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Characterization of non-coding mRNA in Epstein-Barr virus

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Till Erik och Tove

"Man kan inte planera allt"

ABSTRACT

Epstein-Barr virus (EBV) is a human gammaherpesvirus that infects lymphoid and epithelial cells. The virus is the causative agent of infectious mononucleosis, a self-limiting lymphoproliferative disease, and it is additionally associated with various malignancies including Burkitt's lymphoma, Hodgkin's disease and lymphoproliferative syndromes in immunocompromised individuals. The Epstein-Barr virus nuclear antigen 1 (EBNA1) is the only EBV protein expressed in all known states of EBV latency and in the virus lytic cycle. EBNA1 is required for the replication and maintenance of the EBV episome. The aim of this thesis was to characterize non-coding mRNA in EBV, with the focus on EBNA1 gene regulation.

We identified an internal ribosome entry site (IRES) in the 5' untranslated region (5' UTR) of the EBNA1 mRNA. This element, designated EBNA IRES, promotes cap-independent translation by recruiting ribosomes directly to highly structured internal mRNA regions and was shown to increase EBNA1 protein expression.

EBNA1 expression and regulation in peripheral blood cells from organ transplant patients were characterized by RT-PCR and Southern blotting. These patients are at high risk for developing EBV-associated post transplant lymphoproliferative disease (PTLD). The incidence of EBNA1 expression in samples from PTLD patients was 3-fold higher compared to other transplant recipients. In addition to the normal EBNA1 transcript we found an alternatively spliced transcript in the transplant recipients. This transcript was shown to exclude the EBNA IRES element and will consequently not promote IRES mediated translation.

Nucleotide changes were found in the patient derived EBNA IRES mRNA compared to the EBNA IRES derived from the laboratory EBV strains B95.8 and Rael in one or two positions, respectively. The patient specific sequence significantly decreased the IRES activity in T cells, while the nucleotide changes had no significant impact on the activity in B or in epithelial cells.

The ability of EBNA IRES to bind cytoplasmic proteins was examined with electrophoretic mobility shift assay (EMSA). Protein-RNA complexes were identified, showing that the EBNA IRES interact specificially with cytoplasmic proteins collected from both EBV-positive and -negative cell lines. With EMSA competition experiments we showed that the patient specific EBNA IRES bound more efficient to trans-acting proteins compared to the B95.8-derived EBNA IRES.

In summary, we have provided evidence that IRES activity, alternative splicing of non-coding mRNA and nucleotide changes in the EBV genome are important mechanisms for translational control of EBV latent gene expression.

Keywords: *Epstein-Barr virus, EBNA1, IRES, PTLD, alternative splicing, nucleotide substitution, protein interactions*

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LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to by their Roman numerals in the text:

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

II. **Alternative EBNA1 expression in organ transplant patients**
Malin Å.M. Berggren, Åsa Isaksson, Ulrica Larsson, Folke Nilsson, Ulla Nyström, Tor Ekman, Jane Löfvenmark and Anne Ricksten.
Journal of Medical Virology (2005) 76:378-385

III. **Cell specific internal translation efficiency of Epstein-Barr virus present in solid organ transplant patients**
Åsa Isaksson, Malin Berggren, Kerstin Ekeland-Sjöberg, Tore Samuelsson and Anne Ricksten.
Journal of Medical Virology 79:573-581 (2007)

IV. **Interactions of cellular proteins with the EBV internal ribosome entry site**
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ABBREVIATIONS

BL	Burkitt's lymphoma
bp	Base pair
CAT	Chloramphenicol acetyltransferase
cDNA	Complementary DNA
CSFV	Classical swine fever virus
CMV	Cytomegolvirus
Cp	The promoter in the *Bam*HI C fragment in the EBV genome
cpm	Counts per minute
CTL	Cytotoxic T lymphocyte
DEAE	Diethylaminoethyl
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
DNAse	Deoxyribonuclease
EBER	Epstein-Barr virus-encoded RNA
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
eIF	Eukaryotic initiation factor
EMCV	Encephalomyocarditis virus
EMSA	Electrophoretic mobility shift assay
Fp	The promoter in the *Bam*HI F fragment of the EBV genome
HCV	Hepatits C virus
HD	Hodgkin's disease
HHV4	Human herpesvirus 4
IM	Infectious mononucleosis
IRES	Internal ribosome entry site/segment
ITAF	IRES trans-acting factor
kbp	Kilobase pair
LCL	Lymphoblastoid cell line
LCV	Lymphocryptovirus
LMP	Latent membrane protein
LUC	Luciferase
mRNA	Messenger RNA
NPC	Nasopharyngeal carcinoma
nt	Nucleotide
ORF	Open reading frame
OriP	Origin of replication
PAGE	Polyacrylamidee gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PTB	Polypyrimidin tract binding protein
PTLD	Post transplant lymphoproliferative disease
Qp	The promoter in the *Bam*HI Q fragment of the EBV genome
RIPA	RadioImmunoprecipitation Assay
RNA	Ribonucleic acid
RNAse	Ribonuclease
RT PCR	Reverse transcription PCR
SDS	Sodium dodecyl sulfate
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
Wp	The promoter in the *Bam*HI W fragment of the EBV genome

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INTRODUCTION

The Epstein-Barr virus

In 1964 Epstein and Barr first described the presence of a herpes-like virus in lymphoblast cultures of Burkitt's lymphoma [1]. Subsequently, particles of similar morphology and antigenicity were detected in cultures of other Burkitt tumors, in buffy-coat cell cultures of patients with leukaemia and infectious mononucleosis and of apparently healthy individuals. These particles were later designated as Epstein-Barr virus (EBV) belonging to the herpesviridae family of virus, subfamily gammaherpesviridae and the genus lymphocryptovirus. EBV stores its genetic information in the form of a linear double-stranded DNA molecule with the approximate size of 184 kbp that encodes 80-90 viral proteins, most of them involved in the viral replication and the lytic cycle. Upon infection of B lymphocytes the genome becomes circular via the terminal repeats, reviewed in [2]. More than 90% of the world's population is infected with the virus. Infection in childhood is often asymptomatic, while primary infection in adolescents and adults usually results in the development of infectious mononucleosis (IM).

Growth transformation and immortalization

EBV has the unique ability to effectively transform and immortalize resting B lymphocytes *in vitro* into lymphoblastoid cell lines (LCLs) with continuous and indefinite growth [3]. The LCLs provide a useful, albeit incomplete, *in vitro* model of EBV infection and immortalization. Relative the precursor B lymphocytes, immortalized cells have an enlarged appearance due to increased cytoplasmic volume required to support high rates of RNA and protein synthesis. The immortalized cells contain multiple extrachromosomal copies of the EBV episome and constitutively express a limited number of EBV latent genes. For schematic presentation of the virus life-cycle, see Figure 1. Four different latency programmes (0-III) can be seen in established tumor cell lines, LCLs, and healthy individuals, all of which are distinct from the viral lytic programme.

Latency III
The best characterized latency programme is latency III, or the growth programme. This latency form is the least restricted and is found not only in LCLs but also in patients with infectious mononucleosis and immunocompromised individuals with EBV associated lymphoproliferative malignancies. LCLs express all six nuclear proteins (Epstein-Barr Nuclear Antigens (EBNA) 1-6), the three membrane proteins (LMP-1, 2A and 2B), small nonpolyadenylated RNAs (EBERs) and *Bam*HI A RNAs (a family of spliced polyadenylated RNAs) [4], see Table 1. The EBNA encoding mRNAs are generated by differential splicing of a long transcript expressed from one of the two promoters Cp and Wp located within the *Bam*HI C and W region of the viral genome.

Latency II
In latency programme II, EBNA1 is transcribed from the Qp promoter located in the *Bam*HI Q region and different combinations of the three LMPs are expressed as well as EBERs and *Bam*HI A RNAs. This latency programme is found in nasopharyngeal carcinoma (NPC), T cell lymphomas and in Hodgkin's disease [4].

Latency I
Latency I is present in EBV-positive Burkitt's lymphoma cells and is characterized by the expression of Qp initiated EBNA1, EBERs and *Bam*HI A RNAs.

Latency 0
Latency programme 0 is the most restricted programme which is seen in healthy EBV carriers. EBV expression is down-regulated and limited to the expression of LMP2A, and possibly EBERs and *Bam*HI A RNAs [5].

