TRANSCRIPTIONAL REGULATION OF THE LATENT MEMBRANE PROTEIN 1 GENE BY EPSTEIN-BARR VIRUS NUCLEAR ANTIGEN 2

Lars Palmqvist

Göteborg 2001

Institute of Laboratory Medicine
Department of Clinical Chemistry and Transfusion Medicine
Göteborg University

TRANSCRIPTIONAL REGULATION OF THE LATENT MEMBRANE PROTEIN 1 GENE BY EPSTEIN-BARR VIRUS NUCLEAR ANTIGEN 2

AKADEMISK AVHANDLING

som för avläggande av medicine doktorsexamen vid Göteborgs universitet kommer att offentligt försvaras i föreläsningssal F3, Sahlgrenska universitetssjukhuset, fredag den 7 September 2001, kl. 13.00

av

Lars Palmqvist

Fakultetsopponent är professor Ingemar Ernberg, Mikrobiologiskt och tumörbiologiskt centrum (MTC), Karolinska institutet, Stockolm.

Avhandlingen baseras på följande arbeten:

I. Fåhraeus, R., Palmqvist, L., Nerstedt, A., Farzad, S., Rymo, L. and S. Lain. (1994) Response to cAMP levels of Epstein-Barr virus EBNA2 inducible LMP1 oncogene and EBNA2 inhibition of a PP1-like activity. *EMBO Journal 13: 6041-6051.*

II. Sjöblom, A., Yang, W., Palmqvist, L., Jansson, A. and L. Rymo. (1998) An ATF/CRE element mediates both EBNA2 dependent and EBNA2 independent activation of the Epstein-Barr virus LMP1 gene promoter. *Journal of Virology 72:1365-1376.*

III. Palmqvist, L., Jansson, A. and L. Rymo. Increased histone acetylation and phosphorylation of ATF2 are required for transcriptional activation of the Epstein-Barr virus LMP1 promoter. *Manuscript.*

Transcriptional regulation of the latent membrane protein 1 gene by Epstein-Barr virus nuclear antigen 2.

Lars Palmqvist, Institute of Laboratory Medicine, Department of Clinical Chemistry and Transfusion Medicine, Göteborg University.

The Epstein-Barr virus (EBV) is a ubiquitous human herpes virus, which infects B-cells. This leads to a life-long latent infection, which in most cases is non-pathogenic. However, EBV is etiologically associated with several human malignancies, including endemic Burkitt's lymphoma, nasopharyngeal carcinoma and post-transplant lymphoma. This association is probably linked to the ability of the virus to immortalise B-cells *in vitro* and to induce B-cell proliferation. Mutagenesis of the viral genome has defined a subset of four nuclear proteins (EBNA1, 2, 3, and 6) and one membrane protein (LMP1) required for the immortalisation process. The aim of the present study was to increase our knowledge about the different mechanisms by which the expression of the LMP1 gene is regulated in B-cells focussing on the role played by the virally encoded transactivating factor EBNA2.

The importance of an ATF/CRE site and an Sp site in the proximal part of the LMP1 regulatory sequence (LRS) was established. Mutations of the ATF/CRE and Sp sites decreased both EBNA2-dependent and -independent LMP1 promoter activity in transient transfection experiments. Both cAMP and okadaic acid, an inhibitor of protein phosphatase 1 (PP1) and 2A (PP2A), activated the promoter in an ATF/CRE-dependent fashion. Immunoaffinity experiments showed that EBNA2 co-purified with a PP1-like protein from an EBV-immortalised B-cell line and that a recombinant EBNA2 fusion protein specifically bound and inhibited a PP1-like activity in B-cell extracts. Thus, we conclude that the ATF/CRE and Sp site is important in transactivation of the LMP1 promoter and that the ability of EBNA2 to block the effect exerted by protein phosphatases on a certain transcription factor can be part of the mechanism by which the LMP1 promoter is activated.

Electrophoretic mobility shift assays showed that both the positive Sp1 and the negative Sp3 transcription factor bound to the Sp site. However, overexpression of the Sp1 transcription factor did not add to the activity induced by EBNA2. The heterodimeric transcription factor complexes ATF1/CREB1 and c-Jun/ATF2 bound to the ATF1/CRE site in LRS. The binding of ATF1/CREB1 activated the LMP1 promoter in the absence of EBNA2. The ATF2 and c-Jun transcription factors, on the other hand, did not activate the LMP1 promoter in the absence of EBNA2 but did so in the presence of EBNA2. The activating effect was abolished if the phosphate-accepting amino acid residues Thr69 and Thr71 in ATF2 were mutated. Furthermore, EBNA2 could interact with the c-Jun/ATF2 heterodimer. We conclude that EBNA2 is targeted to the promoter by these factors to transactivate the LMP1 promoter.

Histone acetylation at the LMP1 promoter was studied in two different B-cell lines. Both EBNA2 and the histone deacetylase inhibitor Trichostatin A (TSA) rapidly increased the level of histone acetylation at the LMP1 promoter in resting ER/EB2-5 B-cells. However, only EBNA2 induced LMP1 transcription. This was in contrast to the situation in proliferating P3HR1 B-cells, in which TSA both increased histone acetylation at and activated transcription from the LMP1 promoter. Mutational analysis of LRS showed that the ATF/CRE element was an important mediator of the TSA effect. Our results also suggest that both EBNA2 and TSA require the presence of phosphorylated ATF2 to activate the LMP1 promoter.

Keywords: *Epstein-Barr virus, B-cell, transformation, LMP1, EBNA2, transcription, phosphorylation, histone acetylation.*

ISBN 91-628-4860-7

TRANSCRIPTIONAL REGULATION OF THE LATENT MEMBRANE PROTEIN 1 GENE BY EPSTEIN-BARR VIRUS NUCLEAR ANTIGEN 2

by

Lars Palmqvist

Institute of Laboratory Medicine
Department of Clinical Chemistry and Transfusion medicine
Göteborg University

Göteborg 2001

ISBN 91-628-4860-7

ABSTRACT

The Epstein-Barr virus (EBV) is a ubiquitous human herpes virus, which infects B-cells. This leads to a life-long latent infection, which in most cases is non-pathogenic. However, EBV is etiologically associated with several human malignancies, including endemic Burkitt's lymphoma, nasopharyngeal carcinoma and post-transplant lymphoma. This association is probably linked to the ability of the virus to immortalise B-cells *in vitro* and to induce B-cell proliferation. Mutagenesis of the viral genome has defined a subset of four nuclear proteins (EBNA1, 2, 3, and 6) and one membrane protein (LMP1) required for the immortalisation process. The aim of the present study was to increase our knowledge about the different mechanisms by which the expression of the LMP1 gene is regulated in B-cells focussing on the role played by the virally encoded transactivating factor EBNA2.

The importance of an ATF/CRE site and an Sp site in the proximal part of the LMP1 regulatory sequence (LRS) was established. Mutations of the ATF/CRE and Sp sites decreased both EBNA2-dependent and -independent LMP1 promoter activity in transient transfection experiments. Both cAMP and okadaic acid, an inhibitor of protein phosphatase 1 (PP1) and 2A (PP2A), activated the promoter in an ATF/CRE-dependent fashion. Immunoaffinity experiments showed that EBNA2 co-purified with a PP1-like protein from an EBV-immortalised B-cell line and that a recombinant EBNA2 fusion protein specifically bound and inhibited a PP1-like activity in B-cell extracts. Thus, we conclude that the ATF/CRE and Sp site is important in transactivation of the LMP1 promoter and that the ability of EBNA2 to block the effect exerted by protein phosphatases on a certain transcription factor can be part of the mechanism by which the LMP1 promoter is activated.

Electrophoretic mobility shift assays showed that both the positive Sp1 and the negative Sp3 transcription factor bound to the Sp site. However, overexpression of the Sp1 transcription factor did not add to the activity induced by EBNA2. The heterodimeric transcription factor complexes ATF1/CREB1 and c-Jun/ATF2 bound to the ATF1/CRE site in LRS. The binding of ATF1/CREB1 activated the LMP1 promoter in the absence of EBNA2. The ATF2 and c-Jun transcription factors, on the other hand, did not activate the LMP1 promoter in the absence of EBNA2 but did so in the presence of EBNA2. The activating effect was abolished if the phosphate-accepting amino acid residues Thr69 and Thr71 in ATF2 were mutated. Furthermore, EBNA2 could interact with the c-Jun/ATF2 heterodimer. We conclude that EBNA2 is targeted to the promoter by these factors to transactivate the LMP1 promoter.

Histone acetylation at the LMP1 promoter was studied in two different B-cell lines. Both EBNA2 and the histone deacetylase inhibitor Trichostatin A (TSA) rapidly increased the level of histone acetylation at the LMP1 promoter in resting ER/EB2-5 B-cells. However, only EBNA2 induced LMP1 transcription. This was in contrast to the situation in proliferating P3HR1 B-cells, in which TSA both increased histone acetylation at and activated transcription from the LMP1 promoter. Mutational analysis of LRS showed that the ATF/CRE element was an important mediator of the TSA effect. Our results also suggest that both EBNA2 and TSA require the presence of phosphorylated ATF2 to activate the LMP1 promoter.

Keywords: *Epstein-Barr virus, B-cell, transformation, LMP1, EBNA2, transcription, phosphorylation, histone acetylation.*

LIST OF PUBLICATIONS

The present thesis is based on the following papers. These papers will be referred to in the text by their roman numerals.

I. Fåhraeus, R., Palmqvist, L., Nerstedt, A., Farzad, S., Rymo, L. and S. Lain. (1994) Response to cAMP levels of Epstein-Barr virus EBNA2 inducible LMP1 oncogene and EBNA2 inhibition of a PP1-like activity. *EMBO J. 13: 6041-6051.*

II. Sjöblom, A., Yang, W., Palmqvist, L., Jansson, A. and L. Rymo. (1998) An ATF/CRE element mediates both EBNA2 dependent and EBNA2 independent activation of the Epstein-Barr virus LMP1 gene promoter. *J.Virol. 72:1365-1376.*

III. Palmqvist, L., Jansson, A. and L. Rymo. Increased histone acetylation and phosphorylation of ATF2 are required for transcriptional activation of the Epstein-Barr virus LMP1 promoter. *Manuscript.*

TABLE OF CONTENT

INTRODUCTION

Epstein-Barr virus (EBV) infects over 90 percent of the world's population and persists for the lifetime of the infected person. Both primary infection and viral persistence are normally asymtomatic. However, an accumulating number of both benign and malignant human diseases have been associated with EBV infection. These associations in conjunction with the ability of EBV to induce B-cell proliferation and growth transformation explain the interest in EBV biology over the past three decades.

To understand viral pathogenesis it is necessary to unravel the molecular details involved in viral infection. Viruses have evolved various strategies to take control over the infected cell and direct the cell machinery to produce viral material. During the sometimes millions of years of virus and host co-evolution there has been plenty of time to develop complex and intimate interactions between the two. Studies on latent and persistent viruses, such as EBV, have demonstrated sophisticated strategies to replicate and persist within the host.

Cells in multicellular organisms are directed by extracellular signals, where different signals lead to changes in gene expression and appropriate physiological responses. Viruses have evolved numerous genes that function as signal and transcription regulators. In order to control the fate of the infected cell, they interfere with cellular functions that regulate cell division and growth control. The development of uncontrolled cell growth and malignant disease can be one direct consequence of this.

TRANSCRIPTIONAL REGULATION-AN OVERVIEW

Transcription and gene expression can be controlled at several different levels including transcription initiation, termination of elongation, nuclear RNA processing, mRNA transport and mRNA stability. Protein processing, targeting and stability are also important in the overall regulation of protein production and subsequent cellular responses. However, transcription initiation has turned out to be the major control point in most cases. In the first stage of transcription initiation, there is a relief of repression by remodelling of the structure of the chromatin at the promoter. In the second stage, a preinitiation complex (PIC) is formed

at the promoter which contains RNA polymerase II and the general transcription factors (GTFs).

The chromatin template

Promoter DNA elements

There are at least three features common to most promoters for protein coding genes. The transcription start site or initiation motif (INR), the TATA box and upstream sequences bound by transcriptional regulators. The latter sequences have positive (enhancers) and negative (silencers) effects. Enhancers and silencers are binding sites for regulatory proteins that can act independently of orientation and at long distances from the transcription start site. In addition there are elements termed locus control regions (LCR) that consist of complex arrangements of multiple regulatory elements.

Nucleosomes

The fundamental repeating unit of chromatin is the nucleosome, which is comprised of 146 bp of DNA wrapped 1.65 turns around an octamer of histone molecules, two of each H2A, H2B, H3 and H4. Nucleosomes are coiled or folded into chromatin fibers. Nucleosomes interact with other nucleosomes or additional chromosomal proteins to give this higher-order chromatin stucture. Linker histone H1 has been implicated in this organisation. Nucleosomes repress all genes except those whose transcription is activated by specific positive regulatory mechanisms. They repress by occluding sites of protein binding to DNA, thereby interfering with the interaction of activators, repressors, basal transcription factors and DNA-modifying enzymes.

Activation and repression

Gene-specific transcription is stimulated by the binding of activators to enhancers, where they recruit and regulate the activities of chromatin modifying complexes and the transcriptional apparatus. There is an interplay between activators and repressors and the balance between the two determines whether transcription is initiated or not (figure 1).

Activators

Activators typically consist of two domains: one that binds specific DNA sequences and one that recruits or stimulates the activity of the transcription apparatus. A single activator can bind multiple genes, thus providing a mechanism for co-ordinate control of these genes.

Individual genes are regulated through the action of multiple activators, thereby providing a mechanism for combinatorial control. Multiprotein complexes are formed at enhancers by the binding of multiple transcriptional regulators. This arrangement of multiple activators in a single complex provides the capacity to integrate multiple regulatory input into a single output. Several transcriptional activators, co-activators and general transcription factors have been found to possess histone acetylase transferase (HAT) activity (Brown et al. 2000). Numerous HATs have now been identified and they are often associated with multiprotein complexes. Many DNA-binding transcription factors bind to the co-activators CREB-binding protein (CBP) and the closely related p300, which function in co-operation with additional co-activators (e.g. PCAF) (Vo and Goodman 2001). Both p300 and CBP, but also most of the other co-activators, have HAT activity, indicating that multiple HATs are recruited to promoters for stimulation of transcription (figure 1). HATs can also acetylate substrates other than histones but its role in transcriptional activation is still not fully elucidated.

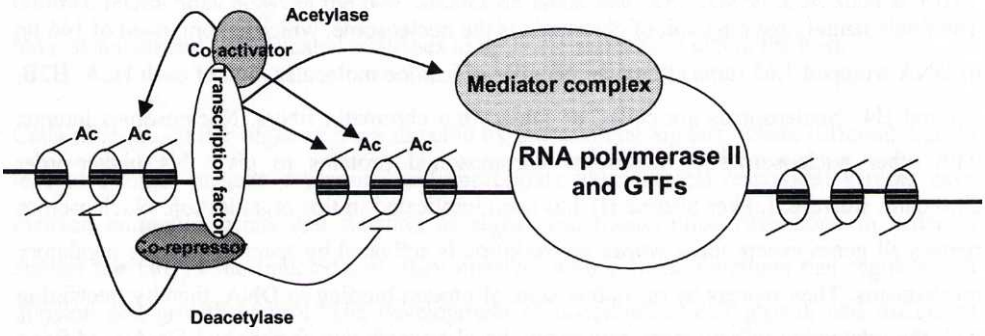

Figure 1. Transcription factors bound to promoters can either be activators or repressors. An activator stimulate transcription by interactions with co-activators, which recruits histone acetylase and direct interactions with the Mediator complex. A repressor interacts with co-repressors, which recruits histone deacetylases.

Repressors

Gene-specific repressors function by binding to activators or by competing for activator binding sites. Many repressors have been found to possess histone deacetylase (HDAC) activity (Ng and Bird 2000). HDACs can repress in a gene- or location-specific manner through their action on chromatin (figure 1). All known HDACs are found in multiprotein complexes. Two mammalian co-repressors, Sin3 and NuRD have been characterised so far. Both complexes contain a core of HDAC1 and HDAC2 and the histone binding protein RbAP46 and RbAP48. Sin3 was originally identified as a co-repressor for the DNA-binding

repressor Mad-Max. However, Sin3 complexes can be recruited by many DNA-binding proteins but also by other co-repressors. Deacetylase activity is also linked to repression mediated by methylated DNA (Razin 1998). DNA methylation of CpG is involved in mammalian gene silencing, where gene-specific methylation correlates inversely with gene activity. Furthermore, artificial demethylation results in gene activation. DNA is methylated by methyltransferases. The methyl-binding protein MeCP2 bind to HDAC containing complexes and form together a very stable repression of promoter activity.

Chromatin modification

Covalent modifications of histones

Possible histon modifications include acetylation, phosphorylation, methylation and ubiquitination. Histone acetylation is the best understood of the modifications in terms of consequences for transcriptional activity. Several lysines in the N-terminal tail of each of the core histones can be reversibly acetylated. Hyperacetylated histones are associated with transcriptionally active DNA regions, wheras hypoacetylated histones are enriched in regions that are transcriptionally silent. The discovery that many transcription factors possess HAT activity has provided the key evidence for a regulatory role of nucleosomes in transcription. The exact mechanisms how histone acetylation facilitates transcription are not known, but it may provide access to DNA for the transcription apparatus and its regulators. The other covalent modifications of histones are poorly understood. Phosphorylation of the N-terminal tail of histone H3 has been linked to increased transcriptional activity.

Noncovalent modifications of histones

Nucleosomes are subjected to conformational remodelling in addition to covalent modifications. Several remodelling complexes have been identified and the best studied is the Swi/Snf complex (Workman and Kingston 1998). All these complexes contain an ATPase subunit that is essential for their activity. Remodelling involves the breaking and reforming of histone-DNA contacts, although the precise mechanism is unknown. Swi/Snf is likely targeted to promoters by DNA-binding transcription activators or the general transcription machinery.

RNA polymerase II and initiation co-factors

Transcriptional activators recruit the RNA polymerase II transcription apparatus to promoters of protein-coding genes. The apparatus contains the RNA polymerase core enzyme, the

general transcription factors (GTFs), and a multisubunit complex called Mediator (Kornberg 1999). The proposed order of events that lead to transcriptional activation are seen in figure 2.

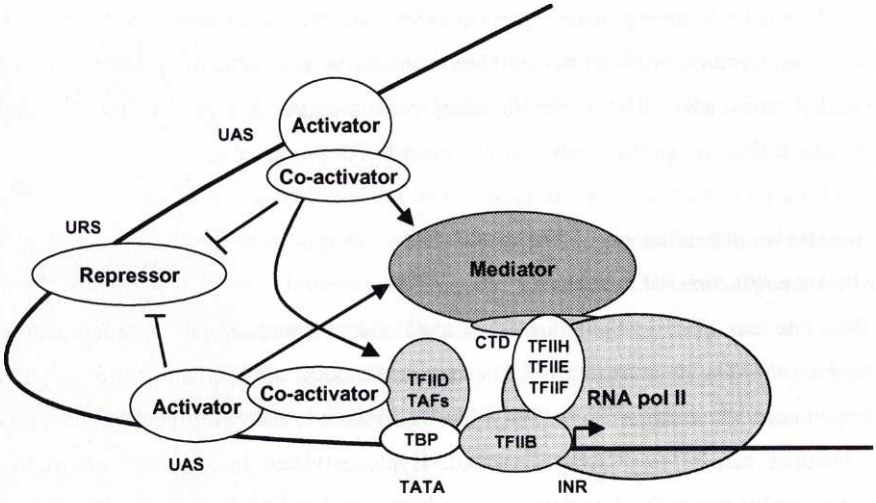

Figure 2. Activators recruit co-activators that counteract the action from repressors and co-repressors. GTFs, RNA polymerase II and mediator assemble at the promoter initiation site (INR). Concerted interactions between transcription factors, co-activators, GTFs and mediator complex then leads to CTD phosphorylation and RNA polymerase initiation, elongation and subsequent elevated transcription.

