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TRANSCRIPTIONAL REGULATION OF THE EPSTEIN-BARR VIRUS LATENT MEMBRANE PROTEIN 1 GENE IN B CELLS

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DISSERTATION ABSTRACT

Transcriptional Regulation of the Epstein-Barr Virus Latent Membrane Protein Gene in B cells

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Epstein-Barr Virus (EBV) is a ubiquitous human B-lymphotrobic herpesvirus that latently infects over 90 percent of the world's population. EBV infection is usually benign in healthy people. However, EBV is the causative agent of infectious mononucleosis, and is strongly associated with an increasing number of human malignancies, including Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease, and immunoblastic lymphomas in immunocomprised people. EBV has the ability to immortalize B cells *in vitro* and stimulate B-cell proliferation. Mutagenesis of the viral genome has defined a subset of five genes required for transformation (EBNA1, -2, -3, -6 and LMP1). The LMP1 gene is of particular interest since it is an oncogene that is involved in the transformation as well as proliferation of B cells latently infected by EBV. The aim of this thesis is to investigate the molecular mechanisms of transcriptional regulation of the LMP1 gene in B cells.

The LMP1 promoter is controlled by both positive and negative transcriptional cis-elements and the gene is inactive without inducers. EBNA2 can overcome the repression of the LMP1 promoter. By deletion analyses, two distinct LMP1 regulatory sequence (LRS) regions, -106 to +40 and -176 to -136, relative to the transcriptional initiation site, were shown to contribute to the EBNA2-responsiveness in LRS. In the distal EBNA2 responsive region, both the octamer motif (bound by a POU domain protein) and the PU box-binding site (bound by a PU.1 factor) were critical for the EBNA2 induction process. Cooperation between the factors binding to these two sites is required for full EBNA2 transactivation, and EBNA2 may be targeted to the LMP1 promoter via interacting with the POU domain protein.

In the promoter-proximal region (LRS-106/+40), mutational analyses showed that both an Sp site and an ATF/CRE site are important in the EBNA2-dependent activation of the LMP1 promoter. Overexpression of Sp1 and ATF1/CREB1 mediates activation of the LMP1 promoter independently of EBNA2 via their respective binding sites, whereas EBNA2-induced activation occurs through a direct contact between EBNA2 and ATF-2/c-Jun heterodimer via the ATF/CRE site.

We have also shown that a silencing element overlaps with a transcriptional enhancer element in an LRS sequence that contains an E-box-homologous motif. Transient co-transfection analyses showed that USF proteins confer EBNA2-independent activity on the LMP1 promoter via the E-box site and that this activation was downregulated by the Max-Mad1-mSin3A factors. In addition to the E-box site, we have also identified an adjacent Ikaros site that binds Ikaros factors, which also functions as a repressive element.

The repression exerted by the factors binding to the E-box site and the Ikaros site was released by an inhibitor of histone deacetylation, Trichostatin A (TSA), indicating that histone deacetylation plays an important role in repression mediated by these factors. EBNA2 relieved the repression through an indispensable response element in the -107/-95 LRS region, which contains a functional AP-2 site. AP-2 factors cooperated with EBNA2 to overcome the repression exerted by the E-box and the Ikaros site binding factors, which led to the activation of the LMP1 promoter in reporter plasmids.

Key words: *Epstein-Barr virus, B-cell, transformation, LMP1, LRS, EBNA2, transcription, transactivation, repression, histone deacytylation*

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I. Anna Sjöblom, Ann Jansson, Weiwen Yang, Sonia Lain, Tina Nilsson and Lars Rymo
PU box-binding transcription factors and a POU domain protein cooperate in the Epstein-Barr virus (EBV) nuclear antigen 2-induced transactivation of the EBV latent membrane protein 1 promoter.
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Manuscript

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ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
AP-1	Activator protein 1
AP-2	Activator protein 2
ATF/CRE	Activating transcription factor/cyclic AMP response element
BCR	B-cell antigen receptor
bHLH	basic helix-loop-helix
BNLF1	*Bam*HI K leftward reading frame 1
BL	Burkitt's lymphoma
bp	base pair
BZLF1	*Bam*HI Z leftward reading frame 1
CAT	Chloramphenicol acetyltransferase
CBF1	C promoter binding protein 1 (same as RBP-Jκ)
CBP	CRE-binding protein
CD	Cluster of differentiation (e.g. CD21)
cDNA	Complementary DNA
Cp	The promoter in the *Bam*HI C fragment of the EBV genome
ChIP	Chromatin immunoprecipitation
CRE	cAMP response element
CTAR	Carboxy terminal activation region
CtBP	C-terminal binding protein
CTD	Carboxy terminal domain
CTL	Cytotoxic T-lymphocyte
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DPE	Downstream promoter element
DS	Dyad symmetry of *oriP*
EBER	Epstein-Barr virus-encoded RNA
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
ED-L1	*EcoRI* D leftward promoter 1
EMSA	Electrophoretic mobility shift assay
ERK	Extra-cellular response kinase
FR	Family of repeats of *oriP*
GTF	General transcription factor
HAT	Histone acetyltransferase
HD	Hodgkin's disease
HDAC	Histone deacetylase
HIV	Human immunodeficiency virus
Ig	Immunoglobulin
IKK	IkappaB-regulatory kinase
IM	Infectious mononucleosis
JAK	Janus kinase
JNK	c-Jun NH_2–terminal kinase
kb	Kilobase pair
LCL	Lymphoblastoid cell line
LCV	Lymphocryptovirus
LMP	Latent membrane protein
LRS	LMP-1 gene regulatory sequence

MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
NIK	NF-κB-inducing kinase
NPC	Nasopharyngeal carcinoma
nt	Nucleotide
NuRD	Nucleosome remodeling and deacetylation
OHL	Oral hairy leukoplakia
ORF	Open reading frame
OriP	Origin of plasmid replication
PAGE	Polyacrylamide gel electrophoresis
P/CAF	p300/CBP-associated factor
PCR	Polymerase chain reaction
PIC	Preinitiation complex
PKA	protein kinase A
PKC	protein kinase C
Pol II	RNA polymerase II
PTLD	Post-transplant lymphoproliferative disorder/disease
Qp	The promoter in the *Bam*HI Q fragment of the EBV genome
RBP-Jκ	Recombination signal binding protein J kappa (same as CBF1)
RNA	Ribonucleic acid
RNase	Ribonuclease
RPA	RNase protection assay
Sp1	Stimulatory protein 1
STAT	Signal transducer and activator of transcription
TAF	TBP-associated factor
TBP	TATA box-binding protein
TNFR	Tumor necrosis factor receptor
TPA	Tetradecanoyl phorbol acetate
TRAF	Tumor necrosis factor receptor-associated factor
TRADD	Tumor necrosis factor receptor-associated death domain protein
TSA	Trichostatin A
Wp	The promoter in the *Bam*HI W fragment of the EBV genome
XLPD	X-linked lymphoproliferative disease

CONTENTS

To Liwei, Bo and Richard

INTRODUCTION

The Epstein-Barr Virus (EBV) is a ubiquitous human herpes virus, infecting over 90 percent of the world's population and persisting for the lifetime in B cells in a latent infection. The virus is usually benign in healthy people. Primary infection usually takes place in the first few years of life and does not result in any recognized disease. However, the dramatic immune response that can occur when primary infection is delayed until adolescence or adulthood, frequently results in infectious mononucleosis. EBV is also associated with several human malignancies derived from B cells, T cells, NK cells, epithelial cells and perhaps other tissues as well, including endemic Burkitt's lymphoma (BL), nasopharyngeal carcinoma (NPC), Hodgkin's disease, gastric carcinomas, certain rare T-cell lymphomas, and immunoblastic lymphomas in immunocomprised people [1].

EBV is remarkable for the efficiency with which it can cause proliferation of the B cells that it infects. *In vitro*, EBV has the ability to establish a latent infection in proliferating B lymphoblasts. However, EBV does not establish latency in a proliferating lymphoblast *in vivo*, but in a resting memory B cell. EBV encodes genes that can cause cell growth and prevent cell death and these are thought to play a role in the establishment of viral persistence because the virus transits in B cells through lymph nodes into the memory B-cell compartment. Only a few of the 85 genes encoded by the virus are necessary for the transforming ability of EBV. The latent membrane protein 1 (LMP1) gene is one of the genes involved in the deregulation of cell growth and is known to be a viral oncogene. The expression of LMP1 is regulated both by general signal transduction pathways and by the EBV-encoded proteins. Detailed knowledge of the transcriptional regulation of the LMP1 promoter will improve the understanding at the molecular level of the roles played by the virus in the pathogenesis of virus-associated lymphomas.

1. Regulation of gene expression

The regulation of gene expression is central to cell differentiation and development. Gene expression can be controlled at six different levels: transcription or RNA synthesis, RNA processing, mRNA transport, mRNA stability, translation or protein synthesis, and protein stability. Eukaryotic genes appear to be regulated primarily at the level of transcription.

Transcription is the process where the information stored within genes is copied into messenger RNAs (mRNAs). Once the primary transcript or pre-mRNA has been formed, a series of processing events occur that lead to the production of the mature transcript: the primary transcript is generally capped at its 5' end by a 7-methylguanylate (*cap structure*) and polyadenylated at its 3' end (*poly A tail*). The primary transcript is spliced to remove introns interrupting the exons. The splicing of pre-mRNA is not a stable process, and alternative splicing can lead to a variety of transcripts encoding distinct protein products, thus increasing the coding capacity of genes. Alternative splicing is often tightly regulated in a cell-type or developmental-stage-specific manner, and it is an important regulatory step in gene expression. Capped/polyadenylated transcripts are generally more stable and associate more easily with ribosomes, ensuring a higher rate of translation. The process of producing the mature transcript is believed to be linked to transport through the nuclear membrane.

In the cytoplasm, the mature RNA transcript becomes associated with ribosomes and a polypeptide chain is produced according to the information coded by RNA, a process known as *translation*. The stability of RNA, *i.e.* how long RNA remains translatable in the cytoplasm, is also another step at which expression levels can be controlled. The protein itself

can also be subjected to post-translational modification, which may influence targeting of the protein for degradation. In this thesis, I will focus on the regulation of gene expression by transcriptional mechanisms.

2. Eukaryotic transcription

Regulation of transcription is a fundamental process in biology. Central to the transcription process is the necessity for an RNA polymerase (Pol) to reach the promoter and transcribe the ensuing DNA sequence into the complementary RNA molecule. RNA polymerases are enzymes that catalyze the formation of 3'-5' phosphodiester binding between ribonucleoside triphosphates. In eukaryotes, there are three different RNA polymerases, RNA polymerases I-III. Each RNA polymerase seems to transcribe a specific class of genes: Ribosomal RNA (rRNA) genes are transcribed by Pol I, transfer RNA (tRNA) genes, the 5S Ribosomal RNA genes and the U6 small nuclear RNA genes are transcribed by Pol III, whereas protein coding genes and most small nuclear RNA genes are transcribed by Pol II, mostly giving rise to mRNA.

2.1 Promoter Structure

Genes are DNA sequences coding for proteins or RNA. Generally, DNA sequences upstream of a gene contain regulatory regions, including regulatory *cis* elements that can be recognized by *trans* factors (transcriptional activators or repressors), which positively or negatively affect the level of transcription. The regulatory elements are highly variable and gene-specific, located in a promoter-proximal position or many kilobases away in a promoter-distal position. For a typical gene, the core promoter is defined as the sequence immediately adjacent to and upstream of the gene that specifies the start and direction of transcription. The core promoter consists of an initiation site (Inr), the TATA box (TATA), downstream promoter element (DPE) and the upstream regulatory regions (Fig. 1). There are two kinds of promoters: TATA containing promoters and TATA lacking promoters. A typical promoter usually contains a TATA box (a sequence rich in the nucleotides A and T) at around 30 base pairs upstream of the transcriptional start site. The LMP1 promoter is also a TATA containing promoter.

