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Malin Berggren

Department of Clinical Chemistry and Transfusion Medicine
Institute of Biomedicine at Sahlgrenska Academy
University of Gothenburg

UNIVERSITY OF GOTHENBURG

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Vadå glömsk? Min hjärna är
inget lager utan en arbetsplats!

- Räpan

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ABSTRACT

Epstein-Barr virus (EBV) is a human herpes virus that infects over 90% of the world population. Once infection has occurred, the virus persists for life in its host, mainly in an asymptomatic, latent stage with only a few active viral genes. In immunosuppressed transplant patients, the virus is sometimes reactivated and may cause cell proliferation with the risk of developing post transplant lymphoproliferative disorder (PTLD).

Epstein-Barr virus nuclear antigen 1 (EBNA1) is important for virus replication and segregation in dividing cells and it is the only viral protein expressed in all dividing B cells and is therefore a key to monitoring the virus and possibly to detect early changes in viral activity.

This thesis focuses on regulation of EBNA1 expression in transplant patients and the discovery of an internal ribosome entry site (IRES) in the 5´ untranslated region of EBNA1 gene. The EBNA IRES enables translation of a downstream gene even if regular cap-dependent translation is impaired. We establish that the EBNA IRES main activity is located within the U exon of the EBV genome. This exon with its IRES function seems to be very important for EBV since it is also part of the EBNA 3, 4 and 6 transcripts (Paper I).

Expression of EBNA1 in peripheral blood is undetectable in a healthy population but may be demonstrable in immunosuppressed individuals. A comparison between transplant patients who were diagnosed with PTLD and transplant patients without these symptoms, showed a more than three fold incidence of EBNA1-expression in blood from PTLD patients. Detection of EBNA1 in peripheral blood may therefore be used in risk evaluation for post transplant lymphoproliferative disease among transplant recipients. We also found that the EBNA IRES is sometimes deleted in the process of mRNA alternative splicing in transplant patients, as we discovered from EBNA1 expression analysis. These transplant patients express both regular and alternatively spliced EBNA1 mRNA. This finding implicates a new model for EBV translational regulation through the deletion of an IRES element (Paper II).

Further we recognized a lung transplant recipient, with no sign of PTLD, who persistently expressed EBNA1 in peripheral blood. This patient has a rare underlying autoimmune disease called hypocomplementemic urticarial vasculitis syndrome (HUVS). HUVS is closely related to systemic lupus erythematosus, which has previously been proposed to be associated with EBV via autoimmune, cross-reactive antibodies against EBNA1. In this case study, we explored the possibilities for a similar connection between HUVS and EBV and found increased antibody response to EBNA1 epitopes in the patient serum when compared to sera from a matched transplant control and healthy blood donors. This is the first study of EBV expression in the HUVS context and further studies are needed to investigate the role of EBV in this disease (Paper III).

In paper IV, the activity of EBNA IRES during lytic induction was investigated. Two reporter vectors were designed, with and without the EBNA IRES, and were stably transfected into EBV-positive B cell lines representing three different types of latency. The transfected cells were induced to enter EBV lytic phase and the effects of the EBNA IRES on the reporter gene expression was studied at the protein and RNA levels. The results showed a 2-3 fold protein expression in induced cells transfected with the EBNA IRES compared with the induced cells with the vector lacking EBNA IRES. These data point to the potential of EBNA IRES activity during lytic EBV infection.

PUBLICATIONS

This thesis is based on the following papers:

I. Epstein-Barr virus U leader exon contains an internal ribosome entry site
Isaksson Å, **Berggren M** and Ricksten A. *Oncogene (2003) 22, 572-581.*

II. Alternative EBNA1 expression in organ transplant patients
Berggren M, Isaksson Å, Larsson U, Nilsson F, Nyström U, Ekman T, Löfvenmark J, Ricksten A. *J Med Virol (2005) 76(3):378-385.*

III. EBNA1 expression in a lung transplant recipient with hypocomplementemic urticarial vasculitis syndrome
Berggren M, Heinlen L, Isaksson Å, Nyström U, and Ricksten A. *J Med Virol (2007) 79(7):963-969.*

IV. EBNA IRES mediates translation during lytic induction of Epstein-Barr virus
Berggren M, Jasinska A, Isaksson Å and Ricksten A. *Manuscript 2008*

TABLE OF CONTENT

ABBREVIATIONS

APC	Antigen presenting cell
BL	Burkitt's lymphoma
cDNA	Complementary DNA
CTL	Cytotoxic T lymphocyte
Cp	The promoter in the *Bam*HI C fragment in the EBV genome
DNA	Deoxyribonucleic acid
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
eIF	Eukaryotic initiation factor
EMCV	Encephalomyocarditis virus
E2F	An eukaryotic transcription factor family
Fp	The promoter in the *Bam*HI F fragment of the EBV genome
GC	Germinal center
HD	Hodgkin's disease
HLA	Human leukocyte antigen (a.k.a. MHC)
HUVS	Hypocomplementemic urticarial vasculitis syndrome
IE	Immediate early
IFN	Interferon
IL	Interleukin
IM	Infectious mononucleosis
IR	Internal repeat
IRES	Internal ribosome entry site/segment
IRF	Interferon regulating factor
ITAF	IRES trans-acting factor
LMP	Latent membrane protein
LUC	Luciferase
mRNA	Messenger RNA
NK cell	Natural killer cell
NPC	Nasopharyngeal carcinoma
nt	Nucleotide
OriP	Origin of replication
OriPI	Part of OriP with family of repeats
PCR	Polymerase chain reaction
PTLD	Post transplant lymphoproliferative disease
Qp	The promoter in the *Bam*HI Q fragment of the EBV genome
Q-PCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcription PCR
snRNP	Small nuclear ribonuclear particle
TCR	T cell receptor
T_H cell	T helper cell
TR	Terminal repeat
UTR	Untranslated region
Wp	The promoter in the *Bam*HI W fragment of the EBV genome

INTRODUCTION

The Epstein-Barr virus (EBV) is a DNA virus of the γ herpes virus family and is one of the most common human viruses, estimated to infect over 90% of the world population. Infection is to a vast majority asymptomatic when infecting children, but may cause infectious mononucleosis (IM) with self-limiting lymphoproliferation in adolescents and adults. The virus persists for life in the host, with occasional reactivation of lytic cycle and viral shedding. EBV has evolved together with the human immune system and mimics and eludes many of this system's features in order to maintain infection. To avoid activation of the immune system and to promote co-existence with the host, it has an intricate system with complex gene regulation and different latency programs. Most infected individuals will therefore never recognize the EBV infection, but in some cases it will contribute to the development of different lymphomas and carcinomas like Burkitt's lymphoma (BL), Hodgkin's disease (HD), nasopharyngeal carcinoma (NPC) or lymphoproliferative disease in immunosuppressed individuals. It is the oncogenic and immune escaping features that make EBV such an interesting virus to study. Indeed, a great deal about cancer development, viral infection, immune response, gene regulation, molecular mechanisms etc. has been learned from EBV research since the discovery of the virus in the 60ies.

EBV INFECTION

EBV is mainly spread via saliva and infects epithelial cells in the naso- and oro-pharynx. From the epithelial layer it is passed to naïve B cells that border follicles in the tonsil lymph nodes. Upon infection, the naïve B cell is activated and differentiates into a lymphoblast that migrates into the follicle, starts to proliferate and form a germinal center (GC). The infected lymphoblasts interact with T cells and some of them become memory B cells. The memory B cells enter the blood circulation and pass through secondary lymphoid tissue where they may encounter a specific T helper cell that mediates plasma cell differentiation. The activated memory B cell then divides into one memory cell and one cell that becomes a plasma cell that migrates to the lymphoepithelium and enters lytic cycle. Infectious virus is released and the cell dies [Thorley-Lawson, 2005]. For a schematic presentation of the EBV infection see figure 1.

Most of the EBV genes are expressed during lytic phase while the latent infection is accompanied only by the expression of a few latent viral genes. The driving force of EBV research is mainly the relation between EBV and cancer and the evasion of immune response that is indeed intriguing. Studies of the mechanisms behind EBV latency have lead to and are still leading to revelations regarding the human immune system. The EBV lytic cycle has been less studied than the latent infection, probably because it is the latent genes that are the most prominently expressed during EBV-associated malignancies. There are however studies that suggest that NPC, BL and HD are preceded by reactivation of lytic EBV replication suggesting a role in disease etiology [Chan et al., 1991; Mueller et al., 1991].