The RNA polymerase core enzyme

The RNA polymerase core enzyme has 12 subunits. The largest subunit contains a C-terminal domain (CTD) that consists of tandem repeats of a consensus heptapeptide sequence. The function of the CTD is closely associated with the phosphorylation status of the domain. RNA polymerases found at initiation start sites have dephosphorylated CTDs, while elongating polymerases contain heavily phosphorylated CTDs. The switch in CTD phosphorylation status appears to cause the RNA polymerase cofactors switch seen in the transition between initiation of transcription and elongation, where the preinitiation complex is replaced by the factors needed for elongation.

The general transcription factors

However, the RNA polymerase requires additional factors for promoter recognition and initiation. The GTFs, designated TFIIB, TFIID, TFIIE, TFIIF and TFIIH mediate this. Altogether they comprise 23 subunits. The TFIID complex is responsible for promoter

recognition and contains the TATA-binding protein (TBP) and TBP-associated factors (TAFs). They create a context for interaction with TFIIB, which positions the polymerase on the promoter. TFIIH contains an ATP-dependent helicase that unwinds the promoter around the start site to trigger initiation and a kinase activity capable of phosphorylating CTD. Transcriptional activators can bind to GTFs, but these interactions are not sufficient for transcriptional activation in a pure *in vitro* transcription system. Obviously co-activators are also needed.

The mediator complex

This mediator complex, which contains at least 20 subunits, was originally isolated from the yeast Saccharomyces cerevisiae, as an activity needed for transcriptional activation *in vitro*. Later studies have identified Mediator-like complexes also in human cells and demonstrated an essential role for the Mediator in the transcription of nearly all RNA pol II dependent genes. The Mediator appears responsible for integrating diverse regulatory signals and acting as an interface between activators and the RNA polymerase.

SIGNAL TRANSDUCTION

To respond to extracellular signals cells have receptor molecules. These fall into two broad categories; cell surface receptors and cytoplasmic receptors that act on changes in the extracellular environment, respectively. Multiple intracellular signalling pathways then mediate the response to modulate the activity of transcription factors, transcriptional co-regulators and chromatin-modifying factors, that lead to changes in gene expression (figure 3).

Protein phosphorylation

Protein phosphorylation is the most frequently used mechanism in signal transduction and also the best understood. More than 10% of the proteins in a typical mammalian cell are thought to be phosphorylated. Phosphates are transferred from ATP molecules to target molecules by protein kinases and are taken off by protein phosphatases. Both protein kinases and protein phosphatases are involved in the intricate regulation of cell signalling. Protein phosphorylation can regulate distinct transcription factor function by at least five different mechanisms (Karin and Hunter 1995; Montminy 1997; Whitmarsh and Davis 2000).

(1) Controlling cellular localisation.

The heterodimeric transcription factor nuclear factor-κB (NF-κB) is regulated by the cytoplasmic inhibitor IκB. IκB is phosphorylated in response to signals produced by different stimuli. This leads to degradation of IκB and the release and translocation of NF-κB to the nucleus. NF-κB binding sites have been identified in a large number of promoters and enhancers and include genes involved in immune function, inflammatory response, growth control and cell death. Another example is the Notch signalling pathway, which plays an important role in cell fate decisions. Activation of the Notch receptor leads to proteolytic cleavage of the receptor at the inner side of the cell membrane. The intracellular domain (Notch-IC) is then translocated to the nucleus where it activates genes by interacting with the Jk recombination signal binding protein (RBP-Jκ).

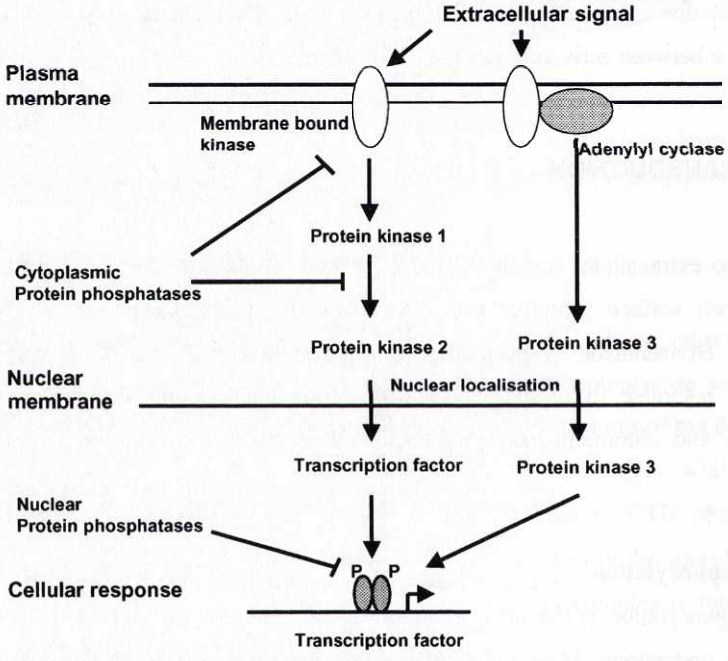

Figure 3. A schematic figure over possible intracellular signalling pathways in the cell from the plasma membrane to the nucleus and transcriptional response. Protein kinases and phosphatases act at all the different levels in the signalling cascade. Increase phosphorylation can both lead to activation or repression of transcription.

(2) Targeting transcription factors for degradation.

The transcription factors ATF2 and c-Jun are targeted for degradation depending on their phosphorylation state. Phosphorylation by the activated c-Jun N-terminal kinase (JNK) or the mitogen-activated protein (MAP) p38 kinase protects them from degradation. JNK and p38 are members of the stress activated protein kinases (SAPK) family and they respond to cellular insults or injury and can induce growth arrest.

(3) Modulating protein-protein interactions.

Many transcription factors contain phosphorylation-dependent activation domains, and sometimes repression domains. The precise mechanisms by which phosphorylation activates or represses transcriptional activity are not fully understood. The affinity for co-activators, co-repressors or basal transcription factors is probably affected. The CREB/ATF and Jun/Fos (AP-1) family of transcription factors are among the most studied. The cAMP-response element binding protein (CREB) is the prototype transcription factor. Several kinases in different signalling pathways can phosphorylate CREB. Phosphorylation of CREB allows the recruitment of the co-activator CREB-binding protein (CBP), which is also regulated through phosphorylation. This interaction with CBP is proposed to link CREB with components of the basal transcription complex in order to activate transcription.

(4) Regulating DNA binding.

The DNA binding activity of the transcription factor c-Jun is regulated by both direct and indirect phosphorylation. Phosphorylation of c-Jun near the C-terminal DNA bindning domain inhibits DNA-binding. Phosphorylation of the N-terminal transactivation domain by JNK cause a conformational change in c-Jun, which in turn facilitates dephosphorylation of the C-terminal residues and results in increased DNA binding.

(5) Modifying chromatin structure.

Remodelling complexes and chromatin modifying enzymes are under signalling dependent control. The chromatin remodelling complex Swi/Snf is inactivated by phosphorylation during mitosis resulting in a repressive chromatin structure and is activated by dephosphorylation when cells exit mitosis. The balance between histone acetyltransferases (HATs) and histone deacetylases (HDACs) is controlled by signal transduction pathways. The activity of specific HATs is increased or decreased by phosphorylation.

THE EPSTEIN-BARR VIRUS

Classification and genetics

EBV is a DNA virus and a member of the gamma-herpesvirus family and the prototype virus in the lymphocryptovirus subfamily based on its ability to latently infect B-cells. It is the most extensively studied virus in this group and the entire genome DNA sequence of the B98-5 strain of the virus was established already in 1984 employing a library of cloned restriction fragments created in Gothenburg (Baer et al. 1984). The virus has a linear double-stranded DNA genome of about 172 000 base pairs which encodes approximately 85 proteins, most of them involved in viral replication and lytic cycle. A limited set of genes is involved in the latent stage of infection. These genes code for six nuclear antigens (EBNA1 to 6), three latent membrane proteins (LMP1, 2A and 2B), a complex family of alternative spliced transcripts (BARTs) and two small noncoding nuclear RNAs (EBER1 and 2). Reviewed in (Kieff 1996). There are two EBV types infecting the human population, formerly designated as types A and B, but are now referred to as EBV-1 and EBV-2. There are no clear differences between EBV-1 and EBV-2 in terms of efficiency in infecting humans *in vivo* or in ability to cause disease. However, EBV-2 transforms B-cells less efficiently *in vitro* than does EBV-1 and B-lymphocytes infected by EBV-2 grow less well in reduced serum concentration and low cell density. These differences are primarily determined by differences in the EBNA2 coding gene (Cohen et al. 1989).

Host range and virus propagation

Humans are the natural host of EBV infection. EBV is an orally transmitted agent and enters the host through the oropharynx. Infiltrating B-cells in the epithelium are believed to be the prime target. The C3d complement receptor molecule (CD21) is the virus receptor in the human host. CD21 expression is largely restricted to mature B-lymphocytes, explaining the predominant B-cell tropism of EBV. Glycoproteins in the viral envelope potentiate the binding of the virus to the target cell and the fusion of the virus with the host cell membrane. Following fusion, viral DNA is released into the cell, becomes circular and is transcribed and replicated in the nucleus, where it persists as multiple episomal copies. A co-ordinate expression of the latent gene products under the control of the transcription factor EBNA2 then induces growth transformation and cell proliferation of the infected B-cell. The virus then probably uses the normal differentiation pathway of B-lymphocytes to gain access to and persist within the long-lived memory B-cell compartment. Usually, EBV remains latent

hereafter and viral replication is activated sporadically in a small percentage of the B-cells. Several findings suggest that only the B-cell compartment is needed for viral persistence. First, patients with X-linked agammaglobulinemia, which lack mature B-cells, are not infected by EBV (Faulkner et al. 1999). Second, EBV can be eradicated in bone marrow-transplant recipients who have received therapy that ablates their hemopoietic cells but not their oropharyngeal cells (Gratama et al. 1988). And third, acyclovir treatment abolishes shedding of EBV from the oropharynx but does not affect the number of infected B-cells in the circulation (Ernberg and Andersson 1986).

EBV's remarkable success relies on efficient spread from infected to non-infected individuals. Virus shedding normally occurs from the oropharynx, with transmission via infectious saliva. Memory B-cells normally migrate to mucosal surfaces to fulfil their role in combating infection and this is probably how EBV-positive B-cells reach the lymphoepithelium in the oropharynx. A controversial question is whether the viral replication takes place in these B-cells or in some other cell type in the oropharynx. Since EBV replicates so poorly in established B-cell lines *in vitro* it has been thought that EBV requires an epithelial site of replication. EBV infection of epithelial cells in oral hairy leukoplakia (OHL) results in an efficient viral replication and release of viral particles (Greenspan et al. 1985). However, recent re-examination of the available evidence has questioned whether epithelial cells are involved at all in normal EBV infection (Faulkner et al. 2000). Epithelial cells do not normally express the CD21 receptor molecule and they are only infected with great difficulty *in vitro* in contrast to the ease and reproducibility by which B-cells can be infected. *In situ* hybridisation techniques have consistently failed to identify infected epithelial cells in normal individuals, whereas both latent and replicating EBV can be detected in intra-epithelial B-cells (Niedobitek et al. 1992; Karajannis et al. 1997; Niedobitek et al. 1997).

This means that B-cells but not epithelial cells are essential for persistence and that there is no clear evidence for viral replication in epithelial cell *in vivo*. Thus, it seems reasonable to assume that infected B-cells are the sole site of EBV persistence, replication and spread in the normal healthy host. However, it should be pointed out that this does not mean that EBV is incapable of infecting cell types other then B-cells *in vivo*. EBV associated diseases of epithelial origin, such as nasopharyngeal carcinoma (NPC) and oral hairy leukoplakia (OHL) contain both EBV DNA and viral gene products and there is little doubt that EBV plays a role

in the pathogenesis of these diseases. But how and when EBV enters these cells is still an open question.

Immune response to EBV infection

Both a humoral and cellular mediated immunity is seen after EBV infection. Although the finding of antibodies directed against viral structural proteins and the EBNAs are important for diagnostic use, these antibodies do not seem to protect from new infection or control the latent stage of EBV infection. A MHC class I restricted cellular immune response has been found to be more important for the control of EBV infection (Rickinson and Moss 1997). Primary CD8+ cytotoxic T-cells (CTL) control over-growth of transformed and proliferating B-cells during the primary infection, assisted by primary CD4+ T-cells and natural killer (NK) cells. Memory CTLs stand for the long-term surveillance of EBV infection. The importance of this continued CTL response is evident in situations of T-cell immunosuppression (e.g. allograft recipients and AIDS patients) where lymphoproliferative disease or EBV positive lymphomas develop with high frequency. Both the primary and memory CTL response are markedly skewed toward HLA allele-specific epitopes derived from the EBNA3, 4 and 6 subset of latent proteins, with reactivities to other antigens being much less frequent. These EBNAs, together with EBNA2, are down-regulated by the virus after the initial steps of latent infection and this is probably necessary to escape the host's immuno-surveillance and to persist in the memory B-cell compartment.

Persistence in healthy individuals

Infected B-cells present several different viral expression patterns depending on stage in infection and localisation in the body. Both latent and lytic infection can be detected in tonsils from healthy infected individuals. Tonsillar IgD positive naive B-cells express the EBNA2-dependent lymphoblastoid phenotype, characteristic of primary infection (Joseph et al. 2000). Tonsillary germinal center centroblasts and centrocytes as well as memory B-cells are IgD negative and express EBNA1 initiated from the *Bam* Q-promoter (Qp), LMP1 and LMP2 but not EBNA2 (Babcock and Thorley-Lawson 2000; Babcock et al. 2000). In the peripheral blood, the virus resides latently in resting B-cells that are restricted to the IgD negative memory subset (Babcock et al. 1998). These cells however only express LMP2A or no latent genes at all (Qu and Rowe 1992; Babcock et al. 2000). EBNA1 can only be detected sporadically (Chen et al. 1995). The question is how these different expression patterns are linked to each other. One possible scenario is that the initial step in infection with expression

of the full set of latent gene products is absolutely necessary to efficiently activate the naive B-cell and induce a polyclonal differentiation toward a proliferating blast, without appropriate antigen stimulation. The activated B-cell blast then switches to a surface IgD negative memory B-cell in order to exit the cell cycle. This is normally the only way for a B-cell to survive after activation since it can not dedifferentiate back to a naive B-cell. The other option is programmed cell death when antigen stimulation becomes limiting. The restricted expression seen in the memory B-cells is limited to the viral products that are needed for maintenance of the viral episome (EBNA1) and to enhance cell survival (LMP1) or to block signals that might activate the viral lytic cycle (LMP2A). A schematic figure over the EBV life cycle *in vivo*, modified from (Babcock et al. 1998; Babcock et al. 2000), is seen in figure 4, with the proposed stages in infection and possible associated diseases.

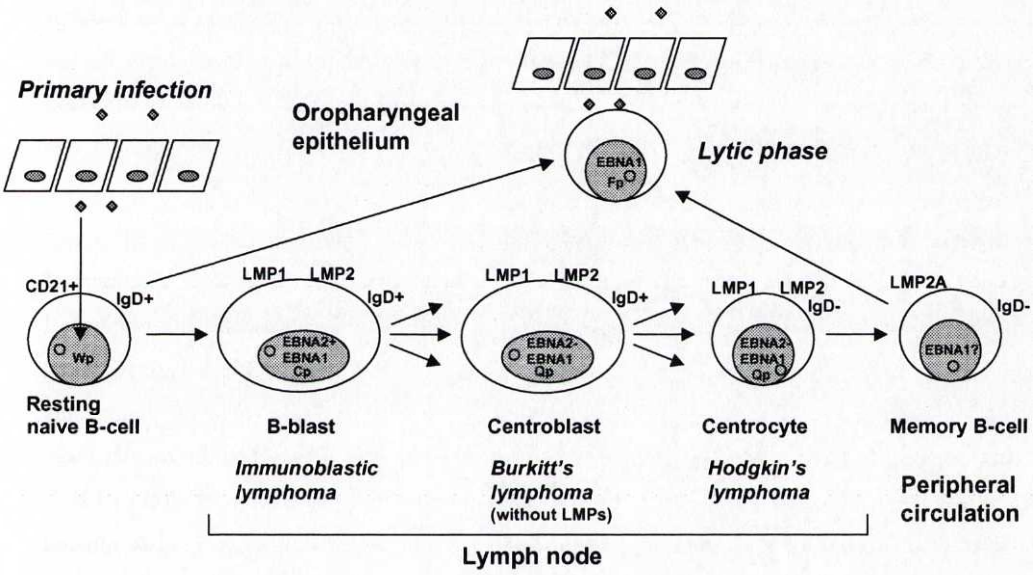

Figure 4. The Epstein-Barr virus life cycle. B-cells are after infection either transformed to proliferating lymphoblasts or enters the lytic phase. The infected lymphoblasts mature into IgD negative memory B-cells and the expression of latent genes are down-regulated. The viral promoter usage in the different stages is indicated. The lytic phase is probably activated in the memory B-cells if and when they enter the oropharynx. The known EBV-positive lymphomas and their proposed origin according to their latent gene expression are also shown.

EBV-associated diseases

EBV is associated with an increasing number of both benign and malignant diseases, with some of the most studied listed in table 1. All are not discussed in the text. Reviewed in (Rickinson and Kieff 1996; Baumforth et al. 1999; Cohen 2000). The ability of EBV to induce cell proliferation and transformation is thought to be the main cause, in conjunction with specific genetic or environmental factors or immunological changes of the host, for its association with disease.

Infectious mononucleosis

Whereas most EBV primary infections of infants and children are asymtomatic or only give non-specific symptoms, infections of adolescents and adults frequently result in infectious mononucleosis (IM). The disease is self-limiting but the convalescence may be very long. The reason why some adults get symptoms from the primary infection and others do not, including children, is not clear. If IM is an atypical state of infection where the virus infects B-cells other than the naive B-cells or simply is an amplified version of the asymtomatic infection remains to be determined.

Lymphoproliferative disease and immunoblastic lymphomas

The ability of EBV to cause malignant disease is most clearly indicated by the development of lymphoproliferative disease and immunoblastic B-cell lymphoma in patients with congenital or acquired immunodeficiency. This includes patients with the rare genetic disease X-linked lymphoproliferative disease (XLPD), recipients of organ or bone marrow transplants undergoing immunosuppressive treatment and patients that have developed AIDS. All these patients have impaired T-cell immunity and are unable to control the proliferation of EBV-infected B-cells. They present symptoms of IM or localised or disseminated lymphoproliferation in lymph nodes, liver, lung, kidney, bone marrow, central nervous system or the small intestine. XLPD patients often get fatal IM upon primary EBV infection, while the minority that survive have a greatly increased risk of developing lymphoma or agammaglobulinemia. All allograft transplanted patients have a elevated risk of developing post-transplant lymphoproliferative disorder (PTLD), although the incidence varies with the organ transplanted and the anti-rejection treatment, from a few percent in bone-marrow transplants up to ten to fifteen percent in cardiac transplants. The risk is particularly high after the use of the immunosuppressive agent cyclosporine A, which inhibit CTL response. Lymphoproliferative disease is a relatively frequent but late manifestation in AIDS patients.

