The interactome of the Epstein-Barr virus nuclear antigen 5 suggests novel roles in RNA and protein metabolism

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Front page: MCF7 cell with staining of EBNA5 (red), luciferase in endoplasmic reticulum (green), and DNA (blue).

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till Mikael och Elin

Det krävs ett helt nytt sätt att tänka för att lösa de problem vi skapat med det gamla sättet att tänka – Albert Einstein

ABSTRACT

Epstein-Barr virus (EBV) is a human herpes virus that infects B and epithelial cells of the oropharynx. EBV is transmitted by saliva and establishes a lifelong latency in over 90% of the world's population. During latency, the virus exists predominantly as multicopy episomes in the nuclei of memory B cells. If these memory B cells are activated they can produce EBV virions and the person may shed the virus. Most people are exposed to the virus as children, when the disease produces no noticeable symptoms or only flu-like symptoms. Latent infection is associated with several malignancies, including Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma, and lymphoproliferative disorders in the immunosuppressed patients. A compromised immune system and an aberrant EBV latent gene expression are thought to play roles in the aetiology of EBV malignancies. EBV immortalizes human B cells through expression of at least 12 viral genes, which include the EBNA5 protein. Elucidation of EBNA5 functions has been guided by identification of interacting cellular and viral proteins. The functions of these cofactors implicate EBNA5 as a potential modulator of apoptosis, cell cycle processes, and transcriptional pathways. This thesis will add to the knowledge about how EBNA5 contributes to EBV biology. By using luciferase and CAT as model proteins to study EBNA5-regulated gene expression in lymphoid cells, we found that EBNA5, at high but still biologically relevant levels, acted as a promiscuous repressor of gene expression from transfected plasmids. However, EBNA5 was more selective in the regulation of chromosomal genes; only two out of 588 human genes, thereof the pro-apoptotic BNIP3 gene, were down-regulated. The decrease in expression from transfected plasmids was partly explained by the ability of EBNA5 to inhibit luciferase pre-mRNA 3´-end cleavage and polyadenylation. To gain further insight into molecular pathways in which EBNA5 may have a regulatory role, we searched for cellular targets for EBNA5. By using an improved tandem affinity purification procedure, we identified 147 novel interaction partners of which 37 were validated with independent methods. The validated proteins could be grouped into three main classes depending on their biological function: I) protein folding and degradation, II) pre-mRNA processing, and III) ribosome biogenesis. We further showed that EBNA5 is part of high molecular complexes. Of particular interest, we verified interaction between EBNA5, Hsp70, and BAG2, a co-chaperone with the ability to inhibit ubiquitinylation, and as a consequence, protein degradation of Hsp70 clients. Further studies on the effect of EBNA5 on luciferase activity demonstrated a correlation between luciferase activity and level of soluble luciferase protein. In addition, substantial amounts of insoluble luciferase had accumulated in the nuclei. Insolubilisation was accompanied by translocation of luciferase, EBNA5, Hsp70, and BAG2 to the nucleoli and to a small number of nuclear foci. An EBNA5 mutant (ΔCR3) was neither able to induce a similar translocation nor able to associate with BAG2. Therefore, we identified BAG2 as a major target of EBNA5. Moreover, over-expression of Hsp70 blocked EBNA5-induced insolubilisation of luciferase. Our results indicate that EBNA5 is likely to play a regulatory role in the decision between degradation and folding.

Keywords: *Epstein-Barr virus, EBNA5, chaperone, proteasome, nucleolus*

PUBLICATIONS

This thesis is based on the following papers:

I. Epstein-Barr virus nuclear antigen 5 inhibits pre-mRNA cleavage and polyadenylation.
Dufva, M., **Flodin, J.**, Nerstedt, A., Rüetschi, U., and Rymo, L.
Nucleic Acids Res. (2002) May;30(10):2131-2143

II. Identification of intracellular proteins associated with the EBV-encoded nuclear antigen 5 using an efficient TAP procedure and FT-ICR mass spectrometry.
Forsman, A., Rüetschi, U., **Ekholm, J.**, and Rymo, L.
Journal of Proteome Research (2008) Jun;7(6):2309-2319

III. Epstein-Barr virus nuclear antigen 5 is a multi-functional protein with a possible role in the chaperone-mediated protein folding and ubiquitin-proteasome degradation systems.
Ekholm, J., Forsman, A., Kashuba, E., Andersson, M. K., Rüetschi, U., and Rymo, L.
In manuscript

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ABBREVIATIONS

BAG2	BCL2-associated athanogene 2
BCR	B cell receptor
BL	Burkitt's lymphoma
cDNA	complementary DNA
CHIP	c terminus of Hsp70-interacting protein
CR	conserved region
Cp	the EBV C promoter
dsDNA	double stranded deoxyribonucleic acid
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
FT-ICR	fourier transform ion cyclotron resonance
GC	germinal centre
HD	Hodgkin's disease
HnRNP	heterogeneous ribonucleoprotein
Hsp	heat shock protein
IM	infectious mononucleosis
IR	internal region
kbp	kilobase pair
LCL	lymphoblastoid cell line
LMP	latent membrane protein
LUC	luciferase
MHC	major histocompatability complex
mRNA	messenger RNA
MS	mass spectrometry
MS/MS	tandem mass spectrometry
m/z	mass-to-charge ratio
NPC	nasopharyngeal carcinoma
PTLD	post transplant lymphoproliferative disease
RNA	ribonucleic acid
TAP	tandem affinity purification
Wp	the EBV W promoter

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Chapter 1
EBV biology

1.1 Discovery of Epstein-Barr virus (EBV)

In 1957, Denis Burkitt served as a surgeon in a hospital in Kampala, Uganda. He noticed several boys with swellings of the jaws and one of them also suffered from abdominal tumours. After further research into records relating to these tumours, he found that swelling of the jaws often correlated with tumours at unusual sites and concluded that these tumours were derived from a single tumour complex. In 1958, Denis Burkitt published the first paper on this tumour which would then be called Burkitt's lymphoma. Burkitt's pioneering work attracted the attention, respect, and friendship of a scientist called Anthony Epstein. In 1964, Epstein managed to develop the first B lymphoid cell line EB1, from biopsy samples received from Burkitt. Further examination of this cell line with the electron microscope showed that these cells contained "virus, like herpes". Epstein, together with Yvonne Barr and Bert Achong published their finding in 1965. In collaboration with the Henle lab, Epstein confirmed that EBV was a new herpes virus (1).

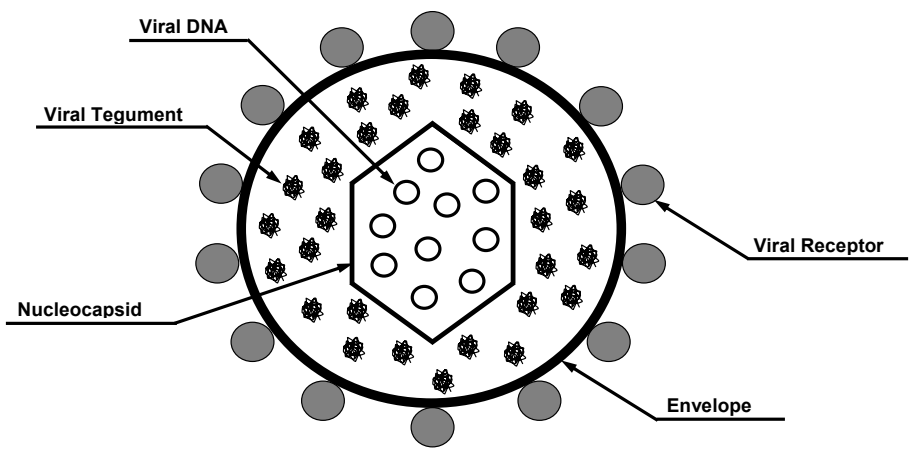

Figure 1. Schematic presentation of herpesvirus.

1.2 Classification of Epstein-Barr virus (EBV)

Group: Group I (double stranded DNA)
Family: Herpesviridae
Subfamily: Gammaherpesvirinae
Genus: Lymphocryptovirus
Species: Human herpesvirus 4

1.3 Viral morphology and virus infection

A virus is an infectious agent too small to be seen directly with a light microscope which is why scanning and transmission electron microscopes are used to visualize virions (virus particles). Viruses are not cells and can only replicate inside the cells of another organism. A complete virus particle consists of nucleic acids surrounded by a protective protein coat called a capsid. The EBV genome is collinear and consists of 175 kilobase pairs of DNA. The capsid is made from proteins encoded by the viral genome and its icosahedral shape serves as the basis for morphological distinction (Fig. 1). The capsid is formed from identical protein subunits called capsomers and the EBV virion contains 162 capsomeres. In addition to DNA and a capsid, the EBV particle has a tegument and an envelope. The tegument is a cluster of proteins that lines the space between the envelope and capsid of many enveloped viruses. It generally contains proteins that aid in viral DNA replication and evasion of the immune response, typically with inhibition of signaling in the immune system and activation of interferons. The tegument is usually released into the cytoplasm shortly after infection. The envelope typically is derived from portions of the host cell membranes (phospholipids and proteins), but includes some viral glycoproteins. The viral envelope helps the virus particle to enter the host cells. Glycoproteins on the surface of the envelope serve to identify and bind to receptor sites on the host membrane. The viral envelope then fuses with the host membrane. Gp350/220 is the most highly abundant glycoprotein in the EBV envelope. Gp85 is the next most abundant glycoprotein. Gp350/220 binds to complement receptor type 2 (CR2)/CD21 on B cells. Binding of the gp350/220 to CD21/CR2 mediates the initial EBV attachment to B cells (2). Viral internalization into B cells depends on additional interactions between the gp85-gp25-gp42 viral glycoprotein complex and the MHC II cell surface receptor (3). After fusion of the virus with the host cell membrane, EBV is endocytosed into vesicles, and subsequently released into the cell. Inside the nucleus, the EBV DNA becomes circular and persists as multiple episomal copies. CD21 is part of the B-cell co-receptor which after binding to complement fragment C3d or to EBV increases signal transduction through the B cell receptor (antigen receptor).