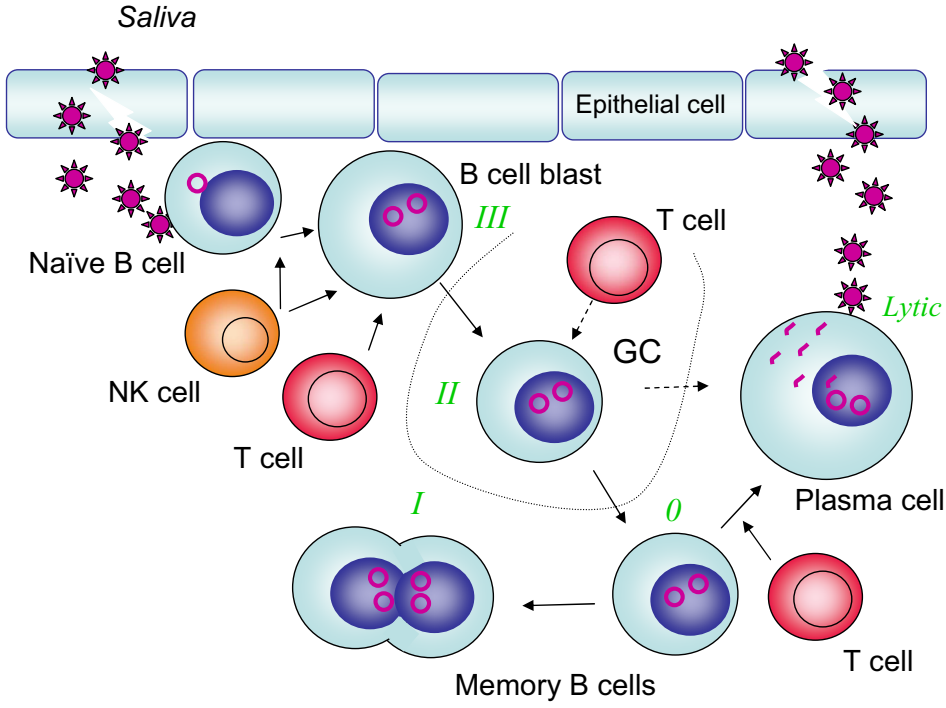

Figure 1
EBV virus particles are spread via saliva to infect epithelial cells in the oro-/naso-pharynx. The infected epithelial cells spread the virus to B cells in the tonsils and the viral DNA is released and forms a circular episome. Interaction with NK cells and T cells allows the infected B cell to differentiate into a B cell blast that moves into the lymphoid follicle and proliferates in a germinal center (GC). Latent viral gene expression and T cell interaction enable development of short lived plasma cells with EBV lytic infection or memory B cells that are latently infected with EBV. Memory B cells circulate in blood and passes through lymph nodes until they encounter a T cell that recognizes the presented, specific antigen and activates the B cell for antibody production, which triggers the EBV lytic phase. The plasma cell migrates towards the lymph epithelium and the virus is spread into the saliva. EBV expression during different stages of B cell differentiation is marked with green, roman numbers I-III, 0 and lytic, respectively.

Different patterns of latent expression are observed in the various stages of B cell differentiation, as listed in table 1. The EBV latent proteins and a summary of EBV lytic cycle are briefly described below.

Table1. Definitions of latency in relation to EBV protein expression and B cell differentiation.

	Host cell biology	Gene expression
Latency III	B cell blast	EBNA1-6, LMP1, LMP2
Latency II	B cell in germinal center	EBNA1, LMP1 and 2
Latency I	Dividing memory B cell	EBNA1
Latency 0	Non-dividing memory B cell	none

EBV latent proteins

EBNA1

Epstein-Barr virus nuclear antigen (EBNA) 1 is a protein that is necessary for replication of the viral genome and partitioning of the replicates during host cell mitosis. It is expressed in all infected cells except from memory B cells that are not dividing. EBNA1 has a DNA binding region and may function both as an activator and repressor of gene transcription. EBNA1 binds to the origin of replication (oriP) in the viral episome, which enhances activation of other latent genes and enables co-segregation of the viral genome in dividing B lymphocytes [Chadburn et al., 1997; Yates et al., 1985]. It also exert auto-repression through negative feedback on its own promoter Qp, located in the BamHI restriction fragment Q [Sample et al., 1992].

EBNA2

EBNA2 is essential for immortalization of B cells [Cohen et al., 1989; Hammerschmidt and Sugden, 1989]. It is the first latent gene to be expressed after primary infection and it activates transcription of EBV latent genes together with EBNA5. It is expressed during latency III when the cell is newly infected and reaches steady state within 24 h after infection [Kieff and Rickinson, 2001]. EBNA2 is involved in the switch from the EBV latent promoter Wp to Cp (located in BamHI restriction fragment W and C respectively) [Woisetschlaeger et al., 1991] and the up-regulation of latent membrane protein (LMP) via interaction with cellular DNA-binding proteins, RBP-Jκ in particular [Abbot et al., 1990; Johannsen et al., 1995].

EBNA3, 4 and 6

EBNA3, 4 and 6 (a.k.a. EBNA3A, B and C respectively) are closely related genes with partial sequence similarities and the same gene structure [Kieff and Rickinson, 2001]. They associate with cellular proteins and may work as transcriptional regulators [Robertson et al., 1996]. EBNA6 is able to activate the LMP1 promoter [Allday et al., 1993] and all three are able to bind RBP-Jκ and thereby disrupt its binding to EBNA2 and inhibit the trans-activating

function of EBNA2 [Cludts and Farrell, 1998; Radkov et al., 1997]. They may also interact with cellular cell cycle proteins and deregulate their function [O'Nions and Allday, 2004].

EBNA5

EBNA5 (a.k.a. EBNA-LP) varies in size due to variation of repeats in the coding region [Dillner et al., 1986]. It cooperates with EBNA2 in LMP1 promoter activation [Harada and Kieff, 1997; Peng et al., 2005] and may also have a function in transformation efficiency [Mannick et al., 1991]. There is accumulating evidence of multifunction of EBNA5, although the function in EBV biology is not yet fully understood.

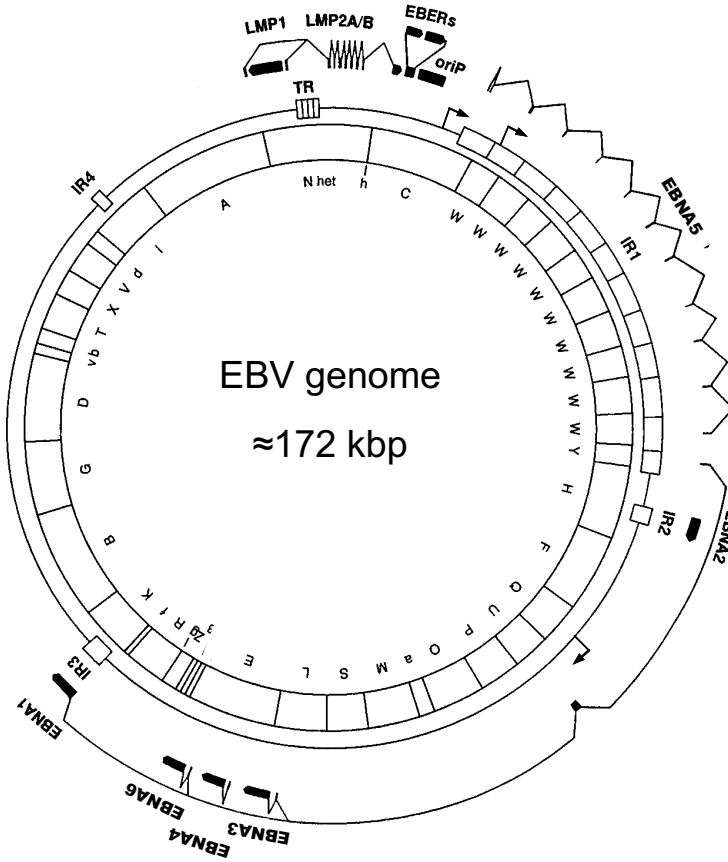

Figure 2
The EBV genome Bam HI restriction fragments are nominated alphabetically in respect to size. Position of the latent genes are marked with black boxes outside the circle and spliced as indicated with thin lines connecting the boxes. Terminal repeat (TR) and internal repeats (IR) are indicated with white boxes and EBV promoters, named after the restriction fragment they are positioned in Cp, Wp and Qp are indicated with black arrows. Modified from published figure in [Osato and Imai, 1996].

LMP1

LMP1 is a functional homologue to the human co-stimulatory protein CD40 and is a constitutively active receptor for tumor necrosis factor ligands. Interaction with ligands induces B cell proliferation and protects from apoptosis through activation of several signaling pathways that up-regulates anti-apoptotic proteins and provides growth signals [Hammerschmidt and Sugden, 1989; Soni et al., 2007]. LMP1 is essential for EBV immortalization and may on its own transform cells *in vitro* and is therefore considered an oncogene. LMP1 is expressed in most EBV related malignancies except Burkitt's lymphoma.

LMP2

The LMP2 gene encodes two related proteins LMP2A and 2B [Longnecker and Kieff, 1990]. LMP2A blocks the B cell receptor (BCR) signal transduction, inhibits activation of virus lytic cycle and protects from apoptosis [Longnecker et al., 2000; Miller et al., 1995]. The function of LMP2B is less clear. It shares the trans-membrane domain with 2A but lacks the B cell receptor-like cytoplasmic domain.

EBV lytic cycle

Reactivation of lytic infection is caused by extra-cellular signals. The most widely suggested is antigen stimulation of the B cell receptor. BCR cross linking leads to the expression of the two immediate early (IE) genes coding for transcription factors [Gold, 2002; Takada and Ono, 1989; Tovey et al., 1978]. The IE proteins trans-activate the viral early genes and alter host cell cycle processes [Cox et al., 1990; Feederle et al., 2000]. The early genes encode proteins responsible for replication, such as the viral DNA polymerase [Daibata and Sairenji, 1993]. Late genes produce structural proteins for packaging of the viral genome into virions ready to infect new cells [Israel and Kenney, 2005].