AIDS lymphoma often involves the central nervous system, which is a very rare event in other forms of immunoblastic lymphoma. Virtually all cases of immunoblastic lymphomas are EBV positive with the exception of the AIDS related ones where some 75% to 80% appear to be EBV-linked. Immunoblastic lymphomas may be polyclonal, oligoclonal or monoclonal at diagnosis. It has been proposed that these lymphomas exhibit an unrestricted EBV latent gene expression but there are indications that the heterogeneity within this group is more complex with respect to EBV gene expression (Cen et al. 1993).

Burkitt's lymphoma

Burkitt's lymphoma (BL) is a high-grade malignant lymphoma of B-cells. There are three different forms recognised, endemic, sporadic and AIDS related. All BL contain a chromosomal translocation involving chromosomes 8 and 14, 22 or 2, where t8;14 is the most common. These translocations result in the positioning of the c-*myc* oncogene near the immunoglobulin heavy-chain or light-chain leading to abnormal regulation of c-*myc*. The endemic form shows the strongest and most consistent association with EBV. It is the most common childhood tumour in equatorial regions of Africa and Papua New Guinea and at least 95% of the tumours are EBV positive. It is believed that EBV and malaria infection together cause the increase in incidence of endemic BL, where malaria is thought to diminish the T-cell control of proliferating EBV-infected B-cells and thereby increase the probability of chromosomal translocations. Tumour tissue samples from these patients usually contain EBV gene products, but these are limited to EBNA1 and the non-translated EBV early RNAs (EBERs). The EBV genome is monoclonal, indicating that the tumours arise from a single EBV-infected cell. They also express high levels of CD10 and CD77, a phenotype resembling that of centroblasts in germinal centres of lymph nodes. The other forms of BL have a much weaker association with EBV infection. The AIDS related BL typically tends to appear earlier in the progression toward AIDS than the AIDS related lymphoproliferative and immunoblastic lymphoma and before the host immune function is radically impaired. Approximately 30% to 40% of the AIDS-BL are EBV positive. In the sporadic form only about 20% of the tumours are EBV positive and the incidence of this type of lymphoma is 50 to 100-fold less than that of the endemic form.

Nasopharyngeal carcinoma

Nasopharyngeal carcinoma (NPC) is an epithelial tumour that, like BL, is characterised by marked geographic and population differences in incidence. It is particularly common in the

Chinese populations in Southeast Asia, where it accounts for nearly 20% of all malignant neoplasm. In contrast to BL, EBV is present in all undifferentiated NPC regardless of geographic location or ethnic background of the patient. Just like BL, NPC contains clonal EBV episomes suggesting that the tumours arise from a single EBV-infected cell. In spite of this strong association with EBV the aetiology of the disease is poorly understood. The raised incidence in specific populations suggests that genetic, cultural or dietary factors rather than environmental carcinogens contribute to the development of this disease. NPC patients do not have impaired CTL response and the viral gene expression is restricted to EBNA1, EBERs and LMP1 and LMP2 expressed independently of EBNA2.

Hodgkin's disease

Epidemiological studies originally suggested a possible role for EBV in the aetiology of Hodgkin's disease (HD) with an increased risk of HD following IM. EBV has been detected in 40% to 60% of HD cases and EBV genome localises to the malignant component of HD, the Reed-Sternberg cells. The viral genomes are monoclonal. The expression pattern has the same restriction as NPC. The association of HD with EBV seems to be age-related. HD in children and older adults are usually EBV-associated, whereas HD in young adults is less frequently EBV-positive. Although the incidence of HD is relatively low this tumour is not geographically restricted, making its association with EBV significant in world health terms.

Table 1. EBV-associated diseases

Disease	Comments	EBV genome + (%)	Type of latency
Infectious mononucleosis	Primary infection in adolescents.	>90	III
Lymphoproliferative disease and Immunoblastic lymphoma	Immunocompromised patients. XLPD, AIDS or post-transplant	80-100	III
Burkitt's lymphoma	Endemic	97	I
	Sporadic	10–80	
	AIDS	30–40	
Hodgkin's disease	Mixed cellularity	>80	II
	Nodular sclerosing	30–40	
	Lymphocyte predominant	<10	
Nasopharyngeal carcinoma	Undifferentiated	100	II
	SCC	40	
T-cell lymphoma	Nasal	100	II
	Others	10–40	
Oral hairy leukoplakia	Viral replication in tongue epithelium of HIV-positive persons.	100	Mixed lytic and latent expression

Growth transformation and immortalisation

Most of our knowledge of the latent stage of EBV infection is based on studies of B-cells infection *in vitro*, lymphoblastoid cell lines and established EBV positive tumour cell lines. Considerable efforts have been made to infect and transform other cell types *in vitro* with little or no success. The presence or absence of the CD21 receptor only in part explains these difficulties. Cell type specific factors probably determine whether the EBV genome is going to be expressed at all after infection, but also if the lytic or latent route is chosen, since EBV gene expression is known to be tightly regulated by cellular transcription factors.

In vitro infection of B-cells with EBV is highly effective and results in the outgrowth of infected blasts that can be established as a lymphoblastoid cell line (LCL) with continuos and indefinite growth. This transformation and immortalisation is achieved by a seemingly low number of viral gene products (table 2) (Kieff 1996). Five of these have been shown to be essential (EBNA1, 2, 3, 6 and LMP1) for initiation of B-cell transformation through genetic analysis of recombinant viruses. In addition, EBNA5 has been shown to significantly improve the outgrowth of transformed cells. EBNA1, EBNA2 and LMP1 are also required for maintenance of transformation. One should however bear in mind that functional redundancy among the non-essential genes might permit the deletion of one but not several genes simultaneously. EBV display several distinct gene expression programmes, according to the host cell. At least three different latency programmes can be seen in established tumour cell lines and LCL, all of which are distinct from the lytic cycle. They are referred to as latency I, II and III (Rickinson and Kieff 1996).

Latency III

The best-characterised latency programme is latency III, or growth program of latency, found in LCLs after infection of B-cells *in vitro*. These cells are probably equivalent to those produced *in vivo* upon initial infection but are also seen in a number of EBV associated diseases; infectious mononucleosis, X-linked lymphoproliferative disease, post-transplant and AIDS related lymphoproliferative disease and immunoblastic lymphoma. All the latent gene products are expressed in this latency. The EBNA2 transactivated *Bam* C-promoter (Cp) is used to express the EBNAs. Expressions of the LMPs are EBNA2 dependent.

Latency I

This latency is found in EBV positive Burkitt's lymphoma and is restricted to expression of the EBNA1 protein, a complex of transcripts from *Bam HI* A (BARTs) and non-translated small RNAs (EBERs). EBNA1 expression is initiated from the *Bam* Q-promoter (Qp). This latency resembles to some extent the resting state in which infected memory B-cells escape the immune response.

Latency II

This latency is found in Hodgkin's disease (HD), T-cell lymphomas and nasopharyngeal carcinoma (NPC). The viral expression is limited to EBNA1 expressed from the Qp, LMPs, EBERs and BARTs. The expressions of the LMPs are EBNA2 independent.

Functions of the EBV latent gene products

EBNA5

EBNA5, also called EBNA-LP, is together with EBNA2 the first viral genes expressed after primary infection of B-cells. EBNA5 is not absolutely necessary for EBV mediated B-cell transformation but greatly enhances the efficiency of the process (Mannick et al. 1991). EBNA5 can interact with the cell cycle regulators and tumour suppressor gene products p53 and Rb *in vitro* (Szekely et al. 1993) but there is no evidence that their functions are modified by EBNA5 *in vivo* (Allday et al. 1995; Inman and Farrell 1995). EBNA5 is involved in transcriptional regulation but the precise mechanism by which this is achieved is not clear. EBNA5 stimulates the EBNA2 activation of the LMP1 promoter in several different assays, but also the LMP2 and the C-promoter (Harada and Kieff 1997; Nitsche et al. 1997). This EBNA5 dependent co-stimulation might be mediated through the CBF1/RBP-Jk binding site (Harada and Kieff 1997). EBNA5 has also been implicated in transcriptional repression. The effect might be mediated through inhibition of pre-mRNA cleavage and polyadenylation (Dufva et al. unpublished investigation).

EBNA2

The essential role of EBNA2 in growth transformation was first evident in studies of the EBV infected BL line, P3HR1. The P3HR1 virus was found to be non-transforming because of a deletion that removed all the EBNA2 coding regions and the last two exons of EBNA5. The transforming ability could only be retained by EBNA2 (Cohen et al. 1989; Hammerschmidt and Sugden 1989). This means that EBNA2 is required for initiation of B-cell transformation

but EBNA2 is also essential for maintenance of transformation (Kempkes et al. 1995c). EBNA2 is the major determinant of the differences between EBV-1 and EBV-2 in ability to induce transformation (Cohen et al. 1989). The EBV-1 (B95-8 strain) EBNA2 consists of 487 amino acids whereas the EBV-2 (AG876 strain) EBNA2 consists of 443 amino acids. Sequence comparison between the closely related baboon herpes virus papio (HVP), EBV-1 and EBV-2 has identified eight conserved regions in the EBNA2 coding gene (Ling et al. 1993b) (figure 5). EBNA2 is a phosphoprotein phosphorylated on serines and threonines. The EBNA2 C-terminus is a substrate for casein kinase 2 *in vitro* (Grässer et al. 1992).

EBNA2 is one of the first viral genes expressed after infection and EBNA2 coordinates the cascade of events leading to B-cell growth transformation. EBNA2 is a specific transcriptional activator of both viral and cellular genes. The *Bam* W promoter (Wp) is initially used to express the EBNAs during B-cell transformation. This promoter is not dependent on viral gene products for its activation. However, shortly after infection the promoter usage is switched from the Wp to the Cp promoter by EBNA2 (Woisetschlaeger et al. 1991). Cp is then regulated by EBNA2 (Sung et al. 1991; Jin and Speck 1992). EBNA2 also upregulates the LMP1 (Abbot et al. 1990; Wang et al. 1990b) and the LMP2 promoters (Zimber-Strobl et al. 1991). In addition, several cellular genes are up-regulated by EBNA2, including the B-cell activation marker CD23 (Wang et al. 1987), the complement and EBV receptor CD21 (Cordier et al. 1990) and the proto-oncogenes c-*fgr* (Knutson 1990) and c-*myc* (Kaiser et al. 1999). EBNA2 also down-regulate the expression of the immunoglobulin heavy-chain locus (Igμ) gene (Jochner et al. 1996). EBNA2 is involved in cell cycle regulation. Growth arrest occurs at G_1 and G_2 stages of the cell cycle when EBNA2 is withdrawn in B-cells conditional for EBNA2 function (Kempkes et al. 1995c). EBNA2 and EBNA5 can co-operate to cause G_0 to G_1 transition in the cell cycle during the transformation of resting B-cells (Sinclair et al. 1994). This is probably achieved by up-regulation of cyclin D2 expression. However, this induction is a secondary event, which require *de novo* protein synthesis (Kaiser et al. 1999). S phase entry from G_1 can also be regulated by EBNA2 since the proto-oncogen c-*myc* facilitates G_1 to S transition and the c-*myc* gene is a direct target for EBNA2 activation (Kaiser et al. 1999).

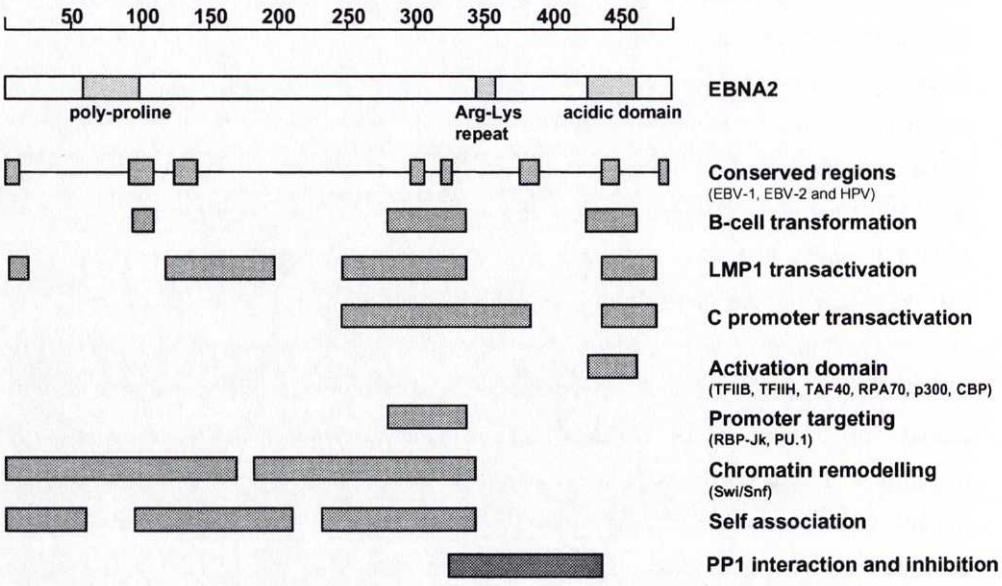

Figure 5. Functional domains in the EBNA2 protein.

Deletion analysis and directed mutagenesis of EBNA2 have identified essential regions for B-cell transformation *in vitro* (Cohen et al. 1991) and transactivation of the Cp and LMP1 promoters (Cohen and Kieff 1991; Sjöblom et al. 1995b) (figure 5). The EBNA2 C-terminal domain (aa. 426-462) is required for both B-cell transformation and for transactivation. A core motif in this domain (aa. 449-462) can be substituted by the VP16 acidic activation domain (Cohen and Kieff 1991; Cohen 1992). This EBNA2 domain has been found to interact with components of the RNA polymerase II transcription machinery. These are TFIIB, TAF40, TFIIH, RPA70 and indirectly TFIIE via a protein called p100 (a et al. 1995; Tong et al. 1995b; Tong et al. 1995c; Wu et al. 1996). The acidic domain of EBNA2 also interacts directly with the transcriptional co-activators CBP and p300 and indirectly with PCAF (Jayachandra et al. 1999; Wang et al. 2000), indicating that EBNA2 can recruit both basal transcription factors and histone acetylase transferases (HATs) to activate transcription. EBNA2 can also interact with the chromatin modulating human Swi/Snf complex and target it to promoters through binding with the hSNF5/Ini1 subunit (Wu et al. 1996; Wu et al. 2000). This interaction involves amino acids 1-170 and 182-344 in EBNA2 (figure 5). The targeting seems to be dependent on presence of RBP-Jk binding sites in the promoter (Wu et al. 2000).

EBNA2 lacks sequence specific DNA-binding ability and the participation of cellular DNA binding proteins is necessary for targeting of EBNA2 to specific promoters. The region between amino acid 280 to 337 in EBNA2, also required for B-cell transformation and transactivation, seems to mediate promoter targeting of EBNA2. This part of EBNA2 interacts with the DNA-binding protein Jk recombination signal binding protein (RBP-Jκ) (Ling et al. 1993a; Grossman et al. 1994; Henkel et al. 1994; Waltzer et al. 1994; Zimber-Strobl et al. 1994), also referred to as Cp-binding factor 1 (CBF1). Amino acids 310 to 336 of EBNA2 are sufficient for this interaction and a short conserved motif, PPWWPP (aa. 317-322), probably mediates the interaction (Yalamanchili et al. 1994). The RBP-Jκ DNA-binding site was originally identified by virtue of its ability to confer EBNA2 inducibility to the Cp, CD23 and LMP2A promoters (Zimber-Strobl et al. 1993; Ling et al. 1994) but most EBNA2 responsive promoters, including the LMP1 promoter, seem to contain one or more RBP-Jκ sites. RBP-Jκ is a transcriptional repressor. The repression on promoter activity is probably mediated by interaction with the RBP-Jκ binding protein SKIP and recruitment of a co-repressor complex containing CIR, SMRT, Sin3A, SAP30 and the histone deacetylases HDAC1 and HDAC2 (Hsieh et al. 1999; Zhou et al. 2000). The current model for EBNA2 transactivation through the RBP-Jκ site is that EBNA2 both binds to RBP-Jκ and SKIP to replace this co-repressor complex (Hsieh and Hayward 1995; Zhou et al. 2000). The RBP-Jκ protein is expressed ubiquitously and has been highly conserved throughout evolution. Mutation of the RBP-Jκ gene results in embryonic lethality in such diverse species as mouse and fruit fly. RBP-Jκ is a component in the Notch signalling pathway and binds to the intracellular domain of the receptor Notch (Notch-IC). Ligand mediated activation of Notch results in cleavage and release of its intracellular domain, which translocate to the nucleus and indirectly binds to DNA through its interaction with RBP-Jκ. Thus, EBNA2 seems to mimic activated Notch. Accordingly, Notch-IC can substitute for EBNA2 in transactivation of cellular and viral promoters to some extent (Höfelmayr et al. 1999; Strobl et al. 2000) and Notch-IC can partially replace EBNA2 function in B-cell immortalisation and maintenance of B-cell proliferation (Gordadze et al. 2001; Höfelmayr et al. 2001). However, it is interesting to note that Notch-IC was unable to or could only with great difficulty induce LMP1 expression in these experiments.

Regarding the LMP1 promoter, a PU box motif and an adjacent octamer motif in the enhancer region have been shown to be just as or more important than the RBP-Jk site in EBNA2 transactivation. The Ets-related PU.1/Spi-1 and Spi-B transcription factors and an unidentified POU-domain transcription factor bind these motifs respectively (Laux et al. 1994a; Johannsen et al. 1995; Sjöblom et al. 1995a; Sjöblom et al. 1995b). The Ets family of transcription factors regulate a wide variety of cellular genes and are important for B-cell development and differentiation. The same EBNA2 domain responsible for RBP-Jk binding might be involved in binding to the PU.1 transcription factor and subsequent targeting to the LMP1 promoter (Johannsen et al. 1995). However, there are data suggesting that the EBNA2 PU.1 interaction is indirect, probably mediated by the POU-domain binding protein (Sjöblom et al. 1995a).

There appears to be a requirement for several EBNA2 molecules to enable all the interactions with co-activators and transcription factors that are necessary for appropriate promoter regulation. Several lines of evidence are consistent with the possibility that EBNA2 is present in a multimeric form *in vivo* (Grässer et al. 1991; Tsui and Schubach 1994) and that this is an important feature for transactivation and transformation. Essential parts of EBNA2 have also been shown to be involved in self association (aa.1-60, 96-210 and 232-344) (Tsui and Schubach 1994; Harada et al. 2001).

EBNA3, 4 and 6

The EBNA3, 4 and 6 proteins are encoded by genes adjacent to each other in the EBV genome and are in an alternative nomenclature also called EBNA3A, 3B and 3C respectively. EBNA3 and EBNA6 are essential for growth transformation whereas EBNA4 is not (Tomkinson et al. 1993). EBNA3 is dispensable in maintenance of transformation (Kempkes et al. 1995b). Information on the role of EBNA6 in maintenance of transformation is not available. EBNA3 and EBNA6 have been shown to participate in transcriptional regulation of LMP1 (Wang et al. 1990a; Allday et al. 1993) and all three EBNAs bind the transcription factor RBP-Jk and inhibit transcriptional activation of EBNA2-responsive promoters (Le Roux et al. 1994; Radkov et al. 1997) by preventing EBNA2- RBP-Jk complexes from binding to their cognate RBP-Jk binding sites (Robertson et al. 1996; Waltzer et al. 1996). EBNA6 also interacts with a histone deacetylase (HDAC1), indicating that the repression of transcription could be mediated through histone deacetylation (Radkov et al. 1999). One function of the EBNA3, 4 and 6 proteins may thus be to counterbalance and fine-tune the action of EBNA2 on different promoters.