1.4 Viral life cycle and immune response

EBV enters their host through saliva into the crypts of lymphoepithelial structures such as the tonsils in the oral cavity. EBV crosses the epithelial barrier, which is often only one cell thick, immediately above the bed of lymphocytes below. There is evidence that B cells are necessary to establish an EBV infection (4) and that the hematopoietic system is the only site of viral latency *in vivo* (5). Although the role of the oropharyngeal epithelium as a site of virus replication is firmly established in AIDS patients with oral hairy leukoplakia (OHL) (6), its role in the life cycle of the virus in normal healthy individuals is controversial. Epithelial cells do not express the virus receptor CD21 and their ability to be infected with cell-free virus is limited. It has however been shown that B cells decorated with surface-bound virus particles act as efficient transfer vehicles for epithelial cells. Epithelial cells can be readily infected by co-culture with virus-producing B cell lines but only poorly by cell-free virus (7). Transfer of virions from B cells to epithelial cells involves the gp85 and gp110 viral glycoproteins but is independent of gp42. A model has been proposed in which naïve B cells transfer virus particles to epithelial cells independently of initial virus replication in the naïve B cells. The infected epithelial cells then initiate replication of the virus to infect other naïve B cells. Epithelial cells therefore represent an amplification step in the viral life cycle (8). EBV infection of naïve B cells drives them to differentiate into B blasts through expression of the growth programme under the regulation of EBV latency III gene products EBV nuclear antigens (EBNAs)1-6, LMP1, LMP2A, LMP2B, and EBERs (9) (Table I). Primary infection of B cells results in latent infection with no virus production but with expansion of B blasts in extrafollicular areas of tonsillar lymphoid tissue and the appearance of large numbers of infected cells in the blood. Expression of EBV proteins elicit a very potent cytotoxic T cell response that keeps infected B cells under control in healthy individuals (10). Both lytic- and latent antigen specific $CD8^+$ T cells are found in the blood in large numbers with dominance for the former. $CD4^+$ T cells specific for EBV are also found but in much smaller quantities (11). EBNAs 3, 4 and 6 elicit a strong immune response, whereas EBNAs1, 2, and 5, as well as LMP1, are weak immunogens. EBNA1 contains a Gly-Ala repeat domain that blocks proteasome degradation (12,13) and inhibits translation of mRNA (14). Despite the vigorous immune response, the virus is not completely eliminated. The activated B blasts that have survived migrate into follicles and form germinal centres. Germinal centres (GCs) are sites of normal B-cell proliferation, selection and maturation into anti-body producing plasma cells or memory cells. It has been debated whether expansion of infected B blasts occurs in germinal centres and whether infected cells undergo a germinal centre reaction. However, recent evidence supports clonal expansion (15,16) and selection and maturation in GCs (17). Normally, survival of B cells requires high-affinity binding of antigen and reception of stimulatory signals from helper T cells and dendritic cells in the GC.

The issue of whether EBV-infected cells must bind antigen and receive T cell help in order to become B blasts and survive has not been resolved. It is known however that EBV infected blasts switch from the latency III growth programme to the default programme (latency II programme) by turning off EBNA2-6. Expression of LMP1 (a CD40 homologue) and LMP2A (a B-cell receptor homologue) in the absence of EBNA2 provide the requisite survival signals and provide the surrogate T cell help and B cell receptor signals that the B cells require for becoming memory B cells. LMP2A and LMP1 have additional tasks in the GC; LMP2 induces B cells to form a germinal centre in the mucosal follicle; LMP1 and LMP2 can drive immunoglobulin gene mutation and isotype switching (18,19). EBNA1 is also expressed in the germinal centre to maintain replication of the EBV episome (20). B cells thus use the germinal centre to gain lifelong access into the pool of memory B cells. It is not known exactly why infected cells become exclusively memory cells (and not plasma cells). One possibility is that LMP1, by mimicking a constitutive active CD40 receptor, contributes to this selection (17,21). After selection in the GC, B cells leave through the efferent lymphatics and enter the circulation. In the peripheral circulation, the infected memory B cells shut down all EBV expression to escape from immune surveillance (latency 0) but express EBNA1 occasionally (latency I) to allow replication of the EBV episome before cell division. As a result, healthy individuals harbour between 1 and 10 latently infected B cells per one million peripheral blood mononuclear cells. Upon encountering an antigen or when receiving a differentiation signal, the memory B cells differentiate into plasma cells which express the EBV lytic genes and release virions onto mucosal surfaces. This will eventually lead to a new round of infection of naïve B cells. However, this time, tonsillar lytic and latent antigen specific memory $CD8^+$ T cells are enriched in the tonsils.

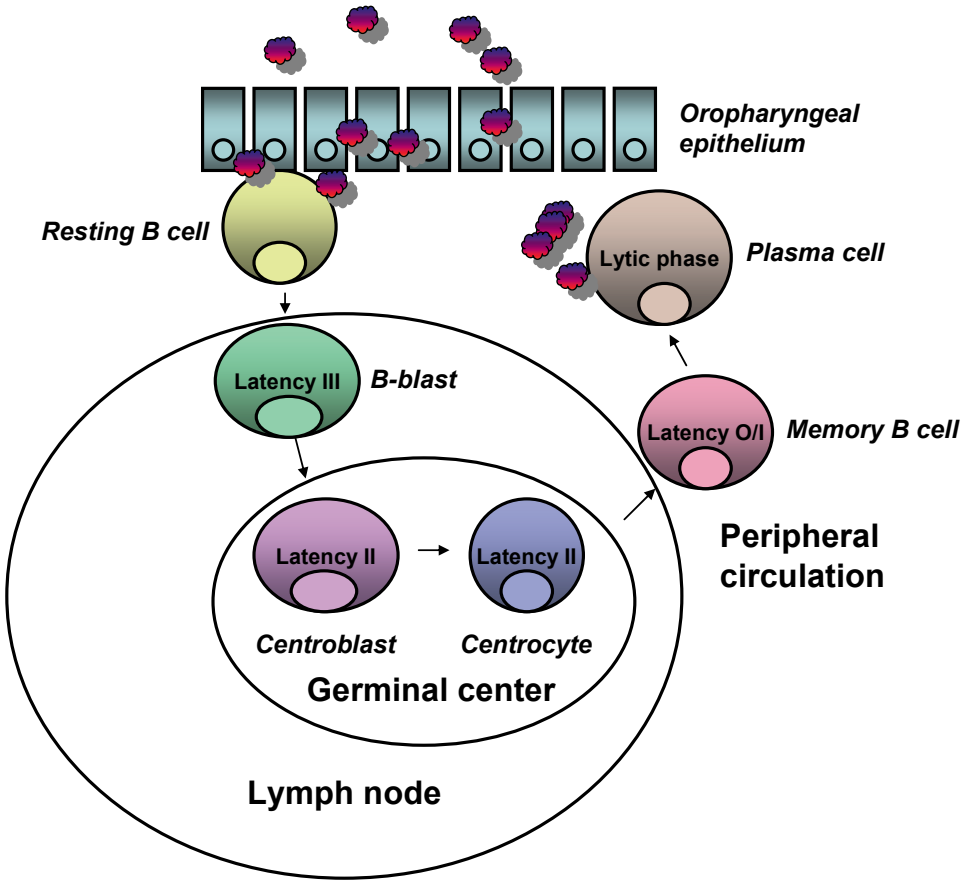

Figure 2. Viral life cycle.

Latency	Genes expressed	EBV-associated diseases, examples
0	none	
I	EBNA1	Burkitt´s lymphoma
II	EBNA1, LMP1, LMP2A	Hodgkin´s disease, Nasopharyngeal carcinoma
III	EBNAs1-6, LMP1, LMP2A, LMP2B	Infectious mononucleosis, Lymphoproliferative disease

Table 1. Latency programmes in EBV.