EBV GENE REGULATION

The genes of the EBV genome are expressed differently during different stages of viral infection and biological phases of the host cell. After infection the virus enters the latent stages where the viral strategy is to transform the infected B cells to become latently infected memory B cells. The majority of memory B cells remains latently infected while a portion of the cells, at various time points enters lytic cycle. The lytic phase is optimized to spread the virus to a new host. To minimize immunogenic exposure, it is of greatest importance to the virus to express the right amount of the right genes at the right time.

Transcriptional regulation

EBV latent gene transcription is regulated by different promoters, DNA methylation of promoter regions and negative and positive feedback from viral proteins in cooperation with host cell transcription factors [Speck, 2005]. During transcription the transcript is awarded a methyl-guanosine cap in the 5′ end and a poly-adenylated (poly (A)) tail in the 3′ end. These signals are needed for transportation to the cytoplasm and as a recognition signal to the ribosome. The pre-mRNA is often produced by one promoter but is alternatively spliced to facilitate coding for different genes.

Splicing

Splicing is the mechanism whereby transcribed introns are excluded from the pre-mRNA. It takes place directly after transcription and involves assembly of spliceosomes. The spliceosomes are complexes consisting of small nuclear ribonucleoparticles (snRNPs), which are different proteins associated with small RNAs. Splice donor and acceptor sites are recognized by snRNPs, which forms 3D structures of the pre-mRNA and catalyze excision of the introns and ligation of the exons. Together with the snRNPs there are other protein factors acting as splice enhancers and regulators of alternative splicing [Graveley, 2000]. The splice donor and acceptor sites are well-conserved sequences whereas intron sequences are in general not well-conserved. The 5′ splice donor site is covering the 3′ end of the first exon and the 5′ end of the intron. This site is cut by interaction of the spliceosome complex before cutting of the 3′ splice acceptor site in the 3′ end of the intron and the 5′ end of the second exon. The exons are then fused to produce the processed mRNA that will be transported out of the nucleus to be accessible for translation [Kramer, 1996]. EBV uses alternative splicing extensively since one promoter often is used to produce several different mRNAs. Alternative splicing may be accounted for by the protein composition of the spliceosome and other regulating proteins such as poly-pyrimidine binding protein that regulates alternative splicing by binding to poly-pyrimidine tracts upstream the spliced intron [Wagner and Garcia-Blanco, 2001]. The interacting proteins are known to vary in a tissue specific or developmentally regulated fashion. A schematic picture of alternative splicing is shown in figure 3.

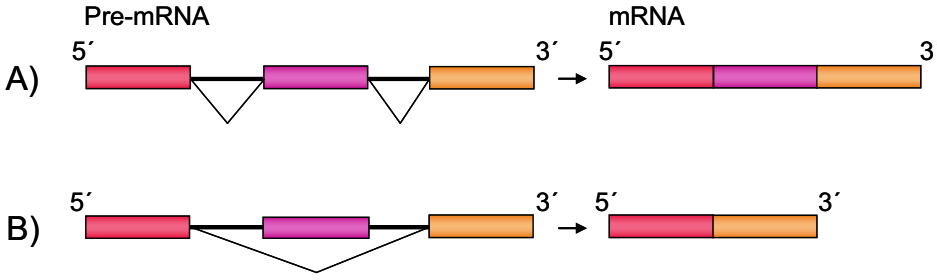

Figure 3
Alternative splicing of a transcript with three exons (colored boxes). The newly transcribed pre-mRNA is separated by two intron-sequences (black lines). In figure A the pre-mRNA is spliced (thin V-shaped lines) between the first and second exon and between the second and third exon. This results in an mRNA consisting of all three exons. In figure B the whole segment between the 3´end of exon one and the 5´end of exon three is excised by alternative splicing. The resulting mRNA only consists of exon one and three.

Auto-regulation

Upon primary infection of resting B cells, EBV uses the latent W promoter to produce all the EBNAs. Wp is located in the IR1 repeat region of EBV (see figure 2) and is therefore present in multiple copies that are thought to give EBNA transcription a jump start in the newly infected cell. EBNA5 and EBNA2 with their coding sequences situated closest to the promoter are the first detected proteins. EBNA2 up-regulates expression of LMP1 via interaction of cellular proteins and EBNA5 enhances the effect [Johannsen et al., 1995].

EBNA2 and EBNA5 have synergistic effects on the switch to the C promoter by interacting with cellular proteins at a binding site upstream the C promoter [Woisetschlaeger et al., 1991]. EBNA1 bind as homo-dimers to binding sites in the EBV oriP and they function together as an enhancer of the Wp, Cp and LMP promoters that are located in the vicinity [Gahn and Sugden, 1995; Nilsson et al., 1993; Puglielli et al., 1996; Reisman and Sugden, 1986].

Cp is the main promoter after the first 24 hours of latency III and is, as well as Wp, able to produce transcripts to all EBNAs and is also regulated by all EBNA proteins. EBNA1, EBNA2 and EBNA5 up-regulates Cp, whereas EBNA3 and 6 repress Cp activity via binding to cellular proteins [Cludts and Farrell, 1998; Radkov et al., 1997]. EBNA1 is transcribed from the Q promoter in latency I and II [Nonkwelo et al., 1996; Rowe et al., 1986].

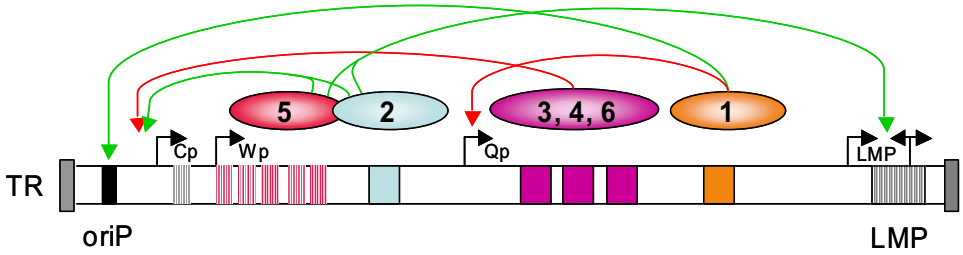

Figure 4
EBNA 1-6 proteins regulate EBV latent gene expression by enhancing (green arrows) or repressing (red arrows) promoter activity. EBNA1 (1) binds to the origin of replication (oriP) which enhances C and LMP promoter activity. EBNA1 also exerts negative feedback on the Q promoter (Qp). EBNA2 (2) and EBNA5 (5) work together to enhance Cp and LMP promoters. EBNA3, 4 and 6 (3, 4, 6) repress Cp activation.

Translational regulation

Protein synthesis is also a highly regulated process that post-transcriptionally regulates the protein levels in the cytoplasm. Once the mRNA is transcribed, processed and exported from the nucleus, the translation into protein is regulated by the presence or absence of elements that activate/inactivate translation or degrade the newly synthesized mRNA. Translation is always dependent on the presence of ribosomes and a number of initiation factors.

Cap-dependent translation

Cap-dependent translation is a process in which the eukaryotic initiation factors (eIF) 3, eIF1A and eIF2 make a 43S complex together with guanine triphosphate, methionine-tRNA and the ribosomal 40S subunit. The 43S complex attaches to the cap at the 5´ end of the mRNA transcript with the aid of the eIF4F protein adaptor complex consisting of four eIFs and the poly(A)-binding protein (PABP) that ties the ends of the mRNA together (see figure 5A). The translation complex moves along the mRNA until an appropriate initiation codon is encountered and the ribosomal 60S subunit is recruited [Gingras et al., 1999]. The majority of mRNAs require a 5´ cap structure in order to be translated, but there are also other mechanisms that may operate simultaneously or during inhibition of regular cap-dependent translation. The 5´ leader sequences of mRNA may form secondary structures that enable the ribosome to dock without the need of all canonical initiation factors. Such structures are called internal ribosome entry sites (IRES) [Jang et al., 1990].

Internal ribosome entry sites

IRESs are cis-acting elements that recruit the 40S ribosomal complex closer to the AUG translation initiation codon and thus allowing translation to be cap-independent.

The IRES function resembles the function of the Shine-Dalgarno sequence in prokaryotes, which is the signal for initiation of protein synthesis located upstream of the first AUG initiation codon [Shine and Dalgarno, 1975] although the translation process appears more complicated. Docking of the 40S ribosome subunit to an IRES does not demand all the

canonical initiation factors involved in cap-dependent translation and may therefore function under conditions where cap-dependent translation is inhibited by modification of these factors (figure 5B). Examples of this are: mitosis, viral infection, heat shock and cell differentiation [Cho et al., 2007; Fernandez et al., 2002; Komar and Hatzoglou, 2005; Kozak, 2005; Pyronnet et al., 2000].

