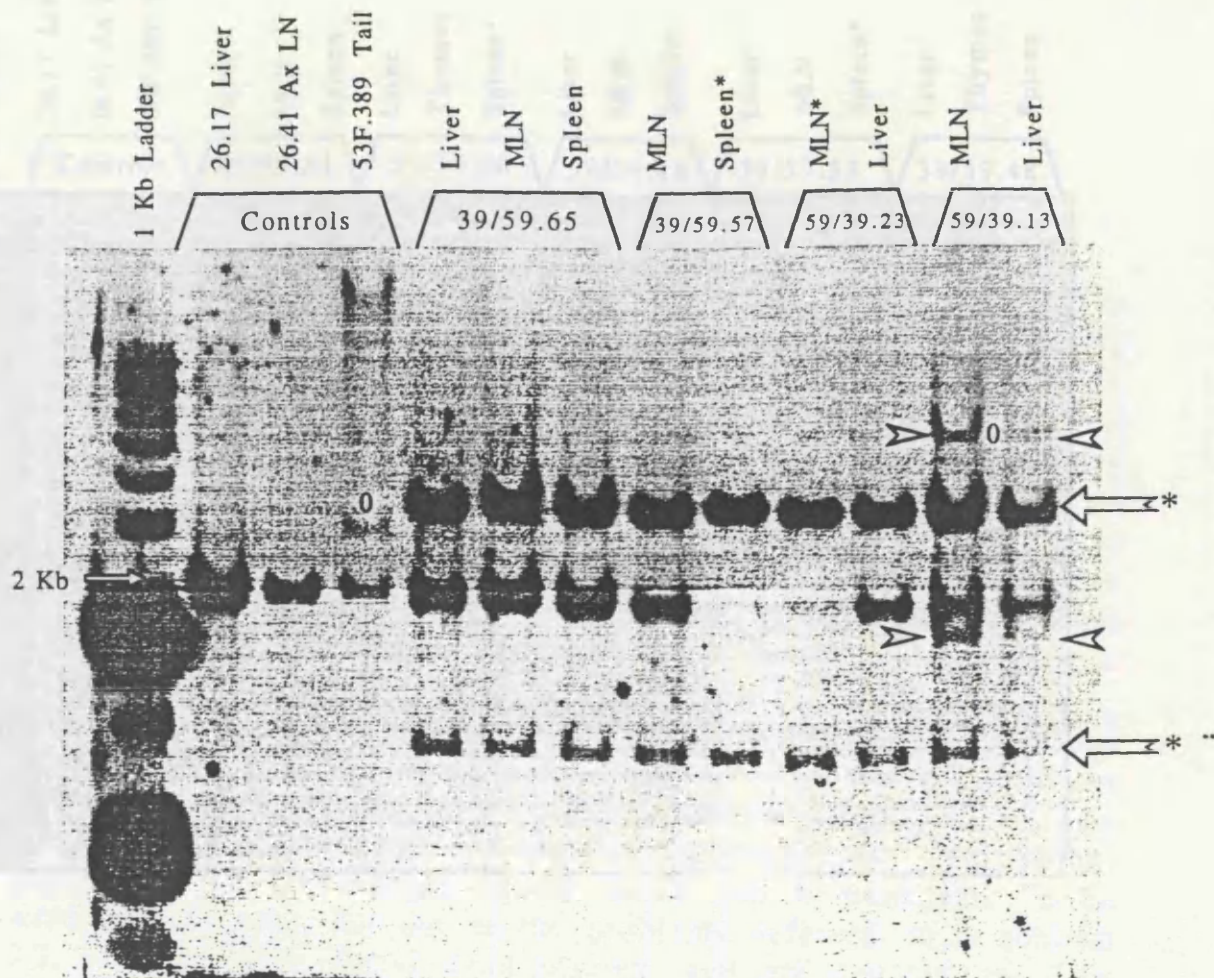
Figure 5.3

Figure 5.3:- TcR re-arangements

A T cell Receptor Jβ2 probe was used to analyse the murine TcR locus for re-
arrangements. The germ line 2.0kb *Eco* R1 TcR-Jβ2 fragment is present in all of
the samples because of the presence of normal cells within the tumour mass.
However, sample 59/39.13 contains extra bands representative of IgH
rearrangements.

KEY:-

*⟹ = Extra bands from contaminated loading dye

▷ = TcR re-arrangements

* = underloaded

o = possible partial digestion patterr

145
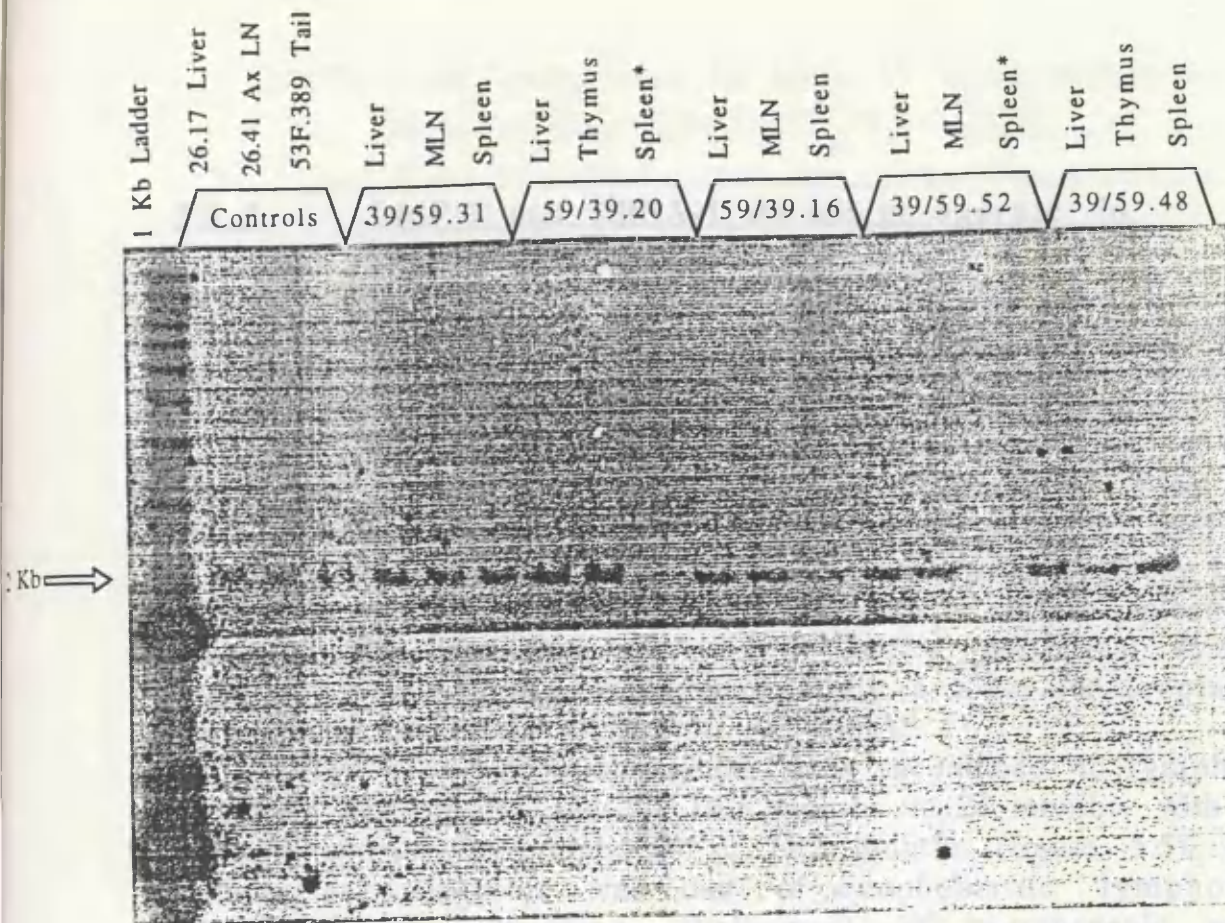
Figure 5.4

A T cell Receptor Jβ2 probe was used to analyse the murine TcR locus for rearrangements. The germ line 2.0kb *Eco* R1 TcR-Jβ2 fragment is the only band present indicating that nor rearrangements of this locus have taken place in these tumours.

KEY:-

*= underloaded

146

5B.3 LMP-1 and EBNA-1 do not co-operate in lymphomagenesis.

52% of the cross 39/59 F1 mice were transgenically positive for both LMP-1 and EBNA-1 as determined by southern blotting of DNA extracted from tail and other tissues (See appendix 3). Both line 39 and 59 independently induce monoclonal B cell lymphoma of notably different gross presentation. Line 39 present with lymphomas of the MLN plus spleen (60%) with no single case where either the MLN or spleen was not involved. Line 59 on the other hand present with lymphomas of the spleen in 100% of cases accompanied by liver and/or peripheral lymph node involvement. The MLN is very rarely a target for neoplastic involvement in line 59 (Wilson *et al.*, 1996).

16/19 (84%) of bi-transgenic mice succumbed to lymphoid neoplasia between the ages of 16.5 months to 24 months, with an average latency to lymphoma of 20.2 months (figure 5.5). The pathological diagnosis was that of lymphoblastic lymphoma, generally with multi-organ involvement. 40 bi-transgenic mice were born in total, but due to the problems referred to in section 5.2, 11 mice were found dead/missing and are denoted by ? in appendix 3. A further 10 mice were sacrificed before 22 months of the study period due to ill health not lymphoma related. These 21 mice were not included in the final totals.

The general symptoms noted for the bi-transgenic mice were similar to those previously described for line 39. The mice had either an obviously distended abdomen or a hunched sickly appearance. 13/16 (81%) bi-transgenic mice had MLN involvement, with 12/16 (75%) having spleen involvement. 100% of mice had lymphoma involving either MLN or spleen as was the case for line 39. Therefore MLN and spleen are again the two major tissues targeted for neoplastic alterations. This therefore differs from the line 59 situation where there is not usually a major involvement of the MLN (Wilson *et al.*, 1996). In addition, liver (31%) and peripheral lymph nodes (44%) were also involved in some lymphoma cases in these bi-transgenic mice. The only differences between the tumour incidence in line 39 and the bi-transgenic mice were seen in the thymus and peyer's patches. 25% of the bi-transgenics had enlargement of the thymus (as seen in line 59), whereas no mice from line 39 showed this phenotype. However, 20% of line 39 mice displayed involvement of the peyer's patches, a symptom not observed in either the bi-transgenics or line 59 mice. The gross phenotype therefore seems to reflect a combination of line 59 and line 39

Death from lymphoma in Line 39 mice versus bi-transgenic LMP-1/EBNA-1mice
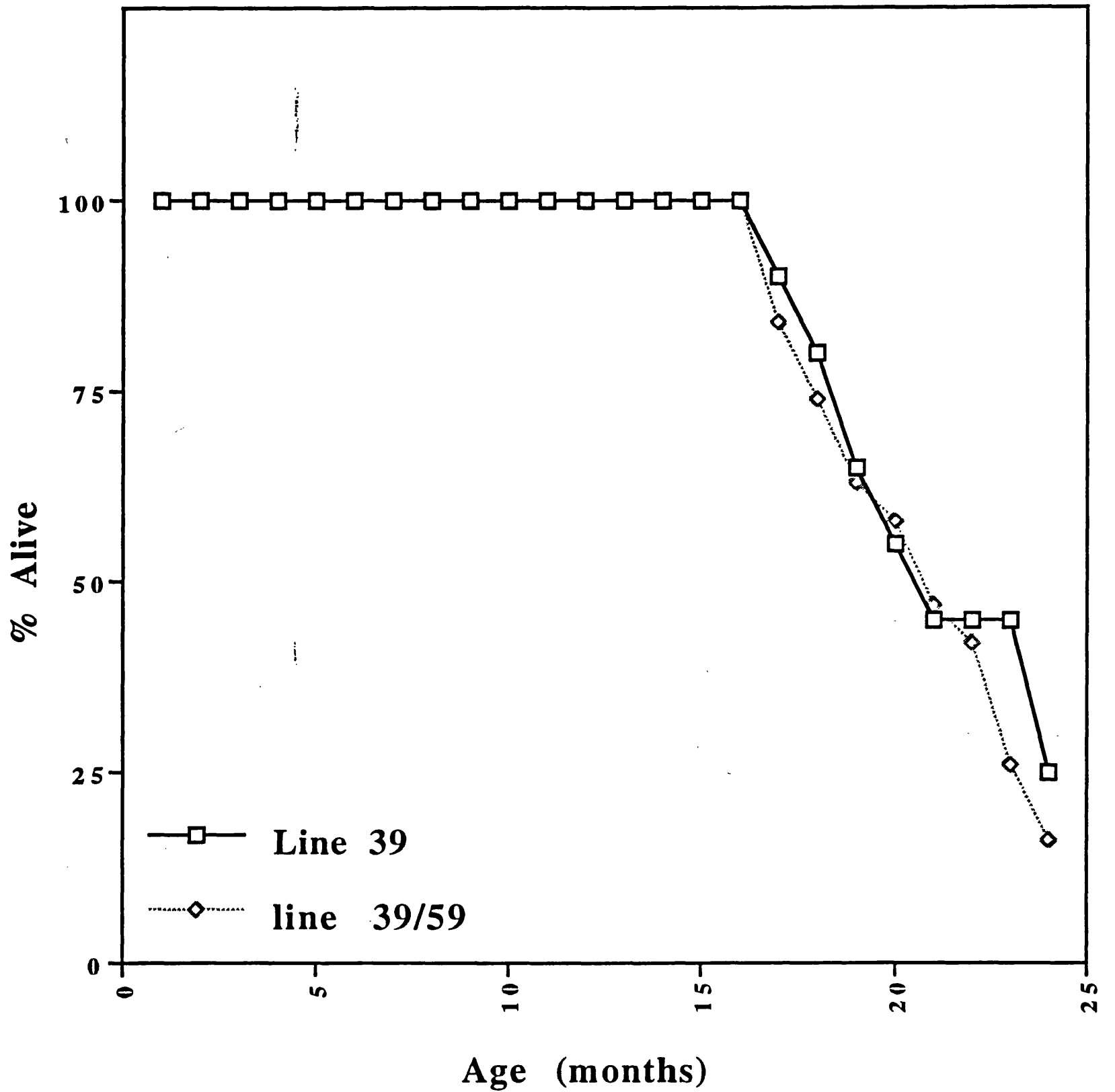
Figure 5.5:-

The incidence of lymphoma in line 39 mice compared to the bi-transgenic line 39/59 mice. There is no acceleration of tumorigenesis when both transgenes are expressed.