2.2 Basal transcription machinery

Eukaryotic RNA polymerases cannot function alone and they require an enormous array of co-factors, termed general transcription factors (GTFs), to achieve transcription. The ability of pol II to initiate transcription from a given point and at a defined frequency is dependent upon interactions of DNA-binding proteins with the core promoter and the regulatory region. Every core promoter requires the GTFs, which include TFIID, TFIIA, TFIIB, TFIIF, TFIIE, and TFIIH [2].

Mechanistic studies using purified eukaryotic systems have divided the process of RNA pol II transcription into distinct steps or stages. Among these steps are promoter recognition, formation of a preinitiation complex, initiation, promoter clearance, elongation, termination, and reinitiation [3]. *In vitro*, the GTFs and Pol II can be assembled onto a promoter in an ordered stepwise pathway [3]. The first basal factor to bind the core promoter is TFIID, a large multi-subunit complex composed of TBP and several tightly bound associated factors TAFs. TBP sits as a saddle on the TATA element and causes a major bend in the DNA. This brings sequences both upstream and downstream of the TATA box into a closer proximity to each other [4]. After the binding of TFIID at the TATA box, TFIIA, TFIIB, TFIIF-pol II, TFIIE and finally TFIIH bind in a stepwise assembly, forming a pre-initiation complex (PIC). Although the GTFs and Pol II can be assembled at the promoter in an ordered stepwise pathway, considerable evidence has accumulated that transcriptional initiation may normally

involve core promoter binding by a pre-assembled holoenzyme complex containing Pol II and many or all of GTFs that are essential for initiation [5].

The assembly of the initiation complex is followed by PIC activation, where the double-stranded DNA forms a "bubble" near the PIC (promoter opening/melting) and transcription is then initiated. The transcription initiation is defined by the formation of the first phosphodiester bond, whereas the promoter clearance is the point where the polymerase leaves the initiation complex to start transcript elongation. After initiation, escape into elongation is concomitant with phosphorylation of the C-terminal domain (CTD) of pol II. The elongation phase continues until the transcribing polymerase terminates transcription, usually 0.5-2 kb past the termination/polyadenylation signal sequence. The various phases of transcription are associated with alternative GTFs. Some components of the PIC remain associated with the elongation complex (TFIIF), whereas other factors (TBP or TFIID) remain at the promoter after promoter clearance or are sequentially dissociated (TFIIB, TFIIE, TFIIH). At or after the termination stage, dephosphorylation of the CTD by a CTD-specific phosphatase returns the polymerase to a stage capable of PIC assembly.

2.3 Transcription factors

The expression levels of genes transcribed by Pol II are predominantly controlled by a complex system of the specific transcription factors that interact with each other and with the *cis* DNA regulatory elements located distal of the core promoter. Most of these transcription factors are DNA-binding proteins composed of two functional domains: a specific DNA-binding domain and a regulatory domain. The activities of the domains can be independently regulated by a number of mechanisms: oligomerization; action of inhibitory molecules; ligand binding; protein-protein interactions; and post-translational modifications such as phosphorylation or dephosphorylation. These changes can alter the DNA-binding characteristics, the activation ability, or the sub-cellular localization of a transcription factor. The transcription factors are classified according to the features of their protein structure, which account either for their interaction to DNA or for their formation of multimers, often dimers. Transcription activators can also be grouped according to the protein composition in their activation domains. Transcription activators function through interacting with components of the PIC directly or via co-regulators.

2.4 Chromatin modification and transcription regulation

The active state of a gene is determined not only by transcription factors but also proteins that modify the structure of chromatin. The purpose of the chromatin remodeling proteins is to alter the nucleosome architecture such that genes become exposed to or hidden from the transcriptional machinery. Genomic DNA is packed into fundamental units known as nucleosomes. A nucleosome contains approximately 146 base pairs of DNA wrapped around an octamer of histone proteins (consisting of two copies of each histone protein H2A, H2B, H3 and H4). All genomic DNA is further packed together in highly ordered structures (termed chromatin) with the help of histone protein H1. The chromatin is an environment in which transcription initiates inefficiently, because the histone proteins are too strongly associated with DNA and prevent transcription factors and the transcription machinery from gaining access to promoters. Therefore, an alteration of chromatin structure is often necessary and a first step for transcriptional initiation. Two highly conserved mechanisms for altering chromosome structure have been identified: 1. the movement of nucleosomes along DNA, which is carried out by ATP-dependent chromatin remodeling complexes such as the Swi/Snf complex and NuRD complex; and 2. the post-translational modification of core histones including methylation, phosphorylation and acetylation. Histone acetylation is probably the

best understood of these covalent modifications where hyper-acetylation by histone acetyl transferases (HATs) leads to increased transcription of particular genes and hypo-acetylation of histones by histone deacetylases (HDACs) has an opposite effect. Many studies have identified several large, multisubunit enzyme complexes responsible for the targeted acetylation/deacetylation of histones, as well as ATP-dependent chromatin remodeling complexes (reviewed in [6, 7]).

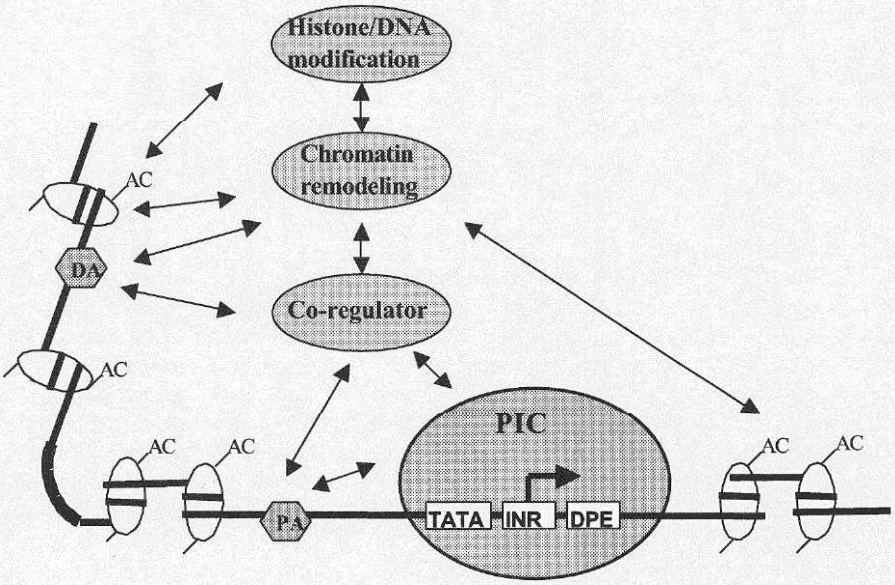

Figure 1. The regulatory network of transcription is assembled by multisubunit complexes, including the transcription machinery, co-regulatory complexes, and transcription factors. The first step for transcription regulation is to alter the nucleosomal architecture where gene activation or repression demands structural changes at the chromatin level. Chromatin modifying activities can be targeted to promoters to alter chromatin structure and thereby modulate the accessibility for interacting proteins. Activation of transcription is controlled by coactivator complexes that mediate activation of transcription by activators located in a promoter-proximal position (PA) or many kilobases away in a promoter-distal position (DA). Also, the composition and sequence of the core promoter directs the formation of the pre-initiation complex (PIC) and defines the start site of transcription. This involves the interplay of basal transcription factors and RNA polymerase with distinct core promoter elements such as TATA box (TATA), Initiator (Inr), and downstream promoter element (DPE) to position the pre-initiation complex. Modified from [8].

3. The Epstein-Barr Virus

The Epstein-Barr Virus (EBV), a B-lymphotropic human herpesvirus, is one of the most common viruses that infect humans. Over 90 percent of humans carry this γ1-herpesvirus for lifetime (reviewed in [9]). Two different EBV types are found in the human population: type 1 and type 2 (type A and B). Only a few of the genes differ between these two virus types. *In vitro* studies suggest that the type 1 virus is more efficient in transforming B cells than the type 2 virus.

3.1 EBV Replication

The EBV genome, which is encased within a nucleocapsid surrounded by the viral envelope in the virion, consists of a linear 172-kb DNA molecule (reviewed in [9]). The DNA is segmented by internal repeats (IR) and unique (U) regions, which have been characterized by *BamHI* restriction endonuclease fragments (from A-Z), depending on fragment size. After infecting the cells, the linear EBV genome becomes circular at its cohesive terminal repeats (TR), forming an episome. The virus exists predominantly in this circular form in infected cells. EBV is sometimes integrated into the host genome at some, but not any specific integration site. The virus replicates once during each cell cycle using the replication machinery of the host. The DNA synthesis starts in a region called *ori*P, the *origin of plasmid replication*. It is composed of the *family of repeats* (FR) and the *dyad symmetry* (DS) regions [10, 11]. The DS sequence contains the initiation site for replication whereas the FR part is required for the replication process and also functions as a transcriptional enhancer. Induction of lytic replication is spontaneously activated in only a small percentage of latently infected B cells from *orilyt*, the *origin of lytic replication* [12].

3.2 EBV Infection

EBV infects a variety of cell types, mainly the mucosal epithelial cells and B cells. The infection of epithelial cells is a primarily lytic infection with production of virus, whereas the B-cell infection is a predominantly latent infection. EBV can also infect T lymphocytes and even smooth muscle cells since the virus is found in some T-cell lymphomas and smooth-muscle tumors in transplant recipients (reviewed in [13]).

The EBV life cycle starts when the virus primarily infects epithelial cells in the oropharynx (Figure 2). Infection of B-lymphocytes with EBV occurs in the oropharyngeal lymphoid organs. Active viral replication takes place in mucosal lymphoid tissue in the oropharynx, and released virus is transmitted via the saliva. Before the virus enters the B cell, the major envelope glycoprotein, gp350/220, binds to the viral receptor, the CD21 molecule (the C3d complement receptor) [14], on the surface of the B cell. Other factors in addition to CD21 are important for infection. The major-histocompatibility-complex (MHC) class II molecule serves as a cofactor for infection of B cells [15]. Cross-linking of surface molecule by the viral glycoproteins provides the necessary signals to trigger the resting B cells to leave the resting state (G0) and enter the G1 state in the cell cycle [16]. During this time, the earliest expressed latent proteins EBNA2 and EBNA5 (or EBNA leader protein, LP) are induced from the Wp promoter that is present in multiple copies in the viral genome. EBNA2 and EBNA5 drive the cells through the first G1 and the transcriptional activator EBNA2 activates promoters necessary to produce the full range of latent proteins. At this point EBNA transcription switches from Wp to Cp [17], a promoter that works optimally in B lymphoblasts. The latent proteins block terminal differentiation and drive the B cells to proliferate. The proliferation of EBV-infected B cells in primary infection is accompanied by the development of a vigorous cytotoxic T-lymphocyte (CTL) response directed against both latent and lytic viral proteins (reviewed in [1]). The EBV-infected B cells can differentiate through a germinal center reaction as centroblasts and centrocytes that express EBNA1 and LMPs, but not EBNA2 whose functions are believed to drive cell proliferation and to block cell differentiation. These cells exit into the peripheral circulation as resting memory B cells that are transcriptionally quiescent for viral latent genes and, thus, not recognized by the immune system [18]. Only a small percentage of the circulating B cells are infected by the virus, ranging from 2 to 60 cells per million [19].

The role of the lymphoblastoid form of latency is proposed to provide EBV with access to the memory B cell compartment where the virus can persist for the life time of the infected host without being cleared by the immune response (reviewed in [20]). During persistence, latently infected memory B-cells transit through mucosal lymphoid tissue where they may become activated. A few of these activated cells exit the cell cycle to replenish the pool of infected memory cells, but most cells migrate into the mucosal epithelium, terminally differentiate and release infectious virus.

