RBP-J DEPENDENT AND INDEPENDENT SIGNALLING OF EBNA-2

A Thesis Submitted for the Degree of Doctor of Natural Sciences

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Mojim roditeljima

(To my parents)

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LIST OF ABBREVIATIONS

AIDS	acquired immune deficiency syndrome
ATF/CRE	activating transcription factor/ cAMP response element
ATP	adenosine triphosphate
AUF1	AU-rich element- and poly(U)-binding and degradation factor, identical to CBF2
BAC	bacterial artificial chromosome
BALF4	BamHI A fragment leftward open reading frame number 4
BL	Burkitt's lymphoma
BZLF1	BamHI Z fragment leftward open reading frame number 1
Cam	chloramphenicol
CAT	chloramphenicol acetyl transferase
cAMP	cyclic adenosine monophosphate
CBF1	Cp promoter binding factor 1, identical to RBP-J
CBF2	Cp promoter binding factor 2, identical to AUF1
CBP	CREB-binding protein
ChIP	chromatin immunoprecipitation
CIR	CBF1 interacting factor
CMV	cytomegalovirus
CoR	co-repressor complex
CR	conserved region
DEAD	D-E-A-D (Asp-Glu-Ala-Asp)
dNTP	3' deoxyribonucleoside-5'-triphosphate
DIG	digoxygenin
DMSO	dimethyl sulphoxide
DP103	DEAD box binding protein 103
DTT	dithiothreitol
EBER	Epstein-Barr virus encoded RNA
EBNA	Epstein-Barr Virus nuclear antigen
EBNA-LP	Epstein-Barr Virus nuclear antigen leading protein
EBV	Epstein-Barr Virus
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetra-acetic acid
ER	oestrogen receptor
EBNA-2 REs	EBNA-2 responsive elements
FACS	fluorescence assisted cell sorting
FCS	foetal calf serum
FITC	fluorescein-isothiocyanat
Gal	galactosidase
GC	germinal centre
GFP	green fluorescence protein
GRU	green Raji units
H1	histone 1
HA	hemagglutinin
HD	Hodgkin's disease
HDAC	histone deacetylase
HHV4	human herpes virus 4
HIV	human immunodeficiency virus
HRP	horse radish peroxidase
IM	infectious mononucleosis
ISRE	interferon stimulated response element
Kan	kanamycin

kb	kilobase(s)
LB	Luria-Bertani
LCL	lymphoblastoid cell line
LMP	latent membrane protein
LSB	low salt buffer
Luc	luciferase
NK cells	natural killer cells
Notch-IC	Notch-intracellular
NPC	nasopharyngeal carcinoma
oct	octamer
OHL	oral hairy leukoplakia
oriLyt	origin of lytic replication
oriP	origin of plasmid replication
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCAF	p300/CBP-associated factor
PCR	polymerase chain reaction
PE	phycoerythrin
PEG	polyethylene glycol
PP1	protein phosphatase-1
RBP-J	recombination signal binding protein-J, identical to CBF1
RLU	relative light units
RNA	ribonucleic acid
rpm	rounds per minute
RPA70	replication protein A 70
RT	room temperature
SAP	SLAM-associated protein
SDS	sodiumdodecylsulphate
SSC	sodium chloride-sodium citrate buffer
SKIP	ski-interacting protein
SLAM	signalling lyphocyte activation molecule
SMN	survival motor neuron protein
TAE	tris-acetate-EDTA buffer
TAF	TATA box binding protein associated factor
TF	transcription factor
TR	terminal repeats
Tris	tris-hydroxymethyl-aminomethan
vol.	volume
v/v	percentage volume to volume
w/v	percentage weight to volume
XLP	X-linked lymphoproliferative disorder

1. Introduction

1.1. EBNA-2, an important tool of Epstein-Barr Virus

1.1.1. Epstein-Barr virus

Epstein-Barr virus (EBV), or human herpesvirus 4 (HHV4) belongs to the genus Lymphocryptovirus within the subfamily of Gammaherpesviruses. Common features of these viruses are their lymphotropism, their ability to establish a latent infection in host cells and to induce proliferation of the latently infected cells. More than 90% of the world's population carry EBV as a harmless and life-long infection of B-cells (Kieff, 2001).

1.1.2. B-cells are essential for establishment and persistence of EBV infection

In healthy individuals EBV persists latently in circulating memory B-cells (Babcock and Thorley-Lawson, 2000; Miyashita et al., 1995). However, in EBV associated malignancies and other disorders the virus is detected not only in B-cells but also in epithelial cells and more rarely in T and NK cells (Kieff, 2001).
While infection of T- and NK-cells is probably a result of specific circumstances, epithelial cells were suggested to be the first cells that are infected with EBV and thus to participate in the EBV life cycle. The virus cannot be detected reliably in epithelial cells of immunocompetent carriers but infection of epithelial cells might be necessary for efficient infection of B-cells (Borza and Hutt-Fletcher, 2002).
Nevertheless, accumulating evidences indicate that B-cells are essential for the establishment and maintenance of EBV infection. So, it was demonstrated that persistent EBV infection of patients is not maintained when the haematopoietic system is irradiated before transplantation of seronegative bone marrow. Thus, the epithelial sites of the patient are not sufficient to maintain persistent infection. Also, patients with X-linked agamma-globulinaemia, a disorder characterised by a defect in

B-cell maturation, are resistant to EBV infection (Bornkamm and Hammerschmidt, 2001; Faulkner et al., 2000).

1.1.3. EBNA-2 takes part in the initial proliferating phase of EBV life cycle in B-cells

EBV infects B-cells by binding to the cellular surface molecule CD21 (Nemerow et al., 1987; Tanner et al., 1987). Upon infection, the virus drives the resting B-cells into proliferation by expressing the so-called growth programme. The growth programme is a latency programme during which no viral particles are being produced (see figure 1.1.) (Babcock et al., 2000). The growth programme is characterised by expression of six nuclear antigens, including EBNA-2 (EBNA-1, -2, -3A, -3B, -3C and -LP), and three membrane proteins (LMP-1, -2A and -2B). Thus, the EBV induced proliferation enables the virus to increase the number of infected cells not only by infection of new cells, but also by proliferation of infected cells.

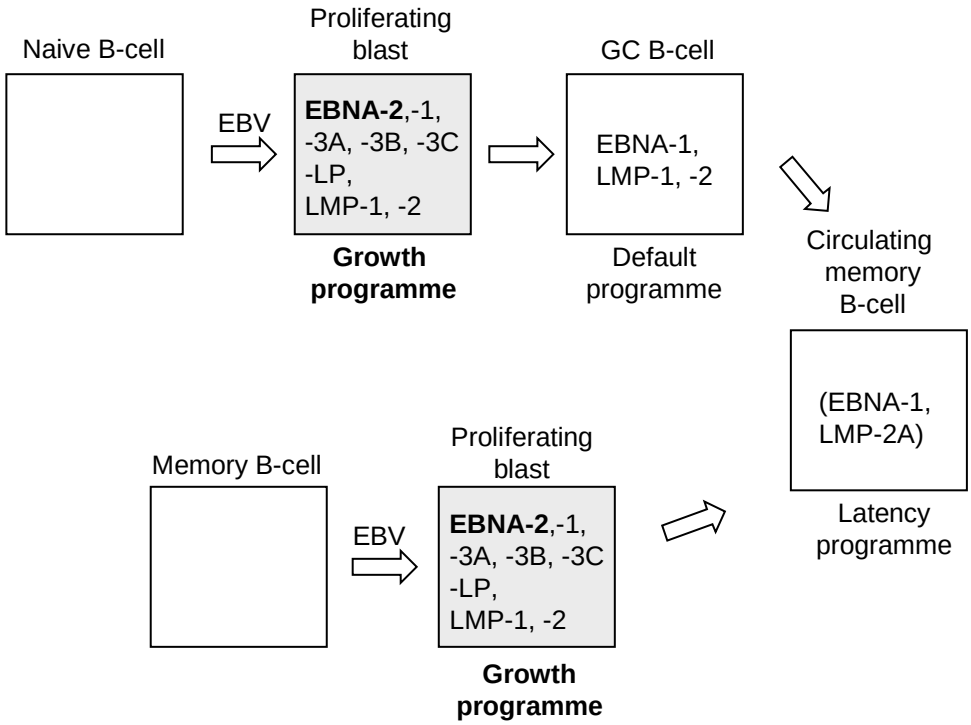

Figure 1.1.
EBV induces proliferation of infected B-cells. The B-cell type and differentiation stage are indicated at the top of boxes. The expressed viral genes and corresponding viral programme are indicated in and beneath the boxes, respectively. For details see text 1.1.3. and 1.1.4. (GC, germinal centre).

The primary B-cell target of EBV is not clear. Both naïve and memory B-cells were shown to be infected with EBV and driven into proliferation by the growth programme in which EBNA-2 is expressed (Babcock et al., 2000; Kurth et al., 2000).

1.1.4. EBV guides proliferation and differentiation of B-cells by multiple latency programmes

The growth programme in which EBNA-2 is expressed and by which EBV enlarges the pool of infected cells is a vulnerable phase of the EBV life cycle, since the expressed latent proteins that drive B-cell proliferation are also highly immunogenic and induce a strong cytotoxic T-cell response (Callan et al., 1996; Callan et al., 1998).

The growth programme is reduced to the so-called default programme found in germinal center (GC) B-cells in which only LMP-1, -2A and -2B are expressed (see figure 1.1.) (Babcock et al., 2000). It was suggested that these viral genes are able to substitute the physiological signals provided by cognate antigen and T-helper cells that are required for a differentiation of a naïve B-cell into a memory B-cell (Thorley-Lawson, 2001). The order and mechanism by which the silencing of the viral genes is accomplished during the transition from the growth to the default programme is not known, but, this is presumably a successive process (reviewed in Middeldorp et al., 2003).

After proliferation and differentiation of B-cells, guided by the growth and default programmes, the viral expression profile is reduced to the latency programme which is found in circulating memory B-cells, the place of viral persistence. In the latency programme none, if any latent gene is expressed, which enables the virus to escape the immune system (Babcock et al., 1998; Miyashita et al., 1995). Finally, the EBV life cycle in B-cells is completed by reactivation of the lytic cycle in resting memory B-cells, presumably via physiological signals such as antigen recognition (Babcock et al., 1998).

In summary, EBNA-2 expression *in vivo* is restricted to a short window of the initial phase of the EBV life cycle in B-cells, when infected B-cells are activated and driven to proliferation.

1.1.5. *In vitro* EBV infection of B-cells: a model to study EBV latent genes

In vitro any resting B-cell can be infected with EBV. The *in vitro* EBV infection is very efficient and results in B-cell proliferation leading to an establishment of so-called lymphoblastoid cell lines (LCLs). The *in vitro* cultivation of lymphocytes from seropositive individuals also leads to outgrowth of LCLs (Rickinson, 2001).
In comparison to primary B-cells, LCL cells are large, activated cells that are irregular in shape and strongly adhere to each other and form large clumps. These phenotypic changes, which accompany B-cell immortalisation, are associated with an entry of the cells into the cell cycle and initiation of continuous proliferation with a doubling time of 20-30 hours.
The only latency programme found in LCLs is the growth programme, which includes the EBNA-2 expression next to other five nuclear (EBNA-1, -3A, -3B, -3C and -LP) and three membrane proteins (LMP-1, -2A and -2B) and small nonpolyadenylated (and therefore non-coding) RNAs EBER1 and 2 (Rickinson, 2001). Thus, the *in vitro* EBV-infection of B-cells is considered to resemble the initial phase of the EBV life cycle, the growth programme, when B-cells are activated and driven into proliferation.

1.1.6. EBNA-2, the central regulator of transcription in latently infected B-cells

EBNA-2 is one of the first genes expressed in EBV infected B-cells *in vitro* (Alfieri et al., 1991; Rooney et al., 1989). It is essential for the establishment of latent infection, growth transformation and maintenance of the immortalised phenotype (Cohen et al., 1989; Hammerschmidt and Sugden, 1989; Kempkes et al., 1995b; Kempkes et al., 1996).
The EBNA-2 protein is localised in the nucleus and is associated with chromatin and nuclear matrix, but it is also present in the nucleoplasma (Grasser et al., 1993; Petti et al., 1990). EBNA-2 activates transcription of both viral and cellular genes. After initial expression of EBNA-2 and EBNA-LP from the Wp promoter EBNA-2 takes over the transcriptional regulation of most latent genes by inducing the Cp, LMP-2A and bi-directional LMP-1/-2B promoters (see figure 1.2.) (Sung et al., 1991; Wang et al., 1990; Woisetschlaeger et al., 1990; Zimber-Strobl et al., 1993).

EBNA-2 upregulates transcription of the cellular proto-oncogene *c-myc*, CD23 and CD21, BLR2/EBI1 and type I interferon and downregulates the immunoglobulin µ gene (Burgstahler et al., 1995; Calender et al., 1987; Cordier et al., 1990; Jayachandra et al., 1999; Jochner et al., 1996; Kaiser et al., 1999; Kanda et al., 1999; Wang et al., 1987). Some EBNA-2 target genes, such as *c-myc* and LMP-1, induce their own target genes leading to a complex transcription cascade downstream of EBNA-2 (Fernandez et al., 2003; Oster et al., 2002). The list of EBNA-2 target genes is still not complete and a systematic search for direct EBNA-2 target genes using a conditional system (Kempkes et al., 1995a) is on the way (Šantak, PhD thesis, Schlee, PhD thesis).

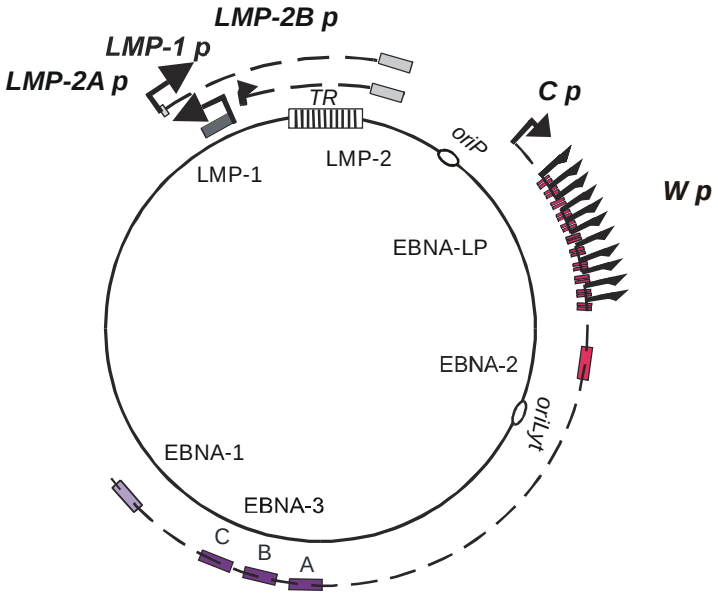

Figure 1.2.
Schematic presentation of the EBV genome in the circularised form as present in EBV immortalised B-cells. EBNA-2 induces transcription from the viral Cp, LMP-1, -2A and -2B promoters and thereby regulates expression of LMP-1, -2A, -2B, EBNA-LP, -1, -2, -3A, -3B and -3C. The transcription start sites and resulting RNAs are depicted with arrows and dashed lines, respectively. Terminal repeats (TR), origin of replication (oriP) and lytic origin of replication (orilyt) are *cis*-elements that enable viral replication and packaging.

1.1.7. EBNA-2 associated disorders

EBNA-2 is expressed in infectious mononucleosis (IM), which is considered to be a clinical form of a primary EBV infection. IM is a self-limiting disease with symptoms

ranging from mild transient fever to several weeks of pharyngitis, lymphadenopathy and general malaise (Rickinson, 2001).

EBNA-2 is also expressed in fatal B-cell lymphoproliferative disorders, which are seen in different groups of immunologically compromised patients. They include post transplant lymphoproliferative disorders, a severe complication in transplant recipients treated with immunosuppressive drugs, and AIDS-related lymphoma. Common to all these tumours is that most of the proliferating B-cells display a lymphoblastoid phenotype resembling B-cells immortalised by EBV *in vitro*.

EBNA-2 expression is also detected in the X-linked lymphoproliferative syndrome (XLP), or fatal mononucleosis that is caused by hereditary mutations in the gene encoding the signalling lymphocyte activation molecule (SLAM)-associated protein (SAP). Individuals that have inherited this trait are usually asymptomatic, but upon primary EBV-infection their immune response becomes overreactive because of the non-functional SAP protein. The SAP protein is a T-cell protein that binds to SLAM protein expressed on both B- and T-cells and acts as a negative regulator of the SLAM induced signalling. Although reports show that the EBV infection triggers the fatal phenotype of XLP, it is not certain that abnormalities in the immune control of EBV particularly are responsible for the disease development (Macsween and Crawford, 2003).

1.1.8. EBV associated diseases

Next to B-cell lymphomas in immunosupressed individuals, where EBNA-2 is expressed, there is a number of EBV associated diseases in which no EBNA-2 expression can be detected.

Burkitt's lymphoma (BL) and Hodgkin's disease (HD) show the strongest association with EBV among lymphoid diseases. EBV expression in HD is reduced to EBNA-1, LMP-1 and -2 and in BL only to EBNA-1 (Rickinson, 2001). So, the viral expression profiles used by EBV as well as the degree of EBV association with a particular disease vary. Early phases of these diseases are not known and possible involvement of other viral expression profiles cannot be excluded. Recent studies of BL indicate that in the initial phases all EBNAs might be active and subsequently turned off (Kelly et al., 2002).

The most prominent epithelial disorders associated with EBV include nasopharyngeal carcinoma (NPC) and oral hairy leukoplakia (OHL). NPC shows a characteristic racial and geographical distribution with the highest incidence among males in Southern China and natives of the Arctic region indicating that numerous factors play a role in the genesis of NPC. In NPC the EBNA-1, LMP-1 and -2A are expressed. OHL is an opportunistic disease in HIV patients in which lesions of tongue epithelium are caused by EBV replication.

1.2. EBNA-2 signalling

1.2.1. EBNA-2 responsive elements

EBNA-2 responsive elements (EBNA-2 REs) have been defined in the viral LMP-1/-2B, -2A and Cp promoters and the cellular CD23 promoter (Jin and Speck, 1992; Laux et al., 1994b; Sung et al., 1991; Tsang et al., 1991; Wang et al., 1987; Zimber-Strobl et al., 1993). The minimal EBNA-2 RE in the LMP-2A and the bidirectional LMP-1/-2B promoters, which are sufficient for conferring EBNA-2 inducibility to a heterologous promoter, encompass 81 bp and 140 bp, respectively (Laux et al., 1994b; Tsang et al., 1991; Zimber-Strobl et al., 1993). EBNA-2 RE from the Cp and CD23 promoter convey EBNA-2 responsiveness only as oligomers (Ling et al., 1994; Ling et al., 1993).

Common to all these defined EBNA-2 REs is the presence of at least one binding site for the cellular DNA-binding protein RBP-J (Tun et al., 1994). RBP-J binds directly to EBNA-2 and is able to tether EBNA-2 to the LMP-2A and Cp promoter (Henkel et al., 1994; Ling et al., 1993; Zimber-Strobl et al., 1993). The mechanism of EBNA-2 transactivation mediated by RBP-J is described in detail in 1.3.4.

However, further analysis conducted on viral promoters showed that EBNA-2 REs are more complex than expected. Therefore, the simple model by which only RBP-J cognate sequences are necessary to direct EBNA-2 to responsive promoters is not sufficient. In the LMP-1 promoter the PU.1 binding site has been unambiguously shown to be vital for the EBNA-2 response, but attempts to coimmuoprecipitate EBNA-2 and PU.1 from cellular extracts have been unsuccessful (Johannsen et al.,

1995; Laux et al., 1994a; Sjoblom et al., 1995a). The interferon stimulated response element (ISRE) and the octamer and ATF/CRE sites in the LMP-1 promoter also contribute to the EBNA-2 responsiveness (see figure 1.3.) (Sjoblom et al., 1995a; Sjoblom et al., 1995b; Sjoblom et al., 1998). The octamer site is recognised by the Oct factors, which belong to a larger family of transcription factors designated POU domain proteins (Phillips and Luisi, 2000; Remenyi et al., 2002). The ATF/CRE sequence motif belongs to one of the major classes of regulatory elements that participate in transcriptional regulation induced by extracellular signals (Hai and Hartman, 2001). ISRE is recognised by interferon regulatory factors (IRF) (Nguyen et al., 1997; Tanaka and Taniguchi, 2000).

The Cp EBNA-2 REs bind AUF1 (also designated as CBF2) in addition to RBP-J (Fuentes-Panana and Ling, 1998; Fuentes-Panana et al., 2000). AUF1 is a cellular sequence-specific DNA binding protein that regulates EBV and cellular promoters (Brys and Maizels, 1994; Tolnay et al., 1997; Tolnay et al., 1999). Although an interaction of AUF1 with EBNA-2 could not be demonstrated, the AUF binding site was shown to significantly contribute to the transactivation mediated by EBNA-2 (Fuentes-Panana et al., 2000).

In summary, EBNA-2 REs described in different viral and cellular promoters include RBP-J binding site(s) but display a remarkable diversity in dependence on additional *cis*-elements. This indicates that EBNA-2 transactivation includes different transactivation mechanisms.

1.2.2. The LMP-2A promoter as a model for RBP-J dependent signalling of EBNA-2

The LMP-1 and LMP-2A promoters are well characterised viral promoters that are strongly inducible by EBNA-2. The LMP-2A promoter contains two RBP-J binding sites at position -219 to -227 bp and -244 to -251 bp relative to the transcription start site, both of which are included in the minimal EBNA-2 RE and can be occupied by the RBP-J protein (Zimber-Strobl et al., 1993) (see figure 1.3.). Binding of RBP-J to both motifs in the LMP-2A promoter is also a prerequisite for the detection of EBNA-2 in the protein/DNA complex (Meitinger et al., 1994). Promoter reporter assays demonstrated the importance of both RBP-J binding sites for the EBNA-2 mediated

transactivation of the LMP-2A promoter although downstream promoter sequences are also required for EBNA-2 inducibility (Meitinger et al., 1994). However, additional cellular factors involved in transactivation of the LMP-2A promoter by EBNA-2 have not yet been identified.

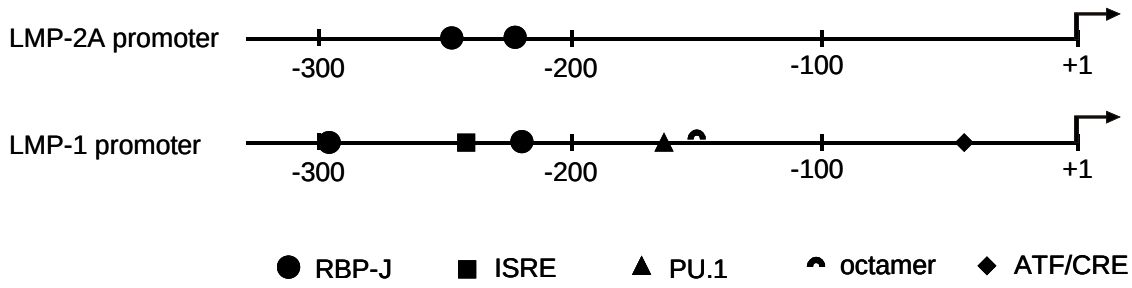

Figure 1.3.
Binding sites for transcription factors in the LMP-2A and LMP-1 promoters that are suggested to participate in transactivation by EBNA-2. Numbers below the promoters indicate positions relative to the transcription start site (+1). See text 1.2.1., 1.2.2. and 1.2.3. for details on the transcription factor binding sites in the LMP-2A and LMP-1 promoter, respectively.

The importance of the RBP-J binding sites for transactivation of the LMP-2A promoter by EBNA-2 was also confirmed by experiments done by Guido Bommer (unpublished data). He constructed dominant negative inhibitors of the RBP-J pathway derived from the Notch protein. Notch is a cellular protein that like EBNA-2 cannot bind to DNA directly (see 1.3.5.). The isolated RBP-J binding domain of Notch can bind to RBP-J and was used to compete with EBNA-2 for binding to RBP-J (Tamura et al., 1995). Guido Bommer demonstrated that in the presence of this dominant negative inhibitor of the RBP-J pathway, EBNA-2 could not transactivate the LMP-2A promoter.

1.2.3. The LMP-1 promoter as a potential model for the RBP-J independent signalling of EBNA-2 in B-cells

The LMP-1 promoter contains two potential RBP-J binding sites at position -298 to

-290 bp and -223 to -213 bp relative to the transcription start site (figure 1.2.). The promoter-distal end of the LMP-1 EBNA-2 RE has been mapped to either -234 bp or -214 bp relative to the transcription start site, thus including either the proximal or none of the RBP-J binding sites (Fahraeus et al., 1990; Tsang et al., 1991). Mutational analysis of the RBP-J binding sites in the LMP-1 promoter also led to controversial conclusions regarding the contribution of these sites to transactivation by EBNA-2. While Laux and Johannsen showed by site-directed mutagenesis that transactivation of the LMP-1 promoter by EBNA-2 is mediated by both RBP-J and PU.1 (Johannsen et al., 1995; Laux et al., 1994a; Laux et al., 1994b), the group of Rymo demonstrated by site-directed mutagenesis and DNase footprint analysis that RBP-J binding sites are not essential for induction of the LMP-1 promoter by EBNA-2 (Fahraeus et al., 1990; Fahraeus et al., 1993; Sjoblom et al., 1995b)

RBP-J binds to the EBNA-2 RE of the LMP-1/-2B with low affinity and the presence of EBNA-2 in protein/DNA complexes at the EBNA-2 RE of LMP-1/-2B could not be demonstrated (Laux et al., 1994a).

EBNA-2 proteins lacking the RBP-J binding domain (see 1.3.4.) or containing mutated tryptophan residues crucial for the EBNA-2 binding to RBP-J could still activate the LMP-1 promoter (Sjoblom et al., 1995b; Yalamanchili et al., 1994).

To determine whether EBNA-2 uses RBP-J to target the LMP-1 promoter Guido Bommer applied the same strategy as in the LMP-2A promoter study (see 1.2.2.). Parts of the RBP-J binding domain of the cellular Notch protein served as dominant negative inhibitors to block binding of EBNA-2 to RBP-J. In contrast to the LMP-2A promoter whose transactivation by EBNA-2 was completely abolished in the presence of the dominant negative Notch mutants, EBNA-2 could still activate the LMP-1 promoter.

1.3. The EBNA-2 protein

1.3.1. EBNA-2 regions important for B-cells immortalisation and transactivation

The EBNA-2 domains important for growth transformation were defined by linker insertion and deletional analyses of the EBNA-2 open reading frame (ORF) in the

past (Cohen et al., 1991; Harada et al., 1998; Tong et al., 1994; Yalamanchili et al., 1994). However, a common consensus scheme integrating all published knowledge has not been evolved until today.

Mutated EBNA-2 ORFs have been compared to wild-type (wt) EBNA-2 for their ability to rescue transformation after transfection into cells infected with the P3HR1 EBV strain. The EBNA-2 ORF (and two adjacent exons of EBNA-LP) is deleted in the P3HR1 EBV strain and this virus is unable to transform cells. When P3HR1 infected cells are transfected with a wt EBV DNA fragment that spans the site of the EBNA-2 deletion, homologous recombination between the transfected DNA and the P3HR1 EBV genome results in a wt EBV genome which can transform primary lymphocytes.

In the described system EBVs containing a deletion in one of the following three specific EBNA-2 regions were unable to induce B-cell growth. These EBNA-2 regions include proline codons 89-95 at the amino terminus and codons 281-336 and 426-462 at the carboxy terminus of EBNA-2. The domains map to several evolutionarily conserved regions (CR) found in EBNA-2 proteins from human and primate lymphocryptoviruses (Peng et al., 2000). The proline residues are part of the CR3 region and were lately found to be dispensible for maintenance of EBV-mediated immortalisation (Gordadze et al., 2002). The role of the proline residues in EBV immortalisation is probably to prevent hyperactivation of viral genes, such as LMP-1, which is cytostatic for B-cells when overexpressed (Gordadze et al., 2002). Region 281-336 include the conserved regions CR5 and CR6 that mediate binding to the cellular proteins SKIP and RBP-J (see 1.3.4.) and are able to deplete PU.1 from cellular extracts (Johannsen et al., 1995). EBNA-2 codons 426 to 462 encompass CR8 and encode an acidic domain that recruits the transcription machinery (see 1.3.3.).

Besides for lymphocyte transformation the mutant EBNA-2 genes were also assayed for the LMP-1 transactivation since LMP-1 is a transforming oncogene in rodent fibroblasts and is also required for B-cell immortalisation (Dirmeier et al., 2003; Kaye et al., 1993; Kilger et al., 1998). Mutations that diminished or abolished lymphocyte transformation also diminished or abolished the LMP-1 transactivation in transient transfections (Cohen et al., 1991; Harada et al., 1998; Tong et al., 1994; Yalamanchili et al., 1994). Nevertheless, cells transformed by recombinant EBV carrying EBNA-2 genes with diminished transforming ability express high levels of LMP-1, CD23 and CD21. These findings suggest that there is a selective pressure for

the lymphocytes to express these genes in order to get immortalised by EBV. Furthermore, these results indicate that the transforming and transactivating functions of EBNA-2 might not be separable.

A comparative study of deletion mutants identified regions of EBNA-2 involved in transactivation of the LMP-1 and Cp promoters (Sjoblom et al., 1995b). Two domains of EBNA-2 defined by deletion of amino acids 247-337 and 437-476 were found to be important for the activation of both promoters, while two different domains corresponding to residues 4-18 and 118-198 were required solely for the LMP-1 promoter indicating that the LMP-1 and Cp promoters are activated by different EBNA-2 transactivation mechanisms.

1.3.2. EBNA-2 associated proteins that bind specifically to DNA

EBNA-2 associated proteins that specifically bind to DNA are a potential link between EBNA-2 and promoters. Thus, the EBNA-2 regions that mediate the contact to these proteins can be considered as promoter targeting domains. This group of EBNA-2 associated proteins includes RBP-J, PU.1, Spi-B, c-Jun/ATF and Nur77, but the only DNA binding protein shown to directly bind and target EBNA-2 to promoters until now is RBP-J. The transactivating mechanism of EBNA-2 mediated by RBP-J is explained in detail in 1.3.4.

PU.1 and Spi-B are ets family proteins important for the regulation of macrophage- and B-cell specific promoters that recognise the PU.1 binding site (reviewed in Oikawa and Yamada, 2003). Although a direct contact between PU.1 or Spi-B and EBNA-2 could not be demonstrated, a broad EBNA-2 region (residues 310-376) has been suggested to mediate the PU.1 contact (Johannsen et al., 1995). The PU.1 binding site is crucial in EBNA-2 mediated transactivation of the LMP-1 promoter (Johannsen et al., 1995; Laux et al., 1994a; Sjoblom et al., 1995a). Binding of EBNA-2 to the c-Jun/ATF heterodimer, which recognises the ATF/CRE site, was not characterised further (Sjoblom et al., 1998).

EBNA-2 binding to Nur77 is mediated by the residues 123-147 and was first described in the context of apoptosis blockage (Lee et al., 2002). But, Nur77 is also a transcription factor that binds to specific DNA motifs (Katagiri et al., 2000; Philips et

al., 1997). Whether the DNA binding of Nur77 is also exploited by EBNA-2 is still an open question.