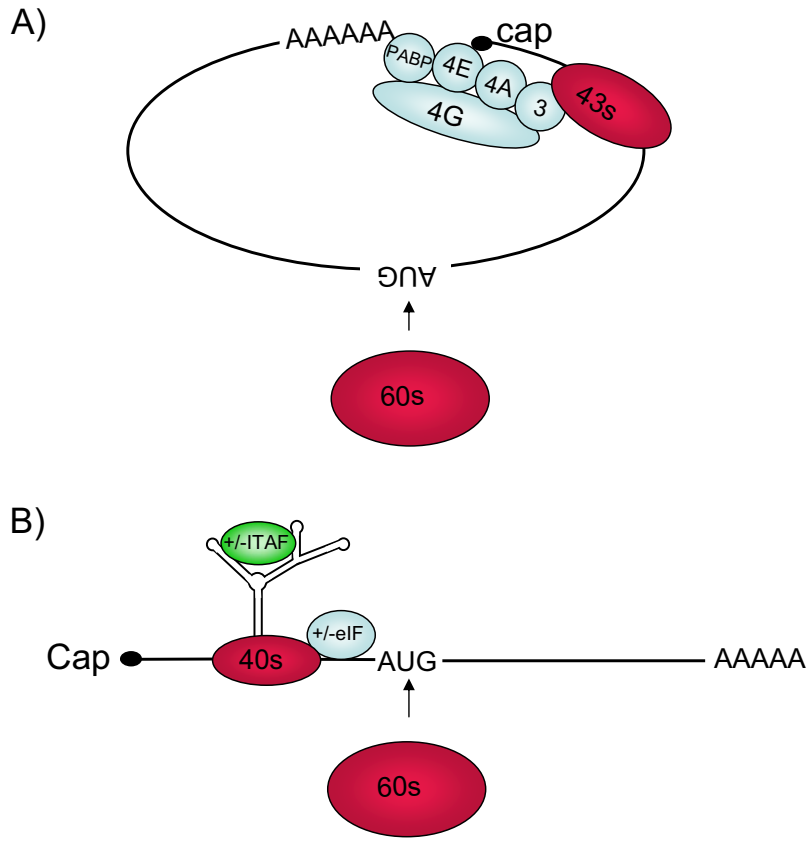

Figure 5
Cap-dependent translation initiation (A) and internal ribosome entry (B) are two mechanisms for translation initiation. ***A)*** *Many eukaryotic initiation factors: eIF3 (3), eIF4A (4A), eIF4E (4E), eIF4G (4G) and poly (A)-binding protein (PABP) are needed to connect the cap and the 43S complex (containing the 40S ribosomal subunit) and connect the far ends of the mRNA to promote a favorable translation. The 43S complex scans the mRNA until it finds the correct AUG site where the ribosomal 60S subunit is recruited and translation starts.* ***B)*** *An internal ribosome entry site forms a secondary structure that may recruit the 40S subunit in a position closer to the AUG initiation codon. Different IRES may or may not need eIFs or additional factors called IRES trans-activating factors (ITAFs) to initiate translation at the closely situated AUG site.*

IRESs are not a homogenous group of sequences or structures even though there are a few conserved motifs found in IRESs from different viral species [Lopez de Quinto and Martinez-

Salas, 1997]. In addition to some of the eIFs, there are other factors called IRES trans-acting factors (ITAFs) that are reported to be important for cap-independent translation for some IRESs. ITAFs are thought to function as chaperones stabilizing or modeling the RNA structure. Polypyrimidine tract binding protein has been identified in several cellular IRES initiation complexes and the RNA-binding protein La and the cold shock protein "upstream of N-ras", are functional ITAFs of both cellular and viral IRESs [Costa-Mattioli et al., 2004; Meerovitch et al., 1993; Spriggs et al., 2005]. The initiation factors needed for internal translation initiation varies between different species and IRES structures and has to be determined for each IRES individually.

The first IRESs were identified in encephalomyocarditis virus [Jang et al., 1988] and polio virus [Pelletier and Sonenberg, 1988], and have since been characterized in other viruses, such as flavi-, retro-, hepato- and herpes viruses [Griffiths and Coen, 2005; Isaksson et al., 2003; Low et al., 2001; Reynolds et al., 1995; Tsukiyama-Kohara et al., 1992]. However, cellular IRES elements have also been identified in mRNAs encoding proteins such as oncogenes, translational initiation factors, transcription factors, growth factors and survival proteins [Nanbru et al., 1997; Stoneley et al., 1998; Stoneley et al., 2000; van der Velden and Thomas, 1999].

EBNA1 regulation

EBNA1 is perhaps the most complexly regulated EBV latent gene since it is active in all stages of infection. It uses three alternative promoters Cp, Wp, and Qp for latent expression and a fourth, Fp, for lytic expression. The three latent promoters are used during different stages of latent infection. Qp is a TATA-less promoter that resembles eukaryotic housekeeping promoters that are active in many different cell types [Schaefer et al., 1995b]. It drives as main promoter transcription of EBNA1 during latency I and II and is moreover shown to be constitutively active in a variety of EBV-positive tumors and cell lines, irrespectively of the activities of other promoters or of viral latency [Tao et al., 1998]. There are many regulatory elements in the Qp region. EBNA1 has a low affinity binding site called the Q locus downstream of the promoter which enables negative feedback on EBNA1 transcription [Sample et al., 1992; Schaefer et al., 1997b]. Overlapping this site are two E2F-binding sites [Sung et al., 1994]. E2F is cell cycle dependent and may hinder EBNA1 repression at cell cycle entry when EBNA1 is important for viral replication [Davenport and Pagano, 1999]. Upstream of Qp there is an interferon regulatory factor (IRF) binding site where different IRFs bind and activate or down-regulate expression [Nonkwelo et al., 1997; Schaefer et al., 1997a; Zhang and Pagano, 1997; Zhang and Pagano, 1999]. There are also STAT binding sites that activate Qp via JAK or STAT binding [Chen et al., 1999].

During latency III, EBNA1 is expressed mainly from the W and C promoters. Due to promoter location, EBNA1 transcripts differ in the length of their 5´ UTRs but the U leader exon is common to all transcripts and spliced directly to the EBNA1 coding exon, see figure 6. Wp/Cp initiated EBNA1 transcripts contain several untranslated exons, which are rich in G and C nucleotides. According to the classical cap-dependent scanning model long leader sequences are expected to impair translation initiation by preventing the ribosome scanning from the capped mRNA 5´ end [Vagner et al., 2001]. We have however detected an internal ribosome entry site, the EBNA IRES, in the U exon that enables translation initiation much closer to the AUG initiation codon in all latencies and during lytic induction *in vitro* (paper I

and IV). The EBNA IRES function is regulated by alternative splicing that includes or excludes the U exon and the EBNA IRES (see figure 6), which was shown in peripheral blood from transplant patients (paper II).

Another post-transcriptional regulation lies in the glycine-alanine (Gly-Ala) repeat region of EBNA1. This repeat retards the translation efficiency, inhibits proteasomal degradation and decrease HLA antigen presentation [Levitskaya et al., 1997; Tellam et al., 2007; Yin et al., 2003].

In conclusion, to minimize immune recognition in memory B cells it is important to keep EBNA1 synthesis as low as possible while it must be readily initiated upon host cell division and B cell differentiation. To achieve this, EBNA1 expression is extensively controlled pre- and post-transcriptionally.

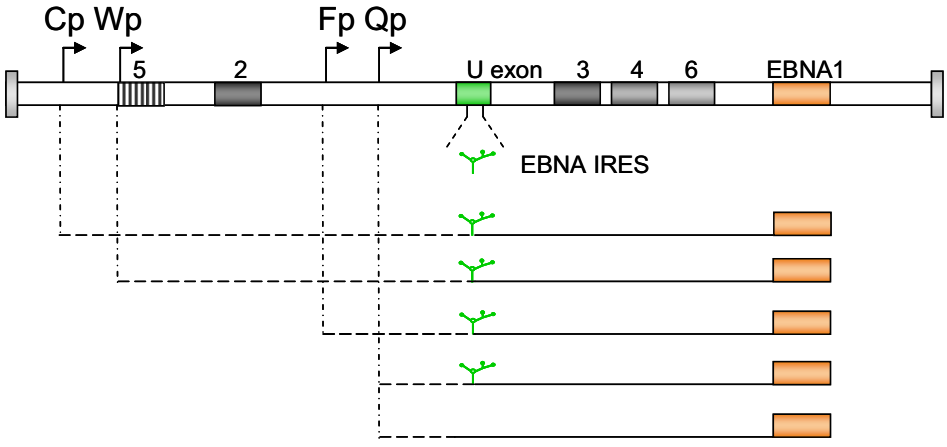

Figure 6
EBNA1 transcription is initiated from four different promoters used during different phases of infection. The 5´ untranslated region varies in length and splicing (indicated by dashed lines) but the U exon (green box) is included in most transcripts (green IRES structure). When the U exon is included, all transcripts are spliced as indicated with a thin black line, directly from the U exon to the coding region of EBNA1 (orange box). Although the primary transcript spans the entire sequence from the promoter to the coding sequence for EBNA1, EBNA2-6 (2-6), the coding exons of other EBNAs are all excluded from the final EBNA1 mRNA.

EBV VERSUS THE IMMUNE SYSTEM

Innate immunity

When EBV infects a B cell, this cell starts to express interferons (IFN) α and β. The IFNs enhance antigen processing in the cell, activate natural killer (NK) cells and inhibit viral transcription. The activated NK cells also produce IFNα and in addition IFNγ, which inhibits proliferation and transformation of the infected cell [Gao et al., 1999; Garner et al., 1984; Lotz et al., 1986]. Within 24 h of primary infection the first latent EBV nuclear antigens EBNA2 and EBNA5 reach steady state. EBNA2 and 5 confer resistance towards the anti-proliferative effect of IFNα [Aman and von Gabain, 1990] and trans-activate other latent genes for example LMP1 that protects the cell from IFNα-induced apoptosis [Henderson et al., 1991]. NK cells are normally able to kill infected cells but EBV overcomes this function and manages to keep the host cell alive. The innate immune defense is the first and only response to viral infection until the adaptive immune defense is fully activated after approximately 5 days.

