Mechanism of superior B cell transformation by type 1 Epstein-Barr virus

by

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Abstract

The Epstein-Barr virus (EBV) is characterised by the ability to infect human B lymphocytes *in vitro* and to transform and immortalise them into lymphoblastoid cell lines (LCLs). EBV strains can be divided into type 1 and type 2, according to the sequence of the viral EBNA-2 protein. Type 1 strains exhibit a superior capacity to efficiently transform B cells and this was mapped to the type 1 EBNA-2 protein. Substitution of the serine residue located in the transactivation domain (TAD) of type 2 EBNA-2 by the equivalent aspartate-442 of type 1 protein was previously shown to confer a type 1-like cell growth phenotype to type 2. In the present study, a stronger ability to transactivate gene expression was demonstrated for type 1 EBNA-2 TAD in comparison to type 2 EBNA-2 and this was determined by aspartate-442. This residue is located next to the interaction site of the transcriptional repressor BS69 with EBNA-2. BS69 inhibits gene transactivation by type 1 and type 2 EBNA-2 but a higher BS69 affinity for type 2 EBNA-2 protein has been shown in this study. Selective EBNA-2 activation of target promoters is also involved in the differential induction of target genes by type 1 and type 2 EBNA-2. Biotinylated oligonucleotide pulldown assays showed that efficient type 1 EBNA-2 binding at the LMP-1 promoter is dependent on the PU.1 and octamer motifs. Greater binding of type 1 EBNA-2 at the differentially regulated promoters, in conjunction with its stronger transactivation ability, conferred by aspartate-442, may determine the higher induction of the LMP-1 and cellular genes required for cell growth proliferation and therefore result in superior B cell transformation by type 1 EBV.

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Declaration of Originality

All the work described and presented in this thesis was the result of my own research work (except where otherwise indicated) and does not form part of any other thesis. This work was carried out at Imperial College London (Section of Virology, Faculty of Medicine) under the supervision of Professor Paul J Farrell.

Paulo Correia

Copyright Declaration

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“Porque essas honras vãs, esse ouro puro,
“Verdadeiro valor não dão à gente:
Melhor é merecê-los sem os ter
que possuí-los sem os merecer.”

Luís de Camões

“Adoramos a perfeição, porque não a podemos ter; repugná-la-iamos, se a tivéssemos. O perfeito é desumano, porque o humano é imperfeito.”

Fernando Pessoa

“Valeu a pena? Tudo vale a pena
Se a alma não é pequena.
….
Quem quer passar além do Bojador
Tem que passar além da dor.
Deus ao mar o perigo e o abismo deu,
Mas nele é que espelhou o céu”

Fernando Pessoa

“It has often proved true that the dream of yesterday is the hope of today and the reality of tomorrow.”

Robert H. Goddard

Abbreviations

ºC	degrees Celsius
A	alanine
aa	amino acid
AID	activation-induced cytidine deaminase
APS	ammonium persulphate
ATP	adenosine triphosphate
BAC	bacterial artificial chromosome
BART	*Bam*HI rightward transcript
BCR	B cell antigen receptor
BGH	bovine growth hormone
BL	Burkitt's lymphoma
BMP	bone morphogenetic protein
bp	base pairs
BRAM	BMP receptor-associated molecule
BS69	BS69/ZMYND11
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
CD	cluster of differentiation
$CdCl_2$	cadmium chloride
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
ChIP-seq	ChIP-sequencing
CMV	cytomegalovirus
Cp	C promoter
CR	conserved region
CR	complement receptor
CRE	cyclic AMP response element
C-terminus	carboxyl terminus
CTAR	C-terminal activating region
D	aspartate
DBD	DNA-binding domain
DEPC	diethylpyrocarbonate

DMEM	Dulbecco’s modified eagle’s medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DS	type 1 D442S
DTT	dithiothreitol
E	glutamate
EBER	Epstein-Barr virus encoded RNAs
EBV	Epstein-Barr virus
EBNA	Epstein-Barr virus Nuclear Antigen
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EICE	ETS-IRF composite element
EMSA	electrophoretic shift mobility assay
ER	endotoxin removal
ER	oestrogen receptor
ETS	E26 transformation-specific
F	phenylalanine
FBS	fetal bovine serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GRU	green Raji units
g	gram
G	glycine
gp	glycoprotein
GST	glutathione S-transferase
H	histidine
h	hour
HAT	histone acetyltransferase
HDAC	histone deacetylase
HEK	human embryonic kidney
HL	Hodgkin’s lymphoma
HRP	horseradish peroxidase
I	Isoleucine
IC	intercellular domain
Ig	immunoglobulin

IL	interleukin
IM	infectious mononucleosis
IRF	interferon regulatory factor
IVT	*in vitro* translated
JNK	c-Jun N-terminal kinase
K	lysine
kb	kilo bases
kDa	kilo Dalton
L	leucine
LB	Luria Bertani
LCL	lymphoblastoid cell line
LMP	latent membrane protein
LP	leader protein
LRS	LMP-1 regulatory sequence
M	molar
MT	metallothionein
µg	microgram
µl	microlitre
ml	millilitre
µM	micromolar
mM	millimolar
min	minute
miRNA	micro RNA
mRNA	messenger RNA
ms	milliseconds
MYND	MYeloid translocation protein, Nervy and DEAF-1
N	asparagine
NaCl	sodium chloride
NaOH	sodium hydroxide
NF-kB	nuclear factor kappa B
ng	nanogram
N-ICD	Notch intracellular domain
NK	natural killer
NLS	nuclear localisation signal
NPC	nasopharyngeal carcinoma

OCT	octamer
OriLyt	origin of lytic replication
OriP	origin of latent replication
P	proline
pBS	pBlueScript plasmid
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pmol	picomole
PMSF	phenol methyl sulfonyl fluoride
PPR	polyproline repeat region
R	arginine
RBP-Jk	recombination signal-binding protein J kappa
RD	randomised
RIPA	radio immunoprecipitation assay
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	room temperature
RT-PCR	reverse transcription-PCR
S	serine
SA	type 2 S442A
SAD	self-association domain
SCID	severe combined immunodeficiency
SD	type 2 S442D
SDM	site-directed mutagenesis
sec	seconds
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SP	specific
T1	type 1
T2	type 2
TAD	transactivation domain
TBE	Tris-borate EDTA
TE	Tris-EDTA
TEMED	NNN'N'-tetramethylethylenediamine
TNF	tumour necrosis factor

TR	terminal repeats
TRADD	TNF receptor-associated death domain
TRAF	TNF receptor-associated factor
V	Volt
v/v	volume:volume ratio
w/v	weight:volume ratio
Y	tyrosine

1 Introduction

1.1 The Epstein-Barr virus

1.1.1 The first human tumour virus

Epstein-Barr virus (EBV) was discovered by Sir Anthony Epstein, Bert Achong and Yvonne Barr, after its isolation from a cultured African Burkitt's lymphoma (BL) cell line derived from a biopsy of a BL patient in 1964. The presence of the herpesvirus-like particles in BL cells was observed by electron microscopy [1]. This endemic lymphoma affecting children in equatorial Africa had been previously described in 1958 by Dennis Burkitt and proposed to have a viral-related cause, closely associated with malaria [2, 3]. The distinct characteristics of EBV were determinant to include this virus in the herpesvirus family [4]. EBV was also classified as a B lymphotropic oncogenic virus due to its capacity to transform resting B lymphocytes *in vitro* causing continuously proliferation of infected B cells and generating lymphoblastoid cell lines (LCLs) [5, 6]. This observation was fundamental to define EBV as the first candidate human tumour virus. Apart from BL, EBV has subsequently been associated with a plethora of human malignancies, which include Hodgkin's Lymphoma (HL), post-transplant lymphoproliferative disease (PTLD) and X-linked lymphoproliferative disease (XLPD). The capacity to infect other cell types besides B lymphocytes, such as T, NK and epithelial cells, has contributed to association of EBV with additional malignancies: Peripheral T cell lymphoma, nasal T or natural killer (NK) cell lymphomas, gastric carcinoma and nasopharyngeal carcinoma [7-9]. In fact, approximately 1.5% of malignancies worldwide are associated with EBV. In addition, EBV is the main cause of the benign disease infectious mononucleosis [9]. Although EBV exhibits a strong growth transforming capacity, more than 95% of the human adult population worldwide is infected and carries this ubiquitous virus asymptomatically under almost perfect immune control and the development of tumour/malignancy associated with infection by EBV is rare in an epidemiological perspective [7]. Nevertheless, it is arguably the most potent human tumour virus and the oncogenic potential is well established as demonstrated by EBV transformation of B cells *in vitro* into immortalised lymphoblastoid cell lines [10]. The mechanism of B cell transformation by EBV will be discussed in sections 1.2.4 and 1.3.1.

1.1.2 The *Herpesviridae* family

EBV is also designated as human herpesvirus 4 (HHV-4) and is a member of the herpesvirus family, *Herpesviridae*, a family of large double-stranded DNA viruses, which is included in the recently classified order *Herpesvirales* [11]. The host range of *Herpesviridae* family members comprise mammals, birds and reptiles, but other members of the order, *Malacoherpesviridae* and *Alloherpesviridae*, infect molluscs, fish and frogs, respectively [11].

Members of the *Herpesviridae* family can be further classified into three distinct subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*, according to their biology and DNA genome sequence [11] (Figure 1.1). The *Alphaherpesvirinae* subfamily includes the neurotropic members Herpes simplex viruses 1 and 2 (HSV-1 and HSV-2) and Varicella-zoster virus (VZV). These viruses are characterised by a short reproductive cycle, rapid dissemination in cell culture and by infecting a variable range of hosts. The *Betaherpesvirinae* subfamily members, such as human cytomegalovirus (HCMV) and human herpesviruses 6 and 7 (HHV-6 and HHV-7), are defined by a long reproductive cycle and a slow spread in cell culture, and are restricted to mammal infection. The *Gammaherpesvirinae* subfamily host range is confined to a specific taxonomic family or order to which the natural host belongs. These viruses are lymphotropic and can replicate in lymphoblastoid cells but also in epithelial and fibroblast cells *in vitro* and can establish lifelong infections in their hosts. Gammaherpesviruses may promote oncogenic effects and also contribute to the development of malignancies but this is a rare outcome [7]. The Gammaherpesvirinae subfamily is composed of four distinct genera: *Lymphocryptovirus* (LCV), *Rhadinovirus* (RDV), *Macavirus* and *Percavirus*. EBV belongs to this subfamily and is the prototypical virus of the genus *Lymphocryptovirus*. The natural hosts of LCV are primates but EBV is the only human pathogen of this genus. RDV Kaposi's sarcoma-associated virus (KSHV), another oncogenic herpesvirus, is the only known human RDV [7, 12]. EBV can be also classified into type 1 and type 2 strains (see section 1.4).

Only 8 viruses out of the approximately 140 members of the *Herpesviridae* family can infect humans and the severity of the disease is variable, from asymptomatic to life-threatening and cancer. Herpesvirus can establish lifelong persistent infections in their host. Lifelong infection is achieved by establishment of latent infections in long-lived cells, with evasion of immune surveillance [7].

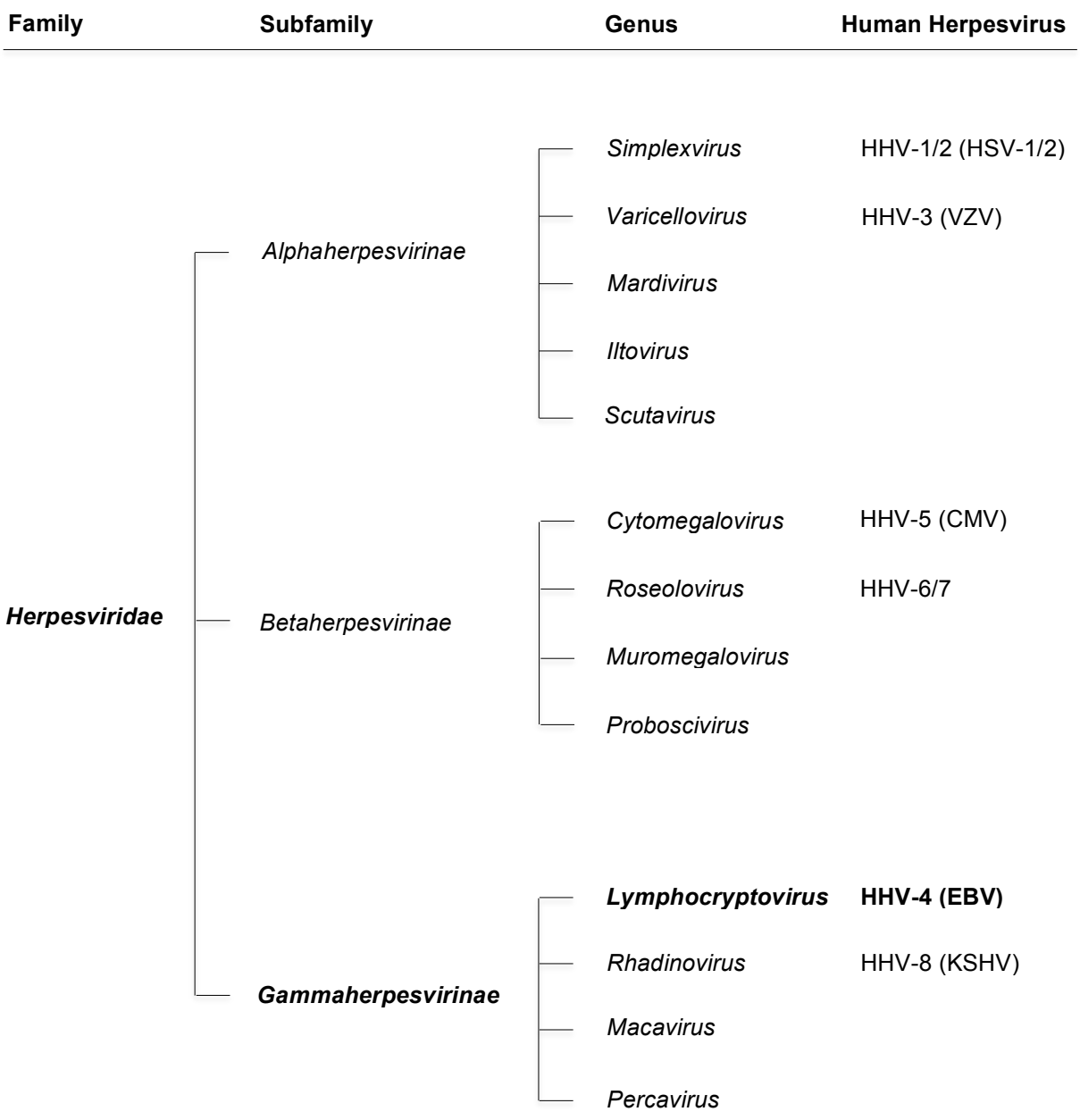


Figure 1.1 *Herpesviridae* taxonomy

According to the classification of viruses established by the International Committee on Taxonomy of Viruses (ICTV 2015).

1.1.3 The EBV virion and genome

The structure of the EBV virion is similar to the structure of other herpesviruses. EBV contains a linear, double-stranded DNA genome wrapped on a protein core. This structure is protected and encased in an icosahedral nucleocapsid about 100-110 nm in diameter composed of 162 capsomers (12 pentavalent and 150 hexavalent capsomers). A protein tegument with viral and cellular proteins including actin, tubulin and cofilin separates the nucleocapsid from the lipid envelope that coats the virus and contains many viral glycoproteins (gp) on the outer surface, important for cell tropism and receptor recognition. These include gp350/220, gp42, gH, gB and gp150 [12, 13].

The size of the EBV genome is around 172 kb and encodes approximately 85 viral proteins and many non-coding functional RNAs (EBER RNAs, BART miRNAs and BHRF1 miRNAs). The gene organisation and genome characteristics are common in the LCV genomes: there are several identical tandem terminal repeats (TR) of 0.5 kb at each terminus of the genome [14] and other internal direct repeats of 3 kb (IR) that include the Wp latency promoter and the unique sequence domains U_S (short) and U_L (long). The U_S and U_L sequences contain almost all of the genome coding capacity [7]. The TRs are crucial for genome circularisation into an episome upon B cell infection by EBV [14].

EBV was the first herpesvirus to have its complete genome sequenced [15]. This was carried out by sequencing an *Eco*RI and *Bam*HI fragment library of the B95-8 EBV strain isolated from a patient with infectious mononucleosis [16]. The restriction fragments were designated alphabetically according to their size (*Bam*HI-A is the largest fragment) and EBV genes were named based on their position and orientation on each *Bam*HI fragment (e.g. BWRF1 is the first rightward open reading frame (ORF) starting in the *Bam*HI-W fragment). Sequencing of the B95-8 strain was supplemented by sequencing the 13.6 kb deleted region in B95-8 strain from the Raji strain in 1990 [17] and further complemented with minor corrections in 2003 [18]. In addition, other strains have since been sequenced, such as AG876, derived from an African BL cell line [19, 20], GD1 [21], GD2 [22], HKNPC1 to HKNPC9 [23, 24], Akata, Mutu, C666-1, M81, Raji in a total of 22 additional EBV strains [25-28]. More recently, 71 EBV genomes have been sequenced in a process that included target enrichment of EBV genome DNA and next-generation sequencing methods, with *de novo* assembly of contigs followed by sequencing using the conventional Sanger method to join the contigs [29].

1.2 The EBV life cycle

1.2.1 Primary infection

Primary infection by EBV usually occurs early in childhood causing a mild or most frequently an asymptomatic infection. However, when the initial infection takes place in adolescence or in adulthood, the virus can be the etiological agent of a self-limiting infectious mononucleosis (IM) defined by symptoms as pharyngitis, fever or lymphadenopathy due to an exacerbated host T cell response to the viral infection [7, 9]. This different susceptibility of adults and children to EBV may be determined by distinct NK activity against EBV [30]. After the initial infection, the virus establishes a lifelong latent infection in the memory B cell compartment of the host in 1 out of 10^5-10^6 cells [7]. EBV is normally transmitted through saliva but can be acquired from blood or transplants [31].

As a member of the herpesvirus family, EBV is able to alternate between two distinct phases in its life cycle: the lytic infection and the latent infection. The lytic phase is characterised by active viral replication and production of new infectious viral progeny, but the latent phase is defined by the absence of active viral replication and by viral dormancy. EBV primarily infects human B lymphocytes and some epithelial cells although other cell types such as T and NK cells are rarely reported to be also infected. Primary infection takes place in the oropharyngeal tissues but the cell type that is initially infected is not fully understood. Primary infection of epithelial cells occurs when EBV establishes lytic replication in the mucosal tissues of the oropharynx. Primary infection of naïve B cells occurs when EBV crosses the tonsillar epithelium and then establishes latent infection of B lymphocytes in the Waldeyer's ring in the tonsils. The dual tropism model for EBV primary infection at the oropharyngeal tissues has been proposed by Faulkner and colleagues [32]. Accordingly, the virus is transmitted orally and initially infects the oropharynx epithelial cells, establishing a primary localised focus of lytic replication and productive infection, where the viral population is amplified, accompanied by shedding of virions in the oropharynx and spreading to the adjacent lymphoid tissue. This would promote secretion of new infectious virus and the concomitant infection of B lymphocytes allowing the establishment of latent infection. Alternatively, EBV might initially infect resting B cells of the Waldeyer's ring in the adjacent lymphoid tissues, which would transfer the virus to epithelial cells in close proximity, where

the virus would then be amplified to be secreted or to infect new naïve B cells [32]. This model is supported by the observation that EBV virion particles shed from B cells infect epithelial cells more efficiently and EBV released from epithelial cells is better at infecting B cells [33]. Infection of epithelial cells *in vitro* by co-culture with virus-producing B cell lines is also more efficient than infection with cell-free viral particles [34]. In addition, in the absence of mature B cells (in the case of X-linked agammaglobulinaemia patients) there is no EBV infection, indicating that B cells are required for productive infections. In this study, it was also determined that epithelial cells could be infected by viral particles bound to the B cell surface, which functions as a transfer vehicle to facilitate epithelial infection since it is not dependent on initial viral replication in B cells [35]. Changes observed in the glycoprotein content of the membrane-derived viral lipid envelope according to host cells support the dual tropism model [33]. The relevance of the oropharyngeal epithelium is also strongly supported by the observation that the M81 EBV strain infected epithelial cells much more efficiently than the B95-8 virus [36] and also by the strong association of EBV infection with the epithelial cell malignancies including NPC and gastric carcinoma, and with the non-malignant oral hairy leukoplakia in immunocompromised patients (AIDS) [37-40].

1.2.2 Cell attachment and viral entry

The primary B lymphocyte receptor for EBV is the CD21 protein on the surface of the cell, also designated as complement receptor 2 (CR2), which is recognised and bound by the viral envelope glycoprotein gp350/gp220 [41]. This interaction triggers CD21 receptor aggregation in the plasma membrane and also a tyrosine kinase signal transduction via CD19 that leads to NF-kB activation and cell cycle entry [42-44]. CD21-negative B cells were shown to be infected through EBV binding to the complement receptor 1 (CR1 or CD35), which is expressed earlier in B cell development [45]. Binding of a second viral glycoprotein gp42 to the human leucocyte antigen class II molecule (HLA class II), as a co-receptor [46], triggers the fusion of viral envelope with the cell membrane and viral entry in a process that depends on the glycoprotein complex gH/gL and also on gB [47-49]. The complex gH/gL is thought to act as a regulator that triggers gB-mediated fusion after gp42 binding to HLA class II molecules [50]. Subsequently, in the cytoplasm the virion nucleocapsids are released and carried on microtubules to nuclear pores [51]. The viral linear genome is thus transported to

the B lymphocyte nucleus where it circularises and the viral genome is then maintained in the nucleus as a covalently closed extrachromosomal episome [7].
In epithelial cells, since there is no CD21 or HLA class II molecules on the cell surface, EBV entry does not require gp350/220 and gp42 [52]. The viral BMRF2 protein is able to mediate interaction with the cellular β1 integrins [53-56]. The fusion of the viral envelope is triggered through binding of the viral gH/gL complex to αvβ6/8 integrins [52]. This is supported by the infection efficiency of virions lacking gp350/220 glycoproteins [35]. The EBV virion expresses three-part gH/gL/gp42 and two-part gH/gL glycoprotein complexes, which confer the ability to infect either B cells or epithelial cells [57]. The EBV gp42 protein determines the viral tropism, promoting the infection of B cells and inhibiting the infection of epithelial cells, through high or low levels on the virion, respectively [33]. The excess of viral gp42 inhibits EBV entry into epithelial cells [58], interacting with the gH-KGD motif implicated in integrin receptor binding and therefore potentially masking the integrin-binding site on gH/gL [59]. Alternate replication of EBV in B cells creates EBV particles that express more gH/gL complexes and replication in epithelial cells produces virions that express more gH/gL/gp42 determining the dual tropism of EBV [33].

1.2.3 EBV lytic infection

The lytic infection is characterised by productive secretion of new infectious viral particles that either are shed in the saliva and infect new humans hosts or infect other naïve B cells in the same host. This reactivation occurs when the memory B cells, the site where EBV latent infection is established, are differentiated into plasma cells [60, 61]. This differentiation upon recognition of a specific antigen by memory B cells or cytokine treatment (e.g. TGF-β) involves the cellular XBP-1 transcription factor [62], which induces the activation of the Zp promoter of EBV and the expression of the lytic transactivator BZLF1 [63], a checkpoint for the initiation of the replicative cycle [64]. The location of these plasma cells in the tonsillar epithelium allows efficient shedding of the new virus particles into the saliva [60, 61].
The genes expressed in the lytic cycle include the immediate early, early and late lytic genes [7]. Expression of immediate early genes BZLF1 and BRLF1, which are involved in induction of the lytic cycle, is triggered by signalling transduction through the B cell receptor (BCR) [65, 66]. BZLF1 is a viral transactivator protein responsible for triggering expression of lytic genes and downregulation of latent genes, culminating in cell death and release of infectious virions

[64]. It initiates BRLF1 expression and also upregulates its own expression, determining the switch from latency to the lytic cycle [67]. BZLF1 protein is a bZIP sequence-specific transcription factor similar to c-FOS and C/EBP [68]. BZFL1 and BRLF1 induce expression of early genes, such as the viral DNA polymerase (BALF5) [69] and a thymidine kinase [70] to carry out the viral DNA replication from the lytic origin of replication (OriLyt) in conjunction with other immediate early and early gene products [7]. The late lytic genes encode viral structural proteins, which include glycoproteins, tegument proteins and the major capsid protein BcLF1. The viral replicated DNA is packaged into nucleocapsids in the cell nucleus, which pass to the cytoplasm through the nuclear membrane by forming vesicles that contain virions with envelope. The vesicles fuse with the cell plasma membrane and the viral particles are exocytosed [7].

1.2.4 EBV latent infection

1.2.4.1 EBV latent infection *in vivo*

Herpesviruses are characterised by their capacity to establish and maintain a latent infection in their hosts. This persistent infection is defined by suppression of viral replication and by viral dormancy, allowing its immune evasion in the host. EBV establishes latency in the B cell pool that constitutes the long-term reservoir for the virus *in vivo.* Approximately 1 out 10^5-10^6 cells in the peripheral blood memory B cell compartment is latently infected with EBV. The EBV colonisation of the B cell compartment *in vivo* follows a model originally proposed by Thorley-Lawson and Gross [71]. The process of B lymphocyte infection and establishment of EBV latency mimics the natural differentiation pathway of antigen-activated B cells, giving the virus access to its site of latent infection. EBV steers infected cells through the various stages of B cell differentiation and finally enters a cell type suitable for long-term persistence and periodic reactivation (Figure 1.2).

The naïve B cells infected by EBV in the Waldeyer's ring in the tonsils proliferate as activated B blasts, which are similar to antigen-activated B lymphocytes in terms of the phenotype and morphology of their cell surface [72]. In normal immune responses, resting B cells proliferate to activated B blasts, when the BCR recognises an antigen and become activated by antigen-specific T-helper cells (T_H) via CD40L/CD40 interaction [73]. Activated B blasts

proliferating by EBV infection express all EBV latent genes (Table 1.1) and this viral expression program is defined as latency III or growth-program, which promotes the activation and proliferation of the cells. This is accomplished through the expression of two viral latent membrane proteins (LMPs), LMP-1 and LMP-2A, which constitute a functional homologue of the CD40 receptor in B lymphocytes and also mimic a constitutively-activated BCR, respectively [74, 75]. Upon activation, B blasts migrate to nearby primary follicles to form germinal centres (GC) and the viral transcriptional program is switched to latency II or default-program to enable the B blast to differentiate into memory B cells. This is accompanied by clonal expansion, rearrangement of immunoglobulin (Ig) genes by somatic hypermutation and isotype switching. Latency II is characterised solely by the expression of LMPs and EBNA-1 protein. LMPs are essential to provide cell survival signals, in the absence of antigen-mediated signals, required to prevent apoptosis of the latently infected B cells. The EBNA-1 protein is for EBV DNA replication and to maintain the viral genome in the cells [76-78]. Latently infected memory B cells leave the cell cycle and enter the peripheral circulation; they constitute the reservoir of EBV latency and viral persistence, in which the virus is not cleared by the immune system [77, 79]. These memory B cells latently infected with EBV are characterised by the silencing of viral protein expression in a program referred to as latency 0 or latency-program, which is thought to allow immune evasion and therefore lifelong persistence in the host [71, 79]. Nevertheless, BART miRNAs and EBERs were reported to be expressed in memory B cells [80]. Memory B cells might sporadically undergo division, in order to maintain their numbers, in a process not dependent on EBV [71]. In the cells carrying the virus, expression of EBNA-1 is triggered and permits the division of the viral genome along with the cell. This expression program is called latency I or EBNA-1-only transcription program [79]. Memory B cells in peripheral circulation might move to mucosal sites in the oropharynx and tonsils and then commit to differentiation into antibody-producing plasma cells. If they were carrying the virus, reactivation of lytic viral replication occurs and infectious virus will be produced. Hence, these viral particles will infect new naïve B cells or be shed into the saline and secreted to the exterior to infect additional hosts [61].

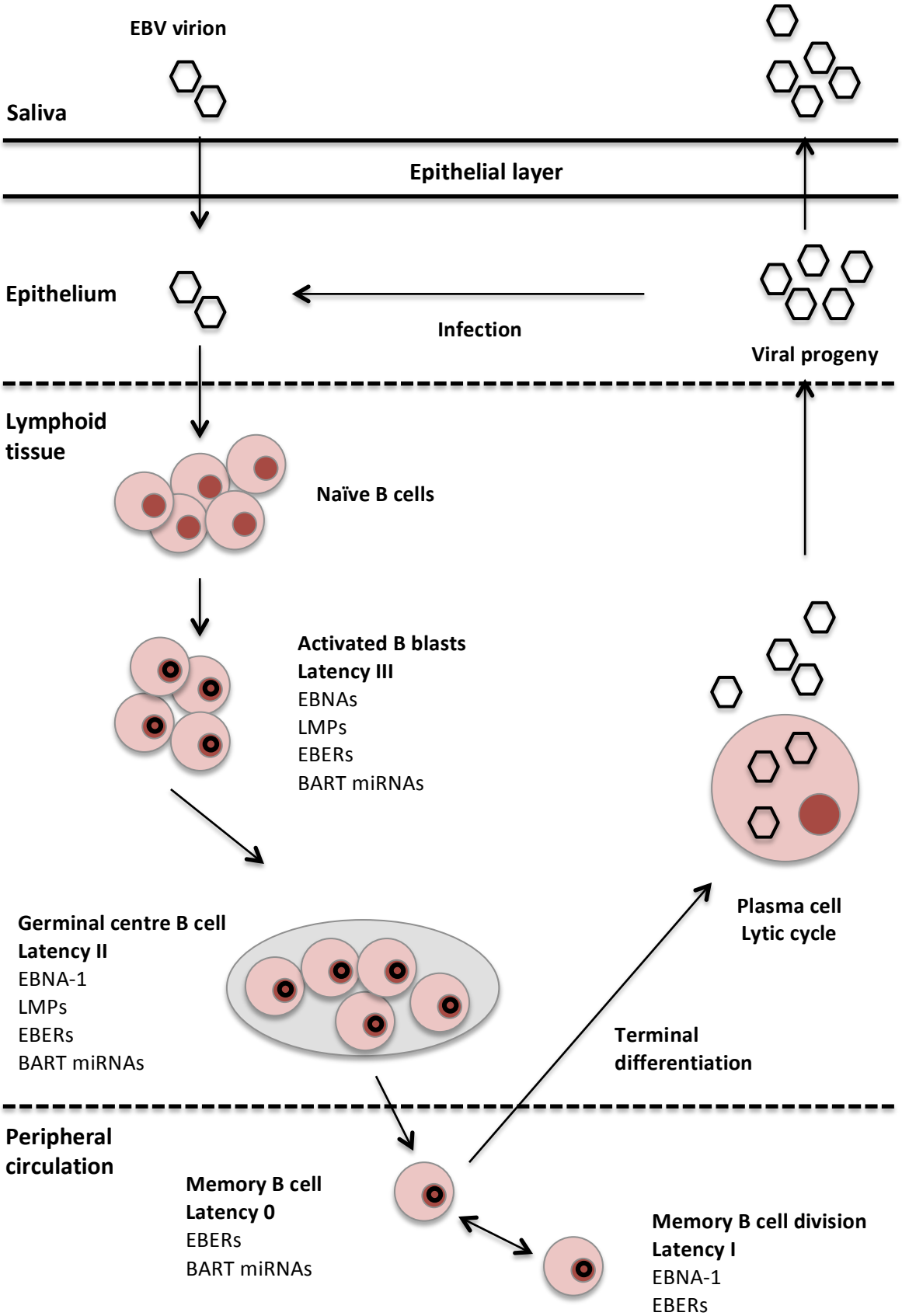

EBV virion
Saliva
Epithelial layer
Epithelium
Infection
Viral progeny
Lymphoid tissue
Naïve B cells
Activated B blasts
Latency III
EBNAs
LMPs
EBERs
BART miRNAs
Plasma cell
Lytic cycle
Germinal centre B cell
Latency II
EBNA-1
LMPs
EBERs
BART miRNAs
Terminal differentiation
Peripheral circulation
Memory B cell
Latency 0
EBERs
BART miRNAs
Memory B cell division
Latency I
EBNA-1
EBERs

Figure 1.2 Model for establishment of EBV persistent latent infection *in vivo*

EBV exploits the normal differentiation pathway of B lymphocytes in the human host. EBV transmitted orally through the saliva, infects human naïve B cells in the Waldeyer's ring of the tonsils. EBV triggers expression of the viral expression growth-program or latency III to activate B lymphocytes and induces B cell proliferation and differentiation into B blasts. These activated B blasts migrate and form germinal centres to differentiate into memory B cells. In the germinal centre B lymphocytes the default-program or latency II is activated to provide survival signals that allow the cell to leave the germinal centre as memory B cell. In resting memory B cells the viral expression latency-program or latency 0 is activated and no viral proteins are expressed, which allows the viral to evade detection by the immune system and persist in the host. When these memory B cells occasionally divide they express the EBNA-1-only program. Memory B cells eventually move to the Waldeyer's ring in the tonsils and differentiate into plasma cells to initiate the lytic cycle and triggers viral replication. The viral progeny may be shed into the saliva for dissemination into new hosts or may infect new B cells. Adapted from [81].

Transcriptional program	Latent gene products expressed	Function	Differentiation state of EBV-infected B cell	EBV-associated malignancy
Latency III or Growth program	EBNA-1 (Cp) EBNA-2 EBNA-3A EBNA-3B EBNA-3C EBNA-LP LMP-1 LMP-2A LMP-2B EBER1/2 BART miRNAs	B cell activation	Naïve B cell	Post-transplant lymphoproliferative disorder (PTLD) / Immunoblastic lymphoma
Latency II or Default program	EBNA-1 (Qp) LMP-1 LMP-2A LMP-2B EBER1/2 BART miRNAs	B cell differentiation into memory B cells	Germinal centre B cells	Nasopharyngeal carcinoma (NPC) Hodgkin's lymphoma (HL)
Latency I or EBNA-1-only	EBNA-1 (Qp) EBER1/2 BART miRNAs	Replication and segregation of viral genome into daughter cells	Peripheral circulation memory B cell undergoing cell division	Burkitt's lymphoma (BL) Gastric carcinoma (GC)
Latency 0 or Latency program	EBER1/2 BART miRNAs	Viral persistence in the host	Peripheral circulation memory B cell	-

Table 1.1 Latency and transcriptional programs of EBV B cell infection *in vivo* and relation with EBV-associated malignancies

1.2.4.2 EBV transcriptional programs during B cell infection *in vivo*

Upon EBV infection of B lymphocytes *in vivo*, to establish latent persistence in the memory B cell compartment, EBV adopts different transcriptional programs referred to as latency 0, I, II and III [82, 83] (Table 1.1). These expression programs are defined by a specific pattern of gene expression, which characterises the different stages of EBV infection *in vivo*. The EBV expression patterns are also observed in different EBV-associated cancers. These distinct B cell tumours correspond in cell type and location to different stages of normal B lymphocyte development [73]. Latency III or growth-program is defined by the expression of all the latent genes of EBV, which include the six Epstein-Barr virus nuclear antigens (EBNAs): EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C and EBNA-LP), initiated from the viral Wp and Cp promoters, the three latent membrane proteins, LMP-1, LMP-2A and LMP-2B, the two small non-polyadenylated and non-coding RNAs (EBER1/2), the family of spliced polyadenylated *Bam*HI-A rightward transcripts (BARTs) and several miRNAs (Table 1.1). This viral program characterises lymphoblastoid cell lines (LCLs) originated from EBV-mediated transformation of resting B cells *in vitro* and is also present in recently-infected naïve B cells *in vivo* [72, 84]. Similarly, latency III is also found in immunosuppression-related lymphoproliferations, which include the polymorphic post-transplant lymphoproliferative disorder (PTLD) [71, 85, 86].

Latency II or default-program is characterised by the expression of EBNA-1, LMP-1, LMP-2A, LMP-2B and BARTs. EBNA-1 is expressed from the viral Qp promoter because Wp and Cp promoters are not active and other EBNAs are not transcribed [71, 79, 82, 83, 87-89]. This expression program is present in Hodgkin's lymphoma (HL), undifferentiated nasopharyngeal carcinoma (NPC), T cell lymphomas and gastric carcinomas and also in EBV-infected B cells that form the germinal centres after activation [71, 76, 79, 83, 90].

Latency I or EBNA-1-only transcription program is common in the EBV-associated Burkitt's lymphoma and in gastric carcinoma and the viral expression is limited to EBERs, BARTs and EBNA-1 from the Qp promoter. *In vivo*, peripheral circulation memory B cells undergoing cell division also display this transcription program (Table 1.1) [82, 91, 92].

Apart from these three distinct forms of latency, an alternative expression program was also associated with 15% of endemic BL cases, in which the EBNAs, except for EBNA-2, but not the LMPs are expressed [93]. This program is referred to as "Wp restricted" because EBNAs are expressed from the Wp promoter. EBNA-2 expression is abrogated due to the deletion of the EBNA-2 locus. All these expression programs can take place in B lymphocytes but other cell types only display latency I or II (such as epithelial cells in NPC). EBV infection in B cells

is initiated with the growth program depending on the transcription factor BSAP/Pax5, which activates the Wp promoter, but this does not occur in epithelial cells [94, 95].

1.2.4.3 EBV latent infection *in vitro*

EBV is characterised by the capacity to efficiently infect primary B lymphocytes *in vitro* and to transform them into continuously proliferating lymphoblastoid cell lines (LCLs) [5-7, 10]. LCLs are latently infected by EBV and contain the viral genome persisting as an episome. The viral expression program latency III or growth program is activated in the cells [82], resembling the initial stages of *in vivo* infection. The B cell-like round and regular morphology is lost in LCLs and their surface phenotype is identical to that of activated B blasts, with high levels of B cell activation markers CD23, CD30, CD39 and CD70. Different cell-adhesion molecules are also expressed, such as LFA1, LFA3 and ICAM, contributing to cell clustering observed in these cells [7, 82, 87, 96]. Upon B cell infection *in vitro*, around 3-10% of the cells are transformed into LCLs [7] and the latency III program is required to maintain their continuous proliferation and their survival. This viral transcriptional program is defined by the expression of six EBV nuclear antigens (EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C and EBNA-LP), three latent membrane proteins (LMP-1, LMP-2A and LMP-2B) and many non-coding small RNAs (EBERs and BART miRNAs) [7, 97, 98].

The infection of B lymphocytes *in vitro* starts with EBV attachment and entry into the cell that is followed by uncoating (described in section 1.2.2) and transport of the viral linear genome to the B cell nucleus where it circularises 12-16 h after infection [7]. Transcription of the viral EBNA-2 and EBNA-LP genes initiated from the W promoter by the host cell RNA polymerase II is triggered [98-100] and EBNA-2 and EBNA-LP proteins are detected 12-24 h post-infection reaching steady state LCL levels after 24-32 h [98]. The EBNA-2 protein is a potent activator of transcription and in conjunction with EBNA-LP activates transcription from the C promoter, which produces a unique long transcript that generates the mRNAs for all the other EBNAs after alternative splicing [7, 101-105]. Transcription from Wp is then shut down and viral transcription is controlled by the Cp promoter [106, 107]. Expression from the bidirectional LMP-1/-2B and the LMP-2A promoters is also triggered by EBNA-2 [108-112] (Figure 1.3) as well as many cell genes fundamental for B lymphocyte survival and continuous proliferation [113-115]. EBNA-2-mediated induction of these target genes is augmented by the cooperative role of EBNA-LP [116-122]. Activation of the Cp and LMP-1 promoters by EBNA-2 and EBNA-LP is assisted by EBNA-1 through regulation of enhancer

elements located within the OriP origin of replication, thereby promoting an increase in transcription of latent genes [107, 123-125]. In addition, EBNA-1 also participates in viral replication and in segregation of chromosomes into the progeny cells. The EBNA-3 proteins were shown to compete with EBNA-2 for RBP-Jk-binding essential for the activation of the Cp promoter, therefore reducing activation of the Cp promoter mediated by EBNA-2 [126, 127]. The LMP-2A promoter is uniquely regulated by the EBNA-2 protein [112, 128] but the LMP-1/-2B divergent promoter is responsive to EBNA-2, EBNA-LP and EBNA-3A [109, 110, 118, 129, 130]. The two non-polyadenylated RNAs (EBERs) are expressed 36 h post-infection to reach normal LCL levels at 72 hours [98]. The other EBNAs and LMPs reach constant normal LCL levels around 48-72 h post-infection [98]. These viral latent gene products induce cell proliferation and allow the cells to enter the cell cycle, which is accompanied by a G_0 to G_1 transition and upregulation of the cell cycle regulator cyclin D2, promoted by EBNA-2 and EBNA-LP acting cooperatively [116]. The cells divide every 20-30 hours, causing the telomeres to progressively shorten their length after each cell division and a critical length is finally reached around 100-150 divisions. The majority of the cells perish and only the stabilisation of the telomeres associated with high telomerase activity promotes survival and immortalisation of the transformed cells [131, 132]. The lymphoblastoid cell line (LCL) system constitutes a good working *in vitro* model of B cell infection by EBV and of the transforming capacity and potential of the virus.

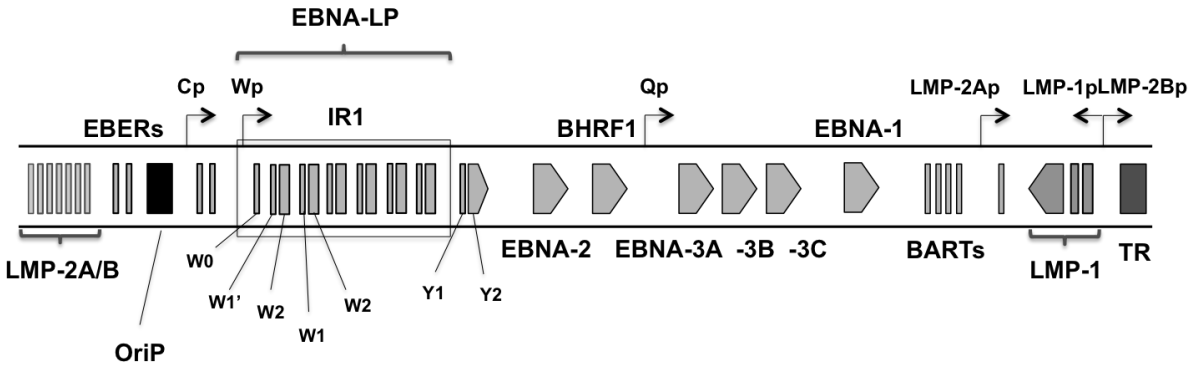


Figure 1.3 Schematic of EBV genome and genes important in latent infection

The coding exons of the EBV nuclear antigens (EBNA-1, EBNA-2, EBNA-3A, -3B, -3C and EBNA-LP) and latent membrane proteins (LMP-1, LMP-2A and -2B) and the respective locations are represented in the diagram. EBNA-LP repeated coding exons (W0, W1', W1 and W2) are located in the *Bam*HI-W internal repeat (IR1). The unique Y1 and Y2 EBNA-LP exons are shown. The terminal repeats (TR) and the OriP latent origin of replication are also shown. Each promoter is represented by an arrow (Cp, Wp, Qp, LMP-2Ap and the divergent LMP-1p/LMP-2Bp).

1.2.4.4 EBV latent gene products involved in B cell transformation *in vitro*

The process of B cell transformation into LCLs is dependent on products of the EBV latent genes. This has been determined using recombinant EBV viruses, in which one of the latent genes was deleted, to investigate the function of each gene in B lymphocyte infection and transformation into LCLs. EBNA-2, LMP-1 and EBNA-3C are fundamental in this context [74, 133-137]. EBNA-2 is essential for B cell transformation since it is a potent transcriptional activator that induces many viral and cell genes required for cell proliferation (in section 1.3). LMP-1 is responsible for the activation of cell signalling pathways, such as NF-kB activation and for triggering the c-Jun N-terminal kinase (JNK) pathway. These signalling pathways are required to provide the anti-apoptotic signals essential for B lymphocyte transformation and for survival of the continuously-proliferating B cells (see section 1.5). EBNA-3C is responsible for repressing different genes, which include the pro-apoptotic protein BIM (BCL-2-interacting mediator of cell death) and $p16^{INK4a}$, allowing the infected cells to evade apoptosis and to be transformed into LCLs and permitting cell proliferation [138-142]. EBNA-3A had initially been shown to be necessary [137], but B lymphocyte transformation was demonstrated, albeit with reduced efficiency, in the absence of EBNA-3A [143]. EBNA-3B is expendable for the EBV-mediated B cell growth transformation [144, 145]. EBNA-LP cooperates with EBNA-2 in gene transcription and also greatly improves the outgrowth of latently-infected B cells [133, 146]. The requirement for the LMP-2 gene products in B cell transformation is not completely clear. Recombinant EBV carrying no LMP-2 genes was capable of transforming B cells *in vitro* as efficiently as wildtype EBV, indicating that those genes are not essential in the context of this process [147-151]. Conversely, other studies determined that the LMP-2 gene products could indeed improve the efficiency of B cell transformation [152, 153]. EBNA-1, as already mentioned, is required to maintain the viral episome in the dividing infected cells [154, 155]. The two small non-polyadenylated RNAs (EBER-1/-2) were found not to be necessary for B cell infection and transformation in reports using P3HR1 and B95-8 EBV genetic background [156, 157], but EBER-2 was shown to be essential for efficient transformation of B cells when an AKATA EBV genome was used [158]. Additionally, a recent report has determined that an EBER-deletion EBV and the wildtype EBV exhibited equal capacity to infect immunodeficient mice reconstituted with a human hematopoietic system and to establish persistent infections [159]. The BHRF1 miRNAs have been shown to increase the efficiency of transformation of B lymphocytes into LCLs *in vitro* [160]. Although the process of B cell transformation requires several latent genes, maintenance of cell proliferation also depends on some of these genes, such as EBNA-2, LMP-1, EBNA-3A and EBNA-3C [74, 136, 161, 162]. EBNA-3A was initially

shown not to be essential for cell growth maintenance, but can confer a selective advantage to proliferating cells [163].

1.2.5 EBV and human cancer

The lymphomagenic potential of EBV is indicated by its ability to infect B cells *in vitro* and to efficiently transform and immortalise them into LCLs [5, 6], highly relevant to lymphomas arising in immunosuppressed people. Around 1-2% of human malignancies are thought to be EBV associated and approximately 200,000 are newly diagnosed every year [164], including Burkitt's lymphoma (BL), Hodgkin's lymphoma (HL), X-linked lymphoproliferative disease (XLPD), post-transplant lymphoproliferative disease (PTLD), peripheral T cell lymphoma (PTCL), nasal T cell lymphoma or natural killer (NK) cell lymphoma, gastric carcinoma (GC) and nasopharyngeal carcinoma (NPC) [7]. Thus, the presence of EBV in various stages of B-cell development and its ability to infect specific epithelial cells can lead to the development of these distinct lymphomas and carcinomas. In addition, the viral expression patterns found in EBV-associated malignancies are similar to the viral expression programs defining distinct stages of the virus-driven B lymphocyte differentiation, indicating the exploitation of the B cell differentiation pathway by EBV can favour the emergence of these malignancies at different points of the normal B cell pathway (Table 1.1). However, the function played by EBV in each malignancy is not always clear.

EBV is associated with around 95% of endemic BL cases. A common gene translocation is constantly present in this cancer: the chromosomal translocation of the *c-MYC* oncogene into the immunoglobulin (Ig) gene, which is thought to take place during isotype switching and Ig gene rearrangement in the germinal centre B cell [165]. This gene translocation promotes the high constitutive expression of the c-MYC protein characteristic of BL cells and contributes to the proliferation of these cells [166-168]. Deregulated high c-MYC levels are also associated with apoptotic cell death through induction of BIM (BCL-2-interacting mediator of cell death). BL cell proliferation promoted by high c-MYC levels needs be complemented by inhibition of apoptotic cell death caused by c-MYC translocation. BL association with EBV could relate to protection from c-MYC induced apoptosis through expression of the latency III program prior to germinal centre entry or expression of the latency II program in the germinal centre [142, 169]. Some EBV latent gene products were shown to mediate protection from the apoptotic

process. EBNA-3C was shown to repress the pro-apoptotic protein BIM, which is activated by c-MYC, enabling EBV-infected B cells to evade apoptosis [142]. This is accomplished by methylation of the BIM promoter [139]. The *BHRF1* gene encoding a BCL-2 homologue also confers resistance to apoptosis in Wp-restricted BL cells [138, 170, 171]. The EBERs were also associated with some protection from apoptosis [172, 173].

EBV-positive BL cells are characterised by the expression of the latency I program similar to dividing memory B cells, in which only EBNA-1 is expressed and none of the latent proteins promoting cell transformation and proliferation is produced. Endemic EBV-positive BL could emerge in the presence of a *c-MYC* gene translocation in an EBV-infected germinal centre B cell due to deregulated AID expression [174]. The cell proliferates due to triggered c-MYC expression and does not complete its differentiation into memory B cell [71]. In fact, EBV-positive BL cells display a conjunction of two different phenotypes exhibited by germinal centre B lymphocytes (the cellular phenotype [175]) and also by memory B cells (identical Ig gene hypermutations [176]). Alternatively, EBV infection could also promote cell proliferation by expanding the population of infected cells where the *c-MYC* translocation can occur and enhance survival and proliferation of the translocation-positive cells at risk of apoptosis. It is also possible that EBV infection might protect BL cells, which have increased expression of c-MYC, from apoptosis, providing survival signals through the LMPs. Additionally, latency I expression program conferred slightly increased protection from apoptosis, in comparison to EBV-negative BL clones [170, 177]. Apart from the *c-MYC* gene translocation, mutations of the p53 and RB pathways can be present in BL cells, contributing to BL pathogenesis [178, 179]. Additionally, a recent report has identified mutations in *TCF3* and in *ID3* genes in 40% and 70% of endemic and sporadic BL cases, respectively [180]. In addition, the *CCND3* gene was found to be mutated in 30% of sporadic BL and only in 2% of the endemic BL [180]. TCF3 is a transcription factor and ID3 its negative regulator. Mutations in these genes are associated with increased TCF3 activity, increasing PI3K signalling and Akt phosphorylation. This is important to prevent apoptosis and suggests a fundamental role for this pathway in BL pathogenesis [180].

EBV is associated with around 40% of the HL cases in immunocompetent people worldwide. HL or Reed-Sternberg tumour cells are defined by large and sporadically bi-nucleated cells similar to latently-infected germinal centre B cells [181]. In fact, Reed-Sternberg tumour cells exhibit the latency II viral expression program as the latently-infected germinal centre B cell and also carry identical hypermutation of the Ig genes [181, 182]. This indicates that the HL cells might emerge from an EBV-infected germinal centre B cell. Reed-Sternberg cells are

also defined by high expression of LMP-1 and LMP-2A proteins, which could be important to provide survival signals to the EBV-positive HL cells [181]. However, it is thought that further mutations preventing differentiation into memory B cells are required to promote oncogenesis [71]. The translocation of the major histocompatibility complex (MHC) class II transactivator *CIITA/MHC2TA* is very frequent in HL cells [183]. The HLA class I alleles were also related to EBV-positive HL as well as the *FCGR2A/CD32* locus and might contribute to oncogenesis in HL [184, 185].

PTLD could emerge in immunosuppressed patients subsequent to transplantation. Most of these lymphomas are associated with EBV (90%) and are similar to *in vitro* EBV-transformed LCLs since the latency III viral program is activated. In the immunocompromised host with an impaired cytotoxic T lymphocyte response, it is likely that EBV could infect a naïve B cell and trigger its proliferation with no differentiation into a memory B cell. This could be associated with additional mutations in order to generate a lymphoma. Infection of germinal centre B cells or memory B cells by EBV in immunosuppressed hosts was also suggested as a likely cause for PTLD development [71].

EBV infection is associated with approximately 90% of NPC cases [165]. EBV expresses the latency II program and LMP-1 is frequently detected in this tumour, indicating that LMP-1 plays an important role in NPC pathogenesis [186]. LMP-1, in cooperation with LMP-2A, was found to promote development of carcinoma in transgenic mice, increasing the frequency of epithelial papillomas and carcinomas, but did not initiate malignant growth [187]. LMP-1 is also found to induce expression of the anti-apoptotic B cell lymphoma 2 (BCL-2) protein, which is frequently overexpressed in NPC [188]. The increased expression of BCL-2 protein has also been associated with EBERs expression [189, 190]. Expression of EBV miRNAs is indeed upregulated in NPC malignancies and could contribute to NPC pathogenesis [191]. Similar to BL and HL tumours, sequence analysis of 128 NPC cases revealed a distinct mutational signature for NPC [192]. This includes the frequently mutated or deleted genes: *PIK3CA*, the tumour suppressor *TP53*, oncogenes *KRAS* and *NRAS*, cell-cycle regulator *CDKN2A*, the deubiquitinating enzyme *BAP1* and the nucleosome remodelling component *ARID1A* (10% of the cases) [192-194]. Recent sequencing has shown that NPC tumours have either LMP-1 expression or mutation of genes that result in activation of NF-kB signalling, suggesting that NF-kB activation is part of the mechanism [195].

Approximately 10% of the gastric cancer cases (GC) are EBV-positive. A specific mutational pattern also characterises GC tumours. Mutations in *PIK3CA* (80%), *ARID1A* (55%) and in *ERBB2* genes have been detected in EBV-positive GC and also in NPC, suggesting that the previous mutations could be important for development and progression of these epithelial malignancies [192, 196, 197]. The gene encoding the anti-apoptotic protein BCOR was also found to carry mutations in EBV-positive GC cells . High expression of the insulin-like growth factor 1 (IGF1) was only detected in GC cells infected with EBV, indicating a possible role in EBV-positive GC pathogenesis. In fact, IGF1 expression has been shown to be induced by the EBERs [198].

The different geographical incidence of these specific malignancies worldwide suggests that environmental factors, such as co-infection with other infectious agents, social status and the genetic predisposition, the immune status of the infected host (immune suppression) and the different EBV strains can promote EBV-driven tumours and can determine the pathogenesis [199]. However, the persistence of EBV genomes in the cells of these malignancies, even in people with normal immune responses, indicates that EBV genomes are important for growth of these malignant cells.

1.3 The Epstein-Barr Nuclear Antigen 2 (EBNA-2)

1.3.1 EBNA-2 is essential for B cell transformation

Epstein-Barr virus nuclear antigen 2 (EBNA-2) is the major transcriptional activator of EBV in the latent cycle and is fundamental for transformation and proliferation of primary B cells into LCLs *in vitro* [7]. The first evidence of EBNA-2 importance arose from the inability of the EBV P3HR1 strain, which contains a deletion of the EBNA-2 open reading frame and of part of the EBNA-LP gene, to cause B lymphocyte transformation *in vitro* [200-204]. Complementation of this deletion by recombination with the wildtype gene or with fragments carrying the EBNA-2 gene confirmed the crucial importance of EBNA-2 when the immortalisation competence was restored [133, 134]. Kempkes and colleagues confirmed the function of the EBNA-2 protein during EBV-mediated B cell transformation using the EREB2.5 system, characterised by the conditional activity of EBNA-2 [136]. The EREB2.5 system was created fusing the open reading frame (ORF) of the oestrogen receptor hormone binding domain (ER) to the ORF of EBNA-2 (ER-EBNA-2). This protein was used to complement the EBNA-2 deletion in P3HR1 virus and infect primary B cells. An LCL was established, EREB2.5 LCL, carrying the P3HR1 EBV genome and an OriP plasmid expressing the ER-EBNA-2 fusion protein. This mini-EBV OriP plasmid also contains the full-length EBNA-LP, which is deleted in the P3HR1 genome [136]. The EBNA-2 activity in EREB2.5 cells is therefore strictly dependent on oestrogen present in the cell culture medium. When oestrogen is available in the medium, it binds to the oestrogen receptor, the ER-EBNA-2 protein localises to the nucleus and is able to mediate gene activation and cell proliferation. In the absence of oestrogen, the ER binds heat-shock proteins and is sequestered in the cytoplasm, where EBNA-2 cannot access its target genes [205]. Half of the cells die by apoptosis and proliferation of the rest is terminated and they enter a quiescent state. Cells are arrested in G_1 and G_2 phases of the cell cycle upon removal of oestrogen but growth arrest in G_1 phase can be reversed upon addition of oestrogen and cells re-enter the cell cycle. The reversible EREB2.5 LCLs growth arrest and re-entering the cell cycle upon oestrogen starvation and re-induction confirmed that EBNA-2 is required for initiation and for maintenance of EBV-driven proliferation [136]. The EREB2.5 LCL system has been used to study the EBNA-2 functions and target genes in the context of EBV-mediated B cell infection in cell culture [206-210].

1.3.2 Structure and functional domains of the EBNA-2 protein

The viral EBNA-2 protein is characterised by forming large nuclear granules and localises to the nucleoplasm, where it associates with the chromatin and the nuclear matrix fractions [211, 212]. The sequence of the EBNA-2 protein defines the EBV strain types (type 1 and type 2, reviewed in section 1.4) since EBNA-2 is the most divergent locus between type 1 and type 2 strain genomes, exhibiting approximately 56% identity at the amino acid level [213]. EBNA-2 protein encoded by the prototype type 1 B95-8 strain has 487 amino acids, whereas EBNA-2 encoded by the prototype type 2 AG876 strain is composed of 454 amino acids [214]. The comparison of the amino acid sequences of EBNA-2 alleles from EBV type 1 or type 2 and baboon and rhesus macaque lymphocryptoviruses defined, according to sequence identity and similarities, nine evolutionary conserved regions (CR1 to CR9) in the primary structure of EBNA-2 (Figure 1.4). In addition, different functional domains of EBNA-2 can be also defined (Figure 1.5) [214, 215]. A large divergent stretch (diversity region) mediates the separation of two groups of conserved regions in the EBNA-2 protein; the group at the N-terminus (CR1 to CR4) and the C-terminal group (CR5 to CR9). At the N-terminus, CR1/2 and CR3/4 are separated by a polyproline repeat region (PPR), whose size is variable depending on the EBV strain [214]. Similarly, the diversity region is also variable in length and in its sequence in different EBNA-2 proteins and homologues. A poly-arginine-glycine motif (RG) separates CR6 and CR7. The RG motif is less conserved in the EBNA-2 protein of lymphocryptoviruses and functions as a nuclear localisation motif in cooperation with the C-terminal nuclear localisation signal (NLS) in CR9 [214, 216]. The RG region was also considered to be a modulator of EBNA-2 activity, and also binds to poly(G) DNA and histone H1 *in vitro* [217]. Two N-terminal self-association domains (SAD1 and SAD2), flanking the PPR, mediate EBNA-2 homotypic association [129]. CR6 is involved in the interaction with the cellular factor RBP-Jk via the conserved residues tryptophan-323 and tryptophan-324 [218-222]. The cell protein SKIP, a co-factor of RBP-Jk, is bound by CR5 and facilitates the formation of the EBNA-2/RBP-Jk protein complex [223]. The transactivation domain of EBNA-2 (TAD) was mapped to the CR8 region and is involved in transcriptional activation of target genes [216]. A second N-terminal activation domain has also been mapped to the SAD1 motif [224]. These two regions specifically interact with EBNA-LP but the CR7 and RG were also shown to be involved in EBNA-LP cooperative function [119, 224]. EBNA-2 is essential for B cell transformation and some of these functional domains are crucial in this context (section 1.3.3).

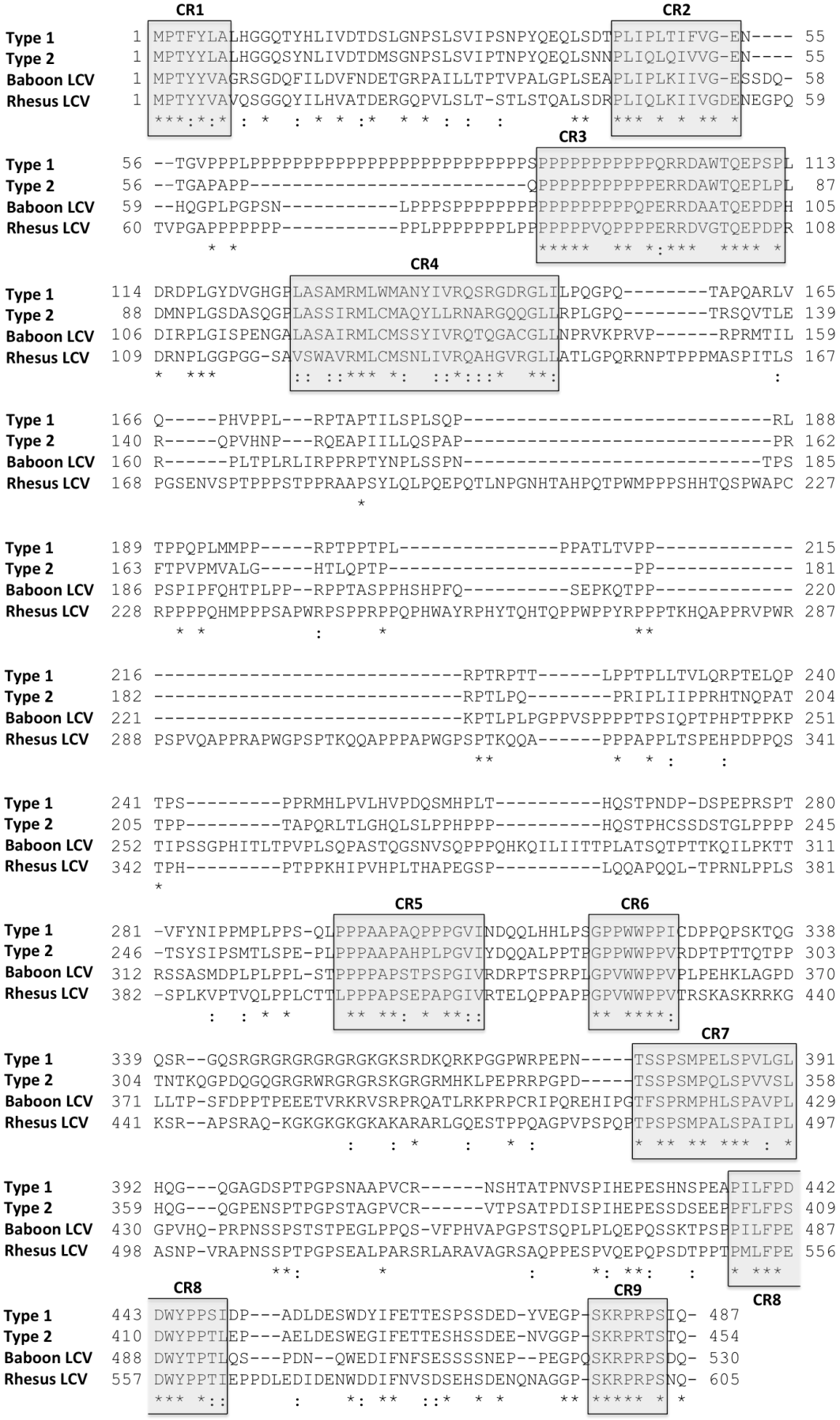

CR1
CR2
CR3
CR4
CR5
CR6
CR7
CR8
CR9
CR8
Type 1
Type 2
Baboon LCV
Rhesus LCV

Figure 1.4 Alignment of the amino acid sequences of EBNA-2 alleles from EBV type 1 or type 2 and baboon and rhesus macaque lymphocryptoviruses

Comparison of the amino acid sequences of EBNA-2 alleles from EBV type 1 (B95-8), type 2 (AG876) and EBNA-2 sequences from baboon and rhesus macaque LCVs defined nine evolutionary conserved regions (CR1 to CR9) in the primary structure of EBNA-2, based on sequence identity and similarities. These conserved regions are represented by grey boxes. Asterisks indicate amino acid residues that are identical between the sequences. The dotted lines mark amino acid residues with overall similarity. From [215].

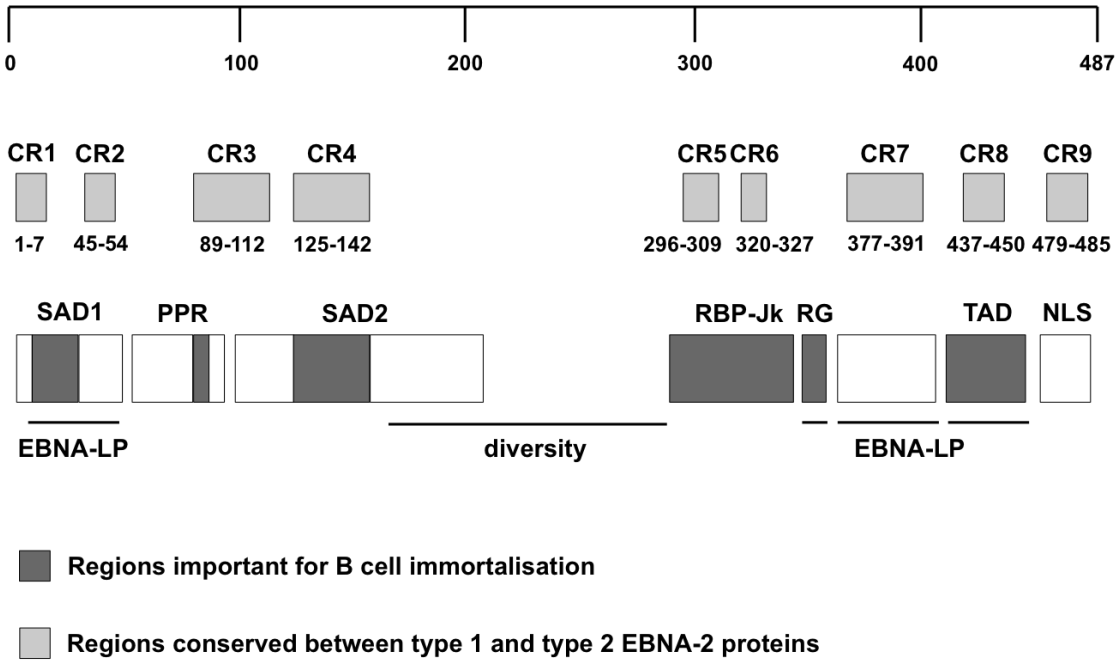


Figure 1.5 Representation of EBNA-2 structure and functional domains

EBNA-2 is composed of nine conserved regions (CR1 to CR9) represented by light grey boxes. The functional domains of EBNA-2 can be identified in the structure of EBNA-2: self-association domains 1 and 2 (SAD1 and SAD2); the polyproline region (PPR); the poly-arginine-glycine motif (RG); the region responsible for the interaction with RBP-Jk (RBP-Jk); the transactivation domain (TAD) and also the nuclear localisation signal (NLS). The region interacting with EBNA-LP is also indicated. The dark grey sections represent the regions important for B cell immortalisation (figure adapted from [209]).

1.3.3 B cell transformation requires important EBNA-2 domains

The discovery of the EBV strain P3HR1, which fails to immortalise B cells, contributed to the functional and genetic analysis of the role of EBNA-2 in B cell transformation. Earlier studies showed that infection of the Burkitt's lymphoma line Raji with P3HR1 viruses generated viral genomes, in which recombination events had occurred between the Raji and the incoming P3HR1 genomes. This recombination had led to the formation of immortalising viruses, in which the deleted EBNA-2 region in P3HR1 virus had been restored [201, 225]. The early experiments were confirmed, with the advent of recombinant DNA methods, using DNA complementation experiments with the cloned EBNA-2 gene, which proved that EBNA-2 is essential for B cell growth transformation [134]. Mutational analysis using EBV recombinant viruses allied to the *trans*-complementation EREB2.5 system, used to test whether specific EBNA-2 mutants can maintain B cell proliferation in an LCL conditional for EBNA-2, determined EBNA-2 regions or domains essential for growth transformation [136, 208]. Four EBNA-2 domains were initially found to be important for B cell transformation and for LMP-1 transactivation by EBNA-2 in transient reporter assays, indicating that these two functions could be closely related [226]. In the EBNA-2 N-terminal end, the amino acids 1-58 and 97-210, included in SAD1 and SAD2 (self-association domains) respectively, are essential for transformation [129, 227, 228]. The regions CR6 and CR5, which mediate binding to RBP-Jk and SKIP, and the transactivation domain (TAD) are absolutely required for B cell transformation [216, 227, 229]. The capacity to activate gene transcription by EBNA-2 is fundamental for growth transformation [229]. The relative importance of the N-terminus or the polyproline region was distinct depending on the system used to study their function. Deletion of amino acids 3-30, included in CR1, was not compatible with B cell proliferation in the *trans*-complementation system but LCLs carrying deletions of the N-terminal end comprising CR1 and CR2 plus most of the polyproline region could by generated using recombinant viruses. Conversely, the polyproline region was found to be dispensable for transformation in the EREB2.5 *trans*-complementation system, while a minimal number of seven prolines was critical for growth transformation when B lymphocytes were infected with recombinant viruses [230-232]. The poly-arginine-glycine (RG) motif is important for greater efficiency of B cell transformation. Deletion of RG motif leads to loss of EBNA-2 chromatin association and to reduction of LMP-1 gene transactivation mediated by EBNA-2, but it is compatible with B lymphocyte growth transformation [217]. Small deletions within the central part of the protein (amino acids 101-126 or 116-145 or 146-231 or 235-284) did not

negatively affect the efficiency of B cell transformation, whereas deletion of the region, comprising all the previous small deletions, abolished cell growth transformation [227, 233, 234]. In contrast, the CR7 region, which is important for EBNA-LP cooperation with EBNA-2, is not required for B lymphocyte immortalisation. Deletion of the casein kinase II (CKII) site SS469, located in the region between CR8 and CR9, abrogates hSNF5/Ini1 binding to EBNA-2 and compromises B cell growth transformation [235, 236].

1.3.4 EBNA-2-mediated transactivation of target genes

1.3.4.1 EBNA-2 does not interact directly with target promoters

EBNA-2 is a potent transcriptional activator but cannot bind directly to DNA to mediate gene transactivation and gains access to the target promoters using adaptor proteins, which confer DNA contact. The best studied DNA adaptor of EBNA-2 is the DNA-binding protein RBP-Jk or CBF1 [221, 222, 226, 237]. In the absence of EBNA-2, RBP-Jk functions as a transcription repressor, recruiting a co-repressor complex to target promoters. That complex includes SMRT/N-CoR, CIR, SKIP, Sin3A, SAP30 and HDAC1 and interferes with histone acetylation of target gene chromatin, repressing transcriptional activation [238]. In the presence of EBNA-2, binding to RBP-Jk relieves this repression and the EBNA-2 TAD activates gene expression. RBP-Jk was found to be important in EBNA-2 transactivation of the viral LMP-1/-2B, LMP-2A and Cp promoters and the CD21 and CD23 cellular promoters [239-241]. However, at the LMP-1 promoter, the RBP-Jk site was shown to have weak RBP-Jk binding [110, 239, 240] and other factors, such as PU.1/Spi-1, Spi-B, AP-2, POU-domain protein, EBF1 and ATF/CREB factors, are important to confer EBNA-2 responsiveness at the LMP-1 promoter [239, 242-245]. The Cp and CD21 promoters were also found to be regulated by AUF1/hnRNP D binding [246, 247].

1.3.4.2 EBNA-2 resembles the intracellular domain of human Notch (N-ICD)

The RBP-Jk factor is a common interaction partner of EBNA-2 and the intracellular domain of Notch (N-ICD), indicating that EBNA-2 could be the viral homologue of the cell Notch. N-ICD

also interacts with RBP-Jk, replacing the co-repressor complex of histone deacetylases (HDACs), to transactivate gene transcription and therefore resembles EBNA-2 actions [248]. Binding of EBNA-2 and Notch to the RBP-Jk protein is mutually exclusive since there is biochemical and genetic evidence that they bind the same minimal repression domain in RBP-Jk [249]. Accordingly, the WWP motif in CR6 of EBNA-2 that binds to RBP-Jk is similar to the WFP sequence in N-ICD [7]. Mutation of the WW amino acids of EBNA-2 impaired the expression from the Cp and LMP-1 promoters and the virus was not capable of transforming B cells [234].

The Notch pathway is an evolutionarily conserved signalling pathway that plays an essential role in cell fate determination during development, tissue homeostasis and maintenance of stem cells. Notch was also implicated in T lymphopoiesis and in regulation of multiple stages of B cell development [250-252]. Deregulation of Notch signalling has been associated with congenital defects and several human malignancies, suggesting that EBNA-2 can contribute to B cell transformation by usurping the Notch signalling pathway. EBNA-2 is regarded as a functional homologue of the activated Notch receptor, but it is constitutively active and functions independently of a cell surface ligand. In fact, in EBNA-2-negative BL cell lines, Notch is able to induce expression of EBNA-2 target genes that include LMP-2A and Cp (carrying RBP-Jk binding sites) albeit to a lower extent than EBNA-2 [253, 254]. However, other genes, such as LMP-1, were not upregulated by Notch, likely because the regulation of this promoter involves additional factors (PU.1/Spi-1) and not only the RBP-Jk adaptor protein as mentioned above. Accordingly, Notch was shown only to transiently maintain proliferation of EBV-transformed LCLs in the absence of EBNA-2 [208, 255]. LCL growth is partially rescued by the activated Notch, either when the LMP-1 gene expression is not dependent on EBNA-2 or in the presence of high levels of Notch expression [208, 256]. EBNA-2 and N-ICD were also shown to downregulate immunoglobulin M (IgM) expression, indicating that Notch has both positive and negative regulatory functions [256]. Analysis of the effect of EBNA-2 and N-ICD on genome-wide transcription in EBV-infected B cell lines revealed strong differences in the regulation of target genes, with only a small group of genes being concordantly regulated [256]. EBNA-2 was more efficient at transactivating genes associated to cell proliferation and survival, whereas Notch was prone to induce genes involved in development and cell differentiation [256]. Apart from the LMP-2A and Cp promoters found to be induced by Notch, a recent report has shown that Notch signalling was able to modulate the expression of other EBV latent genes. Specifically, expression of the LMP-1 gene mediated by EBNA-2 was found to be inhibited upon activation of the Notch

signalling pathway [257]. Taken together, these observations could indicate that EBNA-2 exploits and deregulates the activated Notch signalling pathways, complementing this activity with additional viral mechanisms, such as gene activation independently of RPB-Jk, to carry out B lymphocyte transformation and cell proliferation. The requirement for other cell factors than RBP-Jk for LMP-1 transactivation by EBNA-2, such as the PU.1/Spi-1 factor, could determine the incapacity of the activated Notch receptor to transactivate LMP-1 gene expression.

1.3.4.3 EBNA-2 interacts with cellular and viral proteins

Once tethered to the DNA promoters, activation of transcription by EBNA-2 is mediated by the transactivation domain (TAD) [216], located in CR8 (Figure 1.5), that recruits additional co-activating proteins, such as the histone acetyltransferases PCAF and p300/CBP [258] and other cellular transcription factors. These include TFIIB and TAF40, components of the transcription initiation complex [259], TFIIH, a factor involved in promoter clearance [260] and the cellular co-activator p100 that interacts with the general transcription factor TFIIE and thereby bridges STAT6/RNA polymerase II interactions [261]. EBNA-2 TAD shares structural similarities with the transactivation domain of herpesvirus simplex protein VP16 and functional similarities, since the core fragment of EBNA-2 TAD (amino acids 453-466) can be replaced by an acidic fragment of VP16 TAD with no loss of function. In addition, a chimeric EBV carrying this 14-amino acid fragment from the VP16 TAD could transform B lymphocytes as efficiently as the wildtype EBV [229]. Both TADs were also shown to recruit similar transcription factors to mediate gene activation.

EBNA-2 interacts with EBNA-LP to cooperatively enhance gene expression [116-118]. This interaction mainly occurs through the EBNA-2 TAD and SAD1, but interaction with CR7 and the RG motif also modulates transcriptional activation mediated by EBNA-2 [119, 224]. EBNA-2 cooperation with EBNA-LP was earlier reported to promote cell cycle entry and induction of cyclin D2 [116]. Recently, EBNA-LP was shown to displace the transcriptional repressors N-CoR and RBP-Jk from cell gene promoters facilitating EBNA-2 binding and gene activation [262]. EBNA-LP was also found to bind at similar regions of the genome as EBNA-2 and also the EBNA-2 binding sites were enriched for EBNA-LP-associated transcription factors, which confirms the intimate cooperation between these two viral proteins in gene activation [263]. Conversely, the viral EBNA-3 proteins inhibit EBNA-2-

mediated gene expression via RBP-Jk, by competing with EBNA-2 for RBP-Jk binding [264]. EBNA-2 also interacts with human SNF5/Ini, a component of the human SWI/SNF chromatin remodelling complex. This interaction occurs through the less conserved regions of EBNA-2 flanking CR5 and specifically relies on the amino acid motifs IPP288 and DQQ315 as well as on the CKII phosphorylation of the SS469 motif adjacent to the EBNA-2 TAD [235, 236, 265]. This process of chromatin remodelling is important to facilitate chromatin access for initiation and regulation of transcription. The interaction of EBNA-2 with hSNF5/Ini1 could potentially have an additional function, consisting of interfering with the growth-suppressing activities of hSNF5/Ini1, when forming heterotrimeric complexes with GADD34 and PP-1 [266]. The CR5 region of EBNA-2 is also involved in binding to the Ski-interacting protein (SKIP), which binds to RBP-Jk and promotes EBNA-2 binding to RBP-Jk [223].

The CR7 and CR8 domains of EBNA-2 were shown to interact with the BS69/ZMYND11 cell protein and this binding was shown to repress transcriptional activation mediated by EBNA-2 (reviewed in section 1.6) [267, 268]. The EBNA-2 protein was also found to interact with the DEAD-box protein DP103 (Gemin3/DDX20) through CR4 and CR4 flanking regions (aa 121-216), a putative RNA helicase [269]. The methylated poly-RG motif of EBNA-2 recruits the survival motor neuron (SMN) protein, which is involved in RNA splicing and directly interacts with DP103 and can enhance LMP-1 promoter activation by EBNA-2 [270, 271]. Additionally, EBNA-2 also interacts with Nur77, a nuclear protein that acts as a transcriptional factor and is involved in cell apoptosis. EBNA-2 protects cells from apoptosis by retaining Nur77 protein in the nucleus upon apoptotic stimuli [272, 273]. Similarly, Notch-ICD was also shown to bind Nur77, p300/CBP, PCAF and SKIP, indicating that RBP-Jk binding is not the only common interacting partner between EBNA-2 and Notch.

1.3.5 EBNA-2 cellular target genes

EBNA-2 is a strong transcriptional activator, which is involved in regulation of the viral Cp and LMP promoters as well as of numerous cellular target genes involved in the process of B cell transformation and proliferation. The cell responsive genes have been investigated using two complementary approaches: expression of the EBNA-2 protein in established EBV-negative B cell lines or in the context of the EBV-transformed lymphoblastoid cell lines. The limitation of the first approach resides on the absence of other EBV proteins that can

contribute to the regulation of the target genes. Different reports have identified a plethora of cell target genes actively induced by EBNA-2. These include the oncogenes *c-FGR* and *c-MYC* [274, 275], the cell cycle regulator cyclin D2 [116], the transcription factor RUNX3 [276], the B cell markers CD21 and CD23 [277, 278], the receptor FcRH5 [279] and other genes, such as the cyclin dependent-kinase 5 subunit 1 CDK5R1, *DNASE1L3* or *MFN1* [114]. The chemokines CCL3 and CCL4 [114], the interleukin receptor IL-18R [280], the chemokine receptor CCR7 [281] and TNF-α, LT-α and IL-16 [276, 282] have been also shown to be regulated by EBNA-2.

Some of the cellular target genes upregulated by the EBNA-2 protein are also targets of the activated Notch-ICD. Specifically, the basic helix-loop-helix proteins (bHLH) that include the hairy/enhancer of split (HES) family (HES-1 gene [120]) and the hairy-related transcription factor (HERP) family (HERP1 and HERP2 genes [114]). The expression of BATF was also found to be induced by EBNA-2 and by the activated Notch. BATF encodes a member of the AP-1 family of transcription factors that inhibits cell growth and is likely related to viral latency by repressing induction of lytic cycle by downregulation of BZLF1 expression [283]. EBNA-2 protein was also found to actively downregulate expression of target responsive genes. The downregulation of immunoglobulin M (IgM) expression and of the proteins BCL6, TCL1A and CD79 and CD79B by EBNA-2 revealed that this viral protein could also interfere with the B cell and the germinal centre phenotype [114, 281, 284]. Additionally, expression of activation-induced cytidine deaminase (AID) was also shown to be inhibited by EBNA-2 [285].

Genome wide screens and microarray analysis have identified an extensive number of genes that are regulated by EBNA-2 using the previous approaches, revealing the pleotropic nature of this viral transcriptional activator [113-115, 206, 207]. One of these studies used the DG75 BL cell line, RBP-Jk-negative, to determine whether the activation of a group of EBNA-2 target genes was dependent on RBP-Jk. The EBNA-2 activated genes studied in this work were, in fact, dependent on RBP-Jk [281]. Further reports have also identified genes directly activated by EBNA-2 by using cells, in which EBNA-2-mediated induction of those genes RNA was not dependent on *de novo* synthesis of intermediate genes [206]. The EREB2.5 LCL, conditional for EBNA-2 function [136] (section 1.3.1), was used since EBNA-2 activation did not depend on protein synthesis. The cell genes modulated by the EBNA-2 protein can either be direct targets or genes activated by the cascade of events triggered by viral or cell genes that are primarily induced by EBNA-2. Some of the EBNA-2 target genes include the p55α subunit of PIK3R1, c-MYC, CD21, CD23, RUNX3 and FcRH5 [113, 279]. These

multiple studies have identified numerous EBNA-2 target responsive genes [113-115, 206, 207], whose products display distinct and variable functions and can be organised into different categories such as: proteins involved in the endocytic pathway and recycling; transcription factors important for cell cycle regulation and that control the apoptotic process (STAT6, P1K3R1 and GADD45β); chemokines, cytokines and chemokine and interleukin receptors (CXCR7, CCR7, IL-18R); proteins that compose the Notch signalling pathway (HEY1); transcription factors associated with B cell activation and further differentiation; proteins involved in gene transcription and in RNA processing and proteins fundamental for cytoskeletal remodelling and cell adhesion.

1.4 The EBV types

1.4.1 Type 1 and type 2 classification of EBV strains

EBV strains can be organised into type 1 or type 2 (also known as type A and type B) based on the sequence of the EBNA-2 gene, which was the first major variation identified in EBV [213, 286]. EBNA-2 defines the viral types because it is the most variable locus in the EBV genome and is characterised by 70% identity at the nucleotide sequence but only 56% similarity at the amino acid level between the types [287]. This variation is strikingly high if compared to the extremely low degree of divergence of the genome. This organisation into type 1 and type 2 is also linked to the sequence variation of the viral latent genes EBNA-3A, EBNA-3B, EBNA-3C and EBNA-LP, but the sequence dissimilarity is lower in these genes [288-290]. A recent report confirmed by principal-component analysis on single-nucleotide polymorphisms in a set of genomes studied, that the type 1/type 2 classification is almost entirely dependent on EBNA-2 and the EBNA-3 genes [29] although few intertypic recombinants have also been identified [29, 291, 292]. Sequence variation has also been observed for other genes, which include the genes BZLF1 (lytic cycle transactivation), BRRF2 (tegument) and BNLF2a (linked to immune evasion); BDLF3 and BLLF1 encoding the glycoproteins gp150 and gp350/200; EBNA-1 and LMP-1 in spite of no association with type 1/type 2 classification [29, 293-295]. The prototypical type 1 EBV is the B95-8 strain and had been isolated from a North American infectious mononucleosis case, while the prototypical type 2 strain is the AG876 EBV, which was isolated from a Burkitt's lymphoma case in Central Africa [213].

The major biological difference between type 1 and type 2 strains is that type 1 EBV is able to transform human B lymphocytes into LCLs *in vitro* much more efficiently than type 2 EBV strains [296]. LCLs generated by B cell infection by type 1 strains exhibit an increased growth rate and reach a higher cell density when compared to LCLs transformed by type 2 EBV strains [296]. This difference was attributed to EBNA-2 [134] using a recombinant type 2 P3HR1 strain, in which a type 1 EBNA-2 coding sequence was introduced in the EBNA-2 locus. The recombinant type 2 virus expressing type 1 EBNA-2 protein acquired the B cell transformation phenotype of a type 1 strain [134]. Introduction of type 1 EBNA-3A, EBNA-3B and EBNA-3C gene sequences however did not alter the ability of type 2 P3HR1 to transform

B lymphocytes [288, 297]. Interestingly, the transforming ability exhibited by type 1 EBV strain *in vitro* correlated with higher tumour formation frequency after intraperitoneal inoculation of SCID mice with type 1 and type 2 transformed LCLs. When type 1 EBV and a type 2 P3HR1 strain carrying the type 1 EBNA-2 protein sequence were used to generate LCLs prior to inoculation, the rate of tumour formation was equivalent, confirming the crucial role of the EBNA-2 protein [298, 299].

1.4.2 Geographical distribution and disease association

The EBV types exhibit a distinct geographical distribution. Type 1 strains are ubiquitous and generally predominant worldwide. Specifically, type 1 EBV strains are prevalent in European, American and Chinese populations, where type 2 EBV is only detected in a small percentage of healthy hosts in these populations [300]. Type 2 strains are predominantly present in sub-Saharan Africa, where the prevalence of type 1 and type 2 EBV strains is approximately the same [301]. Type 2 EBV appears to be relatively abundant where malaria, chronic infections or immunodeficiency are present, such as in Central Africa and Papua New Guinea. Type 2 strains were observed in a significant percentage of healthy hosts in Kenya (24%) and New Guinea (21%) [301-304]. Apart from the higher frequency of tumour formation by type 1 LCLs in SCID mice and despite the clear distinct geographical distribution of both viral types, the association of each EBV type with type-specific diseases has not been determined. It is assumed that the geographical distribution of the two viral types in EBV-related diseases only reflects the general prevalence in those areas [305] and no evident relation between the two types and specific disease was observed [305]. Accordingly, type 1 and type 2 EBV were both detected in endemic EBV-positive BL cell lines in Central Africa, where the EBV strains are almost equally abundant. In contrast, the sporadic EBV-positive BL cases, in the regions where type 1 is more prevalent, were almost all associated with type 1 EBV [301, 302]. Many different studies were carried out to determine the frequencies of type 1 and type 2 EBV in distinct geographical areas or populations but some of these studies consisted of recovering virus from the spontaneous LCL outgrowth *in vitro*, using peripheral blood mononuclear cells (PBMCs). This method probably led to an underestimation of the true prevalence of type 2 virus in the general Caucasian population, since the lower transforming-efficiency type 2 EBV could be rescued less efficiently [306]. Moreover, in the case of co-resident EBV strains, the type 1 EBV with greater *in vitro* transforming ability would be selected. In fact, the utilisation

of a type-specific polymerase chain reaction directly on PBMCs to study EBV type prevalence revealed that the prevalence of type 2 virus in HIV-infected individuals was about 50% to 62%, independently of the degree of immunodeficiency [307]. The higher prevalence of type 2 EBV in HIV-infected and in other immunosuppressed patients (transplant recipients), in comparison with healthy individuals has been reported in different studies [308, 309]. This increased prevalence of type 2 is directly related to the higher detection of type 2 EBV in EBV-positive malignancies in these individuals [310-315]. This increased detection of type 2 virus in the immunocompromised individuals has been explained by an increased exposure to the virus combined with deficient EBV-specific cellular immunity, which leads to long-term carriage of multiple EBV genotypes [305]. The immunocompromised individuals were indeed found to more commonly harbour both types of EBV [315]. Additionally, it is possible that the immunodeficiency condition favours the occurrence of higher viral loads, which would favour detection of type 2 EBV in these individuals [316]. In immunocompetent hosts, the low viral loads might not allow detection of type 2 EBV and therefore it might introduce a strong bias in the determination of the prevalence and geographical distribution of EBV types [302, 305]. However, a study comparing the prevalence of type 2 EBV infection in two distinct groups of HIV-infected individuals, specifically HIV-infected haemophiliacs and HIV-infected homosexuals challenged the previous hypothesis. The frequency of the type 2 virus detected in HIV-positive haemophiliacs was lower than the rates of type 2 viral infection in HIV-positive homosexuals and was comparable to the type 2 EBV frequency in healthy individuals in the same geographical area [317], indicating that the immunodeficiency status *per se* is not solely responsible for the increased type 2 EBV detection in western populations and acquisition of type 2 EBV might be due to opportunity for exposure. This hypothesis was further supported by a report showing that HIV-negative homosexual men exhibited a high prevalence of type 2 and dual infection with type 1 EBV (39%), when compared to HIV-negative heterosexual men (6%). A group of HIV-positive homosexual individuals was also assessed and the frequency was 67% [318]. This could suggest that type 2 EBV has become endemic in the homosexual community although the real prevalence in the western populations has yet to be determined.

The almost equal prevalence of type 2 and type 1 EBV strains in the regions of sub-Saharan Africa is still a matter of debate. One interesting hypothesis is that type 2 EBV infection might be promoted in hosts where chronic immune activation is present, such as in the case of co-infections with *Plasmodium falciparum*, the causative agent of malaria, or with other agents. It has been hypothesised that the attenuated transforming ability of type 2 EBV associated to

an immunosuppressive condition (HIV or malaria) might be required for type 2 to be capable of maintaining infection of B lymphocytes and causing transformation, in these regions where the immune system is always subject to a constant pressure [319, 320].

1.4.3 Type 1 EBV more efficiently transforms B cells into LCLs *in vitro*

The clearest biological difference between type 1 and type 2 EBV strains is that type 1 virus transforms and immortalises human B lymphocytes *in vitro* much more efficiently than type 2 EBV [296]. This distinct transforming ability was attributed to the EBNA-2 protein [134] (section 1.4.1). A recent work from this laboratory, using the EBV-transformed EREB2.5 LCL with conditional EBNA-2 function [136] (in section 1.3.1), determined that type 1 EBNA-2 was able to maintain the normal growth of the cells but the type 2 EBNA-2 protein was not able to sustain cell growth [206]. The EREB2.5 growth assay was used to investigate the differential capacity of type 1 EBV to promote cell growth when compared to type 2. The regions of type 1 EBNA-2 protein responsible for the higher ability to sustain LCL growth were determined by testing the ability of different chimeric type 1/ type 2 EBNA-2 proteins to maintain cell growth in the EREB2.5 assay [209]. The C-terminal region of type 1 EBNA-2 was sufficient to confer the ability to sustain cell growth when introduced into a type 2 EBNA-2 protein. Specifically, the type 1 EBNA-2 domains poly-arginine-glycine (RG) motif, the CR7 domain and the TAD (acidic transactivation domain of EBNA-2) were the minimum type 1 sequences required to confer the capacity to maintain LCL growth to type 2 [209].

These studies also determined that the reduced capacity of type 2 EBNA-2 protein to sustain cell proliferation correlated with weaker induction of a small number of EBNA-2 target genes. Specifically, 10 genes out of around 300 EBNA-2 target genes were found to be more strongly induced by type 1 EBNA-2 than by type 2 [206]. This reduced group includes the genes IL-1β, ADAMDEC1, CCL3L3, MARCKS and the most differentially regulated cellular gene, CXCR7 (Figure 1.6). The viral LMP-1 gene was one of the genes more strongly induced by the type 1 EBNA-2 protein [206, 209]. Accordingly, a type 2 EBNA-2 protein carrying the C-terminal end of type 1 EBNA-2 also acquired the type 1-ability to induce expression of CXCR7 and LMP-1, which are essential for cell proliferation [209]. This higher induction of LMP-1 and CXCR7 by type 1 EBNA-2 was observed in different cell systems

and upon infection of primary B cells with BAC-derived EBV expressing type 1 or type 2 EBNA-2 proteins [206, 209]. A differential induction of target genes might determine the higher B lymphocyte transformation activity of type 1 EBV strains when compared to type 2 strains.

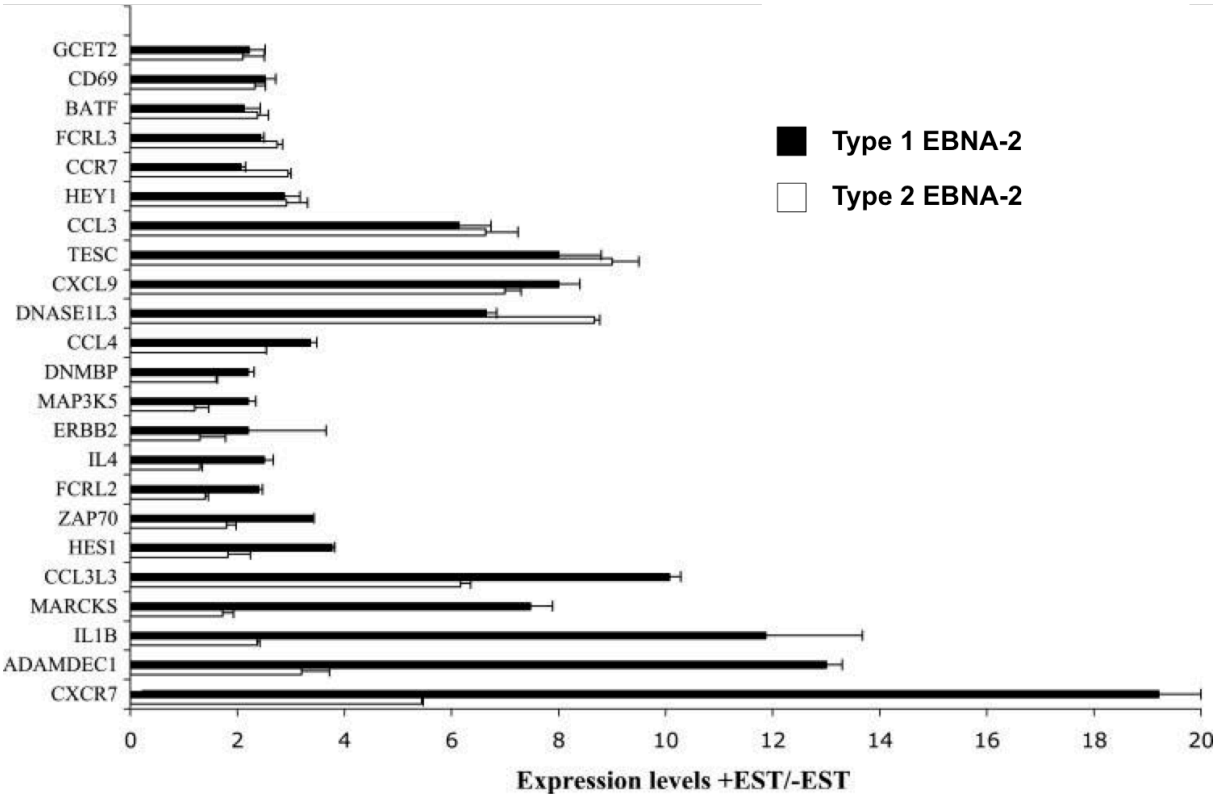


Figure 1.6 A restricted number of genes is differentially induced by type 1 and type 2 EBNA-2

Summary of comparative microarray expression profiling of different genes by type 1 (black) and type 2 (white) EBNA-2. Figure from [206].