Regions in the primary structure of EBNA-2 that mediate the described contacts are depicted in figure 1.4.

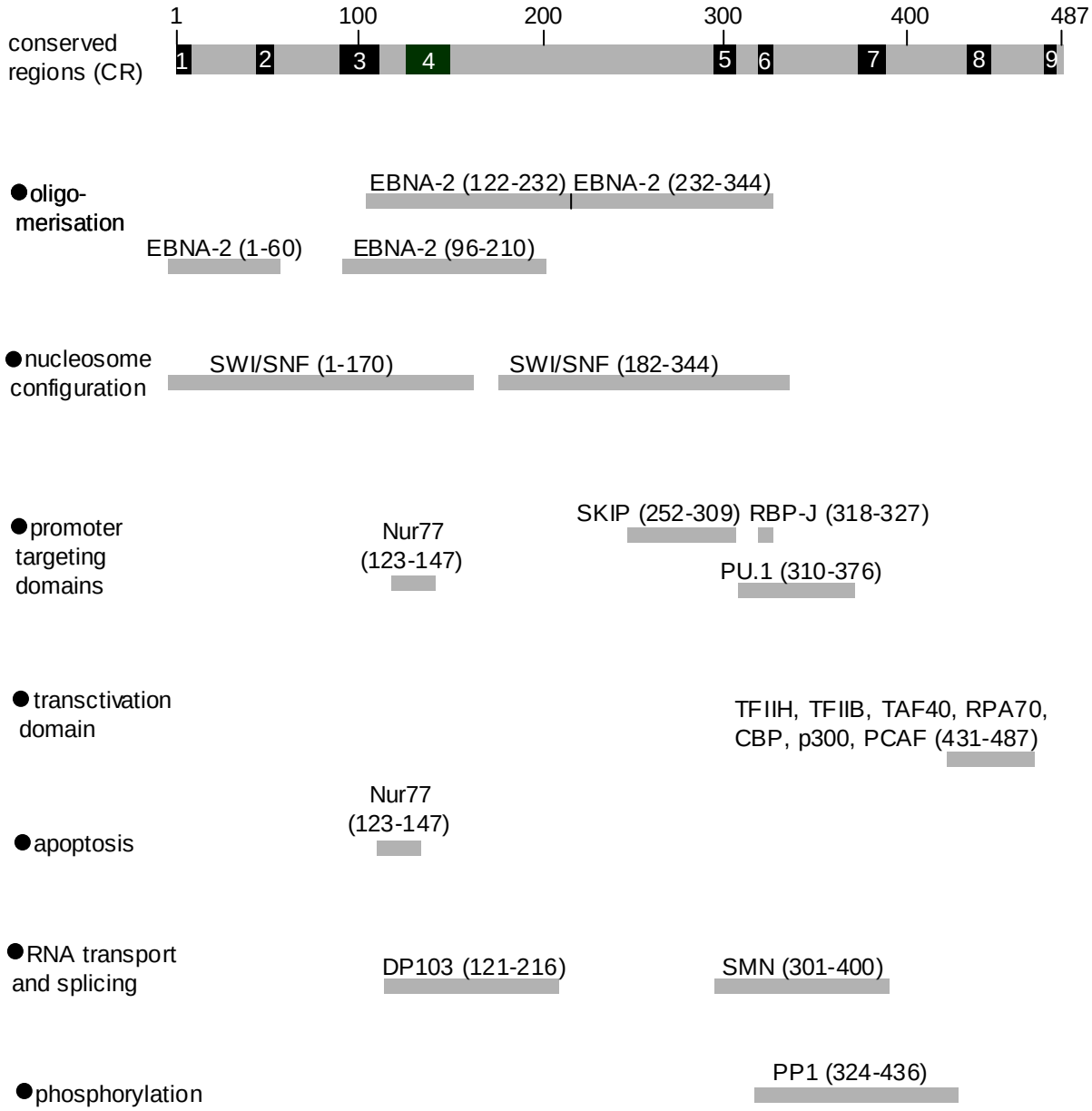

Figure 1.4.
EBNA-2 associated proteins. A schematic presentation of the primary structure of the EBNA-2 protein is depicted on the top of the scheme. The scheme indicates aminoacid positions within the primary structure. Nine conserved regions (CR1-9) are marked with boxes. EBNA-2 associated proteins are grouped according to their functions, which is briefly described on the left edge of the scheme. EBNA-2 residues involved in protein interactions are indicated in brackets.

1.3.3. Further EBNA-2 associated proteins

Additional EBNA-2 functions beside the targeting to specific DNA sequences can be deduced from a number of cellular proteins shown to associate with EBNA-2. Association with hSNF5/Ini1, a member of the family of chromatin remodelling proteins, and histone H1 indicates that EBNA-2 might have an impact on chromatin architecture (Grasser et al., 1991; Khochbin, 2001; Martens and Winston, 2003; Sauder et al., 1996; Wu et al., 1996).

EBNA-2 activates transcription by recruiting the cellular transcription machinery through the transactivation domain that encompasses CR8 (Cohen, 1992). TFIIB, TFIIE, TFIIH, TAF40, RPA70 and histone acetyltransferases p300, CBP and PCAF are recruited to the EBNA-2 transactivation domain (Tong et al., 1995a; Tong et al., 1995b; Tong et al., 1994; Wang et al., 2000).

EBNA-2 might also play a role in RNA transport and splicing since EBNA-2 interactions with DP103, a DEAD box protein and RNA helicase, and SMN, a protein required for assembly of snRNP, were reported (Barth et al., 2003; Grundhoff et al., 1999; Meister et al., 2002; Voss et al., 2001; Yan et al., 2003).

Nuclear localisation signals are localised in the central (residues 341-355) and carboxyl terminal part (CR9) of the EBNA-2 protein (Cohen et al., 1991; Ling et al., 1993).

EBNA-2 exerts its function probably as a dimer or oligomer. Two EBNA-2 regions have been shown to be responsible for multimerisation of EBNA-2: CR1/CR2 and CR3/CR4 (Harada et al., 2001; Tsui and Schubach, 1994).

EBNA-2 has been suggested to be a regulator of phosphorylation, due to its interaction with a protein phosphatase 1 (PP1)-like activity (Fahraeus et al., 1994). The protein phosphatase-1 (PP1) is one of the major classes of protein serine/threonine phosphatases, and has been found in all eucaryotic cells examined to date (Cohen, 2002).

1.3.4. The EBNA-2 transactivation mechanism mediated by RBP-J

Until now RBP-J is the only cellular protein proven to be a direct link between EBNA-2 and DNA and thus enabling EBNA-2 to reach one group of target promoters

(Henkel et al., 1994; Laux et al., 1994b; Ling et al., 1993; Zimber-Strobl et al., 1993). RBP-J is a ubiquitously expressed protein that is highly conserved in evolution with homology in *Drosophila melanogaster* and *Caenorhabditis elegans* (Tun et al., 1994). RBP-J binds to the motif C/GTGGGAA and represses transcription by tethering a histone deacetylase (HDAC) corepressor complex to the promoter (Dou et al., 1994; Hsieh and Hayward, 1995; Kao et al., 1998; Waltzer et al., 1995). The corepressor proteins SMRT, CIR, SAP30, HDAC1 and HDAC2 have been shown to be components of this complex (Hsieh et al., 1999; Kao et al., 1998). Histone deacetylases modify chromatin making it inaccessible to the transcription machinery und thus repressing transcription (Marks et al., 2003; Yang and Seto, 2003).

EBNA-2 activates transcription by binding to the repression domain of RBP-J, replacing the corepressor complex and recruiting the cellular transcription machinery. The EBNA-2/RBP-J binding is stabilised by an additional protein, SKIP, which directly contacts both EBNA-2 and RBP-J and is important for effective EBNA-2 transactivation (Ling and Hayward, 1995; Zhou et al., 2000).

Two adjacent EBNA-2 regions are involved in binding to SKIP and RBP-J and together constitute the RBP-J promoter targeting domain. Through the CR5 region EBNA-2 contacts SKIP while two tryptophan residues in CR6 are crucial for binding to RBP-J (Ling and Hayward, 1995; Ling et al., 1993). Mutation of these two tryptophan residues in CR6 not only abolishes the RBP-J binding but also results in a nonimmortalising phenotype of EBV carrying this EBNA-2 mutant (Yalamanchili et al., 1994). Deletion of the CR5 region from EBNA-2 in the context of the EBV genome also resulted in a nonimmortalising mutant EBV (Harada et al., 1998). These data suggest that the RBP-J signalling is crucial for EBNA-2 function.

1.3.5. The Notch signalling: the cellular pathway converging to RBP-J

By binding to RBP-J EBNA-2 mimics the cellular Notch protein. Notch is a large transmembrane protein that is highly conserved from invertebrates to vertebrates. In B-cells Notch can regulate and suppress differentiation (Kuroda et al., 2003). Notch is associated with several human neoplasms and is oncogenic in some animal model systems (reviewed in Allenspach et al., 2002).

Notch signalling is activated upon binding of Notch to its ligands that are positioned on neighbouring cells. Ligand binding induces cleavage of Notch in its transmembrane region and the intracellular region of Notch (Notch-IC) directly translocates to the nucleus where it activates its target genes (reviewed in Allman et al., 2002). Notch-IC replaces the corepressor complex bound to RBP-J and activates transcription through an intrinsic transactivation domain and thus by a mechanism very similar to EBNA-2 (Hsieh et al., 1996). RBP-J residues bound by Notch-IC and EBNA-2 overlap partially (Fuchs et al., 2001).

EBNA-2 and Notch share the RBP-J signalling, but their functions are only partially overlapping. There are promoters containing RBP-J binding sites, which can be activated solely by either activated Notch (Notch-IC) or EBNA-2 indicating that different additional elements guide the transcriptional activity of both proteins (Dumont 2000, PhD thesis). Furthermore, neither Notch nor EBNA-2 can replace each other functionally in all cellular systems (Gordadze et al., 2001; Hofelmayr et al., 2001; Sakai et al., 1998).

1.4. Goal of the project

EBNA-2 is the central viral transcription factor in the EBV driven immortalisation of B-cells that modulates transcription from both viral and cellular promoters. It is one of the first viral proteins expressed in B-cells after EBV infection. Thus, unravelling EBNA-2 signalling is crucial in order to understand B-cell immortalisation by EBV. Moreover, the knowledge of early transcriptional events in B-cell immortalisation induced by EBNA-2 could find applications in the prevention and therapy of EBV infection that causes lymphoproliferative disorders in immunosupressed individuals.

EBNA-2 does not contain a DNA binding domain. Instead of binding to DNA directly, EBNA-2 uses cellular proteins as anchors to reach target promoters. One of these proteins is the cellular protein RBP-J that binds to a specific DNA sequence. Many analysed EBNA-2 responsive elements contain RBP-J binding site(s) that were mostly described as necessary for or strongly contributing to EBNA-2 transactivation. Still, there are indications that EBNA-2 can exert its function(s) by RBP-J

independent mechanism(s). The goal of the project is to characterise and dissect known and novel EBNA-2 signalling pathways.

In order to reach this goal, the following working packages were defined:

1) **Study of transactivation of the LMP-1 promoter by EBNA-2**
   A mutational analysis of the LMP-1 promoter was conducted in order to test the relative contribution of transcription binding sites to EBNA-2 signalling.

2) **Study of the promoter targeting domains in EBNA-2 protein**
   The EBNA-2 gene was genetically analysed in order to characterise the RBP-J binding domain that targets EBNA-2 to one group of promoters and to search for additional promoter targeting domain(s) within the EBNA-2 protein.

3) **Detection of the DNA regions bound by EBNA-2 *in vivo***
   A chromatin immunoprecipitation protocol using EBNA-2 specific antibodies was established in order to identify DNA regions in the chromatin bound by EBNA-2 *in vivo*.

4) **Characterisation of EBNA-2 mutants in context of the complete viral genome**
   The characterised EBNA-2 mutants were inserted into recombinant EBV and their influence on the immortalisation efficiency of B-cells by EBV and maintenance of the immortalised phenotype were studied.

2. Results

2.1. The mutational analysis of the LMP-1 promoter

EBNA-2 signalling on the LMP-1 promoter is discussed controversially in the literature. While the PU.1 binding site in the LMP-1 promoter was unambiguously shown to be vital for EBNA-2 signalling, the contribution of the RBP-J binding sites to EBNA-2 transactivation of the LMP-1 promoter was not clear (see 1.2.3.). We were interested in whether RBP-J dependent or RBP-J independent signalling of EBNA-2 is responsible for LMP-1 promoter activation.

The analysis of the LMP-1 promoter was performed in two steps. First, the basal activity of the LMP-1 promoter was defined and compared to a set of LMP-1 promoter mutants, which carry non-functional or completely lack the RBP-J binding site(s) due to successive shortening of the promoter sequence. In the second step, the same set of LMP-1 promoter constructs was cotransfected with EBNA-2 expression plasmids and promoter activities were determined.

The experimental system is based on a reporter gene assay. Each LMP-1 promoter was cloned upstream of a luciferase gene whose expression can be monitored by measuring the luciferase activity.

Sjoblom et al. have already done a detailed genetic analysis of the LMP-1 promoter in which binding sites for specific transcription factors were mutated by purine/ pyrimidine conversion (Sjoblom et al., 1995). The LMP-1 promoter reporter constructs contained the chloramphenicol acetyl transferase (CAT) gene, which we replaced by the luciferase gene in order to achieve a more quantitative and sensitive assay. One additional LMP-1 luciferase construct (-327) was kindly provided by Gerhard Laux (Laux et al., 1994b). A schematic overview of the LMP-1 reporter constructs used in this study is shown in figure 2.1.1.

2.1.1. The RBP-J binding sites in the LMP-1 promoter exert repression

The LMP-1 promoter contains two RBP-J consensus binding sites. RBP-J is a cellular DNA binding protein, which recruits a corepressor complex (for details see

1.3.4.). Thus, the presence of the RBP-J binding site(s) in the promoter indicates that the promoter might be repressed in the absence of an activator.

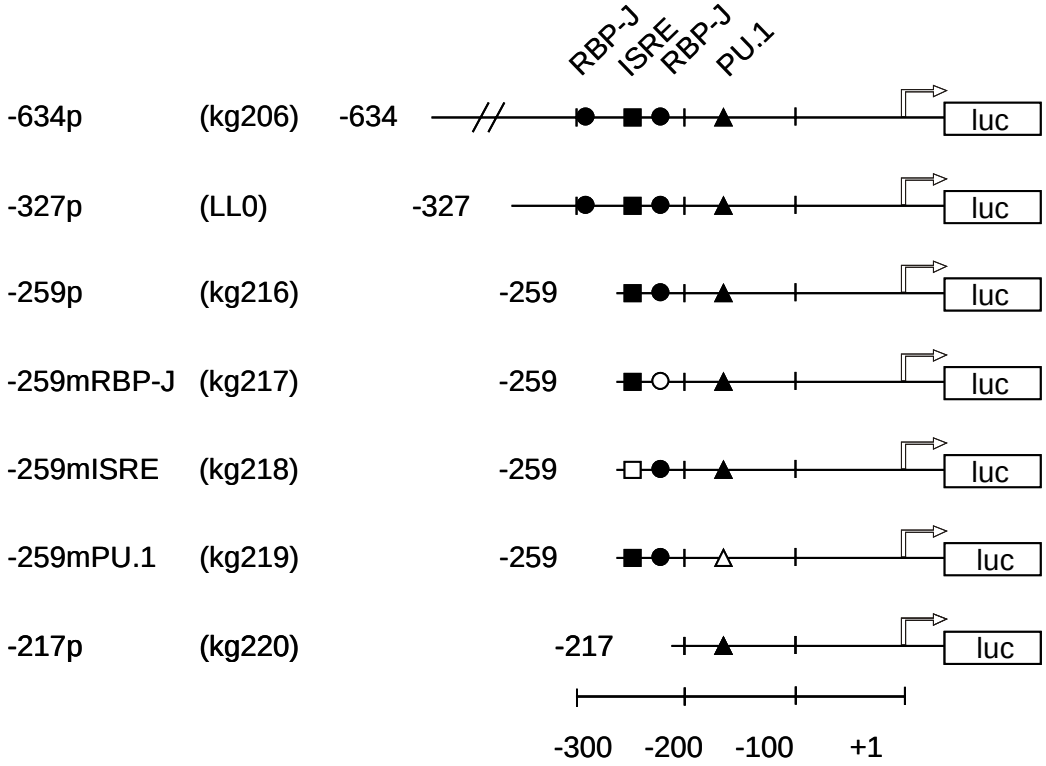

Figure 2.1.1.
Schematic overview of the LMP-1 reporter constructs used in this study. ISRE (quadrant) and binding sites for RBP-J (circle) and PU.1 (triangle) are depicted in black (wild type) and white (mutated). The LMP-1 promoters were cloned upstream the luciferase gene (luc). Numbers indicate the base pair position relative to the transcription start site (bent arrow). Working designations of the LMP-1 reporter constructs are depicted in brackets.

To test the degree of repression mediated by the two RBP-J binding sites in the LMP-1 promoter, wild type LMP-1 promoters of different lengths and LMP-1 promoters mutated for the RBP-J binding sites were compared for their basal activity in the absence of EBNA-2 (figure 2.1.2.).

The LMP-1 reporter constructs were transiently transfected into the cell line BL41P3HR1, a Burkitt's lymphoma cell line infected with an EBV strain lacking the EBNA-2 open reading frame. The expression levels of the luciferase gene under the control of different LMP-1 promoters are indicated as fold activation relative to the

longest wild type (wt) LMP-1 promoter used in these experiments (-634p), the activity of which was set to 1.

The luciferase gene under control of the wt LMP-1 promoter shortened at the 5' end (-327p) that still contains both RBP-J binding sites showed activity of 0.7 fold relative to -634p (lanes 1 and 2). Another successively shortened version of the wt LMP-1 promoter (-259p), which encompasses only one RBP-J binding site showed the same weak activity of 0.7 fold (lane 3).

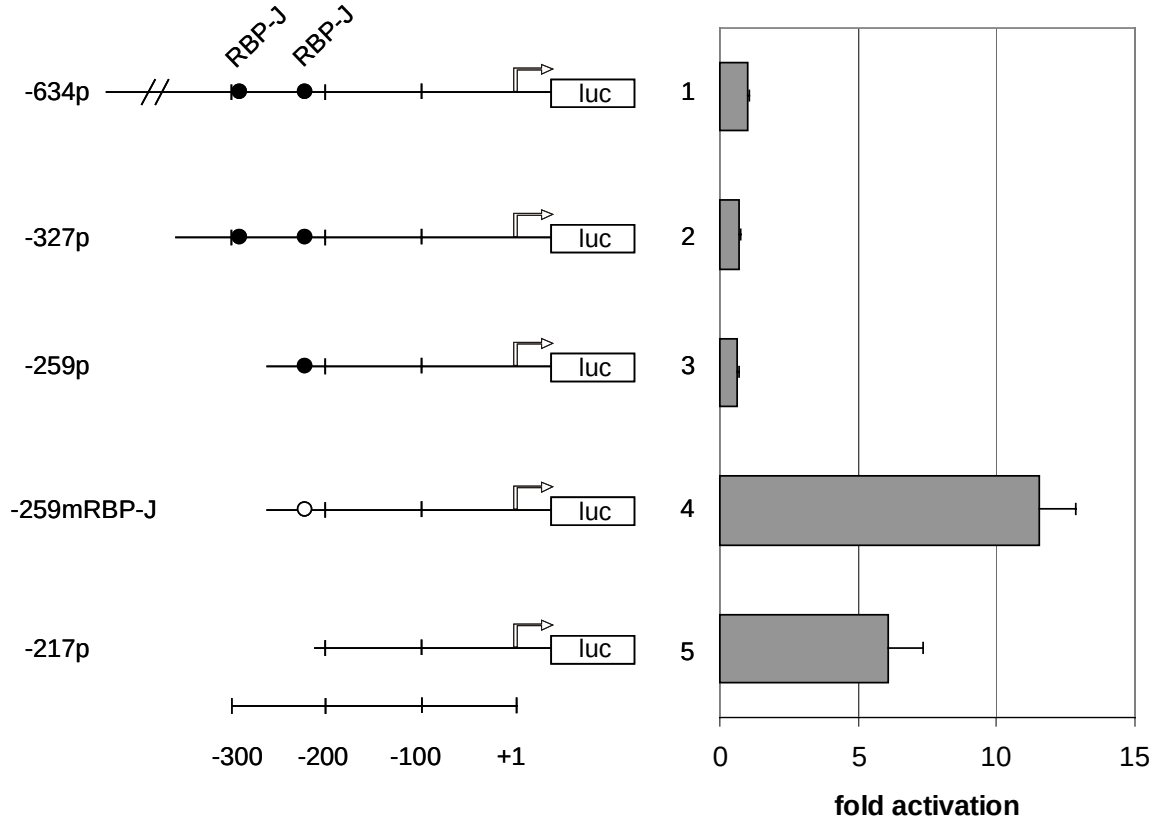

Figure 2.1.2.
Repressive role of the RBP-J binding sites in the LMP-1 promoter. $1x10^7$ BL41P3HR1 cells were transfected with 10µg of the respective reporter construct and 10µg of the pSG5 vector. 2µg of a CMVβGal reporter construct were included in each transfection. Cellular extracts were tested for luciferase activity 2 days after transfection. The luciferase activity was normalised against the galactosidase activity. The experiment was performed 3 times in triplicates. The results of one representative experiment are shown as fold activation of the promoter construct, compared to the -634p. The mean values of triplicates with standard deviations as error bars are shown. The LMP-1 reporter constructs are presented schematically with numbers indicating the promoter length relative to the transcription start site (bent arrow). Only the RBP-J binding sites that are relevant for these experiments are depicted. Wild type and mutated RBP-J binding sites are depicted as black and white circles, respectively.

When the remaining RBP-J binding site in -259p was mutated (-259mRBP-J) or the LMP-1 promoter was shortened to the length encompassing no RBP-J binding sites (-217p), the luciferase expression was drastically upregulated. The -259mRBP-J and -217p reporter constructs exhibited 11.5 and 6 fold activation (lanes 4 and 5).

2.1.2. EBNA2WW325FF activates the LMP1 promoter lacking the RBP-J binding sites

Our reanalysis of the LMP-1 promoter showed that the RBP-J binding sites in the LMP-1 promoter have a strong repressive role and that the proximal RBP-J binding site alone is able to exert repression on the LMP-1 promoter. As the next step we wanted to analyse whether the RBP-J binding sites are necessary for transactivation of the LMP-1 promoter by EBNA-2.

We transiently coexpressed the same set of LMP-1 promoter constructs as in chapter 2.1.1. with an EBNA-2 expression plasmid in BL41P3HR1 cells and determined the promoter activities (figure 2.1.3.).

Mostly in the literature, transactivation of a reporter construct by a protein is described as fold activation compared to the state of the reporter construct in the absence of the protein. In our case, presentation of the data as a ratio of luciferase activity in the presence or absence of EBNA-2, was not feasible, since the basal activity of the reporter construct varied significantly. Thus, we present the activities of the LMP-1 reporter constructs as luciferase activity normalised to galactosidase activity as relative light units (RLU).

Wt LMP-1 reporter constructs, -634p and -327p, which differ in promoter length but still contain both RBP-J binding sites, showed a low basal activity of 2.4 and 1.6 RLU (lanes 1 and 4), and were transactivated by wt EBNA-2 to 84 and 89 RLU (lanes 2 and 5), respectively.

The third LMP-1 reporter construct (-259p) is shortened to encompass only one RBP-J binding site and was activated by wt EBNA-2 to 31 RLU (lane 8), while the basal activity was similar to the longer LMP-1 reporter constructs (2 RLU, lane 7). The -259mRBP-J reporter construct contains only one mutated RBP-J binding site and showed a much higher basal activity of 27 RLU (lane 10) but also a much higher transactivation by wt EBNA-2 i.e. 144 RLU (lane 11).

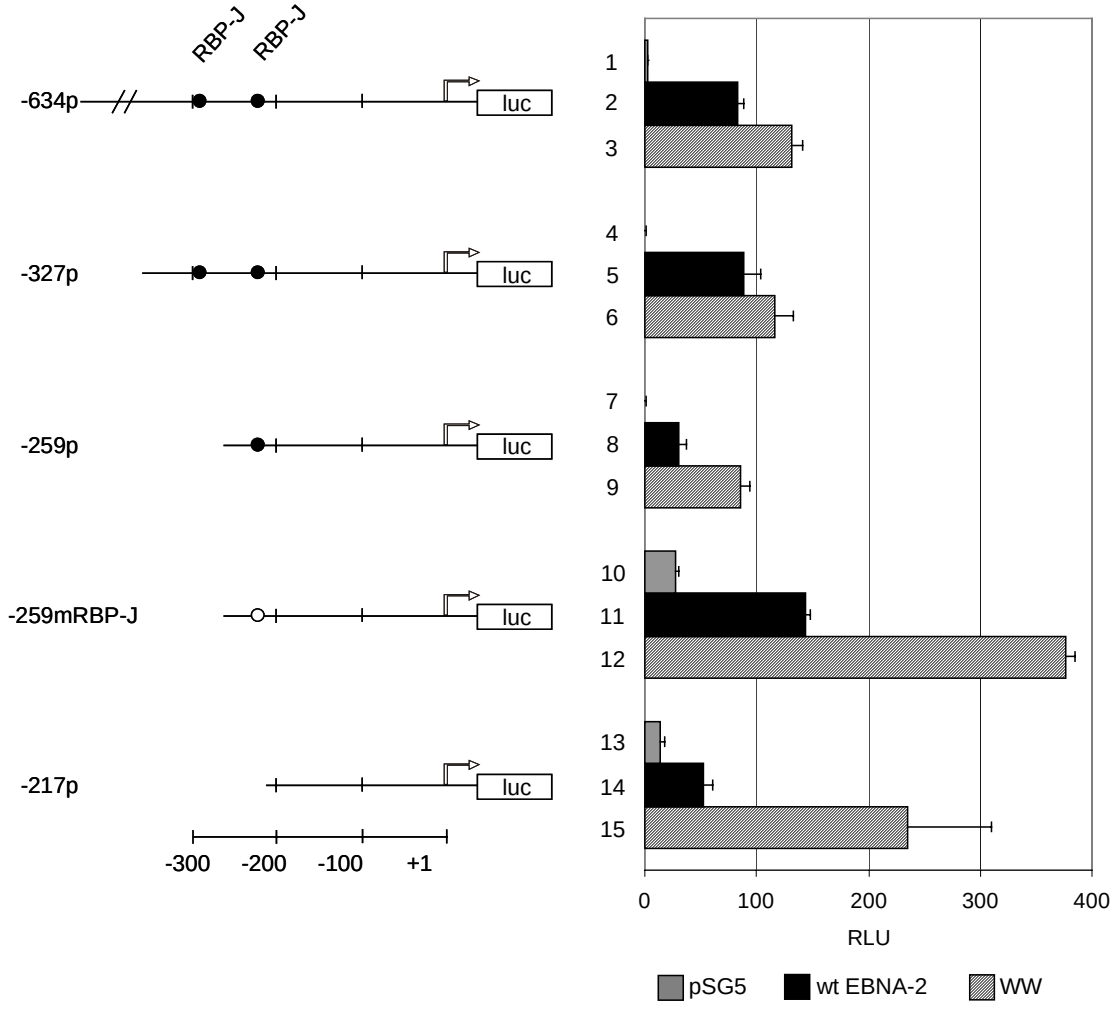

Figure 2.1.3.
The WW mutant activates the LMP-1 promoter lacking the RBP-J binding sites. $1x10^7$ BL41P3HR1 cells were transfected with 10µg of the respective reporter construct and 10µg of the EBNA-2 expression plasmid. 2µg of CMVβGal reporter construct were included in each transfection as a normalisation control. Cellular extracts were tested after 2 days for luciferase activity. The experiment was performed 3 times in triplicates. The results of one representative experiment are shown as relative light units of the luciferase activity normalised to galactosidase activity. The mean values of triplicates with standard deviations as error bars are shown. The LMP-1 reporter constructs are presented schematically with numbers indicating the promoter length relative to the transcription start site (bent arrow). RBP-J binding sites are depicted with black (wild type) and white (mutated) circles.

The shortest LMP-1 reporter construct (-217p) that encompasses no RBP-J binding site showed activity of 14 RLU (lane 13) in the absence and 52 RLU (lane 14) in the presence of wt EBNA-2.

In order to determine whether the RBP-J binding sites are used by EBNA-2 for LMP-1 promoter transactivation, we used a parallel approach to the LMP-1 promoter genetic analysis. Simultaneously to cotransfection of LMP-1 reporter constructs and the wt EBNA-2 expression plasmid, we also cotransfected LMP-1 reporter constructs and the expression plasmid for the EBNA-2WW325FF mutant (WW). This mutant contains two point mutations in the CR6 region that are sufficient to abolish binding to RBP-J (Ling et al., 1993; Yalamanchili et al., 1994). The point mutations in the WW mutant have no impact on the expression level in comparison to wt EBNA-2 (figure 2.1.4.).

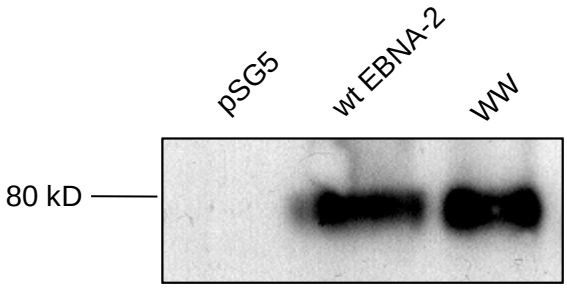

Figure 2.1.4.
Expression of wt EBNA-2 and the WW mutant in DG75 cells. 1x10^7 DG75 cells were transfected with 10µg EBNA-2 expression plasmid or empty vector. Two days after transfection cells were lysed in 100µl ELB buffer. 50µg protein/lane were loaded on a 10% denaturating polyacrylamide gel. The Western blot was stained with an EBNA-2 specific antibody (R3 hybridoma supernatant, 1:5).

We compared promoter activities in the presence of the wild type or the mutated EBNA-2 protein in transiently transfected BL41P3HR1 cells (figure 2.1.3.).

Wt EBNA-2 transactivated the -634p and -327p 84 and 89 RLU (lanes 2 and 5), respectively. The WW mutant transactivated the -634p and -327p stronger than wt EBNA-2 (but not always, see page 32), i.e. to 131 and 116 RLU (lanes 3 and 6). The same tendency of stronger transactivation by the WW mutant in comparison to wt EBNA-2 was seen in the remaining LMP-1 reporter constructs. The wild type LMP-1

promoter -259p was activated by EBNA-2 and the WW mutant to 31 and 86 RLU, respectively (lanes 8 and 9). The -259mRBP-J was activated by wt EBNA-2 and the WW mutant to 144 and 376 RLU (lanes 11 and 12), respectively. Finally, wt EBNA-2 and the WW mutant activated the shortest LMP-1 reporter construct, -217p, to 52 and 235 RLU (lanes 14 and 15).

2.1.3. The repressive role of the RBP-J binding site versus the activating role of the PU.1 binding site in transactivation of the LMP-1 promoter by EBNA-2

RBP-J can have a dual function in a promoter: it might act as a repressor or as a transcription factor depending on the factors bound to it (see 1.3.4.). We could demonstrate that the RBP-J binding sites in the LMP-1 promoter have primarily a repressive role and are not essential for EBNA-2 mediated activation of the LMP-1 promoter (see chapters 2.1.1 and 2.1.2.).