1.5 EBV and disease

The ability of EBV proteins (especially latent gene products LMP1, LMP2A, and EBNA2) to induce hyperproliferation, introduce gene mutations, inhibit apoptosis and increase expression of proto-oncogenes, leads to an increased risk of uncontrolled growth and tumour development. Even though EBV has evolved to minimize the risk that an infected cell will proliferate out of control, tumours sometimes develop. The tumours that may develop through EBV infection are associated with specific stages in the EBV life cycle, i.e. exhibit a specific latency programme. Certain tumours develop as a result of failure of the cells to differentiate into memory cells, others develop as a result of opportunistic growth in the absence of immune response.

1.5.1 Benign tumours

Infectious mononucleosis

Most people are exposed to the virus as children, when the disease produces no noticeable or only flu-like symptoms. Adolescents and young adults infected by EBV often develop Infectious mononucleosis. The acute phase of Infectious mononucleosis (IM) is characterized by peripheral blood B cells expressing the latency III genes, high-level shedding of virus particles in the throat and a strong cytotoxic T cell response. Later on, B cells become resting memory cells which do not express EBV genes (latency 0). The most common symptoms of IM include fever, fatigue, sore throat, and swollen lymph nodes. Other syndromes include splenomegaly, hepatomegaly, and hepatitis. Infectious mononucleosis is usually a self-limited, although sometimes prolonged illness. In most cases no specific treatment is necessary. Treatment is directed toward the relief of symptoms. Available antiviral drugs have no significant effect on the overall outcome of IM. People who have had IM can continue to shed virus particles in their saliva during reactivations of the viral infection throughout their lifetime.

1.5.2 Malignant tumours

Posttransplant lymphoproliferative diseases/disorders

Posttransplant lymphoproliferative diseases/disorders (PTLD) are B cell lymphomas occurring in immunosuppressed patients in 0.2% of patients within one year of organ transplant. Over 80% are associated with EBV infection. PTLD-derived tumours share characteristics with Immunoblastic lymphomas in AIDS patients and X-linked lymphoproliferative syndrome. These lymphomas are derived from a mixture of B cell types (22) and possibly from cells that are unable to differentiate into resting memory B cells and that proliferate indefinitely

because of a suppressed immune response. These cells express the latency III programme.

Hodgkin's disease

Hodgkin´s disease (HD) is associated with EBV in 40% of cases. HD is characterized by the presence of malignant Hodgkin/Reed Sternberg cells. These cells express the latency II programme and are possibly derived from germinal centre cells because they contain hypermutated immunoglobulin genes with a pattern of mutations characteristic for germinal centre cells. HD is most common in the USA, Canada, and northern Europe, and is least common in Asian countries. HD is most common in early adulthood (15-30 years) and in late adulthood (>55 years).

Burkitt's lymphoma

Burkitt's lymphoma (BL) was the first human tumour to be linked to a viral infection (23). BL is a common tumour among children in Central Africa, Papua New Guinea, and Bahia (Brazil). There are three different forms of Burkitt's lymphoma; endemic, sporadic and AIDS related. All BLs carry a chromosome translocation that results in upregulation of the c-myc oncogene. The involvement of EBV in disease progression is not obvious because of the low incidence of EBV in forms other than endemic BL. In addition, BL cells express the latency I programme (EBNA1 only) and none of the growth-promoting genes are expressed. However, recent evidence shows that EBNA1 inhibits apoptosis in BL cells (24). With endemic BL, the association of EBV with primary tumours in Central Africa is almost 100% while association with sporadic BL is only 20%, and AIDS related BL 30%. Based on the restricted geographic distribution of BL, several co-factors for the onset of endemic BL have been proposed. Malaria infection causes increased risk of developing BL and this has been supposed to be attributable to the immunosuppressive effect of malaria (25).

Nasopharyngeal carcinoma

Nasopharyngeal carcinoma (NPC) is an epithelial tumour that arises from the epithelial cells that cover the surface and line the nasopharynx. NPC is always associated with EBV. Both genetic and environmental factors contribute to the tumourigenesis of NPC. Several linkage analyses studies suggested the association of susceptibility HLA haplotypes with NPC development. The consumption of certain salted food and exposure to nitrosamines and polycyclic hydrocarbons contribute to tumourigenesis (26). NPC is very common in Southern China and Southeast Asia where it represents 20% of all cancer cases. In Western countries on the other hand, NPC accounts for only 0.25% of all malignant neoplasm. EBV expression is restricted to the latency II programme.

1.6 Latent EBV gene products and some of their functions

Epstein-Barr virus nuclear antigen 2 and -5 (EBNA2 and EBNA5) are the first viral genes expressed after infection and are crucial for viral gene expression (latency III genes) as well as expression of cellular genes encoding proteins that promote cellular growth and proliferation. After *in vitro* infection of naïve B cells, transcription is initiated from the W promoter (Wp) of the EBV genome, leading to the expression of EBNA2 and EBNA5 (27). Within 48 hours there is a switch in promoter usage from Wp to the C promoter (Cp) leading to the expression of EBNAs1-6. EBNA genes are expressed in a conserved temporal sequence. Multiple isoforms of the EBNA5 protein are generated during infection (28). As a result of alternative splicing, isoforms contain 1-7 copies of the W_1W_2 repeat linked to the unique Y1- and Y2-encoded domains (27,29). EBNA5 is encoded by the exons of the internal repeat 1 (IR1) and the BamHI Y viral fragment of the EBV genome. The six Epstein-Barr virus (EBV) latency nuclear antigens (EBNAs) are expressed from a long common primary transcript. For EBNAs other than EBNA5, the IR1 constitutes a 5´-UTR and alternative splicing dictates the specific translation initiation codon for each EBNA (Figure 3) (30).

EBNA1

EBNA1 is essential for the replication and maintenance of the EBV episome (31). EBNA1 up-regulates two key EBV latency promoters: the BamHI C promoter (Cp) (32-35) and the LMP1 promoter (36).

EBNA2

EBNA2 plays an important role in switching between the W and C promoters. One cis-element in Cp is positively regulated by EBNA2 (37). The LMP1 and LMP2 promoters are also activated by EBNA2. EBNA2 lacks sequence specific DNA-binding ability and is recruited to promoters by its interaction with the DNA-binding protein Jκ recombinant signal binding protein (RBP-Jκ) (38). EBNA2 induces transcription of LMP1 via other transcription factors as well (39-42). Furthermore, expression of CD21, CD23, and cellular proto-oncogenes *c-myc* and *c-fgr* is up-regulated by EBNA2, consistent with its ability to induce cell proliferation and inhibit apoptosis (35).

EBNA3, 4, and 6 (EBNA3A, -3B, and -3C)

EBNA3 and -6 are essential for B-cell immortalization, EBNA4 is not. All three EBNAs can repress EBNA2-mediated transactivation of the EBV LMP2 promoter (43) and EBNAs3 and 6 repress C promoter activity through an EBNA2-independent, RBP-Jκ-dependent mechanism (44,45). In contrast, EBNA6 co-activates the LMP1 promoter with EBNA2 (46). All three EBNAs bind to the

human 20S proteasome but appear to be remarkably stable in actively proliferating lymphoblastoid cell lines (47).

EBNA5 (EBNA-LP)

EBNA5, in combination with EBNA2, induce C promoter-driven expression of the different EBNAs. In addition, EBNA5 and EBNA2 also up-regulate expression of LMP1 (48-50) and cyclin D2 (51), which correlates with increased proliferation of infected cells. It has been shown that EBNA5 is important for efficient immortalization *in vitro* (52). So far, EBNA5 has been reported to associate with EBNA2 (30) but not directly with cis-elements in promoter DNA. The co-stimulatory effect of EBNA5 on transcription is supposed to be mediated by Hsp72 (53) and Sp100 (54). The level of EBNA5 expression is highest during the first two days of EBV infection but then decreases to levels found in established lymphoblastoid cell lines (LCLs) (55,56). EBNA5 is diffusely distributed in the whole nucleoplasm during the initial phase of infection, and appears in PML nuclear bodies as well by the end of the first day. Co-localization between EBNA5 and PML in non-blast cells does not occur during normal growth conditions. In DG75 for example, transfected EBNA5 is expressed as fine speckled nuclear dots (56) and does not co-localize with PML (57). However, co-localization has been observed in p14ARF positive nuclear inclusions in MCF7 cells co-transfected with EBNA5 and p14ARF DNA (58). In addition, proteasome inhibition in cells caused relocation of EBNA5, Hsp70 (59), PML, proteasomes, and Sp100 to the nucleoli (57). Other stressful stimuli like heat shock and metabolic stress, induced by high cell density, caused the reversible translocation of EBNA5 and Hsp70 to the nucleolus (60). The EBNA5 W_1W_2 repeat sequence contains three domains (CR1, -2, and -3) that are conserved among several nonhuman primate lymphocryptoviruses (LCVs). These domains have been shown to be important for the co-activating function of EBNA5 with regard to promoter activation (48,61-63). The ability of EBNA5 to co-operate with EBNA2 is up-regulated by phosphorylation of serine residue 36 in the W_2 domain (the position number is relative to the first amino acid in W_1) which is a potential target of p34cdc2 kinase (61,64,65). In addition to its role in regulating promoter activity, EBNA5 has been suggested to be involved in proteasome activities consistent with the co-localization of EBNA5 and proteasome components in the cell (57,58). EBNA5 binds to several proteins involved in protein folding and proteasomal degradation, including Hsp70 (66,67), Hsp27 (68), BAG2 (69), and subunit 2 of the 19S regulatory complex of the 26S proteasome .