Adaptive immunity

EBV is most visible to the immune system during lytic phase when the most EBV proteins are expressed and during early infection latency III. While the innate immunity works on the infected cells, the process of antigen presentation has started. Viral proteins are digested into peptides that are presented by human leukocyte antigen (HLA) molecules on the cell surface of antigen presenting cells. The peptide-HLA complex may be recognized by the adaptive immune system via T cell receptors on the cell surface of a CD8+ or a CD4+ T cell. The CD4+ T cell will enhance activation of CD8+ cells and induce EBV antibody production in B cells that will differentiate into plasma cells. Both CD4+ and CD8+ T cell produce IFNγ to enhance activation of the immune response and to inhibit proliferation of infected cells (figure 7A). Both CD8+ and CD4+ cells are able to kill infected B cells, but this procedure is largely inhibited during EBV lytic infection. Many of the expressed viral proteins function to down-regulate the expression of both HLA-I and -II and thus make it harder for the T cell receptors to find the presented antigen. CD4+ T cells polarize to T helper (T_H) cells of type 1 or 2. Polarization of T_H1 or 2 is depending on the antigen that is presented and on the cytokine milieu (figure 7B). During a viral infection the immune defense favors T_H1 polarization. T_H1 cells produce IFNγ and IL-2 that activate macrophages, inhibit viral replication and promote T cell proliferation. There are however studies showing that cytokines produced by T_H2 cells are over-expressed in HD and Aids-related malignant non-Hodgkin lymphomas [Herbst et al., 1996; Kapp et al., 1999]. This might be through the inhibition of T_H1 polarization by IL-10 that has been shown to be up-regulated by LMP1 *in vitro* [Lambert and Martinez, 2007].

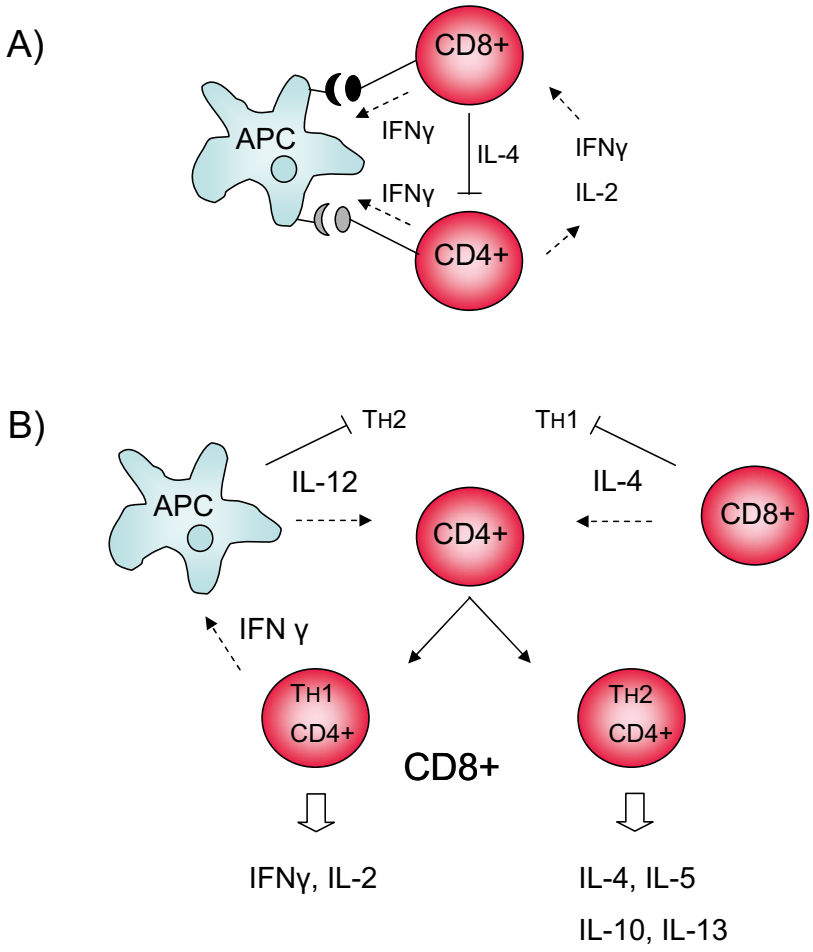

Figure 7
A) Adaptive immunity against EBV peptides presented by HLA class I (black crescent) and HLA class II (gray crescent) on the surface of an antigen presenting cell (APC). A CD4+ T cell with specific T cell receptor (TCR) (gray oval) bind to the HLA-II complex and release IL-2 and IFNγ, which together with the binding of the HLA-I complex (black oval) induces CD8+ T cell proliferation and differentiation into a cytotoxic T lymphocyte (CTL). The active CTL produces cytotoxic enzymes, IFNγ and IL-4. When enough IL-4 is produced by CTLs, CD4+ is down-regulated and thereby the activation of CD8+ cells. B) T helper cell polarization is depending on antigen, TCR interaction, co-stimulation of membrane proteins and cytokine milieu. IL-12 inhibits T_H2 but stimulates T_H1 polarization, which lead to production of IFNγ and IL-2 that inhibit viral replication, activate macrophages, induce apoptosis and promote T cell proliferation, which is favorable for fighting viral infections. T_H2 polarization is influenced by cytokines produced by CD8+ cells and T_H2 cells. They produce anti-inflammatory cytokines (IL-4, -10 and -13) that inhibit T_H1 and macrophage activation and stimulate B cell proliferation and antibody production (IL-4 and -5). T_H2 polarization is not favorable for fighting viral infections.

EBV ASSOCIATED DISEASES

The majority of EBV infections occurs in childhood and is asymptomatic. The reason for this is still not clear but could be related to the relatively immature immune system in children or exposure to lower doses. Because of the co-existence of EBV and the host immune system, it is not surprising that the EBV related diseases are largely caused by imbalances or defaults in the immune system. The virus may cause the imbalance but it is mostly malfunction of the host that keeps the immune defense from conquering an EBV associated disease. For example, Burkitt's lymphoma, Hodgkin's disease and Nasopharyngeal carcinoma are all associated with loss of antiviral T_H1 polarization either due to absence of protective cytotoxic T_H1 responses or presence of T_H2 favoring cytokines, allowing the outgrowth of tumor cells [Münz, 2005].

Infectious mononucleosis

Infectious mononucleosis is a disease that occurs in adolescents or young adults that have not been EBV infected as children. The primary EBV infection causes proliferation of B cells and is spread throughout the body to lymph nodes, spleen and liver. Proliferation of the infected B cells and EBV-specific CD8+ T cells cause most of the symptoms that are: fever, lymphadenopathy, splenomegaly and liver dysfunction [Vetsika and Callan, 2004]. IM patients have elevated serum levels of IL-10 [Taga et al., 1995] which suppress T_H1 polarization and may contribute to the inefficient immune response seen in IM patients. IM is however a self-limiting disease where 2-4 weeks of disease is a normal duration before proliferating cells are cleared [Vetsika and Callan, 2004].

Burkitt's lymphoma

Burkitt's lymphoma was the first cancer found to be associated with virus [Epstein et al., 1964]. BL is caused by a translocated, deregulated c-myc gene, [Klein and Klein, 1986; Leder, 1985], which by itself may produce the tumors. However, EBV is present in > 95% of the endemic BL that are found in Africa in geographical areas where malaria is common. One theory behind this co-existence is that the adaptive immune response to *Plasmodium falciparum* is T_H2 based [Good and Doolan, 1999; Riley, 1999] and the T_H2 cytokine milieu may be responsible for the decrease in T_H1 which diminishes EBV-specific immune control in the malaria patients [Whittle et al., 1984].

Hodgkin's disease

Hodgkin's disease exhibit unusual Hodgkin Reed-Sternberg cells in the germinal centers of lymph nodes. Up to 40% of the tumors are EBV-infected and there is association between HD and high titers of EBV-antigen before onset of the disease. There is also an increased incidence of the disease amongst patients who have had IM [Kapatai and Murray, 2007]. However, it is still debated if EBV may be a causative agent of HD or just a passenger in a memory B cell that has developed into a tumor cell.

Nasopharyngeal carcinoma

Nasopharyngeal carcinoma is different from the other described malignancies in the way that it affects epithelial cells. Unlike when the virus enters lytic phase and is spread to epithelial cells, these infected cells express latency II genes including LMP1 and 2, which are thought to contribute to malignancy. NPC is most common in Southern China and Southeast Asia, which indicates either genetic predisposition, environmental factors or differences between EBV-strains. Some strains are in fact under-represented in NPC [Edwards et al., 2004] and some HLA haplotypes over-represented. High antibody titers of both immunoglobulin G and A against many EBV antigens is a hallmark of NPC [Henle and Henle, 1975; Sam et al., 1989].

X-linked lymphoproliferative disease

X-linked lymphoproliferative disease is a rare and fatal immunodeficiency [Purtilo et al., 1975]. This disease is due to a gene mutation that leads to impaired regulation of IFNγ and reduced NK cell activity in males suffering from this disease [Parolini et al., 2000; Sumegi et al., 2000]. There is also evidence for the mutation causing defects in long term B cell memory [Crotty et al., 2003] leading to inability to differentiate EBV infected lymphoblasts into resting memory B cells. The result is masses of proliferating B cells that infiltrate tissues throughout the body accompanied by T cells and macrophages that try to defeat them.