To emphasise the difference between repressive and transactivating roles of binding sites for transcription factors in the LMP-1 promoter we compared the ability of EBNA-2 to transactivate the LMP-1 reporter constructs that contain mutations in ISRE and the RBP-J and PU.1 binding sites (see 1.2.1.) in transient experiments (figure 2.1.5.).

As in chapter 2.1.1. and 2.1.2. we present the activity of reporter constructs as relative light units (RLU) of the luciferase activity normalised to the RLU of the galactosidase activity.

All four LMP-1 reporter constructs included in this analysis encompassed the LMP-1 promoter region to -259 bp but differed in mutations for ISRE and the RBP-J and PU.1 binding sites. The LMP-1 reporter construct containing a mutated RBP-J binding site (-217mRBP-J) showed an elevated basal activity of 27 RLU (lane 4) while the other LMP-1 reporter constructs including wt and mutated constructs (-259mISRE and –259mPU.1) had similar basal activity ranging in average from 1 to 3 RLU (lanes 1, 7 and 10). Deletion of the RBP-J binding site and ISRE had a positive influence on the EBNA-2 transactivation since the corresponding LMP-1 reporter constructs were activated by wt EBNA-2 to 144 and 79 RLU (lanes 5 and 8), respectively, while the wtLMP-1 promoter was activated by wt EBNA-2 to 30 RLU (lane 2).

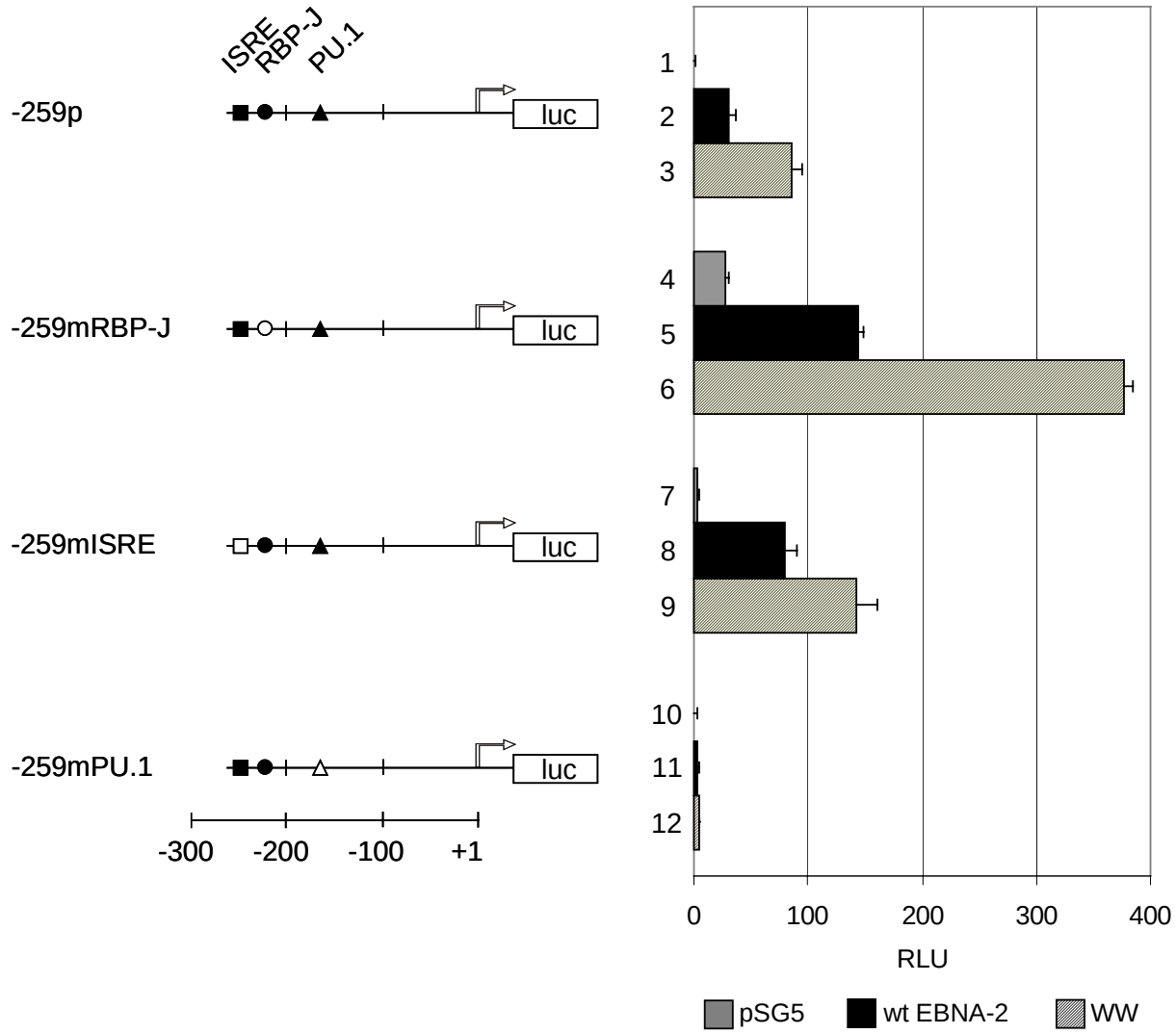

Figure 2.1.5.
Repressive role of the RBP-J binding site versus the transactivation role of the PU.1 binding site in the EBNA-2 transactivation of the LMP-1 promoter. $1x10^7$ BL41P3HR1 cells were transfected with 10µg of the respective reporter constructs and 10µg of the EBNA-2 expression plasmid. 2µg of the CMVβGal reporter construct were included in each transfection as normalisation control. Cellular extracts were tested 2 days after transfection for luciferase activity. All experiments were performed 3 times in triplicates. The results of one representative experiment are shown as relative light units of the luciferase activity normalised to galactosidase activity. The mean values of triplicates with standard deviations as error bars are shown.The LMP-1 reporter constructs are presented schematically with numbers indicating the promoter length relative to the transcription start site (bent arrow). ISRE (quadrant) and the RBP-J (circle) and PU.1 (triangle) binding sites are marked black (wild type) or white (mutated).

The WW mutant transactivated the wtLMP-1 promoter -259p very efficiently with 86 RLU (lane 3) as well as the mutated LMP-1 reporter constructs -259mRBP-J (376 RLU, lane 6) and -259mISRE (143 RLU, lane 9). In contrast deletion of the PU.1 binding site in the -259mPU.1 construct abolished transactivation ability of both wt EBNA-2 and the WW mutant (4 RLU, both lane 11 and 12).

Summary of chapter 2.1.:

Our mutational analysis of the LMP-1 promoter confirmed that the PU.1 binding site is important for transactivation of the LMP-1 promoter by EBNA-2. In contrast, RBP-J binding to the LMP-1 promoter leads to repression and EBNA-2 binding to RBP-J is not required for transactivation. These results imply that EBNA-2 transactivates the LMP-1 promoter preferentially by an RBP-J independent mechanism.

2.2. Characterisation and dissection of EBNA-2 signalling by genetic analysis

As discussed in the introduction, EBNA-2 is a multifunctional oncoprotein whose contribution to the EBV immortalisation of B cells is mainly due to transactivation of viral and cellular genes. Published work by others suggests that EBNA-2 can get access to promoters by more than one mechanism (see 1.2.1 and 1.3.1.). In order to identify and characterise these mechanisms we decided first to characterise the known EBNA-2 promoter targeting domain i.e. the RBP-J promoter targeting domain in detail.

In the second step we performed a genetic analysis of EBNA-2 with the goal to find domains within the EBNA-2 protein, which might target EBNA-2 to promoters by novel mechanisms. EBNA-2 constructs used in this work are described in table 2.2.1. The two viral promoters, LMP-2A and LMP-1, served as promoter models for RBP-J dependent or independent EBNA-2 signalling, respectively (figure 2.2.1.). The EBNA-2 mutants were characterised by their ability to transactivate the luciferase reporter constructs of the LMP-1 and LMP-2A promoters.

Table 2.2.1.
Overview of the EBNA-2 constructs used in this work with indicated mutations and working designations.

Designation	Mutation	Working designation
wt EBNA-2	-	VA32
CR4del	deleted residues 117-146	pkg423
WW	WW325FF	VA54
HAEBNA-2	HA tag	pkg172
HAdelCR5/6A	HA tag + deleted residues 281-333	pkg95
HAdelCR5/6B	HA tag + deleted residues 293-333	pkg99
HAdelSphI	HA tag + deleted residues 248-382	pkg112
HAE2RAM	deleted residues 281-333 (kg95) + 55 residues of RAM	pkg118
HAdelCR7	deleted residues 377-387	pkg170
HACR5-9	deleted residues 2-284	pkg169

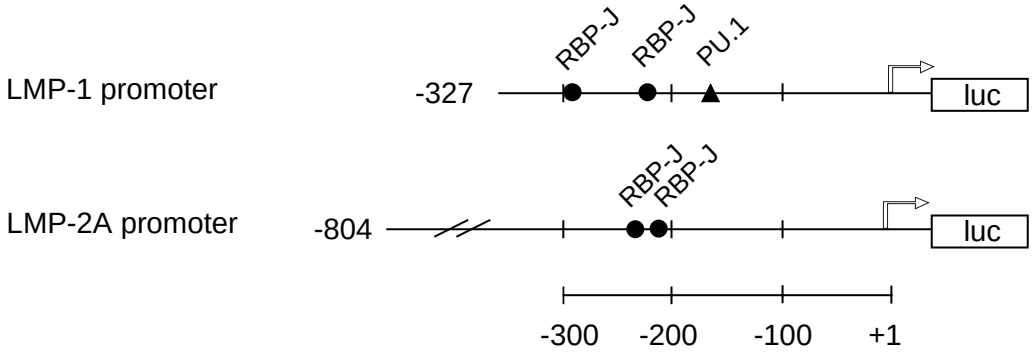

Figure 2.2.1.
Schematic presentation of the LMP-1 and LMP-2A luciferase (luc) reporter constructs. RBP-J (circles) and PU.1 (triangle) consensus binding sites are depicted, as well as base pair positions relative to the transcription start site (+1). Although both promoters contain two RBP-J binding sites, EBNA-2 activates only the LMP-2A promoter via the RBP-J binding sites, while the PU.1 binding site is crucial for the LMP-1 transactivation by EBNA-2.

2.2.1. The RBP-J promoter targeting domain of EBNA-2 as a potential multifunctional domain

The RBP-J promoter-targeting domain of EBNA-2 consists of two separated regions CR5 (residues 296-309) and CR6 (residues 320-327). We generated a panel of EBNA-2 mutants by deleting the regions CR5 and CR6 plus flanking regions. All mutants were epitope-tagged by fusing the hemagglutinin gene (HA) tag to the open reading frame (ORF) of EBNA-2 at the amino terminus.

The mutants HAdelCR5/6A, HAdelCR5/6B and HAdelSphI carry deletions encompassing the residues 281-333, 293-333 and 248-382, respectively, and were expressed at similar levels in transfected DG75 cells (figure 2.2.2.).

The ability of the EBNA-2 mutants to transactivate the LMP-1 and LMP-2A promoters was assayed by transfection experiments in BL41P3HR cells (figure 2.2.3.). Wt EBNA-2 and HAEBNA-2 activated transcription from the LMP-1 promoter construct 24 and 21 fold compared to the empty vector, respectively (lanes 1 and 2). The LMP-2A promoter construct was induced 40 fold by wt EBNA-2 (lane 6) and 115 fold by HAEBNA-2 (lane 7).

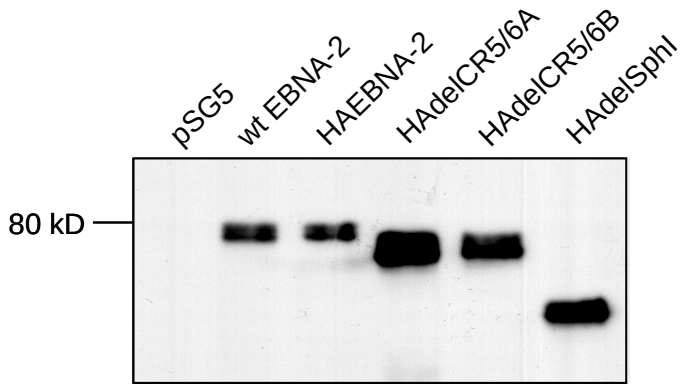

Figure 2.2.2.
Expression of the EBNA-2 proteins in DG75 cells. 1x10^7 DG75 cells were transfected with 10µg EBNA-2 expression plasmid. Two days after transfection, cells were lysed in 100µl ELB buffer. 50µg protein were loaded per lane and Western blot analysis with an EBNA-2 specific antibody (R3 hybridoma supernatant, 1:5) was performed.

As shown in figure 2.2.2, no differences in protein amount could be detected between wt EBNA-2 and HAEBNA-2 and we are not able to explain the selective impact of the HA tag on the EBNA-2 transactivation of the LMP-1 and LMP-2A promoter constructs.

The LMP-1 promoter could still be activated by EBNA-2 proteins lacking the RBP-J promoter-binding domain. HAdelCR5/6A and HAdelCR5/6B activated the LMP-1 promoter to 7 and 12 fold, respectively (lanes 3 and 4). However, deletion of broader regions flanking the RBP-J promoter targeting domain in the HAdelSphI mutant resulted in a complete loss of the transactivation ability (1 fold activation, lane 5).

HAdelCR5/6A, HAdelCR5/6B and HAdelSphI transactivated the LMP-2A promoter construct 2, 4 and 1 fold (lanes 8, 9 and 10). Thus, in contrast to the activation of the LMP-1 promoter by EBNA-2, the deletion of the RBP-J targeting domain abolished the ability of EBNA-2 to transactivate the LMP-2A promoter.

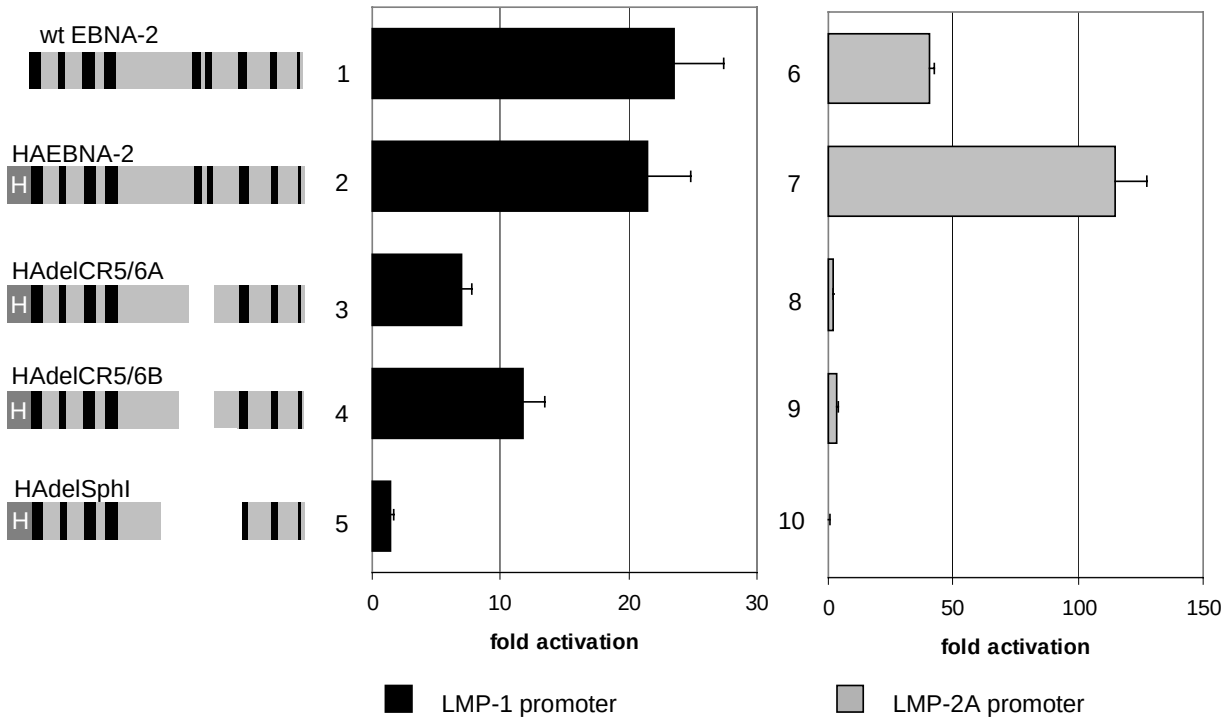

Figure 2.2.3.
The RBP-J promoter targeting domain of EBNA-2 that consist of CR5 and CR6 is crucial for the LMP-2A promoter activation but not for the LMP-1 promoter activation. $1x10^7$ BL41P3HR1 cells were electroporated with 10µg reporter construct and 10µg EBNA-2 expression plasmid. 2µg of the CMVßGal construct were included in each transfection to normalise transfection efficiency. Two days after transfection, cells were lysed and a luciferase assay was performed. The activation of the LMP-1 and LMP-2A promoters is given as fold activation compared to the vector alone. The results are given as the mean of three independent experiments with standard deviations given as error bars. Each experiment was performed in triplicates (H, HA tag).

2.2.2. The RBP-J binding region of Notch functionally replaces the intrinsic RBP-J binding domain of EBNA-2 only in the activation of the LMP-2A promoter

As shown in chapter 2.2.1. EBNA-2 proteins deleted for CR5 and CR6, could still activate the LMP-1 promoter but with a significantly lower efficiency in comparison to wt EBNA-2. In contrast, the point mutations in the CR6 region (the WW mutant), which abolish binding to RBP-J, did not diminish LMP-1 transactivation. These results

suggested, that other functions than RBP-J signalling might reside within the CR5 and CR6 regions. As discussed before, a broad region (residues 314-380) that includes CR5 and CR6 was suggested to target EBNA-2 to the PU.1 binding site (Johannsen et al.1995).

In order to test this hypothesis we performed a domain swap experiment. The RBP-J binding domain of EBNA-2 was replaced by the RBP-J binding region of Notch, termed RAM. Both proteins HAdelCR5/6A and HAE2RAM could be strongly expressed in DG75 cells and their protein expression level even exceeded the expression of wt EBNA-2 or HAEBNA-2 (figure 2.2.4.).

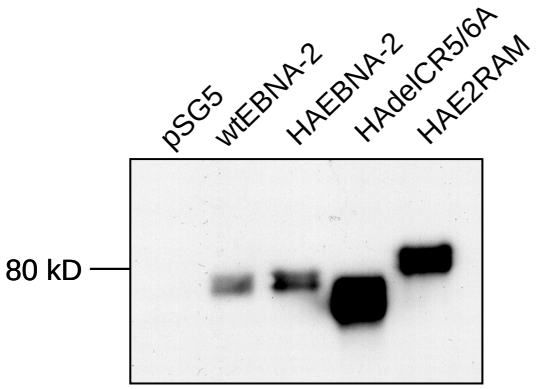

Figure 2.2.4.
Expression of the EBNA-2 proteins in DG75 cells. 1x10^7 DG75 cells were transfected with 10µg EBNA-2 expression plasmid. Two days after transfection, cells were lysed in 100µl ELB buffer. 50µg protein was loaded per lane and Western blot analysis with an EBNA-2 specific antibody (R3 hybridoma supernatant, 1:5) was performed.

The constructed EBNA-2 mutants were tested for transactivation of the LMP-1 and LMP-2A promoters (figure 2.2.5.).

HAEBNA-2 activated the LMP-1 and LMP-2A promoter 21 and 115 fold, respectively (lane 1 and 4). The EBNA-2 mutant lacking the CR5 and CR6 regions (HAdelCR5/6A) and therefore being unable to bind to RBP-J could still activate transcription from the LMP-1 promoter 7 fold (lane 2), while the LMP-2A transactivation was strongly reduced (2 fold, lane 5).

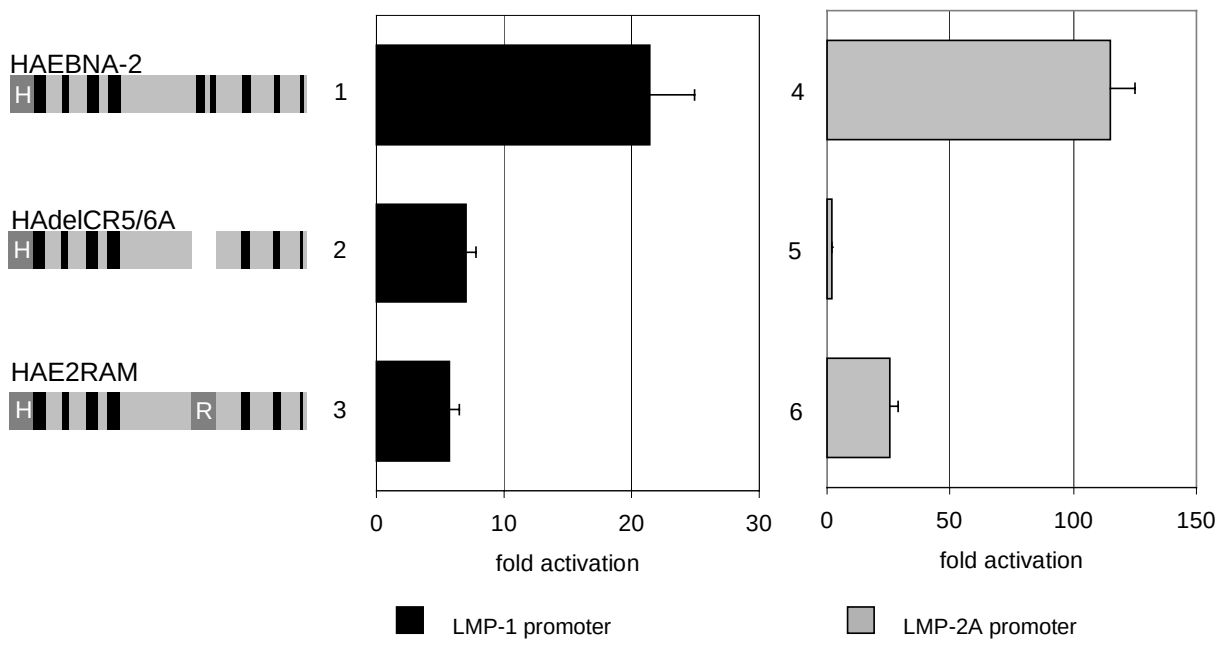

Figure 2.2.5.
The intrinsic RBP-J promoter targeting domain of EBNA-2 can be functionally replaced by the RBP-J binding region (RAM) of the Notch protein. $1x10^7$ BL41P3HR1 cells were electroporated with 10µg reporter construct and 10 µg EBNA-2 expression plasmid. 2µg of the CMVßGal construct were included in each transfection to normalise transfection efficiency. Two days after transfection, cells were lysed and a luciferase assay was performed. Activation of the LMP-1 and LMP-2A promoters is given as fold activation relative to the vector alone. The results are given as the mean of three independent experiments with standard deviations given as error bars. Each experiment was performed in triplicates (H, HA tag, R, RAM).

However, when the RAM domain was inserted in the position of the CR5/CR6 deletion of the HAdelCR5/6A protein, the resulting chimeric protein HAE2RAM activated transcription from the LMP-2A promoter 26 fold (lane 6). The LMP-1 promoter was 6 fold induced by HAE2RAM (lane 3), which was very similar to induction of the LMP-1 promoter by HAdelCR5/6A (compare lanes 2 and 3). Thus, reinsertion of an RBP-J binding domain could reconstitute the LMP-2A transactivation partially, while the LMP-1 promoter activation was unchanged. These results indicate that a secondary function distinct from RBP-J signalling and relevant for the LMP-1 promoter activity might reside within the CR5/6 region of the EBNA-2 protein.

2.2.3. Two EBNA-2 mutants preferentially activate either the LMP-1 or the LMP-2A promoter

As suggested by Sjoblom et al. the EBNA-2 residues 118-198 might be important for transactivation of the LMP-1 promoter (Sjoblom et al., 1995). This EBNA-2 region includes the evolutionary conserved region (CR) 4 (residues 127-151). We generated an EBNA-2 mutant that lacks almost the entire CR4 region (residues 117-146) and compared the activity of this mutant, termed CR4del, to the WW mutant. The WW mutant is known to be defective in RBP-J signalling as described in the previous chapter. Both EBNA-2 mutants are expressed at similar levels in transfected DG75 cells (figure 2.2.6.).

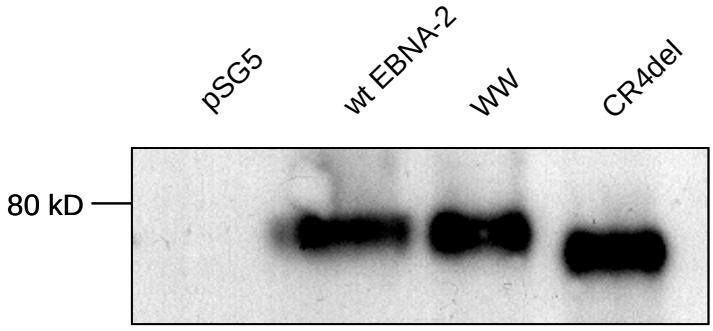

Figure 2.2.6.
Expression of the EBNA-2 proteins in DG75 cells. 1x10^7 DG75 cells were transfected with 10µg EBNA-2 expression plasmid. Two days after transfection, cells were lysed in 100µl ELB buffer. 50µg protein were loaded per lane and Western blot analysis with an EBNA-2 specific antibody (R3 hybridoma supernatant, 1:5) was performed.

The activities of the EBNA-2 mutants were measured on the LMP-1 and LMP-2A luciferase reporter constructs in transient transfections in BL41P3HR1 cells (figure 2.2.7).

Wt EBNA-2 activated the LMP-1 promoter 46 fold relative to the expression vector without the EBNA-2 gene (lane 1). The WW mutant induced the LMP-1 promoter 34 fold (lane 5), while CR4del was significantly impaired in LMP-1 transactivation and activated transcription from the LMP-1 promoter construct 21 fold (lane 3).

In comparison to LMP-1, the LMP-2A promoter construct was activated stronger by wt EBNA-2 (70 fold, compare lanes 1 and 2). WW activated the LMP-2A reporter construct only 3 fold (lane 6). Thus, the weak activation of the LMP-2A reporter construct clearly differed from the strong LMP-1 promoter activation by WW (compare lines 5 and 6). CR4del induced transcription from the LMP-2A promoter construct 74 fold (lane 4). Taken together, WW was strongly impaired in the LMP-2A promoter transactivation, while CR4del was affected in the LMP-1 transactivation.

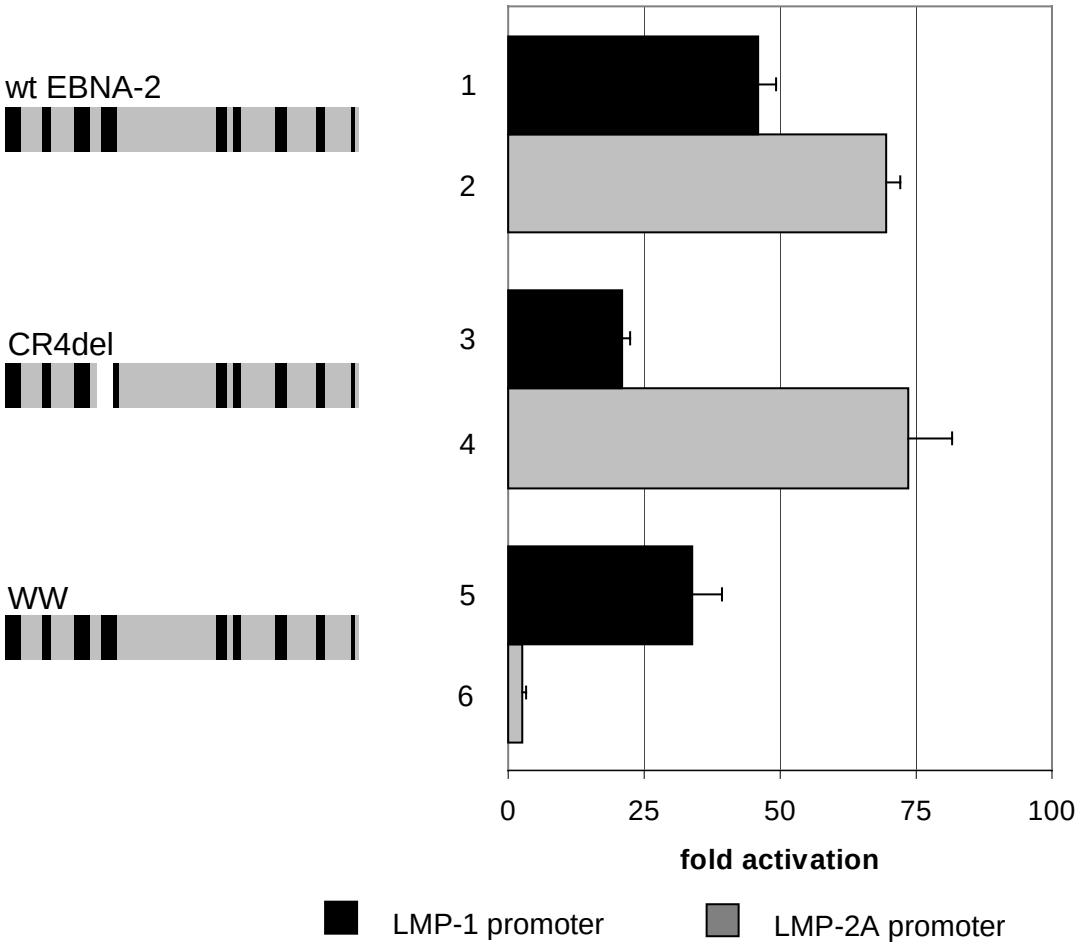

Figure 2.2.7.
The WW mutant and the CR4del mutant specifically activate either the LMP-1 or the LMP-2A promoter. $1x10^7$ BL41P3HR1 cells were electroporated with 10µg reporter construct and 10µg EBNA-2 expression plasmid. 2µg of the CMVßGal construct were included in each transfection to normalise transfection efficiency. Two days after transfection, cells were lysed and a luciferase assay was performed. Activation of the LMP-1 and LMP-2A promoters is given as fold activation respective to the vector alone. The results are given as the mean of three independent experiments with standard deviations given as error bars. Each experiment was performed in triplicates.

2.2.4. EBNA-2 mutants HAdelCR7 and HACR5-9

The conserved region (CR) 7 of EBNA-2 has also been described as an EBNA-2 region involved in LMP-1 transactivation (Sjoblom et al., 1995). We generated an EBNA-2 mutant deleted for CR7 and fused to an HA tag at the amino terminus (HAdelCR7). The HAdelCR7 was expressed to the same extent as the wild type protein upon transfections into DG75 cells (figure 2.2.8.)