LMP1

LMP1 is a potent oncogene, being able to transform cells in the absence of other EBV genes. LMP1 stimulates cell proliferation and protects cells from apoptosis. LMP1 acts as a constitutively active TNF receptor (TNFR)/CD40 homologue (68). LMP1 is expressed at different stages of the viral life cycle. During acute infection of naïve B cells, LMP1 acts by promoting B cell blast activation. In germinal centres, LMP1 ensures survival by virtue of its ability to resemble an active CD40 molecule and by its ability to induce host expression of IL10, IL6, and CD23, resulting in growth and differentiation, increased expression of MHC II, and inhibition of cytotoxic T cell responses.

LMP2

LMP2A can rescue B cells from apoptosis and augment a weak BCR (B cell receptor) signal. LMP2A like LMP1 and EBNA1, is expressed during latency II and III and is thus expressed in malignant tumours from patients with Hodgkin's disease (HD) and NPC. LMP2A inhibits lytic reactivation from latency (70). LMP2A (in combination with LMP1 and EBNA1) ensure temporal activation and proliferation of memory B cells in the tonsillar lymph nodes to ensure long-term survival of memory B cells in the absence of antigen stimulation and T cell help. LMP2A induces B cells to form a germinal centre in the mucosal follicle; LMP1 and LMP2A can drive immunoglobulin gene mutation and isotype switching (18,19). LMP2B is an integral membrane protein and is thought to modulate the function of LMP2A (71).

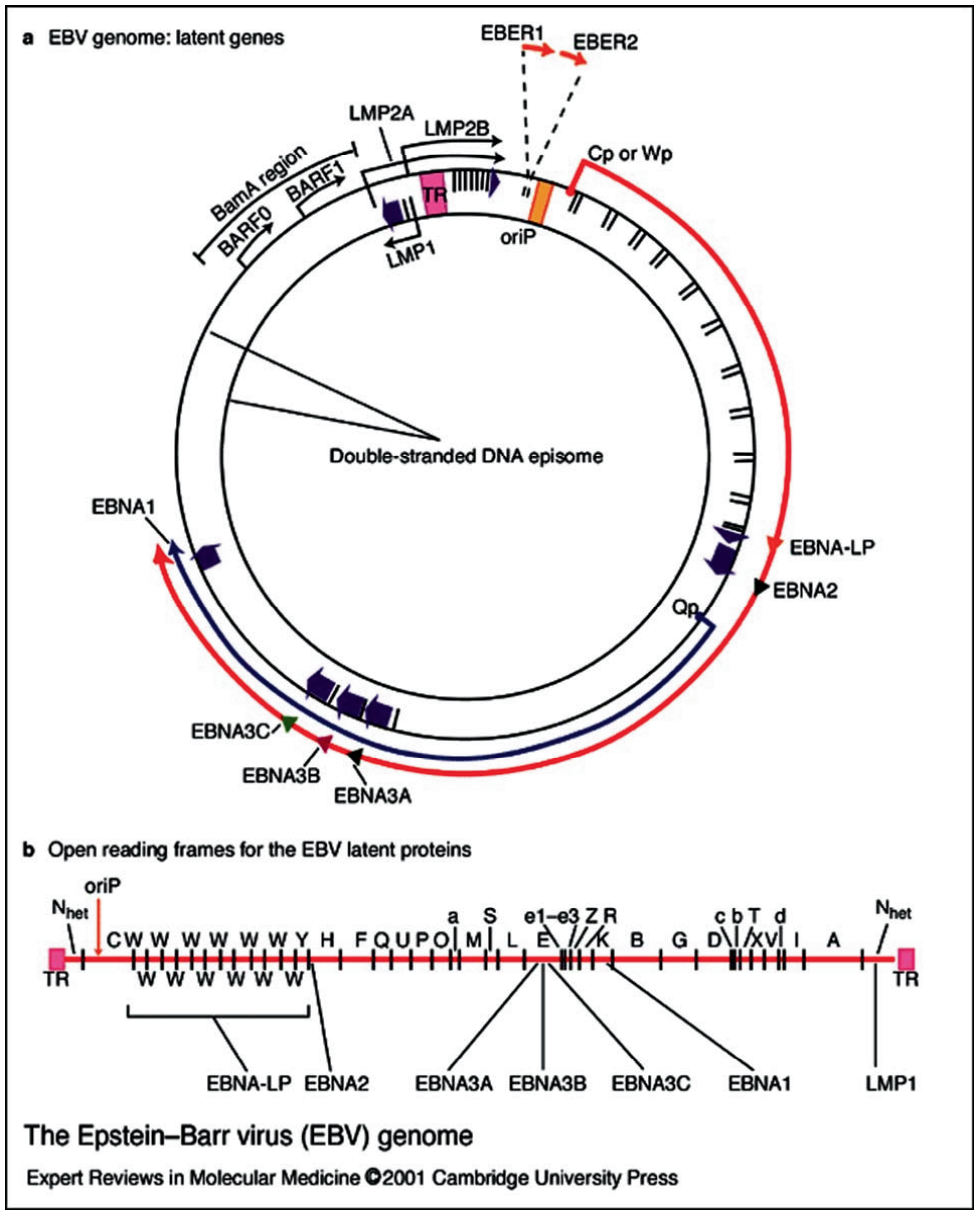

Figure 3. The Epstein-Barr virus genome. *Reprinted from Cambridge University Press ISSN 1462-3994*

Chapter 2
Posttranscriptional regulation

2.1 RNA processing

In eukaryotes, messenger RNA precursors (pre-mRNAs) are transcribed in the nucleus from the genomic DNA by RNA polymerase II (polII). These pre-mRNAs must undergo extensive co-transcriptional processing before they can be transported to the cytoplasm for translation into proteins. The processing events include capping, where the guanosine at the 5′-end of the pre-mRNA is methylated; splicing to remove the intronic sequences of the pre-mRNA; and cleavage and polyadenylation at the 3′-end (72-75). The processing of pre-mRNA 3′-ends has crucial functional importance in eukaryotes. Firstly, it promotes the transport of mRNAs from the nucleus to the cytoplasm (76). Secondly, it promotes the stability of mRNAs (77,78). In the cytoplasm, mRNAs are degraded from the 3′-end first, indicating the importance of protecting the 3′-end (77). The addition of the poly(A) tail and subsequently the binding of poly(A) binding protein (PABP) has been shown to prevent degradation in mammalian cells (79). Thirdly, 3′-end processing enhances the translation of mRNAs into proteins. The poly(A) tail and poly(A)-binding protein (PABP) interact with the methyl cap at the 5′-end to promote translation (77,78,80,81). Finally, 3′-end processing is intricately coupled to the transcription and splicing machineries (82-86). The 3′-end processing complex interacts with transcription factors and the C-terminal domain (CTD) of polII to help control transcriptional initiation and elongation, and a proper poly (A) signal is essential for transcriptional termination. It is now accepted that all RNA processing events are co-transcriptional and interdependent. The 3′-end cleavage and polyadenylation reaction is directed by sequence elements within the untranslated region of the pre-mRNA (*cis* elements). One is the polyadenylation signal (PAS) which is highly conserved and constitutes the hexamer sequence AAUAAA (Figure 4). Another is the downstream element (DSE) which is less conserved and has been observed in two forms; a GU-rich element that has a sequence of YGUGUUYY (Y=pyrimidine) (87,88) and a U-rich sequence (UUUUU) (87,89). However, pre-mRNAs can have neither, one, or both of these sequences (90). The cleavage site is positioned between the PAS and the DSE, and an optimal cleavage site is preceded by the nucleotide sequence CA (91). An auxiliary upstream element, located upstream of the PAS, does not have a consensus sequence, but often consists of a U-rich element (UUUU). The efficiency of cleavage and polyadenylation is enhanced by the presence of this auxiliary element (73). Most auxiliary upstream elements enhance expression of intronless genes, which are normally expressed at lower levels than transcripts that contain introns (92). A large number of proteins are required for 3’-end processing. A complex of proteins called cleavage and

polyadenylation specificity factor (CPSF) binds to the PAS. Yet another complex; cleavage stimulation factor (CstF), is composed of different polypeptide subunits and binds to the DSE. CstF interacts with bound CPSF, forming a loop in the RNA. Binding of CFI (cleavage factor I) and CFII (cleavage factor II) help stabilize the complex. Binding of poly(A) polymerase (PAP) then stimulates cleavage at the PAS. CPSF-73 has been reported to be the endonuclease (93). The cleavage factors are then released, as is the downstream RNA cleavage product, which is rapidly degraded. Bound PAP then adds ≈12 adenine nucleotides at a slow rate to the 3′-hydroxyl group generated by the cleavage reaction. Binding of poly(A)-binding protein II (PABII) to the initial short poly(A) tail accelerates the rate of addition by PAP. After 200–250 A residues have been added, PABII signals PAP to stop polymerization. In addition to their role in polyadenylation reactions, CstF and CF factors bind to TFIID to help in transcription initiation, and also bind to polII CTD to help in transcriptional elongation and termination.

