

**Regulation of the Epstein-Barr Virus Latent Membrane
Protein 1 Expression**

Pegah Johansson

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GÖTEBORG UNIVERSITY

Institute of Biomedicine
Department of Clinical Chemistry and
Transfusion Medicine

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“Satisfaction of one's curiosity is one of the greatest sources of happiness in life.”

Linus Pauling

*To Janne for his continuous love and support
and to my parents for their encouragement and belief in me*

Abstract

Epstein-Barr virus (EBV) is a probably the most effective and successful human virus, infecting more than 90% of the world's adult population. As with the other members of the herpesvirus family, EBV establishes latent infection in its host and persists life-long. EBV infection is generally harmless in children but can cause infectious mononucleosis (IM) in young adults. EBV is associated with a number of human malignancies including Burkitt's lymphoma (BL), Hodgkin's lymphoma (HL), nasopharyngeal carcinoma (NPC), nasal T/NK lymphoma (NL), peripheral T cell lymphoma, gastric carcinoma, and lymphoproliferative diseases in immunocompromised patients. A compromised immune system and an aberrant EBV latent gene expression are thought to be important players in the aetiology of EBV malignancies. EBV is one of the most potent transforming agents in vitro and immortalizes B cells into lymphoblastoid cell lines (LCLs).

Latent membrane protein 1 (LMP1) is the main EBV oncogene, which is critically involved in immortalisation and proliferation of LCLs, and is associated with most EBV malignancies. LMP1 functions as a constitutively active tumour necrosis factor receptor (TNFR) and upregulates anti-apoptotic and pro-survival proteins through the activation of cellular signalling pathways. Thus, inappropriate expression of LMP1 is probably a central process in EBV associated tumourigenesis. The aim of this PhD project was to delineate the regulation of LMP1 gene expression in response to cellular factors.

The LMP1 protein expression is regulated differently according to the expression pattern of the other EBV latent proteins as well as the cell type in which it is expressed in. In latency III infected B cells all of the EBV latent proteins are expressed, and LMP1 expression is driven by the viral transcription factor EBNA2. The EBNA2 protein lacks DNA binding ability itself, and requires cellular factors (adaptors) to be recruited to promoters. In latency II cells that represent most EBV tumours and different cell-type hosts, a more limited set of EBV latent proteins are expressed, and LMP1 expression occurs in the absence of EBNA2. Regardless of the mode of expression and cell type, LMP1 transactivation is critically dependent on cellular proteins.

In the course of this investigation, a new EBNA2 adaptor was identified that bound an AP-2 site in the LMP1 promoter and mediated the relief of promoter repression and activation of the LMP1 promoter.

We also report EBNA2-independent upregulation of the LMP1 promoter in response to upregulation of the p38 kinase pathway. The p38 signalling pathway activates the ATF1-

CREB heterodimer that has been previously shown as an activator of LMP1 transcription. The binding of ATF1-CREB to a CRE site is a central event in LMP1 regulation both in the presence and absence of EBNA2.

Additionally, we showed the presence of a mutation in the LMP1-CRE site of the P3HR1 EBV variant. This mutation led to a reduced binding efficiency of ATF1-CREB to the CRE site and a two fold reduction of LMP1 promoter activity. This finding together with reports from other groups indicate that sequence variations in the CRE site of LMP1 are evolutionary selected, probably to modulate the expression levels of the protein.

Our results also indicate that the NF- κ B dimers, p50-p65 and p50-p50, bind an NF- κ B site in the LMP1 promoter and activate transcription independently of EBNA2. The EBNA2-independent activation of LMP1 transcription by NF- κ B suggests that this signalling pathway may play a role in LMP1 activation in latency II infected B cells. Since the NF- κ B pathway is activated by LMP1, a positive autoregulatory loop in LMP1 activation may exist. The positive autoregulation of LMP1 is supported by reports from other groups.

Finally, we showed that histone acetylation and modulation of the chromatin structure of the LMP1 promoter are involved in the activation of LMP1 transcription. We hypothesise a model whereby the EBNA2 is recruited through interaction with several EBNA2 adaptors at the promoter and mediates activation. Alternatively, several transcriptional activators such as NF- κ B factors and ATF1-CREB bind the promoter in the absence EBNA2 and cooperatively activate the promoter. In both cases factor-binding to the promoter leads to the recruitment of histone acetylases and chromatin remodelling enzymes to the LMP1 promoter to facilitate transcription.

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Abbreviations

ADP	adenosine diphosphate
ARF6	ribosylating factor 6
ATF	activating transcription factor
AUF1	AU-rich element RNA binding protein 1
BCR	B cell receptor
BL	Burkitt's lymphoma
bp	base pair
CBF	C-promoter binding protein
ChIP	Chromatin immunoprecipitation assay
COX	Cyclooxygenase
Cp	C promoter
CREB	cAMP response element-binding protein
CTAR	C-terminal activating region
DNMT	DNA methyltransferase
EBER	Epstein-Barr virus encoded RNA
EBNA	Epstein-Barr virus nuclear antigen
EBNA2RE	EBNA2 response element
EBV	Epstein-Barr virus
ED-L1	EcoR I D leftward promoter 1
EGFR	epidermal growth factor receptor
EMSA	Electromobility shift assay
ERK	extracellular signal-regulated kinase
FGF	fibroblast growth factor
FR	family of repeats
GAP	GTPase-activating proteins
GC	germinal centre
GEF	guanine nucleotide exchange factors
HAT	histone acetyltransferase
HBP	high mobility group-box protein
HDAC	histone deacetylase
HIF	Hypoxia-Inducible Factor
HL	Hodgkin's lymphoma
HMT	histone methyltransferase
HRS	Hodgkin/Reed Sternberg
HSP	heat shock protein
IKK	I- κ B kinase
I- κ B	Inhibitor κ B
IM	Infectious mononucleosis
IL	Interleukin
IR	Internal repeat
IRAK	interleukin-1 β receptor associated kinase
IRF	interferon regulatory factor
JNK	c-Jun NH ₂ -terminal kinase
kb	kilobase pair
kDa	kiloDalton
LCL	lymphoblastoid cell line

LMP	latent membrane protein
LRS	LMP1 regulatory sequence
LSP1	lymphocyte-specific protein 1
MAPK	mitogen-activated protein kinase
MAPKAPK2 (MK2)	MAP kinase-activated protein kinase 2
MAPKK (MAP2K)	MAPK kinase
MAPKKK (MAP3K)	MAPKK kinase
MBD	Methyl-CpG-binding domain
MEFs	mouse embryonic fibroblast
MEF2A	myocyte enhance factor 2A
MEF2C	myocyte enhance factor 2C
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MNase	micrococcal nuclease
mRNA	messenger RNA
MSK	mitogen- and stress-activated protein kinase
NIK	NF- κ B inducing kinase
NFAT	nuclear factor of activated T cells
NF- κ B	Nuclear Factor κ B
NL	nasal NK/T cell lymphoma
NPC	nasopharyngeal carcinoma
OriP	origin of replication
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PI3K	phosphatidylinositol 3-kinase
PIC	preinitiation complex
PKB	protein kinase B
PRAK	p38 regulated/activated kinase
PTLD	post-transplant lymphoproliferative disease
Qp	Q promoter
RBP-J κ	J κ recombination signal binding protein
RIP	receptor-interacting protein
RTK	receptor protein tyrosine kinase
SAP1	SRF accessory protein 1
SAPK	stress activated protein kinase
SH2	Src homology 2
SMN	survival motor neuron
TES	transformation effector site
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
TRADD	TNFR associated death domain
TRAF	TNFR associated factor
TR	terminal direct repeat
L1-TR	Leftward promoter 1 terminal repeat
u-PA	urokinase-type plasminogen activator
VEGF	vascular endothelial growth factor
Wp	W promoter

Background

Epstein-Barr Virus: Discovery

The discovery of Epstein-Barr virus was brought about by a British surgeon, Denise Burkitt, during his medical service in Uganda. Burkitt described for the first time a highly unusual childhood lymphoma that was characterized by large swellings of the jaws (Burkitt, 1958), which eventually became known as Burkitt's Lymphoma (BL). Burkitt reported that the distribution of BL depended on climatic factors such as temperature and rainfall. The epidemiology of BL caught the attention of Anthony Epstein. He postulated the aetiology to be an oncogenic virus, and following discussions with Burkitt began investigating BL tumour biopsies (Epstein et al., 1966). Ultimately, Epstein-Barr virus was identified from the first BL-derived continuous cell line (EB1) using electron microscopy (Epstein et al., 1964). Interestingly enough, this cell line was produced from the outgrowth of B cells from one of the biopsies due to a delay in its transport (Epstein, 2005). Hence, EBV was the first human tumour virus to be discovered.

EBV has since been classified as a gamma herpesvirus, and belongs to the genus *lymphocryptovirus* (Kieff & Rickinson, 2001). Seroepidemiological research by Werner and Gertrude Henle showed that EBV infection is ubiquitous and widely spread in all human populations, infecting more than 90% of the adult population (Henle & Henle, 1969; Henle et al., 1970). The Henles also discovered EBV to be the causative agent for infectious mononucleosis (IM) (Henle et al., 1968).

EBV primary infection and persistence

EBV infection during early childhood is asymptomatic. However, infection by EBV during adolescence or adulthood results in IM. The general mode of transmission is through saliva. The events following in vivo EBV infection are still not fully understood. Based on the in vitro studies of EBV infection and some in vivo data a model has been constructed for EBV infection. Primary infection of infiltrating B-lymphocytes occurs in the epithelium of the naso- and oro-pharyngeal mucosa of the upper respiratory tract (Tao et al., 1995). EBV entry to B cells is mediated by the interaction of its glycoprotein gp350/220, with the CD21 B cell

receptor (Fingeroth et al., 1984; Nemerow et al., 1985) and gp42/gH/gL with the major histocompatibility complex (MHC) class II on the B cell (Li et al., 1997). Recently it has been reported that after binding to primary B cells, the Epstein-Barr virions may remain on the B cell surface and from there transfer efficiently to CD21-negative epithelial cells (Shannon-Lowe et al., 2006) thereby infecting both cell types.

EBV infection in epithelial cells generally leads to activation of its lytic cycle and virus replication (Lemon et al., 1977; Lemon et al., 1978; Sixbey et al., 1983). In B cells however, EBV does not replicate and instead establishes latent infection (Thorley-Lawson, 2005a). During latent infection only a limited subset of viral genes are expressed, which comprise six EBV nuclear antigens (EBNAs 1 to 6) and three latent membrane proteins (LMP1, LMP2A and LMP2B) as well as two EBV non-polyadenylated RNAs (EBERs) (Kieff & Rickinson, 2001). Different patterns of latent protein expression is observed in B cells and tumour cells infected by EBV and the patterns of EBV gene expression appear to be entwined with normal B cell biology and development (Tao et al., 2006) (Figure 1). Upon virus infection of B cells all latency genes are expressed and lead to B cell proliferation (Figure 1). This is referred to as the growth program or latency III. The activation of B cells by the virus mimics antigen activation of B cells. Binding of antigen to the surface of a naive B cell causes the cell to differentiate into an activated blast and migrate into the follicle to form a germinal centre (Liu & Arpin, 1997) (Figure 1). It is proposed that EBV activated B cells also pass through a germinal centre (GC) reaction where a more restricted set of EBV genes are expressed (Babcock & Thorley-Lawson, 2000). This expression pattern is designated the default program or latency II, and exhibits EBNA1, LMP1 and LMP2A as well as EBER expression. In GC normal B cells undergo isotype switching and mutation of immunoglobulin genes. Based on the affinity of antigens binding to the surface of these B cells and a signal from a T-helper cell, some of the GC B cells survive and leave the GC as memory B cells. EBV infected B cells in the GC are also thought to undergo isotype switching (He et al., 2003) and immunoglobulin gene mutation (Casola et al., 2004) and eventually differentiate into memory B cells in the absence of antigen binding and T cell signal. EBV infected memory B cells express no latent genes, (referred to as the latency program or latency 0). These cells only express EBNA1 during B cell replication (EBNA1 only program or latency I) to insure the EBV genome is also replicated and passed on to the daughter cells (Thorley-Lawson, 2005b). Thus, the timely and coordinated expression of latent genes in B cells drives them to develop and enter memory B cells in the absence of extracellular stimuli. EBV infected B cells expressing latent proteins are efficiently detected by specific T cells