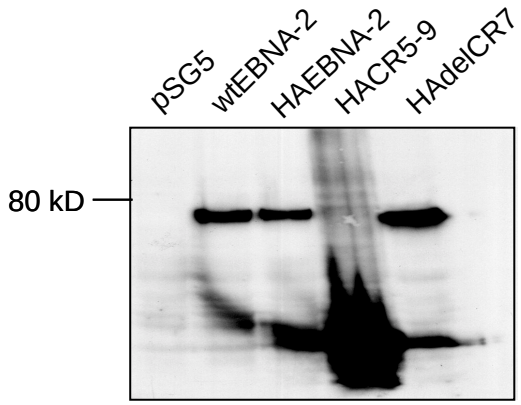

Figure 2.2.8.
Expression of the EBNA-2 proteins in DG75 cells. 1x10^7 DG75 cells were transfected with 10µg EBNA-2 expression plasmid. Two days after transfection, cells were lysed in 100µl ELB buffer. 50µg protein were loaded per lane and Western blot analysis with an EBNA-2 specific antibody (R3 hybridoma supernatant, 1:5) was performed.

Wt protein and the HAdelCR7 mutant were compared for their ability to transactivate the LMP-1 and LMP-2A promoters (figure 2.2.9.). In three independent experiments transactivation of the LMP-1 promoter by HAdelCR7 varied from 18% to 85 % relative to wt EBNA-2 transactivation (lanes 1-3). The inconsistencies, seen in the three independent experiments, are most likely due to technical problems. However, within the time frame of this PhD thesis these technical problems could not be solved and thus the HdelCR7 mutant was omitted from further studies.
The LMP-2A promoter transactivation by HAdelCR7 was constant and ranged from 115% to 140% relative to wt EBNA-2 transactivation of the LMP-2A promoter (lanes 4-6).

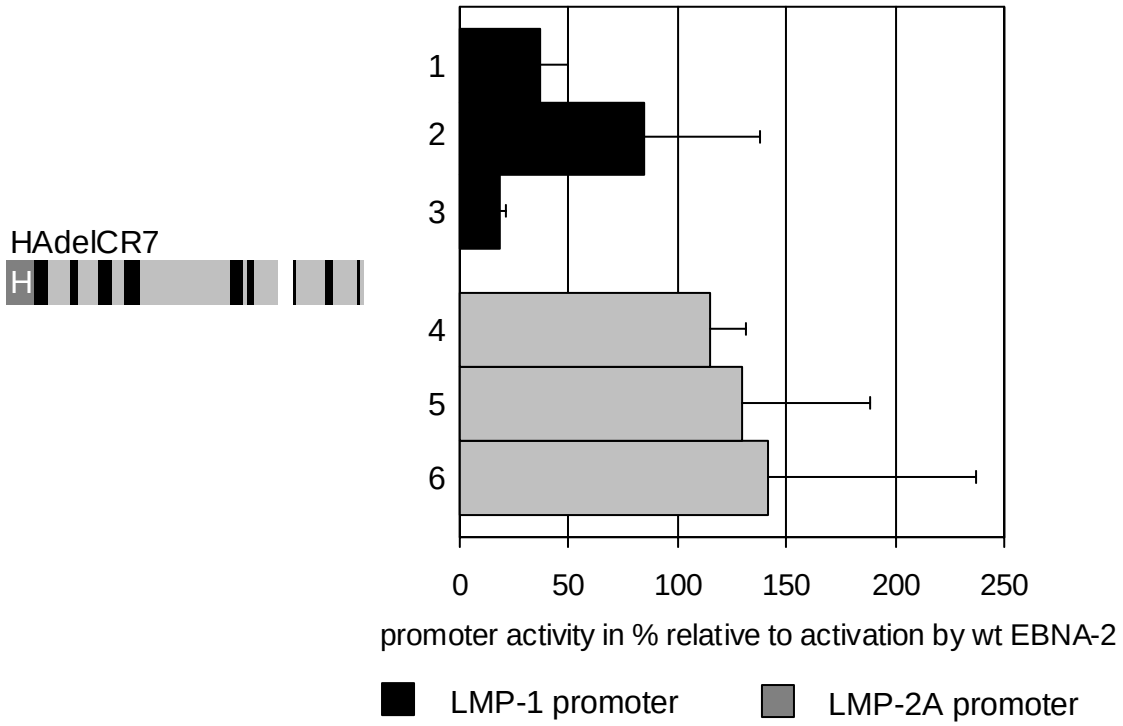

Figure 2.2.9.
HAdelCR7 shows no specificity in the activation of the LMP-1 and LMP-2A promoters. $1x10^7$ BL41P3HR1 cells were electroporated with 10µg reporter construct and 10µg EBNA-2 expression plasmid. 2µg of the CMVßGal construct were included in each transfection to normalise transfection efficiency. Two days after transfection, cells were lysed and a luciferase assay was performed. Triplicates of three independent experiments are shown as percentage relative to the transactivation by wt EBNA-2 with standard deviations given as error bars (H, HA tag).

In order to generate an RBP-J pathway specific EBNA-2 mutant we also cloned the carboxy terminus of EBNA-2 encompassing the conserved regions CR5 to CR9 and fused the HA tag at the amino terminus (HACR5-9). HACR5-9 thus contained the RBP-J promoter targeting domain as well as the transactivation domain and was much stronger expressed than the wild type protein (figure 2.2.8.). Nevertheless, HACR5-9 was not able to transactivate either the LMP-1 or LMP-2A promoter or an artificial reporter construct containing six RBP-J binding sites (6xRBP-J) in front of the minimal globin promoter (figure 2.2.10.)

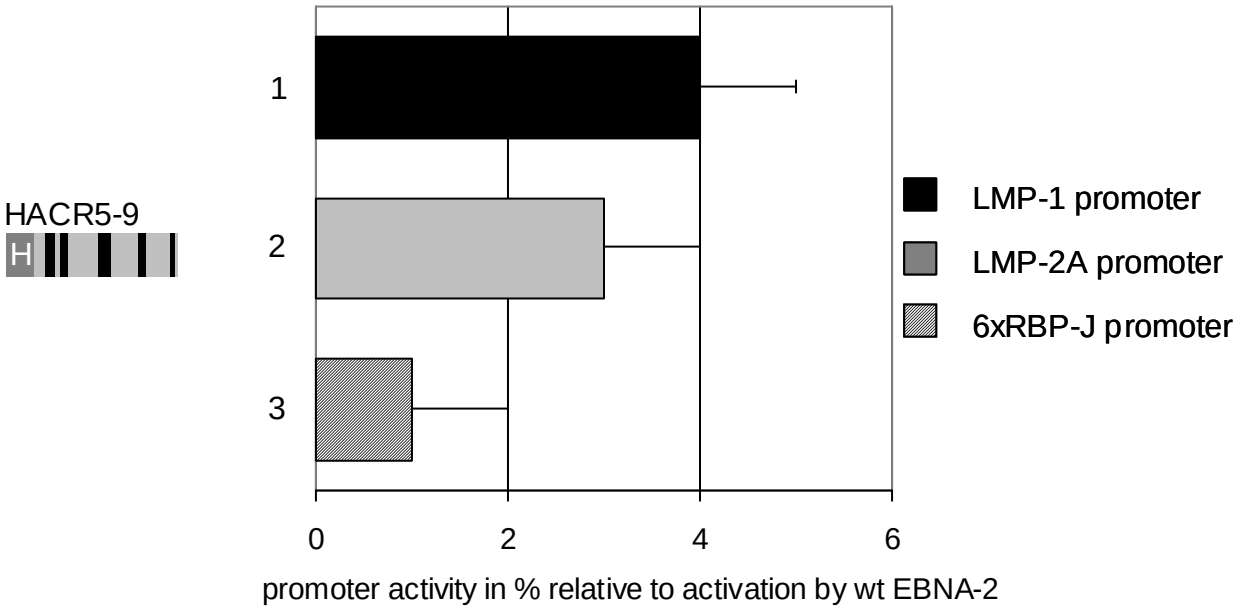

Figure 2.2.10.
The HACR5-9 mutant is unable to activate the LMP-1, LMP-2A and 6xRBP-J reporter construct. $1x10^7$ BL41P3HR1 cells were electroporated with 10µg reporter construct and 10µg EBNA-2 expression plasmid. 2µg of the CMVßGal construct were included in each transfection to normalise transfection efficiency. Two days after transfection, cells were lysed and a luciferase assay was performed. Triplicate results from one of three representative experiments are shown as percentage relative to the transactivation by wt EBNA-2 with standard deviations given as error bars (H, HA tag).

Summary of chapter 2.2.:

a) *the RBP-J binding domain of EBNA-2 can mediate transcription through protein(s) other than RBP-J*
b) *the RAM domain, the RBP-J binding domain of the cellular Notch protein, can partially replace the intrinsic RBP-J binding domain of EBNA-2 and restore activation of the LMP-2A promoter by EBNA-2, however, it does not contribute to the activation of the LMP-1 promoter*
c) *the WW and CR4del mutants preferentially activate RBP-J independent and RBP-J dependent signalling*
d) *so far, no further EBNA-2 mutants were identified, which unequivocally activate RBP-J dependent or independent signalling preferentially.*

2.3. Immunoprecipitation of EBNA-2 protein/DNA complexes in the context of chromatin

EBNA-2 transactivation was studied until now using *in vitro* methods such as promoter reporter and gel retardation assays. These techniques enable the identification of both, *cis* and *trans* elements involved in EBNA-2 dependent transactivation. However, it is questionable whether protein/DNA interactions formed during these assays correspond to interactions formed in the context of chromatin that assembles on the native DNA sequences in living cells.

There are accumulating data that chromatin has a tremendous influence on regulation of transcription and EBNA-2 interactions with chromatin proteins or proteins that influence chromatin structure have been reported (Berger, 2002; Varga-Weisz, 2001 and chapter 1.3.3.).

We established the chromatin immunoprecipitation (ChIP) protocol with EBNA-2 specific antibodies in order to study EBNA-2/DNA interactions *in vivo*. Here we demonstrate the binding of EBNA-2 to viral and cellular promoters *in vivo*.

2.3.1. Three EBNA-2 specific antibodies immunoprecipitate EBNA-2 bound to DNA *in vivo*

Three viral promoters, LMP-1, LMP-2A and Cp, are well known to be induced by EBNA-2 in LCLs (see 1.1.6.) and thus were the appropriate starting point for the establishment of the EBNA-2 specific ChIP protocol. The 721 cell line that was established from primary B-cells immortalised with the EBV strain B95.8 was chosen as an LCL model cell line.

Shortly, nuclei were prepared from 721 cells grown in log phase, treated with 1% formaldehyde to crosslink the chromatin and subsequently lysed. The resulting chromatin was sonicated until most of the DNA fragments were less than 2.5-3 kb in size. 25µg DNA were used per immunoprecipitation. EBNA-2 containing protein/DNA complexes were immunoprecipitated with three EBNA-2 specific antibodies R3, 1E6 and R11. 1E6 binds to residues 438-453 of EBNA-2 while R3 and R11 recognise residues 450-464 (Kremmer et al., 1995). The chromatin cross-link was reversed by

proteinase K treatment. A schematic overview of the ChIP protocol is depicted in figure 2.3.1.

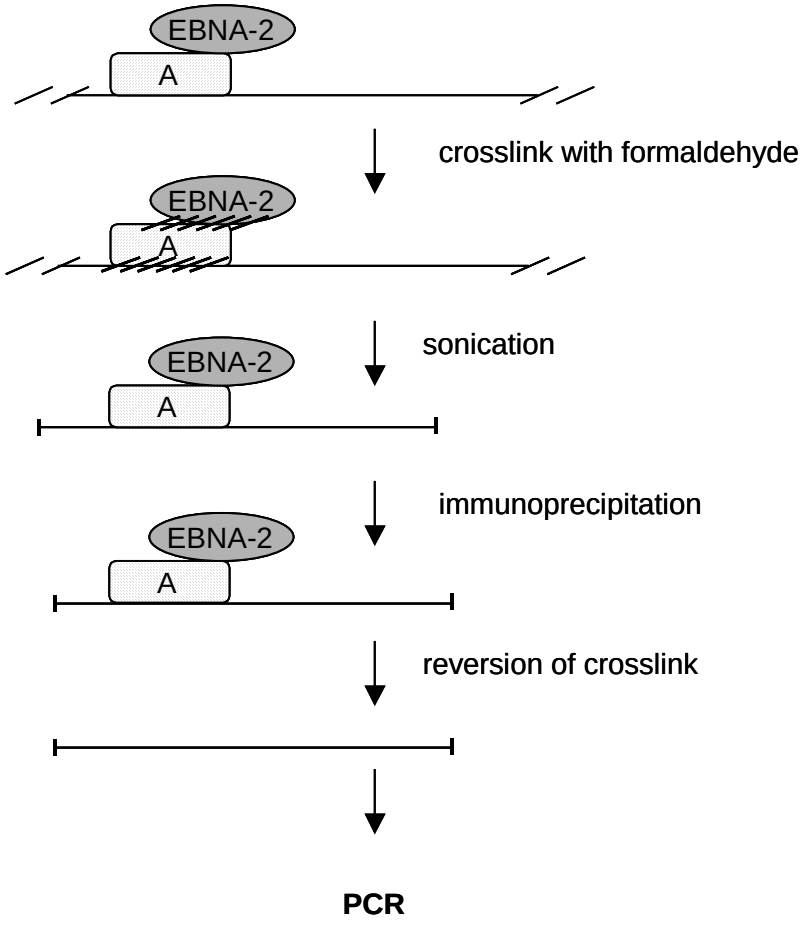

Figure 2.3.1.
Schematic overview of the ChIP method. The protein/DNA complexes are crosslinked in the chromatin context. The chromatin is fragmented and precipitated with a specific antibody, in this case with an EBNA-2 specific antibody. After reversion of crosslink, specific fragments are detected by a PCR reaction (A, anchor protein).

The precipitated DNA served as a template for PCR using primers specific for the LMP-1 promoter (figure 2.3.2.). A PCR reaction without a template was used as negative control (lane 1) and genomic DNA isolated from 293 cells stably transfected with the recombinant EBV was used as positive control for PCR (lane 2). Immunoprecipitation without antibody resulted in no detectable signal (lane 3). The EBNA-2 specific antibodies specifically precipitated EBNA-2 bound to the LMP-1 promoter either applied separately (lanes 4, 5 and 6) or as a mixture (lane 7), whereas the immunoprecipitation with the mixture of EBNA-2 specific antibodies did not enlarge the pool of the precipitated DNA (compare lanes 4, 5, 6 with lane 7).

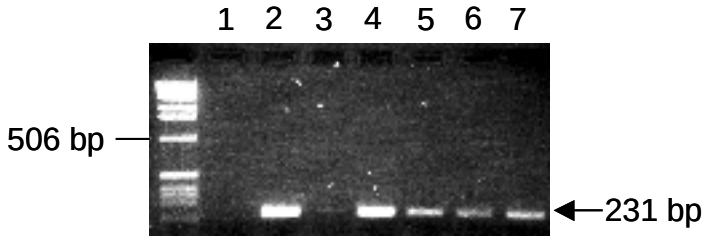

Figure 2.3.2.
Three EBNA-2 specific antibodies precipitate a cross-linked fragment of the LMP-1 promoter. Nuclei were isolated from 721 cells, cross-linked with formaldehyde and lysed. Chromatin was sonicated and immunoprecipitated without an antibody (lane 3) and with three EBNA-2 specific antibodies either separately (R3, 1E6, R11 in lanes 4, 5, 6, respectively) or mixed (R3+1E6+R11, lane 7). A fragment from the LMP-1 promoter was amplified by PCR and detected on a 2% agarose gel stained with ethidium-bromide. A PCR reaction without a template was used as negative control (lane 1) and genomic DNA isolated from 293 cells stably transfected with the recombinant EBV was used as positive control for PCR (lane 2).

2.3.2. EBNA-2 binds to latent viral promoters *in vivo*

After establishing the chromatin immunoprecipitation protocol with three EBNA-2 specific antibodies, we continued to work with only one EBNA-2 specific antibody (R3) and included an isotype control in the immunoprecipitation step. The positions of the PCR fragments amplified from the LMP-1, LMP-2A and Cp promoter relative to the regions of the viral genome are depicted in the figure 2.3.3.

Figure 2.3.4. shows binding of EBNA-2 to the LMP-1 (A), LMP-2A (B) and Cp (C) promoters. A negative control for PCR that contained no template is seen in lane 1. The detected fragments specific for each viral latent promoter corresponded in size to fragments amplified from genomic DNA isolated from 293 cells stably transfected with recombinant EBV (lane 2). Immunoprecipitation without an antibody (lane 3) or with an irrelevant antibody (lane 4) resulted in no detectable viral specific fragment, while fragments containing parts of the LMP-1, LMP-2A and Cp promoters were immunoprecipitated with the EBNA-2 specific antibody (lane 5).

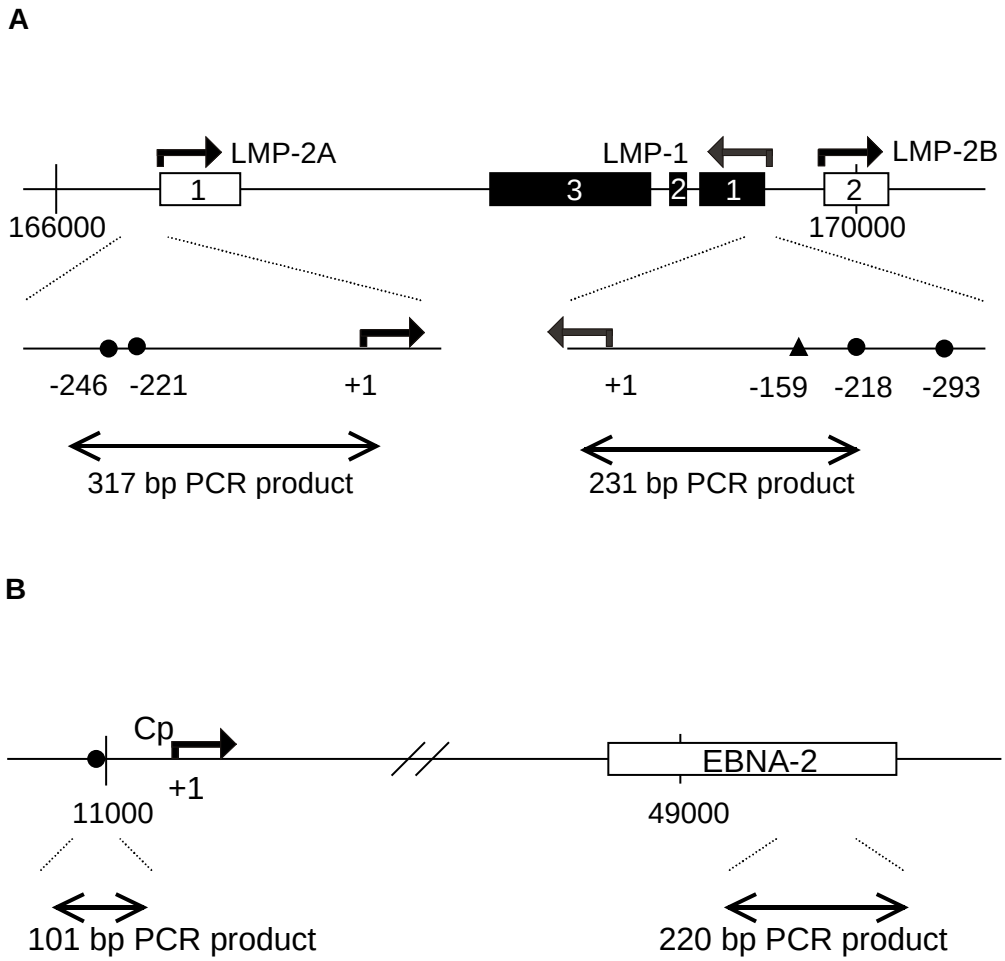

Figure 2.3.3.
Schematic presentation of the PCR fragments amplified from the chromatin immunoprecipitated with EBNA-2 specific antibodies. Schematic presentation of parts of the B95.8 strain genome with depicted nucleotide positions (Baer et al., 1984). Bent arrows show transcription start sites of the LMP-1, -2A, -2B (A) and Cp (B) promoters. Coding exons for the LMP-1 (black boxes), LMP-2A (white boxes) and EBNA-2 genes are indicated. The proximal parts of the LMP-1 and -2A promoters are enlarged in the lower panel and positioned with binding sites for RBP-J (dots) and PU.1 (triangle) relative to the transcription start site. The positions and sizes of the DNA fragments detected in PCR are designated below the enlarged proximal parts of the promoters.

To demonstrate the specificity of the DNA fragments coimmunoprecipitated with EBNA-2 specific antibody, we performed a PCR using primers specific for an EBV region assumed not to bind the EBNA-2 protein. We chose the EBNA-2 gene itself (figure 2.3.3. B). The detection of the EBNA-2 gene fragments immunoprecipitated with the EBNA-2 specific antibody is shown in figure 2.3.4.D. A negative control for PCR that contained no template is shown in lane 1. Amplification of genomic DNA isolated from 293 cells harbouring the recombinant EBV resulted in a DNA fragment

of the expected size (lane 2). Immunoprecipitation without an antibody, with an irrelevant antibody or an EBNA-2 specific antibody resulted in no detectable bands (lanes 3, 4, 5).

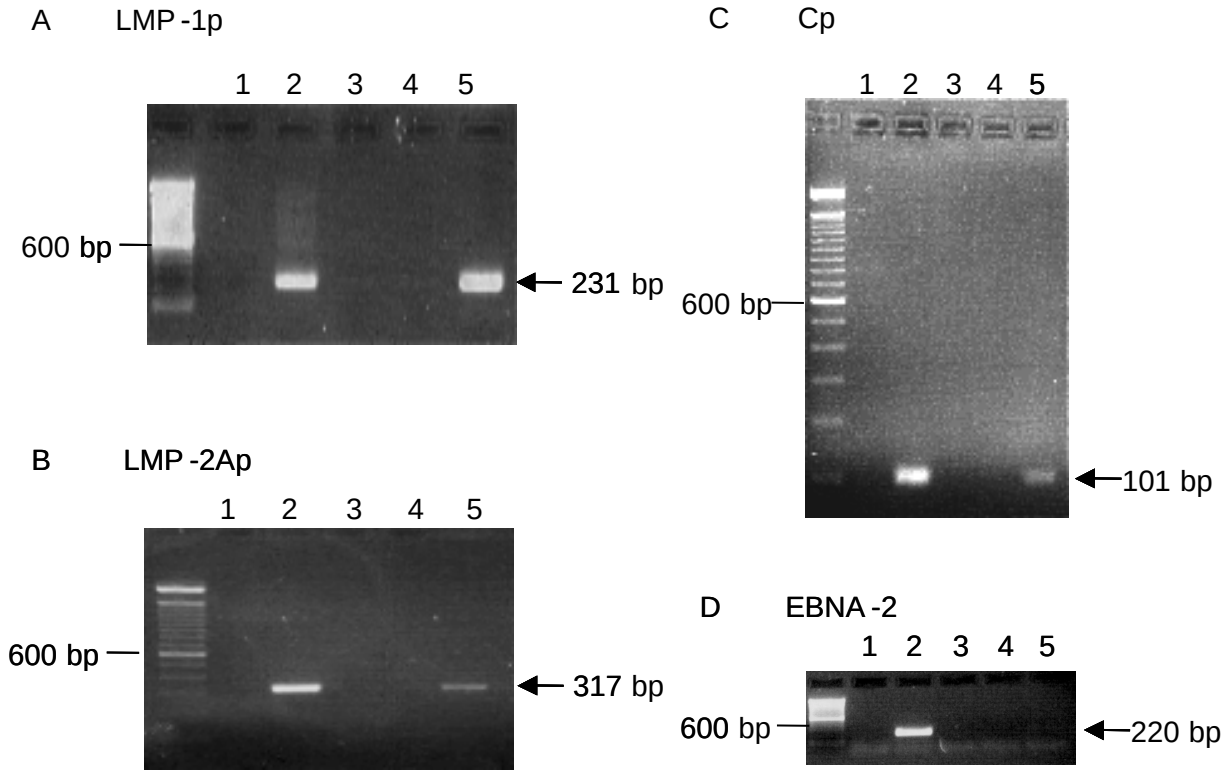

Figure 2.3.4.
EBNA-2 binds to the LMP-1, LMP-2A and Cp promoters *in vivo* but not to the EBNA-2 gene. Nuclear extracts of 721 cells were cross-linked with formaldehyde and lysed. Isolated chromatin was fragmented and immunoprecipitated without an antibody (lane 3), with an irrelevant (lane 4) or EBNA-2 specific antibody (lane 5). Fragments from the LMP-2A (A), LMP-1 (B), Cp (C) promoters and the EBNA-2 gene (D) were amplified by PCR and detected on a 2% agarose gel and stained with ethidium-bromide. A PCR reaction without a template was used as negative control (lane 1) and genomic DNA isolated from 293 cells stably transfected with the recombinant EBV was used as positive control for PCR (lane 2).

2.3.3. EBNA-2 binds to the CD23 promoter *in vivo*

CD23 is a B-cell surface antigen that functions as a low-affinity immunoglobulin E receptor, and a soluble cleavage product acts as an autocrine growth factor

(Kijimoto-Ochiai, 2002). CD23 was one of the first cellular genes shown to be up regulated by EBNA-2 (Calender et al., 1987; Cordier et al., 1990; Rickinson et al., 1987; Wang et al., 1987). Induction of CD23 is closely related to the EBV driven immortalisation of B lymphocytes since only EBV-infected B-cells expressing this protein become immortalised (Azim and Crawford, 1988; Thorley-Lawson and Mann, 1985). The EBNA-2 responsive region in the CD23 promoter was mapped from -89 to -275 bp relative to the mRNA start (Wang et al., 1991). The region includes one RBP-J consensus binding site that was suggested to mediate EBNA-2 transactivation of the CD23 promoter (Ling et al., 1994) (see figure 2.3.5).

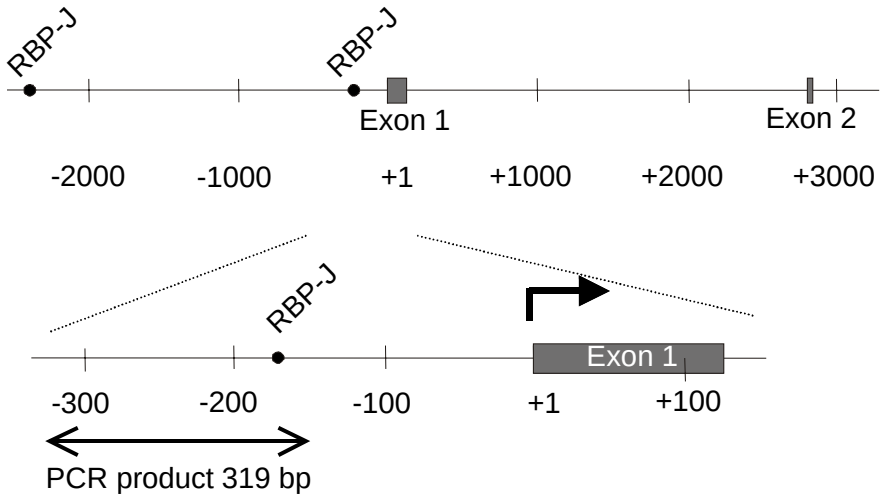

Figure 2.3.5.
Schematic presentation of the 5' region of the CD23 gene including the promoter and the first two exons (indicated with boxes). The transcription start site (bent arrow) and positions relative to the transcription start site are indicated. The positions of the RBP-J binding sites are depicted as black circles. The enlarged proximal part of the promoter is underlined with the DNA fragment detected by PCR using immuprecipitated chromatin.

We wanted to prove binding of EBNA-2 to the CD23 promoter *in vivo* by using the ChIP method (figure 2.3.6.). A reaction without a template served as a negative (lane 1) and genomic DNA isolated from 293 cells stably transfected with EBV served as positive control for PCR (lane 2). Immunoprecipitation without an antibody (lane 3) or with an irrelevant antibody (lane 4) resulted in no PCR product while

immunoprecitation with an EBNA-2 specific antibody resulted in the fragment specific for the CD23 promoter (lane 5).

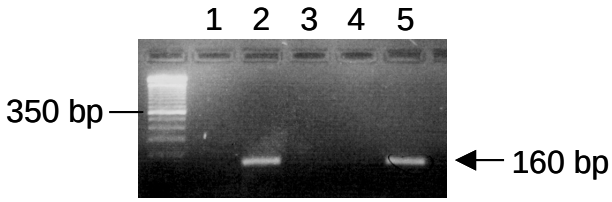

Figure 2.3.6.
EBNA-2 binds to the cellular CD23 promoter *in vivo*. Nuclear extracts of 721 cells were cross-linked with formaldehyde and lysed. Isolated chromatin was fragmented and immunoprecipitated without an antibody (lane 3), with an irrelevant (lane 4) or EBNA-2 specific antibody (lane 5). PCR using primers specific for the CD23 promoter was performed using the DNA fragments obtained by immunoprecitation from chromatin and primers specific for the CD23 promoter. PCR negative (no template, lane 1) and positive control (genomic DNA as template, lane 2) are shown. Amplified fragments were detected on a 2% agarose gel stained with ethidium-bromide.

Summary of chapter 2.3:

Chromatin immunoprecipitation can be applied to detect EBNA-2/DNA complexes in the context of the viral LMP-1, LMP-2A and Cp promoter as well as the cellular CD23 promoter.

2.4. Construction of recombinant Epstein- Barr viruses

Since our previous results had shown that the CR4del and WW mutants activate RBP-J dependent or independent signalling, recombinant EBVs expressing either mutant have been generated and functionally tested.

The technology of recombinant EBV facilitates introduction of any possible mutation into the EBV genome (Delecluse et al., 1998). In this chapter the procedure of recombinant EBV production, from introduction of specific EBNA-2 mutations into the viral genome to the production of virions, is briefly outlined and in figure 2.4.1 schematically presented.

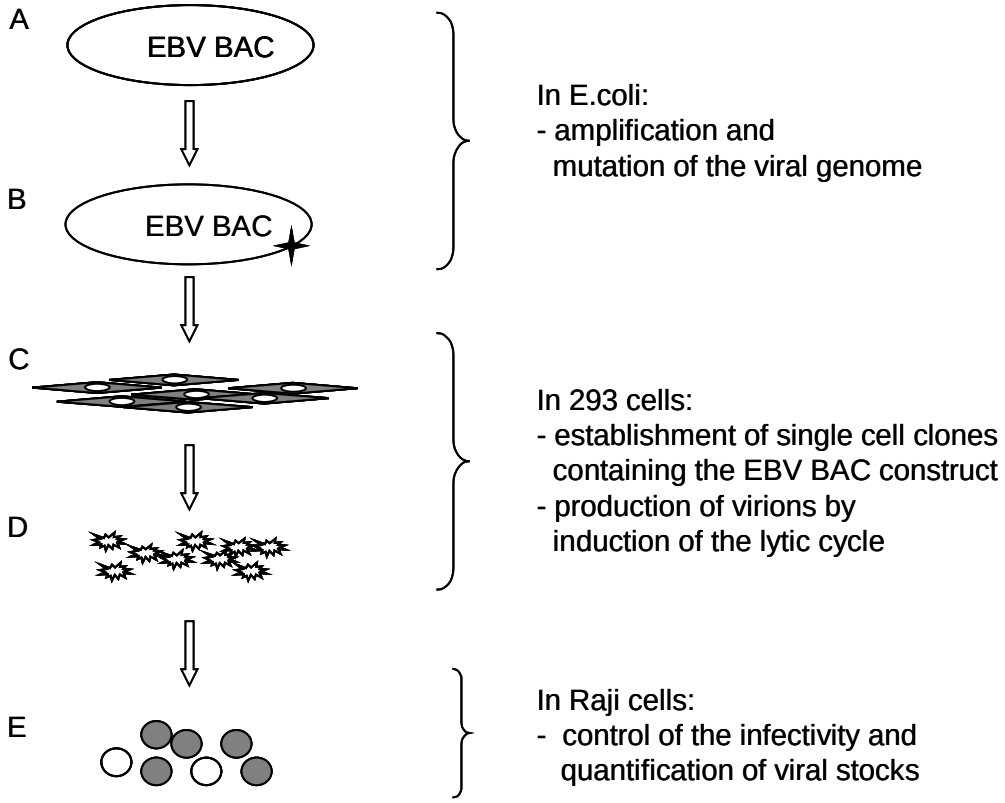

Figure 2.4.1.
Procedure for the production of recombinant EBV. The bacterial artificial chromosome (BAC) is stably maintained in E. coli under chloramphenicol selection (A). After introduction of the desired mutation (black star) into the EBV genome (B), the BAC DNA is isolated and transfected into 293 cells (C). Single 293 cell clones are expanded under hygromycin selection. Viral particles (D) are synthesised after induction of the lytic cycle in the 293 stable cell lines. The viral titre is determined by infection of Raji cells (E).

