Mikrobiologisk och Tumörbiologisk Centrum
Karolinska Institutet
Stockholm

Modulation of cellular and viral functions in Epstein-Barr virus infected cells

Marta P. Imreh

Stockholm 2002

"The association of EBV with different human tumors outnumbers the tumor association of any other human DNA virus. At the same time, it does not cause any disease in a majority of the carriers. This apparent paradox seems to be the result of interaction of the host immune/surveillance and viral gene expression, to achieve a successful coexistence."

George Klein

(The Paradoxal Coexixtence of EBV and the Human Species, 1994)

Abstract

The aim of the study was to investigate the interplay of cellular and viral genes in Epstein-Barr virus (EBV) infected cells. Two aspects of this interaction have been investigated.

The first aspect is related to the expression of major histocompatibility complex (MHC) class I molecules in virus infected malignant cells. MHC class I expression plays a key role in the regulation of immune responses and is frequently altered in human tumors. We analyzed the mechanisms of this MHC class I downregulation in Burkitt's lymphomas (BLs) that arise in HLA A11 positive individuals by comparing five pairs of BL lines and Epstein-Barr virus (EBV) transformed lymphoblastoid cell lines (LCL) derived from the normal B cells of the same individuals. The cell lines were compared for the presence of HLA A11 gene and A11-specific mRNA, the reactivity to IFNα treatment, the restoration of HLA A11 expression by transfection or hybridization, the activity of HLA A11 promoter driven chloramphenicol acetyl transferase reporter gene (pA11CAT). Our results suggest that genetic defects and lack of transcription factors may contribute to the selective down-regulation of HLA A11 in BL cells. Furthermore we analysed the involvement of EBV LMP1 in the process. The BL phenotype-dependent transcriptional defect observed for the HLA A11 promoter was shown to involve other MHC class I promoters including HLA A2. Since the virus LMP1 was shown to be mainly responsible for changing the phenotype of BL cells towards LCL-like characteristics, we have tested how the class I promoter activity is affected by expression of LMP1. We can conclude, that expression of LMP1 correlates with activation of the HLA A11 promoter and up-regulation of several components of the transcription factor family NFκB/Rel.

The second aspect of this thesis deals with viral strategies that promote escape from immune recognition. EBNA1 is expressed in all EBV associated malignancies and is the only viral protein expressed in BL. Endogenously expressed EBNA1 is not recognized by MHC class I-restricted CTLs. This phenomenon is correlated with the presence of an internal glycine-alanine repeat (GAr) in the structure of the protein. The GAr generates a *cis*-acting inhibitory signal that interferes with antigen processing and MHC class I restricted presentation by inhibiting the generation of antigenic peptides by ubiquitin-proteasome/dependent proteolysis. The mechanism of action of the GAr was investigated using as model a known target of the ubiquitin-poteasome system, the NFκB inhibitor IκBα. Insertion of a minimal GA repeat of eight amino acids in different positions of IκBα was sufficient to prevent tumor necrosis factor (TNFα) induced ubiquitin-proteasome dependent degradation, and decrease its basal turnover *in vivo*. The chimeras are phosphorylated and ubiquitinated in response to TNFα but than released and failed to associate with the proteasome. This explains how functionally competent IκBα is protected from proteasomal disruption and identifies the GAr as a regulator of proteolysis. The analysis of the impact of length and amino acid composition on the capacity of various repeat sequences to inhibit the TNFα-induced and physiological turnover of IκBα demonstrated that inhibition is achieved by insertion of octamer peptides containing 3 hydrophobic amino acids, interspersed by no more then three consecutive glycines. The inhibitory activity was abolished by increasing the length of the spacer to four glycines, by elimination of the spacers, or by substitution of a single hydrophobic residue with a polar or charged residue. These findings suggest a model where inhibition of proteolysis requires the interaction of at least three alanine residues of the GAr in a beta-strand conformation with adjacent hydrophobic binding pockets of a putative receptor.

The multifunctional EBNA1 protein is involved also in the replication of the EBV genome as an extra chromosomal element, and plays important role in the maintenance of the viral episome and is the key transcriptional regulator of latent viral gene expression including its own transcriptional regulation. We have investigated whether presence of the GAr modulates the functions of EBNA1. The GAr containing EBNA1 protein has an extremely slow turnover in virus infected cells while a GAr deleted EBNA1 is degraded by the ubiquitin-proteasome system. To assess the contribution of protein stability on EBNA1 functions, destabilized variants were generated and studied for their capacity to promote the maintenance of oriP containing plasmids and regulate the viral Qp promoter. Both functions were strongly influenced by EBNA1 stability suggesting the GAr plays an important role in modulating the function of EBNA1 in virus infected cells.

ISBN: 91-7349-171-3

Abbreviations

BL	Burkitt's Lymphoma
CTL	cytotoxic T-lymphocyte
EBNA	EBV-encoded nuclear antigen
EBV	Epstein-Barr virus
GAr	glycine-alanine repeat
HLA	human leukocyte antigen
LCL	lymphoblastoid cell line
LMP	latent membrane protein
MHC	major histocompatibility complex
NFκB	nuclear factor kappa B
oriP	origin of plasmid replication
Qp	Q promoter
TNFα	tumor necrosis factor α

Papers

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

1. Imreh MP, Zhang QJ, de Campos-Lima PO, Imreh S, Krausa P, Browning M, Klein G, Masucci MG. Mechanisms of allele-selective down-regulation of HLA class I in Burkitt's lymphoma. (1995) Int J Cancer. 62:90-96.

2. Imreh MP, de Campos Lima PO, Sharipo A and Masucci, MG. The Epstein-Barr virus Latent Membrane Protein (LMP1) enhances the expression of HLA class I genes in Burkitt's lymphoma cells by activating the NFκB/Rel family of transcription factors (submitted)

3. Sharipo A, **Imreh** M, Leonchiks A, Imreh S, Masucci MG. Aminimalglycine-alanine repeat prevents the interaction of ubiquitinated IkappaB alpha with the proteasome: a new mechanism for selective inhibition of proteolysis. (1998) Nature Medicine. 8:939-944.

4. Sharipo A, **Imreh** M, Leonchiks A, Branden C, Masucci MG. cis-Inhibition of proteasomal degradation by viral repeats: impact of length and amino acid composition. (2001) FEBS Letters 499 (1-2):137-142.

5. Imreh MP, Heessen S, Dantuma NP, Sharipo A, Masucci MG. Modulation of EBNA1activity by the glycine-alanine repeat (manuscript)

CONTENTS

Aims

The scope of the study was to investigate the **interplay of cellular and viral genes in EBV infected cells.** The general objectives of this thesis were the following:

- To investigate the mechanisms and molecular basis of MHC class I down-regulation in Burkitt's lymphomas that arise in HLA A11 positive individuals characterized by selective loss/down-regulation of the HLA A11 polypeptide.

- To study the EBV LMP1-1 produced modulation of MHC class I expression by testing how the class I promoter activity is affected by expression of LMP1.

- To investigate the mechanisms of prevention of TNFα induced ubiquitin-proteasome dependent degradation by EBNA1 GAr of different lengh and position in IκBα model system.

- To investigate the impact of the length and amino acid composition on the capacity of various viral repeat sequences to inhibit the TNFα-induced and physiological turnover of IκBα.

- To investigate whether the presence of the GAr modulates the functions of EBNA-1 compared with destabilized variants, the promotion of the maintenance of oriP containing plasmids and the regulation the EBV Qp promoter.

1. The Epstein-Barr virus (EBV)

1.1 Taxonomy and virus structure

EBV is a member of the genus Lymphocryptovirus that belongs to the lymphotropic Gammaherpesvirinae subfamily of the family of Herpesviridae. The EBV has a double stranded approximately 170Kb large DNA genome. Its protein core is wrapped with DNA and its icosahedral nucleocapsid contains 162 capsomers. Glycoprotein spikes constituted mainly of the glycoprotein gp350/220 form the outer envelope. The tegument is a protein layer between the nucleocapsid and the envelope (reviewed in Young *et al.*, 1999).

1.2 Pathogenicity

The Epstein/Barr (EBV) virus with a dual tropism for lymphoid and epithelial cells is one of the most potent transforming agents. It persists in most individuals as a lifelong asymptomatic infection. More than 90% of the world's population is infected with EBV. The infection is usually benign in healthy people, where the virus persists life long in B cells as a latent infection. However, specific genetic or environmental factors, or immunological changes (like in HIV or malaria) can result in cancers in which EBV appears to play a role. EBV is remarkable for the efficiency with which it can cause proliferation of the infected B cells and the ability to influence cell growth and death by modulating cellular genes. It has been known for 30 years that EBV is the etiologic agent of acute infectious mononucleosis (IM) and it is closely associated with the genesis of Burkitt's lymphoma and undifferentiated nasopharyngeal carcinoma (NPC). EBV is also implicated in a variety of other malignancies such chronic active EBV infection, EBV associated hemophagocystic syndrom, T-cell lymphoma, NK cell leukemia/lymphoma, Hodgkin's disease, pyothorax-associated B-cell lymphoma, smooth-muscle tumors, gastric carcinoma and in lymphoproliferative diseases in immunocompromised hosts (Rickinson and Kieff, 1996).

1.3 Target tissues

The classical targets for EBV infection are epithelial cells and B lymphocytes. **Epithelial cells** are infected during infectious mononucleosis (IM), oral hairy leukoplakia (OHL) and nasopharingeal carcinoma (NPC). Infectious mononucleosis (IM) is observed mainly when the infection is delayed until adolescence or later. The transmission occurs by the oral route through productive infection of the oropharingeal epithelium and the EBV DNA is present in the exfoliated cells. The detection of lytic EBV infection in oral hairy leukoplakia (OHL) supports also the idea that the oropharyngeal epithelium is the site of lytic infection. Asymptomatic primary infection usually occurs early in life, is a self limiting benign lymphoproliferative disease. After primary infection, the virus establishes in the **B cell compartment** a life long latent infection. There it may also replicate which allows the spread to new hosts. In healthy carriers the virus is detected in different types of B cells, resting B cells and blasts while in the IM patients it is present in the tonsils in fully differentiated plasma cells (Thorley-Lawson and Babcock, 1999). In healthy carriers no viral product is detected in epithelial cells but only one cell per million peripheral blood lymphocytes was detected positive for EBV DNA with the highly sensitive method of semiquantitative PCR (Chen *et al*., 1995). The positive cells are memory B cells according to the surface expression of immunoglobulins (Babcock *et al*., 1998). In this case there is maybe no need of epithelial cells for EBV infection and persistence, because the EBV can adapt its biology to reside in normal B cells. According to this hypothesis, *in vivo* the EBV persists in normal B cells that provide the habitat for the virus to maintain its life cycle (Masucci and Ernberg, 1994) (Thorley-Lawson and Babcock, 1999). The virus genome is present in a restricted population of resting CD23 negative memory B cells. The RNA coding EBER1, LMP2A is always present. In some cases EBNA1 and BamHI rightward transcripts (BARTs) can be detected by cell sorting and RT PCR in $CD19^+$ but not in $CD23^+$ cells (Chen *et al*., 1999). Memory cells in the tonsil express the EBNA1 (from the Qp promoter), LMP1 and LMP2a genes but do not express the

immunogenic and growth-promoting EBNA2 or the other EBNAs. LMP1 and LMP2a are produced to provide surrogate rescue and survival signals that are needed to allow latently infected memory cells to persist, and EBNA1 is produced to allow replication of the viral episome (Babcock and Thorley-Lawson, 2000).

There is now convincing evidence that not only epithelial cells and B lymphocytes but peripheral T cells are also targets of *in vivo* infection by EBV (Franchini *et al*., 2000; Kanegane *et al*., 1999; Yoshioka *et al*., 2001). Within the hematopoietic system, EBV infects also monocytic, granulocytic and natural killer lineages (Knecht *et al*., 2001). Neutrophyls can be infected *in vitro* and *in vivo*, EBV induces apoptosis of these cells during infectious mononucleosis (Larochelle *et al*., 1998). In monocytes and macrophages EBV initiate replicative lytic cycle (Savard *et al*., 2000) (Shimakage *et al*., 1999). EBV further infects human endothelial cells (Jones *et al*., 1995) and follicular dendritic cells (Lindhout *et al*., 1994).

1.4 Immune responses to EBV infection

Humoral and cellular immune responses play a role in the recovery from primary EBV infection and maintenance of the state of virus carrier, but humoral immunity is not sufficient to control an established EBV infection nor to prevent superinfection with an additional virus strain (Yao *et al*., 1996). A large panel of evidence suggests that cell mediated immune responses are having the key role in immune responses directed to EBV infection.

NK (natural killer) cells limit the number of virus infected cells early in the acute phase of EBV infection;

CTLs (cytotoxic T lymphocytes) are directed towards EBV antigenes. Accordingly, the virus has devoted a proportion of its genome towards avoiding host defences. The virus markedly limits its gene expression during latency in the B-cells, produces proteins that inhibit apoptosis and antigen processing and expresses a cytokine and a soluble cytokine receptor (reviewed in Cohen, 2000).

During acute infection CTLs are directed towards both latent and lytic viral antigens (Steven, 1997). Some of these $CD8^+$ cells have an activated/memory

phenotype. CTLs are also the most important limiting factors during the convalescent phase of EBV infection and in healthy EBV seropositive carriers. Large mono or oligoclonal expansions of $CD8^+$ cells are observed during acute infectious mononucleosis. Most of these expanded cells are specific for viral antigens. Recent studies show that oligoclonal-like $CD8^+$ cell expansions are also present in some healthy EBV-carriers (Silins *et al.*, 1998).

Two genetic defects create special situations in EBV related pathology. The X linked lymphoproliferative disease (XLPD) is an example for the critical role of T cells in controlling EBV infected B cells. Boys with this genetic defect have a normal response to most childhood infections but develop fatal lymphoproliferative disorders after infection with EBV. The defect is associated with the absence of a functional SAP (also called SH2D1A or DSHP) gene. The gene codes for an SH2-domain containing protein that interacts with SLAM (signalling lymphocyte-activator molecule) on B cells. The patients suffer from uncontrolled EBV induced B cell proliferation. In the second syndrome, boys with X-linked agammmaglobulinemia have mutations in the gene encoding Bruton's tyrosine kinase that results in inability to produce immunoglobulins and mature B cells. The patients are never infected with EBV and not even their peripherial blood mononuclear cells can be ever infected *in vitro*. This special case indicates that mature B cells are required for EBV infection. The patient's having no evidence for the immune response to EBV (reviewed in Cohen, 2000).

1.5 Viral and cellular events during EBV infection

1.5.1 Early events in viral infection

The first step in EBV infection of B cells is the interaction between the gp350/220 envelope and the surface molecule CD21, the receptor for the C3d complement component on the lymphocyte. The binding of gp350/220/CD21 is followed by aggregation in the plasma membrane and internalization of the virus in cytoplasmic vesicles. The nucleocapsid and tegument are released in the cytosol by fusion of the envelope with the membrane of the vesicle. The linear viral DNA

circularises and persists as an episome, an extrachromosomal plasmid that replicates together with the cellular DNA (Rickinson and Kieff, 1996).

1.5.2 Lytic infection and antigens

During lytic infection the viral DNA is amplified via the origin of lytic replication (ori Lyt). The lytic cycle is characterised by the expression of 80 RNA species, described after their time of appearance as immediate early (IE), early (E) and late (L).