Post transplant lymphoproliferative disease

Post transplant lymphoproliferative disease is a heterogeneous group of diseases which based on histology and clonality may be classified into four major categories according to the world health organization. It is a complication of immunosuppressive treatment after transplantation, which leads to decreased T cell function, uncontrolled proliferation of B cells and eventually lymphomas. Risk factors include total immune suppression and induction therapy [Birkeland and Hamilton-Dutoit, 2003; Caillard et al., 2005; Opelz and Dohler, 2004], EBV sero-negative recipients of EBV-positive allografts [Walker et al., 1995] and recently, published congenital cytokine polymorphisms was recognized as risk factors [Bakker et al., 2007; Lee et al., 2006].

Not all lymphomas in PTLD are EBV-positive. EBV is present in 60-80% of all PTLD cases and in 100% of PTLDs presenting within the first year after transplantation [Thompson and Kurzrock, 2004]. Other evidence for the pathogenic roll of EBV in PTLD is the strong association between EBV DNA load before onset of PTLD, monoclonal EBV-infection in tumors, treatment with EBV-specific cytotoxic T cells reduces tumor size, and finally EBV-infected PTLDs express latency III genes that are shown to transform B cells [Capello et al., 2005]. The majority of all PTLDs are of B cell origin but there are cases of T cell or NK cell origin as well [Nalesnik, 2001; Stadlmann et al., 2001].

PTLD diagnosis

PTLD is often presented in a non-specific way and lesions may occur literally anywhere in the body even though lymph nodes, allografts and digestive tracts are the most frequent sites [Bakker et al., 2005]. The early symptoms are often diffuse and easy to misinterpret. Therefore, early markers of proliferation are of greatest importance for successful treatment of

PTLD. Many patients with B cell lymphomas have high EBV DNA levels in peripheral blood which may be used to predict disease development [Riddler et al., 1994]. Although not conclusive, this is widely used to screen patients at risk. Higher accuracy of recognizing patients at risk may be individual monitoring of EBV DNA load together with EBV-specific T cell response where rising load and decreasing/missing T cell response could be indicative [Smets et al., 2002]. However, it needs to be said that to this date there is no standardized protocol for screening patients in risk of developing PTLD.

PTLD treatment

After PTLD diagnosis, there are some alternative treatments available. The primary treatment, that is usually sufficient for early lesions, is reduction of immunosuppressing agents, which allows improvement of T cell response to eliminate the proliferating cells. In combination with this or as secondary treatment it is increasingly common with anti-CD20 monoclonal antibodies. They induce apoptosis in mature B cells without affecting stem cells or plasma cells. This treatment has few side effects and has a response rate similar to that of chemotherapy, which is the choice for further progressed disease that does not respond to primary (or secondary) treatment. Chemotherapy is a common choice even though the mortality is high from treatment–related toxicity.

These are the major therapies used today but there are studies on adoptive T cell therapy, cytokine based therapy and treatment with anti-viral agents that have shown positive results. For a limited number of lesions it is also applicable with surgical excision or local radiation [Svoboda et al., 2006; Taylor et al., 2005].

THE PRESENT INVESTIGATION

In the present investigation we have taken a special interest in the expression and regulation of EBNA1 since it is the only viral gene expressed in all EBV-related malignancies and because EBNA1 is essential for maintaining EBV infection. An interest was directed towards the untranslated region of EBNA1 transcripts. What is the need for these long sequences? We found the U exon extra intriguing, since it is also part of other EBNA 5′ UTRs, and examined its function in extensive cell culture experiments. Furthermore we have had the great opportunity to cooperate with the department of Cardiology and thoracic surgery and the department of Oncology at Sahlgrenska University Hospital to collect clinical samples from transplant patients before and after transplantation. These samples have been of use for both mechanistic EBV studies and clinical research with post transplant lymphoproliferative disease in focus. The main findings from the work included in this thesis are described below.

Discovery of the EBNA IRES

The expression of EBNA1 is controlled at multiple levels. The transcriptional regulation of EBNA1 involves initiation from three alternative promoters, Wp [Ricksten et al., 1988; Sample et al., 1986], Cp [Woisetschlaeger et al., 1990], and Qp [Schaefer et al., 1995b; Tsai et al., 1995], which are used differentially during different phases of infection and establishment of the stages of latency. During the viral lytic cycle EBNA1 mRNA is transcribed from a fourth promoter called the Fp promoter [Nonkwelo et al., 1996; Schaefer et al., 1995a]. The spliced EBNA1 messages are similar in overall structure regardless of whether the transcripts are initiated from the Cp, Wp, Qp or Fp. The open reading frame for EBNA1 is located in the BamHI K exon at the 3′ end of the message and is preceded by a long potentially highly structured 5′ untranslated region derived from several short exons. The EBNA1 transcripts differ in the length of their 5′ UTRs but the U leader exon is common to all four transcripts and spliced directly to the K exon. The U exon is also included in 5′ UTR of the EBNA3, 4 and 6 mRNA transcripts [Sawada et al., 1989]. Our first aim was to investigate the importance of the U exon that is present in all these EBNA transcripts.

With reporter plasmids and *in vitro* transfection assays we found that sequences contained in the 5′ UTR of the Fp and Qp initiated EBNA1 mRNA increased the expression level 4-14 fold in different Burkitt lymphoma cell lines. This observation was done in both EBV-negative and EBV-positive cells and demonstrate that the EBNA1 5′ UTR has a regulatory role in gene expression through transcriptional or post-transcriptional control mechanisms.

The mRNA of the reporter gene transcript becomes longer and more complex in the presence of the 5′ UTR of EBNA1. According to the classical cap-dependent scanning model, long leader sequences are expected to impair translation initiation by preventing the ribosome to scan from the capped mRNA 5′ end [Vagner et al., 2001]. Ribosomes can, however, access mRNA by binding to an internal ribosome entry site. IRESs are cis-acting elements that recruit the translational machinery to an internal initiation codon in the mRNA thus allowing translation to be cap-independent. Many viral, and a number of cellular, mRNAs possess a long and GC-rich 5′ UTR with a potential to form complex secondary structures that make them difficult to translate with a cap-dependent ribosome scanning mechanism. The GC-richness and similarity of structural motifs in the EBNA1 5′ UTR compared to mRNAs with

known IRESs [Lopez de Quinto and Martinez-Salas, 1997; Nateri et al., 2000] led us to investigate if the 5´ UTR of EBNA1 also contains an IRES.

To test this hypothesis a bicistronic reporter system was used. We inserted the EBNA1 5´ UTR downstream of the Q promoter into the bicistronic vector pCAT-LUC between the CAT and the LUC gene. The construct was transfected into one EBV-negative BL cell line and two EBV-positive BL cell lines of latency I and II. The 5´ UTR of EBNA1 stimulated expression of the second cistron 7-20 fold in all cell lines when compared to a control plasmid. To further define the sequences responsible for this activity, a series of 5´ deletions of the EBNA1 UTR was made. The highest activity was seen in a segment starting from U exon nucleotide 36. It was clear from these results that the activity is not dependent on sequences in the Q exon. RNase protection analysis implied that the regulatory activity of 5´ UTR of EBNA1 is performed at post-transcriptional level. This hypothesis was further established by Northern blot analysis, which demonstrated that the bicistronic mRNAs were of the expected sizes. Thus we conclude that the U leader exon in the untranslated region of the of EBNA1 mRNA contains an IRES.

Our data demonstrate that the EBNA1 5´ UTR contains a translational element capable of directing internal ribosome entry and we therefore propose that EBNA1 protein synthesis may be initiated by such a mechanism. This suggests that the EBNA1 protein can be translated during situations when regular cap-dependent translation is reduced. The mechanism whereby the EBNA IRES recruits the translational machinery to the mRNA is not yet investigated but the variation in efficiency between different cell lines implies that cellular- or EBV latency-dependent factors are involved. There are a number of situations where modulation in the levels of EBNA1 protein through internal ribosome entry may be required including viral lytic infection, onset of proliferation and during mitosis where cap-dependent translation is reduced. The expression of EBNA1 mRNA is regulated by the cell cycle during type I latency, while the EBNA1 protein level remains constant [Davenport and Pagano, 1999]. One explanation to this observation is that the EBNA1 protein is stable with a long half-life but post-transcriptional regulation could also be a contributing factor. Our findings of a functional IRES element in the 5´ untranslated region of the EBNA1 gene implicate a novel mechanism whereby EBV regulates latent gene expression. This EBNA IRES element is also contained within the EBNA3, 4 and 6 mRNAs, demonstrating that part of the EBV immortalization process might be regulated by cap-independent translation.

The EBNA IRES is active during lytic induction

EBNA1 transcripts with the EBNA IRES containing U exon are expressed during all stages of EBV infection. EBNA 3, 4 and 6 transcripts that also contain the EBNA IRES are expressed during growth program (latency III) in B cell blasts and during lytic induction of latency I cell lines [Touitou et al., 2003]. The EBNA IRES activity has been studied in transfected EBV-negative cells and EBV-positive cells of latency I and II, where it is proven to increase translation (paper I and II). However, since U exon-containing EBNA1, 3, 4 and 6 transcripts are initiated from the F promoter during lytic cycle [Sample et al., 1991; Schaefer et al., 1991; Touitou et al., 2003; Zetterberg et al., 1999] it is probable that the EBNA IRES has a functional role in all stages of infection.