2.4.1. Introduction of a mutation into the EBNA-2 ORF of the viral genome

The genome of the B95.8 strain of EBV has been cloned onto an F factor-based replicon in E. coli generating a bacterial artificial chromosome (BAC) (Delecluse et al., 1998). The desired mutation is introduced into the EBV genome by the recombination between a linear DNA fragment containing the mutated gene of interest, in this case EBNA-2, and the EBV BAC.

We introduced the CR4del and WW mutations into the EBNA-2 ORF of the EBV genome by the procedure described in detail in 5.1.3. and 5.1.6. During this work we also constructed four additional recombinant viral constructs containing EBNA-2 genes fused with the HA tag or the oestrogen receptor (ER) at the amino terminus. These constructs are to be used in future biochemical studies of EBNA-2 signalling that are beyond the scope of this project. All EBV recombinant constructs that were cloned and used in this work are described in table 2.4.1.

Table 2.4.1.
Designation of the EBV BAC constructs and the brief description of the mutations. Plasmids designated with pkg were constructed during this work while p2089 and p2491 were kindly provided by Prof. Hammerschmidt.

EBV BAC	description
p2089	wild type recombinant EBV
p2491	EBV deleted for the EBNA-2 ORF
pkg447	EBV containing EBNA-2WW325FF
pkg449	EBV containing EBNA-2delCR4
pkg390	EBV containing HAEBNA-2
pkg404	EBV containing HAEBNA-2delCR4
pkg405	EBV containing HAEBNA-2WW325FF
pkg406	EBV containing EREBNA-2WW325FF

The specific EBNA-2 mutations introduced into the EBV genome and relevant for this work are depicted in figure 2.4.2.

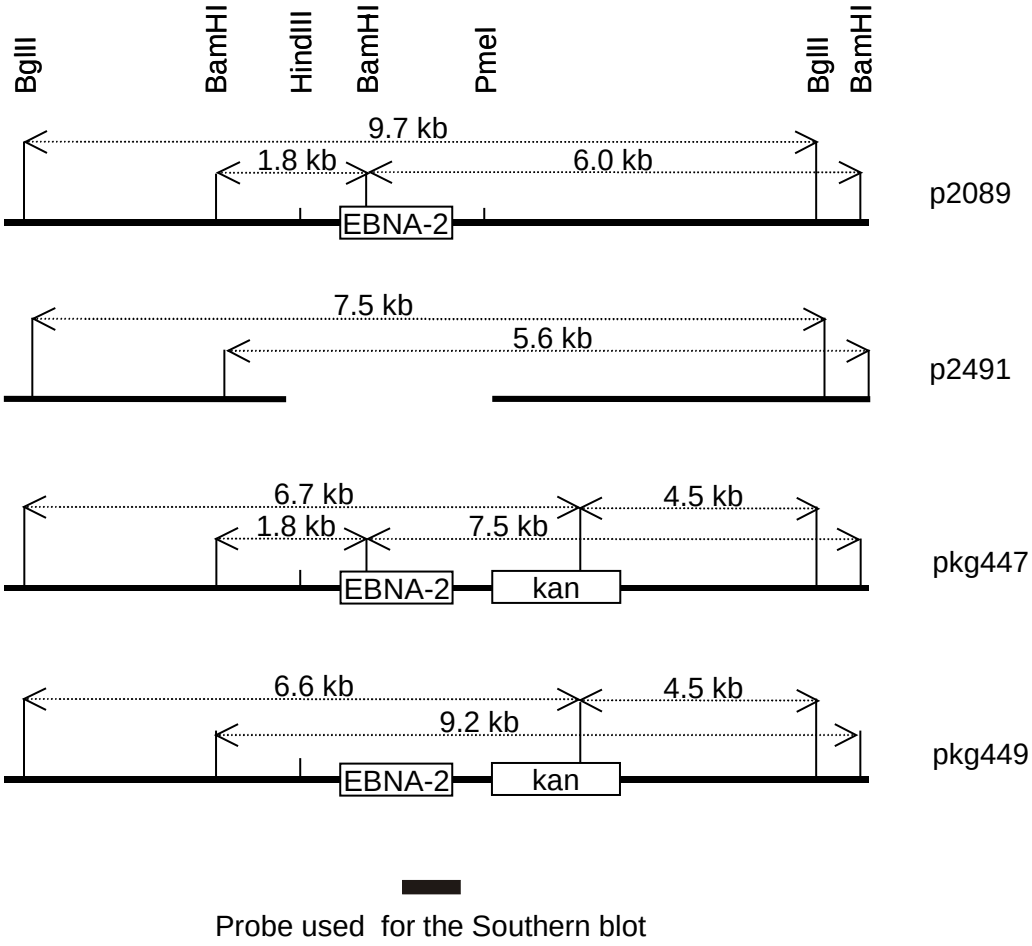

Figure 2.4.2.
Specific mutations in the EBNA-2 gene of EBV recombinant mutants. Relevant restriction sites and the resulting sizes of fragments of the BamHI and BglII restrictions are depicted. In p2491 the region from HindIII to PmeI including the EBNA-2 gene (EBNA-2) is deleted. pkg447 and pkg449 contain the kanamycin cassette (kan) cloned into the PmeI site.

The specific mutations introduced into the viral genome as well as the integrity of the recombinant clones were tested by restriction enzyme digest (figure 2.4.3.) and Southern blot analysis (figure 2.4.4.).

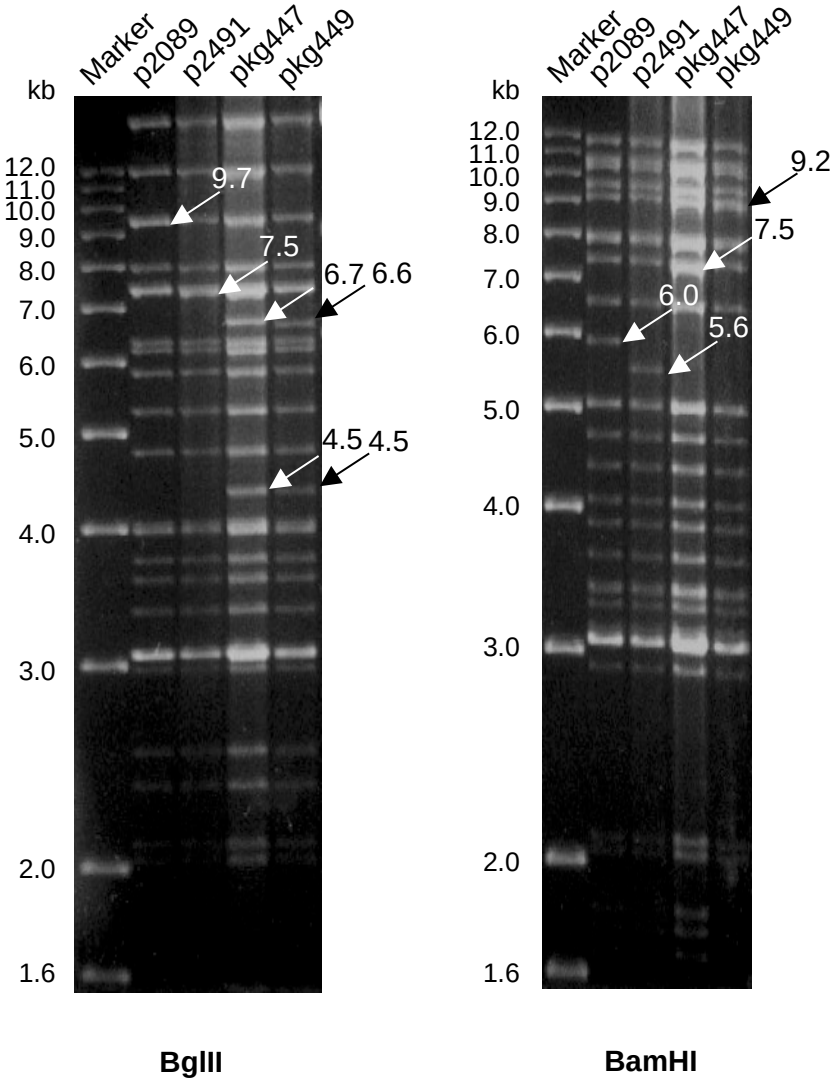

Figure 2.4.3.
Proving the integrity and EBNA-2 specific mutations in the recombinant EBV constructs. 1µg CsCl DNA preparations of the wild type recombinant EBV (p2089), recombinant EBV containing the EBNA-2 deletion (p2491), the WW (pkg447) or CR4del mutant (pkg449) were digested with BglII and BamHI. DNA fragments were separated on a 0.7% agarose gel and stained with ethidium-bromide. EBNA-2 specific fragments are marked by arrows.

2.4.2. Establishment of stable EBV positive 293 cell lines

After recombination and propagation in E. coli, viral DNA was purified by caesium chloride density gradients and transfected into EBV negative 293 cells. GFP expressed from the EBV genome allowed the identification of EBV positive 293 cells. Single cell clones were selected and propagated under hygromycin selection. For

each recombinant virus several 293 stable lines were established and tested for presence of the viral DNA, genomic integrity (figure 2.4.4.) and the ability to support the lytic cycle.

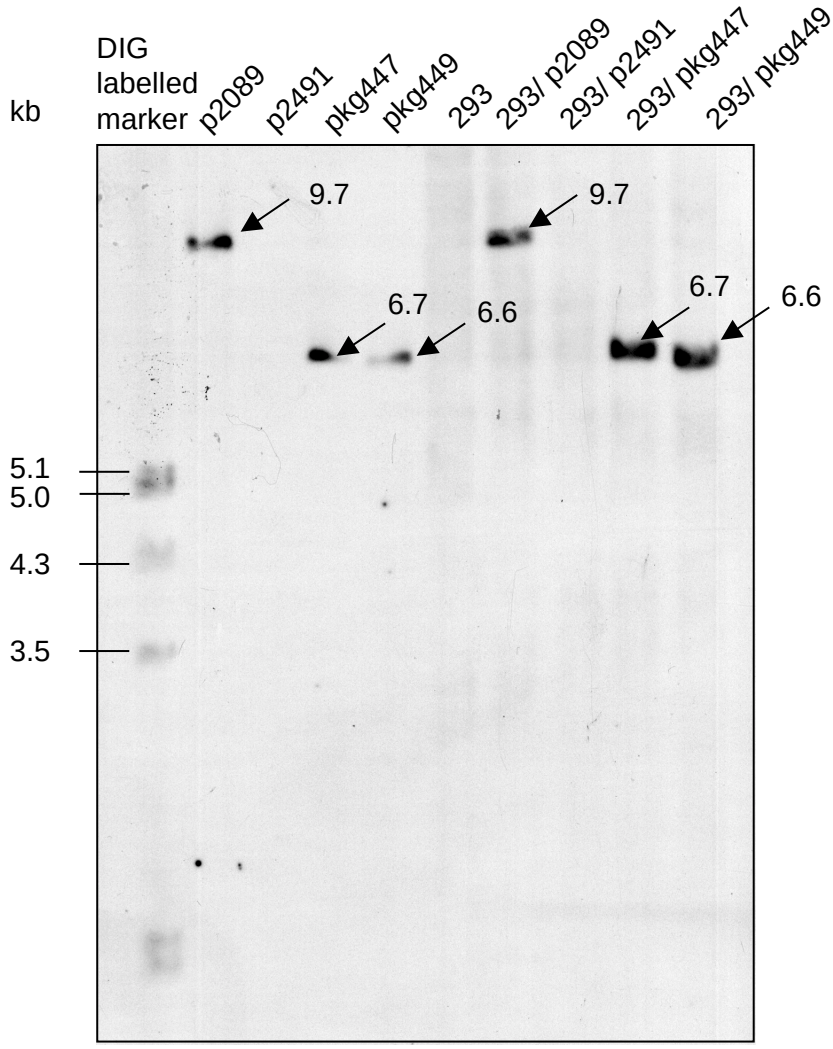

Figure 2.4.4.
Identification of EBNA-2 specific fragments in EBV BAC constructs isolated from DH10B and 293 cells. 50ng of CsCl purified plasmid DNA from DH10B and 10µg of genomic DNA isolated from 293 stable cell lines containing EBV recombinant construct were digested with BglII. A DIG labelled probe specific for the EBNA-2 gene was used to detect EBNA-2 containing fragments in the wild type recombinant EBV (p2089), recombinant EBV lacking EBNA-2 (p2491) or containing the WW (pkg447) or the CR4del mutant (pkg449).

2.4.3. Production and quantification of viral supernatants

In stably transfected 293 cells the EBV genome is present in multiple plasmid copies that are replicated extrachromosomally. The EBV latent phase established in 293 cells is stable and only a small proportion of cells is suggested to support the lytic cycle of EBV spontaneously (Delecluse et al., 1998).

To induce the lytic cycle in the majority of cells, 293 clones were transfected with expression plasmids encoding BZLF1 and BALF4. BZLF1 is the viral inducer of the lytic cycle (Hammerschmidt and Sugden, 1989) and BALF4 is a viral glycoprotein shown to dramatically enhance virus production (Neuhierl et al., 2002).

Three days after transfection of the BZLF1 and BALF4 expression plasmids into 293 stable lines, the culture supernatants were collected and sterile filtered through 0.8μm pore filter. The control of viral infectivity and quantification of the viral supernatants was achieved by superinfection of Raji cells, an EBV positive Burkitt's lymphoma cell line. As in the case of 293 cells, Raji cells containing the recombinant EBV were identified by GFP expression. Quantification of the viral supernatants was assessed by FACS analysis (figure 2.4.5.).

Different viral supernatants considered to be used in the same B-cell infection experiments were quantified in the same Raji superinfection in order to be comparable. Viral titres were expressed indirectly as Green Raji Units (GRU/μl). One GRU corresponds to one green Raji cell detected after infection with recombinant EBV and is presumed to be equivalent to one infectious viral particle.

For example, 78% green Raji cells corresponds to 234 GRU/ μl as follows: 45,000 Raji cells were infected with 150μl recombinant virus. 78% of 45,000 Raji cells is 35,100 GRU in 150 μl or 234 GRU/μl (see equation).

$$\text{GRU/ μl} = \frac{a \times b\,(\%)}{c \times 100}$$

a - number of Raji cells infected

b - GFP positive Raji [%]

c - volume of viral supernatant [μl]

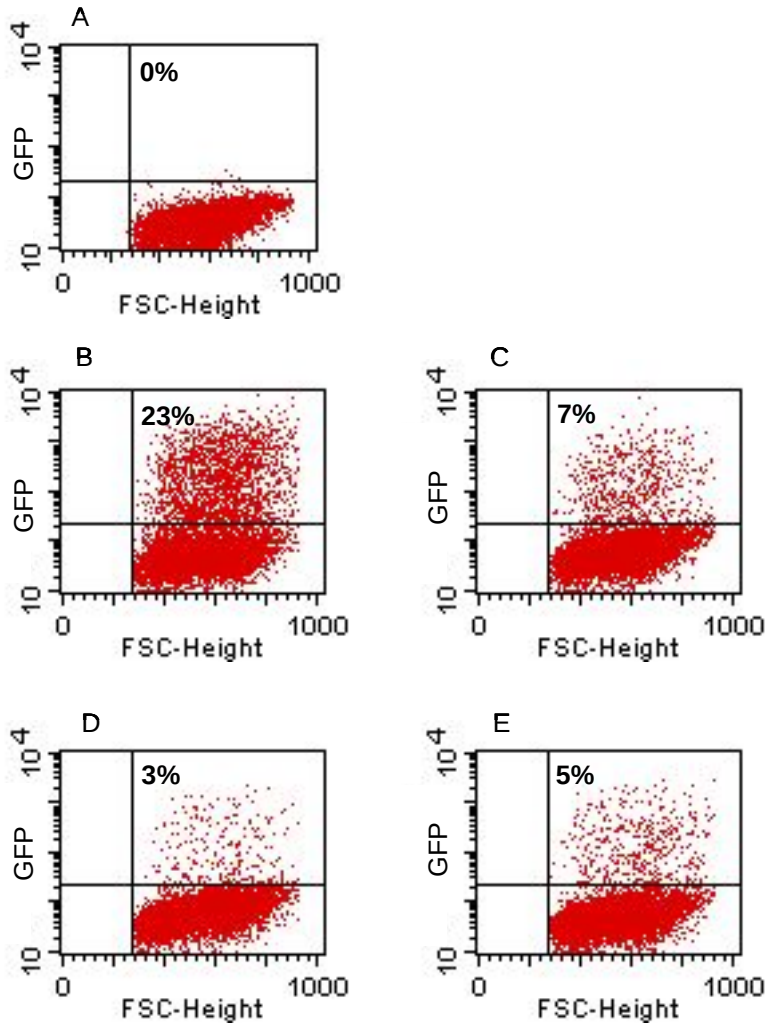

Figure 2.4.5.
Quantification of viral supernatants. Raji cells were infected with recombinant viruses diluted 1:4 and analysed for expression of the green fluorescence protein (GFP) by FACS analysis. GFP is expressed from the genome of the recombinant viruses. Quantification of four viral supernatants in one experiment is shown. A. negative control B. wtEBV C. EBV/delE2 D. EBV/WW E. EBV/CR4del

Summary of chapter 2.4.:

Two new recombinant Epstein-Barr viruses carrying specific mutations in the EBNA-2 gene have been constructed and produced:

a) *kg447 contains the WW mutant that is unable to transactivate via RBP-J*

b) *kg449 contains the CR4del mutant that is diminished in the RBP-J independent signalling*

2.5. Infection of primary B-cells with recombinant EBVs

We characterised WW and CR4del as the EBNA-2 mutants that are impaired in different signalling pathways in transient transfections (see chapter 2.2.). The recombinant EBV system described in chapter 2.4. facilitated the construction and production of recombinant viruses of high titres containing either the WW or CR4del mutant. We wanted to test whether the recombinant viruses containing the specific EBNA-2 mutant can immortalise primary B-cells and if so, what is the impact of the EBNA-2 mutants on the maintenance of the immortalised phenotype.

2.5.1. Infection of primary B-cells with recombinant EBVs and determination of the immortalisation efficiency

Human lymphocytes were isolated from adenoids and tonsils and depleted of T-cells (see 5.2.7. for details). The lymphocyte population used for the EBV infection experiments described below consisted of 70-90% B- and 2-4% T-cells (see figure 2.5.1.). All EBV supernatants used in the same infection experiment were quantified in one Raji superinfection experiment in order to ensure that comparable titres were applied.

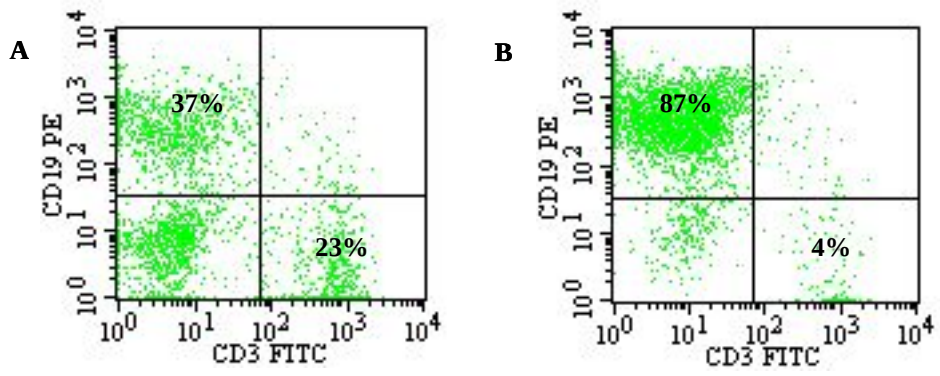

Figure 2.5.1.
The phenotype of the lymphocyte population. Lymphocytes were isolated from adenoids and tonsils and purified on a Ficoll cushion after T-cell rosetting by using sheep erythrocytes. The purified lymphocytes were labelled with an antibody specific for CD19 and coupled to PE and an antibody specific for CD3 and coupled to FITC. The labelled lymphocytes were analysed by FACS to determine the ratio of B- and T-cells, respectively. A representative B-cell isolation and purification from adenoids before (A) and after (B) T-cell depletion is shown.

Primary B-cells were infected with different amounts of recombinant viruses ranging from 0.04 to 360 Green Raji Units (GRU, see chapter 2.4.3.) per well in 96 well plate and plated into 96 well plates coated with irradiated human embryonic fibroblasts which served as feeder cells. Feeder cells support the outgrowth of LCLs probably through soluble factors (Sugden and Mark, 1977). After 4-6 weeks, the number of growth positive cultures was determined (figure 2.5.2.). Once established, the LCLs were kept without feeder cells.

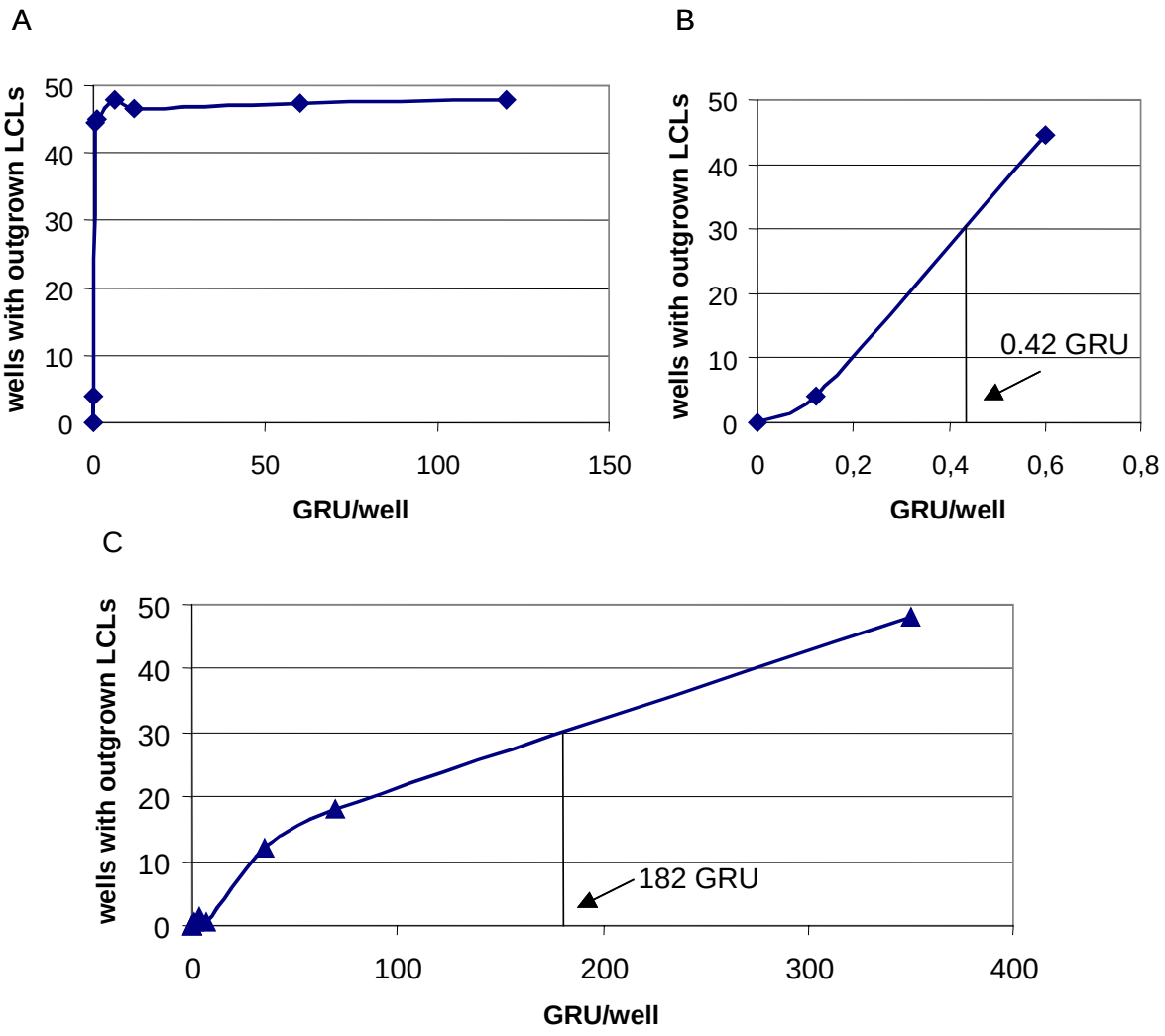

Figure 2.5.2.
Immortalisation efficiency of the wt EBV and EBV/CR4del. 1x10^5 B-cells were infected with serial dilutions of viral supernatants and cultured in groups of 48 microcultures (200µl/well). Titres assayed for B-cell immortalisation ranged from 0.04 to 360 GRU/well. Results are presented as the number of wells positive for growth of LCLs scored after 4 weeks for each group representing a defined virus titre expressed as GRU/well. (A) and (B) are results obtained by infection with wt EBV drawn at different scales. (C) shows the results obtained by EBV/CR4del infection. Arrows indicate the number of GRU required to obtain 63% growing cultures. The mean values of two independent experiments are shown.

Mock infected B-cell cultures showed no spontaneous outgrowth indicating that none of the donors was EBV positive. EBV containing the WW mutant (EBV/WW) or deletion of the EBNA-2 ORF (EBV/E2del) were incapable of immortalising primary B-cells even at 360 GRU used for infection per well (data not shown) while EBV containing the CR4del mutant (EBV/CR4del) immortalised B-cells with low efficiency in comparison to wild type recombinant EBV (wt EBV).

To compare the immortalisation efficiency of wt EBV and EBV/CR4del quantitatively the number of GRU needed to obtain 63% positive wells i.e. 30 positive wells out of 48 wells containing EBV infected B-cells, was determined for a given infection experiment. 0.42 GRU of wt EBV and 128 GRU of EBV/CR4del were necessary to immortalise 63% of the culture in the described experimental system. Thus, 433 times more EBV/CR4del virions were required to immortalise primary B-cells as efficiently as wt EBV. Efficiencies of all recombinant viruses in B-cell immortalisation are presented relative to wt EBV in figure 2.5.3.

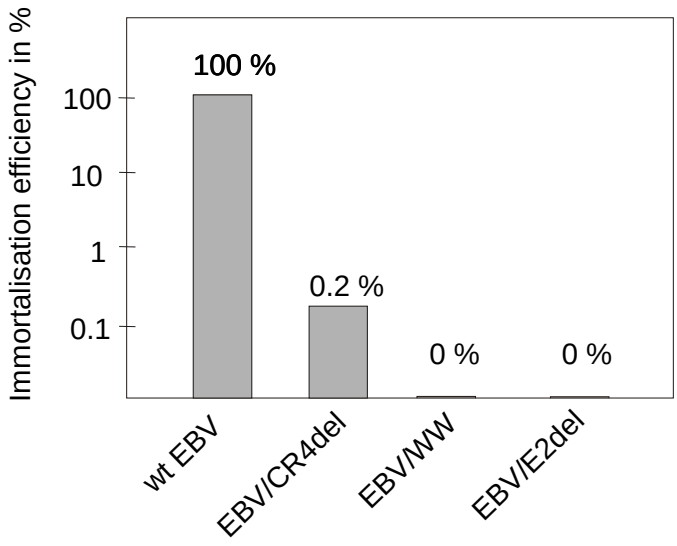

Figure 2.5.3.
Comparison of the recombinant EBV efficiencies in B-cell immortalisation.
B-cells were infected with serial dilutions of wild type EBV (wt EBV), EBV containing the CR4 del mutant (EBV/CR4del), the WW mutant (EBV/WW) or deletion in the EBNA-2 ORF (EBV/E2del). Viral titres required to obtain 63% growing cultures four weeks postinfection are shown relative to the wt EBV titre. The mean values of two independent experiments are presented.

The identity of the specific recombinant virus in the outgrown LCLs could be confirmed by PCR since the CR4del and wild type EBNA-2 genes differ in size by 87 nucleotides (figure 2.5.4.).

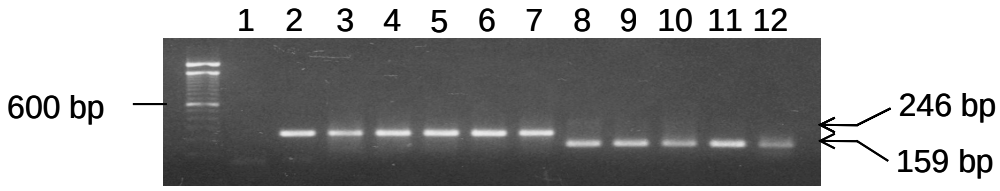

Figure 2.5.4.
Identification of the EBNA-2 gene in LCL immortalised with wt EBV and EBV/CR4del. DNA was isolated from LCLs immortalised with either wt EBV or EBV/CR4del and amplified by PCR using EBNA-2 specific primers. The difference between wt EBNA-2 (lanes 2-7) and the CR4del mutant (lanes 8-12) due to the 87 bp deletion is visible on a 2% agarose gel stained with ethidium-bromide. The negative control is shown in lane 1. Specific fragment sizes are indicated on the right side.

2.5.2. The growth rate of LCL/CR4 is impaired in comparison to LCL/wt EBV

The results described above show that primary B-cells can be immortalised by recombinant EBV containing the CR4del mutant yet with very low efficiency. We established LCLs immortalised with either EBV/CR4del (LCL/CR4) or with wt EBV (LCL/wtEBV) from three donors and compared their growth rate (figure 2.5.5.).
As shown in figure 2.5.5. LCLs immortalised with wt EBV (LCL/wtEBV-A and LCL/wtEBV-B) did not show significant differences in the growth rate while LCLs immortalised with EBV/CR4del (LCL/CR4-B and LCL/CR4-C) revealed small deviations in the growth rate. Until the third day only a small difference in the growth rate of LCL/wtEBV and LCL/CR4 was found. LCL/wtEBV and LCL/CR4 reached on average $3.6x10^5$ and $2.9\ x10^5$ cells/ ml until day 3, respectively. On day 5 LCL/wt EBV reached $5.2x10^5$ cells/ ml while LCL/CR4del reached $2.3x10^5$ cells/ ml indicating that LCL/wtEBV have a growth rate twice as high as LCL/CR4del.