Two viral transactivators initiate the switch from the latency to productive infection. The BZLF1 open reading frame encoded ZEBRA (Zta, Z or EB1) is the key protein in this event (Rickinson and Kieff, 1996). Zebra is a member of the basic leucine zipper family and shares amino acid homology with cellular AP-1 transcription factors including Fos, Fra, Jun-B (Farrell *et al.*, 1989). The early gene product from the open reading frame (ORF) BCRF1 is highly homologous with human and murine IL-10 and can be considered as a viral IL-10 (Burdin *et al.*, 1997). The BCRF1 protein (EBV IL-10 or vIL-10) is detected in the serum in some patients with acute infectious mononucleosis and chronic, active EBV infection. The viral IL-10 shares more than 80% sequence identity with human IL-10. Both human and viral IL-10 inhibits production of interferon (IFN) γ by lymphocytes and IL-12 in macrophages. Viral IL-10 promotes B-cell growth and inhibits maturation of dendritic cells. By inhibiting cytokines witch normally stimulate T cell immunity, and promote B cell growth, viral IL-10 may enhance the survival of EBV in the host. Viral IL-10 is less active than its human homologue, has 1000 times less affinity for binding to the IL-10 receptor than cellular IL-10 (Liu *et al.*, 1997) and less effective in the inhibition of IL-2 production in human cells, in enhancing MHC class II in mouse cells and in costimulating mouse thymocytes.

1.5.3 Latent EBV infection

Classical studies on latent infection are based on *in vitro* immortalized normal B cells. These lymphoblastoid cell lines (LCLs) are growing continuously driven by viral gene

Table 1

The occurrence of different latency types in healthy carriers and EBV associated disorders ***(after Knecht et al., 2001)***

Memory B cells **Burkitt's Lymphoma**	**Latency type I:** *EBER1,2; EBNA1; LMP2A (genes expressed)* • Small lymphocytes in lymphatic organs and peripheral blood. • Small to intermediate–sized lymphoblasts with basophilic and vacuolized cytoplasm. Typical histology with "starry-sky" pattern. Molecularly characterised by Ig/c-Myc translocation t(8;14), t(2;8), or t(8;22).
Hodgkin disease **Angioimmunoblastic lymphadenophaty with dysproteinemia** **Peripheral T-cell lymphoma** **Primary effusion lymphoma**	**Latency type II:** *EBER1,2; EBNA1; LMP1, 2A, 2B (genes expressed)* • Bi- and multinucleated RS cells, $CD15^+$, $CD30^+$. Often non-functional immunoglobulin gene rearrangements • Basophilic B immunoblasts; lymph node histology with polymorphic cytology, vascular proliferation and effacement of nodal architecture. Cytogenetically and molecularly small oligoclonal B and T cell proliferations • Tumor cells of variable size, often with indented or lobated nuclei. Generally prominent T cell receptor gene rearrangement identifiable. • Large immunoblastic to anaplastic tumor cells growing in body cavities as effusions. Contain Kaposi's sarcoma –associated herpesvirus (HHV-8).
Posttransplant lymphomas **AIDS-related lymphomas** **Lymphoblastoid cell lines, LCLs** **Infectious mononucleosis, IM**	**Latency type III:** *EBER1,2; EBNA1-6; LMP1, 2A, 2B (genes expressed)* • Wide range from polymorphic proliferations to monomorphic plasmablastic or B immunoblastic lymphoma. Progression from oligo-to monoclonality. Responds initially to alleviation of immunsupression. • Mainly large-cell B immunoblastic cytology. HIV positive. Often central nervous system localisation. • Medium to large sized lymphoblasts. Few multinucleated cells. • Most EBV positive cells are small lymphocytes (latency type I), but large immunoblasts up to RS cell like cells exist (latency II and III).

products. These cells are similar to those produced upon initial infection *in vivo.* Cell lines established from BL can also readily be grown in the laboratory. In these cells, growth is thought to be driven by a combination of EBV and genetic changes that cause altered expression of cell proto-oncogenes. BL cell lines have been very useful for investigation of different types of EBV infection and the role of EBV in cancer (Wensing and Farrell, 2000)

LCLs transscribe 12 and translate 10 EBV genes. The small EBER1 and EBER2 are abundant non-polyadenilated EBV RNAs (Farrell, 1995). The EBNA1 to EBNA6 are six nuclear antigens and the LMP1, LMP2A and LMP2B are three membrane proteins (Rickinson and Kieff, 1996). Various spliced Bam HI A rightward transcripts (BARTs) (Brooks *et al.*, 1993) and the RK/BARFO (Fries *et al.*, 1997) complete the panel. The EBNA2 and EBNA5 are the first expressed viral proteins after *in vitro* infection.

1.6 Latency types

1.6.1 Classical latency types

Three classical types of EBV latency have been described based on different patterns of EBV gene expression in EBV positive cell lines and EBV associated tumors. (Kerr *et al.*, 1992) (Masucci and Ernberg, 1994)(figure 1). These types of EBV latency are associated with expression of specific cell surface markers (Rowe *et al.*, 1987). The location of the latency genes in different latency states is shown in figure 1. **Latency III** is the best-characterised latency programme and is found found in LCLs. In latency III all latency genes are expressed. The EBNA1 and the other 5 nuclear antigens are expressed from the Wp/Cp promoters (figure 2). This pattern was first described in LCLs and group III BLs, but it is also found in immunoblastic B cell lymphomas and in some patients with infectious mononucleosis. Type III latency cells present an activated phenotype due to the expression of activation markers (CD23) and high levels of adhesion molecules (CD11a, CD54, CD58) associated with MHC class I expression and formation of large clumps in the cell culture.

Latency I is characterised by the expression of the EBNA1 from the Q promoter. Hypermethylation of the viral genome correlates with the silencing of EBNA2/6 and LMP1/2. This type of latency occurs in EBV positive Burkitt's lymphoma biopsies, in the group I BL derived cell lines or in the hybrid cell lines obtained by fusing an LCL with the EBV negative hemopoietic HL60 cell line (Contreras-Brodin *et al*., 1991). High expression of CD10 and CD77 surface molecules and lack of adhesion molecules and activation markers (CD39 and CD23) characterise this type of latency. B cells expressing latency I grow in single cell and resemble germinal center centroblasts or centrocytes (Rowe *et al*., 1987). A newly observed latency, called latency +I, has been described recently in Burkitt's lymphoma. In this variant of latency I, EBNA3 is expressed together with EBNA1 and LMP2 (A. Rickinson, unpublished data).

Latency II is an intermediate program. The EBNA1 is expressed from the Q promoter and various combinations of the three membrane proteins can be expressed (LMP1 and/or LMP2A/B). This type is found in a panel of EBV associated malignancies like Hodgkin`s disease (HD), nasal NK/T cell lymphoma and nasopharyngeal carcinoma (NPC) and in cell hybrids obtained by fusion of LCLs with non-B cell lines (Contreras-Brodin *et al*., 1991).

In a recent study H. Knecht summarises the genes expressed and the forms of EBV latency in different situations (Knecht *et al*., 2001) (table 1).

1.6.2 Alternate nomenclature for latency transcription programs.

Three distinct latency types are described in *in vivo* EBV infected cells. During the **growth programme** (corresponding to latency III) all nine known latent proteins (EBNA1-6, LMP1 and 2A-B) are expressed and their proposed function is the activation of resting B cell to become a proliferating lymphoblast. In the **default programme** (corresponding to latency II) a restricted set of three latent proteins (EBNA1, LMP1, LMP2A) are expressed which provides the necessary survival signals for infected lymphoblasts to differentiate into infected memory cells (Thorley-Lawson, 2001). The **latency programme**, in which few if any latent genes are expressed allows persistence of the virus in resting re-circulating

memory cells in a way that is non-pathogenic and not detectable by the immune system. At present there is a controversy about whether persistence *in vivo* involves no gene expression at all or some expression of LMP2, EBER RNAs and possibly EBNA1 (Babcock *et al.*, 2000).

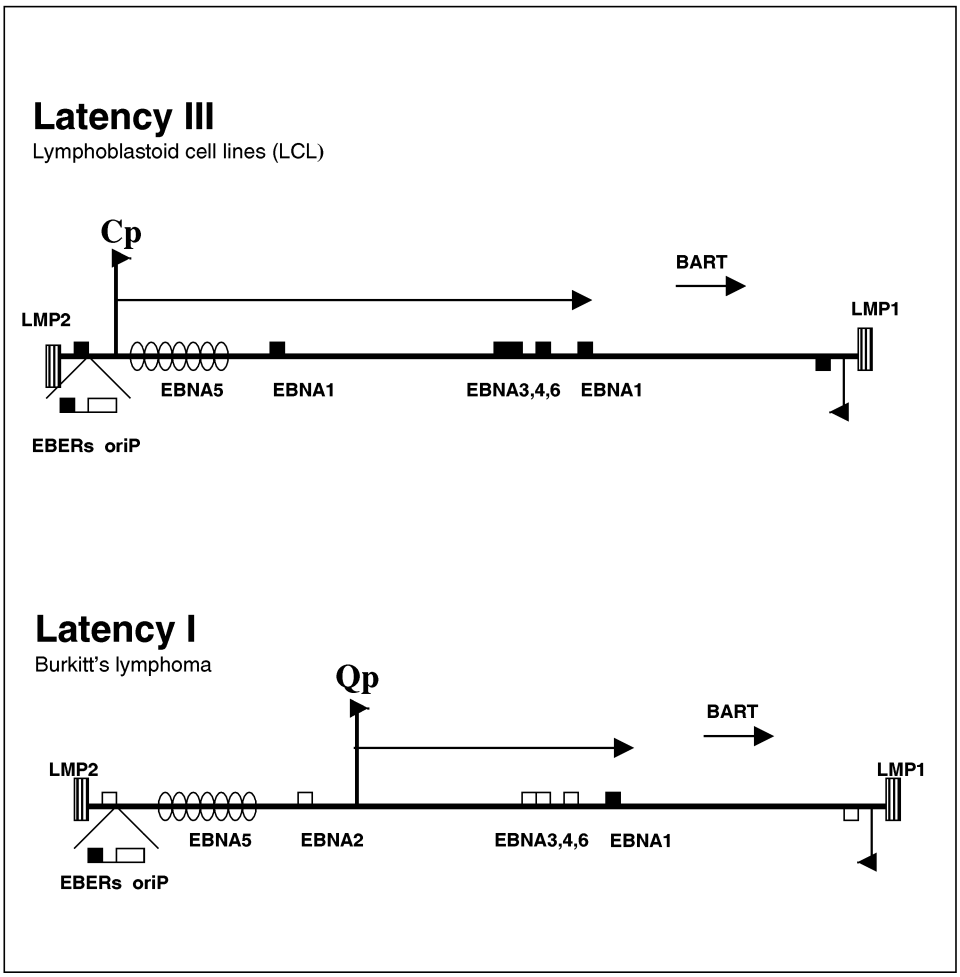

Figure 1

The layout of genes expressed in latency I and III. The positions of the latency genes are shown filled when they are expressed and open when they are not expressed. Transcription of the spliced RNAs that express the EBNA1 genes is from Cp in latency III and Qp in latency I *(adapted after Wensing, 2000).*

1.7 Genes expressed in latency

Alternative nomenclatures for latent genes and their functions are summarized in table 2.

1.7.1 Genes expressed in all latency types.

The EBV encoded RNAs (EBERs) (Randhawa *et al.*, 1994) and the BARTs (Chen *et al.*, 1999; Smith *et al.*, 2000) a family of alternatively spliced rightward BamHI A transcripts belong to this group.

The **EBERs** (EBER1 and EBER2) are two small non-polyadenilated non-coding RNAs (reviewed in (Ambinder and Mann, 1994)) transcribed from BamHI A fragment of the viral genome. They are expressed abundantly except in oral hairy leukoplakia lesions from AIDS patients and some EBV positive hepatocellular carcinomas. Beside other proteins EBERs associate with the interferone inducible serine/threonine protein kinase (PKR) (Zamanian-Daryoush *et al.*, 1999). EBER expression comes after the EBNA and LMP gene expression. The EBERs probably are involved in splicing of viral RNA transcripts.

The BART (BARF0, CST) family is represented by various spliced RNAs transcribed rightward, referred to as *Bam*HI A rightward transcripts (BARTs), BARF0 RNAs or complementary strand transcripts (CSTs). The structure of some of the more abundant splicing patterns has been clarified. In addition to BARF0 ORF important products are made from some of the upstream ORFs in the CST RNAs. Potential biological functions are identified for two of these. The product of the RPMS1 ORF is shown to be a nuclear protein that can bind to the CBF1 component of Notch signal transduction. RPMS1 can inhibit the transcription activation induced through CBF1 by NotchIC or EBNA-2. The protein product of another BART ORF, A73, is shown to be a cytoplasmic protein, which can interact with the cell RACK1 protein. Since RACK1 modulates signaling from protein kinase C and Src tyrosine kinases, the results suggest a possible role for BART products in growth control, perhaps consistent with the abundant transcription of BART RNAs in cancers (Smith *et al.*, 2000).

Table 2

Nomenclature of EBV latent genes and their functions (alternative nomenclature in parentheses)(*modified after Wensing and Farrell, 2000*).

Names	Function
EBNA1	Interference with the antigen presentation EBV plasmid/episome replication and maintenance Transcriptional regulation
EBNA2	Transcripton factor via Notch pathway/RBP-Jκ Transactivator for other EBV genes: LMPs and for cellular genes: bcl-2,CD21, CD23, *c-myc*
EBNA3 (EBNA3A)	Transcription repressor Binds RBP-Jκ
EBNA4 **(EBNA3B)**	Protection from apopthosis: bcl-2 up-regulation Binds RBP-Jκ
EBNA6 **(EBNA3C)**	Transcription repressor Binds RBP-Jκ Effects on cell cycle (CyclineD2)
EBNA5 **(EBNALP)**	Co-operates with EBNA2 to activate transcription Colocalises with Rb protein in LCLs
LMP1	Signal transduction via TRADD/TRAF to NFkB/JNK Up-regulation of cellular genes: CDs, bcl-2, bclx, A20, TAP
LMP2A **(TP-1)**	Blocks Lyn and Fyn, Prevents virus reactivation Interferes with signal transduction
LMP2B **(TP-2)**	unknown
EBERs **EBER1/EBER2**	Associates with PKR (interferone inducible serine/threonine kinase)
BARTs **(BARF0, CST)**	Growth control via Src and Notch pathways RPMS1:transactivator via CBF1/Notchor EBNA2 A7:interacts with RACK1/PKC or Src

1.7.2 EBV proteins expressed in different types of latency

EBNA1 is the only viral protein detected in all EBV-associated malignancies and is expressed in all cells carrying the virus (Yates *et al.*, 1985). Different EBV promoters are used for EBNA1 expression in a cell type and latency type specific manner (Sample *et al.*, 1991; Schaefer *et al.*, 1991; Smith and Griffin, 1992)(figure 2). EBNA1 is a phosphoprotein of more than 600 amino acids and contains a large internal repeat. The glycine alanine repeat (GAr) makes up about one third of the entire protein and varies in length with every viral strain (Gratama *et al.*, 1990). When latently infected B cells are induced to proliferate the EBV genomes undergo DNA replication once per cell cycle and are efficiently partitioned to daughter cells. Only one EBV genome sequence, the latent origin of virus replication (oriP), and only one protein, EBNA1 are required for maintenance (Yates *et al.*, 1985).