It has previously been reported that transfection can be very efficient when using an EBV-based vector carrying the EBNA1 gene and oriP region from the EBV genome [Mazda,

2002]. EBNA1 facilitates the retention of EBV in dividing cells, nuclear localization, transcription and replication of the plasmid DNA through the binding to oriP [Reisman et al., 1985; Wysokenski and Yates, 1989]. This mechanism can be used to express transfected vectors specifically in EBNA1-positive cells if the EBNA1 gene is not included in the vector [Hirai et al., 1997; Reisman et al., 1985]. To investigate EBNA IRES activity during various cell situations, appropriate constructs for transient and stable cell transfection were needed.

In paper IV, we used the oriPI, a part of oriP containing the EBNA1-binding family of repeats, to achieve enhancement of a downstream promoter [Puglielli et al., 1996], in this case Cp. In our constructs Cp drives the expression of a luciferase reporter gene (LUC) with or without the EBNA IRES cloned upstream. The hygromycin-resistance gene was included for selection and propagation of transfected cells to obtain stably transfected cells.

First the constructs were tested in transient transfections in one EBV-negative and three EBV transformed cell lines of latency I-III. Luciferase activity was measured after transfection, and the results from the EBNA IRES-containing vector were compared to the activity of the EBNA IRES-lacking vector. In the EBV-positive cell lines the luciferase activity is 12-17 fold higher in the cells transfected with the EBNA IRES. These results confirm earlier studies on the effect of the EBNA IRES and add proof of EBNA IRES functionality in latency III.

Transient transfection of the EBNA1-negative cell line showed that the expression was truly specific as we could not detect any luciferase activity from either construct. This inactivity could however be surmounted by the co-transfection of an EBNA1-expressing plasmid after which the same pattern as in the EBV-positive cells could be seen (figure 8).

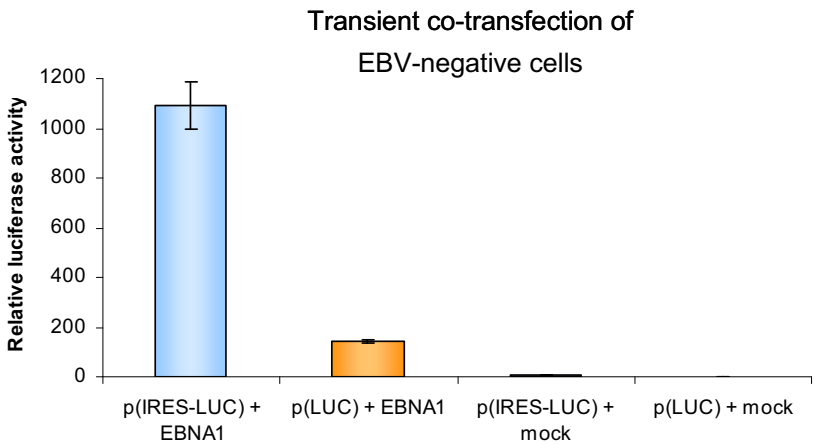

Figure 8
Luciferase activity was measured 48 h after transient plasmid transfections of an EBV-negative BL cell line. The p(IRES-LUC) plasmid contains the EBNA IRES and p(LUC) does not. The cells were co-transfected with an EBNA1-expressing plasmid (EBNA1) or a mock plasmid. The luciferase activity was related to the background activity of a promoter-less control plasmid.

Knowing that the constructs worked and were specific we used these vectors to generate stably transfected cell lines from EBV-positive cell lines of latency I-III, and used them to study EBNA IRES activity during lytic induction. After establishment, the cells were induced to enter lytic cycle or mock induced. After induction and incubation, RNA and protein were harvested and analyzed with luciferase assay, quantitative PCR and Western blot.

Summarized data show that the presence of the EBNA IRES yields higher LUC activity during lytic induction of stably transfected latency I and II cell lines than cells without EBNA IRES. In induced latency I cells the effect of the EBNA IRES was maintained LUC activity after lytic induction compared to the IRES-lacking plasmid where LUC activity was reduced. The effect of the EBNA IRES was most pronounced in induced latency II cells, where induced cells showed a four fold increase in activity compared to non-induced cells. In the latency III cell lines the LUC activity was maintained at the same level after lytic induction in both the cell line with the EBNA IRES-containing construct and the cell line with the EBNA IRES-lacking construct.

We hereby demonstrate that the EBNA IRES is capable of directing internal translation during lytic induction as well as during latent infection of type I, II and III. In addition, ribosome internal initiation was shown to be more effective during lytic induction than in corresponding non-induced cells of latency I and II. This could be because of lack of competition for initiation factors since translation initiation through IRES most commonly uses some, but not all, of the canonical cellular initiation factors used for cap-dependent translation. When cap-dependent translation is down-regulated by restriction in available initiation factors, there might still be other intact initiation factors to entertain IRES activity.
No increased effect of the EBNA IRES on LUC activity was seen after induction of latency III cells. This might be due to the fact that B95-8 already has a proportion of cells in lytic phase and induction did not succeed to push enough additional cells into lytic cycle for it to make a significant difference in reporter assay evaluation. To evaluate the significance of EBNA IRES in latency III cells, further studies are needed.

Alternative splicing of EBNA1 in transplant patients

We describe the detection and characterization of a novel splice variant within the 5´ UTR of the EBNA1 gene in samples from EBNA1-positive organ transplant patients. The alternative splice is shown to delete the EBNA IRES element and indicates the potential of EBV to introduce post-transcriptional regulation.

Together with normal Q promoter-initiated EBNA1 transcripts, an alternatively spliced form of the EBNA1 transcript was expressed in peripheral blood cells in the above mentioned organ transplant patients. This transcript lacks the U leader exon in the 5´ UTR. We have previously identified and characterized a functional internal ribosome entry site in the untranslated U leader exon of EBNA1 (paper I). In this study transfection experiments with EBNA1 coding plasmids followed by Western blot showed that the EBNA IRES promotes cap-independent translation and increases the EBNA1 protein level. The alternative EBNA1 transcript lacking this function is expressed in the majority of the investigated EBNA1-positive patient samples as well as in several latency III cell lines like CBC-Rael, B95-8, Mutu III, and in induced lytic phase P3HR1 and B95-8 [Zetterberg et al., 1999]. Alternative splicing in this form gives EBV the potential to regulate translation of EBNA1 by modifying

the 5´ UTR. These findings indicate a new regulating mechanism for EBNA1 expression *in vivo*.

Alternative splicing in the 5´ UTR has been reported to mediate translational regulation in the human nNOS gene [Newton et al., 2003] and in the glutamine synthetase gene [Shin et al., 2003]. In contrast to our finding, these articles report a repressing translational effect from an additional 5´ UTR exon acquired by alternative splicing.

Expression of the two alternatively spliced EBNA1 mRNAs were not consistent in serial samples from the same patient, but seemed to vary at different time points. It is possible that both transcripts are constitutively transcribed, although not always detected due to either low amounts or PCR competition. The PCR data indicate that the novel EBNA1 transcript is initiated from the Q promoter. This is consistent with previous data suggesting that Qp is the dominant latent EBNA1 gene promoter [Schaefer et al., 1995b; Tsai et al., 1995; Zetterberg et al., 1999] constitutively active in a variety of EBV-positive tumors and cell lines, irrespective of the activities of other promoters or of viral latency [Tao et al., 1998]. Qp-initiated EBNA1 transcripts are known to be down regulated by EBNA1 binding to the Q locus located downstream of the Q promoter [Sample et al., 1992]. The alternative splice may be an additional mechanism for regulating the EBNA1. Alternative splicing as a mean of EBNA1 down-regulation may be an adaptive step to avoid cytotoxic T cell recognition and thereby promote survival and progression of EBV in transplant patients.

The U exon is, except from the EBNA1 transcript, also part of the 5´ non-coding region of the EBNA3, 4 and 6 transcripts. It has also been shown to exist in other transcripts like in a 3.7 kb mRNA transcript with fragments from the W, Y, U, E and K genomic regions [Speck and Strominger, 1985], and upstream the internal repeat leader exons W1 and W2 in lymphoblastoid cell lines [Qu and Rowe, 1995]. The EBNA IRES may therefore be of importance for the regulation of other EBV latent genes as well as EBNA1.

Higher incidence of EBNA1 mRNA in PTLD patients

Post transplant lymphoproliferative disorder is an EBV-associated lymphoid growth that occurs in immunosuppressed organ transplant patients. Lymphomas from these patients often express an EBV latency III pattern with all EBNAs expressed, but at an early stage there might just be EBNA1 expression in the dividing B cell. In paper II we investigate if detectable EBNA1 expression in peripheral blood is associated with an increased risk of PTLD in organ transplant patients.

In order to identify patients at risk for developing PTLD, a sensitive, nested RT-PCR method for detection of EBNA1 gene expression in peripheral blood cells was used. Peripheral blood samples from 60 patients undergoing organ transplantation at Sahlgrenska University Hospital, Göteborg, Sweden, were collected at different time points during post transplant checkups or PTLD treatment. From the patients included in the study, 21 were diagnosed with PTLD and 39 showed no symptoms of lymphoproliferative disease. EBNA1 expression in organ recipients was analyzed and compared with a control group of 24 healthy blood donors. EBNA1 expression was found in 43% of the patients with PTLD and in 18% of the transplanted without symptoms. EBNA1 could not be detected at all in the control group. In conclusion from statistical analysis, there is a significantly higher incidence of EBNA1 expression in peripheral blood cells from organ transplant patients with PTLD than for other

transplant patients (odds ratio: 3.42; 95% CI= 1.02-11.54). A larger cohort and further studies of the on-set of EBNA1 expression in relation to PTLD diagnosis are however needed in order to establish if EBNA1 expression in peripheral blood from transplant patients could be used as a risk factor for PTLD. The EBNA1-positive transplant patients with no symptom of PTLD should be examined regularly for the expression of EBNA1 together with viral load in order to evaluate if they have an increased risk for developing PTLD. The absence of detectable EBNA1 transcripts in some of the patients with diagnosed PTLD might reflect the individual variation of EBV-infected cells, which is also seen in quantitative evaluation of EBV genomic load [Qu et al., 2000] [Wagner et al., 2001].