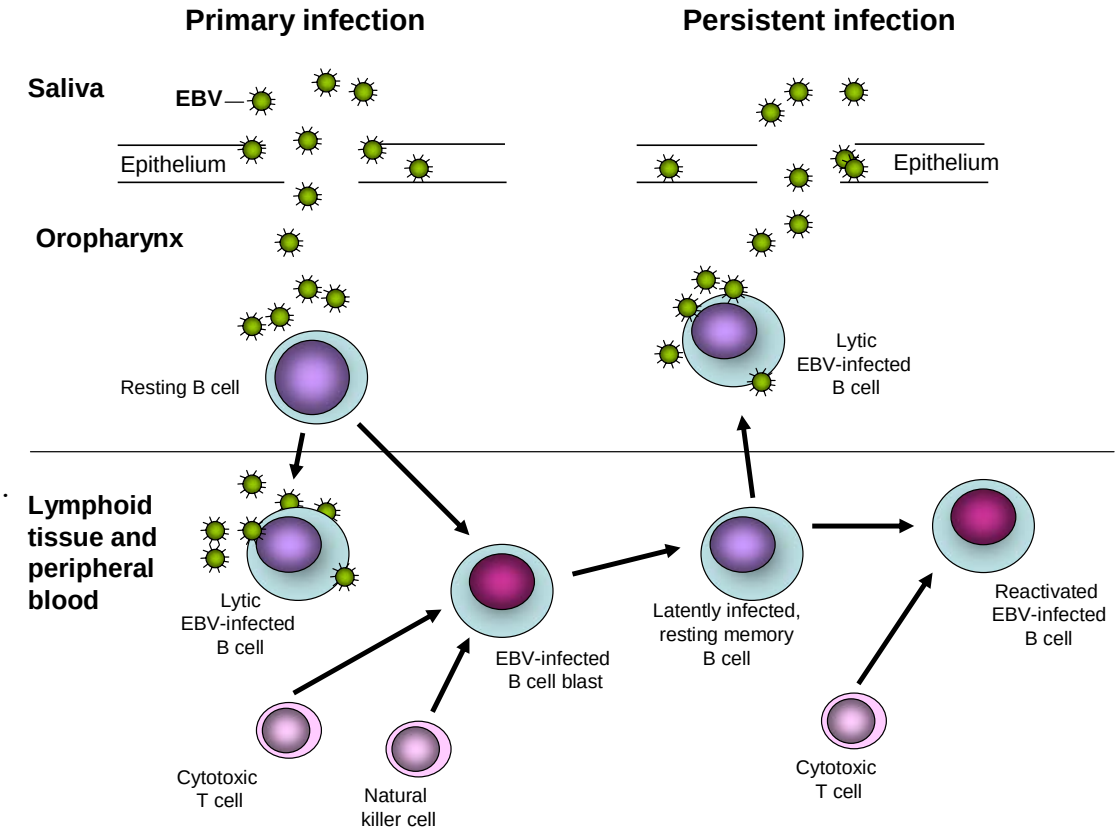

Figure 1. *Model of EBV infection in humans.*
EBV infects B cells or epithelial cells, which in turn infects B cells, in the oropharynx. During primary infection, infected B cells undergo lytic infection with production of virus or expression of the full set of latent viral proteins. The latter are targeted by cytotoxic T cells and natural killer cells. After primary infection, EBV persists in the peripheral blood in latently infected memory B cells, which can undergo EBV reactivation and either be destroyed by cytotoxic T cells or undergo lytic replication in the oropharynx. The latter results in production of virus with shedding of virus into the saliva or infection of epithelial cells with release of virus. Modified from [6].

The lytic programme

In order to survive its host and persist over time the virus must be able to transmit its genome to new hosts. The strategy of lifelong latency in the host is periodically interrupted by lytic reactivation in a fraction of the latently infected B cells, to allow production of free virions for transmission between hosts [7] , Figure 1.

In addition, the lytic programme can also be activated upon infection of naïve B cells. In the viral lytic programme the latent cycle promoters (Wp, Cp, and Qp) are down-regulated and the *Bam*HI F promoter is activated. A minority of the Fp-initiated transcripts are spliced into the EBNA1 open reading frame which maintains EBNA1 expression in lytic cycle [8] [9] [10]. EBNA1 is the only EBNA protein that continues to be synthesized in the viral lytic programme.

Table 1. Latent EBV gene products and their possible functions

Viral gene product	Function
EBNA1	Episome replication and maintenance, transcriptional enhancer
EBNA2	Viral oncogene, transcriptional enhancer
EBNA3 (3A)	Critical for B-cell growth transformation, represses Cp-activity
EBNA4 (3B)	Transactivates cellular genes
EBNA5 (LP)	Cooperates with EBNA2 in the activation of the LMP1 promoter
EBNA6 (3C)	Viral oncogene, regulates LMP1 expression, represses Cp-activity
LMP1	Viral oncogene, transcriptional enhancer
LMP2A and -2B	Inhibits virus lytic cycle, activates cell-survival signals
EBERs	Contribute to efficient growth transformation of B-cells
Bam HI A RNAs	Functions unknown

Table modified from [11]

EBV-associated diseases

Infectious mononucleosis

Primary infection, by oral transmission, is usually asymptomatic or results in non-specific symptoms, but if delayed until adolescence it often presents as infectious mononucleosis (IM). Patients with acute IM shed high titres of infectious virus in the throat from lytic infection at oropharyngeal sites. During early stages of infection EBV-infected peripheral blood cells show a latency programme III expression pattern, with all EBNAs, LMPs, EBERs and *Bam*HI A transcripts expressed [12]. After the acute disease most of the latency transcripts are no longer detectable in resting EBV-infected B cells, which is referred to as EBV latency 0 pattern [13] [14].

Lymphoproliferative disease in immunocompromised individuals

In healthy individuals, EBV-driven B cell transformation is tightly controlled by cytotoxic T cells. Immunocompromised patients are at high risk of developing EBV associated B cell lymphomas, and those that arise in transplant patients are the best studied of these

lymphomas. Post transplant lymphoproliferative disease (PTLD) is commonly described as a wide spectrum of lymphoproliferative diseases and arise as polyclonal or monoclonal lesions within the first year of allografting , when immunosuppression is most severe. PTLD is seen in approximately 10% of all solid organ transplant recipients. The vast majority of PTLD are of B cell origin [15] [16] where over 80% are associated with EBV infection. PTLD of T cell lineage are rare and associated with EBV infection in about 30% of the cases [17]. The majority of recipients of solid organ transplants are EBV seropositive and all receive immunosuppressive treatment but not all of the patients develop EBV-driven PTLD. Additional co-factors are required to promote the development of PTLD, although these are not yet clarified.

The incidence of PTLD after solid organ transplantation is different in children and adults and varies according to the type of transplant. The higher incidence of PTLD in transplanted children is largely due to the development of primary EBV infection after transplantation. EBV seronegative adults who acquire primary EBV infection after transplantation are also at increased risk of developing PTLD. In both children and adults, PTLD is most common after heart and lung transplantation. This is probably due to a more severe immunosuppression in these patients compared to kidney or liver recipients [18].

The initial treatment in all PTLD patients is to reduce the immunosuppressive treatment in order to increase the ability of the host CTLs to eliminate the actively dividing infected lymphocytes, including those which constitute the tumor. If reduced immunosuppression fails to control the disease chemotherapy used to be the second choice of treatment, despite its high mortality due to sepsis and toxicity [18]. Today, there are novel strategies to treat PTLD, which are discussed more in the "Clinical aspects of EBV infection and therapy" section.

Burkitt's lymphoma

There are three different forms of Burkitt's lymphoma (BL), endemic, sporadic and AIDS related. All BLs carry chromosomal translocations involving the placement of the c-myc oncogene under the control of the Ig heavy chain, or less frequently, under the control of one of the light chain loci. This translocation results in deregulated expression of the c-myc gene, reviewed in [19] . Endemic BL is sometimes called "African Burkitt's lymphoma" because it occurs at highest frequencies in children in Equatorial Africa. This high incidence form of BL is found at an annual incidence of 5~10 cases per 100 000 children and coincides with malaria infection. The contribution of malaria to BL development is probably due to multiple immunomodulatory effects of this infection, including release of a B cell mitogen by the malaria parasite and suppression of T cell immunity reviewed in [20]. The EBV association of endemic BL in some parts of Equatorial Africa approaches 100%, which is not the case of the other categories. Sporadic BL is rarely associated with EBV-infection, while AIDS related BL in US or European patients is associated with EBV infection in approximately 30% of the cases [21].