EBNA2 is a transcription transactivating and transformation related factor, which coactivates and coordinates the latent gene expression in latency III. It acts on the EBV promoter Cp for other EBNAs and on LMP1 promoter as well. Cellular genes like bcl-2, c-gfr, CD21, CD23 (a characteristic surface marker of activated B cells) (Calender *et al.*, 1990) and *c-myc* (Kaiser *et al.*, 1999) are transactivated by EBNA2. EBNA2 also regulates transcription of EBV genes such as the latent membrane proteins LMP1, LMP2A and LMP2B (Abbot *et al.*, 1990; Fahraeus *et al.*, 1993). EBNA2 does not associate directly to DNA but associate with adaptor proteins like the Notch pathway related RBP-Jκ (Henkel *et al.*, 1994) EBV mutants lacking the EBNA2 gene cannot immortalise B cells. EBNA2 can interact also with ATF/CRE mediated transcription (Sjoblom *et al.*, 1998) and with the chromatin remodelling factor SNF5/Ini (Wu *et al.*, 1996). **EBNA5** (EBNALP) co-operates with EBNA2 to activate transcription. It is the first viral protein expressed during EBV infection of B cells together with EBNA2 and both are required for induction of cyclin D2 (Sinclair *et al.*, 1994) and the LMP2B and LMP1 promoter (Harada and Kieff, 1997). EBNA5 co-localises with the retinoblastoma protein (Rb) in the nuclei of LCLs (Jiang *et al.*, 1991).

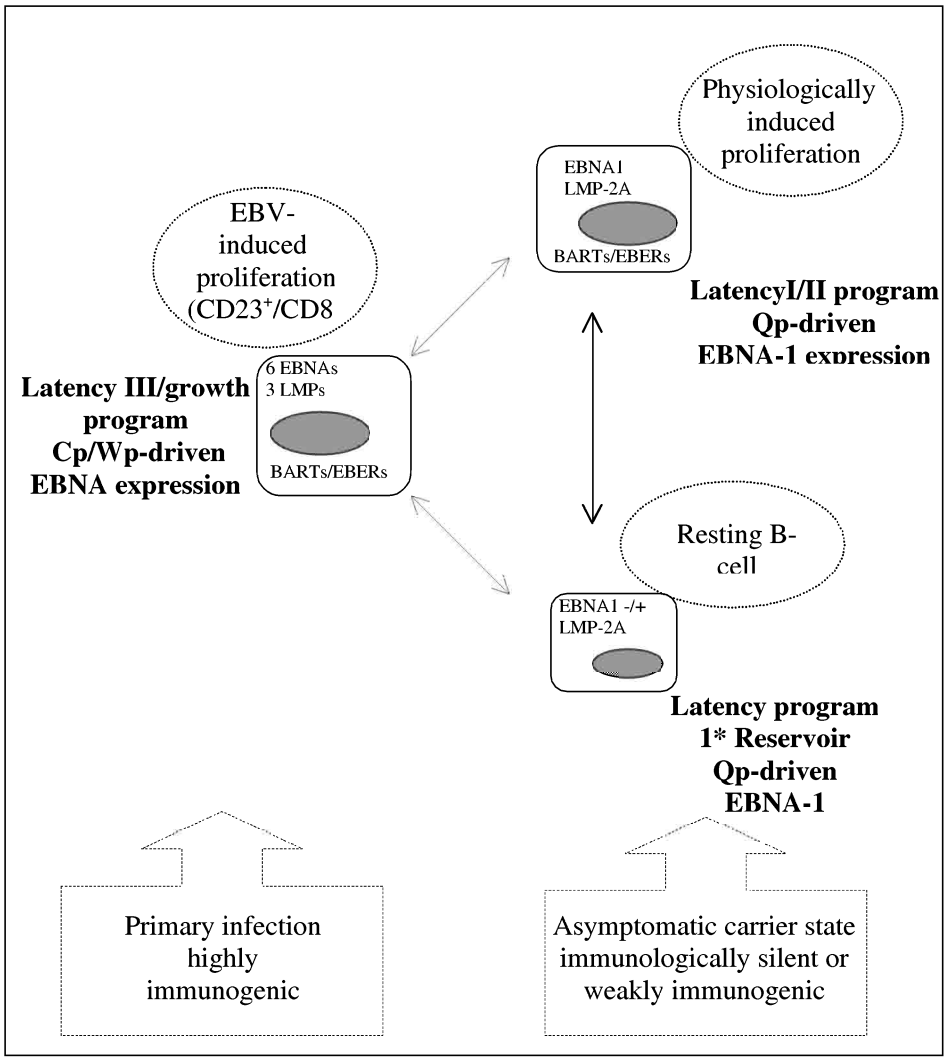

Figure 2

Promoter usage, surface markers and EBV gene expression in different types of latency *(modified after Ruf, 1999)*

EBNA3, EBNA4 and EBNA6, the other 3 EBNAs are encoded by three genes which are adjacent within the EBV genome. EBNA3 (EBNA3A) and EBNA6 (EBNA3C) are required for B-cell immortalization while EBNA4 (EBNA3B) is dispensable. EBNA4 (EBNA3B) can protect the cells from apoptosis by up-regulating bcl-2 (Silins and Sculley, 1995). EBNA6 (EBNA3C) contains a basic leucine zipper domain and functions as a transactivator both for viral (LMP1) (Allday and Farrell, 1994) and cellular genes (CD21, CD23, vimentin) (Allday *et al.*, 1993).

LMP1 is the major modulator of cellular genes during latency. It is encoded by the first ORF (BLNF1 gene) of EBV. The LMP1 protein is composed of a short amino terminus, six transmembrane spanning domains and a long cytoplasmic carboxy-therminal tail which contains the effector part of the protein (reviewed in Farrell, 1998).

LMP1 is oncogenic *in vitro* and *in vivo*. It was shown to transform rodent fibroblasts (Wang *et al.*, 1985), to induce morphological transformation on human keratinocytes (Fahraeus *et al.*, 1990), to inhibit the differentiation of epithelial cells (Dawson *et al.*, 1990) and to be essential for the immortalization of primary B lymphocytes to lymphoblastoid cell lines (Cuomo *et al.*, 1992). For B cell activation the cytoplasmic C-terminal domain but not the N-terminal domain of latent membrane protein 1 of Epstein-Barr virus is essential (Peng-Pilon *et al.*, 1995). Mice transgenic for the LMP1 gene under control of the Ig heavy–chain gene develop disseminated large B cell lymphomas with increasing frequency over time (Kulwichit *et al.*, 1998). LMP1 confers anchorage– and growth factor-independent growth and loss of contact inhibition to fibroblast cell lines, and these changes in cellular phenotype correlate with enhanced tumorigenicity in nude mice (He *et al.*, 2000; Hu *et al.*, 1993).

Several types of cellular genes are upregulated by LMP1 on different cell background: adhesion molecules, bcl-2 and bclx, mcl1, the zinc finger protein A20, the human IL-10, the TAP1 and TAP2 peptide transporter subunits etc. Suppression of LMP-1 expression by antisense oligodeoxynucleotides to LMP-1

inhibits proliferation, promotes apoptosis and suppresses Bcl-2 expression in EBV-transformed B cells (Noguchi *et al*., 2001).

LMP2A and 2B are also transmembrane proteins. They are not essential for transformation of B cells in vitro. LMP2A interferes with signal transduction by interacting with its hydrophilic N-terminal domain with 2 tyrosine kinases Lyn and Fyn. LMP2 may regulate reactivation from latency by interfering with normal B-cell signal transduction processes and in doing so may also provide a survival signal that could be important for viral persistence (Longnecker, 2000).

2. The Burkitt's Lymphoma (BL)

BL is the most common childhood cancer in certain parts of equatorial Africa (Burkitt, 1962) and Papua New Guinea, having an annual incidence of more than 50 cases per million children younger than the age 16. The association of EBV with Burkitt's lymphoma (BL) differs depending on the geographical location. EBV is present in the tumor cells of more than 90% of African BLs, in the regions where malaria is hyperendemic. Histologically the BL is a poorly differentiated malignant lymphoma in which the tumor cells show little variation in size or shape and posses amphophilic cytoplasm with clear vacuoles and non–cleaved nuclei with 2-3 basophilic nucleoli. The tumors are infiltrated by non-malignant hystiocytes, which cause the characteristic "starry sky" appearance. Outside the endemic areas, BL occurs worldwide with a 20-100 times lower frequency. Only 10-20% of the sporadic cases carries EBV (reviewed in Masucci and Klein, 1991). In an EBV positive cancer, all of the tumor cells (with few exceptions) carry EBV in contrast to the very low number of EBV infected cells in non-tumor cells (1 in 100000 B cells in a normal carrier), suggesting a role for EBV in tumorigenesis.

Chromosomal translocations that juxtapose the *c-myc* gene to immunoglobuline gene control elements are a common feature of all subtypes of BLs. The translocation involve the long arm of chromosome 8 (8qp24) and either

chromosome 14, t(8:14) or chromosome 2, t(2:8) or chromosome 22, t(8:22). The translocations bring the *c-myc* oncogene on in the proximity of the immunoglobulin heavy chain gene (or the kappa or the lambda gene) on their respective chromosomes. Activation or deregulation of the oncogene as a consequence of the translocation is believed to be the crucial event in the pathogenesis of BL (reviewed in Masucci and Klein, 1991) and (Potter and Marcu, 1997). Translocation of the c-myc gene to an immunoglobulin locus is a rate-limiting step in the genesis of Burkitt's lymphoma. Its consequences involve a non-immunological and an immunological component. The former acts by preventing the B-cell from leaving the cycling compartment and entering the resting stage when programmed to do so. The latter acts by down-regulation of certain class I polymorphic specificities, adhesion molecules and EBV encoded proteins. The translocation fixes the BL cell in a phenotypic window that can be referred to as "a resting cell that is not resting". Normally EBV-carrying B-blasts face immune controls. Due to it's "resting" rather than B-blast phenotype the BL cell is not rejected by the EBV-specific immune response, however (reviewed in (Klein, 1996)). The importance of Ig/*myc* juxtaposition is illustrated by studies with transgenic mice (reviewed in Potter and Marcu, 1997). Introduction of the *c-myc* oncogene in germline mice resulted in an increase of spontaneous tumors. When the oncogene was fused to an Ig-heavy chain enhancer, B cell tumors arose in nearly all animals (Adams *et al.*, 1985). Deregulation of *c-myc* was however insufficient to induce a fully malignant phenotype because the developing tumors were in all cases monoclonal. In EBV negative tumors the breakpoint in the *c-myc* gene is always very close to the coding region of the gene, whereas in EBV positive tumors this point can be many kilobases away from the *c-myc* gene. Much less specificity of breakpoint seems to be required for tumorigenesis in the presence of EBV, indirectly suggesting a role for EBV in tumorigenensis (Pelicci *et al.*, 1986).

2.1 Cell phenotypes, groups and phenotype changes in BL lines

The ability to maintain continuously growing B-cells *in vitro* has been of crucial importance for studies aiming to characterize normal and tumor related B cells and the stages of normal B cell differentiation represented by the tumor. A compilation of the phenotypic characteristics of Burkitt's lymphoma cell lines is shown in table 3 (reviewed in (Masucci and Klein, 1991)).

Surface marker studies have been focused on three categories of antigens. The first category comprises antigens, such as CD19 and CD20 present and expressed at various levels on all mature B cells with exception of plasma cells. The second category of antigens are associated with Burkitt's lymphoma and generally not expressed by EBV immortalized normal B cells (LCLs). These are the CD10, CD77 and CD38, a differentiation marker usually expressed on plasma cells. The third group of antigens are referred as activation markers, are expressed by antigen and mitogen activated B cells, by LCls and some BL lines. Within this category are the EBV/CR2 receptor, CD21, CD23, CD30, CD39 and the Reed-Sternberg cell marker Ki-24. The classification of the cultured EBV positive BL lines into three groups is based on their growth characteristics and expression of surface markers (Rowe *et al.*, 1986).

Group I BL lines grow as a carpet of single cells and express some differentiation markers but are devoid of activation markers.

Group II BL lines grow as small clumps of cells, and in addition to differentiation markers, acquire one or several of the activation markers.

Group III BL lines are characterised by growth in large clumps**,** loss of some differentiation markers and expression of high levels of activation markers. The group III BL lines resemble EBV transformed LCLs.

Biopsy cells from endemic BLs display a group I phenotype (Roney *et al.*, 1986) (Gregory *et al.*, 1987). The difference between biopsy cells and *in vitro* propagated BL lines is due to the capacity of some EBV positive BLs to drift towards an LCL-like phenotype within the first 20 passages *in vitro* (Gregory *et al.*, 1990). EBV negative BL lines tend to be phenotypically more stable and retain a group I or I/II phenotype even after prolonged *in vitro* propagation.

However, when these lines are converted by infection *in vitro* with EBV they may drift towards a group II or III phenotype, further confirming that the progression is related to the presence of EBV (Masucci and Klein, 1991). The extent of the progression varies between cell lines, and is also dependent on the viral strain used. Notably, cell lines converted by the non-transforming P3HR1 derived viruses tend to undergo a less pronounced phenotypic shift and certain BL lines do not acquire activation markers (Klein *et al.*, 1983). Upon seeding into tissue culture, the EBV positive tumor derived cell lines tend to grow in large clumps (Roney *et al.*, 1986). This change in phenotype and growth pattern of the cells is mediated by a change in the viral gene expression program. EBV positive BL cells *in vivo* express EBNA1 as the only viral antigen in a BL group I pattern (Rowe *et al.*, 1986). Upon prolonged cultivation *in vitro* the cells start to express the whole set of viral genes expressed in EBV immortalized lymphocytes (group III) and as a consequence, start to express a variety of adhesion molecules and activation markers (Rowe *et al.*, 1986; Rowe *et al.*, 1987).

2.2 EBV gene expression in BLs and BL lines

The mechanisms that regulate the expression of EBV genes in different types of B cells are not completely understood. Methylation of the viral DNA plays a role in the sense that both coding and regulatory sequences of EBV genes were found to be highly methylated in BL biopsies and group I BL lines and hypomethylated in LCLs (Ernberg *et al.*, 1989; Masucci *et al.*, 1989), (Jansson *et al.*, 1992). BL lines propagated *in vitro* ranging from typical BL group I to more LCL like group III phenotype showed more moderate or low vial DNA methylation (Minarovits *et al.*, 1991). Sequence specific methylation inhibits the activity of LMP1 and BCR2 enhancer-promoter regions. Clones of the BL line Mutu that differ in expression of LMP1 also show a differential methylation pattern of the LMP1 regulatory sequences. This region is methylated in a group of I clone that does not express LMP1 but is hypomethylated in a LMP1 expressing group III clone (Minarovits *et al.*, 1994). In LCLs the EBV genome is mostly free from CpG metylation, whereas in BL cells, EBV genomes are highly methylated (Salamon *et al.*, 2001).