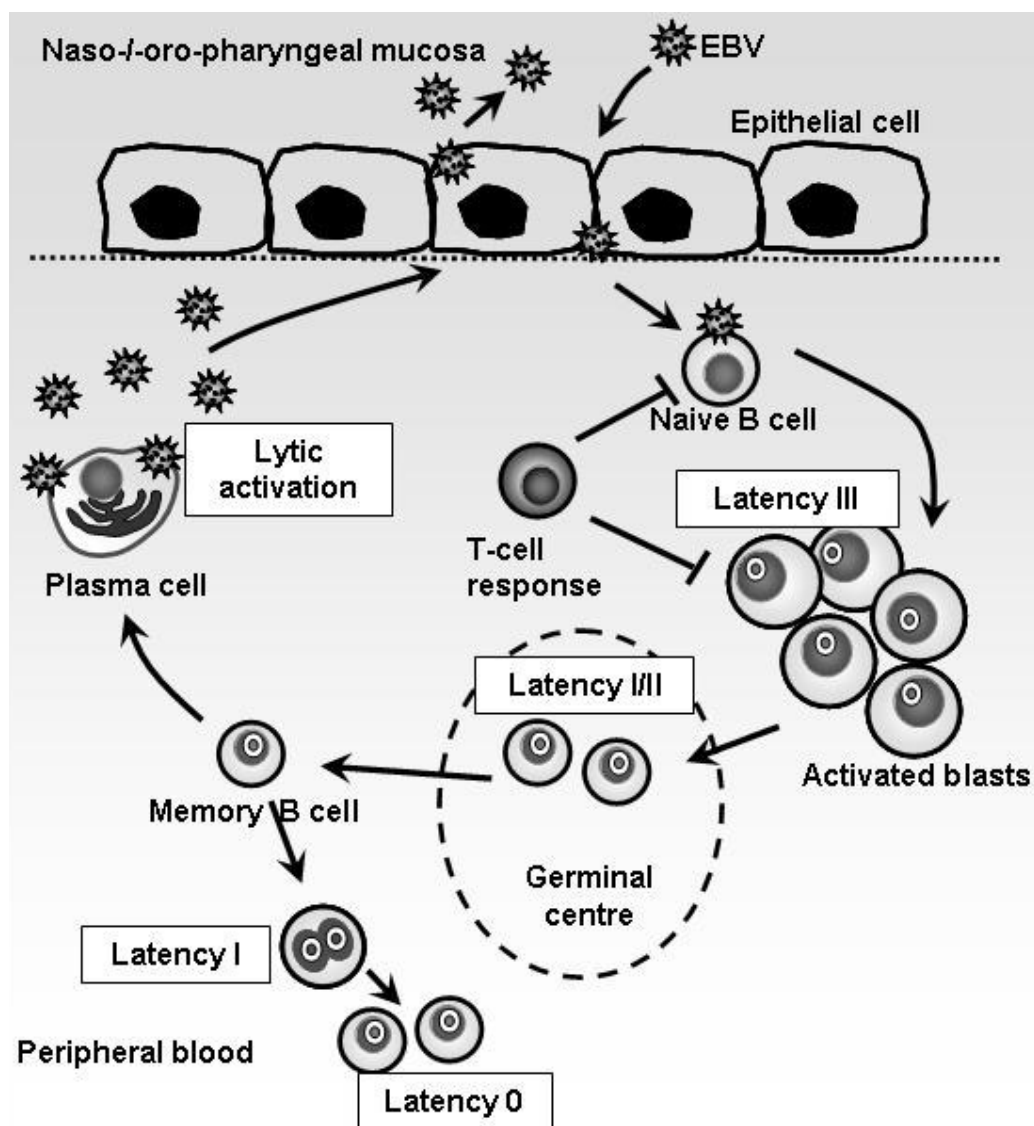


Figure 1. A model for primary EBV infection and persistence in vivo.

The virus enters via the epithelial lining of the oropharynx and gains access to the underlying naive B cells. The viral latent gene expression induces proliferation in infected cells (latency III program of gene expression or the growth program). Many of the EBV infected B cells are killed at this stage by a cytotoxic T-cell response. Some of the cells, however, escape the immune response and undergo a germinal centre (GC) reaction where a more limited set of viral genes are expressed (latency II/I gene expression or the default program). The infected GC cells are then rescued and develop into memory B cells where no EBV gene expression is observed (latency 0 or latency program). During replication of these memory B cells, EBNA1 is expressed to insure that the viral episome is also replicated and passed on to the daughter cells. If the memory B cell is activated by an antigen it differentiates into a plasma cells and EBV switches to its lytic program leading to virus production. Reactivation of the lytic cycle releases new virus that may lead to a new cycle of naive B cell infection. The virus infects epithelial cells, which also leads to the lytic replication of the virus and shedding via saliva.

that control the proliferation of these cells, but the infected memory B cells (latency I/0) escape immune response since they lack immunogenic EBV antigens. EBV infected memory B cells persist life-long in the peripheral blood of the host at a frequency of 1-50 per million B cells. If EBV infected memory B cells differentiate into plasma cells, the virus is released for further infectious spread (Thorley-Lawson, 2005a). A schematic model of the virus's life cycle and persistence in vivo is shown in Figure 1. The fact that EBV can access and persist in memory B cells without causing disease is the key to its success in infecting most of the human population and is evidence of its co-evolution with man. EBV pathogenesis however does arise when the immune system is compromised.

EBV latent genes and expression regulation

Since latent expression programs appear to be central in EBV biology and pathogenesis, the regulation of EBV gene expression has been the focus of many investigations. The EBV genome is a linear double-stranded DNA that is approximately 172 kilobase pairs (kb) (Farrell, 2005). After infection and internalization of the virus, its DNA circularizes to form an episome that localises to the nucleus (Figure 2A). EBV DNA contains a series of terminal direct repeats (TRs) and internal repeat sequences (IRs) (Farrell, 2005).

In EBV positive healthy individuals, EBV latent gene expression is tightly regulated. During early infection of B cells the W promoter (Wp) is the first promoter to be activated (Woisetschlaeger et al., 1990) and drives the expression of all the EBNA proteins (Figure 2). Wp is encoded within the major internal repeat of the virus (IR1) and therefore it is present in multiple copies (Figure 2). The Wp driven EBNA2 protein is a transcriptional activator which is recruited to the C promoter (Cp) located upstream of the Wp. Within a few days, Cp becomes the dominant latent promoter activated by EBNA2 and drives the expression of EBNAs (Woisetschlaeger et al., 1991). The EBNA gene transcripts are produced by alternative splicing from a long transcript originating from Wp or Cp (Figure 2). Every EBNA transcript contains multiple copies of the W exons, which in most cases splice to two or three small exons. Alternative splicing dictates the 3' coding exon present in the mature EBNA gene transcript (Speck, 2005). The EBNA5 protein is encoded by the repeat W exons followed by two unique exons (Figure 2B) (Speck, 2005).

Both Wp and Cp transcription can be detected in peripheral blood mononuclear cells (PBMC) of IM patients (Tierney et al., 1994) but Wp is heavily methylated leading to its repression in PBMC of healthy EBV carriers (Paulson & Speck, 1999). However, in the

absence of Cp activity (for example where Cp is deleted), Wp can substitute for activation of the EBNA_s (Tao et al., 2006). EBNA₁ is transcribed relatively early after infection. Binding of EBNA₁ to the origin of replication (OriP) acts as an enhancer for both Wp and Cp (Speck, 2005). Reporter assays have been used to identify important regulatory DNA sequences in Wp and Cp (regulatory elements) involved in transcription regulation.

Three domains termed UAS 1, 2, and 3 play an important role in Wp activity. Binding sites for YY1, CREB/ATF are involved in Wp activation as well as binding of the B-cell specific activator protein BSAP/Pax5 to the UAS1 domain (Speck, 2005). The function and activity of Wp in other cell types is not clear.

Cp is tightly regulated by EBNA gene products. EBNA₂ upregulates Cp activity through its interaction with the J κ recombination signal binding protein (RBP-J κ) (Kieff & Rickinson, 2001) and the AU-rich element RNA binding protein 1 (AUF1, hnRNPD) (Fuentes-Panana et al., 2000) transcription factors that bind the C-promoter binding protein (CBF) 1 and CBF2 elements in the promoter respectively. EBNA₅ augments EBNA₂ activation through CBF1 while EBNA₃, 4 and 6 repress EBNA₂ activation of Cp through CBF1. Therefore, Cp is autoregulated through the opposite actions of the EBNA_s (Speck, 2005).

In latency I and II, Wp and Cp are not active and EBNA₁ is the only nuclear antigen that is expressed. It is not clear how the expression of other EBNA_s is silenced and what triggers the switch between latency III and II. In latency I and II, EBNA₁ gene expression is driven by the TATA-less Q promoter (Qp). The Qp-initiated EBNA₁ transcript differs to the Wp/Cp driven transcript in that it contains an additional short 5' exon Q (Speck, 2005) (Figure 2). Qp is a cell cycle regulated promoter subject to regulation by the interferon regulatory factor (IRF) family where IRF-2 appears to activate the promoter while IRF-7 represses its activity (Schaefer et al., 1997; Zhang & Pagano, 1997). The E2F transcription factor binding sites in Qp are also thought to mediate promoter activation (Davenport & Pagano, 1999; Sung et al., 1994). EBNA₁ is a potent suppressor of Qp activity itself and may eventually lead to silencing Qp activity in latency 0 B cells where no latent gene expression is observed (Sample et al., 1992; Schaefer et al., 1997). While Cp and Wp have been found hypermethylated in tumour cell lines, the region around Qp has been hypomethylated indicating that methylation plays an important role in EBV latent gene regulation at different stages of its life cycle (Tao et al., 2006).

The LMP1 protein is encoded by three closely spaced exons in the righthand end of the viral genome (Hudson et al., 1985). LMP1 expression is driven by the ED-L1 (EcoRI D

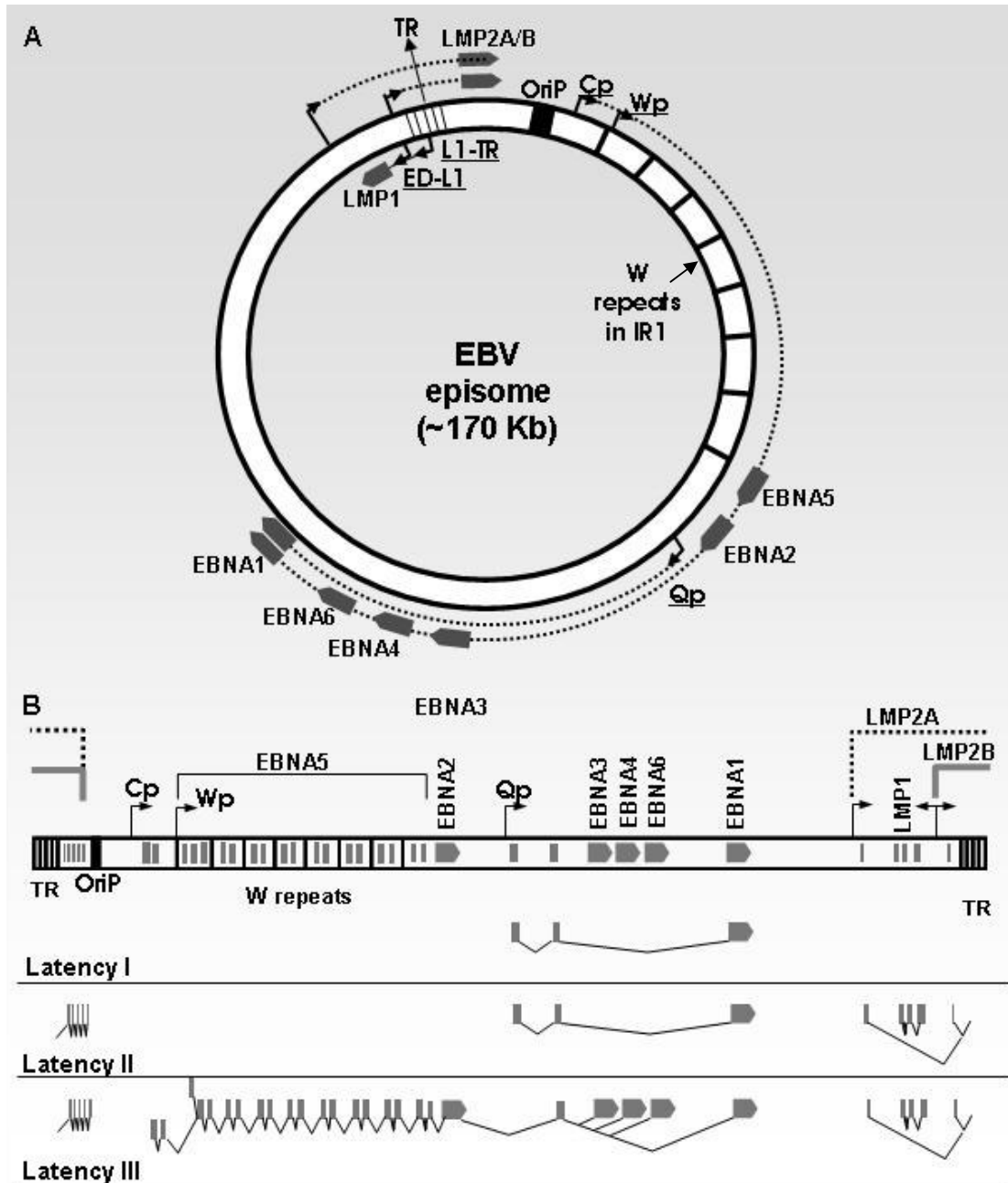


Figure 2. **The Epstein-Barr virus genome.** **A.** A schematic representation of the Epstein-Barr virus (EBV) double-stranded DNA episome. The origin of replication (OriP) is shown in black. The six latent EBV nuclear antigens (EBNA1-6) are produced by alternative splicing of one long primary transcript that initiates from the W promoter (Wp) or the C promoter (Cp) in latency III cells. The Q promoter (Qp) drives the expression of EBNA1 in latency II and I cells. The latent membrane proteins A and B exons are located on either side of the terminal repeat (TR) regions, which means that their expression requires circularisation of the viral DNA. The LMP1 protein is transcribed in the reverse direction relative to the other latent genes. Two promoters can give rise to the LMP1 transcript labelled ED-L1 and L1-TR. The transcription initiation sites are indicated by black arrows and the dotted lines represent the transcripts. The fat grey arrows indicate the direction and the splicing sites of the different latent genes. **B.** Linear diagram of the EBV genome. The latent gene promoters are indicated by black arrows. The coding regions of the latent genes are indicated in grey. In latency I and II EBNA1 is the only EBNA gene, which is transcribed from Qp. The LMP genes are expressed in latency II and III. The alternative splicing of the EBNA genes from one long transcript in latency III is illustrated on the diagram. EBNA genes can be transcribed from either Cp or Wp in this latency. The Wp is encoded within the major internal repeat of the virus (W repeats) giving rise to multiple copies of Wp (only the first one is indicated in the figure).

leftward promoter 1) promoter, also referred to as the LMP1 regulatory sequence (LRS) (Fahraeus et al., 1990a) (Figure 2). In latency III cells EBNA2 activates the LMP1 ED-L1 promoter. During latency II, LMP1 is expressed independently of EBNA2 and must depend on other transcription factors. Latency II pattern of gene expression is observed in tumours of different cell origins, including B cells, NK/T cells, and epithelial cells. LMP1 activation in latency II epithelial cells is complemented by a TATA-less GC box containing promoter referred to as TR-L1 (or ED-L1E) that is located in the terminal repeat of the EBV genome (Figure 2A) (Chen et al., 1992; Sadler & Raab-Traub, 1995). However, it is not clear whether TR-L1 is also involved LMP1 expression in latency II infected B cells. In epithelial cells, EBNA2 is unable to activate the LMP1 ED-L1 promoter (Fahraeus et al., 1993) and different factors bind the promoter region (Johannsen et al., 1995) indicating cell type differences. The current knowledge on the regulation of LMP1 gene expression will be reviewed in more detail in another section of this thesis.