Hodgkin's disease

Hodgkin's disease (HD) is an unusual lymphoma of the human lymphatic system. The malignant Hodgkin/Reed Sternberg cells account for only 1-2% of the total tumor mass, in the middle of a non-neoplastic inflammatory infiltrate [22]. EBV is detected in 30-50% of the HD cases and every malignant cell carries the viral genome. Classic HD is seen

worldwide with a low childhood incidence in western countries, while childhood HD in developing countries is more common.

Nasopharyngeal carcinoma
Carcinomas of nasopharyngeal epithelium show a consistent EBV association and are seen in all parts of the world, although it is particular common in areas of China and south-east Asia with the incidence of around 25 cases per 100 000. The EBV gene expression pattern is the same as seen in HD [23].

Epstein-Barr virus nuclear antigen 1

The Epstein-Barr virus nuclear antigen 1 **(**EBNA1) is a DNA binding protein that binds to the origin of replication (oriP) within the viral genome and allows replication of the EBV DNA episome [24]. After replication, the viral genomes must be segregated to the daughter cells. This mechanism was recently shown to be mediated by the interaction between EBNA1 and the cellular EBNA1-binding protein 2 (EBP2), that joins the EBV genome and the mitotic chromatin leading to efficient segregation of viral episomes [25].

The expression of EBNA1 is controlled at multiple levels. The transcriptional regulation of EBNA1 involves initiation from three alternative promoters, Wp [26 27], Cp [28], and Qp [9 29], 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 [30 31].

The open reading frame for EBNA1 is located in the *Bam*HI K exon at the 3' end of the message and is preceded by potentially highly structured 5' untranslated regions (5' UTRs) 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, Figure 2.

During type I latency, the Q promoter gives rise to EBNA1 expression. Qp is a TATA-less promoter and considered as the EBV "house keeping" promoter. Immediately downstream of the transcriptional start site are two binding sites for the EBNA1 protein. These sites are called the Q locus and binding of EBNA1 to these sites represses Qp activity [32 33]. The transcription factor E2F was shown to bind sequences within the Q locus that partially overlap the two EBNA1 binding sites [34]. This indicates that EBNA1 and E2F control the final outcome of Qp activity. Further, it was shown that the expression of E2F is cell cycle regulated, which suggests that aslo the activity of Qp is cell cycle regulated. Indeed, the level of EBNA1 mRNA peaked in the S-phase, while the lowest level was found in G1-phase. On the other hand, the EBNA1 protein level was unaltered during the cell cycle. The authors speculate that this might be due to its stability and long half-life. Cell cycle-dependent expression of EBNA1 mRNA may ensure that certain level of EBNA1 is maintained at the time when the episome is replicated.

While Qp is negatively autoregulated by EBNA1, transcription initiation from Cp and Wp is strongly upregulated by EBNA1 [33].

The amino-terminal half of the EBNA1 contains a large domain of glycine-glycine-alanine (GGA) repeats. This domain inhibits ubiquitinin-dependent proteasomal degradation of EBNA1 and thereby prevents presentation of EBNA1 derived peptides on the MHC class 1 molecules [35 36]. In patients, cells that express only EBNA1 (EBV latency I programme) can evade EBV-directed immune surveillance by CTL

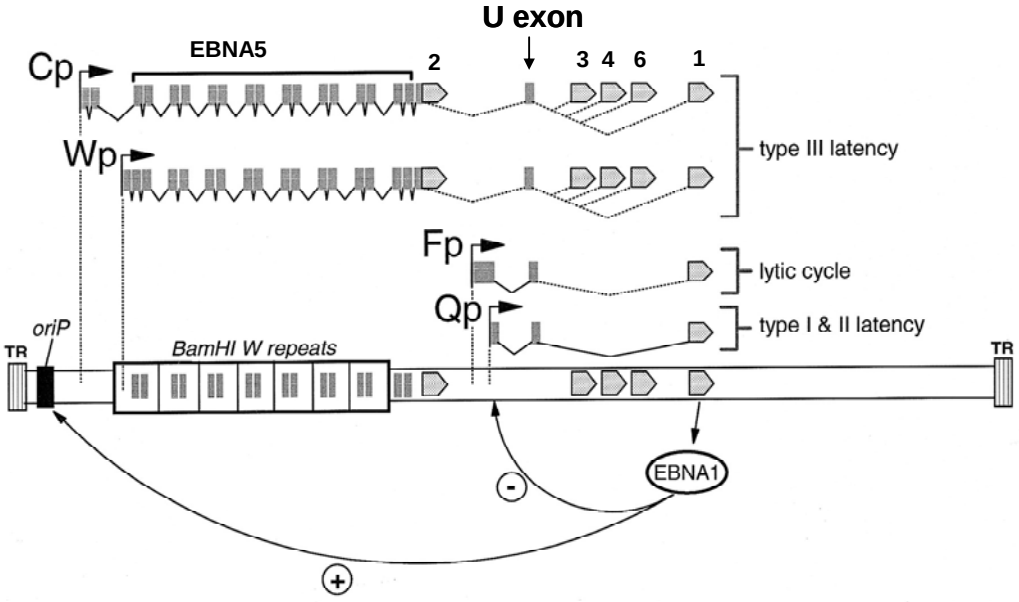

Figure 2. *Schematic representation of the complex splicing pattern seen in EBV infected cells. The numbers refer to the EBNA 1-6 proteins. EBNA1 transcripts are initiated from one of the four promoters; Cp, Wp, Fp or Qp. EBNA1 negatively autoregulates its expression by binding to the Q locus immediately downstream of the Qp promoter, while the Cp and Wp promoters are strongly upregulated by EBNA1. The origin of replication (oriP), when supplied with EBNA1, provides efficient duplication, partitioning and maintenance of the EBV genome.*

Initiation of translation

Protein synthesis (translation) is a critical process in all living cells. The process is highly regulated and involves the recognition of mRNA by a specific subset of eukaryotic initiation factors (eIFs) followed by the recruitment of the ribosome, recognition of the initiation codon AUG and initiation of protein synthesis. This mechanism is known as a linear scanning since it involves movement of the ribosome along the 5' UTR and "scanning" for the initiator codon [37]. An alternative mechanism to the classical cap-dependent initiation is the internal ribosome entry, a process where the ribosomes are directly recruited to highly structured internal mRNA structures closer to the start codon and independent of the cap-structure [38].

Translational control has been shown to be affected in many types of cancers. Several proteins related to growth control have an altered expression in proliferating cancer cells as a result of events that take place during translation initiation, examplified in [39] [40].

Cap-dependent translation initiation

The majority of mRNAs contain a "cap" structure, m^7GpppN (where N is any nucleotide, p is phosphate and m is a methyl group), at the 5' terminus. The cap is specificially bound by the initiation factor eIF4E that associates with two additional initiation factors, eIF4G and eIF4A, to form the eIF4F complex, which attracts the ribosomes to the mRNA [41].

The 5' to 3' migration of ribosomes towards the initiation codon is a process that consumes energy in the form of ATP. The ribosome stops when it binds stably at the initiation codon, which is usually the first AUG triplet in an appropriate sequence context (G/AXXAUGG, where X is any nucleotide) downstream of the 5' cap [42]. Cap-dependent translation is strongly enhanced by polyA-tails, which is achieved by the physical interaction of the polyA-binding protein (PABP) with eIF4G, which tether the ends of the mRNA, Figure 3A.

Over-expression of several components of the translational machinery has been shown to strongly correlate or cause malignant transformation. The cap-binding protein eIF4E is the least abundant initiator factor and is therefore considered to be the rate limiting factor for cap-dependent translation [43]. Over-expression of eIF4E has been found in numerous transformed cell lines and primary cancers. High levels of eIF4E were detected in breast carcinomas, non-Hodgkin's lymphomas and in primary bladder cancer, reviewed in [44].