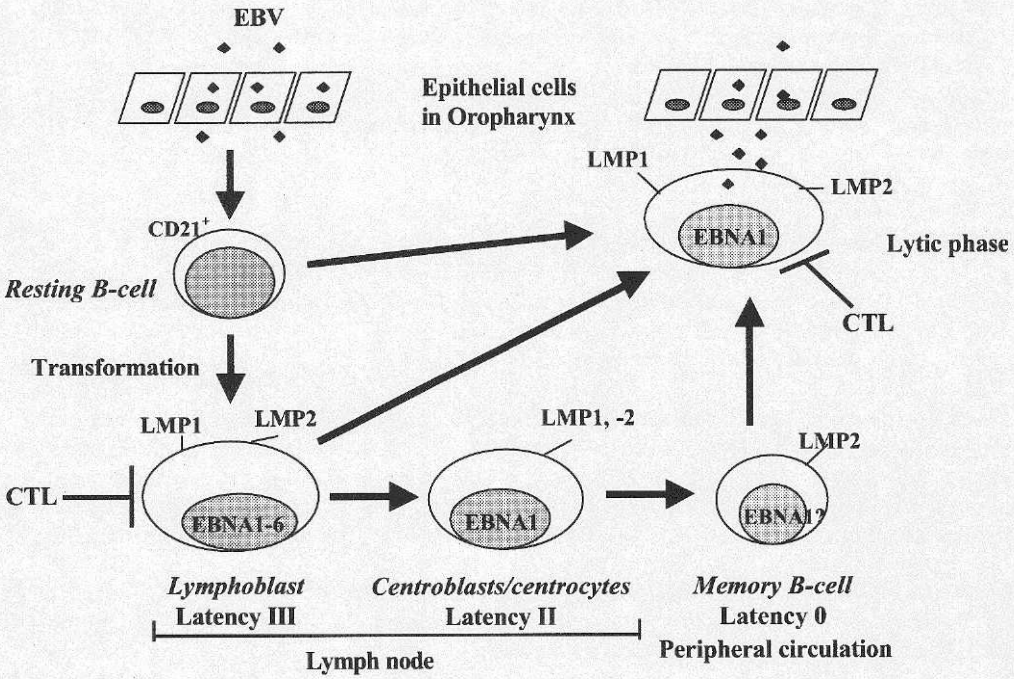

Figure 2. The Epstein-Barr virus life cycle. A detailed description is given in the text. Modified from [18].

3.3 EBV Latency Programs

EBV has been suggested to exist in three different kinds of latency programs (reviewed in [1]). These are all distinct from the lytic cycle. Under the primary infection, EBV will infect resting B cells, and drive them into a latently infected, proliferating lymphoblastoid state. This lymphoblastoid form of latency is referred to as ***latency III***, which is also characteristic of EBV-positive Burkitt's lymphoma (BL) cell lines with what is termed a group III phenotype. This step is considered to be a transient phase *in vivo*, but *in vitro*, these EBV-infected B cells will be immortalized into permanently growing lymphoblastoid cell lines (LCLs). In LCL cells, a limited set of latent gene is expressed, which compromises two small nuclear RNA molecules (EBV-encoded RNAs, EBER1 and 2), six EBV nuclear antigens (EBNA1-6), three membrane proteins (latent membrane proteins LMP1, 2A, 2B) and various spliced *BamHI* A rightward transcripts. Of these EBV-latent genes, only five (EBNA1, EBNA2, EBNA3, EBNA6, and LMP1) are absolutely essential, whereas EBNA5 is important, for B-cell transformation by EBV.

A much more restricted pattern of latency (***latency I***), characterized by the expression of EBNA1 (from Qp promoter), EBERs and the *BamH*I A rightward transcripts, is seen in Burkitt's lymphoma biopsies. While an intermediate program (***latency II***) with expression of the LMPs as well as the EBERs, the *BamH*I A rightward transcripts and EBNA1, is found in germinal center (GC) B cells [18], as well as in EBV-associated malignancies such as Hodgkin's disease, nasopharyngeal carcinoma (NPC), and T/NK-cell lymphomas.

Recently, a form of latent infection was described for infected memory B cells in the peripheral circulation of healthy carriers. This form of infection is referred to as the ***latency 0*** or the true latency program [20]. In this case no viral latent genes are expressed [21], with the possible exception of LMP2A expression whereas EBNA1 expression can be detected sporadically [22-24].

3.4 EBV and diseases

EBV was originally discovered in a cell line derived from a Burkitt's lymphoma by Epstein and Barr in 1964. Since then, EBV has been linked to the pathogenesis of an increasing number of both benign and malignant diseases (reviewed in [1, 13]). The potential of EBV to drive indefinite cell proliferation, in combination with environmental and genetic factors, increases the risk of cellular genetic alterations and consequently malignancy.

Infectious Mononucleosis (IM)

Infectious Mononucleosis is caused by delayed EBV infection of adolescents and adults and is a benign lymphoproliferative disorder. IM is characterized by a proliferation of EBV-infected cells in lymphoid tissues such as tonsils. In IM, the full range of viral proteins characterized of latency III is expressed. However, analysis at the single cell level has revealed a degree of heterogeneity with cells expressing latency I, II, and III patterns being detectable [25]. After several weeks the infection ceases due to a vigorous cytotoxic T-lymphocyte (CTL) response.

Burkitt's lymphoma (BL)

BL was the first human tumor to be linked to a viral infection, and EBV was first discovered in BL cells. BL cells contain a chromosome translocation of the *c-myc* gene (chromosome 8) into one of the immunoglobulin loci (chromosome 14, -2, or -22), leading to abnormal regulation of c-myc expression. Expression of the c-myc in EBV-immortalized B cells results in increased tumorigenicity of the cells. EBV has been demonstrated in virtually all cases of endemic BL; whereas only up to 30% of sporadic cases occurring in Western Europe or in North America are EBV associated. In EBV-positive BL cases, the virus is present in all tumor cells and the viral genomes have been shown to be of monoclonal origin. The expression of EBV-latent genes is limited to the EBERs and EBNA1. Thus, those latent viral proteins that are recognized by EBV-specific CTLs are not present in BL, allowing BL cells to escape EBV-specific immunity.

Hodgkin's disease (HD)

HD is characterized by the presence of a small number of malignant mononuclear Hodgkin and multinuclear Reed-Sternberg cells. EBV has been detected in the Hodgkin and Reed-Sternberg cells of up to 40% of HD cases in western countries and the viral genomes are monoclonal. EBV-gene expression in HD is restricted to the EBERs, EBNA1, LMP1, LMP2A and LMP2B, consistent with a type II latency.

Nasopharyngeal carcinoma (NPC)
NPC occurs with a high incidence in certain parts of Southeast Asia, Northern Africa, and among Alaskan Eskimos, but sporadically in Western Europe and North America. Nearly 100% poorly/undifferentiated NPCs contain EBV genomes and express EBV proteins as type II latency. The EBV episome is present as a monoclonal episome. The expression of LMP1 in NPCs is consistent with the ability of the protein to inhibit terminal differentiation of the epithelial cells. In addition to EBV infection, other factors (such as environmental risk factors and genetic susceptibility) also play a role in the development of NPC.

EBV lymphoproliferative diseases (or Immunoblastic lymphomas)
These can be observed in patients with severe immunodeficiencies, such as AIDS, X-linked lymphoproliferative disease and drug-induced immunosuppression in transplant recipients. These patients have impaired T-cell immunity and are unable to control the increased virus-driven lymphoproliferations of EBV-infected B cells. EBV lymphoproliferative disease can be either monoclonal, oligoclonal, or polyclonal and usually shows a type III latency.

Oral Hairy Leukoplakia
Oral Hairy Leukoplakia occurs in HIV-infected patients as well as in some immuno-suppressed transplant recipients. It is a nonmalignant hyperplastic lesion of epithelial cells. The lesions show active viral replication and expression of lytic viral proteins, but EBNA1 is also detected.

EBV DNA or proteins have been detected in several other cancers, including T/NK-cell lymphoma, peripheral T-cell lymphomas, lymphoblastoid granulomatosis, central nervous system lymphomas in non-immunocompromised patients, smooth-muscle tumors in transplant recipients, and gastric carcinomas (reviewed see [13]).

3.5 EBV-encoded latent proteins
EBV encode a large variety of proteins that enable the virus to survive despite a vigorous immune response. Each of these proteins performs a specific function, and they are expressed in the host cell at different phases according to the need of the virus.

EBNA1
EBNA1 is essential for the replication and maintenance of the EBV episome [26] by binding as dimers to binding sites within EBV origin of plasmid replication, *oriP*. This binding also activates a transcriptional enhancer activity in *oriP* which can activate expression of Cp-initiated viral genes, and the LMP1 gene over a distance of 10 kb [27]. EBNA1 is expressed in all EBV-infected cell lines from different promoters. The amino terminal half of EBNA1 contains a 239-amino acid region of repeating glycine–glycine–alanine residues which inhibits proteasomal degradation and presentation to CTLs [28]. EBNA1 is required for B-cell immortalization and might be oncogenic, since mice carrying an EBNA1 transgene driven by the immunoglobulin heavy chain promoter/enhancer developed B-cell lymphomas at a high frequency [29].

EBNA2
EBNA2 is essential for initiation and maintenance of EBV driven B-cell immortalization [30, 31]. EBNA2 in the absence of other EBV proteins can be oncogenic, as 90% of transgenic mice expressing EBNA2 from the SV40 early enhancer-promoter developed kidney tumors [32]. EBNA2 contributes to transformation by acting as a transcriptional coactivator. EBNA2 regulates expression of cellular genes, such as the B-cell activation markers CD21 and CD23,

as well as the proto-oncogene c-myc [33]. In addition, EBNA2 activates genes encoding EBV nuclear and membrane proteins through regulating the Cp and LMP promoters (reviewed in [9]). EBNA2 cannot bind to DNA directly but interacts with cellular DNA-binding proteins, including RBP-Jκ (also called CBF-1) and PU.1 [34-39], and some other transcription factors presented in this thesis. EBNA2 may contribute to transcriptional activation by recruiting TFIIB, TFIIE, TFIIH, RPA70, and TAF40 basal transcription machinery to the promoter [40-42]. EBNA2 may also facilitate transactivation by interaction with hSNF5/Ini1, components of the SNF-SWI complex, to alter the nucleosome structure of target genes [43]. EBNA2 can also interact with the histone acetytransferases (HATs) CBP, P300 and PCAF in activation of LMP1 promoter [44]. EBNA2 is able to activate promoters through interaction with RBP-Jκ protein, which is also the target of the Notch signaling pathway. EBNA2 is consider to be a biological equivalent of an activated Notch receptor (reviewed in [45]). By molecular genetic analyses, the RBP-Jκ binding domain and the acidic domain of EBNA2 were both required for B-cell immortalization [46, 47].

EBNA5 (also known as EBNA LP)

EBNA5 and EBNA2 are the first viral proteins expressed during EBV infection of B cells. EBNA5 is not required for EBV immortalization of B cells, but its presence enhances transformation [31]. EBNA5 cooperates with EBNA2 to activate transcription of the LMP1 gene [48, 49] and the cyclin D2 gene [16]. Recently, EBNA5 was shown to repress gene expression by inhibition of the polyadenylation process with the result that reporter RNA is not exported to the cytoplasm for translation [50].

EBNA3, -4, -6 (also referred as EBNA-3A, -3B, -3C respectively)

EBNA3, -4, -6 are encoded by three genes that are adjacent within the EBV genome, with a high degree of sequence similarity. Only EBNA4 is dispensable for B-cell immortalization, whereas EBNA3 and -6 are essential. EBNA6 can transform rat embryo fibroblasts and functions as an oncoprotein [51]. All three proteins can bind to RBP-Jκ, preventing RBP-Jκ from binding to DNA, and inhibits EBNA2-mediated transactivation of the LMP2A promoter [52]. All three proteins have been shown to participate in transcriptional regulation of the LMP1 gene as well as several cellular genes (reviewed in [9]). EBNA6 was reported to interact with a histone deacetylase (HDAC1), indicating that the repression of transcription could be mediated through histone deacetylation [53]. EBNA6, but not EBNA3 or -4, was also found to activate transcription from the LMP1 promoter in conjunction with EBNA2 [54]. Like EBNA2, EBNA6 binds directly to PU.1 *in vitro* [54].