Table 3

Phenotypic characteristics of Burkitt's lymphoma cell lines
(adapted after Masucci, 1991)

	Group I	**Group II**	**Group III**
Growth pattern	Single cells	Small clumps	Large clumps
Differentiation markers			
CD10/CALLA	+	+	-
CD77	+	+	-
CD38	+	+	-
Activation markers			
CD21	-/+	+	+
CD23	-	+	+
CD30	-	+	+
CD39	-	+	+
CDw70/Ki-24	-	+	+
Adhesion molecules			
CD11a/CD18 (LFA-1)	-	+	+
CD54 (LFA-2)	-	+	+
CD58 (LFA-3)	-	+	+
EBV antigenes			
EBNA1	+	+	+
EBNA2-6	-	+	+
LMP1-2	-	+	+

2.3 BLs and LCLs. Immunogenicity and immune surveillance

BL cells are charactherized by low immunogenicity. Comparison of paired EBV carrying BL lines and *in vitro* EBV transformed LCLs derived from the same individual demonstrated that the lymphoma cells are often resistant to lysis to the HLA class I antigens restricted, EBV specific CTLs generated by stimulation of lymphocytes from EBV seropositive donors with autologous virus infected cells (Roney *et al.*, 1986) (Torsteinsdottir *et al.*, 1986). Since the T lymphocyte from BL patients maintain the capacity to control the proliferation of autologous infected cells in *in vitro* regression assays (Masucci *et al.*, 1987) the CTL resistance was taken to suggest that escape from immune surveillance may play a role in the pathogenesis of the EBV carrying lymphomas. Various routes of escape may be considered. Burkitt's lymphoma cells may not present the relevant CTL target structure either because the relevant antigen is down-regulated in the tumor or because by mutations or abnormal processing the immunodominant target epitope has been altered. The restriction elements for the CTLs are represented by the MHC class I antigens. In these cell lines the MHC class I frequently is down-regulated or lost. The lack of adhesion molecules that are capable to function as accessory structures in the triggering of CTLs has to be considered also as an escape route from immune surveillance.

The finding that, independently of the EBV carrying status, all BL tumor cells express a similar cell surface phenotype, and that some of the transformation associated viral antigens are down-regulated also in *in vitro* converted BL lines, suggest that the poor immunogenicity of BL cells compared to LCLs may be related to intrinsic properties of the tumor cells (Masucci and Klein, 1991). BL cells are phenotypically more similar to resting than activated B cells in spite of their rapid proliferation rate. This fact and the demonstration that *in vitro* shift towards a more "LCL-like" phenotype correlates with up-regulation of class I antigens, suggests that normal B cells in the same stage of activation/differentiation may exhibit a similar pattern of HLA class I expression. Significant differences were observed when resting and mitogen activated B and

T lymphocytes from HLA A11 positive healthy donors were compared (Torsteinsdottir *et al.*, 1988).

2.4 Allele selective down regulation of HLA class I in BL cell lines

In a panel of BL lines derived from HLA-A11positive individuals the analysis of MHC class I antigen expression has revealed a selective down-regulation of the HLA-A11 allele both in EBV carrying and EBV negative tumors (Masucci *et al.*, 1987). The defect was paralleled by the resistance of the tumor cells to anti-HLA-A11 allospecific and, in the case of EBV carrying tumors, HLA-A11 restricted EBV specific CTLs (Masucci *et al.*, 1987; Torsteinsdottir *et al.*, 1986). Indeed, the down-regulation of HLA-A11 appeared to be sufficient to explain the resistance to lysis since the same BL cells could be lysed by CTLs directed to other HLA class I alleles that were expressed at levels corresponding to LCLs.

Treatment with IFNα or TNFα in some cases increased the sensitivity to CTL lysis (Avila-Carino *et al.*, 1988). In some cases HLA-A11 was re-expressed following *in vitro* EBV conversion of EBV negative BL cell lines (Torsteinsdottir *et al.*, 1988) (Masucci *et al.*, 1989). Seven BL lines, derived from HLA-A11 positive individuals investigated so far have shown a selective down regulation of this allele (table 4). The regularity of this phenomenon suggests that the HLA-A11 defect may play a role in the pathogenesis of the tumor. If so, HLA-A11 positivity could be a risk factor for the development of BL, or, alternatively, analogous allele-selective down regulations may be expected to occur in BLs arising in HLA-A11 negative individuals (reviewed in Masucci and Klein, 1991).

Analysis of HLA class I expression by immunoprecipitation and one dimension isoelectrofocusing (ID-IEF) which allows the resolution of different class I heavy chains by virtue of their distinct isoelectric point revealed allele selective down regulation in 75% (12 out of 16) of BL lines investigated. Within the limits of resolution allowed by the method, losses of one or more class I heavy chain were demonstrated in 50% of these BL lines (6 out 12). In the remaining cell lines certain class I specificities were detected in the autoradiograms but the levels of expression were lower compared to the corresponding LCLs.

Table 4

Defective expression of MHC class I antigens in BL cells
(adapted from Masucci, 1991)

Cell line	EBV	Group	Affected allele	IFN sensitivity
BL 28	-	I	A1; **A11**; Cw7	+,+,-
BL 29	+	I/II	Aw69	+
BL 36	+	II	Cw8, Cw4	-,+
BL 37	+	II	A3; Cw4; Cw5	-,+,+
BL 41	-	I	**A11**; Cw7	-,-
BL 72	+	II	**A11**	+
WW-1-BL	+	II/III	**A11**;	-
WW-2-BL	+	II	**A11**; B39	-,-
Jiyoye M13	+	II	Cw4	+
DH-BL	+	II	**A11**	+
DG 75	-	I	**A11**	-
Mutu	+	I	A1; Cw6; Cw8	-,-,-

In five of the cell lines only single alleles within the HLA-A or HLA-C loci were affected, while in the others both HLA-A and HLA-C specificities were reduced or absent. Only one BL line showed a combined loss of one HLA-A and one HLA-B polypeptide (Masucci and Klein, 1991).

2.5 Correlation between HLA class I antigen expression and cell phenotype.

Almost exclusively the BL lines expressing a group I or I/II phenotype present allele specific defects, while cell lines expressing a group II/III or III phenotype resemble the corresponding LCLs also in their MHC class I antigen expression. The expression of HLA class I eventually may be under the same type of regulatory control that influence the expression of activation/differentiation markers in BL cells (Masucci *et al.*, 1987) (Masucci *et al.*, 1989). A general

increase of W6/32 reactivity and some changes in the reactivity with allele specific monoclonal antibodies was demonstrated in *in vitro* EBV converted BL lines and appeared to be independent of the full expression of all transformation associated viral genes and activation markers (Torsteinsdottir *et al.*, 1988). In contrast, the expression of class I alleles that were selectively down-regulated in the EBV negative parentals was rescued only when *in vitro* conversion had resulted in a significant shift towards more "LCL like" phenotype (Masucci *et al.*, 1989). The relationship between cell phenotype and expression of class I HLA is further supported by the comparison of phenotypically distinct cell clones derived from early in vitro passages of the BL line Mutuku. The clones of the Mutuku BL that had retained a group I phenotype and expressed the virus encoded nuclear antigen EBNA-1 (but not the other transformation associated proteins EBNA2-6 and LMP1), showed a significantly lower expression of class I antigens and a selective down regulation of the HLA A11 allele compared to the clones expressing a group III phenotype. The selective down regulation of one allele in the group I cell lines was not compensated by the up-regulation of the remaining class I alleles. Thus, the β2M/heavy chain ratios of the expressed alleles were similar in sublines expressing a group I and group III phenotype (Masucci and Klein, 1991).

2.6 Mechanisms of HLA A11 related allele down-regulation

As a first approach to the analysis of the mechanisms leading to the allele selective defects, BL cells were treated with IFNα, a lymphokine known to increase class I gene expression by acting on interferon sensitive sequences in the class I promoter region (Sugita *et al.*, 1987). The treatment affected, albeit to different degrees, the expression of the down-regulated class I alleles in BL cells. In general, alleles that were expressed at a lower level in BL than in LCLs were up-regulated by interferon treatment, whereas the treatment could not rescue the expression of alleles that were not detected in the untreated cells. The different response to IFNα cannot be explained by cell line resistance to interferon, since

different alleles within the same cell line responded differently, nor to an allele specific mode of regulation because the same allele could be upregulated by IFN in some of the lines but not in others (Andersson *et al*., 1991).

*In **paper I** we have investigated the mechanisms of HLAA11 down regulation by comparing the HLA genotype, specific mRNA expression and HLA promoter activity in pairs of LCL and BL lines from the same individual. This approach has allowed us to address the molecular mechanisms of this defect.*

Using a PCR method for the identification of HLA A locus alleles from genomic DNA we have confirmed the origin of all five pairs of normal and tumor derived cell lines from HLA A11 positive individuals. Most importantly, the HLA A11 allele was detected in all five BL lines and further Southern blot analysis failed to demonstrate any gross rearrangement of the gene. Thus, selective loss of HLA A11 expression occurred in spite of the presence of the allele in genomic DNA. As already indicated by experiments where the HLA A11 polypeptide was quantitated by immunoprecipitation (Andersson et al., 1991; Masucci et al., 1989b), Northern blot analysis demonstrated that, in all BL lines, down-regulation of HLA A11 expression occurred at the transcriptional level. Comparison of HLA A11 mRNA expression before and after IFN treatment suggests, however, that at least two major types of defects may be involved.

One type of defect, exemplified by the BL28 and BL72 cell lines, can be at least partially restored by IFN treatment suggesting that lack of appropriate transcription factor can be probably involved. Different upstream cis-acting regulatory sequences and nuclear factors contributing to HLA class I transcription have been identified (reviewed in Ting and Baldwin, 1993). In particular, the enhancer A region which displays homology with the κB element of Ig light chain enhancer and the interferon consensus sequence interact with several regulatory proteins such as the NFκB/Rel family members. NFκB/Rel family products are expressed at low levels in BL cells that maintain in vitro the phenotypic characteristics of the in vivo tumor (group I BL lines). Drift towards a more LCL-like phenotype (group III BL lines) is accompanied by qualitative and quantitative changes (Cöster, unpublished data). Earlier observations

demonstrate that class I down-regulation in BL cells is, at least to some extent phenotype related. Cell lines expressing a group I phenotype express 5 to 10 fold lower levels of HLA class I compared to sublines that have drifted towards a group III phenotype ((Torsteinsdottir et al., 1988) and Q-J Zhang unpublished observations). The demonstration of locus and allele selective differences in the regulation of MHC class I gene (Hakem et al., 1991; Soong and Hui, 1991; Soong and Hui, 1992) suggests that certain alleles may be more affected than others. A detailed structural analysis of the HLA A11 promoter region (Blanchet et al., 1991; Blanchet et al., 1994) provides the basis for experimental dissection of this possibility.

The second type of defect, exemplified by the WW1-BL and WW2-BL cell lines correlated with complete absence of HLA A11 specific mRNA and protein and could not be restored by IFN treatment (Andersson et al., 1991). Two lines of evidence indicate that structural defects of the gene, such as minor rearrangements or point mutations that would have gone undetected in our Southern blot analysis, may be involved. HLA A11 expression could be restored in WW1-BL and WW2-BL by transfection of a genomic DNA construct containing the HLA A11 coding sequence and regulatory region. Furthermore, a HLA A11 promoter driven CAT reporter gene was active in these cells confirming that the lack of HLA A11 expression was not due to block of transcription. Our data do not decide whether these abnormalities arise as a result of strong selective pressure, such as a vigorous CTL response, or spontaneously through an intrinsic genetic instability of the tumor cells. It is noteworthy, however, that WW2-BL lacks a second class I allele, HLA B38 (Andersson et al., 1991) although both chromosomes 6 are present in this cell line and major rearrangements of the HLA B locus were not detected by Southern blot analysis.

Analysis of the HLA A11 defect in BL41 revealed an unexpectedly complex phenotype. The failure to express the gene after transfection, and the low activity of the pA11CAT reporter plasmid suggest that transcription factors required for HLA A11 expression are deficient in this cell line. This is likely to be a phenotype associated phenomenon since BL41, as BL28 and BL72, express a group I

phenotype. Indeed, it has been previously reported that a subline of BL41 that had acquired an LCL-like phenotype following in vitro EBV infection, the BL41/95 convertant, also expressed LCL-like levels of HLA A11 (Torsteinsdottir et al., 1988). Interestingly, transfection of the HLA A2 gene was accompanied by high levels of expression in BL41. This finding further supports the possibility that regulation of HLA class I transcription may include allele-specific components. Analysis of HLA A11 expression in BL41xLCL hybrids suggests that lack of transcription factors is not the sole cause of HLA A11 down-regulation in BL41 since reintroduction of adequate transcription machinery in the BL41xRS hybrid did not restore the expression of the endogenous gene. Thus, structural defects may also be involved. Such defects may have been present only in a subpopulation of the original tumor cells since EBV converted sublines obtained from late in vitro passages of the tumor were regularly HLA A11 negative.

In conclusion, our data suggest that multiple defects may contribute to the down-regulation of HLA A11 expression in BL cells. Lack of appropriate transcription factors seems to be a property of the BL cell phenotype. It is tempting to speculate that down-regulation of this allele may not be sufficient to ensure the escape from tumor specific immune responses since cells carrying structural defects appear to enjoy a selective advantage in vivo. It is likely that only extinction of HLA A11 expression as a consequence of genetic alteration may afford full protection (paper I).

3. Modulation of cellular and viral functions by viral gene products

3.1. Modulation of MHC class I expression by LMP1

To achieve persistence in the face of a vigorous host immune responses is a problem that must be solved by the viruses that establish life-long infections. During millions of years viruses have coexisted with their hosts, and have learned how to manipulate host immune control mechanisms. The large list of viral

immune evasion strategies comprise the inhibition of humoral responses, the inhibition and modulation of cytokines and chemokines, the inhibition of apoptosis, inflammation and the evasion of CTLs. Disturbance of TAP function and antigen processing, the inhibition of NK cells by class I homologues and the modulation of MHC class I and class II functions also are frequent strategies (Alcami and Koszinowski, 2000).

We have compared the activity of MHC class I reporter constructs containing the HLA A2 and HLA A11 promoters, in a panel of BL lines with different phenotypic characteristics. Both class I promoters displayed lower activity in BL lines that had maintained the phenotypic properties of the original tumor whereas the activity was high in cell lines that had shifted to the activated B-blast-like phenotype of in vitro EBV transformed LCLs. Thus, transcriptional down regulation appears to play an important role in the low class I expression of phenotypically representative BL cells. This mechanism does not explain the allele-selective and irreversible down-regulations observed in some tumors (Andersson et al., 1991). This is probably caused by genetic changes and/or additional changes that affect allele-specific steps in class I maturation, such as peptide binding or transport. The recent finding that ubiquitin-proteasome/dependent proteolysis is altered in BLs as a consequence of c-myc activation (Gavioli et al., 2001) is particularly interesting in this context since a defect in proteolysis could selectively affect the production of certain MHC class I binding peptides ***(paper II).***

The phenotype dependence of MHC class I promoter activity prompted us to ask whether, similar to other characteristics of BL cells, this could be modulated by expression of the EBV encoded membrane protein LMP1. Our data show that MHC class I can be added to the list of LMP1 regulated cellular genes and conclusively demonstrate that regulation occurs at the level of transcription. Several transcription factors regulate MHC class I expression, including the NFκB/Rel transcription factor which binds to two NFκB responsive elements in the Enhancer A region of the HLA locus (Blanchet et al., 1991; Blanchet et al., 1994) (Soong and Hui, 1991; Soong and Hui, 1992) (Gobin et al., 1998). Two

lines of evidence support the notion that modification of NFκB/Rel activity plays a central role in the up-regulation of MHC class I genes in LMP1 expressing cells. Expression of LMP1 correlated with up-regulation of several components of the transcription factor including the active subunits p50 and p65 as well as for the inhibitory components, p105 and IκBα. The enhanced expression of the inhibitors is probably a consequence of their accelerated turnover. Indeed, phosphorylated IκBα was detected in the LMP1 expressing cells and this correlated with a significantly decreased half-life compared to the LMP1 negative parental line. Increased degradation of the inhibitor results in nuclear translocation of the transcription factors and activation of the target genes including the transcription factor itself and its inhibitors.