DNA from bi-transgenic tumour tissue was extracted as described in the materials and methods in order to investigate cell lineage and clonality of the tumours. The same probes as described in section 5B.2 were utilised. Of the DNA from 12 bi-transgenic tumour samples tested, 8/12 (67%) demonstrated clonal rearrangements of their IgH locus (figure 5.2). The rearranged fragments detected were identical in the neoplastic tissues from a single mouse, demonstrating that the tumours were monoclonal. No rearrangements of the TcRβ locus were detected indicating that the tumours are B cell in origin (figure 5.4).

Therefore, bi-transgenic mice expressing LMP-1 and EBNA-1 in the lymphoid compartment succumb to lymphoblastic lymphoma with phenotypes similar to the transgenic mice of EμLMP-1 line 39.

5C   Discussion of chapter 5.

Expression of EμLMP-1 in line 39 is so low as to be almost undetectable by Northern blot on an extremely long length exposure. Despite this low level of expression, 75% of line 39 mice succumbed to lymphoid neoplasia by 24 months of age. The first observation of neoplastic alterations was at 16.5 months. Thereafter mice steadily developed lymphoid tumours at a rate which gave an average latency to neoplasia of 20.1 months for the 75% of mice affected. Pathological diagnosis revealed that these neoplasia's were consistently lymphoblastic lymphoma, and in many cases encompassed many different organs. 3 cases of histiocytic sarcoma were also noted although their gross appearance did not differ from the lymphoma's.

The tissues affected by these tumours were predominantly mesenteric lymph node and spleen with occasional involvement of other organs. Examination of the murine IgH and TcR loci in these tumours to investigate the cell lineage and clonality of these tumours, indicated that 63% showed clonal rearrangements of their IgH locus. The rearranged fragments were identical in the different tumour tissues from a single mouse and only showed 1 (or occassionally 2) rearranged bands indicating that the tumours were monoclonal (as several bands, even if the same in all the tissues would mean that the tumour was at least oligoclonal in origin). One of the mice which was diagnosed as carrying a histiocytic sarcoma showed rearrangement of the TcR locus and IgH locus. Immunohistochemical analysis of the tumour with reagents which distinguish between B and T cells would reveal if

this tumour was monoclonal or 2 cell types. Apart from this single example, the TcR locus was intact in every other case examined.

Therefore, it would appear that extremely low level expression of LMP-1 in the lymphoid compartment is sufficient to induce a neoplastic conversion to lymphoma. These lymphomas are predominantly B-cell in origin and monoclonal. It is possible that a subset of tumours exists which are histiocytic sarcomas and may show T-cell involvement. Certainly the only rearrangement of the TcR locus noted came from this group of 3 mice. Nonetheless, this is the first example of an *in vivo* role in lymphomagenesis for LMP-1.

Direct comparison can be made with another EBV latently expressed gene which induces lymphoma in transgenic mice. Two lines of EµEBNA-1 mice, lines 26 and 59, also succumb to lymphoma (Wilson and Levine, 1992; Wilson *et al.,* 1996). 100% of line 26 mice develop monoclonal B-cell lymphomas between 4 to 12 months of age. Line 59 mice also develop lymphoma but with a much longer latency with approximately 50% of the mice succumbing between 18 to 24 months. In both cases the tissues involved always include spleen with liver or lymph node involvement. Thus EµLMP-1 line 39 mice succumb to lymphoma at a slightly faster rate than EµEBNA-1 line 59 but with a different bias in organ involvement.

Indeed this B-cell specific oncogenicity by LMP-1 may be highly significant in understanding Hodgkin's Disease. The pathogenesis of HD is poorly understood but it is believed that the multinucleated RS cells of the tumour mass are B-cell in origin (Kieff, 1996). As LMP-1 has been shown to be present in RS cells of EBV positive HD tumours it is conceivable that it is the presence of LMP-1 which drives the B-cell to assume the morphology of an RS cell. This premise is supported by the observation that LMP-1 transfected into HD negative cells gives rise to cells with an appearance very similar to that of RS cells (Knecht *et al.,* 1996). Therefore, EµLMP-1 may be of significant value as a mouse model for EBV positive HD, however cells in those mice do not have an RS cell morphology. High level expression in many tissues of LMP-1 in other EµLMP-1 lines appeared to be toxic. If this problem could be overcome it would be interesting to observe whether 100% of the mice would then succumb to lymphoma and if the latency to lymphoma would reduce.

Two other EBV latently expressed proteins, EBNA-1 and EBNA-2, are consistently detected in HD. EBNA-1 is necessarily present because of it's role of maintaining the viral episome. The recent report from Wilson *et al.,* 1996, have shown that EBNA-1 is oncogenic *in vivo* and therefore might also be involved in the

pathogenesis of HD. Whilst this may indeed be true, EμLMP-1 line 39/EμEBNA-1 line 59 bi-transgenic mice did not show co-operation between EBNA-1 and LMP-1 in lymphomagenesis. This suggests that their functions may overlap using this assay of tumour latency. Crossbreeding with line 26 should be undertaken to confirm this theory.

84% of the bi-transgenic mice developed lymphoid neoplasia with an average latency to lymphoma of 20.2 months. All lymphoid neoplasia's which were pathologically examined proved to be lymphoblastic lymphomas. Similar percentages to line 39 for the individual organ involvement were also observed although slightly more liver and peripheral lymph node involvement was diagnosed in the bi-transgenic group. Almost identical numbers of tumours showed monoclonal rearrangements of the IgH locus as was noted for line 39. Thus LMP-1 and EBNA-1 do not co-operate to accelerate lymphomagenesis, and the tumours arising affect very similar tissues to those seen previously in EμLMP-1 line 39.

Records for several mice were not kept (as detailed) so it is entirely possible and indeed likely that the percentage of mice in both line 39 and the bi-transgenic group which succumb to lymphoma is underestimated. Furthermore, tumours which failed to highlight IgH rearrangements may simply have had a neoplastic component of the tissue too small to detect.

CHAPTER 6:-   Discussion and future work.

6 A

In the transgenic epidermis LMP-1 acts to increase cellular proliferation. It is likely that it is through this action that it functions as a tumour promoter in chemical carcinogen studies. Treatment of mouse dorsal skin with tumour promoters such as TPA results in a variety of events. It is unknown whether one of these occurrences is vital in promoting carcinogenesis, or whether the collection of events as a whole is essential. One such outcome of repeated TPA treatment is persistent general hyperplasia similar to that observed in PyLMP-1 lines 5 and 53. Another event influenced by TPA is the induction and activation of EGFR, a characteristic observed in LMP-1 positive NPC biopsies. Thus LMP-1 may mimic some of the actions of the strong tumour promoter TPA.

A recent report adds support to the findings presented in this thesis. Anti-sense oligonucleotides to LMP-1 sequence, designed to suppress LMP-1 gene expression, resulted in an inhibition of proliferation of EBV transformed B lymphocytes (Mattia *et al.*, 1997). This suggests that LMP-1 functions to increase proliferation of LCL's as well as the transgenic epithelium.

In these studies, no impairment of the differentiation process was evident, in contrast to an earlier report using the assay of *in vitro* transfection of LMP-1 into an immortalised non-tumorigenic squamous cell carcinoma line SCC12F (Dawson, *et al.*, 1990). These cells showed an impairment in response to differentiation signals. However, this study was conducted *in vitro* on one sub-clone of a cell line which was derived from a carcinoma. Therefore SCC12F may differ in many respects to the situation in a living model such as the PyLMP-1 mice. In the epidermis of transgenic mice, LMP-1 is the only abnormality within these cells, whereas when transfected into SCC12F cells, LMP-1 is arriving at an advanced stage of tumorigenesis and may therefore act in a different manner, or in combination with other abnormal factors. The work described by Dawson and later by Fahraeus *et al.*, 1990, and Zheng *et al.*, 1994a, may well reflect that the cell lines are already immortal, it is difficult to compare the results with the *in vivo* situation in primary epithelium.

It should also be remembered that work with cell lines often gives confusing or contradictory results as evidenced by the different effects induced by LMP-1 when transfected into different epithelial or B-cell lines (section 1D.3). This adds further difficulties to interpreting cell culture experiments. Indeed,

Nicholson *et al.*, 1997, reported that LMP-1 was not inhibiting differentiation in the human epithelial cell line SCC12F *in vitro* and *in vivo* providing contradicting results to Dawson. Certainly, differing levels of LMP-1 expression may contribute to the conflicting results since very high, non-physiological levels can be achieved in some cell lines in culture.

One avenue not explored in chapter 2 was that LMP-1 could be acting to block apoptosis thus causing a build up of cells and the observed PyLMP-1 phenotype, in addition to the increase in proliferation. LMP-1 has been shown to induce apoptosis in a human epithelial cell line (Rhek-1) although high levels of LMP-1 expression were noted (Lu, *et al.*, 1996) and this is only a single report. Furthermore Wilson *et al* noted that expression of the EμLMP-1 transgene was possibly toxic. With the increase or induction of expression of Bcl-2 (Rowe *et al.*, 1994) and Mcl-2 (Wang *et al.*, 1996) in B-cells, and the induced expression of CD40 (Wang *et al.*, 1988; and Dawson *et al.*, 1990) and A20 (Miller *et al.*, 1995; and Laherty *et al.*, 1992) in both B-cells and epithelial cells following LMP-1 expression, it is possible that LMP-1 confers protection from apoptosis to these cells. Investigating the level of apoptosis in the epidermis of the PyLMP-1 mice might answer such a question.

Taking all of the results into consideration, it appears that LMP-1 operates *in vivo* to increase the rate of cellular proliferation in the epidermis (chapter 2). The results from chapter 3 clearly support this. Papillomas arise as a result of expansion (proliferation) of a single initiated cell. The observation that LMP-1 expression transgenics developed more papillomas than the negative control mice suggests that the PyLMP-1 epidermis displays increased cellular proliferation. That LMP-1 has weak tumour promoting activity and more importantly acts as an effective second stage promoter is further indirect evidence that LMP-1 increases cellular proliferation. This has important implications for the EBV associated malignancy NPC. A proliferating pool of cells might be more at risk of an oncogenic mutation leading to carcinoma than would non proliferating (or slowly proliferating) epithelium. Indeed, Ki67 has also been shown to be increased in NPC (Zheng *et al.*, 1994b) indicating that there is increased proliferation involved in NPC. Furthermore, it is known that proliferative activity and EGFR expression, events induced by LMP-1, are linked to the size and stage of various neoplasm's. Therefore LMP-1 expression in NPC may enhance proliferation and EGFR expression. Although an antibody to EGFR was available for use in immunohistochemical analysis of line 53 dorsal skin, it was a murine monoclonal and problems with background levels of staining proved inhibitory to the analysis of

the staining pattern. Determining the level of expression of EGFR in the PyLMP-1 mice therefore remains to be addressed.

In chapter 3 LMP-1 expression was shown not to be sufficient to initiate carcinogenesis, nor was it able to influence the malignant conversion rate or type of tumours arising. However, one cannot rule out a continued role for LMP-1 induced proliferation at this stage of carcinogenesis. Lymphoid infiltration has been observed in many NPC's (giving rise to the term lymphoepithelioma) and it is possible that LMP-1 is influencing this by activating the lymphocytes. It is also possible that LMP-1 is acting to upregulate other potential oncogenes during mouse skin carcinogenesis to act in concert with LMP-1 or to continue the carcinogenic process.