LMP1

LMP1 is an oncoprotein that is essential for B-cell immortalization. A detailed description of the LMP1 function is given in a later section.

LMP2A and -2B

LMP2A and -2B are integral membrane proteins. LMP2A interferes with signal transduction through interaction of its hydrophilic N-terminal domain with Lyn and Fyn tyrosine kinases [55, 56]. LMP2A blocks signal transduction from surface immunoglobulins. By blocking the switch from latency to lytic cycle, LMP2A may prevent reactivation of the latent virus. In addition to blocking B cell antigen receptor (BCR) signaling, LMP2A can substitute for a BCR-mediated survival signal in B cells lacking membrane immunoglobulin, as expression of LMP2A in transgenic mice allows non-transformed B cells to survive even in the absence of normal BCR signaling [57].

Table 1. EBV-encoded latent proteins and their possible functions

EBV-encoded latent proteins	Function	Required for transformation	Latency
EBNA1	Essential for viral replication and maintenance of the EBV episome, transactivates the LMP1 promoter and Cp via interaction with *OriP*	+	0*, I-III
EBNA2	Viral oncogene, transactivates cellular and other latent viral genes, functional homologue of Notch	+	III
EBNA5 (-LP)	Cooperates with EBNA2 in transactivation, implicated in transcriptional repression by inhibition of the polyadenylation process	+/–[#]	III
EBNA3 (-3A)	Transcriptional regulator	+	III
EBNA4 (-3B)	Transcriptional regulator	–	III
EBNA6 (-3C)	Viral oncogene, transcriptional regulator	+	III
LMP1	Viral oncogene, induces B-cell activation and adhesion, protects cell from apoptosis	+	II-III
LMP2A	Prevents EBV reactivation and lytic cycle, blocks and/or substitutes BCR signals	–	0, II-III
LMP2B	Modulates LMP2A function	–	0, II-III

* EBNA1 expression can be detected sporadically
[#] Not essential but enhances efficiency of B-cell transformation

3.6 Latent Membrane Protein 1

LMP1 is often expressed in EBV-associated cancers, suggesting a role in the initiation or maintenance of transformation. It has been shown *in vitro* that LMP1 is necessary for EBV to immortalize primary B cells [58] and for the resulting LCLs to continue proliferating [59]. The expression of LMP1 transforms rodent fibroblasts [60] and human keratinocyte cell line [61] and induces a phenotype characteristic of B-cell activation in primary B cells [62]. Evidence that LMP1 has the ability to transform cells *in vivo* was provided in a study of transgenic mice expressing LMP1 under the control of the Ig heavy chain promoter [63]. These mice developed B-cell lymphomas, directly demonstrate the oncogenic potential of LMP1.

Structure and Location of Latent Membrane Protein 1

The LMP1 protein is encoded by the *BamHI* N leftward reading frame 1 (BNLF1) in EBV genome. The structure of LMP1 predicted from the ORF in the cDNA was a 386-residue integral membrane protein with six transmembrane segments (Figure 3). A short 23-residue amino terminus and a long 200-residue carboxyl terminus were both predicted to be located on the cytoplasmic side of the membrane (reviewed in [9]). The LMP1 protein sizes range between 57 and 67 kDa due to a variable number of repeats in the LMP1 gene. Other

variations are also found in LMP1 genes from NPC biopsy samples from specific geographical areas and include a point mutation within an *Xho*I restriction site and a 30 bp deletion [64]. The loss of *Xho*I site has no obvious link to LMP1 function, whereas the deletion of 30 bp in the C terminal part of LMP1 correlates with stronger transforming ability of the protein.

LMP1 is concentrated in glycosphingolipid-rich membrane regions, which also mark the clustering sites for G protein-associated receptors. LMP1 clustering is not ligand dependent and no external ligand has been identified that interacts with the three short loops exposed on the surface of immortalized cells. Mutational analyses demonstrate that the amino terminus and the trans-membrane segments of LMP1 mediate the aggregation of LMP1 into patches, whereas clustering of LMP1 in the membrane of infected cells is an essential feature of the immortalizing phenotype of EBV.

Signaling by Latent Membrane Protein 1

The molecular basis for the EBV oncoprotein LMP1 in transformation is its ability to constitutively activate the tumor necrosis factor receptor (TNFR) signal transduction pathway [65]. Aggregation, mediated by the transmembrane domains, causes LMP1 to behave as a constitutively activated receptor [66]. It is the C-terminal of LMP1 that performs the signaling function, the N-terminal domain is only required for its tethering to the cytoskeleton. Whereas LMP1 is capable of transforming immortal rodent cell lines [60], overexpression of LMP1 in B cells results in cytotoxicity [67]. LMP1 acts as a constitutively active TNFR (reviewed in [68]). LMP1 has several effects similar to those seen in the activation of CD40, a member of the TNFR family; even the C-terminal domain of LMP1 and the cytoplasmic domain of CD40 are functionally interchangeable [59]. The C-terminal domain of LMP1 contains a consensus Tumor Necrosis Factor Receptor-associated Factors (TRAFs) binding motif and has been shown to associate with several members of the TRAF family, in particular TRAF1 and TRAF3, and with the Tumor Necrosis Factor Receptor-associated death domain protein (TRADD) (reviewed in [68]). These protein-protein interaction regions are required for both immortalizing activities and signal transduction of LMP1.

One of the principal effects of LMP1 clustering with TRAFs and TRADD is the activation of NFκB regulated genes [69]. The interactions of TRAFs and TRADD with LMP1 are mediated by separate regions within the C-terminal domain termed Transformation Effector Sites (TES) or C Terminal Activation Regions (CTAR) [69, 70] (Figure 3). CTAR1 activates NFκB to a low degree, whereas CTAR2 activates 70-80% of the NFκB activity. Signaling to NFκB from both CTAR1 and CTAR2 requires NFκB-inducing kinase (NIK), IκB-regulatory kinases (IKKα and IKKβ) [71]. In recent years, it has become apparent that LMP1 initiates other signaling pathways in addition to NFκB. CTAR2 activates c-Jun N-terminal kinase (JNK) in a TRADD- and TRAF2-dependent manner (reviewed in [68]). The LMP1 signaling pathway to JNK and NFκB apparently does not overlap downstream of TRAF2, and there is no cross talk between these two pathways since inhibition of one does not affect the other. LMP1 has also been reported to activate several other MAPK family members: the extra-cellular response kinase (ERK) 1, -2 and p38. LMP1, via an intermediate domain between CTAR1 and CTAR2, can also interact with the Janus kinase 3 (JAK3), which in turn activates JAK/STAT pathway. This domain is not required for transformation.

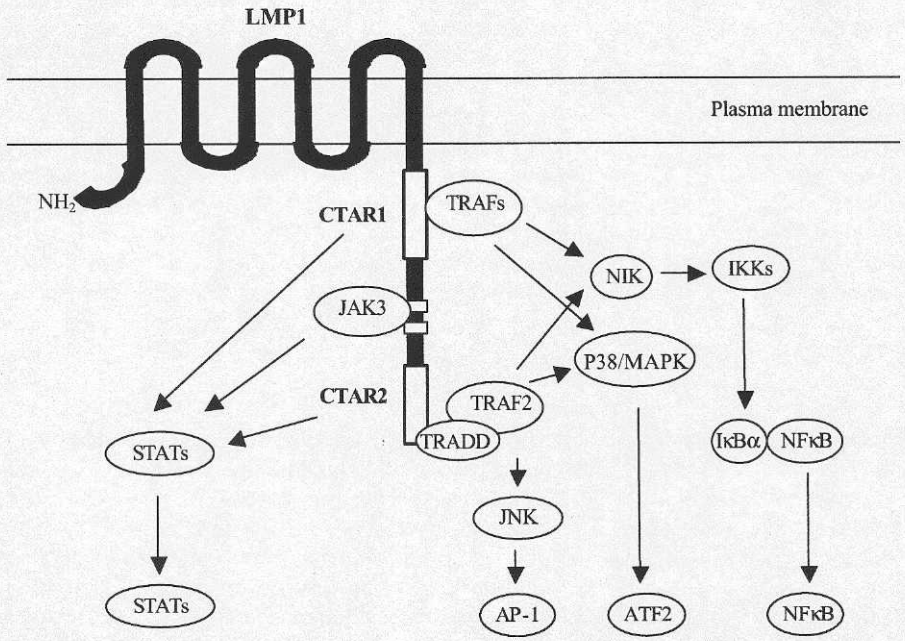

Figure 3. Schematic representation of the signaling pathways engaged by LMP1. LMP1 is an integral membrane with six transmembrane domains, which confers aggregation and oligomerization at the cell membrane. Three C-terminal domains are identified, CTAR1, CTAR2 and CTAR3. CTAR1 binds TRAF1-3, and -5, and activates the NFκB and p38 pathways. CTAR2 binds TRAF2 and TRADD and stimulates the NFκB, p38 and JNK pathways. Both CTAR1 and -2 domains can activate STATs pathways, although an intermediate domain binds of JAK3 and activates JAK/STATs pathways independently of CTAR1/CTAR2. All these signals lead to transcriptional regulation of various cellular genes. Modified from ([68]).

Protection from apoptosis by Latent Membrane Protein 1

One interesting feature of LMP1 is that whereas ectopic overexpression may be cytostatic, the LMP1-expressing cells are protected from apoptosis [72-75]. Several studies have shown that LMP1 expression protects cells from p53-mediated apoptosis. Upregulation of Bcl-2, Mcl-1 and A20 anti-apoptotic genes appears to be the principal underlying mechanism [72, 75, 76]. Transgenic mice expressing LMP1 develop B-cell lymphomas and the tumor cells show increased levels of bcl-2 and A20, suggesting that these genes may have an important role in lymphomagenesis [63].

3.7 The regulation of the LMP1 gene expression in B cells

The expression of LMP1 in EBV-transformed B lymphocytes is tightly regulated by both EBV-encoded proteins and cellular factors that are part of general signal transduction pathways through promoter elements targeted by ubiquitous as well as B-cell-specific proteins. Previous investigations have suggested that the LMP1 gene regulatory sequence (LRS) is composed of both positive and negative transcriptional *cis* elements, and the gene is inactive in the absence of inducers [77-79]. As mentioned above, all the EBNAs are implicated in the regulation of LMP1 gene expression. Among them, EBNA2 is a most potent transactivator of LMP1 gene expression in B cells. Although the regulation of the LMP1

promoter has been extensively investigated, the mechanism by which EBNA2 overcomes the repression of the promoter is still not fully understood.

Promoter usage

There are several different promoters identified in different cell lines to express the LMP1 gene. In B cells, the *EcoRI* D leftward promoter 1 (ED-L1) is the primarily utilized promoter. This promoter was shown to be a latent promoter active in the B cell line B95-8, giving rise to a 2.8-kb long transcript [80]. This thesis focuses on the regulation of this promoter. Another promoter designated ED-L1A was identified and shown to be active in the late lytic phase in B cells resulting in the expression of a truncated LMP1 protein [81]. Cloning of the LMP1 gene from an NPC biopsy from a Chinese identified ED-L1, ED-L1A, and ED-L1B [82]. Another study in a nude mouse-passaged NPC cell line C15 showed that two 3′ coterminal LMP1 mRNAs of 2.8 and 3.5 kb have been detected at equal levels [83]. The 3.5 kb transcript initiates from heterogeneous start sites within the first terminal repeat (TR) of the viral genome. The presence of different promoters will ensure the virus to express the LMP1 gene under different conditions. Furthermore, some EBV strains differ with respect to the LMP1 coding exons and the LMP1 regulatory sequence present in the genes. This indicates that the expression of these different LMP1 genes will vary depending on the cellular context and virus type present in a cell.