2.2 Protein folding and degradation

The protein quality control system determines if an unfolded or misfolded protein is refolded or degraded. The control system consists of chaperones, co-chaperones, proteasomes, and proteins involved in ubiquitinylation and deubiquitinylation. Hsp70 has an intrinsic capacity to fold/refold proteins but Hsp70 can also direct unfolded proteins to the proteasome for proteolysis. Several different co-chaperones can bind to Hsp70. Each co-chaperone is functionally distinct and determines the fate of an unfolded protein associating with Hsp70. Some co-chaperones serve as links to the proteasome and stimulate proteasomal degradation. Other co-chaperones increase Hsp70 folding activity. Molecular chaperones were first recognized for their roles in *de novo* protein folding and the cellular response to stress. Stress could mean for example heat shock, UV irradiation, hypoxia, oxidation, and ATP depletion. There are cytosolic Hsp70 chaperones that bind elongating polypeptides already during translation. After the synthesis is complete, some proteins are released by the chaperones and ready to function. Others remain bound to the Hsp70 molecules that direct them to more specialized folding machines, i.e. TRiC (a ringshaped octamer, homologous to bacterial GroEL) or Hsp90. An overview of chaperones and co-chaperones is presented in Figure 5.

Some of the chaperones and co-chaperones will be discussed due to their involvement with EBNA5.

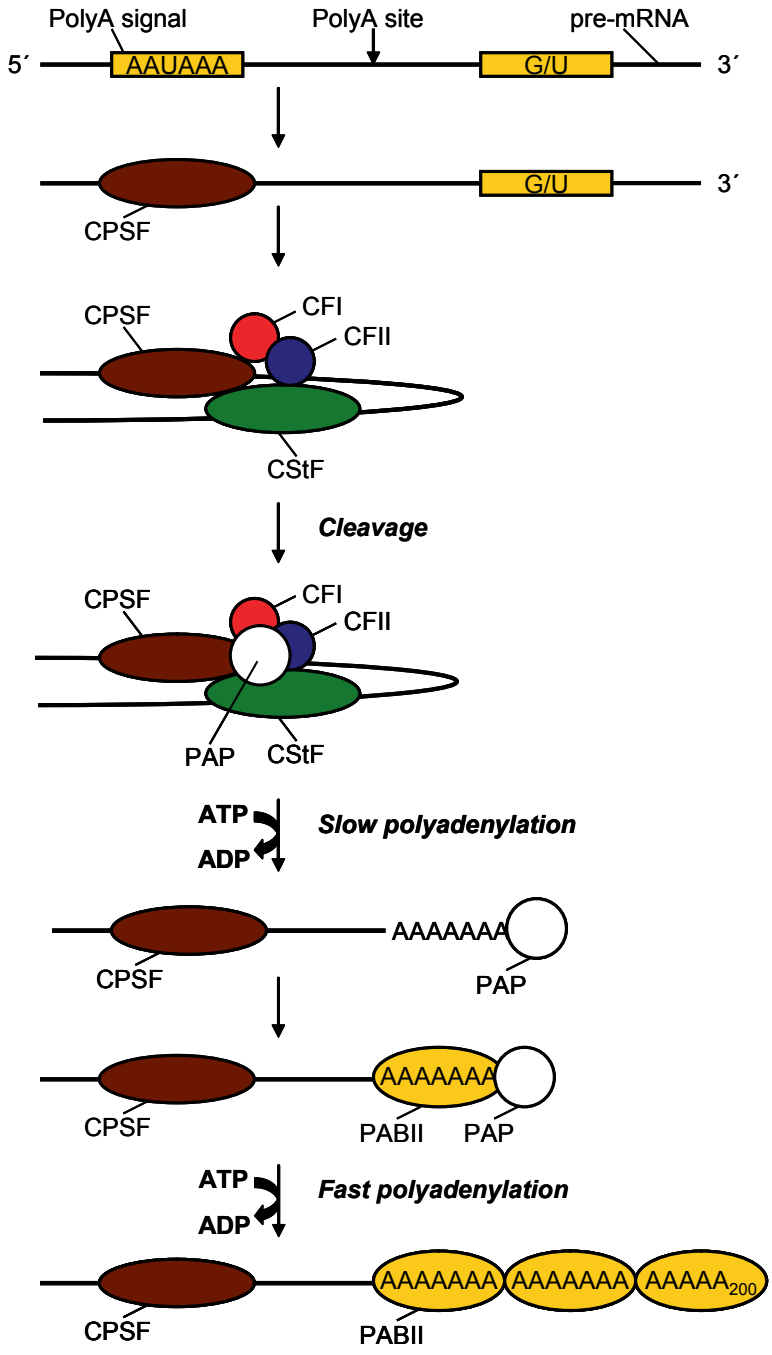

Figure 4. The polyadenylation proccess.

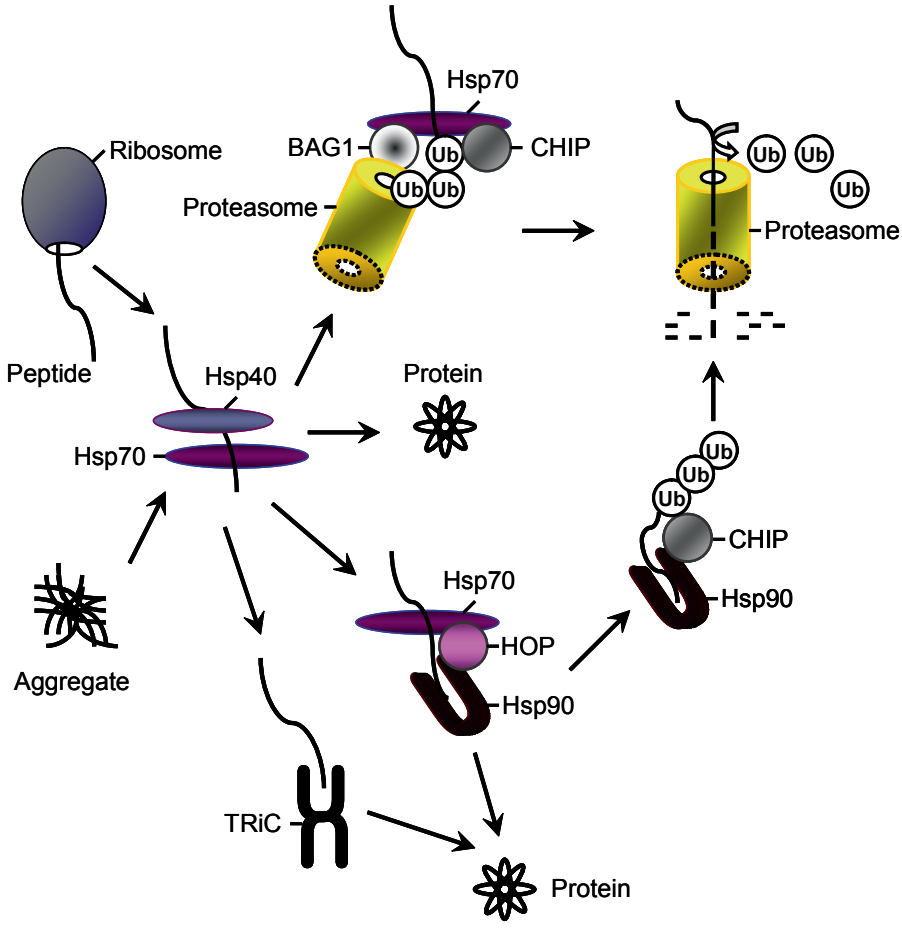

Figure 5. Chaperones, co-chaperones, and the proteasome.

2.2.1 Protein folding

Hsp70 (Heat shock 70 kDa protein)

The principal property of chaperones is to bind unfolded or partially folded polypeptides. During the early stages of folding or when misfolding occurs, the hydrophobic residues of a protein are partially solvent accessible, making the protein vulnerable to aggregation. Association of these hydrophobic protein species with chaperones efficiently suppresses aggregation. Some co-chaperones bind unfolded Hsp70 clients and deliver them to Hsp70. Hsp40 (94), Hsp27 (95), BAG2 (96), and CHIP (97) belong to this group. Hsp40 functions together with Hsp70 to promote protein folding. Hsp70 contains an N-terminal ATPase domain and a C-terminal peptide-binding domain. The peptide-binding domain can be subdivided into a peptide-binding groove and a lid domain. When ATP is bound

to Hsp70, the peptide binding-domain is in an open conformation allowing peptides to bind. Upon hydrolysis of ATP to ADP, catalyzed by Hsp40, the peptide binding-domain switches to a closed conformation. After dissociation of Hsp40, the bound ADP is displaced by a nucleotide exchange factor (which in human can be BAG, HspBP1 and Hsp110). Subsequent binding of ATP to Hsp70 releases the nucleotide exchange factor and induces a conformational change that opens the peptide binding site and the polypeptide can dissociate. In the ATP-bound state, peptides associate and dissociate rapidly from Hsp70, but remain tightly bound in the ADP-bound state. Repeated binding and release is thought to drive the folding of substrate proteins in an iterative process until the native state is achieved and hydrophobic segments are no longer exposed.