The mRNAs for LMP2A and B proteins are transcribed from the same gene and consist of nine exons. However, LMP2A and B are transcribed from different promoters and have unique 5' exons while they share eight common exons (Figure 2B) (Sample et al., 1989). LMP2A and B promoters are near the righthand end of the viral genome so their 3' exons are located at the lefthand end of the viral genome, thus, the expression of LMP2 gene products requires circularization of the viral genome. In the latency III infected B cells, the expression of LMP2A and LMP2B is also regulated by EBNA2 (Kelly et al., 2002; Kieff & Rickinson, 2001). The LMP2B promoter encompasses parts of the LMP1 promoter, and it is thought to be regulated in a similar way as LMP1. Less is known about the regulation of LMP2A in latency II cells. A recent report indicates that LMP2A constitutively activates the Notch pathway in B cells and epithelial cells. The intracellular domain of Notch (Notch-IC) and EBNA2 are functional homologs. It was recently shown that the constitutive activation of the Notch pathway by LMP2A allows the protein to autoregulate its promoter. Expression of LMP2A alone was sufficient to activate its own expression and may explain EBNA2-independent expression of LMP2A (Anderson & Longnecker, 2007). The pattern of EBV gene expression in different latencies is summarized in Table 1.

Table 1. Pattern of EBV gene expression in different latency types (Cohen, 2005).

Latency	EBNA1	EBNA2-6	LMP1	LMP2
I	+	-	-	-
II	+	-	+	+
III	+	+	+	+
0	-	-	-	-

EBV associated diseases

Since its discovery, EBV has been linked to several human diseases and malignancies. The EBV associated malignancies often exhibit gene expression patterns displayed in different stages of EBV life cycle. These include Burkitt's lymphoma (BL), AIDS-Burkitt's lymphoma, Hodgkin's lymphoma (HL), nasopharyngeal carcinoma (NPC), nasal T/NK lymphoma (NL), peripheral T cell lymphoma, gastric carcinoma, primary effusion lymphoma, AIDS-immunoblastic or large cell lymphoma, infectious mononucleosis (IM), chronic active EBV, X-linked lymphoproliferative disease, post-transplant lymphoproliferative disease (PTLD), AIDS-CNS lymphoma, lymphomatoid granulomatosis, and smooth muscle tumour (Cohen, 2005). The pattern of EBV latent gene expression in some of the malignancies is summarized in Table 2.

In vitro, EBV latently infects and immortalizes B cells, establishing lymphoblastoid cell lines (LCLs). In fact, EBV is one of the most potent transforming viruses in tissue culture (Pope et al., 1968).

The association of EBV with several of the tumours mentioned above is well established. However, the exact mechanism of pathogenesis has been more difficult to unravel. Nonetheless, a dysfunctional immune system appears to be a co-factor in most of these diseases. In order to gain more insight into the possible role of EBV in the aetiology of its associated diseases, the function of latent proteins has been the subject of many studies.

Table 2. Pattern of EBV latency in different diseases (Cohen, 2005).

Disease	Latency pattern
Burkitt's lymphoma	I
Hodgkin's lymphoma	II
Nasopharyngeal carcinoma	II
T/NK lymphoma	II
Gastric carcinoma	II
AIDS-immunoblastic lymphoma	II/III
Peripheral T cell lymphoma	II
X-linked and post-transplant lymphoproliferative disease	III
AIDS-CNS lymphoma	III

EBV latent protein function

The presence of different EBV latent genes in tumours associated with the virus, suggests that they may be involved in transformation and growth of these tumours. It should be noted that a large part of latent protein function has been studied in the in vitro LCL models and may not reflect all aspects of protein function.

EBNA1

EBNA1 is a DNA binding nuclear phosphoprotein that associates with chromosomes during mitosis and enables the episomal EBV DNA to replicate and persist in B cells (Yates et al., 1984). EBNA1 is also a transcriptional activator that upregulates both the Cp and LMP1 promoter (Kieff & Rickinson, 2001). A repeated peptide sequence (glycine-glycine-alanine) in the EBNA1 protein appears to inhibit antigen processing (Levitskaya et al., 1995) and therefore protect from endogenous antigen presentation through the MHC class I pathway and immune detection. Some investigations have also suggested that EBNA1 may have oncogenic properties (Wilson et al., 1996) and inhibit p53 induced apoptosis (Kennedy et al., 2003). Transcriptional profiling of EBNA1-expressing carcinoma cells demonstrated that EBNA1 also influences the expression of a range of cellular genes including enhancement of STAT1 expression and repression of TGF- β 1-induced transcription (Wood et al., 2007), functions that may contribute to the development of tumours.

EBNA2

EBNA2 is essential for lymphocyte growth transformation by EBV in vitro (Cohen et al., 1989). In early infection of B cells, EBNA2 together with EBNA5 is sufficient to advance the cells to early G1 phase of the cell cycle (Sinclair et al., 1994). EBNA2 is an acidic phosphoprotein, which localises in large nuclear granules. It functions as a specific transactivator of cellular and viral gene expression, upregulating the viral promoters Cp, LMP1, and LMP2 as well as cellular genes CD23, CD21, and the *c-fgr* and *c-myc* oncogenes. EBNA2 is recruited to promoters by its interaction with the DNA-binding protein RBP-Jκ as well as other DNA binding proteins such as PU.1 (Zetterberg & Rymo, 2005). Induction of the PI3-kinase (PI3K) signalling pathway by EBNA2 may also have a physiological function in the survival of EBV-infected cells (Spender et al., 2006).

EBNA3, 4, and 6

EBNA3, 4, and 6 proteins (alternatively referred to as 3A, 3B, and 3C, or the EBNA3 family) are encoded by three genes that are likely to share a common origin (Kieff & Rickinson, 2001). EBNA3 and 6 are essential in EBV immortalization of B cells in vitro while EBNA4 appears to be dispensable (Robertson et al., 1996). The localisation of EBNA3 protein family resembles that of EBNA2 but the distribution is independent of EBNA2 expression (Petti et al., 1990). EBNA3 and 6 have been shown to associate with the DNA-binding protein RBP-2N, an isoform of RBP-Jκ, thus acting as transcriptional regulators (Krauer et al., 1996). EBNA6 has been shown to upregulate cellular and viral genes including CD21 and LMP1 expression (Kieff & Rickinson, 2001). The EBNA3 family associate with RBP-Jκ and disrupt its binding to EBNA2 and the RBP-Jκ site in promoters, thereby repressing EBNA2 transactivation (Robertson et al., 1996). It has also been shown that repression of the tumour suppressor Bim by EBNA3 and EBNA6, confers resistance to apoptosis induced by cytotoxic drugs in EBV positive cells (Anderton et al., 2007).

EBNA5

EBNA5 is encoded by the leader of each of the EBNA mRNAs, and it is also referred to as EBNA-LP (leader protein). Along with EBNA2, EBNA5 is one of the first viral proteins expressed in infected B cells (Kieff & Rickinson, 2001). Due to the variation of the number of

IR1 repeats among EBV isolates, that are part of the coding region for EBNA5, the protein varies in size between 22 and 70 kDa (Dillner et al., 1986). EBNA5 is phosphorylated at multiple sites and is more associated with the nuclear matrix fraction relative to the other EBNA5s (Petti et al., 1990). EBNA5 non-sense mutant recombinants have been shown to be less efficient in growth transformation suggesting EBNA5 has a role in transformation efficiency (Mannick et al., 1991). EBNA5 together with EBNA2 can activate the expression of cyclin D2, which induces the G0 to G1 transition (Sinclair et al., 1994). Further, EBNA5 preferentially coactivates EBNA2-mediated stimulation of latent membrane proteins (Peng et al., 2005).

LMP2A and 2B

LMP2A and 2B (alternatively named TP1 and TP2 respectively) are integral membrane proteins. LMP2A, colocalises with LMP1 and it is serine and threonine phosphorylated as is LMP1 (Kieff & Rickinson, 2001). LMP2A is a substrate for B-cell src family tyrosine kinases, and it associates with a 70-kDa cellular phosphotyrosine protein (Longnecker et al., 1991). LMP2A mediates the blockage of the B cell receptor (BCR) signal transduction by blocking calcium mobilization, thus inhibiting B cell development (Fruehling & Longnecker, 1997; Miller et al., 1993). This allows the maintenance of EBV latency in lymphoid tissue and prevents activation of the EBV lytic cycle. On the other hand, LMP2A is a B-cell receptor-mimic itself, and is essential for B-cell survival of germinal centre B cells (Mancao & Hammerschmidt, 2007). Additionally, LMP2A can transform epithelial cells partly through activating the PI3K/Akt pathway (Scholle et al., 2000). LMP2B is thought to modulate the aggregating effects of LMP2A and thereby regulate LMP2A function (Longnecker, 2000).

EBERs

In addition to the latent proteins, the two non-polyadenylated RNAs (EBERs) are probably expressed in all the different latencies. They are each about 170 nucleotides in length, transcribed from the same strand of the DNA, and show sequence homology with each other (Arrand & Rymo, 1982). EBERs bind the interferon inducible double-stranded RNA activated protein kinase PKR, which has a role in mediating the antiviral effects of interferon and may be important for viral persistence (Clemens et al., 1994). Transfection of EBERs into the EBV negative Akata BL cell line results in a malignant phenotype that shows resistance to

apoptosis as well as upregulation of the anti-apoptotic Bcl-2 protein (Komano et al., 1999). In situ detection of EBERS is a widely used tool in the detection of EBV in tumour cells.

LMP1

LMP1 is a phosphoprotein comprised of a short cytoplasmic N-terminal domain, six membrane-spanning domains and a long C-terminal cytoplasmic domain (Liebowitz et al., 1986). LMP1 is essential for EBV-dependent immortalisation of B cells, and it has been shown that the transmembrane and C-terminal region of LMP1 are essential for its transformation ability (Kaye et al., 1995). LMP1 is arguably the main EBV oncogene and functions as a constitutively active tumour necrosis factor receptor (TNFR) (Soni et al., 2007). The structure, function and the role of LMP1 in EBV biology is reviewed in the following section in more detail.

Latent membrane protein 1

Oncogenic properties of LMP1

The role of LMP1 as the major EBV oncogene has been well established through several studies. LMP1 is critically involved in the immortalisation of B cells by EBV in vitro (Kaye et al., 1993), and is also required for their continuous proliferation in culture (Kilger et al., 1998). LMP1 expression in rodent and human fibroblasts results in a malignant cell phenotype, and induces loss of contact inhibition and serum and anchorage-independent growth (Baichwal & Sugden, 1988; Fahraeus et al., 1990b; Moorthy & Thorley-Lawson, 1993; Wang et al., 1985). LMP1 expression in EBV negative BLs leads to an LCL phenotype (Cahir-McFarland et al., 2004; Wang et al., 1985; Wang et al., 1988). The ability of LMP1 to transform cells in the absence of other EBV genes, and its expression in most EBV positive tumour types, suggests a functional role for this protein in pathogenesis.

LMP1's transforming properties are due to its ability to upregulate anti-apoptotic proteins and provide growth signals (Soni et al., 2007). To date many LMP1 targets have been identified and continue to be reported. The LMP1 regulation of so many cellular proteins converges on its function in activating several cellular signalling pathways, namely the Nuclear Factor κ B (NF- κ B), c-Jun NH₂-terminal kinase (JNK), p38 kinase, phosphatidylinositol 3-kinase (PI3K), and possibly other pathways (Dawson et al., 2003; Soni et al., 2007). LMP1 protects the cells against apoptosis by upregulating the expression of the anti-apoptotic proteins Bcl-2, Mcl-1, and A20 (Gregory et al., 1991; Henderson et al., 1991; Laherty et al., 1992). LMP1 induces increased cell surface expression of CD23, CD30, CD39, CD44 and cell adhesion molecules LFA1, LFA3 and ICAM1 (Kieff & Rickinson, 2001).