EBNA1

The EBNA1 protein is involved in viral replication and is essential for maintenance of the EBV episome (Yates et al. 1985). EBNA1 is expressed in all established EBV positive cell lines and most EBV positive cells *in vivo*. EBNA1 is a phosphoprotein and binds DNA as a dimer co-operatively to two groups of binding sites within the origin of replication, *oriP*, in the EBV genome. The main role of EBNA1/*oriP* may be to ensure stability of the replicated DNA and segregation into the daughter cells at cell division (Mackey and Sugden 1999). EBNA1 is also an activator of EBNA transcription via *oriP*. The EBNA1 protein contains a large domain of 239 amino acids with a repeated sequence of glycine-glycine-alanine. This domain has been shown to be important during the latent phase *in vivo*. The repeat domain efficiently inhibits the ubiqutin/proteasome-dependent degradation of EBNA1 and thereby prevents presentation of EBNA1 epitopes on MHC class I molecules (Levitskaya et al. 1995). This could explain why EBV infected cells in which viral antigen expression is limited to EBNA1 can escape the hosts immune surveillance mediated by CTLs. Evidence that EBNA1 itself has oncogenic potential is provided by the finding that B-cell lymphomas developed in transgenic mouse lines expressing EBNA1 under the control of the immunoglobulin heavy-chain intron enhancer (Wilson et al. 1996). However, this finding has not been further verified and it is still a controversial issue whether EBNA1 is an oncogene or not.

LMP2A and 2B

The LMP2 gene encodes two distinct proteins, LMP2A and LMP2B, which are produced through alternative splicing. Neither is required for B-cell transformation (Longnecker et al. 1992; Speck et al. 1999). They are integral membrane proteins and LMP2A is a substrate for *src* family tyrosine kinases. LMP2B lacks the amino-terminus, which contains the kinase interacting domain. LMP2A seems to block the signal transduction through the B-cell antigen receptor complex (BCR) (Fruehling and Longnecker 1997). This may inhibit reactivation of the virus from the latent state to the lytic cycle. The function of LMP2B is unclear but it may form complex with and modulate LMP2A function.

LMP1

Genetic studies have revealed that LMP1 is essential for transformation of B-cell *in vitro* and its continuous expression is required for maintenance of LCL growth (Kaye et al. 1993). However, LMP1 alone is not sufficient to sustain B-cell proliferation (Zimber-Strobl et al. 1996). LMP1 is unique among the latent gene products expressed during the immortalisation

process in that it alone induces the classic phenotypic changes associated with EBV transformation in rodent fibroblasts and these cells are tumourigenic in nude mice (Wang et al. 1985). Thus, LMP1 is an oncogene. Transgenic mice with the LMP1 gene under the control of the Ig heavy chain promoter develop lymphomas (Kulwichit et al. 1998). It shares multiple features with the tumour necrosis factor receptor 1 (TNFR1) molecule (Mosialos et al. 1995) and the CD40 molecule (Gires et al. 1997), although they differ strikingly in structure. LMP1 can partially restore the CD40 phenotype in CD40 deficient mice (Uchida et al. 1999) and LMP1 can activate the same signalling pathways as these molecules. However, LMP1 has additional functions absent from the CD40 and TNFR1 molecules (Farrell 1998; Kieser et al. 1999; Kaykas and Sugden 2000).

LMP1 is an integral membrane protein with six transmembrane domains with both the N- and C-terminus at the cytoplasmic side of the cell membrane. LMP1 is phosphorylated on serin and threonine residues and has a short half-life of approximately 2 hours in the plasma membrane (Baichwal and Sugden 1987; Mann and Thorley-Lawson 1987). LMP1 is highly toxic if expressed at high levels (Hammerschmidt et al. 1989). LMP1 aggregates in the plasma membrane with members in the TNFR family and binds to TNFR associated factors (TRAFs) (Devergne et al. 1996), TNF associated death domain protein (TRADD) (Izumi and Kieff 1997) and Janus kinase 3 (JAK3) (Gires et al. 1999). No extracellular ligand has been identified and LMP1 behaves as a ligand-independent and constitutively active signalling molecule. LMP1 has at least three domains important for its functions, referred to as C-terminal activating regions (CTAR), CTAR1 (aa. 186-231), CTAR2 (aa. 351-386) and CTAR3 (aa. 275-280 and 302-307).

At least four signalling pathways are implicated in the function of LMP1 (figure 6). (1) Activation of the transcription factor NF-kB, through CTAR1 and CTAR2 independent of each other (Huen et al. 1995). (2) Activation of the c-Jun N-terminal kinase (JNK) pathway, also known as the stress activated protein kinase (SAPK) pathway, through CTAR2, which leads to transcription factor AP-1 activation (Kieser et al. 1997; Eliopoulos et al. 1999a). (3) Activation of the mitogen activated protein kinase (MAPK) p38 through CTAR1 and CTAR2 independent of NF-kB activation, which leads to transcription factor ATF2 activation. (Eliopoulos et al. 1999b). (4) Activation of the JAK3 kinase in the JAK/STAT (signal transducers and activators of transcription) pathway, through CTAR3, which leads to activation of transcription factor STAT1 and STAT3.

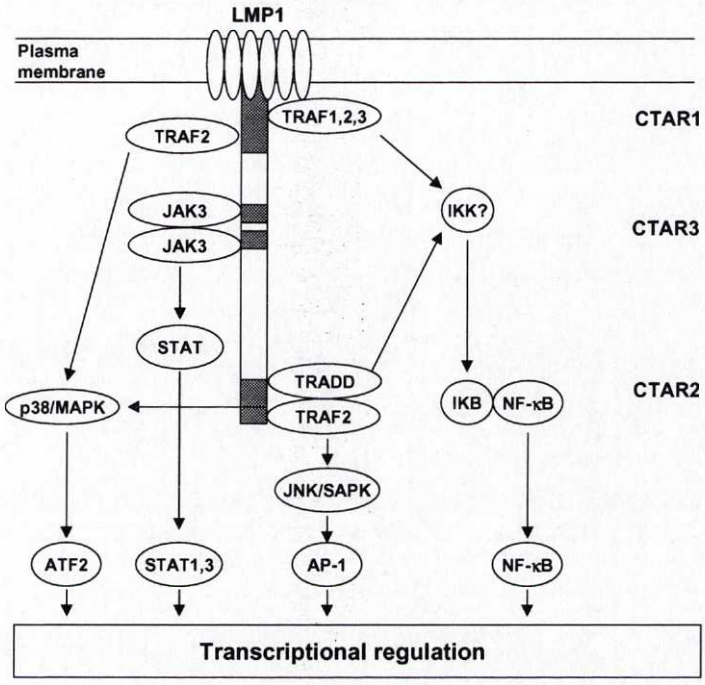

Figure 6. Signalling pathways involved in LMP1 functions. Both NF-κB and p38/MAPK signalling are mediated through CTAR1 and CTAR2 domains via TRAF molecules. JNK/AP1 signalling is mediated through CTAR2 via TRAF and JAK/STAT signalling is mediated through the recently identified CTAR3 domain. The net result of theses signals regulate transcription of various cellular genes and is responsible for many of the pleitropic effects of LMP1. Modified from (Baumforth et al. 1999).

LMP1 up-regulate several cellular genes that are associated with either EBV infection or antigen activation of primary B-cells. Among them are cell surface markers such as CD23, CD39, CD40, CD44 and MHC class II and cell adhesion molecules such as ICAM-1, LFA1 and LFA3 (Wang et al. 1988). LMP1 also induces cytokines with autocrine growth factor activity such as IL-6 and IL-8 (Eliopoulos et al. 1999b). LMP1 has also been shown to protect infected B-cells from programmed cell death or apoptosis. This effect is partly mediated by induction of the antiapoptotic cellular proteins Bcl-2 and A20 (Gregory et al. 1991; Henderson et al. 1991; Laherty et al. 1992).

Table 2. Functions of the EBV latent gene products.

Viral gene/protein	Properties	B-cell transformation	Expressed in latency
EBNA1	Essential for B-cell transformation. Required for viral replication/maintenance. Activates EBNA transcription via *oriP* (Cp/Wp).	+	I-III
EBNA2	Essential for B-cell transformation. Transcriptional activation of EBNA (Cp), LMP1, LMP2B and cellular genes. Associates with sequence-specific DNA-binding factors such as RBP-Jk and PU.1	+	III
EBNA3/EBNA3A	Essential for B-cell transformation. Transcriptional repression of LMP-1 and LMP-2B. Binds RBP-Jk and antagonizes EBNA2/RBP-Jk function.	+	III
EBNA4/EBNA3B	Not essential for B-cell transformation. Transcriptional repression of LMP1 and LMP2B. Binds RBP-Jk and antagonizes EBNA2/RBP-Jk function.	-	III
EBNA6/EBNA3C	Essential for B-cell transformation. Transcriptional repression of LMP1 and LMP2B. Activator of LMP1 and cellular genes. Binds RBP-Jk and antagonizes EBNA2/RBP-Jk function.	+	III
EBNA5/EBNA-LP	Not essential, but enhances efficiency of B-cell transformation. Potentiator of transactivation by EBNA2. Cooperates with EBNA2 during primary infection of resting B cells to induce the G_0 to G_1 transition.	+/-	III
LMP1	Essential for B-cell transformation. Oncogenic potential. Can regulate cell growth, but its main functions is to enhance cell survival. Alters cell phenotype.	+	II-III
LMP2A/TP1	Not essential for B-cell transformation. Affects BCR signalling to inhibit reactivation of virus. Substrate for *src* family tyrosine kinases.	-	II-III
LMP2B/TP2	Not essential for B-cell transformation. LMP2B is an alternatively spliced gene product that is identical to LMP2A except that it lacks the amino-terminus kinase-interacting domain. Its function is unclear, but it may complex with and modulate LMP2A function.	-	II-III
EBER1&2	Not essential for B-cell transformation. Small nuclear RNAs; bind autoantigen La, dsRNA-dependent protein kinase and ribosomal protein L22. Regulation of translation (?).	-	I-III
BARTs	Not essential for B-cell transformation. Complex spliced transcripts, may encode at least two polypeptides (RK-BARF0 and RPMS1). Function unknown	-	I-III

Regulation of the LMP1 gene promoter

LMP1 can be expressed in many different cellular environments and its expression is influenced by both cellular and viral factors. The regulation of the LMP1 gene has been investigated in both B-cells and epithelial cells and several factors important in the regulation have been identified. However, the precise mechanism used by the virus to modulate the expression in different situations is still not fully understood.

Methylation at the LMP1 promoter

DNA methylation has been implicated in regulation of promoter usage in EBV. Methylation of the EBV genome varies depending on the form of latency and the genome is almost unmethylated in LCLs with the unrestricted expression of latent gene products. Conversely, the EBV genome in BL and NPC is highly methylated. The role for DNA methylation in LMP1 regulation has been addressed in some studies. Treatment with the nucleotide analogue 5-Azacytidine results in demethylation in the BL-cell line Rael, which normally only expresses EBNA1. This results in expression of all the EBNAs and the LMP1 protein (Masucci et al. 1989). There are also a correlation between unmethylated DNA at the LMP1 promoter and expression of LMP1 in NPC biopsies, in established BL cell lines and LCLs (Ernberg et al. 1989; Hu et al. 1991; Falk et al. 1998).

Expression in different cell types

EBNA2 is required for LMP1 expression in B-cell lines and during *in vitro* transformation of B-cells (Abbot et al. 1990; Fåhraeus et al. 1990; Wang et al. 1990b). LMP1 can under certain circumstances be expressed independently of EBNA2 in B-cells (Cordier-Bussat et al. 1993) and LMP1 is not always expressed in EBNA2 positive B-cells (Cordier et al. 1990) underscoring the complex nature of LMP1 regulation. EBNA2 dependent LMP1 expression is seen *in vivo* during the initial stages of primary B-cell infection in the normal hosts. However, this requirement for EBNA2 is lifted in the later stages of EBV driven B-cell differentiation towards the persistent stage in the memory B-cell compartment (Babcock et al. 1998; Babcock et al. 2000).

LMP1 expression in epithelial cell lines is independent of EBNA2 (Fåhraeus et al. 1988; Young et al. 1988) and EBNA2 expression is not seen in LMP1 positive epithelial cells (NPC), in T-cells (T-cell lymphomas) or in Reed-Sternberg cells (Hodgkin's), where a latency II expression is present, underlining that cellular or viral factors can substitute for EBNA2

functions in these cells. Epithelial cells are also unresponsive to EBNA2 transactivation of the LMP1 gene, as determined by EBNA2 co-transfection with LRS reporter constructs (Fåhraeus et al. 1993), indicating that these cells lack some important cellular factor needed in EBNA2-dependent activation.

Promoter usage

The LMP1 gene can be expressed from several different promoters. During the latent phase of infection the ED-L1 promoter (*EcoRI* D leftward promoter 1) is primary used in B-cells (Farrell et al. 1983; Fennewald et al. 1984). During the lytic phase the ED-L1A promoter is used instead, which gives rise to a truncated version of LMP1 (Hudson et al. 1985). A few other promoters have also been identified, but these are less active in B-cells and seem to be involved in LMP1 expression in epithelial cells, although it is not ruled out that they can be important in B-cells during some stage of infection. Previous investigations and the present work have been focused on the regulation of the ED-L1 promoter in B-cells.

Regulatory elements

A sequence spanning from +40 to –634 relative to the ED-L1 promoter initiation site has been defined as the LMP1 transcription regulatory sequence (LRS) (Fåhraeus et al. 1990). Studies on LRS in B-cells have established that there is a complex set of both negative and positive cis-acting regulatory elements involved in LMP1 regulation. The region between position –54 and +40 contains a positive transcription element that is constitutively active. The –106 to +40 and –176 to –136 region contribute to EBNA2 responsiveness independently of each other (Fåhraeus et al. 1990; Fåhraeus et al. 1993; Sjöblom et al. 1995a) as well as the region between –234 to –205 (Ghosh and Kieff 1990; Tsang et al. 1991). These regions contain negative regulatory elements that prevent adjacent positive elements from functioning in B-cell. This negative effect must be overridden in order to activate transcription.

Subsequent investigations have identified several of the regulatory elements and the factors binding to them (figure 7). Two sites for the EBNA2 binding factor RBP-Jk have been identified in LRS, where only the more proximal element has been implicated in transcriptional activation (position –223 to –213) (Laux et al. 1994a; Laux et al. 1994b; Johannsen et al. 1995). However, its role in EBNA2 activation of LMP1 is complex. Reporter constructs lacking the RBP- Jκ site can still be activated by EBNA2 (Fåhraeus et al. 1990; Fåhraeus et al. 1993; Johannsen et al. 1995; Sjöblom et al. 1995a; Sjöblom et al. 1995b) and a

reporter construct containing four tandem RBP-Jκ sites is not EBNA2 responsive (Ling et al. 1993a). Furthermore, activated Notch, which is able to activate other RBP-Jk site containing promoters, can not replace EBNA2 in LMP1 activation (Gordadze et al. 2001; Höfelmayr et al. 2001). Two elements in the promoter downstream of RBP- Jκ seem to be more important for EBNA2 transactivation, an octamer motif (position –147 to –139), where a POU-domain protein binds (Sjöblom et al. 1995a), and a PU-box (position –171 to –155), where PU.1/Spi-1 and Spi-B bind (Laux et al. 1994a; Johannsen et al. 1995; Sjöblom et al. 1995a; Sjöblom et al. 1995b). These two elements seem to cooperate to generate EBNA2 responsiveness, possibly through interactions between each other and EBNA2 (Sjöblom et al. 1995a). Additional activating elements in the distal part of LRS do not seem to contribute to EBNA2 responsiveness but might still be important in EBNA2 independent transactivation of the LMP1 gene (Johannsen et al. 1995; Sjöblom et al. 1995a; Sjöblom et al. 1995b). The nature of the observed EBNA2 response in the proximal part of LRS (-106 to +40) (Sjöblom et al. 1995a) and the contribution from these elements in LMP1 regulation has not been investigated previously. This is the subject for this thesis.

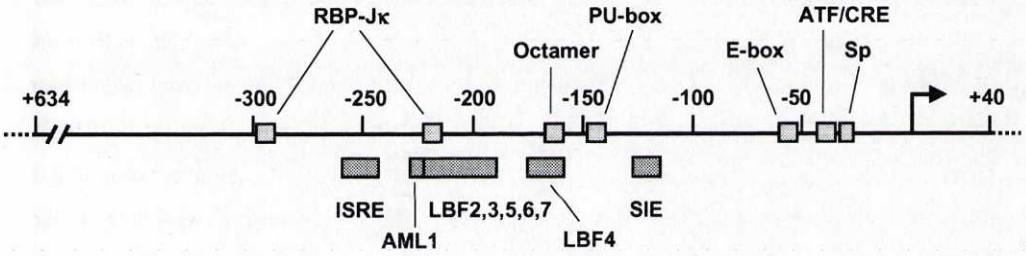

Figure 7. A schematic representation of the LMP1 gene regulatory sequence (LRS) in the B95-8 EBV strain genome. The RBP-Jκ, octamer motif, PU-box, E-box, ATF/CRE and Sp sites are discussed in the text. The ISRE, AML1, SIE and LBF2-7 sites have been identified as protein binding elements but are not believed to participate in EBNA2 response and are not discussed in the text.

THE PRESENT INVESTIGATION

The aim of the present investigation was to increase our knowledge about the mechanisms by which the LMP1 gene is regulated in B-cells and the roles of the EBNA2 transcription factor in particular.

Paper I

The region near the transcription initiation site in the LMP1 transcription regulatory sequence (LRS) (Fåhraeus et al. 1990) contains a putative TATA-box and an activating transcription factor or cAMP responsive element (ATF/CRE) (Ghosh and Kieff 1990). Between these two *cis*-acting elements was a GC-rich sequence observed matching the consensus motif for the binding site of the ubiquitous transcription factor Sp1. The aim of our investigation was to reveal the significance of these two elements and the possible involvement of cAMP in the regulation of the LMP1 promoter.

A 5'-deletion and mutational analyses of the LRS promoter proximal region linked to the CAT reporter gene was performed. The analyses of the ATF/CRE-site in three different LRS constructs (pgLRS-55CAT, pgLRS-259CAT and pgLRS-634CAT) showed that the ATF/CRE-site was important for both EBNA2 dependent and EBNA2 independent activation of the LMP1 promoter in transient transfection experiments in the EBV negative B-cell line DG75. In addition, co-transfection of LRS constructs with a plasmid encoding the CREB antagonist CREMβ caused a significant reduction of LRS-CAT activity, demonstrating that a factor described to bind specifically to ATF/CRE affects LRS promoter activity. On the other hand the deletion analysis including the Sp site (pgLRS(-41)CAT) in the reporter construct did not show any detectable activity and co-transfection of the constitutively active pgLRS(-54)CAT (Fåhraeus et al. 1990; Fåhraeus et al. 1993) with an Sp1 expression vector into the Sp1-negative Schneider cells did not change the activity of the construct. As these result did not support a significant role for the Sp factors in LMP1 regulation our investigation was focused on the ATF/CRE site.