It is noteworthy that the ubiquitin-proteasome system is involved both in the degradation of IκBα (Chen et al., 1995) and in the generation of p50 from its precursor p105 (Palombella et al., 1994). Thus, LMP1 seems to target components of the NFκB/Rel system that are regulated by protein turnover. This could be achieved by accelerating the required targeting steps, such as phosphorylation (Israel, 1995), or by a direct effect on the proteasome or other proteolytic processes. The possibility of a general effect on proteolysis is supported by the demonstration that the degradation of exogenous proteins, such as tetanus toxoid, is significantly enhanced in LMP1 transfected sublines of BL41. This correlates with enhanced presentation of the antigens to MHC class II restricted T-cells (de Campos-Lima et al., 1993).

The upregulation of p50 in LMP1 expressing cells correlated with an increase of p50 containing homo- or heterodimers able to bind NFκB/Rel consensus sequences in EMSA. The relevance of this finding for the transcription of class I genes in BL cells was confirmed in co-transfection experiments where both p50 and RelA (p65) were shown to enhance A11-promoter activity. It is noteworthy that increase of either p50 or p65 levels appeared to be sufficient to rescue the class I transcriptional defect in BLs. Previous reports have suggested that p50 homodimers may have a negative effect on class I expression (Plaskin et al., 1993). The different type of cells used in the experiments, epithelial versus

lymphoid, may explain this discrepancy. In line with this possibility, expression of p50 enhanced the activity of the class I promoter in BL30 but had no effect in the corresponding LCL IARC-139. The pSV40-CAT plasmid was equally active in the cell lines and the p105 and IκBα *inhibitors had similar effects suggesting that the different effect of p50 is not an artefact due to for example to differences in transfection efficiency between the two cell types (paper.*

3.1.1 MHC class I gene expression in tumors

Human MHC antigens are encoded by the HLA complex situated on chromosome 6. Human MHC class I genes encode the highly polymorphic HLA A, B and C (Monaco, 1992). Structurally class I proteins are composed of a "light" β2-microglobulin chain (β2M) (12kDa) that is non-covalently associated with a "heavy" HLA chain (45kD) (Bjorkman *et al.*, 1987).

Expression of HLAs vary considerably, from tissue to tissue even in the absence of pathology (Singer and Maguire, 1990) (Garrido *et al.*, 1993) and may also vary between different alleles, for example expression of HLA-C is always very low.

HLA expression is frequently altered in tumors compared to the tissue from which they originate (Garrido *et al.*, 1993).

Tumors frequently exhibit loss of expression when derived from HLA $^{+}$ tissue but may also upregulate HLA expression when derived from HLA negative or weakly expressing tissues (Fernandez *et al.*, 1991; Lopez-Nevot *et al.*, 1989). In human tumors HLA loss may be as high as 50%, inferring that a reduction on protein levels might offer a survival advantage to the tumor cells (Rees and Mian, 1999).

Several types of HLA class I down regulation have been described, including total (Concha *et al.*, 1991b; Concha *et al.*, 1991a), (Cabrera *et al.*, 1996), locus (Redondo *et al.*, 1991a) (Concha *et al.*, 1991b) (Esteban *et al.*, 1996), allele (Natali *et al.*, 1989) (Blades *et al.*, 1995) (Andersson *et al.*, 1991) (Masucci *et al.*, 1989b), *(paper I)* and haplotype (Torres *et al.*, 1996) related losses.

Down-regulation has been observed in a variety of cancers such as breast (Cabrera *et al*., 1996), (Concha *et al*., 1991b; Concha *et al*., 1991a), (Natali *et al*., 1989) (Kaklamanis *et al*., 1995), laryngeal (Redondo *et al*., 1991b), colorectal (Natali *et al*., 1989), bronchic (Redondo *et al*., 1991a), prostate (Blades *et al*., 1995), cervical (Hilders *et al*., 1995), pancreatic (Torres *et al*., 1996), and oesophageal (Rockett *et al*., 1995) tumors.

The clinical relevance of class I down regulation is reflected in the observation that it is more common in metastases when compared with the primary tumor (Redondo *et al*., 1991b), (Cabrera *et al*., 1996; Hilders *et al*., 1995), (Kaklamanis *et al*., 1995) and in tumors with abnormal cellular DNA content (Redondo *et al*., 1997), suggestive of more aggressive tumors. This increase in metastatic growth with down regulation of class I expression has, in turn, been reported to be associated with decreased patient survival (Concha *et al*., 1991b) (Klein *et al*., 1996).

The defect responsible for altered HLA class I expression can be subdivided into structural, transcriptional and assembly/transport related.

Structural defects were described in the coding region of the light and/or heavy chain coding genes. Disabling mutations in β2M are responsible for total class I loss as a result of failure to assemble the class I molecules for cell surface expression. Classical examples for this situation are the Daudi, a Burkitt's lymphoma (Rosa *et al*., 1983) and the FO-1 a melanoma cell line (D'Urso *et al*., 1991). Total loss of β2M was found also in the colon carcinoma (Momburg and Koch, 1989) (Cabrera *et al*., 1991).

Losses of coding sequences affecting the heavy chain include losses of entire chromosome, chromosome segment or loss of alleles due to mutation in the class I heavy chain coding gene (Garrido *et al*., 1995). Chromosomal non-disjunction or mitotic recombination might underlay the HLA haplotype loss (Browning *et al*., 1993) (Marincola *et al*., 1994; Torres *et al*., 1996). Allelic loss might result from point mutations or deletions (Browning *et al*., 1993) of the HLA class I gene or as

a consequence of chromosomal breakage or somatic recombination (Browning *et al.*, 1996). This type of mechanism for allele selective loss characterises some melanoma (Lehmann *et al.*, 1995) and colon carcinoma lines (Ferrone and Marincola, 1995).

Transcriptional defects involving the *cis* acting promoter/enhancer region of the HLA gene and/or the regulatory *trans* acting nuclear factors have been documented. Cellular modulators acting on this level belong to the family of cytokines, adhesion molecules and hormones. Oncogenes *(c-myc)* and viral products influence transcription by affecting a large panel of cellular pathways (Tortorella *et al.*, 2000). Promoter sequences and mRNA levels in some class I alleles in tumors differ from those of normal cells. Increased *c-myc* transcription interferes with HLA-B transcription in melanomas (Peltenburg and Schrier, 1994; Versteeg *et al.*, 1989) but is not seen in other tumors (Redondo *et al.*, 1991b). In colorectal cancer low expression of transcription factors can induce downregulation (Soong and Hui, 1991). The transcriptionally mediated down regulation frequently can be overdriven by cytokine treatment (Schrier *et al.*, 1991).

Transport and assembly related defects are represented by alterations of the transporter associated with antigen processing (TAPs) function and on the level of MHC encoded LMP2 and LMP7 cathalytic subunits of the proteasome (Cromme *et al.*, 1994) (Kageshita *et al.*, 1999; Seliger *et al.*, 1997; Singal *et al.*, 1996; Yao *et al.*, 2000).
Altered HLA class I expression might reflect multiple simultaneous or serial events resulting in different class I defective phenotypes (Garrido *et al.*, 1997).

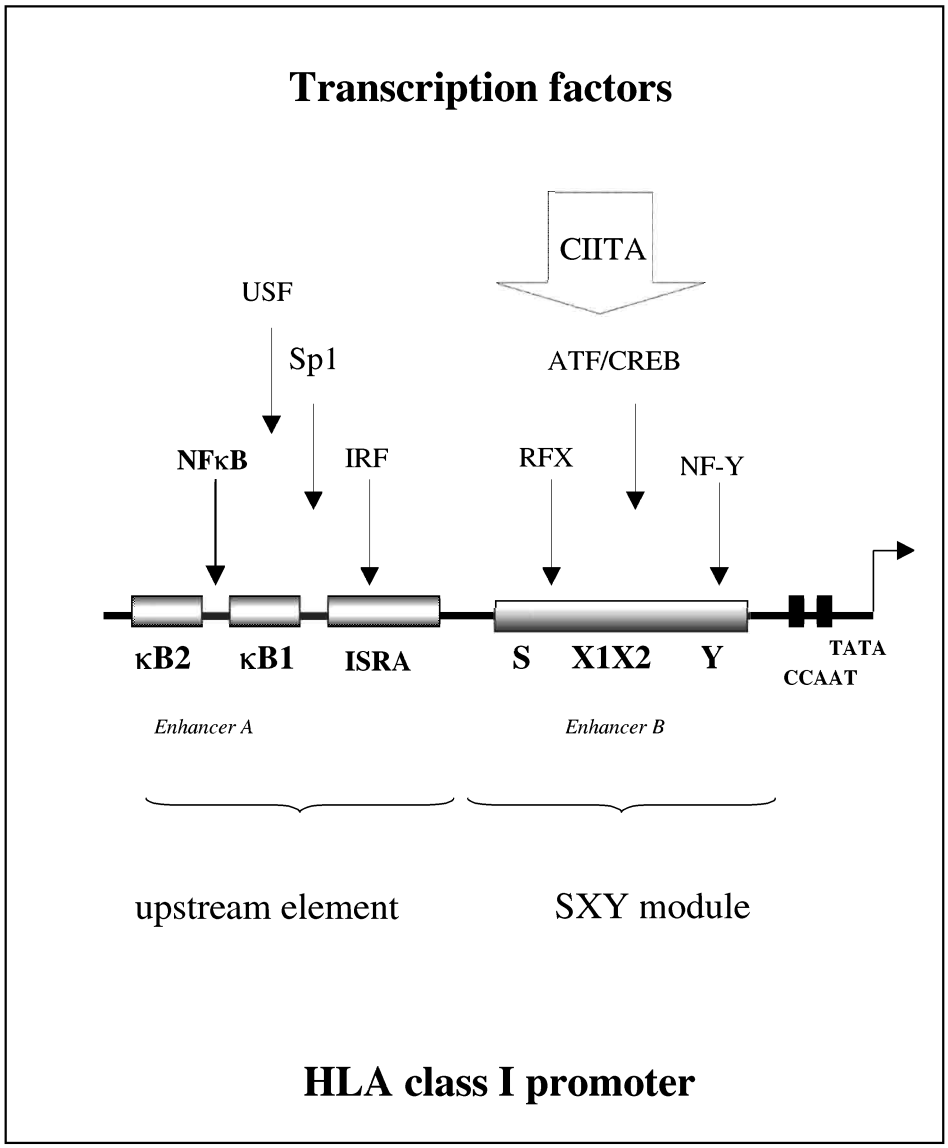

Figure 3

Schematic representation of the MHC regulatory module. Consensus sequences within the MHC class I promoter and the nuclear factors which bind to this elements.

3.1.2 Regulation of MHC class I expression

The promoter/enhancer region of class I genes can be activated/inhibited through several pathways. Various *cis* acting sequences involved in positive and negative regulation of MHC class I genes were identified as well as the *trans* acting proteins that can bind to these sites.

HLA class I expression is highly controlled at the transcriptional level by several conserved regulatory elements in the proximal promoter region. The CCAAT and TATA box are involved in the binding and positioning of the basal transcriptional initiation complex. A series of distal upstream DNA sequences in MHC class I promoter have been shown to play a crucial role in the regulation of MHC class I expression (reviewed in (Le Bouteiller, 1994) (van den Elsen *et al.*, 1998). The regulatory elements in this region can be divided into two regulatory modules an upstream module consisting of the enhancer A and the interferon-stimulated response element (ISRE) and a recently identified SXY module containing regulatory sequences shared by MHC class I, MHC class II and their accessory genes. (Gobin *et al.*, 1998) (van den Elsen *et al.*, 1998).

The upstream module contains binding sites for members of different transcription factor families including the NFκB/rel family of transcription factors (enhancer A), IFR1 and IFR2 (ISRE), and USF1 and USF2 and Sp1. This modul mediates constitutive and cytokine induced routes of transactivation (Gobin *et al.*, 1998; Gobin *et al.*, 1999). The SXY module consists of S, X (comprising X1 and X2 half sites) and Y box sequences that are co-operatively bound by a multiprotein complex of RFX, CREB/ATF and NFY. The γ-IFN induced transcription factor identified as a regulator of MHC class II genes (CIITA), exerts its activity through this multiprotein complex and is also a potent activator of class I genes (Martin *et al.*, 1997) (Figure 3).

These elements exhibit locus and allele specific differences in nucleotide composition. The enhancer A element which compasses two putative NFκB binding sites (κB1 and κB2) is playing the most important role in constitutive and cytokine induced expression of HLA-A genes.

3.1.3 The NFκB/rel family and MHC regulation

This family of transcription factors comprises at least five different members, p50, p52, p65 (Rel-A), c-rel, and RelB that can form either homo- or heterodimers (reviewed in (Thanos and Maniatis, 1995)). The p50 and p52 elements are synthesized as cytoplasmic precursors, p105 and p100. They may act as repressors when bound to target sites as homodimers. Rel-A (p65) and C-rel represent the activators of the NFκB/rel family. The NFκB (p50/p65 heterodimer) is found in two forms, an active DNA binding nuclear form and an inactive cytoplasmic variant. In the cytoplasm, the NFκB is associated with the inhibitor subunit, IκBα. Different stimuli can produce the phosphorylation of IκB, which is usually the first step for NFκB dissociation and nuclear translocation (Figure 4).

Only B-cells and some cells of the monocyte/macrophage lineage exhibit constitutive nuclear NFκB activity. RelB is expressed only in a subset of lymphoid cells (dendritic cells). The κB1 and κB2 sites of the HLA-A locus bind members of NFκB/rel family such as p50, p65 and c-Rel. (Mansky *et al.*, 1994) (Park *et al.*, 1993). These factors interact either as homo- or heterodimers resulting in varying effects on transcription regulation (Mansky *et al.*, 1994) (Gobin *et al.*, 1998). The NFκB mediated transactivation trough enhancer A is the most prominent for the HLA-A locus (Gobin *et al.*, 1998).

The NFκB pathway provides an attractive target to viral pathogens. Activation of NFκB constitutes an obvious target because many of its target genes -growth factors, cytokines and their receptors and protooncogenes- profoundly influence the cell cycle. Some viruses exploit the anti-apoptotic properties of the NFκB family. Several lines of evidence demonstrate that the NFκB family members contribute to human oncogenesis (Rayet and Gelinas, 1999).

Viral oncogene products, including human T-cell leukemia virus type 1 (HTLV-1) tax protein and the Epstein-Barr virus latent membrane protein (EBV LMP1) each act by unique mechanisms to disrupt NFkB regulation and initiate viral transformation (Hiscott *et al.*, 2001).