To investigate this possibility further, the PyLMP-1 transgenic mice were bred with K5-Ha*ras* (provided by K.Brown) transgenic mice (chapter 4). While LMP-1 action has been compared to activated *ras* in tissue culture experiments (Dawson *et al.,* 1990) and has been proposed to upregulate EGFR (which impacts the *ras* pathway) several studies here indicate that the action of LMP-1 and *ras* are separate. For example, LMP-1 increases cellular proliferation 2 to 3-fold whereas *ras* did not induce a significant increase in proliferation. Chapter 3 showed that LMP-1 acts as a tumour promoter, not an initiator whereas *ras* is a tumour initiator not a promoter (although it is mutated further as a result of promotion). Finally, LMP-1 inhibited the K5-Ha*ras* phenotype of sebaceous adenomas (possibly as a result of the hyperplastic phenotype which the K5-Ha*ras* mice do not display). Both the PyLMP-1 and K5-Ha*ras* mice were bred in a C57Bl/6 background, a strain known to be resistant to the effects of chemical carcinogens (DiGiovanni, 1991). Although not fully investigated, there appear to be susceptible ethnic groups to NPC. There may therefore be some correlation between the susceptibility genes involved in man and the suceptibility to chemical carcinogenesis in mouse. Crossing both lines to the susceptible FVB background was initiated but not completed due to time constraints. The results from this cross might provide further information if the C57Bl/6 background masked any co-operative action between the proteins. The promoter used in generating the *ras* mice was a truncated K5 promoter giving rise to hair follicle expression. It is unclear whether LMP-1 is expressed in the hair follicles or not. If the expression patterns are very different any potential co-operation would not be picked up in this particular crossbreed.

p53 is frequently mutated during chemical carcinogen induced mouse skin carcinomas. This tumour suppresser protein is mutated at late stages in the pathway to carcinoma. LMP-1 may

still play a role at this stage (providing proliferation) but in these mice no increase in progression was noted. LMP-1 expressing NPC's grew faster than LMP-1 negative NPC's (Hu *et al.,* 1995) but expression was absent at later stages of NPC, suggesting that LMP-1 acts early on during the disease process but may not be required at later stages. The PyLMP-1 transgenic mice bred with p53 knockout mice showed identical latency and patterns of tumour incidence as seen in p53 knockout mice alone where no skin carcinomas were observed. This suggests that the absence of p53 does not co-operate with LMP-1 in carcinogenesis. Lack of co-operation between LMP-1 and P53 may be because further co-operator(s) acting early are required before p53 loss takes effect. Bi-transgenic LMP/*ras* mice bred with p53 knockouts might provide answers to this point. Furthermore, other viral components are present in the disease state of NPC. EBNA-1, LMP-2 and BARFO might be required in conjunction with LMP-1 before loss of a tumour suppresser might have an effect. p16 is known to be mutated in NPC and loss of this or other tumour suppresser genes in conjunction with LMP-1 (and other viral proteins) might be required for NPC to develop. The LMP-1 variant used to create the transgenic lines involved in this work came from the B95-8 strain of EBV. Using the NPC LMP-1 variant and directing expression to the nasopharynx, then crossing these mice with transgenics where LMP-2 and EBNA-1 are also directed to this region, might provide a more accurate model for NPC. This programme of work is ongoing in the laboratory.

Correlating these results with the disease state of NPC a role for LMP-1 can be hypothesised. LMP-1 has no apparent initiating ability therefore requires this event to occur before it can exert it's effects. However, by increasing proliferation as a primary event, LMP-1 might increase the chances that an initiating event occurs. Once the nasopharynx is initiated, the increased proliferation induced by LMP-1 acts to promote carcinogenesis. When this event has proceeded, other factors come into play to convert the promoted cells to carcinoma. It is interesting to note that the diet of those Southern Chinese prone to NPC includes salted fish. The volatile nitrosamines produced during the preparation of the fish have been shown to have tumour promoting activity (Zou *et al.,* 1994; Zou and Landolph, 1991; Yu *et al.,* 1989). In conjunction with the second stage promotion of LMP-1, this might be sufficient to either induce, or strongly promote carcinogenesis. Alternatively, with other factors present (EBNA-1, LMP-2 etc) including the promotion from the volatile nitrosamines, LMP-1 might act as a strong (1st stage) promoter in the nasopharynx. The epithelial cell type in the nasopharynx might be different to the epithelial cells studied during mouse

skin carcinogenesis allowing LMP-1 to act as a stronger promoter. Creating tri-transgenic (LMP-1/LMP-2/EBNA-1) mice with nasopharyngeal expression and feeding them salted fish or the equivalent nitrosamines might recreate the situation in man in S.E. Asia. If this proves too difficult, treating PyLMP-1 transgenic epidermis with volatile nitrosamines would also provide results to support or contradict the hypothesis that this combination is oncogenic. With the creation of carcinoma cell lines from LMP-1 positive and negative mice, and the creation of B-cell lines from line 39 mice, this project could be taken *in vitro*. Analysis of these cell lines for p16, p53, Ha-*ras* mutations etc. would be an interesting undertaking. These cell lines could also be used to investigate the LMP-1 signalling pathway. If it was intact in these cell lines then it is likely that it is intact *in vivo*. A20, EGFR, NF-κB, CD40 etc. levels could all be analysed both *in vitro* and *in vivo*.

The results from the cross-breeding of PyLMP-1 and the K10-TGFβ1 mice in chapter 4 are more difficult to interpret. Here, almost all of the F1 mice born were LMP-1 negative suggesting that the combination of the two transgenes is lethal *in utero*. Early analysis of embryo's taken from e16 onwards suggests that this lethal effect takes place earlier than e16. TGFβ1 expression is "switched on" at embryonic day15 of mouse development (Byrne *et al.,* 1994). If the bi-transgenics are dying earlier than this time point it may be due to this expression date being brought forward. Indeed LMP-1 has been shown in our laboratory to upregulate expression of K10 3-4 fold at post natal d8. If this upregulation occurs during embryogenesis it is possible that LMP-1 is upregulating the K10 promoter of the TGFβ1 transgenic mice thus indirectly upregulating TGFβ1. Early over-expression of TGFβ1 is known to be lethal to developing embryo's and might explain the death *in utero* of the potential bi-transgenic mice. That 4/192 F1 mice born from one litter were LMP-1 positive and only 2 survived to adulthood suggests some genetic influence allowing these particular mice to escape the lethal effects of TGFβ1.

The results from chapter 5 may have important significance to the study of other EBV associated malignancies, such as HD, and lymphoma in immunosuppressed patients. In chapter 5 I showed that transgenic mice expressing LMP-1 in the B-cell compartment succumbed to long latency monoclonal B-cell lymphomas. In the subset of HD cases where EBV is associated, LMP-1 is expressed and may therefore play a causal role. Indeed, LMP-1 transfection into an EBV negative HD cell line resulted in the appearance of RS morphology cells (Knecht *et al.,* 1996). The results presented here demonstrate that LMP-1 can predispose B-cells to lymphoma. However, cells within the tumour do not adopt the RS morphology.

This might be because other factors, such as EBNA-1 and LMP-2 (both expressed in HD) are not present. However, the bi-transgenic tumours also failed to adopt an RS morphology. Further crossbreeding experiments to generate tri-transgenic LMP-1/LMP-2/EBNA-1 mice are required to generate a closer model for HD. The lack of HD morphology observed in the EμLMP-1 mice may also reflect a difference between mouse and man. In this chapter I also showed that LMP-1 and EBNA-1 do not co-operate in inducing lymphomagenesis. This suggests that the functions of the two proteins overlap in lymphomagenesis, or that a co-operative action does not effect latency to tumour onset (the assay used here) but some other aspect of tumour progression not studied.

    This thesis has therefore shown that LMP-1 acts to increase the rate of cellular proliferation in PyLMP-1 transgenic epidermis. LMP-1 has also been shown to be a mild or second stage tumour promoter. Furthermore, LMP-1 has been shown to predispose EμLMP-1 transgenic mice to B-cell lymphoma's. Expression of the LMP-1 transgenes in both lines of mice is low. It is interesting to speculate what might happen with regards to oncogenicity, if LMP-1 was expressed at higher levels or in a different epithelial cell e.g. mucosal or basal cells. Obviously generation of new transgenic lines where LMP-1 is expressed at higher levels would be interesting.

    The work presented in this thesis indicates that LMP-1 is likely to play a causal role in NPC and certain other EBV associated lymphomas.

CHAPTER 7:- Materials and methods.

7A   MATERIALS.

7A.1   Cells.

EBV negative cell lines:-
Ramos- Derived from an American BL - cat no. 85030802
BJAB- Derived from an African BL - cat no. DSM ACC72

EBV positive cell lines:-
B95-8 - Marmoset peripheral blood lymphocyte cell line derived following infection with B95-8 virus. Cell morphology both lymphocyte and fibroblast - cat no. 85011419
Raji- Derived from an African BL - 85011429
Ramos-EHRB- The Ramos cell line infected *in vitro* with EBV - cat no. 85030804

All cell lines were provided by the European Collection of Animal Cell Cultures(ECACC) except BJAB which was obtained from the German Collection of micro-organisms and cell cultures (DSM).

Carcinoma cell lines were derived from mice treated with carcinogens where the carcinoma was explanted into medium (see methods section 7B.2.1).

7A.2   Antisera.

CD45R/B220	PharMingen, FITC conjugated Rabbit polyclonal anti-mouse CD45R, cat no. 01124A
CD90(Thy.1)	PharMingen, Rabbit polyclonal anti-mouse CD90, cat no. 01021D
CRAF1(M-20)	Santa Cruz Biotechnology, rabbit polyclonal anti-mouse CRAF1, cat no. sc-947
Desmocollin	A kind gift from Prof.D.Garrod, Manchester Univ., rabbit polyclonal anti-bovine desmocollin1
Desmoplakin	Mouse monoclonal anti-Desmoplakins 1+2 (clone 11-5F), a kind gift from Prof.D.Garrod, Manchester Univ
EBNA-1(AZA-2E8)	Monoclonal anti-EBNA1 secreted from a hybridoma cell line (Hearing *et al*, 1985)

EGFR	Sigma, Mouse monoclonal anti-human EGFR, cat no. E2520
Keratin 14	A kind gift from Christina Cheng, NIH, Bethesda, affinity purified Rabbit polyclonal anti-mouse K14, designated K14-AF no.64
Ki67	Dako, Rabbit polyclonal anti-human Ki67, cat no. A0047
LMP(CS1-4)	Dako, Mouse monoclonal anti-LMP, cat no. M0897
LMP	Rabbit polyclonal anti-LMP peptide (Baichwal and Sugden, 1987)
LMP(S12)	Mouse monoclonal anti-LMP(C-terminus) secreted from a hybridoma cell line, (Mann *et al*, 1985).
MB1.2	A kind gift from Dr.B.Chan, Univ. of Western Ontario, Rat polyclonal anti-mouse VLA-β_1 integrin
TNFRII	Santa Cruz Biotechnology, Goat polyclonal anti-mouse TNFR2, cat no. sc-1074

7A.3   Reagents and solutions.

Acrylamide:-
A 30% solution of acrylamide/bis 29:1 (Bio-rad) was used which was de-ionised by stirring with Amberlite beads (Sigma) and filtered through Nalgene 0.45μm filters. Ammonium persulphate (Sigma) was used to initiate polymerisation, catalysed by TEMED (Bio-rad).

Agarose:-	SeaKem LE agarose - Flowgen Low Melting Point (LMP) - Gibco

Autoradiography:-
Kodak XAR was used for most autoradiograms, or alternatively the more sensitive Kodak Biomax MS film.
Film development- X-ograph compactX2
Intensifying screens - Genetic Research Instrumentation(GRI) Ltd.
Cassettes - GRI

Bag sealer:-	Vacuum bag sealer - Salton
Buffered phenol:-	Buffer-saturated phenol pH7.5 - Gibco
Buffers:-	MOPS-E - Boehringer

Tris-base - Boehringer

Camera+film:-
UVP image processor, Mitsubishi video copyprocessor and Mitsubishi thermal paper K65HM.