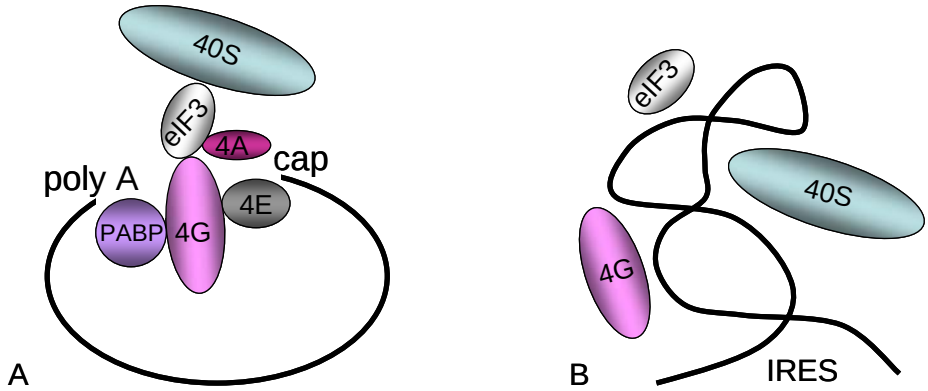

Figure 3. *Simplified models for the recruitment of ribosomes to mRNA during translation initiation. (A) Cap-dependent translation. In this closed-loop model, the eIF4F complex (3-subunit complex composed of eIF4E, eIF4G and eIF4A) interacts with both the 5' end of the mRNA and the polyA tail and recruits the 40S ribosomal subunit via its interaction with eIF3. (B) IRES mediated translation. IRES elements bypass the need for several eIFs and recruits the translational machinery at a position closer to the or directly at the initiation codon.*

IRES mediated translation initiation

Cap-dependent translation initiation is not, however, the only means by which mRNA translation can be initiated. In 1988, it was discovered that translation of uncapped picornaviral mRNA is mediated by an RNA structure which allows assembly of the translational machinery at a position closer to or directly at the initiation codon, the internal ribosome entry segment/site (IRES) [45] [46] [47]. These elements are usually found within relatively long, GC-rich and highly structured 5' UTRs. IRES-mediated translation initiation is strictly dependent on the structural integrity of the IRES. The specific folding of the mRNA is supported by both RNA-protein and long-range RNA-RNA interactions between functional domains. The latter interactions was found, *in vitro*, to be dependent

on RNA concentrations, ionic strength and temperature suggesting that IRES folding is a dynamic process, reviewed in [48].

The exact molecular mechanism of IRES-directed translation is not fully understood, although it is clear that both canonical and non-canonical factors are required. Further, the need for eIFs varies greatly between different IRESs. For example, the EMCV and FMDV IRESs require the presence of several eIFs, whereas the HCV and CSFV IRESs are able to bind the 40S ribosomal subunit in the absence of almost all eIFs, Figure 3A and B, reviewed in [48]. A number of non-canonical factors, also called IRES trans-acting factors (ITAFs) have been reported to be of importance for cap-independent translation. These proteins are thought to act as RNA chaperones, directing and stabilizing the tertiary folding of the mRNA. Among the most frequently studied ITAFs are the polypyrimidine tract binding protein (PTB) [49] [50], the human auto antigen La [51] [52] [53] and the upstream of N-ras protein [54] [50].

Today the existence of viral and cellular IRES elements is well presented, among them are the IRES within the DNA virus Kaposi's sarcoma–associated herpes virus [55] [56] [57], hepatitis virus C [58] [59] the c-myc IRES [60] [61] and the IRES within the p53 gene [62].

AIMS OF THE STUDY

Overall aim

The overall aim with this thesis was to study the molecular biology of EBV infection in human B cells, particularly to characterize untranslated regions in the Epstein-Barr virus genome with the focus on regulation of the EBNA1 gene.

Specific aims

Paper I
To investigate the role of the 5' UTR of the EBNA1 gene, with focus on latent gene expression.

Paper II
To identify patients at risk for developing post transplant lymphoproliferative disease, in reference to EBNA1 expression in peripheral blood cells.
To characterize EBNA1 transcripts in samples from organ transplant patients.

Paper III
To determine the internal translational efficiency of Epstein-Barr virus present in solid organ transplant patients.

Paper IV
To study cellular protein interactions with the EBNA IRES element.

MATERIAL AND METHODS

Clinical material

Peripheral blood samples from patients undergoing organ transplantation at Sahlgrenska University Hospital, Göteborg, Sweden, were collected at different time points during PTLD treatment or post transplant check ups. Patients were all under immunosuppressive treatment with prednisolone, or prednisone together with cyclosporine or tacrolimus when cyclosporine-related side effects were observed. Heart and/or lung transplant recipients were in addition treated with azathioprine or mycophenolate mofetil. Blood samples from healthy blood donors were used as a control group.

Cell lines

Six human cell lines were used. DG75, Rael and P3HR1 are Burkitt's lymphoma derived B cell lines. DG75 is an EBV-negative cell line [63]. The EBV-positive Rael cell line displays latency I phenotype in which EBNA1 is the only detetable viral protein [64]. P3HR1 is an EBV-positive cell line of latency II phenotype and is permissive for viral lytic infection [65]. B95.8 is an EBV-positive marmoset-derived cell line with latency III phenotype [66]. HeLa is an EBV-negative human epidermoid carcinoma cell line [67]. Molt-4 is an EBV-negative T cell line derived from an acute lymphoblastic leukaemia [68]. The lymphoid cells were maintained in RPMI 1640 (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% foetal bovine serum (Sigma) and 1% of penicillin-streptomycin (Sigma). HeLa cells were maintained in RPMI 1640 supplemented with 5% foetal bovine serum and 1% of penicillin-streptomycin. The cells were kept in 37°C in a humidified atmosphere containing 5% CO_2.

Cloning and sequencing

All manipulations involved in vector constructions were carried out by standard procedure [69] and verified by sequencing. Plasmids for generation of the monocistronic luciferase expression vectors, driven by the EBNA1 promoters Fp and Qp, were constructed by cloning PCR-amplified fragments into the pGL3-Basic Luciferase vector (Promega). All bicistronic constructs have the expression vector pIRES (Clontech) as framework, which contains the IRES element from the encephalomyocarditis virus (EMCV). From this vector we constructed a bicistronic vector with the chloramphenichol acetyltransferase (CAT) reporter gene as the first cistron and the luciferase (LUC) gene as the second cistron, Figure 4.

For the immunoblot assay we used vectors driven by the CMV promoter and Qp promoter prior the U exon and the coding K exon for EBNA1. PCR-amplified fragments were inserted into the pcDNAI/Amp vector (Invitrogen AB).

Plasmids for *in vitro* transcribed probe synthesis used in the RNAse protection assay and Northern blot analysis are described previously [70 71]. Plasmids for construction of the various probes used in EMSA experiments have the pGL3-Basic vector as framework.

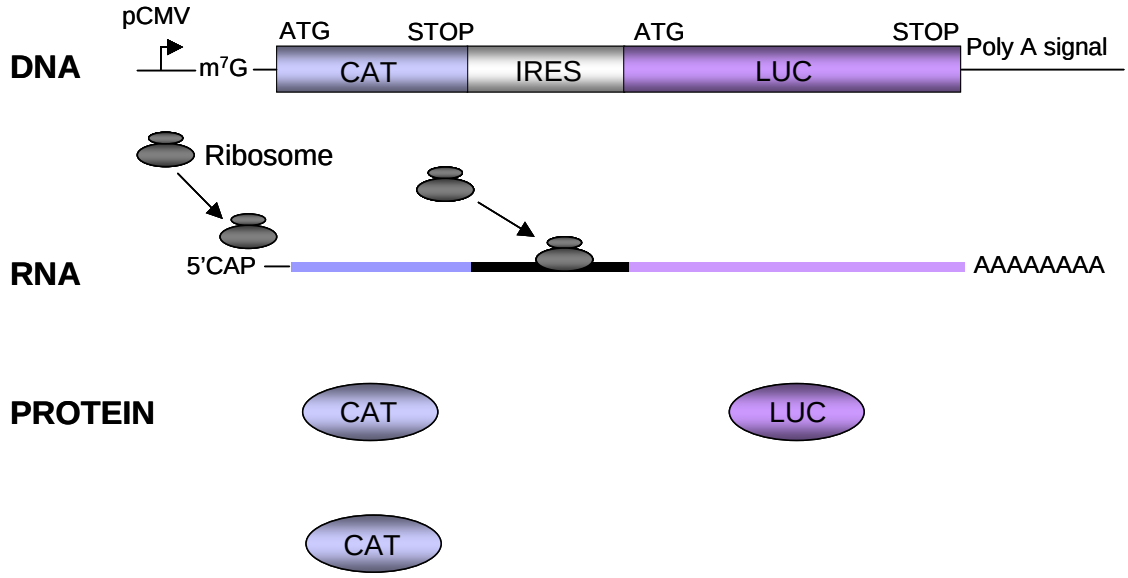

Figure 4. *Schematic representation of the approach to characterize a potential IRES-element. The bicistronic reporter plasmid contains the powerful CMV promoter and the two reportergenes CAT and LUC. Transcription results in one long bicistronic mRNA. The presence of an IRES mediates translation of the LUC gene, in addition to the cap-initiated translation of the CAT gene. However, in the absence of a functional IRES the ribosome drops the mRNA at the stop codon in the CAT gene and subsequently only the CAT protein will be expressed. A ratio between the LUC and CAT activities adjusts for possible differences in transfection activities.*