In a previous study [Brink et al., 2001] it was shown that lymphomas from immunocompromised patients were of both Cp and Qp origin. The present study shows that the majority of the EBNA1 transcripts were initiated from Qp. Low activity from Fp suggest that there is only little shift from latency to lytic program in peripheral blood cells, but the underlying reason for increased EBNA1 expression in these immunosuppressed patients is probably increased B cell proliferation with enhanced EBV genome replication. Increased replication gives a detectable level of EBNA1 in peripheral blood, in contrast to healthy blood donors where the virus remains in latency program (latency 0) and do not express detectable levels of EBNA1 [Miyashita et al., 1997]. In summary, the data presented in paper II shows a more than three fold incidence of EBNA1 positive blood samples from PTLD patients compared to patients with no signs of PTLD, which may be used in risk evaluation for PTLD.

EBNA1 expression in a patient with autoimmune disease

EBNA1 expression in blood is detected only sporadically in the normal, healthy population. As formerly reported in paper II, the majority of transplant patients do not express EBNA1 in peripheral blood, even if there is a three fold incidence in PTLD patients. The patients who do express EBNA1 generally have a variable expression from one time point to the next.

In paper III we report a lung-transplant patient with hypocomplementemic urticarial vasculitis syndrome (HUVS) who expressed EBNA1 in 80% of the samples tested during a period of 91 days after transplantation. This is an incidence four times higher than what is seen in other EBNA1-expressing transplant patients without HUVS or PTLD (paper II). This patient had however no signs of lymphoproliferative disease during or after the 91-day sample collection period.

Evaluation of viral expression in peripheral blood, serum and graft tissue was performed with RT-PCR, Q-PCR, indirect immunofluorescence, anti-peptide assays and *in situ* hybridization. Samples were collected at various time-points up to 91 days post transplantation. The patient expressed EBNA1 in 8/10 of the peripheral blood samples tested during the post transplantation period. After PCR analyses of indicative EBV mRNA, EBNA1 expression was mainly found to be Qp-initiated EBNA1, known to be important for EBV maintenance. Interestingly, EBNA1 was expressed already on the day of transplantation. This was not seen in any of the 60 transplant patients previously screened with this RT-PCR system (paper II). The EBNA1 transcripts seen the day of transplantation in this patient cannot be caused by heavy immunosuppression, which is usually the cause of lymphoproliferative disease and EBV expression in other cases since the patient had not yet fully started immunosuppressive therapy.

There are no previous reports of an association between HUVS and EBV, but there are several studies showing an association with EBV and other autoimmune diseases. For example, multiple sclerosis where possible disease mechanisms are cross-immunization, between microbial and self-antigens, epitope spreading and super-antigens [Haahr and Hollsberg, 2006]. Several studies have implicated EBV in the pathogenesis of systemic lupus erythematosus [James et al., 1997; Poole et al., 2006] and rheumatoid arthritis [Balandraud et al., 2004]. It is also shown that systemic lupus erythematosus (SLE) patients' anti-EBNA1 response is distinct from, and less restricted than, matched normal individuals [McClain et al., 2006]. Additionally, increased levels of EBV DNA as well as increased antibody response against EBV have been reported in Sjögren's syndrome patients [Inoue et al., 1991; Saito et al., 1989; Yamaoka et al., 1988]. These reports all suggest that EBV infection and expression precede the development of autoimmune disease. Poole et al. 2006 present a developmental model which may be applicable for any autoimmune disease, where genetic predisposition combined with EBV infection lead to B cell proliferation, cross-reactive antibodies and the subsequent development of pathogenic auto-antibodies.

Consequently, serum samples from the HUVS patient, one matched lung transplant patient with chronic obstructive pulmonary disease and four healthy EBV-positive blood donors were analyzed for anti-EBNA1 humoral immune responses. Compared to the blood donors, the vasculitis syndrome patient's antibodies bound several EBNA1 epitopes at significantly higher levels and in a wider range of epitopes. About half of the significant epitopes found in the vasculitis syndrome patient coincided with the response seen in the matched transplant patient, but the other half was specific to the vasculitis syndrome patient (figure 9).

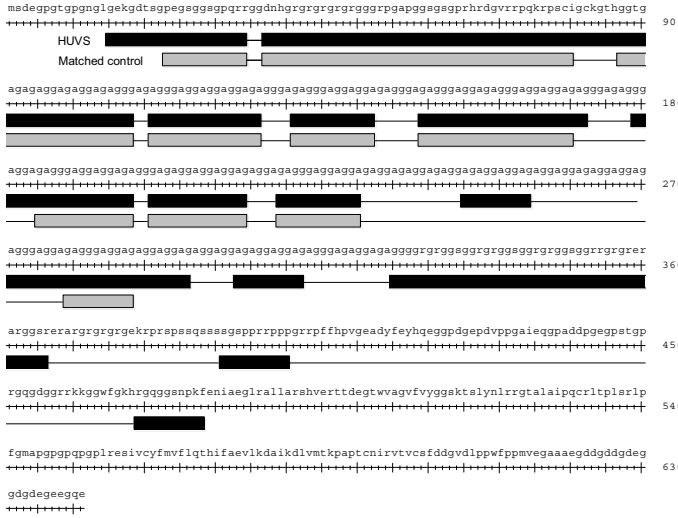

Figure 9
EBNA1 amino acid sequence (1- 641). Black boxes underneath the sequence represent peptides that have more than 2 SD above normal mean response in the HUVS patient. Gray boxes represent the equivalent in a matched control lung transplant patient with a background of chronic obstructive pulmonary disease.

This result may reflect a difference due to the vasculitis syndrome or may reflect individual variation. Serum from the patient on day 0 was compared to serum from day 39, post transplantation. Serum from day 0 gives a stronger signal in the whole Gly-Ala rich region than serum from day 39. This reduction is likely accounted for by the heavy immunosuppression received after transplantation.

Conclusions from analyses on blood, sera and graft tissue from a patient with HUVS are that this patient expresses Qp-initiated EBNA1 in blood more persistently than other lung transplant patients, which is a sign of ongoing B cell proliferation. Evaluation of the patient's medical record show that the persistent EBNA1 expression in this case neither is caused by PTLD nor from reactivation due to heavy immunosuppression since there is evidence for EBNA1 expression at the time of transplantation. The anti-EBNA1 response in the vasculitis syndrome patient is much stronger before than after transplantation as can be expected after immunosuppressive treatment. However, the patient had antibodies against a wider range of EBNA1 epitopes than the normal controls and the matched transplanted control patient. This result is in agreement with what has been shown in SLE patients [McClain et al., 2006].

Although a larger cohort of patients is needed to establish a relationship between the vasculitis syndrome and EBV our case report supports the hypothesis that EBV, and specifically persistent EBNA1 expression, could be involved in the etiology and/or pathogenesis of hypocomplementemic urticarial vasculitis syndrome.

CONCLUSIONS AND FUTURE PERSPECTIVE

This thesis introduces a new regulatory mechanism for EBNA1 expression and suggests a role for non-coding mRNA within EBNA transcripts. The regulation of EBNA1 is growing more and more complex as many control mechanisms work on different levels to keep a low expression level to maintain EBV infection but to limit exposure to the immune system. We have unveiled the importance of the EBV U exon by the discovery of the EBNA IRES that constitutes a, for EBV, new type of translational regulation. The EBNA IRES is not only part of the 5´ UTR of EBNA1 transcripts but also EBNA 3, 4 and 6 and a few other less elucidated transcripts [Touitou et al., 2003; Zetterberg et al., 1999].

We have found a mechanism that regulates the IRES function by alternative splicing (i.e. deletion) of the U exon in EBNA1 transcripts from transplant patients. Furthermore, EBNA1 expression in transplant patients is found to be more abundant in patients suffering from PTLD, which could be used as a risk marker for disease when examining transplant patients.

EBNA1 is also exceptionally expressed in a patient with autoimmune disease, which may reflect a similar relation as seen in other autoimmune diseases where EBV is thought to be a possible actor in disease etiology.

Since EBNA1 is expressed in all EBV-related malignancies, targeting the EBNA1 gene would be useful for elimination of malignant cells. Using an EBNA1-dependent system, like the one we used in the study of IRES in lytic phase, could be a tool for specific expression of apoptosis-inducing genes in EBNA1-expressing cells. We have made a successful pilot study with EBNA1 depending expression of Herpes simplex virus 1 thymidine kinase, which induces apoptosis in transfected, EBNA1-positive cells upon co-treatment with the nucleoside analog Ganciclovir. Over-expression of cellular apoptosis-inducing genes may be an alternative to make use of our findings in a therapeutic setting.

Increased knowledge of EBV-expression in patients with EBV-related diseases, as well as finding what molecular mechanisms regulate EBV gene expression is important to help us find markers for detection and potential anti-viral targets for therapy of these diseases.

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