LMP1 structure

LMP1 protein's functional domains include six transmembrane domains (TM) and two C-terminal cytoplasmic domains referred to as C-terminal activating region 1 and 2 (CTAR1 and 2) or transformation effector site 1 and 2 (TES 1 and 2) (Soni et al., 2007) (Figure 3). The transmembrane domains mediate LMP1's localisation to cholesterol rich lipid rafts in the

membrane, which is important for constitutive aggregation and ligand-independent signalling of LMP1 (Ardila-Osorio et al., 1999; Higuchi et al., 2001). The cytoplasmic CTAR1 and 2 domains interact with molecules that usually mediate signalling by CD40 and other TNFRs in response to TNF ligands (Eliopoulos et al., 1996; Mosialos et al., 1995). LMP1 and CD40 signalling effects on B cells overlap extensively (Uchida et al., 1999). LMP1 is also significantly associated with the cytoskeleton through its C-terminal cytoplasmic domains (Higuchi et al., 2001; Liebowitz et al., 1987). This interaction is thought to mediate stabilization of LMP1 and extending its half life (Liebowitz et al., 1987).

LMP1 signalling

Both CTAR1 and 2 are critical for LMP1 function in latency III LCL outgrowth in vitro (Soni et al., 2007). CTAR1 interacts with TNFR associated factors (TRAFs) and CTAR2 engages TNFR associated death domain proteins (TRADDs) (Cahir-McFarland & Kieff, 2005) (Figure 3). The CTAR1 PQQAT region strongly binds to TRAFs 1, 2, 3, and 5 (Devergne et al., 1996). This results in a strong activation of the NF- κ B inducing kinase (NIK) and inhibitor κ B (I- κ B) kinase α (IKK α) that mediate activation of the non-canonical NF- κ B pathway (Atkinson et al., 2003; Eliopoulos et al., 2003; Luftig et al., 2004; Saito et al., 2003). CTAR2 YYD region weakly binds TRADD and receptor-interacting protein (RIP), the so called 'death domain proteins', without propagating a death signal (Izumi et al., 1999; Izumi & Kieff, 1997). CTAR2 substantially activates IKK β leading to the canonical NF- κ B activation (Eliopoulos et al., 2003; Saito et al., 2003). CTAR1 may also induce the canonical NF- κ B activation in B cells. This was illustrated by LCLs transformed with an LMP1 containing only CTAR1 that exhibited the same complement of nuclear NF- κ B complexes as the wild-type LCLs (Kaye et al., 1999). Therefore both CTAR1 and 2 are involved in NF- κ B activation in B lymphocytes. TRAF6 and interleukin-1 β receptor associated kinase 1 (IRAK1) molecules are also essential for LMP1 activation of NF- κ B and JNK pathways in knock-out mouse embryonic fibroblasts (MEFs), indicating that these factors are indirectly recruited to the LMP1 C-terminal domains (Luftig et al., 2003; Schultheiss et al., 2001; Wan et al., 2004). In recent years LMP1 has also been shown to activate the PI3K/Akt pathway, and this function was mapped to the TRAF-binding domain within CTAR1 and to the residues between CTAR1 and CTAR2 (Dawson et al., 2003; Mainou et al., 2005; Mainou et al., 2007)

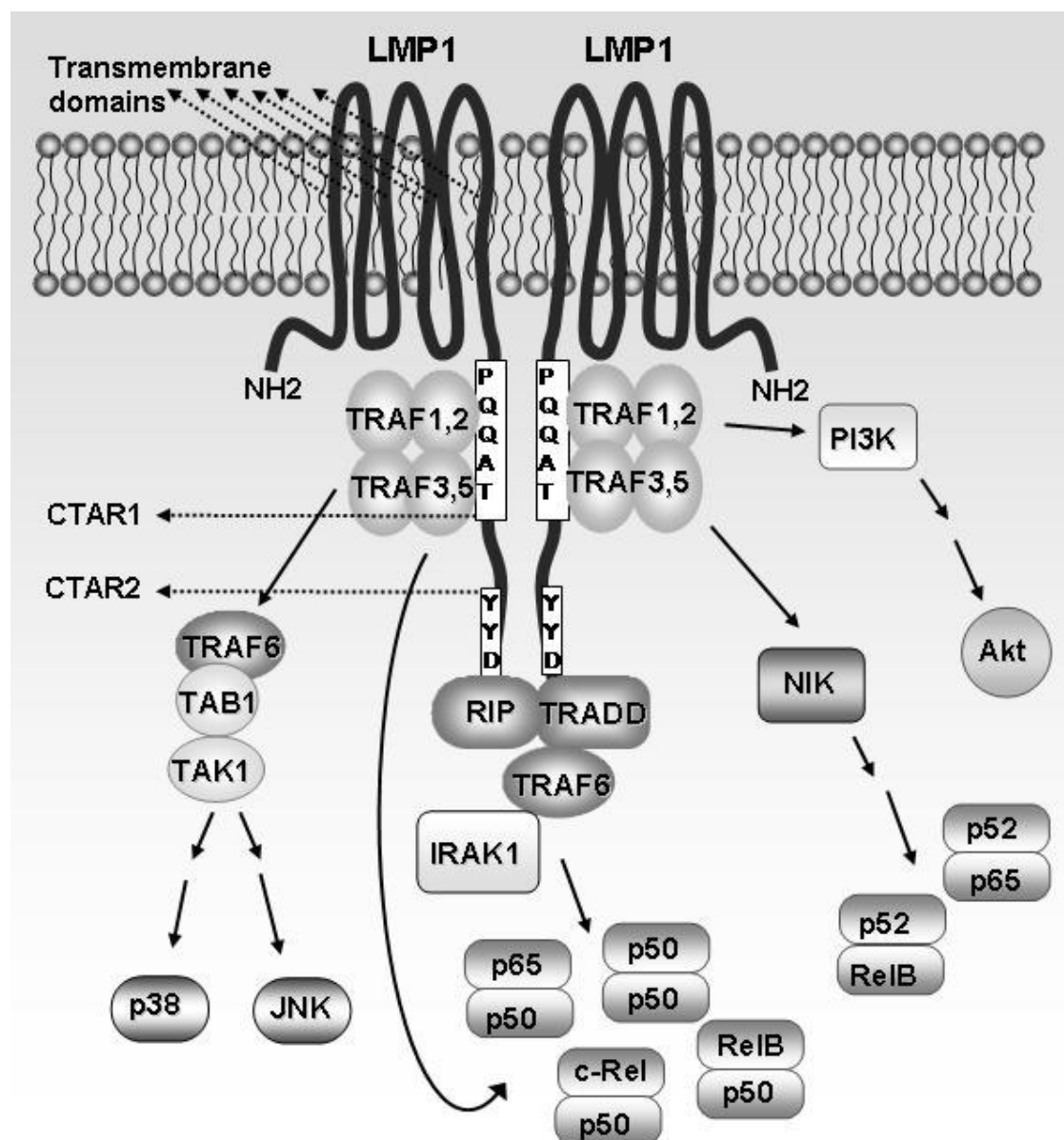


Figure 3. **Signalling pathways activated by LMP1.** LMP1 aggregates in the plasma membrane and functions as a constitutively active tumour necrosis factor receptor (TNFR). The transmembrane domains of LMP1 mediate its aggregation in the plasma membrane. The C-terminal activating region (CTAR) 1 domain of LMP1 interacts with TRAF1-TRAF2 and TRAF3-TRAF5 heterodimers. TRAF binding leads to the activation of the NF-κB inducing kinase (NIK) and the non-canonical NF-κB pathway. This activation results in the nuclear translocation of the NF-κB p52 heterodimers. The CTAR2 domain of LMP1 interacts with the death domain proteins RIP and TRADD, which is thought to mediate the activation of the canonical NF-κB pathway, and nuclear translocation of the different p50 and p65 dimers. IRAK1 and TRAF6 are required for the canonical NF-κB activation by LMP1. CTAR1 also activates the c-Jun NH₂-terminal kinase (JNK) and p38 kinase pathways, and this activation is TRAF6 dependent. The CTAR1 domain can also activate the canonical NF-κB pathway in B cells but the mechanism is not clear.

In epithelial cells, activation of the JNK and p38 pathways is more CTAR2 dependent, while NF- κ B activation is more CTAR1 dependent (Eliopoulos & Young, 1998; Wan et al., 2004). However, epithelial cells do not express TRAF1, and TRAF1 expression enables CTAR1 to activate JNK in epithelial cells (Eliopoulos et al., 2003). Thus, it appears that signalling through CTAR1 and CTAR2 differ according to the context of factors present in the host cells of EBV, and should be taken into consideration when studying downstream effects of LMP1. Figure 3 presents a schematic model of LMP1 activation of the signalling pathways through the recruitment of different molecules.

Overall, despite similarities between TNFRs and LMP1 signalling, there are differences that indicate that LMP1 functions in a unique way that maximises survival and growth signals, without inducing apoptosis (Mainou et al., 2007; Soni et al., 2007). The physiological functions of the main signalling pathways that are activated by LMP1 are briefly reviewed here.

The NF- κ B signalling pathway

The NF- κ B transcription factors are induced by stimuli that trigger inflammation, innate immune responses, adaptive immune responses, secondary lymphoid organ development, and osteoclastogenesis. As such, one of the primary physiological roles of NF- κ B is in the immune system. In particular, NF- κ B family members control the transcription of cytokines and antimicrobial effectors as well as genes that regulate cellular differentiation, survival and proliferation, thereby regulating various aspects of innate and adaptive immune responses. In addition, NF- κ B also contributes to the development and survival of the cells and tissues that carry out immune responses in mammals (Hayden et al., 2006). The NF- κ B pathway generally upregulates anti-apoptotic and anti-oxidizing genes and consequently protects the cells against apoptosis. Therefore, aberrant regulation of the NF- κ B signalling pathway results in an array of different human diseases including cancer (Dutta et al., 2006). NF- κ B functions as a pro-survival factor during negative selection of B cells. Immature B cells display constitutive NF- κ B activity that is downregulated following BCR ligation (Wu, 1996). Decreased NF- κ B activity might then sensitize these cells to proapoptotic signals. The activation of NF- κ B in late B-cell maturation is the result of signalling by both canonical and non-canonical NF- κ B pathways. It is not surprising then that this pathway provides an ideal target for LMP1 in upregulating B cell survival.

The JNK signalling pathway

The JNK or stress activated protein kinase (SAPK) belongs to the mitogen activated protein kinases (MAPKs). MAPKs regulate numerous cellular programs including embryogenesis and proliferation. JNK is activated in response to inflammatory cytokines, environmental stress such as heat shock, ionizing radiation, oxidant stress and DNA damage, DNA and protein synthesis inhibition, and growth factors (Kyriakis and Avruch, 2001). Each MAPK is activated by an upstream MAPK kinase (MAPKK/MAP2K/MEK), which in turn is activated by a MAPKK kinase (MAPKKK/MAP3K/MEKK).

JNK phosphorylates the transcription factors c-Jun, ATF-2, p53, Elk-1, and nuclear factor of activated T cells (NFAT), which in turn regulate the expression of specific sets of genes to mediate cell proliferation, differentiation or apoptosis. JNK proteins are involved in cytokine production, the inflammatory response, stress-induced and developmentally programmed apoptosis, actin reorganization, cell transformation and metabolism (Chen *et al.*, 2001).

Several studies suggest that JNK plays an important role in tumour cells. Ras induced tumourigenecity is suppressed by mutation of the JNK phosphorylation site on c-Jun (Behrens *et. al.*, 2000). JNK expression suppresses growth and induces apoptosis of human tumour cells in a p53 dependent manner (Patapova *et. al.*, 2000). Transformation by the met oncogene is also associated with the activation of the JNK signalling pathway (Rodrigues *et. al.*, 1997).

The p38 kinase pathway

The p38 kinase is another member of MAPKs (Tibbles & Woodgett, 1999). The p38 MAPK responds to a wide range of extracellular stimuli, particularly cellular stress such as UV radiation, osmotic shock, hypoxia, pro-inflammatory cytokines, and less often growth factors. p38 is activated via phosphorylation by its upstream protein kinases. Both the JNK and p38 pathways are activated in response to TNFR signalling (Ichijo, 1999).

The p38 pathway activates several other kinases such as MAP kinase-activated protein kinase 2 (MAPKAPK2 or MK2), p38 regulated/activated kinase (PRAK), mitogen- and stress-activated protein kinase 1 (MSK1). MAPKAPK2 in turn activates various substrates including small heat shock protein 27 (HSP27), lymphocyte-specific protein 1 (LSP1), cAMP response element-binding protein (CREB), transcription factor ATF1, SRF, and tyrosine hydroxylase. Several transcription factors are also activated by p38 directly. Examples include activating transcription factor 1, 2 & 6 (ATF-1/2/6), SRF accessory protein (Sap1),

CHOP, p53, C/EBP β , myocyte enhance factor 2C (MEF2C), MEF2A, MITF1, DDIT3, ELK1, NFAT, and high mobility group-box protein 1 (HBP1). Other important proteins such as tau and keratin 8 have also been reported as substrates for p38 (Cuenda & Rousseau, 2007; Zarubin & Han, 2005).

Consequently, the p38 pathway is involved in the regulation of many cellular functions such as inflammatory responses, DNA-damage induced cell death, regulation of mRNA stability and mRNA translation, differentiation, and cell cycle regulation. Expectedly then, aberrant regulation of the p38 pathway has been reported in tumourigenesis (Cuenda & Rousseau, 2007; Zarubin & Han, 2005).