Sequencing was performed with ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and specific sense and antisense primers for the PCR-products and the plasmid constructs respectively. The sequence products were precipitated, resuspended in Hi-Di Formamide (Applied Biosystems) and further analyzed with the ABI PRISM 3100 Genetic analyzer. Sequences were compared with the published EBV-genome strain B95.8 (GenBank V01555).

Transient transfections and reporter gene assays

Transient transfections of the lymphoid cell lines were performed by electroporation [72] using the BioRad Genepulser (BioRad, Hercules, USA) in the presence or absence of DEAE-dextran. Transfection of epithelial cells was performed by the calcium phosphate-DNA precipitation method essentially as described by [73]. Cells were harvested 48 h post transfection and aliquots of cell lysates were assayed for CAT and LUC activities. The method for measuring CAT activity is described by [27]. The LUC activity was determined with the Luciferase Assay System (Promega) using a TD 20/20 luminometer (Turner Designs Instruments, USA).

PCR and RT PCR

cDNA reactions were performed on total RNA in a reaction mixture consisting of buffer (Invitrogen AB), DTT, random hexamers, dNTPs, RNase inhibitor (Roche BM), and Superscript II (Invitrogen AB). The generated cDNA was analyzed in a PCR followed by a nested or half-nested PCR and products were visualized on agarose gels. cDNA quality controls were performed with PCR on the housekeeping gene GAPDH. The first PCRs and the nested PCRs were carried out in two separate locations and water controls were placed between all patient samples to avoid and to detect possible contamination throughout analysis. To exclude the possibility that an existing Q/U/K transcript is not detected due to PCR-competition, reexamination with primers in U and K was performed on cDNA from those samples where we only detected Q/K transcripts.

Southern Blot

PCR products were transferred from agarose gels to nylon membranes (Hybond-N+, Amersham) by alkaline blotting. To distinguish EBNA1 Q/U/K-spliced transcripts from the alternatively spliced Q/K transcript, hybridization with U-exon and K-exon-specific biotinylated probes were performed separately. Hybridization signals were detected with streptavidin-conjugated peroxidase (Roche diagnostics) and the ECL system (Amersham).

Western Blot

Cells were thawed and lysed in RIPA buffer with protease and phosphatase inhibitors. The protein concentration of the lysates was determined (Bradford protein assay, BioRad) and the total protein amount was standardized by dilution with RIPA buffer. The protein extracts were separated on NuPage Bis-Tris SDS polyacrylamidee gels (Invitrogen life technologies) and blotted to nitrocellulose membranes (Hybond C-extra, Amersham Biosciences). The membranes were blocked and incubated with different antibodies at different concentrations. After repeated washings the membranes were incubated with alkaline phosphatase (AP)-conjugated rabbit anti-human antibody (BioRad), or with HRP-conjugated rabbit anti-goat antibody (BioRad). The proteins were visualized by enhanced chemiluminescence procedures; Immun-Star Chemiluminescent protein detection system (BioRad) or Phototope®-HRP Western blot Detection System (Cell Signaling Technology™), as described by the manufacturers of the reagents. The visualized EBNA1 bands were quantified and compared using the QuantityOne program with a Chemi Doc camera (BioRad).

RNAse protection assay

RNases degrade single-stranded RNA but leave double-stranded RNA intact, i.e double-stranded RNA molecules are protected from degradation. The RNase protection assay (RPA) was used to estimate the relative amounts of mRNA present from different bicistronic vectors. Total RNA was extracted from transfected DG75 cells with TRI REAGENT™ LS reagents as described by the manufacturer of the reagents (Sigma) and treated with DNase (Promega). A ^{32}P-labelled LUC antisense RNA probe was synthesized by in vitro transcription in the presence of [α-^{32}P]CTP (3000Ci/mmol; DuPont NEN) by

standard procedure [69]. RNA molecules were purified by using Chromaspin-100 columns (Clontech). Total RNA and ^{32}P-labelled RNA were incubated, after which single-stranded material was digested by the addition of RNase A and RNase T1. The protected fragments were separated by electrophoresis on a denaturing polyacrylamide gel and visualized by phosphoimage analysis (Molecular Dynamics).

Northern blot

Total RNA was prepared (in the same way as for the RNAse protection assay) and separated on a formaldehyde-containing agarose gel and transferred to Hybond-N membrane (Amersham). The blots were UV cross-linked and hybridized with either the antisense CAT or the antisense LUC-riboprobe in a hybridization buffer. Both probes were synthesized by in vitro transcription. The integrity of the transcripts was visualized by phosphorimage analysis (Molecular Dynamics).

Electrophoretic mobility shift assay

RNA was transcribed *in vitro* from linearized plasmid, using either T7 or Sp6 RNA polymerase, and labelled internally with [α-^{32}P] UTP (3000Ci/mmol; DuPont NEN) by standard procedure [69]. RNA molecules were purified using Chromaspin-100 columns (Clontech). The binding reaction was carried out in binding buffer containing radio labelled RNA and protein extracts, in the presence or absence of excess of unlabelled competitor sequences. After incubation at RT, samples were separated on a non-denaturing polyacrylamidee gel (NuPage, Tris-Acetate, Invitrogen). Gels were visualized by phosphoimage analysis (Molecular Dynamics).

Secondary structure predictions

Prediction of secondary structure was carried out using MFOLD [74] or RNAalifold [75] of the Vienna package, a program that predicts a secondary structure for a set of aligned sequences. For RNAalifold, four unique sequences related to the EBNA IRES, (derived from GenBank entries with accession numbers AF448220.1, M12553.1, AY037858.1 and DQ+16879927) were identified with BLAST and used to produce a multiple alignment.

Statistical data analysis

EBNA1 expression data were compared by the Pearson Chi-square test; odds ratio and 95% confidence interval (CI) were calculated. Analysis was performed using SYSTAT (SPSS, Inc., Chicago, IL).

Variations between the mean translational efficiencies were statistically analyzed. The tests were performed by using SPSS13.0 software (SPSS Inc., Chicago, USA). The significance of the results was determined by one-way ANOVA and post hoc S-N-K (StudenT Newman-Keuls) test. A p-value <0.05 was considered statistically significant.

SUMMARY OF RESULTS

The EBNA1 gene contains an internal ribosome entry site (Paper I)

EBV gene expression is controlled at multiple levels, where the transcriptional regulation is probably the most studied form of regulation. Transcriptional control includes for example the use of several different promoters, with various strength, used differentially during different phases of infection [2]. There is however increasing data on post transcriptional regulation, in general, including alternative splicing, mRNA stability and transport of mRNA from the nucleus to the cytoplasm. To better understand the complex regulation of the EBNA1 gene, we have focused on the study of EBNA1 transcripts and expression both *in vitro* (paper I, III, and IV) and *in vivo* (paper II). In this thesis data is presented that reveals novel mechanisms whereby EBV can control latent gene expression.