Hsp40 (Heat shock protein 40 kDa protein)

Hsp40 increases ATP hydrolysis and thereby stimulates folding and refolding. It has been reported that Hsp40 operates best at a specific Hsp40:Hsp70 ratio. If the molar ratio of Hsp40 to Hsp70 is raised above 1:1, the folding reaction is inhibited (97,98).

Hsp90 (Heat shock 90 kDa protein)

We have verified an interaction between EBNA5 and Hsp90 (paper II). Hsp70 cooperates with Hsp90 in the folding of certain substrates. Substrate transfer from Hsp70 to Hsp90 is mediated by HOP (Hsp organizing protein), an adaptor protein that physically links both chaperones. In eukaryotes, some proteins, including those participating in cellular signalling and regulation, require the assistance of the Hsp90 chaperone in order to finish their folding and to attain a functionally active structure. Client proteins include kinases, transcription factors, signalling molecules, and oncoproteins (99). The sequential and coordinated interaction of the two chaperones with a client protein critically depends on the activity of several cofactors. Upon final activation by external stimuli, for example hormone binding to the steroid receptor signalling molecule, the client is released from Hsp90. In the absence of an activating stimulus, the client protein folds back to the inactive state when released and enters a new cycle of chaperone binding. Hsp90 not only stimulates folding, it can also induce ubiquitin-protesome degradation of clients by binding to the ubiquitin ligase CHIP (C-terminus of Hsp70-interacting protein) (se below). CHIP blocks the interaction of Hsp90 with HOP and cofactors.

Hsp27 (Heat shock 27 kDa protein)

We (paper II) and others (68) have verified an interaction between EBNA5 and Hsp27. Hsp27 belongs to the group of small chaperones that forms large oligomeric structures (95). Hsp27 is able to bind unfolded proteins and inhibit their aggregation. Hsp27 does not possess intrinsic chaperone activity but is

important as a first aid responder in prevention of aggregation, before Hsp70 comes to rescue and folds the substrate (100). Overexpression of Hsp27 enhances refolding of heat-inactivated reporter protein luciferase (101). Hsp27 operates without the need for ATP hydrolysis and is therefore particularly important during stress when ATP often is in limited supply. In addition to its ability to inhibit aggregation, Hsp27 inhibits apoptosis, oxidative damage and rupture of the cytoskeleton (for a review see (102)). During normal growth conditions, Hsp27 molecules are localized in the cytoplasm and exist as large oligomeric structures with a role in aggregate prevention. In contrast, during stress, Hsp27 deoligomerizes and translocates into the nucleus.

BAG2 (Bcl-2-associated athanogene 2)

We (paper II and III) have verified interaction between EBNA5 and BAG2. BAG2 is an Hsp70-associated nucleotide exchange factor and therefore stimulates client release. BAG2 is also important for the delivery of clients to Hsp70 (96). It has been reported that BAG2 inhibits the ubiquitin ligase activity of CHIP, which results in decreased ubiquitinylation, and as a consequence, inhibition of proteasomal degradation of Hsp70 clients (103,104).

2.2.2 Protein degradation

Cells utilize a variety of pathways for the degradation of proteins both during normal protein turnover and as part of their response to proteins that become damaged by environmental stress, are mutated or are extremely toxic. Cytosolic, nuclear and ER proteins are primarily degraded by the ubiquitin-proteasome system (UPS). However, most cells also rely on lysosomal proteolysis pathways, particularly under nutrient-limiting conditions (105). In addition to its role in regulating protein folding, Hsp70 interacts with the UPS via co-factors such as CHIP (C-terminus of Hsp70-interacting protein) (106) and BAG1 (107). BAG2 functions as a negative regulator of CHIP (104).

CHIP (carboxy terminus of Hsc70 interacting protein)

Hsp70 is linked to the UPS by its co-chaperones and in particular CHIP. CHIP interacts with Hsp70 and acts as an ubiquitin ligase for Hsp70 and Hsp90 clients, recruiting ubiquitin conjugating enzyme (Ubc) family members, particularly of the Ubc4/5 family (103,104). This results in increased proteasomal degradation of ubiquitinylated proteins. CHIP also inhibits protein folding by inhibiting the ATPase activity of Hsp70 (108).

BAG1 (Bcl-2-associated athanogene 1)

BAG1 stimulates nucleotide exchange during the Hsp70 ATPase cycle. BAG1 binds simultaneously to Hsp70 and the proteasome and stimulates CHIP-mediated degradation of Hsp70 clients (107,109).

2.2.3 Chaperones and protein aggregation

Stress, molecular crowding and mutations may jeopardize the native folding of proteins. Misfolded and aggregated proteins not only loose their biological activity, but may also disturb protein homeostasis, damage membranes and induce apoptosis. Chaperones favour the native folding of proteins either as “holdases”, sequestering hydrophobic regions in misfolding polypeptides, or as “unfoldases”, forcibly unfolding and disentangling misfolded polypeptides from aggregates. In bacteria, plants and fungi Hsp70/40 act in concert with the Hsp100 (ClpB) unfoldase. In mammalian cells on the other hand, Hsp70/40 is the only known chaperone that can forcibly unfold and neutralize cytotoxic protein conformers. The Hsp70 molecule can exert an unfolding force locally on the misfolded segment, thus destroying the misfolded structures that stabilize aggregates. ADP/ATP exchange triggers Hsp70 dissociation from the ensuing enlarged unfolded peptide loop, which is then allowed to spontaneously refold into a closer-to-native conformation devoid of affinity for the chaperone. Driven by ATP, the cooperative action of Hsp70 and its co-chaperone Hsp40 may thereby gradually convert toxic misfolded protein substrates into non-toxic, natively refolded products. Neuronal cell death is a hallmark of neurodegenerative disease. Neurodegenerative diseases ranging from Alzheimer´s disease and polyglutamine diseases to transmissible spongiform encephalopathies are associated with the aggregation and accumulation of misfolded proteins. In several cases the intracellular and extracellular protein deposits contain amyloid, a fibrillar protein species. Huntington’s disease is characterized by polyglutamine (polyQ) expansions which form SDS-insoluble amyloid fibrils. It has been shown that coexpression of Hsp70 or Hsp40 inhibit the formation of fibrils and large SDS-insoluble polyQ aggregates, resulting in the accumulation of smaller amorphous SDS-soluble inclusions. Thus, the protective activity of Hsp70 does not necessarily include refolding. Modulating the aggregation process or shielding of interaction surfaces of the misfolded polypeptide may be sufficient to decrease cytotoxic effects (110).

2.2.4 The proteasome

Proteins targeted for proteasome degradation are polyubiquitinated. The 26S proteasome is composed of the 20S proteasome, harbouring the proteolytic core, and two 19S regulatory domains. Eukaryotic proteasomes display three major

proteolytic activities: chymotrypsin-like activity (cleavage after hydrophobic aminoacids), trypsin-like activity (cleavage after basic aminoacids), caspase-like activity (cleavage after acidic aminoacids). Ubiquitin needs to be removed from tagged proteins and proteins need to be unfolded before entering the proteolytic core of the proteasome; the regulatory domains perform these tasks. Of particular interest, we note that regulatory subunit S2 interacts with the deubiquitinating enzyme Ubp6. The proteasome plays major roles in several biological processes including cell cycle regulation and immune response. The levels of cell cycle regulators rise and drop during the cell cycle. The proteasome degrades cyclin B during mitosis which is essential for cells to exit mitosis. Another important role applied to the proteasome is degradation of proteins that bind MHC class I molecules and are presented on the cell surface to $CD8^+$ T cells.

2.3 The nucleolus

The nucleolus is a specialized sub-nuclear compartment where rRNA synthesis and ribosome assembly occur. In the nucleolus, rRNAs are synthesized, processed, modified and associated with ribosomal proteins and 5S rRNA before being exported to the cytoplasm. A growing body of evidence suggests that the nucleolus is a specific compartment in which proteins can be sequestered or excluded, in order to transiently stabilize or destabilize them, or locally separate them from their interaction partners. For example, cell-cycle regulators have been identified whose activity is regulated by sequestration in the nucleolus (111). The nucleolus also functions as a stress sensor, responsible for maintaining low levels of p53 during normal conditions but inducing high levels during stress. This is achieved by sequestration of a regulator of p53 (112,113). Two large proteomic analyses have been used to identify nucleolar proteins (114,115). These proteins could be grouped into a small number of functional groups. One of these groups includes proteins involved in RNA metabolism, such as synthesis, processing, editing, export, degradation, and translation. Another group includes chaperones and their related proteins.

2.3.1 Nucleolar proteins

Below is a description of some proteins which are interesting due to their association with EBNA5 and their localization in nucleoli and speckles.