In line with the assumption that LRS contains a functional cAMP responsive element, cAMP was demonstrated to activate the promoter in the absence of EBNA2 in transient transfections in DG75 cells. This effect was dependent on an intact ATF/CRE. cAMP could also increase

LMP1 protein expression in the EBV positive cell lines Cherry and B95-8. However, cAMP treatment of the EBV positive but EBNA2-deficient B-cell line P3HR1 did not stimulate expression of LMP1, indicating that differences in the cellular context influence the ability for LMP1 to be induced by cAMP. In these experiments we also saw that cAMP markedly inhibited DG75 cell proliferation, although it did not kill the cells as determinded by trypan blue exclusion. This was not observed in the Cherry or B95-8 cell lines. Interestingly, DG75 cells stably transfected with an LMP1 expression vector were protected from the inhibitory effect of cAMP on cell proliferation.

It is known that serine/threonin protein phosphateses play an important role in the cAMP signalling pathway and that transcription factors binding to ATF/CRE are phosphoproteins, regulated through phosphorylation of specific serine or threonine residues (Karin and Smeal 1992; Mumby and Walter 1993). To test whether protein phosphatases are involved in regulation of the ED-L1 promoter DG75 cell transfected with LRS reporter constructs were treated with the PP1 and PP2A specific inhibitor okadaic acid (Holmes and Boland 1993). This showed a low but significant increase of LRS activity and this effect was dependent on an intact ATF/CRE. EBNA2 has previously been shown to associate with a protein of 31 kDa (Randahl et al. 1992), which is approximately the size of the catalytic subunit of PP1 and PP2A (Cohen 1989). Furthermore, several other transforming viruses encode transcriptional activators that have been shown to bind and inhibit protein phosphatases (Pallas et al. 1990; Yang et al. 1991; Kleinberger and Shenk 1993). This made us investigate whether EBNA2 could interact with PP1 and PP2A. Cell extracts from the EBNA2 expressing lymphoblastoid cell line Cherry or the EBV negative B-cell line DG75 were fractionated by immunoaffinity chromatography with anti-EBNA2 antibodies as described previously (Dillner et al. 1988). Microcystin labeled with ^{125}I was used for detection. Microcystin interacts specifically with the catalytic subunit of PP1 and PP2A (Holmes and Boland 1993). With this approach we could see that PP1/PP2A activity co-purified with EBNA2 from Cherry extracts. No PP1/PP2A activity was detected in the corresponding fractions from DG75 extracts. Furthermore, recombinant EBNA2 in fusion with the glutathione S-transferase (GST) protein could specifically interact with PP1/PP2A in DG75 cell extracts while GST alone could not. This recombinant EBNA2 could also inhibit a PP1-like activity in nuclear extracts from DG75 cells. The interaction and inhibition were restricted to amino acid 324-436 in the EBNA2 protein, which overlaps with regions in EBNA2 known to be essential in both transformation of B-cells and promoter transactivation (Cohen et al. 1991; Sjöblom et al. 1995). This

suggests that this EBNA2 interaction with and inhibition of a PP1-like activity might play an important role in EBNA2's effect on LMP1 gene transcription, but could of course also be involved in other cellular processes where both EBNA2 and protein phosphatase activity are important.

Paper II

The next step in our investigation was to identify the factors binding to the identified regulatory elements in the proximal part of LRS. However, the first binding study using the electrophoretic mobility shift assay (EMSA) revealed that the specific mutation of the ATF/CRE site used in Paper I prevents the binding of factors both to the Sp site and the ATF/CRE site. To assess the relative contribution of the two binding sites to the promoter activity, new LRS reporter plasmids were created with specific mutations of the Sp and ATF/CRE motifs in pgLRS(-106)CAT and pgLRS(-634)CAT, respectively and co-transfected with EBNA2 in DG75 cells. The results showed that both sites are important for the EBNA2 dependent transactivation of the LMP1 promoter. RNase protection assay (RPA) was performed to confirm that the observed transactivation of the ED-L1 promoter was due to correct initiation.

Factors in DG75 cells binding to the Sp and ATF/CRE sites were then characterised with EMSA. Five specific complexes were identified in DG75 cells. Similar binding patterns were obtained with both EBV-negative and EBV-postive B-cell extracts as well as with extracts from T-cells and epithelial cells. Two complexes were shown to be Sp related and three ATF/CRE related as determined with competition with probes containing either the consensus for Sp or ATF/CRE and LRS probes with either Sp or ATF/CRE mutated. To identify which members in the Sp and ATF/CREB transcription factor families that might be involved in the formation of the complexes, antibody super shift analysis were performed. One of the Sp related complexes was shifted by an anti-Sp1 antibody and two complexes with an anti-Sp3 antibody. One of the Sp3-containing complexes was hidden behind the much stronger band corresponding to the Sp1 complex and therefore became evident only when the anti-Sp1 and anti-Sp3 antibodies were added simultaneously. The analysis of the three ATF/CRE related complexes revealed that they contained two different transcription factor heterodimers. Two complexes were shifted by both an anti-CREB and an anti-ATF1 antibody. The third ATF/CRE related complex was removed by both an anti-ATF2 and an anti-c-Jun antibody and shifted by another anti-c-Jun antibody.

To assess the ability for the different transcription factors that bound Sp and ATF/CRE in LRS to transactivate the LMP1 promoter transient transfection experiments were performed. Expression vectors encoding the different transcription factors were co-transfected with pgLRS(-106)CAT with or without EBNA2. The Sp1 transcription factor induced a low level of activation in absence of EBNA2 but it did not add to the activity of the pgLRS(-106)CAT induced by EBNA2, although mutation of the Sp site largely abolished promoter activity. The Sp3 transcription factor has been shown to function as a repressor of Sp1-mediated transcriptional activation (Hagen et al. 1994). This suggests that Sp1 can activate the LMP1 promoter independently of EBNA2 and that this activity might be modulated by the Sp3 transcription factor and that the integrity of the Sp site is important for EBNA2 dependent activation of the LMP1 promoter. The ATF1 and CREB transcription factors could both separately and together activate the LMP1 promoter in the absence of EBNA2 and this effect was just as strong as the EBNA2 dependent activation of the LMP1 promoter. The effect was ATF/CRE dependent as shown by reduced or abolished activity when the ATF/CRE was mutated. No additional activation was detected when EBNA2 was co-transfected with ATF1, CREB. The ATF2 and c-Jun transcription factors, on the other hand, did not activate the LMP1 promoter in absence of EBNA2 neither alone nor together. Co-expression of either ATF2 or c-Jun together with EBNA2 showed only a weak increase of the promoter activity. However, co-expression of both ATF2 and c-Jun with EBNA2 gave a strong activation in a ATF/CRE dependent fashion.

The EBNA2 dependent activation with ATF2 and c-Jun was in line with the observation that *in vitro* translated EBNA2 abrogated the binding of *in vitro* translated heterodimeric c-Jun and ATF2 to the ATF/CRE in EMSA experiments. EBNA2 did not affect the respective homodimeric forms of ATF2 or c-Jun, suggesting that EBNA2 interacted with a heterodimeric ATF2/c-Jun complex. This notion gained further support by immunoprecipitation experiments. An EBNA2 expression vector was transfected together with expression vectors for c-Jun and ATF2 in DG75 cells. The transfected cells were selected with anti-CD2 antibodies. After lysis of the cells, the proteins were immuno-precipitated with specific antibodies and visualised with immunoblot. The results showed that EBNA2 co-precipitated with both c-Jun and ATF2 when anti-c-Jun or anti-ATF2 antibodies were used. This suggests that the interaction of EBNA2 with ATF2 and c-Jun might be a step in the transactivation of the LMP1 promoter. We have speculated (Paper I) that the ability of

EBNA2 to interact with and inhibit a factor with PP1-like activity might play an important role for the stimulatory effect of EBNA2 on LMP1 gene transcription. Since the identified LRS-ATF/CRE binding factors are regulated through phosphorylation and dephosphorylation we wanted to answer the question if EBNA2 could change the level of phosphorylation of these transcription factors. This experiment was done in DG75 cells transfected with EBNA2 or a control vector after selection of transfected cells. Immunoblots were performed with specific antibodies against phosphorylated ATF2, c-Jun, CREB and ATF1. The assay showed no significant EBNA2-induced changes in the phosphorylation level of any of the tested transcription factors in this cell line. It should, however, be noted that the endogenous level of phosphorylation of ATF2 is high in DG75 cells.

Paper III

In the third paper we moved our focus towards histone acetylation and its possible involvement in LRS regulation. The reason was an investigation in our laboratory showing that elements in the proximal part of the LMP1 promoter were involved in silencing of transcription. Inhibition of histone deacetylase activity with Trichostatin A (TSA) in EBV positive but EBNA2 negative cell lines activated LMP1 protein expression (Sjöblom-Hallén et al. 1999). Furthermore, it became clear from the literature that several transcriptional regulators possess histone acetyltransferase or deacetylase activity or the ability to recruit these activities to promoters resulting in modulation of transcription. In order to study histone acetylation at the LMP1 promoter during EBNA2 activation, we used the ER/EB2-5 cell line and the chromatin immuno-precipitation (ChIP) assay. The lymphoblastoid cell line ER/EB2-5 is conditional for EBNA2 localisation to the nucleus, and the function of EBNA2 is strictly dependent on oestrogen (figure 8). Withdrawal of oestrogen from the medium results in down regulation of LMP1 expression and cell cycle arrest. (Kempkes et al. 1995a). The ChIP assay (Braunstein et al. 1996) with specific antibodies directed against the acetylated forms of histone H3 and histone H4 in combination with a sensitive quantitative PCR allowed us to investigate the effects of EBNA2 and Trichostain A on histone acetylation at the LMP1.

The ER/EB2-5 cells were treated with β-estradiol to activate EBNA2 or with TSA to inhibit deacetylase activity and harvested during a time course of 24 hours. The subsequent ChIP assay showed a clear increase in acetylation level of histone H3 and H4 at the LMP1 promoter

already 30 minutes after either EBNA2 activation or TSA treatment. This increase was independent of *de novo* protein synthesis as determined by cyclohexamid treatment (CHX). RNase protection analysis (RPA) was performed to investigate if increased histone acetylation also resulted in transcriptional activation of the LMP1 ED-L1 promoter. The experiment showed that EBNA2 but not TSA treatment activated transcription of the promoter in resting ER/EB2-5 cells in spite of the observed increase in histone acetylation at the promoter. The results were also in contrast to those obtained previously with the EBV positive but EBNA2 negative BL lines P3HR1 and Daudi, where TSA could induce LMP1 protein expression. ChIP assay and RPA analysis of TSA treated P3HR1 cells verified the latter finding, where both increased histone acetylation at the promoter and induced transcription could be seen. Taken together, the results indicated that histone acetylation precedes transcription of the LMP1 gene in absence of *de novo* protein synthesis but additional events are also needed to activate the LMP1 transcription in resting ER/EB2-5 cells. This is in contrast to the situation in proliferating P3HR1 cells, where inhibition of histone deacetylation alone can induce activation.

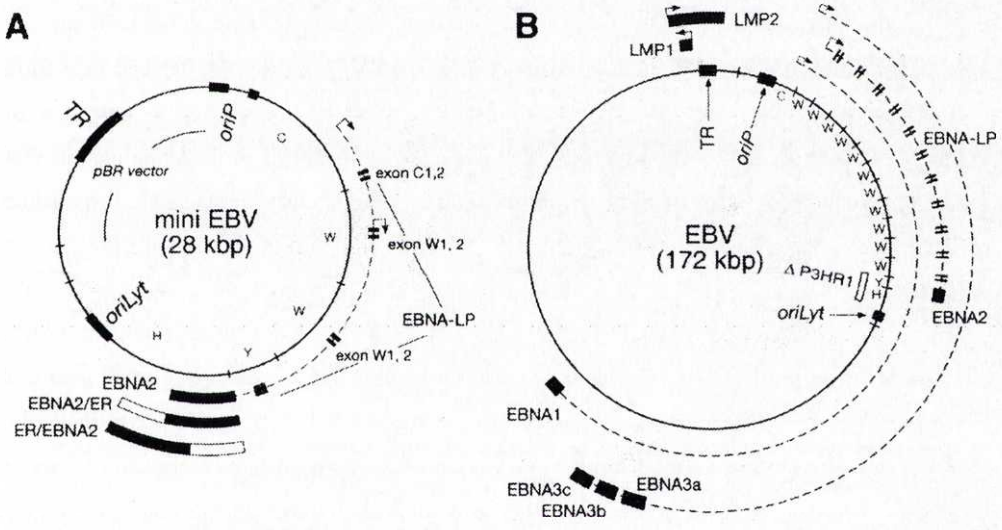

Figure 8. The ER/EB2-5 cell line is established by infection of primary B-cells with the EBNA2-deficient P3HR1 virus strain (B) complemented by an EBNA2-oestrogen receptor fusion construct (A). The function of this ER/EBNA2 fusion protein is strictly dependent on oestrogen. Oestrogen withdrawal leads to inactivation of EBNA2 followed by down regulation of the LMP1 promoter and cell cycle arrest. Reproduced from (Kempkes et al. 1995c). The alternative nomenclature for the EBNA3-6 is used in the figure. See table 2.

To investigate possible common regulatory elements involved in histone acetylation we compared TSA and EBNA2 activation of the LMP1 promoter in a transient transfection assay. This was done in DG75 cells transfected with reporter plasmids containing 5'-end deleted LRS fragments together with EBNA2 expression vector or a control vector. TSA was added 24 hours after transfection and cells were harvested 24 hours later. Both TSA and EBNA2 could activate the promoter when the ATF/CRE was included in the reporter plasmid. A further increase in activity was detected up to –106 relative to the initiation site. The more distal elements in LRS participated only in an EBNA2 dependent fashion. This showed that TSA could up-regulate the LMP1 promoter in a reporter plasmid and that the proximal part of the promoter is sufficient for activation of the LMP1 promoter for both TSA and EBNA2 in proliferating B-cells. Two of the elements in the proximal part of the LMP1 promoter were considered as potential candidates for involvement in regulation of histone acetylation, the ATF/CRE-site described in paper I and II and an E-box element (Sjöblom-Hallén et al. 1999). Mutational analysis of these elements were therefore performed. Reporter constructs with either of these mutations, LRS-634(CREmut)luc or LRS-634(E-boxmut)luc were transfected into DG75 cells together with EBNA2 expression vector or a control vector. TSA was added 24 hours after transfection and cells were harvested 24 hours later. This experiment showed that a mutation in the ATF/CRE decreased both the EBNA2 and the TSA induced activation almost to background level. Mutation in the E-box element did not relieve the repression of transcription from the LMP1 promoter, in the absence of EBNA2 or TSA, indicating that additional histone deacetylase activity associated to other regulatory elements are responsible for the repression of the promoter, e.g. the RPP-Jk response elements (Zhou et al. 2000).

Since the ATF/CRE-site was vital in both EBNA2 and TSA induced activation of LMP1 transcription in proliferating B-cells and as the previously identified factors binding to this element (paper II) are regulated through phosphorylation, we wanted to investigate the phosphorylation status of these factors in resting ER/EB2-5 cells and study them after TSA or β-estradiol stimulation. We used phosphospecific antibodies directed against ATF2 and c-Jun. After β-estradiol treatment and subsequent EBNA2 activation the immuno-blot clearly showed an increase in both phosphorylation and total amount of ATF2 while c-Jun did not show any changes of neither phosphorylation nor total amount of protein. The increase in phosphorylation was evident after approximately 2 hours, which coincide with detectable amounts of transcripts from the ED-L1 promoter as determined with RPA. However, TSA

treatment also led to a weak increase in ATF2 phosphorylation seen approximately after 2 hours, but without detectable amounts of transcripts initiated from the ED-L1 promoter.

To further determine the role for phosphorylation of ATF2 in EBNA2 dependent activation of the LMP1 promoter we performed transient transfection experiments in DG75 cells. A reporter plasmid containing pgLRS(-106)CAT, was co-transfected together with expression vectors for c-Jun, ATF2 or ATF2mut($Ala^{69,71}$) in the presence or absence of EBNA2. This assay showed that mutation of the phosphorylated residues Thr69 and Thr71 in the ATF2 protein decreased its ability to activate the LMP1 promoter.

DISCUSSION

To understand Epstein-Barr virus pathogenesis it is important to unravel the mechanisms involved in B-cell transformation and growth control during infection. The LMP1 is central in this understanding. Several of the viral latent gene products are involved in the regulation of the LMP1 gene, where EBNA2 plays the leading role. The cellular context is obviously important, since a number of cellular factors bind to the LRS and participate in LMP1 regulation (figure 7). In this thesis data are presented that suggest that the proximal part of the promoter is important in both the EBNA2 dependent and independent regulation of the LMP1 gene.

Significance of the Sp element in LMP1 gene regulation

The Sp factor-binding element is one of the most widely distributed promoter elements in cellular and viral genes. Several different Sp protein have been identified (Hagen et al. 1992; Kingsley and Winoto 1992). Mutational analysis and EMSA binding studies showed that an intact Sp element at position –33 in LRS is required for efficient transactivation of the LMP1 promoter, both in absence and presence of EBNA2 (Paper II). The Sp transcription factor family has both activating (Sp1, Sp2 and Sp4) and repressing (Sp3) members. Our data showed that at least Sp1 and Sp3 can bind to the –33 Sp site. Over-expression of the Sp1 factor had only a very small effect on promoter activity in absence of EBNA2 which did not increase in the presence of EBNA2. This was presumably due to the high abundance of endogenous Sp1 protein in DG75 cells. Transfection of Sp1 into the Sp1-negative Schneider cells did not induce additional LRS-derived activity (data not shown). It seems reasonable to

propose that the Sp element is essential for LMP1 gene activity but the site does not seem to be responsible for the EBNA2 responsiveness of the proximal part of the promoter. The balance between Sp1 and Sp3 might be important for determining the final level of LMP1 promoter activity.

Significance of the ATF/CRE element in LMP1 gene regulation

The ATF/CRE motif belongs to one of the major classes of regulatory elements that participate in transcriptional regulation induced by extracellular signals. Transcription regulators belonging to the ATF/CREB family bind to the ATF/CRE site as do members in the Fos/Jun transcription factor family (Hai et al. 1989). The latter factors preferentially bind to AP-1 binding sites or TPA responsive elements (TRE) (Angel and Karin 1991) but the consensus sequence for ATF/CRE differs with only one nucleotide from that of TRE. The ATF/CREB and Fos/Jun families constitute a superfamily where the different members can form cross-family heterodimers and thereby become able to bind to each other's recognition sites (Hai and Curran 1991). The factors activate transcription of target genes in response to a diverse array of stimuli, including peptide hormones and growth factors but are also utilised by viruses to induced modulation of transcription (Flint and Jones 1991; Sheng et al. 1991; Delmas et al. 1994; Mogensen and Paludan 2001). The factors are activated by a variety of protein kinases including protein kinase A (PKA), mitogen-activated protein kinases (MAPKs), stress activated protein kinases (SAPK) and Ca2+/calmodulin-dependent protein kinases (CaMKs). These kinases phosphorylate the transcription factors at particular residues and phosphorylation is necessary to mediate a response.