The EBNA1 gene is the only gene expressed during all types of EBV latencies and in the viral lytic cycle. The spliced EBNA1 messages are similar in overall structure regardless of which of the four promoters (Cp, Wp, Qp and Fp) the transcripts are initiated from. All transcripts are relatively long and contain several spliced exons upstream the coding exon. Although the transcripts differ in the length of their 5' UTRs, the U leader exon is common to all transcripts and spliced directly in front of the coding K exon. The U exon is included not only in EBNA1 transcripts but also in several other transcripts, always as a non-coding exon, for example in the EBNA 3, 4 and 6 transcripts. In addition the U exon has also been shown to exist in a 3,7 kb mRNA transcript with fragments from the W, Y, U, E and K genomic regions [76], and upstream the internal repeat leader exons W1 and W2 in lymphoblastoid cell lines [77]. These observations made us interested in exploring the role of the U exon in EBV gene regulation.

In this study, the effect of the 5' UTR of EBNA1 on gene regulation was first seen in our Fp/Qp-driven reporter plasmids where the U exon was inserted directly upstream of the coding region of the luciferase gene. Transient transfection of Burkitt lymphoma cell lines with these constructs showed an increased expression of the LUC gene in plasmids containing the U exon compared with control plasmids lacking these sequences. This was observed in both EBV-negative and EBV-positive cells. By including the U leader sequence in the constructs the mRNA structure becomes longer, more complex and presumably more difficult for the ribosome to scan and initiate translation. This would actually decrease the expression of the reporter gene. However, we showed that the presence of the U exon mediated an overall higher expression from both Fp- and Qp-driven reporter constructs. These results demonstrate that the EBNA1 5' UTR has a regulatory role in gene expression through transcriptional or post transcriptional control mechanisms.

Despite the afore mentioned technical hitches with long leader sequences, complex mRNAs have been shown to be efficiently translated. Numerous mRNAs have been shown to accomplish translation by a cap-independent mechanism via the presence of an IRES-element [78]. The IRES is a recognition site for translational regulatory factors and allows translation activity in situations where the cap-dependent translation is impeded.

When we analyzed the Qp-initiated EBNA1 transcript we noticed several of the reported features common for IRES-elements including relatively long (225 nt), GC-rich 5' UTR with two potential AUG codons upstream of the initiation start codon in the K

exon, as well as the presence of several GNRA and RAAA motifs (N-nucleotide, R-purine), sequences shown to be essential for IRES activity [79] [80]. These observations led us to investigate whether the 5' UTR of EBNA1 contains an IRES. To test the hypothesis, we constructed bicistronic vectors with the 5' UTR of EBNA1 inserted between the two genes. The use of a bicistronic vector is proclaimed to be the "gold standard" to detect IRES activity. In this study we chose to use the CAT and the LUC reporter genes as the first and second gene in the vector. Since the half-life of the CAT and the LUC proteins are approximately 50 h and 3 h, respectively, we put the CAT gene as the first cistron to achieve the highest sensitivity on the IRES activity in the system. A frequently used bisictronic vector is the Renilla-Firefly vector [55] [81] [82]. The advantage of this vector is the very rapid protein assays, compared to the CAT assay. However, it has been reported that this vector contains a splice donor site in the Renilla gene and might therefore generate aberant transcripts [83].

The bicistronic plasmids were transfected into both EBV-positive and negative cell lines. The 5' UTR of EBNA1 stimulated expression of the second cistron 7-20 fold in all cell lines when compared to the control plasmid. Interestingly, the EBNA1 5' UTR was up to 4-fold more active than the EMCV IRES in the EBV-positive cells, but not in the EBV-negative cells, indicating a cell specific variation in activity.

With RNase protection analysis and Northern blot analysis we demonstrated that i) the relative amounts of RNA present from the various bicistronic constructs were equal and ii) that the transcripts contained both reporter genes in the absence of any signs of cryptic promoters. These results indicate that the U exon mediates post transcriptional regulation. The mechanism whereby the EBNA IRES recruits the translational machinery to the mRNA is not known. The cell specific activity suggests favourable interactions between the EBNA1 mRNA and initiation factors present in the EBV-positive cell lines, which might not be present to the same extent in the EBV-negative cells.

There is 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, whereas the EBNA1 protein level remains constant [34]. One explanation for this observation is that the EBNA1 protein is stable with a long half-life. Maintenance of a constant level of the EBNA1 protein could also be a result from IRES-mediated translation. Furthermore, Fp-initiated lytic transcript containing the U exon which is not spliced to the K-exon is highly expressed upon induction of the virus lytic cycle [10] [30]. Although the intron/exon composition at its 3' end is undefined, this transcript indicates a role for the EBNA IRES during productive viral infection.

In summary, we concluded that the 5' UTR of EBNA1 regulates the expression on the post transcriptional level, through an IRES element. Our findings implicate a novel mechanism whereby EBV regulates latent gene expression. In addition, the presence of the U exon in the EBNA3, 4 and 6 transcripts might indicate that part of the EBV immortalizing process can be regulated through cap-independent translation.

EBNA1 expression and regulation in organ transplant patients (Paper II)

Immunocompromised patients are at high risk of developing B cell lymphomas, and those that arise in transplant patients are the best studied of these lymphomas. Post

transplant lymphoproliferative disease (PTLD) is seen in approximately 10% of all solid organ transplant recipients, where EBV is detected in the majority of the cases [18].

Because elevation of EBV-DNA load in blood is considered to reflect aberrant EBV induced B cell proliferation, much effort has been put in developing methods that might identify patients at risk for developing PTLD by quantitative monitoring of the amount of circulating EBV-DNA in the peripheral blood [84]. This appears to be a promising tool as many transplant recipients with static EBV viral load do not develop PTLD. However, there are some limitations of this approach, and not all patients at risk for PTLD can be identified by EBV-DNA measurements alone. It has been suggested that combined monitoring of EBV-DNA load and low EBV-specific cytotoxic T lymphocytes (CTL) response might better identify the individual patient at risk for PTLD development [85].

In this study we investigated EBNA1 expression in peripheral blood and its potential association with increased risk of PTLD in organ transplant patients. The patients were all transplanted at Sahlgrenska University Hospital, Göteborg, Sweden. A majority of the patients were heart and/or lung recipients, while a few patients were liver or kidney recipients. All patients were under immunsuppressive treatment, as described in Material and Methods. Moreover, we characterized the EBNA1 transcripts in samples from the transplant patients.

A total of 60 organ transplant patients, with or without the diagnosis PTLD, were used in this study. EBNA1 expression in peripheral blood was found in 43% of the patients with PTLD. In the transplanted patients without symptoms, EBNA1 expression was detected in 18%. 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 on 3,42; 95% CI= 1,02-11,54). EBNA1 positive patients without symptoms for PTLD need to 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. To exclude the influences on EBNA1 expression due to different immunosuppression all EBNA1 positive patients were grouped and compared with the EBNA1 negative patients in relation to immunosuppressive treatment. Two treatment strategies were recognized, "standard", with first hand choices of immunosuppression and "alternative", with complications (including adjustments due to PTLD diagnosis) leading to dose adjustment or replacement of immunosuppressive drugs. Among the patients with standard drug treatment 17% were EBNA1-positive and among the group of patients with alternative drug treatment 36% were positive for EBNA1. Statistical analysis showed no significant difference between these groups (P= 0,11; 95% CI = 0,77-8,94).

The absence of detectable EBNA1 transcripts in patients with diagnosed PTLD might reflect different treatment or the individual variation of EBV infected cells, which is also seen in quantitative evaluation of EBV genomic load [86] [87]. Alternatively, specific down regulation of Qp activity by EBNA1 protein might occur [29].

When analyzing promoter activity we showed that the majority of the EBNA1 transcripts initiated from Qp, the EBV "house keeping gene promoter". Low or no detectable activity from the lytic Fp promoter suggest a dominance of latency programme in peripheral blood cells. Consequently, the main reason for increased EBNA1 expression in these immunosuppressed patients is probably an increase in B cell proliferation with enhanced EBV genome replication. Increased EBV genome replication gives a detectable

level of EBNA1 in peripheral blood, in contrast to healthy blood donors where the virus remains in latency program 0 and do not express detectable levels of EBNA1 [5].