Centrifuges:-   Eppendorf centrifuge 5415C
                           Beckman centrifuge model J2-21
                           Heraeus labofuge 400

Chemicals:-   Acetone- Fischer scientific
                           BrdU- Sigma
                           BSA- Sigma
                           Chloroform- Fischer scientific
                           DAB- Sigma
                           DEPC- BDH
                           DePeX- Gurr's
                           DMBA- Sigma
                           Ethanol- Fischer scientific
                           Ethidium bromide- Boehringer
                           Formaldehyde- BDH
                           Formamide- Sigma
                           Gelatin- Sigma
                           Haematoxylin- Sigma
                           Histoclear- National diagnostics
                           Hydrogen peroxide- Sigma
                           Isopropanol- Fischer scientific
                           Methanol- Fischer scientific
                           Mercaptoethanol- Bio-rad
                           PMSF- Sigma
                           Tespa- Sigma
                           TPA- Sigma

Detergents:-   NP-40 - Sigma
                           SDS- Fisons/Sigma
                           Triton-X-100 - Bio-rad
                           Tween 20- Bio-rad

Electrophoresis:-
Power pack- 0-250v; 0.25A, Kikusui electronics
Electrophoresis tank(DNA/RNA)- Kodak international
                                   biotechnologies inc.
Electrophoresis SDS-PAGE tank- Bio-rad Protean IIxi cell and
                                   mini-protean II cell
Electroblotter(DNA/RNA)- TE series TransphorElectroblotter,
                           Hoefer

Electroblotter(protein)- Hoefer

1kb Ladder:-   Gibco

Enzymes:-   Aprotinin- Sigma
                      DNA restriction enzymes- Gibco
                      Exo(-) Klenow- Stratagene
                      Lysozyme- Sigma
                      Proteinase K- Boehringer
                      Trypsin- Sigma

Heating blocks:-   Eppendorf thermomixer 5436

Homogeniser:-   Kinematica polytron

Membranes:-   Biodyne A+B, Pall biosupport membranes

Microscopy:-
Microscopes used were the Olympus CK2 for tissue culture and cell counts, and the Leitz orthoplan for Fluorescence and high power magnification (i.e. x25-x100).
Slides- Superfrost, Menzel-Glazer

Mouse supplies:-   Anaesthetic- Fluothane liquid, Zeneca Ltd
                      Anaesthetising chamber- Fluotec
                      Cauterising iron- International Market
                                      Supply
                      Ear punch- ORME scientific
                      Shaver- Welonda

PCR:-   PCR machine- Appligene oncor crocodile3
                      Oligonucleotides- In-house or Cruachem
                      Taq polymerase- Promega
                      Buffer+MgCl$_2$- Promega
                      dNTP's - Boehringer

Plasmids and Probes:-
EBNA-1 - To detect the EBNA-1 transgene in blotting analysis, a 1Kb *Bst* X1-*Eco* R1 3' EBNA-1 DNA fragment derived from the plasmid pEμEBNA-1 (lab no. 114) was used (Wilson and Levine, 1992). See section 7B.3.5 for methods used to generate and isolate all probe fragments used.

LMP-1 - A *Hind* III- *Eco* R1 3kb fragment from plasmid pPyLMP-1 (lab no. 139) was used to detect the LMP-1 transcript (Wilson *et al*, 1990).

IgH J Region- *Xba* 1- *Xho* 1 900bp fragment from plasmid pEμ(py) Bspt (lab no. 79).

p53 - Kindly provided by Ken Brown at the CRC Beatson Labs.
Eco R1- *Nco* 1 250bp fragment from plasmid pMt T (lab no. 287).

Ras- Kindly provided by Ken Brown at the Beatson CRC Labs.
Sac 1 digest of plasmid BkIII*ras* (lab no. 233) yielding a 2.4kb fragment.

TCR Jβ$_{1+2}$ - Kindly provided by Jim Neil, Vet School, Glasgow.
Jβ1- *Eco* R1- *Bam* H1 3.3kb fragment (Palacios and Famaridis, 1991). Lab no. 463.
Jβ2- *Eco* R1- *Hind* III 750bp fragment from the βJ locus (Palacios and Famaridis, 1991). Lab no. 464.

TGFβ - Kindly provided by Rosemary Akhurst, Medical genetics, Glasgow. *Sac* 1- *Xho* 1 850bp fragment from plasmid pPoly A (lab no. 453).

Radio-isotopes and associated equipment:-
All isotopes were obtained from ICN or Dupont in 0-4^{0}C storage liquid Easytide form respectively. Isotopes used were α^{32}P dCTP, 3000ci/mmol.
Hybridisation- Hybaid midi oven and hybaid hybridisation tubes
Isotope separation- Nuctrap probe purification columns and push
                        column beta shield device, Stratagene
Pipette Tips- Aerosol Resistant Tips(ART), Gibco
Random primers/buffers/klenow- Stratagene Prime-it II kit.
Scintillation counter- Beckman LS1801 liquid scintillation systems
Scintillation fluid- Ecoscint scintillation solution, National
                        Diagnostics

RNA work:-
RNAzol B, Biogenesis, was used for all RNA extractions.   6+12ml Falcon tubes (2063 and 2059 respectively) were used to homogenise tissues and isolate RNA.

Rolling platforms:-   Rolling, rocking and shaking platforms
                        were all from Hoefer.

Slot-blot apparatus:-   Schleicher and Schuell minifold 2

Spectrophotometer:-   Spectronic 601, Milton Roy

<u>Stirrers/Hotplates:-</u>   Fisons

<u>Tissue culture:-</u>
DMEM and RPMI 1640 medium were obtained from Gibco as were
the supplements FCS, L-Glutamine, Penicillin, Streptomycin,   and
Fungizone.
DMSO- Sigma
Freezing container- Nalgene
Incubator- Heraeus
Plasticware- Corning

<u>UV equipment:-</u>
Stratalinker- UV stratalinker 1800, Stratagene
Transilluminator- UVP ultraviolet transilluminator, GRI

<u>Waterbaths:-</u>   Grant

7B   METHODS.

7B.1   Animal procedures.

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

7B.1.1   Breeding and numbering of transgenic mice.

All food, water and bedding requirements were handled by the animal technicians. Mouse pups born to breeding pairs were observed at day3-day10 for appearance of the LMP-1 line 53 phenotype and due notes recorded. The phenotype of hyperplasia is most obvious between 3 and 10 days old as a scurfy/scaly appearance of the skin (Wilson *et al.*, 1990). This provides an additional mechanism for assessing transgenic status, along with subsequent genomic DNA analysis.

At day 21 the mice were weaned and males and females separated. The mice were given a number in consecutive sequence and identified by ear punching. A small biopsy was taken (Tail-tip 0.5-1.0cm) under halothane anaesthesia, which was transferred to an eppendorf containing 700µl of tail solution for subsequent DNA extraction. The mouse tail was cauterised with a hot iron to stop bleeding and sterilise the wound.

7B.1.2   Monitoring health status.

In accordance with procedures as described in the Home Office project licence, all animals subject to the development of neoplasia, were monitored twice weekly for signs of ill health and discomfort. Any animal suffering or deviating from a normal health status was euthenased without delay.

7B.1.3   Mouse lines.

Line Identification	Transgene	Expression	Reference
53	PyLMP-1	Predominantly epidermis and tongue epithelium	Wilson *et al.*, 1990
H	K10-TGFβ1act	Activated TGFβ1 in the suprabasal keratinocytes of the epidermis	Cui *et al.*, 1995
M5	K6-sTGFβ1act	Low level expression in the epidermis. Full activation of TGFβ1 apon induction of hyperplasia	Fowlis *et al.*, 1996
769	K5-Ha*ras*	Epidermis	Bailleul *et al.*, 1990
92	3.7Kb of p53 gene interrupted in exon 5 by a *Pol* II-*neo* expression cassette	p53 fully knocked out- no expression	Donehower *et al.*, 1992
39	EµLMP-1	Weak expression in thymus and B-cell compartment	Wilson *et al.*, 1990
59	EµEBNA-1	B-cells	Wilson and Levine, 1992

7B.1.4   Chemical carcinogenesis treatment of transgenic mice.

All preparations and applications using chemical carcinogens were conducted in a fume hood and the utmost care was exerted at all times. Two pairs of gloves were worn when in contact with the carcinogens and the outer pair was discarded into a specially designated cin-bin for incineration. Aerosol resistant tips (ART) were used to avoid contamination of pipettes and likewise discarded into the specially designated cin-bin. The pipettes used were for carcinogen use only and were never removed from the treatment hood. After treatment the mice remained in the fume hood for 1hr minimum to ensure the chemicals had completely dried. Mice treated with carcinogens were kept in a designated room away from contact with other mice. Gloves were worn whenever treated mice were to be handled, whether they had been recently treated or not.

The two chemicals used in the procedures were 7,12-dimethyl-benz[a]anthracene (DMBA) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Stock solutions were made from anhydrous chemicals using acetone as solvent, and

stored at -20°C. From these, working solutions of 5×10^{-5}M were made and also stored at -20°C.

Four different treatment regimes were employed, but in each case the dose and timing of carcinogen applied, the length of experiment, and the observation and scoring techniques were identical. Mice to be treated had their dorsal skin shaved 24hrs before treatment. The chemical carcinogens diluted in acetone were then applied directly onto the dorsal skin. The acetone helps to spread the carcinogens over the whole dorsal region and also facilitates drying.

Mice were shaved on a Monday and treatment began on the following day. The standard chemical carcinogen treatment protocol involves a single treatment with 200μl DMBA followed by twice weekly treatments with 200μl TPA for 20wks. Thus DMBA was applied on a Tuesday followed one week later by the first TPA application, from then on applied on Tuesdays and Fridays for 20 weeks (Brown and Balmain, 1995)

Records were taken on developing lesions on a weekly basis during the 20wk TPA treatment, and for a further 40wks thereafter. The hair was shaved frequently, carefully avoiding damage to any lesions that had arisen. Papilloma's and carcinoma's were assessed on a measuring scale designed for this experiment and based on the size of the lesion. Thus papilloma's or carcinoma's were designated number 1, 2, 3 or 4 depending on their diameter where 1=<0.2cm; 2=0.2-0.5cm; 3=0.5-1.0cm; and 4=>1.0cm. Weekly counts were taken using this measuring scale.

Mice were euthenased when papilloma's or carcinoma's had reached a size of $2cm^2$ or when the overall papilloma load had reached a maximum Home Office approved limit (as advised by animal house technicians). Tissue samples were removed as described in 1.6 and 2.1 of the methods section.

7B.1.5   Transgenic mice cross-breeding.

Transgenic mice from the LMP-1 expressing line 53 or 39 were bred with other lines of transgenic mice expressing the Ha-*Ras* , TGFβ, and EBNA-1 genes respectively (see methods section 7B.1.3), and also with mice lacking both functional copies of the p53 gene. Offspring from these crosses were observed for alterations in the standard LMP-1 phenotype or any phenotype associated with the line being crossed to, and each crossbred line given a unique identification number system. The mice were treated as previously described in sections 7B.1.1 and 7B.1.2. Any lesions or abnormalities were noted and relevant tissues collected according to the procedure outlined in section 7B.1.6.

7B.1.6   Collection of tissues from transgenic mice.

Animals were euthenased by cervical dislocation or increasing concentrations of CO_2. External lesions were removed by shaving the skin to remove hair and rinsing with ethanol before careful excision with fine scissors and forceps. Part of the lesion was then put into 10% buffered neutral formalin fixative and the rest snap frozen in liquid N_2 for -70^0C storage. Mice with obvious internal abnormalities, or mice that were simply sickly looking were always dissected in the same manner. The fur was wetted with ethanol to exclude loose hair from the samples and then the animal was opened up from throat to stomach leaving the peritoneum intact. The skin was pulled back and the peripheral lymph nodes examined. The peritoneum was then cut to reveal the stomach area and the liver, spleen, intestine, mesenteric lymph node and kidneys examined. The chest cavity was then exposed by cutting through the rib cage and the lungs, heart and thymus examined. Any abnormalities in any of these tissues, or indeed in connective tissue etc., were noted and the lesion removed with scissors and forceps. Again part of the sample was put in fixative and the rest snap frozen. In every case a piece of tail was also frozen for further genomic analysis and confirmation of transgene status if required. In cases where the animal was found dead, a routine examination was undertaken to try to establish the cause of death and due notes recorded. No samples were taken in these cases. Where required the samples in fixative were sent for diagnosis to a pathologist contracted to the Glasgow Vet. School histopathology service.

7B.1.7   Analysis of epidermal proliferation using Bromodeoxyuridine incorporation.

Because Bromodeoxyuridine(BrdU) is a potent carcinogen, all procedures were carried out in the fume hood. Contaminated plastics, needles and mice were kept separate from general waste and later incinerated. Gloves were worn at all times and masks were used when the BrdU powder was weighed out by difference. Once in solution masks were not necessary.
BrdU was dissolved at a concentration of 125mg/ml in water in a glass bijoux by heating the solution to 60^0C. The bijoux was then wrapped in tin foil and stored at 4^0C as the stock solution. From this a working solution of 25mg/ml in sterile saline (0.85% NaCl) was also made and stored wrapped in tin foil at 4^0C. Every

time the working solution was used it was first heated to 60^0C to dissolve any crystals that had formed. Mice to be injected with BrdU had their dorsal skin shaved 24hrs prior to injection. The mice were weighed on an electronic scale and then each mouse was injected intraperitoneally (i.p.) with the equivalent of 2µl BrdU solution per gram of mouse. For 7 day old mouse pups the working solution was further diluted with sterile saline to 2.5mg/ml so that the pups were injected with the equivalent of 20µl BrdU solution per gram of mouse.

When each mouse had reached 1hr post-injection it was euthenased and the appropriate tissues taken into 10% buffered neutral formalin fixative. In most cases these tissues were dorsal skin, tongue and tail (Cui *et al.,* 1995).

7B.2   Cell culture techniques.

7B.2.1   Explantation of carcinomas.