The PI3K pathway

PI3K can be activated by a variety of extracellular growth and mitogenic stimuli involved in a number of cellular processes including cell proliferation, survival, protein synthesis, and tumour growth. PI3K is activated by receptor protein tyrosine kinases (RTKs), and non-receptor protein tyrosine kinases. RTKs interact with the p85 regulatory subunit of PI3K (Jiang & Liu, 2007). In response to growth factors, activated receptors interact with p85 Src homology 2 (SH2) domains, and localise PI3K to the plasma membrane (Cantley, 2002). Upon activation, PI3K generates phospholipids that activate downstream targets with lipid-binding domains. The PI3K targets include Akt (protein kinase B, PKB), Tec family of tyrosine kinases, guanine nucleotide exchange factors (GEF) for Rac, adenosine diphosphate (ADP)-ribosylating factor 6 (ARF6), and GTPase-activating proteins (GAPs) (Engelman et al., 2006; Hennessy et al., 2005; Ward & Finan, 2003). PI3K regulates a number of cellular functions through the activation of Akt (Vivanco & Sawyers, 2002).

Akt regulates multiple cellular functions including metabolism, protein synthesis, cell cycle progression, anti-apoptosis, tumour growth, and angiogenesis through different downstream targets (Jiang & Liu, 2007).

LMP1 function in health and disease

While it has been difficult to elucidate the function of LMP1 in EBV infection in vivo and its contribution to pathogenesis, the study of LMP1 signalling and its targets in vitro has given important leads into the possible role of LMP1 in these functions. Some examples of the proposed function of LMP1 EBV biology and tumour growth are presented here.

In naive B cells infected with EBV, LMP1 is expressed along with other EBV latent genes. As already mentioned LMP1 can mimic CD40 signalling and induce a large range of cell surface markers including activation and adhesion molecules in these cells (Wang et al., 1990). This leads to proliferating lymphoblasts that morphologically and phenotypically resemble antigen activated B blasts (Thorley-Lawson & Mann, 1985). It is thought that this activation of B cells is required for them to form a germinal centre. Generally, in the germinal centre the blasts undergo Ig class switching and randomly mutate their immunoglobulin genes that they eventually express on their surface. These B cells compete for antigen binding and the cells expressing the highest affinity B cell receptor (BCR) survive and can enter the memory B cell reservoir. LMP1 expression together with LMP2A is thought to provide the necessary signals to rescue the germinal centre B cells into the memory B cell compartment, in the absence of BCR signalling (Rastelli et al., 2007). Hence, LMP1 signalling aids EBV in gaining access to the memory B cell reservoir, where the virus can persist life-long (Thorley-Lawson, 2005a). Thus, EBV infected B cells *in vivo* express LMP1 only in the initial stages of infection, and then turn off its expression in order to escape the immune system. It is then conceivable that aberrant expression of the LMP1 oncogene in the absence of a healthy immune system would promote growth transformation.

One of the EBV associated tumours where LMP1 appears to play an important role is nasopharyngeal carcinoma (NPC). NPC is an epithelial tumour characterised by its geographic and population differences in incidence, and is consistently associated with EBV. In NPC tumours a latency II pattern of gene expression is observed and LMP1 and LMP2 are detected in approximately 50% of the tumours (Heussinger et al., 2004; Young et al., 1988). In epithelial cells LMP1 is thought to contribute to growth transformation by inducing the expression of epidermal growth factor receptor (EGFR) (Miller et al., 1995). LMP1 also induces expression of CD40 and interleukin 6 (IL6) and decreases expression of cytokeratins and E-cadherin (Fahraeus et al., 1990b). It also inhibits p53 mediated apoptosis through the induction of A20 expression. Thus, LMP1 is thought to be a key effector molecule in NPC pathogenesis (Tsao et al., 2002).

Another well studied tumour where LMP1 expression is observed is Hodgkin's lymphoma (HL). HL tissues are characterised by the disruption of normal lymph node structure and the presence of malignant Hodgkin/Reed Sternberg (HRS) cells amongst a background of non-neoplastic cell populations (Harris et al., 1994). HRS cells carry somatic Ig mutations indicating that they originate from GC or post-GC B cells (Kanzler et al., 1996). The relative risk of developing EBV positive Hodgkin's lymphoma in individuals with a history of IM is 4

times higher than those with no prior history (Hjalgrim et al., 2003). The particularly high levels of LMP1 expression in HRS cells (Deacon et al., 1993; Murray et al., 1998) suggest that it may contribute to pathogenesis in these cases. LMP1 protects B cells from apoptosis by upregulation of anti-apoptotic genes including Bcl-2, Mcl-1 and A20 through the NF- κ B pathway (Henderson et al., 1991; Laherty et al., 1992; Rowe et al., 1994; Wang et al., 1996). Constitutive activation of several pathways known to be activated by LMP1 is observed in HRS e.g.; NF- κ B (Bargou et al., 1997; Bargou et al., 1996) and AP-1 (Mathas et al., 2002). Hence a model has been proposed where the viral proteins LMP1 and LMP2A provide necessary signals for EBV infected memory B cells to undergo antigen-independent proliferation in GC and together with a co-factor, favour neoplastic transformation

Several lines of evidence suggest that LMP1 expression may also contribute to tumour progression and metastasis, even in cases where EBV infection does not play a central role in the transformation process (Yoshizaki et al., 2005). It has been shown that EBV infection causes an increase in cell ability to transmigrate across a Matrigel barrier, which correlates with the increased mobility of the infected cells (Kassis et al., 2002). The expression of LMP1 is associated with downregulation of intercellular adhesion possibly by downregulation of E-Cadherin and giving the cells an invasive ability (Fahraeus et al., 1992; Farwell et al., 1999). LMP1 expression also correlates with expression of matrix metalloproteinase 9 (MMP-9) (Yoshizaki et al., 1998), the urokinase-type plasminogen activator (u-PA) protein (Kim et al., 2000) and MMP-1 (Lu et al., 2003). These proteins are involved in degrading the extracellular matrix, which allows invasiveness of tumour cells (Yoshizaki et al., 2005). LMP1 is also thought to contribute to angiogenesis by upregulating IL8 expression (Yoshizaki et al., 2001), fibroblast growth factor 2 (FGF-2) (Wakisaka et al., 2002), and COX-2 (Murono et al., 2001) that have been shown to be involved in angiogenesis. Finally LMP1 also induces the hypoxia-inducible factor 1 (HIF1) (Wakisaka et al., 2004). HIF1 is involved in transactivating genes required for tumour progression such as vascular endothelial growth factor (VEGF), glucose transporters and insulin-like growth factor 2, probably through the activation of the PI3K/Akt pathway (Semenza et al., 1999).

In summary it appears that at least two events play a role in EBV associated carcinogenesis; a compromised immune system, and inappropriate activation of EBV latent gene expression, in particular LMP1. Therefore regulation of the LMP1 protein in the presence and absence of EBNA2 has been the focus of the present investigation. In the following sections the current knowledge on LMP1 gene expression mechanism will be reviewed and the outcome of the work carried out in this investigation will be discussed.

LMP1 transcription regulation

General mechanism of transcription regulation

The ability to induce a gene minimally depends on the assembly of the preinitiation complex (PIC) and the recruitment of RNA polymerase to the transcription initiation site. There are two prerequisites for this process. In the presence of accessible DNA, PIC assembly occurs inefficiently in the absence of activators (Pugh, 2000). Therefore, binding of activators that recruit the preinitiation complex to the promoter is the first requirement for transcription initiation. The second obstacle in transcription initiation is the fact that the DNA is packaged into a highly organised and compact nucleoprotein structure known as chromatin. Chromatin consists of DNA wrapped around protein complexes called nucleosomes that comprise of histone subunits. Consequently, chromatin plays a pivotal role in regulating eukaryotic gene transcription by marshalling access of the transcriptional apparatus to genes (Orphanides & Reinberg, 2002). Additionally, methylation of cytosine bases in promoter CpG islands is also associated with a repressed chromatin state and inhibition of gene expression (Bird & Wolffe, 1999). Cytosine methylation inhibits the association of some DNA-binding factors with their cognate DNA recognition sequences and proteins that recognize methyl-CpG can use transcriptional corepressor molecules to silence transcription (Klose & Bird, 2006).

Transcriptional activator proteins must bind to and de-compact repressive chromatin structures to induce transcription (Narlikar et al., 2002). This appears to be a paradox, where the chromatin structure prevents binding of factors to the promoter, and factor-binding to the promoter is required to modify chromatin structure. However, some transcriptional activators can bind the nucleosomal DNA and recruit chromatin modifying factors to the promoter. Transcriptional activators require the cooperation of a diverse family of coregulator proteins (McKenna & O'Malley, 2002) that alter chromatin structure or are themselves chromatin modifying enzymes. Corepressors can also be recruited that antagonise the effect of coactivators. There are two general classes of chromatin remodelling enzymes: ATP-independent nucleosome remodelling complexes that catalyze post-translational modification of histones, and ATP-dependent chromatin remodelling complexes that facilitate access of DNA binding proteins to DNA by repositioning nucleosomes at the promoter or by inducing conformational changes in nucleosomes (Fry & Peterson, 2001).

Four different histone modifiers are implicated in transcriptional regulation including histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases

(HMTs) and histone kinases. Recruitment of HATs and HMTs to promoters results in acetylation and methylation of N-terminal histone tails, which is crucial for the activation of many genes (Narlikar et al., 2002). Hyperacetylation of promoter sequences is usually a hallmark of transcriptionally active genes. Conversely, the recruitment of HDACs by transcriptional repressors leads to the deacetylation of histone tails and transcriptional repression (Narlikar et al., 2002).

The chromatin remodelling activity of the ATP-dependent enzymes can be achieved either by nucleosome sliding along the DNA (Lomvardas & Thanos, 2001) or by inducing a continuous ATP-dependent DNA twist that allows access to DNA sites, even in the absence of histone movement (Gavin et al., 2001; Havas et al., 2001).

DNA methyltransferases (DNMTs) are responsible for introducing cytosine methylation at previously unmethylated CpG sites, and the maintenance of the pre-existing methylation patterns onto the new DNA strand during DNA replication. Methyl-CpG-binding domain (MBD) proteins also mediate silencing of gene expression by targeting chromatin remodelling corepressor complexes to regions containing DNA methylation (Klose & Bird, 2006).

A typical promoter is composed of a myriad of binding sites (regulatory elements) for gene-specific transcription factors, and a core promoter that is composed of a TATA box and /or an initiator element. A typical transcription factor then is generally comprised of several modules including a nuclear localisation domain, a sequence-specific DNA binding domain and subunits that facilitate interactions necessary to recruit and assemble a functional coactivator/repressor (Tjian & Maniatis, 1994). The overall promoter activity is thus ultimately dependent on the cis-acting DNA sequences in the promoter recognised by transcription factors, and on the context of trans-acting active transcriptional factors available in the cell at any time.

The fact that the EBV gene expression is regulated by cellular factors suggests that the EBV DNA is structured and organized in a similar manner as its host's DNA. It has been shown that the episomal EBV DNA is in chromatin structure with the same nucleosome spacing as cellular DNA and contains DNase I hypersensitive sites and DNA methylation indicating its regulation is similar to eukaryotic DNA (Dyson & Farrell, 1985; Shaw et al., 1979).

EBNA2 activation of LMP1 expression

LMP1 expression in latency III infected B cells is predominantly activated by the ED-L1 promoter and is critically dependent on EBNA2 (Kempkes et al., 1995; Wang et al., 1990). This notion is reinforced by the observation that in BL lines where the EBNA2 gene has been deleted there is little expression of the LMP1 gene (Abbot 1990, Murray 1988, Zimmer-strobl 1990, 1991). The mechanism of EBNA2 activation of promoters has been intensely studied by several groups.

In similarity to other transcription factors, EBNA2 contains nuclear localisation motifs and a transactivation domain. EBNA2 contains an acidic transactivation domain that shows structural similarities to the transactivation domain of the herpes simplex viral VP16 protein (Cohen 1992). EBNA2's transactivation domain interacts with the components of the basal transcription machinery (Tong et al., 1995a; Tong et al., 1995b; Tong et al., 1995c), the HATs CBP, p300 and PCAF (Wang et al., 2000), and the ATP-dependent chromatin remodelling protein SWI-SNF (Wu et al., 1996). These interactions are the underlying factor for EBNA2's potent activation of promoters. Unlike most other transcription factors however, EBNA2 does not have a DNA binding domain and needs to be recruited to the promoters by other transcription factors (adaptors) (Kempkes et al., 2005; Zetterberg & Rymo, 2005). The lack of a DNA binding site may appear to be a disadvantage for a transcription factor. However, considering that several different transcription factors can interact with and recruit EBNA2, it seems to serve as an advantage allowing EBNA2 to be recruited to promoters containing different regulatory sequences. Both viral and cellular proteins are transactivated by EBNA2 including LMP1, LMP2, EBNA1-6, CD21, CD23, BLR2, BATF, c-fgr, c-myc, and Ig- μ , some of which have a critical role in EBV biology (Zetterberg & Rymo, 2005). Nonetheless, the lack of a DNA binding site renders EBNA2 totally dependent on cellular transcription factors to mediate its transactivating effects.