2.5.3. LCL/CR4 and LCL/wtEBV express comparable LMP-1 protein levels

CR4del was characterised as an EBNA-2 mutant impaired in the activation of the LMP-1 promoter in transient reporter assays. The recombinant EBV containing the CR4del mutant (EBV/CR4del) immortalised primary B-cells with very low efficiency in

comparison to wt EBV. Also, the resulting LCL/CR4 grew very slowly in comparison to LCL/wtEBV. Since LMP-1 is an important factor in the EBV driven immortalisation of primary B-cells and maintenance of the transformed phenotype, we wanted to determine whether the described phenotype of EBV/CR4 is connected to the LMP-1 expression level.

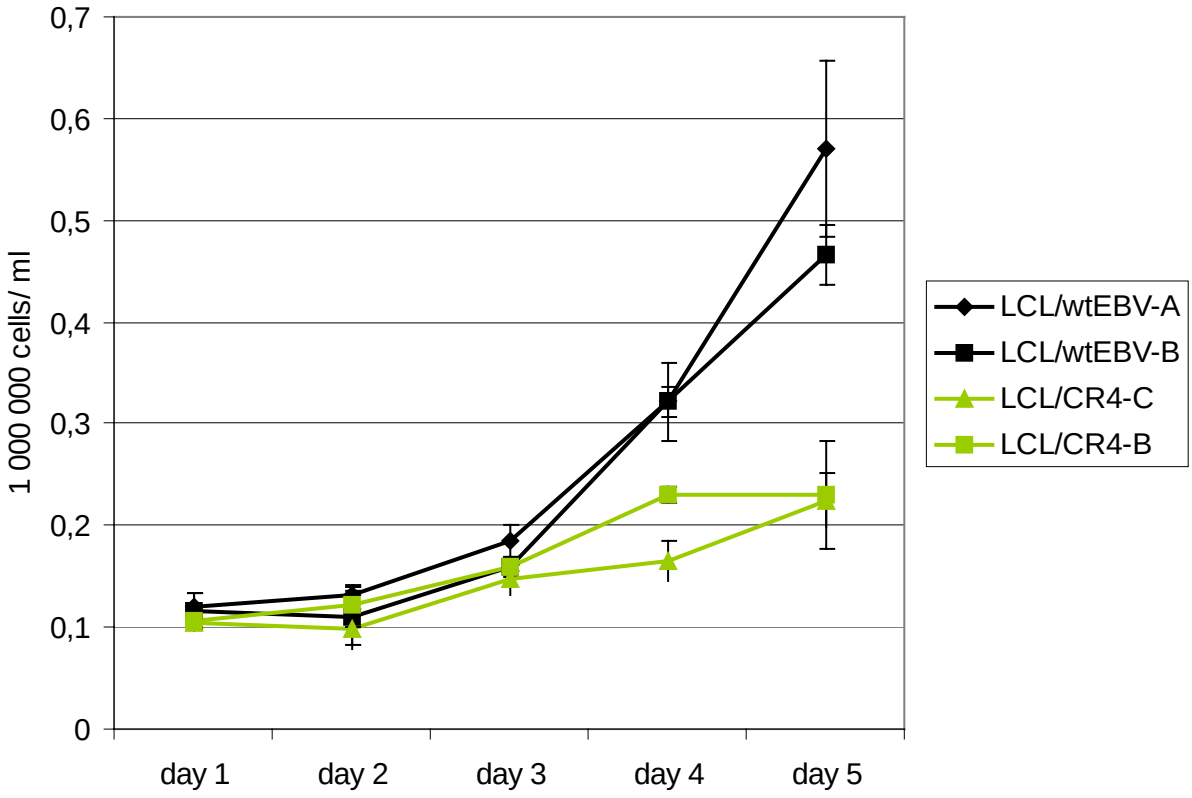

Figure 2.5.5.
LCL/CR4 has an impaired growth in comparison to LCL/wtEBV. Four LCLs originated from three different donors were immortalised with either wt EBV (LCL/wtEBV-A and -B) or EBV/CR4del (LCL/CR4-B and -C) and were seeded at $1x10^5$ cells/ml. The cell number was determined during the following 5 days by counting the living cells. Dead cells were excluded by trypan blue staining. A representative experiment of three done is shown.

As seen in figure 2.5.6. no difference in the LMP-1 protein expression level could be detected in LCL/CR4 and LCL/wtEBV.

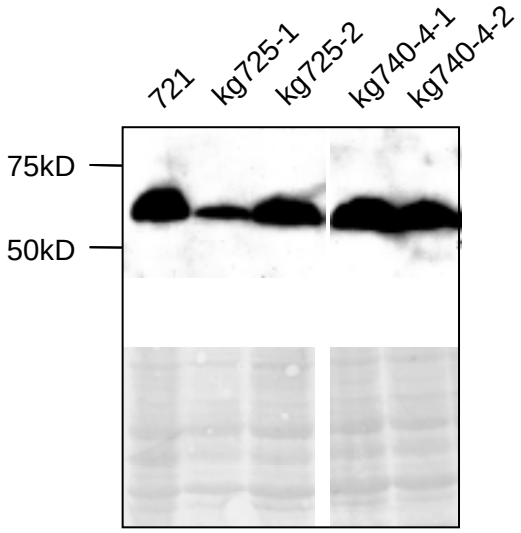

Figure 2.5.6.
LCL/CR4 and LCL/wtEBV express comparable LMP-1 protein levels. 6x10^6 cells of LCL immortalised with either wt EBV (kg740-4-1 and kg740-4-2) or EBV/CR4 (kg725-1 and kg725-2) were lysed in 200µl RIPA buffer. Equal amounts of protein were separated on a 12% SDS gel. LMP-1 protein was detected with the LMP-1 specific antibody (upper panel). The lower panel shows the blot stained with coomasie blue.

2.5.4. DG75 converted with recombinant EBVs do not express LMP-1

The WW mutant was able to induce transcription from the LMP-1 promoter in transient assays, although it cannot bind RBP-J. The *in vivo* study of the signalling that is induced by the WW mutant was not possible since EBV containing the WW mutant was not able to immortalise primary B-cells. We tried to overcome this difficulty by infection of the already established B-cell line DG75 that is EBV negative. The resulting convertants were named DG75/wtEBV, DG75/E2del, DG75/CR4del and DG75/WW. All tested viral samples were able to infect DG75 B-cells confirming that even the non immortalising mutants were functional as indicated by GFP expression post infection (data not shown).

All converted DG75 cell lines expressed the EBNA-2 proteins of the expected sizes i.e. no EBNA-2 as in DG75/E2del (figure 2.5.7., upper panel). In contrast none of the DG75 convertants expressed detectable levels of the LMP-1 protein (figure 2.5.7., lower panel).

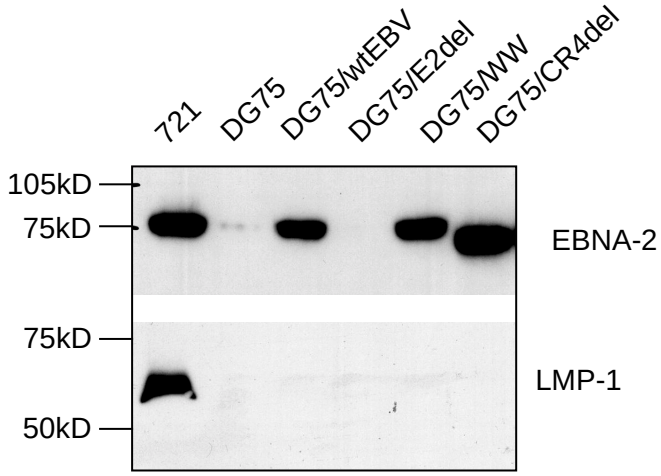

Figure 2.5.7.
DG75 converted with recombinant EBVs express EBNA-2 but no LMP-1. 6x10^6 cells were lysed in 200µl RIPA buffer. Equal amounts of protein were separated on a 12% SDS gel. EBNA-2 and LMP-1 proteins were detected on the same blot with EBNA-2 and LMP-1 specific antibodies. 721 cells immortalised with B95.8 were used as positive control.

Summary of chapter 2.5.:

a) *EBNA-2 and the RBP-J signalling of EBNA-2 are absolutely essential for B-cell immortalisation by EBV. The requirement for EBNA-2 cannot be overcome by viral infection at high virus titres.*

b) *The CR4 region of EBNA-2 strongly influences B-cell immortalisation efficiency and growth rate of the immortalised B-cells. The phenotype of B-cells immortalised by EBV containing the CR4del mutant is not due to reduced LMP-1 expression in the established LCLs.*

3. Discussion

3.1. The LMP-1 promoter: EBNA-2 can induce transcription independently of RBP-J

EBNA-2 is a viral transcription factor that lacks a DNA binding domain. EBNA-2 binds DNA indirectly via an anchor protein(s) and influences transcription from viral and cellular promoters. RBP-J is a ubiquitous DNA-binding protein and one such anchor protein that is able to tether EBNA-2 to DNA. The LMP-1 promoter contains two potential RBP-J binding sites but their role in the transactivation by EBNA-2 was discussed controversially in literature.

We readdressed the role of RBP-J binding sites in the LMP-1 activation by EBNA-2. We conducted a genetic analysis of the LMP-1 promoter and transfected LMP-1 promoter reporter constructs transiently into BL41P3HR1 cells together with an EBNA-2 expression plasmid. We measured the activity of the LMP-1 reporter constructs in the absence and presence of EBNA-2 (chapter 2.1.).

We showed that the LMP-1 reporter constructs that either do not include or contain mutated RBP-J binding site(s) exhibit a high basal activity (figure 2.1.2.). The presence of the intact proximal RBP-J binding site in the LMP-1 reporter construct was sufficient to sustain the repressed state of the LMP-1 promoter. The high basal activity of the LMP-1 reporter constructs prompted us to display the transactivation by EBNA-2 as basic data and not as fold activation i.e. activity of the promoter in the presence of EBNA-2 relative to the promoter's activity in the EBNA-2 absence (figures 2.1.3. and 2.1.5.). When transactivation by EBNA-2 was displayed as basic data, we could show that EBNA-2 activated strongly all LMP-1 reporter constructs irrespective of the presence or integrity of the RBP-J binding sites. We found that EBNA-2 was able to overcome the repression of the LMP-1 promoter caused by RBP-J binding sites and to activate LMP-1 reporter constructs that contained no functional RBP-J binding sites. These data indicate that RBP-J binding sites in the LMP-1 promoter do not contribute to the transactivation by EBNA-2.

In addition to the genetic analysis of the RBP-J binding sites in the LMP-1 promoter we measured the activity of the LMP-1 reporter constructs in the presence of EBNA-2WW325FF (figures 2.1.3. and 2.1.5.). Due to the point mutations, EBNA-

2WW325FF cannot bind to RBP-J (Henkel et al., 1994; Ling et al., 1993; Yalamanchili et al., 1994). In our hands EBNA-2WW325FF was a much stronger transactivator of the corresponding LMP-1 reporter constructs in comparison to the wild type protein. A plausible explanation for this interesting phenotype might be due to the fact that EBNA-2 can bind RBP-J not only in the DNA context but also in solution (Grossman et al., 1994; Henkel et al., 1994). Thus, EBNA-2 might target the LMP-1 promoter in an RBP-J independent manner leaving the RBP-J binding domain free to bind the soluble RBP-J protein. We speculate that soluble RBP-J binds to the DNA-bound EBNA-2 and recruits the corepressor complex. The recruitment of the corepressor complex to EBNA-2 via RBP-J could reduce the overall EBNA-2 effect on the LMP-1 transactivation. Contrary to EBNA-2, EBNA-2WW325FF cannot bind RBP-J and consequently cannot target the corepressive complex to DNA via RBP-J. The consequence is the higher LMP-1 activation by EBNA-2WW325FF in comparison to activation by EBNA-2 (see figure 3.1. for a scheme of the described model).

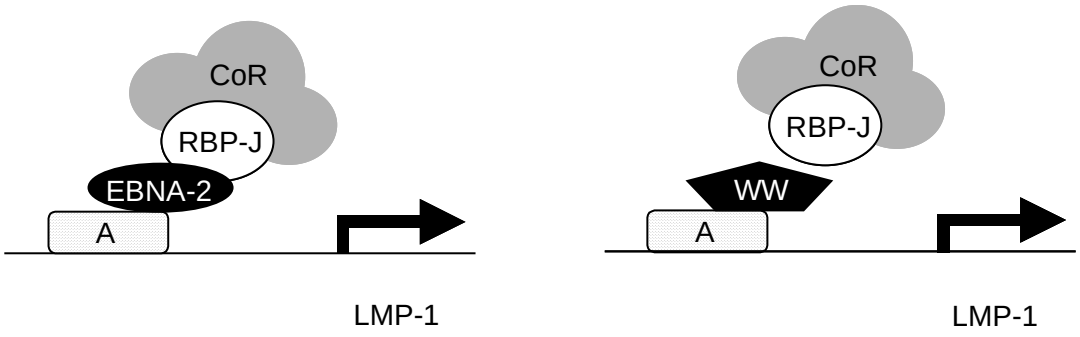

Figure 3.1.
A model for activation of the LMP-1 promoter by EBNA-2. EBNA-2 is tethered to DNA by an anchor protein (A) other then RBP-J but is still able to bind the soluble RBP-J protein. The corepressor complex (CoR) bound to RBP-J impairs the LMP-1 promoter activation by EBNA-2. The WW mutant is not able to bind RBP-J and, indirectly, the corepressor complex and, thus, activates the LMP-1 promoter more efficiently than the wild type protein.

Taken together we could show by two independent approaches, the LMP-1 promoter and EBNA-2 mutational analysis, that RBP-J is not necessary for the LMP-1 activation by EBNA-2.

The necessity of RBP-J binding sites for the LMP-1 transactivation by EBNA-2 had already been questioned (Fahraeus et al., 1993; Sjoblom et al., 1995a; Sjoblom et al., 1995b). Contrary to these and our data a number of genetic analysis of the LMP-1 promoter led to conclusion that RBP-J binding sites significantly contribute to transactivation by EBNA-2 and are necessary for full activation of the LMP-1 promoter by EBNA-2 (Johannsen et al., 1995; Laux et al., 1994a). It is difficult to compare these data with ours since the published results of the LMP-1 promoter transactivation by EBNA-2 are presented as fold activation, thus the activation by EBNA-2 was expressed relative to promoter's basal activity. As we could show, the basal activity of LMP-1 reporter constructs is high if no functional RBP-J binding site is present. The high basal activity of the LMP-1 reporter constructs that lack functional RBP-J binding sites decreases the fold activation by EBNA-2 in comparison to the LMP-1 reporter constructs that contain functional RBP-J binding site(s). This might lead to interpretation that RBP-J binding site(s) is important in the LMP-1 activation by EBNA-2. Thus, interpretation of the data, rather than opposing results of different groups in the field, might have influenced our understanding of the role of RBP-J in the context of the LMP-1 promoter.

Still, there is no doubt that RBP-J binding sites tether EBNA-2 to DNA in other promoters such as in the LMP-2A promoter (see 1.2.2.). It remains to elucidate the mechanisms that enable EBNA-2 to use RBP-J binding sites as anchor sites in some promoters while not in others.

In our genetic analysis of the LMP-1 promoter we also included the reporter constructs that contained mutations in the PU.1 binding site and interferon-stimulated response element (ISRE) (figure 2.1.5.). The mutation of the PU.1 binding site in the LMP-1 promoter completely abolished the transactivation by both wt EBNA-2 and EBNA-2WW325FF. Thus we confirmed that the PU.1 binding site is vital for the LMP-1 transactivation by EBNA-2 (Johannsen et al., 1995; Laux et al., 1994a; Sjoblom et al., 1995a).

Deletion of ISRE led to a higher basal activity of the LMP-1 promoter relative to activity of the wild type LMP-1 promoter as well as to a higher promoter's activity in the presence of wt EBNA-2 and the WW mutant. This indicates that ISRE has a negative impact on the EBNA-2 signalling on the LMP-1 promoter, which is in contradiction to published data showing that ISRE contributes positively to the EBNA-2 signalling (Sjoblom et al., 1995b). Again, this inconsistency between our and

published data might be due to different way of presenting the data. As already mentioned, the LMP-1 promoter containing the mutated ISRE exhibited a slightly higher basal activity in comparison to the wild type LMP-1 promoter. This small difference in the basic activity of wild type and mutated LMP-1 promoter could have a significant influence on the activity of EBNA-2 expressed as the promoter's fold activation and led to controversial conclusions.

The successive deletion of the 5' end of the LMP-1 promoter reveals the presence of additional cis-elements that influence the activation by EBNA-2 (figure 2.1.3.). Especially deletion of -327 to -259 region relative to the transcription start site lowered the EBNA-2 transactivation of the LMP-1 promoter. This indicates the presence of *cis*-elements that positively contribute to the activation by EBNA-2. The deletion of -259 to -217 region had a similar effect, which also indicates the presence of additional EBNA-2 enhancer elements. A detailed genetic analysis of the involved regions in the LMP-1 promoter is required in order to identify these *cis*-elements.

We demonstrated that RBP-J binding sites are not necessary for activation of the LMP-1 promoter, but the RBP-J independent signalling of EBNA-2 on the LMP-1 promoter should also be analysed by *in vivo* studies. For this purpose we constructed a recombinant virus containing EBNA-2WW325FF that cannot bind RBP-J (chapters 2.4. and 2.5.). Although infection of primary B-cells did not result in immortalisation, the virus could still be used to study EBNA-2 signalling. The successful infection of DG75 cells showed that EBV/WW is able to establish latent infection of growing cells. We succeeded in production of viral supernatants of high titre, which could be used to infect large numbers of primary B-cells. The induction of EBNA-2WW325FF target genes might be monitored by a real time PCR in the first days after infection.

3.2. What are the possible roles of the RBP-J binding sites in EBNA-2 activated promoters?

Our data indicate that RBP-J binding sites do not contribute to the LMP-1 transactivation by EBNA-2 in transient transfections. However, we cannot exclude an involvement of RBP-J binding sites in the EBNA-2 activity *in vivo*. The LMP-1 promoter sequences cloned into reporter constructs most probably fail to achieve the complex chromatin structure in transient transfections as they appear in the viral

context of the living cells. Recent data attribute a growing significance of the chromatin structure for transcriptional regulation (Berger, 2002). In contrast to the *in vivo* situation, the effect of chromatin structure is likely to be missing in transient reporter assays.

EBNA-2 interacts with the histone modelling and modifying protein SNF and histone acetylases p300 and CBP (Wang et al., 2000). Recently it was demonstrated that histone H3 and H4 bound to the Cp and bi-directional LMP-1/-2B promoter are hyperacetylated in the presence of functional EBNA-2 (Alazard et al., 2003). Moreover, RBP-J binding sites in the LMP-1/-2B promoter are able to bind RBP-J *in vitro* (Laux et al., 1994b). Taken together, RBP-J binding sites might not be involved in direct transcriptional regulation of the promoter activity, but might mediate the first contact of EBNA-2 to DNA in some promoters. EBNA-2 would then first bind to RBP-J and affect the chromatin structure in the vicinity of the RBP-J binding sites. By changing the chromatin structure into an open state, EBNA-2 would enable binding of other anchor proteins through which it can activate transcription. It is also possible that in some promoters EBNA-2 is not needed for activation of transcription after changing chromatin into an open state. The open chromatin structure might enable the binding of transcription factors, which could activate transcription without a direct involvement of EBNA-2.

The impact of EBNA-2 on the chromatin structure can be now studied with the protocol for chromatin immunoprecipitation established during this work and similar results have been obtained by others (Alazard et al., 2003). With this method the DNA regions that bind EBNA-2 *in vivo* can be identified and then specific chromatin modification in this region can be studied. Recombinant viruses that contain EBNA-2 mutants will be especially helpful to answer this question. It would be interesting to see the effect of EBNA-2WW325FF on transcription since this EBNA-2 mutant cannot bind RBP-J anymore. We also generated a conditional version of this mutant in analogy to already established system with the wild type protein (Kempkes et al., 1995). Thus, chromatin modifications in the presence and absence of the functional EBNA-2 mutant can be investigated.

An additional function of RBP-J binding sites in EBNA-2 activated promoters might be a fine-tuning of the promoter's activity through other proteins. EBNA-3A, -3B and –3C were shown to bind RBP-J and to have either inhibitory or enhancing effects on the EBNA-2 mediated transactivation of the LMP-1 promoter (Le Roux et al., 1994; Lin et

al., 2002; Marshall and Sample, 1995; Robertson et al., 1995; Robertson et al., 1996). A balanced expression of the LMP-1 protein is crucial for the cell's vitality since the effects of the LMP-1 protein on cells range from positive in low levels to toxic in the case of overexpression (Gordadze et al., 2002)

We can imagine that EBNA-3 proteins might influence the EBNA-2 activity via binding to RBP-J in two ways. First, if EBNA-2 activates a promoter via RBP-J, EBNA-3 proteins would compete with EBNA-2 to bind to RBP-J and, thus, would, repress promoter's activation by EBNA-2. Second, EBNA-3 proteins could positively contribute to promoter's transactivation by EBNA-2. In an RBP-J independent promoter EBNA-2 binds to an anchor protein other than RBP-J and activates transcription of the promoter, while EBNA-3 proteins could interact with RBP-J and alleviate repression of the promoter.

3.3. The RBP-J targeting domain of EBNA-2 as a potential binding site for other protein(s)

The RBP-J targeting domain of EBNA-2 is well characterised and shown to be composed of two conserved regions (CR), CR5 and CR6. CR6 mediates the direct contact to RBP-J, while CR5 confers EBNA-2 binding to the SKIP protein. Although RBP-J targets EBNA-2 to DNA, interactions of EBNA-2 with both RBP-J and SKIP are necessary for efficient transactivation.

We deleted CR5 and CR6 in order to abolish binding of EBNA-2 to RBP-J either directly via CR6 or indirectly via CR5. The CR5 and CR6 regions were deleted in three EBNA-2 mutants that differed in the deleted regions flanking the CR5 and CR6 regions. EBNA-2 mutants were compared with the wild type protein in their ability to transactivate the LMP-1 and LMP-2A reporter constructs in transient transfections (figure2.2.3.).

The deletion of the RBP-J targeting domain in all three EBNA-2 mutants led to inability of these mutants to transactivate the LMP-2A promoter. Our data thus confirmed the published results that the EBNA-2 signalling on the LMP-2A promoter is RBP-J dependent (Meitinger et al., 1994; Zimber-Strobl et al., 1993; Zimber-Strobl et al., 1994).

Transactivation of the LMP-1 promoter by EBNA-2 mutants lacking the RBP-J binding domain differed in comparison to the LMP-2A transactivation. Two EBNA-2 mutants that contained narrow deletion of regions flanking CR5 and CR6 transactivated the LMP-1 promoter 30-50% relative to the transactivation by wt EBNA-2. This remaining activity of the EBNA-2 mutants indicates that EBNA-2 can target promoters independently of the RBP-J targeting domain.

However, the loss of the EBNA-2 activity on the LMP-1 promoter reveals that an EBNA-2 function important for the LMP-1 activation was affected. There are two possible explanations for this loss of the EBNA-2 activity on the LMP-1 promoter. First, the RBP-J targeting domain and its binding to RBP-J could still play a role in the activation of the LMP-1 promoter. This is rather unlikely, since our genetic analysis of the LMP-1 promoter showed that RBP-J binding sites do not contribute to the transactivation by EBNA-2. Also, the point mutations in the CR6 region (EBNA-2WW325FF) that abolish the EBNA-2 binding to RBP-J abolished also the LMP-2A activation but had almost no negative effects on the LMP-1 activation. The second possibility is that the RBP-J targeting domain is a multifunctional domain or at least partially overlaps with another promoter targeting domain. The overlapping of RBP-J targeting domain with an additional promoter targeting domain is very likely since the EBNA-2 mutant in which broad regions flanking CR5 and CR6 were deleted (residues 248-382) had lost the ability to transactivate the LMP-1 promoter.

A strong binding candidate for this additional promoter targeting domain is PU.1. While our deletion in EBNA-2 protein that led to loss of the LMP-1 transactivation included residues 248-382, region 314-380 were indicated to mediate the EBNA-2 contact to PU.1 (Johannsen et al., 1995). However, nobody has ever shown that PU.1 binds EBNA-2 directly.

In order to better characterise the RBP-J promoter targeting domain of EBNA-2 we performed the swap experiment discussed in the following section.

3.4. The RBP-J dependent signalling of Notch can partially replace the RBP-J dependent signalling of EBNA-2

The cellular Notch protein uses RBP-J as an anchor protein to indirectly bind to DNA and activate transcription via its transactivation domain in a similar manner to EBNA-

2. The RBP-J binding domain of Notch was characterised as a monofunctional domain that can bind RBP-J also as a peptide isolated from the Notch protein (Tamura et al., 1995).

In order to prove that more functions reside in the RBP-J targeting domain of EBNA-2 we constructed a chimeric EBNA-2 protein. In the chimeric EBNA-2 protein we replaced the intrinsic RBP-J targeting domain by the RBP-J binding region from Notch protein. We compared the chimeric protein, the deletion mutant lacking the RBP-J targeting domain and the wild type protein in their ability to transactivate the LMP-1 and LMP-2A reporter constructs in transient transfections (figure 2.2.5.).

The deletion EBNA-2 mutant lacking the intrinsic RBP-J promoter targeting domain was unable to transactivate the LMP-2A promoter. In contrast to the deletion mutant, the EBNA-2 chimeric mutant was able to bind to RBP-J via the RBP-J binding domain from Notch. The restored ability of the chimeric mutant to bind to RBP-J also restored the transactivation of the LMP-2A promoter. Nevertheless the LMP-2A promoter was transactivated only partially in comparison to the LMP-2A transactivation by the wt EBNA-2.

A possible explanation for the reduced ability of the chimeric EBNA-2 to transactivate the LMP-2A promoter might be inefficient binding of the chimeric mutant to RBP-J. This might be due to structural constraints in the chimeric EBNA-2 protein that impaired either direct or indirect binding to RBP-J. The indirect binding to RBP-J might have been affected by disrupted interaction to SKIP. Namely, the binding of both Notch and EBNA-2 to RBP-J is stabilised by the SKIP protein. SKIP binds to CR5 of EBNA-2 and the ankyrin repeats of Notch. In the chimeric EBNA-2 protein we deleted the RBP-J promoter targeting domain that included CR5 on one hand. On the other hand we incorporated only the RAM domain from Notch and did not include the ankyrin repeats in order to minimise the artificial affects due to incorporation of the long foreign sequence. Thus, the chimeric EBNA-2 could bind to RBP-J but not to SKIP. The missing binding to SKIP might have caused a weaker interaction of EBNA-2 to RBP-J and thereby the weaker transactivation of the LMP-2A promoter. An immunoprecipitation of the chimeric EBNA-2 and RBP-J should be conducted in order to test this possibility.

The EBNA-2 chimeric and deletion mutants showed no significant difference in the ability to transactivate the LMP-1 promoter in comparison to wtEBNA-2. Thus, whether EBNA-2 can or cannot bind to RBP-J played no role for the LMP-1

transactivation. These results confirmed that the RBP-J independent signalling of EBNA-2 is responsible for the activation of the LMP-1 promoter, but the CR5/6 region might contribute to this signalling.

3.5. Two regions of EBNA-2 are critical for promoter targeting

Our data presented in chapter 2.1 and 2.2. indicate that EBNA-2 can activate transcription by an RBP-J independent signalling. We performed a genetic analysis of EBNA-2 with the goal to dissect RBP-J dependent and independent signalling pathways on different EBNA-2 proteins. The four created EBNA-2 mutants were compared in their ability to transactivate either the LMP-1 or LMP-2A reporter construct.

The WW mutant was already described in the genetic analysis of the LMP-1 promoter. We demonstrated that WW could very efficiently transactivate the LMP-1 reporter construct while it was completely inefficient in the activation of the LMP-2A reporter construct in transient transfections (figures 2.1.3. and 2.2.7.). Thus, WW was highly specific for RBP-J independent signalling.

Deletion of residues 117-146 that encompass most of the CR4 region in EBNA-2 protein (CR4del) led to an efficient transactivation of the LMP-2A reporter construct but to an impaired transactivation of the LMP-1 reporter construct (figure 2.2.7.). Thus, the CR4del mutant was impaired in RBP-J independent signalling in contrast to WW.

There are three possibilities by which CR4del might contribute to transactivation of the LMP-1 promoter. The first possibility is that deleted residues 117- 146 are a part of the promoter targeting domain that targets EBNA-2 to an anchor protein other than RBP-J. Residues 123-147 were reported to mediate interaction of EBNA-2 to Nur77 (Lee et al., 2002). Although the interaction of EBNA-2 to Nur77 was first described in context of apoptosis in fibroblasts (Lee et al., 2002), binding of EBNA-2 to Nur77 might also have consequences in EBNA-2 transcriptional signalling. Nur77 is a transcription factor that binds to specific DNA motifs as homo- and heterodimers. Thus, Nur77 is a potential anchor protein for EBNA-2 signalling and other proteins cannot be excluded to mediate the EBNA-2/DNA contact via the CR4 region (Barth et

al., 2003; Grundhoff et al., 1999; Meister et al., 2002; Voss et al., 2001; Yan et al., 2003).

The search in the database revealed that the LMP-1 promoter contains no potential Nur77 binding sites. Nevertheless, the DNA binding specificity of Nur77 is influenced by interaction partners (Winoto and Littman, 2002). Thus, we cannot exclude the possibility that Nur77 binds to the LMP-1 promoter and tethers EBNA-2 to DNA.

The second possibility of the impaired transactivation of the LMP-1 promoter by the CR4del is the abolished binding to DP103 which was shown to act as a coactivator in the EBNA-2 transactivation of the LMP-1 promoter (Voss et al. 2001).

The third possibility is that CR4del might also influence the LMP-1 transactivation by affecting the oligomerisation of EBNA-2, which might be crucial for the activation of the LMP-1 promoter but not for the activation of the LMP-2A promoter. EBNA-2 builds oligomers by either CR1/CR2 or CR3/CR4 contacts (Harada et al., 2001; Tsui and Schubach, 1994). Thus, deletion of CR4 might not effect targeting of EBNA-2 to DNA but might reduce the efficiency of the transactivation by EBNA-2 due to EBNA-2 complexes that depend on EBNA-2 oligomerisation.

In the third EBNA-2 mutant we deleted residues 377-387 that encompass region CR7 (HAdelCR7). This mutant was also reported to specifically influence activation of the LMP-1 promoter (Sjoblom et al., 1995b). We could not confirm this phenotype since in our hands this EBNA-2 mutant showed varying phenotypes in the activation of the LMP-1 reporter construct (figure 2.2.9.). The varying activation of the LMP-1 promoter by HAdelCR7 led to omission of this EBNA-2 mutant from further studies. In order to establish whether CR7 region contributes to the activation of the LMP-1 promoter further transfections are required in order gain reproducible data.

Finally, we constructed the fourth EBNA-2 mutant that consisted of only the carboxy terminus encompassing regions CR5-9. Thus, this EBNA-2 mutant contained the RBP-J promoter targeting domain and the transactivation domain. Surprisingly these regions were neither sufficient to activate the LMP-1 nor the LMP-2A reporter construct (figure 2.2.10.). Moreover, this EBNA-2 mutant was not able to activate transcription from an artificial promoter containing multimerised RBP-J binding sites (figure 2.2.10.). These data indicate that an important EBNA-2 function was abolished. The mutant was synthesised in huge amounts and contained nuclear localisation signals. An immunofluoresce study would show whether this EBNA-2 mutant localises into the nuclear compartments like the wild type protein. An

immunoprecipitation assay of this EBNA-2 mutant and RBP-J would show whether this large deletion lead to inability of EBNA-2 protein to fold properly and bind to RBP-J. Another explanation for the loss of function is that crucial EBNA-2 functions are abolished or missing due to the deletion of the amino terminus.