All stages of the explantation protocol were performed under sterile conditions using 100% EtOH to clean surfaces and equipment and to swab mice. The mouse was sacrificed and swabbed in 100% EtOH. The carcinoma was excised and trimmed of skin and necrotic tissue. The carcinoma was rinsed briefly but thoroughly in EtOH and cut in half. One half of the carcinoma was placed in explant medium <u>Dulbecco's modified Eagle medium(DMEM) containing 87µg/ml Penicillin, 117µg/ml Streptomycin, and 2.9µg/ml Fungizone</u> on ice, and the other half was further split into two, one piece placed in fixative, and the other snap frozen and stored at -70^0C. The carcinoma was transported between animal house and laboratory in the explant medium on ice, where it was transferred to warmed growing medium <u>DMEM containing 20%(v/v) FCS, 2%(v/v) L-Glutamine, 12µg/ml Penicillin, 97µg/ml Streptomycin, and 1.2µg/ml Fungizone</u> in a petri dish in the tissue culture hood. The carcinoma was cut into small pieces using a sterile scalpel and then placed in an F25cm^3 culture flask. The carcinoma pieces were left to adhere to the plastic for 2-5min then 2.5ml of warm growing medium was layered over the lumps of carcinoma carefully avoiding dislodging the carcinoma pieces. The flask was incubated at 37^0C and 5% CO_2 for 7 days, then a further 2.5ml of 20% growing medium was carefully added and the tissue pieces incubated for a further 7 days. By this time outgrowth of adherent cells from the carcinoma was visible. The carcinoma pieces and medium were removed by aspiration and the remaining adherent cells washed

with PBS.A/EDTA. The cells were then trypsinised as described in methods section 7B.2.3, and cultured in 20% growth medium until the cells were confluent. At this point the growth medium was reduced in FCS content to 10%. Cells were expanded to confluence for 4 further passages in F75cm^2 flasks and then stored as frozen stocks as described in methods section 7B.2.4

7B.2.2   Growth of suspension cells.

B-cell lines were grown to be used as controls for various experiments. These were either EBV positive or EBV negative Burkitt's Lymphoma cell lines OR LCL's and were all grown in supplemented RPMI medium.

7B.2.3   Sub-culturing of mammalian cells.

When cells reached 90% confluence they were sub-cultured to allow maintenance of the cell line. In the case of carcinoma cells, the medium was aspirated and the cells were washed in PBS.A/EDTA. Trypsin solution (0.25% trypsin and 0.02% EDTA in dulbeccos buffered saline) was gently layered over the monolayer and incubated at 37^{0}C for 5 minutes to dislodge the cells. Cells were resuspended in fresh medium, pelleted in a Heraeus 400 centrifuge at 1000 rpm/194x g/ rotor no.8172 for 5min, and resuspended in the appropriate medium at the required density. In the case of suspension cells, the cells were pelleted in exactly the same manner and then resuspended in the appropriate fresh medium at the required density (method as suggested by ECACC).

7B.2.4   Storage of frozen stocks.

Epithelial cells grown to 90% confluence were trypsinised and pelleted as before then resuspended in 20% FCS media containing 10% dimethyl sulphoxide (DMSO). 1 ml aliquots were placed in 1.5ml cryovials and placed in Nalgene cryo 1^{0}C freezing container which was placed in a -70^{0}C freezer for 24 hours to ensure gradual chilling of the cells (approximately 1^{0}C/minute). These vials were then transferred to a liquid nitrogen container for long term storage.

7B.2.5   Revival of frozen stocks.

Cells were removed for use when required and quickly thawed by placing in a 37°C water bath. The cells were washed in serum free medium by centrifugation in a Heraeus 400 centrifuge (1000rpm/194x g/rotor no.8172/5min) to remove DMSO, and transferred to a small F25cm² flask containing appropriate medium. Suspension cells were pelleted as before, resuspended in the DMSO free media and thereafter treated as for epithelial cells (method as suggested by ECACC).

7B.3 DNA extraction and manipulation.

7B.3.1 Genomic DNA extraction.

35μl of proteinase K was added to the tail segment or tissue segment in 700μl of tail solution, and incubated at 55°C with shaking in an eppendorf thermomixer overnight (o/n). Samples can then be stored at 4°C indefinitely. DNA was extracted from the sample by adding an equal volume of buffer-saturated phenol pH7.5 and thoroughly mixing by inverting the tube 15-20 times. The samples were centrifuged in an eppendorf benchtop centrifuge at 12,000rpm/12,000x g/rotor 5415c for 15 min. This creates two liquid layers with an interface. The upper aqueous layer containing DNA/RNA was carefully transferred into a fresh eppendorf tube without disturbing the interface, and mixed with an equal volume of 2FC (see materials section 7B.5) by inverting the tube 15-20 times. Again the samples were centrifuged in the eppendorf benchtop centrifuge at 12,000rpm/12,000x g/rotor 5415c for 15 min. The upper layer was carefully transferred into a fresh eppendorf tube and mixed with 100μl of 10M NH4oAc and 750μl of cold absolute EtOH by shaking vigorously by hand for several seconds. The samples were then centrifuged at 10,000rpm/8385x g/rotor 5414c for 2 min to pellet the high molecular weight (HMW) DNA. The supernatant was removed and the pellet washed with 1ml of 70% EtOH. After another 2 min centrifugation at 10,000rpm/8385x g/rotor 5415c, the supernatant was removed and the pellet allowed to air dry. The pellet was then resuspended in 215μl of TE pH 7.6-8.0 by heating to 65°C for 30min, with occasional agitation to help the resuspension process. The samples were then stored at 4°C (Wilson *et al.*, 1990).

7B.3.2 Quantitation, restriction and electrophoresis of DNA.

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15µl of the DNA sample was used for OD_{260} measurement. This allows the DNA concentration to be ascertained, and the samples were then adjusted to 0.33µg/µl where possible to speed subsequent analysis. Samples were then stored at 4^0C indefinitely.

10µg of genomic DNA was routinely digested with restriction enzyme o/n at 37^0C using the recommended buffer. Typically 4µl of enzyme at a concentration of 15units/µl was used in the reactions. The reaction was stopped by heating the mix to 65^0C for 10min. 10% of the volume of loading buffer was added to each reaction and the samples were loaded onto a 0.8-1.0% agarose gel made up in 1xTAE and containing 2µl of 10mg/ml EtBr. 1Kb ladder was also added to one of the wells for subsequent band size evaluation. The DNA samples were electrophoresed in a horizontal electrophoresis chamber at 100v for 5 hrs in 1xTAE buffer and then the DNA was visualised on a short wave UV (280nm) transilluminator to ensure the DNA was intact and had been equally loaded. A photograph was taken for records (Wilson *et al.*, 1990).

7B.3.3   Southern blotting of DNA and specific fragment detection.

The gel slab was then treated with DNA denaturant (see materials section 7B.5) for 45min and then washed 3x15min in 0.5x TAE. The DNA was then transferred onto nylon (Biodyne B) membrane by electroblotting for 4hrs at 75v/1.5A in 0.5xTAE. The nitro-cellulose filter was then UV crosslinked using a stratalinker, and baked at 80^0C for 1hr. The filter was stored at RT or probed directly.

To probe the nylon membranes, the filters were placed in hybridisation tubes with 10ml of Church buffer and the tubes were placed in a hybridisation oven and rotated for 3hrs to o/n at 68^0C to allow full prehybridisation. The ^{32}P labelled DNA fragments used as probes were then denatured by heating to 100^0C for 10min before addition and hybridised o/n at 68^0C. The filters were then stringently washed 4x10min in 2xSSC, 0.1%(w/v) SDS at RT, and then 2x30min in 0.1xSSC, 0.1%(w/v) SDS at 68^0C in a shaking waterbath. The filters were then sealed in polythene bagging, taped into autoradiography cassettes with intensifying screens, and exposed to kodak XAR film at -70^0C. Exposed films were processed in a kodak x-omat (Wilson *et al.*, 1990).

7B.3.4   Slot blot analysis of genomic DNA.

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171

Another technique which was occasionally used to assess the transgene status of DNA extracted from tails was the slot blot technique. This involves identification of a specific gene by hybridisation of a DNA probe fragment to undigested, genomic DNA, and is particularly useful when large numbers of samples need to be tested at once. The technique is also less time consuming than Southern blotting as there are no restriction enzyme digests or gel electrophoresis steps.

10μg of DNA was transferred into an eppendorf tube and the volume made up to 120μl with H_2O. 40μl of 1M NaOH was then added and the tubes vortexed vigorously. The samples were left at RT for 10min and then 160μl of 10xTAE was added. The samples were vortexed once again and then left on ice. The slot blot apparatus was assembled with two pieces of pre-wet 3mm filter paper underlying the nitro-cellulose membrane which had been pre-soaked in 1xTAE for 30min. The samples were loaded without vacuum, one sample per slot, and then the vacuum applied. When the samples had been sucked through, one drop from a Pasteur pipette of 5xTAE was added to each slot. The membrane was then baked, DNA cross-linked, and the blot probed and washed exactly as a Southern blot membrane (Wilson et al., 1990).

7B.3.5 Preparation of ^{32}P labelled DNA probes.

Hybridisation probes for all nylon blotting were made using digested fragments from plasmid DNA. 10μg of the plasmid containing the DNA to be used as a probe was digested with restriction enzymes to generate a suitable DNA fragment. The digested DNA was electrophoresed slowly at 50V for 4hrs in low melting point agarose (LMP-agarose) containing 2μl of 10mg/ml EtBr and made up in 1xTAE. The DNA was visualised on a long wave transilluminator to estimate the DNA content of the desired fragment which was then collected. The fragment was heated to 70^0C to melt the agarose and an estimation of the volume made. From this the fragment concentration was then adjusted to approximately 5ng/μl and stored at -20^0C.

5μl of the DNA fragment (~25ng) was used for each probe reaction. The stratagene Prime-It II kit was used to prepare the probe. 10μl of random 9-mer primers (27 OD units/μl) and 29μl of H_2O was added to the DNA and heated to 100^0C to denature the dsDNA. Then 10μl of 5xdCTP buffer, 5μl of $\alpha^{32}P$ dCTP(3000ci/mmol) and 1μl of Exo(-) klenow were added to the reaction mix and incubated at 37^0C for at least 10mins.

172

Unincorporated nucleotides were removed over Stratagene Nuctrap probe purification columns in the stratagene push column beta shield device using STE according to the Stratagene Prime-it2 random primer labelling kit instruction manual. 1μl of the purified probe DNA was then used to count the specific activity of the probe with counts of >5x10^8 routinely available. The probe could then be store at -20^0C or used directly in which case the probe was heated to 100^0C for 5-10mins to denature the DNA, and then added directly to the hybridisation buffer and nylon filter.

7B.4   Protein manipulation.

7B.4.1   Protein extraction and preparation.

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Protein was extracted from snap frozen tissues (from -70^0C) storage as follows. Pieces of tissue were cut on dry ice using a sterile dissection blade, and the tissues were never allowed to thaw before preparation. The tissue was then homogenised in a 12ml (Falcon 2059) tube using a polytron blade with 2ml of NET:N and the proteinase inhibitors 2% Aprotinin and 1mM PMSF (made up in EtOH) freshly added. Once homogenised, triton-x-100 was added to 0.5% per ml and the samples vortexed hard to thoroughly lyse the cells. The samples were transferred to eppendorf tubes and centrifuged at full speed (14,000rpm/16,000x g/rotor 5415c) in an eppendorf benchtop centrifuge for 10min at 4^0C to pellet the cell debris. The supernatant was removed to a fresh tube on ice. The pellet can be stored at -70^0C if required or further extracted. Since the majority of protein extraction's were designed to isolate LMP-1 it was necessary to further extract the pellet where much of the LMP-1 will be retained after the first extraction with non-ionic detergent. The pellets were vortexed vigorously in 200μl of RIPA buffer containing the ionic detergent SDS to 0.5%. The samples were centrifuged as before to pellet the debris and the supernatants combined with the first non-ionic extract. NET:N was added to each tube to bring the volume to 1ml and to dilute the SDS concentration to approximately 0.1% (Wilson et al., 1990). Protein concentration was measured using the Bio-rad protein assay kit.

7B.4.2   Protein immunoprecipitation.

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Based on the' protein concentration an aliquot was removed to a fresh tube on ice, such that each tube had the same amount of starting protein. All steps were carried out on ice or at 4^0C. The

samples were made up to 1ml with NET:N and 100µl of 50% protein-A-sepharose (Sigma) made up in NET:N pH8.8, was added to each tube and the tubes rotated for 2hrs at 4⁰C. This pre-clear step removes non-specific proteins which may stick to the sepharose beads. The isolation proceeds by adding a volume antibody directed against the protein of interest and rotating the tubes o/n at 4⁰C. When LMP-1 was to be immunoprecipitated for example, 30µl of Rabbit αLMP-1 (See materials section 7A.2) was used. The precipitation step involves adding 30µl of 50% protein-A-sepharose to each tube and rotating for 40min maximum at 4⁰C. The antibody already bound to the protein of interest now also binds to the sepharose beads. The samples were then centrifuged for 1-2 min in an eppendorf benchtop centrifuge (14,000rpm/16,000x g/rotor 5415c) in the cold room to pellet the sepharose and the pellets washed x2 in 1ml NET:N, and 1x in TBS by vigorous vortexing and centrifugation again at 14,000rpm/16,000x g/rotor 5415c/2min/4⁰C. The pellets were then stored at -70⁰C or used directly for SDS-PAGE (Wilson *et al.*, 1990).