Regulation of LMP1 gene expression by DNA methylation

It has been shown that sequence-specific methylation is involved in the regulation of the LMP1 promoter [84]. Methylation of the EBV genome varies in different cell lines. The LMP1 promoter is nearly unmethylated in LCLs and partially methylated in Daudi cells, but fully methylated in latency I Rael cells [85, 86]. Treatment of BL type I cell line Rael with 5-azacytidine, a cytosine demethylation agent, can induce EBNA2-6 and LMP1 expression [87].

Regulation of LMP1 gene expression by PKA and PKC signal pathways

Previous studies have shown that cAMP increases LMP1 expression in the EBV-positive cell lines Cherry and B95-8 via protein kinase A (PKA) pathway [88]. Treatment of BL cell lines with anti-immunoglobulin, n-butyrate or with tetradecanoyl phorbol acetate (TPA) also induces the LMP1 expression in EBV-positive cell lines independent of EBNA2 via the protein kinase C (PKC) signal transduction pathway [89, 90]. These studies indicate that the expression of LMP1 gene can be activated by inducers other than EBNA2.

Regulation of LMP1 gene expression by other EBNAs

As previously mentioned, all the EBNAs are implicated in the regulation of LMP1 gene expression. EBNA1 can activate expression of the LMP1 gene over a distance of 10 kb through binding to *oriP* [27]. Moreover, EBNA2-mediated activation of the LMP1 promoter can be potentiated by EBNA5 [48, 49]. EBNA3, -4, -6 are suggested to repress gene expression of EBNA2-responsive promoters by preventing RBP-Jκ from binding to DNA [52, 91]. EBNA6, but not EBNA3 or -4, was also found to activate transcription from the LMP1 promoter in conjunction with EBNA2 through interacting with PU.1 transcription factor via PU-box element [54].

The LMP1 gene regulatory sequence (LRS)

Extensive studies on the LMP1 gene regulatory sequence (LRS) have been done to characterize the mechanisms involved in regulation of the LMP1 gene expression, and several regulatory elements have been identified [36, 77-79, 92] (Figure 4). A region between -54 to +40 relative to the ED-L1 promoter initiation site contains a constitutively active regulatory

element [77, 78]. Two other positive cis-activating regulatory components (-155 to -147 and -234 to -205) were also defined [79]. Subsequent studies have identified a cellular sequence-specific DNA binding protein RBP-Jκ binding to an RBP-Jκ site between LRS position -223 to -213 [36, 92, 93], and the Ets-related transcription factors PU.1 (Spi-1) and Spi-B binding to one PU box element [36, 92]. Johannsen et al have also identified several LMP1 binding factors (LBF1-7), but their exact nature and functions are still unknown [92]. Later studies have focused on the RBP-Jκ site and its binding factor RBP-Jκ/CBF1, because EBNA2 interacts with RBP-Jκ and stimulates transcription from promoters with this binding site [34-39]. RBP-Jκ binding sites are present in all EBNA2-regulated promoters discovered so far. Thus, this gives rise to an attractive hypothesis that RBP-Jκ may target EBNA2 to EBNA2-responsive elements in EBNA2-regulated promoters as an essential step in EBNA2-induced transactivation. However, this does not seem to be the case for the LMP1 gene, which retains EBNA2 responsiveness even when the RBP-Jκ sites are deleted [47, 77, 78, 92, 94]. This is also consistent with the observation that RBP-Jκ has a much lower affinity for its binding site in the LRS context than for the corresponding sites in the Cp or CD23 promoter [95]. All these results suggest that there must be other factors binding to downstream elements to confer EBNA2-responsiveness on the LMP1 promoter.

The results from our group have previously shown that the LMP1 promoter is controlled by a complex set of both positive and negative transcriptional *cis*-elements, and that EBNA2 can overcome the repression effects, whereas LRS region -214 to +40 is sufficient to mediate this EBNA2-induction [77, 78]. Another investigation from our group has identified the importance of an activating transcription factor/cyclic AMP response element (ATF/CRE) in the proximal part of LRS, and showed that the LMP1 promoter can be activated by cAMP through this ATF/CRE site in the absence of EBNA2 [88].

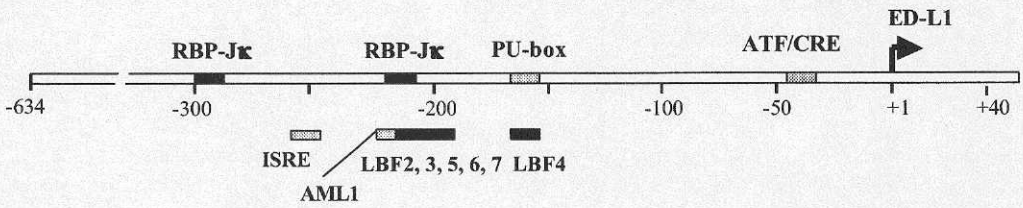

Figure 4. Schematic representation of the identified *cis*-elements in the LMP1 gene regulatory sequence (LRS) of B95-8 EBV DNA origin. The numbers indicate the nucleotide positions relative to the transcription initiation site (+1). A detailed description of the *cis*-elements is discussed in the text.

PRESENT INVESTIGATION

1. Aim of the present study

The expression of the Epstein-Barr virus latent membrane protein 1 (LMP1) is regulated by both EBV-encoded proteins and cellular specific factors. Previous investigations demonstrated that the LMP1 gene regulatory sequence (LRS) is composed of both positive and negative transcriptional *cis* elements, and that the gene is inactive in the absence of the inducers. The EBV nuclear antigen 2 (EBNA2) has been shown to transactivate a number of viral and cellular gene promoters including the LMP1 promoter. The overall aim of the present study is to increase our knowledge about the molecular mechanisms of EBNA2-induced activation of expression of the LMP1 gene in B cells.

2. Methodological Considerations

Reporter Plasmids

Reporter plasmids are commonly used as a tool to investigate the activity of promoters in gene expression. In these plasmids the promoter of interest or a fragment of the regulatory sequence is inserted in front of a gene encoding an enzyme or some other protein, the concentration of which is easily determined. Plasmids containing the chloramphenicol acetyl transferase (CAT) or the luciferase genes have been utilized in the present study of the LMP1 gene promoter. The plasmids are introduced into the eukaryotic cells by transfection. The promoter region will control the expression of the reporter gene and the activity of the promoter will be reflected by the level of reporter protein in the cells or secreted by the cells. However, it is important to keep in mind that the amount of gene product measured at a certain time point is determined by the difference between the rate of synthesis and the rate of degradation both at the mRNA and the protein level. Thus, the protein level is not a direct measure of the rate of transcription initiation, which has to be measured with other methods like nuclear run on assays. There are, however, several advantages with the reporter plasmid system. Since it is easy to create mutations and deletions in the promoter region of interest, reporter gene assays allow a detailed examination of transcription regulatory regions. In addition, there is usually no shortcomings caused by interference by the reporter gene product which might occur with the endogenous system. Furthermore, the assay provides a quantitative measurement of the reporter protein with high sensitivity, and is easy to perform. However, since the level of the reporter protein does not differentiate correct promoter initiation usage of the transcript and may not fully mirror the frequency of transcription initiation during promoter activation, the validity of the method should be verified at the RNA level with methods such as primer extension, S1 RNase mapping or RNase protection. Moreover, it is also important to consider that the protein usually is more stable than the mRNA. The CAT protein has a half-life of approximately 50 hours and will accumulate over time from transfection to harvest. In contrast, the luciferase reporter protein with a half-life of 3 hours will give information on promoter activity around the time of harvest. It is also important to perform the appropriate kinetic control experiments to ensure that the measurements of enzyme activity are done in the linear range of the concentration curve.

DNA-protein binding assay

Eukaryotic genes appear to be regulated primarily at the level of transcription, as mentioned above. Regulation of transcription as a response to environmental signals is a fundamental process in biology. The ability of RNA polymerase II to initiate transcription from a given

nucleotide position in the gene (promoter) and at a defined frequency is dependent upon interactions of sequence specific DNA binding proteins with the core promoter and upstream elements in the regulatory regions. There are several different ways to study the DNA-protein interaction, such as electrophoretic mobility shift assay (also known as gel shift assay), DNase I footprinting analysis and methylation interference assay. In this study, EMSA was chosen to investigate DNA-protein interaction because it is a simple and sensitive method for many applications. This method separates DNA-protein complexes from free DNA by non-denaturing polyacrylamide gel electrophoresis (PAGE), on the basis of differences in charge, conformation and size.

An EMSA experiment consists of several steps: preparation of the DNA probe; preparation of the protein extracts; preparation of the gel; gel electrophoresis and autoradiography. EMSAs are often used, as in the present study, to define the core DNA sequence in a regulatory element essential for binding a transcription factor, to identify the protein binding to a regulatory element by specific antibodies in a supershift assay and to study protein-protein interaction by the use of *in vitro* translated protein (or recombinant proteins or purified proteins) to the EMSA binding mixture.

RNase protection assay

To confirm that the observed transactivation of the reporter plamids was due to transcripts correctly initiated from the LMP1 promoter, the ribonuclease protection assay (RPA) was employed. RPA is a technique used for detection and quantification of specific RNAs, as well as for mapping of transcription initiation and termination sites and intron-exon boundaries. The basic approach of RPA is solution hybridization of an antisense-labeled RNA probe to cellular RNA, followed by treatment with ribonucleases. The enzymes specifically degrade single-stranded RNA while leaving the RNA-RNA hybrids intact. The size of the protected fragment is determined by polyacrylamide gel electrophoresis and visualized by autoradiography. Compared to Northern blot (the standard method to analyze RNA transcripts) and S1 mapping analysis, RPA is more sensitive because solution hybridization is more efficient than filter-based hybridization and can accommodate larger amounts of total or poly(A)-containing RNA samples. RPA is also more tolerant to partially degraded RNA and allows simultaneous detection of multiple mRNA targets. Cytoplasmic RNA was prepared and analyzed by the RNase protection assay (in Paper II) as described previously [96].

3. Results and Discussion

Paper I

PU box-binding transcription factors and a POU domain protein cooperate in the Epstein-Barr virus (EBV) nuclear antigen 2-induced transactivation of the EBV latent membrane protein 1 promoter.

In this report, we confirm and extend our previous observation that the LMP1 promoter can be activated by EBNA2 in the absence of RBP-Jκ-binding sites in the LMP1 gene regulatory sequence (LRS) [77-79]. It demonstrates by transient transfection assays into the EBV-negative B cell line DG75 that two distinct LRS regions, -106 to +40 and -176 to -136, contribute to the EBNA2-responsiveness. Site-directed mutation analysis of the distal EBNA2 responsive region revealed that two discrete *cis*-acting elements were required for full promoter function. These same elements analyzed by electrophoretic mobility shift assay

(EMSA) defined two binding sites recognized by nuclear factors in B cells. An octamer like sequence (-147/-139) contained partially overlapping binding sites for an unidentified transcriptional repressor on the one hand and a factor belonging to the POU domain family but distinct from Oct-1 and Oct-2 on the other. An adjacent purine tract (-170/-156) held a PU.1 binding site, which was also recognized by a related factor (Spi-B protein) [36]. Our further investigation of the probability of EBNA2 interacting with the transcription factors binding to these two sites, however, showed by EMSA that EBNA2 interacts with the POU domain protein. The results suggest that the POU domain protein can assist in the targeting of EBNA2 to the LMP1 promoter. The results of EMSA analysis indicated that the POU domain protein and either of the two PU box-binding proteins bind simultaneously to LRS, creating a ternary complex that might participate in the EBNA2 transactivation of the LMP1 promoter. There were no qualitative differences between EBV-negative and EBV-positive cells regarding transcription factor binding to the octamer motif or to the PU box binding site as revealed by EMSAs.