More recently, work from this laboratory demonstrated that a single amino acid substitution, in which a serine of type 2 EBNA-2 was replaced by the corresponding aspartate in type 1 EBNA-2 (S442D), was sufficient to confer to a type 1-like growth phenotype to type 2 EBNA-

2 protein in the EREB2.5 LCL growth maintenance assay [210]. The residue aspartate 442 is located in the transactivation domain (TAD) of type 1 EBNA-2 protein, suggesting that the higher ability of type 1 EBNA-2 to induce this specific subset of differentially regulated genes could contribute to the superior capacity to transform and immortalise B lymphocytes into LCLs *in vitro* by type 1 EBV.

In the present study, the ability of the acidic transactivation domain (TAD) of type 1 and type 2 EBNA-2 to transactivate gene expression was investigated in order to determine whether a distinct transactivation function by type 1 and type 2 EBNA-2 TAD could account for the type 1/type 2 differential gene regulation. The effect of several amino acid substitutions in the TAD of EBNA-2, including the important serine to aspartate (S442D) mutation, was also assessed in terms of the ability of EBNA-2 to induce gene expression (section 3).

1.5 The Latent Membrane Protein 1 (LMP-1)

1.5.1 The viral oncoprotein LMP-1

The latent membrane protein 1 (LMP-1) is the major EBV oncoprotein and is required for B cell transformation into LCLs *in vitro*, but also to maintain continuous proliferation of the infected LCLs [74, 135, 321, 322]. A recombinant EBV lacking LMP-1 is not able to transform B cells [135]. An earlier study had also shown that LMP-1 could induce transformation of primary rodent fibroblasts and this phenotype was then associated with tumour formation in nude mice [323]. Ectopic expression of LMP-1 in the skin or in the B cell compartment of transgenic mice caused hyperproliferation of the epithelium and increased lymphomagenesis respectively [324, 325]. Effects on epithelial cell differentiation and inflammation were also observed [324-327]. LMP-1 expression has been detected in different EBV-positive malignancies, such as Hodgkin's lymphoma, immunoblastic lymphomas and undifferentiated NPC [9]. LMP-1 is also expressed in LCLs *in vitro* and in the viral expression programs latency III and latency II, during EBV-mediated B lymphocyte differentiation (Table 1.1).

The viral LMP-1 protein resembles a constitutively-active CD40 receptor, which is a member of the tumour necrosis factor receptor (TNFR) superfamily and is essential for B cell survival and apoptosis rescue upon antigen stimulation [322, 328, 329]. Similarly, LMP-1 is required to provide survival signals to activated B lymphocytes upon EBV-induced differentiation [328, 330, 331]. Moreover, LMP-1 also suppresses cell death through the induction of distinct anti-apoptotic proteins, which include BCL-2, MCL-1, BFL-1, A20 and cIAPs [332-336], therefore promoting cell proliferation and protecting them from the apoptotic process. Conversely, the pro-apoptotic factor BAX (the BCL-2-associated protein x), which is an antagonist of BCL-2, is downregulated by LMP-1 [337]. LMP-1 also enhances cell invasiveness through the induction of matrix metalloproteinases expression, suggesting that the viral protein might influence the process of angiogenesis and metastasis in EBV-associated malignancies [338]. LMP-1 was additionally found to induce the expression of cellular factors and adhesion proteins including CD40, ICAM1, CD21, CD23, LFA1, fundamental for B cell transformation [321, 339, 340].

1.5.2 LMP-1 structure and signal transduction

The latent membrane protein 1 is a 63 kDa integral membrane protein with 386 amino acids and is structurally composed of a short cytoplasmic N-terminal domain (amino acids 1-24), six transmembrane domains (aa 25-186), and a long cytoplasmic C-terminal tail (aa 187-386) [7, 341, 342]. This cytoplasmic C-terminal tail contains three C-terminal activation regions (or CTARs): the membrane-proximal CTAR1 and the membrane-distal CTAR2, also referred to as transformation effector sites TES1 and TES2, respectively and the less well characterised CTAR3 located between CTAR1 and CTAR2 [343]. LMP-1 oligomerises on the membrane of the cell through interaction of the transmembrane domains, forming a signalling complex in the absence of ligand. These transmembrane domains and the C-terminal activation regions are required for LMP-1-triggered signal transduction that contributes to efficient B lymphocyte transformation [321]. CTAR1 recruits the members of the tumour necrosis factor (TNF)-associated protein family (TRAFs) since it contains a TRAF-binding motif (PXQXT) via which directly associates with TRAF1, TRAF2, TRAF3 and TRAF5 [328, 340, 344-346]. Binding to TRAFs stimulates the induction of the non-canonical activation of NF-kB through the signalling phosphorylation relay that activates the NF-kB-inducing kinase (NIK), which activates the IkB kinase subunit α (IKKα). This kinase is responsible for phosphorylation of the NF-kB precursor protein p100, which is processed into mature p52 that translocates to the nucleus as an active dimeric NF-kB complex with RelB factor to activate transcription [347, 348]. CTAR2 is responsible for the canonical NF-kB activation via binding to the TNFR-associated death domain (TRADD) [349, 350]. TRAF6 is involved in the signal transduction pathway. The IKKβ kinase is activated and phosphorylates IkBα, which leads to IkBα ubiquitination and degradation, and the p50/p65 mature subunit complexes translocate to the nucleus to activate transcription [348]. CTAR1 and CTAR2 also participate in the activation of the MAPK pathways c-Jun-N-terminal kinase (JNK) and p38 leading to the induction of the AP-1 (dimer of c-Jun and c-Fos proteins) and of ATF2 transcription factors [344, 349, 351-353]. Activation of NF-kB and of the JNK and p38 pathways is fundamental to prevent B cell apoptosis and also to activate transcription of genes crucial for B cell growth transformation and immortalisation [354-356]. Specifically, NF-kB triggers the induction of anti-apoptotic genes, downregulation of pro-apoptotic factors and expression of B lymphocyte markers. The mechanisms of DNA repair and p53-mediated transcriptional activity are also suppressed by LMP-1 through NF-kB [357]. Besides CTAR1 and 2, also the CTAR3 domain was involved in signal transduction and was associated with triggering of JAK3/STAT (Janus kinase 3/Signal

Transducer and Activator of Transcription) signalling cascade [343]. Signal transduction pathways where LMP-1 plays an important function are represented in Figure 1.7.

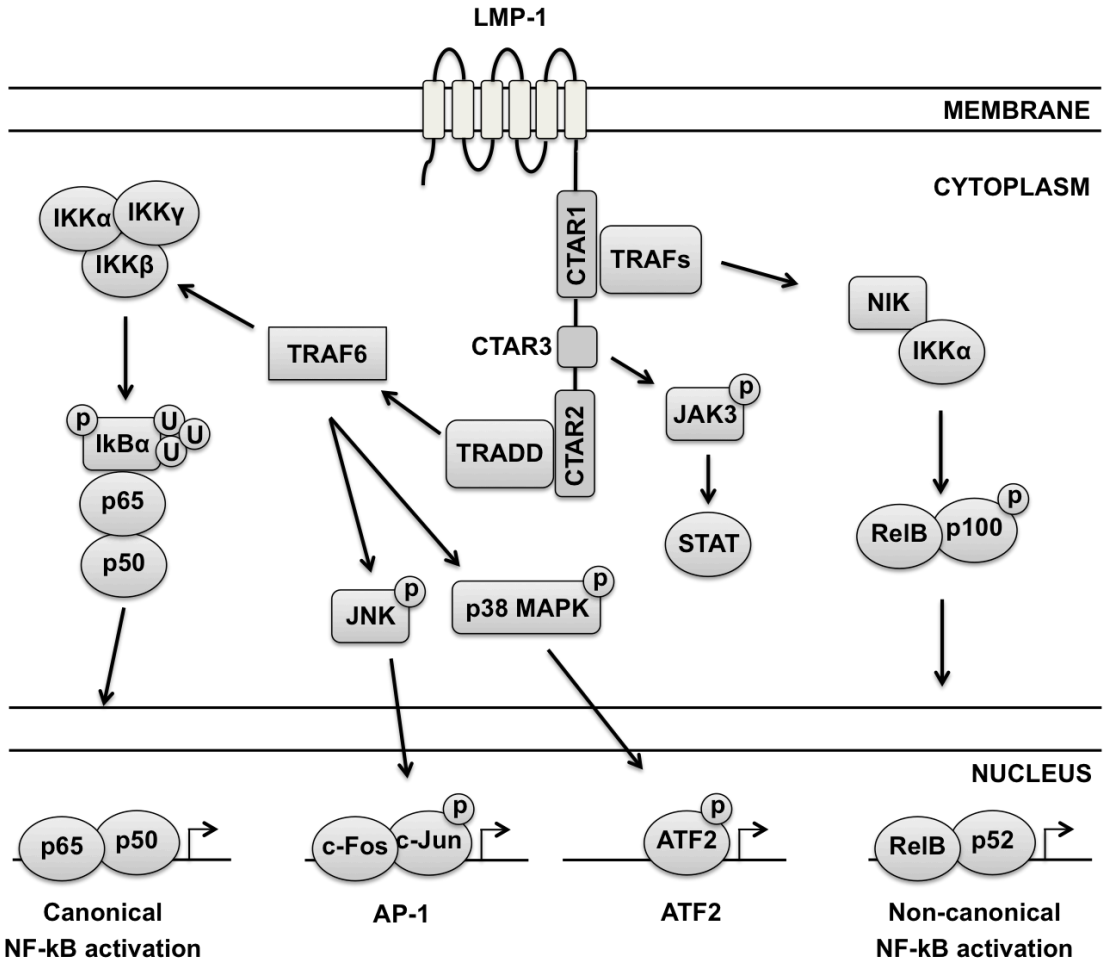


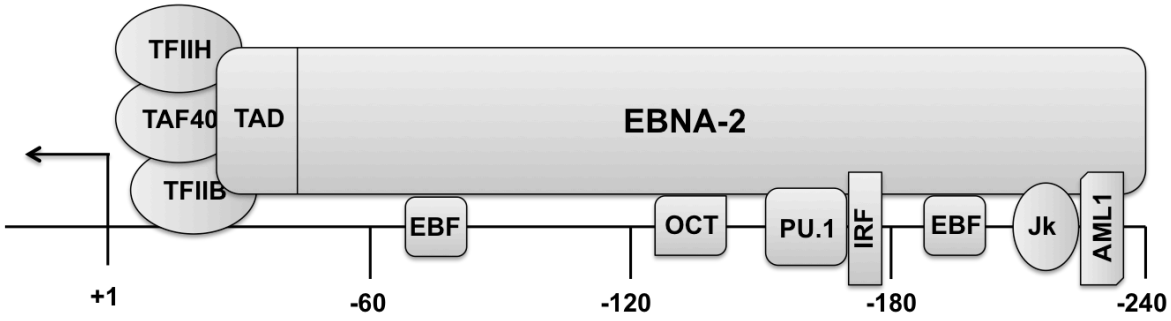
Figure 1.7 Signal transduction mediated by EBV LMP-1

LMP-1 is composed of a short cytoplasmic N-terminal domain, six transmembrane domains and a long cytoplasmic C-terminal tail. This C-terminal tail is composed of three C-terminal activation regions, CTAR1, CTAR2 and CTAR3. CTAR1 and CTAR2 domains are involved in the canonical and non-canonical activation of the transcription factor NF-kB to mediate induction of genes important for B lymphocyte transformation, immortalisation and proliferation. The JNK and p38 MAPK pathways are also activated to stimulate the transcription of additional factors involved in B cell transformation. Adapted from [348, 358].

1.5.3 The LMP-1 promoter

LMP-1 expression can be regulated by two different promoters, a proximal ED-L1p promoter and a distal TR-L1p promoter [359, 360]. The TR-L1p promoter is located within the terminal repeats and mediates LMP-1 expression in latency II transcriptional program in an EBNA-2-independent manner since EBNA-2 is absent in the default program. The TR-L1p promoter is regulated by STAT [361-363]. Various cytokines (such as IL-4, IL-6, IL-10) are involved in the activation of STAT and thereby LMP-1 gene expression [361-366]. In latency III, LMP-1 transcription from the proximal ED-L1p promoter is highly dependent on EBNA-2 [226, 239, 240]. Unlike most EBNA-2 target promoters, the interaction with RBP-Jk in EBNA-2-induced activation of the LMP-1 promoter has a minor role [108] and its regulation requires additional cell co-factors such as PU.1, POU-domain protein, AP-2, SWI-SNF, p300/CBP, ATF/CRE and EBF1 [239, 240, 242-244, 258] (Figure 1.8). The involvement of these cellular factors in the regulation of the LMP-1 ED-L1p promoter by EBNA-2 has been studied extensively for type 1 EBNA-2 but not for type 2. The viral LMP-1 gene is one of the genes differentially regulated by type 1 and type 2 EBNA-2 [206, 209] (section 1.4.3). Comparison of DNA sequences of EBNA-2 binding sites in genes differentially regulated by EBNA-2 types (including LMP-1) identified a conserved sequence motif corresponding to the consensus binding sites for PU.1 and IRF transcription factors, which is not present in EBNA-2 binding sites in equally regulated genes, suggesting that the function of EBNA-2 at the differentially regulated genes might be determined by these cell factors, as at the LMP-1 promoter [210]. In the present study, the mechanism of differential induction of the LMP-1 was studied to investigate the DNA sequences and promoter elements that could be required for the higher LMP-1 induction by type 1 EBNA-2. The interaction of EBNA-2 with the LMP-1 promoter was also addressed to determine the elements at the LMP-1 promoter that recruit EBNA-2 to DNA that could account for the type 1 higher expression of this gene (section 5).

A



B

B95-8 EBV LMP-1 promoter regulatory sequence (-241/+12):

169,047 **169,097**

TTGAGGAAAGAAGGGGGCAGAGCAGTGTGAGAGGCTTATGTAGGGCGGCTA

TSS

169,098 **169,148**

CGTCAGAGTAACGCGTGTTTCTTGGGATGTAGGCCCGGGGGGATTTGCGGG

EBF

169,149 **169,199**

GTCTGCCGGAGGCAGTACGGGTACAGATTTCCCGAAAGCGGCGGTGTGTGT

169,200 **169,250**

GTGCATGTAAGCGTAGAAAGGGGAAGTAGAAAGCGTGTGTTTGTGTTAGAA

octamer PU.1 IRF

169,251 **169,299**

AAGCGGGTCCCCGGGGGGCAAGCTGTGGGAATGCGGTGGCCAAGTGCAA

EBF RBP-Jk AML1

Figure 1.8 The EBV LMP-1 promoter

(**A**) Representation of the interactions of EBNA-2 with factors binding at EBNA-2 responsive elements and cellular transcription factors at the LMP-1 promoter. DNA-interacting proteins recognise promoter elements and recruit EBNA-2 to EBNA-2 responsive elements. EBNA-2 transactivation domain (TAD) recruits cellular factors of the basal transcription machinery to activate transcription. (**B**) The LMP-1 promoter region (-241/+12) of the B95-8 EBV strain. DNA sequence corresponding to the nucleotides 169,047 to 169,299 and is shown in the standard EBV genome orientation. The transcription start site (TSS) is represented by the arrow (+1). Binding sites for regulatory transcription factors are indicated and closely resemble consensus motifs. PU.1 and IRF binding sites constitute an ETS-IRF composite element (EICE). EBF: early B cell factor. IRF: interferon regulatory factor. RBP-Jk (Jk): recombination signal-binding protein J kappa; OCT: octamer/POU-domain protein, AML1: acute myeloid leukemia1.

1.6 The cellular protein BS69/ZMYND11

1.6.1 BS69/ZMYND11 is a transcriptional repressor protein

The cellular protein BS69/MYND (BS69) is composed of several domains, which include an N-terminal PHD (Plant Homo Domain) zinc-finger, a Bromo domain, a PWWP domain and a C-terminal MYND (MYeloid translocation protein 8, Nervy and DEAF-1) domain (Figure 1.9) [267, 367]. BS69 was originally identified as a nuclear protein interacting with the adenoviral protein E1A [368]. E1A is an oncoprotein expressed in the viral replication cycle, which can transform and immortalise rodent fibroblasts in cooperation with other viral products [369-371]. E1A protein was also shown to interact with several cellular proteins that include the pRb family of protein and the p300/CBP to interfere with cell proliferation and differentiation [372-374]. BS69 binds a PXLXP peptide motif (X represents any amino acid) in the E1A protein via the BS69 MYND domain and this interaction inhibited E1A-mediated transcriptional activation of viral and cell genes [267]. This association was also found to stabilise the E1A protein that is unstable in host cells by reducing the ubiquitination of E1A, in a MYND domain-dependent way [375-377].

BS69 was also described as a transcriptional repressor in association with other transcription factors such as c-Myb [378], B-Myb [379], ETS2 [380], the Myc-related MGA factor [267] and the BRCA2 repressor or negative regulator EMSY [381]. BS69 recognises a common PXLXP sequence motif present these proteins. BS69 was also shown to repress transcription through recruitment of N-CoR repressor [367].

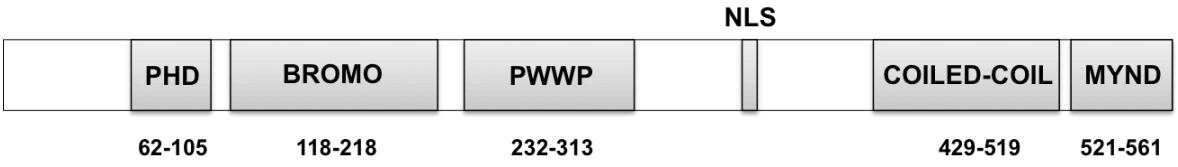


Figure 1.9 The structural domains of the BS69/ZMYND11 protein

BS69 protein is composed of several domains: the N-terminal plant homo domain (PHD); the Bromo domain, the PWWP (proline, tryptophan, tryptophan, proline domain) domain; a nuclear localisation signal (NLS); a coiled-coil domain and the C-terminal MYND (MYeloid translocation protein 8, Nervy and DEAF-1) domain. Amino acid positions delimiting each domain are indicated. Adapted from [268].

1.6.2 BS69/ZMYND11 functions as a chromatin regulator

Some of the protein sequence motifs in BS69 are associated with gene and chromatin-mediated transcriptional regulation, namely the PHD zinc-finger domain [382], present in helicases and in transcriptional activators and Bromo, PWWP and MYND domains [383-385]. The MYND domain functions as a protein-protein interaction surface that mediates BS69 interaction with transcription factors and chromatin [367, 378, 386]. In fact, BS69 was found to associate with chromatin and with mitotic chromosomes. Specifically, BS69 interacts with the ATP-dependent helicase BRG1 [386], a component of the SWI-SNF complex [387]; with the BRG1-related protein brahma [388], the histone deacetylase HDAC1 [389] and the histone-methyltransferase EZH2 [390, 391] and also with the transcription factor E2F6 [392], indicating the BS69 might have a function in chromatin remodelling [386]. Indeed, the Bromo/PWWP domains of BS69 can specifically recognise and associate with histone 3.3 trimethylated at lysine 36 (H3.3K36me) [393], which facilitates the recruitment of MYND-bound transcription factors and of chromatin remodelling factors (EZH2, HDAC1, BRG1) to target loci, thereby repressing gene transcription [394]. ChIP analysis revealed that H3.3K36m3 is enriched at BS69 target genes [395]. This is a highly specific recognition affected by the methyl level on lysine 36 - BS69 binds much less to H3.3K36m0/1/2 than to H3.3K36m3 [396]. Accordingly, it was postulated that BS69 protein regulates gene expression through histone modifications/histone exchange in the transcribed regions, preventing the transition of paused RNA polymerase II to the elongating stage [396, 397]. BS69 was also associated with pre-mRNA processing through H3.3K36m3 [395]. BS69 associates with the chromatin at H3.3K36m3 and interacts with a different set of proteins that are involved in regulation of RNA splicing. Specifically, BS69 associates with the U5 snRNP (small nuclear ribonucleoprotein particle) proteins (EFTUD2, PRPF8 and SNRNP200) and the serine/arginine-rich proteins SRSF1, PNN and ACIN1 [395]. BS69 was shown to regulate the process of intron retention by antagonising the activity of basal splicing machinery [395]. The importance of regulated intron retention is not completely clear but deregulation of intron retention and exon skipping is an important event in different malignancies [398, 399]. Thus, it is possible that BS69, as a candidate tumour suppressor protein might regulate intron retention to inhibit tumorigenesis.

1.6.3 BS69 and EBNA-2

The interaction between BS69/ZMYND11 and EBNA-2 was first described by Ansieau and Leutz in 2002 [267]. EBNA-2 contains two PXLXP peptide motifs (residues 383-387 and 437-441) that are recognised and bound by the MYND domain (residues 521-562) of BS69. This interaction was shown to repress gene transactivation mediated by EBNA-2 [267]. More recently, another report provided further insights into the interaction of the BS69 protein with EBNA-2 using crystallographic studies [268]. A coiled-coil domain that precedes the MYND domain was identified. BS69 forms a homodimer structure through self-association of the coiled-coil domains and two BS69 monomers are brought in close proximity to cooperatively bind the sequentially proximate PXLXP peptide motifs within the CR7 and CR8 regions of the EBNA-2 protein in a synergistic manner, which causes enhanced BS69-EBNA-2 recognition [268] (Figure 1.10). Analysis by isothermal calorimetry titration confirmed that BS69 binding to EBNA-2 was 30-150 fold stronger than binding to either PXLXP motif alone [268]. Residues Q546 and W550 of BS69 MYND domain are important to mediate interaction with the EBNA-2 protein. Mutation of a single residue decreases the affinity of BS69 binding to EBNA-2 but replacing both amino acids by alanine residues largely abolished the interaction. Amino acids P383, L385 and P387 from PXLXP1 and residues P437, L439 and P441 from PXLXP2 were shown to make contact with the BS69 MYND domains [268].

BS69-EBNA-2 interaction is required for BS69 inhibition of EBNA-2-mediated transcriptional activation [267, 268]. BS69 was shown to reduce EBNA-2 transactivation of the target LMP-1 and Cp promoters in reporter assays. This repression was reduced with a Q546A BS69 mutant but completely relieved when the Q546A/W550A double mutation in the MYND domain of BS69 was tested, confirming that BS69 mediates repression of transcriptional activity of EBNA-2 upon binding. Interestingly, ChIP analysis revealed that EBNA-2 increases the amount of BS69 protein bound at the LMP-1 promoter, suggesting that BS69 is recruited to EBNA-2 target promoters inhibiting transcriptional activation [268]. In addition, BS69 was found to reduce the cell viability of EBV-transformed lymphoblastoid cell lines either through direct inhibition of EBNA-2 function or via interference with downstream events relying on the EBNA-2 protein. This reduction in cell viability is dependent on BS69 interaction with EBNA-2 because BS69 Q546A/W550A did not affect LCL viability [268]. BS69 repression of EBNA-2-mediated activation of gene expression and the impairment in LCL proliferation indicates that

BS69 acts as a negative regulator of EBNA-2. All these studies were performed with type 1 EBNA-2.

The viral EBNA-2 protein was initially found to be recruited to the BS69 promoter upon EBV infection of B cells [245], indicating that the tumour suppressor BS69 could be affected in this context. In fact, Harter and colleagues have shown that when EBV infects B lymphocytes the mRNA levels of BS69 are significantly decreased, with a more than 20-fold reduction at day 5 post-infection [268]. This was observed for other EBV-infected cell lines but not for AKATA cells [167], an EBV-positive cell line in which EBNA-2 is not expressed, indicating that EBNA-2 downregulates mRNA and protein levels of BS69 [268]. The downregulation of the tumour suppressor BS69 by EBNA-2 could represent an EBV strategy to evade host defences and cause B lymphocyte transformation and proliferation.

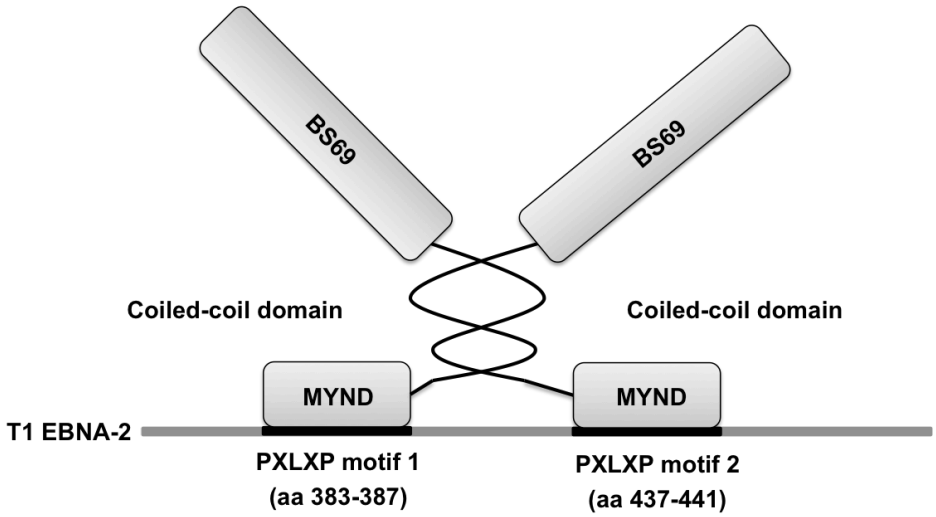


Figure 1.10 Model of BS69 homodimer interaction with PXLXP peptide motifs in type 1 EBNA-2

BS69 forms a homodimer structure through self-association of the coiled-coil domains and two BS69 monomers are brought in close proximity and their MYND domains cooperatively bind the sequentially proximate PXLXP peptide motifs of the EBNA-2 protein (PXLXP motif 1: amino acids 383 to 387 and PXLXP motif 2: amino acids 437 to 441) in a synergistic manner, causing enhanced BS69-EBNA-2 recognition [268].

1.6.4 BS69 interacts with the viral LMP-1 protein

BS69 was found to be a cellular negative regulator of both canonical and non-canonical NF-kB activation mediated by LMP-1 [400, 401]. BS69 protein directly interacts with both CTAR1 and TRAF3 to promote inhibition of CTAR1-mediated NF-kB activation, downregulating the production of inflammatory cytokines (IL-6) [400]. The requirement of TRAF3 for the BS69 repression of NF-kB activation indicated that BS69 forms a protein complex with CTAR1 and TRAF3 to suppress non-canonical activation of NF-kB induced by LMP-1 [400] (Figure 1.11). BS69 was also shown to interact with CTAR2 [401], influencing binding between LMP-1 and TRADD, through its displacement from CTAR2 [400, 401]. BS69 was shown to decrease the formation of CTAR2/TRADD protein complexes and therefore negatively regulates the LMP-1-mediated canonical NF-kB activation [401] (Figure 1.11). LMP-1/CTAR2 was also shown to be involved in the activation of the JNK pathway leading to the upregulation of the AP-1 family of transcriptional factors [402, 403]. In this context, BS69 was found to bind to LMP-1/CTAR2 through its MYND domain and also interacts with TRAF6 via the region comprising the amino acids 274-353. BS69 functions as a scaffold protein or a specific adaptor linking LMP-1 and TRAF6 and this stable complex is required to promote the activation of the JNK pathway mediated by LMP-1 [404]. This suggests that BS69 might have also a function in modulation of inflammation secondary to viral infection.

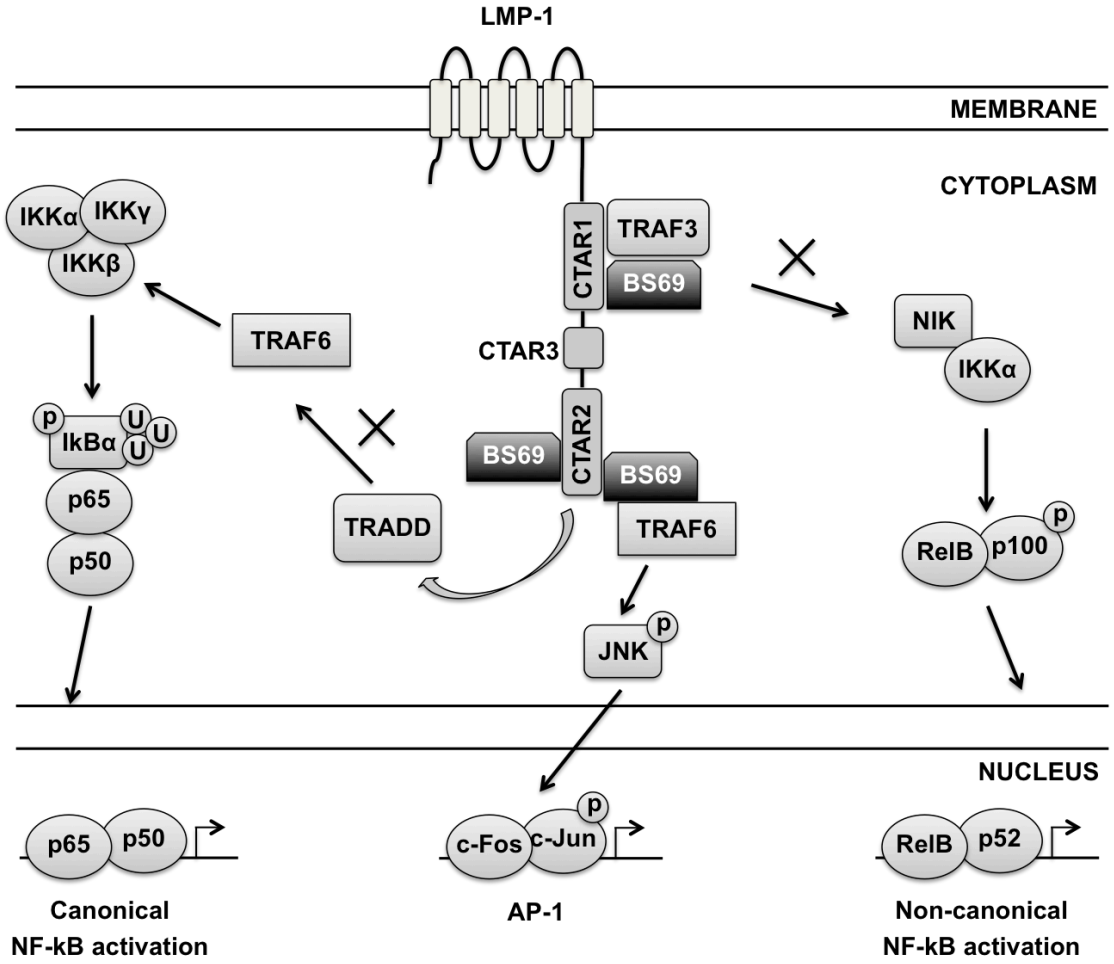


Figure 1.11 BS69/ZMYND11 regulates NF-kB and JNK activation mediated by LMP-1

BS69 protein binds to LMP1/CTAR1 and TRAF3 to negatively regulate non-canonical NF-kB activation and interacts with CTAR2 displacing TRADD from LMP-1. TRADD displacement by BS69 suppresses the canonical NF-kB activation mediated by LMP-1. BS69 functions as an adaptor protein mediating interaction of TRAF6 with LMP1/CTAR2 in the LMP-1-induced JNK activation. Adapted from [400].

The functions described for BS69, such as the capacity to interact with EBNA-2 and repress EBNA-2-mediated gene transactivation, suggest that the BS69 cell protein could also have a specific function in the differential gene regulation by type 1 and type 2 EBNA-2. The location of the important amino acid aspartate 442 of type 1 EBNA-2, shown to be sufficient to confer type 2 EBNA-2 the ability to sustain LCL growth in the EREB2.5 assay also indicated BS69 could indeed play a role in this mechanism. BS69 was shown to recognise the PXLXP motif located immediately before aspartate 442 in the EBNA-2 transactivation domain supporting the previous hypothesis. In this study the transactivation ability of type 1 and type 2 EBNA-2 will be investigated, as a possible mechanism contributing to the differential gene regulation by EBNA-2 types, as well as it will be determined whether BS69 has a specific function in this context. In addition, the mechanism of differential regulation of the viral LMP-1 gene by type 1 and type 2 EBNA-2 will also be addressed.

1.7 Aims of the thesis

The aim of this study is to investigate the mechanism of superior B lymphocyte transformation and LCL growth in response to type 1 Epstein-Barr virus compared to type 2 EBV. The specific objectives investigated successfully were to explore:

1. **Transcriptional activation of target gene promoters mediated by type 1 and type 2 EBNA-2:**

    - Is the transactivation function of the type 1 EBNA-2 transactivation domain (TAD) stronger than the capacity of type 2 EBNA-2 TAD to activate gene expression?
    - Does the serine to aspartate (S442D) mutation in the transactivation domain of type 2 EBNA-2 confer higher transactivation function to type 2 EBNA-2 TAD?

2. **Investigate the function of the cellular protein BS69/ZMYND11 in the regulation of EBNA-2-mediated gene transactivation:**

    - Is the transactivation function of type 1 and type 2 EBNA-2 TAD differentially inhibited or regulated by BS69?
    - Does the BS69 protein have a different affinity for interaction with type 1 or type 2 EBNA-2 proteins?

3. **Differential regulation of the viral LMP-1 gene by type 1 and type 2 EBNA-2:**

    - Is it possible to reconstitute the correct differential LMP-1 gene regulation by type 1 and type 2 EBNA-2 proteins, using reporter assays or small OriP plasmids containing either only the LMP-1 promoter or entire LMP-1 regulatory region?
    - Which EBNA-2-responsive elements (including the PU.1 site) at the LMP-1 are required to recruit the EBNA-2 protein to the LMP-1 promoter?

2 Materials and Methods

2.1 Solutions and chemicals

All chemicals were supplied by BDH (UK), unless otherwise stated.

2.1.1 Solutions used in cell culture protocols

- **1X Phosphate buffered saline (PBS):** 137 mM NaCl, 2.7 mM KCl, 19 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.0**.**
- **0.8% (w/v) ammonium chloride:** 8 g of ammonium chloride (NH_4Cl, Sigma) dissolved in cell culture grade water (Sigma) and made up to 1 litre**.**
- **Cell-freezing solution (10% (v/v) DMSO):** 5 ml DMSO was diluted in 45 ml fetal bovine serum (FBS) (Gibco) and stored at -20ºC.
- **10 mM cadmium chloride:** 0.183 g of cadmium chloride ($CdCl_2$, Sigma) was dissolved in cell culture grade water (Sigma) and made up to 100 ml.

2.1.2 Solutions used in DNA protocols

- **1X Tris-buffered saline (TBS):** 140 mM NaCl, 10 mM Tris-Cl pH 7.5.
- **0.5 M Ethylenediaminetetraacetic acid (EDTA) pH 8.0:** 186.1 g of EDTA was dissolved in 900 ml of dH_2O and pH adjusted with 0.5 M NaOH. The final volume was made up to 1 litre. The solution was autoclaved and stored at room temperature.
- **3 M sodium acetate pH 5.2**: 408.1 g of sodium acetate was dissolved in 900 ml dH_2O. pH was adjusted with acetic acid to 5.2. The solution was made up to 1 l and autoclaved.
- **5 M sodium chloride:** 292.2 g of sodium chloride (NaCl) was dissolved in 1 litre of dH_2O and autoclaved.

- **10X Tris-Borate-EDTA (TBE):** 108 g of Tris base, 55 g of boric acid (Sigma) and 40 mM EDTA pH 8.0 were added to 900 ml of dH_2O. The final volume was made up to 1 litre and the solution was autoclaved. 10X TBE was diluted to 1X TBE in dH_2O.

- **1 M Tris-Cl, pH values between 6.8 and 8.8:** 121.1 g of Tris Base was dissolved in 900 ml of dH_2O and the pH adjusted accordingly using 1 M HCl. The volume of the solution was made up to 1 litre. The solution was autoclaved and stored at room temperature.

- **Luria-Bertani (LB) medium:** 10 g tryptone, 5 g yeast extract and 10 g NaCl dissolved in 900 ml dH_2O and pH adjusted to 7.0 with NaOH. The final volume of the solution made up to 1 litre. The solution was autoclaved and stored at room temperature.

- **Luria-Bertani (LB) agar plates:** 15 g of Bacto-Agar (BD) was dissolved in 1 litre of LB medium and autoclaved before pouring the plates. For antibiotic selection, ampicillin was used at 100 µg/ml and chloramphenicol at 25 µg/ml.

- **Super Optimal broth with Catabolite repression (SOC):** 20 g tryptone, 5 g yeast extract, 2 ml of 5 M NaCl, 2.5 ml of 1 M KCl, 10 ml of 1 M $MgCl_2$, 10 ml of 1 M $MgSO_4$ and 20 ml of 1 M glucose dissolved in 900 ml dH_2O, made up to a final volume of 1 litre and autoclaved.

- **Agarose gels:** 0.8-1.5 g agarose (Invitrogen) according to the size of the DNA fragment to resolve was dissolved in 100 ml 1X TBE by heating up the solution in the microwave. 5 µl of ethidium bromide at 10 mg/ml (Sigma) was added and gel poured.

- **6X agarose gel loading buffer:** 10% (w/v) sucrose, 60 mM EDTA, 10 mM Tris-Cl pH 7.6, 0.03% (w/v) bromophenol blue, 0,03% (w/v) xylene cyanol FF, 60% (v/v) glycerol in dH_2O.

- **Buffer QG (Qiagen):** 5.5 M guanidine thiocyanate (GuSCN), 20 mM Tris-Cl pH 6.6.

- **Buffer PE (Qiagen):** 10 mM Tris-Cl pH 7.5, 80% (v/v) ethanol (C_2H_6O).

- **Buffer EB (Qiagen):** 10 mM Tris-Cl pH 8.5.

- **T4 DNA ligase reaction buffer (NEB**): 50 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$, 1 mM ATP, 10 mM DTT.

- **TA cloning reaction buffer (Invitrogen):** 50 mM Tris-Cl pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 25% (w/v) polyethylene glycol (PEG)-8000.

- **Buffer P1 (Qiagen):** 50 mM Tris-Cl pH 8.0, 10 mM EDTA and 100 µg/ml RNaseA.
- **Buffer P2 (Qiagen):** 200 mM NaOH and 1% (w/v) SDS.
- **Buffer P3 (Qiagen):** 3 M potassium acetate (KCH_3CO_2) pH 5.5.
- **Buffer N3 (Qiagen):** 4.2 M Gu-HCl and 0.9 M potassium acetate (KCH_3CO_2) pH 4.8.
- **Buffer PB (Qiagen):** 5 M Gu-HCl and 30% (v/v) isopropanol.
- **Buffer QBT (Qiagen):** 750 mM NaCl, 50 mM MOPS pH 7.0, 15% (v/v) isopropanol, 0.15% (v/v) Triton X-100.
- **Buffer QC (Qiagen):** 1 M NaCl, 50 mM MOPS pH 7.0 and 15% (v/v) isopropanol.
- **Buffer QN (Qiagen):** 1.6 M NaCl, 50 mM MOPS pH 7.0 and 15% (v/v) isopropanol.
- **TE Buffer:** 10 mM Tris-Cl pH 7.6 or 8.0, 1 mM EDTA pH 8.0 in dH_2O.

2.1.3 Solutions used in RNA protocols

- **10X RQ1 RNase-free DNase buffer (Promega):** 400 mM Tris-Cl pH 8.0, 100 mM $MgSO_4$ and 10 mM $CaCl_2$.

2.1.4 Solutions used in protein protocols

- **Radioimmunoprecipitation assay (RIPA) lysis buffer:** 0.15 M NaCl, 1% (v/v) Nonidet-P40, 0.5% (v/v) deoxycholic acid, 0.1% (w/v) SDS, 50 mM Tris-Cl pH 8.0, 1 mM phenol methyl sulfonyl fluoride (PMSF) (Sigma). The solution was supplemented with a tablet of complete protease inhibitor (CPI) (Roche) was added to 50 ml of RIPA lysis buffer.
- **10% (w/v) SDS:** 100 g of sodium dodecyl sulphate (SDS) was dissolved in 1 litre dH_2O.
- **2X SDS sample buffer:** 120 mM Tris-Cl pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 2% (v/v) β-mercaptoethanol and 0.01% (w/v) bromophenol blue.

- **2X Laemmli buffer (Bio-Rad):** 100 mM Tris-Cl pH 6.8, 4% (w/v) SDS, 200 mM DTT, 0.02% (w/v) bromophenol blue and 20% (v/v) glycerol.
- **1X SDS running buffer for western blot:** 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS dissolved in dH_2O.
- **1X SDS transfer buffer for western blot:** 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, 24% (v/v) ethanol, cooled to 4ºC before use.
- **Coomassie Blue staining solution:** 40% (v/v) methanol, 10% (v/v) acetic acid, 1 g/l Coomassie Brilliant Blue, R-250 (Thermo Scientific), stored at room temperature.
- **Coomassie Blue destaining solution:** 40% (v/v) methanol and 10% (v/v) acetic acid.
- **Gel storage solution:** 5% (v/v) acetic acid.
- **Blocking solution for western blot:** 5% (w/v) skim milk powder (Sigma) in washing buffer for western blot and stored at 4ºC.
- **Washing buffer for western blot:** 0.1% (v/v) Tween-20 (Sigma) in PBS, stored at room temperature.
- **Western blot stripping buffer:** 2% (w/v) SDS, 62.5 mM Tris-Cl pH 6.8 in dH_2O, stored at room temperature. 100 mM β-mercaptoethanol (Sigma) was added prior to use.
- **Buffer A:** 10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM 1,4-dithiothreitol (DTT, Sigma), 1 mM PMSF (Sigma) and 1X complete protease inhibitor cocktail (Roche).
- **Buffer B:** 20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM PMSF, 25% (v/v) glycerol, 1 mM DTT (Sigma) and 1X complete protease inhibitor cocktail (Roche).
- **1 M IPTG:** 2.38 g of isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma) was dissolved in a final volume of 10 ml dH_2O. 1 ml aliquots were stored at -20ºC.
- **Fixative solution:** 40% (v/v) methanol and 10% (v/v) acetic acid.
- **Lysis buffer:** 20 mM Tris-Cl pH 8.0, 150 mM NaCl and 1 mM DTT.
- **Binding buffer:** 20 mM Tris-Cl pH 8.0, 150 mM NaCl, 1 mM DTT and 0.1 mg/ml BSA.

- **2X Binding and Washing Buffer (B&W buffer):** 10 mM Tris-Cl pH 7.5, 2 M NaCl and 1 mM EDTA.
- **D150 buffer:** 10 mM HEPES pH 7.9, 150 mM KCl, 20% (v/v) glycerol, 0.4 mM EDTA, 0.05% (v/v) NP-40, 10 mM β-mercaptoethanol (Sigma), 1 mM PMSF (Sigma) and 1X protease inhibitor cocktail (Roche).

2.2 Cell culture protocols

2.2.1 Cell lines and cell maintenance

The cell lines and respective culture media used in this study are listed in table 2.1.

2.2.1.1 Suspension cell lines

B cells were grown in suspension in RPMI-1640 medium (Gibco) supplemented with 10% (v/v) heat-inactivated (56ºC for 20 min) fetal bovine serum (FBS, Gibco), 200 U/ml penicillin (Gibco) and with 200 U/ml streptomycin (Gibco). For Daudi cells with conditional expression of EBNA-2, cadmium chloride ($CdCl_2$) was added to the medium. Cells were maintained at 37ºC in a humidified incubator with 10% CO_2 and fed 2-3 times a week by 1:3/1:5 dilution with pre-warmed fresh medium.

2.2.1.2 Adherent cell lines

HEK293 cells (human embryonic kidney cells) were cultured as monolayers in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% (v/v) heat-inactivated (56ºC for 20 min) fetal bovine serum (FBS, Gibco), 200 U/ml penicillin (Gibco) and with 200 U/ml streptomycin (Gibco). When confluence was reached, the cells were split by washing with 1X PBS before detaching cells with 0.05% Trypsin-EDTA (Gibco) for 5 min at room temperature. Detached cells were washed in culture medium and seeded at 1:10 dilution using fresh pre-warmed medium in a new cell culture flask.

Cell line	Characteristics	Medium and Selection	Reference
B95-8	BL infected with type 1 B95-8 EBV	RPMI, 10% FBS, p/s	[405]
BJAB	EBV-negative Burkitt's lymphoma (BL) cell line	RPMI, 10% FBS, p/s	[406]
Daudi	BL, EBV-positive, ΔEBNA-2, ΔY1Y2 EBNA-LP	RPMI, 10% FBS, p/s	[407, 408]
Daudi:pHEBo-MT:E2T1	Daudi with MT-controlled type 1 EBNA-2 expression	RPMI, 10% FBS, p/s, hygro	[210]
Daudi:pHEBo-MT:E2T2	Daudi with MT-controlled type 2 EBNA-2 expression	RPMI, 10% FBS, p/s, hygro	[210]
Daudi:pHEBo-MT:E2T2 S442D	Daudi with MT-controlled type 2 S442D EBNA-2 expression	RPMI, 10% FBS, p/s, hygro	[210]
Daudi:puro-OriP-LMP-1p	Daudi with puro-OriP-LMP-1p plasmid	RPMI, 10% FBS, p/s, puro	This study
Daudi:puro-OriP-LMP-1p/pHEBo-MT:E2T1	Daudi with puro-OriP-LMP-1p plasmid and with MT-controlled type 1 EBNA-2 expression	RPMI, 10% FBS, p/s, puro and hygro	This study
Daudi:puro-OriP-LMP-1p/pHEBo-MT:E2T2	Daudi with puro-OriP-LMP-1p plasmid and with MT-controlled type 2 EBNA-2 expression	RPMI, 10% FBS, p/s, puro and hygro	This study
Daudi:pHEBo-LMP-1r	Daudi with pHEBo:Dhet-*Bam*HI-*Eco*RI plasmid	RPMI, 10% FBS, p/s, hygro	This study
Daudi:pHEBo-LMP-1r ΔCTCF	Daudi with pHEBo:Dhet-*Bam*HI-*Eco*RI ΔCTCF plasmid	RPMI, 10% FBS, p/s, hygro	This study
Daudi:pHEBo-LMP-1r ΔTR	Daudi with pHEBo:Dhet-*Bam*HI-*Eco*RI ΔTR plasmid	RPMI, 10% FBS, p/s, hygro	This study
IB4	EBV-positive, lymphoblastoid cell line (LCL)	RPMI, 10% FBS, p/s	[409]
HEK293	EBV-negative, Human Embryonic Kidney	DMEM, 10% FBS, p/s	[410]

Table 2.1 Cell lines used in this study

LCL: lymphoblastoid cell line; BL: Burkitt's lymphoma cell line; RPMI: Roswell Park Memorial Institute 1640 (Gibco); DMEM: Dulbecco's Modified Eagle Medium (Gibco); FBS: fetal bovine serum (Gibco); p/s: 200 U/ml penicillin and 200 U/ml streptomycin (Gibco); Puro: puromycin (concentration 1 µg/ml); Hygro: hygromycin (concentration 300 µg/ml).

2.2.2 Viable cell counts

Cells were stained with trypan blue solution (Sigma) using a 1:2 dilution. The number of live cells was determined by counting those that excluded trypan blue on a haemocytometer slide (Superior Marienfeld, Germany). This procedure was performed by visualising the cells under a CK2 light microscope (Olympus).

2.2.3 Cell freezing

5 x 10^6 cells were pelleted by centrifugation at 335 x *g* for 5 min at 4ºC. Cell pellets were then resuspended in 1 ml of cold 10% (v/v) DMSO in FBS solution and transferred into a cryovial tube (Greiner Bio-One). The cryovials were placed in a Nalgene Cryo 1C freezing container (Thermo Scientific) and then slowly frozen overnight at -80ºC (1ºC *per* min). Cryovials were subsequently transferred into liquid nitrogen for long-term storage.

2.2.4 Cell seeding

Cells stored in a cryovial in liquid nitrogen were thawed rapidly at room temperature and 10 ml of complete medium without selective antibiotics was added to dilute cells and DMSO. DMSO was then removed by centrifugation at 335 x *g* for 5 min at 4ºC. Cell pellet was then resuspended in 10-20 ml of fresh medium without antibiotic selection and transferred into a tissue culture flask. Cells were incubated at 37ºC and when required, antibiotic selection was added after 1-2 days.

2.2.5 Cell harvesting

The number of cells required to carry out downstream experiments was centrifuged at 335 x *g* for 5 min at 4ºC. Cells were washed once or twice using ice-cold 1X PBS. Supernatant was removed by aspiration and the cell pellet was snap-frozen on dry ice and stored at -80ºC.

2.2.6 Cell line transfection

2.2.6.1 Transfection of suspension cell lines using Neon transfection system

Cells to be transfected were diluted 1:3 in fresh culture medium one day before transfection. *Per* each individual transfection, an aliquot of 2 x 10^6 cells was used. Cells were pelleted by centrifugation at 335 x *g* for 5 minutes at 4ºC and washed twice with pre-warmed PBS. Cells were resuspended in 100 µl of Neon resuspension solution R (Invitrogen). Cells suspensions were then mixed with the plasmid DNA (2-12 µg in TE buffer) in microtubes. The mixture was then transferred to a Neon 100 µl transfection pipette tip (Invitrogen). 3 ml Neon electrolytic solution E2 was added to the electroporation chamber and the electroporation pipette tip was then introduced into the electroporation chamber. The Neon transfection protocol 9 was used for electroporation of Daudi and IB4 cells (1400 V of pulse voltage, 30 ms of pulse width and 1 pulse number). The Neon transfection protocol 14 was used to electroporate of BJAB cells (1200 V of pulse voltage, 20 ms of pulse width and 2 pulse number). Cells were transferred into 2 ml of pre-warmed media (RPMI-1640 supplemented with 10% FBS and also 200 U/ml penicillin and with 200 U/ml streptomycin, but no eukaryotic antibiotic selection) in a 6-well plate and incubated at 37ºC. Stable cell lines were generated by adding antibiotic selection after 24 h (300 µg/ml hygromycin and/or 1 µg/ml puromycin) in a final volume of 12 ml. 2 ml aliquots were placed in 6 wells of a 24-well plate and the media with selection was refreshed every 3 days. 3-4 weeks after transfection and when clear cell line outgrowth was observed, cells were transferred to tissue culture flasks and expanded. In transient transfection assays, cells were harvested at 24 h and processed for protein extraction or for luciferase assays.

2.2.6.2 Transfection of adherent cell lines using Lipofectamine™ 2000

Lipofectamine™ 2000 transfections were performed following the manufacturer's instructions (Invitrogen). 5x10^5 HEK293 cells were seeded on each well of a 12-well plate and incubated overnight in 2 ml of DMEM medium supplemented with 10% FBS but without antibiotics. For each transfection, 1600 ng of DNA was combined with 100 µl of OptiMEM I Reduced Serum Medium (Gibco). In a different tube, 4 µl of Lipofectamine™ 2000 (Invitrogen) was mixed with 100 µl of OptiMEM I Reduced Serum Medium and incubated for 5 min. The tube containing the diluted DNA and the tube with Lipofectamine™ 2000 were combined and incubated for additional 20 min at room temperature before adding 200 µl of the mixture to the cells. Cells were incubated at 37ºC for 24 h and harvested and processed for either protein extraction or luciferase assays.

2.3 Nucleic acid protocols

2.3.1 Ethanol precipitation and sterilisation of nucleic acids

1:10 (v/v) of 3 M sodium acetate ($NaCH_3CO_2$) pH 5.2 and 2.5 volumes of 100% ethanol were used to precipitate nucleic acids. Samples were mixed by inversion and incubated at -20ºC for 20 min. Nucleic acids were pelleted by centrifugation at 17,900 x *g* for 10 min. The pellet was washed with 70% ethanol, dried for 5-10 min at room temperature and resuspended in TE buffer.

2.3.2 Nucleic acid quantification

A Nanodrop 1000 spectrophotometer (Fisher BioReagents, UK) was utilised to determine the concentration and purity of nucleic acids by measuring the absorbance of the full spectrum of light (220-750 nm). The concentration was calculated from the absorption of light at the 260 nm wavelength, assuming that an absorbance of 1.0 at this wavelength corresponds to 50 µg/ml for double stranded DNA and 40 µg/ml for RNA. The purity was determined from the A_{260}/A_{280} ratio as proteins absorb light at 280 nm. Pure DNA has a ratio of 1.8 in sterile water and pure RNA has a ratio of approximately 2.0 in 10 mM Tris-Cl pH 7.6.

2.3.3 DNA protocols

2.3.3.1 Extraction of genomic DNA

Genomic DNA of B95-8 cells was extracted using the DNeasy Blood & Tissue Kit (Qiagen). 5 x 10^6 cells were harvested by centrifugation at 300 x *g* for 5 min and resuspended in 200 µl 1X PBS. 20 µl of proteinase K was then added to the resuspended pellet. 200 µl of buffer AL (Qiagen) was added to the tube, mixed thoroughly by vortexing and incubated at 56ºC for 10 min. 200 µl of ethanol was added and mixed again thoroughly by vortexing. The mixture was pipetted into the DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged at 6000 x *g* for 1 min. The DNeasy Mini spin column was placed in a new 2 ml collection tube. 500 µl buffer AW1 (Qiagen) was added and centrifuged at 6000 x *g* for 1 min. The DNeasy Mini spin column was again placed in a new 2 ml collection tube. 500 µl buffer AW2 (Qiagen) was added and the tube centrifuged at 20,000 x *g* for 3 min to dry the DNeasy membrane. The DNeasy column was finally placed in a clean 1.5 ml eppendorf tube. 200 µl buffer AE

(Qiagen) was added directly to the membrane and incubated at room temperature for 1 min. The column was centrifuged for 1 min at 6000 x *g* to elute the DNA.

2.3.3.2 DNA digestion with restriction enzymes

Restriction enzymes supplied by New England Biolabs (NEB) and Promega were utilised to perform DNA restriction digestions. A 10X excess of restriction enzyme was used to digest the required amount of DNA. In the case of double digestions the volume of enzyme in the reaction did not exceed 5% of the total reaction volume. Digestions were incubated for 1-3 h or overnight at the recommended temperature and analysed on an agarose gel of adequate concentration.

2.3.3.3 Removing 5'phosphate groups from DNA fragments

The phosphate group at the 5'-end was removed from the linearised vector before cloning to reduce self-ligation and recircularisation. 1.5 µl (10 U/µl) of calf intestinal phosphatase (CIP) from NEB was added to restriction enzyme digestions and incubated at 37ºC for 45 min.

2.3.3.4 Agarose gel electrophoresis

According to size of the DNA fragments to be separated, 0.8-2% (w/v) agarose (Invitrogen) was dissolved in 1X TBE buffer by heating up in a microwave device. Solution was poured into a gel-casting tray after the addition of 0.5 µg/µl ethidium bromide (Sigma) to allow for UV visualization. DNA samples were mixed with 6X gel loading buffer before loading. Adequate DNA ladders (100 bp or 1 kb) were also loaded on the gel to determine the size of the DNA fragments. Electrophoresis was performed in 1X TBE buffer at 70-100 V to resolve the DNA fragments before visualization in a transilluminator ultraviolet (UV) light source, which run the GeneSnap software (Syngene).

2.3.3.5 DNA extraction from agarose gels

The DNA band corresponding to the fragment of interest was excised from the agarose gel using a scalpel under UV illumination. DNA was extracted from the gel using a QIAquick Gel Extraction kit (Qiagen) according to manufacturer's instructions. The gel slice was dissolved in three volumes of buffer QG for 10 min at 50ºC and the mixture added to a QIAquick spin column and centrifuged at 17,900 x *g* for 1 min to bind the DNA fragments. The column was

washed with 750 µl buffer PE and DNA eluted with 30 µl of buffer EB. The concentration and the purity of the DNA fragment were determined using a Nanodrop 1000 spectrophotometer (Fisher BioReagents) (see section 2.3.2).

2.3.3.6 DNA ligation using T4 DNA ligase

Ligations were carried out using T4 DNA ligase (NEB), using an insert:vector ratio of 3:1. The amount of insert was calculated using the following formula:

ng of insert = 3 x [ng of vector x size of insert (kb) / size of vector (kb)]

Ligations were performed in a final reaction volume of 10 µl using 1 µl of T4 DNA ligase (400 U/µl, NEB) and incubated at 16ºC overnight. A control reaction was carried out only with the vector.

2.3.3.7 TA cloning strategy

The products obtained from PCR amplification were cloned into the pCR2.1 plasmid vector using the TA cloning kit (Invitrogen) according to the manufacturer's instructions. Products were amplified with *Taq* DNA polymerase as described in section 2.3.3.11. Amplification with this enzyme adds a single deoxyadenosine (A) to the 3' ends of PCR products, an activity that is not dependent on the template. The fragment is inserted into a linearised vector (pCR2.1), which has a single 3' deoxythymidine (T) nucleotide overhangs. Ligations were carried out with an insert:vector molar ratio of 3:1. The amount of PCR product required to ligate 50 ng of pCR2.1 vector was determined using the following formula:

ng PCR product = (bp PCR product) x 50 ng pCR2.1 / 3900 bp pCR2.1

Ligations were performed in a final reaction volume of 10 µl with 1 µl of T4 DNA ligase (400 U/µl, NEB) and incubated at 16ºC overnight. A control reaction was carried out only with the vector (section 2.3.3.6). 2 µl of the ligation reaction was used to transform 50 µl of competent *Escherichia coli* XL1 Blue cells (section 2.3.3.9). Positive transformants were grown on LB-agar plates, with appropriate antibiotic selection (ampicillin at 100 µg/ml) and screened by PCR using T7-promoter and M13-reverse oligonucleotide primers (section 2.3.3.11) or by restriction digestion (2.3.3.2). The final PCR products cloned into pCR2.1 were sequenced to validate their identity.

2.3.3.8 Preparing chemically competent bacteria

A single colony of *Escherichia coli* XL1-Blue strain was inoculated in 2 ml of LB broth and incubated overnight at 37ºC with aeration. 1 ml of the culture was diluted into 100 ml of fresh LB broth and incubated at 37º with shaking until the culture reaches an absorbance value of 0.3-0.5 at 600 nm. Bacterial culture was then centrifuged at 2800 x *g* for 15 min at 4ºC using an Allegra X-12 centrifuge (Beckman Coulter) and resuspended in half of the original volume of 50 mM $CaCl_2$ and centrifuged again. The cell pellet was resuspended in 1/10 of the initial volume using 50 mM $CaCl_2$ with 15% (v/v) glycerol. 100 µl aliquots of the cells were prepared and subsequently snap-frozen on dry ice and stored at -80ºC.

2.3.3.9 Transformation of chemically competent bacteria

Previously prepared cells and stored at -80ºC were then thawed on ice. 50 µl of competent bacteria was mixed with 1-5 µl of DNA or of ligation reaction and incubated on ice for 30 min. The mixture was then heat-shocked at 42ºC for 30 sec and incubated again on ice for 5 min. 450 µl of pre-warmed LB medium was added to each transformation and incubated at 37ºC with aeration for 1 h. Bacteria were finally plated onto LB-agar plates with adequate antibiotic selection and incubated overnight at 37ºC.

2.3.3.10 Purification of plasmid DNA from bacterial cultures

2.3.3.10.1 Small-scale preparation of plasmid DNA from bacterial cultures

QIAprep Spin Miniprep Kit (Qiagen) was utilised to prepare plasmid DNA from bacteria on a small scale. Single colonies were picked from LB-agar plates and inoculated into 2 ml of LB broth supplemented with the appropriate antibiotic selection. The cultures were incubated at 37ºC overnight with aeration. 1.5 ml of culture was pelleted by centrifugation (2700 x *g* for 3 min at room temperature). The cell pellets were resuspended in 250 µl of buffer P1 (50 mM Tris-Cl pH 8.0, 10 mM EDTA and 100 µg/mL RNase A) and cells were then lysed by adding 250 µl of buffer P2 (200 mM NaOH with 1% (w/v) SDS) and incubated for 5 min at room temperature. The lysis reaction was neutralised and DNA precipitated following the addition of 350 µl of buffer N3 (4.2 M Gu-HCl and 0.9 M potassium acetate (KCH_3CO_2) pH 4.8). The samples were then centrifuged for 10 min at 17,900 x *g* and the supernatant was applied to a QIAprep spin column. The column was centrifuged at 17,900 x *g* for 1 min, washed with 500 µl of buffer PB (5 M Gu-HCl and 30% (v/v) isopropanol) and then 750 µl of buffer PE (10 mM

Tris-Cl pH 7.5, 80% (v/v) ethanol). The DNA was finally eluted with 35 µl of buffer EB (10 mM Tris-Cl, pH 8.5).

2.3.3.10.2 Large-scale preparation of plasmid DNA from bacterial cultures

EndoFree Plasmid Maxi Kit (Qiagen) was utilised to prepare plasmid DNA from bacteria on a large scale. 250 ml of LB broth with appropriate antibiotic selection was inoculated with 250 µl of overnight culture and incubated at 37ºC for 16 hours. Bacterial culture was then pelleted by centrifugation at 2800 x *g* for 15 min at 4ºC. Cell pellets were resuspended in 10 ml buffer P1 (50 mM Tris-Cl pH 8.0, 10 mM EDTA and 100 µg/ml RNase A) and then lysed for 5 min following the addition of 10 ml buffer P2 (200 mM NaOH with 1% (w/v) SDS). 10 ml ice-cold buffer P3 (3 M potassium acetate (KCH_3CO_2) pH 5.5) was added for neutralisation and to precipitate DNA. The mixture was applied to QIA filter cartridges and incubated for 10 min at room temperature. The filtered lysate was incubated on ice for 30 min after addition of 2.5 ml of buffer ER and poured into a Qiagen-tip 500 column previously equilibrated with 10 ml of buffer QBT (750 mM NaCl, 50 mM MOPS pH 7.0, 15% (v/v) isopropanol, 0.15% (v/v) Triton X-100). The DNA bound on the column was washed twice with 30 ml of buffer QC (1 M NaCl, 50 mM MOPS pH 7.0 and 15% (v/v) isopropanol) and eluted with 15 ml of buffer QN (1.6 M NaCl, 50 mM MOPS pH 7.0 and 15% (v/v) isopropanol). 10.5 ml of isopropanol was added to precipitate the DNA and centrifuged at 2800 x *g*, for 1 h at 4ºC. The DNA pellet was washed with 5 ml of 70% (v/v) ethanol and another identical centrifugation step was carried out. The DNA pellet was air-dried and dissolved in sterile 200-400 µl TE buffer (10 mM Tris-Cl pH 7.6 or 8.0, 1 mM EDTA pH 8.0).

2.3.3.11 Amplification of DNA by polymerase chain reaction (PCR)

Amplification of DNA template was performed by the polymerase chain reaction (PCR) using *Taq* polymerase enzyme (Promega) on the GeneAmp System 9700 (Applied Biosystems):

Reagent	Amount/Volume
5X PCR Green GoTaq Flexi Buffer	10 µl
25 mM $MgCl_2$	4 µl (2.0 mM)
10 mM dNTP mix	1 µl (0.2 mM)
10 µM forward primer	1 µl (0.2 µM)
10 µM reverse primer	1 µl (0.2 µM)
GoTaq DNA polymerase (5 U/µl)	0.25 µl (1.25 U)
DNA template	5 ng plasmid, 2 µg genomic DNA, 2 µl cDNA
Sterile H_2O	made up to a final volume of 50 µl

The cycling parameters for *Taq* polymerase enzyme PCR were adjusted according to the size of the fragment to be amplified and the purpose of the experiment.

Segment	Temperature	Time	Cycles
Initial denaturation	95ºC	3 min	1
Denaturation	95ºC	30 sec	20-35
Annealing	55-60ºC	30 sec	
Extension	72ºC	1 min/kb	
Final Extension	72ºC	5 min	1

2.3.3.12 Site-directed mutagenesis (SDM)

The QuikChange II Site-Directed Mutagenesis kit (Agilent Technologies) was utilised to insert specific point mutations in small plasmids (S2513D substitution in pMAT-OPA-PEST). The complementary oligonucleotide primers containing the desired mutation were designed using the manufacturer's instructions (Agilent Technologies). The primers were 30 bases long and had a melting temperature (T_M) of ≥78ºC, determined using the formula below:

$$\mathbf{T_M = 81.5 + 0.41 \times (\%GC) - 675/n - (\%\ mismatch)}$$

The reaction was performed on the GeneAmp System 9700 (Applied Biosystems) and set up as follows:

Reagent	Amount/Volume
10X reaction buffer	5 µl (1X)
10 mM dNTP mix	1 µl (0.2 mM)
100 ng/µl forward primer	1.25 µl (125 ng)
100 ng/µl reverse primer	1.25 µl (125 ng)
50 ng/µl DNA template	1 µl (50 ng)
PfuUltra HF DNA polymerase (2.5 U/µl)	1 µl (2.5 U)
Sterile H_2O	made up to a final volume of 50 µl

The cycling parameters for *PfuUltra* polymerase enzyme PCR were the following:

Segment	Temperature	Time	Cycles
Initial denaturation	95ºC	30 sec	1
Denaturation	95ºC	30 sec	12
Annealing	55ºC	1 min	
Extension	68ºC	1 min/kb	

Following cycling reaction, the tubes were then placed on ice for 2 min to cool the reaction to ≤37ºC. 1 µl of *Dpn*I restriction enzyme 10 (U/µl) (Agilent Technologies) was added directly to the amplification reaction to digest the parental methylated DNA and incubated for at least 1 h at 37ºC. 1 µl of *Dpn*I-digested DNA was transformed into 50 µl of supercompetent *E. coli* XL1-Blue cells (Agilent Technologies) (see section 2.3.3.9). Positive transformants were then screened by DNA sequencing to confirm that the mutation was successfully introduced.

2.3.3.13 Plasmid vectors

All plasmid vectors used in this study are listed in table 2.2.

2.3.3.13.1 pFR-Luc and pRL-CMV plasmids for luciferase assays

The pFR-Luc reporter plasmid (Agilent Technologies) was used in the transient transfection assays performed in the present study (sections 3.2 and 4.2). Activation of luciferase gene expression by the GAL4 constructs was determined. The pRL-CMV *Renilla* vector (Promega) was also used to carry out the normalisation of the transfection efficiency, according to the expression of *Renilla* luciferase.

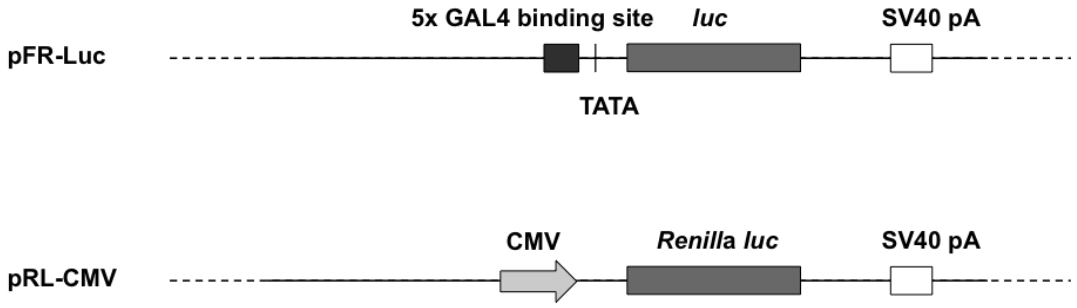


Figure 2.1 pFR-Luc and pRL-CMV plasmids for luciferase reporter assays

CMV: CMV promoter; *luc*: luciferase gene.

2.3.3.13.2 pGL3 Basic vectors containing the LMP-1 promoter

The pGL3b-B95-8-LRS (-634/+40) plasmid vector includes the LMP-1 regulatory sequence (LRS) from the B95-8 EBV genome, cloned upstream of a luciferase gene (*luc*) in the pGL3b Basic vector (Promega). This plasmid construct was kindly provided by Dr. Lars Rymo from Gothenburg University [411]. The LRS comprises nucleotides 169,019 to 169,692 of the B95-8 EBV sequence (Genbank accession number AJ507799), defining the positions -634 to +40 to the transcription initiation site (LRS -634/+40). These vectors were used in the luciferase reporter assays performed in this study (see section 2.6).

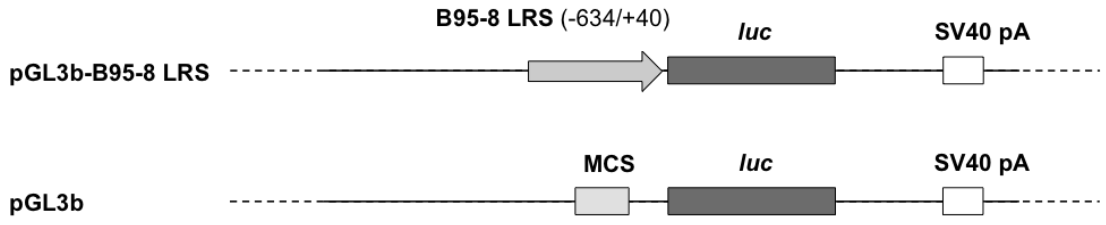


Figure 2.2 pGL3b plasmid containing the LMP-1 promoter region (-634/+40)

MCS: Multiple cloning site; LRS: LMP-1 regulatory sequence; *luc*: luciferase gene

2.3.3.13.3 pcDNA3.1-GAL4-DBD:E2-TAD vectors

The pcDNA3.1-GAL4-DBD:E2-TAD plasmids were generated by cloning the transactivation domain (TAD) of EBNA-2 (amino acids 426-463) into pcDNA3.1-GAL4-DBD vector fused to the GAL4 DNA-binding domain (DBD). pBlueScript plasmids containing the coding sequence of the EBNA-2 proteins and respective mutants were used as template for PCR amplification previous to cloning into pcDNA3.1-GAL4-DBD. The pcDNA3.1-GAL4-DBD:E2-TAD plasmids were used in luciferase assays (section 3.2.1 and section 3.2.2).

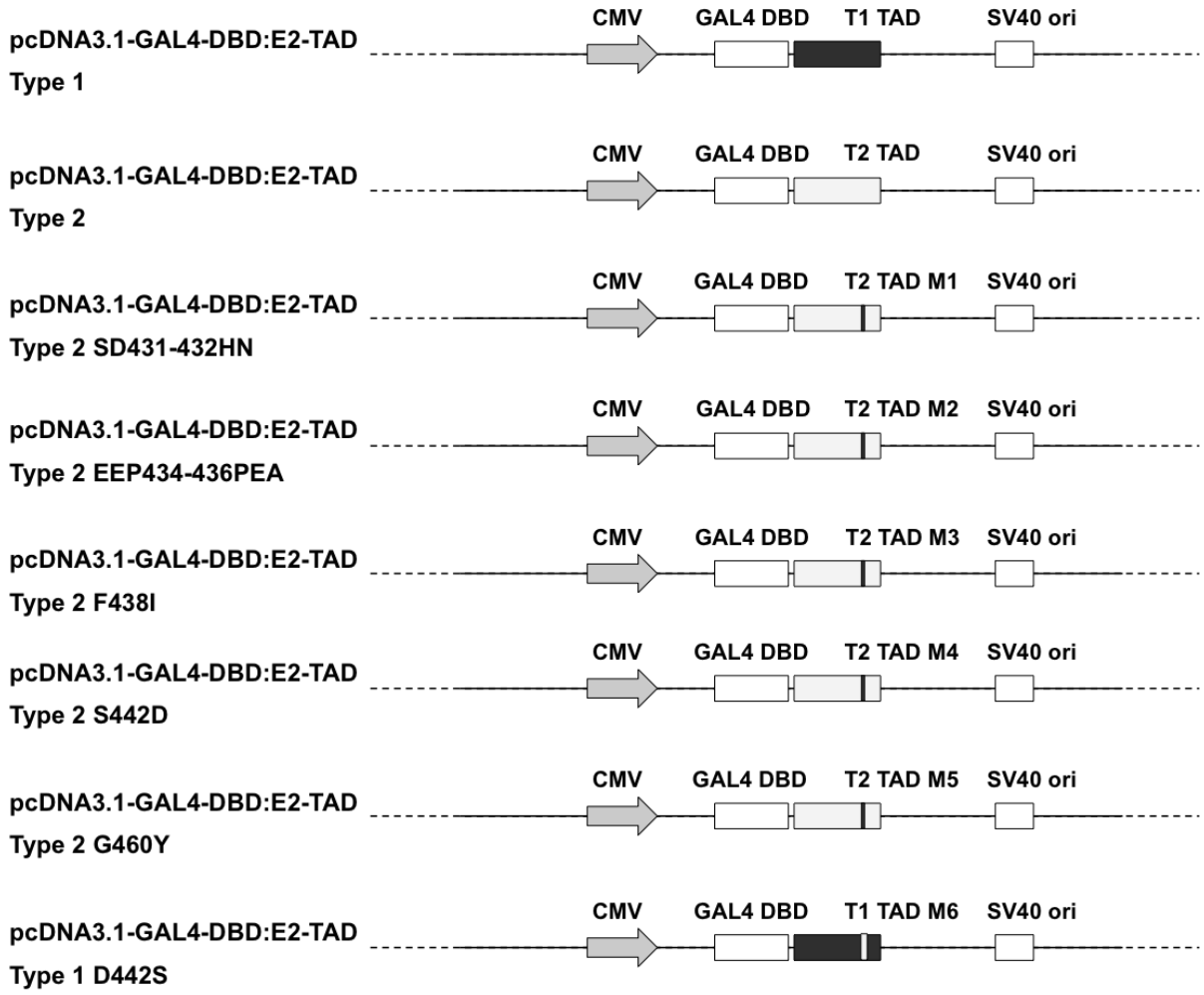


Figure 2.3 pcDNA3.1-GAL4-DBD:E2-TAD plasmid constructs

GAL4-DBD: GAL4-DNA-binding domain; T1: type 1; T2: type 2; TAD: transactivation domain; CMV promoter; M: mutant.

2.3.3.13.4 pcDNA3.1-GAL4-DBD:EBNA-2 vectors

The pcDNA3.1-GAL4-DBD:EBNA-2 plasmids were generated by cloning the EBNA-2 region corresponding to amino acids 334 to 487 into pcDNA3.1-GAL4-DBD vector fused to the DBD of GAL4. pBlueScript plasmids carrying the coding sequence of EBNA-2 (type 1, type 2 and type 2 S442D) were used as template for PCR amplification. pcDNA3.1-GAL4-DBD:EBNA-2 plasmids were used in luciferase assays (section 4.2.1).

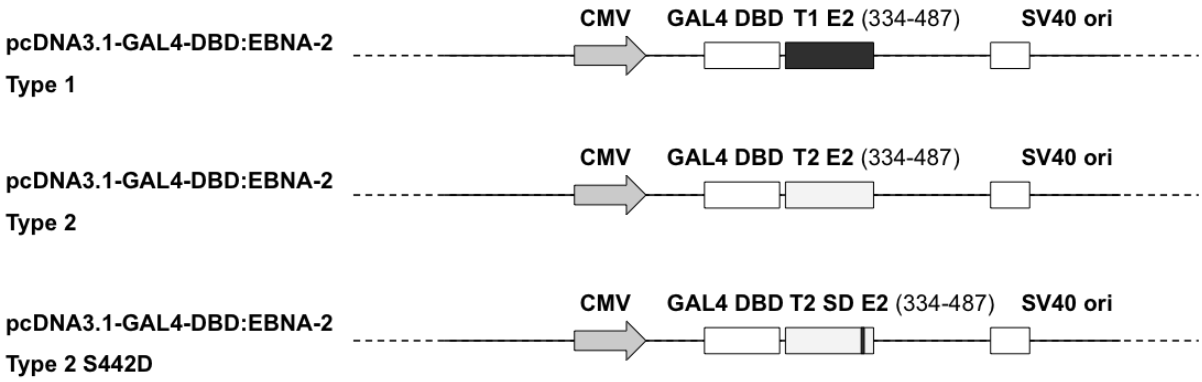


Figure 2.4 pcDNA3.1-GAL4-DBD:EBNA-2 vectors

GAL4-DBD: GAL4-DNA-binding domain; T1: type 1; T2: type 2; T2 SD; type 2 S442D; CMV promoter; M: mutant; E2: EBNA-2.

2.3.3.13.5 pHEBo-OriP plasmids for EBNA-2 expression

The following pHEBo-OriP plasmids were already available in the laboratory [210]. These are expression vectors, in which EBNA-2 protein expression is under control of a metallothionein (MT) promoter. This is a $CdCl_2$-inducible promoter. These vectors also carry the hygromycin and ampicillin resistance genes.

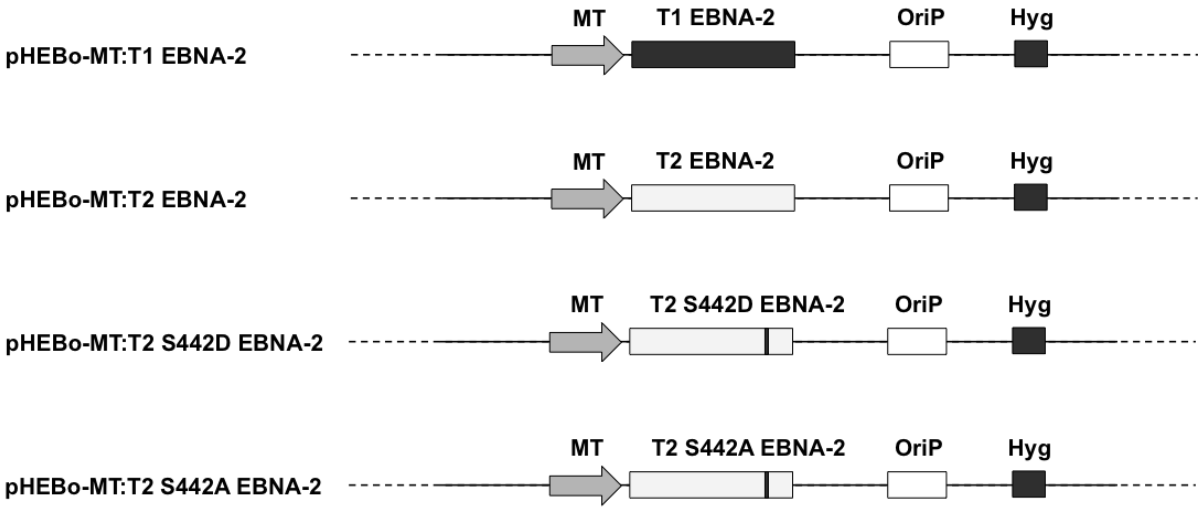


Figure 2.5 pHEBo-OriP plasmids for EBNA-2 expression

MT: $CdCl_2$-inducible metallothionein promoter; OriP: EBV origin of latent replication; Hyg: hygromycin; T1: type 1; T2: type 2.

2.3.3.13.6 pBlueScript plasmids for EBNA-2 *in vitro* translation

The pBlueScript plasmids required for *in vitro* translation of EBNA-2 proteins were available in the laboratory. These plasmids expressed type 1, type 2 and type S442D EBNA-2 proteins under control of the T7 promoter. A pBlueScript vector expressing chimera-2 EBNA-2 protein (a type 2 protein carrying the C-terminal end of type 1 EBNA-2) was also used in this study.

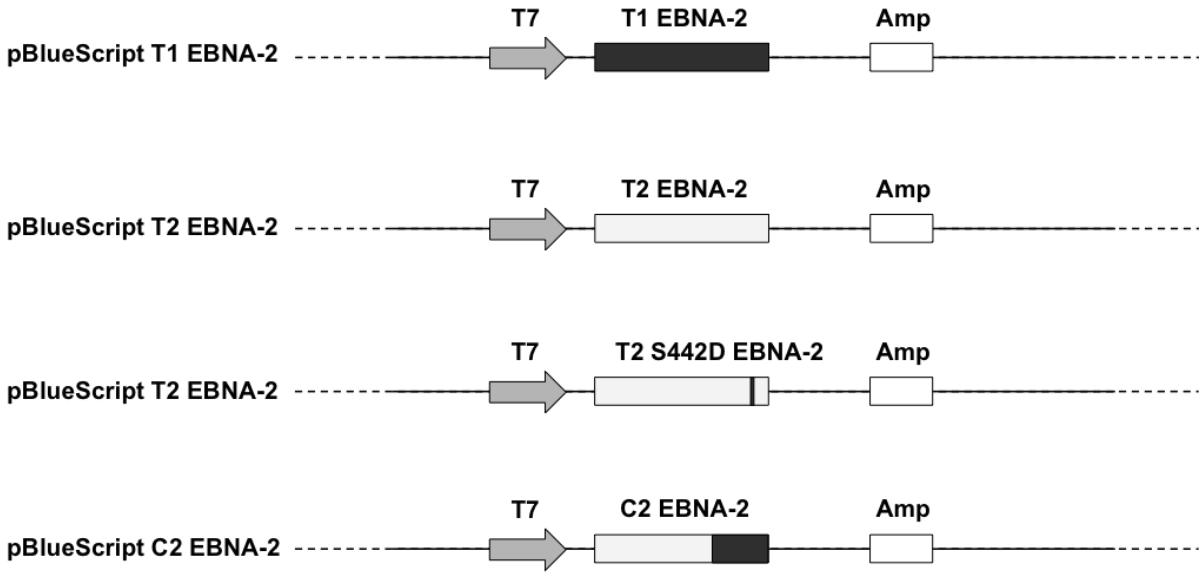


Figure 2.6 pBlueScript plasmids for *in vitro* translation of EBNA-2 proteins

T7: T7 promoter; T1: type 1; T2: type 2; C2: Chimera-2; Amp: ampicillin.

2.3.3.13.7 pcDNA3.1-GAL4-DBD:NOTCH plasmids

The pcDNA3.1-GAL4-DBD:NOTCH plasmids were generated as described in section 3.2.3. The corresponding Notch fragments were cloned into pcDNA3.1-GAL4-DBD vector fused to the GAL4 DNA-binding domain (DBD) and used in further luciferase reporter assays.

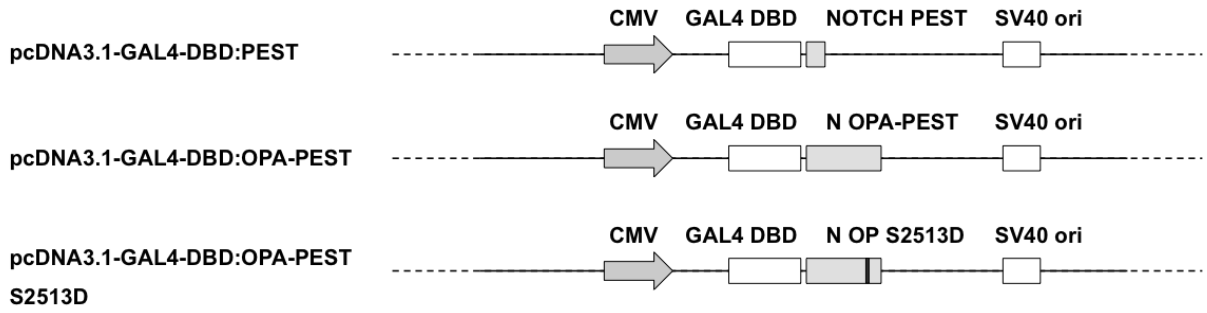


Figure 2.7 pcDNA3.1-GAL4-DBD:NOTCH plasmids

N: Notch; CMV promoter; DBD: DNA-binding domain; OP: OPA-PEST domains

2.3.3.13.8 puro-OriP-LMP-1p (-241/+12) plasmid

The puro-OriP-LMP-1p (-241/+12) plasmid was generated as described in section 5.2.1.2.1.

Figure 2.8 puro-OriP-LMP-1p (-241/+12) plasmid

Puro: puromycin resistance gene; LMP-1p: LMP-1 promoter position -241 to +12 relative to the transcription initiation site; *luc*: luciferase gene; OriP: EBV origin of latent replication.

2.3.3.13.9 pCI-neo-BS69 vectors and pGEX4T1-BS69 plasmid

The pCI-neo-BS69 and pCI-neo-BS69 ΔMYND plasmids were used for expression of BS69 cell protein and of BS69 without the MYND domain, respectively. These expression plasmids were described in a previous report [386]. pGEX4T1-BS69 was used to expression the fusion protein GST-BS69 (amino acids 411-561) and had been previously described in a previous work [267]. These plasmids were kindly provided by Dr. Stéphane Ansieau.

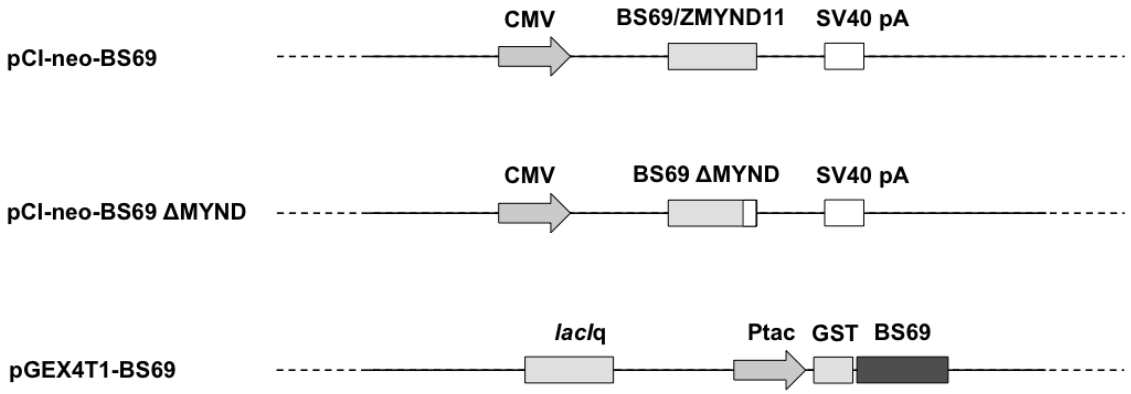


Figure 2.9 pCI-neo-BS69 vectors and pGEX4T1-BS69 plasmid

GST: Glutathione-S-transferase; Ptac-inducible promoter; CMV promoter

Plasmid	Relevant genotype or property	Source
pCR2.1	TA vector for subcloning of PCR products, amp	Invitrogen™
pBlueScript II	cloning vector, amp	Lab collection
pBS-E2T1	pBlueScript II derivative, type 1 EBNA-2 gene, amp	Lab collection
pBS-E2T2	type2 EBNA-2 gene	Lab collection
pBS-E2SD	type 2 EBNA-2 gene with S442D mutation (mut.)	Lab collection
pBS-E2DS	type 1 EBNA-2 gene with D442S mut.	Lab collection
pBS-E2M1	type 2 EBNA-2 gene with SD431-432HN mut.	Lab collection
pBS-E2M2	type 2 EBNA-2 gene with EEP434-436PEA mut.	Lab collection
pBS-E2M3	type 2 EBNA-2 gene with F438I mut.	Lab collection
pBS-E2M4	type 2 EBNA-2 gene with S442D mut.	Lab collection
pBS-E2M5	type 2 EBNA-2 gene with G460Y mut.	Lab collection
pBS-E2C2	chimeric type 2 EBNA-2 with c-terminal end of type 1	Lab collection
pcDNA3.1-GAL4-DBD	pcDNA3.1 der., CMVp-GAL4-DNA-binding domain	Lab collection
pcDNA3.1-GAL4-DBD:E2-TAD-T1	pcDNA3.1-GAL4-DBD derivative, GAL4-DBD fused to type 1 EBNA-2 transactivation domain (TAD)	This study
pcDNA3.1-GAL4-DBD:E2-TAD-T2	pcDNA3.1-GAL4-DBD derivative, GAL4-DBD fused to type 2 EBNA-2 TAD	This study
pcDNA3.1-GAL4-DBD:E2-TAD-SD	pcDNA3.1-GAL4-DBD derivative, GAL4-DBD fused to type 2 S442D EBNA-2 TAD	This study
pcDNA3.1-GAL4-DBD:E2-TAD-SD	pcDNA3.1-GAL4-DBD derivative, GAL4-DBD fused to type 1 D442S EBNA-2 TAD	This study
pcDNA3.1-GAL4-DBD:E2-TAD-M1	pcDNA3.1-GAL4-DBD derivative, GAL4-DBD fused to type 2 SD431-432HN EBNA-2 TAD	This study
pcDNA3.1-GAL4-DBD:E2-TAD-M2	pcDNA3.1-GAL4-DBD derivative, GAL4-DBD fused to type 2 EEP434-436PEA EBNA-2 TAD	This study
pcDNA3.1-GAL4-DBD:E2-TAD-M3	pcDNA3.1-GAL4-DBD derivative, GAL4-DBD fused to type 2 F438I EBNA-2 TAD	This study
pcDNA3.1-GAL4-DBD:E2-TAD-M4	pcDNA3.1-GAL4-DBD derivative, GAL4-DBD fused to type 2 S442D EBNA-2 TAD	This study
pcDNA3.1-GAL4-DBD:E2-TAD-M5	pcDNA3.1-GAL4-DBD derivative, GAL4-DBD fused to type 2 G460Y EBNA-2 TAD	This study
pFR-Luc	luciferase gene downstream of 5x GAL4-binding sites	Agilent™
pRL-CMV	CMV promoter-*Renilla* luciferase gene	Promega™
pcDNA3.1-GAL4-DBD:PEST	pcDNA3.1-GAL4-DBD derivative, GAL4-DBD fused to Notch1-PEST domain (aa 2401-2555)	This study

pcDNA3.1-GAL4-DBD:OPA-PEST	pcDNA3.1-GAL4-DBD derivative, GAL4-DBD fused to Notch1-OPA-PEST domains (aa 2270-2555)	This study
pcDNA3.1-GAL4-DBD:OPA-PEST SD	pcDNA3.1-GAL4-DBD derivative, GAL4-DBD fused to Notch1-OPA-PEST domains w/ S2513D mutation	This study
pHEBo	cloning vector, OriP, Hygromycin	Lab collection
pHEBo-MT:E2T1	type 1 EBNA-2 expression from MT, OriP, Hygro	Lab collection
pHEBo-MT:E2T2	type 2 EBNA-2 expression from MT, OriP, Hygro	Lab collection
pHEBo-MT:E2T2 S442D	type 2 S442D EBNA-2 expression from MT promoter, OriP, Hygro	Lab collection
pHEBo-MT:E2T2 S442A	type 2 S442A EBNA-2 expression from MT promoter, OriP, Hygro	Lab collection
pCI-neo-BS69	BS69 expression from CMV promoter, Neo, amp	[386]
pCI-neo-BS69 ΔMYND	BS69 ΔMYND expression from CMV promoter	[386]
pGEX4T1-BS69	expression of GST-BS69 (aa 411-561) fusion protein	[267]
pcDNA3.1-GAL4-DBD:EBNA-2-T1	pcDNA3.1-GAL4-DBD derivative, GAL4-DBD fused to type 1 EBNA-2 (amino acids 334-487)	This study
pcDNA3.1-GAL4-DBD:EBNA-2-T2	pcDNA3.1-GAL4-DBD derivative, GAL4-DBD fused to type 2 EBNA-2 (amino acids 334-487)	This study
pcDNA3.1-GAL4-DBD:EBNA-2-SD	pcDNA3.1-GAL4-DBD derivative, GAL4-DBD fused to type 2 S442D EBNA-2 (amino acids 334-487)	This study
pGL3b	luciferase reporter vector, amp	Promega™
pGL3b-B95-8-LRS	pGL3b der., LMP-1 regulatory sequence (-634/+40) from B95-8 EBV fused to luciferase gene	Lab collection
puro-OriP-LMP-1p (-241/+12)	LMP-1 regulatory sequence (-241/+12) from B95-8 EBV fused to luciferase gene, OriP, Puromycin	This study
pHC79:Dhet-*Eco*RI	pHC79 der. carrying the Dhet-*Eco*RI DNA fragment from B95-8 EBV genome (nt 159,395-171,823)	Lab collection
pBlueScript:Dhet-*Eco*RI	pBSII der. carrying the Dhet-*Eco*RI DNA fragment from B95-8 EBV genome (nt 159,395-171,823)	This study
pHEBo:Dhet-*Bam*HI-*Eco*RI	pHEBo der. with the Dhet-*Bam*HI-*Eco*RI fragment from B95-8 EBV genome (nt 166,157-171,823)	This study
pHEBo:Dhet-*Bam*HI-*Eco*RI ΔCTCF	pHEBo:Dhet-BamHI-EcoRI derivative with CTCF deletion (deletion of nt 166,515-166,536)	Lab collection
pHEBo:Dhet-*Bam*HI-*Eco*RI ΔTR	pHEBo:Dhet-BamHI-EcoRI derivative with deletion of the terminal repeats (deletion of nt 169,608-171,802)	Lab collection

Table 2.2 Plasmids used in this study

pBS: pBlueScriptII; mut.: mutation; der.: derivative; TAD: transactivation domain; nt: nucleotide; amp: ampicillin, Hygro: hygromycin; OriP: EBV origin of latent replication; Neo: neomycin, Puro: puromycin; MT: metallothionein.

2.3.3.14 Cloning strategies

2.3.3.14.1 Cloning EBNA-2 fragments into pcDNA3.1-GAL4-DBD vector

pBlueScript plasmids carrying EBNA-2 sequences were used as template to PCR amplify the EBNA-2 region to be cloned (amino acids 426-463 or amino acids 334-487) into pcDNA3.1-GAL4-DBD vector fused to the GAL4-DNA-binding domain. The oligonucleotide primers that were used to amplify the desired region carried either *Bam*HI or *Not*I restriction sites at their 5' ends to facilitate further process of cloning into pcDNA3.1-GAL4-DBD vector. The PCR products were first subcloned into pCR2.1 vector using TA cloning strategy (section 2.3.3.7). The pCR2.1 vector carrying the cloned PCR product was then digested with *Bam*HI and *Not*I restriction enzymes and the resultant EBNA-2 fragments were cloned into pcDNA3.1-GAL4-DBD plasmid cut with *Bam*HI and *Not*I. This process of cloning and the screening of positive transformants is extensively described in sections 3.2.1.1, 3.2.2.1 and 4.2.1.2 (Figure 2.10).

2.3.3.14.2 Cloning the LMP-1 promoter region (-241/+12) into puro-OriP vector

The DNA fragment corresponding to the LMP-1 promoter -241/+12 sequence relative to the transcription initiation site was PCR amplified from B95-8 EBV genomic DNA using primers carrying *Xho*I and *Hind*III restriction sites at their respective 5' ends. The resultant fragment was then subcloned into pCR2.1 vector using TA cloning strategy (section 2.3.3.7). pCR2.1 plasmid carrying the cloned PCR product corresponding to the LMP-1 promoter region was then digested with *Xho*I and *Hind*III restriction enzymes and cloned into the final puro-OriP vector cut with *Xho*I and *Hind*III to generate the final puro-OriP-LMP-1p (-241/+12) plasmid. This process of cloning and the diagnostic restriction digestion used to screen the positive transformants is extensively described in section 5.2.1.2.1 (Figure 2.11).