7B.4.3   SDS-PAGE separation of proteins and Western blotting.

SDS-PAGE separation of immunoprecipitated protein was carried out using a 10% running gel at pH 8.8 and 5% stacking gel at pH 6.8 (See frequently used solutions, materials section 7B.5). The sepharose pellets were heated to 95⁰C in 30µl of reducing sample buffer for 5min. The samples were briefly pulsed in the benchtop centrifuge to collect the liquid and the samples stored on ice until the supernatant was loaded onto the gel along with a protein marker standard to allow size determination of the desired protein. The samples were electrophoresed in running buffer at 50V until the samples had "stacked" at the stacking gel and running gel interface, and then electrophoresed at 150V until the dye marker had run off the end of the gel. The proteins were then transferred in transfer buffer onto nylon (Immobilon) membrane (pre-wet with methanol) for 2hrs at 100V/1.5A. The membrane was then submerged in PBS containing 5% non-fat Milk (NFM) o/n at 4⁰C on a rocking platform. This "blocking" step helps to reduce background signal at the later detection step.

The membranes were probed as follows. The primary antibody was diluted in PBS containing 2.5%NFM to a concentration of 1/100 to 1/250 depending on the specificity of the antibody, and added to the membrane for 1hr. All antibody binding and wash steps were carried out on a rocking platform.

The membrane was then washed 3x in Blotto HD and then briefly rinsed in PBS. The secondary antibody, which is HRP conjugated was again diluted in PBS containing 2.5%NFM to a concentration of 1/100, and added to the membrane for 1hr. The wash step was repeated with a further 2x5min PBS wash at the end. The proteins were detected using the Amersham ECL kit reagents where the HRP catalysed oxidation of luminol results in emission of light. Membranes were wrapped in cling film and exposed to kodak XAR film for between 5sec to 10min. The film was developed in a kodak X-omat. The membranes were then stored at -20^0C (Wilson *et al.*, 1990). The membranes could be stripped of the ECL reagents and antibodies using stripping solution (See frequently used solutions, materials section 7B.5), and re-probed.

7B.4.4   Sectioning of paraffin embedded tissues.

Tissues from the BrdU injected mice were left on a rolling platform o/n in fixative, and then washed as follows:- 1x30min in PBS, 1x30min in saline (0.85%NaCl), 2x15min in saline (0.85%NaCl)/100% EtOH, and finally 2x15min in 70% EtOH. All steps were carried out on the rolling platform at 4^0C using sterile solutions. The 70% EtOH was replaced after the last wash with fresh 70% EtOH and the samples sent to the Dermatology department for paraffin embedding.

7μm sections were used for subsequent BrdU staining. Frosted microscope slides were prepared to mount the sections by coating them in 3-aminopropyl triethoxysilane (TESPA). Slides were incubated at RT for 50sec in 2% TESPA (in acetone) followed by 2x50sec washes in acetone and 2x50sec washes in de-ionised water. All steps were carried out in the fume hood. The slides were then baked dry at 42^0C and stored in a slide box at RT until use.

The paraffin embedded tissues were kept in the fridge immediately prior to sectioning as this helps give more cleanly cut sections. The 7μm sections were cut on a microtome and carefully picked up and placed onto a TESPA coated slide using tweezers. 30% EtOH was dribbled over one edge of the section to "flatten" out the paraffin creases. The microscope slide was then gently immersed in a small 37^0C water bath filled with millipore filtered water so that the section floats off. The microscope slide was then carefully positioned under the section and the slide lifted up so that the section adheres to the slide. This procedure results in flat sections which are now adherent to the slide. Slides with sections on them were dried o/n on a 42^0C drying rack and kept thereafter at RT in a slidebox.

7B.4.5   Detection of BrdU incorporated nuclei.

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Sections were placed in a slide rack and immersed for 2x7min in Histoclear to remove the paraffin. The sections were then hydrated by immersing them through 2x100%, 90%, 80%, and 70% ethanol's respectively for 2min each. The sections were then washed by immersing 3x30min in TBS with a stir-bar to circulate the solution. The eventual detection of BrdU is facilitated by the Vectastain elite ABC kit (Vector laboratories) which is based upon an immunoperoxidase detection system. For this reason it was necessary to block endogenous peroxidase activity by immersing the sections in 0.5% H_2O_2 for 30min. The sections were washed again 3x5min in TBS with circulation. The sections were then immersed in warmed (37°C) trypsin solution of 0.1% trypsin and 0.1% CaCl$_2$ made in 50mM Tris pH8.0 for 3min to permeabilise the cell membrane. The sections were washed 3x5min in TBS as before. To minimise background antibody signal, the sections were then blocked with 1% normal horse serum (because the secondary antibody was raised in horse) from the Vectastain kit for 30min. Excess serum was shaken off and 10µl of the undiluted primary anti-BrdU antibody(Sigma) applied to each section. To avoid evaporation of the antibody, each section was covered with a glass coverslip. The anti-BrdU antibody was left for 1hr and then washed off with 3x5min TBS washes (the coverslips fall off during the washing). The secondary antibody, an anti-mouse biotinylated IgG antibody, was prepared as directed in the Vectastain kit and applied for 30min. A much larger volume (200µl) is applied to each section so there is no need for coverslips in this case. Once again the sections were washed 3x5min in TBS. 15min into the secondary antibody step, the ABC complex was prepared according to the Vectastain kit instructions and left at RT for 30min. The two components of the ABC complex are Avidin and biotinylated horseradish peroxidase. Avidin has four binding sites for biotin and because of the dilution's used, at least one of these sites remains unbound and thus able to bind to biotin on the secondary antibody. After the washes the sections were treated with the ABC complex for 1hr, where the avidin:biotinylated HRP complex now binds to the biotinylated secondary antibody through the biotin:avidin high affinity binding sites, and then washed 3x5min in TBS. To visualise individual BrdU stained cells the sections were "developed" with 3,3'-diaminobenzidine tetrahydrochloride (DAB) tablets(Sigma) which is a peroxidase substrate. One 10mg tablet was dissolved in 15ml of TBS and 12µl

of H_2O_2. Slides were placed in the fume hood with sections face up and the DAB stain, applied to each section individually for 1min before being washed off with TBS. The slides were then immersed in freshly filtered undiluted Harris's Haematoxylin(Sigma) for 10sec to counterstain the section, and then washed with tap water. BrdU staining nuclei now appear as brown, with non-staining nuclei blue. The slides were then immersed in Scott's tap water for 1min and rinsed with normal tap water for 1min. The slides were then dehydrated by immersing them through a series of ethanol's from 30% to 50%, 70%, 90% and 100% for 2min each. A further 5min immersion in 100% EtOH was followed by a 2min and then 7min immersion in Histoclear. Finally the slides were mounted with DePeX and a glass coverslip and the edges of the coverslip sealed with clear nail-varnish. Slides were then stored at RT.

The BrdU count is achieved by counting the average number of positively staining cells per field of vision under x312.5 magnification (x25 lens and x12.5 eyepiece). Cell counts excluded areas of hair follicle as these areas are already high in proliferating cells. All counts were done blind i.e. without knowing transgene status.

7B.4.6   Immunofluoresence   of proteins.

Some of the frozen tissues were sent to Dermatology where 7μm thick sections were cut on a cryotome. The sections were put on a gelatin coated slide and the slides stored in a slide box at -20°C. The sections were circled with a wax pen to prevent the subsequent antibody solutions spreading over the slide and thus minimise the volume of antibody required. The sections were "blocked" with wash containing serum from the animal in which the secondary antibody was raised. For example if the secondary antibody was raised in goat, then the sections were blocked with goat wash. This reduces the background signal created by non-specific binding of the secondary antibodies. Following the 30min blocking step the sections are briefly rinsed in PBS and 10μl of the diluted primary antibody added for 1hr in a humidified chamber (Tupperware box with damp paper towel lining and lid closed). The sections were then washed x3 in PBS and the fluorescent conjugated secondary antibody added for 1hr in the humidified chamber. The sections were again washed x3 in PBS and then mounted with vectashield (Vector laboratories), covered with a coverslip and sealed with clear nail varnish. The slides were analysed using a fluorescence microscope and stored at 4°C in the dark (Wilson *et al.*, 1990).

7B.5   Formulation of frequently used solutions.

Church buffer

7%(w/v) SDS
1%(w/v) BSA
1mM EDTA
250mM Na_2HPO_4
pH 7.2

Denaturing solution

0.4M NaOH
0.6M NaCl

2FC

49.45%(v/v) redistilled phenol
49.45%(v/v) chloroform
1%(v/v) isoamyl alcohol
0.1%(w/v) 8-hydroxyquinoline
Saturated with 1M Tris pH8.0

Fixitive

10% buffered neutral formalin:-
NaH_2PO_4
Na_2HPO_4
Formaldehyde

10x Goat wash

1.3M NaCl
70mM $Na_2HPO_4.2H_2O$
30mM $NaH_2PO_4.H_2O$
0.5%(w/v) sodium azide
10%(v/v) Goat serum
2%(v/v) Triton-x-100
0.5%(v/v)Tween 20

10x Loading buffer(DNA)

20%(v/v) Ficoll
0.1M EDTA pH8.0
0.25%(w/v) Bromophenol blue
0.25%(w/v) XC

10x Loading buffer(RNA)

1x MOPS-E
50%(v/v) pure, deionized formamide
17.8%(v/v) formaldehyde

10x Loading dye(RNA)

50%(v/v) glycerol(autoclaved)
0.1%(w/v) Bromophenol blue
0.1%(w/v) XC

Lysis buffer	1%(v/v) Triton-x-100 0.5%(w/v) sodium deoxycholate 0.1%(w/v) SDS 0.1M NaCl 0.05 Tris-HCl pH7.4 0.005M $MgCl_2$
MOPS-E	20mM MOPS 1mM EDTA 5mM NaOAc pH/NaOH/7.0
NET:N	150mM NaCl 5mM EDTA-NaOH pH8.0 50mM Tris-HCl pH8.0 0.05%(v/v) NP-40
PBS	137mM NaCl 2.7mM KCl 4.3mM Na_2PO_4 1.4mM KH_2PO_4 pH/HCl/7.3
PBS.A	1%(w/v) NaCl 0.025%(w/v) KCl 0.14%(w/v) Na_2HPO_4 0.025%(w/v) KH_2PO_4
PBS.A/ EDTA	PBS.A/ 0.54mM EDTA
RIPA buffer	150mM NaCl 50mM Tris-HCl pH7.5 1%(v/v) Triton-x-100 1%(w/v) sodium deoxycholate 0.1%(w/v) SDS
Running buffer(protein)	0.025M Tris 0.2M Glycine 0.1%(w/v) SDS pH8.3
Sample buffer	80mM Tris-HCl pH6.8 2%(w/v) SDS 10%(v/v) glycerol 0.01% Bromophenol blue

179

Fresh 5%(v/v) 2-mercapto-ethanol

Scott's tap water

Magnesium Sulphate 20g/L
Sodium Bicarbonate 2g/L

STE

10mM Tris.Cl pH7.5
10mM NaCl
1mM EDTA

SSC

150mM NaCl
150mM sodium citrate
pH/NaOH/7.5

Stripping solution

100mM 2-mercaptoethanol
2%(w/v) SDS
62.5mM Tris-HCl pH6.7

Tail solution

1%(w/v) SDS
150mM NaCl
10mM Tris-HCl pH7.5
100mM EDTA pH8.0

TAE(50x stock soln.(1L))

242g Tris base
57.1ml Glacial acetic acid
37.2g $Na_2EDTA.2H_2O$
pH ~8.5

TBS

140mM NaCl
20mM Tris
pH/HCl/7.6

TE

10mM Tris-Base
1mM EDTA
pH/HCl/7.6

Transfer buffer(protein)

192mM Glycine
25mM Tris
0.05%(w/v) SDS
20%(v/v) methanol

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Appendix 1:- Table of PyLMP-1 (line 53) transgenic mice crossed with p53-/- (line 92) knockout mice indicating the age of each animal at death, the transgenic and knockout status, and the cause of death.