The J κ recombination signal binding protein (RBP- J κ) alternatively referred to as the C-promoter binding protein (CBF1) is the best studied adaptor protein involved in recruiting EBNA2 to the promoters (Grossman et al., 1994; Henkel et al., 1994; Ling et al., 1993; Waltzer et al., 1994; Zimmer-Strobl et al., 1994). In fact the first identified function of this protein was as a downstream effector molecule of EBNA2 in viral promoter activation. RBP-J κ is a ubiquitously expressed protein that interacts with EBNA2, and binds to the conserved core sequence GTGGGAA present in most viral and cellular EBNA2-responsive promoters (Zetterberg & Rymo, 2005). In the absence of EBNA2, RBP-J κ interacts with a corepressor

complex, comprising SMRT/N-CoR, CIR, SKIP, Sin3A, SAP30 and HDAC1, resulting in transcription repression (Zhou et al., 2000). EBNA2 is thought to activate the promoter by displacing the repressor complex through competition in binding to RBP-J κ and SKIP, and by recruiting proteins with HAT activity (Kao et al., 1998; Zhou et al., 2000).

Despite common belief, the RBP-J κ adaptor protein, while important in EBNA2 activation of promoters, does not play the central role in this process. LCLs generated with EBV containing point mutations in the RBP-J κ site of the C promoter were normal with respect to Cp activity (Evans et al., 1996). A second binding site that has been shown to be activated by EBNA2 also exists in the C promoter (CBF2), which binds the AUF1 adaptor and recruits EBNA2 (Fuentes-Panana & Ling, 1998; Fuentes-Panana et al., 2000). Interestingly, a mutant virus with a deletion encompassing both the RBP-J κ site and CBF2 exhibit greatly reduced transcripts of Cp (Yoo et al., 1997). It is therefore becoming more apparent now that EBNA2 activation of promoters requires more than one EBNA2 recruiting element (EBNA2 responsive element (EBNA2RE)). In the case of LMP1, binding of EBNA2 to the LMP1 promoter via RBP-J κ is not required for the EBNA2 induced activation of the LMP1 promoter in reporter assays (Fahraeus et al., 1990a; Fahraeus et al., 1993; Johannsen et al., 1995; Sjoblom et al., 1995a; Sjoblom et al., 1995b). Indeed, other EBNA2 inducible sequences have been identified in the LMP1 promoter that bind transcription factors including the Ets-related protein PU.1, a POU domain protein, and an AP-2 site binding factor (Jansson et al., 2007b; Johannsen et al., 1995; Laux et al., 1994; Sjoblom et al., 1995a; Sjoblom et al., 1998) and are probably targeting EBNA2 to the promoter.

Other viral and cellular proteins have also been shown to contribute to the EBNA2 dependent activation of EBNA2 responsive promoters through regulating the EBNA2 effect. EBNA5 augments EBNA2 activation of the promoters (Harada & Kieff, 1997). While EBNA3 and EBNA6 are generally thought to repress EBNA2 transactivation (Waltzer et al., 1996). However, EBNA6 seems to mediate activation of the LMP1 promoter together with EBNA2 (Allday & Farrell, 1994; Lin et al., 2002). Furthermore, EBNA2 interaction with the DEAD-box protein DP103, which in turn is complexed to the survival motor neuron (SMN) protein, is thought to coactivate the LMP1 promoter (Voss et al., 2001). It has also been shown that the EBNA1-dependent transcriptional enhancer family of repeats (FR), located in oriP, may have a role in increased expression of LMP1 (Gahn & Sugden, 1995).

EBNA2-independent activation of the LMP1 protein

The LMP1 ED-L1 promoter is activated in the absence of EBNA2 in latency II cells, however the identification of an EBNA2 analogue responsible for this activation has been elusive. Unlike latency III pattern of gene expression that is mostly confined to the B cells, the latency II pattern of EBV gene expression is observed in different cell types including B cells, NK cells, T cells, and epithelial cells, adding to the complexity of LMP1 gene regulation.

So far several transcription factors and transcription factor binding sites (regulatory elements) have been identified that can activate the LMP1 reporter constructs in the absence of EBNA2. A cyclic AMP response element (CRE) element in the proximal promoter region is involved in EBNA2 independent transactivation that is mediated by the CREB-ATF1 heterodimer (Sjoblom et al., 1998). Additionally, binding of USF to an E-box element in the promoter proximal region can also contribute to LMP1 promoter activation. Exogenous overexpression of these factors induces LMP1 promoter activity in reporter assays in the absence of EBNA2. It should be noted that CREB-ATF1 and USF are constitutively active in B cells but they are unable to activate the LMP1 promoter in the absence of EBNA2. Thus, these factors contribute to promoter activation but are not sufficient to do so alone. Another consideration is that these factors also seem to contribute to the activation of the promoter in the presence of EBNA2, even though they are not responsible for recruiting EBNA2. Some groups refer to these factors as basal activators of the LMP1 promoter. These factors seem to be involved in promoter regulation in any latency but are not sufficient to induce activation. Thus EBNA2-independent activator in the context of LMP1 is a vague term used to describe any transcription factor that mediates promoter activity but is not involved in recruiting EBNA2 to do so.

There are additional transcription factors that activate the ED-L1 promoter in an EBNA2-independent manner as described above, but they are not constitutively expressed. These factors include the IRF7 (Ning et al., 2003), the JNK pathway (Goormachtigh et al., 2006), and NF- κ B (Johansson et al., 2007 in manuscript), all of which are known targets of LMP1 itself and are induced in EBV infected cells. The binding of these factors to ED-L1 contributes to both EBNA2-dependent and EBNA2-independent activation of the promoter. The induction of these factors by LMP1 suggests the presence of positive autoregulatory loops in LMP1 expression. However none of these factors alone are indispensable for LMP1 activation and it is not known whether additional activators are required for ED-L1 activation in latency II cells. In other words, it is still not clear whether there is an EBNA2 analogue that

LMP1 expression is critically dependent on in latency II cells. Notably a recent study reported that the critical factor in LMP1 expression in latency II cells is in fact LMP1 itself (Goormachtigh et al., 2006). This finding suggests that the downstream targets of LMP1 that are reported to be involved in ED-L1 activation may in fact cooperate to drive LMP1 expression in the absence of EBNA2.

Another promoter shown to be involved in the expression of LMP1 is the L1-TR (ED-L1E). It is generally presumed that in latency II, LMP1 is expressed predominantly from this promoter, which initiates from a TATA-less GC box approx. 600 bp upstream of ED-L1 (Speck, 2005). The origin of this presumption is however somewhat unclear. While the presence of LMP1 transcripts from this promoter has been elegantly shown in epithelial cells, the relative abundance of this transcript was reported to be equal to ED-L1-originated transcript in a NPC line and NPC biopsies (Sadler & Raab-Traub, 1995). Thus it appears that both LMP1 promoters are involved in driving LMP1 expression in the absence of EBNA2 in epithelial cells. Reporter assays indicate that the L1-TR promoter is activated by Sp1 and Sp3 binding to a GC box as well as STAT transcription factors binding to a STAT site (Chen et al., 2001; Tsai et al., 1999). Since LMP1 is known to activate pathways that can potentially activate STAT signalling, an autoregulatory role has also been proposed for LMP1 activation of this promoter (Chen et al., 2001). L1-TR LMP1 transcripts have also been detected in some LCLs but at much lower levels relative to ED-L1-initiated transcripts (Sadler & Raab-Traub, 1995). The relative levels of ED-L1 and L1-TR initiated LMP1 transcripts in latency II cells of non-epithelial origin has not been investigated

LMP1 promoter repression

Expression of the LMP1 gene is repressed in the absence of an activator. So far several promoter regions have been reported to confer promoter repression of ED-L1 (Fahraeus et al., 1990a; Fahraeus et al., 1993). Binding of a Max-Mad1-mSin3A corepressor complex to an E-box element has been shown to mediate promoter repression in the absence of EBNA2 (Sjoblom-Hallen et al., 1999). The corepressor mSin3A is known to interact with histone deacetylases (HDAC 1 and 2) (Laherty et al., 1997), suggesting a role for histone deacetylation in silencing of the LMP1 gene. Additionally, in the absence of EBNA2, RBP-Jk interacts with a corepressor complex, comprising SMRT/N-CoR, CIR, SKIP, Sin3A, SAP30 and HDAC1, resulting in LMP1 transcription repression (Zhou et al., 2000) and may therefore also play a role in LMP1 promoter repression.

Histone methylation of the LMP1 promoter has also been investigated. In latency III, histone3 lysine4 residue methylation (H3mK4) was enriched while a lower level of methylation of this region was observed in latency I. Conversely, histone H3 K9 methylation (H3mK9), associated with silent heterochromatin, was enriched in the EBNA2 and LMP1 upstream control regions in type I but not type III cells. The correlation of specific methylation patterns of the LMP1 promoter in latency I and III suggest that this mechanism may regulate LMP1 silencing in latency I cells (Chau & Lieberman, 2004).

Several studies have also reported CpG methylation status of the LMP1 promoter region. Methylation of the LMP1 DNA has been observed in latency I BL cells and LMP1 could be activated after treatment with demethylating agents (Li & Minarovits, 2003). Furthermore, DNA methylation of the LMP1 gene correlates with repression of expression in NPC biopsies and silencing of the promoter in a latency I BL (Hu et al., 1991a; Minarovits et al., 1994) although the methylation of the promoter does not inhibit binding of transcription factors to ED-L1 (Takacs et al., 2001).

LMP1 transcription repression can also occur via modulating the activity of the factors that induce promoter activation. EBNA3 and EBNA6 viral proteins have been shown to negatively regulate EBNA2 activation of the C promoter by competing for RBP-J κ binding of EBNA2 (Zimmer-Strobl & Strobl, 2001). This mechanism does not seem to be involved in LMP1 repression and so far only activation of LMP1 by EBNA6 has been reported (Allday & Farrell, 1994; Lin et al., 2002). Hyperphosphorylation of EBNA2 by an EBV kinase however suppresses transactivation of the LMP1 promoter (Yue et al., 2005). A role has been proposed for the downregulation of IRF7 mediated LMP1 expression by IRF5 (Ning et al., 2005). While the NF- κ B factors have been implicated in the repression of the LMP1 promoter by one study (Goormachtigh et al., 2006), the current investigation as well as another report (Johansson et al., 2007 in manuscript; Ning et al., 2003) suggest the opposite role for NF- κ B in LMP1 regulation. Overexpression of the LMP1 protein is cytotoxic (Floettmann et al., 1996; Kaykas & Sugden, 2000; Sandberg et al., 2000) and it is likely that other mechanisms of LMP1 promoter repression also exist. One of these mechanisms may be the evolutionary selection of promoter sequence variations that effect factor binding to the promoter (Jansson et al., 2007a). This will be discussed in more detail in the context of the investigation carried out here.

The present investigation

The current literature on EBV biology strongly suggests that the life cycle and persistence of the virus is critically dependent on the tight regulation of its gene expression programs. Furthermore, the main cause of human disease by the virus also appears to stem from the aberrant regulation of viral gene expression. Despite considerable efforts, the mechanism of EBV gene regulation in vivo and viral pathogenesis is unclear and speculative. It does however appear that cofactors and a compromised immune system often participate in EBV-induced malignancies. Therefore, it is likely that abnormal regulation of cellular factors and environmental stimuli play a significant role in viral gene regulation during the process of tumourigenesis. Since the LMP1 oncogene is associated with most EBV malignancies and is likely to play a causative role, an understanding of LMP1 gene regulation in health and disease is a key factor in elucidating EBV pathogenesis. Hence, the overall aim of this investigation was to gain further knowledge regarding the role of cellular and environmental factors in LMP1 gene regulation.

In order to study regulation of LMP1 gene expression several different aspects of gene regulation were investigated. Firstly, regulatory elements present in the promoter and transcription factors binding to them and modulating promoter activity were investigated. Secondly, the possible involvement of cellular signalling pathways in promoter regulation was examined. Thirdly, the role of chromatin structure in transactivation was studied. Finally, the effect of sequence variations on the level of LMP1 promoter activity was explored. The results of these investigations and the relevance of the outcome are summarized here.

Identification of an EBNA2 response elements

Identification of EBNA2 responsive elements (EBNA2REs) in the LMP1 promoter has always been a major focus in studies of the mechanism of LMP1 expression. This is probably due to the fact that EBNA2 is the only transcription factor known to be critical for LMP1 expression in latency III cells. The presence of different EBNA2REs in the LMP1 promoter has been extensively studied using transient transfection of LMP1 reporter plasmids (Fahraeus et al., 1990a; Fahraeus et al., 1993). The role of the RBP-J κ site as an EBNA2 response element has been established by numerous reports (Kieff & Rickinson, 2001). This site and the neighbouring PU.1 site are presumed to be the fundamental elements responsible

for EBNA2-dependent activation of the LMP1 promoter (Johannsen et al., 1995). Several findings suggest that additional EBNA2REs exist at the LMP1 promoter. Firstly, the LMP1 promoter sequence with a deleted RBP-J κ site and a mutated PU.1 site exhibits EBNA2 inducibility (Sjoblom et al., 1995a). Further, Notch1, which is thought to be the cellular analogue of EBNA2 is unable to maintain LMP1 expression and to maintain cell proliferation (Hofelmayr et al., 2001). Notch transactivates genes by interacting with the RBP-J κ factor. The inability of Notch in transactivating the LMP1 promoter suggests that the EBNA2-dependent activation of the promoter requires EBNA2REs other than RBP-J κ . Thus, other mediators of EBNA2 recruitment exist. Notably, the amount of EBNA2 binding to the LMP1 promoter is reported to be three times higher than that bound to the C promoter as judged from a ChIP analysis (Alazard et al., 2003). Additionally, EBNA2 associates with itself forming multimers that result in high molecular weight complexes (Grasser et al., 1991; Tsui & Schubach, 1994) indicating that the multimer would interact with several EBNA2REs at promoters.