2.3.3.14.3 Cloning the whole LMP-1 regulatory region into pHEBo-OriP plasmid

The whole LMP-1 regulatory region of B95-8 EBV strain (Figure 5.4) was cloned into pHEBo-OriP plasmid. To generate this final construct a two-step cloning procedure was used (Figure 2.12). The pHC79 plasmid vector carrying the Dhet-*Eco*RI DNA fragment of the B95-8 EBV strain genome (nucleotides 159,395-171,823; plus nucleotides 1 and 2, Genbank accession number AJ507799) was digested with *Eco*RI restriction enzyme. The resultant fragment was cloned into pBlueScript vector cut with *Eco*RI, close to a *Bam*HI site present in this plasmid. The new pBlueScript:Dhet-*Eco*RI construct was then digested with *Bam*HI, using the *Bam*HI restriction site carried by the pBlueScript vector and the resultant fragment was finally cloned into pHEBo-OriP previously cut with *Bam*HI. Diagnostic restriction digestions were performed to confirm the positive transformants (Figure 5.5 A and B). The procedure is described in detail in section 5.2.2.1.

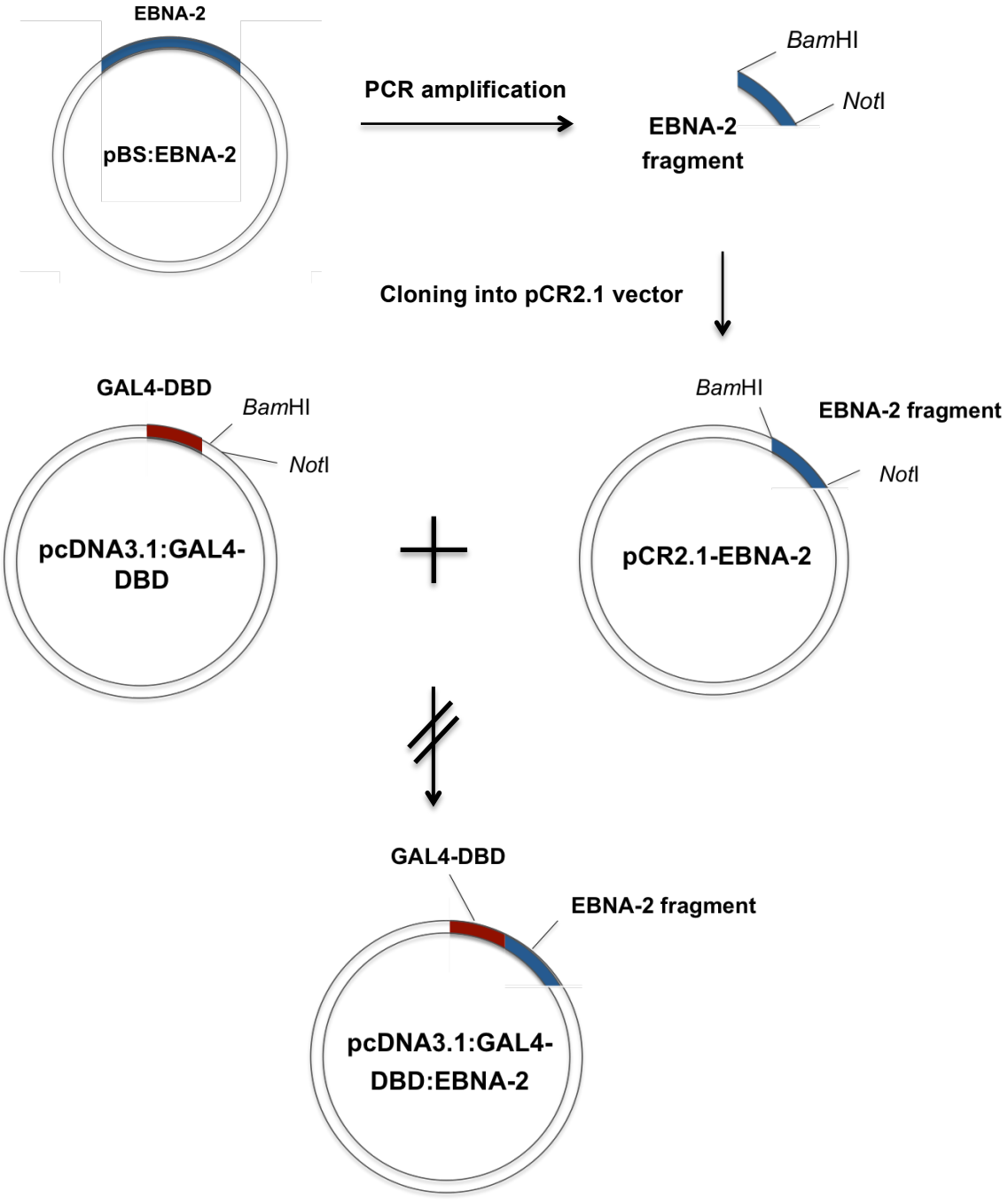


Figure 2.10 Cloning EBNA-2 fragments into pcDNA3.1-GAL4-DBD vector

EBNA-2 fragments (encoding amino acids 426-463 or amino acids 334-487) were PCR amplified using primers carrying *Bam*HI and *Not*I restriction sites at their 5' ends and using as template pBlueScript plasmids containing the EBNA-2 alleles. The PCR products were initially subcloned into pCR2.1 plasmid, which was then digested with *Bam*HI and *Not*I restriction enzymes. The resultant fragment was cloned into the final pcDNA3.1-GAL4-DBD vector also cut with *Bam*HI and *Not*I (fused to the GAL4 DNA-binding domain).

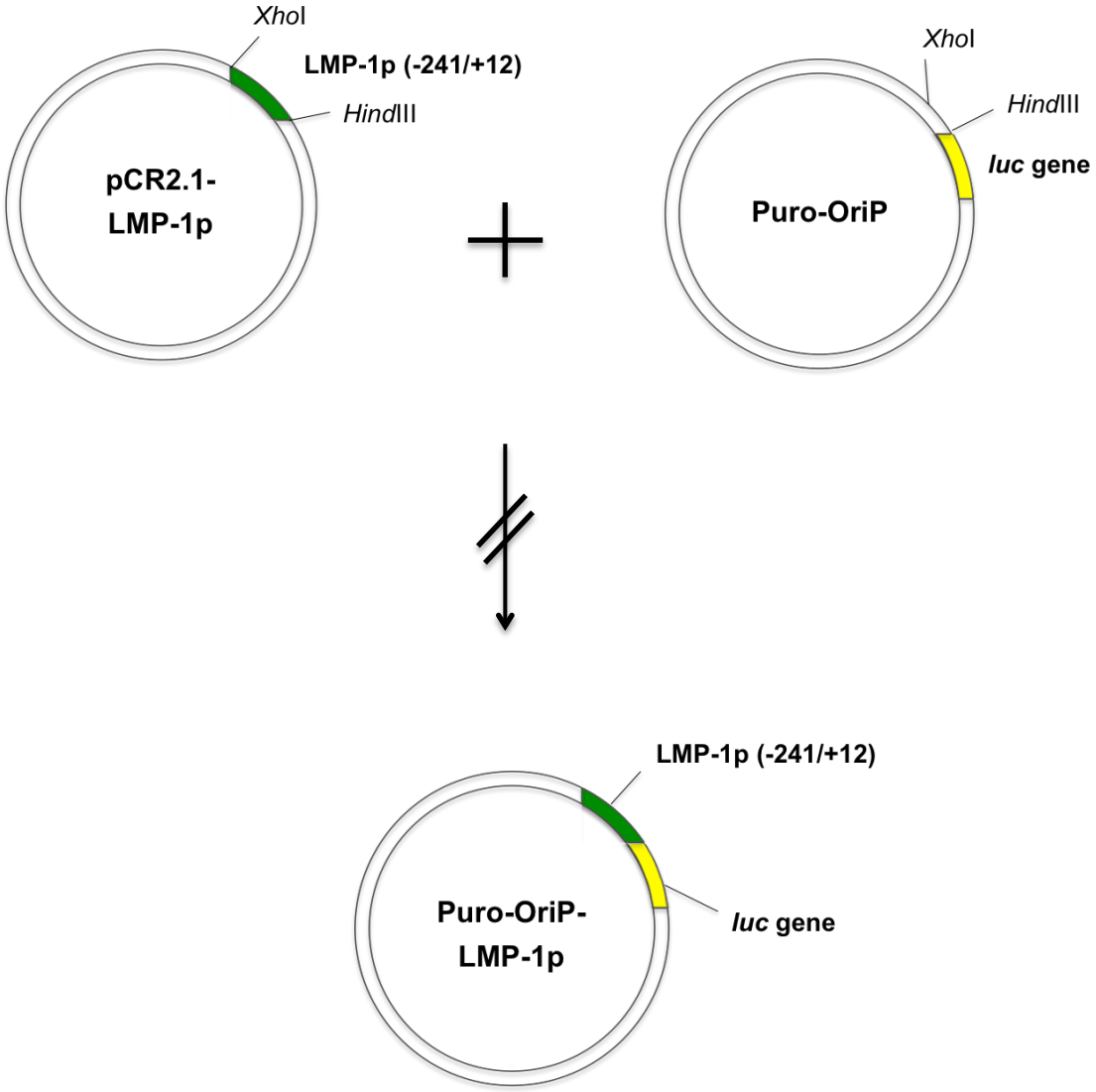


Figure 2.11 Cloning the LMP-1 promoter region (-241/+12) into puro-OriP vector

The LMP-1 promoter sequence that corresponds to the region -241/+12 relative to the transcription initiation site was PCR amplified from B95-8 EBV genomic DNA using oligonucleotide primers carrying *Xho*I and *Hind*III restriction sites at their 5' ends. The PCR product was then subcloned into pCR2.1 vector and finally extracted from this vector by digestion with *Xho*I and *Hind*III and cloned into puro-OriP plasmid fused to a luciferase gene to generate the final construct puro-OriP-LMP-1p (-241/+12).

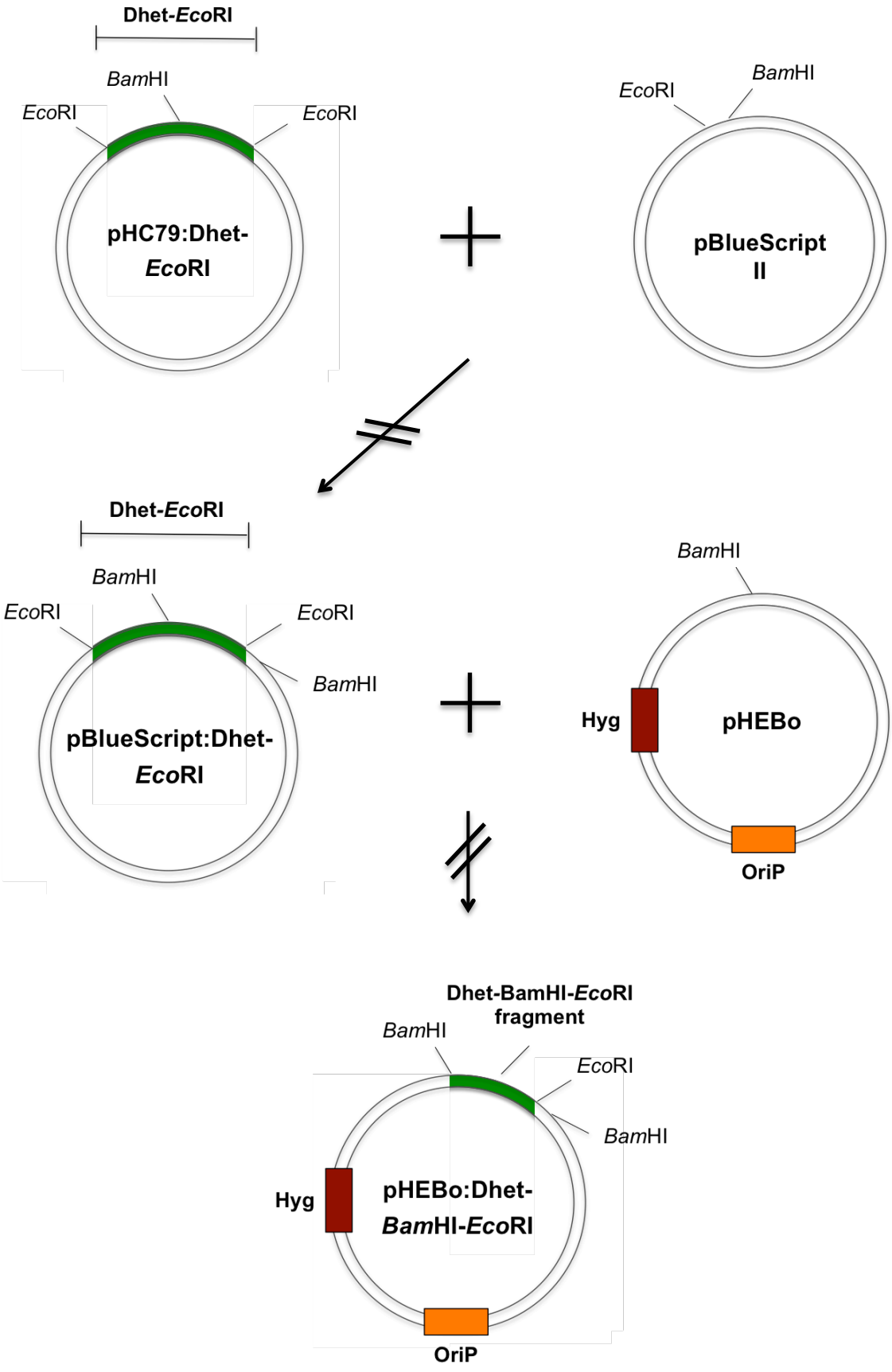

Dhet-EcoRI
BamHI
EcoRI
EcoRI
pHC79:Dhet-EcoRI
EcoRI
BamHI
pBlueScript II
Dhet-EcoRI
BamHI
EcoRI
EcoRI
BamHI
pBlueScript:Dhet-EcoRI
BamHI
Hyg
pHEBo
OriP
Dhet-BamHI-EcoRI fragment
BamHI
EcoRI
BamHI
Hyg
pHEBo:Dhet-BamHI-EcoRI
OriP

Figure 2.12 Cloning the whole LMP-1 regulatory region into pHEBo-OriP vector

Cloning the entire LMP-1 regulatory region into pHEBo-OriP vector required a two-step cloning. The desired LMP-1 region to be cloned (nucleotides 166,157 to 171,823 of B95-8 EBV genome) is located within the *Bam*HI and the downstream *Eco*RI site (site on the right hand side) in pHC79:Dhet-*Eco*RI. This vector carrying the Dhet-*Eco*RI DNA fragment of the B95-8 EBV strain genome (nucleotides 159,395-171,823 plus nucleotides 1 and 2) was digested with *Eco*RI enzyme. The fragment was then cloned into the pBlueScript vector cut with *Eco*RI, close to a *Bam*HI site present in multiple-cloning site in the vector. The new pBlueScript:Dhet-*Eco*RI plasmid construct was digested with *Bam*HI, taking advantage of the *Bam*HI site carried by the pBlueScript vector. The resultant fragment was finally cloned into pHEBo-OriP plasmid previously digested with *Bam*HI.

2.3.4 RNA protocols

2.3.4.1 Total RNA extraction using RNeasy Mini kit

The RNeasy Mini kit (Qiagen) was utilised to extract total RNA according to manufacturer's instructions. The cells were harvested as described before (section 2.2.5) and resuspended in 600 µl of buffer RLT (Qiagen) supplemented with β-mercaptoethanol (10 µl per 1 ml buffer RLT). Lysates were homogenised by passing the lysate at least 5 times through a blunt 20G needle (0.9 mm). 600 µl of 70% ethanol was added to the lysate and mixed well by inversion of the tubes for 10 times. Lysates were transferred to a RNeasy Mini column and centrifuged at 17,900 x *g* for 30 sec. The column was washed with 700 µl of Buffer RW1 (Qiagen) and centrifuged again. The column was finally washed twice with 500 µl of buffer RPE (Qiagen) and centrifuged at 17,900 x *g* for 30 sec and 2 min, respectively. The RNA was eluted with 30 µl of RNase-free water and its concentration was determined as described (section 2.3.2).

2.3.4.2 RNA treatment with DNase

In order to remove any DNA contamination from the extracted RNA, RQ1 RNase-free DNase (Promega) was used prior to reverse transcription (RT). 1 µg of RNA was treated with 1 µl of DNase (1 U/µl) in a reaction volume of 10 µl and incubated at 37ºC for 35 min. The reaction was terminated by adding 1 µl of RQ1 DNase Stop Solution (Promega) and incubating at 65ºC for 10 min.

2.3.4.3 Reverse transcription (RT)

RNA was converted into complementary DNA (cDNA) with the ProtoScript First Strand cDNA Synthesis Kit (NEB). 0.5 µg of DNase-treated RNA was mixed with 2 µl of anchored oligo-dT primer [d(T_{23})VN] (50 µM) to make a total volume of 8 µl and incubated at 70ºC for 5 min to denature the RNA and to remove secondary structures. The samples were transferred to ice and 10 µl of 2X M-MuLV Reaction Mix (Promega) and 2 µl of M-MuLV reverse transcriptase (25 U/µl) was added to the reaction. The mixture was incubated at 42ºC for 1 h for RNA conversion into cDNA and then at 80ºC for 5 min to inactivate the enzyme. 30 µl of RNase-free water was added to the reaction to dilute cDNA to a final volume of 50 µl. The control reaction without M-MuLV reverse transcriptase was also set up to control for total digestion of DNA that was contaminating the extracted RNA.

2.3.4.4 RT-PCR assay

Reverse transcription-PCR (RT-PCR) was performed with GoTaq PCR system (Promega) on GeneAmp System 9700 (Applied Biosystems). The reaction is described in section 2.3.3.11. 2 µl of cDNA prepared in section 2.3.4.3 was used as template for the amplification reaction. EBNA-2 and LMP-1 targets were amplified using 30 cycles, whereas GAPDH was amplified for 20 cycles. The control reaction with no M-MuLV reverse transcriptase (-RT) previously set up was also used as negative control for RT-PCR. The PCR products that were obtained by amplification of LMP-1 were further digested using *Nco*I restriction enzyme (section 2.3.3.2) and analysed by 2% (w/v) agarose gel electrophoresis (section 2.3.3.4).

2.4 Primers

2.4.1 Primers for amplification of EBNA-2 transactivation domain

The following primers were used to amplify the TAD of EBNA-2 (amino acids 426-463):

Type 1 EBNA-2 TAD:
Forward: 5'-CCCGGATCCACATGAACCGGAGTCCCAT-3'
Reverse: 5'-CCCGCGGCCGCTTATCACTCAAAAATGTAATCCCAAC-3'

Type 2 EBNA-2 TAD:
Forward: 5'-CCCGGATCCACATGAACCAGAGTCCTCT-3'
Reverse: 5'-CCCGCGGCCGCTTATCATTCAAAAATGCCCTCCCA-3'

Type 2 EBNA-2 TAD SD431-432HN:
Forward: 5'-CCCGGATCCACATGAACCAGAGTCCCAT-3'
Reverse: 5'-CCCGCGGCCGCTTATCATTCAAAAATGCCCTCCCA-3'

Type 2 EBNA-2 TAD EEP434-436PEA:
Forward: 5'-CCCGGATCCACATGAACCAGAGTCCTCT-3'
Reverse: 5'-CCCGCGGCCGCTTATCATTCAAAAATGCCCTCCCA-3'

Type 2 EBNA-2 TAD F438I:
Forward: 5'-CCCGGATCCACATGAACCAGAGTCCTCT-3’
Reverse: 5'-CCCGCGGCCGCTTATCATTCAAAAATGCCCTCCCA-3’

Type 2 EBNA-2 TAD S442D:
Forward: 5'-CCCGGATCCACATGAACCAGAGTCCTCT-3’
Reverse: 5'-CCCGCGGCCGCTTATCATTCAAAAATGCCCTCCCA-3’

Type 2 EBNA-2 TAD G460Y:
Forward: 5'-CCCGGATCCACATGAACCAGAGTCCTCT-3’
Reverse: 5'-CCCGCGGCCGCTTATCATTCAAAAATGTACTCCCA-3’

Type 1 EBNA-2 TAD D442S:
Forward: 5'-CCCGGATCCACATGAACCGGAGTCCCAT-3’
Reverse: 5'-CCCGCGGCCGCTTATCACTCAAAAATGTAATCCCAAC-3’

All forward and reverse primers include a *Bam*HI and *Not*I restriction sites, respectively.

2.4.2 Primers for amplification of EBNA-2 C-terminal region

The following primers were used to amplify the EBNA-2 C-terminal region (aa 334-487):

Type 1 EBNA-2:
Forward: 5’-CCCGGATCCATCTAAGACTCAAGGCCAG-3’
Reverse: 5’-CCCGCGGCCGCTTATTACTGGATGGAGGGGCGAGGTCT-3’

Type 2 EBNA-2:
Forward: 5’-CCCGGATCCAACTCAGACTCCACCAACA-3’
Reverse: 5’-CCCGCGGCCGCTTATTACTGAGTGGAGGTGCGAGGTCT-3’

Type 2 EBNA-2 S442D:
Forward: 5’-CCCGGATCCAACTCAGACTCCACCAACA-3’
Reverse: 5’-CCCGCGGCCGCTTATTACTGAGTGGAGGTGCGAGGTCT-3’

All forward and reverse primers include a *Bam*HI and *Not*I restriction sites, respectively.

2.4.3 Primers used for screening of positive transformants

Cloning into pCR2.1 vector:
M13-Reverse: 5'-CAGGAAACAGCTATGAC-3'
T7-Promoter: 5'-TAATACGACTCACTATAGGG-3'

Cloning into pcDNA3.1-GAL4-DBD vector:
T7-Promoter: 5'-TAATACGACTCACTATAGGG-3'
BGH-Reverse: 5'-TAGAAGGCACAGTCGAGG-3'

2.4.4 Primers for site-directed mutagenesis of Notch OPA-PEST

Complementary oligonucleotides used were designed as described in section 2.3.3.12.

Notch OPA-PEST – mutation S2513D:
Forward: 5'-CCTTCCTCACCCCGGACCCTGAGTCCCCTG-3'
Reverse: 5'-CAGGGGACTCAGGGTCCGGGGTGAGGAAGG-3'

2.4.5 Primers used for amplification of B95-8 LMP-1 promoter

The following primers were used for amplification of the LMP-1 promoter region (-241/+12) of B95-8 EBV strain:

Amplification of LMP-1 promoter region (-241/+12):
Forward: 5'-CGAAGCTTGAGGAAAGAAGGGGGCAG-3'
Reverse: 5'-GCTCGAGTTGCACTTGGCCACCGCATT-3'

The forward and reverse primers include a *Hind*III and *Xho*I restriction sites, respectively.

2.4.6 Primers used for DNA sequencing

Products cloned into pCR2.1 vector:
M13-Reverse: 5'-CAGGAAACAGCTATGAC-3'
T7-Promoter: 5'-TAATACGACTCACTATAGGG-3'

Products cloned into pcDNA3.1-GAL4-DBD vector:
T7-Promoter: 5'-TAATACGACTCACTATAGGG-3'
BGH-Reverse: 5'-TAGAAGGCACAGTCGAGG-3'

Confirmation of Notch OPA-PEST S2513D mutation:
Forward: 5'-CTCGCAGCACAGCTACTCCT-3'
Reverse: 5'-TGGAGACGCCCTCGGACCAG-3'

2.4.7 Primers used for RT-PCR

LMP-1 gene:
Forward: 5'-GTCATAGTAGCTTAGCTGAACTG-3'
Reverse: 5'-CACAGTGATGAACACCACCACG-3'

Type 1 EBNA-2 gene:
Forward: 5'-AGGGATGCCTGGACACAAGA-3'
Reverse: 5'-TTGTGACAGAGGTGACAAAA-3'

Type 2 EBNA-2 gene:
Forward: 5'-AGGGATGCCTGGACACAAGA-3'
Reverse: 5'-GAAGAGTATGTCCTAAGGCT-3'

GAPDH gene:
Forward: 5'-TGCCTCCTGCACCACCAACT-3'
Reverse: 5'-CGCCTGCTTCACCACCTTC-3'

2.4.8 Oligonucleotides for biotinylated oligonucleotide pulldowns

The following complementary oligonucleotides were initially annealed and then utilised in the biotinylated oligonucleotide pulldowns:

LMP-1 wildtype biotinylated oligonucleotide:
Fw: 5'-GCGGTGTGTGTGTGCATGTAAGCGTAGAAAGGGGAAGTAGAAAGCGTGTG-3'
Rv: 5'-CACACGCTTTCTACTTCCCCTTTCTACGCTTACATGCACACACACACCGC-3'

PU.1 mutant biotinylated oligonucleotide:
Fw: 5'-GCGGTGTGTGTGTGCATGTAAGCGTAGAAAGGCGTAGTAGAAAGCGTGTG-3'
Rv: 5'-CACACGCTTTCTACTACGCCTTTCTACGCTTACATGCACACACACACCGC-3'

EBF/Jk biotinylated oligonucleotide:
Fw: 5'-TGTGTTAGAAAAGCGGGTCCCCGGGGGGCAAGCTGTGGGAATGCGGTGGC-3'
Rv: 5'-GCCACCGCATTCCCACAGCTTGCCCCCCGGGGACCCGCTTTTCTAACACA-3'

These forward (Fw) oligonucleotides carry a biotin label at their 5' end.

Short specific PU.1 competitor:
Fw: 5'-GCGTAGAAAGGGGAAGTAGAAAG-3'
Rv: 5'-CTTTCTACTTCCCCTTTCTACGC-3'

Short randomised competitor:
Fw: 5'-TAGCGTCATGACTACGTGATCAT-3'
Rv: 5'-ATGATCACGTAGTCATGACGCTA-3'

Long specific PU.1 competitor:
Fw: 5'-GCGGTGTGTGTGTGCATGTAAGCGTAGAAAGGGGAAGTAGAAAGCGTGTG-3'
Rv: 5'-CACACGCTTTCTACTTCCCCTTTCTACGCTTACATGCACACACACACCGC-3'

Long randomised competitor:
Fw: 5'-TAGCGTCATGACTACGTGATCATGTCGTAGCGTCATGACTACGTGATCAT-3'
Rv: 5'-ATGATCACGTAGTCATGACGCTACGACATGATCACGTAGTCATGACGCTA-3'

Octamer competitor oligonucleotide:

Fw: 5’-TCCCGAAAGCGGCGGTGTGTGTGTGCATGTAAGCGTA-3’

Rv: 5’-TACGCTTACATGCACACACACACCGCCGCTTTCGGGA-3’

2.5 Protein protocols

2.5.1 Extraction of cellular proteins using RIPA lysis buffer

The cell pellets were initially resuspended in two volumes of RIPA lysis buffer (0.15 M NaCl, 1% (v/v) NP-40, 0.5% (v/v) deoxycholic acid, 0.1% (w/v) SDS, 50 mM Tris-Cl pH 8.0, 1 mM of PMSF (Sigma) with 1X complete protease inhibitor cocktail (Roche)). The cells and the RIPA lysis buffer mixture was incubated at 4ºC for 20 min under rotation and centrifuged for 15 min at 25,000 x *g* (4ºC) to pellet cell debris. Supernatants were then transferred to fresh eppendorf tubes and the protein concentration was determined before storage at -20ºC.

2.5.2 Quantification of protein concentration

The concentration of the protein extracts was determined with the Bio-Rad DC protein assay (Bio-Rad). This was carried out in order to achieve loading of equal amounts of protein in the SDS-PAGE gels for western blotting. A standard curve was initially produced using different concentrations of bovine serum albumin (0, 0.078, 0.156, 0.312, 0.625, 1.25, 2.5 and 5 µg/µl) and their respective absorbance values. 5 µl of protein extract was diluted into 15 µl of RIPA lysis buffer. Reagent S (Bio-Rad) was diluted 1:50 into reagent A (alkaline copper tartrate solution, Bio-Rad) and 100 µl of this solution was added to each sample. 800 µl of reagent B (Folin reagent, Bio-Rad) was added. The samples were mixed by short vortex and incubated at room temperature for 20 min. The bovine serum albumin standards were processed in a similar manner. The concentration of the samples was determined by measuring the values of absorbance at 750 nm using a spectrophotometer (Unicam Heλiosβ) and the values were compared against the standard curve to obtain the protein concentration.

2.5.3 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) has been used to resolve proteins from RIPA protein extracts. The Mini Protean II system (Bio-Rad) was used. Protein separation according to their size was carried out using a 7.5-12% resolving gel (7.5-12% (v/v) acrylamide/bis-acrylamide (29:1) (Bio-Rad), 375 mM Tris-Cl pH 8.8 and 0.1% (w/v) SDS) and a 5% stacking gel (5% acrylamide/bis-acrylamide (29:1), 125 mM Tris-Cl pH 6.8 and 0.1% (w/v) SDS). The gels were polymerised with 10 µl of NNN'N'-tetramethylethylene diamine (TEMED) (National Diagnostics) and also 50 µl of 10% (w/v) ammonium persulphate

(APS) (Sigma) *per* 10 ml of gel volume. Protein samples were then diluted 1:1 using 2X SDS sample buffer (120 mM Tris-Cl pH 6.8, 4% (w/v) SDS, 2% (v/v) β-mercaptoethanol, 20% (v/v) glycerol and 0.01% (w/v) bromophenol blue) and incubated at 95ºC for 5 min. 10-30 µg of protein sample was loaded on the stacking gel along with the Full Range Rainbow Molecular Weight Marker (Amersham) for protein size comparison. Electrophoresis was performed at 150 V for 1 h in 1X SDS running buffer (25 mM Tris base, 192 mM glycine and 0.1% (w/v) SDS).

2.5.4 Coomassie staining

After protein separation by SDS-PAGE, gels were stained using Coomassie blue (40% (v/v) methanol, 10% (v/v) acetic acid, 1 g/l Coomassie Brilliant Blue R-250 (Thermo Scientific)) for 2-4 h and then destained overnight using destaining solution (40% (v/v) methanol, 10% (v/v) acetic acid) with gently agitation. The gel was finally equilibrated in storage solution (5% (v/v) acetic acid) for 1 h.

2.5.5 Western blotting

Protein samples were firstly resolved by SDS-PAGE (section 2.5.3). Resolved proteins were transferred on to an Amersham Protran 0.45 µm nitrocellulose membrane (GE Healthcare) in ice-cold 1X transfer buffer (25 mM Tris base, 192 mM glycine, 0.1% (w/v) SDS and 24% ethanol) at 100 V for 1 h. The membrane and the gel were placed between three layers of 3 mm Whatman paper, a sponge at each side and inserted into a Bio-Rad transfer cassette. The membrane was then saturated in blocking solution (1X PBS with 0.1% (v/v) of Tween-20 (Sigma) and 5% (w/v) of skimmed milk powder (Sigma)) for 1 h at room temperature with shaking. The membranes were incubated overnight with adequate primary antibody diluted in blocking solution, at 4ºC. The membranes were washed three times for 10 min in washing buffer (PBS-T: 1X PBS with 0.1% (v/v) Tween-20) and finally incubated with a secondary antibody conjugated with horseradish peroxidase (HRP) diluted in blocking solution, for 1 h at room temperature. The membranes were washed again three times for 10 minutes using washing buffer. The proteins were finally visualised with the ECL Western Blotting detection reagents (Amersham, GE Healthcare), using the enhanced chemiluminescence method (ECL), which consisted of adding equal volumes of each detection reagent of the kit on to the membranes and revealed by exposure on an autoradiography Super RX X-ray film (Fujifilm, Japan).

2.5.6 Stripping of western blotting membranes for reprobing

Membranes were subjected to stripping to remove the first primary and secondary antibodies from the membranes and to allow for reprobing for a second protein of interest or a loading control. Membranes were incubated with stripping buffer (2% (w/v) SDS, 62.5 mM Tris-Cl pH 6.8 and 0.008% (v/v) β-mercaptoethanol) at 50ºC for 45 min with agitation. The membrane was washed with washing buffer (PBS-T: 1X PBS with 0.1% (v/v) Tween-20) five times for 15 min to remove traces of β-mercaptoethanol. The membrane was again saturated in blocking solution (1X PBS with 0.1% (v/v) of Tween-20 (Sigma) and 5% (w/v) of skimmed milk powder (Sigma)) and incubated with the appropriated antibodies as before.

2.5.7 Antibodies

Antigen	Antibody	Source	Dilution
EBNA-2	PE2 clone, mouse monoclonal[1]	hybridoma cell line (supernatant)	1:100
LMP-1	CS.1-4 clone, mouse monoclonal	Dako	1:500
BS69	BS69 (H-265), rabbit polyclonal	Santa Cruz Biotechnology	1:200
GAL4 (DBD)	RK5C1, mouse monoclonal	Santa Cruz Biotechnology	1:1000
human β-actin	AC-74 clone, mouse monoclonal	Sigma	1:2000
human GAPDH	6C5 clone, mouse monoclonal	Ambion	1:5000
mouse IgG	rabbit polyclonal	Dako	1:1000
mouse IgG	sheep polyclonal, HRP conjugated	GE Healthcare	1:5000
rabbit IgG	goat polyclonal, HRP conjugated	Dako	1:1000

Table 2.3 Antibodies used in this study

DBD: DNA-binding domain; HRP: Horseradish peroxidase.

[1]PE2 antibody equally recognises both EBNA-2 types with the same efficiency [206].

2.6 Luciferase reporter assay

The Dual-Luciferase® Reporter Assay System (Promega) was used to perform the luciferase reporter assays. Cells were harvested 24 h after transfection by centrifugation at 335 x *g* for 5 min at 4ºC and washed with ice-cold PBS. Cells pellets were then lysed using 100 µl of 1X Passive Lysis buffer (PLB, Promega). Two freeze-thaw cycles were performed to achieve a better lysis (20 sec on dry ice and thawing at room temperature for 2 min). Cell debris were removed by centrifugation at 25,000 x *g*, for 1 min at 4ºC and the clear supernatant was then transferred to a fresh tube. 20 µl of each individual lysate was transferred to a well of a white 96-well plate. Using a microplate luminometer (LUMIstar Omega, BMG Labtech) that injects 40 µl of Luciferase Assay Reagent II (LARII, Promega), firefly luciferase of each sample was activated and measured. Then, after 4 sec, the microplate luminometer injected 40 µl of Stop & Glo reagent (Promega), which quenches firefly luciferase and activates *Renilla* luciferase, which was measured. *Renilla* luciferase activity was determined to normalise the values of firefly luciferase activity for transfection and extraction efficiency.

2.7 Protein pulldowns

2.7.1 Preparation of nuclear extracts from Daudi cells

EBNA-2 expression in the Daudi:pHEBo-MT:EBNA-2 cells was induced with 5 µM $CdCl_2$, for 24 hours. Cells were then harvested by centrifugation at 390 x *g* for 5 min at 4ºC and washed twice with ice-cold PBS. Aliquots of at least $4x10^7$ cells were resuspended in 1 ml of buffer A (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM 1,4-dithiothreitol (DTT) (Sigma), 1 mM PMSF (Sigma) and 1X complete protease inhibitor cocktail (Roche)). Cells were again pelleted in eppendorf tubes by centrifugation at 1000 x *g*, for 5 min at 4ºC. Cell pellets were resuspended in 100 µl of buffer A supplemented with 0.1% (v/v) NP-40 to lyse the cells and were incubated on ice for 5 min. Cell lysates were centrifuged at 2700 x *g* for 30 sec at 4ºC and the supernatant was discarded. The nuclear pellet was resuspended in 50 µl of buffer B (20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM PMSF, 25% (v/v) glycerol, 1 mM DTT and 1X complete protease inhibitor cocktail) and samples incubated at 4ºC for 20 min with rotation. Samples were finally centrifuged at 11,600 x *g*, for 10 min, at 4ºC and the supernatants/nuclear extracts were transferred to fresh eppendorf tubes and the protein concentration was determined (see section 2.5.2) before storage at -80ºC.

2.7.2 *In vitro* translation using the rabbit reticulocyte system

The TnT T7 Quick Coupled Transcription/Translation system kit (Promega) was used to carry out *in vitro* translation of EBNA-2 proteins expressed from pBlueScript plasmids (see section 2.3.3.13.6). The reaction mixture containing 2 µl of template (concentration 0.5 µg/µl), 2 µl of L-[S^{35}]-methionine (1 mCi, 10 mCi/ml, Perkin Elmer), 40 µl TnT Quick Master Mix (Promega) and 6 µl of dH_2O was incubated at 30ºC for 60 min. 2 µl of each sample was then diluted in 18 µl of 2X SDS protein sample buffer (120 mM Tris-Cl pH 6.8, 4% (w/v) SDS, 2% (v/v) β-mercaptoethanol, 20% (v/v) glycerol and 0.01% (w/v) bromophenol blue) and samples were resolved by SDS-PAGE (section 2.5.3). The gel was then incubated in fixative solution (40% (v/v) methanol and 10% (v/v) acetic acid) for 30 min at room temperature. Fixative solution was discarded and the gel was soaked in 100 ml of dH_2O with 1% (v/v) glycerol for 15 min at room temperature. The gel was then soaked in Amersham Amplify fluorographic reagent (GE Healthcare) for 45 min at room temperature with agitation. The gel was finally dried using the Bio-Rad 583 Electrophoresis Gel Dryer (Bio-Rad) for 2 hours at 80ºC, under vacuum suction and revealed by exposure on an autoradiography Super RX X-ray film (Fujifilm).

2.7.3 Preparation of lysates expressing GST-tagged proteins

Frozen stock of bacterial strain carrying the plasmid expressing the GST-tagged protein was streaked on LB-agar plate supplemented with ampicillin (100 µg/ml) and grown overnight at 30ºC. Single colony was picked from LB-agar plate and inoculated into 5 ml of LB broth with ampicillin (100 µg/ml) and incubated for 16 h at 30ºC with aeration. 4 ml was transferred into 100 ml of LB broth supplemented with ampicillin (100 µg/ml) and grown at 30ºC with shaking until the culture reached an absorbance value of 0.6-1.0 at 600 nm. The culture was moved to 25ºC and then induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma) for 3-4 h. 500 µl aliquots were collected before and after IPTG induction. Cells were pelleted at 2,800 x *g* for 20 min at 4ºC. Cell pellets were resuspended in 10 ml of Lysis buffer (20 mM Tris-Cl pH 8.0, 150 mM NaCl and 1 mM DTT) and lysed by adding 120 µl of lysozyme (at 10 mg/ml) and incubating on ice for 20 min. Bacterial lysates were sonicated at high speed 3x15 sec pulses in icy water using an Ultrasonic XL2020 Processor (Heat Systems). Lysates were then transferred to eppendorf tubes and centrifuged at 17,900 x *g* for 30 min (at 4ºC) to pellet cell debris. Supernatants that correspond to the soluble protein fraction were transferred into fresh tubes and stored at -80ºC.

2.7.4 Glutathione S-transferase pulldowns

50 µl of 50% Glutathione-Sepharose 4B Bead slurry (GE Healthcare) was washed for three times, using 1 ml of ice-cold Binding buffer (20 mM Tris-Cl pH 8.0, 150 mM NaCl, 1 mM DTT and 0.1 mg/ml BSA). Beads were pelleted by centrifugation at 25,000 x *g* for 1 min. 100 µl of bacterial lysate containing the GST-tagged protein was incubated with the washed beads for 1 h at 4ºC, rotating. Glutathione-Sepharose Beads bound to the GST-tagged protein were washed with ice-cold Binding buffer for six times and pelleted by centrifugation at 25,000 x *g* for 1 min. The complex beads bound to the GST-tagged protein was incubated with nuclear extracts containing EBNA-2 (30 µl, 90 µl or 150 µl, depending on the experiment) or with *in vitro* translated EBNA-2 protein (10 µl of *in vitro* translated (IVT) product plus 20 µl of Binding buffer). The incubation was performed at distinct temperatures (25ºC or 4ºC) and for different times (2, 5, 10, 15, 20, 30 min and 2 h) depending on the experiment. Beads were washed for six times with ice-cold Binding buffer and pelleted by centrifuging at 25,000 x *g* for 1 min. The last traces of Binding buffer were removed and beads were then resuspended in 25 µl of 2X SDS sample buffer (120 mM Tris-Cl pH 6.8, 4% (w/v) SDS, 2% (v/v) β-mercaptoethanol, 20% (v/v) glycerol and 0.01% (w/v) bromophenol blue) and incubated at 95ºC for 5 min. The samples were then loaded onto a 7.5% SDS-PAGE gel (see section 2.5.3). GST pulldowns performed using nuclear extracts of cells expressing EBNA-2 protein as input were analysed by western blotting (section 2.5.5) and the PE2 antibody was used. GST pulldowns using IVT products as input were detected by autoradiography using the same procedure described in section 2.7.2, after resolution by SDS-PAGE.

2.7.5 Biotinylated oligonucleotide pulldowns

2.7.5.1 Binding biotinylated oligonucleotide to magnetic beads

Magnetic Dynabeads M-280 with covalently attached streptavidin on the surface (Invitrogen) were used in the biotinylated oligonucleotide pulldowns. The magnetic beads in solution were firstly resuspended by gently shaking the bottle. 35 µl of beads solution was transferred to an eppendorf tube and spun down for 20 sec at 20,000 x *g*. The eppendorf tube was placed on a magnetic rack for 2 min to allow the beads to settle and the solution was removed without disturbing the beads. The beads were then resuspended in 1 ml of 2X Binding and Washing buffer (B&W buffer: 10 mM Tris-Cl pH 7.5, 2 M NaCl and 1 mM EDTA) and washed for 5 min with rotating at room temperature. The beads were centrifuged for 20 sec at 20,000 x *g*, the tube placed on the magnet for 2 min and the buffer removed. Two washes were carried out.

The beads were resuspended in 50 µl of 2X B&W buffer and mixed with 50 µl of biotinylated oligonucleotide previously annealed (50 pmol). The mixture was incubated for 90 min at RT. The beads with the biotinylated oligonucleotide were centrifuged for 20 sec at 20,000 x *g* and the tube placed on the magnet for 2 min. The beads were washed twice with 1 ml of 2X B&W buffer as before and washed once with 1 ml of D150 buffer (10 mM HEPES pH 7.9, 150 mM KCl, 20% (v/v) glycerol, 0.4 mM EDTA, 0.05% (v/v) NP-40, 10 mM β-mercaptoethanol, 1 mM PMSF (Sigma) and 1X protease inhibitor cocktail (Roche)). The magnetic beads were pulse-spun as before, the tube placed on the magnet and D150 buffer was removed.

2.7.5.2 Incubation of oligonucleotide-beads with nuclear extracts

Nuclear extracts of Daudi:pHEBo-MT cells expressing EBNA-2 were firstly preincubated with sonicated salmon sperm DNA (10 mg/ml stock, Invitrogen) to get a final concentration of 0.4 mg/ml, for 45 min at 4ºC, to reduce unspecific binding to biotinylated oligonucleotide. Nuclear extracts were diluted into nuclear extracts of Daudi cells that do not express EBNA-2 in order to adjust the amount of EBNA-2 protein in the extracts. 50 µl of these nuclear extracts were then diluted with H_2O to make a final input volume of 150 µl. The beads were incubated with this volume of diluted nuclear extract for 45 min at 4ºC with rotating.

2.7.5.3 Washing and elution

After incubation with the nuclear extract, the beads were gently centrifuged at 950 x *g* for 1 min to recover material from the tube lid and the tube placed on the magnetic rack for 2 min. The beads were finally washed three times with 1 ml of ice-cold D150 buffer and centrifuged again to remove the last traces of buffer. The beads were finally resuspended in 25 µl of 2X SDS sample buffer (120 mM Tris-Cl pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 2% (v/v) β-mercaptoethanol and 0.01% (w/v) bromophenol blue) and incubated at 95ºC for 5 min. The samples were loaded onto a 7.5% SDS-PAGE gel (section 2.5.3) and analysed by western blot (section 2.5.5). Immunoblots were probed with the anti-EBNA-2 antibody. This protocol was adapted from reference [412].

3 Transcriptional activation of target gene promoters mediated by type 1 and type 2 EBNA-2

The aim of this chapter is to investigate the ability of the EBNA-2 acidic transactivation domain to mediate gene expression and to determine whether type 1 EBNA-2 displays an enhanced ability to transactivate gene expression when compared to type 2 and if this could account for the differential gene regulation by type 1 and type 2 EBNA-2.

3.1 Introduction

The most important functional difference between type 1 and type 2 EBV strains is related to their distinct ability to transform human B cells *in vitro*. Type 1 EBV strains are characterised by their superior ability to transform and immortalise human B cells into lymphoblastoid cell lines much more efficiently than type 2 strains [296]. This distinct feature was mapped to the EBNA-2 locus because a recombinant type 2 P3HR1 EBV strain carrying a type 1 EBNA-2 sequence acquired a type 1 immortalisation phenotype [134]. EBNA-2 is the most divergent genome locus between the two EBV types, displaying only 56% identity in the amino acid sequence. The EBNA-3 genes co-vary with EBNA-2 but most of the rest of the genome shows only a low level of variation [213]. The EBNA-2 sequence thus normally defines the classification of EBV strains into type 1 or type 2 [7].

EBNA-2 is the major transcriptional activator of latent cycle EBV, essential for transformation of primary B lymphocytes *in vitro*. Upon EBV infection, EBNA-2 drives B cell transformation into LCLs by activating the expression of viral and cellular genes, whose products mediate survival and proliferation of infected cells [7, 113-115, 206]. This activation of target responsive genes is not through direct binding to DNA. Instead, EBNA-2 forms a complex with and is recruited to target promoters by distinct cellular DNA-binding proteins [7]. The most important is RBP-Jk, a transcriptional repressor, which is turned into the transcriptionally active form upon EBNA-2 interaction [220, 226]. RBP-Jk is involved in EBNA-2 activation of the viral LMP-1, LMP-2A, LMP-2B and Cp promoters and the cellular CD21 and CD23 promoters [128, 239-241, 413]. However, other proteins, such as PU.1/Spi-1 [239, 242], ATF/CRE [243] and EBF1 [245], have also been shown to be involved in transactivation of certain promoters by EBNA-2, such as the viral LMP-1 promoter.

Transcriptional activation of target gene promoters by EBNA-2 is mediated by the acidic transactivation domain (TAD) [216]. Once EBNA-2 is tethered to DNA, the TAD interacts with additional co-activating proteins, such as histone acetyltransferases (p300/CBP and PCAF [258]) and factors that compose the cell transcription machinery, namely the factors TFIIH [260], TAF40, TFIIB [259] and the co-activator p100 that interacts with TFIIE [261], to activate gene transcription. Multiple microarray expression profiles of EBNA-2 target genes revealed that about 300 cell genes could be regulated by EBNA-2 [113-115, 206] in addition to the viral Cp and LMP promoters. However, only about 10 of these cell genes are differentially regulated by type 1 and type 2 EBNA-2 and all of these 10 genes are more strongly induced by type 1 [206]. CXCR7 is the most differentially regulated cell gene and is essential for the proliferation of LCLs [206]. The viral LMP-1 gene, which is required for growth transformation by EBV [135], is also induced more strongly by type 1 EBNA-2 than type 2 [206, 209].

These observations suggested that the stronger induction of some genes by type 1 EBNA-2 might account for the enhanced transforming ability of type 1 EBV when compared to type 2. In fact, the EBNA-2 TAD region that mediates gene transactivation was earlier shown to be important for human B cell transformation and immortalisation [227]. Furthermore, previously published studies, developed in this laboratory, have shown that the substitution of the C-terminus end of type 1 EBNA-2, which includes the TAD region, into the type 2 protein was able to confer a type 1 ability to promote LCL growth maintenance in the EREB2.5 cell growth assay [136, 209]. More recently, it was found that an unique amino acid substitution, in which the serine of type 2 EBNA-2 was replaced by the equivalent aspartate in type 1 EBNA-2 (S442D), was sufficient to give a type 1-growth phenotype in the LCL growth maintenance assay [210]. The amino acid aspartate 442 is located in the TAD of type 1 EBNA-2 protein. Together these results suggest that a higher ability to transactivate target responsive genes by type 1 EBNA-2 could contribute to the mechanism responsible for the superior ability to transform and immortalise human B cells into LCLs *in vitro* by type 1 EBV.

In the present study, the ability to mediate gene expression by the TAD of type 1 and type 2 EBNA-2 was investigated in order to determine whether a distinct transactivation function by type 1 and type 2 EBNA-2 could account for the type 1/type 2 differential gene regulation. In addition, the effect of different amino acid substitutions in the EBNA-2 TAD, including the important serine to aspartate (S442D) mutation, was also studied in terms of EBNA-2 ability to transactivate gene expression.

3.2 Results

3.2.1 Comparing the ability of the TAD of type 1 and type 2 EBNA-2 to activate gene expression using GAL4-gene reporter assays

3.2.1.1 Generation of pcDNA3.1-GAL4-DBD vectors expressing the GAL4-DNA-binding domain (DBD)-EBNA-2-TAD fusion constructs

To investigate whether the type 1/type 2 differential gene regulation could be explained by a possible stronger transactivation function by type 1 EBNA-2, the TADs of type 1 and type 2 EBNA-2 were fused to the GAL4 DNA-binding domain (DBD) and then tested for their ability to activate expression of a synthetic promoter containing GAL4 DNA-binding sites located upstream of a luciferase reporter gene. A similar system was originally used to identify the TAD of type 1 EBNA-2 [216]. To generate the GAL4-DBD:E2-TAD constructs, the EBNA-2 region, which comprises the amino acids 426 to 463, was cloned into the pcDNA3.1-GAL4-DBD plasmid fused to GAL4-DBD. This region was chosen because this fragment had been demonstrated to be the strongest activator in CHO cells. When compared to other fragments or to the full-length protein, it was as strong as the herpes simplex VP16 acidic domain in CAT assays performed in EBV-negative B cell lymphoma BJAB cells [216].

Distinct pBlueScript plasmids expressing type 1 and type 2 EBNA-2 were already available in the laboratory. Plasmid vectors carrying either the type 2 EBNA-2 sequence with the amino acid substitution serine to aspartate 442 or the type 1 EBNA-2 sequence with the reciprocal mutation (D442S) had also been previously made (section 2.3.3.13). The plasmids were used as template to PCR amplify the region mentioned above (amino acids 426 to 463), of each of those EBNA-2 proteins, using oligonucleotide primers flanked by *Bam*HI and *Not*I restriction sites (Figure 3.1 A). The PCR products with a size of 140 bp were subcloned into the pCR2.1 vector using the TA cloning strategy (section 2.3.3.7). Transformants were then screened by PCR using T7-promoter and M13-reverse primers. The size of the products obtained for the positive clones is 317 bp (Figure 3.1 B and C).

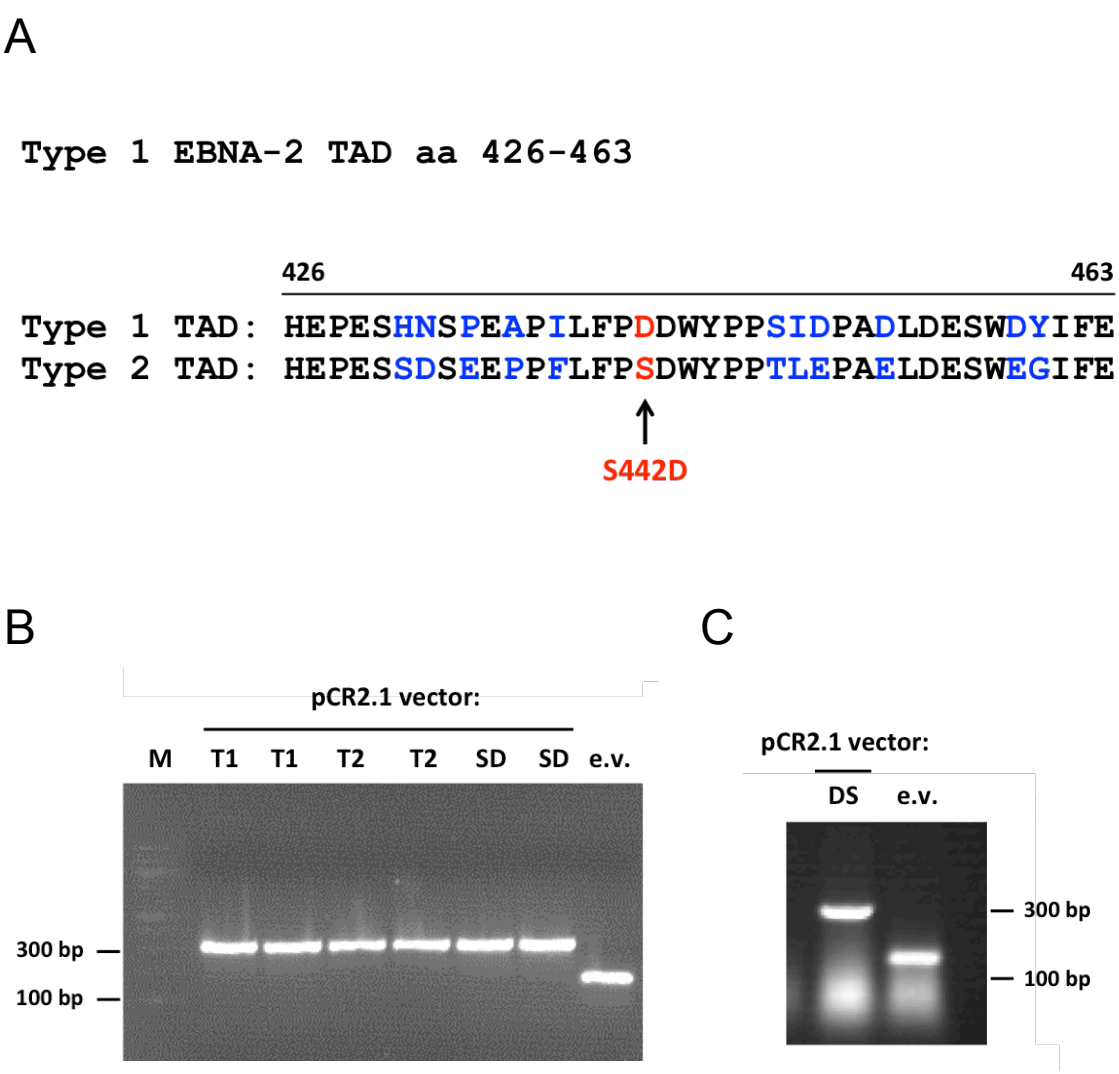


Figure 3.1 Cloning of type 1, type 2, type 2 S442D and type 1 D442S EBNA-2 TAD fragments (amino acids 426-463) into pCR2.1 vector

(**A**) Section of the EBNA-2 protein to be cloned into pCR2.1 vector (amino acids 426 to 463), including the acidic transactivation domain. The amino acid serine 442 of type 2 and the equivalent aspartate of type 1 are represented in red. The other amino acids in this region differing between type 1 and type 2 TADs are represented in blue. (**B**) Representative gel electrophoresis of PCR products obtained from PCR-screening for positive clones of the EBNA-2 TAD fragments (aa 426-463) from type 1 (T1), type 2 (T2), type 2 S442D (SD) and (**C**) type 1 D442S (DS) cloned into pCR2.1 vector. T7-promoter and M13-reverse oligonucleotide primers were used. The empty vector pCR2.1 was used as negative control (e.v.). Expected size of the amplified fragment in positive clones is 317 bp. Expected size for the empty vector is 177 bp. M: 100 bp marker.

The pCR2.1 vectors carrying the cloned fragments were then digested with *Bam*HI and *Not*I enzymes and the resultant EBNA-2 TAD fragments were cloned into pcDNA3.1-GAL4-DBD plasmid cut with *Bam*HI and *Not*I, to generate the pcDNA3.1-GAL4-DBD:E2-TAD plasmid constructs (section 2.3.3.13.3). Transformants were screened by PCR using T7-promoter forward and BGH-reverse oligonucleotide primers (Figure 3.2). The final constructs were sequenced at the Genomics Facility at Imperial College London (Hammersmith Campus) to validate their identity and then compared to the published sequences of EBNA-2.

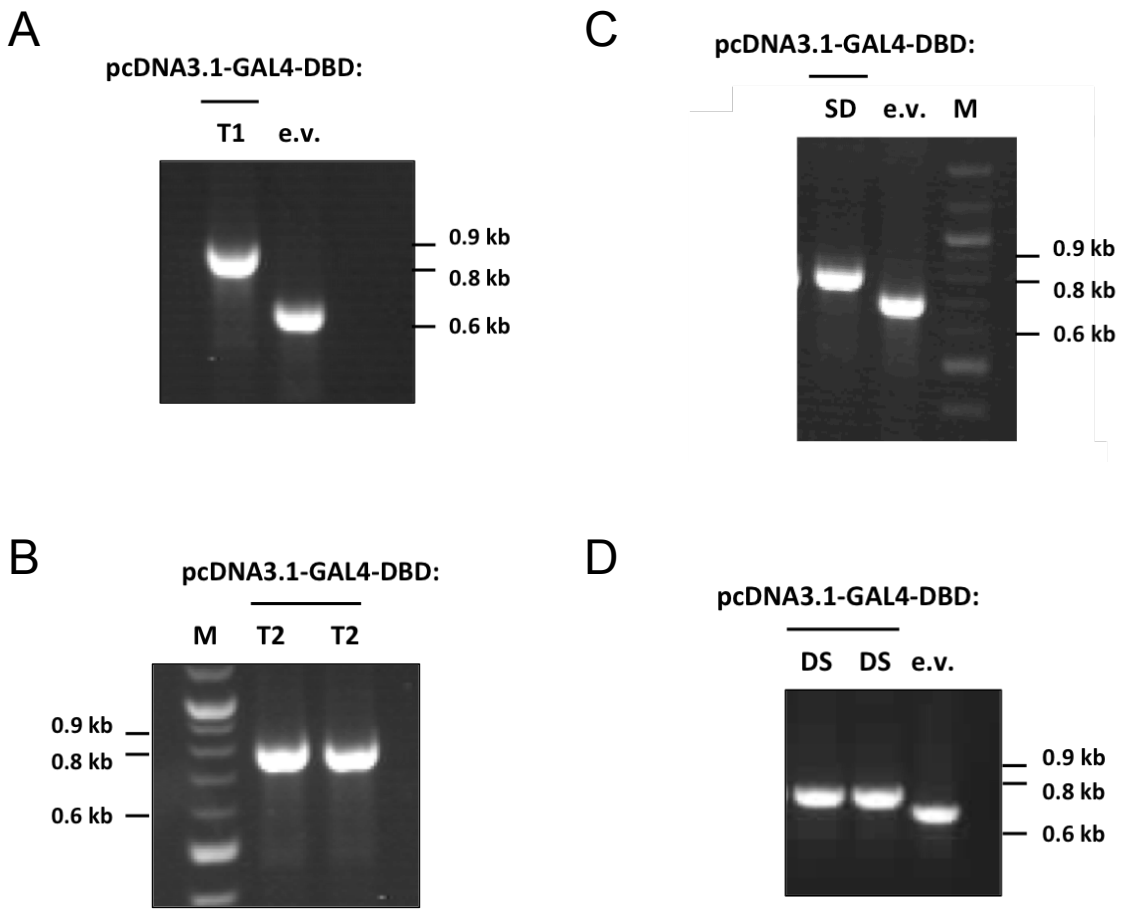


Figure 3.2 Cloning of EBNA-2 TAD fragments (aa 426-463) into pcDNA3.1-GAL4-DBD vector

Representative gel electrophoresis of PCR products obtained from PCR-screening for positive clones of (**A**) type 1 (T1); (**B**) type 2 (T2); (**C**) type 2 S442D (SD) and (**D**) type 1 D442S (DS) EBNA-2 TAD fragments (amino acids 426 to 463) into pcDNA3.1-GAL4-DBD vector. T7-promoter forward and BGH reverse oligonucleotide primers were used. The empty vector pcDNA3.1-GAL4-DBD was used as the negative control (e.v.). Expected size of the amplified fragment in positive clones is 790 bp. The size obtained for the empty vector is 650 bp. M: 100 bp marker.

3.2.1.2 Setting up a transient transfection assay to determine the ability of the GAL4-DBD:E2-TAD fusion proteins to transactivate luciferase gene expression in BJAB and IB4 cells

The GAL4-DBD:E2-TAD fusion constructs were then used in transient transfection luciferase reporter assays in EBV-negative B cell lymphoma BJAB cells [406] and in a lymphoblastoid cell line IB4 [414]. Firstly, the relative amounts of the plasmids carrying the GAL4-DBD:E2-TAD and the pFR-Luc reporter plasmid (section 2.3.3.13) to be transiently transfected were optimised. BJAB cells were cotransfected with either 50 ng or 300 ng of the GAL4-DBD:E2-TAD of type 1 EBNA-2, with distinct amounts of reporter plasmid (50 ng, 250 ng and 500 ng) to achieve a good luciferase expression and therefore determine the conditions to adopt in the reporter assays (Figure 3.3 A). The Neon transfection system was utilised to carry out the transfections into BJAB cells. Transfection settings have been previously optimised for this cell line in the laboratory (1200 V, 2 pulses and a pulse width of 20 ms). Transfection of 300 ng of the GAL4-DBD:E2-TAD plasmid construct with 500 ng of pFR-Luc reporter plasmid led to the strongest luciferase activation within the amounts tested.

A similar procedure was carried out for the IB4 cell line. Cells were cotransfected with either 300 ng or 500 ng of the plasmid expressing GAL4-DBD:E2-TAD type 1 fusion protein, with different amounts of pFR-Luc reporter plasmid (500 ng, 750 ng and 1000 ng), to determine the relative amounts that give good luciferase expression, equivalent to that obtained in the previous cell line. The Neon system was used to transfect the cells using distinct transfection conditions (1400 V, 1 pulse and a pulse width of 30 ms). Transfection of 300 ng of the GAL4-DBD:E2-TAD construct, with 1000 ng of pFR-Luc plasmid led to a luciferase gene expression similar to that observed in BJAB cells (Figure 3.3 B). Therefore, these parameters were used in the further transient transfection reporter assays.

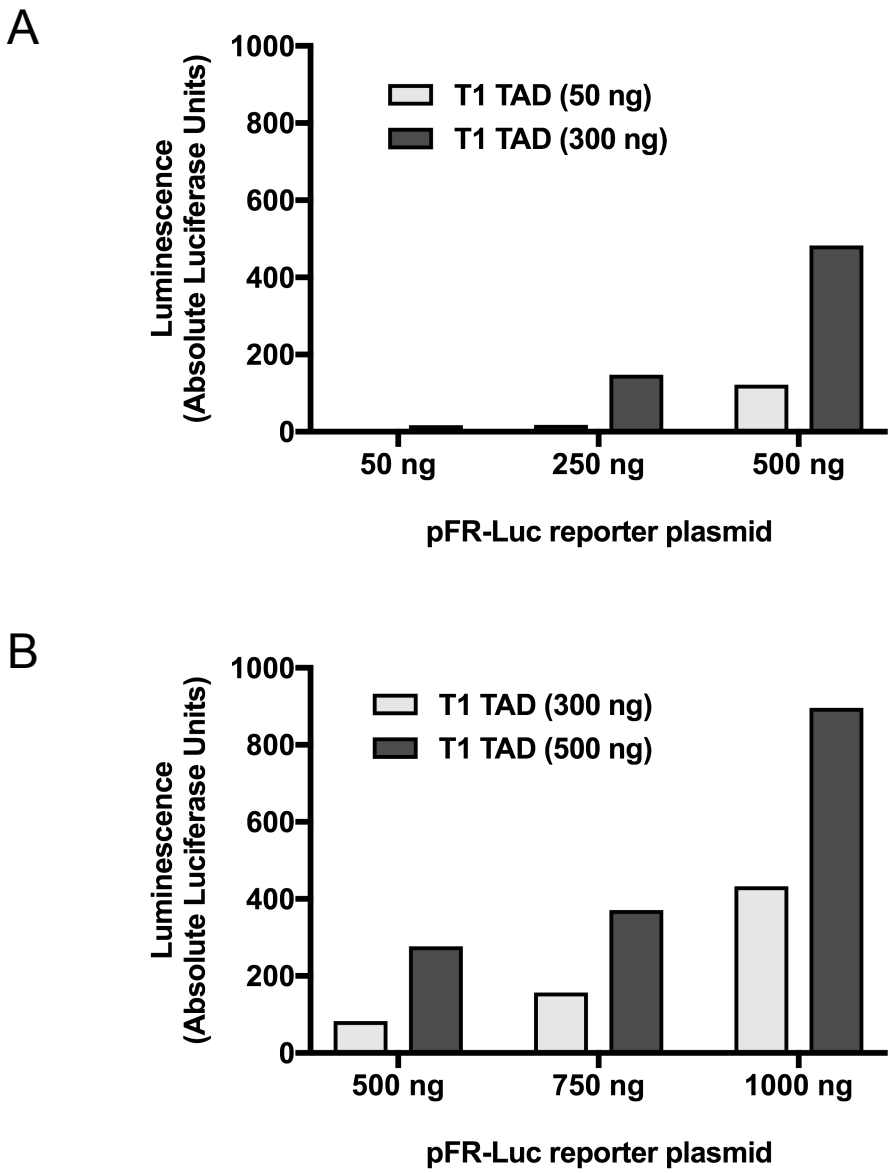


Figure 3.3 Optimisation of the GAL4-DBD:E2-TAD transient luciferase reporter assay

(**A**) Distinct amounts of the pFR-Luc reporter plasmid (50 ng, 250 ng and 500 ng) were cotransfected with 50 ng and 300 ng of the pcDNA3.1-GAL4-DBD:E2-TAD type 1 (aa 426-463) plasmid into EBV-negative B cell lymphoma BJAB cells. (**B**) IB4 cells (LCL) were cotransfected with 500 ng, 750 ng and 1000 ng of pFR-Luc reporter plasmid and with 300 ng or 500 ng of GAL4-DBD:E2-TAD type 1 plasmid to optimise the relative plasmid amount that would be used in the reporter assays. The Neon system was used. At 24 hours post-transfection, cells were harvested and extracts were analysed for absolute luciferase expression. Transfection of BJAB cells with 500 ng of pFR-Luc reporter plasmid and 300 ng of GAL4-DBD:E2-TAD type 1 give similar luciferase expression to that obtained after transfecting IB4 cells with 1000 ng of pFR-Luc reporter plasmid and 300 ng of GAL4-DBD:E2-TAD type 1 construct.

3.2.1.3 Aspartate-442 confers a higher ability to transactivate gene expression to type 1 EBNA-2 transactivation domain in GAL4-gene reporter assays

The mechanism of differential activity of type 1 and type 2 EBNA-2 was then investigated by comparing the strength of transactivation function of type 1 and type 2 EBNA-2 TADs. The type 2 S442D and type 1 D442S EBNA-2 TADs were also tested for their ability to transactivate gene expression because the single serine to aspartate mutation in type 2 was shown to confer a type 1 ability to maintain LCL growth [210].

EBV-negative BJAB cells were transfected with plasmids expressing the GAL4-DBD:E2-TAD fusions (pcDNA3.1-GAL4-DBD:E2-TAD, section 2.3.3.13.3), specifically the TAD of type 1, type 2, type 2 S442D and type 1 D442S EBNA-2 fused to GAL4-DBD. A reporter plasmid carrying five GAL4 DNA-binding sites upstream of a luciferase gene (pFR-Luc, section 2.3.3.13.1) and a vector that expresses *Renilla* luciferase (pRL-CMV, section 2.3.3.13.1) were also cotransfected using the Neon transfection system. The same amount of DNA was used in each individual transfection. At 24 hours post-transfection, cell extracts were analysed for luciferase activity and results were normalised for transfection efficiency according to *Renilla* luciferase values.

The transient reporter assays clearly show that the transactivation function of type 1 EBNA-2 TAD is stronger than that of type 2 EBNA-2 (250-fold and 136-fold activation, respectively) (Figure 3.4 B). However, when the amino acid serine of type 2 TAD was changed by the equivalent aspartate 442 of type 1 (S442D), an increase in luciferase expression was observed and type 2 TAD acquired a transactivation phenotype similar to that of type 1 EBNA-2 TAD (236-fold induction). When the reciprocal substitution was assessed, replacing the aspartate 442 of type 1 TAD by the corresponding serine of type 2 (D442S), type 1 exhibited a reduced ability to activate luciferase expression (135-fold) and a type 2-like phenotype, in terms of transactivation ability, was acquired in type 1 EBNA-2 (Figure 3.4 B). These results indicate that the transactivation ability of type 1 TAD is 2-fold stronger than that of type 2 EBNA-2 TAD and the aspartate 442 of type 1, which was shown to be sufficient to confer a type 1 ability to maintain LCL growth to type 2, also gives a higher ability to transactivate gene expression.

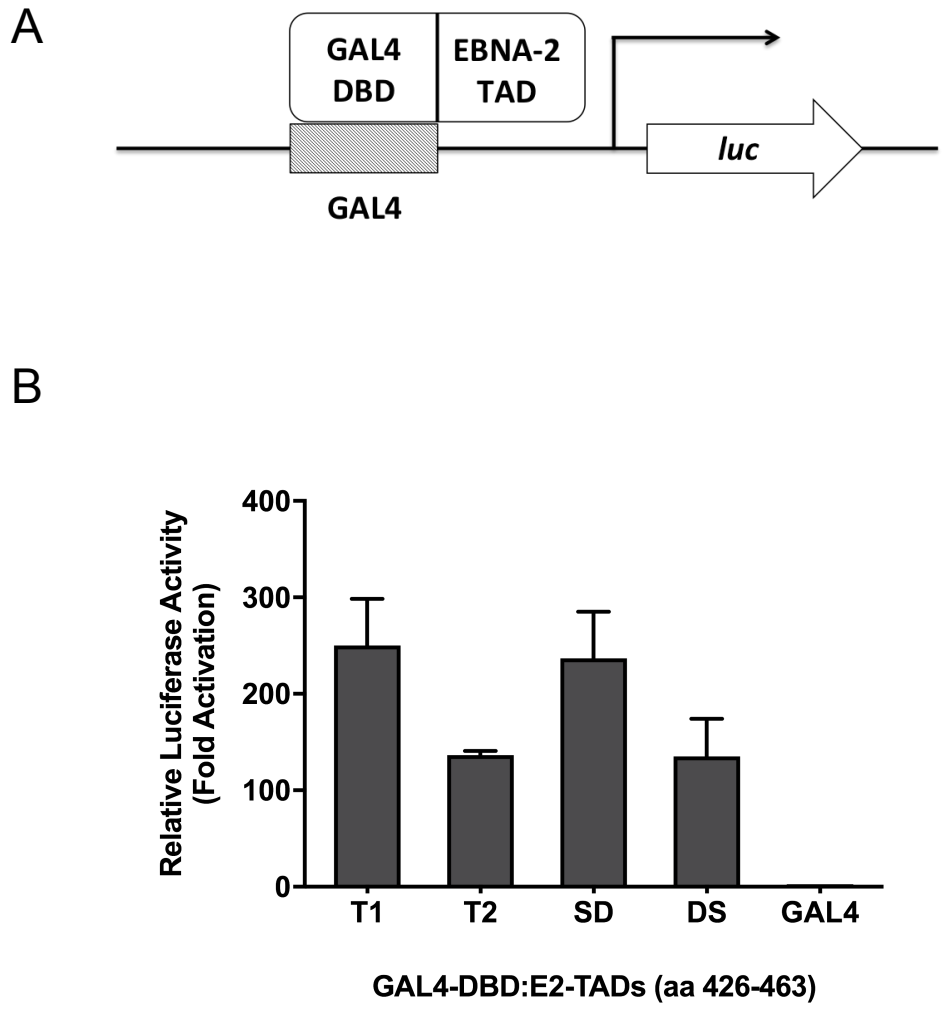


Figure 3.4 Amino acid aspartate-442 gives higher transactivation ability to type 1 EBNA-2

(**A**) Representation of the transactivation domain reporter assay: the GAL4-DBD:E2-TAD fusions bind to GAL4 DNA-binding sites upstream of a luciferase reporter and the EBNA-2 TAD mediates activation of gene expression. (**B**) 2 x 10^6 BJAB cells were cotransfected with 300 ng of type 1 (T1), type 2 (T2), type 2 S442D (SD) and type 1 D442S (DS) GAL4-DBD:E2-TAD constructs; 500 ng of pFR-Luc and 10 ng of pRL-CMV *Renilla* using the Neon transfection system. At 24 hours after transfection, cells were harvested and analysed for luciferase activity. For each sample, luciferase values were normalised for transfection efficiency based on *Renilla* luciferase expression values. Results are presented as luciferase activity relative to the pFR-Luc reporter plasmid with the empty vector pcDNA3.1-GAL4-DBD (GAL4). The results are presented as an average of three independent experiments (n=3). Error bars represent standard deviations.

3.2.2. Determining the effect of different amino acid substitutions in type 2 EBNA-2 TAD on the ability to transactivate gene expression

3.2.2.1 Generation of pcDNA3.1-GAL4 plasmids expressing the GAL4-DBD:E2-TAD constructs carrying different amino acid mutations in type 2 EBNA-2 TAD

Recent work from this laboratory [210] tested which amino acids of type 1 EBNA-2 conferred superior efficiency of LCL growth maintenance when compared to type 2 EBNA-2. Distinct amino acid substitutions in the TAD of type 2 EBNA-2 had been made and tested for their ability to maintain LCL growth in the EREB2.5 LCL growth maintenance assay [210] (Figure 3.5 A). Apart from the serine to aspartate 442 substitution in type 2 (S442D), the other type 1 amino acids replacing type 2 TAD amino acids that were tested in the EREB2.5 growth assay did not rescue cell growth maintenance [210].

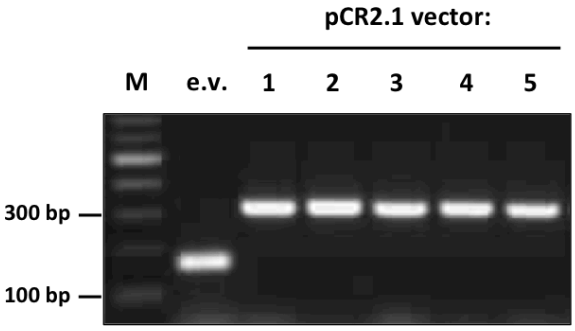
In the previous section of this study, it was demonstrated that the aspartate 442 conferred a stronger transactivation ability to type 1 EBNA-2 TAD (Figure 3.4 B). To determine whether other amino acids that differ between type 1 and type 2 TADs affect TAD function, the additional TAD mutants were also tested for transactivation ability, using the transient transfection reporter assays mentioned above.

Additional GAL4-DBD:E2-TAD constructs were made, using the mutated type 2 TADs (amino acids 426-463), which carry different amino acid substitutions (Figure 3.5 A, mutants 1 to 5). A similar approach was adopted to generate the new GAL4-DBD:E2-TAD constructs (section 3.2.1.1). pBlueScript plasmids, which express type 2 EBNA-2 proteins carrying the mutated TADs, were used as template to PCR amplify these five type 2 TAD mutants. Primers flanked by *Bam*HI and *Not*I restriction sites were used. PCR products were subcloned into pCR2.1 TA vector and transformants screened by PCR using T7-promoter and M13-reverse primers (Figure 3.5 B). The TAD fragments were cloned into the final pcDNA3.1-GAL4-DBD plasmid and positive clones were selected by PCR-screening, using T7-promoter and BGH-reverse oligonucleotide primers (Figure 3.5 C) and finally confirmed and validated by sequencing as before.

A


Type 1 EBNA-2 TAD aa 426-463
426
463
Type 1 TAD: HEPESHNSPEAPILFPDDWYPPSIDPADLDESWDYIFE
Type 2 TAD: HEPESSDSEEPPFLFPSDWYPPTLEPAELDESWEGIFE
1
2
3
4
5
SD431-432HN
EEP434-436PEA
F438I
S442D
G460Y

B


pCR2.1 vector:
M
e.v.
1
2
3
4
5
300 bp
100 bp

C

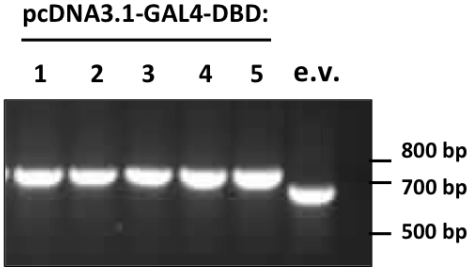

pcDNA3.1-GAL4-DBD:
1
2
3
4
5
e.v.
800 bp
700 bp
500 bp

Figure 3.5 Generation of pcDNA3.1-GAL4-DBD plasmids expressing type 2 GAL4-DBD:E2-TAD constructs carrying different amino acid mutations in type 2 EBNA-2 TAD

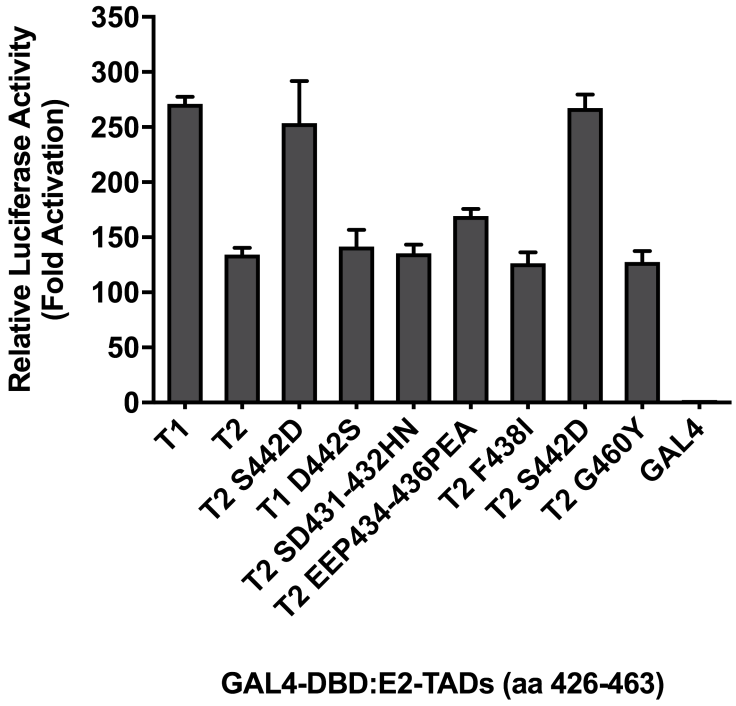
(**A**) Sequence alignment of type 1 and type 2 EBNA-2 TADs (amino acids 426-463). Five different type 2 TAD mutants were cloned into pcDNA3.1-GAL4-DBD plasmid fused to the GAL4-DBD. Amino acids of type 2 TAD were changed for the residue at the corresponding position in the type 1 TAD (shown in red). Substitutions were performed individually (mutants 3, 4 and 5) or for groups (mutants 1 and 2). Amino acids that differed but that were not mutated are represented in blue. (**B**) Representative gel electrophoresis of PCR products obtained from PCR-screening for positive transformants of type 2 TAD mutants cloned into pCR2.1 vector. T7-promoter and M13-reverse primers were used. The empty vector pCR2.1 was used as the negative control (e.v.). Expected size is 317 bp for the positive clones and 177 bp for the empty vector. (**C**) Representative gel electrophoresis of PCR products obtained from PCR-screening for the positive transformants of type 2 TAD mutants (aa 426-463) cloned into pcDNA3.1-GAL4-DBD vector. T7-promoter forward and BGH-reverse primers were used. The empty vector pcDNA3.1-GAL4-DBD was used as the negative control (e.v.). Expected size for the positive clones is 790 bp and for the empty vector is 650 bp. 1: type 2 TAD SD431-432HN; 2: type 2 TAD EEP434-436PEA; 3: type 2 TAD F438I; 4: type 2 TAD S442D and 5: type 2 TAD G460Y. M: 100 bp marker.

3.2.2.2 Aspartate-442 is the single amino acid of the type 1 TAD that confers a transactivation ability to type 2 TAD similar to that of type 1

The GAL4-DBD:E2-TAD fusions carrying different amino acid substitutions in type 2 EBNA-2 TAD were tested for their ability to activate gene expression. A transient transfection reporter assay similar to that performed before (section 3.2.1.2) was carried out using these type 2 TAD mutant constructs (Figure 3.5 A). The results (Figure 3.6 A) showed that, of the TAD mutants tested, only mutant 4, a type 2 TAD in which the serine was replaced by the corresponding aspartate of type 1 (T2 S442D), exhibited a stronger ability to transactivate gene expression, similar to that of type 1 TAD. This construct is independent of the GAL4-DBD:E2-TAD T2 S442D used in section 3.2.1.2 and was made again for the present experiment. These two independently derived type 2 S442D EBNA-2 TAD constructs still displayed the same type 1-like phenotype in terms of luciferase gene expression, showing that the amino acid aspartate-442 confers increased transactivation ability to the TAD of type 2 EBNA-2 (Figure 3.6 A) [210]. The other mutations (T2 SD431-432HN; T2 EEP434-436PEA; T2 F438I and T2 G460Y) did not give enhanced ability to activate gene expression and were about 2-fold weaker than that of the TAD of type 1. This is consistent with these four type 2 TAD mutants not sustaining LCL growth maintenance, in the EREB2.5 assay [210] and suggests that the transactivation ability might indeed be related with the capacity to maintain LCL growth proliferation.

The transactivation ability of the GAL4-DBD:E2-TAD fusion constructs, initially investigated in the EBV-negative B cell lymphoma BJAB cell line was also determined in the lymphoblastoid cell line IB4 [414] (Figure 3.6 B), using the same transient transfection reporter assays. The results obtained support the previous observations in BJAB cells. The activity of type 1 TAD was again found to be approximately 2-fold stronger than that of type 2 TAD. Additionally, S442D substitution increased the transactivation ability of type 2 almost to a level similar to that exhibited by type 1 TAD and the reverse D442S mutation in type 1 reduced its ability to activate luciferase expression and restored a type 2-like phenotype (Figure 3.6 B). The other TAD mutations did not increase the transactivation ability of type 2 TAD, supporting the results obtained in the BJAB cells.

A


Relative Luciferase Activity
(Fold Activation)
350
300
250
200
150
100
50
0
T1
T2
T2 S442D
T1 D442S
T2 SD431-432HN
T2 EEP434-436PEA
T2 F438I
T2 S442D
T2 G460Y
GAL4
GAL4-DBD:E2-TADs (aa 426-463)

B

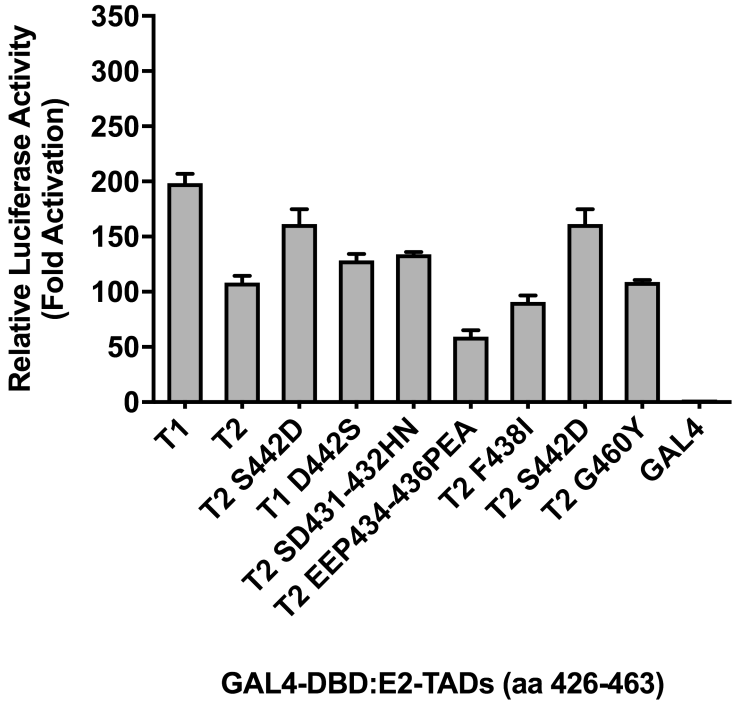
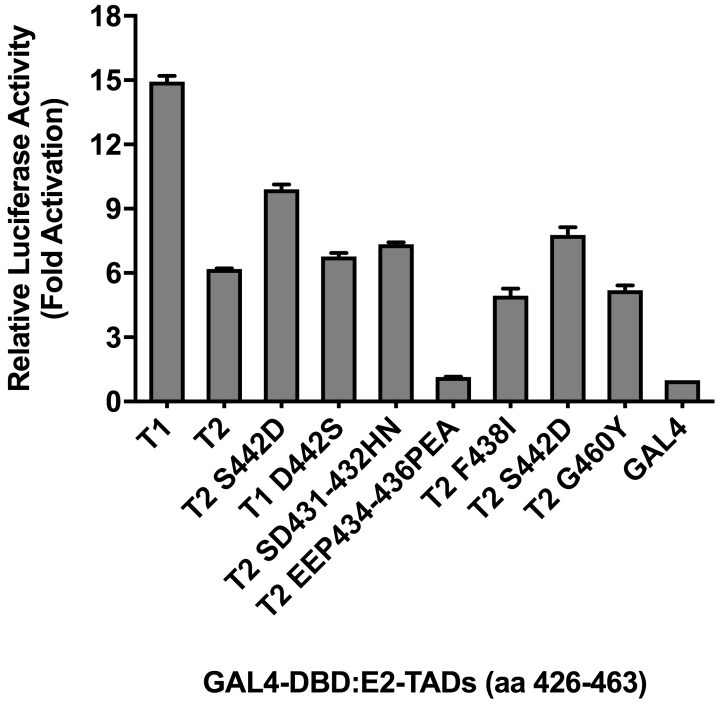

Relative Luciferase Activity
(Fold Activation)
350
300
250
200
150
100
50
0
T1
T2
T2 S442D
T1 D442S
T2 SD431-432HN
T2 EEP434-436PEA
T2 F438I
T2 S442D
T2 G460Y
GAL4
GAL4-DBD:E2-TADs (aa 426-463)

Figure 3.6 Aspartate-442 is the single amino acid of type 1 TAD that confers a transactivation ability to type 2 TAD similar to that of type 1

EBNA-2 TADs (amino acids 426-463) were expressed as fusion proteins with the GAL4 DBD and their ability to activate a synthetic promoter, consisting of GAL4 binding sites located upstream of a reporter luciferase gene, was determined. 2 x 10^6 (**A**) BJAB or (**B**) IB4 cells were cotransfected with 300 ng of one of the GAL4-DBD:E2-TAD plasmid constructs: type 1 (T1); type 2 (T2); T2 S442D; T1 D442S; T2 SD431-432HN; T2 EEP434-436PEA; T2 F438I; T2 S442D or T2 G460Y; (**A**) 500 ng or (**B**) 1000 ng of the pFR-Luc reporter plasmid and 10 ng of pRL-CMV *Renilla* using the Neon transfection system. At 24 hours post-transfection, cells were harvested and analysed for luciferase activity. Luciferase values obtained for each sample were normalised for transfection efficiency according to *Renilla* luciferase expression values. Results are presented as luciferase activity relative to the pFR-Luc reporter vector with the empty plasmid pcDNA3.1-GAL4-DBD (GAL4). Error bars represent three technical replicates. Adapted from [210].

The GAL4-DBD:E2-TAD constructs were all expressed from similar plasmid structures but it was difficult to control for the equivalent expression of the GAL4-DBD:E2-TAD fusions in the transfected BJAB or IB4 cells because the proteins were unstable and could not be detected by Western blotting of BJAB or IB4 cell extracts probed with an anti-GAL4-DBD antibody. Low transfection efficiency of these cell lines might also have contributed to the failure to detect the proteins. However, when the same plasmids were transfected into HEK293 cells, which are characterised by their higher transfection efficiency, and extracts were Western blotted for GAL4-DBD, the protein products were detected, but only the stable GAL4 part of the fusion protein could be observed (Figure 3.7 B). The TAD part of the fusion protein was not detected and was most likely degraded in the cell extracts, leaving the stable GAL4-DBD part. The levels of GAL4 detected were similar for all the proteins except for type 1 and type 2 S442D constructs. Nonetheless, the slightly lower levels of these strongly inducing plasmids were matched by lower levels of actin, indicating that the levels of all proteins are likely similar. Consistent with this observation, the transactivation ability of the GAL4-DBD:E2-TAD fusions was also determined in HEK293 cells, using the same transient transfection reporter assays (Figure 3.7 A). As expected, type 1 TAD displayed an enhanced transactivation ability when compared to type 2 TAD (2-fold stronger). Similarly, S442D conferred an increased ability to activate gene expression to type 2 TAD, but not to type 1 levels. The reciprocal D442S mutation in type 1 reduced its transactivation ability to the levels of type 2. The other substitutions did not give increased ability to activate luciferase expression to type 2 TAD (Figure 3.7 A).

A


Relative Luciferase Activity
(Fold Activation)
18
15
12
9
6
3
0
T1
T2
T2 S442D
T1 D442S
T2 SD431-432HN
T2 EEP434-436PEA
T2 F438I
T2 S442D
T2 G460Y
GAL4
GAL4-DBD:E2-TADs (aa 426-463)

B

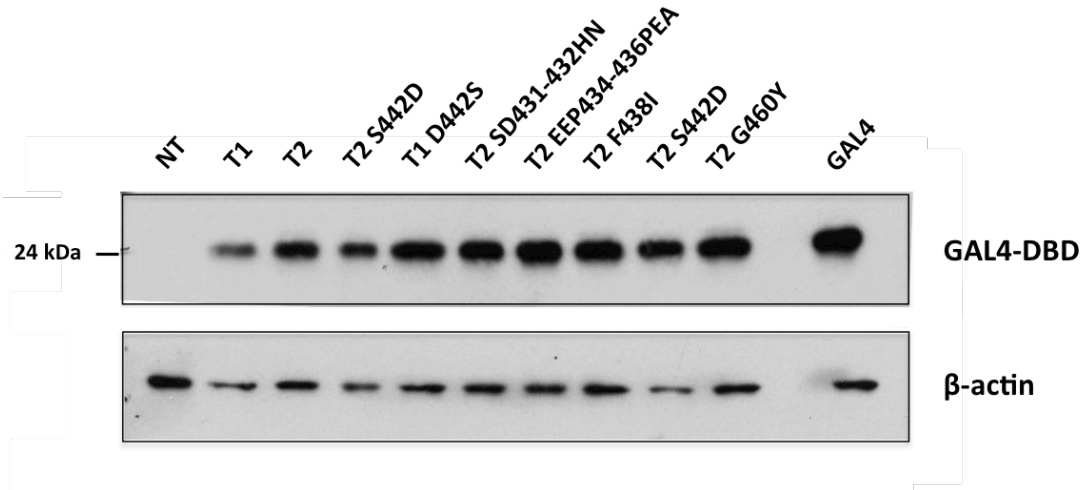

NT
T1
T2
T2 S442D
T1 D442S
T2 SD431-432HN
T2 EEP434-436PEA
T2 F438I
T2 S442D
T2 G460Y
GAL4
24 kDa
GAL4-DBD
β-actin

Figure 3.7 GAL4-TAD reporter assays carried out in high-transfection efficiency HEK293 cells

(**A**) 5 x 10^5 HEK293 cells were cotransfected with 300 ng of one of the GAL4-DBD:E2-TAD plasmid constructs: type 1 (T1); type 2 (T2); T2 S442D; T1 D442S; T2 SD431-432HN; T2 EEP434-436PEA; T2 F438I; T2 S442D or T2 G460Y; 150 ng of pFR-Luc reporter plasmid and 1 ng of pRL-CMV *Renilla* plasmid using the Lipofectamine™ 2000 system. 24 hours post-transfection, cells were harvested and cell extracts were analysed for luciferase activity. Luciferase values were normalised for transfection efficiency based on *Renilla* luciferase expression values. Results are presented as luciferase activity relative to the pFR-Luc reporter vector with the empty plasmid pcDNA3.1-GAL4-DBD (GAL4). Error bars represent three technical replicates. (**B**) Western blot of HEK293 cells transfected with pcDNA3.1-GAL4-DBD plasmids expressing the TAD constructs. Protein extracts were compared by immunoblot analysis using the anti-GAL4-DBD antibody. Immunoblotting analysis for β-actin as protein loading control. NT: non-transfected HEK293 cells; T1: type 1 and T2: type 2.

3.2.3 Investigating the effect of the equivalent serine to aspartate substitution in the TAD of the intracellular domain of human Notch

EBNA-2 and the intracellular domain of human Notch (N-ICD) are often considered functional homologues because both interact with the RBP-Jk protein to regulate gene expression [254, 415]. This cellular factor can mediate transcriptional repression at its target gene promoters by recruiting the histone deacetylase complex. The TAD of EBNA-2 and N-ICD interact with the transcriptional repression domain of RBP-Jk and replace the histone deacetylase complex to activate gene expression [416]. EBNA-2 displays a function similar to that of a constitutively active N-ICD. Previous work has shown that both EBNA-2 and N-ICD were able to activate expression of a luciferase reporter under control of a β-globin minimal promoter composed of six binding sites for RBP-Jk located upstream [417]. Additionally, N-ICD was found to be able to activate expression of EBNA-2 target gene promoters in B cells, such as the Cp and the LMP-2A promoters, although to a lower extent than EBNA-2 [253]. N-ICD was also shown to only transiently maintain B cell proliferation in the absence of EBNA-2. Partial rescue of cell proliferation is only achieved when the LMP-1 gene is expressed independently of EBNA-2 or the activated N-ICD is expressed at very high levels [255].

The intracellular domain of Notch receptor is composed of several domains that have distinct functions: an RBP-Jk associated molecule (RAM) domain, which is involved in N-ICD binding to RBP-Jk [418, 419], a nuclear localisation signal linking the RAM domain to seven ankyrin repeats (ANK domain) involved in different protein-protein interactions [420-422], followed by an additional bipartite nuclear localisation sequence. The C-terminal end of N-ICD consists of a loosely defined transactivation domain (TAD), including a polyglutamine-rich stretch (OPA) conserved between *Drosophila* Notch and mammalian Notch1 and a terminal PEST domain, (Figure 3.8), which contains proline, glutamate, serine and threonine-rich motifs that harbor degradation signals [250], important for Notch protein turnover [423, 424].

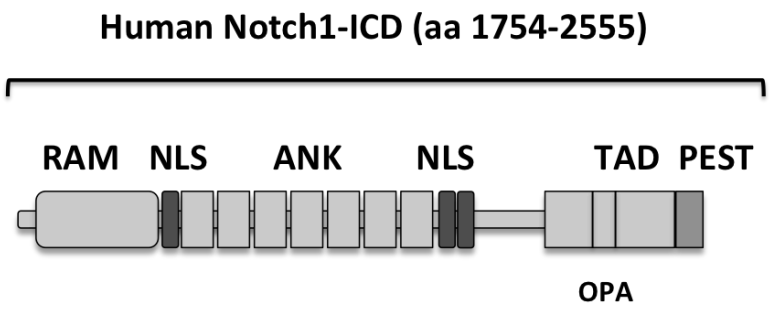


Figure 3.8 Structure of human Notch1 Intracellular domain (Notch1-ICD)

Notch-1 intracellular domain (Notch1-ICD) is composed of several functional domains: the RBP-Jk-associated molecule (RAM) domain linked to the ANK domain by a nuclear localisation signal (NLS). An additional bipartite nuclear localisation signal (NLS) precedes a transactivation domain (TAD), which contains a polyglutamine-rich stretch (OPA) and a C-terminal PEST domain (proline, glutamate, serine and threonine motifs). Adapted from [250, 425].