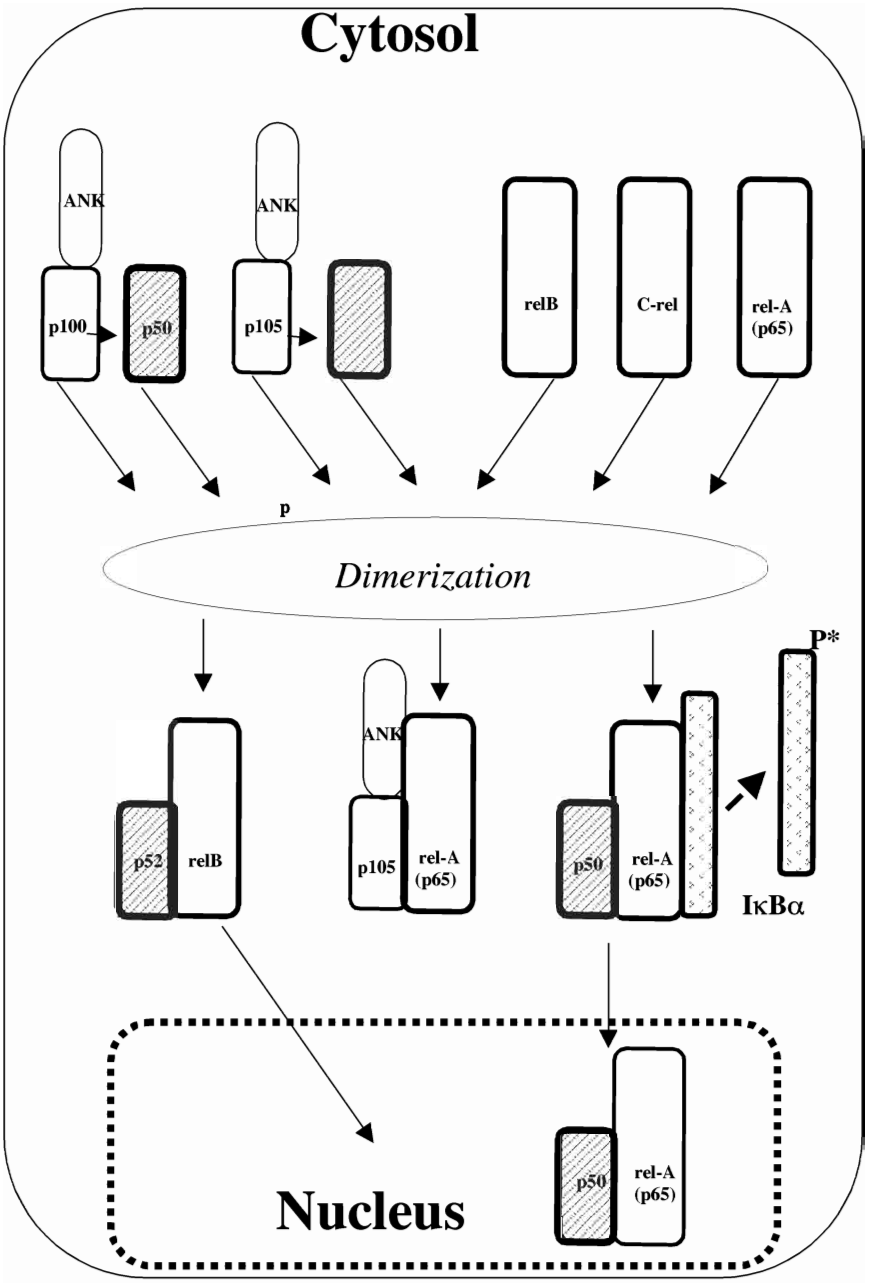

Figure 4
The cellular localization of NFκB/rel family members and heterodimers

3.1.4 LMP1 and MHC regulation

The Epstein-Barr virus (EBV) encoded latent membrane protein (LMP1) plays a crucial role in the long-term persistence of this virus within the cells of the immune system. This protein is not only critical for the transformation of resting B cells by EBV but also displays pleiotropic effects on various cellular proteins expressed in the host cell. LMP1 is the major modulator of cellular genes. These include up-regulation of expression of B cell activation antigens, adhesion molecules and various components of antigen processing pathway.

LMP1 acts like an expression 'switch' which, depending on the stage of EBV infection, maneuvers various pathways that either modulate the immune system towards or against its survival (Pai and Khanna, 2001). The long cytoplasmic carboxy-terminal tail constitutes the effector part of the protein (reviewed in Farrell, 1998). The transforming properties of this oncogene appear to result from multiple interactions with signaling proteins and basic transcription factors (reviewed in (Knecht *et al*., 2001)) (Figure 5). Like CD40 and other members of the tumor necrosis factor (TNF) receptor family, the carboxy–terminal domain of LMP1 is capable of interacting with TNF receptor associated factors (TRAFs) (Mosialos *et al*., 1995) and with TNF receptor-associated death domain (TRADD). The interaction with TRAFs and TRADD has been shown to be essential for the activation NFkB pathway and for immortalization of B lymphocytes. LMP1 acts as a constitutively activated TNF-CD40 family receptor, mimics the B lymphocyte activation antigen CD40 (Gires *et al*., 1997) and is a functional viral homologue of this surface receptor (Kilger *et al*., 1998). LMP1 is a powerful inducer of NFκB mediated transcription (Hammarskjöld and Simurda, 1992) and acts similar to those seen with activation of CD40 on the activation of NFkB/rel family. In *in vitro* Epstein-Barr virus-infected leukaemic B-lymphocytes, LMP1 expression can be induced by antibodies to CD40 and by interleukin 4 (IL4) (Crawford *et al*., 1995). In the B cells, the carboxy terminus of LMP is sufficient for B-cell signaling and immunoglobulin secretion. LMP1 has several effects similar to those seen with activation of CD40: like up-regulation of B7-1, Fas and adhesion molecules, IgM induction.

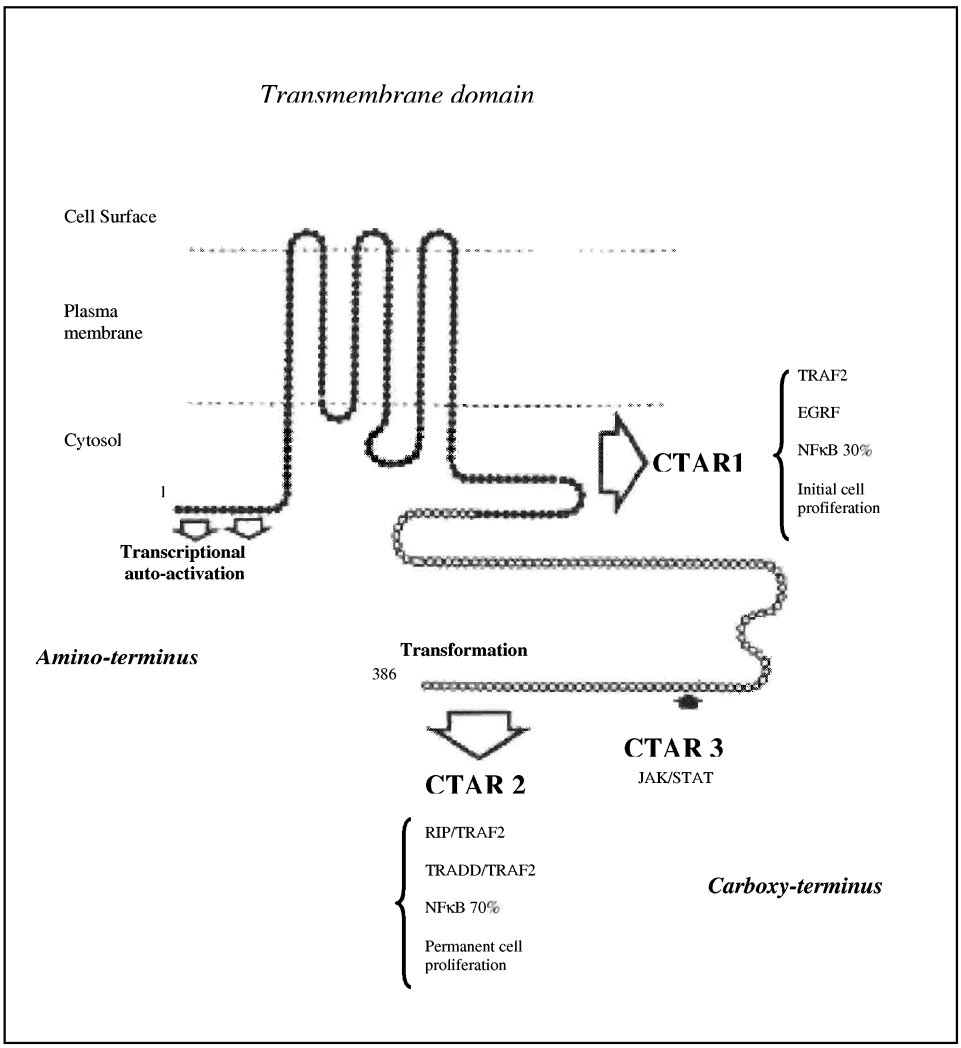

Figure 5
Schematic representation of the LMP1 with functional domains.
CTAR: C-terminal activator region

and IL-6 secretion, activation of stress-activated protein kinase, rescue growth arrest and stimulation of B cell proliferation.

LMP1 mediates activation of nuclear factor kappa B (NFκB) and cell surface phenotype via two effector regions (CTAR1 and CTAR2) in its carboxy-terminal cytoplasmic domain (Huen *et al.*, 1995). Fusion of the amino and hydrophobic domains of LMP1 to the carboxy terminal portion of CD40 produces a chimeric protein. This, like LMP1 constitutively activates NFκB (Hatzivassiliou *et al.*, 1998). This has been shown experimentally by demonstrating that the signalling domains are interchangeable (Gires *et al.*, 1997). The interaction of LMP1with TRAFs (TRAF1, 2, 3 and 5) and TRADD (Izumi and Kieff, 1997) (Mosialos *et al.*, 1995) is cell type specific (Devergne *et al.*, 1998; Miller *et al.*, 1998).

The interaction with TRAFs and TRADD has been shown to be essential for the activation NFkB pathway (Kilger *et al.*, 1998),(Busch and Bishop, 1999) and for triggering AP-1 activity via the c-Jun N-terminal kinase cascade (Kieser *et al.*, 1997). TRAF-2 activates NFκB by targeting the NFkB inducing kinase, followed by activation of IκBα kinases (IKKα and IKKβ) about 30% of LMP1-induced NFκB activation through direct interaction (Brodeur *et al.*, 1997). Most of the LMP1 mediated NFκB activation is dependent on the outermost amino acids of CTAR2 (Brodeur *et al.*, 1997) (Miller *et al.*, 1997). The activation by CTAR2 is trough direct binding of the tumor necrosis factor associated death domain protein (TRADD) which than interacts with TRAF2. CTAR2 also interact with receptor interacting protein, but contrary to TRAF2, receptor interacting protein is not required for NFκB activation. (Izumi and Kieff, 1997) (Izumi *et al.*, 1999). All naturally occurring LMP1 deletion variants show conserved CTARs and fully maintain the capacity to stimulate NFκB mediated transcription (Rothenberger *et al.*, 1997). EBV containing lymphomas, showing co-localisation of LMP1 with TRAF-1 and TRAF-3, and activated forms of NFκB, indicating that LMP1 signalling has a role in the pathogenesis of these lymphomas (Liebowitz, 1998).

3.2 Modulation of MHC class I mediated antigen presentation by EBNA1

3.2.1 MHC and antigenic peptides

The heterogenic membrane protein encoded in the MHC locus serves as a peptide display molecule for recognition by T lymphocytes. The mature MHC class I molecule exists as a trimeric complex comprised of the 43kD type I membrane glycoprotein, the heavy chain, the 12kD soluble β2-microglobulin (β2M) protein, the light chain and the antigenic peptide. The heavy chain supports the peptide grove. The peptides for the MHC class I molecules are generated in the cytoplasm mostly by proteasome (Gaczynska *et al.*, 1993) (Rock and Goldberg, 1999). Peptides of 8-10 residues are translocated from the cytosol into the endoplasmic reticulum (ER) in an ATP dependent manner through the MHC-encoded complex for the transporter associated with antigen processing (TAP). The class I complex exits the ER, proceeds through the Golgi and is displayed at the cell surface, where it may be sampled by CTLs for detection of foreign peptides of viral and pathogenic origin. The interrelationships between proteasomes and viral gene products are very complex (reviewed in (Jarrousse *et al.*, 1999)). During virus infection viral gene products expressed in the cytosol may be targeted for degradation and expressed by class I molecules. In this manner, CTLs can act early to eliminate the infected cell. To remain undetected, viruses may interfere with proteolysis (reviewed in Tortorella *et al.*, 2000).

3.2.2 The ubiqutin/proteasome system plays an important role in a variety of cellular processes, including regulation of the cell cycle, differentiation, modulation of cell surface receptors, DNA repair, transcriptional silencing or enhancing, antigen presentation, and combating viral infections and cancer.

Degradation of a protein by the ubiquitin/proteasome pathway involves two successive steps (Figure 6): conjugation of multiple ubiquitin moieties to the substrate, and degradation of the tagged protein by the downstream 26S.

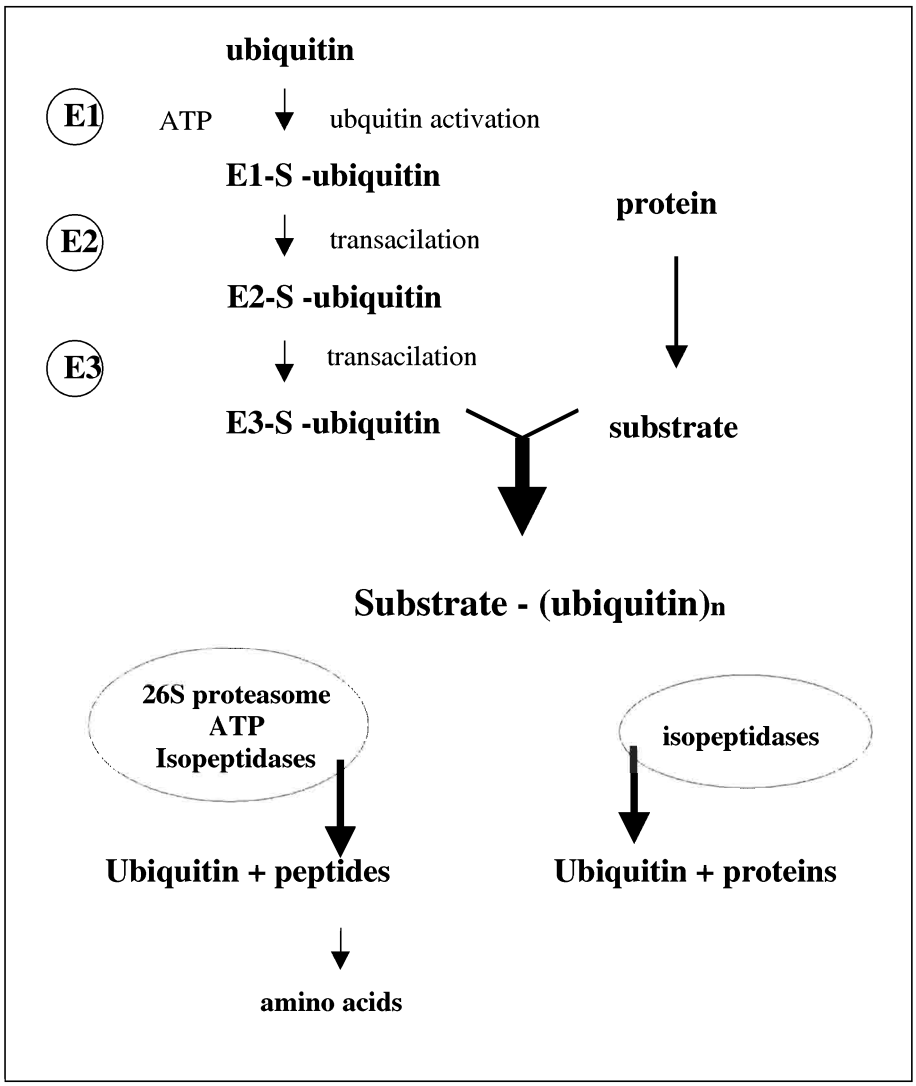

Figure 6
The ubiquitin -proteasome system: proposed sequence of events in the degradation of a protein via the ubiquitin-proteasome pathway *(adapted from Schwartz and Ciechanover , 1999)*

proteasome complex with release of free and re-utilisable ubiquitin (reviewed in Schwartz and Ciechanover, 1999).