Mouse i.d.	D.O.B.	Age	LMP/ p53		Symptoms and appearance of tissues
92.50	19/5/95	1.5	-/	+/-	Sacrificed due to bad cuts from fighting
92.51	19/5/95	?	+/	+/-	Missing, cause of death unknown
92.52	19/5/95	1.5	-/	+/-	Sacrificed due to bad cuts from fighting
92.53	19/5/95	3.5	+/	+/-	Dragging rear legs as if paralysed. Probable osteosarcoma on spine although none visible
92.54	19/5/95	?	+/	+/-	Missing, cause of death unknown
92.55	19/5/95	1.5	-/	+/-	Sacrificed because of LMP-1 negative status
92.56	19/5/95	1.5	-/	+/-	Sacrificed because of LMP-1 negative status
92.57	5/6/95	6	-/	+/-	Hind limb paralysis causing rear legs to drag. Small lump on spine near the middle
92.58	5/6/95	10	-/	+/-	Hind limb paralysis. Osteosarcoma on spine near shoulders
92.59	5/6/95	12.5	-/	+/-	Hind limb paralysis. Nothing obvious on spine but probably osteosarcoma
92.60	5/6/95	11	-/	+/-	Found dead, cause unknown
92.61	5/6/95	8.5	+/	+/-	Hind limb paralysis
92.62	14/7/95	?	-/	?	Sacrificed because of LMP-1 negative status
92.63	14/7/95	?	-/	?	As above
92.64	14/7/95	?	-/	?	As above
92.65	14/7/95	?	-/	?	As above
92.66	14/7/95	?	-/	?	As above
92.67	14/7/95	?	-/	?	As above
92.68	14/7/95	?	-/	?	As above
92.69	18/7/95	9.5	-/	-/-	Swollen abdomen, spleen enlarged/massive
92.70	18/7/95	10	+/	+/-	Dragging hind limbs. Osteosarcoma?
92.71	18/7/95	14	-/	+/-	Dragging hind limbs. Osteosarcoma?
92.72	18/7/95	4	+/	-/-	Cause of death unknown
92.73	18/7/95	8	+/	+/-	Very sickly looking with swollen abdomen. Spleen massive
92.74	18/7/95	?	-/	+/-	Cause of death unkown
92.75	18/7/95	?	-/	+/-	Cause of death unkown

Appendix 1:- Table of PyLMP-1 (line 53) transgenic mice crossed with p53-/- (line 92) knockout mice indicating the age of each animal at death, the transgenic and knockout status, and the cause of death.

Mouse i.d.	D.O.B.	Age (mo)	LMP/ p53		Symptoms and appearance of tissues
92.76	18/7/95	8	+/	+/-	Hind limb paralysis. Osteosarcoma?
92.77	18/7/95	4	-/	-/-	Found dead. Spleen and liver massive
92.78	18/7/95	5	+/	-/-	Liver grossly enlarged
92.79	18/7/95	7	-/	-/-	Swollen abdomen. Enlarged spleen and discoloured liver
92.80	18/7/95	5	+/	-/-	Mouse looked very sickly but had no obvious tumours. Liver very pale, rest normal
92.81	18/7/95	?	-/	+/-	Cause of death unknown
92.82	18/7/95	12	-/	+/-	Lymphoma. Spleen and peripheral lymph nodes enlarged
92.83	18/7/95	14	-/	-/-	As above
92.84	10/9/95	8	-/	-/-	Mouse v.sick. All perLN enlarged. MLN, spleen, liver and thymus all massive
92.85	10/9/95	9	-/	+/-	Poor mobility. Spine rough to touch. Osteosarcoma?
92.86	10/9/95	5.5	-/	-/-	Mouse v.sick. Spleen and MLN lymphomas. Some enlargement of perLN's
92.87	10/9/95	5	-/	-/-	Lymphoma
92.88	10/9/95	9.5	+/	+/-	Hind limb paralysis. Osteosarcoma?
92.89	10/9/95	?	-/	+/-	Cause of death unknown
92.90	10/9/95	7	-/	-/-	Poor mobility-osteosarcoma? Lymphoma of spleen also
92.91	10/9/95	14	-/	+/-	Mouse looked sick but was ok
92.92	10/9/95	10	+/	+/-	Lymphoma
92.93	10/9/95	5	-/	-/-	V.swollen abdomen. Spleen massive, all perLN enlarged
92.94	20/10/95	5	-/	-/-	Hind limb paralysis with severe hunched back- osteosarcoma?
92.95	20/10/95	?	-/	-/-	Sacrificed because LMP1 negative
92.96	20/10/95	?	-/	-/-	As above
92.97	20/10/95	?	-/	-/-	As above
92.98	20/10/95	?	-/	-/-	As above
92.99	20/10/95	?	-/	-/-	As above
92.100	20/10/95	8	+/	+/-	Mouse very hunched up no paralysis. MLN lymphoma
92.101	20/10/95	?	-/	+/-	Sacrificed because LMP1 negative
92.102	20/10/95	?	-/	-/-	As above

Appendix 1:- Table of PyLMP-1 (line 53) transgenic mice crossed with p53-/- (line 92) knockout mice indicating the age of each animal at death, the transgenic and knockout status, and the cause of death.

Mouse i.d.	D.O.B.	Age	LMP/ p53	Symptoms and appearance of tissues
92.103	20/10/95	?	-/ -/+	As above
92.104	26/11/95	·5	-/ -/-	Lump on right hind leg. PLN lymphoma
92.105	26/11/95	5	-/ -/-	Tumour on back below right shoulder
92.106	26/11/95	3	-/ -/-	Difficulty breathing and moving
92.107	26/11/95	7.5	-/ -/-	Large lymphoma
92.108	26/11/95	?	-/ -/-	Cause of death unknown
92.109	26/11/95	4.5	-/ -/-	Mouse hunched and slow moving. Spleen x3, osteosarcoma on spine
92.110	26/11/95	7	-/ -/-	Poor mobility in hind limbs.Spine feels "ribbed". LALN poss.enlarged. Massive thymus
92.111	26/11/95	?	-/ -/-	Cause of death unknown
92.112	26/11/95	?	-/ -/-	As above
92.113	26/11/95	?	-/ -/-	As above
92.114	26/11/95	6	-/ -/-	Tumour above right shoulder. Tumour v.red and bloody. Everything else ok
92.115	26/11/95	7	-/ -/-	Severe hind limb paralysis
92.116	20/12/95	?	?	Cause of death unknown
92.117	20/12/95	?	?	As above
92.118	20/12/95	6.5	?	Surplus
92.119	20/12/95	6.5	?	Surplus
92.120	20/12/95	?	?	Cause of death unknown
92.121	20/2/96	?	?	As above
92.122	20/2/96	4	?	Hind limb paralysis
92.123	20/2/96	?	?	Cause of death unknown
92.124	20/2/96	4	?	Large tumour on neck
92.125	20/2/96	?	?	Cause of death unknown

Mouse i.d.	D.O.B.	Age (mo)	E/L	Symptoms and appearance of tissues	Tissues	By	Pathology diagnosis	Re-ags IgH/TcR
M39/59.1	6/4/93	?	-/+	Spleen enlarged and discoloured with white spots. Other tissues normal	F:- S,L,MLN	?	Marked autolysis and bacterial overgrowth were present. Diagnosis inconclusive due to poor morphology	+/-
M39/59.2	6/4/93	11	-/+	Nothing detected	F:- S,L	UBB	ND	ND
M39/59.4	6/4/93	23	-/+	Sacrificed due to 24 month time point. Nothing detected	F:- S,L P:- S,L	DHC	Marked autolysis and bacterial overgrowth were present. Diagnosis inconclusive due to poor morphology	ND
M39/59.7	6/4/93	22.5	-/+	MLN lymphoma	F:- S,L,MLN,T,Th	?	ND	-/-
F39/59.10	6/4/93	24	-/+	Liver enormous and discoloured. Spleen and MLN slightly enlarged	F:- S,L P:- S,L,MLN	JAC	S:- Red pulp expanded due to marked EMH activity with participation of all cell lineages L:- Extensive EMH MLN:- Congestion, oedema, reactive and inflammatory changes present	ND
M39/59.13	17/5/93	18	-/+	Spleen, MLN, perLN's and kidney all enlarged	F:- ? P:- S,MLN,L,K	?	S:- Grossly enlarged and almost replaced by lymphoblasts. MLN:- Enlarged and replaced by large, pale staining tumour cells including numerous multinucleated giant cells suggesting a histiocytic origin. L:- Foci of granulocytic EMH K:- Several, large lymphoblastic infiltrates were seen in the cortex and pelvis Diagnosis:- Lymphoblastic lymphoma with multi-organ involvement and histiocytic lymphoma.	ND
M39/59.15	17/5/93	?	-/+	?	F:- S,L,Th P:- S,L	DHC	S:- Autolytic L:- Autolytic	ND
F39/59.18	17/5/93	14.5	-/+	Found dead	None	?	ND	ND
F39/59.20	17/5/93	17	-/+	Spleen and perLN massive	F:-? P:- S,perLN,L	?	S:- Enlarged and replaced with lymphoblasts LN:- Enlarged and replaced with lymphoblasts L:- NAD Diagnosis:- Lymphoblastic lymphoma	ND
F39/59.21	17/5/93	18.5	-/+	MLN and perLN tumours	F:- S,MLN P:- S,MLN,Th,L	?	S:- Almost replaced by lymphoblasts showing high mitotic rate and tumour cell necrosis. MLN:- Replaced by tumour cells. Th:- Replaced by lymphoblasts. L:- Heavily infiltrated and some part replaced by tumour cells forming nodular masses. Diagnosis:- Lymphoblatic lymphoma with multi-organ involvement	ND
M39/59.26	26/6/93	24	-/+	Sacrificed due to hunched appearance and poor mobility. MLN red/bloody but normal size	F:- L,MLN,T P:- L,MLN	JAC	L:- Small foci of histocyte/macrophage type cells which appeared to be benign MLN:- Reactive hyperplasia and lymphadenitis	+/-
M39/59.27	26/6/93	21	-/+	MLN red/bloody but normal size	F:- ? P:- S,L,MLN,Th	?	S,L,Th:- NAD MLN:- Reactive changes present	ND
F39/59.30	26/6/93	?	-/+	?	None	?	ND	ND
F39/59.31	26/6/93	20.5	-/+	MLN massive. Spleen enlarged x3	F:- S,L,MLN P:- S,L,MLN	DHC	ND	+/-

F39/59.34	26/6/93	17.5	-/+	MLN tumour	F:- ? P:- S,L,Intestine	DHC	S:- Red pulp enlarged due to marked EMH L:- Dilated and congested blood vessels and centrilobular necrosis of hepatocytes I:- Submucosa was markedly thickened due to infiltration by lymphoblasts showing high mitotic activity **Diagnosis:- Alimentary lymphoblastic lymphoma**	ND
F39/59.35	26/6/93	24	-/+	Spleen massive (x5). MLN lymphoma	F:- S,L,MLN(x3), T P:- S,L,MLN(x3)	JAC	S:- Replaced by pleomorphic lymphoid cells comprising of smaller cleaved cells and medium to large sized lymphoblasts showing high mitotic activity L:- Marked EMH and some tumour cells MLN:- Grossly enlarged and replaced by tumour cells with morphology seen in spleen **Diagnosis:- Mixed cell type of lymphoma with multi-organ involvement**	+/-
F39/59.38	19/7/94	23	-/+	Sacrificed because of bad cuts after fighting. One kidney was very red/bloody and abnormal in shape	F:- S,L,K,T P:- K	JAC	K:- Haemorrhagic necrotic tissue infiltrated by neutrophils	ND
F39/59.46	12/9/94	?	-/+	?	None	?	ND	ND
F39/59.47	12/9/94	14	-/+	Found dead	None	?	ND	ND
M39/59.49	20/10/94	18	-/+	Missing. presumed dead	None	?	ND	ND
M39/59.50	20/10/94	24	-/+	Sacrificed because animal had reached 24months of age. No abnormalities	F:- T	JAC	ND	ND
M39/59.52	20/10/94	16.5	-/+	Sacrificed due to prolapse. Peyers Patches very large. Spleen, MLN and SILN enlarged	F:- S,L,MLN,SILN ,PP,T P:- S,L,MLN,SILN ,PP	JAC	S:- White pulp expanded and replaced by tumour cells. Small areas of red pulp with EMH L:- Foci of EMH MLN:- Replaced by lymphoblasts SILN:- Slight reactive changes but normal architecture PP:- Reactive hyperplasia with germinal centre formation **Diagnosis:- Lymphoblastic lymphoma**	-/-
F39/59.55	20/10/94	24	-/+	Sacrificed because animal had reached 24 months of age. No abnormalities	None	JAC	ND	ND
F39/59.56	20/10/94	?	-/+	?	None	?	ND	ND
F39/59.57	20/10/94	24	-/+	Sacrificed because animal had reached 24 months of age. Massive tumour at one end of spleen. MLN lymphoma. Liver discoloured and 'lumpy'. PP enlarged	F:- S,L,MLN,T P:- S,L,MLN,PP	JAC	S:- Large part of the spleen was replaced with proliferating histiocytes which appeared to be mainly mature type with irregular, indented, vesicular nuclei and abundant cytoplasm. There were numerous multinucleated forms of varying size showing a range of appearances including those of foreign body and Langhans type cells. In some of the histiocytes accumulation of lipid was present. This morphology is similar to the conditions described in humans as 'Histiocytosis X' L:- Several foci of granulocytic EMH. Plasma cells and histiocytic type cells also present MLN:- Marked plasma cell and plasmablast hyperplasia with many Russell bodies PP:- Plasma cell hyperplasia with Russell bodies and increased blast cell activity **Diagnosis:- Histiocytic sarcoma**	-/-
M39/59.61	8/4/95	21	-/+	Sacrificed because animal was slow and hunched. Spleen enlarged x3 with tumour	F:- L,T P:- S	DHC	ND	ND