Comparative analysis of amino acid sequences of the TAD of EBNA-2 and the PEST domain of different N-ICD receptors (Notch1, Notch2 and Notch3) has demonstrated a high level of similarity between the proteins. A common motif of 15 amino acids was identified, in which seven residues were identical. One of these amino acids is the serine of type 2 EBNA-2 TAD that is equivalent to the aspartate 442 of type 1 TAD (Figure 3.9). In this study, the ability of human N-ICD to transactivate gene expression was compared to that of the TAD of type 1 and type 2. The effect of a similar serine to aspartate substitution in the TAD of Notch1-ICD (S2513D) was assessed and compared to the S442D in type EBNA-2.

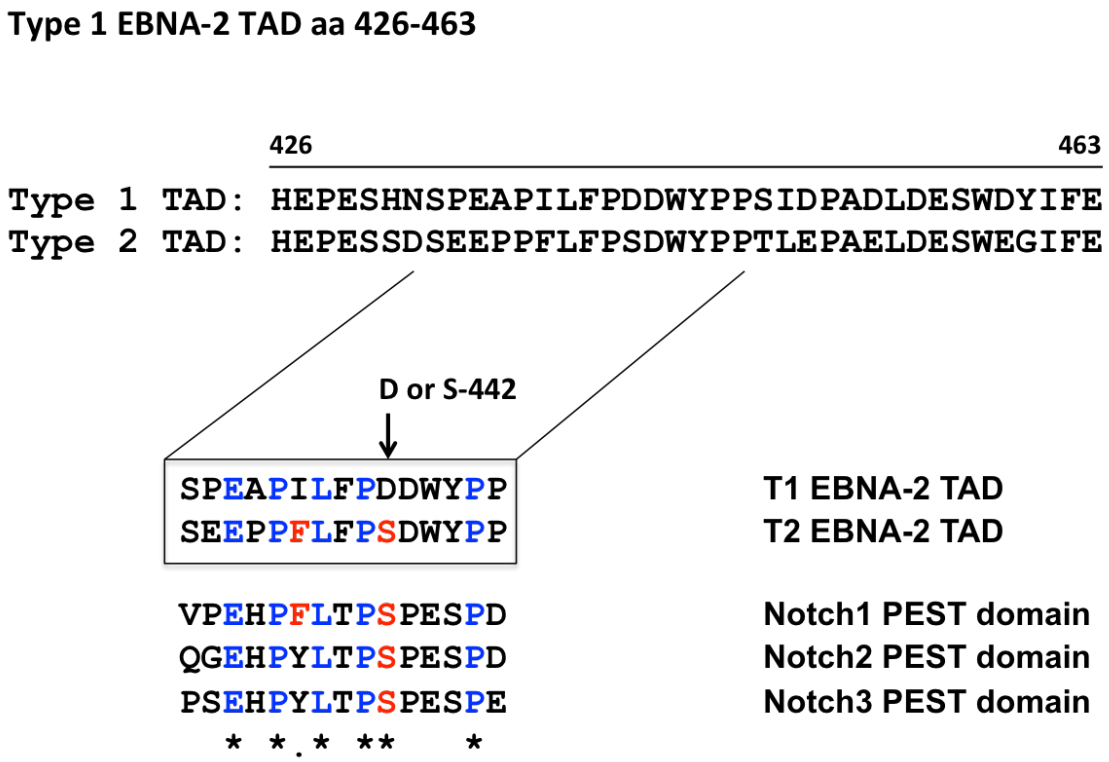


Figure 3.9 Alignment of the sequences of type 1 and type 2 TADs and Notch PEST domains

An amino acid sequence motif comprising 15 residues of type 1 and type 2 EBNA-2 TADs (amino acids 433-447) was compared to a similar peptide motif in the PEST domain of human Notch (amino acids 2504-2518). Amino acids represented in blue are common to EBNA-2 TADs and Notch PEST domains (given by *) in all five sequences. The residues represented in red are common between the Notch PEST domain and the TAD of type 2 EBNA-2. The arrow indicates the position of the amino acid aspartate 442 in type 1 and the serine in type 2 EBNA-2 TAD. Amino acids conserved in all five sequences with similar side chain chemistry (.).

3.2.3.1 Generating GAL4-DBD:PEST domain, GAL4-DBD:OPA-PEST and GAL4-DBD:OPA-PEST S2513D fusion proteins to use in GAL4-reporter assays

Earlier reports using mouse Notch1-ICD have determined that the C-terminal half of N-ICD transactivated twice as strongly as the full-length construct and the TAD of N-ICD was firstly assigned to the fragment between the ANK repeats and the PEST domain and also includes the glutamine-rich region (OPA) [422]. More recent work has determined that the transactivation domain also included the PEST domain, because the fragment without the PEST region was significantly less active in terms of its transactivation potential, indicating that it contributes to the TAD of N-ICD [426]. In addition, studies where these regions were deleted corroborated that the C-terminal protein containing the OPA and PEST sequences was important for the ability of N-ICD to activate gene expression [427, 428].
In the present study, a fragment of human Notch1-ICD (amino acids 2270-2555), equivalent to the region of the mouse Notch1-ICD shown to include the TAD in the work performed by Dumont and colleagues [426] was used to compare the transactivation ability of Notch to that of type 1 and type 2 EBNA-2. A smaller fragment corresponding to amino acids 2401 to 2555 was also tested (Figure 3.10).

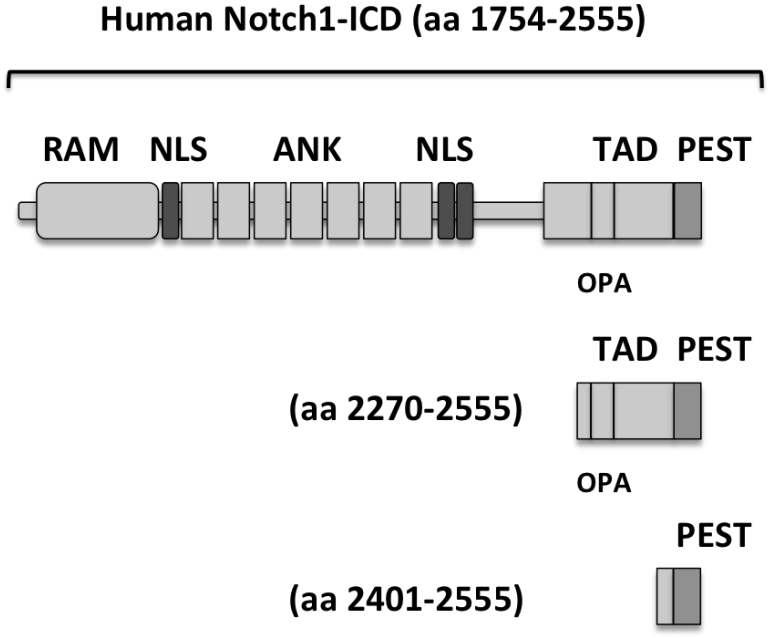
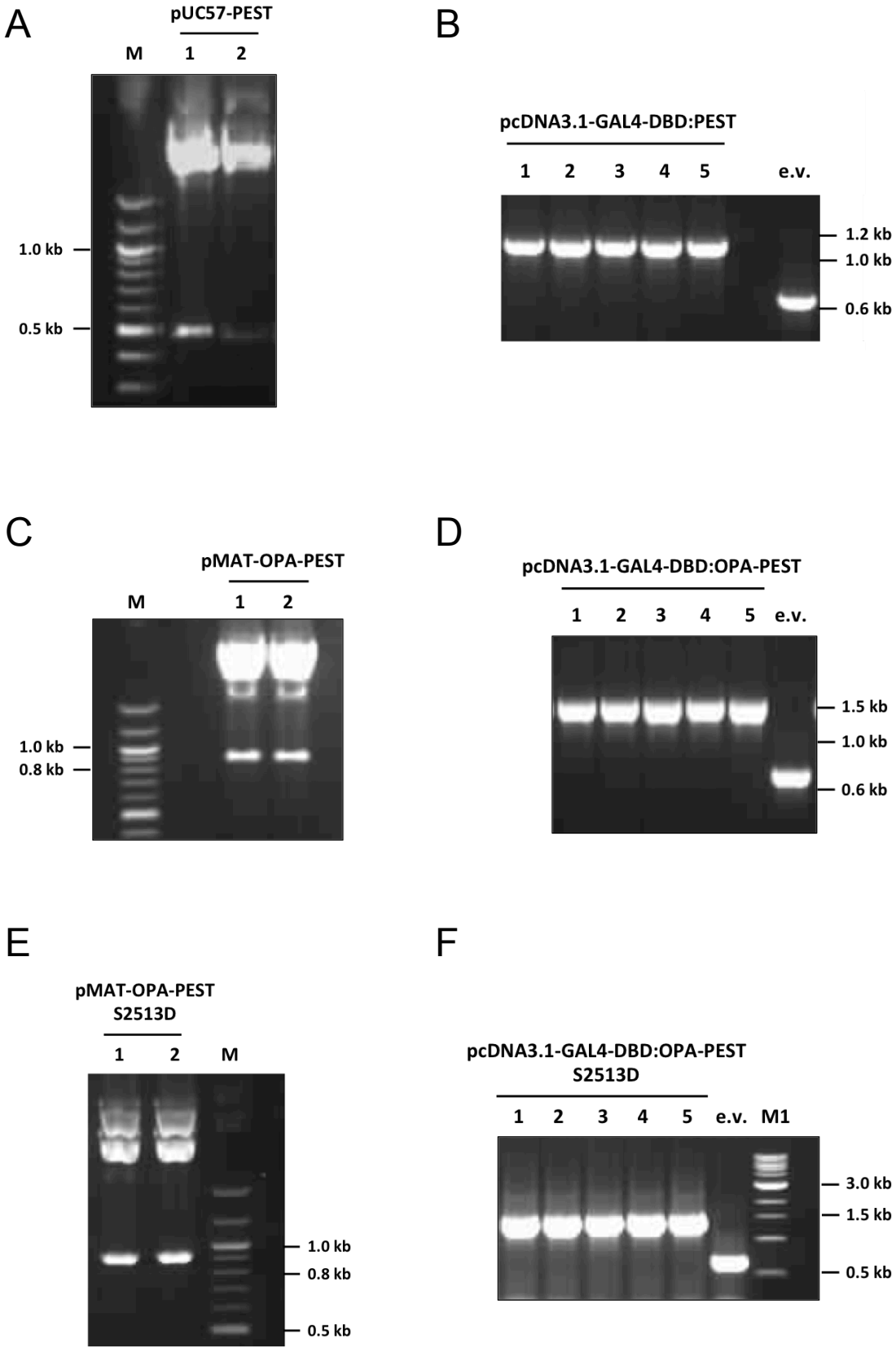


Figure 3.10 Human Notch1-ICD fragments cloned into pcDNA3.1-GAL4-DBD

Two distinct human Notch1-ICD fragments were cloned into pcDNA3.1 plasmids fused to the GAL4-DBD. The Notch1-ICD fragments correspond to the human Notch OPA-PEST domains (amino acids 2270 to 2555) and the Notch PEST domain (amino acids 2401 to 2555). Notch1-ICD comprises the amino acids 1754 to 2555. RAM: RBP-Jk-associated molecule domain; NLS: nuclear localisation signal; ANK: ankyrin repeats; TAD: transactivation domain; OPA: polyglutamine-rich stretch and PEST: proline (P), glutamate (E), serine (E) and threonine (T) motifs.

To generate GAL4-DBD:PEST (amino acids 2401-2555) and GAL4-DBD:OPA-PEST (amino acids 2270-2555) constructs, the fragments were cloned into pcDNA3.1-GAL4-DBD vector fused to the GAL4-DBD. These fragments were initially chemically synthesised in two distinct vectors (pUC57-PEST and pMAT-OPA-PEST plasmids – Invitrogen, UK) and carried *Bam*HI and *Not*I restriction sites flanking their terminal ends to facilitate the excision of the fragments by enzyme digestion. The resultant products of approximately 500 bp and 900 bp (PEST and OPA-PEST fragments respectively) (Figure 3.11 A and C) were then cloned into *Bam*HI-*Not*I cut pcDNA3.1-GAL4-DBD empty vector. Positive clones were screened by PCR, using T7-promoter and BGH-reverse primers (Figure 3.11 B and D) and validated by sequencing.

To create the GAL4-DBD:OPA-PEST fusion construct carrying the S2513D mutation similar to the S442D substitution in type 2 EBNA-2 TAD, Site-Directed Mutagenesis (SDM) reaction was performed using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies). Using the chemically synthesised pMAT-OPA-PEST plasmid as template, SDM-PCR was carried out (section 2.3.3.12). PCR products obtained from the SDM reaction were digested with *Dpn*I enzyme and the amplified plasmid DNA was transformed into competent *Escherichia coli* XL1 Blue cells. The SDM-originated pMAT-OPA-PEST S2513D plasmid was then digested with *Bam*HI and *Not*I enzymes (Figure 3.11 E) and the resultant fragment of around 900 bp was cloned into *Bam*HI-*Not*I cut pcDNA3.1-GAL4-DBD. PCR was used to screen the positive transformants as before (Figure 3.11 F). The final plasmid constructs were subjected to DNA sequencing to confirm whether the specific substitution had been introduced successfully.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pUC57-PEST
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2
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pcDNA3.1-GAL4-DBD:PEST
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2
3
4
5
e.v.
1.2 kb
1.0 kb
0.6 kb
C
pMAT-OPA-PEST
M
1
2
1.0 kb
0.8 kb
D
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1
2
3
4
5
e.v.
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E
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S2513D
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S2513D
1
2
3
4
5
e.v.
M1
3.0 kb
1.5 kb
0.5 kb

Figure 3.11 Cloning PEST (amino acids 2401-2555), OPA-PEST and OPA-PEST S2513D (amino acids 2270-2555) fragments into pcDNA3.1-GAL4-DBD

Notch-ICD PEST (amino acids 2401-2555) and OPA-PEST (amino acids 2270-2555) fragments were chemically synthesised in pUC57 and pMAT vectors respectively, carrying *Bam*HI and *Not*I restriction sites at each end. (**A** and **C**) Digestion of pUC57-PEST and pMAT-OPA-PEST plasmid to excise the corresponding fragments of approximately 500 bp and 900 bp. (**E**) The pMAT-OPA-PEST plasmid was used as template in a SDM reaction carried out to introduce the serine-2513 to aspartate substitution (S2513D). The mutated OPA-PEST S2513D fragment was excised from pMAT vector by restriction digestion with *Bam*HI and *Not*I enzymes. (**B**, **D** and **F**) PEST, OPA-PEST and OPA-PEST S2513D fragments were cloned into pcDNA3.1-GAL4-DBD and positive transformants were screened by PCR using T7-promoter and BGH-reverse primers. Representation of five colonies tested (1-5). Expected size for positive clones is around 1.1 kb for PEST and 1.5 kb for OPA-PEST and OPA-PEST S2513D fragments. The empty vector pcDNA3.1-GAL4-DBD was used as negative control (e.v.) - expected size is around 650 bp. M: 100 bp marker. M1: 1 kb marker.

3.2.3.2 Serine to aspartate substitution (S2513D) in the TAD of human Notch1-ICD gives a higher transactivation ability

The ability of human N-ICD to transactivate gene expression was then compared to that of the TAD of type 1 and type 2. The effect of a serine to aspartate substitution (S2513D) in the TAD of human Notch1-ICD was also assessed using a transient transfection reporter assay similar to that performed in section 3.2.1.3. EBV-negative BJAB cells were transfected with plasmids that express the GAL4-DBD:PEST, GAL4-DBD:OPA-PEST and GAL4-DBD:OPA-PEST S2513D fusion constructs (section 2.3.3.13.7). A luciferase reporter plasmid (pFR-Luc, section 2.3.3.13.1), activated by a synthetic promoter with five GAL4 DNA-binding sites, and a vector that expresses *Renilla* luciferase (pRL-CMV, section 2.3.3.13.1) were also cotransfected using the Neon transfection system. After 24 hours, cell extracts were analysed for luciferase activity and results were normalised for transfection efficiency according to *Renilla* luciferase values.

The results showed that the ability of type 1 and also of type 2 EBNA-2 TAD to activate gene expression is much stronger than the transactivation ability of Notch1-ICD fragments tested in this study. The PEST domain alone displayed a residual ability to activate gene expression (only 3-fold activation), whereas the fragment that includes the OPA-PEST domains (amino acids 2270-2555) exhibited a higher luciferase transactivation (38-fold activation). This region had been shown to display a stronger transactivation potential in earlier studies by Dumont and colleagues [426], but still to a lower extent even when compared to the TAD of the type 2 EBNA-2 (Figure 3.12). Remarkably, when the serine-2513 in the OPA-PEST fragment was replaced by an aspartate, in an amino acid substitution identical to the S442D in type 2 TAD, there was an approximately 2-fold increase in the ability of OPA-PEST to activate luciferase expression (62-fold activation). A similar 2-fold increase had been observed for the S442D mutation in type 2 TAD, when compared to the wildtype type 2 TAD. (Figure 3.4 B)

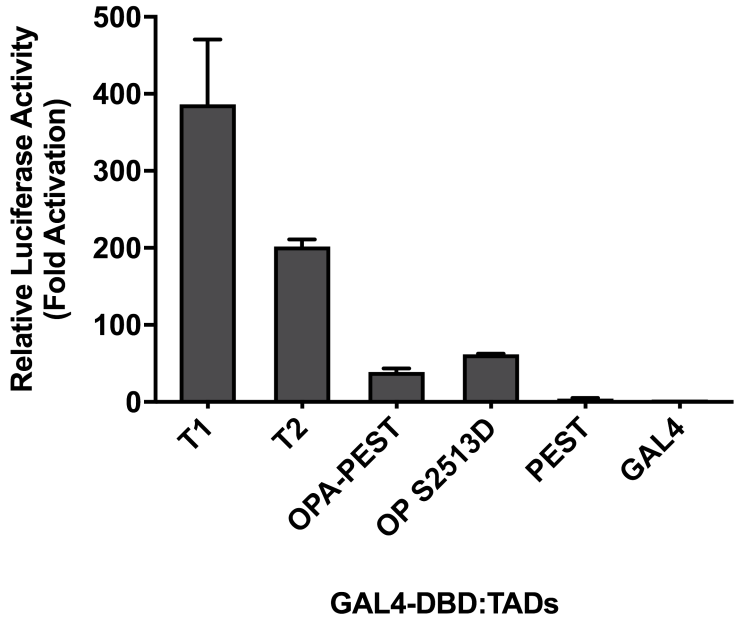
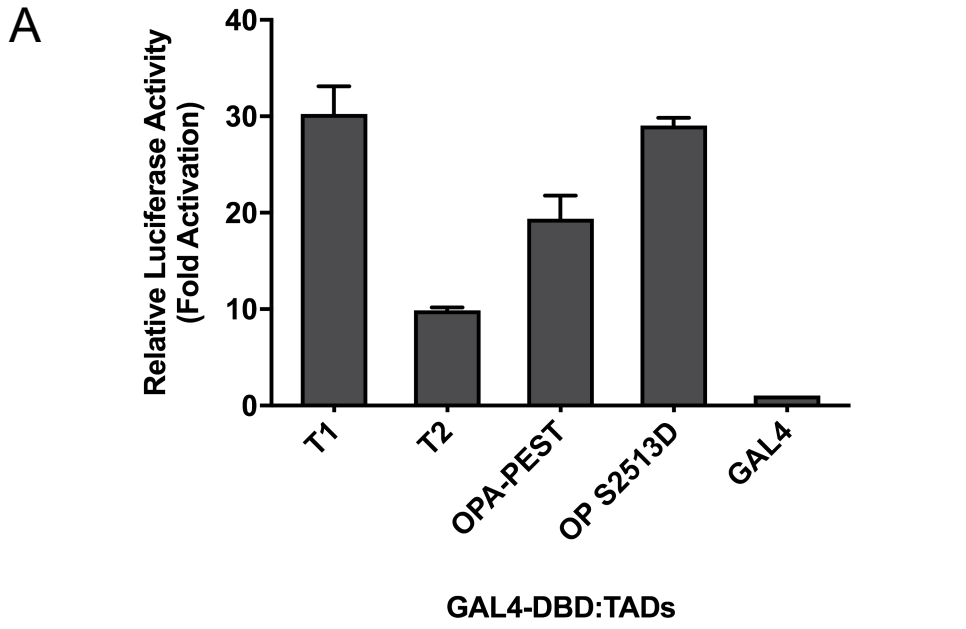


Figure 3.12 Serine to aspartate substitution (S2513D) in the TAD of human Notch1-ICD confers higher transactivation ability to Notch OPA-PEST

2 x 10^6 BJAB cells were cotransfected with 300 ng of type 1 GAL4-DBD:E2-TAD (T1), type 2 GAL4-DBD:E2-TAD (T2), GAL4-DBD:PEST (PEST), GAL4-DBD:OPA-PEST (OPA-PEST) and GAL4-DBD:OPA-PEST S2513D (OP S2513D) expressing plasmids; 500 ng of pFR-Luc reporter and 10 ng of pRL-CMV *Renilla* using the Neon transfection system. At 24 hours after transfection, cells were harvested and analysed for luciferase activity. For each sample, luciferase values were normalised for transfection efficiency based on *Renilla* luciferase values. Results are presented as luciferase activity relative to the pFR-Luc reporter plasmid with the empty vector pcDNA3.1-GAL4-DBD (GAL4). Error bars represent three technical replicates.

These results were confirmed in HEK293 cells in order to control for the expression levels of the GAL4-DBD constructs. Again the S2513D mutation in the OPA-PEST fragment increased its ability to transactivate luciferase gene expression (Figure 3.13 A). Western blot analysis indicated that these GAL4-DBD fusions are unstable and likely degraded in the cell extracts as mentioned above for the GAL4-DBD:E2-TAD fusions. This observation was confirmed when extracts containing either GAL4-DBD:OPA-PEST or GAL4-DBD-OPA-PEST S2513D fusion proteins were probed with the anti-GAL4-DBD antibody. The full-length fusion construct is detected (top band of around 52 kDa), but almost completely degraded, leaving only the stable GAL4-DBD part of the fusion protein (approximately 24 kDa) (Figure 3.13 B).



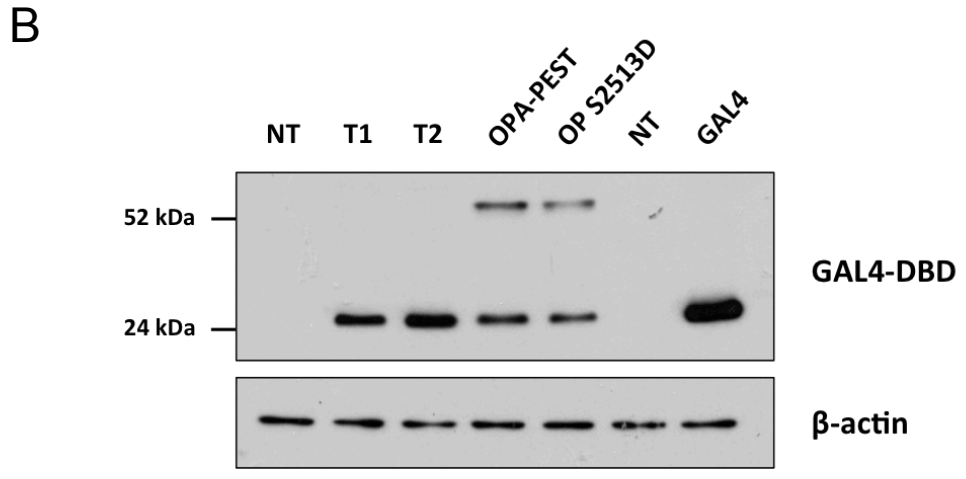


Figure 3.13 S2513D substitution in OPA-PEST fragment of human Notch1-ICD confers stronger ability to activate a luciferase reporter in HEK293 cells

(**A**) 5 x 10^5 HEK293 cells were cotransfected with 300 ng of type 1 GAL4-DBD:E2-TAD (T1), type 2 GAL4-DBD:E2-TAD (T2), GAL4-DBD:OPA-PEST (OPA-PEST) or GAL4-DBD:OPA-PEST S2513D (OP S2513D) expressing plasmids; 150 ng of pFR-Luc reporter plasmid and 1 ng of pRL-CMV *Renilla* plasmid using the Lipofectamine™ 2000 system. 24 hours post-transfection, cells were harvested and cell extracts were analysed for luciferase activity. Luciferase values were normalised for transfection efficiency based on *Renilla* luciferase expression values. Results are presented as luciferase activity relative to the pFR-Luc reporter vector with the empty plasmid pcDNA3.1-GAL4-DBD (GAL4). Error bars represent three technical replicates. (**B**) Western blot analysis of HEK293 cells transfected with pcDNA3.1-GAL4-DBD plasmids expressing the respective fusion constructs. Protein extracts were compared by immunoblot analysis using the anti-GAL4-DBD antibody. Immunoblotting analysis for β-actin as protein loading control. NT: non-transfected HEK293 cells, T1: type 1; T2: type 2; OP S2513D (OPA-PEST S2513D).

Collectively, the results indicated that both the S442D substitution in type 2 EBNA-2 TAD and the S2513D mutation in Notch1-ICD OPA-PEST region of the TAD increased approximately 2-fold their ability to activate gene expression in GAL4-DBD reporter assays in the cell lines tested. This corroborates the importance of the aspartate of type 1 TAD to confer a superior transactivation function when compared to type 2 TAD and might also suggest an identical underlying mechanism explaining this increase in gene activation.

3.2.4 Determining the effect of a serine to alanine mutation (S442A) in the viral LMP-1 gene transactivation by type 2 EBNA-2 TAD

The amino acid serine to aspartate substitution in type 2 EBNA-2 TAD (S442D) and in the Notch1-ICD OPA-PEST TAD (S2513D) increased their transactivation function around 2-fold. This observation suggested that a similar mechanism could be governing this increase in the ability to activate gene expression. A possible physiological phosphomimetic mechanism has been considered. To address this hypothesis, expression of the viral LMP-1 gene in the EBV-positive BL Daudi cell line [407, 408] was determined for a type 2 EBNA-2 protein carrying a serine to alanine substitution (T2 S442A) to prevent potential phosphorylation of the amino acid serine 442. Different pHEBo plasmid vectors expressing type 1, type 2, type 2 S442D or type 2 S442A EBNA-2 proteins under control of an inducible metallothionein (MT) promoter, previously made by Claudio Elgueta and Stelios Tzellos, were already available to use in the laboratory (section 2.3.3.13.5: pHEBo-MT:E2T1, pHEBo-MT:E2T2, pHEBo-MT:E2T2 S442D and pHEBo-MT:E2T2 S442A).

Daudi cells were transfected with these plasmids using the Neon transfection system. EBNA-2 expression from the MT promoter was induced with $CdCl_2$ 5 hours after transfection. At 24 hours post-transfection, total cell protein extracts were made using RIPA extraction method (section 2.5.1) and analysed by immunoblotting and probed with the PE2 antibody to detect EBNA-2 expression and with the anti-LMP-1 antibody (section 2.5.7). The results showed, as expected, that type 1 EBNA-2 activates LMP-1 expression more strongly than type 2 EBNA-2 (Figure 3.14). The viral LMP-1 gene is one of the genes differentially regulated by type 1 and type 2 [209, 210]. The S442D substitution confers a higher LMP-1 transactivation to type 2, but still less that LMP-1 induction by type 1. Strikingly, serine to alanine substitution in type 2 (T2 S442A), which has been performed to impede a potential phosphorylation of the serine 442, did not prevent LMP-1 transactivation by type 2 EBNA-2. In fact, the S442A mutation slightly increased type 2 EBNA-2 ability to induce LMP-1 expression (Figure 3.14). This observation suggests that the mechanism underlying the serine to aspartate (S442D) phenotype in type 2 EBNA-2 is unlikely to involve a phosphomimetic substitution of the serine 442, which would work as a constitutively phosphorylated residue.

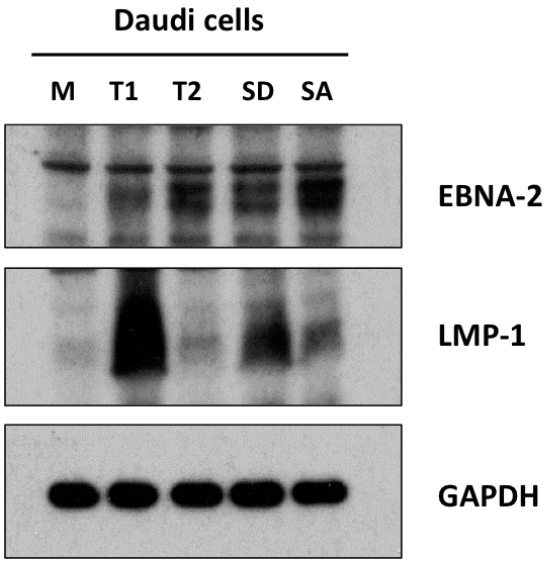


Figure 3.14 Effect of a serine to alanine (S442A) substitution in LMP-1 gene expression by type 2 EBNA-2 in EBV-positive Daudi cells

EBV-positive Daudi cells were transfected with 5 µg of pHEBo-MT:E2T1 (T1, type 1 EBNA-2), pHEBo-MT:E2T2 (T2, type 2 EBNA-2), pHEBo-MT:E2T2 S442D (SD, type 2 EBNA-2 S442D) and pHEBo-MT:E2T2 S442A (SA, type 2 EBNA-2 S442A) using the Neon transfection system. EBNA-2 expression from the metallothionein promoter was induced with 5 µM $CdCl_2$ at 5 hours after transfection. Cells were harvested at 24 post-transfection and protein extracts were obtained by RIPA lysis. Expression levels of the EBNA-2 proteins were determined by western blotting analysis using the PE2 anti-EBNA-2 antibody that equally recognises both types. LMP-1 expression levels were assessed using the anti-LMP-1 antibody (CS1-4 antibody). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) immunoblot analysis ensured that equal amounts of proteins were loaded on the gel. M: mock transfected cells.

3.3 Discussion

The most relevant characteristic that distinguishes type 1 from type 2 EBV strains is that type 1 EBV transforms and immortalises human B cells into lymphoblastoid cell lines *in vitro* much more efficiently than type 2 strains [296]. This different capacity was assigned to the EBNA-2 locus, which defines the EBV viral types, when a recombinant type 2 P3HR1 EBV strain that carried a type 1 EBNA-2 sequence acquired a type 1 immortalisation phenotype [134]. Type 1 and type 2 EBNA-2 exhibit only approximately 56% identity at the amino acid level [213, 214], constituting the most divergent locus of the genome between the EBV viral types. Previously published studies have determined the regions or domains of type 1 EBNA-2 responsible for superior B cell transformation, using the EREB2.5 *trans*-complementation assay [136, 206, 208, 209, 230, 231]. The minimum type 1 sequences initially found to be required to confer to type 2 EBNA-2 the capacity to maintain growth of EREB2.5 LCL cells were the RG, CR7 and TAD domains located in the C-terminal end of the EBNA-2 protein [209]. However, a recent report from this laboratory demonstrated that a single amino acid of type 1 EBNA-2 protein could determine type-specific EBNA-2 function. The substitution of the serine in type 2 EBNA-2 by the equivalent aspartate 442 of type 1 (S442D) was sufficient to confer to type 2 EBNA-2 the ability to maintain cell growth in the EREB2.5 assay [210]. This amino acid aspartate 442 lies in the transactivation domain of type 1 EBNA-2 protein, suggesting that a stronger ability to mediate transactivation of target genes expression by type 1 EBNA-2 could contribute to the mechanism underlying the superior capacity to transform and immortalise human B cells into LCLs *in vitro* by type 1 EBV.

In the present study the transactivation function of the TAD of type 1 and type 2 EBNA-2 was assessed by fusing the EBNA-2 TADs to the GAL4-DBD and measuring the ability to activate a synthetic promoter with GAL4 DNA binding sites in luciferase reporter assays. This system had been initially used to identify the TAD of type 1 EBNA-2 [216]. The results obtained in this chapter showed that indeed the transactivation ability of type 1 TAD is 2-fold stronger than that of type 2 EBNA-2 (Figure 3.4 B), as opposed to an earlier study that had found that type 1 and type 2 EBNA-2 TADs exhibited similar ability to activate gene transcription using CAT assays [229]. Remarkably, the serine to aspartate substitution that had been able to confer to type 2 EBNA-2 (S442D) the ability to sustain LCL growth in the EREB2.5 assay [210], also increased the transactivation function of the TAD of type 2 EBNA-2 to a type 1-like level. On the contrary, the reciprocal D442S change in type 1 TAD reduced its

transactivation function and an ability to activate gene expression similar to that of type 2 EBNA-2 TAD was restored (Figure 3.4 B). Different amino acids substitutions in the TAD of type 2 EBNA-2 were tested (Figure 3.5 A) for their ability to increase the transactivation function of type 2 TAD but none of these mutations promoted an augmented transactivation ability (Figure 3.6). Interestingly, these amino acid changes also did not increase LCL growth in the EREB2.5 assay [210]. These observations confirmed that aspartate 442 is the unique amino acid that conferred an increased transactivation function to the TAD of type 2 EBNA-2 among the amino acids that were tested, indicating that the stronger ability to activate gene expression of type 1 EBNA-2 is mediated by aspartate 442. Therefore, the higher activation of EBNA-2 target genes, which are required for B cell transformation and immortalisation into LCLs, is related to the superior transforming ability by type 1 EBV. However, this small 2-fold difference detected in reporter assays seemed to be insufficient to explain the distinct gene regulation by type 1 and type 2 EBNA-2 and other contributing mechanism might be involved. In fact, this is supported by the previous microarray analysis performed in this laboratory demonstrating that the differentially regulated genes exhibited a 4 or 5-fold stronger induction by type 1 EBNA-2 [206]. Another report confirmed a differential gene upregulation by type 1 greater than the 2-fold observed in this study [209]. Therefore, this suggests that the higher induction of these genes cannot be only by explained by the modest increase in the transactivation ability of the TAD of EBNA-2 determined by aspartate 442 in type 1. Also it did not explain why only a few genes are differentially regulated by the EBNA-2 types.

EBNA-2 is a crucial protein required for B cell transformation [133, 134] and this capacity is linked to its ability to activate expression of viral and cell genes mediated by the TAD [216, 429, 430]. In fact, the TAD of EBNA-2 was earlier shown to be essential for B cell immortalisation, because viruses lacking this region are transformation-incompetent [216, 227]. Once EBNA-2 is tethered to DNA, the TAD recruits many transcriptional factors, histone acetyltransferases and chromatin remodelling complexes. Specifically, the transcriptional factors TAF40, TFIIB [259], TFIIH [260], the co-activator p100 that interacts with TFIIE [261] and also the histone acetyltransferases p300/CBP and PCAF [258] associate with the TAD of EBNA-2 but also with other human and viral TADs, such as those of the human tumor suppressor protein p53 and the TAD of Herpes simplex VP16 acidic domain to which EBNA-2 TAD is similar [214, 229]. A recent report characterised the interaction with EBNA-2 TAD and transcription factor TFIIH. The amino acids 448-471 from EBNA-2 TAD mediate the interaction between type 1 EBNA-2 and the Tfb1/p62 subunit of

TFIIH and the hydrophobic residues W458, I461 and F462 were shown to be important [431]. Similarly, the KIX domain of p300/CBP utilises the same region of EBNA-2 for interaction [431]. Therefore, the activation of target genes by EBNA-2 relies on these protein interactions mediated by the TAD. Accordingly, a W458T change in the TAD of EBNA-2 that disrupted binding to TFIIB, TFIIH, TAF40 and p300/CBP caused a reduction of the transactivation function [258-260]. Thus, it can be argued that a distinct ability of type 1 and type 2 EBNA-2 TADs to recruit these transcription factors might also contribute for their different transactivation ability and for the differential type 1/type 2 gene regulation. It is possible that the acidic residue aspartate 442 of type 1 EBNA-2 could foster the protein to protein interaction function of the TAD and stabilize the assembly of transcription complexes with the mentioned transcriptional factors, contributing for the higher transactivation function of type 1 EBNA-2. However, the recent identification of the ETS/IRF composite motif at the EBNA-2 binding sites at the LMP-1 and other cellular gene promoters that are more strongly activated by type 1 EBNA-2 [210], suggested that specific factors that cooperate with EBNA-2, such as PU.1, are required as key determinants for the differential gene regulation by type 1 and type 2 EBNA-2. This is supported by the differential LMP-1 expression by the EBNA-2 types, which mostly requires other transcription factors (PU.1) than RBP-Jk [239, 240, 242]. These transcription factors however must not be required for most EBNA-2 target genes since most genes are equally regulated by type 1 and type 2 EBNA-2. This is supported by the absence of the ETS/IRF motif at the EBNA-2 binding at equally regulated genes [210]. For this reason it is not likely that the general factors of the RNA polymerase II transcriptional machinery are able to determine the differential gene regulation by type 1 and type 2 EBNA-2. However, the mechanism by which the aspartate 442 mediates this effect remains to be determined.

Previous work from this laboratory had hypothesised that cooperation between EBNA-2 and EBNA-LP could explain the differential gene regulation by type 1 and type 2 EBNA-2 [209]. In fact, EBNA-LP was shown to enhance expression of viral and cell genes mediated by EBNA-2 [116-120]. The EBNA-2 TAD domain was found to directly interact with EBNA-LP [119, 224] but this interaction does not involve tryptophan 458 residue. The mechanism of cooperation with EBNA-LP is not entirely clear although a role for histone deacetylase 4 was proposed [432]. It possible that EBNA-LP cooperation allows recruitment of transcription factors by EBNA-2 TAD, therefore determining the distinct transactivation function by type 1 and type 2 EBNA-2. Although a different ability to induce gene expression has been observed for type 1 and type 2 TADs (Figure 3.4 B) in transient transfection reporter assays

performed in EBV-negative BJAB cells that do not express EBNA-LP, only a slight 2-fold difference was detected. This could have suggested that EBNA-LP was required for a stronger type 1/type 2 difference in terms of transactivation function. However, when the same experiment was carried out in the EBV-positive IB4 cell line (an LCL), a similar 2-fold difference in luciferase activation was still detected (Figure 3.6 B), even in a context of EBNA-LP expression. Another possibility is that the RG and CR7 domain elements of EBNA-2, which have also been shown to be involved in cooperation with EBNA-LP [217], are required for a stronger differential gene expression and the GAL4-TAD constructs made for this study did not carry these EBNA-2 elements. Indeed, these domains were found to be able to downregulate the ability of EBNA-2 TAD to activate gene transcription, when GAL4 fusions were used, indicating that these EBNA-2 elements can modulate the EBNA-LP cooperative function [224]. Although EBNA-LP was not involved in the slightly different transactivation function of type 1 and type 2 TADs observed in this study, it is plausible that its cooperative function could contribute to the mechanism of differential regulation of target genes by type 1 and type 2 EBNA-2, mediating a greater difference in the ability to transactivate gene expression. In this context, it had been proposed that the EBNA-LP type would be important and that type 1 EBNA-LP might cooperate more efficiently with type 1 EBNA-2 than with type 2 EBNA-2 [209]. However, the presence of both type 2 EBNA-LP and type 2 EBNA-2 in the EREB2.5 LCL growth assay did not confer to type 2 EBNA-2 the ability to sustain cell growth [209]. In addition, co-expression of type 2 EBNA-2 with either type 1 or type 2 EBNA-LP still resulted in a lower transactivation of the LMP-1 gene by type 2 EBNA-2, compared to LMP-1 induction by type 1 EBNA-2 when is co-expressed with either type 1 or type 2 EBNA-LP [209]. The weaker LMP-1 transactivation by type 2 EBNA-2 is not affected by the EBNA-LP type. Nevertheless, EBNA-LP cooperative function was found to be important to enhance the expression of viral and cell genes required for B cell transformation mediated by EBNA-2 as already mentioned before and this may contribute to the type 1/type 2 EBNA-2 differential gene induction.

EBNA-2 is often considered the viral homologue of the human Notch intracellular domain (N-ICD) because both interact with RBP-Jk to mediate gene expression [254, 415]. The TAD of EBNA-2 and N-ICD replace the histone deacetylase complex recruited by RBP-Jk to activate gene expression [416]. The Notch signalling pathway controls important cell fate decisions in neurogenesis and T cell development [433, 434]. Deregulation of the Notch pathway is often associated with human malignancies and was found to be oncogenic in some animal models [435]. Accordingly, EBNA-2 is regarded as a constitutively active Notch receptor and it has

been suggested that EBNA-2 takes over the Notch signalling pathway as a likely mechanism by which promotes B cell transformation and immortalisation. Earlier works have also shown that N-ICD was able to induce some EBNA-2 target promoters carrying RBP-Jk binding sites, such as the Cp promoter and LMP-2A gene, but to a lower extent when compared to EBNA-2 [253, 254]. In the present study the ability of N-ICD transactivation domain to activate gene expression was determined and compared to that of EBNA-2. The TAD of N-ICD is loosely defined but it had been initially mapped to the fragment between the ANK repeats and the PEST domain, which includes the glutamine-rich OPA region [422], but then the OPA-PEST fragment was shown to display stronger ability to activate gene expression, indicating that both domains constitute the TAD of N-ICD [426]. An equivalent TAD fragment was used in this study in GAL4 reporter assays (Figure 3.10). The results have indeed confirmed that the transactivation function of N-ICD TAD is much lower, even when compared to that of type 2 EBNA-2 in EBV-negative BJAB cells (Figure 3.12), supporting the lower induction of EBNA-2 responsive genes by N-ICD detected in previous reports [256]. A comparative analysis of the amino acid sequences of EBNA-2 TADs and the PEST domains of Notch1 to 3 (Figure 3.9) revealed a common motif of 15 amino acids, in which seven residues were identical between the type 2 EBNA-2 TAD and the PEST domain of Notch1. Interestingly, one of the residues is the serine 442 of type 2 EBNA-2. Therefore, the effect of a serine to aspartate change in the TAD of N-ICD (S2513D), equivalent to the S442D substitution in EBNA-2 was investigated in this study. Similarly, the S2513D change in the N-ICD also increased the ability of the TAD of N-ICD to transactivate gene expression around 2-fold (Figure 3.12). This increase in transactivation function observed in both cases after replacing the conserved serine residue by an aspartate might suggest that a similar mechanism is involved in this increase.

A possible phosphomimetic mechanism for the S442D substitution has been considered. It is well established that EBNA-2 phosphorylation could be important for its function [211, 436]. Previous reports have determined that EBNA-2 is phosphorylated at the serine 243 by the cdc2/Cyclin B1 kinase but also by the EBV lytic cycle *BGLF4* gene-encoded serine/threonine protein kinase. This residue serine 243 is included in a consensus motif recognised by these kinases and its phosphorylation was associated with reduced transactivation of the Cp and the LMP-1 promoters [437-439]. Additionally, phosphorylation of EBNA-2 by casein kinase II was found to be important for B cell growth transformation. Phosphorylation of the consensus casein kinase II site close to the TAD (serine 469) is essential for EBNA-2 binding to the host chromatin remodeling hSNF5/Ini1 factor, component of the human SWI/SNF complex [235, 236]. An additional phosphorylation process, in this case of the co-activator p100, interacting

with TFIIE and composing the protein complex involved in gene transactivation mediated by EBNA-2 is also important. Phosphorylation of p100 by Pim kinases (Pim-1 and Pim-2) was shown to enhance transactivation of the LMP-1 promoter by EBNA-2 [440, 441]. Analysis of the Notch PEST domains (Figure 3.9) revealed that the amino acid serine 2513 precedes a proline residue. Thus, this S/P motif, indeed, constitutes a consensus phosphorylation site for proline-directed kinases that include the mitogen-activated protein kinases (MAPKs) and the Cyclin-dependent kinases (CDKs) [442, 443], indicating that the amino acid serine 2513 in N-ICD can indeed be phosphorylated and a phosphomimetic mechanism can explain the 2-fold increase in the transactivation ability after the serine to aspartate substitution (Figure 3.12). In fact, different studies have determined that Notch is regulated by various post-translational modifications that include phosphorylation [444]. N-ICD was shown to be phosphorylated by several kinases in multiple sites, such as at the ankyrin domain and PEST domains, affecting the transactivation function and protein stability of N-ICD [445], downregulating or enhancing N-ICD signalling [446-449]. Interestingly, the Cyclin C-dependent CDK8, CDK3 and CDK19 were shown to phosphorylate the PEST domain of Notch1-ICD at the amino acids threonine 2511, serine 2513 and serine 2516 [450, 451], confirming the serine 2513 that is equivalent to serine 442 of EBNA-2 is phosphorylated in N-ICD. However, phosphorylation of residues at the PEST domain is associated with protein turnover and targeting for ubiquitination [451-456]. Conversely, in the TAD of type 2 EBNA-2, the amino acid serine 442 in not followed by a proline residue (Figure 3.9) and therefore it does not lie in any consensus phosphorylation site commonly recognised by either the MAP kinases or the Cyclin-dependent kinases [457]. Although a phosphomimetic mechanism seemed unlikely here, the analysis of the 15 amino acid motif of type 2 EBNA-2 (Figure 3.9), which includes the serine 442 using the Phospho Motif search tool, where 170 serine/threonine phosphorylation-based motifs were catalogued was performed [458]. The inserted motif matched with three potential phosphorylation motifs that had been previously reported in the literature. Specifically, the serine in these motifs was phosphorylated by a DNA-dependent protein kinase (PSX motif, where X depicts any amino acid) [459]; G protein-coupled receptor kinase 1 (XSXXXP motif) [460] and the casein kinase II (SXXY motif, where Y needs to be phosphorylated for the enzyme to recognise the motif) [461]. Although these three similar motifs were once reported to be phosphorylated does not imply the serine 442 is also phosphorylated. This hypothesis was further investigated in this study by assessing the effect of a serine to alanine change (S442A) in LMP-1 gene activation by EBNA-2 (Figure 3.14) in Daudi cells. The Daudi endogenous LMP-1 gene expression was more strongly induced by type 1 EBNA-2 than by type 2 as expected, since LMP-1 is one of the genes differentially regulated by type 1 and type 2 EBNA-2 [209, 210]. Additionally, the

S442D substitution in type 2 EBNA-2 conferred increased ability to transactivate LMP-1 gene expression, to levels similar to those observed for type 1 EBNA-2 protein [210]. Remarkably, the serine to alanine substitution in type 2 EBNA-2 (S442A), which had been made to avoid a potential phosphorylation of the serine 442, did not abrogate or prevent LMP-1 activation by type 2 EBNA-2. Intriguingly, the S442A mutation slightly increased type 2 EBNA-2 ability to induce LMP-1 expression (Figure 3.14), suggesting that the molecular mechanism underlying the serine to aspartate (S442D) phenotype is unlikely to involve a phosphomimetic change of the serine 442, which would work as a constitutively phosphorylated amino acid.

The mechanism by which the amino acid aspartate 442 mediates the higher ability to activate gene expression by type 1 EBNA-2 and therefore the superior B cell transforming capacity of type 1 EBV remains to be determined. However, this amino acid and the equivalent serine in type 2 EBNA-2 were found to be located immediately next to the C-terminal EBNA-2 PXLXP peptide motif, which is recognised by the MYND domain of the cell protein BS69/ZMYND11 [267]. This protein function as a transcriptional repressor and was found to bind to EBNA-2. The function of BS69/ZMYND11 in the regulation of EBNA-2-mediated gene transactivation was studied in great detail in the next chapter to determine whether it can play a role in the differential gene regulation by type 1 and type 2 EBNA-2.

4 Function of the cell protein BS69/ZMYND11 in the regulation of EBNA-2-mediated gene transactivation

The aim of this chapter is to investigate whether the BS69/ZMYND11 protein plays any role in the regulation of EBNA-2-mediated gene transactivation and in the type 1/type 2 EBNA-2 differential ability to activate viral and cell gene expression, which is important for the distinct capability of type 1 and type 2 EBV to transform resting B cells into LCLs *in vitro*.

4.1 Introduction

The cell protein BS69/ZMYND11 (BS69) contains several different domains: an N-terminal PHD zinc-finger [382], a Bromodomain [383], a PWWP domain [384] and coiled-coil domain [268] that precedes the C-terminal MYND domain [385] (Figure 1.9). This structure displays motifs resembling other proteins that are involved in chromatin and gene regulation.

In fact, BS69 has been found to function as a transcriptional repressor in association with a variety of transcription factors, such as c-Myb [378], B-Myb [379], ETS2 [380], MGA [267], EMSY [381] and the corepressor N-CoR [367] through the BS69 MYND domain. Additionally, BS69 was recently found to be associated with chromatin and gene regulation via recognition of histone H3.3 trimethylated at lysine 36 (H3.3K36me3), by its Bromo-PWWP domains. This interaction facilitates the recruitment of MYND-bound transcription factors and chromatin remodelling factors, such as EZH2 [390, 391], HDAC1 [389], BRG1 [386], components of the SWI-SNF complex [387], and E2F6 [392] and the repression of active genes. BS69 was also linked to transcriptional elongation, tumor suppression and pre-mRNA splicing [395-397].

BS69 was originally found to interact with the adenoviral protein E1A via its MYND domain, which recognises and binds to a PXLXP peptide motif (where X corresponds to any amino acid) in E1A. This interaction between BS69 and E1A was demonstrated to repress gene activation mediated by the viral E1A protein [368]. Similarly to E1A, the BS69 MYND domain has been shown to interact with the EBV-encoded EBNA-2 protein by recognising its C-terminal PXLXP peptide motif and BS69 also repressed transcriptional transactivation by EBNA-2, in reporter assays using GAL4 fusion proteins [267].

A recent study by Harter and colleagues has unravelled the crystal structure of the coiled-coil domain in tandem with the MYND domain of BS69 interacting with an EBNA-2 peptide. It has been demonstrated that BS69 forms a homodimer to bind EBNA-2 through its two sequential

PXLXP motifs, in an enhanced BS69-EBNA-2 recognition, in a synergistic manner [268]. In addition, this work has also revealed that EBNA-2 interacted with and downregulated BS69 expression at both mRNA and protein levels in EBV-positive B cell lines [268]. Furthermore, BS69 was revealed as a potential inhibitor of EBNA-2 since BS69 expression led to inhibition of transcriptional activation by EBNA-2 and also to the impairment of growth and proliferation of LCLs, which showed decreased viability when BS69 is present. As expected, these effects are abolished when the interaction between BS69 and EBNA-2 is disrupted [268].

In addition to the EBNA-2 protein, BS69 was also shown to interact with the EBV-encoded LMP-1 protein (section 1.6.4), which is required for B cell transformation into LCLs [400, 401, 404]. BS69 was initially shown to interact with LMP-1/CTAR2 domain via its MYND domain, acting as a scaffold protein between LMP-1 and TRAF6 proteins, forming a complex required for LMP-1-mediated JNK activation [404].

Moreover, BS69 was also found to be involved in the NF-kB signalling pathway. BS69 binds the LMP-1/CTAR1 domain and cooperates with the cell protein TRAF3 to negatively regulate the activation of the non-canonical NF-kB pathway mediated by LMP-1 [400]. Additionally, BS69 interacts with LMP-1/CTAR2 domain and supresses activation of the canonical NF-kB signalling pathway by displacing TRADD from CTAR2 [401].

LMP-1 is required for B cell transformation into LCLs *in vitro* and activation of these signalling pathways by LMP-1 through either CTAR1 or CTAR2 is essential for EBV-mediated B cell immortalisation and for the long-term growth of EBV-transformed B cells [340, 344, 346, 349, 352, 353, 462]. This suggests that BS69, which takes part in the regulation of these pathways that are important for B cell transformation, might also be an important player in the type 1/type 2 differential gene regulation crucial for superior B cell transformation by type 1 EBV.

The results obtained in the previous section of this study and recently published data by this laboratory [210] have demonstrated that the single amino acid aspartate 442 is important to confer higher transactivation ability and superior LCL growth maintenance to type 1 EBNA-2. Intriguingly, the amino acid aspartate 442 and the corresponding serine in type 2 EBNA-2 are located immediately next to the C-terminal EBNA-2 PXLXP peptide motif that is recognised by the MYND domain of BS69 [267].

These results suggested that BS69 might play a fundamental role in type 1/type 2 EBNA-2 differential gene regulation and, consequently, in the superior B cell transformation by type 1 EBV. In this study, the function of BS69 in the regulation of EBNA-2 transactivation of gene expression was investigated, as well as the ability of BS69 to interact with either type 1 or type 2 EBNA-2 proteins.

4.2 Results

4.2.1 Regulation of EBNA-2-mediated gene expression by BS69 in GAL4-gene reporter assays in EBV-negative BJAB cells

4.2.1.1 BS69 does not repress gene transactivation mediated by GAL4-EBNA-2 constructs (amino acids 426-463) in GAL4-gene reporter assays

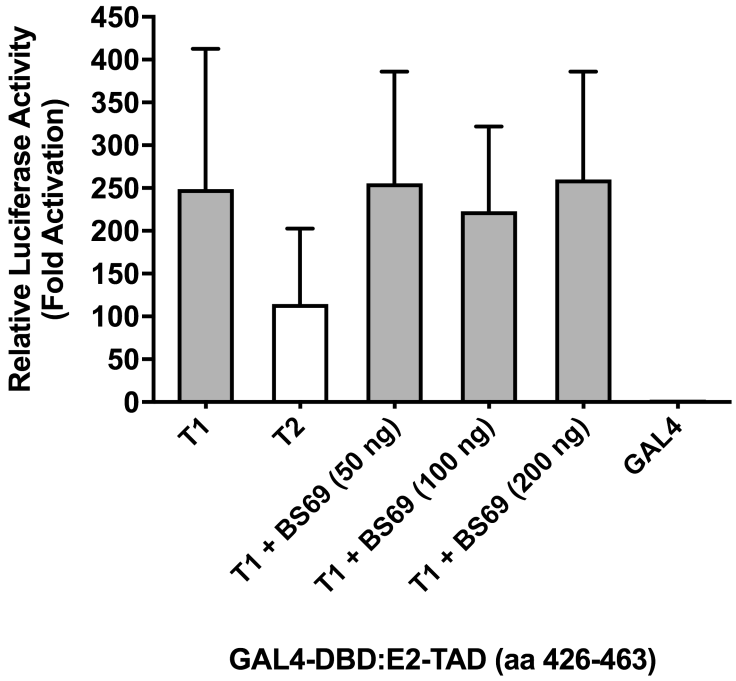
A recent study by Harter and colleagues [268] has shown that BS69 forms a homodimer to interact with EBNA-2. The MYND domains of two BS69 proteins recognise and bind to two sequential PXLXP peptide motifs present in the EBNA-2 protein. In addition, it was previously demonstrated that via this interaction BS69 repressed transcriptional activation mediated by EBNA-2 [267, 268]. However, in those studies the effect of BS69 on the EBNA-2 protein was not investigated for both type 1 and type 2 EBNA-2 proteins, which can exhibit a distinct ability to transactivate gene expression (section 3.2.1).
In order to confirm the previous observations by Ansieau and Leutz [267], transient luciferase reporter assays, which have already been established in the previous chapter (section 3.2.1), were performed. A BS69 expression vector (pCI-BS69) was used to confirm whether BS69 repressed gene activation mediated by the GAL4-DBD:E2-TAD construct, which comprises the amino acids 426 to 463 from the EBNA-2 protein.

EBV-negative BJAB cells were cotransfected with plasmids expressing the GAL4-DBD:E2-TAD fusion protein (pcDNA3.1-GAL4-DBD:E2-TAD, see section 2.3.3.13.3), an expression plasmid for BS69 (pCI-BS69), a luciferase reporter plasmid carrying five GAL4 DBD sites upstream of a luciferase gene (pFR-Luc, section 2.3.3.13.1) and a vector expressing *Renilla* luciferase (pRL-CMV, section 2.3.3.13.1) using the Neon transfection system. The total amount of plasmid DNA used was the same in each individual transfection. 24 hours after transfection, cell extracts were analysed for luciferase activity and results were normalised for transfection efficiency based on *Renilla* luciferase values.
No difference in luciferase expression was detected, even when different amounts of plasmid expressing BS69 were transfected into the cells, in comparison to luciferase gene activation

by type 1 EBNA-2 TAD in the absence of BS69 (Figure 4.1 A). Thus, these results were not consistent with the previously reported by Ansieau and Leutz [267], although the same BS69 expression plasmid and equal amounts of plasmids expressing the GAL4 construct and the BS69 protein have been used in the present experiment.
The ability of BS69 to repress EBNA-2-mediated gene transactivation was then assessed using a higher amount of BS69 expression plasmid *per* transfection (1 µg as opposed to 200 ng). Moreover, the effect on the other GAL4-DBD:E2-TAD constructs (type 1, type 2 and type 2 S442D) was also investigated.

Consistent with what was observed in the previous chapter (section 3.2.1), using the same luciferase reporter assays and the same GAL4-DBD:E2-TAD constructs (aa 426-463); type 1 EBNA-2 TAD activated luciferase gene expression 2-fold stronger than type 2. Additionally, the serine to aspartate substitution (S442D) in type 2 TAD conferred a type 1-like phenotype to type 2 (Figure 4.1 B). However, when the BS69 expression plasmid was transfected there was no repression of gene transactivation by type 1, type 2 and type 2 S442D EBNA-2 TADs. In fact, there was a modest increase in luciferase gene activation by type 1 EBNA-2 TAD when BS69 is expressed (Figure 4.1 B). In the case of either type 2 or type 2 S442D TAD, there was no difference in luciferase activation when BS69 was transfected. These results suggest that BS69 does not repress gene activation mediated by the GAL4-DBD:E2-TAD (amino acids 426-463) constructs.

A


Relative Luciferase Activity
(Fold Activation)
450
400
350
300
250
200
150
100
50
0
T1
T2
T1 + BS69 (50 ng)
T1 + BS69 (100 ng)
T1 + BS69 (200 ng)
GAL4
GAL4-DBD:E2-TAD (aa 426-463)

B

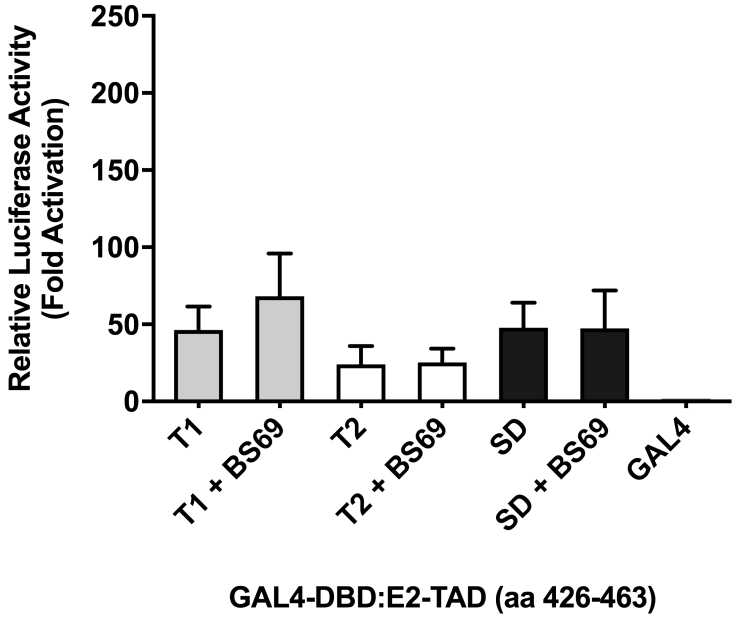

Relative Luciferase Activity
(Fold Activation)
250
200
150
100
50
0
T1
T1 + BS69
T2
T2 + BS69
SD
SD + BS69
GAL4
GAL4-DBD:E2-TAD (aa 426-463)

Figure 4.1 BS69 expression does not cause repression of luciferase transactivation mediated by GAL4-DBD:E2-TAD constructs (amino acids 426-463)

EBNA-2 fragments (aa 426-463) were expressed as fusion proteins with the GAL4 DBD and activate luciferase reporter gene expression by binding to GAL4 binding sites in the reporter plasmid gene promoter. (**A**) 2 x 10^6 BJAB cells were cotransfected with 300 ng of either type 1 (T1) or type 2 (T2) GAL4-DBD:E2-TAD constructs, 500 ng of pFR-Luc, 10 ng of pRL-CMV *Renilla* and 50 ng, 100 ng or 200 ng of pCI-BS69 (BS69 expressing vector) plasmids with the Neon transfection system. (**B**) 2 x 10^6 BJAB cells were cotransfected with 300 ng of type 1 (T1), type 2 (T2) or type 2 S442D (SD) GAL4-DBD:E2-TAD constructs, 500 ng of pFR-Luc, 10 ng of pRL-CMV *Renilla* and 1 µg of pCI-BS69 plasmids using the Neon transfection system. The empty vector pcDNA3.1-GAL4-DBD was used to achieve equivalent amounts of DNA *per* transfection. At 24 hours post-transfection cells were harvested and analysed for luciferase activity. For each sample, luciferase values were normalised for transfection efficiency based on *Renilla* luciferase expression values. Results represent the luciferase activity relative to that of pFR-Luc reporter plasmid with empty vector pcDNA3.1-GAL4-DBD (GAL4). The results are presented as an average of two independent experiments (n=2). Error bars represent standard deviations.

This discrepancy between the present results and previous observations [267] has raised the hypothesis that these GAL4-DBD:E2-TAD (aa 426-463) constructs would not be subject to repression by BS69. In fact, in the previous work, the full EBNA-2 protein had been fused to GAL4-DBD to perform the reporter assays, in which BS69 repressed EBNA-2 transactivation. Additionally, as mentioned before, BS69 homodimer via the two MYND domains interacts with two PXLXP motifs present in the EBNA-2 protein. These PXLXP motifs correspond to amino acids 383-387 (PXLXP 1) and 437-441 (PXLXP 2) (Figure 4.2) [268].

The GAL4-DBD:E2-TAD (aa 426-463) constructs used in this study do not include the first motif, suggesting that both motifs could be required for BS69-mediated repression.

Interestingly, sequence analysis of type 1 and type 2 EBNA-2 protein sequences performed in this study has revealed that type 2 EBNA-2 carries a third PXLXP peptide motif (PXLXP 3, Figure 4.2) that has not been described yet and can also potentially function as an additional site to be recognised by BS69 MYND domain.

Figure 4.2 Alignment of type 1 and type 2 EBNA-2 protein sequences (amino acids 334 to 487)

Amino acids shown in blue correspond to the PXLXP peptide motifs (PXLXP 1 and PXLXP 2) in both type 1 and type 2 EBNA-2 proteins (P is proline, L is leucine and X denotes any amino acid). Sequence analysis identified a third PXLXP motif only present in type 2 EBNA-2 protein (PXLXP 3, in red). The arrow depicts the position of amino acid 442 in type 1 EBNA-2, which is located immediately next to PXLXP 2 peptide motif.

4.2.1.2 Generation of pcDNA3.1-GAL4-DBD vectors expressing the constructs GAL4-DBD:EBNA-2 (amino acids 334-487)

To determine whether BS69 was able to repress gene activation mediated by GAL4-EBNA-2 constructs that included both PXLXP 1 and PXLXP 2 peptide motifs; new pcDNA3.1-GAL4-DBD:EBNA-2 constructs were made. In addition, these plasmids would be used to determine whether BS69 can play a function in regulation of differential gene activation by type 1 and type 2 EBNA-2. To generate pcDNA3.1-GAL4-DBD:EBNA-2 plasmids, the EBNA-2 region comprising the amino acids 334 to 487 was cloned into pcDNA3.1-GAL4-DBD plasmid fused to the GAL4 DNA-binding domain. This region was chosen since it includes both PXLXP peptide motifs (Figure 4.2).

Different pBlueScript plasmid vectors expressing type 1 EBNA-2, type 2 EBNA-2 and type 2 S442D EBNA-2 (section 2.3.3.13.6) were already available in the laboratory. These pBS-EBNA-2 plasmids were used as template to PCR amplify the region that corresponds to the amino acids 334 to 487 in type 1 EBNA-2 protein, using oligonucleotide primers flanked by *Bam*HI and *Not*I restriction sites (section 2.4.2). The PCR products of approximately 500 bp were subcloned into pCR2.1 vector through a TA cloning strategy (section 2.3.3.7). Transformants were then screened by *Bam*HI/*Not*I digestion of purified plasmids (Figure 4.3).

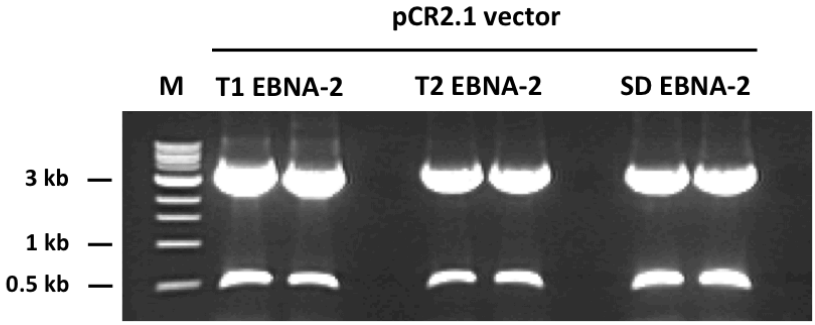


Figure 4.3 Cloning of type 1, type 2 and type 2 S442D fragments (aa 334-487) into pCR2.1

A representative *Bam*HI/*Not*I digestion indicating that type 1 (T1 EBNA-2), type 2 (T2 EBNA-2) and type 2 S442D (SD EBNA-2) EBNA-2 fragments corresponding to the amino acids 334 to 487 were successfully subcloned into pCR2.1 vector. DNA restriction digestion to screen for the presence of 0.5 kb DNA fragments. M: 1 kb marker.

The final pcDNA3.1-GAL4-DBD:EBNA-2 plasmids (see section 2.3.3.13.4) were generated by digesting the previous pCR2.1 vectors and cloning the resultant EBNA-2 *Bam*HI-*Not*I fragments into pcDNA3.1-GAL4-DBD vector cut with *Bam*HI and *Not*I. Transformants were screened by PCR, using T7-promoter forward and BGH reverse primers (Figure 4.4) and the final constructs were then sequenced to validate their identity and compared to the published EBNA-2 sequences.

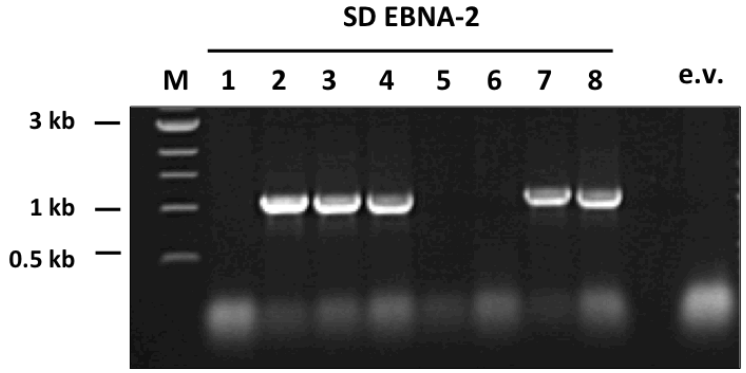


Figure 4.4 Cloning of type 2 S442D fragments (aa 334-487) into pcDNA3.1-GAL4-DBD vector

Representative gel electrophoresis of PCR products obtained from PCR-screening for positive clones of type 2 S442D fragments (aa 334-487) cloned into pcDNA3.1-GAL4-DBD vector. T7-promoter forward and BGH reverse oligonucleotide primers were used. The empty vector pcDNA3.1-GAL4-DBD was used as negative control (e.v.). Eight colonies from transformants were tested: colonies 2-4, 7 and 8 were positive for the presence of type 2 S442D (SD EBNA-2) EBNA-2 fragments (amino acids 334-487), successfully cloned into pcDNA3.1-GAL4-DBD vector. Expected size of the amplified fragment in positive clones is 1.1 kb. M: 1 kb marker. The same procedure was performed for type 1 and type 2 EBNA-2 fragments (aa 334-487).

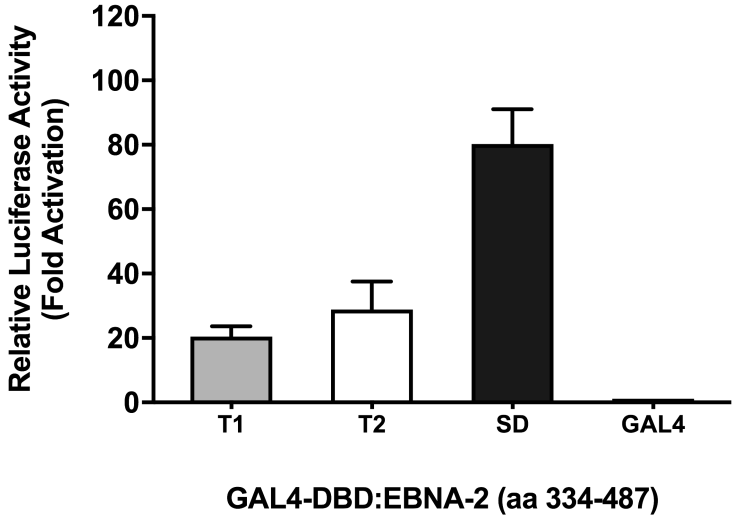
4.2.1.3 BS69 represses transactivation of gene expression by type 1, type 2 and type 2 S442D GAL4-DBD:EBNA-2 (amino acids 334-487)

The results obtained in section 4.2.1.1 suggested that BS69 might require both PXLXP peptide motifs in EBNA-2 to mediate repression of gene transactivation by EBNA-2. To address whether BS69 protein was able to repress gene expression activated by the new GAL4-DBD:EBNA-2 constructs (amino acids 334-487), carrying both PXLXP peptide motifs, a transient transfection assay similar to that performed with the GAL4-DBD:E2-TAD (amino acids 426-463) (section 4.2.1.1) was carried out.

The constructs were initially tested for their ability to transactivate gene expression and also their behaviour compared to that of the GAL4-DBD:E2-TAD (aa 426-463) constructs. It has been previously observed that the ability of type 1 EBNA-2 TAD to activate gene expression is 2-fold stronger than that of type 2. Additionally, when the serine of type 2 is replaced by the corresponding aspartate of type 1 (S442D mutation), type 2 TAD acquires a type 1-like ability to transactivate gene expression (section 3.2.1) [210]. However, when the larger constructs, which include the amino acids 334 to 487, were tested, there was no difference in the ability to transactivate gene expression by type 1 and type 2 EBNA-2 (type 2 construct is a slightly stronger activator: 20 and 28-fold induction). In contrast, type 2 S442D GAL4-DBD: EBNA-2 exhibited an approximately 4-fold stronger ability to activate gene expression than type 1 or type 2 GAL4-DBD:EBNA-2 (Figure 4.5 A), which is distinct from what was observed with the smaller GAL4-DBD:E2-TAD (aa 426-463) constructs. Nevertheless, this observation is not entirely surprising because the system originally used to identify the TAD of type 1 EBNA-2 had revealed that different C-terminal EBNA-2 fragments, which include the TAD, could exhibit distinct transcriptional activator abilities in CAT assays [216]. Indeed, the fragment comprising the amino acids 426 to 462 had been demonstrated to be the strongest activator when compared to other fragments and the full-length protein [216].

The GAL4-DBD:EBNA-2 (amino acids 334-487) constructs were then cotransfected with the BS69 expressing plasmid (pCI-BS69, section 2.3.3.13.9) into BJAB cells and BS69 effect on the transactivation ability was analysed. The results revealed that BS69 represses activation of gene expression by type 1 GAL4-DBD:EBNA-2 construct around 6-fold. Additionally, there was also a 4-fold and 7-fold repression of gene transactivation by type 2 and type 2 S442D GAL4-DBD:EBNA-2 constructs, respectively (Figure 4.5 B).

A


Relative Luciferase Activity
(Fold Activation)
120
100
80
60
40
20
0
T1
T2
SD
GAL4
GAL4-DBD:EBNA-2 (aa 334-487)

B

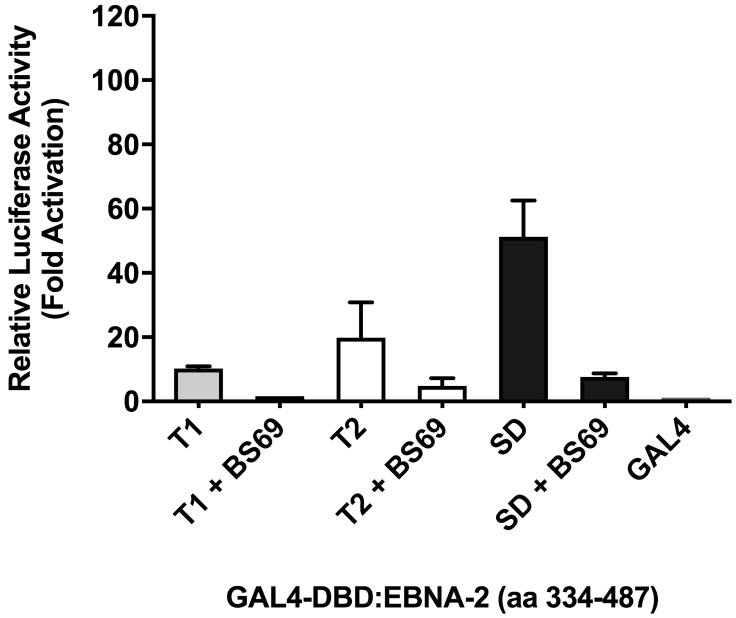

Relative Luciferase Activity
(Fold Activation)
120
100
80
60
40
20
0
T1
T1 + BS69
T2
T2 + BS69
SD
SD + BS69
GAL4
GAL4-DBD:EBNA-2 (aa 334-487)

Figure 4.5 BS69 expression represses gene transactivation mediated by type 1, type 2 and type 2 S442D GAL4-DBD:EBNA-2 (amino acids 334-487)

(A) EBNA-2 fragments (amino acids 334-487) were expressed as fusion proteins with the GAL4-DBD and activated luciferase reporter gene expression by binding to GAL4 binding sites in the reporter plasmid gene promoter. 2 x 10^6 BJAB cells were cotransfected with 300 ng of either type 1 (T1), type 2 (T2) or type 2 S442D GAL4-DBD:EBNA-2 constructs (amino acids 334-487), 500 ng of pFR-Luc, 10 ng of pRL-CMV *Renilla* and **(B)** 1 µg of BS69 expressing plasmid (pCI-BS69) using Neon transfection system. The empty vector pcDNA3.1-GAL4-DBD was used to achieve equivalent amounts of DNA *per* transfection. At 24 hours post-transfection cells were harvested and analysed for luciferase activity. For each sample, luciferase values were normalised for transfection efficiency according to *Renilla* luciferase expression values. Results are represented as the luciferase activity relative to the pFR-Luc reporter plasmid with the empty vector pcDNA3.1-GAL4-DBD (GAL4). **(A)** Results are represented as an average of three (n=3) and **(B)** two independent experiments (n=2). Error bars represent standard deviations.

Consistent with the previously published reports [267, 268], BS69 protein was able to repress gene transactivation mediated by type 1 EBNA-2. However, those studies had not shown the inhibitory role of BS69 in the transcriptional activation mediated by type 2 EBNA-2. Indeed, in this present study, it has been clearly determined that BS69 also represses transactivation of gene expression by type 2 and type 2 S442D EBNA-2 proteins (Figure 4.5 B), showing that this effect is not restricted to type 1 EBNA-2.

Together the results corroborate the inhibition of EBNA-2-mediated transcriptional activation by BS69 protein and suggest that both PXLXP peptide motifs present in the EBNA-2 protein required for transcriptional repression by BS69. However, these observations did not reveal any function of BS69 that could explain the type 1/type 2 differential gene regulation and the possible importance of amino acid aspartate 442 of type 1 EBNA-2 [210].

4.2.1.4 Expression of BS69 ΔMYND substantially increases transactivation of gene expression mediated by either GAL4-DBD:EBNA-2 (aa 334-487) or GAL4-DBD:E2-TAD (aa 426-463)

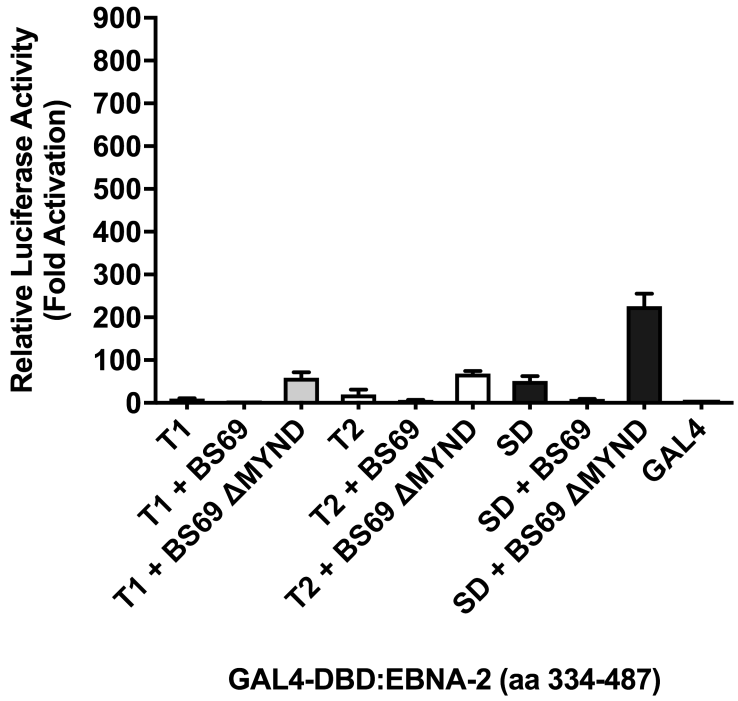
As reported in previous works performed by Ansieau and Leutz [267], the C-terminal MYND domain of BS69 is required for the interaction with EBNA-2 via the PXLXP peptide motifs and therefore is essential for BS69-mediated repression of gene transactivation by EBNA-2 [268]. In addition, mutations in the essential amino acid residues composing the MYND domain led to disruption of the interaction with EBNA-2 and abolished BS69 repression of transcriptional activation mediated by EBNA-2 [268].

In order to confirm the role of the MYND domain in BS69 repression of gene transactivation mediated by the GAL4-DBD:EBNA-2 (amino acids 334-487) constructs used in this study, a plasmid expressing the BS69 ΔMYND mutant was cotransfected (pCI-BS69-ΔMYND) with the EBNA-2 plasmids. As expected, when BS69 ΔMYND is expressed, there is no inhibition of gene transactivation by type 1, type 2 or type 2 S442D EBNA-2 (Figure 4.6 A). However, a substantial increase in luciferase expression (3- to 6-fold stronger) was detected when the BS69 ΔMYND was present, compared to that detected when the cells were only transfected with the EBNA-2 constructs. Hence, BS69 ΔMYND not only does not repress transactivation of gene expression by EBNA-2, as opposed to BS69; but also promotes an increase in gene activation, suggesting that it relieves some repressor mechanism that might be acting on the GAL4-DBD:EBNA-2 constructs (Figure 4.6 A).

As determined in section 4.2.1.1, BS69 does not inhibit gene transactivation mediated by the GAL4-DBD:E2-TAD (amino acids 426-463). Therefore, the effect of BS69 ΔMYND on these constructs was tested to investigate whether BS69 ΔMYND expression would also cause an increase in luciferase activation. Strikingly, cotransfection of BS69 ΔMYND mutant caused a large increase in luciferase gene transactivation by type 1, type 2 and type 2 S442D GAL4-DBD:E2-TAD (14-, 12- and 10-fold stronger activation, respectively) (Figure 4.6 B).

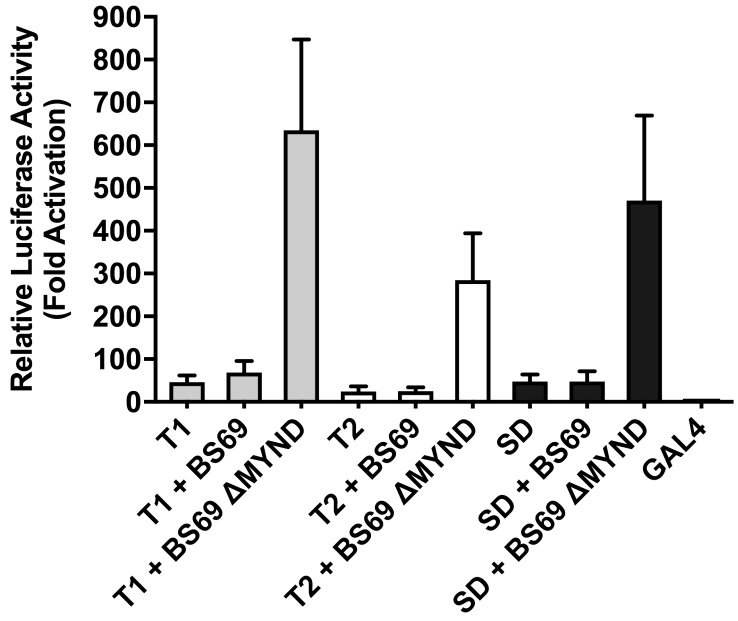
This effect of the BS69 ΔMYND mutant although more pronounced is similar to that observed for the larger GAL4-DBD:EBNA-2 constructs, suggesting an identical underlying mechanism causing this effect. Remarkably, in the presence of BS69 ΔMYND, despite the increase in luciferase gene expression, type 1 EBNA-2 transactivation ability is still approximately 2-fold stronger than that of type 2 and the serine to aspartate substitution (S442D) almost confers a type 1-like phenotype to type 2 EBNA-2 (Figure 4.6 B), as was determined in the section 3.2.1 of this study for these GAL4-DBD:E2-TAD constructs.

A



GAL4-DBD:EBNA-2 (aa 334-487)

B



GAL4-DBD:E2-TAD (aa 426-463)

Figure 4.6 Expression of BS69 ΔMYND mutant substantially increases gene transactivation mediated by type 1, type 2 and type 2 S442D GAL4-EBNA-2 constructs

(**A**) EBNA-2 fragments were expressed as fusion proteins with the GAL4-DBD and activated luciferase reporter gene expression by binding to GAL4 binding sites in the reporter plasmid gene promoter. 2 x 10^6 BJAB cells were cotransfected with 300 ng of either type 1 (T1), type 2 (T2) or type 2 S442D GAL4-DBD:EBNA-2 (aa 334-487) or (**B**) GAL4-DBD:E2-TAD (aa 426-463) constructs, 500 ng of pFR-Luc, 10 ng of pRL-CMV *Renilla* and 1 µg of BS69 (pCI-BS69) or 1 µg of BS69 ΔMYND (pCI-BS69-ΔMYND) expressing plasmids, where indicated, using the Neon transfection system. The empty vector pcDNA3.1-GAL4-DBD was used to achieve equivalent amounts of DNA *per* transfection. At 24 hours post-transfection cells were harvested and analysed for luciferase activity. For each sample, luciferase values were normalised for transfection efficiency according to *Renilla* luciferase expression values. Results are presented as luciferase activity relative to the pFR-Luc reporter plasmid with empty vector pcDNA3.1-GAL4-DBD (GAL4). Results are presented as an average of two independent experiments (n=2). Error bars represent standard deviations.

These previous observations left open the possibility that BS69 ΔMYND could be causing this increase in gene expression *per se* and not in an EBNA-2-dependent manner. To explore this, the plasmid expressing BS69 ΔMYND was transfected with the reporter plasmid (pFR-Luc) into BJAB cells and tested for luciferase expression in the absence of any GAL4-DBD-EBNA-2 construct. As expected, the expression of BS69 ΔMYND alone did not cause an increase in luciferase gene activation (Figure 4.7). This suggests that the higher luciferase gene transactivation occurs in an EBNA-2-dependent manner and that BS69 ΔMYND might affect the regulation of gene activation by EBNA-2.

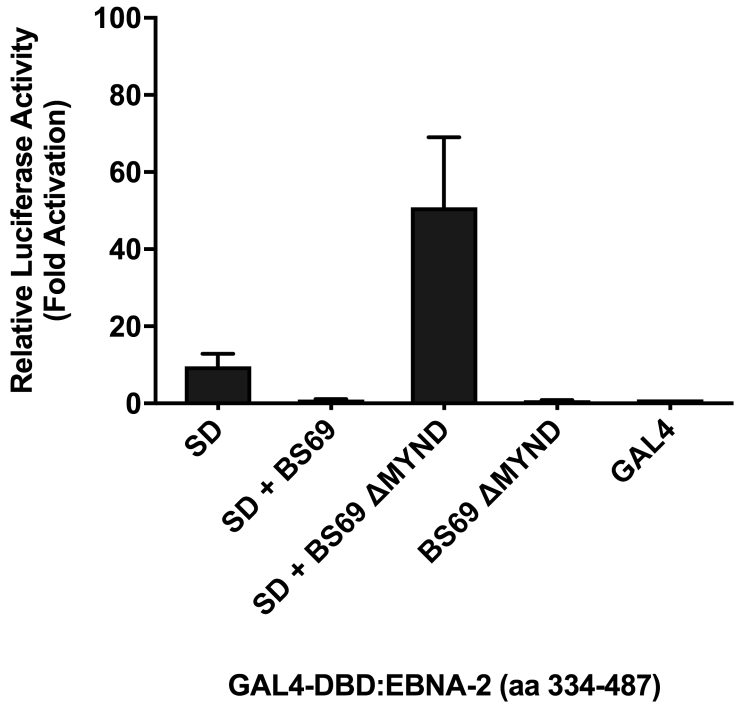


Figure 4.7 Expression of BS69 ΔMYND *per se* does not activate luciferase gene expression

2 x 10^6 BJAB cells were cotransfected with 300 ng of type 2 S442D GAL4-DBD:EBNA-2 (aa 334-487) (SD), 1 µg of pCI-BS69 (BS69) and 1 µg of pCI-BS69-ΔMYND (BS69 ΔMYND), where indicated, 500 ng of pFR-Luc reporter and 10 ng of pRL-CMV *Renilla* plasmids using the Neon system. The empty vector pcDNA3.1-GAL4-DBD was used to achieve equivalent amounts of DNA *per* transfection. At 24 hours post-transfection cells were harvested and analysed for luciferase activity. Luciferase values were normalised for transfection efficiency based on *Renilla* luciferase expression values. Results are presented as luciferase activity relative to the pFR-Luc reporter plasmid with empty vector pcDNA3.1-GAL4-DBD (GAL4). Results represent an average of two independent experiments (n=2). Error bars represent standard deviations.

4.2.1.5 Effect of BS69 and BS69 ΔMYND proteins on the ability of human Notch OPA-PEST domains to transactivate gene expression in GAL4 reporter assays

EBNA-2 is regarded as a viral functional homologue of a constitutively active Notch receptor, because both EBNA-2 and Notch receptor intracellular domain (N-ICD) interact with RBP-Jk to activate gene expression [254, 415]. These observations were supported by the results obtained in the previous section of this study (section 3.2.3.2), where it was shown that the OPA-PEST domains of human Notch fused to the GAL4-DBD can activate luciferase gene expression, but to a lower extent than type 2 EBNA-2 TAD (Figure 3.12). Additionally, comparative analysis of amino acids sequences of the TAD of EBNA-2 and the PEST domains of Notch receptors revealed a high level of similarity. In a common motif of 15 amino acids, seven residues were identical. One of those amino acids is the serine of type 2, equivalent to the aspartate 442 of type 1 EBNA-2 (Figure 3.9). Accordingly, it was also shown that replacing the serine-2513 by an aspartate residue in the PEST domain (S2513D), similarly to the S442D substitution in the TAD of EBNA-2, also increased approximately 2-fold the transactivation ability of Notch (Figure 3.12).

Importantly, this comparative analysis of amino acid sequences has also revealed that the PEST domain of Notch also carries a PXLXP peptide motif, which corresponds to the C-terminal PXLXP2 of EBNA-2 and therefore constitutes a consensus site potentially recognised by the cell protein BS69 (Figure 3.9). For this reason, it was investigated whether BS69 and/or BS69 ΔMYND expression could also affect gene transactivation by Notch-ICD. These BS69 and BS69 ΔMYND expressing plasmids were cotransfected with either the construct GAL4-DBD:OPA-PEST (domains of Notch) or the construct GAL4-DBD:OPA-PEST with the serine 2513 to aspartate (S2513D) substitution in the PEST domain of Notch, which were described previously (section 3.2.3).

The results showed that BS69 expression does not inhibit luciferase gene transactivation by GAL4-DBD:OPA-PEST domains in either case and suggest that the S2513D mutation, which is the amino acid next to the PXLXP motif in the PEST domain, does not promote any change in BS69 effect (Figure 4.8). BS69 ΔMYND expression did not cause any repression of Notch transactivation ability but in both cases there was a modest but equal increase in luciferase gene expression (~2-fold increase). This effect is much less than the substantial increase in gene transactivation observed for the EBNA-2 constructs, caused only by BS69

ΔMYND expression (Figure 4.6). These results suggest that BS69 protein does not repress gene transactivation by GAL4-DBD:OPA-PEST (aa 2270-2555) Notch domains but can potentially enhance it.

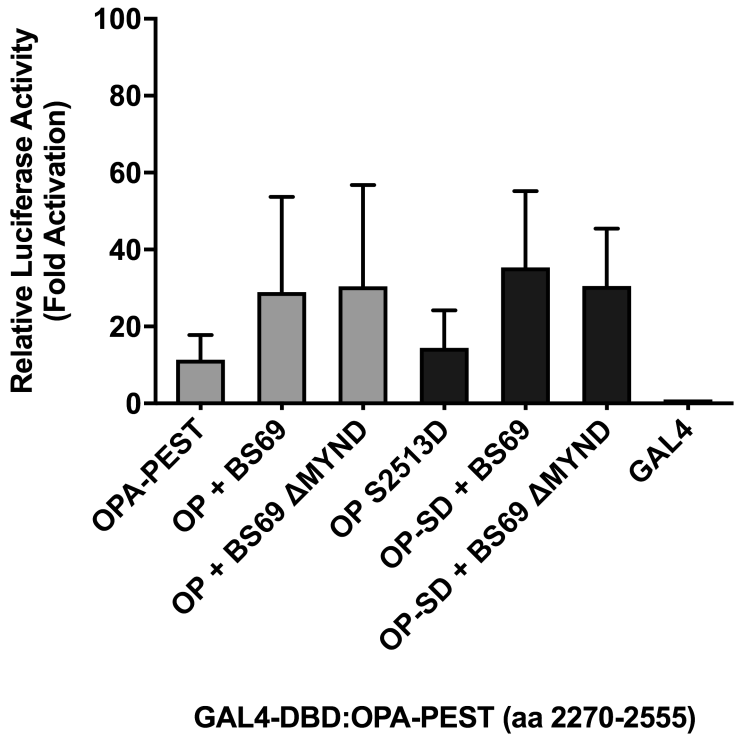


Figure 4.8 Effect of BS69 and BS69 ΔMYND proteins on the ability of human Notch-1 OPA-PEST domains to transactivate gene expression

2 x 10^6 BJAB cells were cotransfected with 300 ng of either GAL4-DBD:OPA-PEST (OPA-PEST, OP) or GAL4-DBD:OPA-PEST S2513D (OP S2513D, OP-SD) (amino acids 2270-2555), 1 μg of pCI-BS69 (BS69) and 1 μg of pCI-BS69-ΔMYND (BS69 ΔMYND), where indicated, 500 ng of pFR-Luc reporter and 10 ng of pRL-CMV *Renilla* plasmids using the Neon system. The empty vector pcDNA3.1-GAL4-DBD was used to get equivalent amounts of DNA *per* transfection. At 24 hours post-transfection cells were harvested and analysed for luciferase activity. Luciferase values were normalised for transfection efficiency based on *Renilla* luciferase expression values. Results are presented as luciferase activity relative to the pFR-Luc reporter plasmid with empty vector pcDNA3.1-GAL4-DBD (GAL4). Results are represented as the average of two independent experiments (n=2). Error bars represent standard deviations.

4.2.2 Regulation of EBNA-2-mediated gene expression by BS69 in LMP-1 promoter-gene reporter assays in EBV-positive Daudi cells

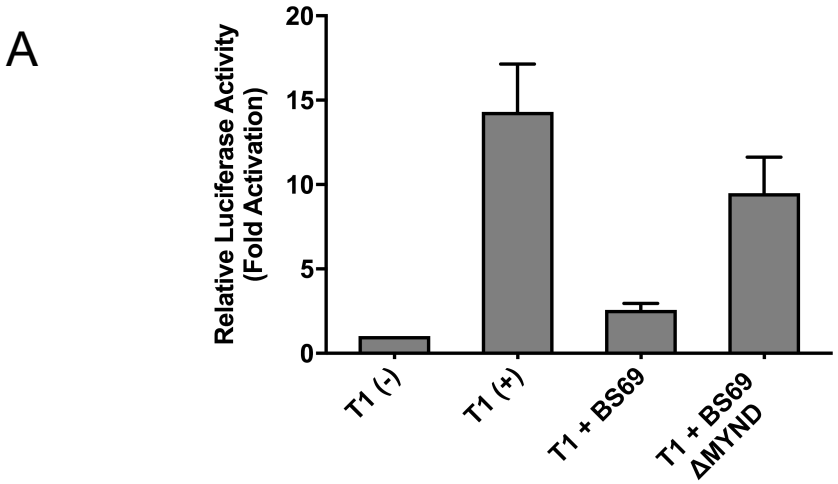
4.2.2.1 BS69 represses transactivation of the viral LMP-1 promoter by either type 1 or type 2 EBNA-2 proteins in LMP-1 promoter-reporter gene assays

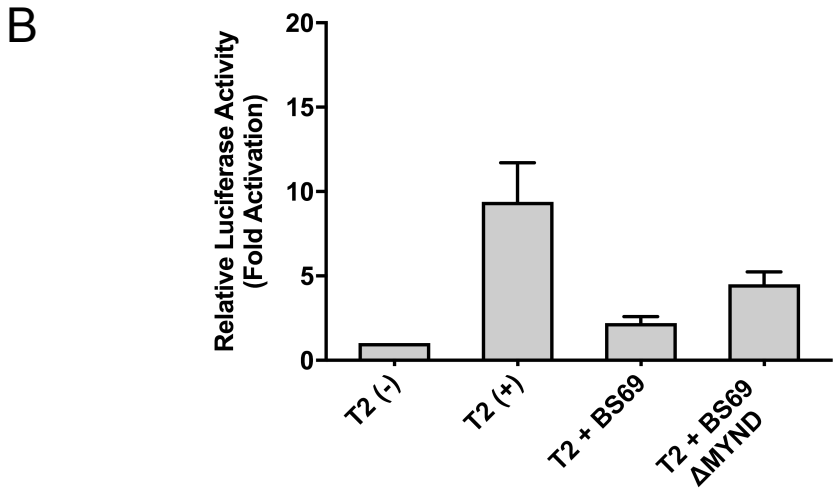
In the previous section, GAL4-gene reporter assays were used to demonstrate that BS69 cell protein represses luciferase gene transactivation by type 1 and type 2 EBNA-2. However, the GAL4-DBD:EBNA-2 constructs used in those experiments exhibited a different behaviour in terms of luciferase gene activation, depending on the size of the EBNA-2 fragment fused to the GAL4-DBD. Therefore, these observations allied to the utilisation of a synthetic promoter containing GAL4 DNA-binding sites suggested that these reporter assays did not constitute the most adequate system to determine whether BS69 could account for the differential gene regulation by type 1 and type 2 EBNA-2.
In order to further investigate the function of the BS69 cell protein in the regulation of EBNA-2-mediated gene expression, a distinct reporter assay consisting of the viral LMP-1 promoter sequence from the B95-8 EBV strain (LMP-1 regulatory sequence -634/+40, LRS) was used in this study. This LMP-1-promoter-luciferase reporter assay had previously been developed by Jansson and colleagues [411] and had shown that this LRS region could be regulated by type 1 EBNA-2 in DG75 cells.