The sequence at position -147/-139 in LRS with a partial identity to the octamer motif suggested that a member of the Oct family of transcription factors might be responsible for the effect of this LRS element on LMP1 promoter activity. We have, however, not been able to demonstrated binding of Oct-1 and Oct-2 factors, which are expressed in B cells, to this site with specific antibodies. The Oct factors belong to a large family of transcription factors designated as POU domain proteins, characterized by a unique bipartite DNA-binding domain (the POU domain). The POU domain includes a variant homeodomain (POU-H) domain and a conserved POU-specific (POU-S) domain connected by a variable linker. Both subdomains contain helix-turn-helix motifs that directly associate with the two components of bipartite DNA-binding sites (ATGCAAAT) (review see [97]). Using an antibody against the POU-specific domain we demonstrate that both EBV-positive and EBV-negative B-cells contain a distinct factor that binds to the octamer motif in the context of surrounding LRS sequences. If LRS sequences upstream of position –146 in the binding site were deleted, the POU domain protein was replaced by an unidentified factor, suggesting that the octamer motif-carrying sequence contain two overlapping protein-binding sites. Interestingly, the binding of the unidentified factor correlated with a complete loss of EBNA2-responsiveness. Deletion of further 2 bp resulted in loss of binding of the unidentified factor, which correlated to the restitution of EBNA2-responsiveness to almost the same level as when the POU domain protein was bound. It is, however, not immediately obvious what role this putative silencer might play in the regulation of the LMP1 promoter activity, since the binding of the POU domain protein to an unmutated octamer motif always dominated over that of the unidentified repressor in both EBV-positive and EBV-negative cells. Repeated attempts to isolate the putative silencer of the LMP1 promoter by purification were unsuccessful.

What role do the octamer and PU box elements play in the induction of the LMP1 promoter by EBNA2? Evidence reported by several groups strongly suggests that the EBNA2 protein does not bind directly to a specific DNA motif but acts via protein-protein interaction with the regulatory region of affected promoters. There are several possible candidates for the targeting of EBNA2 to the LMP1 promoter, including RBP-Jκ (also called CBF-1) at the RBP-Jκ site [34-39], Ets-related PU.1 factor at the PU box [36, 92], the POU domain protein at the octamer motif (this investigation), as well as several other transcription factors binding to sites in the promoter-proximal region (Paper II and IV in this thesis). The PU-box and PU.1 could not convey EBNA2 responsiveness by themselves since a reporter plasmid containing only the PU.1-binding site but not the octamer motif (pLRS(-181/-145)TKCAT) was not induced by EBNA2. Furthermore, a series of reporter plasmids derived from pLRS(-106)(-

181/-145)CAT, which contains the PU box but not the octamer sequence, showed the same level of EBNA2 responsiveness irrespective of whether the PU box was mutated or not. On the other hand, the POU domain protein and its binding site seemed to be instrumental in mediating the EBNA2 effect since a -160/-136 LRS fragment conveyed responsiveness to the basal TK promoter. These results were in line with EMSA experiments with *in vitro* translated EBNA2, which suggested that EBNA2 interacts with the POU domain protein. The results are further strengthened by previous DNaseI footprinting experiments indicating that EBNA2 may modulate the binding of the POU domain protein to the octamer motif [94].

In conclusion, our findings are consistent with the notion that efficient EBNA2-mediated transactivation of the LMP1 promoter requires the cooperation of multiple factors binding to different DNA *cis* elements. We have identified a POU domain protein with the ability to target EBNA2 to the promoter. Functional cooperation between the POU domain protein and the PU.1 factor may contribute to the B cell-specific activation of the LMP1 promoter.

Paper II

An ATF/CRE element mediates both EBNA2-dependent and EBNA2-independent activation of the Epstein-Barr virus LMP1 promoter

Our studies reported in Paper I reinforced the notion that elements in the promoter proximal −106/+40 part of LRS play an essential role in the EBNA2 induced activation of the LMP1 promoter. Furthermore, a previous investigation from our group suggested that an activating transcription factor/cyclic AMP response element (ATF/CRE) in this region of LRS is essential for the regulation of the LMP1 promoter and mediates activation of the LMP1 promoter by cAMP in the absence of EBNA2 [88]. Thus, the objective of our studies reported in Paper II was to further define the regulatory elements and transcription factors in the proximal part of LRS that contribute to the LMP1 promoter activity in general and EBNA2-induced activation in particular. In Paper II, we established the importance of the ATF/CRE element and an adjacent Sp site by site-directed mutational analysis. We showed that both EBNA2-dependent and -independent activation of the promoter occur through the ATF/CRE site but are mediated by separate sets of transcription factors. Electrophoretic mobility shift assays using specific antibodies revealed that the ATF-1, CREB-1, ATF-2 and c-Jun factors bind to the ATF/CRE site as ATF-1/CREB-1 and ATF-2/c-Jun heterodimers whereas the Sp1 and Sp3 factors bind to the Sp site. To determine the ability of these transcription factors with regard to transactivation of the LMP1 promoter, transient transfection experiments were performed. Expression plasmids for the different factors were transfected into EBV-negative DG75 lymphoid cells together with the pgLRS(-106)CAT reporter plasmid and an EBNA2 expression vector or control plasmid. Mutation of the Sp site largely abolished promoter activity both in the presence and absence of EBNA2. The Sp1 factor activated the unmutated reporter plasmid in the absence of EBNA2 but did not increase the activity level induced by EBNA2. Overexpression of ATF-1 and CREB-1 in the cells demonstrated that the homodimeric as well as the heterodimeric forms of the factors transactivate the LMP1 promoter in an EBNA2-independent manner and the level of activation was just as high as that obtained by EBNA2. The effect was ATF/CRE site dependent. The ATF-2 and c-Jun factors, on the other hand, did not activate the LMP1 promoter in the absence of EBNA2, neither alone nor together. Coexpression of either ATF-2 or c-Jun with EBNA2 produced only a slight increase of promoter activity. However, coexpression of ATF-2 and c-Jun with EBNA2 resulted in a strong promoter activation that depended on an intact ATF/CRE site. These results were in line with our observation that addition of *in vitro* translated EBNA2 to a

mixture containing *in vitro* translated ATF-2 and c-Jun abrogated the binding of the heterodimeric factor complex to the ATF/CRE site-containing DNA probe in EMSA experiments. Notably, EBNA2 did not affect the binding of the respective homodimeric complexes of ATF-2 or c-Jun, suggesting that EBNA2 interacted only with the heterodimeric factor complex. This notion gained further support by the results obtained by immunoprecipitation of complexes in extracts of DG75 cells, which had been co-transfected with expression plasmids for ATF-2, c-Jun and EBNA2. Both anti-c-Jun and anti-ATF-2 antibodies pulled down complexes that contained EBNA2 and the two transcription factors. Taken together, these results suggest that the interaction between the c-Jun/ATF-2 complex and EBNA2 is an inherent step in the transactivation of the LMP1 promoter.

Our studies demonstrate that an Sp element at position -33 in LRS is required for efficient EBNA2-dependent and EBNA2-independent activation of the LMP1 promoter and that the Sp1 and Sp3 transcription factors bind to this site. Sp1 is a well-known transcriptional activator of different sets of genes of both viral and cellular origin. The limited effect of overexpression of the Sp1 protein on the activity of the promoter in the absence of EBNA2 in our transfection experiments may well be explained by high level of Sp1 in DG75 cells that diminishes the relative contribution of the exogenously added protein. Overexpression of Sp1 into the Sp1-negative Schneider cells did not induce additional LRS-derived activity (data not shown). The Sp3 transcription factor has been shown to function as a repressor of Sp1-mediated transcriptional activation [98]. Multiple Sp3-containing complexes similar to those observed in our EMSA analyses have previously been found in another system [99]. We suggest that the stimulatory effect of Sp1 on the LMP1 promoter is independent of EBNA2 but is a prerequisite for EBNA2-induced activation of LMP1 promoter. The interaction of Sp1 and Sp3 factors with their binding site in LRS might constitute an EBNA2-independent regulatory system in which the balance between the positively acting Sp1 and the negatively acting Sp3 factors is one of the factors that determines the final level of activity of the LMP1 promoter.

In this paper, we also establish the importance of the ATF/CRE element by mutational analysis and show that both EBNA2-dependent and -independent activation of the promoter occur via this site but are mediated by separate sets of factors. EMSA with specific antibodies showed that the ATF-1, CREB-1, ATF-2 and c-Jun factors bind to the site as ATF-1/CREB-1 and ATF-2/c-Jun, since the homomeric forms of the factors were not detected. The presence of two ATF-1/CREB-1 complexes with different mobilities in the electrophoreto-grams might be explained by the previous observation that phosphorylation drastically changes the conformation of ATF-1 and, as a consequence, the electrophoretic mobility of the corresponding EMSA complex [100]. It should be noted, however, that overexpression of the factors in the cells by transfection with expression vectors under conditions that favored the formation of the homodimeric forms showed that these were as efficient in inducing promoter activity as was the heterodimeric form. Previous studies have shown that cAMP via protein kinase A pathway increases LMP1 expression in the EBV-positive cell lines Cherry and B95-8 [88], and that treatment of BL cell lines with anti-immunoglobulin or tetradecanoyl phorbol acetate (TPA) induces the LMP1 expression independently of EBNA2 via the protein kinase C (PKC) signal transduction pathway [90]. It has been shown that the transactivating function of CREB-1 and ATF-1 is stimulated by phosphorylation of the factors via the PKA and PKC pathways [101, 102]. Thus, it is conceivable that EBNA2-independent promoter activation through the PKC pathway and the PKA pathway is mediated by the binding of a heterodimeric complex between the ATF-1 and CREB-1 factors to the ATF/CRE site.

The results of the present study lend strong support to the notion that EBNA2 can activate the LMP1 promoter via a mechanism that is different from the ATF-1/CREB-1 pathway and involves the binding of the ATF-2 and c-Jun factors as a heterodimer to the ATF/CRE site. EBNA2 is required for the activation and, judging from the EMSA and coimmunoprecipitation experiments, seems to make a direct contact with the c-Jun/ATF-2 dimer complex. Thus, the question arises of how this interaction may lead to promoter interaction. Does EBNA2 induce a modification of the ATF-2/c-Jun complex and/or its binding site or change the concentration of the factors in the cell nucleus in a way that favors promoter activation through the activating domains of ATF-2 and c-Jun? Or is EBNA2 recruited to the LMP1 promoter through protein-protein interactions with the ATF-2/c-Jun dimer to bring the EBNA2 transactivating domain in the correct position for a productive contact with one or several factors belonging to the basal transcription machinery? The possibility also exists that the interaction between EBNA2 and the c-Jun/ATF-2 dimer decreases the affinity of this complex for the ATF/CRE site, leading to an increased binding of the ATF-1 and CREB-1 factors and activation of the LMP1 promoter through this pathway. However, the fact that overexpression of ATF-2 and c-Jun in the presence of EBNA2 has a pronounced activating effect argues against such a hypothesis. With regard to the first alternative, we have not detected any change in the phosphorylation status or the levels of ATF-2 and c-Jun in parallel with the EBNA2-induced activation of the LMP1 promoter. Furthermore, it has been demonstrated in several studies that the C-terminal acidic domain of EBNA2 is required for transcriptional transactivation by EBNA2 [94, 103, 104]. It has also been shown that the activating domain of EBNA2 makes physical contact with several general transcription factors, including TFIIB, TFIIE, TFIIH, RPA70, and TAF40 [40-42]. Thus, it seems possible that EBNA2, at least in the context of the -107/+40 part of LRS, functions in a manner analogous to the transcriptional coactivators CBP (CREB-binding protein) and the adenovirus E1A-associated cellular protein p300 with regard to ATF/CRE site. Neither CBP nor p300 by itself binds to DNA, but they can be recruited to promoter elements by interaction with a multitude of sequence-specific activators. CBP can activate transcription through the C-terminal part of the protein, and the activation domain has been shown to interact with components of the basal transcription machinery [105]. Thus, CBP and p300 are transcriptional coactivators that provide a crucial link between transcriptional activators stimulated by signaling cascades and initiation of transcription. EBNA2 seems to function through a similar mechanism.