ID	Date	Age	Status	Cause of death / notes	F/P	Code	Pathological diagnosis	IgH/TcR
M39/59.62	8/4/95	14	-/+	Sacrificed due to bad ulceration under chin. Kidney poss. enlarged. Other tissues normal	F:- S,L,K,T,Ulceration P:- S,K,Ulceration	JAC	ND	ND
F39/59.66	8/4/95	20	-/+	Spleen slightly enlarged with white spots on it	F:- S,L P:- S,L	DHC	ND	ND
M39/59.67	10/6/95	?	-/+	?	/	/	/	/
M39/59.68	10/6/95	12	-/+	Sacrificed because of badly cut and infected tail. Everything normal	F:-S,L,T	JAC	ND	ND
M39/59.69	10/6/95	?	-/+	?	/	/	/	/
F39/59.70	10/6/95	?	-/+	?	/	/	/	/
F39/59.71	10/6/95	?	-/+	?	/	/	/	/
M59/39.13	30/10/94	20	-/+	Sacrificed because of poor mobility. Spine hunched and rough to touch. Massive MLN lymphoma. SILN slightly enlarged. Spleen slightly enlarged and discoloured. Liver poss. enlarged	F:- S,L,MLN,T,Gut P:- S,L,MLN,SILN,Gut	JAC	S:- Partially replaced by histiocytic/histioblastic type cells showing many multinucleated giant cells, phagocytic activity and high mitotic rate L:- Large areas of EMH and other areas infiltrated by tumour cells of the morphology described for spleen MLN:- Replaced by histiocytic/histioblastic type cells SILN:- Numerous neutrophils were present indicating a lymphadenitis Diagnosis:- Histiosarcoma	*+/+
M59/39.14	30/10/94	19	-/+	Sacrificed due to prolapse. Massive MLN lymphoma. PP slightly enlarged. Spleen normal size but discoloured	F:- S,L,MLN,T P:- S,MLN,PP	JAC	S:- White pulp expanded and replaced by medium to large lymphoblasts MLN:- Loss of architecture, replaced by lymphoblasts PP:- Grossly enlarged, neoplastic with large lymphoblasts Diagnosis:- Lymphoma	+/-
M59/39.19	15/12/94	20	-/+	Sacrificed due to poor mobility. Everything normal	F:- S,L,T P:- S,L.	MED	ND	ND
M59/39.23	4/2/95	19	-/+	Sacrificed because mouse looked v.sick. Spleen normal size but with 'lumpy' areas. MLN enlarged x2. Thymus poss. enlarged	F:- S,L,MLN(x2),Th,T P:- S,MLN	JAC	S:- Loss of architecture due to replacement by pleomorphic large blast cells with indented, irregular, vesicular nuclei containing multiple nucleoli and fairly abundant eosinophilic cytoplasm. MLN:- Enlargement due to loss of architecture caused by replacement by tumour cells with the morphology described for spleen. Many mitotic figures, giant and multinucleated cell forms and evidence of phagocytic activity Diagnosis:- Histiocytic sarcoma	?/-

Appendix 3:- Table of EμLMP-1 (line 39) mice crossed with EμEBNA-1 (line59) showing the transgenic status of each mouse, it's age at death, cause of death, pathological diagnosis, and IgH/TcR re-arrangements

Mouse i.d.	D.O.B.	Age (mo)	E/L	Symptoms and appearance of tissues	Tissues	By	Pathology diagnosis	Re-args IgH/TcR
M39/59.3	6/4/93	?	+/+	?	None	?	ND	ND
M39/59.5	6/4/93	22.5	+/+	Huge MLN lymphoma.	F:- S,L,MLN,T P:- S,L,MLN	?	Marked autolysis and bacterial overgrowth were present. Diagnosis inconclusive due to poor morphology.	+/-
M39/59.6	6/4/93	11	+/+	Nothing detected	F:- S,L	UBB	ND	ND
M39/59.8	6/4/93	?	+/+	?	None	?	ND	ND
F39/59.9	6/4/93	23	+/+	Ovary(?), CLN and spleen enlarged. Other tissues normal	F:- S,O P:- S,O,CLN	DHC	Marked autolysis and bacterial overgrowth were present. Diagnosis inconclusive due to poor morphology	ND
F39/59.11	6/4/93	?	+/+	?	None	?	ND	ND
M39/59.12	17/5/93	?	+/+	?	None	?	ND	ND
M39.59.14	17/5/93	23	+/+	Sacrificed due to 24 month time point. Nothing detected	F:- S,L,SILN P:- S,L	DHC	Marked autolysis and bacterial overgrowth were present. Diagnosis inconclusive due to poor morphology.	ND
M39/59.16	17/5/93	22.5	+/+	MLN enlarged, all other tissues normal	F:- S,L,MLN,T P:- S,L,MLN	DHC	ND	-/-
F39/59.17	17/5/93	24	+/+	Swollen stomach which turned out to be full bladder- urinary tract blockage?	F:- S,L,T P:- S,L	JAC	S:- NAD L:- Small foci of EMH	ND
F39/59.19	17/5/93	?	+/+	?	F:- S,L	DHC	ND	ND
F39/59.22	17/5/93	22	+/+	Spleen massive and virtually white. Liver enlarged and pale. MLN enlarged	F:- S,L,MLN,T P:- S,L,MLN	DHC	ND	-/-
F39/59.23	17/5/93	?	+/+	?	None	?	ND	ND
M39/59.24	26/6/93	?	+/+	?	F:- S P:- S,L	?	S,L:- NAD	ND
M39/59.25	26/6/93	?	+/+	?	None	?	ND	ND
M39/59.28	26/6/93	24	+/+	MLN massive and bloody. Two other gut assoc. tumours. White lump at one end of spleen but spleen normal size.	F:- L,MLN(x3),T P:- S,MLN(x3)	JAC	S:- White pulp expanded and replaced by lymphoblasts showing high mitotic rate MLN:- Gross enlargement due to replacement by tumour cells Diagnosis:- Lymphoblastic lymphoma with multi-organ involvement	-/-
F39/59.29	26/6/93	20	+/+	?	None	?	ND	ND
F39/59.32	26/6/93	?	+/+	?	None	?	ND	ND
F39/59.33	26/6/93	20.5	+/+	Liver enlarged x2 and pale cf. to normal. Spleen enlarged x3, pale with white spots. Tumour on one end of spleen. MLN and CLN enlarged also.	F:- S,L,MLN P:- S,L,MLN,CLN	DHC	ND	-/-
M39/59.36	19/7/94	24	+/+	Sacrificed because animal was 24 months old. No abnormalities	F:- S,L,T P:- S,L	MED	ND	ND
F39/59.37	19/7/94	24	+/+	Sacrificed because animal was 24 months old. Stomach full of murky red fluid. MLN poss. enlarged and red/bloody. Spleen enlarged x2 and discoloured	F:- S,MLN,T P:- S,MLN	JAC	S:- White pulp markedly expanded and replaced by large, pleomorphic lymphoblasts showing high mitotic activity and tumour cell necrosis. MLN:- Infiltrated by pleomorphic lymphoblasts Diagnosis:- Lymphoblastic lymphoma	+/-

ID	Date	Age	Status	Cause of death / notes	F/P	Code	Pathological diagnosis	IgH/TcR
M39/59.39	12/9/94	18	+/+	Sacrificed because of poor mobility. All organs normal	F:- S,L,T,Spine P:- S,L,Spine	MED	S:- Expanded white pulp showing reactive follicular hyperplasia	ND
M39/59.40	12/9/94	18	+/+	Sacrificed because of poor mobility. All tissues normal	F:- S,L,T P:- S,L	JAC	S:- Reactive hyperplasia of the white pulp L:- NAD	ND
M39/59.48	20/10/94	18	+/+	Spleen massive and very pale. Thymus enlarged x2	F:- S,L,CLN,SILN,Th,T P:- S,L,SILN	MED	S:- Enlarged SILN:- Loss of architecture. Replaced by medium sized lymphoblasts Diagnosis:- Lymphoma	+/-
M39/59.51	20/10/94	19	+/+	Animal sickly but nothing abnormal noted	F:- S,L,T P:- S,L	JAC	ND	ND
M39/59.53	20/10/94	20.5	+/+	Sacrificed due to lump on muzzle which was red and soft to touch. Liver massive with lymphoma	F:- L,T,Muzzle lump P:- L,Muzzle lump	JAC	L:- Focal necrosis probably due to 'Tyzzer's disease', which is commonly seen in mice, caused by an intracellularly growing bacteria Haemangioma-type lesions also present Skin:- Capillary haemangioma (benign lesion) Diagnosis:- Haemangioma	ND
F39/59.54	20/10/94	20.5	+/+	Sacrificed due to swollen stomach. Spleen massive (x10). MLN enlarged	F:- S,MLN,T,Intestine P:- S,MLN,Intestine	JAC	S:- Enlarged and replaced by a pleomorphic population of atypical cells MLN:- Enlarged and replaced with pleomorphic atypical cells Diagnosis:- Lymphoma	ND
M39/59.60	8/4/95	21	+/+	Sacrificed because animal was slow and hunched. Heart pale but otherwise rest ok	F:- S,L,T P:- S,L,H	DHC	ND	ND
M39/59.63	8/4/95	17	+/+	Paralysed rear legs. All tissues normal	None	JAC	ND	ND
M39/59.64	8/4/95	20	+/+	Mouse looked sickly but all tissues were normal	F:- S,L P:- S,L	DHC	ND	ND
F39/59.65	8/4/95	17	+/+	Sacrificed due to massively swollen stomach. All perLN's enlarged. MLN enlarged and red/bloody. Spleen massive (x10) and discoloured. Liver massive (x5) and discoloured	F:- S,L,MLN,perLN'S,T P:- S,L,MLN	JAC	S:- Completely replaced by large pleomorphic lymphoblasts showing high mitotic rate L:- Heavily infiltrated and partially replaced by tumour cells which were forming tumour masses MLN:- Replaced by lymphoblasts with morphology described for spleen Diagnosis:- Lymphoblastic lymphoma	+/-
M59/39.1	24/9/94	18.5	+/+	Found dead	None	?	ND	ND
M59/39.12	30/10/94	18.5	+/+	Sacrificed because of poor mobility. All organs normal size although spleen had tumour on one end	F:- S,L,T,Spleen tumour P:- S,L,Spleen tumour	MED	S:- Reactive white pulp showing follicular hyperplasia	ND
F59/39.15	30/10/94	10	+/+	Sacrificed because of carcinoma. Liver normal size but pale. Spleen enlarged x2	F:- S,L,T,Carcinoma P:- S,L,Carcinoma	JAC	S:- Red pulp expanded due to EMH L:- Foci of EMH Skin:- Squamous cell carcinoma	ND

M59/39.16	15/12/94	16.5	+/+	Sacrificed due to hind limb paralysis. All perLN's enlarged. Thymus enlarged. Spleen massive and white. MLN massive.	F:- S,L,perLN's,Th,Kidney tumour, Gut tumours. P:-As above plus spine	JAC	S:- Loss of architecture, replaced by lymphoblasts L:- Small foci of EMH perLN's:- All enlarged and replaced by lymphoblasts Th:- Replaced by lymphoblasts Diagnosis:- Lymphoblastic lymphoma	+/-
M59/39.20	15/12/94	20	+/+	Sacrificed due to poor mobility. Thymus massive	F:- S,L,Th,T P:- S,L,Th	MED	ND	+/-
M59/39.22	4/2/95	19	+/+	Spleen massive (x20), bi-lobed and full of white pulp. MLN and CLN enlarged	F:- S,L,CLN,T P:- S,L,CLN	MED	ND	+/-
F59/39.24	4/2/95	19	+/+	Found dead. MLN and liver lymphomas. PerLN slightly enlarged	None	JAC	ND	ND
F59/39.25	4/2/95	18	+/+	Sacrificed because mouse looked v.sick. Massive MLN lymphoma. Spleen, liver and thymus enlarged	F:- S,L,MLN,Th,T P:- S,L,MLN,Th	JAC	S:- Almost entirely replaced by lymphoblasts and only small foci of EMH were recognisable MLN:- Grossly enlarged and replaced by sheets of large lymphoblasts showing high mitotic rate and tumour cell necrosis. Diagnosis:- Lymphoblastic lymphoma	ND
F59/39.27	4/2/95	16.5	+/+	Sacrificed because of ulceration on neck. MLN and spleen lymphomas	F:- S,L,MLN,T,Ulceration P:- S,MLN,Ulceration	JAC	S+MLN:- White pulp of both was markedly enlarged with some loss of architecture. Both contained a variety of cell types including plasma cells and atypical cells which possibly represented a grossly exaggerated reactive process turning into a neoplasia	+/-