EBV-positive Daudi cells were cotransfected with plasmids expressing either type 1 or type 2 EBNA-2 under control of a metallothionein-inducible promoter (pHEBo-MT:E2T1 and pHEBo-MT:E2T2, section 2.3.3.13.5), an LMP-1 promoter-luciferase reporter plasmid carrying the LMP-1 regulatory sequence (LRS region -634/+40) from the B95-8 EBV strain (pGL3b-B95-8-LRS, section 2.3.3.13.2), a vector expressing either BS69 (pCI-BS69) or BS69 ΔMYND (pCI-BS69-ΔMYND, section 2.3.3.13.9) and a plasmid expressing *Renilla* luciferase (pRL-CMV, section 2.3.3.13.1), using the Neon transfection system. EBNA-2 expression from the metallothionein (MT) promoter was induced with $CdCl_2$ 5 hours post-transfection. 24 hours after transfection, cell extracts were analysed for luciferase activity and results were normalised for transfection efficiency based on *Renilla* luciferase reference values (Figure 4.9).

The results showed that expression of the full-length EBNA-2 protein from the MT promoter with $CdCl_2$ caused an induction of LMP-1 promoter-luciferase transactivation by type 1 (14-fold) and by type 2 EBNA-2 (9-fold activation), in comparison to LMP-1 promoter-luciferase reporter activation when EBNA-2 expression is not induced. As expected, when the plasmid expressing BS69 is also transfected, there was an approximately 5-fold repression of LMP-1 promoter-luciferase transactivation by type 1 and type 2 EBNA-2, indicating that BS69 is able to inhibit activation of the viral LMP-1 promoter by EBNA-2 and not just synthetic promoters with GAL4 DNA-binding sites. This is consistent with a recently published report showing that BS69 downregulates the EBNA-2-mediated transcriptional activation of the viral LMP-1 and Cp promoters [268]. Nonetheless, it was first demonstrated in the present study that BS69 also inhibits transactivation of the viral LMP-1 promoter by type 2 EBNA-2 (Figure 4.9 B) and not only by type 1. Expression of BS69 ΔMYND led to a higher transactivation of luciferase in both cases, when compared to BS69 expression, but not to the same extent as when EBNA-2 was expressed in the absence of either BS69 or BS69 ΔMYND, suggesting that the MYND domain might be required for BS69-mediated repression of EBNA-2 transactivation ability.
Interestingly, in this LMP-1-promoter-luciferase reporter assay, BS69 ΔMYND expression did not cause a substantial increase in EBNA-2 transactivation ability as that observed for both GAL4-DBD:E2-TAD (aa 426-463) and GAL4-DBD:EBNA-2 (aa 334-487) constructs (Figure 4.6).

Western blot analysis with the PE2 antibody, performed to examine the levels of expression of both types of EBNA-2 proteins upon transfection of the pHEBo-MT:E2T1 and pHEBo-MT:E2T2 plasmids and further induction with $CdCl_2$, revealed that the protein levels of type 1 EBNA-2 were decreased when BS69 expressing vector is cotransfected (Figure 4.9 C) but not when BS69 ΔMYND is expressed. However, this reduction in the levels of type 1 EBNA-2 protein when BS69 is present was not observed for type 2 EBNA-2 (Figure 4.9 D).
Taken together these results indicate that BS69 represses transactivation of the viral LMP-1 promoter by type 1 and type 2 EBNA-2 in a similar manner, but does not explain the different gene regulation by type 1 and type 2. Interestingly, an effect on type 1 EBNA-2 protein levels by BS69 was observed. This reduction in the protein levels of type 1 but not in type 2 EBNA-2 suggests that BS69 might play a role in the type 1/type 2 differential regulation.


A
Relative Luciferase Activity
(Fold Activation)
20
15
10
5
0
T1 (-)
T1 (+)
T1 + BS69
T1 + BS69
ΔMYND


B
Relative Luciferase Activity
(Fold Activation)
20
15
10
5
0
T2 (-)
T2 (+)
T2 + BS69
T2 + BS69
ΔMYND

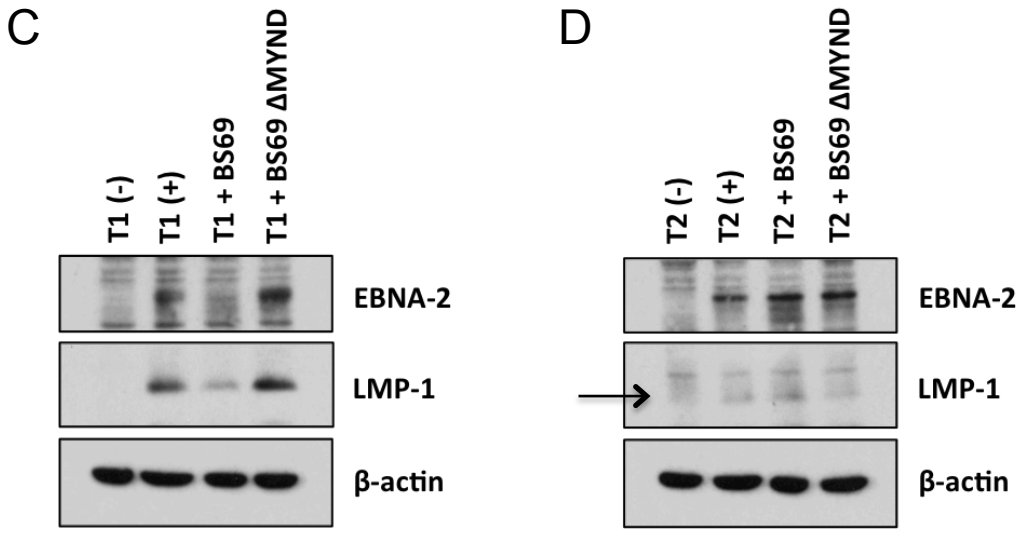

C
T1 (-)
T1 (+)
T1 + BS69
T1 + BS69 ΔMYND
EBNA-2
LMP-1
β-actin
D
T2 (-)
T2 (+)
T2 + BS69
T2 + BS69 ΔMYND
EBNA-2
LMP-1
β-actin

Figure 4.9 BS69 inhibits transactivation of the viral LMP-1 promoter by either type 1 or type 2 EBNA-2 proteins in LMP-1 promoter-reporter gene assays

2 x 10^6 EBV-positive Daudi cells were cotransfected with (**A**) 4 µg of either pHEBo-MT:E2T1 (T1, type 1 EBNA-2) or (**B**) pHEBo-MT:E2T2 (T2, type 2 EBNA-2) and 4 µg of pCI-BS69 (BS69), 4 µg of pCI-BS69-ΔMYND (BS69 ΔMYND), where indicated, 4 µg of pGL3b-B95-8-LRS (-634/+40) luciferase reporter and 50 ng of pRL-CMV *Renilla* plasmids using Neon transfection system. The empty vector pGL3b was used to get equivalent amounts of DNA *per* transfection. 5 hours after transfection cells were induced with 5 µM $CdCl_2$, except for the non-induced Daudi cells [T1 (-) and T2 (-)]. At 24 hours post-transfection cells were harvested and analysed for luciferase activity. Luciferase values were adjusted for transfection efficiency according to *Renilla* luciferase expression values. Results are then represented as the luciferase activity relative to the transfected but non-induced cells. **(A)** Results are represented as an average of three (n=3) or **(B)** two individual experiments (n=2). Error bars represent standard deviations. (**C** and **D**) Protein expression levels of type 1 (T1) and type 2 (T2) EBNA-2 were compared by western blotting analysis using PE2 anti-EBNA-2 antibody at 24 hours post-transfection. Expression levels of LMP-1 were also assessed with the CS1-4 antibody. β-actin immunoblot analysis ensured that equal amounts of proteins were loaded on the gel.

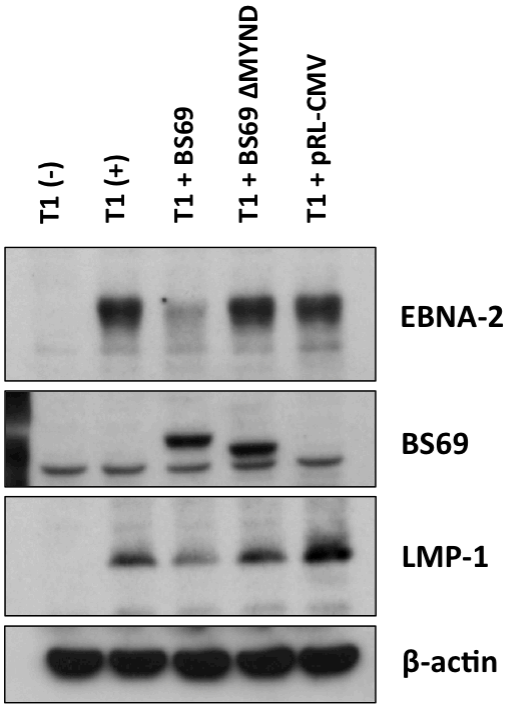
4.2.2.2 Expression of the Daudi endogenous LMP-1 gene activated by type 1 EBNA-2 and the levels of type 1 EBNA-2 protein are affected by BS69

Following the previous results showing that BS69 expression inhibited the transactivation of the viral LMP-1 promoter-luciferase reporter by either type 1 or type 2 EBNA-2, it was then investigated whether BS69 also repressed the expression of the endogenous LMP-1 gene. The aim was to test for an inhibitory role of BS69 in the EBNA-2-mediated activation of an EBNA-2 target responsive promoter, without using luciferase gene reporters. The viral LMP-1 gene is one of the genes differentially regulated by type 1 and type 2 EBNA-2 [206, 209] and is essential for transformation and proliferation of B cells latently infected by EBV [74, 135, 463].

As in the previous experiment in section 4.2.2.1, EBV-positive Daudi cells, lacking the EBNA-2 locus, were cotransfected with pHEBo plasmids expressing either type 1 or type 2 EBNA-2 from a $CdCl_2$-inducible MT promoter (pHEBo-MT:E2T1 and E2T2, section 2.3.3.13.5) and a plasmid expressing either BS69 (pCI-BS69) or BS69 ΔMYND (pCI-BS69-ΔMYND, section 2.3.3.13.9) using the Neon transfection system. EBNA-2 expression was induced 5 hours after transfection using $CdCl_2$. Protein extracts were made 24 hours post-transfection using RIPA extraction method (section 2.5.1) and analysed by western blotting to detect EBNA-2, LMP-1 and also BS69 expression levels.

The results showed that as expected, after induction of type 1 EBNA-2 expression with $CdCl_2$ there is activation of the endogenous LMP-1 gene in Daudi cells. No LMP-1 expression could be detected when there is no induction of EBNA-2 expression and LMP-1 induction by type 1 is stronger than by type 2 EBNA-2 (Figure 4.10). Although there was a lower level of LMP-1 when BS69 was co-expressed with type 1 EBNA-2, there was less EBNA-2 (or the EBNA-2 was degraded) therefore it was not possible to determine an inhibitory effect of BS69 in this experiment (Figure 4.10 A). It was noteworthy that the BS69 ΔMYND did not cause induction of LMP-1 with type 1 or type 2 EBNA-2, indicating that the levels of LMP-1 in these cells are not determined by the type of interaction that was revealed in the GAL4-activation domain experiments described earlier.

A


T1 (-)
T1 (+)
T1 + BS69
T1 + BS69 ΔMYND
T1 + pRL-CMV
EBNA-2
BS69
LMP-1
β-actin

B

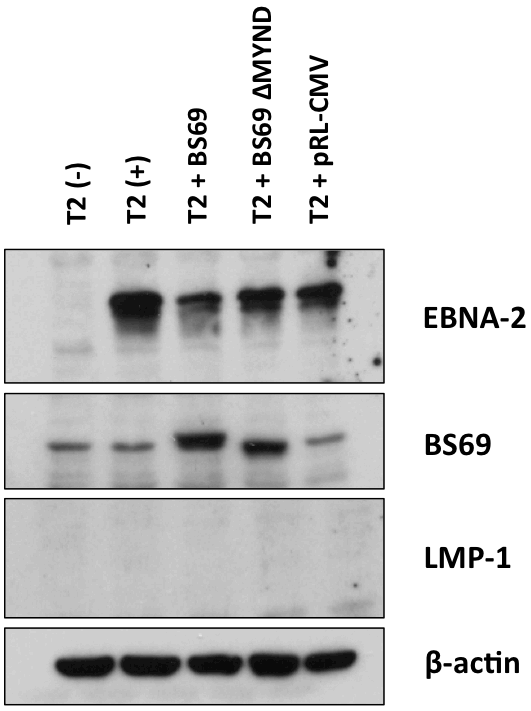

T2 (-)
T2 (+)
T2 + BS69
T2 + BS69 ΔMYND
T2 + pRL-CMV
EBNA-2
BS69
LMP-1
β-actin

Figure 4.10 Expression of the Daudi endogenous LMP-1 gene activated by type 1 EBNA-2 and the levels of type 1 EBNA-2 protein are affected by BS69 expression

EBV-positive Daudi cells were transfected with 4 µg of either (**A**) pHEBo-MT:E2T1 (T1, type 1 EBNA-2) or (**B**) pHEBo-MT:E2T2 (T2, type 2 EBNA-2) and 4 µg of pCI-BS69 (BS69), 4 µg of pCI-BS69-ΔMYND (BS69 ΔMYND) or 4 µg of pRL-CMV plasmids, where indicated, using the Neon system. At 5 hours after transfection cells were induced with 5 µM $CdCl_2$ for EBNA-2 expression, except for the non-induced Daudi cells (-). Cells were harvested 24 hours post-transfection and protein extracts were obtained by RIPA lysis. Expression levels of type 1 and type 2 EBNA-2 were determined by western blotting analysis using the PE2 anti-EBNA-2 antibody that equally recognises both types. Expression levels of LMP-1 and BS69 were also assessed with the CS1-4 anti-LMP-1 antibody and BS69 (H-265) anti-BS69 antibody, respectively. β-actin immunoblot analysis ensured that equal amounts of proteins were loaded on the gel.

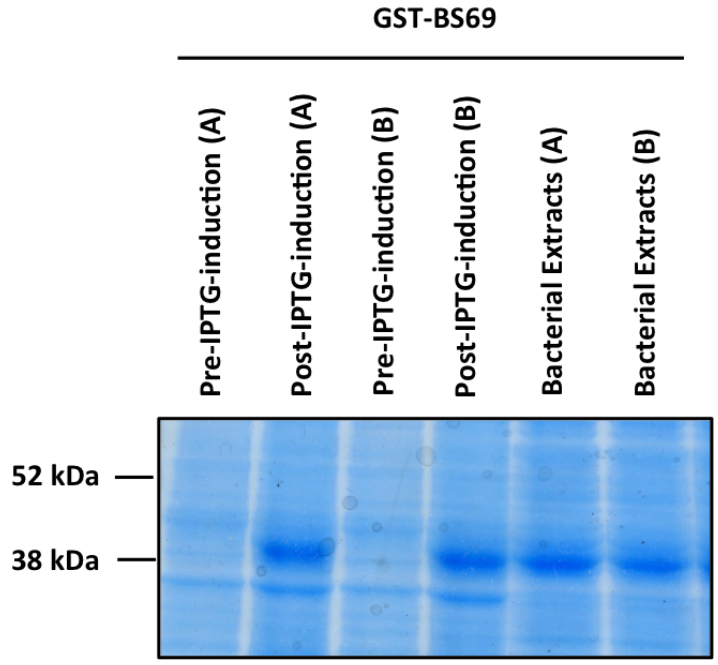
4.2.3 Comparing type 1, type 2 and type 2 S442D EBNA-2 binding to BS69 protein using glutathione S-transferase (GST) pulldowns

EBNA-2 was previously shown to interact with the BS69 cell protein [267]. BS69 forms a homodimer and two MYND domains recognise and synergistically bind two sequential PXLXP peptide motifs present in the EBNA-2 protein [268]. However, this interaction was only demonstrated for type 1 EBNA-2 but not for type 2 EBNA-2. Interestingly, one of the PXLXP motifs (PXLXP 2) in EBNA-2 is located immediately before the amino acid aspartate-442 in type 1, suggesting that this residue that was shown to be important in the transactivation ability of EBNA-2 and in the superior LCL growth maintenance by EBV type 1 EBNA-2 [210], might be associated with a differential BS69 interaction with both EBNA-2 types, relevant to the distinct type 1/type 2 EBNA-2 regulation of gene expression.

4.2.3.1 Establishing a GST pulldown assay to compare type 1, type 2 and type 2 S442D EBNA-2 binding to BS69 protein

In order to address the hypothesis that BS69 interacts differentially with type 1 or type 2 EBNA-2 and that the amino acid in position 442 might affect this interaction, glutathione S-transferase (GST) pulldown assays were performed using a GST-BS69 fusion as bait.
The fragment containing the last 150 amino acids (aa 411-561), including the MYND domain of BS69, had been cloned into the pGEX4T1 vector, fused to a GST tag. This construct had been previously used to confirm BS69 binding to E1A and EBNA-2 proteins [267] and was kindly provided by S Ansieau. This plasmid was transformed into *Escherichia coli* strain BL21 (DE3) to induce protein expression and to make bacterial extracts containing GST-BS69 (aa 411-561) fusion protein (section 2.7.3). A vector expressing a different GST-tagged fusion protein (GST-RAB11B) was also transformed into competent bacteria to make a negative control protein for the GST pulldown assays.
SDS-PAGE was used to analyse protein samples obtained from bacterial extracts were the fusions proteins have been expressed (Figure 4.11). The sizes of these GST-tagged proteins are approximately 44 kDa for GST-BS69 (aa 411-561) and 50 kDa for GST-RAB11B.

A


GST-BS69
Pre-IPTG-induction (A)
Post-IPTG-induction (A)
Pre-IPTG-induction (B)
Post-IPTG-induction (B)
Bacterial Extracts (A)
Bacterial Extracts (B)
52 kDa
38 kDa

B

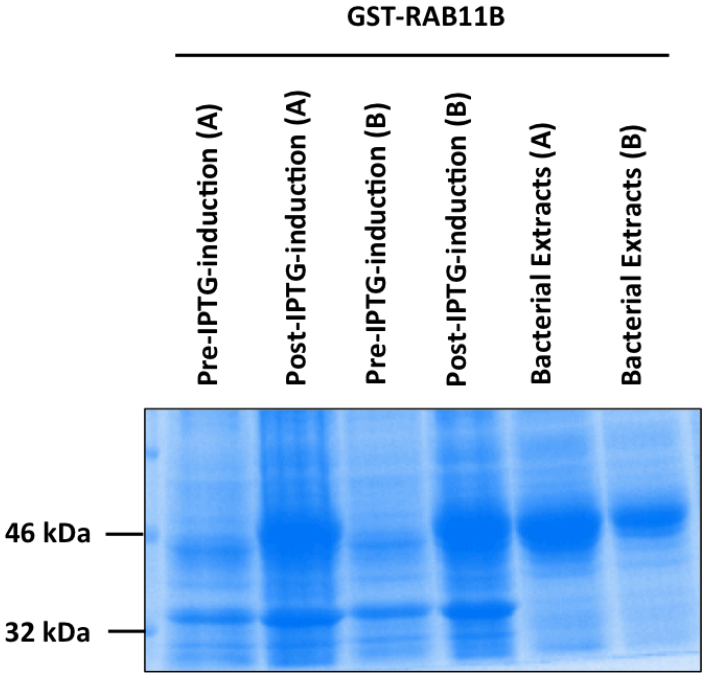

GST-RAB11B
Pre-IPTG-induction (A)
Post-IPTG-induction (A)
Pre-IPTG-induction (B)
Post-IPTG-induction (B)
Bacterial Extracts (A)
Bacterial Extracts (B)
46 kDa
32 kDa

Figure 4.11 Coomassie staining of SDS-PAGE gel demonstrating expression of GST-BS69 (aa 411-561) and GST-RAB11B fusion proteins in bacterial extracts

Escherichia coli BL21 (DE3) strain was transformed with plasmids expressing either (**A**) GST-BS69 (pGEX4T1-BS69-411-561) or (**B**) GST-RAB11B (pGEX4T1-RAB11B) fusion proteins. Protein samples were collected pre- and post-induction with isopropyl-β-D-1-thiogalactopyranoside (IPTG). Bacterial cell extracts were made and 20 µl aliquots were resolved by SDS-PAGE using a 10% acrylamide gel. The gel was then stained using Coomassie blue staining. The GST-BS69 and GST-RAB11B proteins have molecular weights of approximately 44 kDa and 50 kDa, respectively.

These GST fusion proteins were used as bait and incubated with nuclear extracts of EBV-positive Daudi cells that express either type 1, type 2 or type 2 S442D EBNA-2 proteins, in order to confirm that type 1 EBNA-2 could be pulled down by GST-BS69 using this assay and to determine whether type 2 and type 2 S442D EBNA-2 also interacted with BS69.

The three distinct stably transfected Daudi cell lines carrying a pHEBo plasmid expressing either type 1, type 2 or type 2 S442D EBNA-2, under control of a MT promoter inducible with $CdCl_2$ were previously generated in the laboratory by Claudio Elgueta and Stelios Tzellos. Before making the nuclear extracts used to perform the GST pulldown assays, the cell lines were induced with $CdCd_2$ for EBNA-2 expression.

Western blot analysis of nuclear extracts from these Daudi:pHEBo-MT cell lines expressing the different EBNA-2 types revealed that EBNA-2 expression was not the same and type 1 EBNA-2 was highly expressed when compared to type 2 or type 2 S442D (Figure 4.12 A). However, equal expression of type 1, type 2 and type 2 S442D EBNA-2 was required to establish comparisons regarding BS69 binding to each EBNA-2 type. To get equal amounts of each EBNA-2 protein to be used as input in the GST pulldown assays, the nuclear extracts were diluted with nuclear extracts of Daudi cells in which EBNA-2 expression had not been induced. The process to finally to obtain equal amounts of EBNA-2 required a considerable amount of experimentation to achieve (Figure 4.12 B).

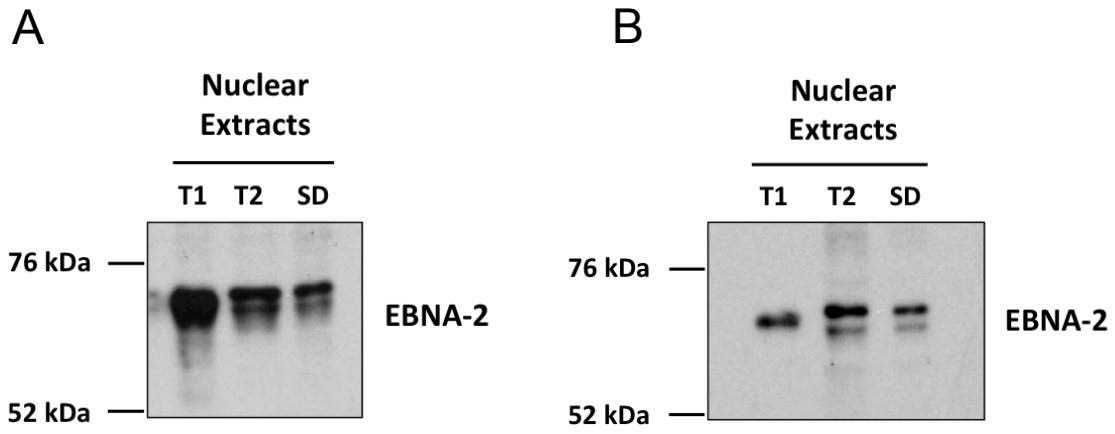


Figure 4.12 Optimisation of the amounts of type 1, type 2 and type 2 S442D EBNA-2 proteins in nuclear extracts to be used in GST-BS69 pulldown assays

Daudi:pHEBo-MT:EBNA-2 cell lines were treated with 5 μM $CdCl_2$ to induce expression of type 1 (T1), type 2 (T2) and type 2 S442D (SD) EBNA-2 proteins. At 24 hours after induction cells were harvested and lysed to make nuclear extracts. Expression levels of EBNA-2 were assessed by immunoblotting analysis using the PE2 antibody (anti-EBNA-2 antibody), which equally detects all EBNA-2 types. (**A**) Different EBNA-2 expression was detected in the nuclear extracts from the three distinct cell lines. (**B**) Nuclear extracts were diluted using nuclear extracts of non-induced cells to achieve equal amounts of each EBNA-2 type. The EBNA-2 proteins expressed by these cell lines display almost the same size (approximately 72 kDa) since the polyproline repeat region was equalised in all EBNA-2 alleles.

The nuclear extracts expressing type 1, type 2 and type 2 S442D EBNA-2 and the bacterial extracts expressing the GST-tagged proteins, were used in a GST pulldown assay. The fusion proteins GST-BS69 (aa 411-561) and GST-RAB11B were bound to glutathione beads and then incubated with nuclear extracts, containing type 1 EBNA-2 protein from Daudi:pHEBo-MT cells (section 2.7.1). Samples obtained from each individual pulldown reaction were then analysed by SDS-PAGE and the immunoblots were probed with the PE2 antibody (Figure 4.13). The previous observation by Ansieau and Leutz, in which BS69 was able to interact with EBNA-2 [267] was confirmed. Additionally, the amount of EBNA-2 protein pulled down by GST-BS69 is dose-dependent. The higher EBNA-2 amount used as input, the more is the amount of EBNA-2 pulled down by BS69 (Figure 4.13), indicating that the assay is not saturated with GST-BS69 protein.

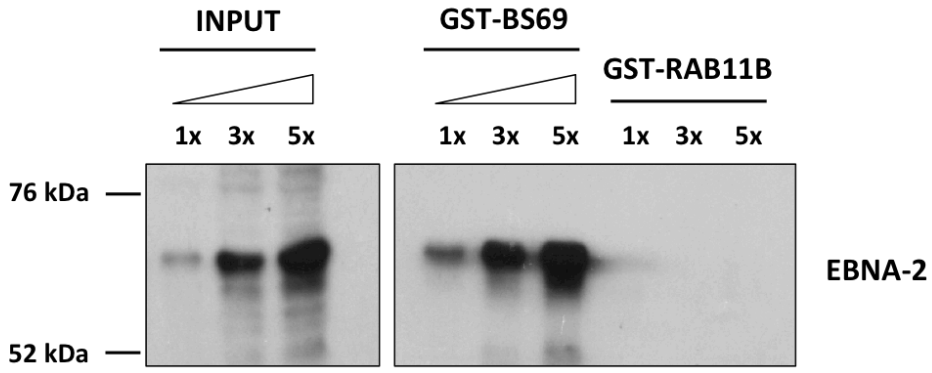


Figure 4.13 GST pulldown assay confirming type 1 EBNA-2 interaction with BS69 cell protein in a dose-dependent manner

Nuclear extracts of Daudi:pHEBo-MT:E2T1 cells expressing type 1 EBNA-2 protein were incubated with bacterial lysates expressing GST-BS69 (aa 411-561) or GST-RAB11B fusion proteins for 2 hours at 4ºC. Inputs with distinct amounts of EBNA-2 protein were used (1x, 3x and 5x) in these pulldown reactions. Each pulldown reaction was washed and the beads pelleted before being resuspended with protein sample buffer and resolved on a 7.5% SDS-PAGE gel. EBNA-2 bound to GST-tagged proteins was assessed by immunoblotting analysis with the PE2 antibody (anti-EBNA-2 antibody).

4.2.3.2 BS69 protein interacts with type 2 and type 2 S442D EBNA-2 proteins in GST pulldown assays and not only with type 1 EBNA-2

The GST pulldown assay was then used to determine whether type 2 EBNA-2 also interacted with BS69 protein and to investigate whether there are differences in BS69 binding to either EBNA-2 type.

Nuclear extracts of Daudi:pHEBo-MT cells expressing type 1, type 2 or type 2 S442D EBNA-2 proteins were incubated with the GST-BS69 (aa 411-561) and GST-RAB11B fusion proteins bound to glutathione beads for 2 hours as mentioned above. These initial results showed that BS69 also interacts with type 2 and type 2 S442D EBNA-2 proteins (Figure 4.14). However, it was not possible to determine whether there is a differential BS69 binding to each EBNA-2 type, perhaps due to the long incubation time used and also because the inputs utilised are not exactly equal to allow comparisons between each EBNA-2 protein binding to BS69. As expected, GST-RAB11B, the negative control did not pulldown any EBNA-2 protein type.

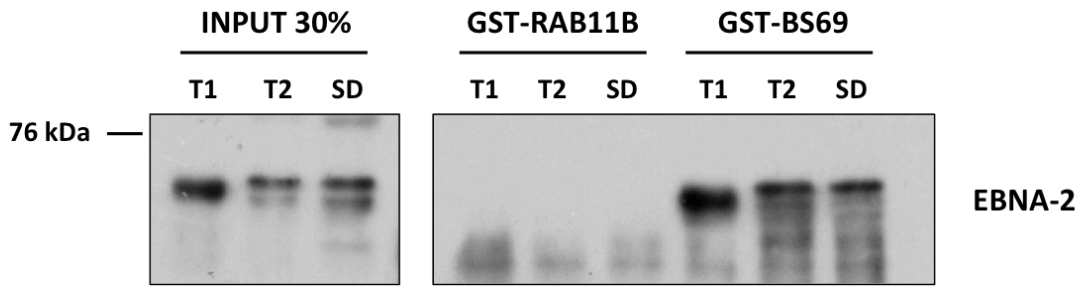


Figure 4.14 GST pulldown assay showing that BS69 protein pulled down type 1 EBNA-2, type 2 and type 2 S442D EBNA-2 proteins

Nuclear extracts of Daudi:pHEBo-MT:EBNA-2 cells expressing type 1 (T1), type 2 (T2) or type 2 S442D (SD) EBNA-2 proteins were incubated for 2 hours at 4ºC with bacterial lysates expressing the either the fusion proteins GST-BS69 (aa 411-561) or GST-RAB11B, previously bound to glutathione beads. Each pulldown reaction was thoroughly washed using binding buffer and the beads pelleted previous to resuspension with protein sample buffer. The samples were resolved on a 7.5% SDS-PAGE gel. EBNA-2 bound to GST-tagged proteins was assessed by immunoblotting analysis with the PE2 antibody (anti-EBNA-2 antibody).

4.2.3.3 GST pulldown assay suggesting that BS69 has a higher affinity for type 2 EBNA-2 than for type 1 EBNA-2

To investigate whether BS69 binds differentially to each EBNA-2 type, a timecourse experiment was carried out. Nuclear extracts of Daudi:pHEBo-MT cells expressing type 1, type 2 or type 2 S442D EBNA-2 proteins were incubated with the GST-BS69 (aa 411-561) fusion for 2 min, 5 min, 15 min and 30 min. GST-RAB11B was used as negative control (incubation time 30 min) (Figure 4.15).

The results showed that after 2 min incubation there is no detection of BS69 interaction with any EBNA-2 type, indicating that the incubation time was too short to allow EBNA-2 binding to GST-BS69. Strikingly, after 5 min and 15 min incubation, BS69 pulled down more type 2 and type 2 S442D EBNA-2 proteins than type 1 EBNA-2, suggesting that BS69 might exhibit a higher affinity for type 2 EBNA-2. However, the substitution of the serine in type 2 EBNA-2 by the corresponding amino acid aspartate 442 of type 1 EBNA-2 did not change the ability of type 2 EBNA-2 to be pulled down by BS69. At 30 min incubation, type 1, type 2 and type 2 S442D EBNA-2 proteins are equally pulled down by BS69. Throughout the timecourse, type 1 EBNA-2 binding to BS69 increased and reached saturation at 30 min. For this reason, no differences in EBNA-2 binding were detected in the previous experiment with the two hour-incubation time.

Together these data obtained in this study first demonstrated that BS69 protein might exhibit a higher affinity for type 2 EBNA-2 protein. The S442D substitution in type 2 did not make any difference in terms of EBNA-2 binding to BS69 in spite of being located immediately next to PXLXP peptide motif 2 (Figure 4.2), which is a motif recognised by BS69. The amino acid aspartate 442 in type 1 EBNA-2, which had been shown to be important for the higher ability to activate gene expression and for the superior ability to maintain LCL growth, does not account for the different BS69 ability to bind type 1 and type 2 EBNA-2. Therefore, there is indeed a differential type 1/type 2 effect in terms of BS69 binding but this did not explain the type 1/type 2 differential gene regulation, since type 2 S442D EBNA-2 is equally bound by BS69.

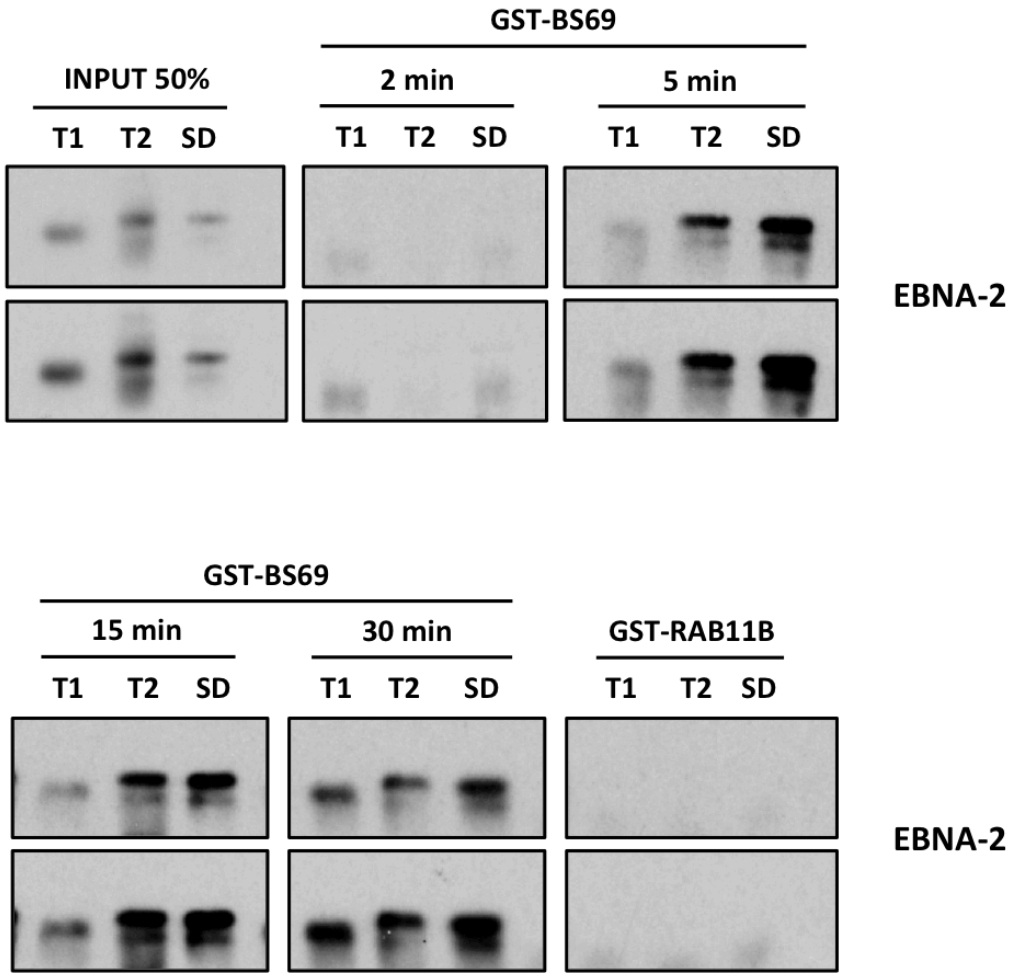


Figure 4.15 GST pulldown assay showing that BS69 protein exhibits a higher capacity to bind type 2 and type 2 S442D EBNA-2 than type 1 EBNA-2

In a timecourse experiment nuclear extracts of Daudi:pHEBo-MT:EBNA-2 cells expressing type 1 (T1), type 2 (T2) or type 2 S442D (SD) EBNA-2 proteins were incubated for 2 min, 5 min, 15 min and 30 min at 4ºC, with bacterial lysates expressing the GST-BS69 (aa 411-561) fusion, previously bound to glutathione beads. GST-RAB11B was used as a negative control and incubated for 30 min with the nuclear extracts. Each pulldown reaction was thoroughly washed using binding buffer and the beads pelleted previous to resuspension with protein sample buffer. The samples were resolved on a 7.5% SDS-PAGE gel. EBNA-2 bound to GST-tagged proteins was assessed by immunoblotting analysis using the PE2 antibody (anti-EBNA-2 antibody). The EBNA-2 proteins expressed by these cell lines display almost the same size (72 kDa) since the polyproline repeat region was equalised in all EBNA-2 alleles.

4.2.3.4 GST pulldown assay using *in vitro* translated EBNA-2 proteins showed no difference in binding to BS69

To test whether cell modifications to EBNA-2 or other proteins in the nuclear extracts might affect the interaction with BS69, EBNA-2 was also prepared by *in vitro* translation. The required pBlueScript plasmids expressing type 1, type 2 and type 2 S442D EBNA-2 proteins, under control of a T7 promoter (section 2.3.3.13.6), were already available in the laboratory. A pBlueScript vector that expresses chimera-2 EBNA-2 protein (a type 2 protein carrying the C-terminal end of type 1 EBNA-2) [209] was also used (Figure 4.16 A). These plasmids were used for *in vitro* translation of [S-35] methionine-labelled EBNA-2 proteins with the rabbit reticulocyte system (section 2.7.2) (Figure 4.16 B).

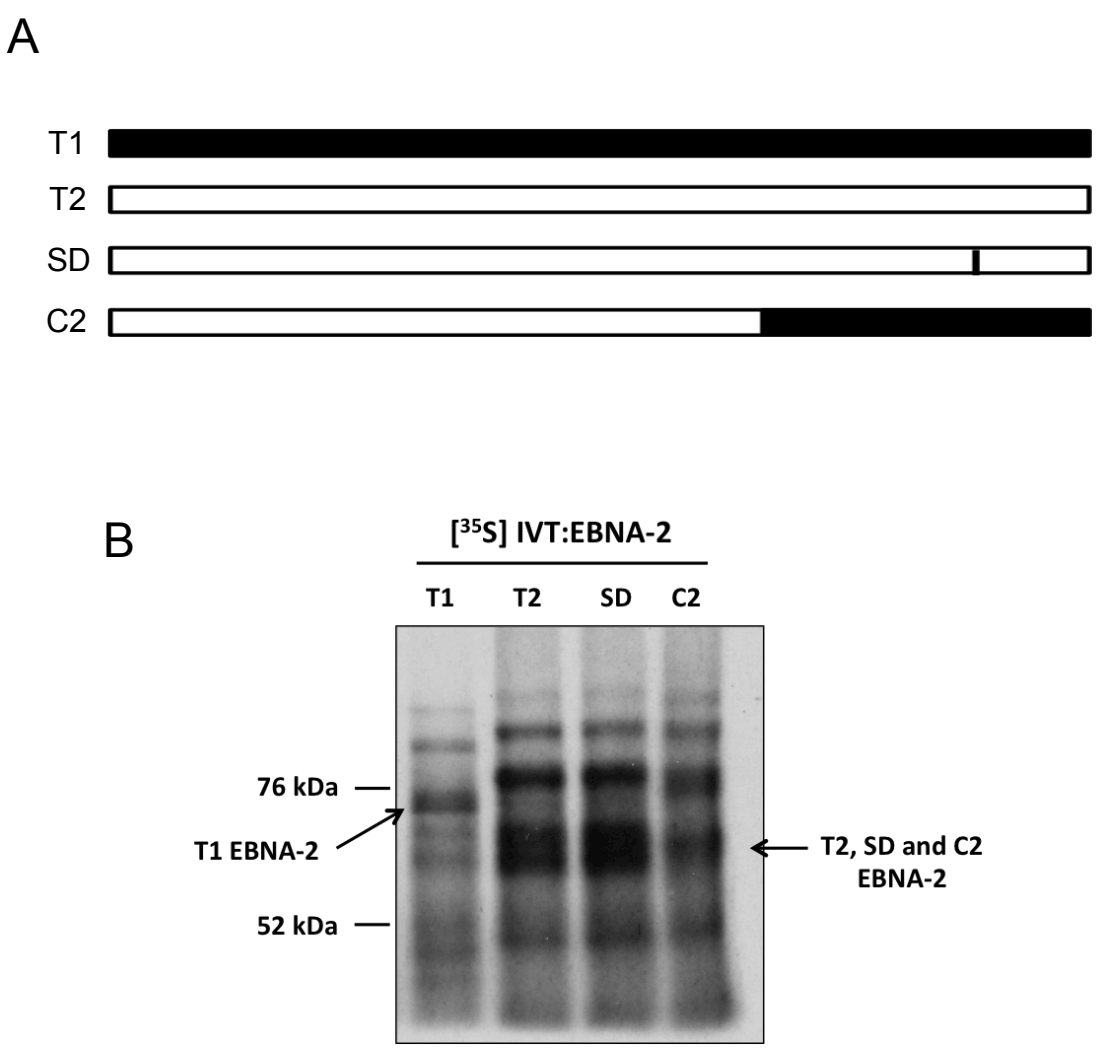


Figure 4.16 Type 1, type 2, type 2 S442D EBNA-2 and chimera-2 EBNA-2 proteins were *in vitro* translated and [S-35] methionine-labelled using the rabbit reticulocyte system

(**A**) Panel of EBNA-2 proteins that were *in vitro* translated and [S-35] methionine-labelled for this study. Black and white indicate type 1 and type 2 sequences, respectively. T1: type 1 EBNA-2; T2: type 2 EBNA-2; SD: type 2 S442D EBNA-2 and C2: chimera-2 EBNA-2 proteins. (**B**) *In vitro* translated proteins were resolved on a 7.5% SDS-PAGE gel and detected by autoradiography. Expected sizes of the EBNA-2 proteins are approximately 80 kDa for type 1 EBNA-2 and 72 kDa for type 2 and type 2 S442D EBNA-2, as well as for chimera-2 EBNA-2 protein. The size of type 1 EBNA-2 is larger than that of type 2 EBNA-2 since the EBNA-2 alleles cloned into the pBlueScript vectors were not equalised for the size of the polyproline repeat region.

Several attempts were made to change the transcription and translation conditions in order to obtain a single *in vitro* translated (IVT) EBNA-2 product (Figure 4.16 B). The expected size for type 1 EBNA-2 is around 80 kDa and the size of EBNA-2 proteins with type 2-backbone (type 2, type 2 S442D and chimera-2) is approximately 72 kDa. In contrast to the EBNA-2 proteins expressed by Daudi:pHEBo-MT:EBNA-2 cells, the EBNA-2 alleles cloned into the pBlueScript vectors have not been equalised for the length of the polyproline repeat region. For this reason, a chimeric EBNA-2 protein was also used in this study (Chimera-2, C2). Chimera-2 is a type 2 EBNA-2 protein carrying the C-terminal region of type 1 EBNA-2 spanning from the WWPP motif (codons 323 to 326) to the end of the protein that was swapped at homologous amino acid positions into the type 2 protein [209]. This C-terminal region of type 1 includes both PXLXP motifs recognised by BS69 protein.

These [S35]-labelled EBNA-2 proteins were used in the GST pulldown assay using GST-BS69 (aa 411-561) fusion protein immobilised on glutathione-agarose beads as bait, as in the section 4.2.3.3. The purpose of this experiment was to demonstrate a direct interaction between the EBNA-2 proteins and BS69 and also reproduce the previous GST pulldown experiment in the absence of all the other proteins present in the Daudi:pHEBo-MT:EBNA-2 nuclear extracts used above.

The results indicated that the different [S35]-labelled EBNA-2 proteins when incubated for 20 min with GST-BS69 (aa 411-561) are strongly pulled down by BS69, confirming the previous results with the EBNA-2 proteins expressed in nuclear extracts. However, no differences in BS69 binding to type 1, type 2, type 2 S442D and Chimera-2 EBNA-2 proteins were detected (Figure 4.17 A). GST-RAB11B was used as a negative control and EBNA-2 protein was not pulled down by RAB11B or by the beads. Each pulldown experiment was also loaded on a second 7.5% SDS-PAGE and stained with Coomassie blue stain and the presence of equal amounts of the GST-BS69 protein *per* each individual experiment was confirmed (Figure 4.17 B).

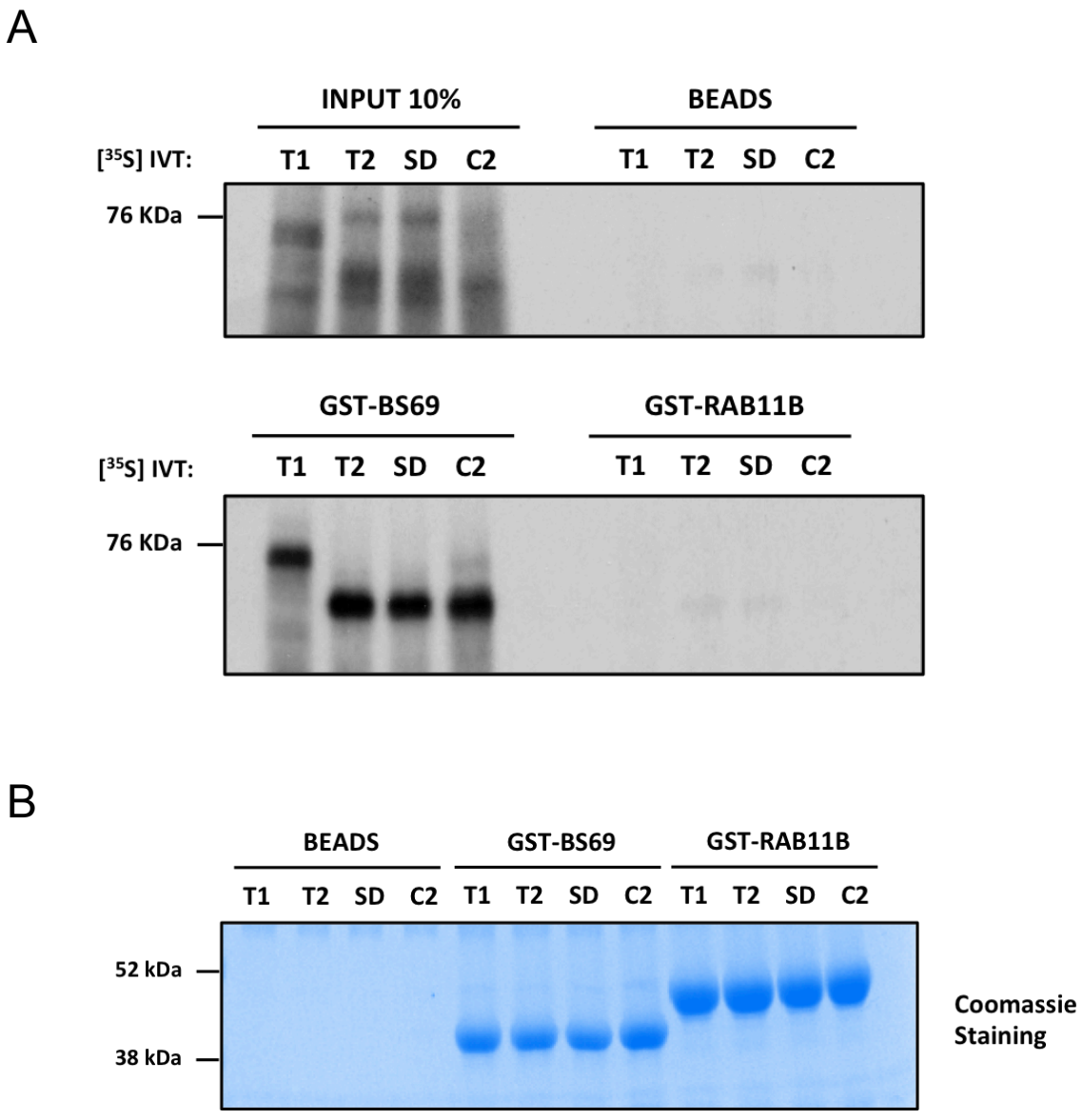


Figure 4.17 GST pulldown assay showing that *in vitro* translated [S^{35}] methionine-labelled type 1, type 2, type 2 S442D and chimera-2 EBNA-2 proteins are equally bound by BS69 (aa 411-561)

(**A**) *In vitro* translated [S^{35}] methionine-labelled type 1 (T1), type 2 (T2), type 2 S442D (SD) and chimera-2 (C2) EBNA-2 proteins were incubated with the GST-BS69 (aa 411-561) and GST-RAB11B fusion proteins immobilised on glutathione-agarose beads, for 20 min at 25ºC. Samples were resolved on a 7.5% SDS-PAGE gel and detected by autoradiography. Expected sizes of EBNA-2 proteins are around 80 kDa for type 1 EBNA-2 and 72 kDa for type 2 and type 2 S442D EBNA-2, as well as for chimera-2 protein (**B**) Samples were also resolved on a parallel 7.5% SDS-PAGE gel to control for equal amounts of GST-BS69 and GST-RAB11B fusion proteins *per* pulldown experiment. The SDS-PAGE gel was stained using Coomassie blue staining. The GST-BS69 and GST-RAB11B proteins have molecular weights of approximately 44 kDa and 50 kDa, respectively.

The previous observation suggested that possibly the duration of the incubation of the [S35]-labelled EBNA-2 proteins with the GST-BS69 protein was not sufficiently short to reveal any differences in BS69 binding to type 1 and type 2 EBNA-2, as was previously observed for the EBNA-2 proteins expressed in nuclear extracts of Daudi:pHEBo-MT:EBNA-2 cells.

To address this point, shorter incubations times (5 min and 10 min) were used, as well as different incubation temperatures (25ºC and 4ºC) to determine whether BS69 exhibits a distinct affinity to type 1 and type 2 EBNA-2 [S35]-labelled proteins, as demonstrated for the EBNA-2 proteins expressed in Daudi cells. However, when type 2 EBNA-2 was compared to chimera-2 (type 2 protein carrying the C-terminal region of type 1 that comprises both PXLXP peptide motifs recognised by BS69) no evident differences in BS69 binding were detected. In addition, type 2 S442D EBNA-2 exhibited a similar binding to BS69 (Figure 4.18) and type 2 binding to BS69 was not directly compared to type 1 in this experiment.

The results in Figure 4.17 have shown that type 1 and type 2 EBNA-2 proteins bind directly to BS69 but no differences in BS69 binding when the *in vitro* translated [S35]-labelled EBNA-2 proteins were incubated with BS69. In Figure 4.18, distinct experimental settings were tested but type 2 was not compared to type 1 EBNA-2 but only to chimera-2. Hence, the previous results observed in section 4.2.3.3 for the proteins present in nuclear extracts of Daudi cells could not be reproduced when using the cell-free synthesised EBNA-2 proteins. Although not fully tested since direct type 1 EBNA-2 binding to BS69 was not compared to that of type 2, it is possible that these proteins might lack any appropriate protein modification or a third partner present in the nuclear extracts, which could be required to cause the differential type 1/type 2 binding to BS69 observed for the EBNA-2 proteins expressed by Daudi cells.

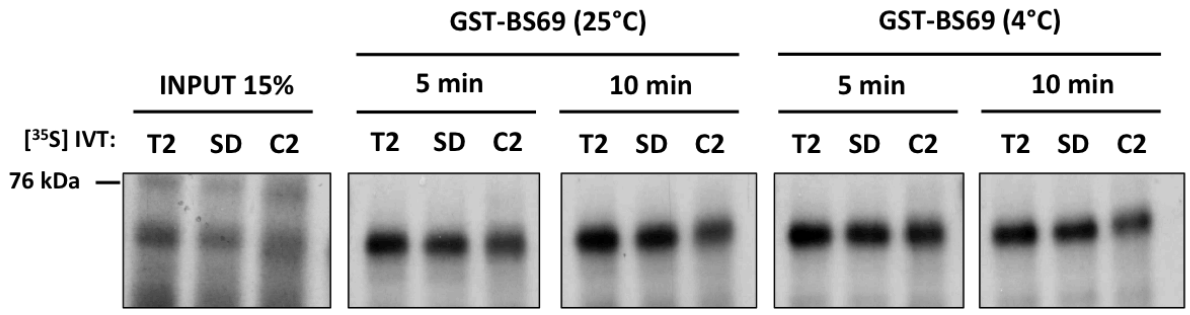


Figure 4.18 GST pulldown assay showing that *in vitro* translated [S^{35}] methionine-labelled type 2, type 2 S442D and chimera-2 EBNA-2 proteins are equally bound by GST-BS69 (aa 411-561), irrespective of incubation time and temperature

In vitro translated [S^{35}] methionine-labelled type 2 (T2), type 2 S442D (SD) and chimera-2 (C2) EBNA-2 proteins were incubated with GST-BS69 (aa 411-561) fusion protein immobilised on glutathione-agarose beads, for different incubation times (5 min and 10 min) and at distinct temperatures (4ºC and 25ºC). Each GST pulldown reaction was thoroughly washed with binding buffer and the beads pelleted previous to resuspension with protein sample buffer. Samples were resolved on a 7.5% SDS-PAGE gel and detected by autoradiography. Expected sizes of EBNA-2 proteins are 72 kDa for type 2 and type 2 S442D EBNA-2, as well as for chimera-2 protein.

4.3 Discussion

In the present study, the function of BS69 in the regulation of EBNA-2 transactivation of gene expression was investigated. For this, the GAL4-DBD:E2-TAD (aa 426-463) constructs, that were previously used to compare the transactivation function of type 1 and type 2 EBNA-2 (in section 3.2.1), were used again to determine the effect of BS69 expression on their ability to activate a synthetic promoter with GAL4 DNA-binding sites controlling luciferase expression. The results showed that BS69 did not repress gene transactivation mediated by these GAL4 constructs (Figure 4.1), contrary to a previous study where BS69 was shown to downregulate EBNA-2-mediated transcriptional activation [267]. In the previous report, the full-length EBNA-2 protein had been fused to the GAL4-DBD to perform the reporter assays and BS69 was able to repress EBNA-2-mediated transactivation function [267] but my initial GAL4 constructs contained only the amino acids 426 to 463 of EBNA-2 TAD and were not subject to repression by the BS69 protein. The GAL4-DBD:E2-TAD (aa 426-463) constructs did not contain the EBNA-2 peptide motif PXLXP1 (aa 383-387), which was shown to be involved in EBNA-2 recognition by BS69 homodimer [268], suggesting that both PXLXP peptide motifs are necessary for BS69 inhibition of gene induction by EBNA-2. To address this point, GAL4-DBD:EBNA-2 constructs, which comprise the EBNA-2 region from the amino acids 334 to 487 and therefore also include the peptide motif PXLXP1 (aa 383-387), were generated (Figure 4.2). Indeed, in the presence of both PXLXP peptide motifs, there was BS69 inhibition of gene transactivation mediated by either type 1 or type 2 EBNA-2, when the plasmid expressing BS69 protein was cotransfected (Figure 4.5 B). An earlier report, where the TAD of EBNA-2 was identified, had shown that different EBNA-2 fragments could exhibit distinct ability to transactivate gene expression [216]. The TAD EBNA-2 fragment (aa 426-462) had been shown to be a stronger activator when compared to other EBNA-2 fragments and to the full-length protein perhaps because it lacked the PXLXP1 motif and could not be subject to BS69-mediated repression.

In an attempt to demonstrate that the repression of gene activation was due to BS69 protein, a plasmid expressing BS69 ΔMYND mutant, a BS69 protein without the MYND domain, was cotransfected. As expected, there was no repression of gene transactivation mediated by the GAL4-EBNA-2 (aa 334-487) constructs because BS69 ΔMYND cannot interact with EBNA-2 and binding of the BS69 MYND domains to EBNA-2 PXLXP motifs is required for repression of EBNA-2-mediate gene transactivation [267, 268]. The requirement of BS69 interaction via

the MYND domain to mediate its repressor function was also observed in previous works for other proteins [367, 368, 378]. Although no repression was detected in the presence of BS69 ΔMYND mutant, a substantial increase in gene transactivation was observed (Figure 4.6 A), suggesting that the dominant-negative mutant BS69 ΔMYND protein relieves some repressor mechanism that might be acting on the GAL4-EBNA-2 (aa 334-487) constructs. Accordingly, it is possible that the levels of the endogenous BS69 protein might be sufficient to repress gene transactivation by the GAL4 fusions and the presence of BS69 ΔMYND could displace BS69 from EBNA-2 by the formation of homodimers with the endogenous BS69 protein, preventing its binding to EBNA-2 since BS69 ΔMYND cannot bind to EBNA-2. Therefore, an increase in gene transactivation due to alleviation of repression mediated by the endogenous BS69 was detected. Interestingly, this increase in gene activation upon cotransfection of BS69 ΔMYND was also detected for the GAL4-TAD (aa 426-463) constructs, which contain only the PXLXP2 peptide motif and had not been shown to be repressed by BS69 protein (Figure 4.6 B). This increase caused by BS69 ΔMYND mutant was much more pronounced than that observed in the GAL4-EBNA-2 (aa 334-487) constructs. This observation suggested that despite having only one PXLXP peptide motif, the GAL4-TAD (aa 426-463) fusions might also be repressed by BS69. This was supported by a recent work confirming that BS69 was able to bind EBNA-2 peptides encompassing either PXLXP1 or PXLXP2 motifs with a dissociation constant (K_d) of 7.4 μM and 34.9 μM, respectively, when isothermal titration calorimetry was used [268]. The binding affinity of the BS69 protein for an EBNA-2 fragment encompassing both PXLXP motifs was shown to be 30-150 fold stronger than the binding of BS69 to either motif alone (K_d of 0.24 μM) [268]. Therefore, BS69 can bind one of the PXLXP motifs alone although with a lower affinity, suggesting that the endogenous BS69 might also repress gene transactivation by the GAL4-TAD (aa 426-463). This is supported by an earlier report showing the mutation of a single PXLXP motif in EBNA-2 did not abrogate BS69 binding [267]. Therefore, in a similar manner, expression of BS69 ΔMYND dominant-negative mutant might relieve this repression (Figure 4.6 B). In addition, the absence of gene transactivation inhibition when the wildtype BS69 protein was coexpressed might indicate the levels of the endogenous BS69 protein could be sufficient to bind to and saturate binding to a single PXLXP peptide motif and no further repression is observed in the GAL4-TAD (aa 426-463) constructs. Interestingly, after derepression of gene transactivation when BS69 ΔMYND was cotransfected, type 1 EBNA-2 TAD still exhibited a 2-fold stronger ability to transactivate gene expression than that of type 2 and the S442D change increased transcription activation of type 2 (Figure 4.6 B), as observed in the previous chapter (see section 3.2.1). Conversely, when using the GAL4-EBNA-2 (aa 334-487) fusions, the transactivation ability of type 1 was

identical to that of type 2 EBNA-2 (Figure 4.5 A and Figure 4.6 A). The S442D substitution in type 2 however conferred stronger transactivation function than that of either type 1 or type 2 (Figure 4.5 A). The discrepant behaviour of the GAL4-EBNA-2 fusions in terms of the ability to transactivate gene expression indicated that this system is not the most adequate to study the differential gene regulation by type 1 and type 2 EBNA-2 and the function played by the amino acid aspartate 442 in this context. Although it has been shown that BS69 could indeed affect EBNA-2 ability to activate gene expression by repressing this activation process, it was not possible to determine whether BS69 plays a role in the distinct gene induction by type 1 and type 2 EBNA-2.

The two MYND domains of a BS69 homodimer recognise two sequentially proximate EBNA-2 PXLXP peptide motifs located in the CR7 and CR8 domains, respectively. These regions of EBNA-2 were shown to play a role in its B cell transformation function. CR7 was found to be important for cooperative coactivation of EBNA-2 by EBNA-LP protein [119, 224], whereas CR8 includes the TAD of EBNA-2, which mediates transcriptional activation through recruitment of the cellular basal transcriptional machinery and histone acetyltransferases [258-261]. It is very likely that interactions of BS69 protein with the PXLXP motifs located in these domains might affect cooperation with EBNA-LP and the binding of EBNA-2 with basal transcriptional machineries and histone acetyltransferases and thereby inhibiting the transactivation function of EBNA-2.

The effect of BS69 protein on the EBNA-2 functional homologue N-ICD was also investigated using the fusions GAL4-DBD:OPA-PEST domain of N-ICD generated in the previous chapter (see section 3.2.3). Cotransfection of BS69 did not cause repression of gene transactivation by the TAD of N-ICD (Figure 4.8). This is consistent with a previous report that demonstrated that expression of BS69 strongly reduced reporter activation through E1A or EBNA-2 but not through the active form of Notch [267]. When the peptide sequence of Notch1 PEST domain was analysed (Figure 3.9), a PXLXP peptide motif was identified, corresponding to PXLXP2 in EBNA-2. This motif in Notch is indeed a consensus motif recognised by BS69, suggesting that this protein binds to human Notch1. However, it was not possible to determine whether BS69 bound to Notch and there is no evidence in the literature that demonstrates the Notch1 interacts with the cell protein BS69. Therefore, the presence of this PXLXP peptide motif in Notch1 might not sufficient for BS69 interaction. Similarly, to other proteins that carry PXLXP peptide motifs, the human TATA-box binding protein contains a PXLXP motif at its C-terminal

end but failed to bind BS69, suggesting that additional structural constraints are required for specific MYND domain/PXLXP interaction [267].

In order to circumvent the limitations of using GAL4 gene reporters, in which different EBNA-2 fragments fused to GAL4-DBD exhibited distinct transactivation function depending on their sizes, allied to the utilisation of a synthetic promoter containing GAL4 DNA-binding sites, the role of BS69 in EBNA-2-mediated gene expression was investigated using a distinct reporter assay (section 4.2.2). Cotransfection of BS69-expressing plasmid demonstrated that BS69 is able to inhibit transactivation of the viral LMP-1 promoter fused to a luciferase gene by either type 1 or type 2 full-length EBNA-2 proteins (Figure 4.9 A and B) in EBV-positive Daudi cells. This is consistent with a recent report by Harter *et al*, showing that BS69 repressed EBNA-2-mediated transcriptional activation of the viral LMP-1 and Cp promoters [268]. Although first demonstrated in the present study that BS69 inhibited transactivation of gene expression by type 2 EBNA-2, again no differential function of BS69 on type 1 and type 2 EBNA-2-mediated induction of the LMP-1 promoter was detected.

The inhibitor role of BS69 in the EBNA-2-mediated activation of an EBNA-2 target promoter, was also assessed without using reporter assays. Specifically, it was determined whether the activation of the endogenous LMP-1 gene of the EBV-positive Daudi cell line by EBNA-2 was subject to repression by BS69. LMP-1 is differentially induced by type 1 and type 2 EBNA-2, as previously determined [209, 210]. Accordingly, when EBNA-2 expression was induced in Daudi cells, LMP-1 gene was more strongly transactivated by type 1 EBNA-2 than by type 2 (Figure 4.10 A and B). When BS69 was cotransfected into Daudi cells there was an inhibition of LMP-1 induction but this was accompanied by decreased levels of type 1 EBNA-2 protein that could account for the lower induction of LMP-1. Therefore, this reduced activation might not be due to the role of BS69 as a repressor protein (Figure 4.10 A). Intriguingly, this strong reduction of type 1 EBNA-2 levels when BS69 was cotransfected was consistently observed in different experiments (Figure 4.9 C and Figure 4.10 A) and was not detected for the type 2 EBNA-2 protein (Figure 4.9 D and Figure 4.10 B). This suggested that when overexpressed, BS69 might downregulate the levels of type 1 EBNA-2 in a MYND domain-dependent effect because this reduction was not detected in the presence of BS69 ΔMYND. Further analysis will be required to test this hypothesis. Similarly, a recent study has shown that BS69 ectopically expressed was able to block LCL proliferation mediated by EBNA-2, in a mechanism depending on the interaction of BS69 with EBNA-2 [268]. Conversely, it had been revealed that EBNA-2 is recruited to the BS69 gene promoter upon EBV infection of B

cells [245] and that EBNA-2 also downregulates the mRNA and the protein levels of BS69 in LCLs and in EBV-infected B cells when EBNA-2 expression is triggered [268]. BS69 downregulation had also been reported in a previous study, in which the HCA587 protein was found to regulate BS69 expression at a post-transcriptional level by promoting its proteosomal degradation through enhancement of BS69 ubiquitination [464]. In fact, these effects are not entirely surprising taking into account the emerging role of BS69 as a tumor suppressor candidate, which is frequently deleted in many human cancers [367, 368, 378, 465]. BS69 was also found to interact with the LMP-1 protein and to negatively regulate NF-kB activation mediated by LMP-1 [400, 401]. Overexpression of the cancer-testis antigen HCA587/MAGE-C2 was shown to enhance LMP-1-induced IL-6 production by degradation of BS69 [464]. Therefore, it is very likely that EBNA-2 downregulates BS69 levels as a strategy to evade host defenses and to promote B cell proliferation. Although many effects have been described involving BS69 and EBNA-2 proteins, none of these identified a specific function of BS69 in the type 1/type 2 EBNA-2 differential gene regulation.

BS69 repressed both type 1 and type 2 EBNA-2-mediated gene transactivation, so the capacity of BS69 to bind EBNA-2 was investigated. Previous works have revealed that BS69 was able to interact with type 1 EBNA-2 protein [267, 268]. The hypothesis addressed in this study was that a differential interaction of BS69 with type 1 and type 2 EBNA-2 proteins might account for the distinct regulation of EBNA-2 target responsive genes. Accordingly, a stronger binding of BS69 to the type 2 EBNA-2 protein would allow a higher inhibition of gene transactivation by type 2. In contrast, the interaction with less affinity with type 1 EBNA-2 would promote a stronger gene induction by type 1. In fact, GST pulldowns, in which nuclear extract of Daudi cells expressing either type 1 or type 2 EBNA-2 proteins was incubated with the GST-BS69 fusion protein for different incubation times demonstrated that BS69 was able to pull down more type 2 EBNA-2 than type 1 protein. This was observed for the incubation times of 5 and 15 min. After 30 min both proteins were equally pulled down by BS69 because saturation was achieved and the differences in BS69 binding were no longer detected. This suggests that BS69 might indeed have a stronger affinity to type 2 EBNA-2 protein (Figure 4.15). The results showed that BS69 differentially interacts with type 1 and type 2 EBNA-2 proteins and suggest that BS69 might indeed have a function in the type 1 type 2 differential gene regulation.

A recent report provided further evidence for the function of BS69 in determining the different transactivation potential of two homologous ETS transcription factors, ETS1 and ETS2 [466],

which share 55% amino acid similarity [467]. ETS1 and ETS2 compete for the same binding sites in the genome and were found to have distinct transactivation function due to a specific protein-protein interaction between the N-terminus of ETS2 and the repressor BS69. In fact, both ETS factors carry a PXLXP motif at their N-terminal end but only ETS2 can bind BS69, which indicates binding might be determined by other sequence differences [380, 466]. ETS2 interaction with BS69 therefore results in reduced transactivation function [466]. Interestingly, a previous study reported that ERK (extracellular signal-regulated kinase) phosphorylation of the threonine 72 residue, included in the ETS2 PXLXP motif, relieved BS69 interaction with the ETS2 factor [380]. Similarly, this observation might suggest that replacing the serine 442 of type 2 EBNA-2 by the corresponding aspartate of type 1, preceded by PXLXP2 motif in EBNA-2, would cause a reduction in the affinity of BS69 interaction with type 2 EBNA-2 and therefore contribute for an increased transactivation function and for the ability to sustain cell growth as determined before (section 3.2.1 and [210]). However, GST pulldown assays have revealed that the S442D change did not affect the ability of type 2 EBNA-2 to bind BS69 with higher affinity than type 1 (Figure 4.15). In fact, BS69 interaction with type 2 S442D EBNA-2 was similar to that with type 2 EBNA-2. Nevertheless, it would be interesting to determine the effect of a D442S or D442A mutation in type 1 EBNA-2 binding to BS69 and assess whether these mutations increase BS69 binding. It might be possible that the S442D change in type 2 protein indeed slightly decreases the BS69 affinity to type 2 EBNA-2 but this effect could not be detected in the GST pulldown assays. Alternatively, it might be possible that this higher BS69 affinity to type 2 and type 2 S442D EBNA-2 when compared to type 1 could reflect the presence of a third PXLXP motif (PXLXP3) in type 2 EBNA-2 sequence (Figure 4.2). This peptide motif, which was identified in the present study, is a consensus BS69 site that could be potentially recognised by this protein. Hypothetically, the stronger binding to type 2 EBNA-2 might also stabilise type 2 protein but not type 1 and therefore a reduction in type 1 EBNA-2 protein levels was detected when BS69 was overexpressed (Figure 4.9 C and Figure 4.10 A). Interestingly, BS69 was associated with ubiquitin ligase activity via its PHD finger domain and was found to reduce ubiquitination of the adenoviral E1A protein therefore promoting its stabilisation in a MYND domain-dependent manner [377].

The EBNA-2 protein undergoes several post-translational modifications that include different patterns of phosphorylation and arginine methylation of the RG repeat [211, 212, 271, 439, 468]. In order to determine whether cell modifications to EBNA-2 or other proteins in Daudi nuclear extract might affect the interaction with BS69, *in vitro* translated EBNA-2 proteins were used to assess the ability of BS69 to bind type 1 and type 2 EBNA-2. The results

revealed that no differences in BS69 binding were observed for type 1, type 2 and type 2 S442D EBNA-2. A chimeric type 2 EBNA-2 protein, carrying the C-terminal end of type 1 including both PXLXP peptide motifs, was used to test EBNA-2 proteins with similar sizes and to facilitate *in vitro* translation because the length of the polyproline repeat region is smaller in type 2 EBNA-2 N-terminal end. Nevertheless, equal BS69 binding to each EBNA-2 protein (not directly tested for type 1 EBNA-2, only for chimera-2 protein) was detected even when shorter incubation times and distinct temperatures were tested in an attempt to reveal different binding. This suggests that likely some post-translational modifications absent in the *in vitro* translated protein might be required to generate the differential ability of BS69 to bind type 1 and type 2 EBNA-2. Alternatively, a third protein partner present in the Daudi nuclear extract could be necessary to condition the distinct type 1/type 2 binding to EBNA-2.

EBNA-3 proteins were shown to inhibit RBP-Jk-dependent transcriptional activation of the EBNA-2 responsive promoters Cp and LMP-2A by competing with the EBNA-2 protein for the RPB-Jk binding sites at these promoters [126, 127, 264, 469, 470]. Interestingly, type 1 EBNA-3A and type 1 EBNA-3C were also found to contain a PXLXP peptide motif at their N-terminus, in contrast to the type 2 proteins. Inês Costa in our laboratory (working under my supervision) investigated the capacity of BS69 to bind EBNA-3A and EBNA-3C proteins. The N-terminus fragment of each protein was synthesised using *in vitro* translation and incubated with GST-BS69 fusion, as carried out in the present study for EBNA-2 and it was determined whether BS69 could pulldown EBNA-3A and EBNA-3C proteins. Strikingly, type 1 EBNA-3A/3C proteins were found to interact with BS69. Type 2 proteins that do not carry the PXLXP peptide did not seem to interact with BS69 and only a weak unspecific binding was detected (Figure 4.19). Intriguingly, the function played by BS69 upon binding to type 1 EBNA-3A/3C and to EBNA-2 proteins still remains undetermined. This observation however could suggest a common mechanism involving both EBNA-3A/3C and EBNA-2 proteins and BS69. Accordingly, it might be possible that type 1 EBNA-3A/EBNA-3C proteins indeed bind BS69, outcompeting type 1 EBNA-2 interaction with BS69 but not type 2 EBNA-2, contributing to a differential EBNA-2 binding to BS69 that was not observed when *in vitro* translated EBNA-2 proteins were used.

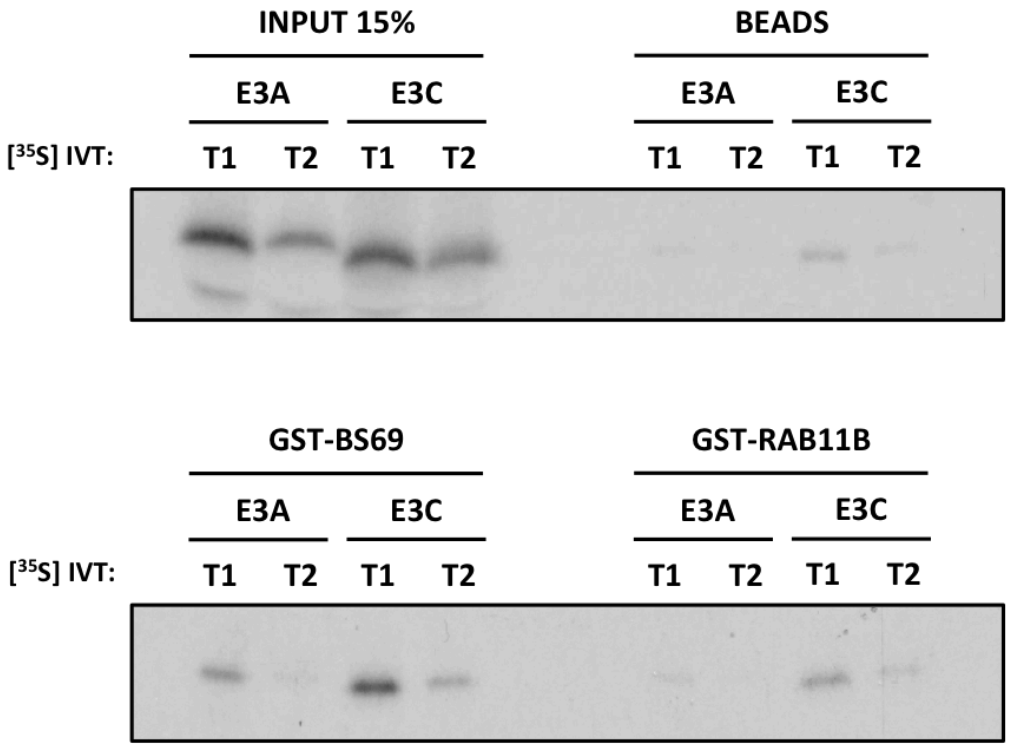


Figure 4.19 GST pulldown assay showing that *in vitro* translated [S^{35}] methionine-labelled type 1 EBNA-3A and EBNA-3C interact with BS69

In vitro translated [S^{35}] methionine-labelled type 1 (T1) and type 2 (T2) EBNA-3A (E3A) and EBNA-3C (E3C) proteins were incubated with the GST-BS69 (aa 411-561) and GST-RAB11B fusion proteins immobilised on glutathione-agarose beads, for 20 min at 25ºC. Pulldown samples were resolved on a 12% SDS-PAGE gel and detected by autoradiography. Conducted by Inês Costa.

Apart from the PXLXP motifs detected in type 1 EBNA-3A and EBNA-3C proteins, analysis of the amino acid sequence of the PU.1 transcriptional factor also revealed the presence of two of these PXLXP peptide motifs. PU.1 factor belongs to the family of the ETS factors, such as the already mentioned ETS1 and ETS2 factors and was shown to be required for LMP-1 transactivation by EBNA-2 [239, 242]. Although the presence of the PXLXP motifs in the amino acid sequence does not imply BS69 binding it would be interesting to determine whether this factor is bound by BS69 and what its role could be in the differential gene induction by type 1 and type 2 since LMP-1 is one of the differentially regulated genes.

In summary, the data reported in this chapter demonstrates that the BS69 protein represses gene activation mediated by either type 1 and type 2 EBNA-2 and BS69 was found to possibly have a stronger affinity to type 2 EBNA-2 protein than to type 1. The D442 found to be important to give higher transactivation function and capacity to sustain LCL to type 1 EBNA-2 was shown not to relieve BS69 binding to type 2 EBNA-2. Additionally, many different effects that involve BS69 and EBNA-2 were observed and described, suggesting that BS69 might have a function in EBNA-2 activity. However, a specific role in the differential gene induction by type 1 and type 2 was not identified and the phenotype involving the S442D amino acid change, which is located immediately after PXLXP2 in EBNA-2, could not be explained by the data obtained in the present study.

5 Differential regulation of the viral LMP-1 gene by type 1 and type 2 EBNA-2

The aim of this chapter is to investigate the differential regulation of the viral LMP-1 gene by type 1 and type 2 EBNA-2. The different ability to transactivate LMP-1 gene expression by type 1 and type 2 EBNA-2 and their interaction with the LMP-1 gene promoter were the focus of this chapter.

5.1 Introduction

The viral LMP-1 gene is required for transformation of primary B cells into LCLs *in vitro* [135] and also to continuously maintain proliferation of EBV-infected LCLs [74]. The LMP-1 protein activates several cellular signalling pathways eliciting potent oncogenic effects in LCLs [349, 350, 471-473]. LMP-1 gene expression can be initiated from two distinct promoters, a proximal (ED-L1p) and a distal (TR-L1p) promoter [359, 360]. Transcription from the ED-L1p promoter in latency III B cells or in LCLs is highly dependent on EBNA-2, which constitutes the strongest activator of LMP-1 expression [109, 474].

EBNA-2 does not bind directly to DNA to mediate gene transactivation, instead it is tethered to responsive promoters by cell DNA-binding proteins [7]. Regulation of the viral LMP-1 gene by EBNA-2 is complex and different cellular protein factors are involved, which include RBP-Jk, PU.1 [239, 240, 242], AP-2 [244], POU-domain protein [242], p300/CBP [258], SWI-SNF, ATF/CREB [243] and the DP103/SMN complex [270]. Although the activation of other EBNA-2 responsive promoters is dependent on the RBP-Jk/EBNA-2 interaction, such as the Cp promoter, RBP-Jk has a less important role in LMP-1 transactivation by EBNA-2 [108, 242, 475, 476]. The main cofactors involved in EBNA-2-mediated induction of LMP-1 promoter are PU.1 [239, 242], ATF/CREB [243] and EBF1 [245]. However, the involvement of these cellular factors in the regulation of the LMP-1 ED-L1p promoter by EBNA-2 has largely been studied for type 1 but not for type 2 EBNA-2.

The viral LMP-1 gene is one of the few genes that is differentially induced by type 1 and type 2 EBNA-2 [206, 209]. The activation of LMP-1 by EBNA-2 through these other cell cofactors suggests that the other differentially regulated genes could also be activated in a similar manner and they could account for the superior transforming ability of type 1 EBV.

Recently published work from this laboratory has, in fact, shed some light on the differential gene regulation by type 1 and type 2 EBNA-2 and on the most likely importance of the PU.1 cell factor in LMP-1 activation mediated by EBNA-2 [210]. Analysis of DNA sequences of the EBNA-2 binding regions in the cell gene promoters differentially regulated by type 1 and type 2 identified one enriched sequence element comprising overlapping binding sequences for E26 transformation-specific (ETS) transcription factors (PU.1) and interferon regulatory factor (IRF) [210]. This motif is similar to the ETS-IRF composite element (EICE) present in the IgL (λ) enhancer site [477] and to the PU.1 binding sequence that mediates LMP-1 activation by EBNA-2 [239, 242]. This enriched motif was not identified in EBNA-2 binding sites in the cellular gene promoters equally regulated by type 1 and type 2 EBNA-2, suggesting that this element may explain the type 1/type 2 differential gene regulation. Furthermore, this indicates that the differentially induced genes are regulated by EBNA-2 in a similar manner to that of LMP-1.

Additionally, ChIP-qPCR assays performed by this laboratory [210], have also revealed that type 1 EBNA-2 protein exhibited an increased binding at the EBNA-2 binding site at the LMP-1 promoter when compared to type 2 EBNA-2. Interestingly, the serine to aspartate (S442D) mutation in type 2 EBNA-2 improved the ability to bind at the LMP-1 promoter but to a lower extent when compared to type 1 [210]. This was also observed for the EBNA-2 binding site at the CXCR7 gene, which is more strongly induced by type 1 EBNA-2 than by type 2. Equally regulated genes did not display any difference in type 1 and type 2 EBNA-2 binding at those sites. Taken together, these observations suggest that the higher transactivation function, the greater binding of type 1 EBNA-2 at target responsive elements and the interaction with cell cofactors that may mediate increased binding could confer increased expression of the LMP-1 gene and the other cell genes differentially regulated by type 1 and type 2 EBNA-2, leading to a superior transforming ability of type 1 EBV.

In the present study, the mechanism of differential induction of the viral LMP-1 gene by type 1 and type 2 EBNA-2 was studied in order to investigate the DNA sequences or promoter elements that are important for the higher LMP-1 expression by type 1 EBNA-2. The interaction of the EBNA-2 protein with the LMP-1 promoter was also studied to determine the cellular cofactors and promoter elements that mediate EBNA-2 recruitment at the LMP-1 promoter and that could account for the superior induction by type 1 EBNA-2.

5.2 Results

5.2.1 Transcriptional regulation of LMP-1 gene promoter by type 1 and type 2 EBNA-2 in promoter-gene reporter assays

LMP-1 had been demonstrated to be more rapidly and strongly induced by type 1 EBNA-2 in EREB2.5 LCLs, AK31, Daudi and P3HR1 BL cell lines and in the early infection of primary B cells with BAC-derived EBV expressing type 1 or type 2 EBNA-2 proteins [206, 209]. In this study, promoter-gene reporter assays were used to attempt to reconstitute this differential regulation of the LMP-1 gene by type 1 and type 2 EBNA-2.

5.2.1.1 Transcriptional regulation of the B95-8 EBV LMP-1 regulatory sequence (LRS) by type 1 and type 2 EBNA-2 in EBV-positive Daudi cells using transient transfection reporter assays

LMP-1 expression from the LRS was compared for type 1 and type EBNA-2, using promoter-luciferase reporter assays to recapitulate the differential LMP-1 regulation. A reporter plasmid carrying the LMP-1 promoter sequence from B95-8 EBV strain, defined as positions -634 to +40 to the transcription initiation site (LRS -634/+40), regulating a luciferase gene was used. This reporter assay had been previously developed in earlier studies [411] to show regulation of this LRS -634/+40 by EBNA-2 and to demonstrate that BS69/ZMYND11 mediated repression of LMP-1 promoter expression by EBNA-2 (section 4.2.2.1).

Daudi:pHEBo-MT cell lines, which correspond to EBV-positive Daudi cells stably transfected with the pHEBo plasmids expressing type 1, type 2 or type 2 S442D EBNA-2 proteins, under control of a MT promoter, were used in this study (pHEBo-MT:E2T1, pHEBo-MT:E2T2 and pHEBo-MT:E2T2 S442D, section 2.3.3.13.5). These cells were transiently transfected with the LMP-1 promoter-luciferase plasmid carrying the LMP-1 regulatory sequence (pGL3b-B95-8-LRS -634/+40, section 2.3.3.13.2) and with the vector expressing *Renilla* luciferase (pRL-CMV, section 2.3.3.13.1), using the Neon transfection system. EBNA-2 expression from the MT promoter was induced with $CdCl_2$ at 5 hours post-transfection. At 24 hours after

transfection, total cellular extracts were analysed for luciferase activity and the results were normalised for transfection efficiency based on *Renilla* luciferase reference values.

The results showed that induction of type 1 EBNA-2 expression from the $CdCl_2$-inducible MT promoter in Daudi:pHEBo-MT cells, indeed activated the LMP-1 promoter-luciferase reporter (approximately 16-fold activation). Expression of the LRS (-634/+40)-luciferase construct was also observed for type 2 and type 2 S442D EBNA-2 proteins. However, this LMP-1 induction was similar for all EBNA-2 proteins that were tested and no great differences were observed between LRS induction activated by type 1, type 2 and type 2 S442D EBNA-2 (Figure 5.1).

These observations presented in this section demonstrate that this transient LMP-1 promoter luciferase reporter assay carried out in Daudi cells expressing EBNA-2 does not recapitulate the differential regulation of the viral LMP-1 gene by type 1 and type 2 EBNA-2 proteins that had been previously observed in the EBV genome using other systems [206, 209, 210]. Accordingly, the lack of adequate chromatinisation of the promoter region, which is only exhibited by the LMP-1 gene in the EBV genome but not in the promoter-luciferase reporter constructs; associated with the absence of post-translational histone modifications and other epigenetic marks may contribute to explain why the differential LMP-1 regulation was not recapitulated.

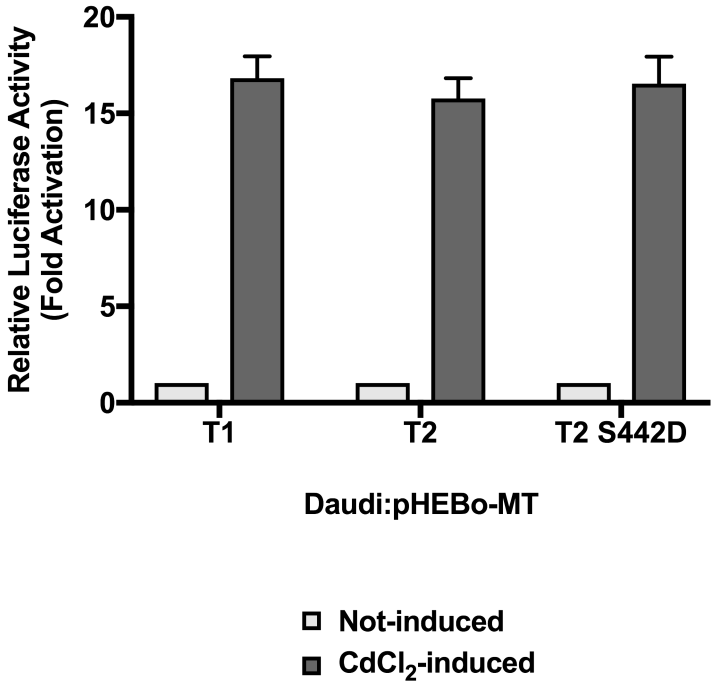


Figure 5.1 Transcriptional regulation of the B95-8 LRS (-634/+40) by type 1, type 2 and type 2 S442D EBNA-2 in Daudi:pHEBo-MT cells

2 x 10^6 EBV-positive Daudi:pHEBo-MT cells were cotransfected with 4 µg of pGL3b-B95-8-LRS (-634/+40) luciferase reporter and 50 ng of pRL-CMV *Renilla* plasmids using the Neon system. 5 hours post-transfection cells were induced for EBNA-2 expression with 5 µM $CdCl_2$ (dark grey bars), except for the not-induced cells (light grey bars). At 24 hours after transfections, cells were harvested and analysed for luciferase activity. Luciferase values were normalised for transfection efficiency according to the *Renilla* luciferase expression values. Results are presented as luciferase activity relative to the transfected, but not-induced cells (light grey bars). Error bars represent three technical replicates. T1: Type 1 EBNA-2; T2: Type 2 EBNA-2; T2 S442D: Type 2 S442D EBNA-2.

5.2.1.2 Transcriptional regulation of an LMP-1 promoter-reporter gene construct in a stably chromatinised OriP plasmid in an EBV-positive Daudi cell line

5.2.1.2.1 Generation of an OriP plasmid carrying an LMP-1 promoter-luciferase fusion

To address the previous hypothesis and determine whether the differential LMP-1 regulation by type 1 and type 2 EBNA-2 could be observed in a stably chromatinised LMP-1 promoter-luciferase reporter plasmid, the promoter-gene construct was cloned into an OriP plasmid to allow the vector to replicate in EBV-positive cells and to ensure that progeny cells receive the reporter plasmid [478].

B95-8 EBV genomic DNA was used as template to PCR-amplify the fragment corresponding to the -241/+12 region relative to the transcription initiation site of the LMP-1 promoter. This region was selected since it comprises important LMP-1 promoter regulatory elements, such as the RBP-Jk binding site, the PU.1-box, the octamer sequence and the AML1 binding site. In addition, this region includes the sequences responsible for EBNA-2 responsiveness and the minimal LMP-1 EBNA-2 response element (-236/-145) [239, 242] (Figure 5.2 A). Primers carrying *Xho*I and *Hind*III restriction sites at their respective 5' ends were used to amplify the 253 bp product. This fragment was subcloned into pCR2.1 vector using TA cloning strategy (section 2.3.3.7). Positive transformants were screened by PCR using T7-promoter and M13-reverse primers (Figure 5.2 B). The product size obtained for the positive clones is 430 bp.

The pCR2.1 plasmid carrying the cloned LMP-1 promoter fragment was digested with *Xho*I and *Hind*III restriction enzymes and the resultant fragment was cloned into puro-OriP plasmid cut with *Xho*I and *Hind*III to generate the final puro-OriP-LMP-1p (-241/+12) plasmid (section 2.3.3.13.8). Positive clones were screened using *Xho*I/*Hind*III, *Hind*III/*Bam*HI and *Bam*HI/*Eco*RI diagnostic restriction digestions and compared to the empty puro-OriP vector (Figure 5.2 C). The final construct was sequenced for identity validation and also compared to the published sequences.

A

B95-8 EBV LMP-1 promoter regulatory sequence (-241/+12):

169,047 **169,097**

TTGAGGAAAGAAGGGGGCAGAGCAGTGTGAGAGGCTTATGTAGGGCGGCTA

← TSS

169,098 **169,148**

CGTCAGAGTAACGCGTGTTTCTTGGGATGTAGGCCCGGGGGGATTTGCGGG

EBF

169,149 **169,199**

GTCTGCCGGAGGCAGTACGGGTACAGATTTCCCGAAAGCGGCGGTGTGTGT

169,200 **169,250**

GTGCATGTAAGCGTAGAAAGGGGAAGTAGAAAGCGTGTGTTTGTGTTAGAA

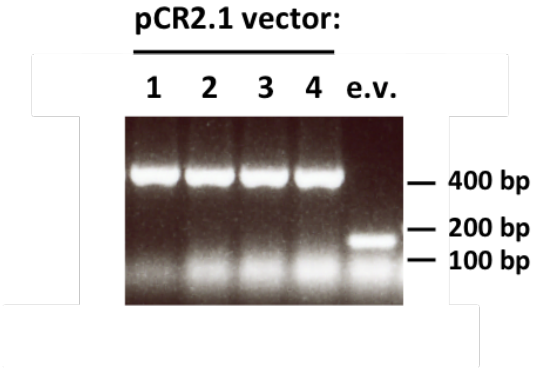
octamer PU.1 IRF

169,251 **169,299**

AAGCGGGTCCCCGGGGGGCAAGCTGTGGGAATGCGGTGGCCAAGTGCAA

EBF RBP-Jk AML1

B



C

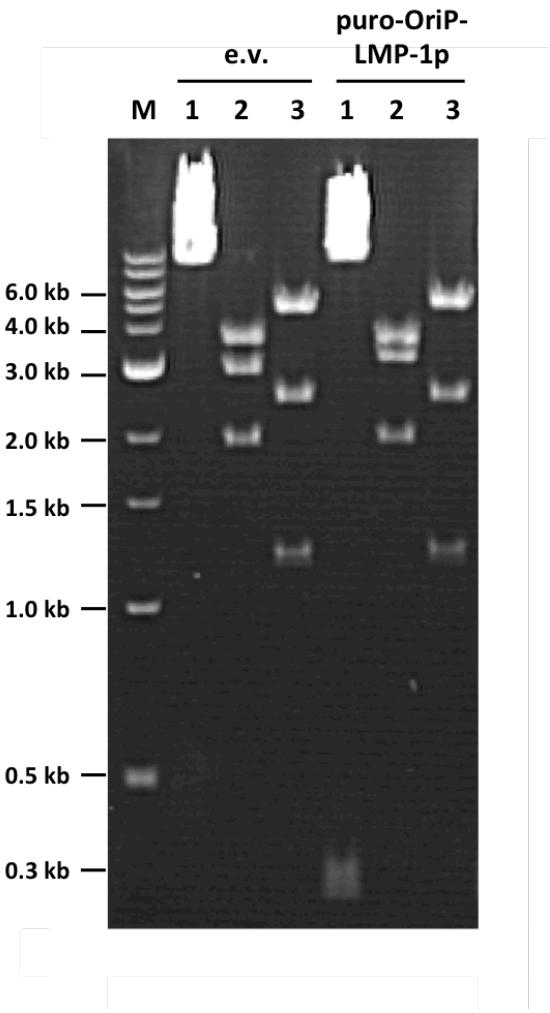


Figure 5.2 Generation of a puro-OriP plasmid carrying the B95-8 EBV LMP-1 promoter region (-241/+12) regulating a luciferase reporter gene

(**A**) The LMP-1 promoter regulatory region (-241/+12) was cloned into a puro-OriP plasmid. Sequence corresponding to nucleotides 169,047 to 169,299 is shown in the standard EBV genome orientation. The transcription start site (TSS) is represented by the arrow. Binding sites for regulatory transcription factors are indicated and closely resemble consensus motifs. PU.1 and IRF binding sites constitute an ETS-IRF composite element (EICE). EBF: early B cell factor. IRF: interferon regulatory factor. RBP-Jk: recombination signal-binding protein J kappa. AML1: acute myeloid leukemia1. (**B**) Representative gel electrophoresis of products obtained from PCR-screening for positive clones of the LMP-1 promoter region cloning into pCR2.1 vector. T7-promoter and M13-reverse oligonucleotide primers were used.

The empty vector pCR2.1 was used as negative control (e.v.). Expected size of the amplified fragment in positive clones is 430 bp. Expected size for the empty vector is 177 bp. 1-4: four representative positive clones. (**C**) Representative diagnostic restriction digestions indicating that the LMP-1 promoter sequence has been successfully cloned into puro-OriP plasmid. The empty vector puro-OriP was used as a negative control (e.v.). **1**: *Xho*I/*Hind*III restriction digest - positive clones: 8.7 kb (vector backbone) and 0.25 kb (LMP-1 promoter insert); empty vector: 8.7 kb (vector backbone). **2**: *Hind*III/*Bam*HI restriction digest - positive clones: 3.7 kb, 3.25 kb and 2.0 kb; empty vector: 3.7 kb, 3.0 kb and 2.0 kb. **3**: *Bam*HI/*Eco*RI restriction digest - positive clones: 5.25 kb, 2.4 kb and 1.3 kb; empty vector: 5.0 kb, 2.4 kb and 1.3 kb.