Paper III

Silencing of the Epstein-Barr virus (EBV) Latent Membrane Protein 1 gene by the Max/Mad1/mSin3A modulator of chromatin structure

Paper I and other previous investigations have presented evidence demonstrating that the promoter proximal part of LRS contains a negative transcriptional *cis* element with a major role in EBNA2-mediated regulation of LMP1 gene expression in B cells [77-79, 106]. In the present study, we have focused on a sequence immediately upstream of the ATF/CRE element that contains a potential E-box site involved in silencing of the LMP1 gene. E-box sites bind proteins that belong to the basic helix-loop-helix (bHLH) family of transcription factors, which regulate the expression of different cellular functions in various differentiated cell types. The specific objective of the study was to define the role of the E-box site in the EBNA2 responsiveness of the LMP1 promoter and to determine its relation to the previously identified negative element in the promoter-proximal LRS region. To analyze the role of the E-box containing region in the regulation of LMP1 promoter activity, we generated a series of

LRS-CAT reporter plasmids containing 5' deletion mutations of the LRS from position -107 to -40 and introduced them into the EBV-negative DG75 B-cell line together with an EBNA2 expression plasmid or an empty control plasmid. The results indicated that the reporter plasmids could operationally be divided into three categories according to the pattern of CAT expression and the length of the LRS insert. We interpreted the results as follows. The properties of the reporter plasmids in category 1 with the shortest LRS fragments are due to the effect of the stepwise inclusion of an EBNA2-dependent positive regulatory element. As demonstrated in Paper II this element is an ATF/CRE site, and the activating effect is mediated by an ATF-2/c-Jun heterodimer. In plasmids belonging to category 2, an EBNA2-independent positive element and an overlapping negative element have been included in the reporter plasmids, with the positive effect dominating in those that contain the shorter LRS inserts and the negative effect dominating in the longer LRS inserts. In the third category in which the region between -67 and -107 has been added, EBNA2 responsiveness of the reporter plasmid was reconstituted to the same level of activation as that obtained with pgLRS(-50)CAT. This suggests that the -106/-67 LRS region contains elements that participate in the EBNA2-induced alleviation of the repressor effect on the LMP1 promoter. Taken together, the results suggest that a repressor element and an EBNA2-independent enhancer element overlap with an E-box-homologous motif at position -56 to -51 in the LRS. To characterize the pattern of transcription factor binding to the E-box-containing region, we performed EMSAs with DG75 nuclear extracts and the relevant oligonucleotide probes. The factors binding to the E-box region were identified by antibody supershift analysis using a panel of commercially available antibodies against transcription factors. In summary, the results demonstrated that the USF, Max, Mad1, mSin3A, E12 and E47 transcription factors are present in DG75 cells and interact with the E-box motif-containing sequence in the promoter proximal part of the LRS. To assess whether USF transcription factors can activate the LMP1 promoter in an EBNA2-independent manner, reporter plasmids containing the −106/+40 LRS region were cotransfected with expression vectors for human USF1 and/or mouse USF2a into DG75 cells. Transfection of either USF1 or USF2a, resulting in the dominant generation of homodimeric forms, transactivated LMP1 promoter-containing reporter plasmids in an E-box-dependent manner. Cotransfection of the pgLRS(-106)CAT reporter plasmid with expression vectors for USF2a, Max and Mad1 showed that Max-Mad1 repressed the activity of the LMP1 promoter in an E-box-dependent manner. Cotransfection with the mSin3A vector was not necessary because of the abundance of this protein in the cells. Thus, the results were compatible with the notion that USF factors activate the LMP1 promoter in an EBNA2-independent manner via the E-box region and that this activation can be downregulated by the Max-Mad1-Sin3A factors.

It is now well established that Max-Mad1-mSin3A functions as a repressor by recruiting deacetylases to the promoter, thereby leading to chromatin remodeling and loss of transcription factors access to the nucleosome-associated promoter sequences [107]. To analyze whether the expression of LMP1 from the endogenous EBV genomes was affected by an increase of the level of histone acetylation, we performed TSA (trichostatin A, the deacetylase inhibitor) induction experiments in three EBV-positive cell lines, Rael, P3HR-1, and Daudi. Interestingly, inhibition of deacetylation activity with TSA induced expression of the endogenous LMP1 gene in the EBV-transformed cell lines P3HR-1 and Daudi, suggesting that the LMP1 promoter can be regulated via Max-Mad1-mSin3A recruitment of deacetylase to the promoter, leading to core histone deacetylation and modulation of chromatin structure. However, TSA cannot induce LMP1 expression in highly methylated Rael cells, indicating that other regulatory mechanisms also exist to override the TSA effects. The results also

revealed the appearance of significant levels of BZLF1 in P3HR-1 and Daudi cells, indicating that the lytic cycle was induced in these cells but not in Rael cells.

In the present study, we show that a silencing element overlaps with a transcriptional enhancer element in an LRS sequence that contains an E-box-homologous motif. Mutation of the putative repressor-binding site relieved the repression both in a promoter-proximal and a complete LRS context, indicating a functional role of the repressor in LMP1 gene regulation. This confirms our previous reports that the proximal region of the LMP1 promoter contains a negative *cis* element with a major role in EBNA2-mediated regulation of LMP1 gene expression in B-lymphoid cells (Paper I).

A number of proteins belonging to the bHLH family of transcription factors, including transcriptional repressor complex Max-Mad1-mSin3A and transcriptional activator USF, as well as E12 and E47, bound in a sequence-specific manner to the E-box-containing sequence. The Max protein is thought to play an essential role in the function of this biologically important group of transcription factors by being a partner in complex formation with Myc or Mad1 to Mad4 or with itself (review in [108]). Myc-Max heterodimers, regarded as the biologically active form of Myc, transactivate genes involved in cell proliferation and apoptosis which contain the specific E-box sequence. Max itself is thought to be transcriptionally inert. Myc-Max heterodimers are favored over homodimers when the two proteins are at equilibrium, since both the Myc and Max proteins preferentially heterodimerize. The Max-Mad dimeric molecules are repressors of Myc-Max-mediated transcriptional activation through competition for the same E-box site (review in [108]). However, we were unable to demonstrate binding of Myc to the LRS E-box site by supershift experiments. Instead, our results demonstrated that USF proteins confer EBNA2-independent activity to the LMP1 promoter via the E-box. The USF proteins represent the larger part of the LRS E-box binding activity in the B cells investigated in our study. Interestingly, this group of transcription factors, while being ubiquitously expressed, is involved in the expression of several tissue-specific or developmentally regulated genes [108]. The factors are encoded by two distinct genes (the USF1 and USF2 genes) and exist in the form of homomeric and heteromeric dimers able to bind to specific E-box sites. *In vivo*, four combinations of the different USF proteins are prevalent, with the most common species being heterodimers between USF1 and USF2a isoforms [109]. Here we show that the E-box is a transcriptional enhancer of the LMP1 promoter, which is activated by the USF proteins in an EBNA2-independent way. We have so far not identified the specific members of the USF factor family that interact with the E-box site in LRS. However, the quantitative dominance of the most slow-moving USF complex in the EMSA suggests that it corresponds to the USF1-USF2a heterodimer. Transfections under conditions that favor the formation of either the homomeric or the heteromeric form of USF1 and USF2a suggested that all dimer combinations were equally effective in the transactivation of the LMP1 promoter.

The silencing effect of the E-box motif involved not only competition in binding to the E-box motif between Max-Mad1-mSin3A and USF factors, but also the modulation of chromatin structure by recruitment of deacetylases to the promoter. The repressor complex Max-Mad1-mSin3A has been shown to function as a repressor by recruiting histone deacetylases (HDACs) to the promoter, thereby lowering the level of acetylated histones in the surrounding chromatin structure [107]. Acetylation of histone is thought to destabilize nucleosomes and facilitate access of regulatory factors to DNA [110]. Inhibition of deacetylation activity by trichostatin A induced expression of the endogenous LMP1 gene in EBV-transformed cells (P3HR-1 and Daudi), suggesting that the LMP1 promoter can be regulated via Max-Mad1-

mSin3A recruitment of deacetylase to the promoter. This TSA-induced expression of LMP1 seems to be a direct effect on core histones in the LMP1 promoter region, because of the observation that treatment with TSA activated the LMP1 promoter in reporter plasmids transfected into DG75 cells (Paper IV). The difference between Rael and the other cell lines regarding the sensitivity to TSA might be due to differences in the methylation pattern of the LMP1 promoter region, as the LMP1 promoter is only partially methylated in Daudi cells but is fully methylated in Rael cells [85, 86]. It has also been shown that sequence specific methylation is involved in the regulation of the LMP1 promoter [84].

Our initial observation of EBNA2-induced transactivation of the LMP1 promoter suggested that one important element of the process was the overriding of the effect of a negative element in the proximal part of the promoter regulatory sequence. The mechanism for this action was, however, not clarified [77, 106]. The identification of the Max-Mad1-mSin3A complex as the likely mediator of the repressor effect and the assumption that repression occurs through the recruitment of deacetylases open up a number of possible options for EBNA2-induced reversal of repression. In one model, the balance between the binding of the Max-Mad1-mSin3A complex and USF is influenced by EBNA2 in favor of USF. This could be achieved via several conceivable mechanisms. In this way the recruitment of deacetylases to the promoter would be impeded. However, comparative DNA binding studies of proteins in EBV-negative and EBV-positive cells reveal that factor binding patterns in the E-box region are indistinguishable from each other, which would be an argument against this hypothesis. In a second model, EBNA2 abolishes the repressive effect of Max-Mad1-mSin3A by affecting histone acetylation in a more direct manner. Several transcription factors, including Gcn5, CBP, p300 and TAFII250, have been shown to possess histone acetyltransferase activity [111]. EBNA2 might have a similar catalytic activity or in some indirect way be able to recruit histone acetyltransferase activity to the LMP1 promoter. Under the assumption that EBNA2 confers acetyltransferase activity, one might also speculate that acetylation of nonhistone proteins, such as high-mobility-group proteins or transcription factors, is important for transcriptional regulation of the LMP1 promoter and contributes to the transactivating function of EBNA2. Another conceivable way for EBNA2 to counteract deacetylation and overcome Max-Mad repression would be to recruit the SWI-SNF complex to the promoter. The complex remodels chromatin by an ATP-dependent mechanism, creating a chromatin structure that is more accessible for protein interactions and thereby for induction of transcription. It has, in fact, been shown that EBNA2 can interact with the SWI-SNF complex [43].

Paper IV

Cooperation between Epstein-Barr virus Nuclear Antigen 2 (EBNA2) and AP-2 factors relieves Max-Mad1 and Ikaros factor-mediated repression of the LMP1 promoter in B cells

Our previous work showed that repression of the LMP1 promoter is partially mediated by a Max-Mad1-mSin3A complex bound to an E-box element at position -56 to -51 in LRS (Paper III). The data also suggested that a second negative regulatory element was present in the adjacent, -67/-59, part of LRS. The repression exerted by the two elements was relieved by EBNA2 when elements in the LRS region between -106 and -67 were included. The aim of the present study was to investigate the functional significance of the putative transcription factor binding sites in the -107 to -52 region of LRS, which in addition to E-box element encompasses an Ikaros site at position -67/-59, an NF-κB site at -89/-79, and two AP-2 sites

at -80/-72 and -103/-95, respectively. The role of these sites in EBNA2-induced relief of LMP1 repression was investigated using a series of luciferase reporter plasmids carrying LRS fragments with 5'-end deletions. The plasmids were transfected into the EBV-negative B-cell line DG75 together with the EBNA2 expression plasmid or the empty control vector. Since the repression of LMP1 promoter seemed to be due to the recruitment of histone deacetylases (HDACs) by the repressive elements, the ability of Trichostatin A (TSA), an inhibitor of histone deacetylation, to activate reporter plasmids was also investigated and compared with that of EBNA2. The results showed that the Ikaros site added substantially to the inhibition exerted by the E-box element on promoter activity. Inclusion of the putative AP-2 and NF-κB elements in the LRS plasmid did not increase EBNA2 inducibility (pLRS(-95)LUC). In contrast, TSA induced approximately the same level of activity in all of the reporter plasmids, showing that TSA unlike EBNA2 could overcome the repression caused by the inclusion of the E-box and Ikaros elements in the plasmids lacking the AP-2 element.