We have established the importance of an ATF/CRE motif in the regulation of the LMP1 promoter by both mutational and functional analysis (Paper I-III). Mutations of the ATF/CRE decreased both EBNA2 dependent and independent LMP1 promoter activity in transient transfection experiments in DG75 cells. This was also observed after transfection of the ATF/CRE binding antagonist CREMβ. This effect was dependent on an intact ATF/CRE sequence. The LMP1 promoter was activated by cAMP both in transiently transfected cells and in EBV positive LCLs. The location of the ATF/CRE site at position –41 and the high turnover of the LMP1 gene product correlate well with properties of other cAMP regulated genes (Roesler et al. 1988). Studies on signalling transduction in the epithelial cell line HeLa have shown that the LMP1 promoter can be activated by the PKA pathway in the absence of EBNA2 (A. Jansson *et al.*, unpublished investigation), indicating that PKA is sufficient for

activation of the LMP1 gene in epithelial cells. It is previously known that increase in cAMP levels results in growth retardation in some transformed B-cell lines (Friedman 1982). This was also seen in DG75 cells in our assays. Interestingly, LMP1 expression seemed to confer resistance to the inhibition of proliferation by cAMP. This might be relevant *in vivo* due to the importance of the cAMP signalling pathways in differentiation and survival of B-cells, where increased cAMP levels lead to apoptosis in resting B-cells as well as in germinal centre B-cells (Knox et al. 1993; Newell et al. 1993). Possibly, EBV needs to respond to such apoptotic signals by up-regulation of the LMP1 gene and the ATF/CRE site would provide a direct link between cell signal and viral response.

EMSA binding studies of the ATF/CRE site in LRS showed that a heterodimers consisting of CREB and ATF1 subunits could bind to the element (paper II). CREB and ATF1 are both regulated by PKA and PP1 or PP2A (Hagiwara et al. 1993; Wadzinski et al. 1993). However, over-expression of CREB and ATF1 showed that both factors induced LMP1 promoter activity in DG75 cells in the absence of EBNA2. Co-expression of EBNA2 gave no additional effect. This suggests that these factors are not responsible for the EBNA2 responsiveness. Instead, it is probable that they are involved in EBNA2 independent activation of LMP1 expression in both B-cells and epithelial cells. Previous investigations have shown that activation of the protein kinase C (PKC) pathway by cross-linking of cell surface immunoglobulins (Rowe et al. 1992), treatment with the phorbol ester TPA or n-butyrate (Rowe et al. 1987; Contreras-Salazar et al. 1990) can induce LMP1 expression in BL-cell lines in the absence of EBNA2. The PKC pathway normally activates transcription through transcription factors binding to TRE sites but CREB and ATF1 binding to ATF/CRE can be regulated by PKC (Xie et al. 1993; Xie et al. 1996). This is compatible with the notion that activation of the PKC pathway might activate LMP1 expression via binding of the CREB and ATF1 transcription factors to ATF/CRE in LRS. We conclude that both the PKA and PKC signalling pathways are involved in LMP1 gene regulation and that at least PKA but possibly also PKC utilises the CREB and ATF1 transcription factors and the ATF/CRE site for activation. These reaction pathways seem to be EBNA2 independent under most circumstances.

Our EMSA binding studies of the ATF/CRE also revealed that a heterodimer between ATF2 and c-Jun could bind to the element (paper II). In contrast to the CREB and ATF1 factors, this heterodimeric factor complex did not induce LMP1 promoter activity in the absence of

EBNA2. However, in the presence of EBNA2, the heterodimeric complex induced a pronounced activating effect on the LMP1 promoter. Furthermore, EMSA and co-immunoprecipitation experiments supported the notion that EBNA2 makes a direct contact with c-Jun and ATF2 as a heterodimer complex (Paper II). EBNA2 interacts with several other transcription regulatory factors that can bind to the LMP1 promoter. Notably, EBNA2 presumably exists as a dimer or multimer (Grässer et al. 1991; Tsui and Schubach 1994). The interacting factors include the RBP-Jk (Ling et al. 1993a; Grossman et al. 1994; Henkel et al. 1994; Waltzer et al. 1994; Zimber-Strobl et al. 1994), the Ets-related PU.1 factor (Laux et al. 1994a; Johannsen et al. 1995; Sjöblom et al. 1995a; Sjöblom et al. 1995b), and an unidentified member of the POU-domain protein family (Sjöblom et al. 1995a). These multiple interactions between DNA-binding transcription factors and EBNA2 are probably needed to form a stable multiprotein complex at the promoter. This give EBNA2 the opportunity to recruit additional co-factors required for transcriptional activation. The latter factors include the co-activators CBP, p300, PCAF (Jayachandra et al. 1999; Wang et al. 2000), members in the RNA polymerase II preinitiation complex (a et al. 1995; Tong et al. 1995b; Tong et al. 1995c; Wu et al. 1996) and the chromatin remodelling machinery (Wu et al. 1996; Wu et al. 2000). In conclusion, our data supports the idea that EBNA2 also utilises the ATF2 and c-Jun factors and the ATF/CRE site for the activation of the LMP1 promoter and that the EBNA2 responsiveness of the promoter in part arises from this element.

EBNA2 and protein phosphorylation in LMP1 gene regulation

In a series of experiments the possible interaction between EBNA2 and protein phosphatase 1 (PP1) or PP2A and ability of EBNA2 to inhibit the phosphatase activity in B-cells were investigated (Paper I). Several transforming viruses encode transcription factors that interact with protein phosphatases (Pallas et al. 1990; Yang et al. 1991; Kleinberger and Shenk 1993; Sontag et al. 1993), showing that viruses have evolved strategies for affecting the response of the infected host cell by modulating the activity of cellular protein phosphatases. We found that EBNA2 bound to and inhibited the activity of a factor displaying a PP1-like activity in the cells. The amino acid residues 324-436 in the EBNA2 protein were sufficient for both binding and inhibition of the PP1-like activity. The enzymatic activity inhibited by EBNA2 was also inhibited by the PP1 specific inhibitor-1 peptide. However, the 324-436 EBNA2 fragment did not inhibit partially purified PP1 from rabbit skeletal muscle (data not shown). This indicates that a targeting factor is present in the DG75 nuclear extracts or that post-translational modifications of the EBNA2 polypeptide are required. The amino acids in

EBNA2 responsible for the interaction and the inhibition of the PP1-like activity partly overlap with domains in EBNA2 important for B-cell transformation and transactivation of the Cp and LMP1 promoter (figure 5). The activation of LRS-derived CAT activity by okadaic acid demonstrates the involvement of PP1 or PP2A activity in the repression of the LMP1 promoter. Therefore, it is tempting to speculate that one of the functions of EBNA2 in the activation of LMP1 expression is to diminish the effect exerted by the protein phosphatases on a certain transcription factor.

Transfection of EBNA2 in DG75 cells did not reveal any effects of EBNA2 on the phosphorylation status of CREB, ATF1, ATF2 or c-Jun (paper II). It should be noted that at least ATF2 was already highly phosphorylated also in the absence of EBNA2 in the cells. However, analysis of the phosphorylation status of these factors during EBNA2 activation of ER/EB2-5 cells (paper III) demonstrated a low level of phosphorylated ATF2 in resting ER/EB2-5 cells which increased significantly after EBNA2 induction. TSA treatment also led to a weak increase in ATF2 phosphorylation in ER/EB2-5 cells apparent after 2 hours. However, the TSA induced increase was only transient, phosphorylation of ATF2 decreased after 4 hours and was below the detection level after 8 hours (data not shown). A low level of phosphorylated ATF2 in the cells might possibly lead to a low activity of the ED-L1 promoter and explain the absence of transcripts initiated from this promoter in ER/EB2-5 cells after TSA treatment. We have also analysed the phosphorylation level of ATF2 in P3HR1 cells. The level of phosphorylated ATF2 turned out to be high both in the untreated P3HR1 cells and cells incubated with TSA for 24 hours (data not shown). Furthermore, mutation of the phosphate-accepting amino acid residues Thr69 and Thr71 in ATF2 abolished the ability of the corresponding expression vector to induce LRS-derived CAT activity in transfected DG75 cells. Taken together, our data strongly support the notion that phosphorylation of ATF2 is required for transcription of LMP1 in B-cells. This might be an effect mediated by EBNA2 via inhibition of PP1, but further studies are necessary to confirm such a notion. We were not able to determine if the phosphorylation of ATF2 depended on *de novo* protein synthesis, since cyclohexamid (CHX) treatment itself increased ATF2 phosphorylation (data not shown) probably due to activation of the p38/MAPK pathway (Zinck et al. 1995). The fact that LMP1 also can activate the p38/MAPK pathway and subsequently induce ATF2 phosphorylation (Eliopoulos et al. 1999b), indicates that an auto-regulatory loop might be involved in LMP1 regulation.

It is also possible that the EBNA2 inhibition of PP1 instead influences other transcription factors at the LMP1 promoter (e.g. RNA pol. II CTD or the PU.1 transcription factor) or other viral or cellular promoters involved in other functions, such as cell cycle regulation and growth control. It was recently reported that EBNA2 can induce $p21^{WAF1}$ expression by promoting p53 phosphorylation (Lin et al. 2000). Both PP1 and PP2A have been shown to dephosphorylate p53 and decrease p53 DNA-binding activity (Takenaka et al. 1995). Notably, a deletion in the EBNA2 region that interacts with PP1 could not activate the $p21^{WAF1}$ promoter (Lin et al. 2000).

Histone acetylation and LMP1 gene regulation

Repression of gene expression is accomplished by several different mechanisms, including covalent modifications like histone deacetylation, and non-covalent modifications of the nucleosome structure that leads to a more restricted chromatin structure. The repression must be removed in order to activate transcription. At least two different elements in LRS have been implicated in silencing of the LMP1 promoter through recruitment of histone deacetylase complexes. The RBP-Jk sites in the distal part of the promoter and an E-box motif (position –56 to –51) in the proximal part of the promoter. The repression mediated by RBP-Jκ on promoters is probably mediated by a co-repressor complex containing CIR, SMRT, Sin3A, SAP30 and the histone deacetylases HDAC1 and HDAC2 (Hsieh et al. 1999; Zhou et al. 2000). The connection between the E-box motif and silencing of the LMP1 promoter has been investigated in our laboratory (Sjöblom-Hallén et al. 1999). Our experiments showed that a Max-Mad1-Sin3A complex binds to the E-box site and probably recruits a co-repressor complex containing HDAC1 and HDAC2. Accordingly, inhibition of histone deacetylase activity with the specific inhibitor Trichostatin A (TSA) activated LMP1 protein expression in the EBV positive cell lines P3HR1 and Daudi that do not express EBNA2 (Sjöblom-Hallén et al. 1999). However, TSA did not activate LMP1 expression in the Rael BL-line. The EBV genome in this cell line is known to be heavily methylated and treatment with the demethylating agent 5-azacytidine induced LMP1 expression (Masucci et al. 1989; Sjöblom-Hallén et al. 1999). Upregulation of LMP1 might to some extent be a secondary effect since 5-azacytidine also upregulated EBNA2 (Masucci et al. 1989).

In paper III we investigated histone acetylation at the LMP1 promoter using the ER/EB2-5 cell line which is conditional for EBNA2 expression (Kempkes et al. 1995a) (figure 8). An advantage of this conditional cell system is the possibility to obtain synchronised and resting

B-cells before the activation of EBNA2 is initiated. The activation leads to induction of EBNA2 responsive genes including the LMP1 promoter, generating a system that to some extent resembles a primary EBV infection. This system have been used in several investigations and has proved to be very useful to unravel details of the mechanisms involved in EBNA2 and LMP1 functions (Kempkes et al. 1995c; Jochner et al. 1996; Kempkes et al. 1996; Kaiser et al. 1999; Gordadze et al. 2001; Spender et al. 2001).

In our studies with the synchronised and resting ER/EB2-5 cells, estrogen-induced EBNA2 activation or TSA treatment almost immediately increased acetylation of the H3 and H4 histones at the LMP1 promoter. However, only EBNA2 was able to induce LMP1 transcription. This was in contrast to the results obtained with proliferating P3HR1 cells, where TSA both increased histone acetylation at the LMP1 promoter and induced LMP1 transcription. This shows that inhibition of histone deacetylation can substitute for EBNA2 with regard to LMP1 expression in proliferating EBV transformed, B-cells not expressing EBNA2, but not in resting B-cells. Thus additional mechanisms for the repression of LMP1 expression must exist in resting B-cells as compared with dividing cells. Resting cells have presumably a much more condensed chromatin structure. The fact that EBNA2 can bind to a subunit of the human Swi/Snf ATPas dependent chromatin remodelling complex suggests that EBNA2 might recruit this machinery to promoters in order to relieve repression (Wu et al. 1996; Wu et al. 2000). Presumably, TSA can not activate this system, since TSA is a rather specific inhibitor of HDACs (Marks et al. 2000). Notably, P3HR1 cells are able to proliferate in the absence of EBNA2, partly due to the presence of a deregulated c-myc gene. It is known that TSA down-regulates and EBNA2 upregulates the normal wild type c-myc gene, which is the one present in ER/EB2-5 cells (Van Lint et al. 1996; Kaiser et al. 1999). These differences suggest that the proliferative phenotype of the cells is a prerequisite for the ability of TSA to activate the LMP1 gene.

A deletion series of LRS to identify elements involved in TSA activation showed that full responsiveness was reached with a construct containing a short segment of the proximal part of the promoter. Mutational analysis of the regulatory elements in LRS showed that TSA activation required an intact ATF/CRE just like EBNA2. However, mutation of the E-box did not eliminate the requirement for EBNA2 or TSA. The absence of such an effect can be explained by the fact that additional histone deacetylase activity associated to other regulatory

elements is responsible for the repression of the promoter, such as the RBP-Jκ response element (Zhou et al. 2000)).

All the transcription factors that can bind to the CRE/ATF element in LRS, interact with co-activators such as CBP, p300 and PCAF and these interactions are required for activation of the promoter, reviewed in (Vo and Goodman 2001). Since CBP, p300, PCAF and ATF2 possess intrinsic HAT activity and have been shown to interact directly or indirectly with EBNA2 (Paper II; Jayachandra et al. 1999; Wang et al. 2000), they could all be involved in the observed increase of histone acetylation at the LMP1 promoter. It has also been shown that over-expression of ATF2/c-Jun and p300 enhance the ability for EBNA2 to up-regulate LMP1 expression (Paper II; Wang et al. 2000). In the case of p300, this is probably accomplished by bridging effects from other transcription factors to the promoter, since the effect was not dependent on the HAT activity of p300 (Wang et al. 2000). This indicates that the histone acetylation at the LMP1 promoter must be achieved by some other transcription factor. The increased phosphorylation of ATF2 seen during activation of resting ER/EB2-5 cells suggested that ATF2 could be one candidate for this. However, increased histone acetylation was seen before the observed increase of phosphorylated ATF2 in the ER/EB2-5 cells. This indicates that at least the early phase of histone acetylation at the LMP1 promoter is performed by some other histone acetyltransferase.

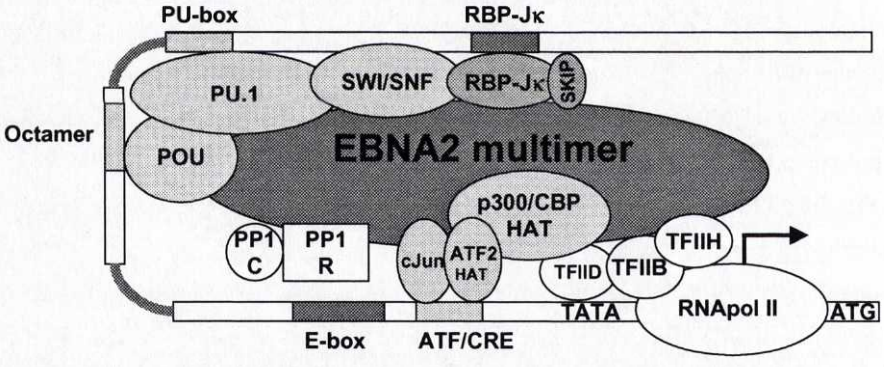

Figure 9. Proposed model for EBNA2 activation of the LMP1 ED-L1 promoter. EBNA2 makes direct contact with transcription factors bound to LRS (RBP-Jκ/SKIP, PU.1/POU protein and c-Jun/ATF). EBNA2 then recruits additional complexes (p300/CBP, Swi/Snf and GTFs) to remodel the chromatin structure and to allow binding of the RNA polymerase II, which leads to activated transcription. EBNA2 might also influence the phosphorylation status of some of the factors involved through inhibition of PP1 (e.g. ATF2) to facilitate transcription.

Concluding remarks

The proposed model for regulation of the LMP1 gene in B-cells is that at least two elements, the E-box and RBP-Jk binding sites, are involved in the silencing of the promoter through recruitment of histone deacetylase activity. However, additional mechanisms are also involved in the repression, since inhibition of histone deacetylase is not sufficient to activate transcription of LMP1 in resting B-cells and some BL-cell lines. These mechanisms probably include both a more condensed chromatin structure and DNA-methylation.

Multiple elements in the LMP1 enhancer and promoter region have been implicated in the activation of the LMP1 gene and have been extensively investigated. However, our current understanding of their relative contribution in the different stages in EBV infection and in malignant disease is still incomplete. Our own results and those of other investigators suggest that at least three regions of LRS and the factors binding to them are needed for appropriate EBNA2 activation: the RBP-Jk binding site, the PU-box and octamer motif and the ATF/CRE region. EBNA2 probably needs to make direct contact with some or all of the factors binding to these elements to create a stable multiprotein complex at the promoter and to remove repressor complexes. EBNA2 also recruits other factor complexes needed for remodelling of the chromatin structure and transcription initiation (figure 9).

The requirement for different regulatory factors both in EBNA2 dependent and independent activation of the LMP1 gene probably varies depending on cell cycle and differential stage but also the cellular context as a whole. The challenge for the future is to understand the combinatorial action of all these different factors and how they influence the expression of the LMP1 gene both in normal infection and in malignant disease. The current development of new techniques where many gene products, both at RNA and protein level, can be studied simultaneously might be the tool to unravel the intricate mechanisms involved.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to my supervisor professor Lars Rymo for his continuos support and guidance and for providing excellent laboratory facilities.

I want to thank all present and former colleagues in the research group for good companionship through out the years and for creating a stimulating atmosphere. A special thanks to Ann Jansson, since this thesis probably would never have been completed if it had not been for her enthusiastic support.

I also want to thank to all other friends and colleagues at the department of Clinical Chemistry and at the department of Medical Biochemistry, where it all started ages ago, for a pleasant time.

Specials thank to Claes Gustafsson and Kerstin Palmqvist for proofreading the manuscript. Writing, especially in English, is a troublesome business.

Finally, to Eva-Lotta, August and Viktor for your endurance, your love and for reminding me that there are more important things in life than work.

REFERENCES

Abbot, S. D., M. Rowe, et al. (1990). "Epstein-Barr virus nuclear antigen 2 induces expression of the virus- encoded latent membrane protein." J Virol **64**(5): 2126-34.

Ait-Si-Ali, S., S. Ramirez, et al. (1998). "Histone acetyltransferase activity of CBP is controlled by cycle- dependent kinases and oncoprotein E1A." Nature **396**(6707): 184-6.

Allday, M. J., D. H. Crawford, et al. (1993). "Epstein-Barr virus (EBV) nuclear antigen 6 induces expression of the EBV latent membrane protein and an activated phenotype in Raji cells." J Gen Virol **74**(Pt 3): 361-9.