The puro-OriP-LMP-1p plasmid was transfected into EBV-positive Daudi cells to generate a stable cell line in which the reporter plasmid is properly chromatinised. The Neon transfection system was used and adequate antibiotic selection was added to ensure the maintenance of the plasmid (puromycin, 1 µg/ml). The Daudi:puro-OriP-LMP-1p cell line was then generated (section 2.2.6.1).

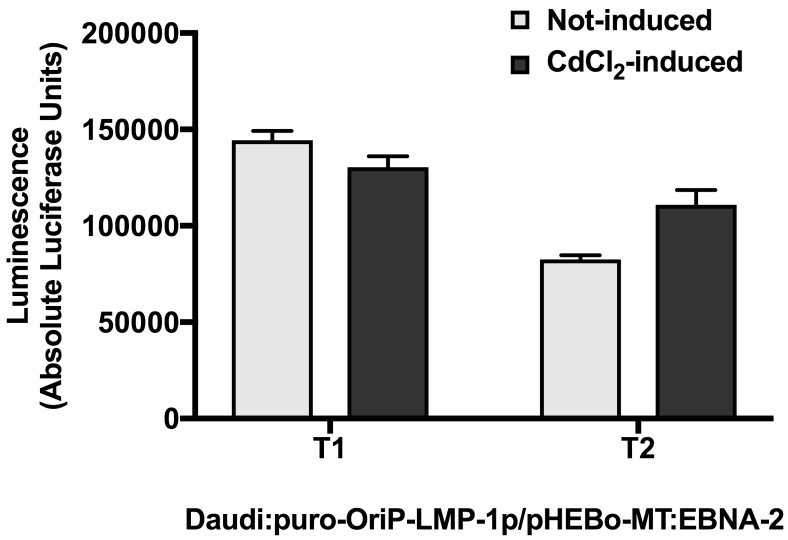
5.2.1.2.2 The LMP-1 promoter-luciferase gene reporter construct carried by a stably chromatinised OriP plasmid in the Daudi cell line does not recapitulate the differential LMP-1 regulation by type 1 and type 2 EBNA-2

Daudi:puro-OriP-LMP-1p cells were transiently transfected with pHEBo plasmids expressing either type 1 or type 2 EBNA-2 proteins (pHEBo-MT:E2T1 and pHEBo-MT:E2T2, see section 2.3.3.13.5) and with the vector expressing *Renilla* luciferase (pRL-CMV, section 2.3.3.13.1) using the Neon transfection system. EBNA-2 expression from the MT promoter in pHEBo plasmids was induced with $CdCl_2$ at 5 hours post-transfection. At 24 hours after transfection, cellular extracts were analysed for luciferase activity and the results were normalised for transfection efficiency according to *Renilla* luciferase reference values.

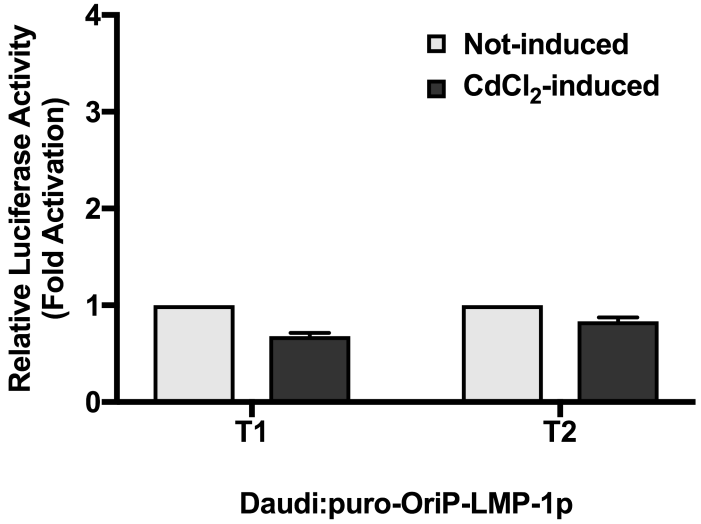
The results showed that LMP-1 promoter activation by type 1 and type 2 EBNA-2 is identical when the stably chromatinised puro-OriP-LMP-1p reporter plasmid was tested. Surprisingly, the LMP-1 promoter-luciferase construct was constitutively activated, even when expression of EBNA-2 protein from the pHEBo plasmid had not been induced with $CdCl_2$ (Figure 5.3 B). This observation was then confirmed in western blotting analysis performed to assess EBNA-2 protein expression using the anti-EBNA-2 antibody PE2, which revealed that no EBNA-2 is expressed when there is no induction with $CdCl_2$ (Figure 5.3 C).

This observation left open the possibility that the pHEBo plasmids expressing either type 1 or type 2 EBNA-2 also needed to be stably chromatinised to give correct regulation of the LMP-1 promoter-luciferase reporter. To investigate this hypothesis, Daudi:puro-OriP-LMP-1p cells were transfected with either pHEBo-MT:E2T1 or pHEBo-MT:E2T2 plasmids to create stable cell lines carrying one of the pHEBo plasmids and the puro-OriP-LMP-1p reporter. Adequate antibiotic selection was added in order to maintain both plasmids (hygromycin at 300 µg/ml and puromycin at 1 µg/ml). Daudi:puro-OriP-LMP-1p/pHEBo-MT:EBNA-2 cell lines were then generated (section 2.2.6.1). These cell lines were induced with $CdCl_2$ for 24 hours to activate EBNA-2 expression. At 24 hours after induction, cellular extracts were analysed for luciferase activity. Again no difference in LMP-1 promoter induction by type 1 and type 2 EBNA-2 was observed and luciferase gene expression was detected even when EBNA-2 expression was not induced (Figure 5.3 A).

A


Not-induced
CdCl2-induced
Luminescence
(Absolute Luciferase Units)
200000
150000
100000
50000
0
T1
T2
Daudi:puro-OriP-LMP-1p/pHEBo-MT:EBNA-2

B


Not-induced
CdCl2-induced
Relative Luciferase Activity
(Fold Activation)
4
3
2
1
0
T1
T2
Daudi:puro-OriP-LMP-1p

C

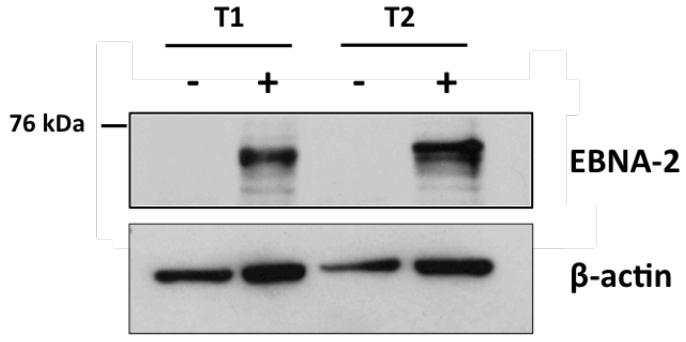

T1
T2
-
+
-
+
76 kDa
EBNA-2
β-actin

Figure 5.3 Stably chromatinised puro-OriP plasmid carrying B95-8 LMP-1 promoter region (-241/+12) did not recapitulate the differential LMP-1 gene regulation by type 1/type 2 EBNA-2

(**A**) 2 x 10^6 Daudi cells carrying the stably chromatinised puro-OriP-LMP-1p reporter plasmid and one of the pHEBo-MT:EBNA-2 plasmids expressing either type 1 (T1) or type 2 (T2) EBNA-2 proteins were induced for EBNA-2 expression from the MT promoter with 5 µM $CdCl_2$ for 24 hours ($CdCl_2$-induced). Cells were then harvested and analysed for luciferase activity (dark bars). Daudi cells that have not been induced with $CdCl_2$ were also analysed for luciferase activity (Not-induced, light grey bars). (**B**) 2 x 10^6 Daudi cells with the stably chromatinised puro-OriP-LMP-1p reporter plasmid were transiently transfected with 5 µg of either pHEBo-MT:E2T1 (T1, type 1 EBNA-2) or pHEBo-MT:E2T2 (T2, type 2 EBNA-2) and with 50 ng of pRL-CMV *Renilla* plasmids using the Neon transfection system. At 5 hours after transfections, cells were induced for EBNA-2 expression with 5 µM $CdCl_2$ ($CdCl_2$-induced, dark bars), except for the not-induced cells (Not-induced, light grey bars). 24 hours after transfections, cells were harvested and analysed for luciferase activity. Luciferase values were normalised for transfection efficiency based on *Renilla* luciferase expression values. Results are presented as luciferase activity relative to the transfected, but not-induced cells (light grey bars). Error bars represent three technical replicates. (**C**) Expression levels of type 1 (T1) and type 2 (T2) EBNA-2 proteins were compared by western blotting analysis after transiently transfection of the pHEBo-MT:EBNA-2 plasmids, expressing either type 1 or type 2 EBNA-2, into the Daudi cell line with the stably chromatinised puro-OriP-LMP-1p reporter. EBNA-2 expression from the MT promoter in pHEBo plasmid was induced with $CdCl_2$ (+) or not induced (-). EBNA-2 levels were assessed using the anti-EBNA-2 antibody PE2 that equally detects both EBNA-2 types. β-actin immunoblot analysis ensured that equal amounts of proteins were loaded on the gel.

Collectively, these observations indicate that this LMP-1 promoter-luciferase reporter plasmid did not recapitulate the differential LMP-1 gene regulation by type 1 and type 2 EBNA-2. This OriP derivative plasmid that was transfected into EBV-positive Daudi cells, even when stably chromatinised, was not able to exhibit the correct LMP-1 regulation observed when the whole EBV genome was used in previous reports [209, 210]. Therefore, these results have raised the hypothesis that a larger LMP-1 promoter sequence than those used in previous promoter luciferase gene reporter assays (LRS, -634/+40 and -241/+12) is required to recapitulate the differential regulation of the LMP-1 gene promoter by type 1 and type 2 EBNA-2.

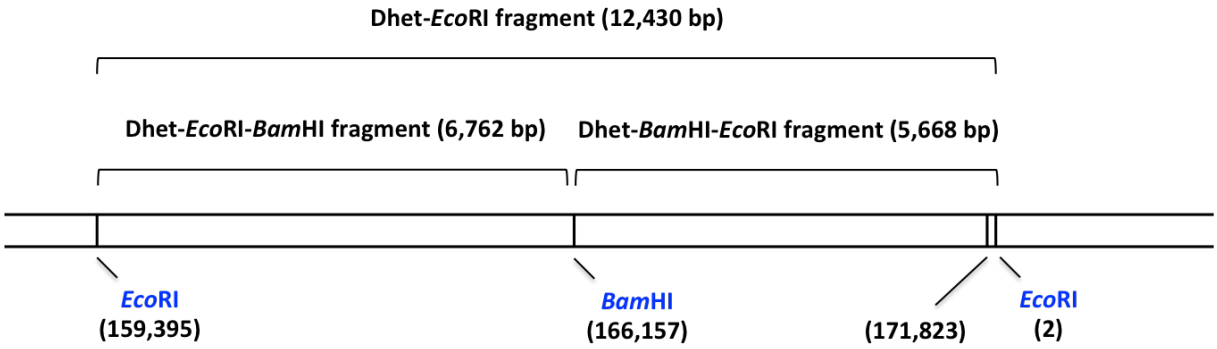
5.2.2 Transcriptional regulation of LMP-1 gene promoter by type 1, type 2 EBNA-2 using the whole LMP-1 regulatory region

5.2.2.1 Generation of a pHEBo-OriP plasmid vector carrying the whole LMP-1 regulatory region

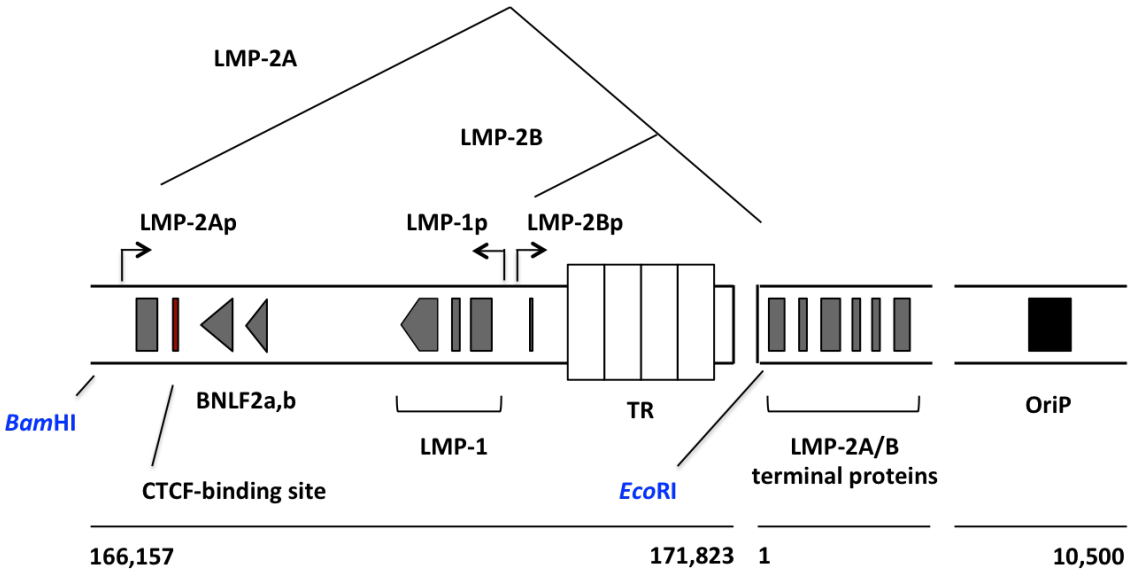
The previous LMP-1 promoter-luciferase gene reporter assays (sections 5.2.1.1 and 5.2.1.2) did not successfully recapitulate the differential regulation of the LMP-1 promoter by type 1 and type 2 EBNA-2. LMP-1 was shown to be much more strongly induced by type 1 EBNA-2, in other systems, in which the whole EBV genome was used [209, 210], suggesting that a region larger than the LMP-1 promoter sequence or the LMP-1 gene (ORF) itself is required to reproduce the differential type 1/type 2 regulation. To address this hypothesis a distinct approach was adopted, consisting of cloning the whole LMP-1 regulatory region into an OriP plasmid and assessing expression of the LMP-1 gene induced by type 1 and type 2 EBNA-2 proteins. This larger LMP-1 regulatory region includes the LMP-1 gene and promoter, the LMP-2A and LMP-2B promoters, the EBV genome terminal repeats and also a binding site for the CCCTC-binding factor CTCF (Figure 5.4 B), which could be required to confer a distinct LMP-1 gene regulation by type 1 and type 2 EBNA-2.

To generate a pHEBo-OriP plasmid vector carrying the whole LMP-1 regulatory region a two-step cloning procedure was used as described previously (section 2.3.3.14). Firstly, a pHC79 plasmid vector, which carried the Dhet-*Eco*RI DNA fragment from B95-8 EBV strain genome (nucleotides 159,395 to 171,823; plus nucleotides 1 and 2, in Genbank accession number AJ 507799) (Figure 5.4 A), was digested with *Eco*RI restriction enzyme. The resultant fragment (12,430 bp) was cloned into pBlueScript vector cut with *Eco*RI, close to a *Bam*HI site present in the vector. Positive transformants were then screened by *Bam*HI digestion to confirm that the Dhet-*Eco*RI genome fragment had been successfully cloned into pBlueScript (Figure 5.5 A). The generated pBlueScript:Dhet-*Eco*RI plasmid construct was then digested with *Bam*HI; taking advantage of the *Bam*HI restriction site carried by the pBlueScript plasmid vector and the resultant fragment of around 5.7 kb was finally cloned into pHEBo-OriP vector (~7 kb) cut with *Bam*HI. Transformants were screened by *Bam*HI digestion to ensure that the fragment had ligated correctly (Figure 5.5 B). Positive clones carried the Dhet-*Bam*HI-*Eco*RI genome fragment cloned into pHEBo (around 5.7 kb), which includes the LMP-1 regulatory sequence and not the Dhet-*Eco*RI-*Bam*HI fragment of approximately 6.7 kb (Figure 5.4 and 5.5 B).

A


Dhet-*Eco*RI fragment (12,430 bp)
Dhet-*Eco*RI-*Bam*HI fragment (6,762 bp)
Dhet-*Bam*HI-*Eco*RI fragment (5,668 bp)
*Eco*RI
(159,395)
*Bam*HI
(166,157)
(171,823)
*Eco*RI
(2)

B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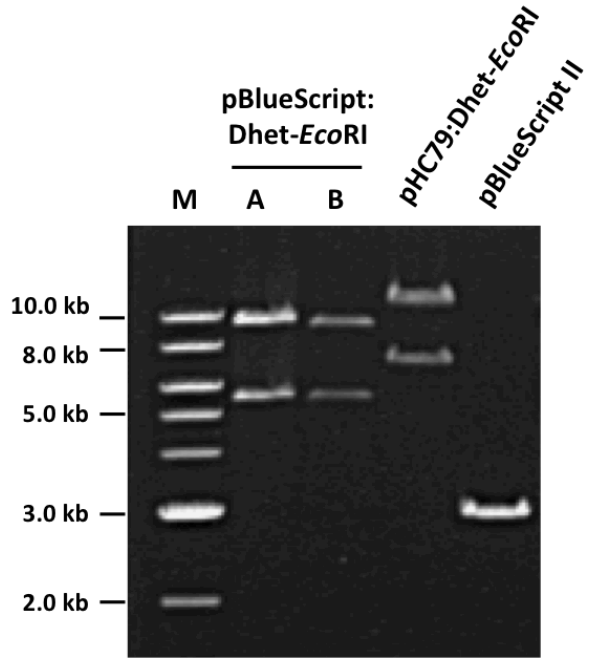
LMP-2B
LMP-2Ap
LMP-1p
LMP-2Bp
*Bam*HI
BNLF2a,b
CTCF-binding site
LMP-1
TR
*Eco*RI
LMP-2A/B
terminal proteins
OriP
166,157
171,823
1
10,500

Figure 5.4 The EBV B95-8 strain genome fragment Dhet-*Bam*HI-*Eco*RI carries the whole LMP-1 regulatory region to be cloned into pHEBo-OriP plasmid

(**A**) The Dhet-*Eco*RI B95-8 genome fragment lies between two *Eco*RI restriction sites from nucleotides 159,395 to 2 (Genbank accession number AJ507799) and its size is around 12.4 kb. This fragment comprises two smaller pieces defined by an additional *Bam*HI site (Dhet-*Eco*RI-*Bam*HI and Dhet-*Bam*HI-*Eco*RI fragments). Their sizes are approximately 6.7 kb and 5.7 kb, respectively. The position of the last nucleotide of the viral genome is also represented (171,823). (**B**) The Dhet-*Bam*HI-*Eco*RI fragment, including the LMP-1 regulatory region to be cloned into pHEBo-OriP vector. This fragment comprises the LMP-1 promoter and respective gene, the LMP-2A and LMP-2B promoters, the viral genome terminal repeats (TR), the regulatory element CCCTC-binding factor CTCF site (in red) and the BNLF2a and b genes. The terminal exons of the LMP-2A and LMP-2B proteins are not included in this fragment cloned into pHEBo-OriP vector.

A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Dhet-EcoRI
M
A
B
pHC79:Dhet-EcoRI
pBlueScript II
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8.0 kb
5.0 kb
3.0 kb
2.0 kb

B

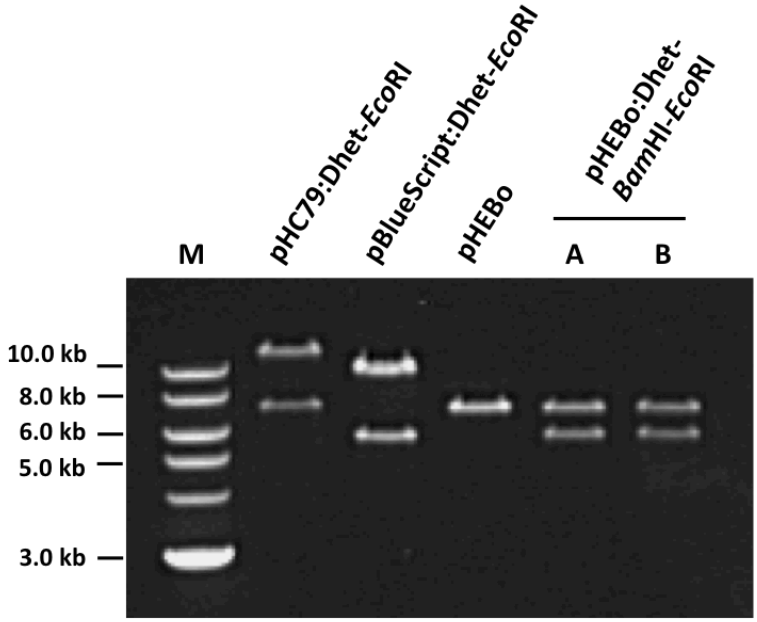

M
pHC79:Dhet-EcoRI
pBlueScript:Dhet-EcoRI
pHEBo
pHEBo:Dhet-BamHI-EcoRI
A
B
10.0 kb
8.0 kb
6.0 kb
5.0 kb
3.0 kb

Figure 5.5 Cloning of the EBV B95-8 fragment Dhet-*Bam*HI-*Eco*RI into pHEBo-OriP plasmid

(**A**) Representative diagnostic restriction digestion with *Bam*HI indicating that the Dhet-*Eco*RI fragment has been successfully cloned into pBlueScript vector. The empty plasmid pBlueScript was digested as a negative control (pBlueScript II; 3 kb) and also the original pHC79:Dhet-*Eco*RI vector (11.8 kb and 7.1 kb). Expected sizes for positive transformants digested with *Bam*HI enzyme are 9.7 kb and 5.7 kb. Two individual clones carrying pBlueScript:Dhet-*Eco*RI plasmid were tested (clone A and clone B). (**B**) Representative diagnostic restriction digestion with *Bam*HI restriction enzyme indicating that the Dhet-*Bam*HI-*Eco*RI fragment (nucleotides 166,157-2) has been successfully cloned into pHEBo vector. The empty pHEBo vector (pHEBo, 6.9 kb); the original pHC79:Dhet-*Eco*RI vector (11.8 kb and 7.1 kb) and the pBlueScript:Dhet-*Eco*RI plasmid (9.7 kb and 5.7 kb) were used as the digestion controls. Expected sizes for the positive transformants digested with *Bam*HI are 6.9 kb (pHEBo vector backbone) and 5.7 kb (Dhet-*Bam*HI-*Eco*RI fragment). Two individual clones carrying pHEBo:Dhet-*Bam*HI-*Eco*RI plasmid were tested (clone A and clone B). The size of the Dhet-*Bam*HI-*Eco*RI fragment (5.7 kb) after *Bam*HI digestion confirms that this piece was cloned into pHEBo-OriP vector and not the Dhet-*Eco*RI-*Bam*HI fragment (6.7 kb). M: 1kb ladder.

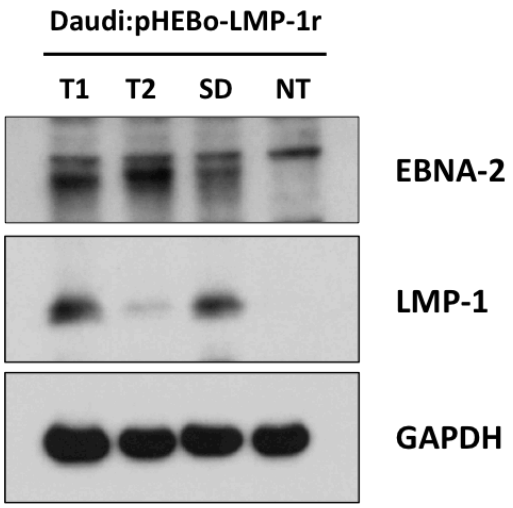
The pHEBo:Dhet-*Bam*HI-*Eco*RI plasmid was then transfected into EBV-positive Daudi cells to generate a stable cell line, in which this pHEBo-OriP plasmid is chromatinised. The Neon transfection system was used and appropriate antibiotic selection was added to ensure the maintenance of the plasmid (hygromycin at 300 µg/ml). The Daudi:pHEBo-LMP-1r cell line, carrying the pHEBo:Dhet-*Bam*HI-*Eco*RI plasmid, was made as described previously (section 2.2.6.1).

Using the pBlueScript:Dhet-*Eco*RI plasmid, Claudio Elgueta generated distinct mutants in the LMP-1 regulatory region and cloned them into pHEBo-OriP vector. Deletions of the CCCCT-binding factor CTCF site and of the genome terminal repeats were carried out to investigate whether these elements could account for the differential regulation of LMP-1 gene by type 1 and type 2 EBNA-2. Two additional constructs were generated: pHEBo:Dhet-*Bam*HI-*Eco*RI ΔCTCF and pHEBo:Dhet-*Bam*HI-*Eco*RI ΔTR. These plasmids were transfected into Daudi cells to generate two distinct stable cell lines as performed for wildtype pHEBo:Dhet-*Bam*HI-*Eco*RI plasmid. The Daudi:pHEBo-LMP-1r ΔCTCF and Daudi:pHEBo-LMP-1r ΔTR cell lines were generated (section 2.2.6.1).

5.2.2.2 Transcriptional regulation of LMP-1 by type 1 and type 2 EBNA-2 using a stably chromatinised pHEBo-OriP plasmid vector carrying the whole LMP-1 regulatory region

Daudi:pHEBo-LMP-1r cells, which carry the stably chromatinised pHEBo:Dhet-*Bam*HI-*Eco*RI plasmid, were transiently transfected with pHEBo plasmids expressing type 1, type 2 or type 2 S442D EBNA-2 proteins (section 2.3.3.13.5, plasmids pHEBo-MT:E2T1, pHEBo-MT:E2T2 and pHEBo-MT:E2T2 S442D), using the Neon transfection system. EBNA-2 expression from the metallothionein promoter was induced with $CdCl_2$ at 5 hours after transfections. 24 hours post-transfection, total protein extracts were analysed by western blotting. Immunoblotting for EBNA-2 and for LMP-1 proteins was performed. The results showed that LMP-1 induction by type 1 EBNA-2 is indeed stronger than that by type 2 but it is not clear whether the LMP-1 protein is coming from the plasmid or the endogenous EBV genomes in the Daudi cells. The S442D mutation in type 2 EBNA-2 confers a higher ability to induce LMP-1 expression similar to that of type 1 EBNA-2 (Figure 5.6 A). Comparing the Daudi EBV LMP-1 protein to the B95-8 EBV LMP-1 gene product it was observed that their sizes were very similar and for this reason is difficult to adequately resolve both proteins (Figure 5.6 B). To determine whether the LMP-1 gene from pHEBo:Dhet-*Bam*HI-*Eco*RI vector was being induced, Daudi and Daudi:pHEBo-LMP-1r cells were transiently transfected with the pHEBo plasmids expressing type 1, type 2 or type 2 S442D EBNA-2 proteins. Protein extracts were analysed by western blotting after loading on an SDS-PAGE gel in an alternate order to permit the observation of both LMP-1 proteins (Figure 5.6 C). These initial results suggested that both the endogenous Daudi LMP-1 and the B95-8 LMP-1 genes are induced in Daudi:pHEBo-LMP-1r cells after EBNA-2 expression as opposed to Daudi cells, which do not carry the pHEBo:Dhet-*Bam*HI-*Eco*RI plasmid and therefore only a single and smaller LMP-1 band is detected (Figure 5.6 C).

A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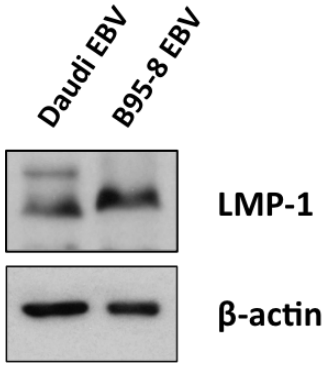
T1
T2
SD
NT
EBNA-2
LMP-1
GAPDH

B


Daudi EBV
B95-8 EBV
LMP-1
β-actin

C

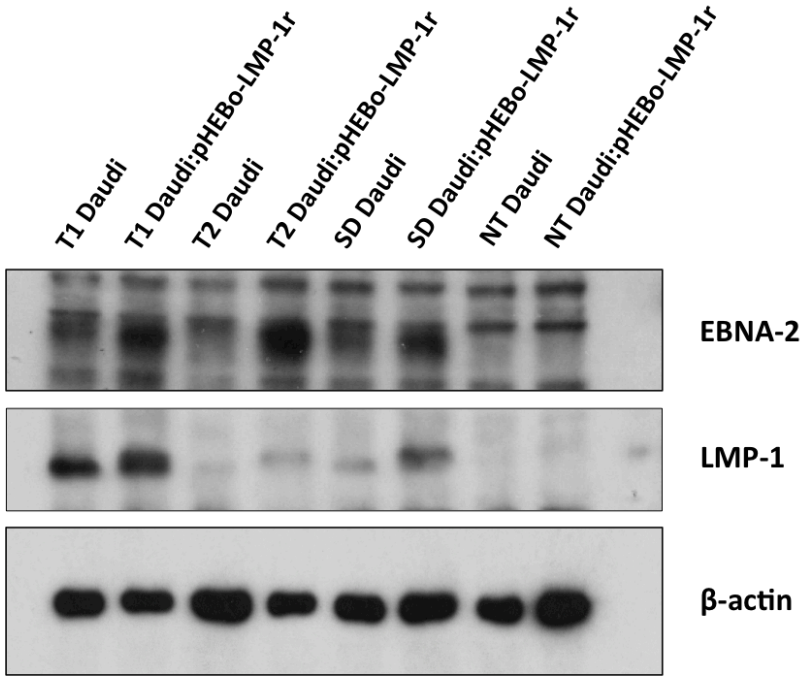

T1 Daudi
T1 Daudi:pHEBo-LMP-1r
T2 Daudi
T2 Daudi:pHEBo-LMP-1r
SD Daudi
SD Daudi:pHEBo-LMP-1r
NT Daudi
NT Daudi:pHEBo-LMP-1r
EBNA-2
LMP-1
β-actin

Figure 5.6 Transcriptional regulation of LMP-1 gene by type 1 and type 2 EBNA-2 using a stably chromatinised pHEBo-OriP plasmid vector carrying the whole LMP-1 regulatory region

(**A**) 2 x 10^6 Daudi:pHEBo-LMP-1r cells were transiently transfected with 6 µg of EBNA-2-expressing plasmids: pHEBo-MT:E2T1 (T1, type 1 EBNA-2), pHEBo-MT:E2T2 (T2, type 2 EBNA-2) or pHEBo-MT:E2T2 S442D (SD, type 2 S442D EBNA-2) using the Neon transfection system. At 5 hours after transfections cells were induced with 5 µM $CdCl_2$ for EBNA-2 expression from the MT promoter. Cells were harvested 24 hours post-transfection and protein extracts were obtained by RIPA lysis. Non-transfected Daudi:pHEBo-LMP-1r cells (NT) were also used. (**B**) 2 x 10^6 Daudi cells were transfected with pHEBo-MT:E2T1 and EBNA-2 expression was induced with 5 µM $CdCl_2$. 2 x 10^6 B95-8 LCL cells and the Daudi cells were harvested 24 hours post-transfection and protein extracts were obtained by RIPA lysis. (**C**) 2 x 10^6 Daudi:pHEBo-LMP-1r and Daudi cells were transiently transfected with 6 µg of pHEBo-MT:E2T1 (T1, type 1), pHEBo-MT:E2T2 (T2, type 2) or pHEBo-MT:E2T2 S442D (SD, type 2 S442D) plasmids using Neon transfection system. At 5 hours after transfection cells were induced with 5 µM $CdCl_2$ for EBNA-2 expression from the MT promoter. Cells were harvested at 24 hours after transfection and protein extracts were obtained by RIPA lysis. Protein extracts obtained in each case were analysed by immunoblotting to determine expression levels of type 1, type 2 and type 2 S442D EBNA-2 using the PE2 anti-EBNA-2 antibody. Expression levels of LMP-1 were determined with the CS1-4 anti-LMP-1 antibody. GAPDH or β-actin immunoblot analysis ensured that equal amounts of proteins were loaded on the gel.

A different approach was then devised to assess the induction of the B95-8 LMP-1 gene from pHEBo:Dhet-*Bam*HI-*Eco*RI and of the Daudi LMP-1 gene. A reverse-transcriptase PCR (RT-PCR) assay was used to separately detect expression of both LMP-1 genes and then determine LMP-1 regulation by type 1 and type 2 EBNA-2 when the pHEBo:Dhet-*Bam*HI-*Eco*RI plasmid was used.

5.2.2.3 Setting up a RT-PCR assay to investigate the transcriptional regulation of LMP-1 by type 1 and type 2 EBNA-2 in the pHEBo:Dhet-*Bam*HI-*Eco*RI vector

In order to investigate the transcriptional regulation of the LMP-1 gene by type 1 and type 2 EBNA-2, using the stably chromatinised pHEBo:Dhet-*Bam*HI-*Eco*RI plasmid, which contains the whole LMP-1 regulatory region (Figure 5.4 B), an RT-PCR assay was established. Daudi:pHEBo-LMP-1r cells carrying the pHEBo:Dhet-*Bam*HI-*Eco*RI plasmid were transiently transfected with the pHEBo plasmids, which express type 1, type 2 or type 2 S442D EBNA-2 proteins as performed before. EBNA-2 expression from MT promoter was induced with $CdCl_2$ at 5 hours post-transfection. Total RNA was extracted from cells 24 hours post-transfection. RNA was then converted into cDNA and analysed by RT-PCR for LMP-1 mRNA (see section 2.3.4.4). Oligonucleotide primers utilised for specific amplification of LMP-1 mRNA converted into cDNA are represented in Figure 5.7. The technical impossibility of designing primers that would selectively amplify only the LMP-1 mRNA obtained from expression of the B95-8 LMP-1 gene carried by the pHEBo:Dhet-*Bam*HI-*Eco*RI plasmid required a distinct approach to distinguish it from the LMP-1 mRNA obtained from the Daudi endogenous gene. This consisted of converting total cell RNA into cDNA and analyse by RT-PCR for total LMP-1 mRNA, which would amplify both Daudi LMP-1 and B95-8 LMP-1 genes. To distinguish the amplification products obtained from each of the LMP-1 gene mRNAs, a single restriction site (*Nco*I), present only in the B95-8 LMP-1 gene was used (Figure 5.7). After enzyme digestion, the amplified product that was not digested corresponds to the expression of the Daudi LMP-1 endogenous gene (Figure 5.8 A, dig. LMP-1 panel, top band) whereas the digested product is the RT-PCR-amplified product obtained from expression of the B95-8 LMP-1 gene, carried by the pHEBo:Dhet-*Bam*HI-*Eco*RI vector, after induction mediated by EBNA-2 (Figure 5.8 A, dig. LMP-1 panel, bottom band). The amplification products of both LMP-1 gene mRNAs that is obtained before *Nco*I digestion is also shown (Figure 5.8 A, n.d. LMP-1 panel). Total cDNA was also analysed by RT-PCR for EBNA-2 and GAPDH mRNA as controls. An additional positive control was also performed. The pHEBo:Dhet-*Bam*HI-*Eco*RI plasmid was used as template in the same amplification reaction and the resultant PCR product was also digested with *Nco*I enzyme to demonstrate full digestion of the amplified products and also to rule out the possibility of incomplete digestion of the amplified B95-8 LMP-1 mRNA, when compared to the Daudi LMP-1 product (Figure 5.8 B). This RT-PCR assay was established and used to investigate LMP-1 regulation by type 1 and type 2 EBNA-2 proteins in Daudi cell lines, which

carry the stably chromatinised pHEBo:Dhet-*Bam*HI-*Eco*RI plasmid, including the whole LMP-1 regulatory region (Figure 5.4 B).

Alignment of Daudi and B95-8 LMP-1 sequences (exon 167,702-168,507 from B95-8 genome)

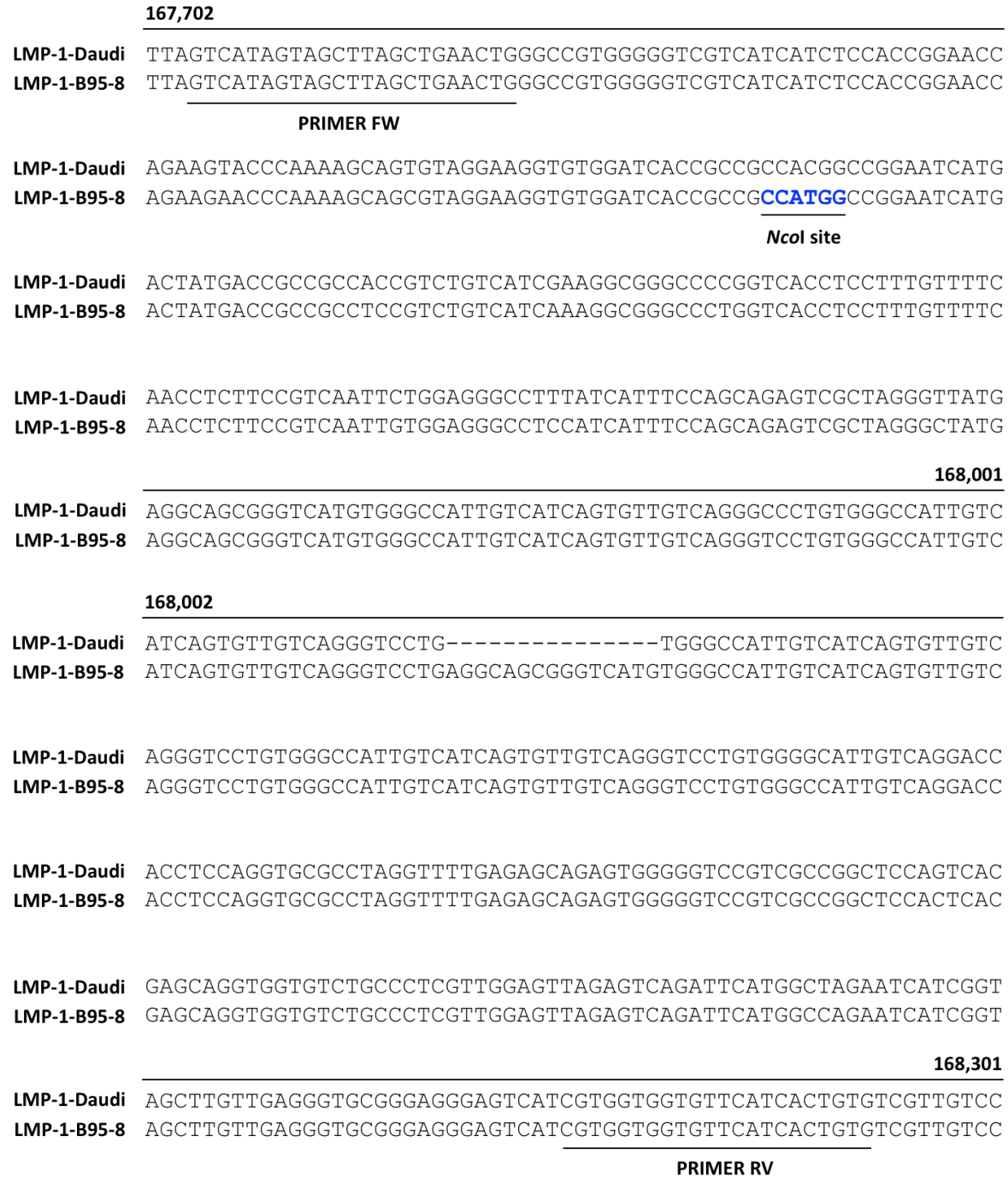


Figure 5.7 Alignment of Daudi EBV LMP-1 and B95-8 EBV LMP-1 gene sequences, nucleotide 167,702 to 168,301 (included in the LMP-1 exon 167,702-168,502 from B95-8 genome)

Sequence alignment of Daudi EBV LMP-1 and B95-8 EBV LMP-1 gene sequences corresponding to the exon 167,702-168,502 from B95-8 EBV genome. Region to be amplified in the RT-PCR assay for LMP-1 mRNA. Oligonucleotide primers used are represented (PRIMER FW: primer forward and PRIMER RV: primer reverse). The unique *Nco*I restriction site (in blue) that was used to distinguish between the amplified products derived from Daudi LMP-1 or B95-8 LMP-1 mRNAs is also represented.

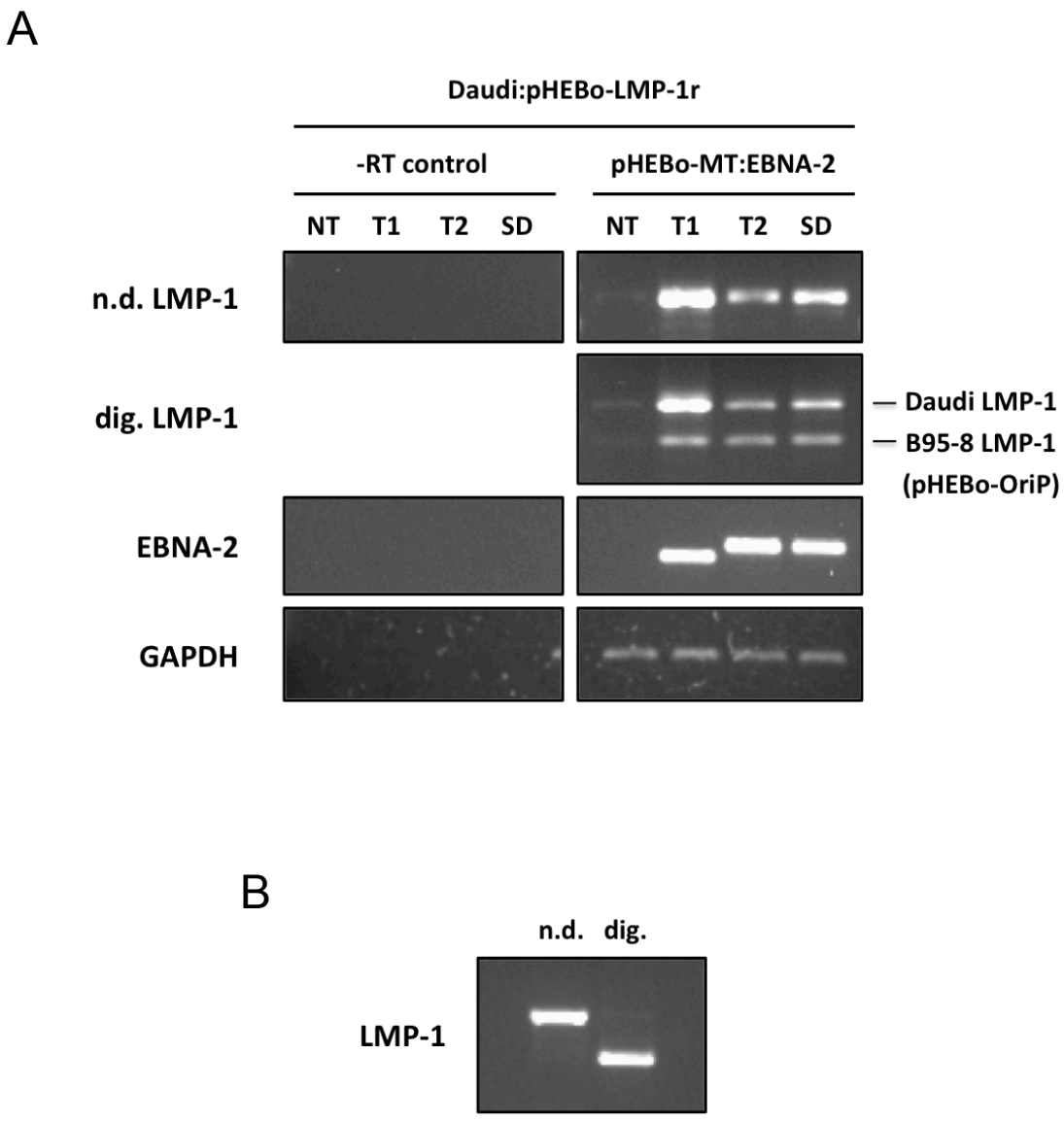


Figure 5.8 RT-PCR assay used to investigate the transcriptional regulation of LMP-1 gene by type 1 and type 2 EBNA-2 in the pHEBo:Dhet-*Bam*HI-*Eco*RI vector

(**A**) Daudi:pHEBo-LMP-1r cells were transiently transfected with 6 µg of EBNA-2-expressing plasmids: pHEBo-MT:E2T1 (T1, type 1 EBNA-2), pHEBo-MT:E2T2 (T2, type 2 EBNA-2) or pHEBo-MT:E2T2 S442D (SD, type 2 S442D EBNA-2) using the Neon system. At 5 hours after transfections cells were induced with 5 µM $CdCl_2$ for EBNA-2 expression from the MT promoter. Cells were harvested 24 hours post-transfection. Non-transfected Daudi:pHEBo-LMP-1r cells were also used (NT). Total cell RNA was extracted and subject to RT-PCR analysis for LMP-1, EBNA-2 and GAPDH. Amplification product obtained from total LMP-1 mRNA (n.d. LMP-1) was then digested with *Nco*I restriction enzyme (dig. LMP-1) to only digest the PCR products amplified from B95-8 LMP-1 mRNA, transcribed from the pHEBo:Dhet-*Bam*HI-*Eco*RI plasmid, carrying the whole B95-8 LMP-1 regulatory region (dig. LMP-1 panel, bottom band) and not the PCR products amplified from Daudi endogenous LMP-1 gene, which does not have a *Nco*I restriction site (dig. LMP-1 panel, top band). (**B**) The pHEBo:Dhet-*Bam*HI-*Eco*RI plasmid was used as template in the same PCR reaction. The resultant amplification product was then digested with *Nco*I to demonstrate that the RT-PCR product obtained from the B95-8 LMP-1 mRNA is fully digested. n.d.: not-digested; dig.: digested; -RT: no reverse transcriptase or no template negative control.

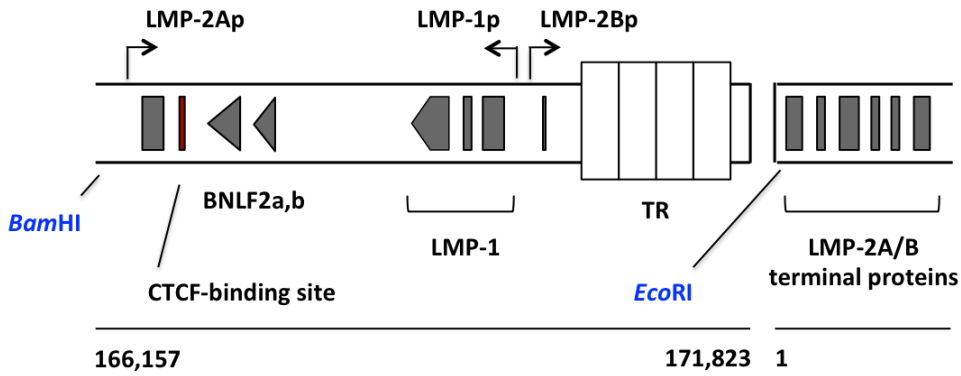
5.2.2.4 RT-PCR assay to investigate the transcriptional regulation of LMP-1 by type 1 and type 2 EBNA-2 in the pHEBo:Dhet-*Bam*HI-*Eco*RI plasmid showed no differences in LMP-1 mRNA expression levels

The RT-PCR assay established in the previous section was then used to determine whether the stably chromatinised pHEBo:Dhet-*Bam*HI-*Eco*RI plasmid, which carries the whole LMP-1 regulatory region, was able to reproduce the differential LMP-1 gene regulation by type 1 and type 2 EBNA-2 proteins that was observed in distinct systems, when the whole EBV genome was used [209, 210].

Daudi:pHEBo-LMP-1r cells carrying the pHEBo:Dhet-*Bam*HI-*Eco*RI plasmid were transiently transfected with the pHEBo plasmids, which express type 1, type 2 or type 2 S442D EBNA-2 proteins as before. EBNA-2 expression from MT promoter was induced with $CdCl_2$ at 5 hours post-transfections. Total cell RNA was harvested from cells at 24 hours after transfection and was then retro-transcribed into cDNA and analysed by RT-PCR for total LMP-1 mRNA. PCR amplified products were then digested with *Nco*I as explained in section 5.2.2.3 to distinguish between LMP-1 expressed from Daudi endogenous gene or from B95-8 gene, carried by the pHEBo:Dhet-*Bam*HI-*Eco*RI plasmid (Figure 5.9 A). The results showed that there was mRNA transcription from the induction of the B95-8 LMP-1 gene by type 1, type 2 and type 2 S442D EBNA-2 (Figure 5.9 B – dig. LMP-1 panel, bottom band). However, since this technique (RT-PCR) is not a quantitative method, it was not possible to observe differences in LMP-1 gene expression or its differential regulation by the EBNA-2 types. This is consistent with the result obtained in the process of optimisation of the RT-PCR assay (Figure 5.8 A). Interestingly, for the endogenous Daudi LMP-1 gene (Figure 5.9 B – dig. LMP-1 panel, top band), the intensity of the band corresponding to LMP-1 induction by type 2 EBNA-2 is weaker than those from Daudi LMP-1 gene activation by type 1 and type 2 S442D EBNA-2 (Figure 5.8 A and 5.9 B – dig. LMP-1 panel, top bands). A stronger activation of the endogenous Daudi LMP-1 gene by type 1 EBNA-2 would be consistent with the distinct LMP-1 regulation determined when other systems were utilised in previous reports.

Protein extracts of Daudi:pHEBo-LMP-1r transiently transfected cells were also analysed by western blotting analysis of EBNA-2 and LMP-1 levels (Figure 5.9 C). Again LMP-1 induction by type 1 EBNA-2 is stronger than by type 2. However, as mentioned before, western blotting analysis does not allow adequate resolution of the endogenous Daudi and the B95-8 LMP-1 proteins in order to assess their expression levels.

A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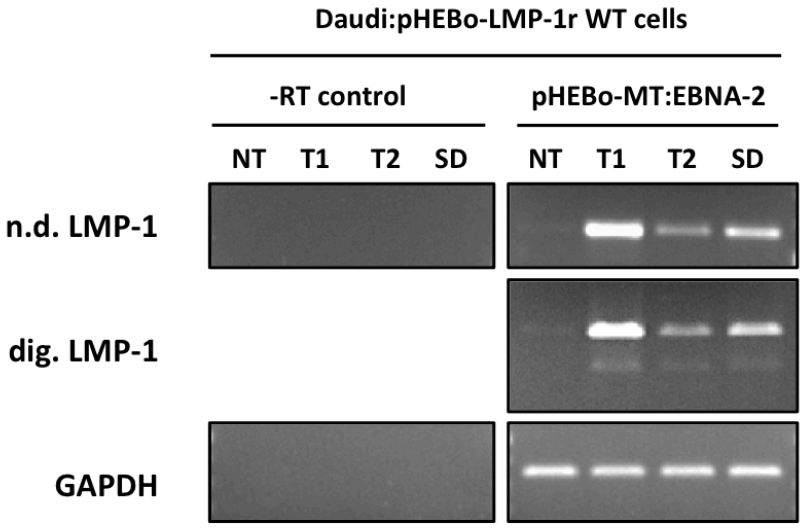
LMP-2Bp
BamHI
BNLF2a,b
CTCF-binding site
LMP-1
TR
EcoRI
LMP-2A/B
terminal proteins
166,157
171,823
1

B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-RT control
pHEBo-MT:EBNA-2
NT T1 T2 SD
NT T1 T2 SD
n.d. LMP-1
dig. LMP-1
GAPDH

C

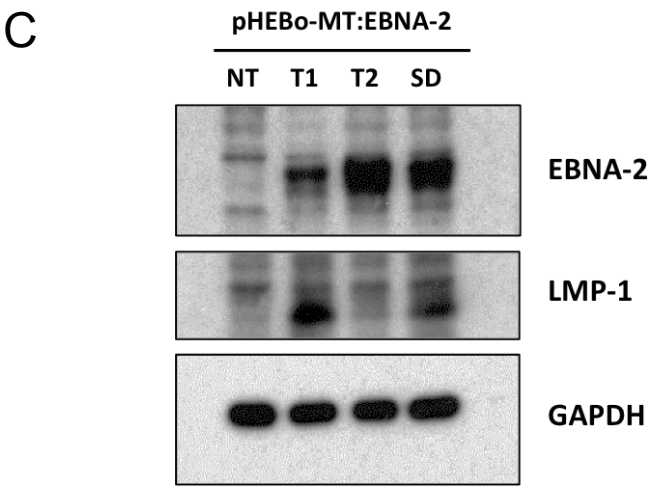

pHEBo-MT:EBNA-2
NT T1 T2 SD
EBNA-2
LMP-1
GAPDH

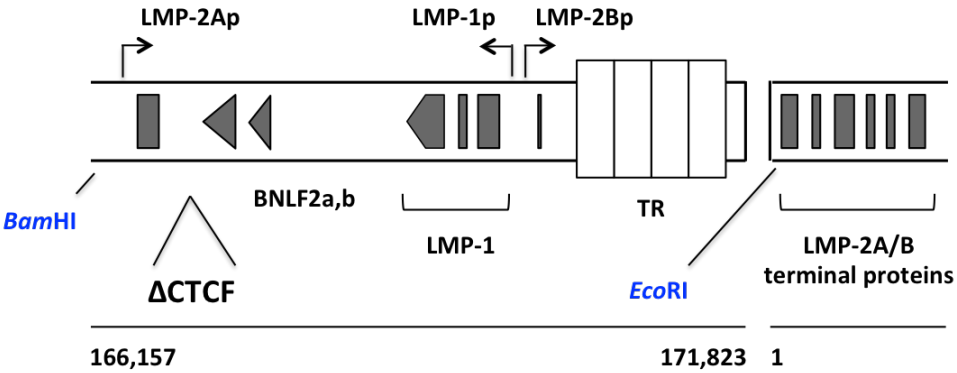
Figure 5.9 Expression of the B95-8 EBV LMP-1 gene carried by the pHEBo:Dhet-*Bam*HI-*Eco*RI plasmid that comprises a larger LMP-1 regulatory region by type 1 and type 2 EBNA-2

(**A**) The Dhet-*Bam*HI-*Eco*RI fragment (limited by a *Bam*HI and *Eco*RI restriction sites, in blue) includes the whole LMP-1 regulatory region cloned into pHEBo:Dhet-*Bam*HI-*Eco*RI vector. The region includes the LMP-1 promoter and respective gene, the LMP-2A and LMP-2B promoters, the terminal repeats (TR), the regulatory element CTCF-binding site and the BNLF2a and b genes. The terminal exons of the LMP-2A and LMP-2B proteins are not included in the Dhet-*Bam*HI-*Eco*RI fragment that has been cloned. 2 x 10^6 Daudi:pHEBo-LMP-1r cells carrying a stably chromatinised pHEBo:Dhet-*Bam*HI-*Eco*RI vector were transiently transfected with 6 µg of one of the EBNA-2-expressing plasmids: pHEBo-MT:E2T1 (T1, type 1 EBNA-2), pHEBo-MT:E2T2 (T2, type 2 EBNA-2) or pHEBo-MT:E2T2 S442D (SD, type 2 S442D EBNA-2) using the Neon transfection system. EBNA-2 expression from the MT promoter was induced with 5 µM $CdCl_2$ at 5 hours after transfections. Cells were harvested 24 hours post-transfection and total cell RNA and proteins were then extracted and analysed by (**B**) RT-PCR or (**C**) Western blot, respectively. Non-transfected Daudi:pHEBo-LMP-1r cells were also analysed (NT). (**B**) Total cell RNA was converted into cDNA and this was analysed by PCR using LMP-1 specific primers. Product amplified from total LMP-1 mRNA (n.d. LMP-1) was then digested with *Nco*I restriction enzyme (dig. LMP-1) to solely digest the PCR products amplified from B95-8 LMP-1 mRNA, which was transcribed from the pHEBo:Dhet-*Bam*HI-*Eco*RI plasmid, carrying the whole B95-8 LMP-1 regulatory region (dig. LMP-1 panel, bottom band) and not the PCR products amplified from the Daudi endogenous LMP-1 mRNA, which does not carry a *Nco*I restriction site (dig. LMP-1 panel, top band). Total cell RNA was also subject to RT-PCR analysis for GAPDH as an internal control. (**C**) Protein samples were analysed for expression of EBNA-2 and LMP-1. GAPDH immunoblotting analysis ensured that equal amounts of proteins were loaded on the gel. n.d.: not-digested; dig.: digested; -RT: no reverse transcriptase or no template negative control.

Although the pHEBo:Dhet-*Bam*HI-*Eco*RI plasmid was not able to recapitulate the differential LMP-1 gene regulation by type 1 and type 2 EBNA-2 proteins using this assay, the effect of deletion mutants in this region was assessed. Specifically, deletion of the binding site for the CCCTC-binding factor CTCF and deletion of the EBV genome terminal repeats were carried out in the pHEBo plasmid comprising the LMP-1 regulatory region (Figure 5.9 A). Previous reports determined that disruption of the CTCF-binding site caused a deregulation of LMP-1 transcription, which was characterised by a decrease in LMP-1 mRNA [479]. In addition, CTCF was found to be required for transcriptional regulation of LMP-1 and deletion of this site was associated with increase of H3K9me3 and CpG DNA methylation at the LMP-2A and LMP-1 promoter region [479], suggesting the involvement of the CTCF factor in type 1/type 2 regulation of the LMP-1 gene. The CTCF factor was shown to bind to the LMP-1 and LMP-2A regulatory region at the position within the first intron of LMP-2A and the LMP-1 3' untranslated region [480]. Earlier works on the EBV genome terminal repeats have determined that apart from the ED-L1p, the transcription of LMP-1 gene mRNA could be initiated from the TR-L1p distal promoter, which is located in the first terminal repeat [359, 360, 481]. Additionally, it has also been shown that LMP-1 expression from this promoter varies inversely to the number of terminal repeats and the transcription from TR-L1p predominates at lower TR number [482]. Accordingly, the terminal repeat region could also be involved in the LMP-1 regulation by type 1 and type 2 EBNA-2.

Daudi:pHEBo-LMP-1r ΔCTCF or Daudi:pHEBo-LMP-1r ΔTR cells carrying the pHEBo:Dhet-*Bam*HI-*Eco*RI ΔCTCF or pHEBo:Dhet-*Bam*HI-*Eco*RI ΔTR vector were transiently transfected with the pHEBo plasmids, which express type 1, type 2 or type 2 S442D EBNA-2 proteins as before. EBNA-2 expression was induced with $CdCl_2$ at 5 hours after transfections. Total cell RNA was harvested from cells at 24 hours after transfections, reverse transcribed into cDNA and analysed by RT-PCR for total LMP-1 mRNA. Products amplified by PCR were then digested with *Nco*I enzyme as explained in section 5.2.2.3 to distinguish between LMP-1 expressed from Daudi endogenous gene or from B95-8 gene (Figure 5.10 B and 5.11 B). The results showed that there was transcription of the B95-8 LMP-1 gene after activation by type 1, type 2 and type 2 S442D EBNA-2 despite the deletion of the CTCF-binding site or of the genome terminal repeats (Figure 5.10 B and 5.11 B – dig. LMP-1 panel, bottom band). In either case, for the endogenous Daudi LMP-1, the band that corresponds to LMP-1 activation by type 1 EBNA-2 is stronger than that from Daudi LMP-1 gene induction by type 2 EBNA-2 (Figure 5.10 B and 5.11 B – dig. LMP-1 panel, top band) as observed before for the wild-type Daudi:pHEBo-LMP-1r cells (Figure 5.9 B – dig. LMP-1 panel, top band).

A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LMP-2Bp
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terminal proteins
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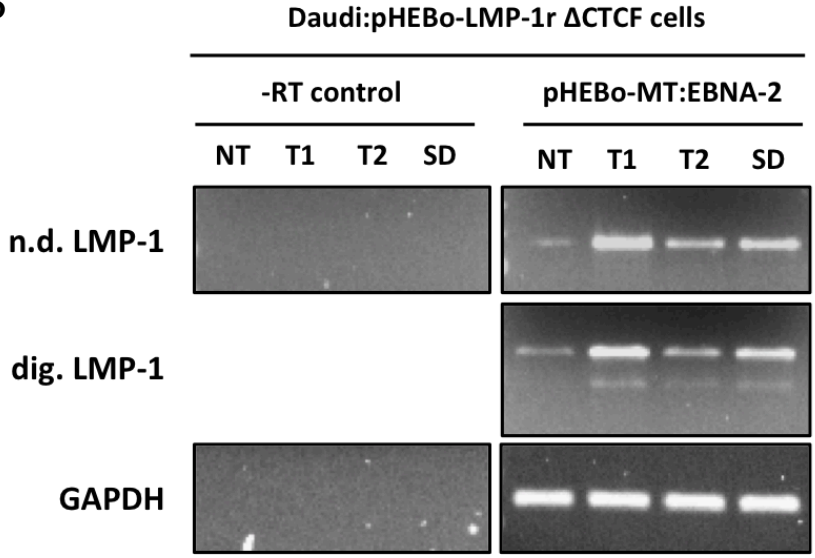
EcoRI
166,157
171,823
1

B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NT T1 T2 SD
n.d. LMP-1
dig. LMP-1
GAPDH

C

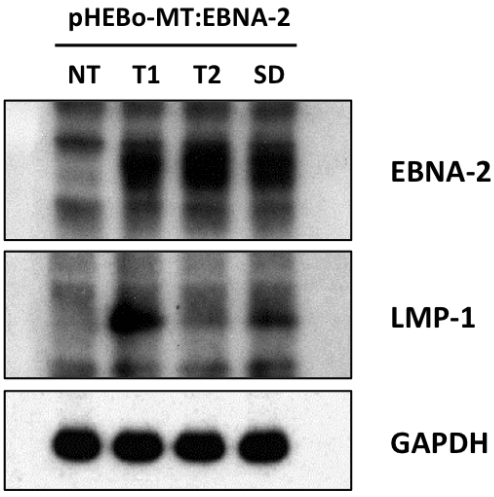
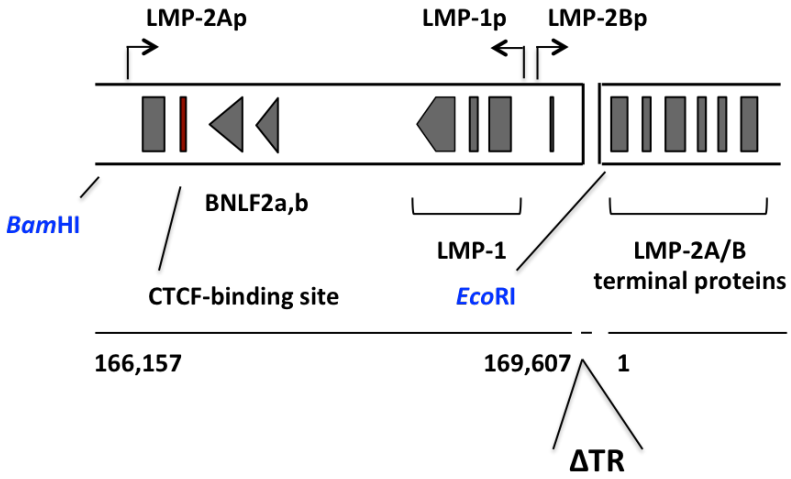

pHEBo-MT:EBNA-2
NT T1 T2 SD
EBNA-2
LMP-1
GAPDH

Figure 5.10 Transcriptional regulation of B95-8 LMP-1 gene carried by the pHEBo:Dhet-*Bam*HI-*Eco*RI ΔCTCF plasmid by type 1 and type 2 EBNA-2 when the CTCF-binding site was deleted

(**A**) The Dhet-*Bam*HI-*Eco*RI fragment (limited by a *Bam*HI and *Eco*RI restriction sites, in blue) includes the whole LMP-1 regulatory region cloned into pHEBo:Dhet-*Bam*HI-*Eco*RI vector. The CTCF-binding site has been deleted (nucleotides 166,515 to 166,536) before cloning into the pHEBo-OriP plasmid. 2 x 10^6 Daudi:pHEBo-LMP-1r ΔCTCF cells carrying a stably chromatinised pHEBo:Dhet-*Bam*HI-*Eco*RI ΔCTCF vector were transiently transfected with 6 µg of one of the EBNA-2-expressing plasmids: pHEBo-MT:E2T1 (T1, type 1 EBNA-2), pHEBo-MT:E2T2 (T2, type 2 EBNA-2) or pHEBo-MT:E2T2 S442D (SD, type 2 S442D EBNA-2) using the Neon transfection system. EBNA-2 expression from the MT promoter was induced with 5 µM $CdCl_2$ at 5 hours after transfections. Cells were harvested 24 hours post-transfection and total cell RNA and proteins were then extracted and analysed by (**B**) RT-PCR or (**C**) Western blot, respectively. Non-transfected Daudi:pHEBo-LMP-1r ΔCTCF cells were also analysed (NT). (**B**) Total cell RNA was reverse transcribed into cDNA and this was analysed by PCR using LMP-1 specific primers. Product amplified from total LMP-1 mRNA (n.d. LMP-1) was then digested with *Nco*I restriction enzyme (dig. LMP-1) to solely digest the PCR products amplified from B95-8 LMP-1 mRNA, which was transcribed from the pHEBo:Dhet-*Bam*HI-*Eco*RI ΔCTCF plasmid, carrying the whole B95-8 LMP-1 regulatory region (dig. LMP-1 panel, bottom band) and not the PCR products amplified from the Daudi endogenous LMP-1 mRNA, which does not carry a *Nco*I restriction site (dig. LMP-1 panel, top band). Total cell RNA was also subject to RT-PCR analysis for GAPDH as an internal control. (**C**) Protein samples were analysed for expression of EBNA-2 and LMP-1. GAPDH immunoblotting analysis ensured that equal amounts of proteins were loaded on the gel. n.d.: not-digested; dig.: digested; -RT: no reverse transcriptase or no template negative control.

A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166,157
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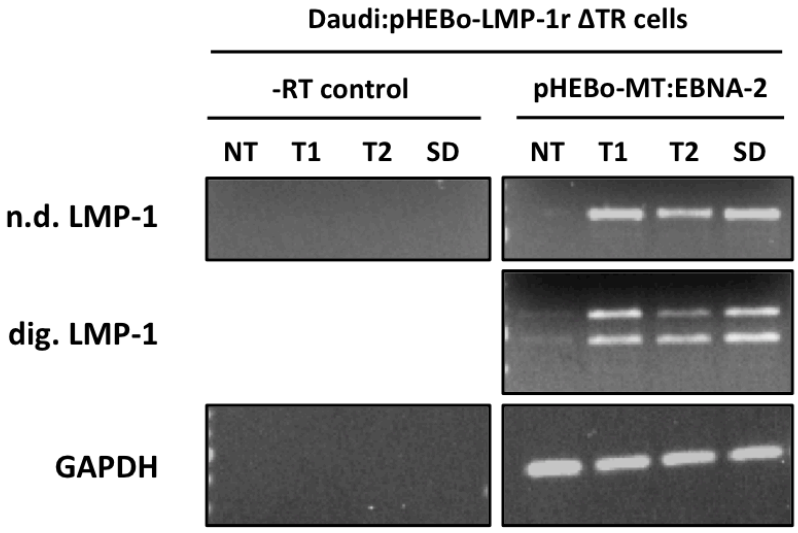
ΔTR

B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n.d. LMP-1
dig. LMP-1
GAPDH

C

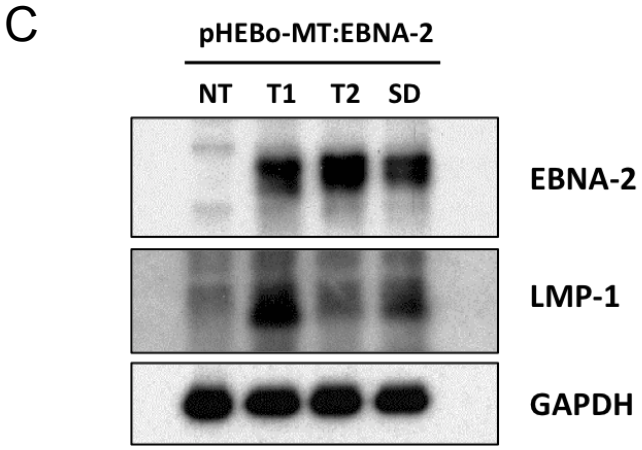

pHEBo-MT:EBNA-2
NT T1 T2 SD
EBNA-2
LMP-1
GAPDH

Figure 5.11 Transcriptional regulation of B95-8 LMP-1 gene carried by the pHEBo:Dhet-*Bam*HI-*Eco*RI ΔTR plasmid by type 1 and type 2 EBNA-2 when the EBV genome terminal repeats were deleted

(**A**) The Dhet-*Bam*HI-*Eco*RI fragment (limited by a *Bam*HI and *Eco*RI restriction sites, in blue) includes the whole LMP-1 regulatory region cloned into pHEBo:Dhet-*Bam*HI-*Eco*RI vector. The EBV terminal repeats (TR) of the viral genome have been deleted (nucleotides 169,608 to 171,802) before cloning into the pHEBo-OriP plasmid. 2 x 10^6 Daudi:pHEBo-LMP-1r ΔTR cells carrying a stably chromatinised pHEBo:Dhet-*Bam*HI-*Eco*RI ΔTR vector were transiently transfected with 6 µg of one of the EBNA-2-expressing plasmids: pHEBo-MT:E2T1 (T1, type 1 EBNA-2), pHEBo-MT:E2T2 (T2, type 2 EBNA-2) or pHEBo-MT:E2T2 S442D (SD, type 2 S442D EBNA-2) using the Neon transfection system. EBNA-2 expression from the MT promoter was induced with 5 µM $CdCl_2$ at 5 hours after transfections. Cells were harvested 24 hours post-transfection and total cell RNA and proteins were then extracted and analysed by (**B**) RT-PCR or (**C**) Western blot, respectively. Non-transfected Daudi:pHEBo-LMP-1r ΔTR cells were also analysed (NT). (**B**) Total cell RNA was reverse transcribed into cDNA and this was analysed by PCR using LMP-1 specific primers. Product amplified from total LMP-1 mRNA (n.d. LMP-1) was then digested with *Nco*I restriction enzyme (dig. LMP-1) to solely digest the PCR products amplified from B95-8 LMP-1 mRNA, which was transcribed from the pHEBo:Dhet-*Bam*HI-*Eco*RI ΔTR plasmid, carrying the whole B95-8 LMP-1 regulatory region (dig. LMP-1 panel, bottom band) and not the PCR products amplified from the Daudi endogenous LMP-1 mRNA, which does not carry a *Nco*I restriction site (dig. LMP-1 panel, top band). Total cell RNA was also subject to RT-PCR analysis for GAPDH as an internal control. (**C**) Protein samples were analysed for expression of EBNA-2 and LMP-1. GAPDH immunoblotting analysis ensured that equal amounts of proteins were loaded on the gel. n.d.: not-digested; dig.: digested; -RT: no reverse transcriptase or no template negative control.

Collectively, these results suggest that the pHEBo:Dhet-*Bam*HI-*Eco*RI plasmid, which carried a larger LMP-1 regulatory region and not just the LMP-1 promoter sequence, was not able to successfully reproduce the differential LMP-1 gene regulation mediated by type 1 and type 2 EBNA-2, observed when whole genome regulation was investigated in previous reports. The expression of the B95-8 LMP-1 gene in the pHEBo:Dhet-*Bam*HI-*Eco*RI plasmid was induced by type 1, type 2 and type 2 S442D EBNA-2 but the RT-PCR assay established in this study did not allow the detection of differences in B95-8 LMP-1 gene activation. However, in every experiment performed in sections 5.2.2.3 and 5.2.2.4, a distinct band intensity was observed for the endogenous Daudi LMP-1 gene after induction by type 1 and type 2 EBNA-2 proteins. Specifically, the intensity of the band, which corresponds to Daudi LMP-1 expression induced by type 1 EBNA-2, was stronger than that of the band corresponding to induction by type 2. This would be consistent with a differential regulation of the LMP-1 gene by type 1 and type 2 EBNA-2 observed in other systems, which used the full-length EBV genome to demonstrate the distinct LMP-1 gene activation by the EBNA-2 types. Despite the unsuitability of the RT-PCR assay to detect differences in B95-8 LMP-1 gene induction by type 1, type 2 and type 2 S442D EBNA-2, it could also be proposed that either the LMP-1 regulatory region cloned into the pHEBo-OriP plasmid is not sufficient and a larger sequence must be required or a distinct type 1/type 2 regulation of the LMP-1 gene could only be observed when the whole genome is used.

The ΔTR and ΔCTCF deletions in the LMP-1 regulatory region have been also investigated to determine whether any of these regions could account for the differential LMP-1 regulation by type 1 and type 2 EBNA-2 proteins. However, since this distinct LMP-1 regulation was not successfully reproduced using the pHEBo-OriP plasmid, the function played by these regions was not adequately determined. Alternative approaches must be adopted to investigate what is determining the type 1/type 2 gene regulation and the molecular mechanism controlling the distinct LMP-1 gene expression by type 1 and type 2 EBNA-2.

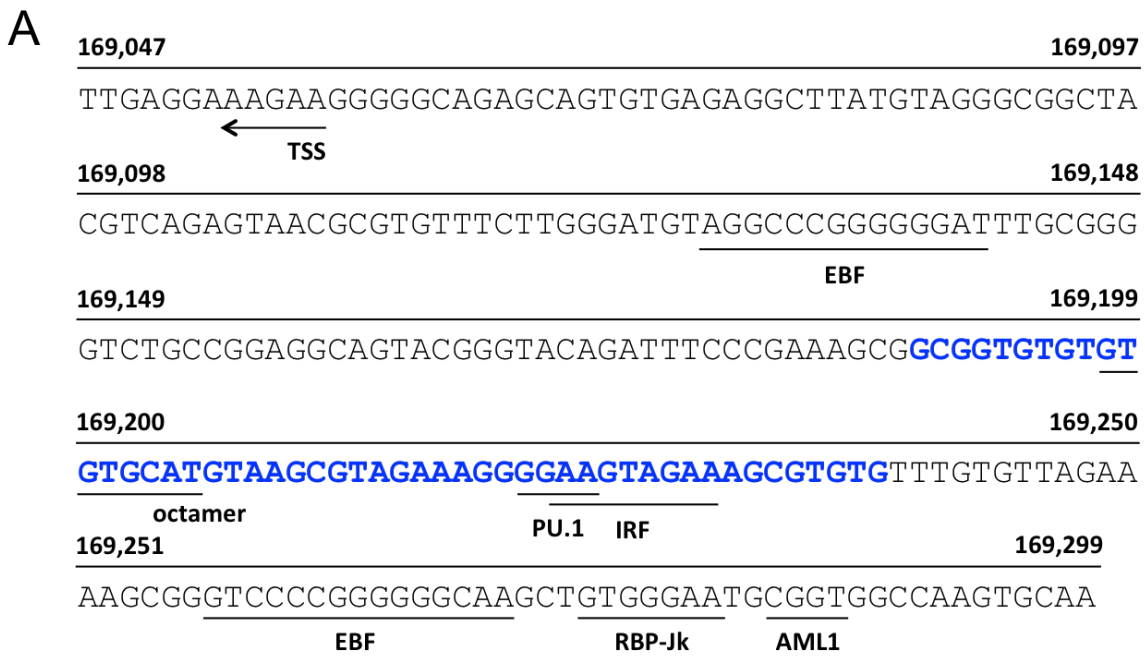
5.2.3 Investigating the interaction of type 1 and type 2 EBNA-2 with the LMP-1 promoter using biotinylated oligonucleotide pull-downs

5.2.3.1 Type 1 EBNA-2 binding at the LMP-1 promoter is dependent on the PU.1 transcriptional factor binding site and on the octamer motif

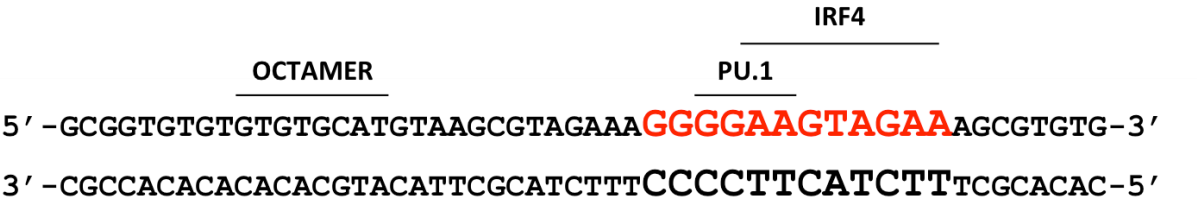
ChIP-qPCR assays performed in this laboratory demonstrated that type 1 EBNA-2 displayed an increased ability to interact with the EBNA-2 binding site at the LMP-1 gene promoter when compared to type 2 EBNA-2. Moreover, the S442D substitution in type 2 EBNA-2 improved its capacity to bind at the LMP-1 promoter but it was still weaker than type 1 EBNA-2 binding [210]. LMP-1 is one of the genes differentially regulated by type 1 and type 2 EBNA-2. Analysis of DNA sequences of EBNA-2 binding sites in cellular gene promoters differentially induced by type 1 and type 2 EBNA-2 proteins (and also in the viral LMP-1 gene promoter) has identified one enriched sequence element, which corresponds to overlapping binding sequences for an ETS transcription factor (PU.1) and for interferon regulatory factor (IRF) (ETS-IRF composite element) [210]. This motif is similar to the binding sequence for the PU.1 factor, which mediates LMP-1 activation by EBNA-2 [239, 242]. Interestingly, this enriched sequence was not identified in EBNA-2 binding sites at the cellular gene promoters regulated equally by type 1 and type 2 EBNA-2, suggesting that this element could account for the distinct EBNA-2 binding at target promoters and therefore for the differential gene induction by type 1 and type 2 EBNA-2.

In the present study the mechanism of EBNA-2 interaction with target responsive promoters was investigated. Specifically, the importance of the PU.1 binding motif to EBNA-2 binding at the LMP-1 promoter was addressed. To accomplish this aim a binding assay was developed (previous attempts using EMSAs had given no complexes containing EBNA-2). Biotinylated oligonucleotides carrying sections of the LMP-1 promoter were used to search for binding motifs or DNA regulatory sequences included in the promoter, which can bind with affinity and specificity the EBNA-2 protein present in nuclear extracts of Daudi cells expressing EBNA-2 (described in section 2.7.5). The oligonucleotides labelled with biotin comprised a 50 bp section of the LMP-1 promoter, which included the PU.1 binding site, the IRF4 motif and the octamer sequence (Figure 5.12 A). Two different oligonucleotides were used: biotinylated oligonucleotide LMP-1wt, carrying the PU.1 binding site consensus sequence (GGAA) and biotinylated oligonucleotide PU.1mut, in which the ETS motif was modified (CGTA) with two

nucleotide substitutions (Figure 5.12 B and C). Their capacity to pulldown EBNA-2 was then investigated.

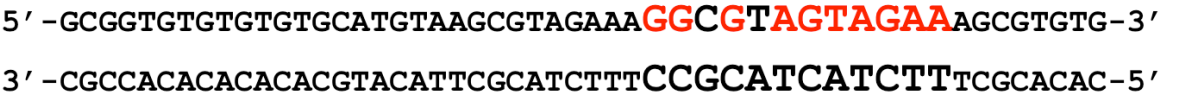


B



LMP-1 wildtype biotinylated oligonucleotide

C



PU.1 mutant biotinylated oligonucleotide

Figure 5.12 Oligonucleotides with 5' biotin label designed to investigate interaction of EBNA-2 with the LMP-1 promoter in pull-down assays

(**A**) The LMP-1 promoter regulatory region (-241/+12) of B95-8 EBV (nucleotides 169,047 to 169,299). Section of the LMP-1 promoter (represented in blue) used to design the biotinylated oligonucleotides for pull-down assays. Transcription start site (TSS) is shown. Binding sites for regulatory transcription factors are indicated and closely resemble consensus motifs. PU.1/IRF binding sites and the octamer motif are included in the designed oligonucleotide. Regulatory sequence elements not included in the biotinylated oligonucleotides: EBF: early B cell factor; RBP-Jk: recombination signal-binding protein J kappa and AML1: acute myeloid leukemia1. (**B**) LMP-1 wildtype biotinylated oligonucleotide: carries the conserved consensus sequence of the PU.1 factor binding site (GGAA). (**C**) PU.1 mutant biotinylated oligonucleotide: PU.1 binding sequence has two nucleotide substitutions (CGTA).

Each biotinylated oligonucleotide was bound to magnetic beads linked to streptavidin and the complex was then incubated with nuclear extracts of Daudi:pHEBo-MT cells, expressing type 1 EBNA-2 protein. Proteins that were pulled down by each biotinylated oligonucleotide bound to the beads were removed from the total nuclear extracts by magnetic separation. Samples obtained from each individual pulldown reaction were then analysed by SDS-PAGE and the immunoblots were probed using the anti-EBNA-2 PE2 antibody.

The results showed that the biotinylated oligonucleotide carrying the consensus sequence of the PU.1 binding motif (LMP-1wt – GGAA) was able to pulldown much more type 1 EBNA-2 protein than the oligonucleotide that included a disrupted PU.1 binding site (PU.1mut – CGTA) (Figure 5.13). This observation supported previous work on the importance of PU.1 for LMP-1 induction by EBNA-2 [239, 242], since the absence of the PU.1 consensus sequence caused an impairment in type 1 EBNA-2 binding. In fact, it was first demonstrated in the present study that EBNA-2 is indeed able to interact with this small section of the LMP-1 promoter. Moreover, EBNA-2 tethering to this limited region carried by the oligonucleotide is most likely to require the cellular PU.1 factor. Earlier reports had shown that the PU.1 factor could bind at the LMP-1 promoter [239, 240] but the formation of a protein complex involving EBNA-2 binding at this promoter region had not yet been demonstrated in previous approaches.

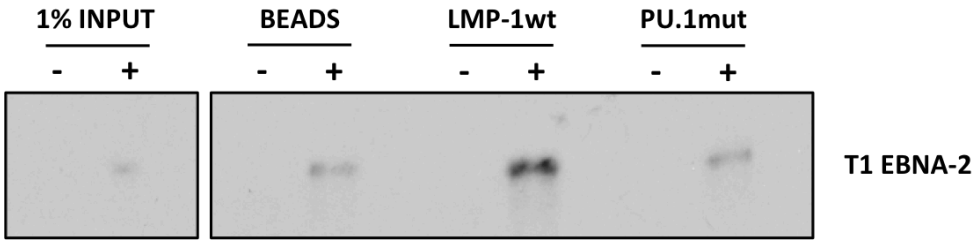


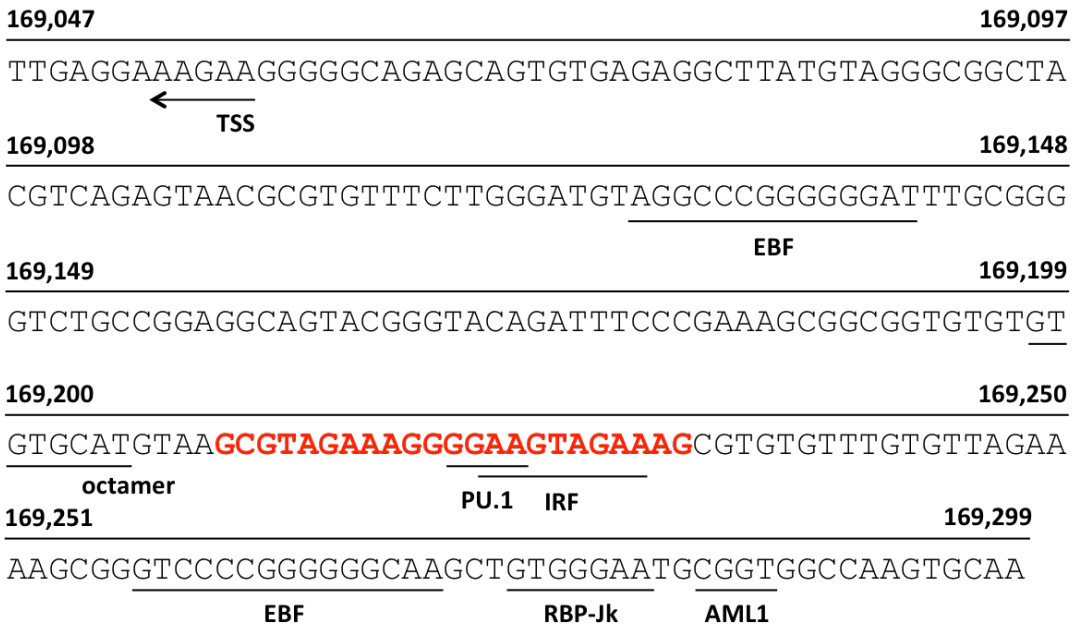
Figure 5.13 Type 1 EBNA-2 protein is pulled down by biotinylated oligonucleotide carrying the consensus sequence of the PU.1 binding site (GGAA) at the LMP-1 promoter

Biotinylated oligonucleotides, corresponding to a 50-bp section of the LMP-1 promoter, which includes the binding sites for PU.1 and IRF4 and the octamer motif, were immobilised on streptavidin-magnetic beads. The oligonucleotide-beads complex was incubated with nuclear extracts of Daudi:pHEBo-MT cells, in which expression of type 1 EBNA-2 had been induced with 5 μM $CdCl_2$ (+) or not induced (-). Samples obtained from each individual pull-down reaction were analysed by western blotting analysis and probed for EBNA-2 using the PE2 anti-EBNA-2 antibody. The LMP-1 wildtype biotinylated oligonucleotide (LMP-1wt) carried the consensus PU.1 binding site (GGAA), while the PU.1 mutant biotinylated oligonucleotide (PU.1mut) carried a disrupted PU.1 binding site, with two nucleotide substitutions (CGTA). 50 pmol of biotinylated oligonucleotide was used *per* pull-down reaction. T1: type 1 EBNA-2.

Having shown that type 1 EBNA-2 protein was pulled down by this 50 bp oligonucleotide that carried a section of the LMP-1 promoter encompassing the PU.1/IRF composite element and the octamer binding sequence (Figure 5.12 B), the specificity of EBNA-2 binding and also the importance of the PU.1 site for this interaction was further assessed. This pull-down reaction was performed again adding a specific competitor oligonucleotide with no biotin label but 10-fold more concentrated (Figure 5.14 B) than the biotinylated oligonucleotide.

This was to determine whether the competitor oligonucleotide, which carries the sequences for PU.1 and IRF4 binding sites, was able to compete for EBNA-2 binding with the biotinylated oligonucleotide. An additional pull-down reaction using a randomised competitor was also performed as a negative outcompetition reaction control (Figure 5.14 C).

A



B

Specific (SP) PU.1 competitor oligonucleotide (no biotin label)

C

5′-TAGCGTCATGACTACGTGATCAT-3′
3′-ATCGCAGTACTGATGCACTAGTA-5′

Randomised (RD) competitor oligonucleotide (no biotin label)

Figure 5.14 Competitor oligonucleotides used in the biotinylated oligonucleotide pull-downs

(**A**) The LMP-1 promoter regulatory region (-241/+12) of B95-8 EBV (nucleotides 169,047 to 169,299). Section of the LMP-1 gene promoter (in red) used to design the specific competitor oligonucleotide for the pull-down assay with competition. (**B**) Specific (SP) competitor oligonucleotide with no biotin label that encompasses the conserved sequence of PU.1 (GGAA) and IRF4 binding sites. (**C**) Randomised (RD) competitor oligonucleotide used in the pull-down assay with competition as a negative control.

The results showed again that the biotinylated oligonucleotide with the conserved PU.1 site pulled down more type 1 EBNA-2 that the oligonucleotide with the mutated PU.1 binding site (Figure 5.15). However, when the specific competitor oligonucleotide that encompasses this region of the LMP-1 promoter (Figure 5.14 B) was added to the pull-down reaction, EBNA-2 binding to the biotinylated oligonucleotide (LMP-1wt) was not outcompeted and the pulled down EBNA-2 was not reduced as observed for the negative outcompetition control, a randomised competitor oligonucleotide carrying no PU.1 sites. These observations suggest that either the reduced length of specific competitor did not permit the assembly of an EBNA-2 complex that would allow it to be pulled down using this assay or the octamer sequence, absent in the competitor, but included in the PU.1 biotinylated oligonucleotide, is required for the formation of the protein complex that recruits EBNA-2 to this region of the LMP-1 gene promoter.

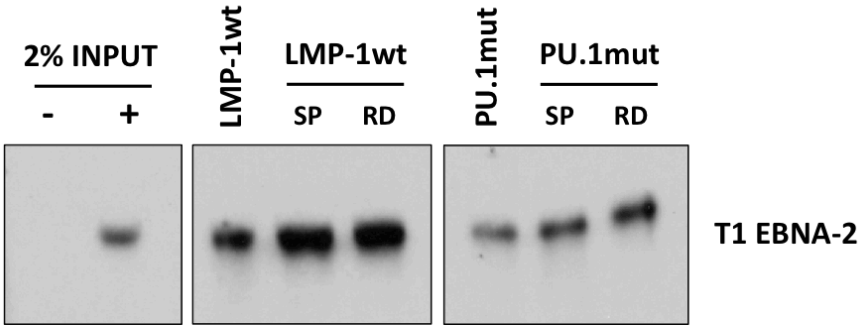


Figure 5.15 Competitor oligonucleotide carrying the PU.1/IRF composite element sequence did not outcompete type 1 EBNA-2 protein pull-down by the LMP-1wt biotinylated oligonucleotide

50 pmol of biotinylated oligonucleotides, carrying a 50-bp section of the LMP-1 gene promoter, which includes the binding motifs for PU.1 and IRF4 factors and the octamer sequence, were immobilised on streptavidin-magnetic beads. Biotinylated oligonucleotide-beads complex was incubated with nuclear extracts of Daudi:pHEBo-MT cells, in which expression of type 1 EBNA-2 had been induced with 5 μM $CdCl_2$. The LMP-1 wildtype biotinylated oligonucleotide (LMP-1wt) carrying the consensus PU.1 binding site (GGAA) and the PU.1 mutant biotinylated oligonucleotide (PU.1mut) carrying a disrupted PU.1 binding site, with two nucleotide substitutions (CGTA), were used in the pull-down reactions. Where indicated, this pull-down reaction was competed by adding 500 pmol of either specific (SP) or randomised (RD) competitor. Samples obtained from each pull-down reaction were analysed by western blotting and immunoblots probed for EBNA-2 using the PE2 anti-EBNA-2 antibody. T1: type 1 EBNA-2.

To address the requirement of the octamer sequence for EBNA-2 binding, the pull-down reaction was carried out using a longer specific competitor oligonucleotide with no biotin label carrying the PU.1 and IRF4 sites as the shorter competitor but also the octamer motif (Figure 5.16 B). This competitor oligonucleotide, with no biotin label, is similar to the LMP-1wt biotinylated oligonucleotide (Figure 5.12 B) but was added to the pull-down reaction 10-fold more concentrated as done before for the shorter competitor. The longer randomised competitor oligonucleotide was also used as negative control in a pull-down reaction with no outcompetition (Figure 5.16 C). The results showed that when the longer specific competitor oligonucleotide, encompassing the octamer sequence and the PU.1/IRF composite element, was added to the pull-down reaction, there was a reduction in the amount of EBNA-2 pulled down, when compared to bound EBNA-2 when the randomised competitor was added. Thus, the longer specific competitor oligonucleotide was able to outcompete EBNA-2 binding to the wildtype biotinylated oligonucleotide LMP-1wt (Figure 5.17), suggesting that the octamer motif is required to recruit EBNA-2 to this region of the LMP-1 promoter. When the shorter competitor oligonucleotide that does not contain the octamer motif was used (Figure 5.14 B), there was no reduction in EBNA-2 binding (Figure 5.15). Additionally, the results demonstrated again that the wildtype biotinylated oligonucleotide, carrying a conserved PU.1 binding sequence, binds more EBNA-2 protein than the biotinylated oligonucleotide PU.1mut, which carries a disrupted PU.1 motif (Figure 5.17), in this case in the presence of a randomised competitor oligonucleotide (LMP-1wt, RD and PU.1mut, RD).

A

B

Specific (SP) PU.1 competitor oligonucleotide (no biotin label)

C

5′-TAGCGTCATGACTACGTGATCATGTCGTAGCGTCATGACTACGTGATCAT-3′
3′-ATCGCAGTACTGATGCACTAGTACAGCATCGCAGTACTGATGCACTAGTA-5′

Randomised (RD) competitor oligonucleotide (no biotin label)

Figure 5.16 Competitor oligonucleotides used in the biotinylated oligonucleotide pull-downs

(**A**) The LMP-1 promoter regulatory region (-241/+12) of B95-8 EBV (nucleotides 169,047 to 169,299). Section of the LMP-1 gene promoter (in red) used to design a specific competitor oligonucleotide for the pull-down assay with competition. (**B**) Specific (SP) competitor oligonucleotide with no biotin label that encompasses the conserved sequence of PU.1 (GGAA) and IRF4 binding sites and the octamer motif. (**C**) Randomised (RD) competitor oligonucleotide used in the pull-down assay with competition as a negative control.