Consistent with these data a CRE site adjacent to the TATA box in the ED-L1 promoter was found to be an EBNA2 responsive element (Sjoblom et al., 1998). It has also been shown that a major part of LMP1 promoter repression is mediated by the binding of the Mad-Max-Sin3A complex to the E-box element (Sjoblom-Hallen et al., 1999). The repression conferred by these factors can only be alleviated in the presence of EBNA2 and is mediated by yet another EBNA2 responsive element located in the -67 to -106 region of the LMP1 regulatory sequence (LRS) relative to the transcription start site (+1).

As a result, one of the goals of this study was to define the EBNA2RE in the -67 to -106 region of LMP1, identify the factor/s binding to it and investigate the mechanism of repression relief mediated by this element in response to EBNA2. To define the regulatory element involved in this process, transient transfection of LMP1 promoter in the context of reporter plasmids was used. Reporter assays are a useful tool in studying regulatory elements as they allow investigation of promoter activity after introduction of mutations in these elements or deletions. Deletion and mutation analysis of LMP1 reporter plasmids indicated the presence of an EBNA2 response element located at position -95 to -103 of LRS (Jansson et al., 2007b). This element appeared to be an AP-2 factor binding site according to database searches. Electromobility shift assay (EMSA) was used to characterise factor binding properties of this site. It was confirmed that the factors binding this site were AP-2 site binding factors as competition with an AP-2 consensus sequence successfully abolished

binding to this site. However, the factors binding to the site could not immunologically be defined as AP-2 proteins as they did not react with the commercially available anti-AP-2 antibodies. The very low levels of AP-2 gene products both at RNA and protein levels in B cells also indicate that AP-2 is unlikely to be the mediator of EBNA2 activation of this site. Thus, we hypothesize that the factor binding to the LMP1 AP-2 site in B cells is not a previously identified member of the AP-2 family, but it binds to the AP-2 site in the LMP1 promoter in B cells and activates the promoter in response to EBNA2 (Jansson et al., 2007b). We refer to this factor as the AP-2 site-binding factor. The identification of this factor is currently under investigation.

Identification of transcription factors modulating the LMP1 promoter

We have previously reported several regulatory elements in LRS that are involved in its regulation together with or independently of EBNA2 (Zetterberg & Rymo, 2005). The identification of these factors has been primarily carried out using EMSA. However, as it was the case with the AP-2 site-binding factor, it is not always possible to identify proteins binding to DNA in EMSA. Another disadvantage of EMSA is that the DNA sequences used as probe are relatively short and may not be able to assemble the complexes formed in vivo. To overcome these problems, a DNA affinity purification combined with a sensitive mass spectrometry method was used to identify proteins that bind the LMP1 promoter region corresponding to positions -202 to -1 of LRS. The LMP1 promoter sequence was used as a bait, and specifically pulled-down the NF- κ B transcription factors p50 (NF- κ B1) and p65 (RelA) from a nuclear extract (Johansson et al., 2007 in manuscript). Database searches indicated the presence of an NF- κ B site in the proximal LMP1 promoter at position -79 to -89. EMSA then confirmed the binding of NF- κ B dimers p50-p65 and p50-p50 to this site. Chromatin immunoprecipitation assay (ChIP) using an anti-p50 antibody confirmed the binding of p50 to the LMP1 promoter in vivo in LCLs. The LRS NF- κ B binding sequence seems to be conserved in different EBV isolates. Introduction of a mutation in this site hampered EBNA2 dependent activation of the promoter significantly in reporter assays. It was also demonstrated that overexpression of the NF- κ B factors leads to a large increase in promoter activity independently of EBNA2. While it is not possible to correctly estimate the relative activating ability of different factors in reporter assay, LMP1 promoter induction in response to NF- κ B factors was very strong, showing even higher activity than obtained with EBNA2 in a similar system. Specific inhibition of p65 activity using siRNA, significantly

decreased endogenous LMP1 levels in a latency III LCL. The effect of p50 siRNA silencing on LMP1 is currently under investigation. Our data suggest that the NF- κ B factors do not convey EBNA2 responsiveness, but contribute to promoter activity in the presence of EBNA2. This would mean that NF- κ B may also be involved in EBNA2 independent activation of the ED-L1 promoter in latency II cells. This notion is supported by the responsiveness of the LMP1 promoter to NF- κ B factors in epithelial cells (Johansson et al., 2007 in manuscript).

NF- κ B transcription factors are comprised of homo- and heterodimers of five proteins: p50, p52, c-Rel, RelA/p65, and RelB (Hoffmann et al., 2006). These transcription factors are related through an N-terminal DNA-binding/dimerization domain, called the Rel homology domain, through which they can form homodimers and heterodimers. These dimers bind to a variety of related target DNA sequences called NF- κ B sites to modulate gene expression. The primary mechanism by which NF- κ B activity is regulated is controlled through the regulation of its ability to bind DNA. This function is carried out by a family of proteins referred to as inhibitor of κ B (I κ B) proteins that bind NF- κ B dimers and prevent their nuclear localisation. Two major signalling pathways have been characterised that result in NF- κ B activation via distinct kinases and I κ B proteins; the canonical (classical) and the non-canonical (alternative) pathway (Hoffmann et al., 2006). Both these pathways are activated by LMP1 leading to the nuclear translocation of p52-p65, p52-RelB, p50-p65, p50-RelB, p50-c-Rel heterodimers as well as the p50-p50 homodimer (Kaye et al., 1999). Activation of NF- κ B by LMP1 occurs both in B cell and epithelial cells expressing LMP1 as indicated by EMSA analysis (Herrero et al., 1995; Paine et al., 1995). Therefore, activation of the LMP1 promoter by NF- κ B factors implicates the presence of an autoregulatory loop that may contribute to LMP1 promoter activation in both B cells and epithelial cells. Interestingly, the NF- κ B pathway plays a central role in the immune system and controls the transcription of cytokines and antimicrobial effector molecules. Many other viruses also induce NF- κ B activity and contain NF- κ B binding sites in their viral promoters, turning the hosts defence machinery against itself (Pahl, 1999). A positive autoregulatory circuit between LMP1 and NF- κ B is a conceivable mechanism to insure sustained viral gene expression. The results of this study suggest that NF- κ B factors may be involved in LMP1 regulation in both latency types II and III.

Regulation of the LMP1 promoter activity by cellular signalling pathways

Since both EBNA2 dependent and independent activation of LMP1 gene expression is fully dependent on cellular transcription factors, it is likely that the signalling pathways that regulate these transcription factors would have a role in regulating LMP1 gene expression levels. Due to the fact that the CRE element was shown to be a central mediator of LMP1 activation in the presence and absence of EBNA2, the signalling pathways regulating factors binding to this site were investigated. The heterodimeric transcription factors CREB-ATF1 and c-Jun-ATF2, were reported to bind the CRE element in the LMP1 promoter previously (Sjoblom et al., 1998). Additional studies failed to confirm the binding of the c-Jun-ATF2 heterodimer to the LMP1 promoter and indicated that the activation mediated by the LMP1 CRE is mediated by the CREB-ATF1 heterodimer (Jansson et al., 2007a). CREB and ATF1 are expressed ubiquitously and can bind DNA in their inactive state (Mayr & Montminy, 2001). These factors become transcriptional activators when they are phosphorylated on specific residues (Ser133 in CREB (Gonzalez & Montminy, 1989) and Ser63 in ATF1 (Shimomura et al., 1996)). Generally, the phosphorylation of these transcription factors is regulated by upstream kinases in response to a wide range of extra-cellular stimuli (Mayr & Montminy, 2001). We investigated the role of a range of kinases in the regulation of the LMP1 promoter. Our results showed that a high level of active p38 MAPK correlates with LMP1 upregulation. In accordance, inhibition of p38 phosphorylation using the specific inhibitors of its phosphorylation or siRNA silencing of p38 led to a downregulation of LMP1 expression both at RNA and protein level. Thus, the p38 kinase pathway appears to be involved in the regulation of LMP1 expression.

The p38 kinase can induce CREB and ATF1 phosphorylation by an indirect phosphorylation cascade, through its substrates MAPKAPK-2 (Tan et al., 1996) and MSK1 (Deak et al., 1998). In this study, the level of CREB and ATF1 phosphorylation correlated with p38 phosphorylation and LMP1 expression levels. Hence, the CREB-ATF1 heterodimer is a good candidate in mediating p38 regulation of LMP1 expression through the CRE site in the LMP1 promoter. Notably, other mechanisms of LMP1 promoter regulation by p38 may exist.

Interestingly, LMP1 is phosphorylated on serine and threonine residues (Kieff & Rickinson, 2001). The nascent, non-ionic LMP1, which is not phosphorylated, has a half-life of less than 2 h, before it is converted to a more stable cytoskeleton-associated phosphorylated form (Kieff & Rickinson, 2001) with a reported metabolic half-life of

approximately 5 h (Baichwal & Sugden, 1987). Therefore other mechanisms of LMP1 regulation by p38 such as post-translational modification of LMP1 is conceivable and cannot be excluded by this study.

Overall, upregulation of LMP1 by the p38 MAPK may have at least two biological implications. Upregulation of LMP1 by the stress-activated p38 may allow EBV positive cells to evade stress-induced apoptosis as LMP1 upregulates anti-apoptotic proteins. This could be a useful mechanism for survival not only during EBV infection but also in LMP1 expressing tumours that need to escape apoptosis induced by their extra-cellular environment. As reviewed in the previous section, LMP1 activates the p38 MAPK pathway which yet again presents the possibility of a positive autoregulatory loop, allowing maintained expression of LMP1 in tumours. Since our study suggests that p38 upregulation of LMP1 is independent of EBNA2, this may even be a mechanism in LMP1 expression regulation in latency II EBV infected cells where LMP1 is expressed independently of EBNA2.

The effect of sequence variation on LMP1 promoter activity

In many human diseases, and in particular carcinogenesis, genetic variations are a major source of disease predisposition. Since LMP1 expression is associated with most EBV tumours, the effect of variations in the LMP1 promoter sequence on gene expression was investigated. Several mutations were detected in the P3HR1 virus strain relative to the prototype EBV strain B95-8. One of these mutations was located in the CRE site of the LMP1 promoter. A mutation in this site has also been reported for other strains of EBV by others. The EBV strains CAO and C15 are NPC-derived virus isolates and contain a two base pair substitution in the CRE site (Chen et al., 1992; Hu et al., 1993). This variation has also been reported for virus isolates from several HL, IM, and asymptomatic carriers referred to as group D (Sandvej et al., 2000; Zhou et al., 2001). The Rael and Raji viral strains contain a C to G substitution (Takacs et al., 2001) in the same position as the C to A substitution in the CRE site in P3HR1.

The CREB-ATF1 heterodimer binding efficiency for the CRE variants relative to the B95-8 sequence was greatly diminished as indicated by EMSA. The lower amount of CREB-ATF1 binding to the P3HR1 sequence correlated with a 40% reduction in LMP1 promoter activity in reporter assays (Jansson et al., 2007a). Thus it appears that variations in the LMP1 CRE site are evolutionary selected to downregulate the LMP1 expression levels.

There are two major types of EBV isolates referred to as A and B or types 1 and 2. These two EBV types are mostly identical but show allelic polymorphism (50-80 % identity). There is also minor heterogeneity and polymorphisms within each virus type (Chen et al., 1992; Hu et al., 1991b). However, generally it seems that the distribution of tumour virus subtypes is geographic and does not exhibit tumour-specific association (Khanim et al., 1996; Tao et al., 1998). The P3HR1 cell line is a subline of the type 2 Jijoye BL and has an African origin like the Raji and Rael cell lines (Epstein et al., 1966; Hinuma et al., 1967; Klein et al., 1972; Pulvertaft, 1965). The NPC-derived EBV isolates CAO and C15 have an Asian origin. The phylogenetic tree based on the LMP1 sequence constructed by Kanai et al. showed that the Asian EBV isolates contain large differences compared with the African BL isolates (Kanai et al., 2006) suggesting independent selection for the CRE site sequence variants. Stable, genetic variations in CRE sites have also been described for several other promoters (Mitchison, 2001). It has been hypothesized that the activity of some elements such as the CRE site can be changed to a lower level by subtle sequence variations that modulate the level of expression of genes important for cell survival (Mitchison, 2001).

Different factors may be involved in the selection of EBV variants with lower LMP1 expression levels. It has been shown that a high level of LMP1 expression can induce cytostasis (Floettmann et al., 1996; Kaykas & Sugden, 2000; Sandberg et al., 2000). LMP1 can also inhibit the activity of viral and cellular promoters in the absence of cytostasis (Narbonnet & Mariame, 2006). Downregulation of LMP1 expression to a steady state level where cytostasis is prevented and cell survival is promoted may be one of the factors leading to the selection of these CRE variants. Since our results show that upregulation of the ATF1-CREB factors by the p38 kinase increases LMP1 expression, another advantage of a CRE with lower affinity for these transcription factors may be that activation of stress-induced signalling pathways would not lead to an upregulation of LMP1 expression. The ability of the latently infected B cells to maintain a low level of the immunogenic LMP1 despite extra-cellular signalling may be of advantage in EBV biology.