3.6. EBNA-2 and the RBP-J signalling of EBNA-2 are absolutely required for immortalisation of B-cells by EBV

EBNA-2 was the first EBV gene shown to be indispensable for B-cell immortalisation by EBV due to a spontaneously occurred deletion in the EBV genome under *in vitro* conditions (Miller et al., 1974). The immortalisation phenotype of this mutated EBV could be restored by providing the EBNA-2 gene either in *cis* by a recombinational repair of the deletion (Cohen et al., 1989) or in *trans* by episomally expressed EBNA-2 (Hammerschmidt and Sugden, 1989). The recombinational repair of this deletion with the EBNA-2WW325FF mutant also showed that RBP-J signalling of EBNA-2 is important for the immortalisation of B-cells by EBV (Yalamanchili et al., 1994).

We readdressed the question of the necessity of EBNA-2 and the RBP-J signalling of EBNA-2 for the EBV driven immortalisation of B-cells with the newly developed technology of recombinant EBV (Delecluse et al., 1998). The technology of recombinant EBV brings a new quality into the EBV study since it enables production of genetically identical viral particles in highly concentrated viral titres.

We constructed a recombinant EBV that contained EBNA-2WW325FF and Prof. Hammerschmidt kindly provided the recombinant EBV lacking the EBNA-2 ORF (chapters 2.4. and 2.5.). We succeeded in producing viral supernatants of highly concentrated recombinant EBVs with which we infected primary B-cells isolated from adenoids or tonsils. However, we could not established LCLs immortalised with these viruses. Thus, we confirmed that EBNA-2 and the RBP-J signalling of EBNA-2 are absolutely required for the EBV driven immortalisation of B-cells.

Open questions are still how does the RBP-J signalling of EBNA-2 contribute to immortalisation of B-cells by EBV? Although infection of primary B-cells with the recombinant EBV containing EBNA-2WW325FF does not result in LCLs, experiments of infected primary B-cells might give some answers. Primary B-cells infected with high titres of recombinant EBV could be analysed for RNA synthesis. Thus we could

determine whether EBNA-2WW325FF can induce expression of LMP-1 and other EBNA-2 target genes *in vivo.* Direct target genes would be identified by the established protocol for chromatin immunoprecipitation (chapter 2.3.). We tried to address this question by establishing DG75 convertants with the corresponding recombinant EBV. Unfortunately, DG75 convertants did not express LMP-1.

3.7. The CR4 deletion in EBNA-2 strongly influences the immortalisation efficiency of the recombinant virus and affects the growth rate of the established LCLs

The deletion in the EBNA-2 gene that encompassed the CR4 region had an interesting phenotype in transient reporter assays. The CR4del mutant was impaired for the activation of the LMP-1 promoter (figure 2.2.7.). Since our data indicate that EBNA-2 induces the LMP-1 promoter independently of RBP-J, the CR4del mutant might be affected in RBP-J independent signalling. We constructed a recombinant EBV containing the CR4del mutant in place of the wild type EBNA-2 gene in order to study the effect of the CR4del mutant *in vivo* (chapters 2.4. and 2.5.). We produced viral supernatants of high titres and infected primary B-cells isolated from adenoids and tonsils with the mutated recombinant virus.

In our hands the recombinant virus containing the CR4del mutant could immortalise primary B-cells with 0.2% efficiency relative to the wild type recombinant virus (figures 2.5.2 and 2.5.3.). A low efficiency of a similar EBNA-2 deletion mutant was also reported with a system that does not enable quantification of the immortalisation efficiency (Cohen et al., 1991).

The LCLs immortalised with the recombinant EBV containing the CR4del mutant showed an impaired growth that was dependent on cell density in comparison to the LCLs immortalised with the wild type recombinant EBV. Although the CR4del mutant was impaired in the activation of the LMP-1 promoter under *in vitro* studies, the LCLs established with the recombinant virus containing the CR4del mutant expressed the LMP-1 protein as efficiently as the LCL established with the wild type recombinant virus. This result raises two interesting questions. The first question concerns the reason for the inconsistency between the activation of the LMP-1 promoter by the CR4del mutant under *in vitro* conditions and the LMP-1 expression under *in vivo*

conditions. The second question involves the reason(s) for the impaired growth of the LCLs containing the mutated EBV despite the high expression of LMP-1, which is considered to be one of the most important EBNA-2 target genes.

A few possible explanations for the inconsistency between the *in vitro* and *in vivo* studies might be constraints of the *in vitro* system, which we used for the study of the EBNA-2 signalling. We studied the effect of the CR4del mutant on activation of the LMP-1 reporter construct that encompassed -327 bp relative to the transcription start site. The activation of the -327 LMP-1 reporter construct by the CR4del mutant was impaired in BL41P3HR1 cells. The flanking regions, which were not included in the reporter construct, might significantly contribute to the LMP-1 activation in LCLs and overcome the negative impact of the CR4del mutant on activation of the LMP-1 promoter in the *in vitro* conditions. Moreover, not only *cis*-elements might have been missing in our *in vitro* studies but also *trans*-elements. We studied the effect of the CR4del mutant on activation of the LMP-1 reporter construct in the absence of other viral proteins. However, in LCLs EBNA-2 is co-expressed with other viral nuclear antigens. Especially, EBNA-3 family proteins and EBNA-LP were reported to influence the activation by EBNA-2, both positively and negatively (Harada and Kieff, 1997; Lin et al., 2002; Nitsche et al., 1997; Yokoyama et al., 2001). Thus, EBNA-LP and EBNA-3 proteins might have overcome the negative phenotype of the CR4del mutant in the activation of the LMP-1 promoter by positively influencing the transcription.

We also cannot exclude the possibility that BL41P3HR1 and LCL might differ in expression of cellular proteins that could influence the EBNA-2 signalling.

However, high levels of the LMP-1 expression might also be a consequence of divergent selection and compensation processes in B-cells that took place after the EBV infection. In B-cells infected with recombinant EBV containing the CR4del mutant, the initial expression level of the LMP-1 protein might have been below the optimal one due to the inefficient activation of the LMP-1 promoter by the CR4del mutant. The optimal expression level of the LMP-1 protein is pivotal for the EBV driven immortalisation of B-cells. Thus, only B-cells that could drive expression of the LMP-1 protein to a sufficient level would survive and form LCLs. The selection pressure for specific levels of LMP-1 expression might have influenced the infected B-cells in several ways.

First, additional genetic changes in some B-cells, either effecting transcriptional or posttranslational events, might have supported a higher expression level of the LMP-1 protein and thereby enable the outgrowth of these cells into LCLs. Second, LMP-1 is able to regulate its own expression via positive autoregulatory loops either including STAT or interferon regulatory factor signalling (Chen et al., 2003; Ning et al., 2003). Thus, once expressed, LMP-1 might sustain its own expression.
Still, despite the high expression level of the LMP-1 protein, LCL immortalised with the recombinant virus containing the CR4del mutant have impaired growth in comparison to LCL immortalised with the wild type EBV. This indicates that important EBNA-2 functions were affected due to the CR4 deletion. As already mentioned before, residues deleted in CR4del affect transcriptional activity of EBNA-2 and might influence apoptosis. Thus, a screening of EBNA-2 target genes in LCL immortalised with the recombinant virus containing CR4del and performing apoptosis assays might give the answers to these questions. The established ChIP protocol (chapter 2.3.) could be used to characterise the DNA binding properties of the CR4del mutant in comparison to wt EBNA-2.

3.8. Outlook

EBNA-2 is a multifunctional viral oncogene that is essential for the EBV driven immortalisation of B-cells. The main contribution of EBNA-2 to the immortalisation of B-cells by EBV is due to the transcriptional regulation of viral and cellular genes. Our data indicate that EBNA-2 uses at least two independent signalling pathways. The RBP-J dependent pathway is absolutely required for the immortalisation of B-cells by EBV. The RBP-J independent pathway strongly influences the viral ability to establish and maintain immortalised B-cells.
Study of EBNA-2 target genes downstream of particular signalling pathways would help to better understand the role of EBNA-2 in the EBV driven immortalisation of B-cells. The constructed recombinant EBVs containing EBNA-2 mutants and the established ChIP protocol might be especially helpful since these tools enable identification of direct target genes of specific EBNA-2 mutants.

4. MATERIAL

4.1. Bacterial strains

E. coli DH5α	F-, *lac*l-, *rec*A1, *end*A1, *hsd*R17, Δ(*lac*ZYA-*arg*F), U169, F80d*lac*ZΔM15, *sup*E44, *thi*-1, *gyr*A96, *rel*A1; available in KMOLBI, used for common cloning
E. coli DH10B	F-, *mcr*A, (*mrr-hsd*RMS-*mcr*BC), Φ80d*lac*ZΔM15, Δ*lac*X74, *deo*R, *rec*A1, *end*A1, *ara*D139, Δ(*ara*, *leu*)7697, *gal*U, *gal*K, λ-, *rps*L, *nup*G, kindly provided by Prof. Dr. Hammerschmidt, used for propagation and recombination of EBV BAC

4.2. Cell lines

4.2.1. Basic cell lines

BL41P3HR1	obtained after infection of the EBV-negative Burkitt's lymphoma cell line BL41 with the virus strain P3HR1 (Bornkamm et al., 1982; Miller et al., 1974).
DG75	EBV negative Burkitt's lymphoma cell line (Ben-Bassat et al., 1977).
293	human embryonic epithelial kidney cell line (Graham et al., 1977).
Raji	EBV positive human Burkitt's lymphoma cell line (Pulvertaft, 1964).
WI38	primary human fibroblast cell line (ATCC).
721	lymphoid cell line immortalised with the EBV strain B95.8, (B Sugden, Madison, USA)

4.2.2. 293 cell lines established in this work by stable transfection with the indicated EBV BAC:

kg480-2	p2089 (wt EBV)
kg480-5	p2089 (wt EBV)
kg481-2	p2491 (EBV deleted in EBNA-2 ORF, EBV/E2del)
kg463-5	pkg447 (EBV containing EBNA-2WW325FF, EBV/WW)
kg640-4	pkg449 (EBV containing EBNA-2delCR4, EBV/CR4del)

4.2.3. Lymphoblastoid cell lines established in this work by immortalisation with the indicated recombinant EBV:

kg740-4-1 to -10	immortalised with wt EBV
kg763-1 to -5	immortalised with wt EBV
kg725-1 to -10	immortalised with EBV/CR4del
kg763-6 to -10	immortalised with EBV/CR4del

4.2.4. DG75 convertants established in this work by infection with the indicated recombinant EBV:

DG75/wtEBV	wt EBV
DG75/E2del	EBV deleted in EBNA-2 ORF
DG75/WW	EBV containing EBNA-2WW325FF
DG75/CR4del	EBV containing EBNA-2delCR4

4.3. Material for bacterial and eucaryotic cell culture

Bacto-tryptone	Invitrogen, UK
Bacto-yeast extract	Invitrogen, UK
bacto-agar	Invitrogen, UK
amipicillin	Sigma-Aldrich, USA
chloramphenicol	Sigma-Aldrich, USA
kanamycin	Sigma-Aldrich, USA
RPMI - 1640 medium	Invitrogen, Germany
Foetal calf serum	Biochrom KG, Germany
L-glutamine	Invitrogen, Germany
Penicillin/Streptomycin	Invitrogen, Germany
Amphoterin	Invitrogen, Germany
HEPES	Invitrogen, Germany
Trypsin	Invitrogen, Germany
Lipofectamine	Invitrogen, Germany
Ficoll-Paque	Amersham Pharmacia Biotech, Germany
sheep blood	Oxoid GmbH, Germany

4.4. Plasmids

4.4.1. Existing plasmids used during this work

HA554	kindly provided by Dr. A. Rappel
p554	(Hammerschmidt and Sugden, 1989)
p554-4	(Kempkes et al., 1995)
pBSNER	kindly provided by PD Dr. B. Kempkes
pSG5	SV40 promoter eucaryotic expression plasmid, Stratagene
pBluescript® II (+) (pBS)	Stratagene
pCP15	(Cherepanov and Wackernagel, 1995)

pKD46	(Datsenko and Wanner, 2000)
LL0	(Laux et al., 1994)
LMP-2A	(Zimber-Strobl et al., 1991)
CMVβ Gal	available in KMOLBI
VA32 (pSG5EBNA-2)	kindly provided by W. Schubach
VA54 (pSG5EBNA-2WW325FF)	kindly provided by W. Schubach
pgLRS-634	(Sjoblom et al., 1995)
pgLRS-259 CAT	(Sjoblom et al., 1995)
pgLRS-259 M1 CAT	(Sjoblom et al., 1995)
pgLRS-259 M2 CAT	(Sjoblom et al., 1995)
pgLRS-259 M4 CAT	(Sjoblom et al., 1995)
pgLRS-217 CAT	(Sjoblom et al., 1995)
p2089	kindly provided by Prof. Hammerschmidt
p2491	kindly provided by Prof. Hammerschmidt
p509 (BZLF1)	kindly provided by Dr. B. Neuhierl
p2670 (BALF4)	kindly provided by Dr. B. Neuhierl
pGB103 (RAM)	kindly provided by G. Bommer
Ga981-6 (6xRBP-J)	(Minoguchi et al., 1997)

4.4.2. Plasmids produced for this work

EBNA2 constructs in pBS

pkg1	HAEBNA-2
pkg58	HAdelCR5/6a
pkg59	HAdelCR5/6b
pkg64	HAEBNA-2delSph
pkg72-1	HAEBNA-2delCR4
pkg92	HARAM
pkg147	HAdelCR7
pkg148	HACR5-9
pkg192D1	HAEBNA-2WW325FF
pkg193	HAEBNA-2delCR4 with a kanamycin expression cassette
pkg194	HAEBNA-2 with a kanamycin expression cassette
pkg232	HAEBNA-2WW325FF with a kanamycin expression cassette
pgk299	HAEBNA-2 with a kanamycin expression cassette and an upstream EBV homology sequence to the first XhoI site
pkg309	HAEBNA-2delCR4 with a kanamycin expression cassette
pkg310	HAEBNA-2WW325FF with a kanamycin expression cassette
pkg379	HAEBNA-2WW325FF with an upstream EBV homology sequence to the first KpnI site
pkg410	EBNA-2delCR4
pkg435	EBNA-2WW325FF with a kanamycin expression cassette
pkg436	EBNA-2delCR4 with a kanamycin expression cassette

EBNA-2 constructs in pSG5

pkg95	HAdelCR5/A
pkg99	HAdelCR5/6B
pkg112	HAdelSph

pkg118	HARAM
pkg169	HACR5-9
pkg170	HAdelCR7
pkg172	HAEBNA-2
pkg423	EBNA-2delCR4

EBNA-2 constructs in other vectors

pkg344	EREBNA-2WW325FF with a kanamycin expression cassette in BSNER
pkg373	EREBNA-2WW325FF with a kanamycin expression cassette in p554

Luciferase reporter constructs

pkg206	-634 LMP-1p
pkg203-26A	luciferase gene without a promoter
pkg216	-259 LMP-1p
pkg217	-259mRBP-J LMP-1p
pkg218	-259mISRE LMP-1p
pkg219	-259mPU.1 LMP-1p
pkg220	-217 LMP-1p

EBV BAC constructs

pkg390	HAEBNA-2
pkg404	HAEBNA-2delCR4
pkg405	HAEBNA-2WW325FF
pkg406	EREBNA-2WW325FF
pkg447	EBNA-2WW325FF
pkg449	EBNA-2delCR4

4.5. Oligonucleotides

Origins of complement sequences are listed in brackets.

kg5 (EBNA-2):	5´-CCT CTA CGC CCG ACA GCA-3´
kg9 (EBNA-2):	5´-GGA CTC CGG TTC ATG TAT TG-3´
kg38 (RAM23):	5´-CGG GAT CCG CAT CTA GAA AG-3´
kg39 (RAM23):	5´-CGG BAT CCC CCT CTA GTC-3´
kg43 (EBNA-2):	5´-CCT CTA CGC CCG ACA GCA CCC-3´
kg44 (EBNA-2):	5´-GGA AGA TCT AGT CGG GGA CCG TGG TTC TG-3´
kg45 (EBNA-2):	5´-GGA AGA TCT GGG TAA TGG CAT AGG TGG AA-3´
kg46 (EBNA-2):	5´-GGA AGA TCT TCT AAG ACT CAA GGC CAG AGC-3´
kg47 (EBNA-2):	5´-GGA CTC CGG TTC ATG TAT TGG TG-3´
kg134 (EBNA-2):	5´-GCG AAG ACC GAG GAC GTT TGG CTC TGG TCT CCA AGG TCC ACC-3´
kg137 (EBNA-2):	5´-CCG AAT TCA TGA TTC CAC CTA TGC CAT TAC CCC CC-3´
kg138 (EBNA-2):	5´-GGT GGC GGC CGC CCG GCT GC-3´
kg542 (LMP-2Ap)	5´-AAT GGG GGG GTG GCA TTG-3´

kg543 (LMP-2Ap)	5´-CTG AGA AAC AAG GCG AGA GGG-3´
kg551 (LMP-1p)	5´-TGT CAG GAG CAA GGC AGT TGA G-3´
kg552 (LMP-1p)	5´-GGA CCC GCT TTT CTA ACA CAA AC-3´
kg553 (Cp)	5´-CCA GCC AGA GAT AAT GTC ACA AG-3´
kg554 (Cp)	5´-TCT ACT GTT CCA AAG ATA GCA CTC G-3´
kg572 (CD23p)	5´-GCC GTC CTT CTA ACC CAA GAG-3´
kg573 (CD23p)	5´-AAG CAG CAA GTT CCC ACA GG-3´
kg760 (EBNA-2)	5´-AGG GAT GCC TGG ACA CAA GAG-3´
kg761 (EBNA-2)	5´-TGA CAG AGG TGA CAA AAT GGT GG-3´

4.6. Antibodies

Primary antibodies

anti-EBNA-2 (rat IgG2a, polyclonal), R3	(Kremmer et al., 1995)
anti-EBNA-2 (rat IgG2a, polyclonal), R1E6	(Kremmer et al., 1995)
anti-EBNA-2 (rat IgG2a, polyclonal), R11	(Kremmer et al., 1995)
anti-LMP-1 (mouse IgG1, polyclonal), CS1-4	Dako (Hamburg)
anti- canine antibody (ML3) (used as the isotype control in ChIP)	kindly provided by Dr. Kremmer

Conjugated antibodies

Goat anti-rat, IgG-HRP	Santa Cruz Biotechnology, USA
Sheep anti-mouse, Ig-HRP	Amersham Pharmacia Biotech, Germany
Mouse anti-DIG antibody, AP conjugated	Roche Diagnostics GmbH, Germany
anti-human CD3 FITC	Dianova, Germany
anti-human CD19 PE	Dianova, Germany

4.7. Probe for Southern blot analysis

The EBNA-2 specific probe was DIG labelled by PCR using the oligonucleotides kg5 and kg9 and the plasmid kg1 as template.

4.8. Enzymes

Alkaline shrimp phospatase	Roche Diagnostics GmbH, Germany
Klenow fragment	Roche Diagnostics GmbH, Germany
Mung Bean Nuclease	New England Biolabs, USA
Proteinase K	Sigma-Aldrich GmbH, Germany
Pwo DNA polymerase	Roche Diagnostics GmbH, Germany
Taq DNA polymerase	PeqLab, Germany
RNAse A	Sigma-Aldrich GmbH, Germany
T4-DNA ligase	New England Biolabs, USA
Restriction enzymes	New England Biolabs, USA; Roche Diagnostics GmbH, Germany

4.9. Other molecular biology and chemical reagents

dNTP	Roche Diagnostics GmbH, Germany
DIG DNA labelling mix	Roche Diagnostics GmbH, Germany
Complete protease inhibitor coctail	Roche Diagnostics GmbH, Germany
Full range Rainbow protein marker	Amersham Bioscience, Sweden
DNA ladders	Invitrogen, UK
Agarose	Invitrogen, UK
Ethidium-Bromide	Merck KG, Germany
luciferin	Roche Diagnostics GmbH, Germany
Acetyl Coenzym A	Sigma-Aldrich,USA
bovine serum albumine	New England Biolabs, USA
Arabinose	Sigma-Aldrich,USA
CsCl	ICN, USA
Scintillation solution	Merck KG, Germany

Other chemical reagents were purchased from Merck KG, Germany, Sigma-Aldrich, USA and ICN, USA

4.10. Kits and other material

NucleoSpin Plasmid kit	Machery Nagel, Germany
Jet Star kit	Genomed, Germany
QIAquick Gel Extraction kit	Qiagen, Germany
QIAquick PCR Purification Kit	Qiagen, Germany
ECL™	Amersham Bioscience, Sweden
3MM-paper	Whatman Ltd., Kentucky, USA
Electroporation cuvettes, 2 mm, 4 mm	Bio-Rad Laboratories, UK
Hybond N+ nylon membrane	Amersham Bioscience, Sweden
Immobilion P, PVDF membrane	Millipore GmbH, Germany
Uni-Filter 96 GF/C	Packard Biosciences GmbH, Germany
Filter, 0.8 µm	Renner GmbH, Germany
Filter , 0.45 µm, 0.2 µm	Sartorius, Germany
X-ray film (Biomax MS)	Eastman Kodak Co, USA
White Nunc F96 MicroWell™ Plates	Nalge Nunc International, Denmark

Plastic laboratory wares were purchased from NUNC GmbH, Germany, Greiner GmbH, Germany, Becton-Dickinson GmbH, Germany and Eppendorf, Germany.

4.11. Laboratory equipment

Gel electrophoresis units (DNA)	PeqLab, Germany
Gel electrophoresis units (proteins)	Amersham Bioscience, Sweden
Centrifuge, 6K10	Sigma Centrifuges GmbH, Germany
Centrifuge 2K15	Sigma Centrifuges GmbH, Germany
Centrifuge 5415D	Eppendorf, Germany
Centrifuge RC5C	Sorvall Instruments, USA
Spectrophotometer	Biometra GmbH, Germany

Bacteria incubator	Heraeus Instruments, Germanys
Bacteria shaker C25	New Brunswick Scientific, USA
Flow hood work bench	Heraeus Instruments, Germanys
Mammalian cell incubator	Heraeus Instruments, Germanys
Electroporator	Bio-Rad Laboratories, USA
Microlumat LB 96P	Berthold, Germany
Ultracentrifuge L7-55, L8-70M	Beckman, USA
PCR System 9600	Perkin Elmer, Germany
X-ray film developer	Eastman Kodak Company, USA
Flow cytometer	Beckton-Dickinson GmbH, Germany
Speed-vac, concentrator SVC100	Savant, USA
Thermomixer 5436	Eppendorf, Germany
UV transilluminator	PeqLab, Germany
Water purification system	Millipore GmbH, Germany
Ultrasonic Cell Disrupter, Sonifier®II	Branson, Germany

5. Methods

The standard protein and molecular biology techniques used in this work were performed according to the protocols described (Sambrook and Russell, 2001).

5.1. Bacterial cell culture

5.1.1. Maintenance and propagation of bacteria

Bacteria were cultured either on LB-agar plates in a bacterial incubator or in liquid LB-medium in a bacteria shaker at 37°C overnight. For liquid cultures 200-400ml LB medium wer inoculated with a single bacteria colony picked from an agar plate. Transformed bacteria were selected by adding the appropriate antibiotics (ampicillin 50μg/ml, kanamycin 25μg/ml, chloramphenicol 30μg/ml). For long-term storage, the pellet of 5ml of a dense bacteria culture was resuspended in 1.5ml 50% v/v glycerol in LB medium and stored at -80°C.

LB-medium: 20mM $MgSO_4$; 10mM KCl; 1% (w/v) Bacto-Tryptone; 0.5% (w/v) Bacto-yeast extract; 0,5% (w/v) NaCl
LB-agar: LB-medium, 1.5% (w/v) Bacto-agar

5.1.2. Preparation of competent bacteria

An LB-plate was inoculated with a probe from a bacterial stock and grown overnight at 37°C. A single colony was used to inoculate 2.5ml of LB medium, which was then incubated overnight in a loose-capped vessel, with shaking. 1ml of this overnight culture was used to inoculate 300ml of LB-medium containing 20mM $MgSO_4$ and 10mM KCl. Bacteria were grown in a 1l flask, with shaking, at 37°C until the absorbance at 600nm reached 0.3. Bacteria were kept on ice 10-20min. and pelleted at 4000rpm 10min. at 4°C. The medium was discarded and the bacteria re-suspended in 90ml of ice-cold TFB1. The bacteria were incubated further 10min. on

ice before centrifugation (as above), and re-suspended in 12ml of ice-cold TFB2. Aliquotes of 200µl bacteria were snap-frozen in liquid nitrogen and stored at -80°C.
NOTE: All vessels and pipettes must be pre-chilled.

TFB1: 30mM CH_3CHOOK; 10mM$CaCl_2$, 50mM $MnCl_2$;100mM RbCl; 15% glycerol. Adjust to pH 5.8 with 1M CH_3COOH; sterile filter (0.2 µm).
TFB2: 10mM MOPS, pH7; 75mM $CaCl_2$; 10mMRbCl; 15% glycerol. Adjust to pH 6.5 with 1M KOH; sterile filter (0.2 µm).

5.1.3. Preparation of electrocompetent DH10B with the induced λRed system

DH10B containing p2491 were transformed by TSS transformation (5.1.5.) with the pKD46 plasmid and grown over night at 30°C on plates containing 30µg/ml chloramphenicol (cam) and 50µg/ml ampicillin (amp). A single colony of DH10B/p2491/pKD46 was used to inoculate 5ml of LB-medium containing 30µg/ml cam and 50µg/ml amp, which was then incubated at 30°C overnight in a loose-capped vessel, with shaking. The following day, two times 2ml of the overnight culture were used to inoculate two times 200ml of LB-medium contaning 30µg/ml cam and 50µg/ml amp. Bacteria were grown in a 1l flask at 180rpm and 30°C until the absorbance at 600nm (A_{600}) reached 0.3 (ca 7 h). Arabinose (10% w/v solution) was added to a final concentration of 0.1% to the first flask at A_{600} 0.046 and to the second one at A_{600} 0.189. The λRed system driven by the arabinose inducible promoter was thus expressed for roughly 3 and 1h, respectively.
The bacteria were incubated on ice for 15min and pelleted at 7000rpm at 4°C for 10min. The medium was discarded and the bacteria resuspended in 10ml of ice-cold 10% glycerol. After addition of 200ml of ice-cold 10% glycerol the bacteria were pelleted as above. The washing was repeated twice. The supernatant was discarded and the bacteria were resuspended in a total volume of 900µl of ice-cold 10% glycerol. Aliquotes of 60µl bacteria were snap-frozen in liquid nitrogen and stored at -80°C.
NOTE: All vessels and pipettes must be pre-chilled.

Arabinose solution: Stock: 10 % w/v in H_2O; working concentration: 0.1%

5.1.4. Heat shock transformation

100µl competent bacteria were defrozen on ice, mixed with 10-100ng of plasmid DNA or 10µl of ligation mixture and incubated on ice for 30min. Cells were subjected to heat shock at 42°C for 2min and subsequently returned to ice for 1min. 400µl LB-medium were added to the cells and incubated at 37°C for 1h. 100µl of the bacterial suspension were plated onto LB-agar plates containing the appropriate antibiotic. The LB-agar plates were incubated at 37°C for 16-18h.

5.1.5. TSS transformation

The TSS transformation was an alternative method for a quick transformation of DH10B containing recombinant EBV (p2491) with the plasmid pKD46 to avoid the time consuming preparation of competent bacteria.
A single colony of DH10B containing p2491 was used to inoculate 2.5ml of LB medium, which were then incubated overnight in a loose-capped vessel, with shaking. 100µl of this overnight culture were used to inoculate 10ml of LB-medium contaning 30µg/ml chloramphenicol. Bacteria were grown in a 250ml flask at 180rpm and 37°C until the absorbance at 600nm reached 0.3. 1.5ml of the bacteria suspension were decanted into a microfuge tube and centrifuged at maximum speed for 30sec. The medium was removed from the pellet by aspiration with a disposable tip. The pellet was resuspended in 100µl TSS-solution after addition of 0.1µg of pKD46. The mixture was incubated on ice for 30min. For phenotypic expression the bacteria were supplemented with 500µl LB-medium containing 30µg/ml chloramphenicol and 50µg/ml ampicillin and then incubated at 30°C with vigorous shaking for 2h. The bacteria were plated on LB-agar plates containing 30µg/ml chloramphenicol and 50µg/ml ampicillin and incubated at 30°C over night.

TSS-solution: 10% PEG800 (or 3350), 5% DMSO, 50 mM $MgCl_2$ or $MgSO_4$

5.1.6. Electroporation and homologous recombination in DH10B

60µl of electrocompetent bacteria (DH10B containing p2491 and pKD46) were defrozen on ice, mixed with 5µl of DNA fragment (see 5.3.1.) and transferred to a 0.2mm pre-chilled cuvette. Bacteria were electroporated at 2.5kV, 200Ω and 25µF and transferred to a 1.5ml microfuge tube containing 1ml LB-medium. After 1.5-2h incubation at 37°C with vigorous shaking the bacteria were plated onto LB-agar plates containing 30µg/ml cam and 25µg/ml kan and incubated at 42°C over night.

5.1.7. Isolation of plasmid DNA

Single colonies obtained from transformation of bacteria with ligation reactions were used to inoculate 5ml LB containing the appropriate antibiotic, and grown overnight at 37°C with vigorous shaking. 1.5ml of the overnight culture was decanted into a microfuge tube and centrifuged at maximum speed for 30min in a microfuge. The medium was removed from the pellet by aspiration with a disposable pipette tip. The cell pellet was resuspended in 200µl of alkaline lysis solution I by vigorous vortexing, ensuring complete dispersal of bacterial cells. 200µl of freshly prepared alkaline lysis solution II was added, and mixed gently by inverting the tube 5 times before incubation at room temperature for 5min. 200µl of solution III were added and then mixed by inverting the tube, before centrifugation at maximum speed for 5min. The resulting supernatant was transferred to a microfuge tube containing 900µl of absolute ethanol to precipitate the plasmid DNA, and centrifuged at 15000rpm for 10min. The supernatant was decanted, the pellets dried on air for 10-15min and then re-suspended in 100µl TE buffer containing 10µg/ml RNase A. 2-3µl of this DNA solution were cleaved with restriction enzymes (0.5-1 units of enzyme/1µg DNA) for at least 2h before separation on agarose gels.
Large quantities of plasmids were purified from 300ml cultures using anion exchange columns (Jetstar, Genomed) according to the manufactor's protocol.

Solution I: 50mM Tris-Cl, pH8; 10mM EDTA
Solution II: 0.2M NaOH; 1 % SDS
Solution III: 3M CH_3CHOOK

5.1.8. Isolation and purification of recombinant EBV DNA

Single colonies of DH10B transformed with recombinant EBV were used to inoculate 6ml LB containing 30μg/ml cam, and grown overnight at 37°C with vigorous shaking. Small scale plasmid preparation was conducted out of 4-6ml of overnight culture basically as described in 5.1.7. with additional strong shaking after addition of the solution II.