When examining the EBNA1 transcripts in peripheral blood samples from the organ transplant patients, an alternatively spliced 5' UTR was found. We showed that the U leader exon of the 5' UTR of the EBNA1 gene is deleted by alternative splicing. Intact donor/acceptor splice sites in the flanking Q and K exons indicate that the EBNA1 open reading frame is unaltered. Alternative splicing in the 5' UTR has been reported to mediate translational regulation in the human nNOS gene [88] and in the glutamine synthetase gene [89]. In contrast to ours, these articles report that an additional 5' UTR exon with a repressing translational effect is acquired by alternative splicing. The alternatively spliced EBNA1 transcript detected in this study is lacking the U exon and the EBNA1 protein will consequently not be regulated by IRES mediated translation. The Q/K splice was detected together with the normal Q/U/K spliced EBNA1 in most of the patients. However, in some patients only the Q/K spliced EBNA1 transcript was detected. The Q/K spliced transcript is also expressed in several EBV-positive, latency III cell lines CBC-Rael, B95.8, Mutu III, and in induced lytic phase P3HR1 and B95.8 [10]. Expression of the two alternative EBNA1 mRNAs in the organ transplant patients were not consistent in every sample from the same patient, but seemed to vary at different time points perhaps reflecting different stages in EBV reactivation. The expression pattern of the two splicing variants in the PTLD patients and the transplant patients without PTLD does not differ significantly. Hence, the alternative splice seems to have a general regulating function of EBNA1 expression. It is possible that both transcripts are constitutively transcribed, although not always detected due to either low amounts or PCR competition.

As mentioned above the majority of the EBNA1 transcripts detected in the patients were Qp-initiated, which are known to be down regulated by EBNA1 binding to the Q locus located downstream of the Q promoter [32]. Thus, a possible mechanism of the Q/K expression is to reduce translation efficiency of EBNA1 protein and thereby reduce feedback regulation of the Qp activity. 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 [90].

Our study emphasizes the complex regulation of EBNA1 gene expression and provides a new model of translational regulation involving alternative splicing and deletion of the EBNA IRES in the 5' UTR of the EBNA1 gene. The biological significance of the alternatively spliced EBNA1 transcript could be to reduce the translation efficiency of EBNA1 in order for the virus to keep EBNA1, the EBV "house keeping gene", in a steady state.

Cell specific IRES activity (Paper III)

IRES-elements have been shown to exhibit cell type specific activities. For example, the c-myc IRES is active in a wide range of cell types, albeit with different efficiencies [91]. Also, the c-myc IRES activity was shown to be repressed in vivo in adult transgenic mice, while it is active in embryos with strong tissue specificity [92]. A number of groups have reported that a single nucleotide substitution within an IRES element can change the secondary structure and alter the IRES activity. A single substitution in the FMDV IRES and in the c-myc-IRES was shown to increase the IRES activity [93] [94], while a substitution

in the IRES element of the Connexine-32 gene was shown to abolish the IRES function [95]. For the IRES element present in the Hepatitis C virus it was reported that a two-nucleotide substitution resulted in a significant impairment of the IRES activity [96].

In the third paper, we analyzed the EBNA IRES mRNA sequence in samples derived from organ transplant recipients and from well-known EBV strains. In addition, we investigated the impact of found sequence variations on the IRES activity in transient transfection experiments.

When we examinined the EBNA IRES mRNA derived from seven EBNA1 expressing organ transplant patients, two nucleotide changes (G to A and C to T) in the mRNA, relative to the corresponding DNA sequence, were found in samples from all patients. This disparity was not found when we analyzed the mRNA and DNA sequences from the B cell lines B95.8, Rael and P3HR1.

The sequence differences in the viral DNA and mRNA might be due to infection with multiple EBV strains, although no signs of multiple infection were detected by sequence analysis of DNA or cDNA patient samples. The sensitivity of the sequence analysis used was confirmed by plasmid titrations to be 10-20%. Consequently, low abundance of an EBNA1 expressing EBV strain will be difficult to detect. To further examine the possibility of infection by multiple EBV strains, we cloned the EBNA IRES DNA sequence from three different patients and sequence analyzed 48-50 clones from each patient. The sequences were identical in all clones from all three patients (data shown in paper IV). Even though the results point toward infection by a single EBV strain, the presence of several strains cannot be excluded. However, assuming that the patients are infected by only one strain, RNA editing might instead be the explanation for the sequence discrepancy. Bourara et al. reported that human immunodeficiency virus-type 1 (HIV-1) mRNA undergoes the same two nucleotide changes (G to A and C to T) by the RNA editing mechanism [97]. This was suggested to be an additional mechanism for controlling viral gene expression through post transcriptional modifications of mRNAs. They speculated that RNA-editing might play a role in the modulation of HIV-1 gene expression. However, further investigations are needed to clarify the mechanism of the nucleotide changes found in the EBV-positive organ transplant patients.

To functionally investigate the importance of the observed nucleotide changes, three bicistronic vectors were constructed, in which the EBNA IRES cDNA from organ transplant patients, Rael and B95.8 were inserted, respectively, between the two reporter genes. Transient transfections of six human cell lines showed a significant impairment in translational activity in the T cell line Molt-4, with the patient specific vector compared with the Rael and B95.8 deduced vectors. Furthermore, the previously reported nucleotide substitution seen in Rael at position 67585 [98] [99], showed a lower translational activity compared with the sequence found in B95.8. In contrast, comparable IRES activities were seen from the constructs in B and epithelial cells.

The transfection results suggest that one of the nucleotide changes (G to A) occurs at a position located in a region important for the IRES activity and cell specificity. The mechanism of EBNA IRES mediated translation is not yet known, but the affinity of a protein binding in a sequence specific manner to that region might be sensitive to a single nucleotide change as observed in the patient material. Another alternative, as suggested by the secondary structure model, is that the nucleotide substitution gives rise to structural changes. Hence, the substitution is expected to disrupt a local helical structure or possibly to change the global structure even more extensively. Such structural changes are likely to

affect the binding of one or more proteins. In line with this hypothesis it was previously reported that a single nucleotide change in the c-myc IRES is likely to influence the secondary structure [94] and that the IRES-protein binding pattern is affected by this change [100].

In conclusion, the patient specific EBNA IRES sequence and its influence on the translational activity reported here, might illustrate new strategies utilized by the EBV to adapt to the immune control in patients with EBV associated diseases.

Cellular protein interactions with the EBNA IRES (Paper IV)

In the fourth paper we turned our interest to the protein-binding capability of the EBNA IRES element. The exact molecular mechanism by which the host translational machinery recognizes IRESs is so far unknown, although the major determinant of IRES function is the three-dimensional RNA fold rather than its primary sequence. Different IRES-elements have different requirements for the use of eukaryotic initiation factors. The EMCV IRES requires almost all factors like the cap-dependent initiation mechanism, while the HCV IRES can bind 40S subunits in the absence of any eIFs, reviewed by [101]. In addition to the requirement of eIFs, the efficiency of IRESs is augmented by non-canonical factors known as IRES trans-acting factors, ITAFs. Among the most frequently studied ITAFs are the polypyrimidine tract binding protein (PTB) [49 50], the human auto antigen La [51 52 53] and the upstream of N-ras (unr) protein [54 50].

Here, we investigated sequences within the EBNA IRES important for cellular protein interactions. Nondenaturing EMSAs were used for identification of protein-RNA complexes. Data obtained from the experiments demonstrated that patient derived EBNA IRES transcripts were able to bind cytoplasmic proteins collected from both EBV-positive and negative cell lines, which indicated that the involved proteins are most likely not EBV encoded. By using the full length and two deleted EBNA IRES probes we established that both regions in the 5' and 3' end of the transcript were necessary for specific protein interactions. In paper III we demonstrated that sequence variations within the EBNA IRES contributed to different translational efficiencies in transfection experiments [102]. We extended these observations further in paper IV and investigated the effect of the sequence variation on binding of cytoplasmic proteins. EMSA competition experiments were performed with P3HR1 and Molt-4 cytoplasmic extracts and an excess of unlabelled full length EBNA IRES probe from the organ transplant patients and the B95.8 strain, respectively. The binding pattern seen with the two different cell extracts were similar, indicating that the same set of proteins were involved in binding. The experiment showed that the proteins involved were not EBV encoded, since the cell line P3HR1 is EBV-positive, whereas Molt-4 is EBV-negative. Using excess unlabelled RNA transcripts we observed that the patient derived IRES were able to form RNA-protein complexes more efficiently than the B95.8 IRES. Paulin et al. reported analogous data regarding the c-myc IRES. In that study a patient-derived mutant form of the c-myc IRES showed enhanced binding of proteins compared to wild type c-myc IRES [100]. It was speculated that the single nucleotide change combined with the altered binding capacity could modify the initiation of translation through the IRES.