EMSA revealed that Ikaros transcription factors bound to an Ikaros consensus sequence adjacent to the previously defined E-box element. The subtype of the Ikaros factors could not be defined due to lack of specificity of the antibody that recognized the common C-terminal domain present in all Ikaros isoforms. The apparent involvement of the E-box and Ikaros elements in LMP1 promoter repression was assessed by mutation analysis. The results revealed that the E-box and Ikaros sites both function as negative *cis* elements and act in concert to repress LMP1 promoter activity. The Ikaros gene family encodes zinc finger DNA-binding proteins that, based on genetic disruptions in mice, are essential for lineage determination and control of proliferation in the lymphoid system (reviewed in [112]). Ikaros has many isoforms derived from an alternatively spliced pre-mRNA, and those isoforms work in concert with related proteins called Ailos and Helios. They have been reported to function both as repressors and activators of transcription depending on the cellular context. In lymphocytes, most Ikaros proteins are present in a 2-MDa nucleosome remodeling and deacetylation (NuRD) complex that contains 10-12 Ikaros molecules and several other proteins including Mi-2β, an ATP-dependent chromatin remodeler, and HDACs [113]. The Ikaros–NuRD complex has potent chromatin remodeling activity *in vitro* and can deacetylate histones. Smaller amounts of Ikaros are found associated with the SWI-SNF remodeling complex and with the co-repressors mSin3 and the C-terminal binding protein (CtBP), further linking Ikaros function to chromatin remodeling and deacetylation [114, 115]. The results of the TSA induction experiments reported in Paper IV and of unpublished chromatin immunoprecipitation assays (ChIP) (Palmqvist et al., in manuscript) supported the notion that the predominant causative factor in the repression of the LMP1 promoter is hypoacetylation of lysine residues in the N-terminal tails of nucleosomal histones. Thus, in the simplest model for the formation of the repressed chromatin structure of the LMP1 gene, HDACs are targeted to the core promoter via interaction with Max-Mad1-mSin3A bound to the E-box and with Ikaros-NuRD bound to the Ikaros site. The recruited complexes then promote deacetylation of nucleosomal histones and the formation of a more condensed chromatin configuration that does not permit transcription. However, other mechanisms like DNA methylation are also involved in the repression, as inhibition of deacetylation activity by TSA is not sufficient to activate LMP1 expression in BL cell line Rael (Paper III) and resting B cells (Palmqvist et al., in manuscript).

The pLRS(-107)Luc plasmid, that contains an AP-2 element at LRS position -103/-95, was activated by EBNA2, suggesting that EBNA2-mediated relief of repression depended on the presence of an AP-2 site. The AP-2 element was shown to bind the AP-2α, AP-2β and AP-2γ factors in EMSA analyses. Mutations that disrupted AP-2 binding to this site abolished

EBNA2-induced activation of the promoter in reporter plasmids, supporting the notion that the AP-2 site is important in EBNA2-mediated transactivation of the LMP1 promoter. Inhibition of HDACs by TSA induced activation of the mutated plasmids. Overexpression of AP-2 activated the LMP1 promoter in reporter plasmids in the absence of EBNA2 expression. However, a much higher activity was observed in the presence of high levels of both EBNA2 and AP-2 in the context of the pgLRS(-107)CAT reporter plasmid. Taken together our results support the notion that cooperation between EBNA2 and AP-2 might play an important role in the activation of LRS. The EBNA2 and the AP-2 factors might act in concert to recruit HATs to the promoter and induce acetylation of specific histones, leading to release of repression and activation of the LMP1 promoter. It should be noted, however, that the level of AP-2α, AP-2β and AP-2γ in the EBV-negative DG75 B cell line is very low as judged from western immunoblotting experiments using commercial antibodies against these factors. We cannot exclude that some other member of the AP-2 transcription factor family acting via the AP-2 element is responsible for the basal cooperative activity with EBNA2 in LMP1 promoter transactivation. On the other hand it is quite obvious from our overexpression experiments that increasing concentrations of AP-2 increases the activity of the LMP1 promoter at a constant EBNA2 level in a cooperative manner.

The AP-2 proteins are encoded by a family of related genes, AP-2α [116], AP-2β [117, 118], and AP-2γ [118]. The three AP-2 proteins differ in their N-terminal transcription activation domains, but show a high degree of conservation (75-85%) within their DNA binding and dimerisation domains and bind as homo- or heterodimers to a GC-rich element with the consensus sequence GCCNNNGGC [119, 120]. Generally, the transactivating properties of the AP-2 proteins are regulated by phosphorylation through the PKA and PKC cellular signaling pathways [121, 122]. AP-2 was first characterized as a transactivating protein in HeLa cell nuclear extracts [116]. The AP-2 family has since been shown to be involved in several other vital cellular functions including cell proliferation, cell death, and programmed gene expression both during embryonic morphogenesis and adult cell differentiation (reviewed in [123]). The involvement of AP-2 in different cellular functions is attributed to its ability to interact with many different cellular and viral proteins, including the retinoblastoma tumor suppressor protein, the oncoprotein c-Myc, the transcription factor YY1, the simian virus 40 large T antigen, the adenovirus E1A protein, and the human T-cell leukemia virus type I tax protein, Moreover, AP-2 transcription factors interact with members of the CBP/p300-interacting transactivator with ED-rich tail (CITED) family of proteins. CITED2 and CITED4 bind CBP and p300 with high affinity and function as coactivators for all isoforms of AP-2 [124, 125]. It is well established that CBP/p300 possess intrinsic HAT activity [126] and are recruited to gene promoters by diverse DNA-bound transcription factors including AP-2. Targeted nucleosomal histone acetylation by such a protein complex modifies the chromatin structure rendering promoters more accessible to transcription factors. Thus, AP-2 as a DNA-binding factor would have the capacity to recruit HATs to the LMP1 promoter via CITED and overcome HDAC mediated repression. Different models can be proposed for the molecular mechanism underlying the cooperation between EBNA2 and AP-2 in the transactivation of the LMP1 core promoter. Like AP-2, EBNA2 has been shown to interact, directly or indirectly, with CBP/p300 [44]. This, coupled to our observation that the transactivating effect of EBNA2 on the LMP1 core promoter is totally dependent of the presence of AP-2, suggests that EBNA2 functions as a coactivator of AP-2 in a way analogous to that of the CITED factors. Hypothetically, EBNA2 on the one hand interacts with AP-2 targeted to the core promoter via the AP-2 binding site and on the other with CBP/p300. In this way HAT activity is linked to the core promoter region, locally favoring acetylation of nucleosomal histones and chromatin remodeling. A different mechanism for the

EBNA2-induced activation of the LMP1 promoter could also be conceived exploiting the known ability of EBNA2 to inhibit protein phosphatase 1-like activity [88], thereby increasing the level of phosphorylated AP-2 at the promoter. The phosphorylated AP-2 bound to the AP-2 site might then interact with the CITED4 coactivator and recruit CBP/p300 to the promoter. Notably, the two mechanisms would not have to be mutually exclusive. Furthermore, this line of argument could also be applied to the reported, EBNA2-independent, activation of the LMP1 promoter by TPA and cAMP [88, 89]. The transactivating properties of the AP-2 proteins are known to be regulated by phosphorylation through the PKA and PKC cellular signaling pathways [121, 122] which are activated by TPA and cAMP. Consequently, a cellular signal that leads to increased phosphorylation of AP-2 may under certain conditions also lead to increased expression of LMP1 through the AP-2–CITED4–CBP/p300 pathway.

4. Conclusion

In conclusion, we propose a model for transcriptional regulation of the LMP1 gene in B cells (Fig. 5). The LMP1 gene is inactive in the absence of the inducers. The repression mechanisms of the LMP1 promoter include histone deacetylation and DNA methylation. Our results indicate that HDACs are targeted to the LMP1 promoter via interaction with Max-Mad1-mSin3A bound to the E-box and with Ikaros-NuRD bound to the Ikaros site. The RBP-Jκ sites in the distal region of LRS may also be involved in silencing of the LMP1 promoter through recruitment of HDACs.

The expression of LMP1 can be induced by both EBNA2-dependent and -independent mechanisms. Our investigations indicate that Sp1, ATF-1/CREB-1, USF and AP-2 transcription factors can transactivate the LMP1 promoter in the absence of EBNA2, through Sp, ATF/CRE, E-box and AP-2 elements at promoter proximal region, respectively. Three LRS regions are required for EBNA2-dependent transactivation of the LMP1 promoter, including the ATF/CRE element, the octamer motif and PU-box site, and the RBP-Jκ sites. An AP-2 element in the -103/-95 LRS region also participates in the EBNA2-induced relief of the repression of the LMP1 promoter. EBNA2 is able to interact with ATF2/c-Jun heterodimer, the POU domain protein, PU.1 and RBP-Jκ, to transactivate the LMP1 promoter. Several EBNA2 molecules appear to be required in close proximity to enable interactions among some or all of the transcription factors that are necessary for appropriate promoter regulation. The EBNA2 multimer formed via self-association could enable coordinated assembly of multiple factors, including transcription factors, coactivators, histone acetyltransferases, and the basal transcriptional machinery, at the LMP1 promoter. By bridging among multiple transcriptional regulators, EBNA2 may play a central role in regulation of the LMP1 gene (Fig. 5).

While the results of this study allow a better understanding of the regulation of the LMP1 promoter activity, their relevance to promoter activation in the *in vivo* situation, i.e. in the context of the physiological promoter, the viral episome and EBV transformation of B cells, is not clear. Still, several lines of evidence from this study support a similar scheme for the endogenous relief of repression and activation of the LMP1 promoter. The future challenge is to study chromatin remodeling and the possible function of EBNA2 as a modulator of the nucleosome position at the LMP1 promoter.

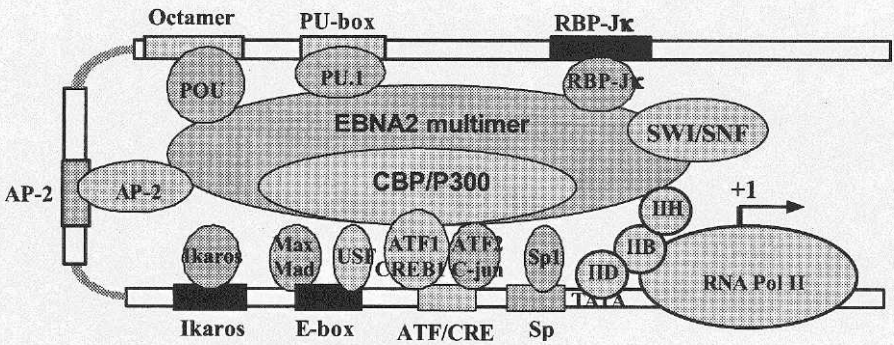

Figure 5. Hypothetical model for the transcriptional regulation of the LMP1 gene. The EBNA2 is recruited to the LMP1 promoter via interacting with the ATF2/c-Jun heterodimer, the POU domain protein, PU.1 and RBP-Jκ. EBNA2 then recruits multiple complexes (including CBP/P300, SWI/SNF, and the basal transcriptional machinery) to remodel the chromatin structure and to activate the LMP1 gene transcription.

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