Allday, M. J., A. Sinclair, et al. (1995). "Epstein-Barr virus efficiently immortalizes human B cells without neutralizing the function of p53." Embo J **14**(7): 1382-91.

Angel, P. and M. Karin (1991). "The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation." Biochim Biophys Acta **1072**(2-3): 129-57.

Babcock, G. J., L. L. Decker, et al. (1998). "EBV persistence in memory B cells in vivo." Immunity **9**(3): 395-404.

Babcock, G. J. and D. A. Thorley-Lawson (2000). "Tonsillar memory B cells, latently infected with epstein-barr virus, express the restricted pattern of latent genes previously found only in epstein-barr virus-associated tumors." Proc Natl Acad Sci U S A **97**(22): 12250-5.

Babcock, J. G., D. Hochberg, et al. (2000). "The expression pattern of Epstein-Barr virus latent genes in vivo is dependent upon the differentiation stage of the infected B cell." Immunity **13**(4): 497-506.

Baer, R., A. T. Bankier, et al. (1984). "DNA sequence and expression of the B95-8 Epstein-Barr virus genome." Nature **310**(5974): 207-11.

Baichwal, V. R. and B. Sugden (1987). "Posttranslational processing of an Epstein-Barr virus-encoded membrane protein expressed in cells transformed by Epstein-Barr virus." J Virol **61**(3): 866-75.

Baumforth, K. R., L. S. Young, et al. (1999). "The Epstein-Barr virus and its association with human cancers." Mol Pathol **52**(6): 307-22.

Braunstein, M., R. E. Sobel, et al. (1996). "Efficient transcriptional silencing in Saccharomyces cerevisiae requires a heterochromatin histone acetylation pattern." Mol Cell Biol **16**(8): 4349-56.

Brown, C. E., T. Lechner, et al. (2000). "The many HATs of transcription coactivators." Trends Biochem Sci **25**(1): 15-9.

Cen, H., P. A. Williams, et al. (1993). "Evidence for restricted Epstein-Barr virus latent gene expression and anti-EBNA antibody response in solid organ transplant recipients with posttransplant lymphoproliferative disorders." Blood **81**(5): 1393-403.

Chen, F., J. Z. Zou, et al. (1995). "A subpopulation of normal B cells latently infected with Epstein-Barr virus resembles Burkitt lymphoma cells in expressing EBNA-1 but not EBNA-2 or LMP1." J Virol **69**(6): 3752-8.

Cohen, J. I. (1992). "A region of herpes simplex virus VP16 can substitute for a transforming domain of Epstein-Barr virus nuclear protein 2." Proc Natl Acad Sci U S A **89**(17): 8030-4.

Cohen, J. I. (2000). "Epstein-Barr virus infection." N Engl J Med **343**(7): 481-92.

Cohen, J. I. and E. Kieff (1991). "An Epstein-Barr virus nuclear protein 2 domain essential for transformation is a direct transcriptional activator." J Virol **65**(11): 5880-5.

Cohen, J. I., F. Wang, et al. (1991). "Epstein-Barr virus nuclear protein 2 mutations define essential domains for transformation and transactivation." J Virol **65**(5): 2545-54.

Cohen, J. I., F. Wang, et al. (1989). "Epstein-Barr virus nuclear protein 2 is a key determinant of lymphocyte transformation." Proc Natl Acad Sci U S A **86**(23): 9558-62.

Cohen, P. (1989). "The structure and regulation of protein phosphatases." Annu Rev Biochem **58**: 453-508.

Contreras-Salazar, B., B. Ehlin-Henriksson, et al. (1990). "Up regulation of the Epstein-Barr virus (EBV)-encoded membrane protein LMP in the Burkitt's lymphoma line Daudi after exposure to n-butyrate and after EBV superinfection." J Virol **64**(11): 5441-7.

Cordier, M., A. Calender, et al. (1990). "Stable transfection of Epstein-Barr virus (EBV) nuclear antigen 2 in lymphoma cells containing the EBV P3HR1 genome induces expression of B- cell activation molecules CD21 and CD23." J Virol **64**(3): 1002-13.

Cordier-Bussat, M., A. Calender, et al. (1993). "Expression of the Epstein-Barr virus (EBV) latent membrane protein is tightly regulated, independently of EB nuclear antigen 2 and of EBV integration or copy number." Virus Res **27**(1): 55-69.

Delmas, V., C. A. Molina, et al. (1994). "Complexity and versatility of the transcriptional response to cAMP." Rev Physiol Biochem Pharmacol **124**: 1-28.

Devergne, O., E. Hatzivassiliou, et al. (1996). "Association of TRAF1, TRAF2, and TRAF3 with an Epstein-Barr virus LMP1 domain important for B-lymphocyte transformation: role in NF-kappaB activation." Mol Cell Biol **16**(12): 7098-108.

Dillner, J., V. Wendel-Hansen, et al. (1988). "Purification and characterization of the Epstein-Barr virus nuclear antigen 2 using monoclonal antipeptide antibodies." Int J Cancer **42**(5): 721-7.

Eliopoulos, A. G., S. M. Blake, et al. (1999a). "Epstein-Barr virus-encoded latent membrane protein 1 activates the JNK pathway through its extreme C terminus via a mechanism involving TRADD and TRAF2." J Virol **73**(2): 1023-35.

Eliopoulos, A. G., N. J. Gallagher, et al. (1999b). "Activation of the p38 mitogen-activated protein kinase pathway by Epstein-Barr virus-encoded latent membrane protein 1 coregulates interleukin-6 and interleukin-8 production." J Biol Chem **274**(23): 16085-96.

Ernberg, I. and J. Andersson (1986). "Acyclovir efficiently inhibits oropharyngeal excretion of Epstein-Barr virus in patients with acute infectious mononucleosis." J Gen Virol **67**(Pt 10): 2267-72.

Ernberg, I., K. Falk, et al. (1989). "The role of methylation in the phenotype-dependent modulation of Epstein-Barr nuclear antigen 2 and latent membrane protein genes in cells latently infected with Epstein-Barr virus." J Gen Virol **70**(Pt 11): 2989-3002.

Falk, K. I., L. Szekely, et al. (1998). "Specific methylation patterns in two control regions of Epstein-Barr virus latency: the LMP-1-coding upstream regulatory region and an origin of DNA replication (oriP)." J Virol **72**(4): 2969-74.

Farrell, P. J. (1998). "Signal transduction from the Epstein-Barr virus LMP-1 transforming protein." Trends Microbiol **6**(5): 175-7; discussion 177-8.

Farrell, P. J., A. Bankier, et al. (1983). "Latent and lytic cycle promoters of Epstein-Barr virus." Embo J **2**(8): 1331-1338.

Faulkner, G. C., S. R. Burrows, et al. (1999). "X-Linked agammaglobulinemia patients are not infected with Epstein-Barr virus: implications for the biology of the virus." J Virol **73**(2): 1555-64.

Faulkner, G. C., A. S. Krajewski, et al. (2000). "The ins and outs of EBV infection." Trends Microbiol **8**(4): 185-9.

Fennewald, S., V. van Santen, et al. (1984). "Nucleotide sequence of an mRNA transcribed in latent growth- transforming virus infection indicates that it may encode a membrane protein." J Virol **51**(2): 411-9.

Flint, K. J. and N. C. Jones (1991). "Differential regulation of three members of the ATF/CREB family of DNA- binding proteins." Oncogene **6**(11): 2019-26.

Friedman, D. L. (1982). Regulation of the cell cycle and cellular proliferation by cyclic nucleotides. Handbook of experimental pharmacology. J. W. Kebabian and J. A. Nathanson, Springer Verlag. **58:** 151-188.

Fruehling, S. and R. Longnecker (1997). "The immunoreceptor tyrosine-based activation motif of Epstein-Barr virus LMP2A is essential for blocking BCR-mediated signal transduction." Virology **235**(2): 241-51.

Fåhraeus, R., H. L. Fu, et al. (1988). "Expression of Epstein-Barr virus-encoded proteins in nasopharyngeal carcinoma." Int J Cancer **42**(3): 329-38.

Fåhraeus, R., A. Jansson, et al. (1990). "Epstein-Barr virus-encoded nuclear antigen 2 activates the viral latent membrane protein promoter by modulating the activity of a negative regulatory element." Proc Natl Acad Sci U S A **87**(19): 7390-4.

Fåhraeus, R., A. Jansson, et al. (1993). "Cell phenotype-dependent control of Epstein-Barr virus latent membrane protein 1 gene regulatory sequences." Virology **195**(1): 71-80.

Ghosh, D. and E. Kieff (1990). "cis-acting regulatory elements near the Epstein-Barr virus latent-infection membrane protein transcriptional start site." J Virol **64**(4): 1855-8.

Gires, O., F. Kohlhuber, et al. (1999). "Latent membrane protein 1 of Epstein-Barr virus interacts with JAK3 and activates STAT proteins." Embo J **18**(11): 3064-73.

Gires, O., U. Zimber-Strobl, et al. (1997). "Latent membrane protein 1 of Epstein-Barr virus mimics a constitutively active receptor molecule." Embo J **16**(20): 6131-40.

Gordadze, A. V., R. Peng, et al. (2001). "Notch1ic partially replaces ebna2 function in b cells immortalized by epstein-barr virus." J Virol **75**(13): 5899-912.

Gratama, J. W., M. A. Oosterveer, et al. (1988). "Eradication of Epstein-Barr virus by allogeneic bone marrow transplantation: implications for sites of viral latency." Proc Natl Acad Sci U S A **85**(22): 8693-6.

Greenspan, J. S., D. Greenspan, et al. (1985). "Replication of Epstein-Barr virus within the epithelial cells of oral "hairy" leukoplakia, an AIDS-associated lesion." N Engl J Med **313**(25): 1564-71.

Gregory, C. D., C. Dive, et al. (1991). "Activation of Epstein-Barr virus latent genes protects human B cells from death by apoptosis." Nature **349**(6310): 612-4.

Grossman, S. R., E. Johannsen, et al. (1994). "The Epstein-Barr virus nuclear antigen 2 transactivator is directed to response elements by the J kappa recombination signal binding protein." Proc Natl Acad Sci U S A **91**(16): 7568-72.

Grässer, F. A., S. Gottel, et al. (1992). "Phosphorylation of the Epstein-Barr virus nuclear antigen 2." Biochem Biophys Res Commun **186**(3): 1694-701.

Grässer, F. A., P. Haiss, et al. (1991). "Biochemical characterization of Epstein-Barr virus nuclear antigen 2A." J Virol **65**(7): 3779-88.

Hagen, G., S. Muller, et al. (1992). "Cloning by recognition site screening of two novel GT box binding proteins: a family of Sp1 related genes." Nucleic Acids Res **20**(21): 5519-25.

Hagen, G., S. Muller, et al. (1994). "Sp1-mediated transcriptional activation is repressed by Sp3." Embo J **13**(16): 3843-51.

Hagiwara, M., P. Brindle, et al. (1993). "Coupling of hormonal stimulation and transcription via the cyclic AMP- responsive factor CREB is rate limited by nuclear entry of protein kinase A." Mol Cell Biol **13**(8): 4852-9.

Hai, T. and T. Curran (1991). "Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity." Proc Natl Acad Sci U S A **88**(9): 3720-4.

Hai, T. W., F. Liu, et al. (1989). "Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers [published erratum appears in Genes Dev 1990 Apr;4(4):682]." Genes Dev **3**(12B): 2083-90.

Hammerschmidt, W. and B. Sugden (1989). "Genetic analysis of immortalizing functions of Epstein-Barr virus in human B lymphocytes." Nature **340**(6232): 393-7.

Hammerschmidt, W., B. Sugden, et al. (1989). "The transforming domain alone of the latent membrane protein of Epstein- Barr virus is toxic to cells when expressed at high levels." J Virol **63**(6): 2469-75.

Harada, S. and E. Kieff (1997). "Epstein-Barr virus nuclear protein LP stimulates EBNA-2 acidic domain- mediated transcriptional activation." J Virol **71**(9): 6611-8.

Harada, S., R. Yalamanchili, et al. (2001). "Epstein-Barr Virus Nuclear Protein 2 Has at Least Two N-Terminal Domains That Mediate Self-Association." J Virol **75**(5): 2482-2487.

Henderson, S., M. Rowe, et al. (1991). "Induction of bcl-2 expression by Epstein-Barr virus latent membrane protein 1 protects infected B cells from programmed cell death." Cell **65**(7): 1107-15.

Henkel, T., P. D. Ling, et al. (1994). "Mediation of Epstein-Barr virus EBNA2 transactivation by recombination signal-binding protein J kappa." Science **265**(5168): 92-5.

Holmes, C. F. B. and M. P. Boland (1993). "Inhibitors of protein phosphatase-1 and -2A; two of the major serine/threonine protein phosphatases involved in cellular regulation." Curr Opin Struct Biol **3**: 934-943.

Hsieh, J. J. and S. D. Hayward (1995). "Masking of the CBF1/RBPJ kappa transcriptional repression domain by Epstein-Barr virus EBNA2." Science **268**(5210): 560-3.

Hsieh, J. J. D., S. Zhou, et al. (1999). "CIR, a corepressor linking the DNA binding factor CBF1 to the histone deacetylase complex." Proc Natl Acad Sci U S A **96**(1): 23-28.

Hu, L. F., J. Minarovits, et al. (1991). "Variable expression of latent membrane protein in nasopharyngeal carcinoma can be related to methylation status of the Epstein-Barr virus BNLF-1 5'-flanking region." J Virol **65**(3): 1558-67.

Hudson, G. S., P. J. Farrell, et al. (1985). "Two related but differentially expressed potential membrane proteins encoded by the EcoRI Dhet region of Epstein-Barr virus B95-8." J Virol **53**(2): 528-35.

Huen, D. S., S. A. Henderson, et al. (1995). "The Epstein-Barr virus latent membrane protein-1 (LMP1) mediates activation of NF-kappa B and cell surface phenotype via two effector regions in its carboxy-terminal cytoplasmic domain." Oncogene **10**(3): 549-60.

Höfelmayr, H., L. J. Strobl, et al. (2001). "Activated Notch1 Can Transiently Substitute for EBNA2 in the Maintenance of Proliferation of LMP1-Expressing Immortalized B Cells." J Virol **75**(5): 2033-2040.

Höfelmayr, H., L. J. Strobl, et al. (1999). "Activated mouse notch1 transactivates epstein-barr virus nuclear antigen 2-regulated viral promoters." J Virol **73**(4): 2770-80.

Inman, G. J. and P. J. Farrell (1995). "Epstein-Barr virus EBNA-LP and transcription regulation properties of pRB, p107 and p53 in transfection assays." J Gen Virol **76**(Pt 9): 2141-9.

Izumi, K. M. and E. D. Kieff (1997). "The Epstein-Barr virus oncogene product latent membrane protein 1 engages the tumor necrosis factor receptor-associated death domain protein to

mediate B lymphocyte growth transformation and activate NF- kappaB." Proc Natl Acad Sci U S A **94**(23): 12592-7.

Jayachandra, S., K. G. Low, et al. (1999). "Three unrelated viral transforming proteins (vIRF, EBNA2, and E1A) induce the MYC oncogene through the interferon-responsive PRF element by using different transcription coadaptors." Proc Natl Acad Sci U S A **96**(20): 11566-11571.

Jin, X. W. and S. H. Speck (1992). "Identification of critical cis elements involved in mediating Epstein- Barr virus nuclear antigen 2-dependent activity of an enhancer located upstream of the viral BamHI C promoter." J Virol **66**(5): 2846-52.

Jochner, N., D. Eick, et al. (1996). "Epstein-Barr virus nuclear antigen 2 is a transcriptional suppressor of the immunoglobulin mu gene: implications for the expression of the translocated c-myc gene in Burkitt's lymphoma cells." Embo J **15**(2): 375-82.

Johannsen, E., E. Koh, et al. (1995). "Epstein-Barr virus nuclear protein 2 transactivation of the latent membrane protein 1 promoter is mediated by J kappa and PU.1." J Virol **69**(1): 253-62.

Joseph, A. M., G. J. Babcock, et al. (2000). "Cells Expressing the Epstein-Barr Virus Growth Program Are Present in and Restricted to the Naive B-Cell Subset of Healthy Tonsils." J Virol **74**(21): 9964-9971.

Kaiser, C., G. Laux, et al. (1999). "The proto-oncogene c-myc is a direct target gene of epstein-barr virus nuclear antigen 2." J Virol **73**(5): 4481-4.

Karajannis, M. A., M. Hummel, et al. (1997). "Strict lymphotropism of Epstein-Barr virus during acute infectious mononucleosis in nonimmunocompromised individuals." Blood **89**(8): 2856-62.

Karin, M. and T. Hunter (1995). "Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus." Curr Biol **5**(7): 747-57.

Karin, M. and T. Smeal (1992). "Control of transcription factors by signal transduction pathways: the beginning of the end." Trends Biochem Sci **17**(10): 418-22.

Kawasaki, H., L. Schiltz, et al. (2000). "ATF-2 has intrinsic histone acetyltransferase activity which is modulated by phosphorylation." Nature **405**(6783): 195-200.

Kaye, K. M., K. M. Izumi, et al. (1993). "Epstein-Barr virus latent membrane protein 1 is essential for B- lymphocyte growth transformation." Proc Natl Acad Sci U S A **90**(19): 9150-4.

Kaykas, A. and B. Sugden (2000). "The amino-terminus and membrane-spanning domains of LMP-1 inhibit cell proliferation." Oncogene **19**(11): 1400-10.

Kempkes, B., M. Pawlita, et al. (1995a). "Epstein-Barr virus nuclear antigen 2-estrogen receptor fusion proteins transactivate viral and cellular genes and interact with RBP-J kappa in a conditional fashion." Virology **214**(2): 675-9.

Kempkes, B., D. Pich, et al. (1995b). "Immortalization of human B lymphocytes by a plasmid containing 71 kilobase pairs of Epstein-Barr virus DNA." J Virol **69**(1): 231-8.

Kempkes, B., D. Spitkovsky, et al. (1995c). "B-cell proliferation and induction of early G1-regulating proteins by Epstein-Barr virus mutants conditional for EBNA2." Embo J **14**(1): 88-96.

Kempkes, B., U. Zimber-Strobl, et al. (1996). "Epstein-Barr virus nuclear antigen 2 (EBNA2)-oestrogen receptor fusion proteins complement the EBNA2-deficient Epstein-Barr virus strain P3HR1 in transformation of primary B cells but suppress growth of human B cell lymphoma lines." J Gen Virol **77**(Pt 2): 227-37.

Kieff, E. (1996). Epstein-Barr virus and its replication. Fields Virology. B. N. Fields, D. M. Knipe and P. M. Howley. Philadelphia, PA, Lippincott-Raven Publishers. **2:** 2343-2396.

Kieser, A., C. Kaiser, et al. (1999). "LMP1 signal transduction differs substantially from TNF receptor 1 signaling in the molecular functions of TRADD and TRAF2." Embo J **18**(9): 2511-21.

Kieser, A., E. Kilger, et al. (1997). "Epstein-Barr virus latent membrane protein-1 triggers AP-1 activity via the c-Jun N-terminal kinase cascade." Embo J **16**(21): 6478-85.

Kingsley, C. and A. Winoto (1992). "Cloning of GT box-binding proteins: a novel Sp1 multigene family regulating T-cell receptor gene expression." Mol Cell Biol **12**(10): 4251-61.