The **proteasome** is a large multisubunit protease that is found in the cytosol, both free and attached to the endoplasic reticulum (ER) and in the nucleus of all eukaryotic cells. The proteasome can constitute up to 1% of all protein content in eukaryotic cells. The ubiquitous presence in these cellular compartments support it's central role in protein turnover in the cell. Proteasomes recognise, unfold and digest protein substrates that have been marked for degradation by the attachment of ubiquitin chains. The 26S proteasome is composed of a catalytic core, the 20S subunit, capped at each end by regulatory subunits, the 19S regulators, that are essential for the recognition and unfolding of polyubiquitinated substrates (Bochtler *et al.*, 1999). The pathway involves an enzymatic cascade through which multiple ubiquitin molecules are covalently attached to the target protein, which is then degraded by the 26S proteasome, a highly conserved ~ 2.5 MDa complex built from 31 different subunits. The 26S proteasome processes target protein substrate in an ATP-dependent fashion to yield 3-23 amino acid long oligopeptides and free ubiquitin molecules. Most of these oligopeptides are further rapidly degraded by a set of downstream proteases. Some of the peptides are translocated through the ER membrane and exported on MHC class I molecules to the cell surface, where they are presented to CTLs.

EBV interferes with MHC class I presentation via viral gene products operating during lytic cycle and latency. The BCRF1 lytic gene product, the viral-IL-10 downregulates TAP, causes downregulation of MHC class I at the cell surface (Zeidler *et al.*, 1997), and inhibits T cell proliferation and NK cell activity.

3.2.3 The EBNA1 protein

EBV-associated malignancies that express only EBNA1 escape virus-specific immune surveillance since this antigen is not a target for CTL recognition. EBNA1 is an interesting example how viruses exploit the ubiquitin/proteasome system to escape immune surveillance, most likely resulting in persistence and/or exacerbation of the infection. Against EBNA-1 expressed in all infected B cells

under latency, specific CTLs are generated during T-cell development but they fail to be activated during an EBV infection, suggesting that the appropriate EBNA1 derived peptides are not presented by class I molecules. It is not possible to detect presentation of EBNA1-encoded peptides by cells synthesising amounts of protein at level more than sufficient for presentation of determinants from other proteins.

The internal glycine-alanine repeat (GAr) domain of EBNA1 acts as a *cis*-acting inhibitor of MHC class I-restricted presentation. Insertion of the domain downstream of the immunodominant human leukocyte antigen HLA A11 restricted epitope in EBNA4 was shown to prevent recognition of the chimeric protein by EBV-specific CTLs. Conversely, the epitope was presented in the context of an EBNA1 deletion mutant lacking the GAr but not in the context of the native, GAr containing protein (Levitskaya *et al.*, 1995). EBNA4 was efficiently degraded in an ATP/ubiquitin/proteasome-dependent fashion whereas EBNA1 was resistant to degradation. Processing of EBNA1 was restored by deletion of the GAr domain whereas insertion of GAr of various lengths and in different positions prevented the degradation of EBNA4 without appreciable effect on ubiquitination. The results suggest that the repeat may affect MHC I restricted responses by inhibiting antigen processing via the ubiquitin/proteasome pathway (Levitskaya *et al.*, 1997).

3.2.4 The glycine-alanine repeat (GAr) in EBNA1 protein can prevent proteasomal proteolysis. This phenomenon is unique regarding viral proteins. No other known viral repeat can interfere with protein degradation. Eukaryotic cells present only one more resembling example, the exceptional case of the glycine-rich region "stop" signal in the middle of the p105 precursor molecule of p50 subunit of the NFκB (Ciechanover *et al.*, 2001).

Restricted information is available about diversity and structural-functional implications of amino acid repeat patterns in protein sequences. All the protein sequences from SWISS-PROT database were analyzed for occurrence of single amino acid repeats, tandem oligo-peptide repeats, and periodically conserved

amino acids. Single amino acid repeats of glutamine, serine, glutamic acid, glycine, and alanine seem to be tolerated to a considerable extent in many proteins. Tandem oligo-peptide repeats of different types with varying levels of conservation were detected in several proteins and found to be conspicuous, particularly in structural and cell surface proteins. It appears that repeated sequence patterns may be a mechanism that provides regular arrays of spatial and functional groups, useful for structural packing or for one to one interactions with target molecules. (Katti *et al.*, 2000)

Very few examples of glycine-alanine repeat are known. Spiders produce a variety of silks, and a gene family from the spider Araneus diadematus was found to encode silk-forming proteins (fibroins) with different proportions of amorphous glycine-rich domains and crystal domains built from poly(alanine) and poly(glycine-alanine) repeat motifs. Spiders produce silks of different composition by gland-specific expression of this gene family, which allows for a range of mechanical properties according to the crystal-forming potential of the constituent fibroins (Guerette *et al.*, 1996).

3.2.5 GAr transferability and other repeats

An interesting feature of the repeat is the capacity to act as a transferable element a property shared with many other known protein degradation signals. The repeat can be transferred both to viral and cellular proteins. The GAr has been successfully transferred to viral EBNA4 protein (Levitskaya *et al.*, 1997), to GFP a jellyfish (Aquorea victoria) produced green fluorescent protein, (Dantuma *et al.*, 2000), to IκBα, the inhibitor of transcription factor family NFκB *(paper III)* and to p53 tumor suppressor gene (Heessen *et al.*, 2002).

It has been demonstrated that the deletion of GAr from its natural protein, EBNA1 targets such a protein for ubiquitin/proteasomal degradation showing the role of the repeat in the protection of EBNA1 itself. The insertion of the repeat abolished the presentation of epitopes from an another EBV protein, **EBNA4** (Levitskaya *et al.*, 1997).

*The glycine-alanine repea t(GAr) prevented tumor necrosis factor (TNFα) induced degradation of the inhibitor **IκBα**, confirming that the phenomenon is not restricted to viral proteins only. The GAr containing IκBα was ubiquitinated efficiently, indicating that the repeat acts downstream of this proteasome-targeting step. The impact of the position and the length of inserted repeat on the degradation of IκBα has been also studied. Insertion of a minimal GA repeat of eight amino acids in different positions of IκBα was sufficient to prevent tumor necrosis factor (TNFα) induced ubiquitin-proteasome dependent degradation and to decrease the basal turnover of the protein in vivo. The chimeras were phosphorylated and ubiquitinated in response to TNFα released from NFκB but failed to associate with the proteasome. This explains how functionally competent IκBα is protected from proteasomal disruption and identifies the glycine-alanine repeat as a regulator of proteolysis. All these results strongly demonstrated the capacity of GAr to protect specifically the protein of interest from proteasomal degradation **(paper III)**.*

Synthetic glycine-alanine polypeptides have no stable conformation in solution and the presence of the repeat did not influence the folding and thermal stability of IκBα chimeras (Leonchiks *et al.*, 1998). These results, together with the finding that even an 8 amino acid long GAr is sufficient to protect IκBα from degradation, suggest that the repeat may interact with a yet unidentified component of the proteasomal degradation pathway.

***Other repeat peptides** have been analyzed in order to define the length and amino acid composition required to inhibit the TNFα-induced and physiological turnover of IκBα also. Inhibition of TNFα-induced degradation was achieved by insertion of octamer peptides containing three hydrophobic amino acids, alanine or valine, interspersed by no more then three consecutive glycines. The inhibitory activity was abolished by increasing the length of the spacer to four glycine, by elimination of the spacers, or by substitution of a single hydrophobic residue with a polar, serine, or charged, aspartic acid, residue. While the turnover of IκBα was increased from 30 min to more then 12 h by insertion of a GAr octamer, a serine containing octamer was inactive but the effect could be partially*

reconstituted by insertion of three consecutive repeats. These findings suggest a model where inhibition of proteolysis requires the interaction of at least three alanine residues of the GAr in a beta-strand conformation with adjacent hydrophobic binding pockets of a putative receptor ***(paper IV)***.

An obvious question was, whether the GAr mediated inhibition of antigen processing and presentation is restricted to the EBV EBNA1 or can be observed also in other lymphocryptoviruses (LCV). Most humans and Old World non-human primates are infected for life with EBV related gammaherpesviruses. Given the high degree of biologic conservation among LCVs, the strategy essential for viral persistence may well be conserved among viruses of this subgroup. Human, rhesus and baboon LCVs possesses a GAr or GAr like domain, but the baboon and rhesus LCV EBNA1 repeats fail to inhibit antigen processing and presentation as determined by using *in vitro* CTL assays. (Blake *et al*., 1999). This suggest that EBNA1 GAr produced inhibition of antigen processing and presentation may be unique for human and other mechanisms may be important for immune evasion during other primate LCV infection. The cynomolgus monkey EBV EBNA-1 sequence also shows a relatively high homology with human EBV. The functionality of the serine containing repeat of this EBNA1 like molecule is not yet tested (Ohara *et al*., 2000).

3.2.6 Functional inactivation by GAr

The inhibitory activity of the GAr has been also investigated by using **GFP** based reporters. Targeted proteolysis by N end rule or ubiquitin-fusion degradation signals (UFD) resulted in various degrees of destabilization. Degradation of the green fluorescent protein substrates was inhibited on insertion of a 25-aa GAr, but strongly destabilized reporters were protected only partially (Dantuma *et al*., 2000). Increasing the length of the repeat has enhanced the GAr mediated protection. However, reporters containing the strongly destabilized Ub-R and UFD signals were degraded even in the presence of a 239-aa GAr. In accordance, insertion of a powerful degradation signal relieved the blockade of proteasomal degradation in EBNA1. These findings suggest that the turnover of natural

substrates may be finely tuned by GAr-like sequences that counteract targeting signals for proteasomal destruction (Dantuma *et al.*, 2000).

Functional inactivation of the tumor suppressor protein **p53** by accelerated ubiquitin/proteasome-dependent proteolysis is a common event in tumor progression. Chimeras of p53 containing GAr domains of different lengths and positions within the protein are protected from proteolysis induced by the ubiquitin ligases murine double minute 2 (Mdm2) and HPV-E6-associated protein but are still ubiquitinated and retain the capacity to interact with the S5a ubiquitin-binding subunit of the proteasome. The GAr chimeras transactivate p53 target genes, induce cell cycle arrest and apoptosis, and exhibit improved growth inhibitory activity in tumor cells with impaired endogenous p53 activity (Heessen *et al.*, 2002).

An interesting observation is that human $CD8^+$ CTL clones recognizing EBNA1-specific peptides evidenced that full-length EBNA1 is not presented when expressed endogenously in target cells, whereas the GAr-deleted form is presented efficiently. However, when supplied as an exogenous antigen, the full-length protein can be presented on HLA class I molecules by a TAP-independent pathway; this may explain how EBNA1-specific CTLs are primed *in viv*o (Blake *et al.*, 1997).

The GAr-mediated proteasomal block on EBNA1 reversed by specifically targeting of this antigen for rapid degradation results in enhanced $CD8^+$ T cell-mediated recognition *in vitro* and *in viv*o. Cotranslational ubiquitination combined with N-end rule targeting enhances the intracellular degradation of EBNA1, thus resulting in a dramatic reduction in the half-life of the antigen. Using DNA expression vectors encoding different forms of ubiquitinated EBNA1 for *in vivo* studies revealed that this rapid degradation, remarkably, leads to induction of a very strong CTL response to an EBNA1-specific CTL epitope. Furthermore, this targeting also restored the endogenous processing of HLA class I-restricted CTL epitopes within EBNA1 for immune recognition by human EBV-specific CTLs (Tellam *et al.*, 2001).

4 Modulation of viral gene expression

4.1 Modulation of EBV genes by EBNA1

4.1.1 Feature of oriP and EBNA1

oriP is one of two viral components required for the replication and maintenance of the EBV genome. The oriP includes two essential elements termed the family of repeats (**FR**) and the dyad symmetry (**DS**) element, separated by approximately one kilobase pair of DNA (Leight and Sugden, 2000)(Figure 7).

The **FR** contains 20 imperfect copies of a 30–base pair repeat. EBNA1 dimers bind site specific and with high affinity to a 16 base pair palindrome within each repeated element. FR in conjunction with EBNA1 provides a retention/maintenance function and transcriptional enhancement to plasmids containing this sequence (Middleton and Sugden, 1994). FR can also act to enhance transcription from viral and heterologous promoters (Sugden and Warren, 1989). The **DS** contains four low affinity EBNA1 binding sites, two of which are located within a 65-base pair extended palindrome or dyad for which the element was named (Ambinder *et al*., 1990). DS has several notable features. The dyad has the potential to form a cruciform structure and other herpesviruses (for i.e. HPV or herpesvirus papio) also contain dyad symmetry regions, which may contribute in *cis* to support replication. Foot printing analysis demonstrates that these sites are occupied throughout most of all cell cycle.

EBNA1 is the only known EBV encoded protein to function at oriP. The protein derived from prototypical B95-8 strain of EBV is 641 amino acid length. The different regions of the EBNA 1 protein are having different role in his functionality (figure 7). The **GAr**, the sequence which conferees stability to the protein has a position in the amino-terminal half while the other regions are important to bind of the EBNA1 molecules to each other (dimerization) or to bind the EBNA1 to viral and/or eukariotyic DNA. The **NLS** is a nuclear localisation sequence defined initially by its ability to target a cytoplasmic protein to the nucleus is located between the amino acids 379-386 (Ambinder *et al*., 1991).

The **DBD/DD,** the DNA site specific binding and dimerization domain is located in the carboxy-terminus. The structure of this domain(Bochkarev *et al.*, 1996) resembles the DBD of E2 protein from bovine papilloma virus. Carboxy-terminal to DBD/DD is a 34 amino acid acidic tail. The role of this region is not clear. The **LR1/LR2** are two redundant linking regions on the both sites of the GAr. DNA linking refers to the ability of EBNA1 dimers which are bound to DNA to associate with other EBNA1 dimers which are bound to DNA, thereby linking the DNAs or looping the intervening DNA sequences (Goldsmith *et al.*, 1993) (Laine and Frappier, 1995) (Mackey *et al.*, 1995). Besides binding DNA, EBNA1 is able to bind RNA *in vitro* including the EBER1. The role of EBNA1 binding *in vivo,* if any is not understood.

4.1.2 The role of EBNA1 in the replication of oriP plasmids

EBNA1 clearly provides a maintenance function to plasmids containing **FR** (Middleton and Sugden, 1994). EBNA1 and oriP plasmids both associate with methaphase chromosomes and EBNA1 are facilitating the maintenance of plasmids containing FR (Marechal *et al.*, 1999). Leight and Sudgen envision a model by which EBNA1 facilitate maintenance. According to this model EBNA1 associates with an oriP plasmid via its DBD/DD and homes this plasmid to a chromosome via heterotypic interactions in which the two LRs of EBNA1 associate with a protein bound to the chromosomal DNA. Cellular proteins are involved in this process, perhaps EBP2 which associates with the LR2 region of EBNA1(Shire *et al.*, 1999) or the *kid* which associate with the spindle apparatus and binding oriP (Zhang and Nonoyama, 1994).

4.1.3 The role of EBNA1 in transcriptional activation

Viral origins of replication such SV40 or polyoma virus contain enhancer elements which positively regulate transcription from neighboring promoters and may contribute to the regulation of DNA synthesis.