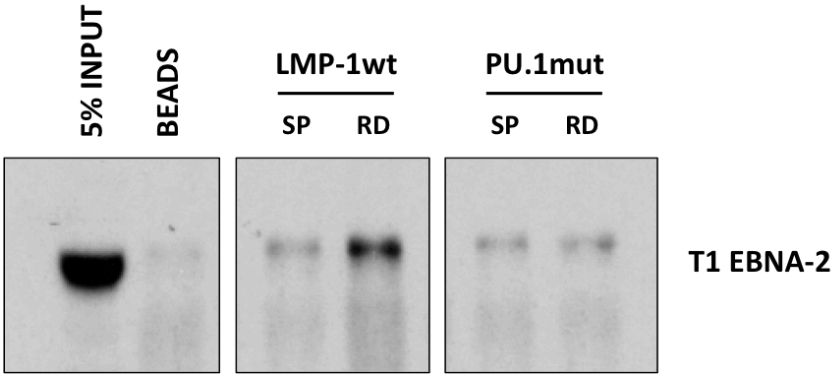


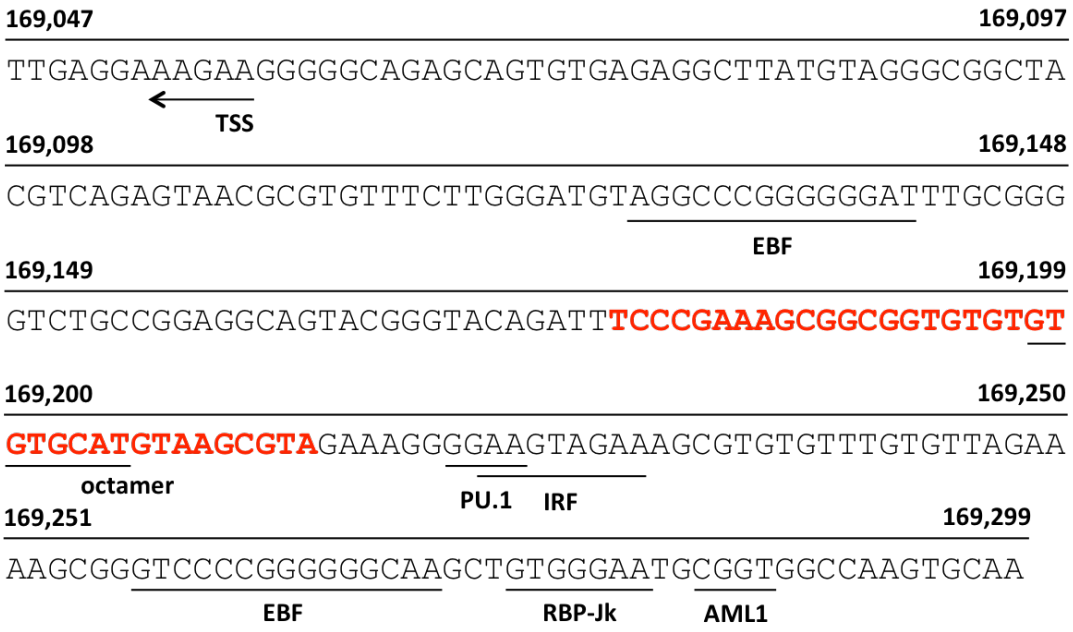
Figure 5.17 Competitor oligonucleotide carrying the PU.1/IRF composite element motif and the octamer sequence outcompeted EBNA-2 binding by the LMP-1wt biotinylated oligonucleotide

50 pmol of biotinylated oligonucleotides, carrying a 50-bp section of the LMP-1 gene promoter, which includes the binding sites for PU.1 and IRF4 and the octamer motif, were immobilised on streptavidin-magnetic beads. Biotinylated oligonucleotide-beads complex was incubated with nuclear extracts of Daudi:pHEBo-MT cells, in which expression of type 1 EBNA-2 protein had been induced using $CdCl_2$. The LMP-1 wildtype biotinylated oligonucleotide (LMP-1wt) carrying the consensus PU.1 binding site (GGAA) and the PU.1 mutant biotinylated oligonucleotide (PU.1mut) carrying a disrupted PU.1 binding motif, with two nucleotide substitutions (CGTA) were used in the pull-down reactions. Where indicated these pull-down reactions were competed by adding 500 pmol of the longer specific (SP) or longer randomised (RD) competitors. Pulled down samples were subjected to western blotting analysis and immunoblots probed for EBNA-2 using the PE2 anti-EBNA-2 antibody. T1: type 1 EBNA-2. 5% of input was loaded on the gel. Beads alone were incubated with nuclear extracts as a negative control.

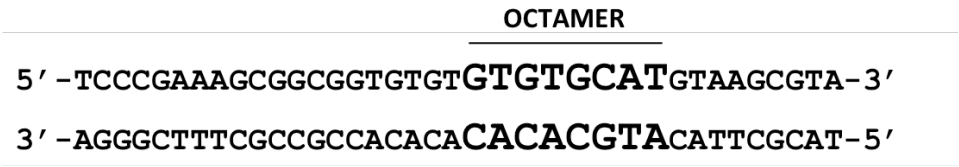
To further demonstrate the importance of the octamer motif in type 1 EBNA-2 binding to the LMP-1 promoter region, an additional competitor oligonucleotide that only encompasses the octamer motif (Figure 5.18 A and B), was used to determine whether it was able to compete for EBNA-2 binding with the LMP-1 wildtype biotinylated oligonucleotide (LMP-1wt) (Figure 5.12 B). The addition of the octamer competitor oligonucleotide (OCT) caused a pronounced reduction of EBNA-2 binding to the biotinylated oligonucleotide and less EBNA-2 is pulled down (Figure 5.18 C). Strikingly, the competitor oligonucleotide carrying only the octamer motif and no PU.1/IRF binding motif was able to outcompete EBNA-2 binding, suggesting that the cell transcription factor binding to the octamer motif is also involved in EBNA-2 recruitment to the LMP-1 promoter. This is consistent with previous works claiming that the POU-domain factor, which binds to the octamer sequence, targeted EBNA-2 to the LMP-1 promoter [242]. Additionally, PU.1 and POU domain factors had been found to be involved in LMP-1 induction by EBNA-2 [239, 242, 483]. Taken together, these results indicate that the PU.1 and POU-domain factors, which bind to PU.1-box and octamer sequence respectively, are important for type 1 EBNA-2 binding at the LMP-1 promoter.

A

B95-8 EBV LMP-1 promoter regulatory sequence (-241/+12):



B



Octamer (OCT) competitor oligonucleotide (no biotin label)

C

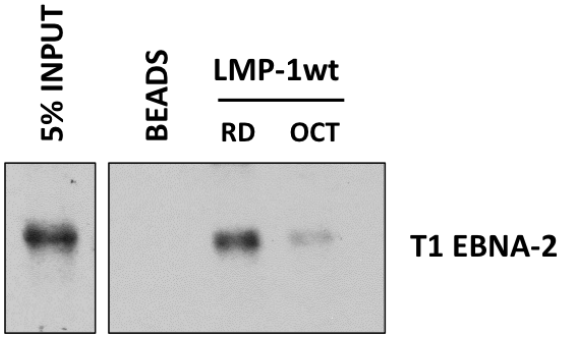


Figure 5.18 Octamer competitor oligonucleotide carrying the octamer sequence outcompeted EBNA-2 protein binding by the LMP-1 wildtype biotinylated oligonucleotide

(**A**) The LMP-1 promoter regulatory region (-241/+12) of B95-8 EBV (nucleotides 169,047 to 169,299). Section of the LMP-1 gene promoter (in red) used to design a specific competitor oligonucleotide for the pull-down assay with competition. (**B**) Octamer (OCT) competitor oligonucleotide no biotin labelled that encompasses the octamer motif of the LMP-1 promoter. This competitor oligonucleotide does not carry the PU.1/IRF composite element sequence. (**C**) 50 pmol of biotinylated oligonucleotide, carrying a 50-bp section of the LMP-1 gene promoter, which includes the binding sites for PU.1 and IRF4 and the octamer sequence, was immobilised on streptavidin-magnetic beads. Biotinylated oligonucleotide-beads complex was incubated with nuclear extracts of Daudi:pHEBo-MT cells, in which expression of type 1 EBNA-2 protein had been induced using $CdCl_2$. The biotinylated oligonucleotide LMP-1wt carrying the consensus PU.1 binding site was used in the pull-down reactions. Where indicated, this pull-down reaction was competed by adding 500 pmol of either the longer randomised (RD) competitor or the octamer (OCT) competitor oligonucleotides. Samples obtained from each individual pull-down reaction were analysed by western blotting and immunoblots probed for EBNA-2 using the PE2 anti-EBNA-2 antibody. T1: type 1 EBNA-2. Beads alone were incubated with nuclear extracts as negative control.

5.2.3.2 Type 2 EBNA-2 protein also binds to biotinylated oligonucleotide that encompasses the octamer motif and the PU.1/IRF4 binding sites

The distinct binding of type 1 and type 2 EBNA-2 at the LMP-1 promoter was hypothesised to also account for the differential induction of this gene. The identification of the enriched motif containing the PU.1/IRF composite element motif in EBNA-2 binding sites at the differentially regulated genes but not at the equally regulated genes suggested that the different binding at the LMP-1 promoter could be due to the requirement of the PU.1 factor to activate the LMP-1 gene. Accordingly, type 1 EBNA-2 would interact with this gene promoter mediated by PU.1 and its involvement would lead to a weaker interaction of type 2 EBNA-2 with this and other gene promoters, which are differentially regulated by type 1 and type 2 EBNA-2 proteins. In the previous section, it was demonstrated that type 1 EBNA-2 interacts with this region of the LMP-1 promoter and required the PU.1 binding site and the octamer motif (section 5.2.3.1).

The ability of type 2 EBNA-2 to bind to the same region of the LMP-1 promoter was also then investigated using the wildtype biotinylated oligonucleotide LMP-1wt that encompasses the PU.1/IRF4 binding sites and the octamer motif (Figure 5.12 A and B). This oligonucleotide was bound to magnetic beads linked to streptavidin and the complex was incubated with nuclear extracts of Daudi:pHEBo-MT cells, expressing type 2 EBNA-2 protein. Samples obtained in each individual pull-down reaction were analysed by SDS-PAGE and the immunoblots were probed using the anti-EBNA-2 antibody. Different amounts of input were used to ensure that the absence of EBNA-2 binding was not due to low quantity of protein available in the input.

The results showed that type 2 EBNA-2 was in fact able to be pulled down by the biotinylated oligonucleotide carrying only this small 50 bp-section of the LMP-1 promoter, which includes the PU.1/IRF binding sites and the octamer sequence (Figure 5.19). However, type 2 EBNA-2 binding was only detected when the biotinylated oligonucleotide was incubated with reaction inputs containing higher amounts of EBNA-2 protein, suggesting that type 2 EBNA-2 binding is less efficient than that of type 1 EBNA-2. Comparative analysis of type 1 and type 2 EBNA-2 binding at this region was not carried out in this study, but it is very likely that type 1 EBNA-2 binds more strongly at the region of the LMP-1 promoter as demonstrated in ChIP assays performed in recently published work from this laboratory [210]. Although the present study has shown that type 2 EBNA-2 was able to interact with this region of the LMP-1 gene

promoter, comparative type 1 and type 2 EBNA-2 binding needs to be performed to confirm their different ability to bind at this region.

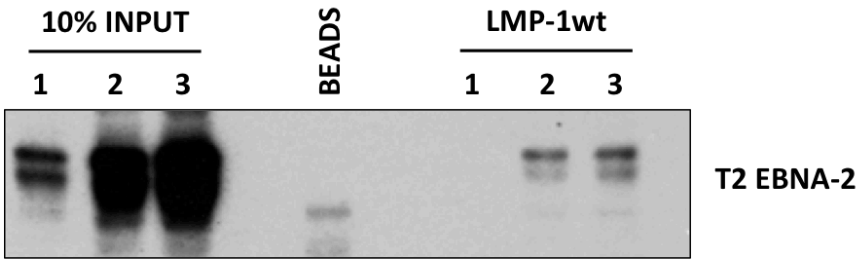


Figure 5.19 Type 2 EBNA-2 protein is pulled down by biotinylated oligonucleotide carrying the consensus sequence of the PU.1 binding site (GGAA) at the LMP-1 promoter

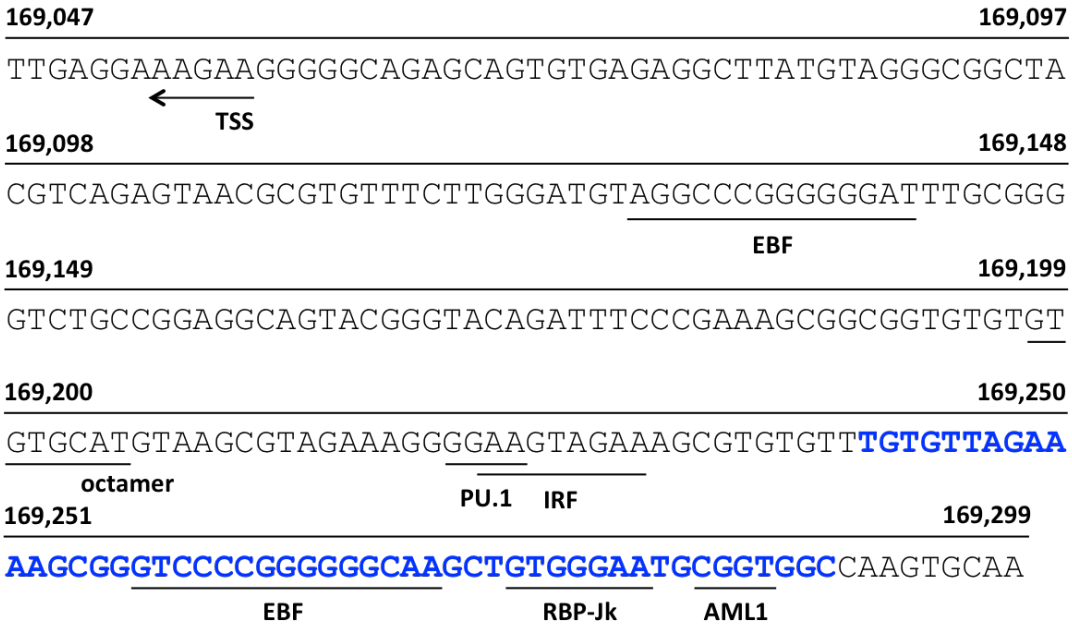
50 pmol of the biotinylated oligonucleotide, corresponding to a 50-bp section of the LMP-1 promoter, which includes both the binding sites for PU.1 and IRF4 and the octamer sequence, was immobilised on streptavidin-magnetic beads. The oligonucleotide-beads complex was then incubated with nuclear extracts of Daudi:pHEBo-MT cells, in which expression of type 2 EBNA-2 had been induced with 5 µM $CdCl_2$. Different amounts of nuclear extracts were used as input for the pull-down reaction (1: 50 µl of nuclear extracts; 2: 100 µl of nuclear extracts and 3: 150 µl of nuclear extracts). The LMP-1 wildtype biotinylated oligonucleotide (LMP-1wt), carrying the PU.1 binding site was used in the pull-downs. Samples that were obtained from each individual pull-down reaction were analysed by western blotting and probed for EBNA-2 using the PE2 anti-EBNA-2 antibody. T2: type 2 EBNA-2. 10% of each input was loaded on the gel. Beads with no biotinylated oligonucleotide were incubated with input 3.

5.2.3.3 Type 1 EBNA-2 protein binds to the biotinylated oligonucleotide that encompasses only the EBF1 and the RBP-Jk binding motifs

LMP-1 transcriptional activation from the ED-L1p promoter dependent on EBNA-2 was found to require distinct cell factors such as PU.1 or the POU-domain factor [239, 242] but also EBF1. This transcription factor was found to interact with the LMP-1 promoter. Mutation of the EBF1 binding motif caused a decrease in LMP-1 activation mediated by EBNA-2 [245]. Nonetheless, EBNA-2 was found to interact with a small section of the LMP-1 promoter, which only carries the PU.1/IRF binding sequences and the octamer motif (see section 5.2.3.1) and both were found to be important for EBNA-2 binding. To investigate whether EBNA-2 interacted with the LMP-1 promoter in the absence of both these motifs, a biotinylated oligonucleotide spanning only the EBF and RBP-Jk binding sites, important for LMP-1 transactivation, was then bound to magnetic beads. The complex was incubated with nuclear extracts of Daudi:pHEBo-MT cells, expressing type 1 EBNA-2 protein. Samples obtained in the pull-down reactions were analysed by SDS-PAGE and the immunoblots were probed using the anti-EBNA-2 antibody.

The results indicate that EBNA-2 was pulled down by the biotinylated oligonucleotide that did not carry either the PU.1 or the octamer motifs, suggesting that EBNA-2 is able to bind to the section of the LMP-1 promoter containing only the EBF and RBP-Jk sites. However, this was only observed when the input with higher amounts of EBNA-2 protein was used (Figure 5.20 C). As observed for type 2 EBNA-2 (Figure 5.19), when comparing the EBNA-2 pulled down with the amount available in the input, only a small fraction is bound, suggesting that EBNA-2 binding at this LMP-1 region is less strong or with less affinity than that to the oligonucleotide carrying the PU.1 and octamer motifs. Comparative analysis between type 1 EBNA-2 binding to the biotinylated oligonucleotide carrying the PU.1 and octamer motifs and type 1 EBNA-2 binding to the biotinylated oligonucleotide containing the EBF and RBP-Jk will be required to confirm this is the case. Additionally, a beads control to rule out binding to the beads without biotinylated oligonucleotide (not shown in Figure 5.20 C) would be essential to confirm type 1 EBNA-2 binding at this region of the LMP-1 promoter, which includes the EBF and RBP-Jk sites. In this case, it is also possible that EBNA-2 could be recruited to the LMP-1 promoter with different efficiency by the cellular transcription factors that interact with this promoter although adequate LMP-1 transactivation dependent on EBNA-2 requires the assembly of a protein complex formed by the EBNA-2 and all the participant cell factors.

A



B

EBF/Jk biotinylated oligonucleotide

C

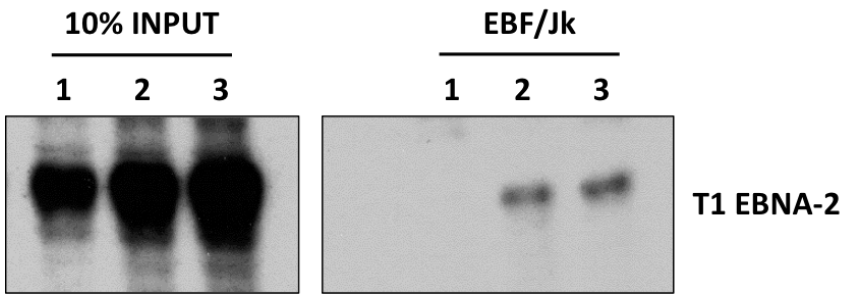


Figure 5.20 Type 1 EBNA-2 protein is pulled down by biotinylated oligonucleotide carrying the sequence of the binding sites for EBF1 and RBP-Jk transcriptional factors

(**A**) The LMP-1 promoter regulatory region (-241/+12) of B95-8 EBV (nucleotides 169,047 to 169,299). Section of the LMP-1 promoter (represented in blue) used to design the biotinylated oligonucleotide for the pull-down assay. Binding sequences for regulatory transcription factors are indicated and closely resemble consensus motifs. Sequence elements that are included in the biotinylated oligonucleotide: EBF: early B cell factor; RBP-Jk: recombination signal-binding protein J kappa and AML1: acute myeloid leukemia1. PU.1/IRF binding sites and the octamer motif were not included in the designed oligonucleotide. (**B**) EBF/Jk biotinylated oligonucleotide: carries the conserved consensus sequence of the EBF1 factor, RBP-Jk and AML1 binding sites. (**C**) 50 pmol of EBF/Jk biotinylated oligonucleotide, corresponding to a 50-bp section of the LMP-1 promoter, was immobilised on streptavidin-magnetic beads. The oligonucleotide-beads complex was then incubated with nuclear extracts of Daudi:pHEBo-MT cells, in which expression of type 1 EBNA-2 had been induced with 5 μM $CdCl_2$. Different amounts of nuclear extracts were used as input for the pull-down reaction (1: 50 μl of nuclear extracts; 2: 100 μl of nuclear extracts and 3: 150 μl of nuclear extracts). Samples that were obtained from each individual pull-down reaction were analysed by western blotting and then probed for EBNA-2 using the PE2 anti-EBNA-2 antibody. T1: type 1 EBNA-2. 10% of each input was loaded on the gel.

In the present study it was first demonstrated that EBNA-2 binds at the LMP-1 promoter. These results indicated that the PU.1/IRF4 binding sequences and the octamer motif are important for type 1 EBNA-2 interaction with the LMP-1 promoter. The type 2 EBNA-2 protein was also able to bind this small region of the LMP-1 encompassed by the biotinylated oligonucleotide. Comparison between type 1 and type 2 EBNA-2 binding at this region was not carried out but it is possible that the affinity of type 1 EBNA-2 to this section including the PU.1 motif is stronger than that of type 2, as demonstrated before. This mechanism of distinct EBNA-2 binding at the differentially regulated promoters mediated by PU.1/IRF composite element motif, such as the LMP-1 gene, could account for the stronger gene transactivation mediated by type 1 EBNA-2 and therefore for the superior transforming ability of type 1 EBV.

5.3 Discussion

The most important testable difference between type 1 and type 2 EBV is that type 1 strains transform and immortalise B lymphocytes into LCLs *in vitro* much more efficiently than type 2 strains [296]. This different ability to establish LCLs was attributed to the EBNA-2 locus [134] and has been associated with the higher capacity of type 1 EBNA-2 to induce target cellular and viral genes required for transformation of B lymphocytes [206, 209, 210]. Only 10 out of approximately 300 genes were identified as genes more strongly induced by type 1 EBNA-2 than by type 2 [206]. The viral LMP-1 gene is one of the genes differentially regulated by type 1 and type 2 EBNA-2 [206, 209, 210] and is required for B cell transformation and to maintain cell proliferation [74, 135]. The higher induction of LMP-1 expression by type 1 EBNA-2 was demonstrated in different systems, which include distinct cell line backgrounds, Daudi, AK31, P3HR1 BL cells and the EREB2.5 LCL, but also in the early infection of primary B cells using BAC-derived EBV expressing type 1 or type 2 EBNA-2 proteins [206, 209]. This higher induction however cannot solely be explained by the 2-fold stronger transactivation function of type 1 EBNA-2 transactivation domain, as previously discussed in section 3 of this work. In fact, recent work from this laboratory showed that type 1 EBNA-2 protein binds more strongly at the EBNA-2 binding site at the LMP-1 promoter when compared to type 2 EBNA-2 protein. The interaction with the equally regulated genes however was similar, indicating that binding to target promoters could contribute for the distinct gene induction [210]. It is well established that LMP-1 induction by type 1 EBNA-2 requires many host cellular factors, which include not only the DNA-binding protein RBP-Jk [108, 220, 221, 226, 476] but also PU.1 [239, 240, 242, 476], ATF/CREB [243], POU-domain protein [242] and EBF1 [245]. Therefore, it is likely that a distinct recruitment of these co-activating proteins or transcriptional factors at the LMP-1 promoter might, in fact, determine the differential gene induction by type 1 and type 2 EBNA-2. Interestingly, when the DNA sequences of EBNA-2 binding sites at differentially regulated genes were compared, LMP-1 included, a conserved motif containing the consensus binding sites for PU.1 and IRF transcription factors was identified. The same motif was not present in EBNA-2 binding sites at equally regulated genes, suggesting that the function of EBNA-2 at the differently induced genes could be determined by EBNA-2 association with these cellular factors [210].

In this study, the differential regulation of the LMP-1 promoter by type 1 and type 2 EBNA-2 proteins was investigated using promoter-luciferase reporter assays (see section 5.2.1). The

main objective was to determine whether this distinct LMP-1 induction, observed in other cell systems, could be fully reconstituted using the LMP-1 promoter fused to a luciferase reporter, in order to further determine the promoter elements or sequences responsible for the distinct LMP-1 activation. The first approach consisted of using the LMP-1 promoter sequence of the B95-8 EBV strain, defined as positions -634 to +40 relative to the transcription initiation site, fused to a luciferase gene reporter. Transient transfections of this reporter plasmid into three different Daudi cell lines, expressing either type 1, type 2 or type 2 EBNA-2 resulted in similar induction of the LMP-1 promoter-gene reporter (around 16-fold activation) and therefore the pronounced differential LMP-1 regulation by type 1 and type 2 was not observed (Figure 5.1). The results suggested that the lack of proper chromatinisation of the LMP-1 regulatory region in the reporter plasmid and the absence of post-translational histone modifications and other epigenetic marks could have determined the equal regulation of the LMP-1 promoter by type 1 and type 2 EBNA-2. Genome chromatinisation and epigenetic markers have been shown to be important in the regulation of viral expression. For example, the EBV lytic genes were found to be silenced upon chromatinisation of the EBV genome [484]. To test this hypothesis of a possible requirement for an adequate chromatinisation of the LMP-1 promoter-luciferase reporter, the LMP-1 promoter region controlling a luciferase gene expression was cloned into the puro-OriP vector, which was then stably transfected and chromatinised into the Daudi cell line and LMP-1 induction was determined after type 1 and type 2 EBNA-2 expression (Figure 5.3). Although the reporter plasmid carrying the LMP-1 promoter region was then adequately stably chromatinised there was no differential LMP-1 induction by type 1 and type 2 EBNA-2 proteins and the promoter was constitutively active, even in the absence of EBNA-2 (Figure 5.3). It is possible that a larger regulatory sequence, comprising not only the LMP-1 regions: -634/+40 and -241/+12 tested in this study, might be required to recapitulate the differential regulation of LMP-1 induction by type 1 and type 2 EBNA-2, which is observed for the whole EBV genome in several cell systems as previously determined [206, 209, 210]. Interestingly, the promoter sequences used in the reporter assays did not include the binding site for the cell chromatin factor CTCF, which binds at the LMP-1 regulatory region, in the overlapping first intron of the LMP-2A gene and the 3' untranslated region (UTR) of LMP-1. Mutation of the CTCF-binding site or depletion of the CTCF factor were shown to result in deregulation of gene expression. In addition, CTCF was also shown to be required for proper transcriptional regulation of LMP-1, LMP-2A and LMP-2B and loss of CTCF was associated with a switch from euchromatic to heterochromatic epigenetic marks at the LMP-1 promoter region [479]. CTCF and cohesins are important for epigenetic patterning of the viral chromosome and in the coordination of the histone modification and DNA methylation in viral gene expression

programs [485]. For these reasons, it is very likely that the CTCF-binding site in the vicinity of the LMP-1 promoter is necessary for the differential induction of the LMP-1 gene by type 1 and type 2 EBNA-2 and indicates that the promoter-reporter assays performed in the present study are not adequate to investigate LMP-1 regulation.

In order to circumvent the limitations of the previous reporter assays, a distinct approach was adopted, consisting of using the whole LMP-1 regulatory region cloned into an OriP plasmid to determine LMP-1 gene induction by type 1 and type 2 EBNA-2 proteins. This larger region included the LMP-1 gene and promoter, the LMP-2A and LMP-2B viral promoters, the EBV genome terminal repeats and also the aforementioned CTCF-binding site (Figure 5.4 B). The plasmid construct was stably chromatinised in the Daudi cell line and LMP-1 expression was assessed by western blotting analysis after transient transfection of a plasmid that expressed either type 1 or type 2 EBNA-2. A stronger LMP-1 induction was indeed observed for type 1 EBNA-2 (Figure 5.6 A), but because these cells carry not only the pHEBo-OriP plasmid with the whole LMP-1 regulatory region but also endogenous Daudi EBV genomes, it was difficult to determine whether the differential LMP-1 induction had been established either in the OriP plasmid or in the Daudi EBV genome. The size of the LMP-1 protein from the Daudi genome is slightly smaller than the LMP-1 protein coming from the pHEBo-OriP plasmid (B95-8 EBV) (Figure 5.6 B). Therefore, western blotting analysis was not adequate to assess whether the OriP construct, containing the whole LMP-1 regulatory region, could recapitulate LMP-1 gene regulation and a different methodology was used. This consisted of using an RT-PCR assay to separately evaluate the induction of both LMP-1 genes, which come from the endogenous Daudi EBV genomes and from the stably chromatinised pHEBo-OriP vector (section 5.2.2.3). The results revealed that the LMP-1 gene carried by the pHEBo-OriP plasmid was activated by type 1, type 2 and also type 2 S442D EBNA-2 (Figure 5.9 B), but using this method was not possible to determine differences in B95-8 LMP-1 mRNA levels induced by the EBNA-2 types. Interestingly, the band corresponding to the induction of the Daudi endogenous LMP-1 gene activated by type 1 EBNA-2 displayed a stronger intensity than that from Daudi LMP-1 activation by type 2 EBNA-2 protein (Figure 5.9 B, digested LMP-1, top bands). A differential induction of the Daudi endogenous LMP-1 gene would be consistent with the stronger LMP-1 activation by type 1 EBNA-2 observed in previous reports, when the whole EBV genome had been used [206, 209, 210]. Besides the limitations of the RT-PCR assay, it might be possible that the LMP-1 regulatory sequence cloned into the pHEBo-OriP plasmid is not sufficient and a larger sequence might be required to permit differential type 1/type 2 EBNA-2 regulation of LMP-1. Interestingly, the whole LMP-1 regulatory region cloned into the pHEBo-OriP plasmid

does not include the terminal exons of the LMP-2A and LMP-2B proteins (Figure 5.4 B) and the LMP-2B protein is expressed from the LMP-2Bp divergent promoter, also responsible for initiation of LMP-1 expression. It might be possible that the terminal exons of the LMP-2A and -2B proteins are required to generate the differential LMP-1 gene regulation by type 1 and type 2 EBNA-2. Alternatively, it is also likely that the distinct LMP-1 gene regulation could only be observed when the whole genome is used. Mutations in the LMP-1 regulatory sequence cloned into the pHEBo-OriP plasmid had been made in the laboratory to assess their contribution for the differential LMP-1 regulation by type 1 and type 2 EBNA-2 proteins. Specifically, a deletion of the CTCF-binding site (Figure 5.10 A) and a deletion of the EBV genome terminal repeats (Figure 5.11 A). However, since the wildtype LMP-1 region cloned into pHEBo-OriP plasmid did not successfully reconstitute the correct differential LMP-1 gene regulation, their plausible function in this context was not adequately determined. Induction of the LMP-1 gene from these mutant regulatory sequences by type 1 and type 2 EBNA-2 was however investigated but no different activation could be detected using this RT-PCR assay (Figure 5.10 B and Figure 5.11 B). Again, in both cases, it was possible to detect a stronger intensity of the band corresponding to the Daudi endogenous LMP-1 gene activation by type 1 EBNA-2 when compared to type 2. Therefore, it would be interesting to use progressively larger EBV genome regions, including the LMP-1 sequence, cloned into an OriP plasmid, when technically possible, to determine whether the differential LMP-1 gene induction could be recapitulated. Alternatively, an approach consisting of using recombinant viruses, in which distinct elements are disrupted such as the CTCF-binding site, the genome terminal repeats or the PU.1 binding site, could be important to evaluate whether the differential regulation, observed when the whole genome is used, could be affected by any of these elements.

In this study the interaction of EBNA-2 with the LMP-1 promoter was also investigated using biotinylated oligonucleotide pull-down assays (see section 5.2.3). Utilisation of a biotinylated oligonucleotide carrying a 50 bp-section of the LMP-1 promoter encompassing the PU.1/IRF motif of this gene promoter (LMP-1wt) and also an identical biotinylated oligonucleotide with the disrupted PU.1/IRF motif (PU.1mut) revealed that the wildtype oligonucleotide was able to pull-down much more type 1 EBNA-2 than the mutated oligonucleotide (Figure 5.13). This indicated that the PU.1-binding site is important for EBNA-2 interaction with the LMP-1 gene promoter. Earlier reports have shown that the PU.1 factor was important for LMP-1 activation by type 1 EBNA-2 [239, 242], but it was first demonstrated in the present study interaction of EBNA-2 at the LMP-1 promoter since previous attempts with EMSA had given no complexes containing EBNA-2. In fact, the PU.1 transcription factor had been shown to bind at the LMP-

1 promoter, at the PU.1 consensus binding site, but formation of a protein complex with the EBNA-2 protein had not been demonstrated [239]. The observation that the PU.1-binding site is important for the EBNA-2 interaction with the LMP-1 gene promoter strongly supports the hypothesis suggesting that the PU.1/IRF motif, which was identified in EBNA-2 binding sites at the genes differentially regulated but not at the equally regulated genes, is crucial for the higher induction by type 1 EBNA-2 [210]. It is possible that the PU.1/IRF motif promotes a stronger binding of type 1 EBNA-2 with the LMP-1 gene promoter than that of type 2 EBNA-2 protein. In fact, ChIP-qPCR assays carried out in this laboratory showed that type 1 EBNA-2 exhibited an increased ability to interact with the EBNA-2 binding site at the LMP-1 promoter, when compared to that of type 2 EBNA-2 protein [210].

The interaction of type 2 EBNA-2 with the biotinylated oligonucleotide carrying the consensus PU.1/IRF-binding sites was also investigated. The results showed that type 2 EBNA-2 was also able to interact with this 50 bp-section of the LMP-1 promoter. However, type 2 EBNA-2 was only detected in the pull-downs when reaction inputs containing higher amounts of type 2 EBNA-2 protein were used, suggesting that type 2 EBNA-2 interaction is less efficient than that of type 1 EBNA-2 (Figure 5.19). Comparative binding of type 1 and type 2 EBNA-2 at the LMP-1 promoter section encompassed by the biotinylated oligonucleotide was not performed in the present study but, as already mentioned, this was observed in a recent report [210]. Nonetheless, the interaction of type 2 EBNA-2 with this small section of the LMP-1 promoter was first demonstrated in the present study.

The biotinylated oligonucleotide encompassing the 50 bp-section of the LMP-1 promoter that included the PU.1/IRF motif also carried the octamer sequence or binding site for the POU-domain protein (Figure 5.12 B). This sequence had been previously shown to be important for EBNA-2 transactivation of the LMP-1 promoter [242]. In fact, mutation or deletion of the POU-domain binding site (octamer sequence) at the LMP-1 promoter caused a pronounced reduction of the EBNA-2-mediated transactivation of LMP-1 expression [242]. The same had been observed for PU.1-binding site. These observations suggested that the factor binding to the octamer sequence could be important for EBNA-2 interaction with the LMP-1 promoter. Interestingly, when the interaction of EBNA-2 with the wildtype biotinylated oligonucleotide (LMP-1wt) was competed using a competitor oligonucleotide encompassing only the PU.1 motif and not the octamer sequence (Figure 5.14 A and B), there was no reduction in EBNA-2 protein binding to the LMP-1wt biotinylated oligonucleotide (Figure 5.15). In contrast, when the octamer sequence was included in the competitor oligonucleotide (Figure 5.16 A and B)

the reduction in the amount of pulled down EBNA-2 protein was very pronounced (Figure 5.17). Therefore, these observations might indicate that the octamer sequence or the POU-domain factor are important for complex formation at the LMP-1 promoter and for EBNA-2-mediated transactivation of LMP-1. Additional evidence of its importance was obtained when EBNA-2 binding to the wildtype biotinylated oligonucleotide LMP-1wt was competed with a competitor oligonucleotide encompassing only the octamer sequence and not the PU.1/IRF sequence motifs (Figure 5.18). Addition of this competitor oligonucleotide promoted a great reduction in EBNA-2 binding to LMP-1wt (Figure 5.18 C), confirming the relevance of the octamer site. It would be interesting to use the same oligonucleotide sequence but biotin-labelled in order to determine whether this LMP-1 promoter region (Figure 5.18 A and B), which does not include the PU.1/IRF motif, is indeed capable of binding and pulling down the EBNA-2 protein.

The POU-domain protein family includes the factors Oct-1 and Oct-2 [486]. A different factor belonging to the POU-domain family binds to the octamer sequence at the LMP-1 promoter [242] and is important for LMP-1 expression by EBNA-2. A recent report revealed that EBV carrying mutations either in the POU-domain factor or PU.1 protein-binding sites exhibited lower B lymphocyte transformation efficiencies, in comparison to the wildtype virus [483]. The same was not observed for mutations in the RBP-Jk-binding site, which did not alter the efficiency of B lymphocyte transformation [483]. This observation and the results presented in this study, showing that EBNA-2 could interact with a section of the LMP-1 gene promoter that only encompasses the octamer motif and the PU.1/IRF-binding site but not the RBP-Jk site (Figure 5.12 and Figure 5.13), support previous reports indicating that RBP-Jk plays only a minor role in the activation of ED-L1p promoter [108, 239, 240, 242].

The early B-cell factor (EBF) binding site was also shown to be important for LMP-1 gene activation by EBNA-2 since mutation of this site associated with a reduction in expression of LMP-1 [245]. Interestingly, introduction of mutations into the EBF-binding sites in the LMP-1 promoter of a recombinant EBV also associated with reduced B cell transformation efficiency [483]. In addition, knockdown of EBF1 transcription factor was shown to cause a reduction of LMP-1 protein expression in LCLs [487]. Together, these suggest that EBF has an important function in LMP-1 transcription. The capacity of the EBNA-2 protein to bind to a section of the LMP-1 promoter, which includes the EBF1-binding site was also investigated in this study. A biotinylated oligonucleotide that encompasses the EBF1 and the RBP-Jk-binding sites, but not the octamer or PU.1/IRF motifs, was able to bind type 1 EBNA-2 in the pull-down assays

(Figure 5.20). However, when the pulled down EBNA-2 is compared with the reaction input, only a small fraction is bound. This might suggest that EBNA-2 binding at this region is less efficient than the EBNA-2 binding to the biotinylated oligonucleotide encompassing the PU.1 and octamer motifs, although direct comparison is needed to confirm this is the case. In this experiment, a negative beads control (not shown in Figure 5.20) is also necessary to rule out EBNA-2 binding to the beads with no biotinylated oligonucleotide. The main purpose of this experiment consisted of testing whether EBNA-2 could also bind to a section of the LMP-1 promoter, which did not span the octamer and the PU.1/IRF motifs. The relative contribution of EBF1 for EBNA-2 binding was not determined because the biotinylated oligonucleotide also included the RBP-Jk and AML1 (RUNX1) binding sites (Figure 5.20), and the EBNA-2 binding at this region could only reflect the presence of the RBP-Jk site.

Taken together, these results suggest that the PU.1-binding site and octamer sequence are important for type 1 EBNA-2 interaction with the LMP-1 promoter. It is likely that these motifs might determine the stronger interaction of type 1 EBNA-2 protein with the LMP-1 promoter sequence, when compared to type 2 EBNA-2 binding at this site, as previously demonstrated by ChIP-qPCR assays [210]. Accordingly, it is possible that the interaction of type 2 EBNA-2 with the LMP-1 sequence that includes the PU.1 and octamer sites could take place with less affinity than that for type 1 EBNA-2. Additionally, type 1 EBNA-2 binding to the EBF/Jk oligo might suggest that EBNA-2 could be recruited to the LMP-1 promoter with distinct efficiency by the cell transcription factors that bind at the LMP-1 promoter region, but adequate LMP-1 gene expression is only achieved upon assembly of a protein complex composed by EBNA-2 and all the transcription factors. Therefore, a mechanism of differential EBNA-2 binding at the LMP-1 gene promoter and at the other differentially regulated promoters, could contribute for the higher gene induction by type 1 EBNA-2 and, consequently for the superior transforming ability of type 1 EBV.

6 Final Discussion: summary and conclusions

The major aim of this study was to investigate the mechanism of efficient transformation of B lymphocytes into lymphoblastoid cell lines *in vitro* carried out by type 1 EBV in comparison to the poor transforming ability of type 2 EBV strains. The greater transforming capacity of type 1 EBV was associated in previous studies with the stronger induction of the viral LMP-1 and cellular CXCR7 genes, which are required for B lymphocyte immortalisation and proliferation [206, 209]. Apart from these genes, eight additional genes were shown to be more strongly induced by the potent viral transactivator EBNA-2 from type 1 EBV than by type 2 EBNA-2, indicating that a higher transactivation function of type 1 EBNA-2 could contribute to explain the differential regulation of these EBNA-2 responsive genes. Accordingly, the first specific objective of the present thesis was to investigate the transcriptional regulation of target gene promoters mediated by type 1 and type 2 EBNA-2 and also to determine if the transactivation function of type 1 EBNA-2 was stronger than the capacity of type 2 EBNA-2 to activate gene expression.

Transcriptional activation of target gene promoters mediated by type 1 and type 2 EBNA-2

Transient transfection gene-reporter assays that consisted of fusing the transactivation domain (TAD) of type 1 and type 2 EBNA-2 proteins to the GAL4 DNA-binding domain and determining their ability to activate luciferase gene expression, clearly demonstrated that the transactivation function of type 1 EBNA-2 TAD was 2-fold stronger than the ability to activate gene expression by the type 2 EBNA-2 TAD. This stronger ability of type 1 EBNA-2 TAD to induce gene expression was observed in the EBV-negative BL-B cell line BJAB but also in the EBV-positive lymphoblastoid cell line IB4.

The functional EREB2.5 LCL growth assay had previously been used to identify the regions or domains of type 1 EBNA-2 responsible for the higher B lymphocyte transformation and proliferation [209]. The C-terminal region of type 1 EBNA-2 protein was initially demonstrated to be sufficient to complement the deficiency of type 2 EBNA-2 in LCL proliferation [209]. Specifically, the CR7, RG and TAD domains of type 1 EBNA-2 protein were identified as the minimum sequences required. However, recent work from this laboratory demonstrated that a single amino acid of type 1 was sufficient. When the serine of type 2 EBNA-2 was replaced by the corresponding aspartate 442 of type 1 (S442D substitution), type 2 EBNA-2 acquired the ability to rescue LCL growth in the EREB2.5 assay [210]. This amino acid substitution

also conferred type 1-like levels of LMP-1 and CXCR7 expression. Strikingly, the reverse mutation, replacing the aspartate 442 by a serine in type 1 EBNA-2, promoted the opposite effect [210]. Interestingly, this amino acid is located in the transactivation domain of EBNA-2, suggesting that it could be responsible for the higher transactivation function of type 1 EBNA-2 protein. Determining the effect of the serine to aspartate change in the transactivation function of type 2 EBNA-2 constituted another specific objective of the present thesis. In this study, it was demonstrated that the S442D mutation in the type 2 EBNA-2 transactivation domain conferred higher ability to activate gene expression to type 2 EBNA-2 and a type 1-like phenotype was acquired in terms of transactivation function. The reciprocal substitution in type 1 EBNA-2, replacing the aspartate 442 by the equivalent serine of type 2, reduced the transactivation function of type 1 EBNA-2 and a type 2 phenotype was acquired. Therefore, the results presented in this thesis clearly demonstrated that the transactivation domain of type 1 EBNA-2 exhibited a 2-fold stronger ability to transactivate gene expression, which is conferred by aspartate-442. It was the only amino acid of type 1 EBNA-2, from all amino acid residues tested in this study, that was capable of conferring type 2 EBNA-2 a type 1-like transactivation function. Similarly, aspartate 442 had been the unique amino acid to confer type 2 EBNA-2 the ability to rescue LCL growth proliferation in the EREB2.5 assay [210].

Function of the cellular protein BS69/ZMYND11 in the regulation of EBNA-2-mediated gene transactivation

The amino acid aspartate 442 was found to be located immediately after a PXLXP peptide motif (amino acids 437 to 441) in the CR8 of type 1 EBNA-2 protein. This PXLXP motif and a second PXLXP peptide motif (amino acids 383 to 387) in type 1 EBNA-2 protein had been shown to interact with the cellular protein BS69 via its MYND domain [268]. BS69 forms a homodimer and two MYND domains recognise these two sequentially proximate PXLXP peptide motifs. BS69 interaction with EBNA-2 was associated with the inhibition of EBNA-2-mediated activation of gene expression [267, 268]. The proximity of the aspartate 442 to the PXLXP peptide motif (aa 437-441), suggested that the BS69 cell protein could have a role in the differential gene regulation by type 1 and type 2 EBNA-2. Therefore, the next specific objective of this thesis was to investigate the function of the cellular protein BS69/ZMYND11 in the regulation of the EBNA-2-mediated gene transactivation and to determine whether the transactivation function of type 1 and type 2 EBNA-2 was differentially inhibited or regulated by the BS69 protein. The results presented in this thesis demonstrated that BS69 represses gene transactivation mediated by type 1 and type 2 EBNA-2 transactivation domains, when fused to GAL4-DBD in GAL4-reporter assays. Moreover, BS69 inhibits transactivation of the

viral LMP-1 promoter by either type 1 or type 2 EBNA-2 proteins, in LMP-1 promoter-gene reporter assays. In fact, the results suggested that BS69 equally inhibits type 1 and type 2 EBNA-2-mediated activation of target gene expression and a specific function of BS69 in the differential gene regulation by type 1 and type 2 EBNA-2 was not identified in this thesis, as well as any specific role in determining the phenotype or the importance exhibited by the amino acid aspartate 442 despite the intimate proximity to the PXLXP peptide motif recognised by BS69.

As mentioned above, BS69 interacts with type 1 EBNA-2 but this interaction had not been described for the type 2 EBNA-2 protein. Another specific objective of this report was to determine whether the BS69 cell protein also interacted with type 2 EBNA-2 and, in that case, to investigate whether BS69 had a different capacity to bind type 1 and type 2 EBNA-2 proteins. The results presented in this thesis suggested that the cell protein BS69 displays a higher ability to interact with type 2 EBNA-2 than with the type 1 protein. In fact, it was shown in this study that type 2 EBNA-2 protein could also interact with BS69. The distinct interaction however is not dependent on the amino acid aspartate 442, since a type 2 EBNA-2 carrying the serine to aspartate mutation (S442D) displayed an affinity to BS69 similar to that of type 2 EBNA-2 and higher than type 1 EBNA-2. The significance of this differential interaction is not understood but this might involve an additional PXLXP peptide motif in type 2 EBNA-2 protein increasing BS69 binding to type 2 protein and not a mechanism contributing for the differential gene regulation by type 1 and type 2 EBNA-2. The similar effect of BS69 on the gene transactivation mediated by type 1 and type 2 EBNA-2 and the similar ability of BS69 to bind type 2 EBNA-2 and type 2 S442D EBNA-2 could suggest that this is the case but it may also be that we have not yet found the correct assay to reconstitute the differential regulation of gene expression. Future work to further investigate the plausible role of BS69 in gene regulation by EBNA-2 could include analysis by isothermal titration calorimetry (ITC) of BS69 interaction with type 1, type 2 and type 2 S442D EBNA-2 protein to confirm the data obtained using the pull-down assays and also perform siRNA of BS69 expression to knockdown BS69 activity and determine its effect on the expression levels of the differentially regulated genes by type 1 and type 2 EBNA-2.

Differential regulation of the viral LMP-1 gene by type 1 and type 2 EBNA-2

The differential regulation of a small set of genes by type 1 and type 2 EBNA-2 is associated with the higher capacity of type 1 EBV to transform B lymphocytes into LCLs *in vitro* and to maintain their proliferation. Approximately 300 genes are regulated by EBNA-2 but only 10 are more strongly induced by type 1 EBNA-2 than by type 2. The LMP-1 gene is included in

the group of genes differentially regulated by type 1 and type 2 EBNA-2 proteins [206, 209, 210]. Another specific objective of this thesis was to determine whether it was possible to recapitulate the correct differential LMP-1 gene regulation by type 1 and type 2 EBNA-2, using reporter assays or small OriP plasmids containing either only the LMP-1 promoter or the whole LMP-1 regulatory region. The different approaches utilised in this report showed that the higher induction of LMP-1 by type 1 EBNA-2 protein could not be reconstituted using these methods. Utilisation of an LMP-1 promoter-luciferase reporter assay showed an equal LMP-1 promoter induction by type 1, type 2 and type 2 S442D EBNA-2. Similarly, when the whole LMP-1 regulatory sequence was cloned into an OriP plasmid and then the expression of the LMP-1 gene was determined by RT-PCR, no evident differences in gene induction by type 1, type 2 or type 2 S442D EBNA-2 proteins were observed. The stronger induction of the LMP-1 gene by type 1 EBNA-2 had been observed in distinct cell systems, in approaches using the full-length EBV genome with adequate chromatinisation of the LMP-1 promoter sequences or in the context of B cell infection with BAC-derived EBV expressing either type 1 or type 2 EBNA-2 proteins [209]. The results obtained in this thesis suggested that the differential LMP-1 induction might only be recapitulated when the full-length EBV genome is present, or when a regulatory sequence even larger than those tested in this study is used or possibly when genome modifications found in established EBV-infected cell lines are present (for example DNA methylation).

The mechanism governing the specific differential regulation of the small number of genes important for B lymphocyte transformation is not fully understood. ChIP-based approaches demonstrated that type 1 EBNA-2 protein interacted more strongly with the specific promoter elements of the differentially regulated genes LMP-1 and CXCR7, compared to type 2 protein binding at these gene promoters [210]. The S442D change in type 2 EBNA-2 protein partially increased the capacity of type 2 to interact with these promoter elements. Conversely, type 1 and type 2 EBNA-2 proteins equally bind to promoters of equally regulated genes. Analysis of DNA sequences of the EBNA-2-binding sites at the differentially regulated genes identified a conserved motif, corresponding to the consensus binding site for the PU.1/Spi-1 factor and for the interferon regulatory factor (ETS-IRF composite element: EICE) [210]. This sequence motif was not identified in the EBNA-2-binding sites at the equally regulated gene promoters, suggesting that this motif is required to promote a stronger interaction of type 1 EBNA-2 with the differently activated genes. The LMP-1 promoter contains the consensus PU.1/IRF motif. Indeed, induction of LMP-1 expression by EBNA-2 was shown to involve not only the PU.1 factor but also other transcription factors already mentioned. The final specific objective of

this work was to determine the EBNA-2-responsive elements (PU.1-binding site included) at LMP-1, which are required to recruit the EBNA-2 protein to the LMP-1 promoter. Using biotinylated oligonucleotide pull-downs carrying short sequences of the LMP-1 promoter, it was here first demonstrated in this thesis EBNA-2 binding/presence in complexes formed at this section of the LMP-1 promoter, whereas other previous attempts, such as EMSAs, failed to demonstrate the presence of EBNA-2 in those protein complexes bound at the LMP-1 gene promoter. The results obtained in the present work demonstrated that the POU-domain factor-binding site or octamer motif and the PU.1-binding site are important for type 1 EBNA-2 protein binding at the LMP-1 gene promoter, because the absence of the octamer motif or disruption of the PU.1 site strongly impaired type 1 EBNA-2 protein interaction with the LMP-1 promoter. Type 2 EBNA-2 was also found to bind the same LMP-1 gene promoter section carrying the octamer sequence and also the PU.1-binding site although apparently with lower efficiency, supporting the observation that type 2 EBNA-2 binds more weakly at the EBNA-2-binding site in the LMP-1 promoter than type 1 [210]. Similarly, type 1 EBNA-2 seemed to be capable of interacting with a different section of the LMP-1 gene promoter that only included the EBF and RBP-Jk sites but not the PU.1-box and octamer motif, but with lower efficiency. Future work might include the utilisation of different biotinylated oligonucleotides spanning not only other regions of the LMP-1 promoter, which could include other promoter elements, but also oligonucleotides encompassing EBNA-2-binding sites at other differentially regulated genes such as the CXCR7 gene, in order to establish a similar mechanism of stronger induction of these genes by type 1 EBNA-2. It would be also interesting to investigate/identify other cellular factors that might be present in the EBNA-2 complexes at the LMP-1 and other genes that might not be identified by the consensus binding sites at the moment.

Taken together, the previously published data and the results obtained in this thesis suggest that the superior transforming ability of type 1 EBV is determined by the higher induction of a small number of genes by type 1 EBNA-2. The viral LMP-1 gene is included in the genes that are more strongly induced by type 1 than by type 2 and it was shown to be required for B cell transformation and also to maintain continuous cell proliferation [74, 135, 206, 209, 210]. The stronger induction of this small set of genes by type 1 EBNA-2 is likely to be determined by its stronger interaction with the EBNA-2-binding sites at these differentially regulated genes [210]. At the LMP-1 gene promoter, the POU-domain protein binding site (octamer motif) and the PU.1 factor binding sequence were found to be important for type 1 EBNA-2 interaction with this gene promoter (chapter 5, section 5.2.3). In fact, it is possible that these elements

might determine a greater binding of type 1 EBNA-2 protein at the LMP-1 and at the other differentially regulated gene promoters since the EBNA-2-binding sites at the gene promoters that are regulated equally do not exhibit the ETS/IRF (PU.1/IRF) composite element. This specific sequence element is only enriched in the EBNA-2-binding regions at this reduced number of genes distinctly induced by the EBNA-2 types [210]. Accordingly, it is possible that the presence of the PU.1-binding motif determines the weaker interaction of type 2 EBNA-2 with these gene promoters when compared to type 1 (observed in ChIP-qPCR assays [210]) and therefore contributes to the weaker induction of these genes by type 2 EBNA-2. This greater binding of type 1 EBNA-2 at the LMP-1 and at the other differentially induced gene promoters, in combination with the stronger transactivation function exhibited by type 1 EBNA-2 TAD (acid transactivation domain), which is mediated by the amino acid residue aspartate-442 (D442) (chapter 3, section 3.2.1), and with a plausible influence on cellular factors that recruit EBNA-2 at the target promoters, is likely to determine the augmented expression of these genes by type 1 EBNA-2 and therefore the superior ability to transform B lymphocytes into LCLs *in vitro* and the increased cell proliferation mediated by type 1 EBV. In this context, the serine to aspartate substitution (S442D) in the type 2 EBNA-2 TAD confers a higher ability to transactivate target gene expression (chapter 3, section 3.2.1) and also an increased capacity of type 2 S442D EBNA-2 to bind at the LMP-1 gene promoter [210], when compared to type 2 EBNA-2. This supports the hypothesis of the combination of a stronger transactivation function allied to a greater gene promoter binding of type 1 EBNA-2 as the mechanisms contributing for the higher transforming ability of the type 1 viruses. A specific function played by the cellular BS69/ZMYND11 protein in the differential gene regulation by the EBNA-2 types stills remains to be determined although BS69 recognises and binds a PXLXP peptide motif in EBNA-2 that immediately precedes the amino acid aspartate-442. In addition, BS69 interacts more strongly with type 2 EBNA-2 than with type 1, but the S442D mutation in type 2 however did not alter this distinct binding to the EBNA-2 types (chapter 4, section 4.2.3) in this report and a plausible role for the BS69 protein was not unravelled.

What is the biological context or significance of type 1 and type 2 EBV?

EBV infection *in vivo* is characterised by the ability of the virus to infect resting B cells in the Waldeyer's ring of the tonsils and to induce their proliferation into activated B blasts and their differentiation into memory B cells, where the virus establishes a latent infection, defined by a long-term persistence and by periodic lytic reactivation [71, 77]. During the process, the virus exploits the normal differentiation pathway of B lymphocytes in the human host by promoting

their proliferation and by steering the virus-infected cells through the various stages of B cell differentiation. The observation that type 1 EBV strains are capable of transforming B cells *in vitro* into LCLs much more efficiently than type 2 strains could suggest that type 1 EBV might exhibit a better ability to establish persistence in the B cell compartment *in vivo*, in the human host, conferred by a great capacity to induce proliferation of B cells into activated B blasts via adequate expression of the viral growth-program (latency III) and posterior differentiation into memory B cells, the virus reservoir during latent infection. Therefore, it might be possible that the superior transforming ability of type 1 EBV exhibited *in vitro*, likely promoted by the higher ability to induce a small number of genes required for B cell transformation, could relate to a better capacity to drive differentiation of EBV-infected B lymphocytes in the human host and then establish persistence. The different geographical distribution and prevalence of the EBV types seems to favour the previous conjecture. Accordingly, type 1 EBV strains are generally predominant worldwide, such as in the American, European and Chinese populations, where type 2 EBV strains are only present in a small percentage of the healthy individuals of these populations [300]. This might indeed suggest that type 1 is better at establishing persistence and colonising the human host. Type 2 EBV however exhibits a higher prevalence in specific areas (sub-Saharan Africa), where it is as equally abundant as type 1 strains [301], indicating that an additional variable characterising these regions might contribute for type 2 EBV to be able to successfully colonise human hosts and to overcome its poor transformation potential. In fact, these regions, where type 2 EBV is more predominant, are areas where malaria is a holoendemic disease [488]. The etiological agent is the parasite *Plasmodium falciparum* and the holoendemic form is characterised by a high prevalent level of infection beginning early in life, affecting most of the child population and by repeated exposure events and infections in the population. Malaria infection has been reported, along with EBV, as an important factor in the development of the endemic Burkitt's lymphoma [489]. In addition, *P. falciparum* infection is a B cell mitogen contributing for their proliferation *in vitro* and was also associated with the constant antigenic stimulation of B lymphocytes that favours their infection by EBV [489-493]. Moreover, in the presence of cultured *P. falciparum* extracts, the occurrence of spontaneous EBV-transformed LCLs from peripheral blood lymphocytes *ex vivo* is augmented [493]. The previous observations have raised the possibility that the attenuated or the poor transforming potential of type 2 EBV might be successfully overcome in the presence of other infectious agents, such as *P. falciparum* infection, when there is chronic immune activation or a chronic stimulation of the B cell compartment, allowing type 2 strains to establish persistent infection as efficiently as type 1 EBV in these areas where the immune system is subject to a constant pressure. Furthermore, previous reports have suggested that type 1 EBV strains are better at

the transformation of resting B cells than of activated B cells [494]. Conversely, type 2 EBNA-2 might confer type 2 strains the capacity to establish persistence after infection of activated B cells in human hosts with a chronically-stimulated immune system, facilitating type 2 EBV-mediated B cell differentiation. Therefore, it would be interesting to determine, as future work, the capacity of type 2 strains to infect B lymphocytes *in vitro* artificially activated with CD40L and IL-4 to investigate whether these strains could efficiently originate LCLs *in vitro* and to unravel the significance of the type 1 and type 2 EBV.

In future work, it would be also interesting to generate a recombinant EBV-BAC virus, which would express a type 2 EBNA-2 protein carrying the serine to aspartate substitution (S442D), in order to determine the ability of this virus to infect B cells and to transform and immortalise them into LCLs *in vitro*. This unique amino acid in EBNA-2 was shown to determine superior B lymphoblastoid cell line growth maintenance by type 1 EBNA-2 and also this single S442D mutation in type 2 EBNA-2 conferred the ability to sustain LCL growth in the EREB2.5 assay [210]. Therefore, the effect of this amino acid mutation (S442D) in type 2 virus could be then investigated to determine its effects upon infection of B lymphocytes and in its transformation potential. Given that the previous reports consisted of infecting primary B lymphocytes using a BAC-derived EBV using a type 1 viral genome background, which then carried either type 1 or type 2 EBNA-2 coding sequences [209], it would be useful to generate a type 2 EBV-BAC from the prototype type 2 EBV AG876 strain and also produce the amino acid mutation S442D in the EBNA-2 protein, in this genetic background, to study its functional features and properties upon infection of resting B cells *in vitro*.

The biotinylated oligonucleotide pulldowns utilised in this study allied to the observation that the PU.1/IRF motif was enriched at the EBNA-2-binding regions in the differentially regulated genes by type 1 and type 2 (such as in the viral LMP-1 gene) might suggest that this binding motif might be required to determine the greater type 1 EBNA-2 interaction at these genes in comparison to type 2 protein and, consequently might promote a stronger induction of these gene promoters by type 1. In this context, it would be interesting to generate a recombinant virus in which the PU.1-binding motif at the LMP-1 gene promoter is deleted/mutated in order to determine whether this mutated type 1 EBV-BAC virus retains the capacity to transform B cells *in vitro* and to compare the transformation efficiency with the wildtype type 1 EBV-BAC, with type 1 virus expressing type 2 EBNA-2 and with a type 2 EBV. LMP-1 expression upon infection of resting B lymphocytes with this set of viruses could be also determined by qPCR

to investigate whether the PU.1 motif is important to contribute for the differential induction of the LMP-1 observed in different systems [209, 210].

The Daudi cell system had been used as a system to demonstrate a differential activation of the LMP-1 gene with inducible type 1, type 2 and type 2 S442D EBNA-2 protein expression, from the pHEBo-MT plasmids in a previous study from this laboratory [210]. A similar system could be used to investigate the differential LMP-1 activation by the EBNA-2 types. A P3HR1 EBV-BAC, which lacks the EBNA-2 locus, could be stably transfected into an adequate EBV-negative B cell line (e.g. DG75, BJAB with EBNA-1), followed by transient transfection of the pHEBo-MT plasmids to then express type 1, type 2 or type 2 S442D EBNA-2 proteins. LMP-1 induction would be determined by either Western blotting or qPCR analysis. In the case of a successful recapitulation of LMP-1 induction by the EBNA-2 types, it would be interesting to mutate different regions of the P3HR1 EBV-BAC that include the PU.1-binding motif and the octamer sequence (POU-domain protein interaction motif) at the LMP-1 gene promoter, or to delete other regions, such as the CTCF-binding site or the genome terminal repeats as carried out for the pHEBo:Dhet-*Bam*HI-*Eco*RI plasmid (section 5.2.2). These mutated viruses would be used to create new stable cell lines and the role of these altered regions/sequences in determining the distinct LMP-1 induction would be assessed upon transient transfection of the vectors expressing each of the EBNA-2 types.

The biotinylated oligonucleotide pulldown assays first demonstrated the interaction of EBNA-2 at the LMP-1 promoter and permitted the determination of promoter elements that might be important for type 1 EBNA-2 interaction (PU.1-box, octamer motif) (chapter 5, section 5.2.3). Other future work that could be carried out might not only include new pulldown assays using distinct biotinylated oligonucleotides encompassing different responsive elements/sequences of the LMP-1 promoter and as well as promoters of other genes differentially induced by type 1 and type 2 EBNA-2 as aforementioned, but also the determination of the components that constitute the protein complex recruiting EBNA-2 at the LMP-1 promoter. The eluted samples after the pulldowns reactions could be tested by Western blotting using different antibodies that would recognise specific cofactors that possibly are included in the protein complex. In addition, samples could also be analysed by mass spectrometry to identify unknown proteins in the EBNA-2 complex established at the target responsive promoters.

Finally, to further shed some light on the biological significance of the type 1 and type 2 EBV *in vivo*, the utilisation of the most recently developed humanized mice to model EBV infection

might constitute an important working tool to understand the effects of this strain variation on the development of the EBV-associated diseases. These humanized mice have been used to recapitulate characteristics of human EBV infection that include pathogenesis, latent infection and immune responses [495]. In these mice, EBV causes a B lymphocyte lymphoproliferative disease similar to PTLD in immunosuppressed patients subsequent to transplantation [496]. Distinct EBV mutants with specific gene deletions have been tested in humanized mice and their effects on lymphomagenesis have been studied [497, 498]. In a similar way, it would be interesting to infect these humanized mice with type 1 and type 2 EBV strains to determine the possibly distinct effects of each EBV type *in vivo* and also to investigate the frequency of the development of EBV-associated malignancy in those mice and determine whether it can correlate with the ability to transform human B cells into LCLs *in vitro.* Additional aspects of B lymphocyte infection, the difference in establishment of latent infection and also the induced immune responses could be also investigated. The association of each EBV type with type-specific diseases still remains to be determined and the superior ability of type 1 EBV strains to promote efficient B cell growth transformation into LCLs *in vitro*, when compared to type 2 persists as the clearest example of functional variation in the EBV types.

7 References

1. Epstein MA, Achong BG, Barr YM: **Virus Particles in Cultured Lymphoblasts from Burkitt's Lymphoma**. *Lancet* 1964, **1**(7335):702-703.
2. Burkitt D: **A sarcoma involving the jaws in African children**. *Br J Surg* 1958, **46**(197):218-223.
3. Burkitt D: **A children's cancer dependent on climatic factors**. *Nature* 1962, **194**:232-234.
4. Epstein MA, Henle G, Achong BG, Barr YM: **Morphological and Biological Studies on a Virus in Cultured Lymphoblasts from Burkitt's Lymphoma**. *J Exp Med* 1965, **121**:761-770.
5. Henle W, Diehl V, Kohn G, Zur Hausen H, Henle G: **Herpes-type virus and chromosome marker in normal leukocytes after growth with irradiated Burkitt cells**. *Science* 1967, **157**(3792):1064-1065.
6. Pope JH, Horne MK, Scott W: **Transformation of foetal human keukocytes in vitro by filtrates of a human leukaemic cell line containing herpes-like virus**. *Int J Cancer* 1968, **3**(6):857-866.
7. Kieff E, Rickinson A: **Epstein-Barr virus**. In: *Fields' virology.* Edited by Fields BN, Knipe DM, Howley PM, 5th edn. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins; 2007: p 2603-2654.
8. Kenney S: **Theodore E. Woodward Award: development of novel, EBV-targeted therapies for EBV-positive tumors**. *Trans Am Clin Climatol Assoc* 2006, **117**:55-73; discussion 73-54.
9. Crawford DH: **Biology and disease associations of Epstein-Barr virus**. *Philos Trans R Soc Lond B Biol Sci* 2001, **356**(1408):461-473.
10. Nilsson K, Klein G, Henle W, Henle G: **The establishment of lymphoblastoid lines from adult and fetal human lymphoid tissue and its dependence on EBV**. *Int J Cancer* 1971, **8**(3):443-450.
11. Davison AJ, Eberle R, Ehlers B, Hayward GS, McGeoch DJ, Minson AC, Pellett PE, Roizman B, Studdert MJ, Thiry E: **The order Herpesvirales**. *Arch Virol* 2009, **154**(1):171-177.
12. Pellett PE, Roizman B, Iancu CV: **The Herpesviridae: a brief introduction**. In: *Field's Virology.* Edited by Fields BN, Knipe DM, Howley PM, 5th edn. Philadelphia: Wolters Kluwer Health/Lippincott, Williams & Wilkins; 2007: p 2479-2500.

13. Johannsen E, Luftig M, Chase MR, Weicksel S, Cahir-McFarland E, Illanes D, Sarracino D, Kieff E: **Proteins of purified Epstein-Barr virus**. *Proc Natl Acad Sci U S A* 2004, **101**(46):16286-16291.
14. Bankier AT, Deininger PL, Satchwell SC, Baer R, Farrell PJ, Barrell BG: **DNA sequence analysis of the EcoRI Dhet fragment of B95-8 Epstein-Barr virus containing the terminal repeat sequences**. *Mol Biol Med* 1983, **1**(4):425-445.
15. Baer R, Bankier AT, Biggin MD, Deininger PL, Farrell PJ, Gibson TJ, Hatfull G, Hudson GS, Satchwell SC, Seguin C *et al*: **DNA sequence and expression of the B95-8 Epstein-Barr virus genome**. *Nature* 1984, **310**(5974):207-211.
16. Miller G, Lipman M: **Release of infectious Epstein-Barr virus by transformed marmoset leukocytes**. *Proc Natl Acad Sci U S A* 1973, **70**(1):190-194.
17. Parker BD, Bankier A, Satchwell S, Barrell B, Farrell PJ: **Sequence and transcription of Raji Epstein-Barr virus DNA spanning the B95-8 deletion region**. *Virology* 1990, **179**(1):339-346.
18. de Jesus O, Smith PR, Spender LC, Elgueta Karstegl C, Niller HH, Huang D, Farrell PJ: **Updated Epstein-Barr virus (EBV) DNA sequence and analysis of a promoter for the BART (CST, BARF0) RNAs of EBV**. *J Gen Virol* 2003, **84**(Pt 6):1443-1450.
19. Dolan A, Addison C, Gatherer D, Davison AJ, McGeoch DJ: **The genome of Epstein-Barr virus type 2 strain AG876**. *Virology* 2006, **350**(1):164-170.
20. Pizzo PA, Magrath IT, Chattopadhyay SK, Biggar RJ, Gerber P: **A new tumour-derived transforming strain of Epstein-Barr virus**. *Nature* 1978, **272**(5654):629-631.
21. Zeng MS, Li DJ, Liu QL, Song LB, Li MZ, Zhang RH, Yu XJ, Wang HM, Ernberg I, Zeng YX: **Genomic sequence analysis of Epstein-Barr virus strain GD1 from a nasopharyngeal carcinoma patient**. *J Virol* 2005, **79**(24):15323-15330.
22. Liu P, Fang X, Feng Z, Guo YM, Peng RJ, Liu T, Huang Z, Feng Y, Sun X, Xiong Z *et al*: **Direct sequencing and characterization of a clinical isolate of Epstein-Barr virus from nasopharyngeal carcinoma tissue by using next-generation sequencing technology**. *J Virol* 2011, **85**(21):11291-11299.
23. Kwok H, Tong AH, Lin CH, Lok S, Farrell PJ, Kwong DL, Chiang AK: **Genomic sequencing and comparative analysis of Epstein-Barr virus genome isolated from primary nasopharyngeal carcinoma biopsy**. *PLoS One* 2012, **7**(5):e36939.

24. Kwok H, Wu CW, Palser AL, Kellam P, Sham PC, Kwong DL, Chiang AK: **Genomic diversity of Epstein-Barr virus genomes isolated from primary nasopharyngeal carcinoma biopsy samples**. *J Virol* 2014, **88**(18):10662-10672.
25. Lei H, Li T, Hung GC, Li B, Tsai S, Lo SC: **Identification and characterization of EBV genomes in spontaneously immortalized human peripheral blood B lymphocytes by NGS technology**. *BMC Genomics* 2013, **14**:804.
26. Lin Z, Wang X, Strong MJ, Concha M, Baddoo M, Xu G, Baribault C, Fewell C, Hulme W, Hedges D *et al*: **Whole-genome sequencing of the Akata and Mutu Epstein-Barr virus strains**. *J Virol* 2013, **87**(2):1172-1182.
27. Santpere G, Darre F, Blanco S, Alcami A, Villoslada P, Mar Alba M, Navarro A: **Genome-wide analysis of wild-type Epstein-Barr virus genomes derived from healthy individuals of the 1,000 Genomes Project**. *Genome Biol Evol* 2014, **6**(4):846-860.
28. Tso KK, Yip KY, Mak CK, Chung GT, Lee SD, Cheung ST, To KF, Lo KW: **Complete genomic sequence of Epstein-Barr virus in nasopharyngeal carcinoma cell line C666-1**. *Infect Agent Cancer* 2013, **8**(1):29.
29. Palser AL, Grayson NE, White RE, Corton C, Correia S, Ba Abdullah MM, Watson SJ, Cotten M, Arrand JR, Murray PG *et al*: **Genome diversity of Epstein-Barr virus from multiple tumor types and normal infection**. *J Virol* 2015, **89**(10):5222-5237.
30. Azzi T, Lunemann A, Murer A, Ueda S, Beziat V, Malmberg KJ, Staubli G, Gysin C, Berger C, Munz C *et al*: **Role for early-differentiated natural killer cells in infectious mononucleosis**. *Blood* 2014, **124**(16):2533-2543.
31. Odumade OA, Hogquist KA, Balfour HH, Jr.: **Progress and problems in understanding and managing primary Epstein-Barr virus infections**. *Clin Microbiol Rev* 2011, **24**(1):193-209.
32. Faulkner GC, Krajewski AS, Crawford DH: **The ins and outs of EBV infection**. *Trends Microbiol* 2000, **8**(4):185-189.
33. Borza CM, Hutt-Fletcher LM: **Alternate replication in B cells and epithelial cells switches tropism of Epstein-Barr virus**. *Nat Med* 2002, **8**(6):594-599.
34. Imai S, Nishikawa J, Takada K: **Cell-to-cell contact as an efficient mode of Epstein-Barr virus infection of diverse human epithelial cells**. *J Virol* 1998, **72**(5):4371-4378.
35. Shannon-Lowe CD, Neuhierl B, Baldwin G, Rickinson AB, Delecluse HJ: **Resting B cells as a transfer vehicle for Epstein-Barr virus infection of epithelial cells**. *Proc Natl Acad Sci U S A* 2006, **103**(18):7065-7070.

36. Tsai MH, Raykova A, Klinke O, Bernhardt K, Gartner K, Leung CS, Geletneky K, Sertel S, Munz C, Feederle R *et al*: **Spontaneous lytic replication and epitheliotropism define an Epstein-Barr virus strain found in carcinomas**. *Cell Rep* 2013, **5**(2):458-470.
37. Sixbey JW, Nedrud JG, Raab-Traub N, Hanes RA, Pagano JS: **Epstein-Barr virus replication in oropharyngeal epithelial cells**. *N Engl J Med* 1984, **310**(19):1225-1230.
38. Andersson-Anvret M, Forsby N, Klein G, Henle W, Biorklund A: **Relationship between the Epstein-Barr virus genome and nasopharyngeal carcinoma in Caucasian patients**. *Int J Cancer* 1979, **23**(6):762-767.
39. Burke AP, Yen TS, Shekitka KM, Sobin LH: **Lymphoepithelial carcinoma of the stomach with Epstein-Barr virus demonstrated by polymerase chain reaction**. *Mod Pathol* 1990, **3**(3):377-380.
40. Temple RM, Zhu J, Budgeon L, Christensen ND, Meyers C, Sample CE: **Efficient replication of Epstein-Barr virus in stratified epithelium in vitro**. *Proc Natl Acad Sci U S A* 2014, **111**(46):16544-16549.
41. Nemerow GR, Mold C, Schwend VK, Tollefson V, Cooper NR: **Identification of gp350 as the viral glycoprotein mediating attachment of Epstein-Barr virus (EBV) to the EBV/C3d receptor of B cells: sequence homology of gp350 and C3 complement fragment C3d**. *J Virol* 1987, **61**(5):1416-1420.
42. Sinclair AJ, Farrell PJ: **Host cell requirements for efficient infection of quiescent primary B lymphocytes by Epstein-Barr virus**. *J Virol* 1995, **69**(9):5461-5468.
43. Spender LC, Cannell EJ, Hollyoake M, Wensing B, Gawn JM, Brimmell M, Packham G, Farrell PJ: **Control of cell cycle entry and apoptosis in B lymphocytes infected by Epstein-Barr virus**. *J Virol* 1999, **73**(6):4678-4688.
44. Tanner J, Weis J, Fearon D, Whang Y, Kieff E: **Epstein-Barr virus gp350/220 binding to the B lymphocyte C3d receptor mediates adsorption, capping, and endocytosis**. *Cell* 1987, **50**(2):203-213.
45. Ogembo JG, Kannan L, Ghiran I, Nicholson-Weller A, Finberg RW, Tsokos GC, Fingeroth JD: **Human complement receptor type 1/CD35 is an Epstein-Barr Virus receptor**. *Cell Rep* 2013, **3**(2):371-385.
46. Li Q, Spriggs MK, Kovats S, Turk SM, Comeau MR, Nepom B, Hutt-Fletcher LM: **Epstein-Barr virus uses HLA class II as a cofactor for infection of B lymphocytes**. *J Virol* 1997, **71**(6):4657-4662.

47. Li Q, Turk SM, Hutt-Fletcher LM: **The Epstein-Barr virus (EBV) BZLF2 gene product associates with the gH and gL homologs of EBV and carries an epitope critical to infection of B cells but not of epithelial cells**. *J Virol* 1995, **69**(7):3987-3994.
48. Haan KM, Lee SK, Longnecker R: **Different functional domains in the cytoplasmic tail of glycoprotein B are involved in Epstein-Barr virus-induced membrane fusion**. *Virology* 2001, **290**(1):106-114.
49. Connolly SA, Jackson JO, Jardetzky TS, Longnecker R: **Fusing structure and function: a structural view of the herpesvirus entry machinery**. *Nat Rev Microbiol* 2011, **9**(5):369-381.
50. Stampfer SD, Heldwein EE: **Stuck in the middle: structural insights into the role of the gH/gL heterodimer in herpesvirus entry**. *Curr Opin Virol* 2013, **3**(1):13-19.
51. Nemerow GR, Cooper NR: **Early events in the infection of human B lymphocytes by Epstein-Barr virus: the internalization process**. *Virology* 1984, **132**(1):186-198.
52. Chesnokova LS, Hutt-Fletcher LM: **Fusion of Epstein-Barr virus with epithelial cells can be triggered by alphavbeta5 in addition to alphavbeta6 and alphavbeta8, and integrin binding triggers a conformational change in glycoproteins gHgL**. *J Virol* 2011, **85**(24):13214-13223.
53. Tugizov SM, Berline JW, Palefsky JM: **Epstein-Barr virus infection of polarized tongue and nasopharyngeal epithelial cells**. *Nat Med* 2003, **9**(3):307-314.
54. Xiao J, Palefsky JM, Herrera R, Berline J, Tugizov SM: **EBV BMRF-2 facilitates cell-to-cell spread of virus within polarized oral epithelial cells**. *Virology* 2009, **388**(2):335-343.
55. Xiao J, Palefsky JM, Herrera R, Tugizov SM: **Characterization of the Epstein-Barr virus glycoprotein BMRF-2**. *Virology* 2007, **359**(2):382-396.
56. Xiao J, Palefsky JM, Herrera R, Berline J, Tugizov SM: **The Epstein-Barr virus BMRF-2 protein facilitates virus attachment to oral epithelial cells**. *Virology* 2008, **370**(2):430-442.
57. Chesnokova LS, Ahuja MK, Hutt-Fletcher LM: **Epstein-Barr virus glycoprotein gB and gHgL can mediate fusion and entry in trans, and heat can act as a partial surrogate for gHgL and trigger a conformational change in gB**. *J Virol* 2014, **88**(21):12193-12201.
58. Borza CM, Morgan AJ, Turk SM, Hutt-Fletcher LM: **Use of gHgL for attachment of Epstein-Barr virus to epithelial cells compromises infection**. *J Virol* 2004, **78**(10):5007-5014.

59. Sathiyamoorthy K, Hu YX, Mohl BS, Chen J, Longnecker R, Jardetzky TS: **Structural basis for Epstein-Barr virus host cell tropism mediated by gp42 and gHgL entry glycoproteins**. *Nat Commun* 2016, **7**:13557.
60. Crawford DH, Ando I: **EB virus induction is associated with B-cell maturation**. *Immunology* 1986, **59**(3):405-409.
61. Laichalk LL, Thorley-Lawson DA: **Terminal differentiation into plasma cells initiates the replicative cycle of Epstein-Barr virus in vivo**. *J Virol* 2005, **79**(2):1296-1307.
62. Iwakoshi NN, Lee AH, Glimcher LH: **The X-box binding protein-1 transcription factor is required for plasma cell differentiation and the unfolded protein response**. *Immunol Rev* 2003, **194**:29-38.
63. Sun CC, Thorley-Lawson DA: **Plasma cell-specific transcription factor XBP-1s binds to and transactivates the Epstein-Barr virus BZLF1 promoter**. *J Virol* 2007, **81**(24):13566-13577.
64. Kenney S, Kamine J, Holley-Guthrie E, Lin JC, Mar EC, Pagano J: **The Epstein-Barr virus (EBV) BZLF1 immediate-early gene product differentially affects latent versus productive EBV promoters**. *J Virol* 1989, **63**(4):1729-1736.
65. Bryant H, Farrell PJ: **Signal Transduction and Transcription Factor Modification during Reactivation of Epstein-Barr Virus from Latency**. *J Virol* 2002, **76**(20):10290-10298.
66. Amon W, Binne UK, Bryant H, Jenkins PJ, Karstegl CE, Farrell PJ: **Lytic cycle gene regulation of Epstein-Barr virus**. *J Virol* 2004, **78**(24):13460-13469.
67. Flemington E, Speck SH: **Autoregulation of Epstein-Barr virus putative lytic switch gene BZLF1**. *J Virol* 1990, **64**(3):1227-1232.
68. Farrell PJ, Rowe DT, Rooney CM, Kouzarides T: **Epstein-Barr virus BZLF1 trans-activator specifically binds to a consensus AP-1 site and is related to c-fos**. *EMBO J* 1989, **8**(1):127-132.
69. Lin JC, Sista ND, Besencon F, Kamine J, Pagano JS: **Identification and functional characterization of Epstein-Barr virus DNA polymerase by in vitro transcription-translation of a cloned gene**. *J Virol* 1991, **65**(5):2728-2731.
70. Young LS, Murray PG: **Epstein-Barr virus and oncogenesis: from latent genes to tumours**. *Oncogene* 2003, **22**(33):5108-5121.
71. Thorley-Lawson DA, Gross A: **Persistence of the Epstein-Barr virus and the origins of associated lymphomas**. *N Engl J Med* 2004, **350**(13):1328-1337.

72. Babcock GJ, Hochberg D, Thorley-Lawson AD: **The expression pattern of Epstein-Barr virus latent genes in vivo is dependent upon the differentiation stage of the infected B cell**. *Immunity* 2000, **13**(4):497-506.
73. Parham P: **The development of B lymphocytes**. In: *The Immune System.* 2nd Edition edn: Garland Science; 2005.
74. Kilger E, Kieser A, Baumann M, Hammerschmidt W: **Epstein-Barr virus-mediated B-cell proliferation is dependent upon latent membrane protein 1, which simulates an activated CD40 receptor**. *EMBO J* 1998, **17**(6):1700-1709.
75. Caldwell RG, Wilson JB, Anderson SJ, Longnecker R: **Epstein-Barr virus LMP2A drives B cell development and survival in the absence of normal B cell receptor signals**. *Immunity* 1998, **9**(3):405-411.
76. Babcock GJ, Thorley-Lawson DA: **Tonsillar memory B cells, latently infected with Epstein-Barr virus, express the restricted pattern of latent genes previously found only in Epstein-Barr virus-associated tumors**. *Proc Natl Acad Sci U S A* 2000, **97**(22):12250-12255.
77. Thorley-Lawson DA: **Epstein-Barr virus: exploiting the immune system**. *Nat Rev Immunol* 2001, **1**(1):75-82.
78. Roughan JE, Torgbor C, Thorley-Lawson DA: **Germinal center B cells latently infected with Epstein-Barr virus proliferate extensively but do not increase in number**. *J Virol* 2010, **84**(2):1158-1168.
79. Hochberg D, Middeldorp JM, Catalina M, Sullivan JL, Luzuriaga K, Thorley-Lawson DA: **Demonstration of the Burkitt's lymphoma Epstein-Barr virus phenotype in dividing latently infected memory cells in vivo**. *Proc Natl Acad Sci U S A* 2004, **101**(1):239-244.
80. Chen H, Smith P, Ambinder RF, Hayward SD: **Expression of Epstein-Barr virus BamHI-A rightward transcripts in latently infected B cells from peripheral blood**. *Blood* 1999, **93**(9):3026-3032.
81. Thorley-Lawson DA, Allday MJ: **The curious case of the tumour virus: 50 years of Burkitt's lymphoma**. *Nat Rev Microbiol* 2008, **6**(12):913-924.
82. Rowe M, Rowe DT, Gregory CD, Young LS, Farrell PJ, Rupani H, Rickinson AB: **Differences in B cell growth phenotype reflect novel patterns of Epstein-Barr virus latent gene expression in Burkitt's lymphoma cells**. *EMBO J* 1987, **6**(9):2743-2751.

83. Rowe M, Lear AL, Croom-Carter D, Davies AH, Rickinson AB: **Three pathways of Epstein-Barr virus gene activation from EBNA1-positive latency in B lymphocytes**. *J Virol* 1992, **66**(1):122-131.

84. Joseph AM, Babcock GJ, Thorley-Lawson DA: **Cells expressing the Epstein-Barr virus growth program are present in and restricted to the naive B-cell subset of healthy tonsils**. *J Virol* 2000, **74**(21):9964-9971.

85. Thomas JA, Hotchin NA, Allday MJ, Amlot P, Rose M, Yacoub M, Crawford DH: **Immunohistology of Epstein-Barr virus-associated antigens in B cell disorders from immunocompromised individuals**. *Transplantation* 1990, **49**(5):944-953.

86. Oudejans JJ, Jiwa M, van den Brule AJ, Grasser FA, Horstman A, Vos W, Kluin PM, van der Valk P, Walboomers JM, Meijer CJ: **Detection of heterogeneous Epstein-Barr virus gene expression patterns within individual post-transplantation lymphoproliferative disorders**. *Am J Pathol* 1995, **147**(4):923-933.

87. Gregory CD, Rowe M, Rickinson AB: **Different Epstein-Barr virus-B cell interactions in phenotypically distinct clones of a Burkitt's lymphoma cell line**. *J Gen Virol* 1990, **71 (Pt 7)**:1481-1495.

88. Schaefer BC, Strominger JL, Speck SH: **Redefining the Epstein-Barr virus-encoded nuclear antigen EBNA-1 gene promoter and transcription initiation site in group I Burkitt lymphoma cell lines**. *Proc Natl Acad Sci U S A* 1995, **92**(23):10565-10569.

89. Nonkwelo C, Skinner J, Bell A, Rickinson A, Sample J: **Transcription start sites downstream of the Epstein-Barr virus (EBV) Fp promoter in early-passage Burkitt lymphoma cells define a fourth promoter for expression of the EBV EBNA-1 protein**. *J Virol* 1996, **70**(1):623-627.

90. Rowe M, Kelly GL, Bell AI, Rickinson AB: **Burkitt's lymphoma: the Rosetta Stone deciphering Epstein-Barr virus biology**. *Semin Cancer Biol* 2009, **19**(6):377-388.

91. Neri A, Barriga F, Inghirami G, Knowles DM, Neequaye J, Magrath IT, Dalla-Favera R: **Epstein-Barr virus infection precedes clonal expansion in Burkitt's and acquired immunodeficiency syndrome-associated lymphoma**. *Blood* 1991, **77**(5):1092-1095.

92. Niedobitek G, Agathanggelou A, Rowe M, Jones EL, Jones DB, Turyaguma P, Oryema J, Wright DH, Young LS: **Heterogeneous expression of Epstein-Barr virus latent proteins in endemic Burkitt's lymphoma**. *Blood* 1995, **86**(2):659-665.

93. Kelly G, Bell A, Rickinson A: **Epstein-Barr virus-associated Burkitt lymphomagenesis selects for downregulation of the nuclear antigen EBNA2**. *Nat Med* 2002, **8**(10):1098-1104.

94. Klein E, Kis LL, Klein G: **Epstein-Barr virus infection in humans: from harmless to life endangering virus-lymphocyte interactions**. *Oncogene* 2007, **26**(9):1297-1305.

95. Tierney R, Nagra J, Hutchings I, Shannon-Lowe C, Altmann M, Hammerschmidt W, Rickinson A, Bell A: **Epstein-Barr virus exploits BSAP/Pax5 to achieve the B-cell specificity of its growth-transforming program**. *J Virol* 2007, **81**(18):10092-10100.

96. Gregory CD, Murray RJ, Edwards CF, Rickinson AB: **Downregulation of cell adhesion molecules LFA-3 and ICAM-1 in Epstein-Barr virus-positive Burkitt's lymphoma underlies tumor cell escape from virus-specific T cell surveillance**. *J Exp Med* 1988, **167**(6):1811-1824.

97. Wensing B, Farrell PJ: **Regulation of cell growth and death by Epstein-Barr virus**. *Microbes Infect* 2000, **2**(1):77-84.

98. Alfieri C, Birkenbach M, Kieff E: **Early events in Epstein-Barr virus infection of human B lymphocytes**. *Virology* 1991, **181**(2):595-608.

99. Woisetschlaeger M, Strominger JL, Speck SH: **Mutually exclusive use of viral promoters in Epstein-Barr virus latently infected lymphocytes**. *Proc Natl Acad Sci U S A* 1989, **86**(17):6498-6502.

100. Allday MJ, Crawford DH, Griffin BE: **Epstein-Barr virus latent gene expression during the initiation of B cell immortalization**. *J Gen Virol* 1989, **70 (Pt 7)**:1755-1764.

101. Bodescot M, Perricaudet M: **Epstein-Barr virus mRNAs produced by alternative splicing**. *Nucleic Acids Res* 1986, **14**(17):7103-7114.

102. Speck SH, Pfitzner A, Strominger JL: **An Epstein-Barr virus transcript from a latently infected, growth-transformed B-cell line encodes a highly repetitive polypeptide**. *Proc Natl Acad Sci U S A* 1986, **83**(24):9298-9302.

103. Sample J, Hummel M, Braun D, Birkenbach M, Kieff E: **Nucleotide sequences of mRNAs encoding Epstein-Barr virus nuclear proteins: a probable transcriptional initiation site**. *Proc Natl Acad Sci U S A* 1986, **83**(14):5096-5100.

104. Woisetschlaeger M, Yandava CN, Furmanski LA, Strominger JL, Speck SH: **Promoter switching in Epstein-Barr virus during the initial stages of infection of B lymphocytes**. *Proc Natl Acad Sci U S A* 1990, **87**(5):1725-1729.

105. Woisetschlaeger M, Jin XW, Yandava CN, Furmanski LA, Strominger JL, Speck SH: **Role for the Epstein-Barr virus nuclear antigen 2 in viral promoter switching during initial stages of infection**. *Proc Natl Acad Sci U S A* 1991, **88**(9):3942-3946.

106. Puglielli MT, Desai N, Speck SH: **Regulation of EBNA gene transcription in lymphoblastoid cell lines: characterization of sequences downstream of BCR2 (Cp)**. *J Virol* 1997, **71**(1):120-128.

107. Puglielli MT, Woisetschlaeger M, Speck SH: **oriP is essential for EBNA gene promoter activity in Epstein-Barr virus-immortalized lymphoblastoid cell lines**. *J Virol* 1996, **70**(9):5758-5768.

108. Fahraeus R, Jansson A, Ricksten A, Sjoblom A, Rymo L: **Epstein-Barr virus-encoded nuclear antigen 2 activates the viral latent membrane protein promoter by modulating the activity of a negative regulatory element**. *Proc Natl Acad Sci U S A* 1990, **87**(19):7390-7394.

109. Wang F, Tsang SF, Kurilla MG, Cohen JI, Kieff E: **Epstein-Barr virus nuclear antigen 2 transactivates latent membrane protein LMP1**. *J Virol* 1990, **64**(7):3407-3416.

110. Laux G, Dugrillon F, Eckert C, Adam B, Zimber-Strobl U, Bornkamm GW: **Identification and characterization of an Epstein-Barr virus nuclear antigen 2-responsive cis element in the bidirectional promoter region of latent membrane protein and terminal protein 2 genes**. *J Virol* 1994, **68**(11):6947-6958.

111. Tsang SF, Wang F, Izumi KM, Kieff E: **Delineation of the cis-acting element mediating EBNA-2 transactivation of latent infection membrane protein expression**. *J Virol* 1991, **65**(12):6765-6771.

112. Zimber-Strobl U, Suentzenich KO, Laux G, Eick D, Cordier M, Calender A, Billaud M, Lenoir GM, Bornkamm GW: **Epstein-Barr virus nuclear antigen 2 activates transcription of the terminal protein gene**. *J Virol* 1991, **65**(1):415-423.

113. Spender LC, Lucchesi W, Bodelon G, Bilancio A, Karstegl CE, Asano T, Dittrich-Breiholz O, Kracht M, Vanhaesebroeck B, Farrell PJ: **Cell target genes of Epstein-Barr virus transcription factor EBNA-2: induction of the p55alpha regulatory subunit of PI3-kinase and its role in survival of EREB2.5 cells**. *J Gen Virol* 2006, **87**(Pt 10):2859-2867.

114. Maier S, Staffler G, Hartmann A, Hock J, Henning K, Grabusic K, Mailhammer R, Hoffmann R, Wilmanns M, Lang R *et al*: **Cellular target genes of Epstein-Barr virus nuclear antigen 2**. *J Virol* 2006, **80**(19):9761-9771.

115. Zhao B, Maruo S, Cooper A, M RC, Johannsen E, Kieff E, Cahir-McFarland E: **RNAs induced by Epstein-Barr virus nuclear antigen 2 in lymphoblastoid cell lines**. *Proc Natl Acad Sci U S A* 2006, **103**(6):1900-1905.

116. Sinclair AJ, Palmero I, Peters G, Farrell PJ: **EBNA-2 and EBNA-LP cooperate to cause G0 to G1 transition during immortalization of resting human B lymphocytes by Epstein-Barr virus**. *EMBO J* 1994, **13**(14):3321-3328.

117. Harada S, Kieff E: **Epstein-Barr virus nuclear protein LP stimulates EBNA-2 acidic domain-mediated transcriptional activation**. *J Virol* 1997, **71**(9):6611-6618.

118. Nitsche F, Bell A, Rickinson A: **Epstein-Barr virus leader protein enhances EBNA-2-mediated transactivation of latent membrane protein 1 expression: a role for the W1W2 repeat domain**. *J Virol* 1997, **71**(9):6619-6628.

119. Peng CW, Xue Y, Zhao B, Johannsen E, Kieff E, Harada S: **Direct interactions between Epstein-Barr virus leader protein LP and the EBNA2 acidic domain underlie coordinate transcriptional regulation**. *Proc Natl Acad Sci U S A* 2004, **101**(4):1033-1038.

120. Peng R, Moses SC, Tan J, Kremmer E, Ling PD: **The Epstein-Barr virus EBNA-LP protein preferentially coactivates EBNA2-mediated stimulation of latent membrane proteins expressed from the viral divergent promoter**. *J Virol* 2005, **79**(7):4492-4505.

121. Ling PD, Peng RS, Nakajima A, Yu JH, Tan J, Moses SM, Yang WH, Zhao B, Kieff E, Bloch KD *et al*: **Mediation of Epstein-Barr virus EBNA-LP transcriptional coactivation by Sp100**. *EMBO J* 2005, **24**(20):3565-3575.

122. Peng CW, Zhao B, Chen HC, Chou ML, Lai CY, Lin SZ, Hsu HY, Kieff E: **Hsp72 up-regulates Epstein-Barr virus EBNALP coactivation with EBNA2**. *Blood* 2007, **109**(12):5447-5454.

123. Gahn TA, Sugden B: **An EBNA-1-dependent enhancer acts from a distance of 10 kilobase pairs to increase expression of the Epstein-Barr virus LMP gene**. *J Virol* 1995, **69**(4):2633-2636.

124. Reisman D, Sugden B: **trans activation of an Epstein-Barr viral transcriptional enhancer by the Epstein-Barr viral nuclear antigen 1**. *Mol Cell Biol* 1986, **6**(11):3838-3846.

125. Altmann M, Pich D, Ruiss R, Wang J, Sugden B, Hammerschmidt W: **Transcriptional activation by EBV nuclear antigen 1 is essential for the expression of EBV's transforming genes**. *Proc Natl Acad Sci U S A* 2006, **103**(38):14188-14193.

126. Johannsen E, Miller CL, Grossman SR, Kieff E: **EBNA-2 and EBNA-3C extensively and mutually exclusively associate with RBPJkappa in Epstein-Barr virus-transformed B lymphocytes**. *J Virol* 1996, **70**(6):4179-4183.

127. Waltzer L, Perricaudet M, Sergeant A, Manet E: **Epstein-Barr virus EBNA3A and EBNA3C proteins both repress RBP-J kappa-EBNA2-activated transcription by inhibiting the binding of RBP-J kappa to DNA**. *J Virol* 1996, **70**(9):5909-5915.

128. Zimber-Strobl U, Kremmer E, Grasser F, Marschall G, Laux G, Bornkamm GW: **The Epstein-Barr virus nuclear antigen 2 interacts with an EBNA2 responsive cis-element of the terminal protein 1 gene promoter**. *EMBO J* 1993, **12**(1):167-175.

129. Harada S, Yalamanchili R, Kieff E: **Epstein-Barr virus nuclear protein 2 has at least two N-terminal domains that mediate self-association**. *J Virol* 2001, **75**(5):2482-2487.

130. Zhao B, Sample CE: **Epstein-barr virus nuclear antigen 3C activates the latent membrane protein 1 promoter in the presence of Epstein-Barr virus nuclear antigen 2 through sequences encompassing an spi-1/Spi-B binding site**. *J Virol* 2000, **74**(11):5151-5160.

131. Kataoka H, Tahara H, Watanabe T, Sugawara M, Ide T, Goto M, Furuichi Y, Sugimoto M: **Immortalization of immunologically committed Epstein-Barr virus-transformed human B-lymphoblastoid cell lines accompanied by a strong telomerase activity**. *Differentiation* 1997, **62**(4):203-211.

132. Counter CM, Botelho FM, Wang P, Harley CB, Bacchetti S: **Stabilization of short telomeres and telomerase activity accompany immortalization of Epstein-Barr virus-transformed human B lymphocytes**. *J Virol* 1994, **68**(5):3410-3414.