Kleinberger, T. and T. Shenk (1993). "Adenovirus E4orf4 protein binds to protein phosphatase 2A, and the complex down regulates E1A-enhanced junB transcription." J Virol **67**(12): 7556-60.

Knox, K. A., G. D. Johnson, et al. (1993). "Distribution of cAMP in secondary follicles and its expression in B cell apoptosis and CD40-mediated survival." Int Immunol **5**(9): 1085-91.

Knutson, J. C. (1990). "The level of c-fgr RNA is increased by EBNA-2, an Epstein-Barr virus gene required for B-cell immortalization." J Virol **64**(6): 2530-6.

Kornberg, R. D. (1999). "Eukaryotic transcriptional control." Trends Cell Biol **9**(12): M46-9.

Kulwichit, W., R. H. Edwards, et al. (1998). "Expression of the Epstein-Barr virus latent membrane protein 1 induces B cell lymphoma in transgenic mice." Proc Natl Acad Sci U S A **95**(20): 11963-8.

Laherty, C. D., H. M. Hu, et al. (1992). "The Epstein-Barr virus LMP1 gene product induces A20 zinc finger protein expression by activating nuclear factor kappa B." J Biol Chem **267**(34): 24157-60.

Laux, G., B. Adam, et al. (1994a). "The Spi-1/PU.1 and Spi-B ets family transcription factors and the recombination signal binding protein RBP-J kappa interact with an Epstein-Barr virus nuclear antigen 2 responsive cis-element." Embo J **13**(23): 5624-32.

Laux, G., F. Dugrillon, et al. (1994b). "Identification and characterization of an Epstein-Barr virus nuclear antigen 2-responsive cis element in the bidirectional promoter region of latent membrane protein and terminal protein 2 genes." J Virol **68**(11): 6947-58.

Le Roux, A., B. Kerdiles, et al. (1994). "The Epstein-Barr virus determined nuclear antigens EBNA-3A, -3B, and - 3C repress EBNA-2-mediated transactivation of the viral terminal protein 1 gene promoter." Virology **205**(2): 596-602.

Levitskaya, J., M. Coram, et al. (1995). "Inhibition of antigen processing by the internal repeat region of the Epstein-Barr virus nuclear antigen-1." Nature **375**(6533): 685-8.

Lin, C. S., H. H. Kuo, et al. (2000). "Epstein-barr virus nuclear antigen 2 retards cell growth, induces p21(WAF1) expression, and modulates p53 activity post-translationally." J Mol Biol **303**(1): 7-23.

Ling, P. D., J. J. Hsieh, et al. (1994). "EBNA-2 upregulation of Epstein-Barr virus latency promoters and the cellular CD23 promoter utilizes a common targeting intermediate, CBF1." J Virol **68**(9): 5375-83.

Ling, P. D., D. R. Rawlins, et al. (1993a). "The Epstein-Barr virus immortalizing protein EBNA-2 is targeted to DNA by a cellular enhancer-binding protein." Proc Natl Acad Sci U S A **90**(20): 9237-41.

Ling, P. D., J. J. Ryon, et al. (1993b). "EBNA-2 of herpesvirus papio diverges significantly from the type A and type B EBNA-2 proteins of Epstein-Barr virus but retains an efficient transactivation domain with a conserved hydrophobic motif." J Virol **67**(6): 2990-3003.

Longnecker, R., C. L. Miller, et al. (1992). "The only domain which distinguishes Epstein-Barr virus latent membrane protein 2A (LMP2A) from LMP2B is dispensable for lymphocyte infection and growth transformation in vitro; LMP2A is therefore nonessential." J Virol **66**(11): 6461-9.

Mackey, D. and B. Sugden (1999). "The linking regions of EBNA1 are essential for its support of replication and transcription." Mol Cell Biol **19**(5): 3349-59.

Mann, K. P. and D. Thorley-Lawson (1987). "Posttranslational processing of the Epstein-Barr virus-encoded p63/LMP protein." J Virol **61**(7): 2100-8.

Mannick, J. B., J. I. Cohen, et al. (1991). "The Epstein-Barr virus nuclear protein encoded by the leader of the EBNA RNAs is important in B-lymphocyte transformation." J Virol **65**(12): 6826-37.

Marks, P. A., V. M. Richon, et al. (2000). "Histone deacetylase inhibitors: inducers of differentiation or apoptosis of transformed cells." J Natl Cancer Inst **92**(15): 1210-6.

Masucci, M. G., B. Contreras-Salazar, et al. (1989). "5-Azacytidine up regulates the expression of Epstein-Barr virus nuclear antigen 2 (EBNA-2) through EBNA-6 and latent membrane protein in the Burkitt's lymphoma line rael." J Virol **63**(7): 3135-41.

Mogensen, T. H. and S. R. Paludan (2001). "Molecular pathways in virus-induced cytokine production." Microbiol Mol Biol Rev **65**(1): 131-50.

Montminy, M. (1997). "Transcriptional regulation by cyclic AMP." Annu Rev Biochem **66**: 807-22.

Mosialos, G., M. Birkenbach, et al. (1995). "The Epstein-Barr virus transforming protein LMP1 engages signaling proteins for the tumor necrosis factor receptor family." Cell **80**(3): 389-99.

Mumby, M. C. and G. Walter (1993). "Protein serine/threonine phosphatases: structure, regulation, and functions in cell growth." Physiol Rev **73**(4): 673-99.

Newell, M. K., J. VanderWall, et al. (1993). "Ligation of major histocompatibility complex class II molecules mediates apoptotic cell death in resting B lymphocytes." Proc Natl Acad Sci U S A **90**(22): 10459-63.

Ng, H. H. and A. Bird (2000). "Histone deacetylases: silencers for hire." Trends Biochem Sci **25**(3): 121-6.

Niedobitek, G., A. Agathanggelou, et al. (1997). "Epstein-Barr virus (EBV) infection in infectious mononucleosis: virus latency, replication and phenotype of EBV-infected cells." J Pathol **182**(2): 151-9.

Niedobitek, G., H. Herbst, et al. (1992). "Patterns of Epstein-Barr virus infection in non-neoplastic lymphoid tissue." Blood **79**(10): 2520-6.

Nitsche, F., A. Bell, et al. (1997). "Epstein-Barr virus leader protein enhances EBNA-2-mediated transactivation of latent membrane protein 1 expression: a role for the W1W2 repeat domain." J Virol **71**(9): 6619-28.

Pallas, D. C., L. K. Shahrik, et al. (1990). "Polyoma small and middle T antigens and SV40 small t antigen form stable complexes with protein phosphatase 2A." Cell **60**(1): 167-76.

Qu, L. and D. T. Rowe (1992). "Epstein-Barr virus latent gene expression in uncultured peripheral blood lymphocytes." J Virol **66**(6): 3715-24.

Radkov, S. A., M. Bain, et al. (1997). "Epstein-Barr virus EBNA3C represses Cp, the major promoter for EBNA expression, but has no effect on the promoter of the cell gene CD21." J Virol **71**(11): 8552-62.

Radkov, S. A., R. Touitou, et al. (1999). "Epstein-Barr virus nuclear antigen 3C interacts with histone deacetylase to repress transcription." J Virol **73**(7): 5688-97.

Randahl, H., R. Fahraeus, et al. (1992). "Biochemical characterization of Epstein-Barr virus nuclear antigen 2A and an associated ATPase activity." Eur J Biochem **207**(1): 55-9.

Razin, A. (1998). "CpG methylation, chromatin structure and gene silencing-a three-way connection." Embo J **17**(17): 4905-8.

Rickinson, A. B. and E. Kieff (1996). Epstein-Barr virus. Fields Virology. B. N. Fields, D. M. Knipe and P. M. Howley. Philadelphia, PA, Lippincott-Raven Publishers. **2:** 2397-2446.

Rickinson, A. B. and D. J. Moss (1997). "Human cytotoxic T lymphocyte responses to Epstein-Barr virus infection." Annu Rev Immunol **15**: 405-31.

Robertson, E. S., J. Lin, et al. (1996). "The amino-terminal domains of Epstein-Barr virus nuclear proteins 3A, 3B, and 3C interact with RBPJ(kappa)." J Virol **70**(5): 3068-74.

Roesler, W. J., G. R. Vandenbark, et al. (1988). "Cyclic AMP and the induction of eukaryotic gene transcription." J Biol Chem **263**(19): 9063-6.

Rowe, M., H. S. Evans, et al. (1987). "Monoclonal antibodies to the latent membrane protein of Epstein-Barr virus reveal heterogeneity of the protein and inducible expression in virus-transformed cells." J Gen Virol **68**(Pt 6): 1575-86.

Rowe, M., A. L. Lear, et al. (1992). "Three pathways of Epstein-Barr virus gene activation from EBNA1- positive latency in B lymphocytes." J Virol **66**(1): 122-31.

Sheng, M., M. A. Thompson, et al. (1991). "CREB: a Ca(2+)-regulated transcription factor phosphorylated by calmodulin-dependent kinases." Science **252**(5011): 1427-30.

Sinclair, A. J., I. Palmero, et al. (1994). "EBNA-2 and EBNA-LP cooperate to cause G0 to G1 transition during immortalization of resting human B lymphocytes by Epstein-Barr virus." Embo J **13**(14): 3321-8.

Sjöblom, A., A. Jansson, et al. (1995a). "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." J Gen Virol **76**(Pt 11): 2679-92.

Sjöblom, A., A. Nerstedt, et al. (1995b). "Domains of the Epstein-Barr virus nuclear antigen 2 (EBNA2) involved in the transactivation of the latent membrane protein 1 and the EBNA Cp promoters." J Gen Virol **76**(Pt 11): 2669-78.

Sjöblom-Hallén, A., W. Yang, et al. (1999). "Silencing of the epstein-barr virus latent membrane protein 1 gene by the max-Mad1-mSin3A modulator of chromatin structure." J Virol **73**(4): 2983-93.

Sontag, E., S. Fedorov, et al. (1993). "The interaction of SV40 small tumor antigen with protein phosphatase 2A stimulates the map kinase pathway and induces cell proliferation." Cell **75**(5): 887-97.

Speck, P., K. A. Kline, et al. (1999). "Epstein-Barr virus lacking latent membrane protein 2 immortalizes B cells with efficiency indistinguishable from that of wild-type virus." J Gen Virol **80**(Pt 8): 2193-203.

Spender, L. C., G. H. Cornish, et al. (2001). "Direct and indirect regulation of cytokine and cell cycle proteins by EBNA-2 during Epstein-Barr virus infection." J Virol **75**(8): 3537-46.

Strobl, L. J., H. Hofelmayr, et al. (2000). "Activated Notch1 modulates gene expression in B cells similarly to Epstein-Barr viral nuclear antigen 2." J Virol **74**(4): 1727-35.

Sung, N. S., S. Kenney, et al. (1991). "EBNA-2 transactivates a lymphoid-specific enhancer in the BamHI C promoter of Epstein-Barr virus." J Virol **65**(5): 2164-9.

Szekely, L., G. Selivanova, et al. (1993). "EBNA-5, an Epstein-Barr virus-encoded nuclear antigen, binds to the retinoblastoma and p53 proteins." Proc Natl Acad Sci U S A **90**(12): 5455-9.

Takenaka, I., F. Morin, et al. (1995). "Regulation of the sequence-specific DNA binding function of p53 by protein kinase C and protein phosphatases." J Biol Chem **270**(10): 5405-11.

Tomkinson, B., E. Robertson, et al. (1993). "Epstein-Barr virus nuclear proteins EBNA-3A and EBNA-3C are essential for B-lymphocyte growth transformation." J Virol **67**(4): 2014-25.

Tong, X., R. Drapkin, et al. (1995a). "The 62- and 80-kDa subunits of transcription factor IIH mediate the interaction with Epstein-Barr virus nuclear protein 2." Proc Natl Acad Sci U S A **92**(8): 3259-63.

Tong, X., R. Drapkin, et al. (1995b). "The Epstein-Barr virus nuclear protein 2 acidic domain forms a complex with a novel cellular coactivator that can interact with TFIIE." Mol Cell Biol **15**(9): 4735-44.

Tong, X., F. Wang, et al. (1995c). "The Epstein-Barr virus nuclear protein 2 acidic domain can interact with TFIIB, TAF40, and RPA70 but not with TATA-binding protein." J Virol **69**(1): 585-8.

Tsang, S. F., F. Wang, et al. (1991). "Delineation of the cis-acting element mediating EBNA-2 transactivation of latent infection membrane protein expression." J Virol **65**(12): 6765-71.

Tsui, S. and W. H. Schubach (1994). "Epstein-Barr virus nuclear protein 2A forms oligomers in vitro and in vivo through a region required for B-cell transformation." J Virol **68**(7): 4287-94.

Uchida, J., T. Yasui, et al. (1999). "Mimicry of CD40 signals by Epstein-Barr virus LMP1 in B lymphocyte responses." Science **286**(5438): 300-3.

Wadzinski, B. E., W. H. Wheat, et al. (1993). "Nuclear protein phosphatase 2A dephosphorylates protein kinase A- phosphorylated CREB and regulates CREB transcriptional stimulation." Mol Cell Biol **13**(5): 2822-34.

Waltzer, L., F. Logeat, et al. (1994). "The human J kappa recombination signal sequence binding protein (RBP-J kappa) targets the Epstein-Barr virus EBNA2 protein to its DNA responsive elements." Embo J **13**(23): 5633-8.

Waltzer, L., M. Perricaudet, et al. (1996). "Epstein-Barr virus EBNA3A and EBNA3C proteins both repress RBP-J kappa- EBNA2-activated transcription by inhibiting the binding of RBP-J kappa to DNA." J Virol **70**(9): 5909-15.

Van Lint, C., S. Emiliani, et al. (1996). "The expression of a small fraction of cellular genes is changed in response to histone hyperacetylation." Gene Expr **5**(4-5): 245-53.

Wang, D., D. Liebowitz, et al. (1985). "An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells." Cell **43**(3 Pt 2): 831-40.

Wang, D., D. Liebowitz, et al. (1988). "Epstein-Barr virus latent infection membrane protein alters the human B- lymphocyte phenotype: deletion of the amino terminus abolishes activity." J Virol **62**(11): 4173-84.

Wang, F., C. Gregory, et al. (1990a). "Epstein-Barr virus latent membrane protein (LMP1) and nuclear proteins 2 and 3C are effectors of phenotypic changes in B lymphocytes: EBNA-2 and LMP1 cooperatively induce CD23." J Virol **64**(5): 2309-18.

Wang, F., C. D. Gregory, et al. (1987). "Epstein-Barr virus nuclear antigen 2 specifically induces expression of the B-cell activation antigen CD23." Proc Natl Acad Sci U S A **84**(10): 3452-6.

Wang, F., S. F. Tsang, et al. (1990b). "Epstein-Barr virus nuclear antigen 2 transactivates latent membrane protein LMP1." J Virol **64**(7): 3407-16.

Wang, L., S. R. Grossman, et al. (2000). "Epstein-Barr virus nuclear protein 2 interacts with p300, CBP, and PCAF histone acetyltransferases in activation of the LMP1 promoter." Proc Natl Acad Sci U S A **97**(1): 430-5.

Whitmarsh, A. J. and R. J. Davis (2000). "Regulation of transcription factor function by phosphorylation." Cell Mol Life Sci **57**(8-9): 1172-83.

Wilson, J. B., J. L. Bell, et al. (1996). "Expression of Epstein-Barr virus nuclear antigen-1 induces B cell neoplasia in transgenic mice." Embo J **15**(12): 3117-26.

Vo, N. and R. H. Goodman (2001). "CREB-binding protein and p300 in transcriptional regulation." J Biol Chem **276**(17): 13505-8.

Woisetschlaeger, M., X. W. Jin, et al. (1991). "Role for the Epstein-Barr virus nuclear antigen 2 in viral promoter switching during initial stages of infection." Proc Natl Acad Sci U S A **88**(9): 3942-6.

Workman, J. L. and R. E. Kingston (1998). "Alteration of nucleosome structure as a mechanism of transcriptional regulation." Annu Rev Biochem **67**: 545-79.

Wu, D. Y., G. V. Kalpana, et al. (1996). "Epstein-Barr virus nuclear protein 2 (EBNA2) binds to a component of the human SNF-SWI complex, hSNF5/Ini1." J Virol **70**(9): 6020-8.

Wu, D. Y., A. Krumm, et al. (2000). "Promoter-specific targeting of human SWI-SNF complex by Epstein-Barr virus nuclear protein 2." J Virol **74**(19): 8893-903.

Xie, H., T. C. Chiles, et al. (1993). "Induction of CREB activity via the surface Ig receptor of B cells." J Immunol **151**(2): 880-9.

Xie, H., Z. Wang, et al. (1996). "Signaling pathways for antigen receptor-mediated induction of transcription factor CREB in B lymphocytes." Cell Immunol **169**(2): 264-70.

Yalamanchili, R., X. Tong, et al. (1994). "Genetic and biochemical evidence that EBNA 2 interaction with a 63-kDa cellular GTG-binding protein is essential for B lymphocyte growth transformation by EBV." Virology **204**(2): 634-41.

Yang, S. I., R. L. Lickteig, et al. (1991). "Control of protein phosphatase 2A by simian virus 40 small-t antigen." Mol Cell Biol **11**(4): 1988-95.

Yates, J. L., N. Warren, et al. (1985). "Stable replication of plasmids derived from Epstein-Barr virus in various mammalian cells." Nature **313**(6005): 812-5.

Young, L. S., C. W. Dawson, et al. (1988). "Epstein-Barr virus gene expression in nasopharyngeal carcinoma." J Gen Virol **69**(Pt 5): 1051-65.

Zhou, S., M. Fujimuro, et al. (2000). "A role for SKIP in EBNA2 activation of CBF1-repressed promoters." J Virol **74**(4): 1939-47.

Zimber-Strobl, U., B. Kempkes, et al. (1996). "Epstein-Barr virus latent membrane protein (LMP1) is not sufficient to maintain proliferation of B cells but both it and activated CD40 can prolong their survival." Embo J **15**(24): 7070-8.

Zimber-Strobl, U., E. Kremmer, et al. (1993). "The Epstein-Barr virus nuclear antigen 2 interacts with an EBNA2 responsive cis-element of the terminal protein 1 gene promoter." Embo J **12**(1): 167-75.

Zimber-Strobl, U., L. J. Strobl, et al. (1994). "Epstein-Barr virus nuclear antigen 2 exerts its transactivating function through interaction with recombination signal binding protein RBP-J kappa, the homologue of Drosophila Suppressor of Hairless." Embo J **13**(20): 4973-82.

Zimber-Strobl, U., K. O. Suentzenich, et al. (1991). "Epstein-Barr virus nuclear antigen 2 activates transcription of the terminal protein gene." J Virol **65**(1): 415-23.

Zinck, R., M. A. Cahill, et al. (1995). "Protein synthesis inhibitors reveal differential regulation of mitogen- activated protein kinase and stress-activated protein kinase pathways that converge on Elk-1." Mol Cell Biol **15**(9): 4930-8.

På grund av upphovsrättsliga skäl kan vissa ingående delarbeten ej publiceras här.
För en fullständig lista av ingående delarbeten, se avhandlingens början.

Due to copyright law limitations, certain papers may not be published here.
For a complete list of papers, see the beginning of the dissertation.

GÖTEBORGS UNIVERSITET

ISBN 91-628-4860-7