PSF (polypyrimidine-tract binding protein-associated factor) and $p54^{nrb}$ (nuclear RNA-binding protein, 54 kDa)

PSF and $p54^{nrb}$ are multifunctional proteins implicated in many regulatory events within the cell. $p54^{nrb}$ forms a heterodimer with PSF (116). $p54^{nrb}$ shows extensive sequence homology to PSF (117). They seem to function as a pair most of the time. PSF and $p54^{nrb}$ are localized to nuclear paraspeckles and perinucleolar caps.

PSF associates with a protein complex which is thought to function both in splicing and in polyadenylation (118) and $p54^{nrb}$ is clearly important for pre-mRNA 3´-end cleavage (119). $p54^{nrb}$ and PSF bind to the XRN2 protein which associates with processing factors involved in pre-mRNA 3´ cleavage and polyadenylation (120). In EBV-infected cells, there is some evidence of down-regulation of $p54^{nrb}$. Down-regulation of $p54^{nrb}$ was confirmed in three sets of LMP1 expressing NPC cell lines (121).

hnRNP M (heterogeneous nuclear ribonucleoprotein M)

hnRNP M is involved in splicing and is present in both pre-spliceosomal complexes and in mature spliceosomes. hnRNP M has a role in splice site recognition and alternative splicing regulation (122). hnRNP M interacts with $p54^{nrb}$ and PSF and co-localizes with these proteins in paraspeckles (123).

hnRNP K (heterogeneous nuclear ribonucleoprotein K)

hnRNP K has been shown to localize to nucleoli (114,115). hnRNP K is a multifunctional protein involved in many processes, including chromatin remodelling and transcription as well as mRNA splicing, export, and translation (124). Furthermore, hnRNP K interacts with p53 and strongly induces the recruitment of p53 to p21 and HDM2 promoters, two p53 responsive promoters. siRNA against hnRNP K efficiently inhibits transcription of p21 and HDM2 (125). We have verified association between EBNA5 and hnRNP K (paper II) and interestingly, Kasuba and collegues have showed that EBNA5 inhibits p21 expression despite high levels of p53 (*personal communication of unpublished data*, Elena Kashuba, MTC, Karolinska Institutet, Stockholm, Sweden). They have also confirmed that p53 can not bind to the p21 promoter.

Chapter 3
The present investigation

Methods have been described in detail in the attached papers.

3.1 Aims of the present investigation

Overall aim

Despite years of research, the contribution of EBNA5 to transformation is still poorly understood. The present study was undertaken to further advance our understanding of EBNA5 function and provide valuable information toward identification of cellular co-factors that mediate EBNA5 function.

Specific aims

Paper I

Uncover molecular mechanisms involved in EBNA5-mediated repression using transiently expressed reporter genes as a model system.

Paper II

Identification of cellular interaction partners for EBNA5.

Paper III

Uncover molecular mechanisms involved in EBNA5-mediated repression using the reporter expression model system.

3.2 Results and discussion

3.2.1 Results

Paper I – Epstein Barr virus nuclear antigen 5 inhibits pre-mRNA cleavage and polyadenylation.

The well-defined positive effect of EBNA5 and EBNA2 on transcription from viral promoters attracted our attention at first. We found that the ability of EBNA5 to function as a co-activator of EBNA2 very much depended on the expression level of EBNA5; low levels of EBNA5 had a stimulatory effect on reporter gene expression (i.e. enzyme activity) from viral promoters C, W, and LMP1 in an EBV-negative cell line (DG75), whereas high levels of EBNA5 had a repressive effect. Furthermore, we observed that the repressive effect was clearly independent of EBNA2. The ability of EBNA5 to switch from an activator to a repressor (or vice verse) may occur *in vivo* in viral infection since the level of EBNA5 changes in the course of the acute infection. EBNA5 is expressed very early in acute primary infection and the level of expression reaches its maximum within 48 hours after which it declines to the levels found in lymphoblastoid cell lines (LCLs). We found that repression of reporter gene expression was clearly promiscuous both with regards to the promoter and the reporter proteins and not specific for promoter sequences derived from EBV. We prepared and analyzed RNA from cells transfected with the reporter model system and found that EBNA5 decreased the amount of reporter RNA in the cytoplasm. However, EBNA5 did not decrease the amount of total reporter RNA. Accordingly, EBNA5 did not inhibit transcription of reporter genes. Taken together, the results presented in paper I suggest that EBNA5 has the ability to inhibit pre-mRNA 3´ cleavage and polyadenylation. The precise mechanism could not be established. However, EBNA5 did not inhibit cleavage and polyadenylation of RNA molecules in which the canonical polyA signal was either mutated or deleted, suggesting that the polyA signal is important.

Notably, EBNA5 does not seem to indiscriminately inhibit the expression of chromosomal genes. Using cDNA expression arrays including 588 human cDNAs and comparing the expression of these genes in vector- *vs.* EBNA5-transfected cells, we demonstrated that only a minority of the tested genes were repressed. This suggests that some kind of specificity must exist for the choice of target cellular genes. Whether specificity is determined by gene context (plasmid/episomal context *vs*. chromosomal context) or gene expression (high level *vs.* low level) will be the subject for future studies. One of the few chromosomal genes that were down-regulated was BNIP3, a pro-apoptotic gene.

Accordingly, an anti-apoptotic function might contribute to explain why EBNA5 is important for efficient immortalisation of human B cells.

Paper II – Identification of intracellular proteins associated with the EBV-encoded nuclear antigen 5 using an efficient TAP procedure and FT-ICR mass spectrometry.

EBNA5 is a complex protein and its function is still to a large extent unknown. Co-immunoprecipitation as well as co-localization studies have revealed that EBNA5 interacts with multiple proteins, including Hsp70, HA95, DNA-PKc, Hsp27, Sp100, Hax-1, ERR1, p14ARF, α-tubulin, β-tubulin, and prolyl-4-hydroxylase (54,66-68,126-130). To identify additional EBNA5-interacting proteins in human cells, we have developed an improved tandem affinity purification method which produces less back-ground than the original method (131). In the improved version, the calmodulin binding peptide is replaced with one copy of the StrepTagII sequence followed by two Tobacco etch virus (TEV) protease cleavage sites and the IgG-bindning Protein A domains. The modified tag was cloned downstream of the EBNA5 open reading frame in the pCI expression vector, yielding the EBNA5-StrepTAP plasmid. Analysis of proteins co-purifying with EBNA5-StrepTAP or StrepTAP (control) was performed by LC-MS/MS analysis of tryptic digests on a tandem mass spectrometer. Protein dentification was performed by comparing all acquired MS/MS spectra with the complete Swiss-Prot protein sequence database. In parallel, evaluation of possible false positive hits was done with a randomized version of the database. A true hit was defined as ≥2 hits with the non-randomized database (with $p<0,01$ for one peptide and $p<0,05$ for the other) and <2 false hits. In total, 147 novel proteins were identified and 37 were validated by independent methods (immunoprecipitation and split-tag experiments). The identified proteins could be grouped into three main classes depending on their biological function: 1) protein folding and degradation 2) pre-mRNA processing, and 3) ribosome biogenesis. Proteins belonging to the UPS and folding systems include Hsp70, Hsp27, Hsp40, Hsp90, BAG2, and proteasome 26S non-ATPase regulatory subunit. Splicing and pre-mRNA processing factors interacting with EBNA5 include $p54^{nrb}$, PSF, hnRNP K, and hnRNP M.

Paper III - Epstein-Barr virus nuclear antigen 5 is a multi-functional protein with a possible role in the chaperone-mediated protein folding and ubiquitin-proteasome protein degradation systems.

In the previous study (paper I), we found that EBNA5 repressed reporter gene activity in transiently transfected lymphoid cells. EBNA5-mediated inhibition of polyadenylation of reporter mRNA could partly explain this effect. The purpose of this paper was to further advance our understanding of EBNA5 function and uncover molecular mechanisms involved in EBNA5-mediated repression of reporter gene expression. Research into the mechanisms that determine luciferase and CAT expression levels revealed that EBNA5 had a stimulatory effect on reporter gene transcription, whereas no increase in mRNA levels could be detected. Accordingly, EBNA5 inhibited polyadenylation but repression of reporter activity did not result from interference at the transcriptional or the post-transcriptional level. The results further showed that there was a strict correlation between the reporter activity and the amount of NP40-soluble reporter protein. Additionally, substantial amounts of NP40-insoluble reporter protein had accumulated in the nuclei of the EBNA5-transfected cells. A time course study of the accumulation of insoluble luciferase and the disappearance of soluble luciferase showed that the insoluble luciferase accumulated over time, which implies that EBNA5 did not inhibit translation. To measure the half-life of luciferase and to study the impact of EBNA5 on destabilization of luciferase, we performed the time-course experiment in the presence of cycloheximide, a translational inhibitor. The results showed that EBNA5 did not significantly increase the degradation rate of luciferase. However, immunostaining of cells revealed that insolubilisation was accompanied by complete translocation of luciferase from the nucleoplasm to the nucleoli and a number of nuclear foci. EBNA5 was always associated with these nuclear structures. We conclude that EBNA5-induced translocation of proteins to the nucleoli is a conceivable explanation for the repressor effect. Mutational analysis of EBNA5 identified a conserved region in the EBNA5 W_1W_2 repeat domain (CR3) as essential for EBNA5-mediated accumulation of insoluble luciferase and nucleolar localization of luciferase.