The role of chromatin structure in LMP1 promoter activity

During the course of these investigations, several transcriptional factors involved in LMP1 regulation have been identified. The literature regarding the mechanism of promoter activation by these transcription factors have helped in constructing a model for LMP1 activation (Figure 4).

At the RBP-J κ sites, EBNA2 is thought to increase histone acetylation both by displacing the HDACs through competition in binding to RBP-J κ , and by recruiting proteins with HAT activity (Kao et al., 1998). It is likely that the recruitment of EBNA2 to other response elements serves the same function. Consistent with this model, the two EBNA2 inducible sites in the LMP1 reporter plasmids, namely CRE and AP-2 sites were also activated by treatment with TSA, an inhibitor of histone deacetylases (HDACs). Our ChIP results as well as those reported by Alazard et al (Alazard et al., 2003) indicate that the repressed endogenous LMP1 promoter is hypoacetylated at lysine residues in the N-terminal tails of nucleosomal histones and that the same residues become acetylated in the process of EBNA2-induced promoter activation. Furthermore, the ChIP analysis carried out by Alazard *et al* also showed that the increased histone H3 acetylation was not centred on the RBP-J κ element, but extended over the proximal promoter and about 500 bp into the coding region (Alazard et al., 2003). Additionally, TSA treatment of P3HR1 cells results in LMP1 expression (Sjoblom-Hallen et al., 1999). Thus, our results are consistent with the hypothesis that acetylation of specific nucleosomal histones is a major component of EBNA2-induced activation of the endogenous LMP1 promoter, and that EBNA2 functions as a transcriptional coactivator partly by targeting the CBP, p300 and PCAF histone acetylases to the EBNA2REs and overcoming the repression exerted by HDACs at the E-box element. Notably, LMP1 reporter plasmids containing the E-box region are also repressed in epithelial cells (Fahraeus et al., 1993). Therefore, histone acetylation is most likely involved in overcoming LMP1 repression in latency II cells of different origins. At this point it is not possible to say which factors are responsible for mediating histone acetylation of the LMP1 promoter in the absence of EBNA2. It is however known that the CREB-ATF1 and NF- κ B p50-p65 heterodimers also recruit HATs as coactivators to promoter regions (De Cesare & Sassone-Corsi, 2000; Gerritsen et al., 1997). We hypothesize that these factors and possibly other transcription factors at LRS together induce LMP1 expression by increasing histone acetylation at the promoter.

Although we have shown that histone acetylation induced by TSA is able to substitute for EBNA2 in the activation of LMP1 in reporter plasmids as well as LMP1 expression in P3HR1, it is not the minimal requirement for activation of the promoter. This was shown by the fact that in the EBNA2 conditional EREB2.5 cell line, TSA could not activate LMP1 expression in the absence of an active EBNA2 (unpublished data). This finding also suggests that reporter plasmids alone may not provide a complete model for the study of chromatin

remodelling of promoters. Interestingly, the EBNA2 protein also interacts with the ATP-dependent chromatin remodelling complex SWI-SNF and recruits this complex to promoters (Wu et al., 1996; Wu et al., 2000). Therefore it is possible that chromatin remodelling of the LMP1 promoter by SWI-SNF is also required for the activation of the endogenous promoter. It is in fact postulated that generally histone acetylation and the action of ATP-dependent chromatin remodelling complexes are coupled together in the activation of promoters (Fry & Peterson, 2001). We have investigated the nucleosome positioning at the LMP1 promoter using micrococcal nuclease (MNase) digestion and detection of the fragments produced using southern blotting. MNase makes double-stranded DNA cuts at the linker regions between nucleosomes. Our results suggest that the active LMP1 promoter is much more sensitive to digestion by MNase than the inactive LMP1 promoter (unpublished data). Hence, nucleosome remodelling by SWI-SNF is probably another important event in LMP1 promoter activation. It is unknown what factors may be responsible for the recruitment of ATP-dependent chromatin remodelling factors to the promoter in the absence of EBNA2.

Concluding remarks

We have identified new factors that appear to be involved in LMP1 expression in latency III and latency II cells, and also presented some data on the role of chromatin remodelling in this process. None of the factors that have been reported by us or others seem to be critically involved in LMP1 activation in latency II infected cells. To date, only LMP1 itself appears to be required for its own expression in latency II cells (Goormachtigh et al., 2006). Since LMP1 is not a transcription factor this mechanism obviously requires additional cellular and viral transcription factors that are downstream targets of LMP1. NF- κ B is a particularly good candidate for LMP1 upregulation as it is activated by EBV infection prior to LMP1 expression (Sugano et al., 1997), and can be involved in LMP1 gene regulation even at the early stages of infection. It is likely that the NF- κ B factors cannot activate the LMP1 promoter on their own, and require the cooperation of other factors at the promoter as indicated by our finding that reporter plasmids containing the NF- κ B site but lacking the upstream promoter sequences were not activated. In fact, it has been shown that promoter activation by NF- κ B factors is regulated not only by interactions with coactivators but also protein-protein interactions with other factors binding at the promoter (Hoffmann et al., 2006; Kim & Maniatis, 1997; Ten et al., 1993). Taking into consideration that even the EBNA2 dependent activation of the LMP1 promoter requires several EBNA2 responsive elements at

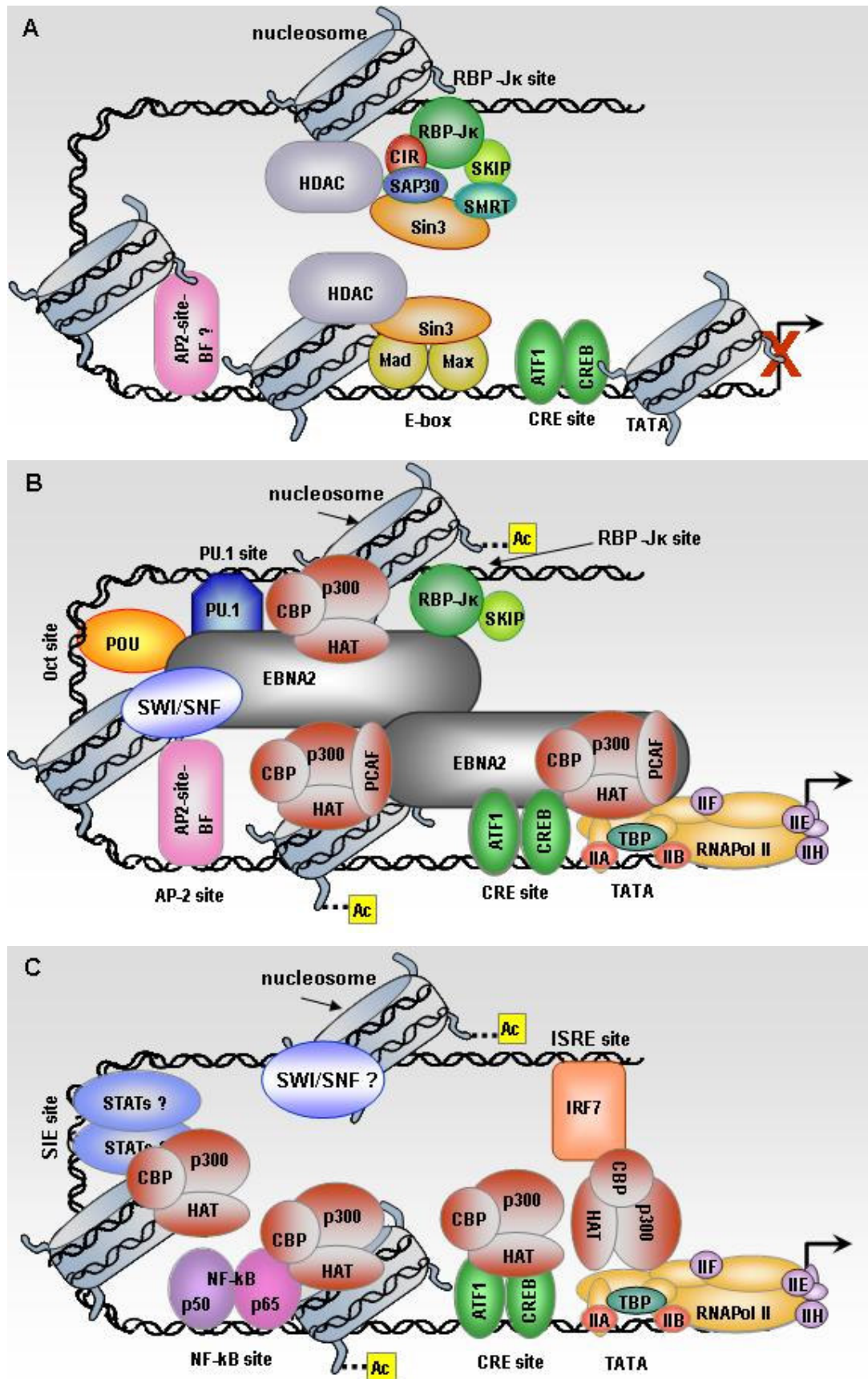


Figure 4. **A schematic model of the LMP1 ED-L1 promoter regulation.** **A.** The LMP1 ED-L1 promoter is repressed in the absence of activators. The repression is thought to be mediated by the recruitment of histone deacetylases (HDAC) to the promoter. HDACs lead to the hypoacetylation of the histone tails at the promoter and a more compact chromatin structure that cannot assemble the transcription initiation machinery. The recruitment of HDACs to the promoter is probably mediated by the RBP-J κ factor binding to the RBP-J κ site and MAD-Max to the E-box in the LMP1 promoter. Both these complexes are known to interact with Sin3 that in turn can recruit HDACs to the promoter. Some of the EBNA2 adaptor proteins may also bind the promoter but are unable to mediate activation in the absence of EBNA2. **B.** EBNA2 dependent activation of the LMP1 promoter occurs through the binding of EBNA2 adaptor proteins RBP-J κ , PU.1, POU domain protein, AP-2 site-binding factor (AP-2 site BF) and ATF1-CREB to the EBNA2 responsive sites in the promoter. The recruitment of EBNA2 by these factors leads to the recruitment of histone acetylase (HAT) complexes p300, CBP and PCAF and the chromatin remodelling factor SWI-SNF to the promoter. Subsequently the histone tails of the nucleosomes become hyperacetylated and remodelled allowing the binding of TBP to the TATA box and assembly of the transcription initiation machinery and eventually gene expression. The interaction of EBNA2 with members of the transcription initiation complex facilitates this process. **C.** In the absence of EBNA2, LMP1 is important for its own expression. Some of the transcription factors that are activated by LMP1 are thought to be involved in the activation of its promoter. These factors that include NF- κ B p50-p65 heterodimer and the p50-p50 homodimer (not shown in the figure), ATF1-CREB heterodimer, IRF7, and possibly the c-Jun-ATF2 heterodimer (not shown), have binding sites in the LMP1 promoter. It has been shown that the cooperation of these factors together leads to histone acetylation, chromatin remodelling and activation of the IFN- β promoter. Therefore, these factors would be able to activate the LMP1 expression through increased histone acetylation and chromatin remodelling of the promoter in the absence of EBNA2. Additionally STAT transcriptional factors can bind an SIE site in the promoter, but their function in the regulation of ED-L1 has not been investigated.

the LMP1 promoter, this is an attractive model (Figure 4B). We propose that LMP1 expression in latency II cells is activated by the cooperative contributions of the transcription factors ATF1-CREB, NF- κ B, IRF7, STATs and possibly c-Jun-ATF-2, all of which are downstream targets of LMP1 and are activated in EBV positive tumours (Chang et al., 2005; Chen et al., 2001; Eliopoulos & Young, 1998; Nepomuceno et al., 2002; Shair et al., 2007; Zhang & Pagano, 1997; Zhang et al., 2004). This would lead to increased histone acetylation and chromatin remodelling at the LMP1 promoter and transactivation (Figure 4C). A similar model has been proposed for the activation of the IFN- β promoter in response to viral infection (Parekh & Maniatis, 1999; Wathelet et al., 1998).

Future directions

The PhD project summarized here has contributed to a better understanding of the molecular mechanism of the LMP1 gene expression. Since both EBNA2 dependent and independent pathways of LMP1 gene expression are dependent on the cellular transcription factors, the continued efforts in the identification of cellular factors involved in this process are relevant in constructing a complete model for this process. This may in fact identify transcription factors whose inappropriate expression/induction maybe the risk factor in the initiation of LMP1 associated tumours. The DNA affinity purification and the availability of the new age sensitive mass spectrometry techniques will be a useful tool in the identification of additional factors binding to the LMP1 promoter. This method can also be used with protein extracts from different cell types to aid identify factors that give rise to cell type differences in the activation of the LMP1 promoter. An EBNA2 affinity protein purification will also aid in building a more complete picture of LMP1 expression as well as other EBNA2 responsive promoters.

Our study also suggests that the NF- κ B pathway may be a candidate for the treatment of EBV malignancies where LMP1 is involved. Such a therapy would downregulate both LMP1 expression as well as its downstream signalling, both of which may be central in LMP1 associated tumours. The use of NF- κ B as a target in cancer treatment has been suggested and several drugs are available that inhibit NF- κ B and exhibit anti-inflammatory and anti-cancer activities (Fabre et al., 2007; Olivier et al., 2006). The NF- κ B pathway has already been proposed as a potential therapeutic target in EBV associated lymphoma (Chuang et al., 2007). Therefore, it is possible that inhibition of NF- κ B in combination with other anti-cancer treatments may present a useful and specific therapy for LMP1 associated tumours.

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