To identify proteins involved in the complexes we used mass spectrometry analysis, which resulted in the identification of proteins with the approximate size of 40-70 kDa. Among these proteins one well-known ITAF, PTB [49 50], together with hnRNP C, Hsp 60,

Hsp 70 and RPS23, were recognized. However, supershift EMSA experiments showed no evidence of specific interactions with any of the proteins mentioned above (data not shown). Future studies including RNA-protein pull-down assays, in combination with mass spectrometry analysis and supershift EMSAs, are needed for identification of the proteins involved in EBNA IRES interactions

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

IRES-elements are important regulators for translation

This thesis demonstrates the presence and function of an IRES element located in an untranslated region within the Epstein-Barr virus genome.

The 5' UTRs of many viral and cellular mRNAs possess complex features predicted to impair ribosome recruitment and linear scanning: i) long leader sequence; ii) stable secondary structures; and iii) potential upstream initiation codons. Nevertheless, these 5' UTRs confer efficient translation. These discoveries argued for an alternative translational mechanism such as the internal entry of ribosomes. Picornaviral mRNAs are naturally uncapped at their 5' end which even more argued for an alternative to the cap-dependent scanning initiation.

The mechanisms that viruses have developed to promote internal entry of ribosomes are examples of very effective hijacking of the translational machinery of the host cell to favour the expression of foreign transcripts. For example, certain picornaviruses encode proteases that cleave eIF4G, thus dissociating the cap-binding activity of the eIF4F complex adaptor from its 40S subunit binding activity, which blocks cap-dependent translation of most host transcripts and enhances IRES-mediated translation of viral mRNAs [103]. There are several other mechanisms that lower the efficiency of cap-dependent translation initiation besides viral infection. Inhibition of cap-dependent translation in mitosis results from a combination of phosphorylation modifications leading to eIF4F complex disruption. It was shown that several viral and cellular mRNAs, for instance HCV [104], c-myc [105] and the ornithine decarboxylase (ODC) [106] IRESs ensure maintained or elevated levels of protein expression even under mitosis. Moreover, during apoptosis there is a reduction in translational initiation caused by the caspase cleavage of factors required for the cap-dependent scanning mechanism. Among other IRESs, it was shown that the IRES included in the vFLIP gene within the Kaposi's sarcoma associated herpesvirus (KSHV) was upregulated during apoptosis [57]. They speculated that this finding might provide a survival advantage for KSHV infected cells.

To facilitate studies of the biological significance of the EBNA IRES in different situations, we have stably transfected EBV-positive P3HR1 cells with an IRES-containing and IRES-lacking luciferase construct, respectively. We have performed experiments to investigate the EBNA IRES activity during induced lytic EBV phase. Lytic cycle was induced by adding the EBV-lytic cycle inducing agent phorbol-12-myristate-13-acetate (PMA). After induction cells were counted, harvested and analyzed with luciferase assay. Preliminary data showed a significant, 3-fold, difference ($p = 0{,}008$) between IRES mediated luciferase expression and cap-dependent translation in lytically induced cells. This indicates that the EBNA IRES is active in lytic phase. Data will be confirmed with quantitation of luciferase mRNA and for further validation. We also plan to quantify endogenous EBNA1 protein levels before and after lytic induction of untransfected EBV-positive cell lines. EBNA1 levels will then be compared to other protein levels that are not under the influence of an IRES, for example EBNA2 or actin.

The IRES activity in lytic phase might be beneficial for the viral exploitation of the host cell translating machinery when proliferating and spreading to new hosts.

The question if the EBNA IRES is utilized in other situations, as mentioned above, remains to be elucidated.

Clinical aspects of EBV infection and therapy

Organ transplantation is today saving patients at increasing rate, although the time after transplantation often is accompanied by serious complications. Severe immunsuppression make the patients especially susceptible to normally harmless viral infections, which under these circumstances might be life-threatening. Efficient antiviral treatments against EBV are missing, which constitute a severe problem for these patients. Several potential ways of treating PTLD have been suggested, but no strategy has so far been completely successful.

Immunosuppression reduction (IR) has been considered standard treatment in established PTLD to allow the immune system to decrease tumor progression. However, this approach accomplishes a decrease in progression, at best, in 50% of the cases [107]. Until recently, patients who failed IR were treated with cytotoxic chemotherapy with high morbidity and mortality. Yet, monoclonal antibody therapy (rituximab) is now frequently applied and widely regarded as the second line of treatment after IR [108]. However, PTLD may recur 4-8 months following rituximab treatment, as this treatment does not restore EBV-specific T cell immunity [107].

Antiviral treatment include the use of agents such as ganciclovir and acyclovir, thymidine kinase inhibitors, which are capable of inhibiting lytic viral replication but have no effect on tumor cells due to the absence of thymidine kinase in latently infected B cells [109]. Other treatment options includes enhancing CTL response and cytokine therapy [110] .

Strategies in order to control IRES activity could prove to be of antiviral therapeutic interest. A future perspective could be to target the EBNA IRES, which is included not only in the EBNA1 transcript but also in the EBNA3, 4, 6 transcripts, with small interfering RNAs, a process in which double stranded RNA targets homologous mRNA for endonucleolytic cleavage and degradation. This mechanism has proved to be efficient for inhibition of hepatitis C virus replication [111].

Another approach that is currently under investigation in our laboratory is the construction and use of EBV-based plasmids for specific gene therapy. EBV-based plasmid vectors are commonly used for non-viral gene therapy. These vectors contain the EBNA1 gene and the EBV oriP element that enable strong and long-term maintenance of transgene expression. For gene therapy against malignancies, EBV plasmid vector encoding the herpes simplex virus 1 thymidine kinase (HSV1-TK) suicide gene in combination with ganciclovir (GCV) administrations has been used successfully for tumor suppression [112]. However, there is a problem with this type of construct where a non-specific general HSV1-TK expression occurs in all treated cells including non-malignant cells.

We have constructed vectors expressing high amounts of HSV1-TK in EBV-infected cells but with no or very low expression in EBV-negative cells. These vectors may be useful for specific therapy against EBV-induced human malignancies. The vectors contain an EBV-specific promoter that drives the TK-expression, the EBV oriP element but without the EBNA1 gene. This ensures that only EBV-infected cells, which express EBNA1, are affected by GCV treatment. Further development of this system was to incorporate the EBNA IRES upstream the HSV1-TK gene in the vector in order to

increase the translation of HSV1-TK in compliance with the previous results from our bicistronic system [98]. To test the potential of EBV/TK vectors for therapeutic applications, functional assays *in vitro* will be used on human B cells representing different EBV latencies. The hygromycin resistance gene is included in the vectors to facilitate selection of transfected cells.

Biological relevance

Increased knowledge of molecular mechanisms regulating EBV latent gene expression will help us to find markers and potential antiviral targets for EBV induced proliferative disease. Our data provide evidence that cap-independent translation through an internal ribosome entry site, alternative splicing of non-coding mRNA and nucleotide changes in the EBV genome are important mechanisms for EBV gene regulation. Since the EBNA IRES element is also included within the EBNA3, 4 and 6 mRNAs, it demonstrates that part of the EBV immortalization process might be regulated by cap-independent translation. Further, the biological relevance of the presence and function of the EBNA IRES might be to fine tune the EBNA1 expression in order for the virus to keep the EBNA1 protein in a steady state.

OVERALL CONCLUSIONS

- Epstein-Barr virus U leader exon, present in EBNA1 transcripts contains an internal ribosome entry site.

- The incidence of EBNA1-positive blood samples is more than 3-fold in samples from PTLD patients compared to patients with no signs of PTLD.

- An alternatively spliced transcript, that excludes the IRES-element, was found in samples from EBV-positive organ transplant patients.

- The patient specific EBNA IRES sequence differs in one and two nucleotide positions compared with the sequence derived from laboratory cell lines.

- The EBNA IRES activity is cell type specific.

- The EBNA IRES binds specifically cellular proteins collected from both EBV-positive and -negative B cell lines, as well as proteins from an EBV-negative T cell line.

- The patient derived EBNA IRES sequence binds proteins more efficiently than the B95.8 derived sequence.

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