In paper II, we identified Hsp70 and BAG2 as significant interaction partners of EBNA5. In this paper (III), we investigated the ability of Hsp70 and BAG2 to induce insolublisation and nucleolar translocation of luciferase. Over-expression of Hsp70 blocked EBNA5-induced accumulation of insoluble luciferase as well as EBNA5-mediated reduction of luciferase activity. Double immunostaining of transfected B lymphocytes demonstrated co-localization of luciferase, EBNA5, and endogenous Hsp70 in the nucleoli. A new technology has recently become available for the study of protein interactions at the single-molecule resolution

(Duolink (Olink Biosciences, Uppsala, Sweden)). Using this *in situ* proximity ligation assay, we identified co-localization between EBNA5 and the co-chaperone BAG2 in the cells including the nucleoli. The ΔCR3-EBNA5 mutant did neither co-localize with BAG2 nor induce translocation of luciferase, Hsp70, or itself to nucleoli, implicating a functional link between nucleolar localization of EBNA5, luciferase, Hsp70, and BAG2 on one hand, and physical association of EBNA5 with Hsp70 and BAG2 on the other.

3.2.2 Discussion

EBNA5 is part of multi-protein complexes that affect several cellular processes but we have limited understanding of the function of EBNA5. Multiple EBNA5-associating factors may contribute to the processing of pre-mRNA and since EBNA5 inhibits mRNA processing, a conceivable hypothesis would be that EBNA5 inhibits the activity of regulatory proteins which participate in and stimulate pre-mRNA processing. $p54^{nrb}$ and PSF are potential mediators of this effect due to their [I)] role in pre-mRNA 3´-end processing and polyadenylation (118,119,132,133) [II)] and their association with EBNA5 (paper II) [III)] as well as their localization in speckles and nucleoli, that is similar to EBNA5 (paper III). $p54^{nrb}$ and PSF only localize to nucleoli during circumstances when RNA transcription is obstructed (114,134). Accordingly, it seems that localization to nucleoli does not correlate with normal activity of $p54^{nrb}$ and PSF. It is well established that the nucleolus can provide a protected environment and can function as a shelter in which proteins can be sequestered for transient stabilization and subsequent repair or refolding, or be kept separated from possible interaction partners for regulatory purposes. Together, these observations support the hypothesis that EBNA5-mediated sequestration of $p54^{nrb}$ and PSF in nucleoli would inhibit pre-mRNA processing. The fact that down-regulation of $p54^{nrb}$ has been confirmed in NPC cell lines (121) is compatible with the notion that down-regulation of $p54^{nrb}$ contributes to the transforming activity of the virus.

Sequestration of other EBNA5-associated factors could also in theory explain why EBNA5 inhibits polyadenylation. The EBNA5-associated factors hnRNP M and -K (paper II) are splicing factors and the importance of a concurrent splicing-polyadenylation system is stated (122,124). It is interesting to note that hnRNP M interacts with PSF and $p54^{nrb}$ (123). In addition, it has been shown that hnRNP K and hnRNP M can localize to the nucleoli (114,134). The ability of EBNA5 to induce sequestration of $p54^{nrb}$, PSF, hnRNP K, and hnRNP M and inhibit their activity will be the subject for future studies.

Sequestration of proteins in nucleoli is a regulatory mechanism which EBNA5 may use for the purpose to either protect target proteins from proteasomal degradation or to inhibit target protein activity. The ability of EBNA5 to cause

translocation of proteins to the nucleoli may have important biological functions in EBV infection. By inhibiting proteasomal degradation, this could decrease the amount of peptides that bind MHC class I molecules and are presented on the cell surface to $CD8^+$ T cells. Consequently, EBNA5 would allow EBV-infected cells to escape from immune surveillance and survive.

Several findings in paper III support the notion that EBNA5 inhibits degradation of proteins. The results in paper II-III are compatible with a hypothetical model in which EBNA5, in synergy with BAG2, inhibits the ubiquitin ligase activity of CHIP. It is known that BAG2 and CHIP can simultaneously bind to Hsp70. It is possible, although not proven, that EBNA5 displaces CHIP, resulting in simultaneous binding of EBNA5 and BAG2 to Hsp70. If we assume that prevention of ubiquitinylation results in increased aggregation and reduced degradation of misfolded proteins, our simplistic model of proteasome inhibition is supported by data that shows that over-expression of BAG2 results in increased accumulation of insoluble luciferase. The ability of EBNA5 to inhibit ubiquitinylation has previously been observed by another group (*personal communication of unpublished data,* Elena Kashuba, MTC, Karolinska Institute, Stockholm). EBNA5 was found to inhibit the ubiquitin ligase activity of MDM2, possibly via direct interaction.

We demonstrated that EBNA5, as well as BAG2, at high levels, stimulated Hsp70 expression or increased Hsp70 stability (paper III). In addition, EBNA5 is tightly bound to Hsp70 (paper II). It has been shown by other groups that Hsp70 is perfectly capable of translocating to the nucleoli by itself (59) whereas luciferase is dependent on Hsp70 for translocation to the nucleoli (135). The Szekely group showed that cells expressing higher levels of EBNA5 tethered Hsp70 to the nucleoli during proteasome inhibition (59). These accumulating data supports the hypothesis that EBNA5 engaged Hsp70 in translocation of luciferase to the nucleoli.

Reduction in the amount of soluble luciferase might result from decreased folding of luciferase. Our and others results argue against this assumption. By using the Duolink proximity ligation assay, we have been able to verify co-localization between EBNA5 and Hsp27 in the cytoplasm and the nucleoli. It is well established that Hsp27 inhibits irreversible aggregation of substrates and delivers them to Hsp70 for folding. Furthermore, Nollen and collegues (135) demonstrated that stress induced nucleolar localization of over-expressed Hsp70 and luciferase, but recovery from stress correlated with translocation of luciferase from the nucleoli to the nucleoplasm and cytoplasm and subsequent refolding. In line with these results, we observed co-localization of luciferase and Hsp70 in the nucleoli. Taken together, these observations argue for a role of EBNA5 in the maintenance of luciferase in the folding competent state. Future investigations will determine whether EBNA5 inhibits the folding activity of Hsp70.

In summary, EBNA5 is a highly dynamic protein that associates with high molecular complexes, shows localisation to multiple and diverse sub-nuclear structures, and shows fluctuations in expression level *in vivo* in EBV infection. Accordingly, this flexibility might contribute to explain why EBNA5 is important for efficient immortalisation of human B cells.

3.2.3 Overall conclusions

- EBNA5 inhibits pre-mRNA processing.
- EBNA5 exerts regulatory functions in the absence of EBNA2.
- EBNA5 is part of multi-protein complexes.
- EBNA5 interacting proteins belong mainly to three functional groups involved in I) RNA processing II) protein folding and degradation III) ribosome biogenesis.
- EBNA5 can localize to different sub-cellular structures.
- Ectopic high level expression of EBNA5 and a reporter protein, expressed from co-transfected plasmids in transient assays results in:

    reduced expression of soluble and active reporter protein in the nuclei

    translocation of reporter protein from the nucleoplasm to the nucleoli and nuclear speckles

    accumulation of EBNA5 in nucleoli and nuclear speckles both in the presence and absence of reporter protein

- EBNA5 interacts with protein components of the protein quality control system that recognize proteins with abnormal structures and either refold them to normal configuration or target them for degradation through the ubiquitin-protesome pathway.

3.3 Future perspectives

The papers and manuscripts presented here have contributed to a better understanding of the molecular mechanisms underlying the actions of EBNA5. Since EBNA5-mediated regulation of RNA processing, protein folding, and degradation is dependent on cellular proteins, the continued efforts in the characterization of cellular factors are relevant in constructing a complete model of these processes. Elena Kashuba and colleagues have established a method for the purification of EBNA5. By virtue of this information, it is now possible to do *in vitro* experiments with EBNA5 and a limited number of proteins to allow functional characterization of individual proteins. We will study ubiquitinylation, refolding and proteasomal degradation in the presence of EBNA5, luciferase, Hsp70, and BAG2. There are several established protocols for the studies of these processes. Furthermore, we will study the impact of EBNA5 on $p54^{nrb}$-PSF-mediated regulation of pre-mRNA polyadenylation. Protocols are available for the study of both polyadenylation *in vitro* and when using a transfection system (132). Additionally, the Duolink *in situ* proximity ligation assay will be used to study co-localization between EBNA5 and $p54^{nrb}$ and PSF and aid in building a more complete picture of these protein interactions. To study the dynamics of EBNA5-induced movements of luciferase, perhaps collaboration could be arranged to be able to use leading-edge technology confocal microscopy methods. By this method it is possible to record living cells using a combination of fluorescence and phase contrast illumination over several hours (up to 24 hours) (136). Additionally, if we will be able to carry out FRAP (fluorescence recovery after photobleaching) and FLIP (fluorescence loss in photobleaching) analysis on EBNA5- and luciferase-transfected cells, we will measure the rate of mobility of luciferase and EBNA5 in different sub-nuclear compartments.

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