133. Hammerschmidt W, Sugden B: **Genetic analysis of immortalizing functions of Epstein-Barr virus in human B lymphocytes**. *Nature* 1989, **340**(6232):393-397.

134. Cohen JI, Wang F, Mannick J, Kieff E: **Epstein-Barr virus nuclear protein 2 is a key determinant of lymphocyte transformation**. *Proc Natl Acad Sci U S A* 1989, **86**(23):9558-9562.

135. Kaye KM, Izumi KM, Kieff E: **Epstein-Barr virus latent membrane protein 1 is essential for B-lymphocyte growth transformation**. *Proc Natl Acad Sci U S A* 1993, **90**(19):9150-9154.

136. Kempkes B, Spitkovsky D, Jansen-Durr P, Ellwart JW, Kremmer E, Delecluse HJ, Rottenberger C, Bornkamm GW, Hammerschmidt W: **B-cell proliferation and induction of early G1-regulating proteins by Epstein-Barr virus mutants conditional for EBNA2**. *EMBO J* 1995, **14**(1):88-96.

137. Tomkinson B, Robertson E, Kieff E: **Epstein-Barr virus nuclear proteins EBNA-3A and EBNA-3C are essential for B-lymphocyte growth transformation**. *J Virol* 1993, **67**(4):2014-2025.

138. Anderton E, Yee J, Smith P, Crook T, White RE, Allday MJ: **Two Epstein-Barr virus (EBV) oncoproteins cooperate to repress expression of the proapoptotic tumour-suppressor Bim: clues to the pathogenesis of Burkitt's lymphoma**. *Oncogene* 2008, **27**(4):421-433.

139. Paschos K, Smith P, Anderton E, Middeldorp JM, White RE, Allday MJ: **Epstein-barr virus latency in B cells leads to epigenetic repression and CpG methylation of the tumour suppressor gene Bim**. *PLoS Pathog* 2009, **5**(6):e1000492.

140. Skalska L, White RE, Franz M, Ruhmann M, Allday MJ: **Epigenetic repression of p16(INK4A) by latent Epstein-Barr virus requires the interaction of EBNA3A and EBNA3C with CtBP**. *PLoS Pathog* 2010, **6**(6):e1000951.

141. Skalska L, White RE, Parker GA, Turro E, Sinclair AJ, Paschos K, Allday MJ: **Induction of p16(INK4a) is the major barrier to proliferation when Epstein-Barr virus (EBV) transforms primary B cells into lymphoblastoid cell lines**. *PLoS Pathog* 2013, **9**(2):e1003187.

142. Paschos K, Parker GA, Watanatanasup E, White RE, Allday MJ: **BIM promoter directly targeted by EBNA3C in polycomb-mediated repression by EBV**. *Nucleic Acids Res* 2012, **40**(15):7233-7246.

143. Hertle ML, Popp C, Petermann S, Maier S, Kremmer E, Lang R, Mages J, Kempkes B: **Differential gene expression patterns of EBV infected EBNA-3A positive and negative human B lymphocytes**. *PLoS Pathog* 2009, **5**(7):e1000506.

144. Chen A, Divisconte M, Jiang X, Quink C, Wang F: **Epstein-Barr virus with the latent infection nuclear antigen 3B completely deleted is still competent for B-cell growth transformation in vitro**. *J Virol* 2005, **79**(7):4506-4509.

145. Tomkinson B, Kieff E: **Use of second-site homologous recombination to demonstrate that Epstein-Barr virus nuclear protein 3B is not important for lymphocyte infection or growth transformation in vitro**. *J Virol* 1992, **66**(5):2893-2903.

146. Mannick JB, Cohen JI, Birkenbach M, Marchini A, Kieff E: **The Epstein-Barr virus nuclear protein encoded by the leader of the EBNA RNAs is important in B-lymphocyte transformation**. *J Virol* 1991, **65**(12):6826-6837.

147. Longnecker R, Miller CL, Miao XQ, Tomkinson B, Kieff E: **The last seven transmembrane and carboxy-terminal cytoplasmic domains of Epstein-Barr**

virus latent membrane protein 2 (LMP2) are dispensable for lymphocyte infection and growth transformation in vitro. *J Virol* 1993, **67**(4):2006-2013.

148. Longnecker R, Miller CL, Tomkinson B, Miao XQ, Kieff E: **Deletion of DNA encoding the first five transmembrane domains of Epstein-Barr virus latent membrane proteins 2A and 2B**. *J Virol* 1993, **67**(8):5068-5074.
149. Kim OJ, Yates JL: **Mutants of Epstein-Barr virus with a selective marker disrupting the TP gene transform B cells and replicate normally in culture**. *J Virol* 1993, **67**(12):7634-7640.
150. Speck P, Kline KA, Cheresh P, Longnecker R: **Epstein-Barr virus lacking latent membrane protein 2 immortalizes B cells with efficiency indistinguishable from that of wild-type virus**. *J Gen Virol* 1999, **80 (Pt 8)**:2193-2203.
151. Longnecker R, Miller CL, Miao XQ, Marchini A, Kieff E: **The only domain which distinguishes Epstein-Barr virus latent membrane protein 2A (LMP2A) from LMP2B is dispensable for lymphocyte infection and growth transformation in vitro; LMP2A is therefore nonessential**. *J Virol* 1992, **66**(11):6461-6469.
152. Brielmeier M, Mautner J, Laux G, Hammerschmidt W: **The latent membrane protein 2 gene of Epstein-Barr virus is important for efficient B cell immortalization**. *J Gen Virol* 1996, **77 (Pt 11)**:2807-2818.
153. Kempkes B, Pich D, Zeidler R, Hammerschmidt W: **Immortalization of human primary B lymphocytes in vitro with DNA**. *Proc Natl Acad Sci U S A* 1995, **92**(13):5875-5879.
154. Lee MA, Diamond ME, Yates JL: **Genetic evidence that EBNA-1 is needed for efficient, stable latent infection by Epstein-Barr virus**. *J Virol* 1999, **73**(4):2974-2982.
155. Yates J, Warren N, Reisman D, Sugden B: **A cis-acting element from the Epstein-Barr viral genome that permits stable replication of recombinant plasmids in latently infected cells**. *Proc Natl Acad Sci U S A* 1984, **81**(12):3806-3810.
156. Swaminathan S, Tomkinson B, Kieff E: **Recombinant Epstein-Barr virus with small RNA (EBER) genes deleted transforms lymphocytes and replicates in vitro**. *Proc Natl Acad Sci U S A* 1991, **88**(4):1546-1550.
157. Gregorovic G, Bosshard R, Karstegl CE, White RE, Pattle S, Chiang AK, Dittrich-Breiholz O, Kracht M, Russ R, Farrell PJ: **Cellular gene expression that correlates with EBER expression in Epstein-Barr Virus-infected lymphoblastoid cell lines**. *J Virol* 2011, **85**(7):3535-3545.

158. Wu Y, Maruo S, Yajima M, Kanda T, Takada K: **Epstein-Barr virus (EBV)-encoded RNA 2 (EBER2) but not EBER1 plays a critical role in EBV-induced B-cell growth transformation**. *J Virol* 2007, **81**(20):11236-11245.

159. Gregorovic G, Boulden EA, Bosshard R, Elgueta Karstegl C, Skalsky R, Cullen BR, Gujer C, Ramer P, Munz C, Farrell PJ: **Epstein-Barr Viruses (EBVs) Deficient in EBV-Encoded RNAs Have Higher Levels of Latent Membrane Protein 2 RNA Expression in Lymphoblastoid Cell Lines and Efficiently Establish Persistent Infections in Humanized Mice**. *J Virol* 2015, **89**(22):11711-11714.

160. Feederle R, Linnstaedt SD, Bannert H, Lips H, Bencun M, Cullen BR, Delecluse HJ: **A viral microRNA cluster strongly potentiates the transforming properties of a human herpesvirus**. *PLoS Pathog* 2011, **7**(2):e1001294.

161. Maruo S, Johannsen E, Illanes D, Cooper A, Kieff E: **Epstein-Barr Virus nuclear protein EBNA3A is critical for maintaining lymphoblastoid cell line growth**. *J Virol* 2003, **77**(19):10437-10447.

162. Maruo S, Wu Y, Ishikawa S, Kanda T, Iwakiri D, Takada K: **Epstein-Barr virus nuclear protein EBNA3C is required for cell cycle progression and growth maintenance of lymphoblastoid cells**. *Proc Natl Acad Sci U S A* 2006, **103**(51):19500-19505.

163. Kempkes B, Pich D, Zeidler R, Sugden B, Hammerschmidt W: **Immortalization of human B lymphocytes by a plasmid containing 71 kilobase pairs of Epstein-Barr virus DNA**. *J Virol* 1995, **69**(1):231-238.

164. Cohen JI, Fauci AS, Varmus H, Nabel GJ: **Epstein-Barr virus: an important vaccine target for cancer prevention**. *Sci Transl Med* 2011, **3**(107):107fs107.

165. Pattle SB, Farrell PJ: **The role of Epstein-Barr virus in cancer**. *Expert Opin Biol Ther* 2006, **6**(11):1193-1205.

166. Dalla-Favera R, Bregni M, Erikson J, Patterson D, Gallo RC, Croce CM: **Human c-myc onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells**. *Proc Natl Acad Sci U S A* 1982, **79**(24):7824-7827.

167. Takada K, Horinouchi K, Ono Y, Aya T, Osato T, Takahashi M, Hayasaka S: **An Epstein-Barr virus-producer line Akata: establishment of the cell line and analysis of viral DNA**. *Virus Genes* 1991, **5**(2):147-156.

168. Brady G, Macarthur GJ, Farrell PJ: **Epstein-Barr virus and Burkitt lymphoma**. *Postgrad Med J* 2008, **84**(993):372-377.

169. Vereide DT, Seto E, Chiu YF, Hayes M, Tagawa T, Grundhoff A, Hammerschmidt W, Sugden B: **Epstein-Barr virus maintains lymphomas via its miRNAs**. *Oncogene* 2014, **33**(10):1258-1264.

170. Kelly GL, Milner AE, Baldwin GS, Bell AI, Rickinson AB: **Three restricted forms of Epstein-Barr virus latency counteracting apoptosis in c-myc-expressing Burkitt lymphoma cells**. *Proc Natl Acad Sci U S A* 2006, **103**(40):14935-14940.

171. Kelly GL, Long HM, Stylianou J, Thomas WA, Leese A, Bell AI, Bornkamm GW, Mautner J, Rickinson AB, Rowe M: **An Epstein-Barr virus anti-apoptotic protein constitutively expressed in transformed cells and implicated in burkitt lymphomagenesis: the Wp/BHRF1 link**. *PLoS Pathog* 2009, **5**(3):e1000341.

172. Nanbo A, Inoue K, Adachi-Takasawa K, Takada K: **Epstein-Barr virus RNA confers resistance to interferon-alpha-induced apoptosis in Burkitt's lymphoma**. *EMBO J* 2002, **21**(5):954-965.

173. Nanbo A, Takada K: **The role of Epstein-Barr virus-encoded small RNAs (EBERs) in oncogenesis**. *Rev Med Virol* 2002, **12**(5):321-326.

174. Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T: **Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme**. *Cell* 2000, **102**(5):553-563.

175. Gregory CD, Tursz T, Edwards CF, Tetaud C, Talbot M, Caillou B, Rickinson AB, Lipinski M: **Identification of a subset of normal B cells with a Burkitt's lymphoma (BL)-like phenotype**. *J Immunol* 1987, **139**(1):313-318.

176. Klein G: **Specific chromosomal translocations and the genesis of B-cell-derived tumors in mice and men**. *Cell* 1983, **32**(2):311-315.

177. Shimizu N, Tanabe-Tochikura A, Kuroiwa Y, Takada K: **Isolation of Epstein-Barr virus (EBV)-negative cell clones from the EBV-positive Burkitt's lymphoma (BL) line Akata: malignant phenotypes of BL cells are dependent on EBV**. *J Virol* 1994, **68**(9):6069-6073.

178. Lindstrom MS, Wiman KG: **Role of genetic and epigenetic changes in Burkitt lymphoma**. *Semin Cancer Biol* 2002, **12**(5):381-387.

179. Farrell PJ, Allan GJ, Shanahan F, Vousden KH, Crook T: **p53 is frequently mutated in Burkitt's lymphoma cell lines**. *EMBO J* 1991, **10**(10):2879-2887.

180. Schmitz R, Young RM, Ceribelli M, Jhavar S, Xiao W, Zhang M, Wright G, Shaffer AL, Hodson DJ, Buras E *et al*: **Burkitt lymphoma pathogenesis and therapeutic targets from structural and functional genomics**. *Nature* 2012, **490**(7418):116-120.

181. Deacon EM, Pallesen G, Niedobitek G, Crocker J, Brooks L, Rickinson AB, Young LS: **Epstein-Barr virus and Hodgkin's disease: transcriptional analysis of virus latency in the malignant cells**. *J Exp Med* 1993, **177**(2):339-349.
182. Kuppers R, Rajewsky K: **The origin of Hodgkin and Reed/Sternberg cells in Hodgkin's disease**. *Annu Rev Immunol* 1998, **16**:471-493.
183. Steidl C, Shah SP, Woolcock BW, Rui L, Kawahara M, Farinha P, Johnson NA, Zhao Y, Telenius A, Neriah SB *et al*: **MHC class II transactivator CIITA is a recurrent gene fusion partner in lymphoid cancers**. *Nature* 2011, **471**(7338):377-381.
184. Kushekhar K, van den Berg A, Nolte I, Hepkema B, Visser L, Diepstra A: **Genetic associations in classical hodgkin lymphoma: a systematic review and insights into susceptibility mechanisms**. *Cancer Epidemiol Biomarkers Prev* 2014, **23**(12):2737-2747.
185. Ghesquieres H, Dogan A, Link BK, Maurer MJ, Cunningham JM, Novak AJ, Larrabee BR, Slager SL, Allmer C, Habermann TM *et al*: **FCGR2A and FCGR3A polymorphisms in classical Hodgkin lymphoma by Epstein-Barr virus status**. *Leuk Lymphoma* 2013, **54**(11):2571-2573.
186. Burgos JS: **Involvement of the Epstein-Barr virus in the nasopharyngeal carcinoma pathogenesis**. *Med Oncol* 2005, **22**(2):113-121.
187. Shair KH, Bendt KM, Edwards RH, Nielsen JN, Moore DT, Raab-Traub N: **Epstein-Barr virus-encoded latent membrane protein 1 (LMP1) and LMP2A function cooperatively to promote carcinoma development in a mouse carcinogenesis model**. *J Virol* 2012, **86**(9):5352-5365.
188. Young LS, Dawson CW, Clark D, Rupani H, Busson P, Tursz T, Johnson A, Rickinson AB: **Epstein-Barr virus gene expression in nasopharyngeal carcinoma**. *J Gen Virol* 1988, **69 (Pt 5)**:1051-1065.
189. Komano J, Takada K: **Role of bcl-2 in Epstein-Barr virus-induced malignant conversion of Burkitt's lymphoma cell line Akata**. *J Virol* 2001, **75**(3):1561-1564.
190. Wong HL, Wang X, Chang RC, Jin DY, Feng H, Wang Q, Lo KW, Huang DP, Yuen PW, Takada K *et al*: **Stable expression of EBERs in immortalized nasopharyngeal epithelial cells confers resistance to apoptotic stress**. *Mol Carcinog* 2005, **44**(2):92-101.
191. Wong AM, Kong KL, Tsang JW, Kwong DL, Guan XY: **Profiling of Epstein-Barr virus-encoded microRNAs in nasopharyngeal carcinoma reveals potential biomarkers and oncomirs**. *Cancer* 2012, **118**(3):698-710.

192. Lin DC, Meng X, Hazawa M, Nagata Y, Varela AM, Xu L, Sato Y, Liu LZ, Ding LW, Sharma A *et al*: **The genomic landscape of nasopharyngeal carcinoma**. *Nat Genet* 2014, **46**(8):866-871.
193. Or YY, Hui AB, To KF, Lam CN, Lo KW: **PIK3CA mutations in nasopharyngeal carcinoma**. *Int J Cancer* 2006, **118**(4):1065-1067.
194. Chou CC, Chou MJ, Tzen CY: **PIK3CA mutation occurs in nasopharyngeal carcinoma but does not significantly influence the disease-specific survival**. *Med Oncol* 2009, **26**(3):322-326.
195. Chung GT, Lou WP, Chow C, To KF, Choy KW, Leung AW, Tong CY, Yuen JW, Ko CW, Yip TT *et al*: **Constitutive activation of distinct NF-kappaB signals in EBV-associated nasopharyngeal carcinoma**. *J Pathol* 2013, **231**(3):311-322.
196. Cancer Genome Atlas Research N: **Comprehensive molecular characterization of gastric adenocarcinoma**. *Nature* 2014, **513**(7517):202-209.
197. Wang K, Yuen ST, Xu J, Lee SP, Yan HH, Shi ST, Siu HC, Deng S, Chu KM, Law S *et al*: **Whole-genome sequencing and comprehensive molecular profiling identify new driver mutations in gastric cancer**. *Nat Genet* 2014, **46**(6):573-582.
198. Iwakiri D, Eizuru Y, Tokunaga M, Takada K: **Autocrine growth of Epstein-Barr virus-positive gastric carcinoma cells mediated by an Epstein-Barr virus-encoded small RNA**. *Cancer Res* 2003, **63**(21):7062-7067.
199. Price AM, Luftig MA: **To be or not IIb: a multi-step process for Epstein-Barr virus latency establishment and consequences for B cell tumorigenesis**. *PLoS Pathog* 2015, **11**(3):e1004656.
200. Rabson M, Gradoville L, Heston L, Miller G: **Non-immortalizing P3J-HR-1 Epstein-Barr virus: a deletion mutant of its transforming parent, Jijoye**. *J Virol* 1982, **44**(3):834-844.
201. Skare J, Farley J, Strominger JL, Fresen KO, Cho MS, zur Hausen H: **Transformation by Epstein-Barr virus requires DNA sequences in the region of BamHI fragments Y and H**. *J Virol* 1985, **55**(2):286-297.
202. Bornkamm GW, Hudewentz J, Freese UK, Zimber U: **Deletion of the nontransforming Epstein-Barr virus strain P3HR-1 causes fusion of the large internal repeat to the DSL region**. *J Virol* 1982, **43**(3):952-968.
203. King W, Dambaugh T, Heller M, Dowling J, Kieff E: **Epstein-Barr virus DNA XII. A variable region of the Epstein-Barr virus genome is included in the P3HR-1 deletion**. *J Virol* 1982, **43**(3):979-986.

204. Rowe D, Heston L, Metlay J, Miller G: **Identification and expression of a nuclear antigen from the genomic region of the Jijoye strain of Epstein-Barr virus that is missing in its nonimmortalizing deletion mutant, P3HR-1**. *Proc Natl Acad Sci U S A* 1985, **82**(21):7429-7433.

205. Picard D, Salser SJ, Yamamoto KR: **A movable and regulable inactivation function within the steroid binding domain of the glucocorticoid receptor**. *Cell* 1988, **54**(7):1073-1080.

206. Lucchesi W, Brady G, Dittrich-Breiholz O, Kracht M, Russ R, Farrell PJ: **Differential gene regulation by Epstein-Barr virus type 1 and type 2 EBNA2**. *J Virol* 2008, **82**(15):7456-7466.

207. Schlee M, Krug T, Gires O, Zeidler R, Hammerschmidt W, Mailhammer R, Laux G, Sauer G, Lovric J, Bornkamm GW: **Identification of Epstein-Barr virus (EBV) nuclear antigen 2 (EBNA2) target proteins by proteome analysis: activation of EBNA2 in conditionally immortalized B cells reflects early events after infection of primary B cells by EBV**. *J Virol* 2004, **78**(8):3941-3952.

208. Gordadze AV, Peng R, Tan J, Liu G, Sutton R, Kempkes B, Bornkamm GW, Ling PD: **Notch1IC partially replaces EBNA2 function in B cells immortalized by Epstein-Barr virus**. *J Virol* 2001, **75**(13):5899-5912.

209. Cancian L, Bosshard R, Lucchesi W, Karstegl CE, Farrell PJ: **C-terminal region of EBNA-2 determines the superior transforming ability of type 1 Epstein-Barr virus by enhanced gene regulation of LMP-1 and CXCR7**. *PLoS Pathog* 2011, **7**(7):e1002164.

210. Tzellos S, Correia PB, Karstegl CE, Cancian L, Cano-Flanagan J, McClellan MJ, West MJ, Farrell PJ: **A single amino acid in EBNA-2 determines superior B lymphoblastoid cell line growth maintenance by Epstein-Barr virus type 1 EBNA-2**. *J Virol* 2014, **88**(16):8743-8753.

211. Grasser FA, Haiss P, Gottel S, Mueller-Lantzsch N: **Biochemical characterization of Epstein-Barr virus nuclear antigen 2A**. *J Virol* 1991, **65**(7):3779-3788.

212. Petti L, Sample C, Kieff E: **Subnuclear localization and phosphorylation of Epstein-Barr virus latent infection nuclear proteins**. *Virology* 1990, **176**(2):563-574.

213. Dambaugh T, Hennessy K, Chamnankit L, Kieff E: **U2 region of Epstein-Barr virus DNA may encode Epstein-Barr nuclear antigen 2**. *Proc Natl Acad Sci U S A* 1984, **81**(23):7632-7636.

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

215. Peng R, Gordadze AV, Fuentes Panana EM, Wang F, Zong J, Hayward GS, Tan J, Ling PD: **Sequence and functional analysis of EBNA-LP and EBNA2 proteins from nonhuman primate lymphocryptoviruses**. *J Virol* 2000, **74**(1):379-389.

216. Cohen JI, Kieff E: **An Epstein-Barr virus nuclear protein 2 domain essential for transformation is a direct transcriptional activator**. *J Virol* 1991, **65**(11):5880-5885.

217. Tong X, Yalamanchili R, Harada S, Kieff E: **The EBNA-2 arginine-glycine domain is critical but not essential for B-lymphocyte growth transformation; the rest of region 3 lacks essential interactive domains**. *J Virol* 1994, **68**(10):6188-6197.

218. Ling PD, Rawlins DR, Hayward SD: **The Epstein-Barr virus immortalizing protein EBNA-2 is targeted to DNA by a cellular enhancer-binding protein**. *Proc Natl Acad Sci U S A* 1993, **90**(20):9237-9241.

219. Ling PD, Hayward SD: **Contribution of conserved amino acids in mediating the interaction between EBNA2 and CBF1/RBPJk**. *J Virol* 1995, **69**(3):1944-1950.

220. Henkel T, Ling PD, Hayward SD, Peterson MG: **Mediation of Epstein-Barr virus EBNA2 transactivation by recombination signal-binding protein J kappa**. *Science* 1994, **265**(5168):92-95.

221. Waltzer L, Logeat F, Brou C, Israel A, Sergeant A, Manet E: **The human J kappa recombination signal sequence binding protein (RBP-J kappa) targets the Epstein-Barr virus EBNA2 protein to its DNA responsive elements**. *EMBO J* 1994, **13**(23):5633-5638.

222. Ling PD, Hsieh JJ, Ruf IK, Rawlins DR, Hayward SD: **EBNA-2 upregulation of Epstein-Barr virus latency promoters and the cellular CD23 promoter utilizes a common targeting intermediate, CBF1**. *J Virol* 1994, **68**(9):5375-5383.

223. Zhou S, Fujimuro M, Hsieh JJ, Chen L, Hayward SD: **A role for SKIP in EBNA2 activation of CBF1-repressed promoters**. *J Virol* 2000, **74**(4):1939-1947.

224. Peng CW, Zhao B, Kieff E: **Four EBNA2 domains are important for EBNALP coactivation**. *J Virol* 2004, **78**(20):11439-11442.

225. Fresen KO, Cho MS, zur Hausen H: **Recovery of transforming EBV from non-producer cells after superinfection with non-transforming P3HR-1 EBV**. *Int J Cancer* 1978, **22**(4):378-383.

226. Grossman SR, Johannsen E, Tong X, Yalamanchili R, Kieff E: **The Epstein-Barr virus nuclear antigen 2 transactivator is directed to response elements by the J kappa recombination signal binding protein**. *Proc Natl Acad Sci U S A* 1994, **91**(16):7568-7572.

227. Cohen JI, Wang F, Kieff E: **Epstein-Barr virus nuclear protein 2 mutations define essential domains for transformation and transactivation**. *J Virol* 1991, **65**(5):2545-2554.

228. Tsui S, Schubach WH: **Epstein-Barr virus nuclear protein 2A forms oligomers in vitro and in vivo through a region required for B-cell transformation**. *J Virol* 1994, **68**(7):4287-4294.

229. Cohen JI: **A region of herpes simplex virus VP16 can substitute for a transforming domain of Epstein-Barr virus nuclear protein 2**. *Proc Natl Acad Sci U S A* 1992, **89**(17):8030-8034.

230. Gordadze AV, Onunwor CW, Peng R, Poston D, Kremmer E, Ling PD: **EBNA2 amino acids 3 to 30 are required for induction of LMP-1 and immortalization maintenance**. *J Virol* 2004, **78**(8):3919-3929.

231. Gordadze AV, Poston D, Ling PD: **The EBNA2 polyproline region is dispensable for Epstein-Barr virus-mediated immortalization maintenance**. *J Virol* 2002, **76**(14):7349-7355.

232. Yalamanchili R, Harada S, Kieff E: **The N-terminal half of EBNA2, except for seven prolines, is not essential for primary B-lymphocyte growth transformation**. *J Virol* 1996, **70**(4):2468-2473.

233. Harada S, Yalamanchili R, Kieff E: **Residues 231 to 280 of the Epstein-Barr virus nuclear protein 2 are not essential for primary B-lymphocyte growth transformation**. *J Virol* 1998, **72**(12):9948-9954.

234. Yalamanchili R, Tong X, Grossman S, Johannsen E, Mosialos G, Kieff E: **Genetic and biochemical evidence that EBNA 2 interaction with a 63-kDa cellular GTG-binding protein is essential for B lymphocyte growth transformation by EBV**. *Virology* 1994, **204**(2):634-641.

235. Kwiatkowski B, Chen SY, Schubach WH: **CKII site in Epstein-Barr virus nuclear protein 2 controls binding to hSNF5/Ini1 and is important for growth transformation**. *J Virol* 2004, **78**(11):6067-6072.

236. Wu DY, Kalpana GV, Goff SP, Schubach WH: **Epstein-Barr virus nuclear protein 2 (EBNA2) binds to a component of the human SNF-SWI complex, hSNF5/Ini1**. *J Virol* 1996, **70**(9):6020-6028.

237. Zimber-Strobl U, Strobl LJ, Meitinger C, Hinrichs R, Sakai T, Furukawa T, Honjo T, Bornkamm GW: **Epstein-Barr virus nuclear antigen 2 exerts its transactivating function through interaction with recombination signal binding protein RBP-J kappa, the homologue of Drosophila Suppressor of Hairless**. *EMBO J* 1994, **13**(20):4973-4982.

238. Lai EC: **Keeping a good pathway down: transcriptional repression of Notch pathway target genes by CSL proteins**. *EMBO Rep* 2002, **3**(9):840-845.

239. Johannsen E, Koh E, Mosialos G, Tong X, Kieff E, Grossman SR: **Epstein-Barr virus nuclear protein 2 transactivation of the latent membrane protein 1 promoter is mediated by J kappa and PU.1**. *J Virol* 1995, **69**(1):253-262.

240. Laux G, Adam B, Strobl LJ, Moreau-Gachelin F: **The Spi-1/PU.1 and Spi-B ets family transcription factors and the recombination signal binding protein RBP-J kappa interact with an Epstein-Barr virus nuclear antigen 2 responsive cis-element**. *EMBO J* 1994, **13**(23):5624-5632.

241. Jin XW, Speck SH: **Identification of critical cis elements involved in mediating Epstein-Barr virus nuclear antigen 2-dependent activity of an enhancer located upstream of the viral BamHI C promoter**. *J Virol* 1992, **66**(5):2846-2852.

242. Sjoblom A, Jansson A, Yang W, Lain S, Nilsson T, Rymo L: **PU box-binding transcription factors and a POU domain protein cooperate in the Epstein-Barr virus (EBV) nuclear antigen 2-induced transactivation of the EBV latent membrane protein 1 promoter**. *J Gen Virol* 1995, **76 (Pt 11)**:2679-2692.

243. Sjoblom A, Yang W, Palmqvist L, Jansson A, Rymo L: **An ATF/CRE element mediates both EBNA2-dependent and EBNA2-independent activation of the Epstein-Barr virus LMP1 gene promoter**. *J Virol* 1998, **72**(2):1365-1376.

244. Jansson A, Johansson P, Yang W, Palmqvist L, Sjoblom-Hallen A, Rymo L: **Role of a consensus AP-2 regulatory sequence within the Epstein-Barr virus LMP1 promoter in EBNA2 mediated transactivation**. *Virus Genes* 2007, **35**(2):203-214.

245. Zhao B, Zou J, Wang H, Johannsen E, Peng CW, Quackenbush J, Mar JC, Morton CC, Freedman ML, Blacklow SC *et al*: **Epstein-Barr virus exploits intrinsic B-lymphocyte transcription programs to achieve immortal cell growth**. *Proc Natl Acad Sci U S A* 2011, **108**(36):14902-14907.

246. Fuentes-Panana EM, Peng R, Brewer G, Tan J, Ling PD: **Regulation of the Epstein-Barr virus C promoter by AUF1 and the cyclic AMP/protein kinase A signaling pathway**. *J Virol* 2000, **74**(17):8166-8175.

247. Tolnay M, Lambris JD, Tsokos GC: **Transcriptional regulation of the complement receptor 2 gene: role of a heterogeneous nuclear ribonucleoprotein**. *J Immunol* 1997, **159**(11):5492-5501.

248. Hayward SD, Liu J, Fujimuro M: **Notch and Wnt signaling: mimicry and manipulation by gamma herpesviruses**. *Sci STKE* 2006, **2006**(335):re4.

249. Hsieh JJ, Hayward SD: **Masking of the CBF1/RBPJ kappa transcriptional repression domain by Epstein-Barr virus EBNA2**. *Science* 1995, **268**(5210):560-563.

250. Kopan R, Ilagan MX: **The canonical Notch signaling pathway: unfolding the activation mechanism**. *Cell* 2009, **137**(2):216-233.

251. Awong G, La Motte-Mohs RN, Zuniga-Pflucker JC: **In vitro human T cell development directed by notch-ligand interactions**. *Methods Mol Biol* 2008, **430**:135-142.

252. Maillard I, Fang T, Pear WS: **Regulation of lymphoid development, differentiation, and function by the Notch pathway**. *Annu Rev Immunol* 2005, **23**:945-974.

253. Hofelmayr H, Strobl LJ, Stein C, Laux G, Marschall G, Bornkamm GW, Zimber-Strobl U: **Activated mouse Notch1 transactivates Epstein-Barr virus nuclear antigen 2-regulated viral promoters**. *J Virol* 1999, **73**(4):2770-2780.

254. Strobl LJ, Hofelmayr H, Marschall G, Brielmeier M, Bornkamm GW, Zimber-Strobl U: **Activated Notch1 modulates gene expression in B cells similarly to Epstein-Barr viral nuclear antigen 2**. *J Virol* 2000, **74**(4):1727-1735.

255. Hofelmayr H, Strobl LJ, Marschall G, Bornkamm GW, Zimber-Strobl U: **Activated Notch1 can transiently substitute for EBNA2 in the maintenance of proliferation of LMP1-expressing immortalized B cells**. *J Virol* 2001, **75**(5):2033-2040.

256. Kohlhof H, Hampel F, Hoffmann R, Burtscher H, Weidle UH, Holzel M, Eick D, Zimber-Strobl U, Strobl LJ: **Notch1, Notch2, and Epstein-Barr virus-encoded nuclear antigen 2 signaling differentially affects proliferation and survival of Epstein-Barr virus-infected B cells**. *Blood* 2009, **113**(22):5506-5515.

257. Rowe M, Raithatha S, Shannon-Lowe C: **Counteracting effects of cellular Notch and Epstein-Barr virus EBNA2: implications for stromal effects on virus-host interactions**. *J Virol* 2014, **88**(20):12065-12076.

258. Wang L, Grossman SR, Kieff E: **Epstein-Barr virus nuclear protein 2 interacts with p300, CBP, and PCAF histone acetyltransferases in activation of the LMP1 promoter**. *Proc Natl Acad Sci U S A* 2000, **97**(1):430-435.

259. Tong X, Wang F, Thut CJ, Kieff E: **The Epstein-Barr virus nuclear protein 2 acidic domain can interact with TFIIB, TAF40, and RPA70 but not with TATA-binding protein**. *J Virol* 1995, **69**(1):585-588.

260. Tong X, Drapkin R, Reinberg D, Kieff E: **The 62- and 80-kDa subunits of transcription factor IIH mediate the interaction with Epstein-Barr virus nuclear protein 2**. *Proc Natl Acad Sci U S A* 1995, **92**(8):3259-3263.

261. Tong X, Drapkin R, Yalamanchili R, Mosialos G, Kieff E: **The Epstein-Barr virus nuclear protein 2 acidic domain forms a complex with a novel cellular coactivator that can interact with TFIIE**. *Mol Cell Biol* 1995, **15**(9):4735-4744.

262. Portal D, Zhao B, Calderwood MA, Sommermann T, Johannsen E, Kieff E: **EBV nuclear antigen EBNALP dismisses transcription repressors NCoR and RBPJ from enhancers and EBNA2 increases NCoR-deficient RBPJ DNA binding**. *Proc Natl Acad Sci U S A* 2011, **108**(19):7808-7813.

263. Portal D, Zhou H, Zhao B, Kharchenko PV, Lowry E, Wong L, Quackenbush J, Holloway D, Jiang S, Lu Y *et al*: **Epstein-Barr virus nuclear antigen leader protein localizes to promoters and enhancers with cell transcription factors and EBNA2**. *Proc Natl Acad Sci U S A* 2013, **110**(46):18537-18542.

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

265. Wu DY, Krumm A, Schubach WH: **Promoter-specific targeting of human SWI-SNF complex by Epstein-Barr virus nuclear protein 2**. *J Virol* 2000, **74**(19):8893-8903.

266. Wu DY, Tkachuck DC, Roberson RS, Schubach WH: **The human SNF5/INI1 protein facilitates the function of the growth arrest and DNA damage-inducible protein (GADD34) and modulates GADD34-bound protein phosphatase-1 activity**. *J Biol Chem* 2002, **277**(31):27706-27715.

267. Ansieau S, Leutz A: **The conserved Mynd domain of BS69 binds cellular and oncoviral proteins through a common PXLXP motif**. *J Biol Chem* 2002, **277**(7):4906-4910.

268. Harter MR, Liu CD, Shen CL, Gonzalez-Hurtado E, Zhang ZM, Xu M, Martinez E, Peng CW, Song J: **BS69/ZMYND11 C-Terminal Domains Bind and Inhibit EBNA2**. *PLoS Pathog* 2016, **12**(2):e1005414.

269. Grundhoff AT, Kremmer E, Tureci O, Glieden A, Gindorf C, Atz J, Mueller-Lantzsch N, Schubach WH, Grasser FA: **Characterization of DP103, a novel DEAD box**

protein that binds to the Epstein-Barr virus nuclear proteins EBNA2 and EBNA3C. *J Biol Chem* 1999, **274**(27):19136-19144.

270. Voss MD, Hille A, Barth S, Spurk A, Hennrich F, Holzer D, Mueller-Lantzsch N, Kremmer E, Grasser FA: **Functional cooperation of Epstein-Barr virus nuclear antigen 2 and the survival motor neuron protein in transactivation of the viral LMP1 promoter**. *J Virol* 2001, **75**(23):11781-11790.
271. Barth S, Liss M, Voss MD, Dobner T, Fischer U, Meister G, Grasser FA: **Epstein-Barr virus nuclear antigen 2 binds via its methylated arginine-glycine repeat to the survival motor neuron protein**. *J Virol* 2003, **77**(8):5008-5013.
272. Lee JM, Lee KH, Farrell CJ, Ling PD, Kempkes B, Park JH, Hayward SD: **EBNA2 is required for protection of latently Epstein-Barr virus-infected B cells against specific apoptotic stimuli**. *J Virol* 2004, **78**(22):12694-12697.
273. Lee JM, Lee KH, Weidner M, Osborne BA, Hayward SD: **Epstein-Barr virus EBNA2 blocks Nur77- mediated apoptosis**. *Proc Natl Acad Sci U S A* 2002, **99**(18):11878-11883.
274. Kaiser C, Laux G, Eick D, Jochner N, Bornkamm GW, Kempkes B: **The proto-oncogene c-myc is a direct target gene of Epstein-Barr virus nuclear antigen 2**. *J Virol* 1999, **73**(5):4481-4484.
275. Knutson JC: **The level of c-fgr RNA is increased by EBNA-2, an Epstein-Barr virus gene required for B-cell immortalization**. *J Virol* 1990, **64**(6):2530-2536.
276. Spender LC, Cornish GH, Sullivan A, Farrell PJ: **Expression of transcription factor AML-2 (RUNX3, CBF(alpha)-3) is induced by Epstein-Barr virus EBNA-2 and correlates with the B-cell activation phenotype**. *J Virol* 2002, **76**(10):4919-4927.
277. Cordier M, Calender A, Billaud M, Zimber U, Rousselet G, Pavlish O, Banchereau J, Tursz T, Bornkamm G, Lenoir GM: **Stable transfection of Epstein-Barr virus (EBV) nuclear antigen 2 in lymphoma cells containing the EBV P3HR1 genome induces expression of B-cell activation molecules CD21 and CD23**. *J Virol* 1990, **64**(3):1002-1013.
278. Wang F, Gregory CD, Rowe M, Rickinson AB, Wang D, Birkenbach M, Kikutani H, Kishimoto T, Kieff E: **Epstein-Barr virus nuclear antigen 2 specifically induces expression of the B-cell activation antigen CD23**. *Proc Natl Acad Sci U S A* 1987, **84**(10):3452-3456.
279. Mohan J, Dement-Brown J, Maier S, Ise T, Kempkes B, Tolnay M: **Epstein-Barr virus nuclear antigen 2 induces FcRH5 expression through CBF1**. *Blood* 2006, **107**(11):4433-4439.

280. Pages F, Galon J, Karaschuk G, Dudziak D, Camus M, Lazar V, Camilleri-Broet S, Lagorce-Pages C, Lebel-Binay S, Laux G *et al*: **Epstein-Barr virus nuclear antigen 2 induces interleukin-18 receptor expression in B cells**. *Blood* 2005, **105**(4):1632-1639.

281. Maier S, Santak M, Mantik A, Grabusic K, Kremmer E, Hammerschmidt W, Kempkes B: **A somatic knockout of CBF1 in a human B-cell line reveals that induction of CD21 and CCR7 by EBNA-2 is strictly CBF1 dependent and that downregulation of immunoglobulin M is partially CBF1 independent**. *J Virol* 2005, **79**(14):8784-8792.

282. Spender LC, Cornish GH, Rowland B, Kempkes B, Farrell PJ: **Direct and indirect regulation of cytokine and cell cycle proteins by EBNA-2 during Epstein-Barr virus infection**. *J Virol* 2001, **75**(8):3537-3546.

283. Johansen LM, Deppmann CD, Erickson KD, Coffin WF, 3rd, Thornton TM, Humphrey SE, Martin JM, Taparowsky EJ: **EBNA2 and activated Notch induce expression of BATF**. *J Virol* 2003, **77**(10):6029-6040.

284. Boccellato F, Anastasiadou E, Rosato P, Kempkes B, Frati L, Faggioni A, Trivedi P: **EBNA2 interferes with the germinal center phenotype by downregulating BCL6 and TCL1 in non-Hodgkin's lymphoma cells**. *J Virol* 2007, **81**(5):2274-2282.

285. Tobollik S, Meyer L, Buettner M, Klemmer S, Kempkes B, Kremmer E, Niedobitek G, Jungnickel B: **Epstein-Barr virus nuclear antigen 2 inhibits AID expression during EBV-driven B-cell growth**. *Blood* 2006, **108**(12):3859-3864.

286. Adldinger HK, Delius H, Freese UK, Clarke J, Bornkamm GW: **A putative transforming gene of Jijoye virus differs from that of Epstein-Barr virus prototypes**. *Virology* 1985, **141**(2):221-234.

287. Tzellos S, Farrell PJ: **Epstein-barr virus sequence variation-biology and disease**. *Pathogens* 2012, **1**(2):156-174.

288. Sample J, Young L, Martin B, Chatman T, Kieff E, Rickinson A, Kieff E: **Epstein-Barr virus types 1 and 2 differ in their EBNA-3A, EBNA-3B, and EBNA-3C genes**. *J Virol* 1990, **64**(9):4084-4092.

289. McCann EM, Kelly GL, Rickinson AB, Bell AI: **Genetic analysis of the Epstein-Barr virus-coded leader protein EBNA-LP as a co-activator of EBNA2 function**. *J Gen Virol* 2001, **82**(Pt 12):3067-3079.

290. Rowe M, Young LS, Cadwallader K, Petti L, Kieff E, Rickinson AB: **Distinction between Epstein-Barr virus type A (EBNA 2A) and type B (EBNA 2B) isolates extends to the EBNA 3 family of nuclear proteins**. *J Virol* 1989, **63**(3):1031-1039.

291. Midgley RS, Blake NW, Yao QY, Croom-Carter D, Cheung ST, Leung SF, Chan AT, Johnson PJ, Huang D, Rickinson AB *et al*: **Novel intertypic recombinants of epstein-barr virus in the chinese population**. *J Virol* 2000, **74**(3):1544-1548.

292. Yao QY, Tierney RJ, Croom-Carter D, Cooper GM, Ellis CJ, Rowe M, Rickinson AB: **Isolation of intertypic recombinants of Epstein-Barr virus from T-cell-immunocompromised individuals**. *J Virol* 1996, **70**(8):4895-4903.

293. Allday MJ, MacGillivray AJ: **Epstein-Barr virus nuclear antigen (EBNA): size polymorphism of EBNA 1**. *J Gen Virol* 1985, **66 (Pt 7)**:1595-1600.

294. Habeshaw G, Yao QY, Bell AI, Morton D, Rickinson AB: **Epstein-barr virus nuclear antigen 1 sequences in endemic and sporadic Burkitt's lymphoma reflect virus strains prevalent in different geographic areas**. *J Virol* 1999, **73**(2):965-975.

295. Lin JC, Lin SC, Luppi M, Torelli G, Mar EC: **Geographic sequence variation of latent membrane protein 1 gene of Epstein-Barr virus in Hodgkin's lymphomas**. *J Med Virol* 1995, **45**(2):183-191.

296. Rickinson AB, Young LS, Rowe M: **Influence of the Epstein-Barr virus nuclear antigen EBNA 2 on the growth phenotype of virus-transformed B cells**. *J Virol* 1987, **61**(5):1310-1317.

297. Tomkinson B, Kieff E: **Second-site homologous recombination in Epstein-Barr virus: insertion of type 1 EBNA 3 genes in place of type 2 has no effect on in vitro infection**. *J Virol* 1992, **66**(2):780-789.

298. Rowe M, Young LS, Crocker J, Stokes H, Henderson S, Rickinson AB: **Epstein-Barr virus (EBV)-associated lymphoproliferative disease in the SCID mouse model: implications for the pathogenesis of EBV-positive lymphomas in man**. *J Exp Med* 1991, **173**(1):147-158.

299. Cohen JI, Picchio GR, Mosier DE: **Epstein-Barr virus nuclear protein 2 is a critical determinant for tumor growth in SCID mice and for transformation in vitro**. *J Virol* 1992, **66**(12):7555-7559.

300. Peh SC, Kim LH, Poppema S: **Frequent presence of subtype A virus in Epstein-Barr virus-associated malignancies**. *Pathology* 2002, **34**(5):446-450.

301. Young LS, Yao QY, Rooney CM, Sculley TB, Moss DJ, Rupani H, Laux G, Bornkamm GW, Rickinson AB: **New type B isolates of Epstein-Barr virus from Burkitt's lymphoma and from normal individuals in endemic areas**. *J Gen Virol* 1987, **68 (Pt 11)**:2853-2862.

302. Zimber U, Adldinger HK, Lenoir GM, Vuillaume M, Knebel-Doeberitz MV, Laux G, Desgranges C, Wittmann P, Freese UK, Schneider U *et al*: **Geographical prevalence of two types of Epstein-Barr virus**. *Virology* 1986, **154**(1):56-66.

303. Yao QY, Rowe M, Martin B, Young LS, Rickinson AB: **The Epstein-Barr virus carrier state: dominance of a single growth-transforming isolate in the blood and in the oropharynx of healthy virus carriers**. *J Gen Virol* 1991, **72 (Pt 7)**:1579-1590.

304. Gratama JW, Oosterveer MA, Weimar W, Sintnicolaas K, Sizoo W, Bolhuis RL, Ernberg I: **Detection of multiple 'Ebnotypes' in individual Epstein-Barr virus carriers following lymphocyte transformation by virus derived from peripheral blood and oropharynx**. *J Gen Virol* 1994, **75 (Pt 1)**:85-94.

305. Gratama JW, Ernberg I: **Molecular epidemiology of Epstein-Barr virus infection**. *Adv Cancer Res* 1995, **67**:197-255.

306. Tierney RJ, Edwards RH, Sitki-Green D, Croom-Carter D, Roy S, Yao QY, Raab-Traub N, Rickinson AB: **Multiple Epstein-Barr virus strains in patients with infectious mononucleosis: comparison of ex vivo samples with in vitro isolates by use of heteroduplex tracking assays**. *J Infect Dis* 2006, **193**(2):287-297.

307. van Baarle D, Hovenkamp E, Kersten MJ, Klein MR, Miedema F, van Oers MH: **Direct Epstein-Barr virus (EBV) typing on peripheral blood mononuclear cells: no association between EBV type 2 infection or superinfection and the development of acquired immunodeficiency syndrome-related non-Hodgkin's lymphoma**. *Blood* 1999, **93**(11):3949-3955.

308. Sculley TB, Apolloni A, Hurren L, Moss DJ, Cooper DA: **Coinfection with A- and B-type Epstein-Barr virus in human immunodeficiency virus-positive subjects**. *J Infect Dis* 1990, **162**(3):643-648.

309. Kyaw MT, Hurren L, Evans L, Moss DJ, Cooper DA, Benson E, Esmore D, Sculley TB: **Expression of B-type Epstein-Barr virus in HIV-infected patients and cardiac transplant recipients**. *AIDS Res Hum Retroviruses* 1992, **8**(11):1869-1874.

310. De Re V, Boiocchi M, De Vita S, Dolcetti R, Gloghini A, Uccini S, Baroni C, Scarpa A, Cattoretti G, Carbone A: **Subtypes of Epstein-Barr virus in HIV-1-associated and HIV-1-unrelated Hodgkin's disease cases**. *Int J Cancer* 1993, **54**(6):895-898.

311. Boyle MJ, Sewell WA, Sculley TB, Apolloni A, Turner JJ, Swanson CE, Penny R, Cooper DA: **Subtypes of Epstein-Barr virus in human immunodeficiency virus-associated non-Hodgkin lymphoma**. *Blood* 1991, **78**(11):3004-3011.

312. Gunthel CJ, Ng V, McGrath M, Herndier B, Shiramizu B: **Association of Epstein-Barr virus types 1 and 2 with acquired immunodeficiency syndrome-related primary central nervous system lymphomas**. *Blood* 1994, **83**(2):618-619.

313. Ometto L, Menin C, Masiero S, Bonaldi L, Del Mistro A, Cattelan AM, D'Andrea E, De Rossi A, Chieco-Bianchi L: **Molecular profile of Epstein-Barr virus in human immunodeficiency virus type 1-related lymphadenopathies and lymphomas**. *Blood* 1997, **90**(1):313-322.

314. Shibata D, Weiss LM, Hernandez AM, Nathwani BN, Bernstein L, Levine AM: **Epstein-Barr virus-associated non-Hodgkin's lymphoma in patients infected with the human immunodeficiency virus**. *Blood* 1993, **81**(8):2102-2109.

315. Borisch B, Finke J, Hennig I, Delacretaz F, Schneider J, Heitz PU, Laissue JA: **Distribution and localization of Epstein-Barr virus subtypes A and B in AIDS-related lymphomas and lymphatic tissue of HIV-positive patients**. *J Pathol* 1992, **168**(2):229-236.

316. Birx DL, Redfield RR, Tosato G: **Defective regulation of Epstein-Barr virus infection in patients with acquired immunodeficiency syndrome (AIDS) or AIDS-related disorders**. *N Engl J Med* 1986, **314**(14):874-879.

317. Yao QY, Croom-Carter DS, Tierney RJ, Habeshaw G, Wilde JT, Hill FG, Conlon C, Rickinson AB: **Epidemiology of infection with Epstein-Barr virus types 1 and 2: lessons from the study of a T-cell-immunocompromised hemophilic cohort**. *J Virol* 1998, **72**(5):4352-4363.

318. van Baarle D, Hovenkamp E, Dukers NH, Renwick N, Kersten MJ, Goudsmit J, Coutinho RA, Miedema F, van Oers MH: **High prevalence of Epstein-Barr virus type 2 among homosexual men is caused by sexual transmission**. *J Infect Dis* 2000, **181**(6):2045-2049.

319. Buisson M, Morand P, Genoulaz O, Bourgeat MJ, Micoud M, Seigneurin JM: **Changes in the dominant Epstein-Barr virus type during human immunodeficiency virus infection**. *J Gen Virol* 1994, **75 (Pt 2)**:431-437.

320. Thompson MP, Kurzrock R: **Epstein-Barr virus and cancer**. *Clin Cancer Res* 2004, **10**(3):803-821.

321. Gewurz BE, Mar JC, Padi M, Zhao B, Shinners NP, Takasaki K, Bedoya E, Zou JY, Cahir-McFarland E, Quackenbush J *et al*: **Canonical NF-kappaB activation is essential for Epstein-Barr virus latent membrane protein 1 TES2/CTAR2 gene regulation**. *J Virol* 2011, **85**(13):6764-6773.

322. Greenfeld H, Takasaki K, Walsh MJ, Ersing I, Bernhardt K, Ma Y, Fu B, Ashbaugh CW, Cabo J, Mollo SB *et al*: **TRAF1 Coordinates Polyubiquitin Signaling to Enhance Epstein-Barr Virus LMP1-Mediated Growth and Survival Pathway Activation**. *PLoS Pathog* 2015, **11**(5):e1004890.

323. Wang D, Liebowitz D, Kieff E: **An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells**. *Cell* 1985, **43**(3 Pt 2):831-840.

324. Wilson JB, Weinberg W, Johnson R, Yuspa S, Levine AJ: **Expression of the BNLF-1 oncogene of Epstein-Barr virus in the skin of transgenic mice induces hyperplasia and aberrant expression of keratin 6**. *Cell* 1990, **61**(7):1315-1327.

325. Kulwichit W, Edwards RH, Davenport EM, Baskar JF, Godfrey V, Raab-Traub N: **Expression of the Epstein-Barr virus latent membrane protein 1 induces B cell lymphoma in transgenic mice**. *Proc Natl Acad Sci U S A* 1998, **95**(20):11963-11968.

326. Moorthy RK, Thorley-Lawson DA: **All three domains of the Epstein-Barr virus-encoded latent membrane protein LMP-1 are required for transformation of rat-1 fibroblasts**. *J Virol* 1993, **67**(3):1638-1646.

327. Hu LF, Chen F, Zheng X, Ernberg I, Cao SL, Christensson B, Klein G, Winberg G: **Clonability and tumorigenicity of human epithelial cells expressing the EBV encoded membrane protein LMP1**. *Oncogene* 1993, **8**(6):1575-1583.

328. Mosialos G, Birkenbach M, Yalamanchili R, VanArsdale T, Ware C, Kieff E: **The Epstein-Barr virus transforming protein LMP1 engages signaling proteins for the tumor necrosis factor receptor family**. *Cell* 1995, **80**(3):389-399.

329. Kehry MR: **CD40-mediated signaling in B cells. Balancing cell survival, growth, and death**. *J Immunol* 1996, **156**(7):2345-2348.

330. Gires O, Zimber-Strobl U, Gonnella R, Ueffing M, Marschall G, Zeidler R, Pich D, Hammerschmidt W: **Latent membrane protein 1 of Epstein-Barr virus mimics a constitutively active receptor molecule**. *EMBO J* 1997, **16**(20):6131-6140.

331. Eliopoulos AG, Rickinson AB: **Epstein-Barr virus: LMP1 masquerades as an active receptor**. *Curr Biol* 1998, **8**(6):R196-198.

332. Henderson S, Rowe M, Gregory C, Croom-Carter D, Wang F, Longnecker R, Kieff E, Rickinson A: **Induction of bcl-2 expression by Epstein-Barr virus latent membrane protein 1 protects infected B cells from programmed cell death**. *Cell* 1991, **65**(7):1107-1115.

333. Laherty CD, Hu HM, Opipari AW, Wang F, Dixit VM: **The Epstein-Barr virus LMP1 gene product induces A20 zinc finger protein expression by activating nuclear factor kappa B**. *J Biol Chem* 1992, **267**(34):24157-24160.

334. Wang S, Rowe M, Lundgren E: **Expression of the Epstein Barr virus transforming protein LMP1 causes a rapid and transient stimulation of the Bcl-2 homologue Mcl-1 levels in B-cell lines**. *Cancer Res* 1996, **56**(20):4610-4613.

335. D'Souza B, Rowe M, Walls D: **The bfl-1 gene is transcriptionally upregulated by the Epstein-Barr virus LMP1, and its expression promotes the survival of a Burkitt's lymphoma cell line**. *J Virol* 2000, **74**(14):6652-6658.

336. Hong SY, Yoon WH, Park JH, Kang SG, Ahn JH, Lee TH: **Involvement of two NF-kappa B binding elements in tumor necrosis factor alpha -, CD40-, and epstein-barr virus latent membrane protein 1-mediated induction of the cellular inhibitor of apoptosis protein 2 gene**. *J Biol Chem* 2000, **275**(24):18022-18028.

337. Grimm T, Schneider S, Naschberger E, Huber J, Guenzi E, Kieser A, Reitmeir P, Schulz TF, Morris CA, Sturzl M: **EBV latent membrane protein-1 protects B cells from apoptosis by inhibition of BAX**. *Blood* 2005, **105**(8):3263-3269.

338. Yoshizaki T, Sato H, Furukawa M, Pagano JS: **The expression of matrix metalloproteinase 9 is enhanced by Epstein-Barr virus latent membrane protein 1**. *Proc Natl Acad Sci U S A* 1998, **95**(7):3621-3626.

339. Calderwood MA, Venkatesan K, Xing L, Chase MR, Vazquez A, Holthaus AM, Ewence AE, Li N, Hirozane-Kishikawa T, Hill DE *et al*: **Epstein-Barr virus and virus human protein interaction maps**. *Proc Natl Acad Sci U S A* 2007, **104**(18):7606-7611.

340. Kaye KM, Devergne O, Harada JN, Izumi KM, Yalamanchili R, Kieff E, Mosialos G: **Tumor necrosis factor receptor associated factor 2 is a mediator of NF-kappa B activation by latent infection membrane protein 1, the Epstein-Barr virus transforming protein**. *Proc Natl Acad Sci U S A* 1996, **93**(20):11085-11090.

341. Eliopoulos AG, Young LS: **LMP1 structure and signal transduction**. *Semin Cancer Biol* 2001, **11**(6):435-444.

342. Li HP, Chang YS: **Epstein-Barr virus latent membrane protein 1: structure and functions**. *J Biomed Sci* 2003, **10**(5):490-504.

343. Gires O, Kohlhuber F, Kilger E, Baumann M, Kieser A, Kaiser C, Zeidler R, Scheffer B, Ueffing M, Hammerschmidt W: **Latent membrane protein 1 of Epstein-Barr virus interacts with JAK3 and activates STAT proteins**. *EMBO J* 1999, **18**(11):3064-3073.

344. Huen DS, Henderson SA, Croom-Carter D, Rowe M: **The Epstein-Barr virus latent membrane protein-1 (LMP1) mediates activation of NF-kappa B and cell surface phenotype via two effector regions in its carboxy-terminal cytoplasmic domain**. *Oncogene* 1995, **10**(3):549-560.

345. Brodeur SR, Cheng G, Baltimore D, Thorley-Lawson DA: **Localization of the major NF-kappaB-activating site and the sole TRAF3 binding site of LMP-1 defines two distinct signaling motifs**. *J Biol Chem* 1997, **272**(32):19777-19784.

346. Devergne O, Hatzivassiliou E, Izumi KM, Kaye KM, Kleijnen MF, Kieff E, Mosialos G: **Association of TRAF1, TRAF2, and TRAF3 with an Epstein-Barr virus LMP1 domain important for B-lymphocyte transformation: role in NF-kappaB activation**. *Mol Cell Biol* 1996, **16**(12):7098-7108.

347. Luftig M, Yasui T, Soni V, Kang MS, Jacobson N, Cahir-McFarland E, Seed B, Kieff E: **Epstein-Barr virus latent infection membrane protein 1 TRAF-binding site induces NIK/IKK alpha-dependent noncanonical NF-kappaB activation**. *Proc Natl Acad Sci U S A* 2004, **101**(1):141-146.

348. Kang MS, Kieff E: **Epstein-Barr virus latent genes**. *Exp Mol Med* 2015, **47**:e131.

349. Izumi KM, Kieff ED: **The Epstein-Barr virus oncogene product latent membrane protein 1 engages the tumor necrosis factor receptor-associated death domain protein to mediate B lymphocyte growth transformation and activate NF-kappaB**. *Proc Natl Acad Sci U S A* 1997, **94**(23):12592-12597.

350. Izumi KM, Cahir McFarland ED, Ting AT, Riley EA, Seed B, Kieff ED: **The Epstein-Barr virus oncoprotein latent membrane protein 1 engages the tumor necrosis factor receptor-associated proteins TRADD and receptor-interacting protein (RIP) but does not induce apoptosis or require RIP for NF-kappaB activation**. *Mol Cell Biol* 1999, **19**(8):5759-5767.

351. Miller WE, Mosialos G, Kieff E, Raab-Traub N: **Epstein-Barr virus LMP1 induction of the epidermal growth factor receptor is mediated through a TRAF signaling pathway distinct from NF-kappaB activation**. *J Virol* 1997, **71**(1):586-594.

352. Kieser A, Kilger E, Gires O, Ueffing M, Kolch W, Hammerschmidt W: **Epstein-Barr virus latent membrane protein-1 triggers AP-1 activity via the c-Jun N-terminal kinase cascade**. *EMBO J* 1997, **16**(21):6478-6485.

353. Eliopoulos AG, Blake SM, Floettmann JE, Rowe M, Young LS: **Epstein-Barr virus-encoded latent membrane protein 1 activates the JNK pathway through its extreme C terminus via a mechanism involving TRADD and TRAF2**. *J Virol* 1999, **73**(2):1023-1035.

354. Mosialos G: **Cytokine signaling and Epstein-Barr virus-mediated cell transformation**. *Cytokine Growth Factor Rev* 2001, **12**(2-3):259-270.

355. Lam N, Sugden B: **CD40 and its viral mimic, LMP1: similar means to different ends**. *Cell Signal* 2003, **15**(1):9-16.

356. Cahir McFarland ED, Izumi KM, Mosialos G: **Epstein-barr virus transformation: involvement of latent membrane protein 1-mediated activation of NF-kappaB**. *Oncogene* 1999, **18**(49):6959-6964.

357. Liu MT, Chang YT, Chen SC, Chuang YC, Chen YR, Lin CS, Chen JY: **Epstein-Barr virus latent membrane protein 1 represses p53-mediated DNA repair and transcriptional activity**. *Oncogene* 2005, **24**(16):2635-2646.

358. Kieser A: **Signal transduction by the Epstein-Barr virus oncogene latent membrane protein 1 (LMP1)**. *Signal Transduction* 2007, **7**:20-33.

359. Chang MH, Ng CK, Lin YJ, Liang CL, Chung PJ, ChenMl, Tyan YS, Hsu CY, Shu CH, Chang YS: **Identification of a promoter for the latent membrane protein 1 gene of Epstein-Barr virus that is specifically activated in human epithelial cells**. *DNA Cell Biol* 1997, **16**(7):829-837.

360. Sadler RH, Raab-Traub N: **The Epstein-Barr virus 3.5-kilobase latent membrane protein 1 mRNA initiates from a TATA-Less promoter within the first terminal repeat**. *J Virol* 1995, **69**(7):4577-4581.

361. Kis LL, Gerasimcik N, Salamon D, Persson EK, Nagy N, Klein G, Severinson E, Klein E: **STAT6 signaling pathway activated by the cytokines IL-4 and IL-13 induces expression of the Epstein-Barr virus-encoded protein LMP-1 in absence of EBNA-2: implications for the type II EBV latent gene expression in Hodgkin lymphoma**. *Blood* 2011, **117**(1):165-174.

362. Kis LL, Salamon D, Persson EK, Nagy N, Scheeren FA, Spits H, Klein G, Klein E: **IL-21 imposes a type II EBV gene expression on type III and type I B cells by the repression of C- and activation of LMP-1-promoter**. *Proc Natl Acad Sci U S A* 2010, **107**(2):872-877.

363. Chen H, Lee JM, Zong Y, Borowitz M, Ng MH, Ambinder RF, Hayward SD: **Linkage between STAT regulation and Epstein-Barr virus gene expression in tumors**. *J Virol* 2001, **75**(6):2929-2937.

364. Kis LL, Takahara M, Nagy N, Klein G, Klein E: **IL-10 can induce the expression of EBV-encoded latent membrane protein-1 (LMP-1) in the absence of EBNA-2 in B lymphocytes and in Burkitt lymphoma- and NK lymphoma-derived cell lines**. *Blood* 2006, **107**(7):2928-2935.

365. Konforte D, Simard N, Paige CJ: **Interleukin-21 regulates expression of key Epstein-Barr virus oncoproteins, EBNA2 and LMP1, in infected human B cells**. *Virology* 2008, **374**(1):100-113.

366. Chen H, Hutt-Fletcher L, Cao L, Hayward SD: **A positive autoregulatory loop of LMP1 expression and STAT activation in epithelial cells latently infected with Epstein-Barr virus**. *J Virol* 2003, **77**(7):4139-4148.

367. Masselink H, Bernards R: **The adenovirus E1A binding protein BS69 is a corepressor of transcription through recruitment of N-CoR**. *Oncogene* 2000, **19**(12):1538-1546.

368. Hateboer G, Gennissen A, Ramos YF, Kerkhoven RM, Sonntag-Buck V, Stunnenberg HG, Bernards R: **BS69, a novel adenovirus E1A-associated protein that inhibits E1A transactivation**. *EMBO J* 1995, **14**(13):3159-3169.

369. Chinnadurai G: **Modulation of oncogenic transformation by the human adenovirus E1A C-terminal region**. *Curr Top Microbiol Immunol* 2004, **273**:139-161.

370. Flint J, Shenk T: **Viral transactivating proteins**. *Annu Rev Genet* 1997, **31**:177-212.

371. Jones N, Shenk T: **An adenovirus type 5 early gene function regulates expression of other early viral genes**. *Proc Natl Acad Sci U S A* 1979, **76**(8):3665-3669.

372. Whyte P, Williamson NM, Harlow E: **Cellular targets for transformation by the adenovirus E1A proteins**. *Cell* 1989, **56**(1):67-75.

373. Brockmann D, Esche H: **The multifunctional role of E1A in the transcriptional regulation of CREB/CBP-dependent target genes**. *Curr Top Microbiol Immunol* 2003, **272**:97-129.

374. Boyer TG, Martin ME, Lees E, Ricciardi RP, Berk AJ: **Mammalian Srb/Mediator complex is targeted by adenovirus E1A protein**. *Nature* 1999, **399**(6733):276-279.

375. Slavicek JM, Jones NC, Richter JD: **Rapid turnover of adenovirus E1A is determined through a co-translational mechanism that requires an aminoterminal domain**. *EMBO J* 1988, **7**(10):3171-3180.

376. Spindler KR, Berk AJ: **Rapid intracellular turnover of adenovirus 5 early region 1A proteins**. *J Virol* 1984, **52**(2):706-710.

377. Isobe T, Uchida C, Hattori T, Kitagawa K, Oda T, Kitagawa M: **Ubiquitin-dependent degradation of adenovirus E1A protein is inhibited by BS69**. *Biochem Biophys Res Commun* 2006, **339**(1):367-374.

378. Ladendorff NE, Wu S, Lipsick JS: **BS69, an adenovirus E1A-associated protein, inhibits the transcriptional activity of c-Myb**. *Oncogene* 2001, **20**(1):125-132.
379. Masselink H, Vastenhouw N, Bernards R: **B-myb rescues ras-induced premature senescence, which requires its transactivation domain**. *Cancer Lett* 2001, **171**(1):87-101.
380. Wei G, Schaffner AE, Baker KM, Mansky KC, Ostrowski MC: **Ets-2 interacts with co-repressor BS69 to repress target gene expression**. *Anticancer Res* 2003, **23**(3A):2173-2178.
381. Ekblad CM, Chavali GB, Basu BP, Freund SM, Veprintsev D, Hughes-Davies L, Kouzarides T, Doherty AJ, Itzhaki LS: **Binding of EMSY to HP1beta: implications for recruitment of HP1beta and BS69**. *EMBO Rep* 2005, **6**(7):675-680.
382. Aasland R, Gibson TJ, Stewart AF: **The PHD finger: implications for chromatin-mediated transcriptional regulation**. *Trends Biochem Sci* 1995, **20**(2):56-59.
383. Jeanmougin F, Wurtz JM, Le Douarin B, Chambon P, Losson R: **The bromodomain revisited**. *Trends Biochem Sci* 1997, **22**(5):151-153.
384. Stec I, Nagl SB, van Ommen GJ, den Dunnen JT: **The PWWP domain: a potential protein-protein interaction domain in nuclear proteins influencing differentiation?** *FEBS Lett* 2000, **473**(1):1-5.
385. Gross CT, McGinnis W: **DEAF-1, a novel protein that binds an essential region in a Deformed response element**. *EMBO J* 1996, **15**(8):1961-1970.
386. Velasco G, Grkovic S, Ansieau S: **New insights into BS69 functions**. *J Biol Chem* 2006, **281**(24):16546-16550.
387. Wang W, Cote J, Xue Y, Zhou S, Khavari PA, Biggar SR, Muchardt C, Kalpana GV, Goff SP, Yaniv M *et al*: **Purification and biochemical heterogeneity of the mammalian SWI-SNF complex**. *EMBO J* 1996, **15**(19):5370-5382.
388. Chiba H, Muramatsu M, Nomoto A, Kato H: **Two human homologues of Saccharomyces cerevisiae SWI2/SNF2 and Drosophila brahma are transcriptional coactivators cooperating with the estrogen receptor and the retinoic acid receptor**. *Nucleic Acids Res* 1994, **22**(10):1815-1820.
389. Marks PA, Miller T, Richon VM: **Histone deacetylases**. *Curr Opin Pharmacol* 2003, **3**(4):344-351.
390. Muller J, Hart CM, Francis NJ, Vargas ML, Sengupta A, Wild B, Miller EL, O'Connor MB, Kingston RE, Simon JA: **Histone methyltransferase activity of a Drosophila Polycomb group repressor complex**. *Cell* 2002, **111**(2):197-208.

391. Kuzmichev A, Nishioka K, Erdjument-Bromage H, Tempst P, Reinberg D: **Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein**. *Genes Dev* 2002, **16**(22):2893-2905.

392. Cartwright P, Muller H, Wagener C, Holm K, Helin K: **E2F-6: a novel member of the E2F family is an inhibitor of E2F-dependent transcription**. *Oncogene* 1998, **17**(5):611-623.

393. Wang J, Qin S, Li F, Li S, Zhang W, Peng J, Zhang Z, Gong Q, Wu J, Shi Y: **Crystal structure of human BS69 Bromo-ZnF-PWWP reveals its role in H3K36me3 nucleosome binding**. *Cell Res* 2014, **24**(7):890-893.

394. Lan F, Shi Y: **Histone H3.3 and cancer: A potential reader connection**. *Proc Natl Acad Sci U S A* 2015, **112**(22):6814-6819.

395. Guo R, Zheng L, Park JW, Lv R, Chen H, Jiao F, Xu W, Mu S, Wen H, Qiu J *et al*: **BS69/ZMYND11 reads and connects histone H3.3 lysine 36 trimethylation-decorated chromatin to regulated pre-mRNA processing**. *Mol Cell* 2014, **56**(2):298-310.

396. Wen H, Li Y, Xi Y, Jiang S, Stratton S, Peng D, Tanaka K, Ren Y, Xia Z, Wu J *et al*: **ZMYND11 links histone H3.3K36me3 to transcription elongation and tumour suppression**. *Nature* 2014, **508**(7495):263-268.

397. Wen H, Li Y, Li H, Shi X: **ZMYND11: an H3.3-specific reader of H3K36me3**. *Cell Cycle* 2014, **13**(14):2153-2154.

398. Eswaran J, Horvath A, Godbole S, Reddy SD, Mudvari P, Ohshiro K, Cyanam D, Nair S, Fuqua SA, Polyak K *et al*: **RNA sequencing of cancer reveals novel splicing alterations**. *Sci Rep* 2013, **3**:1689.

399. Shapiro IM, Cheng AW, Flytzanis NC, Balsamo M, Condeelis JS, Oktay MH, Burge CB, Gertler FB: **An EMT-driven alternative splicing program occurs in human breast cancer and modulates cellular phenotype**. *PLoS Genet* 2011, **7**(8):e1002218.

400. Ikeda O, Miyasaka Y, Yoshida R, Mizushima A, Oritani K, Sekine Y, Kuroda M, Yasui T, Fujimuro M, Muromoto R *et al*: **BS69 cooperates with TRAF3 in the regulation of Epstein-Barr virus-derived LMP1/CTAR1-induced NF-kappaB activation**. *FEBS Lett* 2010, **584**(5):865-872.

401. Ikeda O, Sekine Y, Mizushima A, Oritani K, Yasui T, Fujimuro M, Muromoto R, Nanbo A, Matsuda T: **BS69 negatively regulates the canonical NF-kappaB activation induced by Epstein-Barr virus-derived LMP1**. *FEBS Lett* 2009, **583**(10):1567-1574.

402. Derijard B, Hibi M, Wu IH, Barrett T, Su B, Deng T, Karin M, Davis RJ: **JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain**. *Cell* 1994, **76**(6):1025-1037.

403. Gupta S, Campbell D, Derijard B, Davis RJ: **Transcription factor ATF2 regulation by the JNK signal transduction pathway**. *Science* 1995, **267**(5196):389-393.

404. Wan J, Zhang W, Wu L, Bai T, Zhang M, Lo KW, Chui YL, Cui Y, Tao Q, Yamamoto M *et al*: **BS69, a specific adaptor in the latent membrane protein 1-mediated c-Jun N-terminal kinase pathway**. *Mol Cell Biol* 2006, **26**(2):448-456.

405. Miller G, Shope T, Lisco H, Stitt D, Lipman M: **Epstein-Barr virus: transformation, cytopathic changes, and viral antigens in squirrel monkey and marmoset leukocytes**. *Proc Natl Acad Sci U S A* 1972, **69**(2):383-387.

406. Menezes J, Leibold W, Klein G, Clements G: **Establishment and characterization of an Epstein-Barr virus (EBC)-negative lymphoblastoid B cell line (BJA-B) from an exceptional, EBV-genome-negative African Burkitt's lymphoma**. *Biomedicine* 1975, **22**(4):276-284.

407. Jones MD, Foster L, Sheedy T, Griffin BE: **The EB virus genome in Daudi Burkitt's lymphoma cells has a deletion similar to that observed in a non-transforming strain (P3HR-1) of the virus**. *EMBO J* 1984, **3**(4):813-821.

408. Klein E, Klein G, Nadkarni JS, Nadkarni JJ, Wigzell H, Clifford P: **Surface IgM-kappa specificity on a Burkitt lymphoma cell in vivo and in derived culture lines**. *Cancer Res* 1968, **28**(7):1300-1310.

409. King W, Thomas-Powell AL, Raab-Traub N, Hawke M, Kieff E: **Epstein-Barr virus RNA. V. Viral RNA in a restringently infected, growth-transformed cell line**. *J Virol* 1980, **36**(2):506-518.

410. Graham FL, Smiley J, Russell WC, Nairn R: **Characteristics of a human cell line transformed by DNA from human adenovirus type 5**. *J Gen Virol* 1977, **36**(1):59-74.

411. Jansson A, Johansson P, Li S, Rymo L: **Activity of the LMP1 gene promoter in Epstein-Barr virus-transformed cell lines is modulated by sequence variations in the promoter-proximal CRE site**. *J Gen Virol* 2007, **88**(Pt 7):1887-1894.

412. Atanasiu C, Lezina L, Lieberman PM: **DNA affinity purification of Epstein-Barr virus OriP-binding proteins**. *Methods Mol Biol* 2005, **292**:267-276.

413. Makar KW, Ulgiati D, Hagman J, Holers VM: **A site in the complement receptor 2 (CR2/CD21) silencer is necessary for lineage specific transcriptional regulation**. *Int Immunol* 2001, **13**(5):657-664.

414. Adams A, Lindahl T, Klein G: **Linear association between cellular DNA and Epstein-Barr virus DNA in a human lymphoblastoid cell line**. *Proc Natl Acad Sci U S A* 1973, **70**(10):2888-2892.

415. Hsieh JJ, Henkel T, Salmon P, Robey E, Peterson MG, Hayward SD: **Truncated mammalian Notch1 activates CBF1/RBPJk-repressed genes by a mechanism resembling that of Epstein-Barr virus EBNA2**. *Mol Cell Biol* 1996, **16**(3):952-959.

416. Kao HY, Ordentlich P, Koyano-Nakagawa N, Tang Z, Downes M, Kintner CR, Evans RM, Kadesch T: **A histone deacetylase corepressor complex regulates the Notch signal transduction pathway**. *Genes Dev* 1998, **12**(15):2269-2277.

417. Strobl LJ, Hofelmayr H, Stein C, Marschall G, Brielmeier M, Laux G, Bornkamm GW, Zimber-Strobl U: **Both Epstein-Barr viral nuclear antigen 2 (EBNA2) and activated Notch1 transactivate genes by interacting with the cellular protein RBP-J kappa**. *Immunobiology* 1997, **198**(1-3):299-306.

418. Fortini ME, Artavanis-Tsakonas S: **The suppressor of hairless protein participates in notch receptor signaling**. *Cell* 1994, **79**(2):273-282.

419. Tamura K, Taniguchi Y, Minoguchi S, Sakai T, Tun T, Furukawa T, Honjo T: **Physical interaction between a novel domain of the receptor Notch and the transcription factor RBP-J kappa/Su(H)**. *Curr Biol* 1995, **5**(12):1416-1423.

420. Beatus P, Lundkvist J, Oberg C, Pedersen K, Lendahl U: **The origin of the ankyrin repeat region in Notch intracellular domains is critical for regulation of HES promoter activity**. *Mech Dev* 2001, **104**(1-2):3-20.

421. Ehebauer MT, Chirgadze DY, Hayward P, Martinez Arias A, Blundell TL: **High-resolution crystal structure of the human Notch 1 ankyrin domain**. *Biochem J* 2005, **392**(Pt 1):13-20.

422. Kurooka H, Kuroda K, Honjo T: **Roles of the ankyrin repeats and C-terminal region of the mouse notch1 intracellular region**. *Nucleic Acids Res* 1998, **26**(23):5448-5455.

423. Gupta-Rossi N, Le Bail O, Gonen H, Brou C, Logeat F, Six E, Ciechanover A, Israel A: **Functional interaction between SEL-10, an F-box protein, and the nuclear form of activated Notch1 receptor**. *J Biol Chem* 2001, **276**(37):34371-34378.

424. Oberg C, Li J, Pauley A, Wolf E, Gurney M, Lendahl U: **The Notch intracellular domain is ubiquitinated and negatively regulated by the mammalian Sel-10 homolog**. *J Biol Chem* 2001, **276**(38):35847-35853.

425. Demarest RM, Ratti F, Capobianco AJ: **It's T-ALL about Notch**. *Oncogene* 2008, **27**(38):5082-5091.

426. Dumont E, Fuchs KP, Bommer G, Christoph B, Kremmer E, Kempkes B: **Neoplastic transformation by Notch is independent of transcriptional activation by RBP-J signalling**. *Oncogene* 2000, **19**(4):556-561.
427. Kato H, Taniguchi Y, Kurooka H, Minoguchi S, Sakai T, Nomura-Okazaki S, Tamura K, Honjo T: **Involvement of RBP-J in biological functions of mouse Notch1 and its derivatives**. *Development* 1997, **124**(20):4133-4141.
428. Lu FM, Lux SE: **Constitutively active human Notch1 binds to the transcription factor CBF1 and stimulates transcription through a promoter containing a CBF1-responsive element**. *Proc Natl Acad Sci U S A* 1996, **93**(11):5663-5667.
429. Babcock GJ, Decker LL, Volk M, Thorley-Lawson DA: **EBV persistence in memory B cells in vivo**. *Immunity* 1998, **9**(3):395-404.
430. Herbst H, Dallenbach F, Hummel M, Niedobitek G, Pileri S, Muller-Lantzsch N, Stein H: **Epstein-Barr virus latent membrane protein expression in Hodgkin and Reed-Sternberg cells**. *Proc Natl Acad Sci U S A* 1991, **88**(11):4766-4770.
431. Chabot PR, Raiola L, Lussier-Price M, Morse T, Arseneault G, Archambault J, Omichinski JG: **Structural and functional characterization of a complex between the acidic transactivation domain of EBNA2 and the Tfb1/p62 subunit of TFIIH**. *PLoS Pathog* 2014, **10**(3):e1004042.
432. Portal D, Rosendorff A, Kieff E: **Epstein-Barr nuclear antigen leader protein coactivates transcription through interaction with histone deacetylase 4**. *Proc Natl Acad Sci U S A* 2006, **103**(51):19278-19283.
433. Mumm JS, Kopan R: **Notch signaling: from the outside in**. *Dev Biol* 2000, **228**(2):151-165.
434. Weinmaster G, Kintner C: **Modulation of notch signaling during somitogenesis**. *Annu Rev Cell Dev Biol* 2003, **19**:367-395.
435. Zimber-Strobl U, Strobl LJ: **EBNA2 and Notch signalling in Epstein-Barr virus mediated immortalization of B lymphocytes**. *Semin Cancer Biol* 2001, **11**(6):423-434.
436. Grasser FA, Gottel S, Haiss P, Boldyreff B, Issinger OG, Mueller-Lantzsch N: **Phosphorylation of the Epstein-Barr virus nuclear antigen 2**. *Biochem Biophys Res Commun* 1992, **186**(3):1694-1701.
437. Yue W, Gershburg E, Pagano JS: **Hyperphosphorylation of EBNA2 by Epstein-Barr virus protein kinase suppresses transactivation of the LMP1 promoter**. *J Virol* 2005, **79**(9):5880-5885.

438. Yue W, Shackelford J, Pagano JS: **cdc2/cyclin B1-dependent phosphorylation of EBNA2 at Ser243 regulates its function in mitosis**. *J Virol* 2006, **80**(4):2045-2050.
439. Yue W, Davenport MG, Shackelford J, Pagano JS: **Mitosis-specific hyperphosphorylation of Epstein-Barr virus nuclear antigen 2 suppresses its function**. *J Virol* 2004, **78**(7):3542-3552.
440. Leverson JD, Koskinen PJ, Orrico FC, Rainio EM, Jalkanen KJ, Dash AB, Eisenman RN, Ness SA: **Pim-1 kinase and p100 cooperate to enhance c-Myb activity**. *Mol Cell* 1998, **2**(4):417-425.
441. Rainio EM, Ahlfors H, Carter KL, Ruuska M, Matikainen S, Kieff E, Koskinen PJ: **Pim kinases are upregulated during Epstein-Barr virus infection and enhance EBNA2 activity**. *Virology* 2005, **333**(2):201-206.
442. Schutkowski M, Bernhardt A, Zhou XZ, Shen M, Reimer U, Rahfeld JU, Lu KP, Fischer G: **Role of phosphorylation in determining the backbone dynamics of the serine/threonine-proline motif and Pin1 substrate recognition**. *Biochemistry* 1998, **37**(16):5566-5575.
443. Hashimoto N, Ogashiwa M, Okumura E, Endo T, Iwashita S, Kishimoto T: **Phosphorylation of a proline-directed kinase motif is responsible for structural changes in myogenin**. *FEBS Lett* 1994, **352**(2):236-242.
444. Palermo R, Checquolo S, Bellavia D, Talora C, Screpanti I: **The molecular basis of notch signaling regulation: a complex simplicity**. *Curr Mol Med* 2014, **14**(1):34-44.
445. Lee HJ, Kim MY, Park HS: **Phosphorylation-dependent regulation of Notch1 signaling: the fulcrum of Notch1 signaling**. *BMB Rep* 2015, **48**(8):431-437.
446. Ranganathan P, Vasquez-Del Carpio R, Kaplan FM, Wang H, Gupta A, VanWye JD, Capobianco AJ: **Hierarchical phosphorylation within the ankyrin repeat domain defines a phosphoregulatory loop that regulates Notch transcriptional activity**. *J Biol Chem* 2011, **286**(33):28844-28857.
447. Song J, Park S, Kim M, Shin I: **Down-regulation of Notch-dependent transcription by Akt in vitro**. *FEBS Lett* 2008, **582**(12):1693-1699.
448. Shimizu K, Chiba S, Hosoya N, Kumano K, Saito T, Kurokawa M, Kanda Y, Hamada Y, Hirai H: **Binding of Delta1, Jagged1, and Jagged2 to Notch2 rapidly induces cleavage, nuclear translocation, and hyperphosphorylation of Notch2**. *Mol Cell Biol* 2000, **20**(18):6913-6922.

449. Han X, Ju JH, Shin I: **Glycogen synthase kinase 3-beta phosphorylates novel S/T-P-S/T domains in Notch1 intracellular domain and induces its nuclear localization**. *Biochem Biophys Res Commun* 2012, **423**(2):282-288.

450. Li N, Fassl A, Chick J, Inuzuka H, Li X, Mansour MR, Liu L, Wang H, King B, Shaik S *et al*: **Cyclin C is a haploinsufficient tumour suppressor**. *Nat Cell Biol* 2014, **16**(11):1080-1091.

451. Fryer CJ, White JB, Jones KA: **Mastermind recruits CycC:CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover**. *Mol Cell* 2004, **16**(4):509-520.

452. Garcia-Alai MM, Gallo M, Salame M, Wetzler DE, McBride AA, Paci M, Cicero DO, de Prat-Gay G: **Molecular basis for phosphorylation-dependent, PEST-mediated protein turnover**. *Structure* 2006, **14**(2):309-319.

453. Andersson ER, Sandberg R, Lendahl U: **Notch signaling: simplicity in design, versatility in function**. *Development* 2011, **138**(17):3593-3612.

454. Foltz DR, Santiago MC, Berechid BE, Nye JS: **Glycogen synthase kinase-3beta modulates notch signaling and stability**. *Curr Biol* 2002, **12**(12):1006-1011.

455. O'Neil J, Grim J, Strack P, Rao S, Tibbitts D, Winter C, Hardwick J, Welcker M, Meijerink JP, Pieters R *et al*: **FBW7 mutations in leukemic cells mediate NOTCH pathway activation and resistance to gamma-secretase inhibitors**. *J Exp Med* 2007, **204**(8):1813-1824.

456. Chiang MY, Xu ML, Histen G, Shestova O, Roy M, Nam Y, Blacklow SC, Sacks DB, Pear WS, Aster JC: **Identification of a conserved negative regulatory sequence that influences the leukemogenic activity of NOTCH1**. *Mol Cell Biol* 2006, **26**(16):6261-6271.

457. Ubersax JA, Ferrell JE, Jr.: **Mechanisms of specificity in protein phosphorylation**. *Nat Rev Mol Cell Biol* 2007, **8**(7):530-541.

458. Amanchy R, Periaswamy B, Mathivanan S, Reddy R, Tattikota SG, Pandey A: **A curated compendium of phosphorylation motifs**. *Nat Biotechnol* 2007, **25**(3):285-286.

459. Watanabe F, Teraoka H, Iijima S, Mimori T, Tsukada K: **Molecular properties, substrate specificity and regulation of DNA-dependent protein kinase from Raji Burkitt's lymphoma cells**. *Biochim Biophys Acta* 1994, **1223**(2):255-260.

460. Onorato JJ, Palczewski K, Regan JW, Caron MG, Lefkowitz RJ, Benovic JL: **Role of acidic amino acids in peptide substrates of the beta-adrenergic receptor kinase and rhodopsin kinase**. *Biochemistry* 1991, **30**(21):5118-5125.

461. Meggio F, Marin O, Pinna LA: **Substrate specificity of protein kinase CK2**. *Cell Mol Biol Res* 1994, **40**(5-6):401-409.

462. Izumi KM, Kaye KM, Kieff ED: **The Epstein-Barr virus LMP1 amino acid sequence that engages tumor necrosis factor receptor associated factors is critical for primary B lymphocyte growth transformation**. *Proc Natl Acad Sci U S A* 1997, **94**(4):1447-1452.

463. Kaye KM, Izumi KM, Mosialos G, Kieff E: **The Epstein-Barr virus LMP1 cytoplasmic carboxy terminus is essential for B-lymphocyte transformation; fibroblast cocultivation complements a critical function within the terminal 155 residues**. *J Virol* 1995, **69**(2):675-683.

464. Hao J, Shen R, Li Y, Zhang Y, Yin Y: **Cancer-testis antigen HCA587/MAGE-C2 interacts with BS69 and promotes its degradation in the ubiquitin-proteasome pathway**. *Biochem Biophys Res Commun* 2014, **449**(4):386-391.

465. Mitelman F, Mertens F, Johansson B: **A breakpoint map of recurrent chromosomal rearrangements in human neoplasia**. *Nat Genet* 1997, **15 Spec No**:417-474.

466. Plotnik JP, Hollenhorst PC: **Interaction with ZMYND11 mediates opposing roles of Ras-responsive transcription factors ETS1 and ETS2**. *Nucleic Acids Res* 2017.

467. Watson DK, McWilliams MJ, Lapis P, Lautenberger JA, Schweinfest CW, Papas TS: **Mammalian ets-1 and ets-2 genes encode highly conserved proteins**. *Proc Natl Acad Sci U S A* 1988, **85**(21):7862-7866.

468. Liu CD, Cheng CP, Fang JS, Chen LC, Zhao B, Kieff E, Peng CW: **Modulation of Epstein-Barr virus nuclear antigen 2-dependent transcription by protein arginine methyltransferase 5**. *Biochem Biophys Res Commun* 2013, **430**(3):1097-1102.

469. Radkov SA, Bain M, Farrell PJ, West M, Rowe M, Allday MJ: **Epstein-Barr virus EBNA3C represses Cp, the major promoter for EBNA expression, but has no effect on the promoter of the cell gene CD21**. *J Virol* 1997, **71**(11):8552-8562.

470. Robertson ES, Grossman S, Johannsen E, Miller C, Lin J, Tomkinson B, Kieff E: **Epstein-Barr virus nuclear protein 3C modulates transcription through interaction with the sequence-specific DNA-binding protein J kappa**. *J Virol* 1995, **69**(5):3108-3116.

471. Eliopoulos AG, Young LS: **Activation of the cJun N-terminal kinase (JNK) pathway by the Epstein-Barr virus-encoded latent membrane protein 1 (LMP1)**. *Oncogene* 1998, **16**(13):1731-1742.

472. Eliopoulos AG, Caamano JH, Flavell J, Reynolds GM, Murray PG, Poyet JL, Young LS: **Epstein-Barr virus-encoded latent infection membrane protein 1 regulates the processing of p100 NF-kappaB2 to p52 via an IKKgamma/NEMO-independent signalling pathway**. *Oncogene* 2003, **22**(48):7557-7569.

473. Saito N, Courtois G, Chiba A, Yamamoto N, Nitta T, Hironaka N, Rowe M, Yamamoto N, Yamaoka S: **Two carboxyl-terminal activation regions of Epstein-Barr virus latent membrane protein 1 activate NF-kappaB through distinct signaling pathways in fibroblast cell lines**. *J Biol Chem* 2003, **278**(47):46565-46575.

474. Kempkes B, Pawlita M, Zimber-Strobl U, Eissner G, Laux G, Bornkamm GW: **Epstein-Barr virus nuclear antigen 2-estrogen receptor fusion proteins transactivate viral and cellular genes and interact with RBP-J kappa in a conditional fashion**. *Virology* 1995, **214**(2):675-679.

475. Fahraeus R, Jansson A, Sjoblom A, Nilsson T, Klein G, Rymo L: **Cell phenotype-dependent control of Epstein-Barr virus latent membrane protein 1 gene regulatory sequences**. *Virology* 1993, **195**(1):71-80.

476. Sjoblom A, Nerstedt A, Jansson A, Rymo L: **Domains of the Epstein-Barr virus nuclear antigen 2 (EBNA2) involved in the transactivation of the latent membrane protein 1 and the EBNA Cp promoters**. *J Gen Virol* 1995, **76 (Pt 11)**:2669-2678.

477. Eisenbeis CF, Singh H, Storb U: **Pip, a novel IRF family member, is a lymphoid-specific, PU.1-dependent transcriptional activator**. *Genes Dev* 1995, **9**(11):1377-1387.

478. Kirchmaier AL, Sugden B: **Plasmid maintenance of derivatives of oriP of Epstein-Barr virus**. *J Virol* 1995, **69**(2):1280-1283.

479. Chen HS, Martin KA, Lu F, Lupey LN, Mueller JM, Lieberman PM, Tempera I: **Epigenetic deregulation of the LMP1/LMP2 locus of Epstein-Barr virus by mutation of a single CTCF-cohesin binding site**. *J Virol* 2014, **88**(3):1703-1713.

480. Arvey A, Tempera I, Tsai K, Chen HS, Tikhmyanova N, Klichinsky M, Leslie C, Lieberman PM: **An atlas of the Epstein-Barr virus transcriptome and epigenome reveals host-virus regulatory interactions**. *Cell Host Microbe* 2012, **12**(2):233-245.

481. Gilligan K, Sato H, Rajadurai P, Busson P, Young L, Rickinson A, Tursz T, Raab-Traub N: **Novel transcription from the Epstein-Barr virus terminal EcoRI fragment, DIJhet, in a nasopharyngeal carcinoma**. *J Virol* 1990, **64**(10):4948-4956.

482. Repic AM, Shi M, Scott RS, Sixbey JW: **Augmented latent membrane protein 1 expression from Epstein-Barr virus episomes with minimal terminal repeats**. *J Virol* 2010, **84**(5):2236-2244.

483. Murata T, Noda C, Narita Y, Watanabe T, Yoshida M, Ashio K, Sato Y, Goshima F, Kanda T, Yoshiyama H *et al*: **Induction of Epstein-Barr Virus Oncoprotein LMP1 by Transcription Factors AP-2 and Early B Cell Factor**. *J Virol* 2016, **90**(8):3873-3889.

484. Murata T: **Regulation of Epstein-Barr virus reactivation from latency**. *Microbiol Immunol* 2014, **58**(6):307-317.

485. Knipe DM, Lieberman PM, Jung JU, McBride AA, Morris KV, Ott M, Margolis D, Nieto A, Nevels M, Parks RJ *et al*: **Snapshots: chromatin control of viral infection**. *Virology* 2013, **435**(1):141-156.

486. Ryan AK, Rosenfeld MG: **POU domain family values: flexibility, partnerships, and developmental codes**. *Genes Dev* 1997, **11**(10):1207-1225.

487. Lu F, Chen HS, Kossenkov AV, DeWispeleare K, Won KJ, Lieberman PM: **EBNA2 Drives Formation of New Chromosome Binding Sites and Target Genes for B-Cell Master Regulatory Transcription Factors RBP-jkappa and EBF1**. *PLoS Pathog* 2016, **12**(1):e1005339.

488. Bloland PB, Boriga DA, Ruebush TK, McCormick JB, Roberts JM, Oloo AJ, Hawley W, Lal A, Nahlen B, Campbell CC: **Longitudinal cohort study of the epidemiology of malaria infections in an area of intense malaria transmission II. Descriptive epidemiology of malaria infection and disease among children**. *Am J Trop Med Hyg* 1999, **60**(4):641-648.

489. Rochford R, Cannon MJ, Moormann AM: **Endemic Burkitt's lymphoma: a polymicrobial disease?** *Nat Rev Microbiol* 2005, **3**(2):182-187.

490. Greenwood BM: **Possible role of a B-cell mitogen in hypergammaglobulinaemia in malaria and trypanosomiasis**. *Lancet* 1974, **1**(7855):435-436.

491. Gabrielsen AA, Jr., Jensen JB: **Mitogenic activity of extracts from continuous cultures of Plasmodium falciparum**. *Am J Trop Med Hyg* 1982, **31**(3 Pt 1):441-448.

492. Donati D, Zhang LP, Chene A, Chen Q, Flick K, Nystrom M, Wahlgren M, Bejarano MT: **Identification of a polyclonal B-cell activator in Plasmodium falciparum**. *Infect Immun* 2004, **72**(9):5412-5418.

493. Kataaha PK, Facer CA, Holborow EJ: **Plasmodium falciparum products enhance human lymphocyte transformation by Epstein-Barr virus**. *Clin Exp Immunol* 1984, **56**(2):371-376.

494. Aman P, Gordon J, Lewin N, Nordstrom M, Ehlin-Henriksson B, Klein G, Carstensson A: **Surface marker characterization of EBV target cells in normal blood and tonsil B lymphocyte populations**. *J Immunol* 1985, **135**(4):2362-2367.

495. Fujiwara S, Imadome K, Takei M: **Modeling EBV infection and pathogenesis in new-generation humanized mice**. *Exp Mol Med* 2015, **47**:e135.

496. Yajima M, Imadome K, Nakagawa A, Watanabe S, Terashima K, Nakamura H, Ito M, Shimizu N, Honda M, Yamamoto N *et al*: **A new humanized mouse model of Epstein-Barr virus infection that reproduces persistent infection, lymphoproliferative disorder, and cell-mediated and humoral immune responses**. *J Infect Dis* 2008, **198**(5):673-682.

497. Ma SD, Yu X, Mertz JE, Gumperz JE, Reinheim E, Zhou Y, Tang W, Burlingham WJ, Gulley ML, Kenney SC: **An Epstein-Barr Virus (EBV) mutant with enhanced BZLF1 expression causes lymphomas with abortive lytic EBV infection in a humanized mouse model**. *J Virol* 2012, **86**(15):7976-7987.

498. White RE, Ramer PC, Naresh KN, Meixlsperger S, Pinaud L, Rooney C, Savoldo B, Coutinho R, Bodor C, Gribben J *et al*: **EBNA3B-deficient EBV promotes B cell lymphomagenesis in humanized mice and is found in human tumors**. *J Clin Invest* 2012, **122**(4):1487-1502.