Examination of oriP and EBNA1 indicates that FR too is an enhancer activated by the binding of EBNA1 (Reisman *et al.*, 1985). The EBNA1 binding to FR not

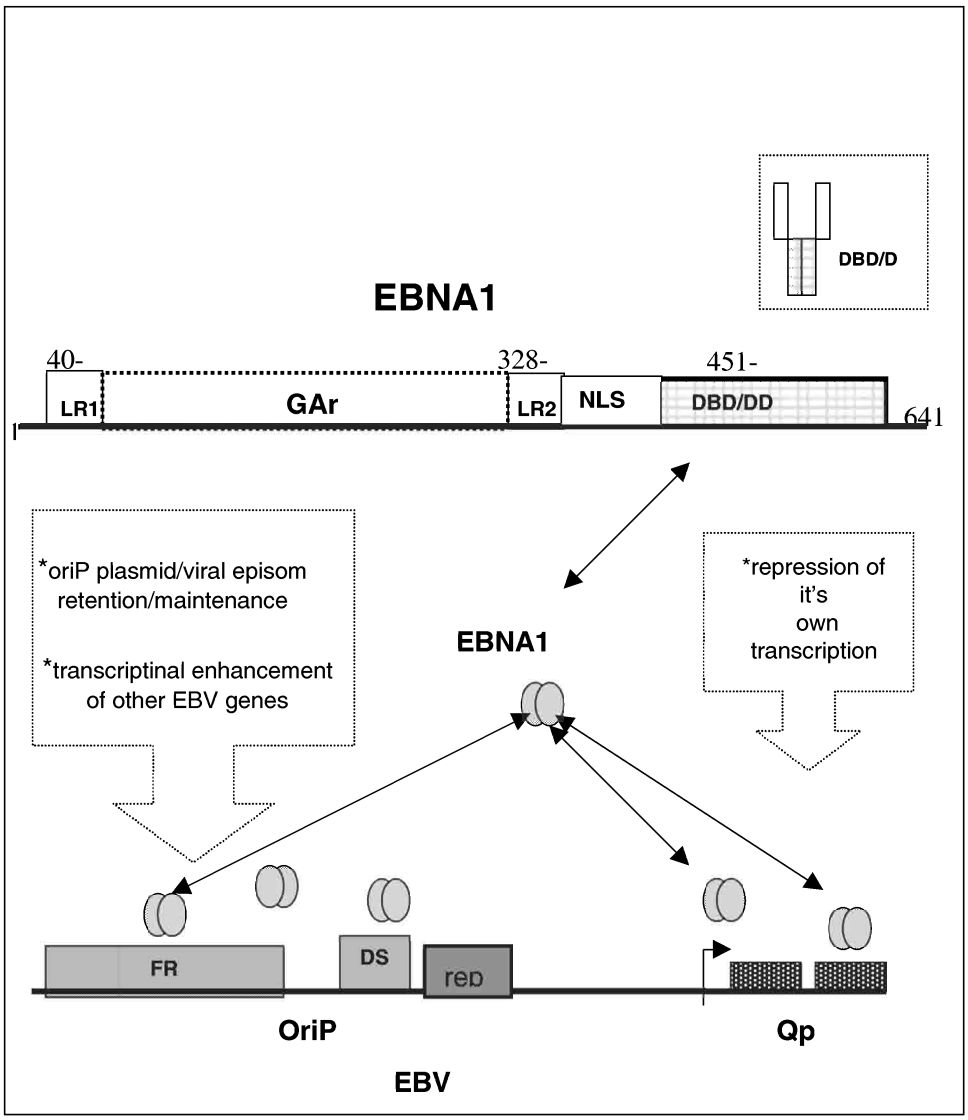

Figure 7

The impact of EBNA1 on the EBV genome

EBNA1: **LR**: Linking region/basic amino acid rich chromosome association domain, **GAr**: glycine-alanine repeat, **NLS**: Nuclear localization signal **DBD/DD**: DNA biding and dimerization domain; **oriP:** **FR**: family of repeats, **DS**: dyad simmertry region/distortion region **REP**: replication start

only enhances transcription from heterologous promoters (Reisman and Sugden, 1986) but enhances transcription from 2 key late promoters in EBV, namely the BamHI C and LMP1 promoters (Sugden and Warren, 1989). Thereby, EBNA1 enhances expression of five latent genes required for the induction and maintenance of proliferation in EBV infected cells.

In addition, EBNA1 binds to two sites within the BamHI Q promoter (Qp) (Ambinder *et al*., 1990) and regulates its own expression in some tumor biopsies and group I Burkitt's lymphoma lines (Schaefer *et al*., 1995b).

The mechanism by which EBNA1 facilitates transcriptional enhancement is obscure but the DBD/DD region are required (Schaefer *et al*., 1995b). Cellular proteins EBP2 and P32/TAP may contribute. Perhaps EBNA1 enhances transcription from an oriP plasmid by binding oriP and relocating the plasmid to a transcriptional activation center within the nucleus (Langle-Rouault *et al*., 1998). EBNA1 may enhance transcription independently of relocation and bound DNAs to the nucleus by proteins acting as transcription factors.

4.1.4 The role of the GAr in EBNA1 function

The GAr can be deleted without affecting the ability of EBNA-1 to support replication and transcription in cell culture (Bornkamm and Hammerschmidt, 2001; Yates *et al*., 1996) nor affecting its ability to immortalize B cells (Blake *et al*., 1999). However, this domain plays an important role during the latent phase of the viral life cycle *in vivo*. Previously has been shown that the GAr efficiently inhibits the ubiquitin/proteasome dependent degradation of EBNA-1, and thereby prevents presentation of EBNA-1 derived antigenic peptides on MHC class I molecules (Levitskaya *et al*., 1997). Thus EBV carrying neoplastic cells have means to bypass EBNA-1-directed immune surveillance mediated by cytotoxic T lymphocytes.

Beside favoring evasion of CTL surveillance, the stabilizing effect of the GAr might serve other purposes that explain the regular detection of long repeat domains in the EBNA1 gene of all EBV isolates and the presence of analogous domains in the EBNA1-like genes of gamma herpesviruses.

Two critical functions of EBNA1, the capacity to maintain the viral plasmid and the regulation of the Qp promoter, are strongly enhanced by the presence of the repeat.

Using a set of EBNA1 recombinant proteins that lack GAr domain, or are targeted for ubiquitin-dependent proteolysis by insertion of specific signals we demonstrate that the stabilizing effect of the GAr is instrumental for at least two functional properties of EBNA1. Thus, destabilized EBNA1 polypeptides showed impaired capacity to promote the maintenance an EBV oriP containing plasmid and failed to repress transcription of an EBV Qp promoter-driven luciferase reporter plasmid. The results suggest that the GAr may fulfil an important regulatory function that is critical for the persistence of EBV infected cells in latently infected hosts ***(paper 5)****.*

Conversely, destabilization of GAr containing EBNA1 by insertion of signals that target the protein for ubiquitin-dependent proteasomal degradation diminished both functions, confirming that resistance to proteolysis is indeed an important prerequisite for numerous activities of this viral protein. It is important to notice, that the GAGS repeat which is unable to protect EBNA1 or the endogenous EBNA1-like proteins from herpesvirus papio, produces MHC class I restricted CTL responses. GAGS is capable of reconstituting the plasmid maintenance and Qp promoter regulation activities of the GAr at least to some extent (Davenport and Pagano, 1999) (Gavioli *et al*., 2001). Thus, partial stabilization appears to be sufficient to promote the regulatory functions of EBNA1 but insufficient for immunoescape. This is probably explained by the great efficiency of the antigen processing and presentation machinery where a very small amount of antigenic peptide presented at the cell surface is sufficient for CTL triggering. This finding has interesting implications in the context of EBV biology and evolution. The different function of the human and primate repeat may reflect a different adaptation of the protecting sequence of the proteolytic machinery expressed in the natural host. For example, species-specific variations may influence the binding of the repeats to chaperones or other regulatory components of the ubiquitin-proteasome pathway. Alternatively, the failure to preserve the

immunoescape promoting activity of the repeat may indicate that this is not a critical function in the context of the biology of virus infection. Indeed, while the selective escape of EBNA1 does not prevent the recognition of EBV transformed blast that express other highly immunogenic viral proteins (Gavioli *et al*., 2001), the non-immunogenic phenotype coupled with endogenous defects in antigen processing may be sufficient to assure the immunoescape of EBNA1-only expressing tumors (Thorley-Lawson and Babcock, 1999).

It remains to be seen how EBNA1 stability per se may contribute to the biology of virus infection. There are at least two situations where resistance to proteolysis could play a critical role. The ubiquitin-proteasome system is an important player in cell cycle control and different components of the system are activated in a temporally regulated fashion during cell cycle progression and cell division. The maintenance of oriP plasmids is an active process, which includes, but is not limited to, events involved in partitioning/segregation of plasmids to daughter cells and retention of these plasmids within the nucleus of the dividing cell. Conceivably, these functions could be critically dependent on the capacity of EBNA1 to avoid proteolysis and persist at constant levels throughout the cell cycle. Protein stability could also be important in conditions of minimal metabolic activity such as in latently infected resting B cells. It is noteworthy that the use of efficient quantitative PCR methods for evaluation of the number of latently infected B lymphocytes in healthy EBV carriers has revealed an interesting discrepancy between the number of virus DNA carrying cells and the number of cells expressing viral transcripts (Thorley-Lawson and Babcock, 1999). Thus, the viral genome appears to be transcriptional silent in at least a proportion of the infected cells. A stable EBNA1 protein could be expressed at significant levels also under these conditions and continues to exert its regulatory functions. One function that could be critically important in this type of virus infected cells is the down-regulation of the Qp promoter that drives transcription of the EBNA1-only messages in resting B cells and EBV carrying tumors. Relatively high levels of EBNA1 may be instrumental for silencing the promoter in resting B cells and many counteract the activity of positive regulators in proliferating tumor cells. A

drop in EBNA1 levels may be the triggering factor that stochastically promotes exit from latency in a subpopulation of resting B cells.

Conclusions and future perspectives

Our studies have shed some more light on the interplay of cellular and viral genes in EBV infected cells. Our data may also be relevant for a better understanding of the role of EBV antigens LMP1 and EBNA1 in EBV related malignancies and illustrates the diversity of mechanisms of MHC related modulation of immune responses.

- Loss of HLA expression has been described in a wide variety of human tumors but analysis of the underlying mechanisms has been limited. Immunological and biochemical methods have previously evidenced a selective down-regulation of HLA A11 in BL lines derived from HLA A11 positive tumor patients. Our approach of comparing HLA genotype, specific mRNA expression and HLA promoter activity in pairs of LCL and BL lines from the same individual has allowed us to address the molecular mechanisms of this defect. Our data suggest that multiple defects may contribute to the down-regulation of HLA A11 expression in BL cells. Lack of appropriate transcription factors seems to be a property of the BL cell phenotype. It is tempting to speculate that down-regulation of this allele may not be sufficient to ensure the escape from tumor specific immune responses since cells carrying structural defects appear to enjoy a selective advantage *in vivo*. It is likely that only extinction of HLA A11 expression as a consequence of genetic alteration may afford full protection.
- The phenotype dependence of MHC class I promoter activity prompted us to ask whether, similar to other characteristics of BL cells, this could be modulated by expression of the EBV encoded membrane protein LMP1. Our data show that MHC class I can be added to the list of LMP1 regulated cellular genes and conclusively demonstrate that regulation occurs at the level

of transcription. Expression of LMP1 is correlated with up-regulation of several components of the transcription factor family NFκB/Rel including the active subunits as well as for the inhibitory components. The enhanced expression of the inhibitors is probably a consequence of their accelerated turnover. Increased degradation of the inhibitor results in nuclear translocation of the transcription factors and activation of the target genes including the transcription factor itself and its inhibitors.

- In the row of viral strategies that promote escape from immune recognition the EBNA1, expressed in all EBV related malignancies occupies a special place. EBNA1 is not recognized by MHC class I restricted CTLs. This phenomenon is correlated with the presence of an internal repeat sequence, the glycine-alanine repeat (GAr) which has been identified as a unique regulator of proteolysis and opens the perspective for a useful tool for the modulation of the proteolysis in normal and malignant cells. We have chosen IκBα as a model to investigate whether and tough which mechanism the GAr domain influences degradation dependent on ubiquitin-proteasome *in vivo*. We observed that insertion of a minimal GAr motif in different positions in IκBα protect the protein from signal-induced degradation dependent on ubiquitin/proteasome, and decreases its basal turnover *in vivo* resulting in constitutive dominant-negative mutants. The chimeras are phosphorylated and ubiquitinated in response to TNFα but are then released from NFκB and fail to associate with the proteasome. This explains how functionally competent IκBα is protected from proteasomal disruption and identifies the glycine-alanine repeat as a new regulator of proteolysis.
- The GAr of the EBV nuclear antigen EBNA1 is the first example of protein domain that acts as a *cis*-inhibitor of ubiquitin/proteasome-dependent proteolysis. An eight amino acid long GAr containing at least 3 alanine residues inhibits the TNFα-induced degradation of IκBα by preventing stable interaction of the ubiquitinated substrate with the proteasome. When we have examined the structural and sequence requirements for the inhibitory effect by comparing the degradation of IκBα chimeras containing different octamer

peptides, we found a clear relationship among the spacing between the three key alanine residues and the inhibitory effect. The inhibition was weaker when the spacer was increased from one to three glycines and disappeared in octamers, which contains a four-glycine spacer. Clearly, much remains to be done to clarify the function of the GAr in the context of EBV infection. In addition, the capacity of the GAr to inhibit proteasomal degradation in *cis* provides an interesting new tool for the selective stabilisation of proteins that are degraded by the ubiquitin/proteasome pathway *in vivo*. Protection of transduced proteins from proteasomal degradation, in order to prolong their half-life or prevent immune recognition, remains an important task for gene therapy.

- Two benefits are likely to be derived from our increased understanding of the mechanisms by which EBNA-1 supports replication and transcription. First, its ubiquitious expression in and essential contribution to EBV-associated tumors makes it the most desirable target from EBV for antiviral therapy. Understanding EBNA-1 will facilitate the development of drugs to inhibit its intrinsic activities. Second, understanding EBNA-1's contributions to the replication of and transcription from oriP plasmids will facilitate the development of powerful non-immunogenic vectors for use in human gene therapy.

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Acknowledgements

This work has been carried out at the Department of Tumor Biology and later at the Microbiology and Tumorbiology Center, Karolinska Institutet, Stockholm, Sweden.

I would like express my gratitude to all those who, in many different ways helped me to complete this work. Supporting me in a variety of aspects in good times and bad times, at the start or in the final faith. It's a very large number of people. To not forget anybody I will mention only the persons decisive to start and finish my thesis. Without their help this thesis never had been reality. I will mention them in chronological order of their impact.

I have to thank to **Stefan** who had the crazy idea to start a new life in Sweden after 40. For this switch and his stubborn and obstinate way of making science. For being my partner and supporter in so many aspects of the life.

To **George and Eva Klein** to accept me from nowhere in the old Tumorbiology and not only for establishing the basis of the tumorbiology and EBV biology which continues today at MTC, but for their friendship and support during all this years.

I would like to thank to **Maria G. Masucci**, for being much more than an excellent supervisor, for her incommensurable patience and tolerance, supporting me and helping me with my personal matters. I whish to express also my sincere gratitude for her generous guidance, corrections and comments. Without her help this thesis would have never been written.

To **Anatoly Sharipo** for being such a special friend, for his precious help, discussions, support and guidance and kind companionship.

To **my family**, my children: Istvan, Gabriella and Beatrice and my brother Gyurci. To my **friends and relatives** all over the world.

I would like to thank for all support, fun and friendship to many people with whom I worked at the old Department of Tumorbiology and the MTC. I would like to thank to former and present members of MGM group, my colleagues at MTC, members of the stuff, the former and present members of the Hungarian community.