For large scale plasmid preparation 6 x 1l of LB-medium containing 30μg/ml cam were inoculated with 1ml overnight culture and grown overnight at 37°C with vigorous shaking. Cells were harvested by centrifugation at 5000rpm for 30min at 4°C.

The cell pellet was resuspended in 80ml solution I. 80ml of freshly prepared alkaline lysis solution II were added, and mixed gently by inverting 5 times before incubation at room temperature for 5min. 40ml of solution III were added and then mixed by inverting. Before centrifugation at 8000rpm at RT for 30min. 200-500μg RNA/l culture were added and incubated at RT for 30min. The resulting supernatant was filtered through gaze into a fresh flask containing 100ml isopropanol. After incubation at RT for 30min the DNA was pelleted by centrifugation at 8000rpm for 30min at RT. The supernatant was decanted and the pellet air-dried for 10-20min. The DNA was dissolved in 10ml 5xTE. Proteinase K was added to a final concentration of 25μg/ml and incubated at 37°C over night. After addition of CsCl (10g CsCl per 10ml DNA solution) the DNA solution was transferred into 35ml ultracentrifuge tubes (Sorvall). The filled tubes were tared with 1.55g/ml CsCl solution and supplemented with 400μl of ethidium bromide (10mg/ml stock solution) per tube. A CsCl gradient was achieved by centrifugation at 35000rpm in a TFT 70.38 rotor at 20°C for 3-4 days. The lowest DNA bands were withdrawn under long wave UV light (365nm) with disposable injection needles (2,1x38 mm, BOVI•VET) and transferred to 3.5ml ultracentrifuge tubes. The tubes were filled up and tared with 1,55g/ml CsCl solution and centrifuged at 35000rpm in a TFT 80.4 rotor at 20°C for 24-48 h. The DNA bands were withdrawn as described above and diluted with the same volume of TE. Ethidium bromide was extracted using n-butanol with one additional extraction after the DNA solution appeared colorless. After addition of 3 volumes of TE to the DNA solution and precipitation with isopropanol, the DNA was dissolved in TE and stored at 4°C.

Solution I: 10mM Tris, 1mM EDTA
Solution II: 0,2M NaOH, 0,5 % SDS
Solution III: 5M $CH_3CHOONa$: CH_3CHOOH = 6:1 (v/v)
5xTE: 50mM Tris, 5mM EDTA, pH 8,5

5.2. Eucaryotic cell culture and analysis of cells

5.2.1. Cultivation of suspension and adherent cells

Burkitt's lymphoma and lymphoblastoid cell lines grow in suspension culture in RPMI containing 10% (v/v) FCS, 2mM L-glutamine and 100U/ml penicillin. For DG75 convertants medium was supplemented with 200μg/ml hygromicin. Primary B-cells were isolated and immortalised in RPMI containing 10% (v/v) FCS, 2mM L-glutamine, 100U/ml penicillin, 0,5μg/ml amphotericin B, 1mM sodium pyruvate and 5mM HEPES. All cells were grown at 37°C in a 5% CO_2, water saturated atmosphere. As soon as cells reached a cell-density of ca $1x10^6$/ml they were split 1:3 to 1:7.
Adherent cells were also grown in RPMI containing 10% (v/v) FCS, 2mM L-glutamine and 100U/ml penicillin at 37°C in a 5% CO_2, water saturated atmosphere. 293 cell lines stably transfected with the EBV BAC constructs were selected and maintained in presence of 100μg/ml hygromycin. To detech from flask-bottom, cells were treated with trypsin for 1-3 min after medium removal, washed with RPMI and transferred to 3-6 times larger flasks. For a co-culture with primary B-cells, WI38 cells at 50% confluency were irradiated with 5000 rad to stop proliferation.
Cells were counted in a Neubauer chamber as 1:1 solution in trypan blue to exclude dead cells.

5.2.2. Storage of cell lines

Cells were split 1:3 one day before storage, pelleted (1200rpm, 10min), resuspended in the storage medium ($1x10^7$ cells/ml) and transferred into 1.5ml cryotubes. Cell stocks were first slowly frozen overnight to -70°C in the freezing container (Nalgene)

containg iso-propanol that enables a freezing rate of -1°C/min before transfer to storage facilities in liquid nitrogen.

5.2.3. Transient transfection by electroporation

$1x10^7$ suspension cells per transfection were centrifuged at 1200rpm for 10min and washed once in sterile PBS. Cells were re-suspended in 250µl of RPMI and mixed with previously prepared DNA in a microfuge tube. The mixture of cells and DNA was transferred into 4mm wide "Gene pulser" cuvettes (Biorad). Cells were electroporated at 230V and 950µF and subsequently transferred into warm RPMI medium containing 10% FCS.

5.2.4. Luciferase assay

Cells were harvested 36-48h after transfection by centrifugation at 1200rpm for 10min and washed once with PBS. The cell pellet was resuspended in 100µl luc-lysis buffer and incubated on ice for 10min. The lysed cells were centrifuged at full speed for 10min at 4°C. The supernatant was transferred to a fresh microfuge tube and either measured for luciferase activity or stored at -80°C.
To measure luciferase activity 10µl of supernatant were transferred into a microtiter plate. The measurement of the luciferase activity was conducted in the Microlumat LB96P, Berthold by automatic addition of 50µl luc-buffer per sample and measurement of bioluminiscence in relative light units (RLU).
To measure galactosidase activity 100µl of reaction buffer A were added to 10µl supernatant in a microtiter plate. The measurement of the luciferase activity was conducted in the Microlumat LB96P, Berthold by automatic addition of 50µl acceleration-buffer S per sample and measurement of bioluminiscence in relative light units (RLU).

5x Luc-lysis buffer: 50% w/v glycerol, 5% w/v Triton X-100, 10mM EDTA, 125mM Tris

Luc-buffer: 20mM Tricin, 1mM 4(MgCO3)xMg(OH)$_2$ x 5 H_2O, 2.67mM $MgSO_4$, 0.1mM EDTA, 33.3mM DDT, 0.27mM acetyl-coenzyme A, 0.53mM ATP, 0.47mM luciferin
Reaction buffer A: 100mM Na-P, 1mM MgCl2, 1% Galacton-Plus
Acceleration buffer S: 0.2M NaOH, 10% Emerald enhancer

5.2.5. Transfection of 293 cells and selection of stable cell clones

293 cells were plated into 6-well plates to 70% confluency one day before transfection. 1μg of CsCl purified EBV BAC DNA per well was transfected with lipofectamine according to the manufactore's instructions. On the following day the cells were transfered and maintained in 140mm cell dishes with RPMI containing 10% (v/v) FCS, 2mM L-glutamine, 100U/ml penicillin/streptomycin and 100μg/ml hygromycin. After 3 to 4 weeks the outgrown cell clones were tested for GFP expression under the fluorescence microscope and expanded separately.

5.2.6. Production of infectious virions and quantification of viral titres

To induce the lytic cycle 293 cell clones stably transfected with EBV BAC constructs were transfected with expression plasmids for BZLF1 (p509, 0.5 μg/well) and BALF4 (p2670, 0.5μg/well) in 6-well plates. Transfections were conducted using lipofectamine according to the manufacturer's instructions. After 3-4 days of incubation at 37°C the supernatants were collected, filtered through 0.8μm filters and frozen at -80°C.
A quantification of the viral supernatants was achieved by infection of 0.3ml Raji cells ($3x10^5$ cells/ml) with 0.3ml viral supernatant in 24 well plates. After 3 days the percentage of green Raji cells was determined by immunofluorescence analysis. The concentration of viral stocks was expressed as the number of Green Raji Units (GRU).

5.2.7. Preparation of primary B-cells

Human primary B-cells were purified from adenoids on a Ficoll cushion. Adenoids were cut with a sterile scalpel to small pieces and minced through a 100µl cell strainer (Falcon). Cells were counted and diluted with sterile PBS to $1x10^7$cells/ml. After addition of 100µl fresh sheep blood per $1x10^8$ cells to rossette T cells, the cell suspension was incubated on RT for 10min and subsequently on ice for 1h. 25ml of the cell suspension were layered on 25ml Ficoll-Paque PLUS and centrifuged at 2200rpm at RT for 45min. The interphase containing mostly B-cells was transferred into a clean tube with a sterile Pasteuer pipette. The B-cell suspension was diluted with 4 volums of sterile PBS and centrifuged at 1800rpm for 10min. Washing was repeated twice with centrifugation at 1600 and 1200rpm, respectively. B-cells were re-suspended in the LCL-medium and used for the experiment or cultivated over night at 37°C.

LCL medium: 1xRPMI, 10% (v/v) FCS, 2mM L-glutamine, 100U/ml penicillin, 0,5µg/ml amphotericin B, 1mM sodium pyruvate and 5mM HEPES.

5.2.8. FACS analysis

$1x10^6$ cells were centrifuged at 1200rpm for 10min. at RT and washed once in PBS. The supernatant was dicscarded and the cells were incubated with 20µl CD3 FITC or CD19 PE for 1h at RT in the dark. The cells were washed twice with PBS, resuspended in 1ml PBS and analysed in FACS.

5.2.9. Infection of primary B-cells with recombinant EBV and determination of the immortalisation efficiency

$1x10^5$ primary B-cells per well in a 96 well plate were infected with different amounts of recombinant EBV and seeded onto irradiated WI38. For each viral dilution 48 wells of infected B-cells were prepared. LCL medium was changed once a week. After 4-6 weeks outgrown wells were counted and clones expanded in the absence

of WI38 feeder cells. Immortalisation efficiency was calculated as the number of GRU needed to obtain 63% of LCL positive wells.

5.3. Methods for manipulation and analysis of DNA

5.3.1. Cloning of recombinant plasmids

EBNA-2 constructs in pBluescript® II (+)

pkg1: the HindIII, NotI fragment containing HAEBNA-2 from p554_HA was cloned into the same sites of pBS

pkg58: pkg1 was cleaved with SphI and ligated with two PCR products (oligonucleotides: kg 43 + kg44, kg46 + kg47) which were synthesised on pkg1 as template. PCR products encompassed each SphI site and contained an introduced BglII site. PCR products were cleaved with SphI and BglII and ligated via BglII to each other and via SphI to pkg1

pkg59: pkg1 was cleaved with SphI and ligated with two PCR products (oligonucleotides: kg 43 + kg45, kg46 + kg47) which were synthesised on pkg1 as template. PCR products encompassed each SphI site and contained an introduced BglII site. PCR products were cleaved with SphI and BglII and ligated via BglII to each other and via SphI to pkg1

pkg64: pkg1 was cleaved with SphI and selfligated

pkg72: pkg1 was cleaved with BstEII and BamHI, filled with Klenow and self-ligated

pkg92: pkg58 was cleaved with BglII and ligated to a PCR product synthesised on pgb103 (kindly provided by Guido Bommer) as template with the oligonucleotides kg38 and kg39. Prior to ligation the PCR product was cleaved with BglII

pkg147: pkg1 was cleaved with EcoRI and NotI and ligated with the PCR product synthesised with the oligonucleotides kg137 and kg138 on pkg1 as template. The PCR product was cleaved with EcoRI and NotI prior to ligation

pkg148: pkg1 was cleaved with BbsI and SphI and ligated to the PCR product synthesised with the oligonucleotides kg43 and kg134 on pkg1 as template. The PCR product was cleaved with BbsI and SphI prior to ligation.

pkg309: the HindIII-SmaI fragment from pCP15 containing the kanamycin expression cassette was blunted Klenow and inserted into the PmeI site of pkg72 (**pkg193**); the XhoI-HindII fragment from p554 containing the EBV region 5' to the EBNA-2 gene was cloned into the same sites of pkg193

pkg310: the BstEII-BbsI fragment from VA54 containing the EBNA-2 gene was inserted into the same sites of pkg1 (**pkg192D1**); the HindIII-SmaI fragment from pCP15 containing the kanamycin expression cassette was blunted Klenow and inserted into the PmeI site of pkg192D1 (**pkg232**); the XhoI-HindII fragment from p554 containing the EBV region 5' to the EBNA-2 gene was cloned into the same sites of pkg232

pkg373: the BstEII-NotI fragment from pkg232 containing the EBNA-2 gene and the kanamycin expression cassette was cloned into the same sites of the pBSNER (**pkg344**); the ClaI-NotI fragment from pkg344 containing the EBNA-2 gene and the kanamycin expression cassette was cloned into the same sites of p554-4

pkg379: the HindIII-SmaI fragment from pCP15 containing the kanamycin expression cassette was blunted with Klenow and inserted into the PmeI site of pkg1 (**pkg194**); the XhoI-HindII fragment from p554 containing the EBV region 5' to the EBNA-2 gene was cloned into the same sites of pkg194 (**pkg299**); in order to extend the homology region for recombination the KpnI-HindIII fragment from 554 containing the EBV region 5' to the EBNA-2 gene was cloned into the same sites of the pkg299

pkg410: pkg72 was cleaved with Bpu10I and XhoI and ligated to the Bpu10I-XhoI fragment from p554

pkg435: the BstEII- BbsI fragment from VA54 containing the carboxy terminus of EBNA-2 was cloned into the same sites in pkg1 (**pkg192D1**); the HindIII- SmaI fragment from pCP15 containing the kanamycin expression cassette was filled with Klenow and inserted into the PmeI site of pkg192D1 (**pkg232**); the XhoI- HindIII fragment from p554 containing the EBV sequence 5' relative to EBNA-2 was cloned into the same sites of kg232 to provide homologous sequences for recombination with p2491

pkg436: the HindIII-SmaI fragment from pCP15 containing the kanamycin expression cassette was filled with Klenow and inserted into the PmeI site of pkg72-1

(**pkg193**); the XhoI-HindIII fragment from p554 containing the EBV sequence 5' to EBNA-2 was cloned into the same sites of kg193 (**pkg309**) to provide homologous sequences for recombination with p2491

EBNA2 constructs in pSG5:
The described fragments containing EBNA-2 were inserted into the MluI and NotI sites of Ga981-20.

pkg95 (HAdelCR5/6a)	Mlu-NotI fragment from pkg58
pkg99 (HAdelCR56b)	Mlu-NotI fragment from pkg59
pkg112 (HAdelSph)	Mlu-NotI fragment from pkg64
pkg118 (HAE2RAM)	Mlu-NotI fragment from pkg92
pkg169 (HACR5-9)	Mlu-NotI fragment from pkg147
pkg170 (HAdelCR7)	Mlu-NotI fragment from pkg148
pkg172 (HAEBNA-2)	Mlu-NotI fragment from pkg1
pkg423 (CR4del)	Mlu-NotI fragment from pkg410

recombinant EBVs and fragments containing the EBNA-2 gene used for recombination

pkg390 (HAEBNA-2):	SacI fragment from pkg379
pkg404 (HAEBNA-2delCR4):	XhoI-NotI fragment from kg309
pkg405 (HAEBNA-2WW325FF):	XhoI-NotI fragment from kg310
pkg406 (EREBNA-2WW325FF):	XhoI-NotI fragment from kg373
pkg447 (EBNA-2'WW325FF):	XhoI-NotI fragment from kg435
pkg449 (EBNA-2delCR4):	XhoI-NotI fragment from kg436

luciferase reporter constructs

pkg206: LL0 was cleaved with HindIII and XhoI, blunted with Klenow and selfligated (**pkg190**); the HindIII fragment from pgLRS-634 containing the -634 LMP-1 promoter was inserted into the HindIII site of pkg190

pkg203-26: LL0 was cleaved with HindIII and XhoI and filled up with Klenow (LL0 vector); the insert originated also from LL0: LL0 was cleaved with HindIII, blunted

with Klenow, subsequently cleaved with XhoI and ligated with the LL0 vector, thereby the multiple cloning site was inverted

pkg203-26A: pkg203-26 was cleaved with BglII and selfligated, thereby in front of the luciferase gene the LMP-1 promoter was eliminated

pkg216: the HindIII-PstI fragment from pgLRS-259 CAT containing the LMP-1 promoter was inserted into pkg203-26A cleaved with HindIII and PstI

pkg217: the HindIII-PstI fragment from pgLRS-259 M1 CAT containing the LMP-1 promoter was inserted into pkg203-26A cleaved with HindIII and PstI

pkg218: the HindIII-PstI fragment from pgLRS-259 M2 CAT containing the LMP-1 promoter was inserted into pkg203-26A cleaved with HindIII and PstI

pkg219: the HindIII-PstI fragment from pgLRS-259 M4 CAT containing the LMP-1 promoter was inserted into pkg203-26A cleaved with HindIII and PstI

pkg220: the HindIII-PstI fragment from pgLRS-217 CAT containing the LMP-1 promoter was inserted into pkg203-26A cleaved with HindIII and PstI

5.3.2. Small scale preparation of DNA from LCL samples for PCR analysis

1.5 ml of $0.5x10^6$ LCL cultures were centrifuged at 1200rpm for 5min. The medium was removed from the pellet by aspiration with a disposable pipette tip. The cell pellet was re-suspended in 25µl of 50mM NaOH and cooked at 95°C for 10 min. The lysed cells were neutralised with 4µ 1M Tris, pH 7 and 1µl of prepared samples were used per PCR reaction a in total volume of 20µl.

5.3.3. Polymerase chain reaction

The standard polymerase chain reaction (PCR) was performed essentially as recommended by the manufacturer. For standard (analytical) applications, *Taq* polymerase was used, whereas the proof-reading DNA polymerase, Pfu, was used for cloning purposes. In brief, 10ng of the DNA template to be amplified were mixed with 5pmol of forward and reverse primers, 0.2mM dNTPs (dATP, dGTP, dCTP, dTTP), and 1U Taq DNA polymerase, in a volume of 20µl 1x polymerase buffer. PCR was performed in a thermocycler. Samples were first denaturated at 95°C for 5min.

before commencement of the amplification stage, and for 30sec between each cycle thereafter. The annealing temperature used corresponded to the calculated melting temperature for the primers used. Elongation was performed at 72°C and the elongation time depended on the length of the expected PCR product. Following the final cycle, a further elongation step of 10min was performed to ensure complete synthesis.

5.3.4. Non-radioactive labelling of DNA fragments

DNA probes were labelled non-radioactively by incorporation of digoxigenin-11-dUTP (DIG) by PCR. Essentially a PCR was performed as described in 5.3.3. with addition of DIG-labelled dNTP mix. The total amount of dNTPs in the PCR mixture was composed of 50% of dNTP (0.2mM) and 50% of 1xDIG DNA Labelling Mix (Roche, 1mM dATP, 1mM dCTP, 1mM dGTP, 0.65mM dTTP, 0.35mM DIG-dUTP). Due to slower incorporation of DIG-labelled dNTP, the elongation time was extended in comparison to PCR reactions using non-labelled dNTPs. The evaluation of PCR-labelled probes was achieved by agarose gel elecrophoresis since labelled PCR products have a significantly greater molecular weight than the unlabelled products.

5.3.5. Isolation of total DNA in large scale

Confluent cultures of 293 cells from 92x17mm cell culture dishes were treated with trypsine and washed with PBS. Cells were pelleted by centrifugation at 1200rpm for 10min. and re-suspended in 3ml proteinase K buffer. After addition of 100µl 20% SDS and 30µl proteinase K (stock 20µg/µl), the cell extract was mixed and incubated at 37°C over night. Following addition of 1ml 5M NaCl the cell extract was vigorously vortexed until the viscosity was lost and incubated on ice for 30min. Cell debris was pelleted by centrifugation at 3000rpm for 15min. The supernatant was decanted into a fresh 15ml tube and mixed with 0,6vol isopropanol. After centrifugation at 4500rpm for 15min, the DNA was pelleted and washed twice with 70% ethanol. The dried pellet was dissolved in TE.

Proteinase K buffer: 10mM Tris/HCl, pH 8,0, 400mM NaCl, 10mM EDTA, pH 8,0

5.3.6. Nonradioactive Southern blot analysis

10μg genomic DNA were digested with resctriction enzymes and separated on a 0.7% agarose gel in 1xTAE at 50-60V over night. DNA bands were visualised under UV after staining with ethidium bromide (50ng/ml in 1xTAE). The DNA was prepared for transfer to a nylon membrane (Hybond, Amersham) by submerging the gel into depurination solution for 10-20min, subsequently into the denaturation buffer for 30-60 min and finally to the neutralisation buffer for 30- 60min with agitation. After setting up the capillary blot, the DNA was transferred onto the nylon membrane over night in 20xSSC. The membrane was washed with 2xSSC and exposed to 1200J UV to cross-link the DNA. After short soaking in 2xSSC, the membrane was prehybridised with prehyb-mix (20ml/100 cm^2) for 30min at 68°C. 2.5ng DIG-labelled probe were denaturated in 50μl H_2O by boiling for 10min and added to freshly replaced prehyb-mix on the membrane. The DIG-labelled probe hybridised to the DNA at 68°C over night. After hybridisation, the membrane was washed 3x20 min in pre-warmed hyb-washing buffer at 65°C. The membrane was washed for 5min in washing buffer1 and incubated in blocking buffer2 for 60min. 1μl of α-DIG-AP conjugate (Roche) was added to fresh blocking buffer and incubated on the membrane for 30min. After washing 4x10min in washing buffer1, the membrane was first equilibrated in substrate buffer4 and then incubated for 5min with the substrate solution (CSPD or CDP* diluted in substrate buffer4, as recommended by manufacturer, Roche, Germany). The membrane was exposed to X-ray film over night.

1xTAE: 40mM Tris-Cl (pH 8.0); 5mM Na-acetate; 1mM EDTA
Depurination solution: 0.25N HCl
Denaturation buffer: 1.5 MNaCl; 0.5N NaOH
Neutralisation buffer: 0.5M Tris-Cl, pH 7.2; 3 MNaCl; pH set to 7.2 with HCl
20xSSC: 3M NaCl; 300mM Na3- citratex2H_2O, pH7.0
Prehyb-mix: 0.25M Na_2HPO_4, pH7.2; 1mM EDTA; 20% SDS; 0.5% blocking reagent
10xblocking reagent: 10%(w/v) blocking powder (Roche, Germany), 0.1M maleic acid; 0.15M NaCl
Hyb-washing buffer: 20mM Na_2HPO_4; 1mM EDTA, 1% (w/v) SDS
Washing buffer 1: 0.1M maleic acid, pH 8.0; 3M NaCl; 0.3%(w/v) Tween-20

Blocking buffer: 0.5% blocking reagent in washing buffer
Substrate buffer 4: 0.1M Tris-Cl; 0.1M NaCl, 50mM $MgCl_2$

5.4. Methods for the analysis of proteins and DNA/protein interactions

5.4.1. Preparation of protein extracts

For the analysis of total cellular protein, DG75 cells were pelleted by centrifugation at 1200rpm for 10min 36-48h after transfection, washed in PBS and resuspended in 100µl ELB lysis buffer. After centrifugation at full speed at 4°C, supernatants were transferred into fresh microfuge tubes and stored at -20°C.
To detect intracellular LMP-1 or EBNA-2 expression $6x10^6$ cells were prepared as described above but lysed in 200µl RIPA buffer.

ELB buffer*: 50mM HEPES, pH7, 250mM NaCl, 1% Igepal, 5mM EDTA
RIPA buffer*: 20mM Tris, pH 7.5, 150mM NaCl, 1% Triton X-100, 0.5 % DOC, 0.1% SDS,
* Buffers were supplemented with complete protease inhibitor coctail (Roche diagnostics, Germany)

5.4.2. Protein quantification

Protein quantification was performed using a dye reagent (Bio-Rad Protein Assay) based on the method of Bradford (Bradford, 1976). 5µl of protein extract diluted 1:3 were mixed with 800µl PBS and 200µl dye reagent and the absorbance at 595nm (A595) was determined. In the same way A595 of serial dilutions of bovine serum albumin from 0.2-5 µg/µl in PBS was determined. In the resulting titration curve showing the protein concentration against A595, the concentrations of protein extracts were determined.

5.4.3. SDS-polyacrylamide gel electrophoresis

Denaturating (SDS) polyacrylamide gel electrophoresis (PAGE) was performed essentially as described (Laemmli, 1970). 10-50µg protein were denaturated by boiling at 95°C for 5min in 1xSDS PAGE loading buffer before separation on an SDS-PAGE gel. Stained marker standard (Rainbow, Amersham) was loaded simultaneously on the same gel for the estimation of the molecular weight. The acrylamide content of SDS-PAGE gels was varied according to the size of the protein being examined (described in detail in Sambrook and Russell, 2001) in conjuction with 5% acrylamid stacking gels. Gels were separated using a constant current of 30mA in 1xSDS running buffer. Following separation, gels were used for Western blotting.

4 x SDS-PAGE loading buffer: 200mM Tris pH 6.8; 8% SDS; 40% (v/v) glycerol; 0.4% bromphenolblue; 400mM DTT; 0.2mM EDTA
SDS-PAGE running buffer: 25mM Tris; 0.2M glycin; 0.1% SDS

5.4.4. Western blotting and immunodetection of proteins

Proteins separated by SDS-PAGE were trasferred to a PVDF membrane in a transfer unit (Hoefer TE 22 Mini Tank Transfer Unit, Amersham) filled with transfer buffer. The blotting sandwich was composed of the gel placed on top of a piece of Whatman 3MM filter paper and a sponge and overlaid with the PVDF membrane, pre-washed in methanol and equilibrated in transfer buffer. The blotting sandwich was completed by the addition of a second layer of filter paper and sponge. Transfer in a cassette assembly was carried out at 400mA for 1h.
Following transfer, the PVDF membrane was incubated in blocking buffer for 1h. The primary antibody was diluted to its correct working concentration in blocking buffer and incubated with PVDF membrane over night at 4°C. The membrane was washed 3x with PBS-T and incubated with a peroxidase-conjugated secondary antibody, specific for the isotype of the primary antibody used for 1h at RT. The secondary antibody was diluted in blocking buffer to its correct working concentration. After 3x washing in PBS-T membrane was layered with ECL solution, following the manufacturer's protocol. Protein-antibody complexes were detected on X-ray film.

Transfer buffer: 25% methanol; 25mM Tris; 0.2M glycin; 0.1% SDS
PBS-T: 1xPBS, 0.05% Tween-20
Blocking buffer: 5mM Tris pH 7.5; 150mM NaCl; 5% fat-free milk powder

5.4.5. Chromatin-binding and immunoprecipitation assay

1-2 x 10^8 721 cells were pelleted at 1200rpm for 10min, washed with PBS and re-suspended in 1ml LSBII. To prepare nuclei 1vol of 0.14% Triton X-100 in LSBII was added to the cell suspension to a final concentration of Triton X-100 of 0.07%. After incubation on ice for 30min chromatin was cross-linked by incubating the nuclei in 1% formaldehyde in LSBII. The cross-linking was stopped by addition of EDTA to a final concentration of 50mM. The nuclei were pelleted at 1500rpm at 4°C for 8min and washed first with 50ml pre-chilled 0.5% Igepal in PBS and then with 50 ml pre-chilled Low Salt Buffer (LSB).

The nuclei were re-suspended in 2-3ml of cold LSBII. After addition of 0.1vol of 17% sarcosyl solution, the nuclei were carefully layered on 40ml cold LSBII containing 0.1M sucrose. The nuclei were pelleted by centrifugation at 4000g at 4°C for10 min. and lysed in 2ml ChIP-lysis buffer. The chromatin was sonicated on ice at 40% amplitude for 2min (1sec impuls, 1sec pause) in the ultrasonic cell disrupter (Sonifier®II, Branson, Germany). The average size of sheared DNA fragments was proved by electrophoresis on 0.7% agarose gels. For immunoprecipitation 25µg chromatin as determined by absorption at 260nm were diluted 10x with dilution buffer and incubated with 60µl of antibody at RT in a microfuge tube for 1h with rollling. 10µl protein G sepharose per immunoprecipitation (Protein G sepharose 4 Fast Flow, Amersham) were washed 3 x in dilution buffer and added to the immunoprecipitation mix. After incubation at RT for 2h with rolling, unspecifically bound chromatin was washed away with 5x1ml RIPA buffer and subsequent by 1ml final wash buffer. The immunoprecipitated DNA was eluted with 2x200µl of elution buffer, extracted with phenol-chloroform and precipitated with ethanol. Pelleted DNA was dissolved in 70µl TE and stored at -20°C.

2µl of the immunoprecipitated chromatin were used for a PCR reaction and specific regions were amplified in 30-35 cycles depending on the primer pair applied.

LSBII: 10mM Hepes, pH7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.1mM EGTA, 1mM ATP, 0.5mM DTT
LSB: 100mM NaCl, 10mM Tris, pH8, 1mM EDTA, pH8, 0.1% Igepal
ChIP lysis buffer: 1% SDS, 10mM EDTA, pH8, 50mM Tris, pH8
Dilution buffer: 1% Triton X-100, 2mM EDTA, pH 8, 150mM NaCl, 20mM Tris, pH8
RIPA: 0.1% SDS, 1% Igepal, 0.5% DOC, 150mM NaCl, 50mM Tris, pH8
Final Wash Buffer: 0.1% SDS, 1% Triton X-100, 2mM EDTA, 500mM NaCl, 20mM Tris, pH8
TE: 10mM Tris, pH8, 1mM EDTA, pH8
Elution buffer: 1% SDS in TEs

6. Summary

EBNA-2 is a multifunctional viral oncogene involved in the immortalisation of B-cells by EBV. EBNA-2 regulates transcription of viral and cellular genes in the proliferative phase of the viral life cycle, which *in vitro* results in the outgrowth of EBV positive B-cells into lymphoblastoid cell lines (LCLs). EBNA-2 transcriptional signalling is mediated by cellular DNA-binding proteins, such as RBP-J and PU.1, since EBNA-2 does not contain its own DNA-binding domain.

In order to better characterise EBNA-2 signalling we conducted a mutational analysis of the viral LMP-1 promoter that is strongly induced by EBNA-2 in the EBV-immortalised B-cells. Our mutational analysis of the LMP-1 promoter confirmed that the PU.1 binding site is important for transactivation of the LMP-1 promoter by EBNA-2, whereas RBP-J binding to the LMP-1 promoter leads to repression and EBNA-2 binding to RBP-J is not required for transactivation. These results imply that EBNA-2 transactivates the LMP-1 promoter preferentially by an RBP-J independent mechanism.

We further characterised EBNA-2 signalling by dissection of promoter targeting domains in the EBNA-2 protein. Two EBNA-2 mutants, the CR4del and WW mutant, preferentially activated RBP-J dependent and independent signalling indicating that EBNA-2 uses at least two separate signalling pathways.

We introduced the characterised EBNA-2 mutants into the EBV genome and produced recombinant viruses carrying specific mutations in the EBNA-2 genes. Primary B-cells were infected with increasing titres of recombinant EBVs lacking the EBNA-2 ORF or carrying the WW or CR4del mutant. Viruses lacking the EBNA-2 ORF or carrying the WW mutant were not able to immortalise primary B-cells even at high viral titres. The CR4 region of EBNA-2 strongly influenced B-cell immortalisation efficiency and growth rate of the immortalised B-cells. These results indicate that EBNA-2 and the RBP-J signalling of EBNA-2 are absolutely essential for B-cell immortalisation by EBV. In contrast, the CR4 EBNA-2 region mediating RBP-J independent signalling is critical, but not absolutely essential for the process of EBV immortalisation.

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